Aquaculture of stalked barnacles

(*Pollicipes pollicipes*)

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Abstract

The stalked barnacle, *Pollicipes pollicipes*, is considered a delicacy on the Iberian Peninsula and has a high market value. Despite being a dangerous activity, increased collection efforts and associated stock shortage have raised awareness of the need for effective conservation and stock management policies. Accordingly, aquaculture has received interest as an alternative to supply the market and for re-stocking programmes. However, knowledge on the aquaculture requirement of this species and applicable production cycles is limited.

Research challenges span the entire *P. pollicipes* life cycle, from adult reproduction to larval settlement. Though adults have been kept in culture, the conditions required for broodstock reproduction and larval release remain poorly studied and larvae have been routinely extracted from wild-collected adults and reared to cyprids. Optimization of larval culture is essential for the production of high-quality larvae and avoidance of high mortality. Furthermore, cyprid settlement on artificial substrata presents a bottleneck to production, with settlement occurring mostly on conspecific adults. The conditions that mediate settlement on preferential substrata have yet to be established. Though juvenile behaviour and growth in the wild have been the subject of ecological studies, research on culture conditions is limited and the influence of environmental factors is poorly understood.

In the present work, the effect of environmental conditions on the behaviour and development of *P. pollicipes* was tested throughout the life cycle to identify optimal culture conditions and assess potential for larger-scale culture. Research focused on broodstock reproductive conditioning, larval culture, larval settlement and juvenile culture and behaviour. Broodstock reproductive conditioning was investigated by monitoring larval production and nauplius quality from adults reared under different temperature regimes. *P. pollicipes* larvae were also cultured under different conditions of temperature, food quality, photoperiod and salinity, and monitored for growth and survival. Larval attachment and metamorphosis on adults were tested for cyprids of different age and batch, and according to environmental factors such as temperature, salinity, hydrodynamics and light conditions. Optimal conditions were then used for investigating settlement on substrata in culture, and natural and artificial structures were

tested for settlement in the wild. Juvenile feeding behaviour and performance in culture were investigated in relation to hydrodynamics, temperature, food quality and quantity.

Results indicated that *P. pollicipes* conditioning requires 28 days after acclimatization, when subjected to increasing temperatures (from 16 ^{o}C to 24 ^{o}C), as found during the breeding season. This could be achieved either by steady increase, or increase with daily temperature oscillations. Larvae were released daily and peak releases ($\geq$ 10000 – 30000 nauplii per tank; $\approx$122 adults per tank) occurred 1 – 2 times during the conditioning period. Higher fecundity was recorded for broodstock reared under steady temperature increase, while lamella maturation and larval production were higher at oscillating temperatures. No differences in larval quality were recorded among temperature regimes.

Larval growth and survival were improved by feeding daily with *Tetraselmis chuii/Skeletonema costatum or Isochrysis galbana/S. costatum* mixed diets, temperatures of 15 – 20 ^{o}C, 24:0 L/D photoperiod and salinity of 20 – 40 psu. Under these conditions, development to the cyprid was achieved within 16 days (20 ^{o}C), with 20 – 30 % survival.

Larval settlement on the adult was higher for older cyprids (6 days), at 20 ^{o}C, with water circulation, light and salinity of 30 – 40 psu to a maximum of 30 – 35 % settlement. Metamorphosis was affected by temperature and cyprid age, with a maximum of 70 – 80 % larvae metamorphosing within a week. Settlement occurred predominantly on the adults, mainly on the capitulum ($\approx$ 60 % of settled larvae), and was greatly reduced on artificial substrata tested in the laboratory (< 3 %) and, in this experiment, was not recorded in the wild. Settlement on natural substrata (e.g. *Chthamalus* sp., *Corallina* sp., rocks) was higher (< 17 % per surface) and in the wild appeared to be related to future survival potential on the substrata. On another occasion, settlement was observed on marine epoxy in the wild, used as a fixative for structures, being comparable to that on adults (2 recruits per cm^{2}).

Juvenile feeding varied with the degree of laboratory conditioning, as unconditioned individuals only responded to water speeds above 23 cm s^{-1} in comparison to above 6 cm s^{-1} for conditioned animals. Those barnacles conditioned in the laboratory prior to testing also showed more frequent prey captures, although live nauplii of *Artemia* sp. promoted the highest feeding rates for both groups. Alterations to juvenile morphology were observed in culture, though reversible after transfer to the wild. Growth and survival were optimized in cultures not subjected to daily tides, at 16:8 L/D

photoperiod, fed daily with excess *Artemia* sp., under which conditions laboratory specimens achieved growth values comparable to the wild.

Due to the limited previous knowledge on *P. pollicipes* rearing, the present study aimed at identifying the conditions required to the culture of this species, over the various production phases. It also aimed at building on ecological studies, towards the understanding of the factors that mediate habitat selection and distribution. The present work has established the initial basis for adult reproduction in culture and provided the first reference values on broodstock performance in captivity. Future studies should focus on investigating the use of different diets and other environmental factors for *P. pollicipes* broodstock conditioning, in order to maximize yields. The use of recirculating systems for larval culture should be investigated, using as a basis the knowledge acquired on environmental conditions and further looking at improving feeding and husbandry protocols. Current reference values on larval growth and survival can be used as a base for future culture optimization. The present work investigated some of the factors affecting larval settlement and has given important clues over key factors mediating settlement and potential substrata to be used for recruitment. However, settlement remains the largest limitation to culture and efforts should build on the current work to target the testing of settlement substrata that afford larval protection post-settlement, as well as the use of chemical attractants to induce settlement. The development of field trials should be a priority as well as further assessing the performance of laboratory-cultured barnacles transferred to the wild. The investigation on the effect of environmental factors on larval and juvenile behaviour provides new data on *P. pollicipes* behaviour in response to factors such as hydrodynamics, towards the understanding of *P. pollicipes* distribution in the wild, and the impact of larval choice in substrata selection. The acquired knowledge on juvenile performance under culture can be used to direct future work on *P. pollicipes* production and tackle expected pitfalls (e.g. changes in morphology). Studies on juvenile growth should be directed at the assessment of economical viability of commercial scale culture, considering both culture options in captivity and in the natural environment.

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What started from a simple idea and a few questions, soon became an overreaching challenge and a rollercoaster ride, between endless questioning, difficulties, experiments and investigations. But, as Fernando Pessoa once said “Tudo vale a pena. Se a alma não é pequena.”.

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Chapter 1. Aquaculture of stalked barnacles (*Pollicipes pollicipes*): state of the art and future challenges

Abstract

Pollicipes pollicipes is an economically important species, considered a delicacy on the Iberian Peninsula, where it commands high market prices. In past decades, the high market demand associated with stock shortages and ecological concerns has raised interest in aquaculture-based production of the species. However, in spite of its potential for culture, few studies have investigated the conditions necessary for production of *P. pollicipes* and have, instead, focused mainly on the ecology of the species.

The present paper synthesises current knowledge surrounding the biology and ecology of *P. pollicipes* with a focus on informing development of sustainable aquaculture for the species. There is also critical analysis of the existing literature describing recommended culture conditions. The main challenges of the various production phases (broodstock reproductive conditioning and spawning, larval rearing and settlement, and juvenile culture) are addressed and potential production cycles are discussed in terms of feasibility, current supporting knowledge and future challenges.

Future research should focus on investigating how to optimize culture conditions, systems and protocols, as well as assessing the feasibility of *P. pollicipes* commercial aquaculture and best culture practices. Current avenues of interest include artificial settlement surfaces as well as synthetic or natural chemical settlement inducers. Notwithstanding its preliminary nature, further development of this work is imperative, to assist the balancing of commercial interests and the ecological preservation of this species.

1.1. Introduction to *P. pollicipes*: taxonomy, ecology and biology

Pollicipes pollicipes (Gmelim, 1970) is a pedunculate barnacle, superorder Thoracica, that inhabits exposed intertidal rocky shores, from the shallow subtidal to the mid-intertidal zone (Barnes, 1996; Cruz & Araújo, 1999; Cruz et al., 2010; fig. 1.1). Thoracican crustaceans (Crustacea, Maxilopoda, Cirripedia; Lin & Hwang, 2006; ITIS, 2010; Bisby et al., 2010) comprise stalked barnacles (order Pedunculata) (e.g. *Pollicipes* spp., *Lepas* spp.) and acorn barnacles (order Sessilia) (e.g. *Balanus* sp., *Semibalanus* sp.) (Anderson, 1994; Pérez-Losada et al., 2008). The genus *Pollicipes* (Newman, 1987) is represented by *P. pollicipes*, found in the east Atlantic, from Senegal to France (Darwin, 1851; Bishop et al., 1957; Stubbings, 1967; Girard, 1982; Newman & Killingley, 1985; Cruz, 1993; De la Hoz & Garcia, 1993; Barnes, 1996) and formerly in southern UK (Hayward and Ryland, 1990); the recently described *P. caboverdensis* sp. nov., endemic to Cape Verde (Quinteiro, 2007; Fernandes et al., 2010; Van Syoc et al. 2010); *P. elegans*, found in the east Pacific, from Peru to Baja California, with a distribution gap on the central american coast (Kameya & Zebballos, 1988; Newman, 1992; Van Syoc, 1994); and *P. polymerus*, found in the east Pacific, from Baja California to the Aleutian Islands (Tarasov and Zevina, 1957; Lewis, 1975a; Poltarukha & Korn, 2006). Morphological, phylogeographical and genetic evidence suggest that *P. polymerus* diverged from an ancestral pollicipedine (55 – 65 myBP) prior to the emergence of the remaining *Pollicipes* species that seem to have radiated later (Van Syoc et al., 1995, 2010; Pérez-Losada et al., 2008). This may explain the considerable morphological similarity among the latter species. *Pollicipes* spp. are also closely related to *Capitulum mitella* (Van Syoc, 1995; Young, 2001; Perez-Losada et al., 2008), which is found in the west Indo-Pacific (Jones et al. 2000; Chan, 2006), and to *Calantica spinosa*, also a native of this region (Van Syoc et al., 2010). The similarities between *Capitulum* and *Pollicipes* are so striking that Darwin (1854) considered them to be the same genus. Nevertheless, morphological and genetic criteria support the current genus-level distinction among these taxa (Van Syoc, 1995), in spite of conflicting positions between authors (e.g. Lin & Hwang, 2006).

Barnacle biology as a field was established by Darwin (e.g. Darwin, 1851, 1854) and followed by Crisp (e.g. Crisp & Southward, 1961; Crisp & Bourget, 1985), Southward (e.g. Southward, 1955ab) and others (e.g. Knight-Jones, 1953), with H. and M. Barnes responsible for extensive work on *Pollicipes* spp. specifically (e.g. Barnes & Reese, 1959, 1960; Barnes, 1996). In spite of the extensive research on the genus *Pollicipes*,

some studies have focused on *P. polymerus* (e.g. Hilgard, 1960; Cimberg, 1981; Hoffman, 1988, 1989; Lauzier, 1999), very few on *P. elegans* (e.g Van Syoc, 1994; Karneya & Zeballos, 1988), with research on *P. pollicipes* developed for the most part by Cruz (e.g. Cruz, 1993, 2000; Cruz et al., 2010) and Molares (e.g. Molares et al., 1994a, 1994b; Molares, 1998), although others have contributed significantly (e.g. Cardoso & Yule, 1995; Norton, 1996; Pavón, 2003; Candeias, 2005; Macho, 2006) (table 1.1).

Subject	Reference	Location
Archaeology	Alvaréz-Fernandez et al. (2010)	Europe
Stock management	Bald et al. (2006)	NE Spain
Archaelogy	Bicho (2004)	Portugal
Stock management, distribution	Borja et al. (2004; 2006a)	NE Spain
Philogeography	Campo et al. (2010)	SW Europe / NW Africa
Larval feeding	Candeias (2005)	Laboratory
Reproduction	Cardoso & Yule (1995)	SW Portugal
Reproduction, growth	Cardoso (1998)	SW Portugal
Stock management	Castro (2004)	SW Portugal
Conservation	Castro & Cruz (2009)	SW Portugal
Stock management, behaviour, reproduction	Cribeiro (2007)	NW Spain
Reproduction	Cruz & Araújo (1999)	SW Portugal
Reproduction	Cruz & Hawkins (1998)	SW Portugal
Growth	Cruz (1993)	SW Portugal
Reproduction, recruitment, growth, distribution	Cruz (2000)	SW Portugal
Recruitment, growth	Cruz et al. (2010)	SW Portugal
Growth, recruitment	Cunha & Weber (2000)	Portugal
Distribution, reproduction	De la Hoz & Garcia (1993)	NW Spain
Stock management	Freire & Garcia-Allut (2000)	NW Spain
Conservation, stock management	Jacinto et al. (2010)	SW Portugal
Stock management	Jesus (2003)	SW Portugal
Adhesion, mobility	Kugle (1993, 1998)	Laboratory
Larval development	Kugele & Yule (1996)	Laboratory
Adult morphology, reproduction	Klepal & Barnes (1978)	Laboratory
Reproduction, recruitment, larval distribution	Macho (2006)	NW Spain
Larval release	Macho et al. (2005)	NW Spain
Gametogenesis, larval growth	Molares et al. (1994ab)	Laboratory/NW Spain
Stock management	Molares & Freire (2003)	NW Spain
Biological cycle, exploitation	Molares (1994, 1998)	NW Spain
Stock management	Molares (2001)	NW Spain
Larval ecology	Molares et al. (2002)	NW Spain
Feeding	Norton (1996)	Laboratory
Stock management	Novo Loureiro (2000)	Spain
Reproduction, recruitment, population dynamics	Pavón (2003)	NW Spain
Stock management	Perez (1996)	NW Spain
Phylogeography	Quinteiro et al. (2007)	SW Europe / NW Africa
Growth, stock management	Sestelo et al. (2009)	NW Spain
Growth, stock management	Sestelo (2002)	NW Spain

Table 1.1 Recent literature focused on *P. pollicipes* biology, ecology and stock management, according to subject area and location of study. References are provided in alphabetical order. Monographs and reviews on superior phyla and similar species were excluded.

Fig. 1.1 *P. pollicipes* in the natural environment. Detail of the clusters of *P. pollicipes* (a) attached to the rock, (b) adults with epiphyte *Ulva* sp., and view of the intertidal rocky shore environment in (c) Cabo Sardão (Portugal) and (d) Sines (Portugal)

P. pollicipes are usually found in rosette-shaped clusters of sessile individuals attached to each other and to the primary substratum. The muscular stalk, attached to the substratum, is covered by a flexible cuticle with calcareous scales. These scales vary in size along the stalk and support the capitulum, which is in turn protected by the capitular plates. The capitulum, furthest from the substratum, contains the thoracic appendages and most of the other organs (Molares, 1993), as shown in Fig. 1.2.

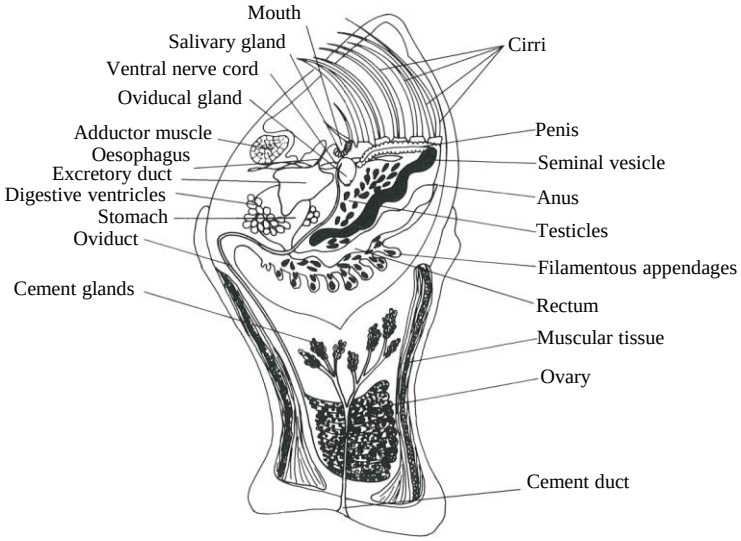


Fig. 1.2 Internal anatomy of *P. pollicipes* (adapted from Molares, 1994).

P. pollicipes are simultaneous hermaphrodites and obligate cross fertilisers. Gregariousness (Knight-Jones & Stevenson, 1950; Knight-Jones, 1951, 1953) has thus probably evolved in these sessile organisms to facilitate mating with conspecifics (fig. 1.3; Cruz & Hawkins, 1998; Molares et al., 1994b; Cruz, 2000; Pavón, 2003).

Fig. 1.3 Clusters of *P. pollicipes*.

Adults produce approximately 30000 – 130000 eggs per brood and have been reported to have 1 to 5 broods per year, during the breeding period generally that occurs from March to October (Girard, 1982; De la Hoz & Garcia, 1993; Molares et al, 1994a; Cardoso & Yule, 1995; Barnes 1996; Cruz & Hawkins, 1998; Cruz & Araújo, 1999; Cruz, 2000; Pavón, 2003; Macho, 2006). Though ovaries and testes have been reported to remain functional year-round (Cardoso & Yule, 1995), various authors (Molares et al., 1994b; Cruz & Hawkins, 1998; Cruz, 2000) have concluded that the female gonad rests outside the breeding season. Embryonic development of *P. pollicipes* occurs inside the mantle cavity of adults, who carry the fertilised eggs about 1 monthm but up to 3 months (Cruz & Araújo, 1999) until hatching and release of first stage nauplii. When released to the water column, nauplii moult through six nauplii, culminating in a specialised stage known as the cyprid (fig. 1.4; Coelho, 1990; Molares et al., 2002). The cyprid is responsible for surface selection and settlement, and therefore the transition from pelagic to benthic life (Nott, 1969; Laggerson & Høeg, 2002). Cyprids of *P. pollicipes* are often observed settled on the peduncle of adults of the same species, or substrata which have been in contact with conspecific adults (Kugele & Yule, 1996; Cruz, 2000; Cruz et al., 2010). This larva-adult interaction is key to survival and thought to be based the larval response to chemical cues from the adults, as well as the surface texture of the adult, as will be discussed later (section 1.3.2.3). Immediately after settlement, *P. pollicipes* cyprids metamorphose into juvenile barnacles, which can be found in the natural environment from July to January (Cardoso & Yule, 1995; Cruz

& Hawkins, 1998; Cruz & Araújo, 1999; Cruz, 2000; Cruz et al., 2010; Molares et al., 2002; Pavón, 2003; Macho et al., 2005; Macho, 2006). Recruitment is dependent upon both cyprid settlement and early juvenile survival (Cruz, 2000; Cruz et al., 2010). Post-settlement mortality has a significant impact on barnacle recruitment, and spat survival can be highly affected by abiotic factors such as temperature, food availability, tidal cycles, desiccation and hydrodynamic conditions (Hoffman, 1989), as well as biotic factors such as predation and competition (Barnes & Reese, 1960; Hoffman, 1984; Cruz et al., 2010). In the laboratory, cyprids have been observed to settle in the capitulum (Kugele & Yule, 1996), while recruits in the wild are often observed on the stalk (Cruz, 2000), with larger juveniles often found close to the base of the stalk, suggesting not only the importance of post-settlement survival, but also hinting at the possible relevance of relocation for survival (Kugele & Yule, 1993).

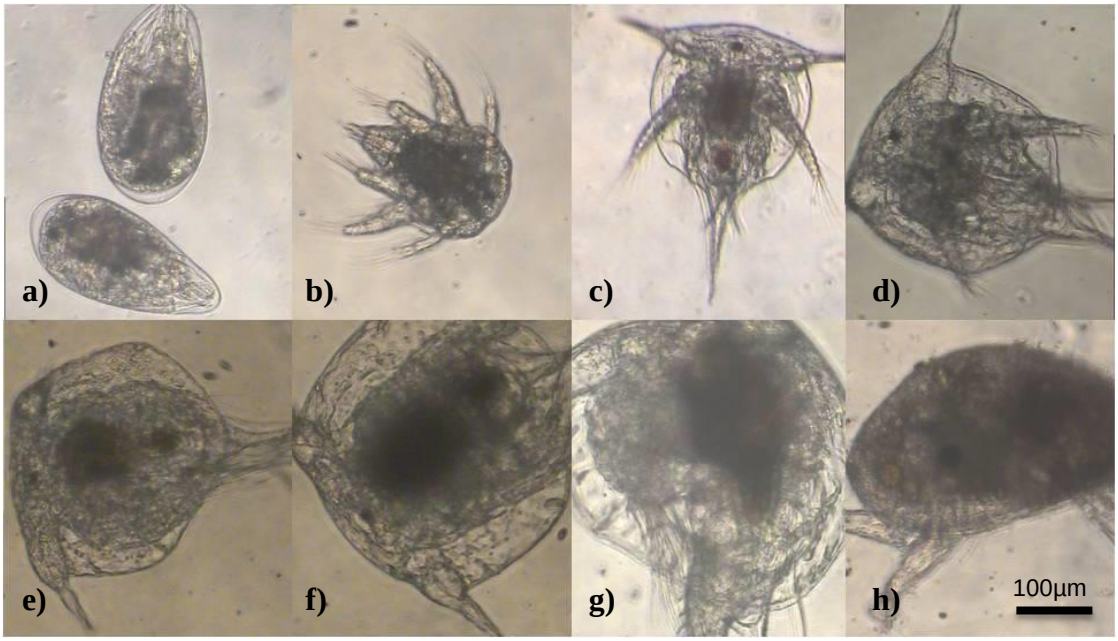


Fig. 1.4 Development stages on *P. pollicipes* larvae (a) embryo, (b) nauplii I, (c) nauplii II, (d) nauplii III, (e) nauplii IV, (f) nauplii V, (g) nauplii VI, and (h) cyprid.

Growth of *Pollicipes* sp. is dependent on food availability and environmental conditions that effect feeding behaviour, as individuals tend to face the water flow, to benefit from food transport (Barnes & Reese, 1959; Lewis, 1981; Norton, 1996; Cribeiro, 2007). Intraspecific competition is reduced by differen ces in feeding behaviour and prey selectivity within age groups. The growth of *P. pollicipes* occurs by periodic moults of the exoskeleton of the prosoma, cirri and thorax, and by the successive accretion of material to the calcareous plates of the capitulum (Crisp & Bourget, 1985; Chaffee & Lewis, 1988; Anderson, 1994; Barnes, 1996).

Adult size is often classified based on the distance between the rostrum and carina capitular plates (RC distance) (Cruz, 2000). Adult size varies between 15 to 65 mm in stalk length and 10 to 28 mm RC (Macho, 2006). Growth averages between 0.11 to 0.66 mm RC per month (Cruz, 1993; Cruz, 2000) and individuals can live from 2 to 6 years in the wild (Coelho, 1990). *P. pollicipes* life cycle is shown in fig. 1.5.

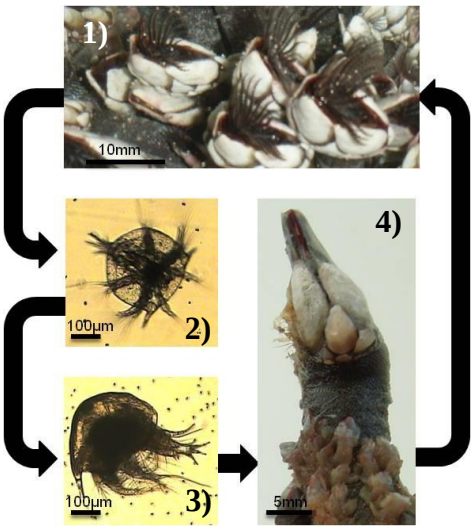


Fig. 1.5 Life cycle of *P. pollicipes*, from (1) adult reproduction and larval release, to (2) early larval development in the wild, to (3) late naupliar stages, metamorphosis to cypris, (4) settlement and juvenile development to mature adults.

1.2. Bio-economy of *P. pollicipes*

1.2.1. Balancing exploitation and conservation

Harvesting of *P. pollicipes*, or the "percebes", as it is commonly known, is an important economic activity in Portugal and Spain (Goldberg, 1984; Bernard, 1988; Cunha & Weber, 2000; Freire & García-Allut, 2000; Molares & Freire, 2003; Bald et al., 2006; Borja et al., 2006a, 2006b). The collection of stalked barnacles is a risky activity and the work is mostly carried out at times when the tide is at its lowest level, though some fisherman also collect the barnacles under apnea during higher tides (Cruz, pers. com.). The removal of *P. pollicipes* from the rocks has to be done manually often in extreme conditions (fig. 1.6). The activity is dependent on season and sea conditions (Cribeiro, 2007) and the fisherman must carefully consider the risks of collecting barnacles in a highly exposed intertidal regime (Pérez, 1996; Novo Loureiro, 2000; Cribeiro, 2007). Jacinto et al. (2010) estimated average collection rates of *P. pollicipes* in the Berlengas Natural Reserve of 16.7 ± 7.1 kg person day^{-1}, while Bernard (1988) when referring to *P. polymerus* estimated 30 – 70 kg person day^{-1} in British Columbia.

Fig. 1.6 Collection of *P. pollicipes* from sites of difficult access and extreme environmental conditions, in Galicia (Spain) (photograph courtesy of Sergio Ramazzoti).

In Portugal, percebes are heavily collected on the SW coast and are vital to local communities (Cruz, 1993; Cardoso & Yule, 1995; Cruz & Araújo, 1999; Cruz, 2000; Jesus, 2003; Cruz et al., 2010; Sousa et al., 2013). However there are no official records of the fisheries production of *P. pollicipes*, since this species is collected individually and sold directly to buyers. Cruz & Araújo (1999) reported that old fisherman considered that the size of the exploitable population has been decreasing in recent decades and Castro (2004) classified this species as highly to fully fished. In Spain, the exploitation of *P. pollicipes*, originally restricted to the NW coast of Galicia, has increased in recent years to the whole North coast (Cribeiro, 2007). Due to the increased harvesting pressure, *P. pollicipes* is considered to be overexploited in Spain (Borja et al., 2000) and though for over a century it has been collected for commercial purposes, the increase in demand since the 1970's has precipitated overfishing (Cribeiro, 2007). Borja et al. (2006a, 2006b) calculated that the overexploitation of *P. pollicipes* in the north of Spain, in two separate sites in the Basque country, had led to a stock decrease of 3 to 12.5 times in comparison to what would have been expected in unexploited conditions. Nevertheless, the real impact of exploitation is difficult to quantify and it is

possible that other factors are influencing stock fluctuations, especially in species where larval transport might be highly dependent on suitable winds and water currents (Cribeiro, pers. com.). Since consecutive years of low larval recruitment and high exploitation can have an impact on the standing stocks and the overall biological community, a more conservative approach towards the conservation of this species may be required.

Concern over the state of natural stocks has led to the implementation of conservation measures and regulation of exploitation in a number of marine reserves (e.g. Gaztelugatxe Marine Reserve in Spain and Berlengas Natural Reserve in Portugal; Borja et al., 2006; Queiroga et al., 2008). Additionally, co-management through negotiation of territorial user rights, the creation of regulatory fisheries associations, definition of quotas and collection periods, establishment of a minimal collection size etc. (see Molares & Freire, 2003 and Borja et al., 2006ab), has helped to establish a framework for the preservation of this species. Several biological studies (e.g. Molares, 1993, 1994; Molares & Freire, 2003) have assisted in the establishment of such regulations and the general opinion is that conservation measures have had a beneficial effect on the slow recovery of the stocks. Similarly, in Portugal, collection is already regulated in some areas (e.g. Berlengas Natural Reserve) and parallel protection measures have been suggested by Castro & Cruz (2009) and Sousa (2013) for other protected areas in the SW of Portugal (e.g. Natural Park of the Sudoeste Alentejano and Costa Vicentina).

1.2.2. Economics and market

Recently, Lopez et al. (2010) provided the first comprehensive review of the economic importance of barnacles. The stalked barnacles are considered to be the most important for consumption, with all Pollicipedidae being currently under exploitation (fig. 1.7). In spite of most of these species supplying local markets, they converge to the south Japanese market, where *C. mitella* is locally consumed, and to the Iberian market, where *Pollicipes* sp. is considered a delicacy (Lopez et al. 2010). In fact, the consumption of *P. pollicipes* in the Iberian Peninsula has deep historical roots, supported by evidence of the exploitation of stalked barnacles in Europe from about 8400 yBP (Bicho, 2004; Alvaréz-Fernandéz et al., 2010; Dean, 2010). The fleshy peduncle of *P. pollicipes*, which has a rich fatty acid profile (Moris & Barnes, 1975), is commonly consumed fresh or cooked in sea water (fig. 1.8). *C. mitella* muscle also has a very high nutritive value (Chen et al., 2009).

Fig. 1.7 Species from Pollicipedidae, belonging to the genus *Pollicipes*, namely (a) *Pollicipes pollicipes* (by Keith Hiscock), (b) *Pollicipes caboverdensis* (from Fernando et al., 2010), (c) *Pollicipes polymerus* (by Genny Anderson) and (d) *Pollicipes elegans* (by Flavia Giglio), as well as *Capitlum*, namely (e) *Capitulumm mitella* (by Artur Buczkowski and Slawomir Boron).

Fig. 1.8 Harvested *P. pollicipes* (a) being sold in fish stand at a local market in Lisbon (Portugal); and (b) cooked for consumption (photograph courtesy of Sebastião Castilho).

P. pollicipes can attain high consumer prices, with 1 kg costing up to 150 € in Iberian restaurants (Goldberg, 1984; Cruz et al., 2010). Current initial market prices fluctuate roughly between 10 € kg^{-1} and 30 € kg^{-1}, though occasionally reaching values over 80 € kg^{-1} (Molares & Freire, 2003; Borja et al. 2006a; Jacinto et al., 2010; fig. 1.6b). Due to the considerable demand for this product in the Iberian market, the harvest of *P. pollicipes* over the past few years averaged 300 to 500 tonnes $year^{-1}$, though in the 70's estimates were as high as 2000 tons $year^{-1}$ (Proverbs, 1979). In the Berlengas Natural Reserve alone, collection has been estimated to vary between 11.0 to 19.9 tonnes $year^{-1}$ (Jacinto et al., 2010). To accentuate the unpredictability of such a market, in Spain the

greatest demand for the product is during the winter months, where exploitation is at its lowest due to weather and sea conditions (Cribeiro, 2007). The high demand of the Spanish market, together with supply problems in recent years, led to the unavoidable opening of the market to other species, *P. polymerus* and *P. elegans*, which are often less expensive (10 € kg^{-1} to 15 € kg^{-1}) (Goldberg, 1984; Bernard, 1988; Bald et al,. 2006). Currently, the limitations inherent to the economical viability of product importation (e.g. impact in quality, transport time) and the already overexploited Iberian stocks suggest that viable alternatives are required to supply the market.

1.2.3. Potential for aquaculture

Though *P. pollicipes* is a species whose desirability is currently limited to the Iberian market, it is interesting to note that data from 2005 show that Portugal and Spain had a per capita fish consumption between 30 to 60 kg $year^{-1}$, considerably above the European average of 20.8 kg $year^{-1}$ (FAO, 2009). Additionally, the contribution of fish to animal protein supply in the Iberian countries averaged more than 10 kg per capita per day, which in Portugal also represents a contribution of fish in more than 20 % of total animal protein (FAO, 2009), leaving *P. pollicipes* well positioned in a market that although its limitations, has huge economic potential.

While still requiring significant optimisation, most production phases, such as reproduction, larval culture and juvenile rearing, have been proved possible in previous studies. The conditions required for reproduction in culture have not been investigated in detail, though various authors (Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007) have maintained broodstock for several months with mating and larval release occurring naturally. In addition, the simplicity of direct embryo extraction from the adults might also be of use to production, despite being labour intensive. Larval culture has been accomplished (Coelho, 1990; Molares et al., 1994b; Kugele & Yule, 1996), but optimal conditions have not yet been identified. The major limitation to culturing this species is undoubtedly larval settlement. In the natural environment, cyprids of *P. pollicipes* are often found settled on or near conspecific adults. While some of the factors determining settlement site selection are well known for cyprids of generalist barnacle species (e.g. *B. amphitrite*; see Clare, 2011), the cues necessary to stimulate settlement of species such as *P. pollicipes* to artificial surfaces are less well understood. Several authors (e.g. Coelho, 1990; Molares, 1994; Molares et al., 1994a) were unable to stimulate settlement of cyprids in the laboratory, while others had success rates of less than 1 % (Kugele & Yule, 1996). Results from field experiments have been equally

disappointing (Coelho, 1991). Future studies are clearly required to develop methods for control of larval settlement and test culture conditions over the post-settlement production phases. In spite of current barriers to continuous culture, *P. pollicipes* is a promising species for aquaculture and success would bring with it economic, social and ecological benefits.

1.3. Production cycle and culture methods of *P. pollicipes*

Few studies have addressed key questions pertaining to broodstock conditioning (Cribeiro, 2007), larval development under culture conditions (Coelho, 1990; Molares, 1994; Molares et al., 1994b; Candeias, 2005; Kugele & Yule, 1996) larval settlement in the laboratory and spat recruitment in nature (Coelho, 1990; Coelho, 1991; Kugele & Yule, 199; Cruz, 2000; Cruz et al., 2010) or juvenile growth (Goldberg, 1984; Cribeiro, 2007). The existing literature has been summarised briefly in Table 1.2.

Subject	Reference	Location
Larval feeding	Candeias (2005)	Laboratory
Larval culture	Coelho (1990)	Laboratory
Spat collection	Coelho (1991)	SW Portugal
Recruitment	Cruz (2000)	SW Portugal
Broodstock reproduction, rearing	Cribeiro (2007)	Laboratory
Juvenile ongrowing	Goldberg (1984)	NW Spain
Juvenile relocation	Kugele & Yule (1993)	Laboratory
Larval morphology, settlement	Kugele & Yule (1996)	Laboratory
Juvenile relocation	Kugele & Yule (2000)	Laboratory
Gametogenesis	Molares et al. (1994a)	Laboratory
Larval development and culture	Molares et al. (1994b)	Laboratory
Larval culture, settlement	Molares et al. (2002)	Laboratory
Juvenile/adult feeding	Norton (1996)	Laboratory

Table 1.2. Literature on *P. pollicipes* with direct application on aquaculture, according to subject area and location of study. References are provided in alphabetical order.

1.3.1. Broodstock conditioning and larval release

Broodstock conditioning in culture is dependent not only on the collection of wild broodstock and successful maintenance through the full reproductive cycle (gonad maturation and recovery, mating, development of lamellae and liberation of nauplii), but also on an understanding of how to control these cycles in the absence of natural stimuli.

1.3.1.1. NATURAL BREEDING CYCLES

Reproduction of *P. pollicipes* in nature has been followed throughout its geographic range (Girard, 1982; De la Hoz & Garcia, 1993; Molares et al, 1994a; Cardoso & Yule, 1995; Barnes 1996; Cruz & Hawkins, 1998; Cruz & Araújo, 1999; Pavón, 2003).

Though the reproductive cycle of *P. pollicipes* in the wild can be followed year round, the breeding period has been observed from April to October, when the percentage of breeding adults ranges from 20 to 75% of the population (Cardoso & Yule, 1995). Though gravid ovaries and seminal vesicles appeared to remain functional throughout the non-breeding season (November to March; Cardoso & Yule, 1995), various authors have established that the female gonad is found in resting stage at this time (Cruz & Hawkins, 1998; Molares, 1994), conditioning reproduction in the wild.

As the female gonad matures, it progresses from a resting ovary (stage 0) to the beginning of gametogenesis (stage I), then to a mature gonad (stage II). After spawning, it regenerates (stage III) before final re-absorption and gonad desintegration (stage IV) (Molares et al., 1994a). Most ovaries are mature and capable of fertilization (stage II) from March to September, with maxima in July and August (Cardoso & Yule, 1995; Cruz & Hawkins, 1998; Molares, 1998; Cruz & Araújo, 1999; Cruz, 2000; Molares et al., 2002; Pavón, 2003; Macho et al., 2005; Cruz et al., 2010). Meanwhile, over 80% of individuals have seminal vesicles filled with spermatozoa throughout the yea, as male gamete volume fraction is maximal in March, before spermatozoa are released from the follicles, entering the seminal vesicle for storage (Molares et al., 1994a). The male gonad progresses from a resting phase (stage 0), through an increase in follicle size and concurrent appearance of spermatogonia, spermatozoa aggregates, spermatocytes and spermatids (stage I) to finally having large aggregates of spermatozoa in the lumen (stage II), ready to be release (Molares, 1994; Molares et al., 1994a).

P. pollicipes are simultaneous hermaphrodites (Cruz & Hawkins, 1998) and at any one time individuals may act as a male or as a female. For this reason, the terminology is of "acting males" or "acting females", in spite of both gonads undergoing simultaneous development (Anderson, 1994). Despite suggestions by several authors that self-fertilization could occur (Darwin, 1851; Barnes, 1996) *P. pollicipes* is considered an obligate cross-fertilizer (Molares et al., 1994b; Cruz & Hawkins, 1998; Cruz, 2000; Pavón, 2003). However, recent studies on the closely related *P. polymerus* show that spermcast mating can be used as an alternative to direct fertilization (Barazandeh et al., 2013). This capability would be especially important for isolated individuals, for which sperm capture was of 100 %, compared to 24 % for individuals within reach of conspecifics.

Charnov (1987) hypothesized that barnacle functional females can probably be fertilized by more than one male. During direct fertilization, the functional male will extend the

penis and look for a functional female, with the oviducal glands distended by a clear fluid, as observed in *B. balanoides* (Walley, 1965). Semen is then deposited inside the mantle cavity. A viscous sac of spermatozoa is ejaculated into the functioning barnacle female mantle cavity where it is deposited as a mass of inactive spermatozoa. This deposition stimulates the female to release oocytes (Walley et al., 1971; Anderson, 1994). In *P. pollicipes*, the oocytes are expelled by the oviducts and deposited into the oviducal atrium forming two ovisacs (Molares et al., 1994). Upon ovoposition, the fluid secretion from the oviducal gland contacts the immotile spermatozoa, causing the spermatozoa mass to break down, simultaneously activating the spermatozoa and allowing the fertilisation of oocytes to take place (Anderson, 1994; Walley et al., 1971). The resulting embryos form two laminar egg lamellae in the sides of the prosoma, with thousands of embryos each enclosed in a membrane, to avoid dispersion inside the mantle cavity (Anderson, 1994). Embryos develop aerated by currents produced by movement of the prosoma (Darwin, 1851; Walker, 1983) until the first stage of free swimming nauplii are released in jets to the water column by the adult (e.g.Lewis,1975).

The beginning and end of the breeding period of *P. pollicipes* are synchronous within populations despite considerable asynchronicity in larval release events during this period (Macho, 2006). Adults may have up to 5 broods per year (Girard, 1982; Molares et al, 1994a; Cardoso & Yule, 1995; Cruz & Araújo, 1999; Cruz, 2000; Macho, 2006), and therefore the gonads recuperate multiple times per season. The female gonad is thought to resume its resting stage from October to February, although Cardoso & Yule (1995) observed gravid gonads all year round. Molares et al. (1994a) concluded unequivocally that *P. pollicipes* ovary redevelopment occurs between larval release events and therefore gonads cannot be continuously ripe. Therefore, the results of Cardoso & Yule (1995) might be explained by the fact that, due the lack of breeding synchrony, few ripe individuals are still possible to be found throughout the year, though histological observations (such as Cruz & Hawkins, 1998; Molares et al., 1994a) allow the clarification over gonadal development cycles in the wild.

1.3.1.2. METHODS FOR OBTAINING LARVAE: EGG MASS EXTRACTION, EARLY STAGE LARVAL COLLECTION AND ARTIFICIAL FERTILISATION

The extraction of egg masses directly from adults of *P. pollicipes* sp. has been the primary method for obtaining embryos and early stage larvae for culture (fig. 1.9). This method consists of sectioning the capitulum from the prosoma, followed by a cut through the carina plate and opening of the capitulum in two halves. This allows easy

access to the two egg lamella, which are found in both sides of the capitulum and can be readily removed and left to hatch.

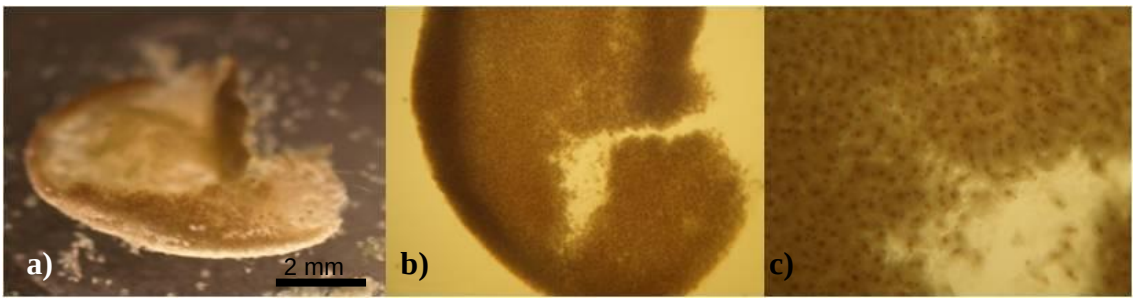


Fig. 1.9 Mature egg lamella of *P. pollicipes*, observed with (a) naked eye, with visibly flimsy texture and darker coloration, (b) 6x magnification, with over ready-to-hatch embryos within the ovisac, and (c) 50x magnification, with visible embryos being released after sectioning of the ovisac, giving place to naupliar hatching.

A less common alternative for obtaining larvae is to collect larvae directly from the water column, although this presents difficulties due to the constantly changing dominance of species in the plankton, as well as separating species of cyprid that all appear very similar. *Chthamalus* spp., for example, can represent over 90 % of the barnacle larvae present in plankton samples during the *P. pollicipes* larval release season (Macho et al., 2005; Macho, 2006).

Alternatively the potential for artificial insemination has been considered, but results from similar species have shown that unfertilized eggs cannot be fertilized by non-ejaculated sperm (Hilgard, 1960; Walley et al., 1971; Lewis, 1975a,). The fertilization seemingly requires spermatozoa already deposited in the mantle cavity, plus fluid from the oviducal gland and oocytes. However, in *C. spinosa* in-vitro fertilization was accomplished using sperm in plump seminal vesicle plus ovulating eggs and oviducal gland fluid, which activates the sperm (Qiu et al., 1994).

The recent discovery of barnacle spermcast mating (Barazandeh et al., 2013), might hold unexplored potential for artificial fertilization in the laboratory. The ease of extraction of egg lamellae has however provided enough larvae to support research studies to date. Due to their seasonal breeding, conditioning of broodstock is essential for the provision of larvae, allowing an extension to the production season and removing the reliance on natural reproductive cycles.

1.3.1.3. BROODSTOCK REPRODUCTIVE CONDITIONING

From an aquaculture perspective, it is essential that an artificial supply of larvae can be established throughout the year from conditioned broodstock. This can be facilitated either by natural mating in culture followed by larval release (induced or natural) or artificial fertilization of previously extracted eggs. While natural larval release in culture represents a low-effort approach to larval collection, providing a continuous supply of larvae without the need for induction events (Kugele & Yule, 1996; Candeias, 2005), yields may be low since larval release is sparse and uncontrolled. Broodstock has been maintained in culture for extended periods with mating occurring naturally and with records of natural, though sparse, larval release (Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007). Natural larval release in aquaculture can be accomplished using reproductively conditioned broodstock and larval release induction allows for controlled larval release events, however, this is dependent on the availability of an efficient induction method and this has yet to be investigated in *P. pollicipes*.

Broodstock conditioning in aquaculture is essential, not only to assure the steady supply of larvae, but also as a tool to improve gamete quality and viability, larval performance and survival, among other factors (Helm et al., 1973; Bayne, 1976; Lannan et al., 1980). Although various authors (Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007) have observed reproduction and larval production in culture, the conditions required for broodstock reproductive conditioning have not been directly investigated. The minimum size for broodstock animals should be 8 mm capitulum height (Cardoso & Yule, 1995) or, following the maximal rostro-carinal plates distance (RC distance) defined by Cruz (2000), 10 mm RC for males and 12.5 mm RC for females. The rearing conditions for *P. pollicipes*, used in the majority of cases, were mostly extrapolated directly from the conditions in the wild. Consequently, the optimal environmental conditions of salinity, photoperiod, light intensity, food and temperature remain unknown. As noted by Barnes (1996), however, in the wild, factors such as light, temperature and food are considered to be among the dominant stimuli for biological cycles. Cribeiro (2007) and Norton (1996) conducted the only studies directly investigating the feeding preferences and feeding behaviour of *P. pollicipes*. The former author also developed the only preliminarily study testing various rearing systems, using different hydrodynamic regimes and optimizing water quality and feeding.

Reproduction and maturation of broodstock are controlled by both endogenous and exogenous factors (Mann 1979b; Newell et al. 1981; Muranaka & Lannan 1984;

Robinson, 1992a; Ruiz et al, 1992a; Lannan et al., 1980; Liu et al., 2008). The maturation and gonadal development of *P. polymerus* observed in the wild are thought to be controlled more by temperature than by food. While changes in temperature seem to trigger gonadal development under sufficient food availability, food is nevertheless essential to gonad regeneration after larval release (Cimberg, 1981; Page, 1983). Although the importance of temperature for brooding activity has also been verified for *P. pollicipes* from field studies (Cardoso & Yule, 1995: Macho, 2006), the correlation between these factors has made it difficult to determine to what extent gonadal development is a function of temperature or food availability (Molares et al., 1994b; Cardoso & Yule, 1995; Cruz & Hawkins, 1998). In the wild, brooding has often been related to plankton availability and coastal upwelling, whose maxima are often recorded during Summer months, in synchrony with the breeding period (Crisp, 1954, Crisp & Davies, 1955; Cardoso & Yule, 1995; Barnes, 1996). In fact, Macho (2006) reported that in natural stocks the number of brooding individuals (with ovisacs) varies from 1 – 3 % in March, to 50 – 80 % in May and maintained during July and August, peaking at 85 %, then decreasing to 50 % in September and 0 % from November to February. This regime is indicative of a breeding cycle that can be maintained for 5 months of the year in the wild. Cardoso & Yule (1995) argued that food is not limiting in the wild, as ripe ovaries and seminal vesicles are present all year round. However, ovaries take demonstrably longer to regenerate at the end of the reproductive season (Molares et al., 1994b; Cruz & Hawkins, 1998), coinciding with the end of upwelling but no major temperature changes, suggesting an important role of food availability in gonad regeneration. Similarly, the gonadal development rate of others, such as bivalves, beginning of gametogenesis and conditioning period are mostly dependent upon temperature (Mann, 1979a; Muranaka & Lannan, 1984; Helm & Bourne, 2004), while diet significantly affects fecundity, gonadal recovery, egg quality and larval viability (Robinson 1992; Utting & Millican, 1997; Helm & Bourne, 2004). So far, no studies have analysed broodstock conditioning in *P. pollicipes* and the only information available is mostly descriptive (Candeias, 2005; Cribeiro, 2007).

1.3.1.3.1. TEMPERATURE

Water temperature on the Atlantic coast of Portugal and Spain, where *P. pollicipes* is more heavily harvested, varies between 9 to 25 ºC, averaging roughly between 10 to 16ºC from November to April and 14 to 24 ºC from May to October (Instituto Hidrográfico, 2012; Meteo Galicia, 2012; Euskalmet, 2012). Studies in the field have

related air temperature (Cardoso & Yule, 1995; Macho, 2006) with *P. pollicipes* breeding, finding no correlation with water temperature. Additionally neither study fully explained the variability in brooding; temperature only accounted for 60% and 15% of the total variability (respectively, Cardoso & Yule, 1995 and Macho, 2006). The tolerances of *P. pollicipes* to temperature, lethal and optimal temperatures, have not been investigated. Results for other barnacles show that each species responds to a specific set of conditioning temperatures (Patel & Crisp, 1960). Temperature is also known to influence cirral activity, essential to respiration and feeding, and maximal activity has been associated to optimal temperatures, which can in turn be related to a species distribution (Southward, 1955; Barnes, 1996). Extrapolating from conditions in the wild to culture conditions must be made with care, since even within the same species wild stocks can vary markedly in terms of the conditions that trigger breeding behaviour. In the wild it has been suggested that physiological races along the latitude of distribution of *P. polymerus* and *C. mitella* are associated with temperature (Hines, 1978; Cimberg, 1981; O'Riordan et al., 2004; Chan, 2006). In fact at higher latitudes *C. mitella* have a significantly shorter breeding period (Chan, 2006) and a similar phenomenon is seen in *P. polymerus* (Cimberg, 1981). Furthermore, in *P. polymerus*, stocks at higher latitudes breed at higher temperatures than those at lower latitudes (Cimberg, 1981). In the wild, brooding individuals of *P. pollicipes* are not commonly found below 14 ºC (south of Portugal) and 11 ºC (north of Spain) (respectively, Cardoso & Yule, 1995; Cruz & Hawkins, 1998 and Macho, 2006). Though these Iberian stocks have never been compared in detail for significant differences in the temperature triggering breeding behaviour, they correspond to stocks located at latitudes with a difference of about 5º (between approximately 47º and 43º N). This is considerably higher than the difference of 2º of latitude, observed by Cimberg (1981) when reporting the existence of two physiological races along the North American Pacific coast.

Notwithstanding differences in the wild, captive conditioning is invariably dependent on the broodstock holding temperature, which can affect gonadosomatic index (GSI), growth and survival in barnacles (Patel, 1959; Patel & Crisp, 1960), as well as conditioning time. Knowledge of the effects of different temperatures on conditioning, in particular on the GSI, allows not only calculation of a regression coefficient between these two factors, but also an estimation of the biological zero (Hahn, 1979); the temperature below which development ceases, supposedly similar to that reported by Cardoso & Yule (1995) and Macho (2006). Most studies on *P. pollicipes* on adult

rearing did not tightly control temperature (e.g. 15 – 21 ºC by Cribeiro, 2007; 15 – 24 ºC by Kugele & Yule, 1996) and failed to reference gonadal development rates or other aspects of the adult reproductive condition. The results of Page (1983) suggest that according to temperature, there are differences in resource allocation that prioritise either growth or reproduction, with higher temperatures privileging reproduction over growth. In fact Cruz (2000) observed that growth rates in the wild decrease from about 0.4 mm RC month^{-1} to about 0.1 mm RC month^{-1} during the breeding season, which is in accordance with the allocation of energetic resources to reproduction instead of growth. The relation of higher temperatures to successful reproduction seems to be a general rule amongst *Pollicipes* spp., as observed from the results in the wild. Extrapolations of conditioning time and optimal temperature range are premature, however, as optimal ranges are still to be established. Nevertheless, conditioning is feasible with this species, bearing in mind that both Kugele & Yule (1996) and Candeias (2005) reported continuous, albeit uncontrolled, natural larval release from captive stocks, while Cribeiro (2007) observed copulating individuals.

1.3.1.3.2. NUTRITION

Nutrition is a major factor in maintaining healthy broodstock. In the wild, it impacts both reproductive fitness and larval production in *P. pollicipes* (Macho, 2006). The cost of feeding broodstock represents around 50 % of total aquaculture costs (Fegan, 2004). The few studies that have investigated the diet of *Pollicipes* sp. in the wild reported the presence of small copepods and cirripede larvae in stomach contents (Barnes, 1959), with small crustaceans constituting the bulk of the diet (Barnes & Reese, 1959; Lewis, 1981). The dietary needs of *P. pollicipes* adults in culture are poorly understood. Norton (1996) developed the only study on their feeding apparatus and feeding patterns.

P. pollicipes can exhibit various types of cirral activity, namely testing (retracted cirri with open capitulum), pumping (cirral rhythmic extension and retraction) and beating (prolonged cirral extension), often with in-curling of one cirrus after the other to transport the captured food items (Norton, 1996), as observed when suitable prey touch the cirral net (fig. 1.10). The acceptability of *Artemia* sp. based diets has been confirmed by various studies (Norton, 1996; Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007) and by the authors' experience with adults and juveniles of *P. pollicipes* kept in culture successfully for almost 3 years while fed a monodiet of *Artemia* sp. nauplii. Norton (1996) further noted that the presence of *Artemia* sp. leads to increased cirral beat rates, supporting a strong feeding response. Though this species is of widespread

use in aquaculture (Légger et al. 1986; Bengtson et al., 1991; Lavens & Sorgeloos, 2000), other potential food sources such as copepods, rotifers and natural plankton can be often more suitable, in terms of nutritional profile, fatty acid content and being natural prey of many cultured species (Shields et al., 1993; McEvoy et al., 1998) and further research is necessary.

Fig.1.10 Adults of *P. pollicipes* actively feeding of *Artemia* sp..

Norton (1996) observed that young adults of *P. pollicipes* (11.9 mm RC) were able to ingest *A. salina* prey up to 6.64 mm in length, while juveniles (5.5 – 6.6 mm RC) could ingest prey up to 3.9 mm. Observations such as these must be considered when feeding mixed populations of *P. pollicipes* in order to provide a variety of prey sizes, thus reducing competition. Howard & Scott (1959) further observed that *Artemia* sp. and *Trigriopus* sp. up to 11 mm in length were ingested by *P. polymerus*, depending on barnacle size. Moreover, the addition of microalgae can stimulate feeding and acts as a dietary supplement, and is therefore a useful addition for many aquaculture-produced species (Muller-Feuga, 2000; Spolaore et al., 2005). Field studies of *P. polymerus* have verified differences in diet and feeding behaviour according to life stage (Lewis, 1981; Barnes & Reese, 1959). Norton (1996), however, argued that differences in diet (of *P. pollicipes*) are due to the size and efficiency of the cirral nets. Barnes (1959) observed that the stomach contents of *P. polymerus* contained crustaceans between 500 to 1000 µm, as well as cirriped nauplii, cyprids and small copepods. Larger juveniles and adults of *P. polymerus* consume phytoplankton, blue-green algae, copepods, polychaete, eggs, and hydroids. Infrequently in bigger individuals, molluscs, large algae, echinoids and shrimp are found (Lewis, 1981). Howard & Scott (1959) also identified cyprids, amphipods, small clams, hydroids and nauplii amongst gut contents. However, for *P. pollicipes* Norton (1996) noted that although algal cells are frequently ingested, these are insufficient to maintain body mass. Cribeiro (2007) concluded that live nauplii of

Artemia sp. were considerably more acceptable and led to higher growth rates than inert diets (e.g. fish meat, cephalopods, mussels, seabass pellet food and frozen *Artemia* sp.).

With regard to quantity, the rule applied to other species (e.g. bivalves) is that reproductively conditioned individuals should consume from 2 to 4 % of their dry meat weight per day (Helm & Bourne, 2004), depending on the specifics of the system and species. Norton (1996) clearly showed that ingestion rates of *P. pollicipes* vary with prey, density and diet. *Tetraselmis chuii* and *Pavlova lutheri* were ingested in negligible quantities, while ingestion rates for *Rhinomonas reticultata*, *Skeletonema costatutum*, *Brachionus plicatilis* varied with density until a plateau was reached. Juveniles of *P. pollicipes* (5.8 mm RC) showed a mean ingestion rate of *A. salina* of 2.14 J ind^{-1} h^{-1}, the equivalent to 58 artemia ind^{-1} h^{-1}. Page (1983) when rearing adults considered 2 nauplii ml^{-1} as a low ration for *P. polymerus* and of 12 nauplii ml^{-1} as high ration, with maximal daily ingestion averaging 8.9 to 9.2 nauplii d^{-1} and daily maintenance requirements of 6.8 cal ind d^{-1}, depending on temperature. *Ad libitum* feeding, as used by Cribeiro (2007), is also an option, though besides the effects on water quality, excess feeding might over-stimulate growth to the detriment of reproduction. The standard is to allow the feeding of individuals daily for a minimal period of 2 h, until food is fully consumed. Additionally, it should be considered that lack of adequate feeding might lead to nutritional deficiencies and consequent effects on external morphology, as observed in other cultured species, such as *Oncorhynchus* sp., *Solea* sp., *Scardinious* sp., among others (Romanov, 1984; Kamler, 2008; Boligno, 2012). Field observations of the morphology of *Pollicipes* sp. more sheltered habitats, and consequently increased competition for food, have showed that the peduncle is often elongated, with fewer calcareous scales and tapers towards the base to bring the capitula into positions where feeding is possible (Barnes & Reese, 1960). Similarly, in culture juveniles may exhibit increased stalk length which is disproportionate to rostro-carinal growth (Cribeiro, 2007).

1.3.1.3.3. HYDRODYNAMICS AND TIDES

The natural distribution of *P. pollicipes* is limited to intertidal sites with high wave exposure (Cardoso & Yule, 1995; Castilla et al., 1998; Cruz & Araújo, 1999; Borja et al., 2006a, 2006b; Cribeiro, 2007; Cruz et al., 2010). As in other *Pollicipes* spp. (Barnes & Reese, 1959, 1960; Meglittsch, 1978; Barnes, 1996; Lauzier, 1999), feeding seems to be dependent upon hydrodynamics and water flow. Highly hydrodynamic rocky shores can have flow rates between 0.5 – 20 m s^{-1} (Denny, 1988). *Pollicipes* spp. orientate

themselves according to wave exposure and microtopographical features (Barnes & Reese, 1960; Meglittsch, 1978; Barnes, 1996; Norton, 1996; Lauzier, 1999). Perception of water flow by *P. pollicipes* was hypothesized by Norton (1996) to be accomplished by the stalk and opercular flap, since the animals can still respond to flow with the capitulum closed. Cirral extension in barnacles is often maintained between a minimal and maximal critical water flow, outside which the cirral net retracts (Southward, 1955; Barnes & Reese, 1959, 1960). Furthermore, older juveniles and adults of *Pollicipes* sp. are unable to maintain active rhythmic cirral activity, and are thus dependent on water currents for feeding (Barnes & Reese, 1960; Lewis, 1981; Cribeiro, 2007). Norton (1996) verified that in *P. pollicipes* cirral beating only occurred in static water. At flow rates above 14 cm s^{-1}, individuals shift to prolonged cirral extension, with beating mostly related to respiration.

For these reasons, high energy systems have been used by various authors (Molares, 1994; Cribeiro 2007) to culture of *Pollicipes* spp.. Successful maintenance of *P. polymerus* for prolonged periods requires a continuous jet of running water, rather than using simple bubbling systems (Barnes & Reese, 1960; Barnes, 1996). Recirculating or flow-through systems have been mainly used (e.g. Kugele & Yule, 1996), although more detailed tank characteristics are often left unmentioned. Since *P. pollicipes* is gregarious and sessile, rearing tanks presumably benefit from larger surface area, rather than increased water column height as long as adequate tank hydrodynamics are maintained. Frequent water renewal and exposure to tidal cycles have been recommended by Cribeiro (2007) for *P. pollicipes*, and were used in practice by Chaffe & Lewis (1988) with *P. polymerus*.

Pollicipes sp. are naturally exposed to air for periods of up to 9 h per day in the natural habitat, and *P. polymerus* can tolerate 5 to 10 h exposure to air at 2 – 36 ºC (Fyhn et al., 1972; Petersen et al., 1974), with recovery even after 40 – 50 % water loss (Fyhn et al, 1972). However, supplying a tidal cycle requires increased technological investment and growth rates in *P. polymerus* are higher when barnacles remain immersed (Hoffman, 1989), presumably due to increased feeding and time for growth. System features and tank hydrodynamics can also affect food dispersion and therefore food availability. However, according to Barnes & Reese (1960), even in the most suitable sites in nature full feeding activity is only achieved during a limited period of the tidal cycle. Knowledge of behaviour in altered hydrodynamic conditions is of considerable importance since it affects both feeding and mating, and remains poorly understood.

1.3.1.3.4. WATER QUALITY AND OTHER ENVIRONMENTAL CONDITIONS

Under suitable hydrodynamic conditions, groups of *P. pollicipes* have been maintained for months without acute mortality (Cribeiro, 2007; Franco, pers. obs.). Nevertheless, water quality is important. In the case of crustaceans, calcium should be available from the water, food or exuviae (Greenway, 2008). Other minerals such as zinc, selenium, magnesium, potassium, phosphorous and copper are also recommended (see Davis & Gatlin, 1996). Despite the apparent tolerance of *P. pollicipes* to poor water quality, other potentially crucial factors such as pH, water hardness, dissolved oxygen (DO), ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-) etc. have not been tested and would provide helpful insight into the adequacy of rearing systems.

Similarly, optimal environmental conditions of salinity, photoperiod and light intensity remain unknown, mostly due to high tolerance of this species to a range of conditions (Cribeiro 2007, Franco pers. obs.). For *P. pollicipes*, salinity from 33 to 37 psu is typically used (e.g. Cribeiro, 2007). Photoperiod and light intensity have been shown to influence maturation, ovopostion, gametogenesis and internal clock regulation in other aquacultured species (Aiken, 1969; Kuo-Cheng Shan, 1974; Nelson, 1986; Paulet & Boucher, 1991; Utting & Millican, 1997). Pedunculate barnacles have a pair of ocelli (Anderson, 1994) identified as eyes in *P. pollicipes* by Pouchet & Jobert (1876), which explains their sensitivity to light. Studies undertaken in this species, have used natural photoperiods (Cribeiro, 2007), though most studies fail to mention which light conditions are used (Kugele & Yule, 1996; Candeias, 2005). The effect of day length on fertilization has been suggested after laboratory observations (Barnes, 1963). However, ideal conditions of photoperiod and light intensity for *P. pollicipes* remain unknown.

1.3.1.4. NATURAL AND INDUCED LARVAL RELEASE

Natural larval release of *P. pollicipes* has not been directly investigated in culture. While maintaining broodstock in the laboratory, however, several authors have reported natural larval release (Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007). Intense cirral contact and mating were observed in populations reared in culture (Cribeiro, 2007; Franco, per. obs.). Kugele & Yule (1996) and Candeias (2005) both report that adults kept in culture were able to produce and release viable broods, providing a continuous supply of larvae. Neither study presented any further detail of conditions under which natural larval release was observed.

Independently of the mode of larval release, broodstock of suitable reproductive condition must be acquired to establish a larval release stock. Biological factors, such as moulting, age, size, and density might also affect larval release capacity, frequency and quality in *P. pollicipes* (Macho, 2006). When moulting occurs in barnacles it may result in the loss of ovisacs or embryos developing in the mantle cavity, so often in temperate species copulation occurs post-moulting (Crisp & Patel, 1960; Barnes, 1989). Additionally, older individuals spawn more frequently than younger individuals (Crisp, 1954; Cruz & Araújo, 1999; Cruz, 2000). Very dense barnacle populations have shown delayed larval release (Crisp & Davies, 1955; Crisp & Patel, 1961) as well as decreased fecundity (Crisp, 1964), as also observed in *P. pollicipes* (Cruz & Araújo, 1999; Cruz, 2000). Other factors, such as embryonic parasites can lead to the loss of batches, compromising reproduction. Though *P. pollicipes* has not been listed as susceptible to protozoan and isopod parasites, these have been observed in cultures kept for prolonged periods in the laboratory (Franco, pers. obs.). Factors affecting larval release and larval release induction require further investigation.

1.3.1.4.1. TRIGGERING FACTORS AND LARVAL RELEASE SYNCHRONY

The breeding activity of most marine organisms is controlled by sea temperature (Thorson, 1946) and although changes in temperature seem to induce the beginning of larval release season, many species lose this synchronicity thereafter (Macho, 2006). Field studies on *P. pollicipes* have focused on the timing of larval release in natural populations (e.g. Macho et al., 2005). Macho (2005, 2006) observed that *P. pollicipes* tend to release their larvae in the water column on the morning high tide, especially during the waxing moon, and to a lesser extent during the full moon. Macho (2006) further suggested that releasing larvae during the day is advantageous, since this induces larval gregariousness and protection from predators as they move through the water column, though the long planktonic phase raises questions over the viability of such an hypothesis. Location within the intertidal range (Cruz & Hawkins, 1998; Cruz & Araújo, 1999; Pavón, 2003) can also affect larval release in *P. pollicipes*. Observations by several authors (Paula, 1989; Saigusa & Kawagaye, 1997) support the suggestion that sunrise and sunset cue the onset of the larval release, whereas in tubeworms larval release has often been related to moon phases (Knight-Jones, 1951). Crisp (1959) suggested that photoperiod could be the main factor controlling larval release synchrony, since the simultaneous larval release of *B. balanoides* occurs annually at

approximately the same time. Decreasing photoperiod could signal the end of breeding activity in *P. pollicipes* (Cardoso & Yule, 1995).

While rearing *P. polymerus* in culture, Lewis (1975b) made observations that appeared to support the existence of food related triggering factors in larval release activity. Adults apparently released jets of hatching nauplii when fed with *Artemia* sp., which is in line with observations various authors (e.g. Crisp, 1962; Crisp and Spencer, 1958; Starr et al., 1991; Clare et al., 1995) regarding larval release in response to food supply. It is, of course, advantageous to synchronize larval release with phytoplankton blooms in the natural setting. *P. pollicipes* is a multiple brooder, larval release asynchronously, with various peaks of breeding (Molares et al., 1994a). By analogy with other barnacle species that have been studied, multiple breeding under natural conditions suggest it is possible to manipulate breeding in culture to give year-round supply, through the control of critical environmental factors.

The reported number of broods in wild of *P. polymerus* varies considerably according to authors, from 1 to 11 (e.g. Hilgard, 1960; Lewis & Chia, 1981; Page, 1984) per breeding season. *P. pollicipes* on the other hand has been reported to have between 1 to 5 broods (Molares et al., 1994a; Cardoso & Yule, 1995; Macho, 2006) per year. Nevertheless, major concerns for aquaculture reside in what degree of larval release synchrony can be achieved in this species. The number of releases by each individual might vary significantly with growth conditions and with the capacity to regenerate the female gonads. The problem of larval release synchronicity has similarities with some aquaculture-grown larviparous mollusc species, such as *Ostrea edulis* and *Tiostrea lutari*. These species cannot be efficiently induced since larvae brood in the mantle cavity and larval release occurs naturally during the conditioning process (Helm & Bourne, 2004). This may also be the case in *P. pollicipes*.

1.3.1.4.2. LARVAL RELEASE INDUCTION PROTOCOLS

Larval release induction is common in aquaculture and holds significant advantages over natural larval release, since it allows the control over when individuals should spawn, concentrating the production of larvae in a single event. However, in spite of the labour/effort involved, induction of larval release can cause individuals to release eggs or larvae prematurely potentially affecting their quality. In fact, differences have been reported between natural and induced larval release of eggs in other species, as spawned eggs can be of inferior size and show distinct fatty acid and aminoacid profiles (e.g Ako et al., 1994). However, this procedure has been widely used. Common larval release

inducing methods, used for fish, bivalve and crustaceans, include thermal cycling (Loosanoff & Davies, 1963; Devauchelle & Mingant, 1991; Utting & Spencer, 1991; Kent et al., 1999; Sarkis et al., 2006), addition of gamete suspension (Loosanoff & Davies, 1963; Helm & Bourne, 2004), hormonal manipulation (Zohar & Mylonas, 2001; Mañanós et al., 2009), chemicals and neurotransmitters (Vaca & Alfaro, 2000; Wongprasert et al., 2006; Mañanós et al., 2009), light (Loosanoff & Davies, 1963; Devauchelle & Mingant, 1991; Pillai et al., 1988; Fong, 1998; Alfaro et al., 2004), organ ablation (Primavera, 1978; Sagi et al., 1997) and stress induction (Thiyagarajan et al., 2003, 2007), among others. In barnacles, eicosanoids also induce release (reviewed by Clare, 2011), as well as limited periods of exposure to air (Franco pers. obs.).

According to Manãnos et al. (2009) the design of a larval release protocol is dependent on having reproductive information regarding sexual differentiation, size at maturation, timing of onset of vitelogenesis and of environmental parameters during the larval release season, endocrinology of reproduction, larval release behaviour, egg size and type, and fecundity. Despite current knowledge, the investigation over the efficiency of the different methods in *Pollicipes* sp. is pending.

1.3.2. Hatchery production of larvae

Establishing a hatchery phase allows not only control over productivity and independence from wildstock, but also improved larval quality both in terms of survival and development time, as well as biochemical profile.

Larval culture methods are intrinsically dependent on the natural larval cycle in the wild. *P. pollicipes* brood their embryos in egg lamellae until the larvae hatch inside the mantle cavity and are released by the adult. After released to the water column, barnacles larvae develop in the water column to a cyprid stage (Anderson, 1994). Unlike the planktotrophic nauplii II to VI, the barnacle cyprid is a non-feeding stage (lecithotrophic, as nauplii I), with a characteristic oval shape, six biramous appendages and frontal antennules modified for surface exploration (Molares, 1994; Anderson, 1994; fig. 1.11). The cypris larva of barnacles shows a strong thigmotropism, as is often seen in bivalve species (Nelson, 1924). Settlement comprises therefore not only the surface selection and attachment, but also the metamorphosis into a juvenile barnacle (Hui & Moyse, 1987; Rittschof et al., 1992). This transition occurs in a matter of hours to days after barnacle attachment (Doochin, 1951; Knight-Jones & Crisp, 1951; Kugele & Yule, 1996). Detailed descriptions of larval morphology and development of *P. pollicipes* can be found in Coelho (1990), Molares (1994) and Kugele & Yule (1996).

Hatchery production of stalked barnacles therefore encompasses all stages from embryo culture to larval settlement. Embryo culture need only be developed if egg lamellae are extracted directly from adult barnacles, since larvae collected from the wild, or from larval release in culture, only need to be reared to the settlement of the cyprid. Embryo culture and larval rearing have been accomplished in the laboratory, however these processes require optimization. Larval settlement, however, remains by far the most significant bottleneck.

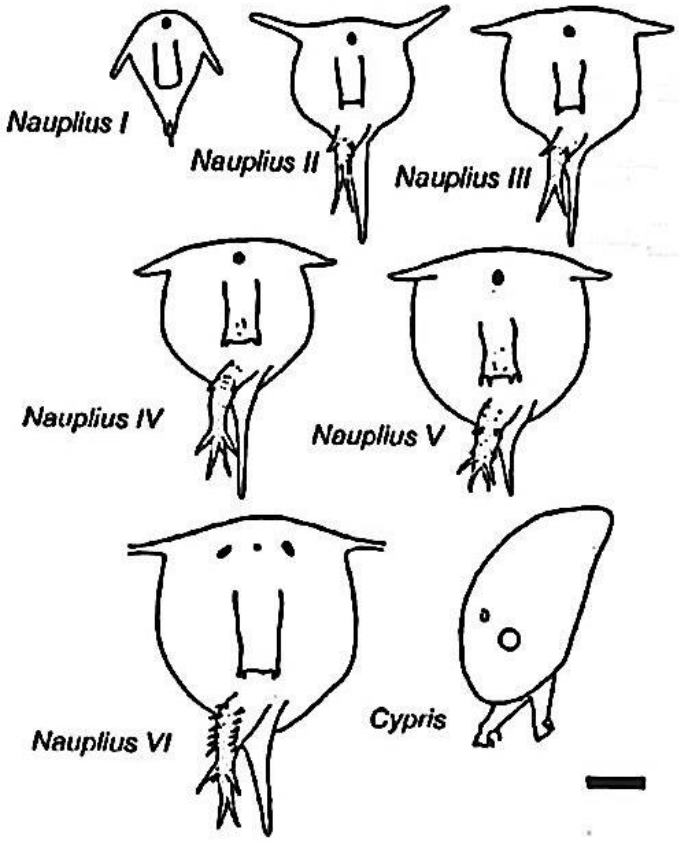


Fig. 1.11 Ventral view of outlines of naupliar stages I to VI and side view of the cyprid of *Pollicipes pollicipes* (from Cruz, 2000). Scale bar of 100 µm.

1.3.2.1. EMBRYO CULTURE

Although it is preferable in theory to allow rearing of embryos and larval release by adults, fertilized eggs of *P. pollicipes* can be extracted and cultured in the laboratory (Coelho, 1990; Molares, 1994; Molares et al., 1994a; Kugele & Yule, 1996; Candeias, 2005). In fact, Batham (1946) reported, when referring to *C. spinosa*, that prematurely liberated specimens take longer to develop (if they develop at all) than nauplii that hatch naturally, and that the shorter the embryonic period, the longer it took for larvae to develop to the cyprid stage. Egg masses removed from the mantle cavity of adults of *P.*

polymerus can be collected and allowed to hatch naturally, though the precocious removal of the lamellae from the mantle cavity can result in partial hatching sporadically over a period of days, with uneven growth among embryos (Lewis, 1975a). According to Lewis (1975a), a fairly reliable method for estimation of the developing embryonic stages is to consider lamella colour and texture, since newly fertilized egg lamellae are pale orange, flimsy and rounded, while mature lamellae have eggs already with naupliar eyes and are dark orange/brown, fattened, crumbling or fragmented. This method is accepted to be reasonably reliable for *P. pollicipes* (Coelho, 1990; Molares, 1994; Molares et al. 1994a), though microscopic observation is considered the most reliable method. Nevertheless, uneven embryo development in *P. pollicipes* might be experienced even if late stage egg masses are collected (Molares, 1994; Molares et al., 1994a) due, most probably, to the rate of aerobic respiration (Crisp, 1959). To minimize this effect, Candeias (2005) stressed the importance of using strong aeration to avoid oxygen depletion and to mechanically rupture the mature lamella membrane to stimulate nauplii release. Though in extracted lamellae, protease enzymes can be used to promote membrane rupture (Burrows et al., 1992), in natural conditions hatching occurs in response to an egg-hatching pheromone (Crisp & Spencer, 1958; Barnes, 1982; Clare et al., 1995). This pheromone was also reported in the prosome of *P. pollicipes* with and without mature ovisacs (Molares et al., 2002).

Embryonic development has been described for *P. polymerus* (Lewis, 1975b; Nussbaum, 1980), *P. pollicipes* (Coelho, 1990; Molares, 1994; Molares et al., 1994a; Kugele & Yule, 1996) and *C. mitella* (Qui et al., 1994a; Lin et al., 2002; Zhang et al., 2009; Rao et al., 2010). An overview of the most relevant results on embryonic development in Pollicipedidae is shown in Table 1.3. Embryonic development of *P. pollicipes* in the wild occurs within 14 to 96 days (Cruz & Araújo, 1999; Macho, 2006) depending on environmental factors such as food availability and temperature (Macho, 2006). The total embryo and larval development time of *P. pollicipes* in the laboratory is about 1.5 to 2 months, with larval development taking 23 to 28 days at 20 °C (Molares, 1994; Molares et al., 1994a; Kugele & Yule, 1996). Temperature manipulation is often used to control development time, in the laboratory, though this can have implications on fitness and survival, and should therefore be carefully considered. Studies on the embryonic development of *C. mitella* are a good example, since development time decreases significantly with increasing rearing temperatures, though survival is higher at lower temperatures (Zhang et al., 2009; Rao et al., 2010).

Nevertheless, for *P. pollicipes* little is known about these effects on embryonic development, despite some studies on temperature effects on larval development (Coelho, 1990).

Species	Embryonic development time	Embryonic hatching	Reference
P. pollicipes	25 days at 20 °C	n.a	Molares et al. (1994a)
P. polymerus	≈30 days at 13 °C	n.a	Hilgard (1960)
	20 – 30 days at 12 – 15 °C	n.a	Lewis (1975a)
	9 – 12 days at 12 – 13 ºC	n.a	Lewis (1975b)
C. mitella	5.5 days at 33 – 36 °C	n.a	Zhang et al. (2009)
	12 days at 18 °C	n.a.	Zhang et al. (2009)
	6 days at 28 ºC	100 % at 27 °C	Rao et al. (2010)
	9 days at 24ºC	52 % at 33 °C	Rao et al. (2010)

Table 1.3. Embryonic development time (days) and embryonic hatching (%), according to temperature, of various Pollicipediade, namely *Pollicipes pollicipes*, *Pollicipes polymerus* and *Capitulum.mitella*. *N.a.* stands for not applicable.

Embryo culture in *Pollicipes spp.* is usually accomplished by extracting the egg lamellae 2 – 6 h after collection of adults from the rocky shore, and incubating them in 100 – 200 ml of autoclaved or filtered seawater (Lewis, 1975b; Coelho, 1990; Molares, 1994; Molares et al., 1994a; Candeias, 2005). Water replacement normally occurs every 2 – 3 d (Lewis, 1975b; Molares 1994; Molares et al., 1994a), though Batham (1946) recommended daily water changes for lamellae of *C. spinosa* to avoid contamination by protozoa, that can dramatically reduce productivity. Candeias (2005) further suggests that a combination of high temperatures, high food concentrations and increased larval densities can lead to the faster accumulation of faecal pellets, water deterioration and protozoan concentration. The optimal pH range for embryonic development in *C. mitella* is 7 – 9, maximizing hatching rate and decreasing development time, and never below 5 (Guo et al., 2011). According to Lewis (1975b), optimal conditions for the development of *P. polymerus* embryos would consist of aeration, drugs at a lower dosage, darkness and lamellae sub-divided into smaller pieces. This is in accordance to what was used by other authors (Coelho, 1990; Molares, 1994; Molares et al., 1994a) when culturing embryos of *P. pollicipes*, using temperatures of 20 – 24 °C and natural salinities, with Coelho (1990) choosing also a natural photoperiod. On this basis, conditions simulating the adult mantle cavity would seem to be the most advantageous, though further studies are necessary. Salinities of 28 – 35 psu and temperatures of 27 – 28 °C (Lin et al., 2002; Rao et al., 2010) are recommended for *C. mitella*, which is in accordance to the natural temperature where this species is found.

1.3.2.2. LARVAL CULTURE

Larval production and cyprid settlement are without doubt the main bottlenecks to aquaculture production of *P. pollicipes*. Main factors governing larval development in laboratory barnacle cultures include feed quantity and quality, temperature, photoperiod, salinity, larval density and water quality. The effects of nutrition and environmental factors on larval growth and survival are broadly unknown for *Pollicipes* spp. and the few studies on this subject fail to mention mortality rates. Studies on the Pollicipedidae have been limited to the works with *C. mitella* (Lin et al., 1994; Qiu et al., 1994a, 1994b; Zhang et al., 2009; Lin et al., 2002; Rao et al., 2010) and *P. polymerus* (Lewis, 1975b). No concerted effort has yet been made to systematically describe the optimal conditions for rearing *P. pollicipes* nauplii to the cyprid stage, although preliminary work on larval holding systems (Molares et al., 2002), rearing diets (Coelho, 1990; Candeias, 2005), effects of temperature (Coelho, 1990), light (Molares et al., 2002) and culture density (Coelho, 1990) have allowed initial larviculture protocols to be devised .

1.3.2.2.1. CULTURE SYSTEMS AND HUSBANDRY PROCEDURES

Culture and settlement methods have been established for several species of barnacle (Keough & Downes, 1982; Brown & Roughgarden, 1985; Gabbott & Larman, 1987; Maki et al., 1988; Qiu & Qian, 1999; Matsumura et al., 2000; Mishra et al., 2001; Khandeparker et al., 2002; Thiyagarajan et al., 2003, Dreanno et al., 2007; Tremblay et al., 2007; Qiu et al., 2008), mainly to satisfy the need for experimental organisms in studies of marine biofouling. Early barnacle production efforts date back to Batham (1946), Knight-Jones (1953), Costlow & Bookhout (1958) and Crisp (1959), followed by others (Barnes & Barnes, 1959; Patel & Crip, 1960). Batham (1946) had first attempted to raise *C. spinosa* through to settlement *in vitro*, followed by Crisp (1959) with *S. balanoides*, which develops indirectly. Attempts with other species have followed varying degrees of success (e.g. Barnes & Barnes, 1959; Patel & Crisp, 1960) with *S. balanoides*, *B. balanoides*, *P. polymerus* and *Lepas anatifera*. Currently the procedures for cultivation of a few barnacle species (e.g. *B. amphitrite*, *B. improvisus*, *E. modestus*) are relatively well established (after Moyse, 1963; Tighe-Ford, 1970), though much remains under investigation.

Optimal culture systems and husbandry procedures are essential for successful larval production. Effects of changing conditions can be monitored easily by measuring larval activity and feeding behaviour, as well as feeding rate, larval size, rate of development, physical condition (e.g. deformation) and mortality rate. Static systems have been used

predominantly for larval culture of *P. pollicipes* in the laboratory. In such systems point aeration assures water movement sufficient for maintaining oxygen levels, while not exposing the larvae to extreme flow (Coelho, 1990; Molares, 1994; Molares et al., 1994a). This is done to minimise contact between larvae and to prevent larva from gathering (Macho et al., 2005; Macho, 2006). Water is normally filtered to 0.2 or 45 µm and UV treated (Coelho, 1990; Molares, 1994; Molares et al., 1994a; Candeias, 2005). The only study focusing specifically upon larval rearing (Molares et al., 2002) aimed to identify the best design for studies of larval behavioural, so it was not directed towards identifying an optimum culture method. These authors used closed systems and concluded that vertical aquariums were preferable to horizontal ones. On the other hand, flow-through and recirculating aquaculture systems (RAS) can often offer better water quality in comparison to static systems, provided that flow is sufficiently weak not to damage larvae. Static systems require periodic water changes, normally every 2 to 3 days or weekly (Lewis, 1975b; Coelho, 1990; Molares, 1994; Molares et al., 1994a; Kugele & Yule, 1996; Candeias, 2005), which despite increasing larval stress and damage, allows for more efficient exclusion of contaminant species (Molares, 1994; Candeias, 2005). Culture cleaning may be achieved by either attracting larvae to a light at the top of the culture while removing a portion of water from the bottom (Lewis, 1975b) and replacing it with clean water (Coelho, 1990), or by filtering the entire culture through an appropriate mesh size and replacing the entire water volume (Kugele & Yule, 1996; Candeias, 2005). It should be noted that the procedure for nauplii collection by attraction to light is commonly used in species that show phototactic behaviour, as is the case for *P. pollicipes* throughout its development (Molares et al., 2002).

The presence of contaminant species is often problematic in *P. pollicipes* culture (e.g. Molares, 1994; Candeias, 2005), especially due to the length of the larval rearing period. Molares (1994) reported acute larval mortality after 28 d of culture for this reason. Candeias (2005) noted that the proliferation of protozoans and bacteria was directly related to culture temperatures, food quantity and larval density. In practical terms, therefore, conical, bottom-opening vessels with aeration were found by Coelho (1990) to be most practicable in terms of ease of cleaning and provided advantageous water circulation.

1.3.2.2.2. NUTRITION AND TEMPERATURE

Feeding and nutritional requirements are of paramount importance in larval culture of *Pollicipes* spp. (Moyse, 1963; Lewis 1975b). Molares et al. (2002) investigated the use of *Isochrysis*, *Tetraselmis* and *Skeletonema* spp., but with unsatisfactory results and high mortality. The ingestion rate has been shown to vary depending on the algal species provided as food (Candeias, 2005). Furthermore, it should be noted that nutritional requirements might change with larval development, as suggested by Kugele & Yule (1996). Apparently healthy larvae, raised on sub-optimal diets may not be competent to settle, as seen with *B. amphitrite* larvae raised on *Dunaliella* sp. and *Mytilus edulis* raised on others than *Chaetoceros calcitrans*, where they both look healthy, but neither will settle (Aldred, pers. com.). A summary of diets tested and routinely used for *P. pollicipes* larval rearing are shown in Table 1.4.

Reference	Positive	Negative
Coelho (1990)	*Tetraselmis suecica* *Isochrysis galbana*	*Skeletonema costatum* *Thalassiosira pseudonana* *Chaetoceros gracilis* *Chlorella* sp.
Candeias (2005)	*Isochrysis galbana* *Skeletonema costatum* *Tetraselmis suecica* *Rhinomonas reticulata*	None (from diets tested)
Kugele & Yule (1996)	*S. costatum* and *R. reticulata* (1:2)	n.a
Stone (1988)	*R. reticulata* to NIII and *T. suecica* from NIV	n.a
Molares (1994)	*Isochrysis galbana*	n.a
Molares et al. (1994)	*Isochrysis galbana*	n.a
Molares et al. (2002)	*Isochrysis galbana*	n.a

Table 1.4. Summary of literature on larval diets shown to sustain or not development to the cyprid stage for Pollicipes pollicipes, according to the respective reference studies. Diets are divided into positive or negative according to larval development being supported or not in culture, on the respective studies. Both monodiets and mixed diets are shown. Abbreviations are as follows, (NIII) nauplii stage III, (NIV) nauplii stage IV, (n.a.) not applicable.

Although mixed diets were thought to result in better development and survival of larvae, this depends largely on the nutritional quality of the algae (Stone, 1989; Vanderploeg et al., 1996). Results from several authors (Coelho, 1990; Candeias, 2005) indicate that monodiets of flagellate species such as *Tetraselmis* sp. and *I. galbana* are sufficient to sustain adequate larval development and survival, as are mixed diets based mainly on flagellates and a complementary diatom species, such as *S. costatum*. Besides food quality and feeding regime, food quantity is often key in culture performance, both in growth and survival, but yet to be studied for *P. pollicipes*. There is no consensus on feeding quantity to be used, and protocols with P. *pollicipes* have ranged from 50 – 100

cells μl^{-1} fed daily or every 2 – 3 days (Molares, 1994; Molares et al., 1994a, 2002; Kugele & Yule, 1996; Candeias, 2005). Candeias (2005) recommends the use of algal densities $\leq$ 500 cells μl^{-1}, when antibiotics are not used, to avoid the deterioration of water quality. However, it should be noted that algal density is not always an adequate measure to compare carbon content available to the larvae, since cell volumes and nutritional profile vary greatly among species (e.g. *I. galbana* 20 μm^3, *S. costatum* 64 μm^3 and *T. suecica* 500 μm^3; Candeias, 2005).

Culture temperature has a major effect on barnacle larval development and survival (Batham, 1946), with higher temperatures often decreasing fitness and survival, but increasing growth rate within a tolerance range (Lewis, 1975b). This is a pattern common to many aquaculture species, from bivalves and crustaceans, to fish (e.g. *Crassostrea virginica*, Davies & Clabrese, 1964; *Solea solea*, Fonds, 1979; *Penaeus semisulcatus*, Kumlu et al., 2000). Although there have been several studies of *P. pollicipes* development in laboratory (Coelho, 1990; Molares, 1994; Molares et al., 1994a; Kugele & Yule, 1996; Candeias, 2005), only Coelho (1990) investigated larval growth at different temperatures. Development from nauplii I to nauplii VI decreased from 20 days at 15 ºC to 9 days at 22 ºC. Mortality was however significantly higher at 22 ºC (Coelho, 1990). Similarly for *C. mitella*, which has a longer larval phase (Qiu et al., 1994b) and is found in temperatures between 15 – 30 ºC (Morton & Wu, 1975; Leung, 2002), higher temperatures decrease development time (Rao et al., 2010; Zhang et al., 2009). Although this species is normally found at slightly higher water temperatures than *P. pollicipes*, Rao et al. (2010) also reports that at the optimal temperature of 27 ºC, survival ranges from 90 – 99 % and metamorphosis from 73 – 81 %. An overview of published larval development time for these species, according to temperature, is shown in Table 1.5. With regard to survival, Candeias (2005) reported 30 % mortality during the first 3 days and an overall mortality of 85.6 % after 14 days at 17.5 ºC. Results from other species also suggest increased mortality prior to the cyprid stage, e.g. 49.2 % for *B. amphitrite* (Costlow & Bookhout, 1959). With regard to *P. pollicipes* larvae, Coelho (1990), while studying the effect of density and temperature on larval rearing, simply referred to a generally high mortality. Nevertheless, it is interesting to note that the results obtained with *P. pollicipes* have been considerably inferior to *C. mitella*, highlighting the need for optimisation. The fact that optimal temperatures remain unknown presents a major gap in the knowledge of optimal conditions for larval rearing of *P. pollicipes*.

Species	Larval development time	Stages	Reference
P. pollicipes	20 days at 15 ºC	NI to NVI	Coelho (1990)
	9 days at 22 ºC	NI to NVI	
	23 – 28 days at 20 °C	NI to C	Molares et al. (1994a)
	14 days at 17.5 ºC	NI to NVI	Candeias, 2005
	21 to 28 days at 15 – 24 ºC	NI to V	Kugele & Yule, 1996
C. mitella	11 days at 24 ºC	NI to C	Rao et al. (2010) and
	7 days at 30 – 31 ºC	NI to C	Zhang et al. (2009)

Table 1.5. Larval development time in culture for *P. pollicipes* and *C. mitella,* according to temperature, with reference to development stages. Development stages are as follows, (NI) nauplii satge I, (NVI) nauplii stage VI and (C) cyprid. *N.a.* stands for not applicable.

1.3.2.2.3. OTHER ENVIRONMENTAL CONDITIONS

Other factors such as density, salinity, photoperiod and light intensity can also potentially have an impact on larval fitness. However, results from the few preliminary studies on *P. pollicipes* were inconclusive (Coelho, 1990).

A low larval density is normally used to maximize growth and survival. Larval density of 1 to 5 larvae ml^{-1} have been used (Coelho, 1990; Molares, 1994; Molares et al., 1994a; Molares et al., 2002), though most studies fail to mention this parameter (Kugele & Yule, 1996; Candeias, 2005). Coelho (1990) made the only attempt to compare the effect of varying larval densities (0.1 to 5 larvae ml^{-1}) on fitness but with inconclusive results, due to abrupt early mortalities.

A salinity of 33 – 34 psu has commonly been used for larval culture of *P. pollicipes* (Coelho, 1990; Molares, 1994; Molares et al., 1994a). However, unnatural salinities have proven beneficial in other species of bivalves, crustaceans and fish (e.g. *Penaeus semisulcatus; Mytilus edulis, Solea solea, Sparus sarba;* Fonds, 1973; Innes & Haley, 1977; Kumlu et al., 2000; Boeuf & Payan, 2001), with different results in terms of growth and survival.

Laboratory cultures of *P. pollicipes* have generally used natural photoperiod (Coelho, 1990) or full day photoperiod (Molares 1994; Molares et al., 1994a), though no studies have been directed at this specifically. In crustaceans there is a high variability in response to photoperiod during larval rearing, as seen when comparing *Strombus pugilis* and *Portunus pelagicus* larvae reared in the dark vs. full light. For the first, survival was higher in the dark but growth rate decreased (Manzano et al., 1998), while for the second, dark photoperiod had negative effects on both parameters (Andres et al., 2010). For others, such as *Jasus edwardsii* the response to photoperiod changes during development (Bermudes & Ritar, 2008), making species specific studies mandatory.

Light is a relevant factor for feeding, in particular when the simple eye structure of the larval stage is highly reliant on absolute illumination, making it more dependent on not only the amount of light provided, but also on light dispersion in the tanks (Naas & Huse, 1996). Molares et al. (2002) observed that all larval stages of *P. pollicipes* are positively phototactic, independent of light intensity, with exception of nauplii II for which results were inconsistent. The effects of light dispersion on larval behaviour as well as the effects of light on feeding remain unstudied however.

1.3.2.3. LARVAL SETTLEMENT IN CULTURE

Research on larval settlement in culture is restricted to Kugele & Yule (1996), who investigated settlement on various substrata, and Molares (2002), who attempted to assess the importance of chemical cues to settlement. On the other hand, studies of *P. pollicipes* recruitment in the wild (Coelho, 1991; Cruz, 2000; Cunha & Webber, 2000) may however provide important clues about factors that control the settlement process in nature. Observations on the settlement of *P. polymerus* and *C. mitella* are limited to the field (Lewis, 1975a ; Hoffman, 1989). However, work on other barnacle species, such as *B. amphitrite* and *E. modestus*, provides interesting background. The possibility of securing larval settlement on a substratum other than the natural one, an adult conspecific, could potentially have major positive implications for commercial culture and conservation.

1.3.2.3.1. LARVAL SETTLEMENT BEHAVIOUR

The barnacle cyprid explores surfaces prior to settlement using a mechanism of temporary adhesion (fig. 1.12), which allows the cyprid to survey a surface's physical and chemical characteristics before attaching permanently (Aldred et al., 2008; Aldred & Clare, 2009). During this exploratory phase, cyprids demonstrate sophisticated discriminatory capabilities (Knight-Jones & Stevenson, 1950). Barnacle cyprids can postpone their settlement for varying periods that differ between species (Wilson, 1932; Knight-Jones, 1953; Krug, 2006) in order to select a substratum that is suitable for settlement. During this period, many surfaces may be explored, rejected and the larva may return to the water column many times before committing to permanent attachment. The duration as a cyprid can range from a few hours to several months, and authors have reported particularly long cyprid phases for larvae reared in culture (Batham, 1946). Once the settlement site has been selected, the barnacle cyprid secrets a second adhesive that permanently fixes it to the substratum (Nott & Foster, 1969; Walker, 1972; Ödling et al., 2006; Phang et al., 2006; Maruzzo et al., 2012). Settlement

in barnacles is influenced by a range of factors from cyprid origin and condition, to environmental factors such as hydrodynamics, light, surface characteristics and the presence of chemical cues, biofilms or conspecifics (Aldred & Clare, 2008). Barnacle settlement, adhesion and surface selection have been investigated extensively by previous authors (Lewis, 1975a, 1978; Crisp, 1984; Bourget, 1988; Aldred & Clare, 2008), although very few studies have focused on the settlement of *Pollicipes* sp. cyprids (Lewis, 1975a ; Hoffman, 1989).

Fig. 1.12 Exploring cyprid of *P. pollicipes*.

Cyprids *P. pollicipes* are known to be highly discriminatory and gregarious, and tend to select as settlement substrata the peduncle of adults of the same species or substrata which have been in contact with conspecific adults (Coelho, 1991; Barnes, 1996; Kugele & Yule, 1996; Cruz, 2010). The larvae of *P. pollicipes* are often found attached between the scales of the adult stalk (Macho, 2006), where they can benefit from extended protection against predators and desiccation. Spat of *P. polymerus* are rarely found on the capitular plates except when under constant immersion, and epiphytes such as algae can significantly reduce settlement (Hoffman, 1989). To date, very little has been accomplished in terms of identifying the stimuli necessary for *P. pollicipes* cyprid settlement to non-conspecific surfaces under culture conditions, and most studies often used as reference refer to knowledge from other barnacle species.

Even on surfaces to which cyprids would naturally settle, settlement rates in the laboratory have never been higher than e.g. 20 % for *P. polymerus* (Lewis, 1975a) and 1 % for *P. pollicipes* (Kugele & Yule, 1996), highlighting the bottleneck presented by larval settlement in culture (fig. 1.13). Settlement in the wild has, however, been observed to occur on surfaces other than the adult, supporting the case that these are not

the only settlement substrata acceptable to cyprids. Studies of *P. pollicipes* larval development in culture often report that no settlement was observed and that a high mortality occurred at the cyprid stage (Molares, 1994; Molares et al., 1994a). However, other species, such as *C. spinosa*, also presented negative results with regard to settlement in early literature (Batham, 1945, 1946) and are now considered relatively trivial to culture and settlement. Of course, unsatisfactory settlement results in culture could easily be a product of cyprid quality, or competency. Evidence for this hypothesis comes in the form of historic references to the absence of lipidic droplets (energy store for the lecithotrophic phase) in the cyprid (Lewis, 1975a; Kugele & Yule, 1996). The presence of lipid in marine larvae strongly reflects larval fitness. In fact, as observed by Tremblay et al. (2007), cyprid settlement is positively related to the abundance of lipid reserves.

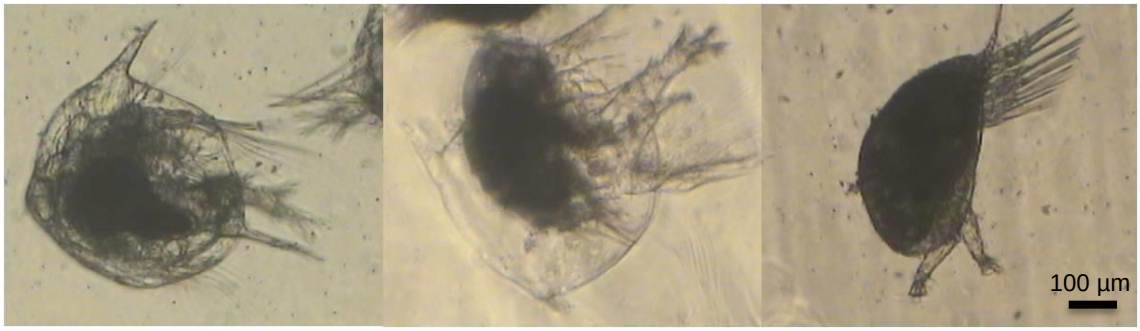


Fig. 1.13 Moulting nauplii VI to cyprid larval stage of *P. pollicipes*.

1.3.2.3.2. FACTORS AFFECTING SETTLEMENT

Factors related to larval culture, such as temperature and feeding (Harder et al., 2001; Thiyagarajan et al., 2002ab; West & Costlow, 2005; Emlet & Sadro, 2006), have been observed to significantly affect cyprid competence in barnacles, impacting directly juvenile quality. The use of inadequate diets during the planktotrophic nauplius phase can therefore reduce barnacle settlement from ~ 70 % to ~ 10 % in non-aged cyprids (Harder et al., 2001). Harder et al. (2001) tested various diets for *B. amphitrite*, providing different lipid content and concluded that settlement success was directly related to lipid reserves of the cyprid, varying both with diet and age. *S. costatum* is therefore often used in culture only as a dietary supplement. A settled cyprid will always attempt to moult to a juvenile barnacle (West & Costlow, 2005), although a minimal oil cell volume is required to ensure successful metamorphosis. Emlet & Sadro (2006) observed that the use of high food rations or low temperatures can lead to the production of consistently larger barnacle cyprids that grow faster as juveniles and

sometimes survive better in the field than juveniles from poorly fed larvae. Furthermore, the barnacle cyprid energy reserves influence the length of cyprid life span, habitat selection, success of metamorphosis and juvenile development (see Thiyagarajan et al., 2002ab). Additionally, cyprid age (i.e. the number of days post-moulting to cyprid) is also known to affect settlement, as cyprids become less selective as they age (Satuito et al., 1977, 1996; Rittschof et al., 1984; Maki et al., 1988; 1992; Holm et al., 2000; Marechal et al., 2012), which can be used to enhance settlement rates. There remains some disagreement regarding the best temperatures for ageing cyprids and the effects on settlement and discrimination, even for model species such as *B. amphitrite* (Rittschof et al., 1984; Crisp, 1988; Pechenik et al., 1993; Kitamura & Nakashima, 1996; Marechal et al., 2012).

Barnacle settlement is known to be influenced by hydrodynamics (Crisp, 1955; Knight-Jones, 1955; Mullineaux & Butman, 1991), light (Daniel, 1957; Crisp & Ritz, 1973; Pawlik, 1992) temperature (Satuito et al., 1996; Marechal et al., 2004), salinity (Dineen & Hines, 1992, 1994ab), cyprid density (Clare et al., 1994), among others. Settlement systems in the laboratory have been shown to benefit from conditions of flow, which maximize larval movement and larva-surface encounters. Crisp (1955) observed that cyprids settled only in flows with midrange shear stresses. Mullineaux & Butman (1991) observed that although cyprids reject surfaces more frequently in fast flow (10 cm s^{-1}) than in slow flow (5 cm s^{-1}), this does not necessarily result in lower settlement at fast flow since contact frequency is higher.

1.3.2.3.3. SETTLEMENT SUBSTRATA AND CHEMICAL CUES TO SETTLEMENT

Settlement is also dependent on the substratum itself and its physicochemical characteristics. In fact, the low settlement of *P. pollicipes* on artificial substrata (Kugele & Yule, 1996) could be strongly related to substrate chemistry, texture or the lack of settlement cues. Recent work has highlighted the importance of surface chemistry and the presence of chemical cues in other barnacle species (Crisp & Meadows 1963, Lewis 1978, Rittschof et al. 1984, 1985; Rittschof 1985; Crisp et al. 1985). For *P. pollicipes* the specificity of cyprids for adult conspecifics might suggest a larval response to the texture of the adult peduncle and/or its chemical nature, and that might be lacking in artificial substrata provided.

1.3.2.3.3.1. CONSPECIFICS AND GREGARIOUSNESS

The presence of conspecifics, either larvae or adults, can also significantly alter the behaviour of cyprids, as barnacle larvae are known to be highly gregarious and respond

positively to the presence of chemical cues left by conspecifics. Gregariousness is a strategy long known to be used by barnacles and other groups during settlement (Knight-Jones & Stevenson, 1950; Knight-Jones, 1951, 1953), as it allows the identification of suitable settlement places, protection and easiness of breeding. Though gregarious species are drawn to settle on conspecifics, some bivalves, such as oyster larvae, have been observed to settle in proximity of conspecifics but on reasonable distance from other larvae, allowing for growing space and decrease competition (Nelson, 1924). Alternatively, some species as *E. modestus* can present changing colonization patterns, with an early colonization of suitable surfaces by groups of larvae, followed by a later colonization of the previously bare areas, creating a surface almost evenly covered (Knight-Jones & Stevenson, 1950), showing that different species can exhibit distinct forms of gregariousness and response to chemical cues.

1.3.2.3.3.2. SIPC AND CYPRID FOOTPRINTS

In recent years, interest in chemical cues to settlement has waned, in particular regarding the main conspecific chemical cues identified from barnacles, the settlement-inducing protein complex (SIPC). Knight-Jones & Crisp (1953) suggested long ago that barnacle cyprids could be responding to surface-bound chemicals while settling, with crude protein extracts from adult barnacles shown to effectively induce cyprid settlement (Knight-Jones, 1953; Crisp & Meadows, 1963; Larman et al., 1982; Yule & Crisp, 1983). In fact, the addition of this "settlement factor" to settlement assays could increase settlement six-fold. Research on several species of barnacles has highlighted the importance of conspecific settlement pheromones, produced by both adults and exploring larvae, in the induction of cyprid settlement (Crisp, 1984; Gabbot & Larman, 1987; Clare, 1995; Clare & Matsumura, 2000; Hadfield & Paul, 2001; Pawlik, 1992; Thiyagarajan, 2010).

The adult SIPC (Matsumura et al., 1998a, 1998b; Clare & Matsumura, 2000) is a water soluble protein originating from the integument of adult barnacles and has been shown to induce cyprid settlement in laboratory experiments (Clare & Yamazaki, 2000; Matsumura et al., 2000). In the barnacle *Balanus amphitrite*, this large glycoprotein of ≈160 kDa has been shown to be related to the α2-macroglubulin family of proteins (Dreanno, 2006a). It is expressed throughout the barnacle life cycle and under denaturating conditions it fragments into 3 major subunits of 76 kDa, 88 kDa and 98 kDa (Matsumura et al., 1998a, 1998b; Dreanno, 2006c). These show remarkable similarities between barnacle species (Kato-Yoshinaga et al., 2000). SIPC has also been

identified in the "footprints" of temporary adhesive deposited by cyprids during surface exploration (Dreanno et al. 2006b). These "footprints" have also been shown to induce settlement in conspecifics cyprids (Clare et al., 1994; Yule & Walker, 1985; Matsumura et al., 1998a). However, in spite of various hypotheses (Knight-Jones, 1953; Crisp & Meadows, 1963; Barnes et al., 1970), the mechanism for detection of such cues by exploring cyprids remains unexplained. As research progresses, other waterborne chemical cues of 32 kDa and ≤1 kDa have been isolated from adult barnacles and have been shown to induce cyprid searching behaviour (Tegtmeyer & Ritschoff, 1988; Clare & Yamazaki, 2000; Endo et al., 2009; Elbourne & Clare, 2010). None of these cues have been identified in *P. pollicipes* and require further investigation. Molares et al. (2002) used the protocol for SIPC extraction and purification described by Matsumura et al (1998b) to isolate the SIPC from *P. pollicipes*, followed by SDS Page and Western Blotting. Molares et al. (2002) reported the existence of this protein complex in *P. pollicipes* while noting a much weaker signal on a Western blot in comparison to the signals of the SIPC extracts of *B. amphitrite*, *E. modestus*, *S. balanoides* and *B. perforatus*. Although mentioning the presence of SIPC in *P. pollicipes,* the inductive capability of this compound has not been validated. Further testing is required to clarify the existence of this protein complex in *P. pollicipes*, supporting Clare et al. (1997) and Molares et al. (2002), and confirm its effects on conspecific cyprid settlement. Molares et al. (2002) also tested the response of *B. amphitrite* cyprids to *P. pollicipes* SIPC in three experiments, although the results allowed for only limited conclusions and require further development of the work. In fact, Kugele & Yule (1996) attempted to induce settlement of *P. pollicipes* larvae using crude extract and concentrated protein extract from conspecifics, but were unsuccessful in spite of having high cyprid numbers. The same was reported in *P. polymerus*, by Lewis (1975a). Nevertheless, it is clear that a conspecific cue, so potent in modulating the settlement of barnacles, could be a very important tool in maximising cyprid settlement for aquaculture.

1.3.2.3.3.3. ARTIFICIAL CHEMICAL INDUCERS

Additionally, settlement-inducing chemical compounds such as 3-isobutyl-1-methylxanthine (IBMX), phorbol 12,13-dibutyrate (PBD), and hydroxyecdysone (20-HE), K^+, Ca^{2+}, γ-aminobutyric acid, choline, acetylcholine, serotonin, L-DOPA, forskolin, caffeine and theophylline among others have been identified to significantly affect settlement in barnacles and bivalve species (Clare et al., 1995; Holm et al., 2000; Dobretsov & Qian, 2003; Yu et al., 2008). However no studies have been conducted for *P. pollicipes* to date.

1.3.2.3.3.4. BIOFILMS

As highlighted by Maki et al. (1988), the presence of certain biofilming species and their respective extracellular products can either induce or inhibit settlement in barnacles (Crisp & Meadows 1963, Tighe-Ford et al. 1970; Maki et al. 1988, 1992), tube-worms (Knight-Jones, 1951) and bivalves (Satuito et al., 1997), among others. These are yet to be investigated for *P. pollicipes*. On the contrary, in field trials with *M. azoricus*, Pham et al. (2011) noted that on recruitment decreased with algal abundance. Similarly, Hudon et al. (1983), while observing the settlement of *B. crenatus* in the wild, found higher recruitment on clean surfaces, free of detritus and diatoms.

1.3.2.3.3.5. SUBSTRATA AND SURFACE PROPERTIES

The barnacle cyprid has long been known to respond to the physical characteristics of substrata (Knight-Jones, 1951; Crisp & Barnes, 1954; Barnes, 1956; Le Tourneux & Bourget, 1988). In fact, cyprids tend to settle preferentially in cracks and pits (Bergeron & Bourget, 1986), which potentially hold survival value by benefiting from protection from predators and desiccation. Lewis (1975a) recorded no settlement of *P. polymerus* in laboratory onto glass, bare rock, stalk epidermis or glass slides, although Hoffman (1988) reported settlement onto artificial substrata in the field. Similarly, Kugele & Yule (1996), while working with *P. pollicipes*, did not see consistent settlement on substrata such as slate in various colours (either with pits, smooth or slotted), as well as Perspex plates roughened on one side, black plastic, sandstone, plastic mesh of 80 µm and epoxy models of adults. These approaches failed even when combined with crude extracts and concentrated protein extracts from conspecifics. Nevertheless, the same authors observed less than 1 % settlement on artificial and natural surfaces. Of the 115 settled cyprids, 93 % settled on live adults, with the remaining 7 % being found on the Perspex plates with crude extract, on mimics with protein extract and on pitted slate, although it is unclear if these assays were multiple or no choice. This evidence collectively, associated with the often observed settlement on adults, has strengthened the belief that settlement in *P. pollicipes* might be an effect of multiple cues, including surface texture. The texture of the adult stalk skin and capitulum should be investigated further (fig. 1.14).

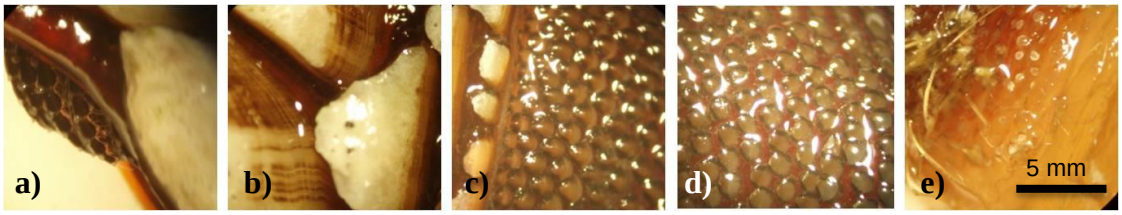


Fig. 1.14 Details of *P. pollicipes* (a) cirri and capitulum opening, (b) capitular plates, (c) base of capitulum and stalk, (d) stalk, (e) stalk base.

1.3.2.3.4. ALTERNATIVE OPTIONS TO SECURE LARVAL SETTLEMENT

Considering that, the only substratum which *P. pollicipes* has been observed to settle consistently is the peduncle of conspecific adults, or substrata that have been in contact with conspecific adults, few alternative strategies are obvious. Since juveniles of *Pollicipes* sp. have the ability to actively relocate (Hoffman, 1981, 1989; Chaffee & Lewis, 1998; Kugele & Yule, 1993, 2000; Cribeiro, 2007), one option could be to use live adults as settlement inducers and expect the juveniles to subsequently relocate themselves from the adult peduncle down towards primary substratum. *Pollicipes* sp. have been observed to form peduncular extensions by basal growth in order to relocate from the adults towards the primary substratum (Chaffee & Lewis, 1988; Hoffman, 1989; Kugele & Yule, 1993, 2000). Hoffman (1981) suggested that it might be maladaptative to remain attached to the adults and advantageous to retain mobility, in order to allow for dispersion and creation of new aggregations. Individuals removed from the substratum retain the capacity to re-attach to a new substratum (Hoffman, 1981). However, it can take a considerable amount of time for a relocating juvenile to reach a primary substratum (mean speed of 50 µm d^{-1}) and the factors that modulate relocation are unknown; neither gravity nor unidirectional flow have been confirmed as directing stimuli (Kugele & Yule, 2000).

A second option could be to use previously colonized substrata, which have been depleted of conspecific adults, as an attractant to juvenile settlement. However, the use of "clean" but previously colonized substrata as a settlement basis for cyprids raises the problem of obtaining such substrata in the first place without collecting directly from the natural habitat. However, use of such platforms would be virtually unlimited after the first collection, assuming that the cleaning of other *P. pollicipes* was not too thorough.

A third option could be to use barnacle extract in conjunction with surfaces and environmental conditions that mimic the natural situation to attempt to induce

settlement to specific structures. With regard to settlement patterns, the response to settlement cues and environmental variables in culture, limited work has been undertaken for *P. pollicipes*. Future studies should focus on verifying the existence of chemical cues to settlement in *P. pollicipes* and testing the response of cyprids to them, and other surface characteristics.

1.3.3. Nursery production of early juveniles

Nursery production, as defined by Claus (1981), is the transition between production in larval hatcheries and grow-out in the wild. The main objective is to obtain juveniles that are of higher fitness than the young post-larvae, facilitating this transition and minimizing costs (Claus, 1981; Wickins & Lee, 2002). Crustacean nursery production has the additional advantage of maximizing the limited ongrowing season in subtropical and temperate climates, allowing for production of early juveniles indoors in colder months, before transfer to the field when temperatures are higher (Wickins & Lee, 2002).

After attachment to a surface, the cyprid metamorphoses to an early spat, or juvenile barnacle, in the adult form. During metamorphosis, the larva undergoes several structural alterations, as described by Anderson (1994) and Cruz (2000). After contact with the substratum, temporary fixation, exploration and permanent fixation by the antennules, the moult occurs with the eye disappearing, the antennules shortening and the anterior mantle cavity gives way to the incipient stalk, which is thickened and further expanded until perpendicular to the substratum forming an apical-basal axis and, subsequently, the rostrum-carina axis (fig. 1.15). Kugele & Yule (1996) found, in *P. pollicipes*, that from a small sample of settled cyprids, 18 % failed to metamorphose and only 64 % of those settled moulted in the first 6 days (fig. 1.16). In the first year of life early juveniles show growth rates of between 0.83 – 1.61 mm RC month^{-1}, with individuals between 9 to 12 months growing to a total of 15.67 mm RC. General growth rates for juveniles (considered ≤ 12.5 mm RC by Cruz, 2000) average between about 0.4 to 0.5 mm RC month^{-1}, depending on season and decreasing with age, with adult *P. pollicipes* growing on average between 0.1 to 0.4 mm RC month^{-1} (Cruz, 2000). Though most studies have assessed growth rate using wild individuals of indeterminate age, field studies using surfaces previously hosting *P. pollicipes* and subsequently recolonized have allowed the estimation of growth rates from individuals of different sizes and of known maximal age. However, these growth rates have been recorded in

field studies (Cruz, 2000; Cunha & Webber, 2000), and there is very limited data from rearing in culture, as previously noted.

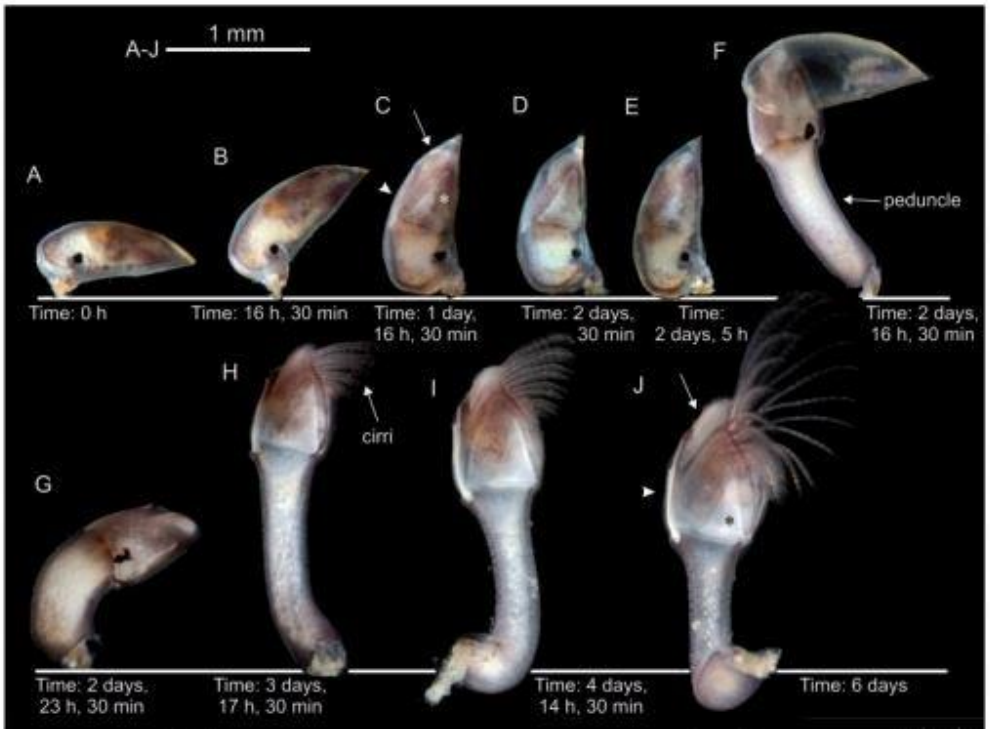


Fig. 1.15 Example of metamorphosis in pedunculated barnacles, namely metamorphosis in *Lepas* sp. (extracted from Høeg et al. 2012). (A–J) Lepas sp. (A) cyprid cemented by the tips of its antennules; the black spot in the 'cypris' body is the compound eye; (B–E) the whole cypris body is raised around the attachment point and there is incipient formation of the shell plate [asterisk, arrowhead, and arrow in (C)]; (F) the peduncle extended and the shedding of the 'cypris' cuticle is visible; (G) early juvenile with the peduncle in a contracted state; (H–J), juvenile with cirri extended for feeding and showing increasingly developed shell plates.

As in other species, nursery production of *Pollicipes* sp. may require a mixing of the conditions of larval production, under less strict conditions, and juvenile production under much controlled conditions. These conditions will not be re-analysed here in detail, as they have been already previously addressed in the respective sections. However, attention should be given to adjusting the feeding and hydrodynamic regime to the specificities of early spat rearing. Though no studies have investigated *P. pollicipes* early spat diet in the wild, studies on early spat of *P. polymerus* (1 – 6 mm RC distance) can be used has a reference. These indicate a wild diet based solely on diatoms, detritus, shells, sand, sponge spicules, barnacle exuvia and crustaceans, while larger juveniles (7 – 14 mm RC distance) feed also on other unicellular phytoplankton, blue-green algae, copepods, polychaetes and to a lesser extent on eggs, hydroids, molluscs and large algae (Lewis, 1981). Water movement is essential for feeding in

Pollicipes spp. (Barnes & Reese, 1959; Lewis, 1981; Cribeiro, 2007). Juveniles of *P. polymerus* have been reported to maintain cirral beating in static conditions, while in turbulent conditions cirral extension occurs in a similar way to the adults (Lewis, 1981).

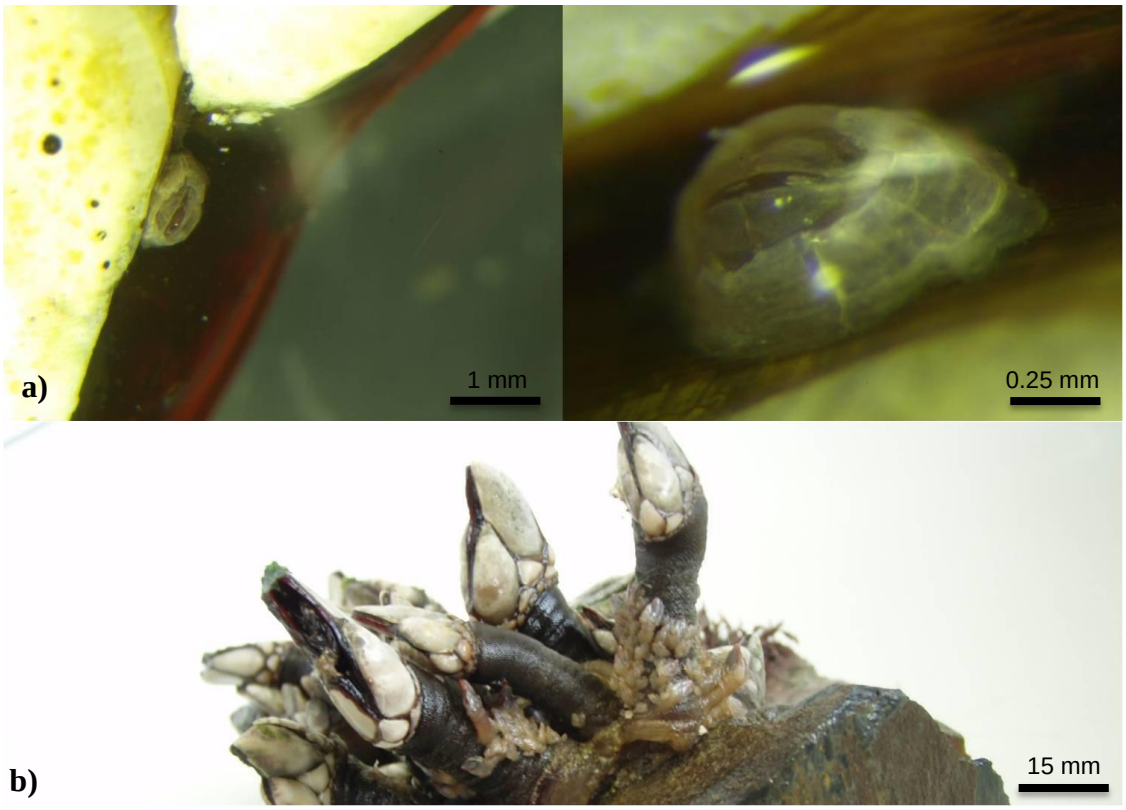


Fig. 1.16 Early juveniles of P. *pollicipes* attached to the adults, with less than a week observed (a) between the capitular plates, and with less than 3 months (b) attached to the adult's stalk.

Besides the main production monitoring factors, such as growth and survival, other production relevant factors, such as external morphology, taste and nutritional profile, should also be considered as they are thought to be mostly dependent on species, nutrition and environmental conditions during rearing. Several authors have suggested that light and photoperiod can significantly impact the *Pollicipes* sp. physiology, with individuals showing distinct stalk colours depending on whether they have been exposed to light (Barnes & Reese, 1960). Therefore, environmental factors that might induce alterations in the appearance and external morphology of *P. pollicipes* should be investigated further, as seen with the case of the "percebe mijão", which holds no market value, due to its stalk elongation and high water content (Cruz, 1993; Cribeiro, 2007). Barnes & Reese (1960) observations, of differences in stalk elongation and number of scales in populations from areas of disticnt hydrodynamic conditions, have also raised the question whether hydrodynamism can affect competition and feeding

capacity and the potential subsequent effects on morphology. Additionally, the use of adequate diets is essential for development and pigmentation (e.g. Barnes & Reese, 1960; Cruz, 2000; Cribeiro, 2007). Food quality has been shown to affect colouration, such as in *Lepas anatifera* whose ovaries become pink when feeding on *Artemia* sp., as opposed to the natural yellow coloration of wild individuals (Patel & Crisp, 1960). Additionally it should be considered that diets, if not from water, must also provide the minerals required for crustacean shell growth (Barnes et al., 1976). Alternatively, as in the culture of several bivalve species (e.g oysters), the end of the grow-out period might require a period of shell thickening.

For *P. pollicipes* a reasonable size for starting grow-out might be ~5 – 7 mm RC, when individuals start resembling the adults in feeding behaviour and are less likely to perish due to adverse environmental conditions. Cruz et al. (2010) reported that juveniles attached to conspecifics were rarely greater than 6 mm RC, which suggests that individuals above this size were independent enough to relocate towards the primary substratum. Considering that Cruz (2000) found that individuals in the wild with a maximal age of 3 months averaged 4.62 mm RC and a growth rate in the first 5 months of between 1.2 - 1.5 mm RC month^{-1}, the expected nursery time might be the order of 4 months if growth rates in culture are similar to those in the wild. Additionally, it should be noted that the nursery phase in crustaceans and molluscs can range from 1 to 4 months (Claus, 1981; Wickins & Lee, 2002). Norton (1996) when maintaining *P. pollicipes* in culture observed maximal growth rates of 0.930 mm RC month^{-1} for older juveniles and 0.509 mm RC month^{-1} for adults in culture, above what was measured by Cruz (1993, 2000) and Goldberg (1984) in the field, which might be due not only to the tide and temperature but also to the different diet, from wild omnivorous individuals to culture reared carnivorous individuals. Results from *P. polymerus* in the wild are in accordance with this, with growth rates of 0.7 – 1.2 mm RC month^{-1} (Paine, 1974), and 1.4 mm RC month^{-1} (first year) and 0.17 mm RC month^{-1} (after the first year) (Lewis & Chia, 1981). However, further studies are required to determine maximal growth rates and the conditions required to attain them, with a focus on the systems and protocols, imperative for maximizing survival and attaining high quality adults.

1.3.4. Juvenile on-growing and collection from the wild

Juvenile grow-out could be achieved using extensive or semi-intensive open systems located off- or on-shore. Results obtained from other barnacle species (Lopez et al., 2010) and grow-out trials with *P. pollicipes* undertaken in the natural environment

(Goldberg, 1984) have pointed to the many advantages of extensive off and on-shore growth systems since costs remain relatively low, taking advantage of natural feeding resources and high water exchange in the wild. Nevertheless, extensive systems in aquaculture often imply little control over culture-related factors, due to unpredictable changes in the weather, environment and nutrition, as well as the effects of predation, competition and disease (Appleford et al., 2003). Considering growth rates in the wild, it has been estimated that individuals of commercial size (RC ≥ 20 mm RC) could be produced within 12 – 24 months post-settlement (Cunha & Webber, 2000; Cruz et al., 2010) when growing in the wild on the SW coast of Portugal and in less than 12 months when growing in off-shore suspended systems in northern Spain (Goldberg, 1984).

Despite huge interest in these production phases, very is known about juvenile growth and survival in culture systems or in the wild (Goldberg, 1984). Similarly, spat collection was only briefly investigated by Coelho (1991) and Cruz (2000). The most relevant commercial studies on barnacle spat collection and ongrowing in the wild have been done in other barnacle species, such as *Austromegabalanus psittacus* (Lopez et al., 2005, 2010, 2012) and *Megabalanus azoricus* (Pham et al., 2008, 2011). The option of placing settlement panels in the natural habitat and collecting the individuals post-settlement has the greatest potential for aquaculture and the use of this method has been tested in other commercially relevant species, with positive results (Lopez et al., 2010, 2012; Pham et al., 2011). The disadvantages of such an approach are that: (a) the period for collection is dependent on natural cycles; (b) larval recruitment is dependent on larval availability and post-settlement survival; and (c) efficient collection methods through artificial substrata and presence of chemical cues for *P. pollicipes* have not been established and recruitment to artificial substrata in the wild remains a bottleneck. However, this method would allow for the collection of recruits (considered to be below 1 mm RC; Macho, 2006), negating the need for settlement and larval production in culture and providing less fragile individuals for grow-out.

1.3.4.1. SPAT COLLECTION

Collection of larvae from the wild using artificial settlement structures is a clear alternative to hatchery production and settlement. In addition, this would take advantage of the natural larval pool and thus mitigate any negative effects from inbreeding. In fact, sourcing seed stock from nature is common in extensive production, where grow-out is mainly field-based (Appleford et al., 2003), and such an approach is not new for

barnacle species (Lopez et al., 2010). Nevertheless, in spite of obvious advantages in terms of practicality and reduced costs, recruitment in nature is a product of (a) larval availability, (b) cyprid selectivity and (c) post-settlement survival.

1.3.4.1.1. LARVAL AVAILABILITY, SELECTIVITY AND POST-SETTLEMENT SURVIVAL

Larval availability is influenced by factors such as seasonality and variability of larval production during the reproductive season, and the impact of environmental factors on larval transport and survival (Shanks, 1986; Farrell et al., 1991; Pineda, 1991; Bertness et al., 1996; Shkedy & Roughgarden, 1997). Larval production varies greatly between breeding seasons, since it is dependent upon suitable environmental conditions for broodstock reproduction and larval development. Larval supply is determined by wind, currents, tides and other hydrodynamic factors (Gaines et al., 1985) and can either facilitate transport on-shore to recruitment areas, or off-shore making settlement to the natural habitat impossible. In fact, spat collection from the wild suffers from many of the fundamental problems of commercial fishing activities such as variable recruitment, low productivity, unpredictable stock size, and difficulties in establishing limits and regulating exploitation (Lucas, 2003). Besides larval availability, cyprid selectivity is another main factor affecting cyprid settlement in nature, as *P. pollicipes* cyprids are very selective in their choice of substratum, as previously discussed.

Additionally, in the wild a distinction must be made between settlement and recruitment. Low recruitment does not necessarily imply low settlement, but could instead be linked to low post-settlement survival (Bernard, 1988; Cruz, 2000). In fact, post-settlement survival in nature is thought to be low (Hoffman, 1989). Post-transplantation survival of *Balanus glandula* spat, for example, was only 43 – 49 % after 52 days (Emlet & Sadro, 2006). Early studies (Barnes & Reese, 1960) suggested that settlement on the adult peduncle could hold a survival value for young larvae, by providing shelter from abrasion and predators as well as avoiding desiccation. This hypothesis is, however, hard to substantiate since it can be difficult to distinguish substrata onto which cyprids did not settle, from others that received settlement but were subsequently denuded of spat. Competition in the field, either for food or for space, is dominated by species such as *Balanus perforatus*, *Chthamalus* sp., *Mytilus* sp., *Corallina officinalis*, *Plocamium cartilagineum*, *Patella* sp., among others (Cardoso & Yule, 1995; Cruz, 2000; Candeias, 2005; Macho, 2006), with species balance constantly changing (Hoffman, 1989). Predation effects have received increased attention in recent years and studies on *M. azoricus* have verified the presence of predatory flatworms

inside dead barnacles (Pham et al., 2011). Predation can significantly affect survival of spat in the field (Connell, 1961, 1970). Several authors reported *P. polymerus* being predated by crabs, seagulls and polychaetes (Bernard, 1988; Hoffman, 1989; Meese, 1993; Wooton, 1994), while Taylor and Morton (1996) mention gastropods as predators of *C. mitella*. Wooton (1994) further observed that the use of protective cages over the barnacle cultures can significantly decrease predation and increase *P. polymerus* coverage from below 20 % to over 60 %. When it is considered that *P. pollicipes* larval settlement on the stalk of the adults can average 15 larvae per adult, with the percentage of adults with recruits being above 80 % (Cruz et al., 2010), this can significantly impact the number of new recruits surviving to older juveniles. Hoffman (1989) observed over 300 spat cm^{-2} of peduncular surface on *P. polymerus*, suggesting that the loss of "nursery individuals" could have a large impact on recruitment. These consequences are similar in the context of barnacle collection for consumption, with a single "percebeiro" collecting up to 60 kg of *P. pollicipes* on a good tide. The average collections are, however, thought to be about 15 kg per harvester per tide (Jacinto et al., 2010), having either way a considerable impact on natural stock recruitment.

1.3.4.1.2. RECRUITMENT ON ARTIFICIAL SUBSTRATA

The results of studies investigating recruitment of *P. pollicipes* onto artificial structures in the wild are not encouraging and raise questions over the extent to which low recruitment is due to high larval selectivity or post-settlement mortality. Only Coelho (1991) and Cruz (2000) directly studied the recruitment of *P. pollicipes* in the wild.

Cruz (2000) tested the efficacy of artificial settlement structures for spat collection using PVC plates, both smooth and rough (vertical or horizontal), provided clean or with crude barnacle extract obtained from crushed conspecifics, but recording no recruitment over short periods of field exposure (e.g. 48h) throughout the summer period. Similarly Coelho (1991) did not find any recruitment on artificial structures. However, in the same study, recruitment was observed on natural surfaces, with 75 % of spat found on *P. pollicipes* adults and the remaining 25 % on non-conspecific natural substrata such as bare rock, calcareous algae, acorn barnacles and mussels (Coelho, 1991). Of course, the failure of these studies could equally be a consequence of a deterrent effect of the material used to fabricate the artificial substrata, rather than discerning by the larvae per se. The artificial substrata Coelho (1991) used to test larval settlement comprised tufnol panels (both smooth or with pits), plastic panels, rubber sheets (with 1.5 mm bumps) and sisal ropes organized in a matrix resembling *P.*

pollicipes clusters. Despite using whole barnacle extract with all of the surfaces, no settlement was recorded during the 6 week study, including on local bare rock previously cleaned of colonizing organisms. Indeed, these control patches were clear of barnacles, even after 7 months. This could be due either to the absence of settlement during that period or simply be in accordance with results from Hoffman (1989), who reported that if colonized surfaces are thoroughly cleaned of stalked barnacles, re-colonization of the same surface would not recur for months. The author seemed to suggest that prior colonization of the substrate is a mandatory requirement for *Pollicipes* sp. (see also Bernard, 1988; Hoffman, 1988; Coelho, 1991). However, *P pollicipes* has been observed to settle on unnatural substrata in the wild, such as epoxy resin, metal nuts and screws, among others (Cruz, pers. com.; Franco pers. obs.) although if in very low numbers. Similarly, studies on *P. polymerus* showed that they are able to settle substrata such as terracotta tiles, fibreglass panels, PVC and water inlet systems (Hoffman, 1988; Stachell & Farrell, 1993; Pineda, 1994).

Could systems currently is use for other barnacle species be applied to *P. pollicipes*? Seed collection of *A. psittacus* from the natural environment, for example, has been developed using suspended cultures with different artificial substrata (PVC tubes and plates), with results showing higher recruitment at depths of 4 – 6 m all year round, but increased in spring and summer (to 0.02 - 0.07 spats cm^{-2}). PVC substrata led to higher recruitment in comparison with other substrata such as expanded polystyrene and felt (bidín) (Lopez et al., 2010). Similarly *M. azoricus* has been grown in suspended culture systems of PVC at depths of 9 – 12 m, with rapid recruitment occurring all year round, peaking from June to October with final densities of 1109 juveniles m^{-2} after 15 months (Pham et al., 2008; 2011). These systems tend to be used both as barnacle seed collectors, i.e. the structures are placed in the wild during the recruitment season and the cyprids then settle and metamorphose to juveniles, as well as barnacle nursery and grow-out systems, all the way up to commercial size.

1.3.4.1.3. SITE SELECTION, TIMING AND CHARACTERISTICS OF COLLECTORS

The specific environmental conditions of the site selected for the placement of the seed collectors is clearly of major significance, since this will affect both cyprid settlement and early juvenile survival. According to site selection, larval supply will change dramatically, significantly affecting recruitment in many cases (Gaines et al., 1985; Young, 1987; Michinton & Sheibling, 1991). This has been verified by Lopez et al. (2012) for *A. psittacus* in Chile. Other production-related factors, such as the timing of

substrate deployment and collection, as well as the method for holding and securing collectors to the benthos should also be considered. Although the depth of the collector can affect collection efficiency (Lopez et al., 2010), this is not always the case (Lopez et al., 2012). On the Iberian coast, *P. pollicipes* larvae settle predominantly onto conspecifics during summer and autumn (Cruz et al., 2010), allowing an extended period for juvenile collection, as the settlement panels can be placed in the field during that period, acting as larval collectors.

Planktonic dominance can significantly affect which species are collected, as settlement panels used as larval collectors are hardly species specific, and several authors have found that the only way to control which species settles is by closely synchronizing the time for deployment and collection of substrata with the dominance period of the target species (Pham et al. 2011). Macho (2006) observed that while larvae of *Chthamalus* sp. can be found mostly in spring and summer, occasionally this range is extended to the end of winter and beginning of autumn, which can coincide with the period of larval release and recruitment of *P. pollicipes*. Additionally the way the substrata are held, to allow not only for settlement but also for post-settlement panel collection, can interfere with their capacity to act as collectors, due to compromises made in terms of optimal site selection and environmental conditions. Due to the requirement for *P. pollicipes* to inhabit high energy environments, deployment sites are not always easy to access and the maintenance of structures is challenging, as observed by Coelho (1991) on-shore and Goldberg (1984) off-shore. Both of these authors report that structures were easily lost, severely affecting the outcome of the studies. The vulnerability to adverse sea conditions of the collection structures, as well as the specifics of the type of system in use, all require future research.

1.3.4.2. JUVENILE ON-GROWING

1.3.4.2.1. CULTURE SYSTEMS

P. pollicipes cultures in open systems could be either on-shore systems, with settlement panels being fixed to the coast, or off-shore systems using suspended ropes or rafts to culture the juveniles. Although both options have advantages, on-shore systems more closely resemble the natural habitat, benefiting from richer waters and coastal upwelling. However, they are subjected to strong wave action, harsh environmental variation and strong predation effects. The competition for coastal space is also to be considered, as it might be an important conditioning affecting system selection.

Goldberg (1984) monitored the growth and survival of transplanted *P. pollicipes* on off-shore systems, however there have been no authoritative accounts since. Information from ecological studies on *P. pollicipes* developed by Cruz (1993, 2000), and others, may nevertheless serve as important reference material for estimating growth periods in the wild. Additionally, other barnacle species are currently cultured in open systems in the wild (e.g. *Austromegabalanus psittacus*, *Megabalanus azoricus*), and the development and optimization of these systems have been documented (Pham et al., 2008, 2010; Lopez et al., 2005, 2012; reviewed by Lopez et al., 2010). Culture systems for bivalves and crustaceans both off- and on-shore include use of various substrata, such as racks and suspended cultures as well as long-lines (Appleford et al., 2003) that may be of potential interest for *P. pollicipes*. The systems in use for *A. psittacus* and *M. azoricus* are based on suspended cultures with the recruits attached to the settlement substrata. In both cases spat are obtained from the wild by placing the collector surfaces in natural recruitment zones. The system tested for *P. pollicipes* by Goldberg (1984) used suspended rope cultures, similar to mussel culture systems, with system loss due to adverse sea conditions leaving a lot of room for improvement and further studies. Lopez et al. (2012) reported far fewer losses from collector systems (300 units per longline), suspended from double longlines (100 m length), at various depths of 4, 5 and 6 m.

1.3.4.2.2. GROWTH AND SURVIVAL

Goldberg (1984) showed however that the growth of transplanted stalked barnacles on floating offshore systems was higher than on the coast, without significant mortalities or effects on length-weight ratio. Cruz et al. (2010) estimated that individuals of commercial size could be produced in about 12 months after settlement. Cunha & Webber (2000) estimated 24 months. *A. psittacus* and *M. azoricus* grow to commercial size in 18 – 24 months (Lopez et al., 2010, 2012; Lopez, 2008) and 17 – 24 months (Pham et al. 2011), respectively, which highlights the prospects of *P. pollicipes* culture.

Although ecological estimations of growth rate have inherent variability (Barnes, 1996), most studies on *P. polymerus* reported growth rates within the range of 10 to 30 mm RC in the first year (Lewis & Chia, 1981; Page, 1986; Bernard, 1988). Some reference values for reported growth in the wild of *P. pollicipes* and *P. polymerus* are given in Table 1.6. For *P. pollicipes*, Cruz (2000) estimated yearly adult and juvenile growth rates of 3.54 and 5.46 mm RC respectively, with values ranging from 0.3 to 9.1 for adults and 0.5 to 13.3 mm RC per year for juveniles. On the other hand, the average growth reported by Goldberg (1984), during a study undertaken during summer months

was 3.22 ± 1.71 mm in nearly 2 months. This is considerably above values reported by Cruz (2000) in natural populations. Goldberg (1984) mentioned that the study period was from June to September which could explain the differences in results between studies, besides the differences in the type of system used. The previous author further reported that juveniles under 10 mm grew 4.55 ± 1.60 mm RC and juveniles above 10 mm grew 2.25 ± 1.02 mm RC, in accordance with Lopez et al. (2010; *A. psittacus*), where growth rate decreased significantly after 18 months. In fact, Cunha & Webber (2000) reported a decrease in *P. pollicipes* growth rate to one sixth of the maximum value, between individuals of 3 – 5 mm RC to 9 – 11 mm RC, with larger individuals (RC ≥ 20 mm) approaching null growth rates.

Species	RC increment (per year)	RC increment (per month)	Reference
P. polymerus	11 – 15 mm RC year^{-1}	n.a	Lewis and Chia (1981)
	15 mm RC year^{-1}	n.a	Bernard (1988)
	18 – 30 mm RC year^{-1}	n.a	Page (1986)
	n.a	5.5 mm RC in 47 days	Hoffman (1988)
	n.a	9 mm RC month^{-1}	Hoffman (1989)
P. pollicipes	5.46 mm RC year $^{-1}$	0.17 – 0.66 mm RC month^{-1}	Cruz (1993, 2000)
	15.7 mm RC year^{-1}	1.3 mm RC month^{-1}	Cruz et al. (2010)
	na	1.61 mm RC month-1	Gooldberg (1984)

Table 1.6. Results from studies conducted in the field on growth (RC increment, mm RC month^{-1} or mm RC year^{-1}) of *P. pollicipes* and *P. polymerus* juveniles, during the first year after settlement.

Seasonal variability also exists, with stalked barnacles showing faster growth in spring and summer, when temperature and food availability are more advantageous (Cruz, 1993; 2000; Cruz et al., 2010). Interestingly, Lopez et al. (2012) reported differences in growth according to substrate used, with larger individuals being found on the tubular collectors, followed by plate collectors and the smallest on the felt or "bidín" collectors, though no explanation is suggested for these findings. Depth was also important, with larger sizes at 4 m than 6 m. Besides growth rate, stock survival is of significant importance for culture. Goldberg (1984) reported mortality averaging 8 % over a growth period of nearly 2 months, equivalent to 4 % mortality month^{-1}, similar to on-coast controls. This constitutes a promising initial result. Mortality was attributed to the handling of individuals, though the results of Cruz (2000) reveal an average natural mortality of 4.0 to 4.6 % month^{-1}, without clear differences among seasons. Studies with other barnacle species cultured in the wild have reported average monthly mortality rates of 20 % to commercial size (Lopez et al., 2010). Additionally, no negatives effects on barnacle external appearance were observed during *P. pollicipes* grow-out in the field (Goldberg, 1984).

1.3.4.2.3. SITE SELECTION AND SYSTEM DESIGN

Similarly to spat collection systems, site selection and system design are important for juvenile grow-out, since tidal level, light, hydrodynamics and currents, productivity, temperature, predation and competition can have a significant impact on growth and survival of the individuals (Hoffman, 1988). In terms of production yield, Lopez et al. (2010) reported that a 100 m long-line could generate between 7 and 10 gross tonnes of product, with growth of individuals representing a 15-fold increase in biomass after 18 months in the field. *Pollicipes* sp. growth varies with tidal level, being greater in conditions of constant immersion (Hoffman, 1988). As previously discussed, tidal cycles have also been reported to positively affect growth (Cribeiro, 2007) and the development of stalk colour is affected by light (Barnes & Reese; 1960), being important considerations for site selection. Furthermore, although more sheltered sites may provide some protection from physical damage/loss, specific hydrodynamic conditions may be necessary for optimal development, with weaker currents limiting feeding and growth in adults (Lewis, 1981; Cribeiro, 2007). Rearing systems placed in areas of upwelling are preferred since, according to Pham (pers. com.), lower water productivity can significantly hinder the growth of others, such as *M. azoricus*. Nevertheless, the west coast of Portugal is of high productivity and upwelling occurs annually in the summer months (Wooster, 1976; Fiuza et al., 1982; Sousa & Bricaud, 1992). Water temperatures in open systems cannot be controlled, but within the range of the natural distribution of *P. pollicipes*, reported summer temperatures range from 19 – 23 ºC, dropping about 10 ºC in winter months (Goldberg, 1984; Cruz, 2000). Predation and competition effects should be considered and the use of protective devices, such as nets and other predator control techniques (Appleford et al., 2003), might be advisable. These were shown by Wooton (1994) to significantly decrease loss to predators in the case of *P. polymerus*.

1.4. Concluding remarks and future directions

In spite of considerable research on the ecology and stock management of *P. pollicipes*, much remains unknown regarding the potential for aquaculture, conditions required for culture and the feasibility of production. However, from the existing research and extrapolation from similar aquacultured species, several production cycles can be considered (fig. 1.17), each with varying degrees of control over production and independence from wild stocks, as well as associated production costs.

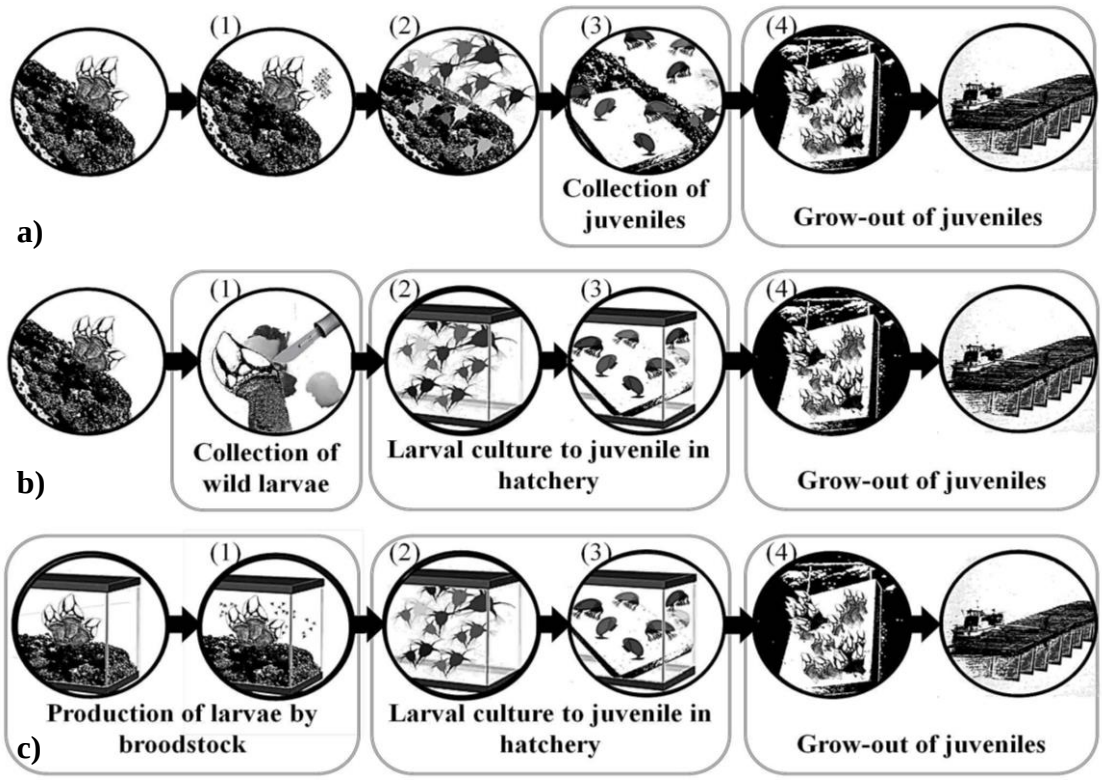


Fig. 1.17 Potential *P. pollicipes* production cycles (a) collection of juveniles, followed by ongrowing in the field; (b) collection of wild adults for embryo extraction, followed by larval rearing and settlement in culture to early juvenile rearing and posterior transfer for ongrowing in the wild; and (c) full production cycle in culture, from broodstock conditioning, to larval release, larval culture, settlement in culture and early juvenile grow-out, followed by grow-out in the field. The various production phases are shown: (1) the production of larvae by broodstock, (2) larval development to cyprid, (3) cyprid settlement or spat collection followed by early juvenile growth, and (4) juvenile grow-out.

One option is to limit the production cycle to collection of juveniles from the wild followed by on-growing in the field (fig. 1.17a). This would minimize production under culture, but would be increasingly dependent upon natural larval availability and environmental conditions. The fact that on the Iberian coast, *P. pollicipes* larval settlement is seasonal facilitates the collection of juveniles with artificial collectors. However, results of Coelho (1991) and Cruz (2000) suggest that further research on substrata preference, structure optimization, deployment sites and timing of collection, as well as identification of settlement cues will be required. The use of adults as settlement substrata for new recruits remains a possibility, but the practicability of this option requires further analysis. The estimates presented by Cruz et al. (2010) on the minimal time to commercial size are encouraging, with results from the preliminary works of Goldberg (1984) suggesting investment in off-shore suspended systems, which use low-cost technology and remove the need for exogenous feeding. Nevertheless, site

selection and system characteristics are of major concern, and though Goldberg (1984) recorded only residual mortality rates, the loss of several culture systems severely impacted culture production. This production cycle, of collection followed by ongrowing, is currently the production model in use for other commercially relevant barnacle species (Pham et al., 2011; Lopez et al., 2012). It presents the highest feasibility in terms of commercial production, given the current state of knowledge for *P. pollicipes* culture, and is only dependent on efficient spat collection from the wild, having the lowest associated production costs.

A second option would be the collection of wild adults for embryo extraction, followed by larval rearing and settlement in culture. After larval settlement, juvenile rearing could proceed in the field as above (fig. 1.17b). This assumes control over larval culture and cyprid settlement in culture, which have to be achieved. Higher production costs would be offset by a greater degree of temporal control over the production cycle, raising the possibility of multiple cycles in a year. Nevertheless, there remains a high degree of dependence on adult reproduction and fecundity during the breeding season, which is naturally variable and accentuated by the effect of breeding asynchronicity. Embryo extraction from the adults is a well-established procedure, and although culture conditions are not fully optimised, it is a possible strategy that provides a large number of larvae. In laboratory studies, several authors have described the larval development of *P. pollicipes* and successfully cultured nauplii to the cyprid stage (Coelho, 1990; Molares et al., 1994; Kugele and Yule, 1996). Concerns have been raised, however, over the likelihood of ever determining conditions necessary to promote settlement of cyprids in culture. Cyprids of this species are particularly discriminating, settling primarily on adult conspecifics and only rarely on other substrata (Kugele & Yule, 1996) and with records of very low cyprid survival in laboratory studies (Coelho, 1990; Molares et al., 2002). The way *P. pollicipes* cyprids respond to the texture of the adult stalk and its chemical cues is still, for the most part, unknown. The lack of assured settlement in culture represents a bottleneck to the viability of this production cycle, which in light of current knowledge prevents its implementation.

A third option would be to develop the full production cycle in culture, from broodstock conditioning to larval release, larval culture, settlement and early juvenile grow-out, and then secure the grow-out in the field (fig. 1.17c). This would permit the highest control over production, allowing for several cycles per year and optimisation of cyprid quality, but incur in increased costs due to the need for supporting the maintenance of adults in

culture and follow-up until early spat. Preliminary studies have confirmed that reproduction can be achieved in culture (Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007). Notwithstanding the ease of culture reported by these authors, the conditions required to achieve this have not been investigated in detail and require a renewed effort to identify the key factors controlling reproductive conditioning. On the other hand, though natural larval release has been reported, it is an unreliable method for collection of larvae, and larval release induction would provide better control over collection events, allowing large quantities of larvae to be obtained at planned intervals to establish consecutive production cycles.

In light of current knowledge, the most viable production cycle might rely upon spat collection and grow-out in the wild, similar to methods employed for *M. azoricus* and *A. psittacus* (Lopez et al., 2010). Accordingly, and as noted by Pham et al. (2010) for *M. azoricus,* the potential of these species for commercial aquaculture resides upon larval settlement on artificial substrata, tolerance to high stocking densities, reduced feeding costs in the wild, high growth rates and high commercial value. Future studies should focus on assessing the economical feasibility of such a production and on optimizing spat collection and systems deployed in the field. Preeminent research areas include the investigation of the use of chemical inducers to settlement (e.g. the SIPC), as well as the mechanisms of settlement and response of cyprids to surface characteristics. The identification of suitable settlement substrata would be a major asset to the culture of this species as, although settlement on artificial substrata has been observed, it remains the main bottleneck to production.

1.5. Project rationale and aims

As previously introduced, despite the high interest on the aquaculture of stalked barnacles (*P. pollicipes*) and its economic importance, current knowledge on rearing conditions and performance under culture is limited. Production challenges encompass the entire life cycle of this species and range from broodstock reproduction, to larval rearing and settlement, as well as juvenile growth in culture. The present work aimed to address the current culture bottlenecks, with a view to improving rearing protocols and increasing knowledge of possible production cycles.

The current research was therefore divided in four components: 1) adult reproduction; 2) larval rearing; 3) larval settlement; and 4) juvenile rearing under culture. From the analysis of existing literature, several production challenges over the various culture phases were identified (table 1.17).

Culture phase	State of the art	Challenges to culture
Adult reproduction	Adults maintained under culture and observed to breed, though larval production was sparse and unreliable (Cribeiro, 2007; Candeias, 2005; Kugele & Yule, 1996).	Lack of knowledge on triggering and optimal culture conditions for reproduction and larval release, as well as reference values for larval production and reproductive performance in culture.
Larval culture	Description of larval stages, with culture done until the cyprid stage, but with acute mortality and production of residual number of cyprids (Molares et al., 1994b; Kugele & Yule, 1996) and with limited number of studies testing culture conditions (Coelho, 1990).	Optimization of culture conditions and improvement of larval quality, with establishment of culture protocols and reference values for growth and survival under culture.
Larval settlement	Low when under culture, occurring almost exclusively in association with the adults, with no data of recruitment on artificial structures in the wild (Coelho, 1991; Molares, 1994; Molares et al., 1994a; Kugle & Yule, 1996).	Lack of knowledge on the factors (environment, substrata and cyprid related) that mediate settlement, effect of conspecific cues and performance of artificial substrata for settlement.
Juvenile culture	Juveniles have been kept under culture and observations made on preferred culture conditions (Cribeiro, 2007). Limited results from transplanted barnacles in the wild (Goldberg, 1984), in spite of several studies of growth of wild populations (e.g. Cruz, 2000; Cruz & Araújo, 2010)	No data on growth and survival under culture and limited knowledge of performance of barnacles transplanted to the wild. Lack of information on culture conditions.

Table 1.17 Production challenges according to production phase and state of the art research.

Culture phase-specific research objectives were therefore devised accordingly to allow both an integrated and phase-specific approach to address these challenges:

1. To compile the existing literature with respect to *P. pollicipes* culture and critically analyse current research gaps and challenges to future production of this species.
2. To investigate the reproduction of *P. pollicipes* broodstock under culture and larval production, and the effect of different temperature regimes, in order to establish a suitable rearing protocol.
3. To investigate a range of environmental and dietary conditions on larval culture performance, with a focus on the effects of temperature, diet, photoperiod and salinity on larval survival and growth.
4. To investigate the conditions required to promote cyprid settlement on adult conspecifics in the laboratory, as well as to evaluate cyprid settlement on artificial and natural substrata in the laboratory and in the field.
5. To investigate the conditions (tidal cycle, photoperiod, temperature, food quantity and quality) for optimisation of juvenile culture, with a focus on growth, survival, external morphology and feeding behaviour, as well as the effects of a finishing grow-out period in the wild.

These objectives were aligned with the proposed overall goal for the present study, which aimed at providing the tools for the aquaculture of the present species, by acquiring reference values of culture performance and investigating the effects of major environmental factors on production traits. It was hypothesised that a better understanding of the effects of key factors on *P. pollicipes* culture performance could assist in the establishment of the culture of this species. Successful completion of the above objectives would allow suggestions for future directions to approach the production of this species and the viability of potential production cycles, in view of current and prospective challenges. Therefore, the goal is to open the door to the aquaculture of stalked barnacles by addressing its main bottlenecks, which will in turn address social concerns regarding collection hazards and also the conservation of natural populations.

Chapter 2. Broodstock reproductive conditioning of stalked barnacles (*Pollicipes pollicipes*)

Abstract

Pollicipes pollicipes (Crustacea; Pedunculata) is considered a delicacy on the Iberian Peninsula where, in recent years, stock shortages associated with high market value have increased interest in the aquaculture potential of this species. The present study investigated the effects of rearing temperature on reproductive conditioning as an alternative to the current protocol of larval extraction. During a 4-week period, broodstock were subjected to temperature regimes characteristic of stable spring temperatures (*spT*), increasing spring to summer temperatures (*sp-suT*) and increasing spring to summer temperatures with daily fluctuations of 1 ºC (*sp-suT2*). During these periods, broodstock were monitored for fecundity, lamella development, lamella maturation, larval release rate, nauplius size and survival over 24 h. Cultured broodstock were fecund at smaller sizes (15.94 ± 0.23 mm RC) than wild-collected individuals (17.71 ± 0.65 mm RC). Fecundity increased significantly in all treatments, from 5.09 ± 0.08 %, and was highest under the *sp-suT* regime (26.67 ± 1.92 %). The lamella development index was higher in treatments with increasing temperatures (*sp-suT2,* 3.15 ± 0.60; *sp-suT*, 2.53 ± 0.47), while *spT* showed values comparable to the initial broodstock (0.33 ± 0.21 to 0.80 ± 0.36) and 0 % of mature lamellae. Increasing temperatures led to higher lamella maturation (*sp-suT*, 32 %; *sp-suT2*, 38 % of mature lamellae) and more frequent spawning peaks, possibly due to faster development rates. The number of nauplii released per tank per day varied according to treatment and time, averaging 4670.90 ± 506.63 nauplii day^{-1}. Due to the low number of larvae released daily, it is suggested that adults might release larvae gradually, as embryos hatch within the mantle cavity. Average release rates increased towards the end of the conditioning period, with releases on peak days ranging from 10000 to 30000 nauplii per tank. For *spT*, peak values were observed on week 3, while *sp-suT* and *sp-suT2* showed peaks of release on weeks 2 and 4, when temperatures averaged 20 and 23 ºC, respectively. Temperature oscillations seemed to favour a shorter interval between peaks of release. In terms of the total number of larvae released, no differences were observed between treatments (128147.39 ± 13548.26 nauplii). There were no differences either in relation to nauplius size (202.89 ± 0.69 µm GW) or 24-h survival (91.56 ± 0.35 %).

Notwithstanding the need for further optimization, broodstock reproductive conditioning can be accomplished and a continuous supply of larvae obtained using the protocols described herein. Future studies should focus on the impact of food quality and photoperiod on reproductive conditioning, as well as the optimization of larval release induction protocols.

2.1. Introduction

Pollicipes pollicipes is a high-value barnacle species, historically subject to an intensive fishery (e.g. Freire & Garcia-Allut, 2000; Molares & Freire, 2003; Bald et al., 2006a; Borja et al., 2006b). In recent years, growing concerns over depletion of natural stocks and the consequent stock protection measures (e.g. Cruz & Araújo, 1999; Borja et al, 2000, 2006a, 2006b; Castro, 2004; Queiroga et al., 2008) have increased interest in the potential of this species for aquaculture, both for ecological reasons and for commercial production. However, there are few published reports that relate to culture conditions of *P. pollicipes* (e.g. Molares et al., 1994; Candeias, 2005; Cribeiro, 2007) and, as such, much remains unknown regarding broodstock conditioning, larval culture, settlement and juvenile grow-out.

P. pollicipes is a pedunculated barnacle species, found in clusters on the Atlantic coast from France to Senegal, in areas of the intertidal exposed to strong wave action, (Stubbings, 1967; Newman & Killingley, 1985; Barnes, 1996; Castilla et al., 1998). These simultaneous hermaphrodites, dependent on cross fertilization, brood their developing eggs inside the mantle cavity (e.g. Molares, 1994b; Cruz, & Araújo, 1999: Cruz, 2000; Pavón, 2003). Adults produce approximately 30000 – 130000 embryos per batch and have been reported to release asynchronously 1 to 5 batches per year during the breeding period, which extends from March to September (Cardoso & Yule, 1995; Cruz & Hawkins, 1998; Molares, 1998; Cruz & Araújo, 1999; Cruz, 2000; Molares et al., 2002; Pavón, 2003; Macho et al., 2005; Cruz et al., 2010). This behaviour seemingly responds to changes in environmental factors, such as food availability and temperature. Functional testes are present all year round (Cardoso & Yule, 1995) and a significant number of adults have mature spermatozoa throughout the year. The female gonad rests from October to January (Molares et al., 1994b; Cruz & Hawkins, 1998), although there are conflicting reports (Barnes, 1992; Cardoso & Yule, 1995). Embryonic development of *P. pollicipes* occurs inside the mantle cavity of adults (Cruz & Araújo, 1999) until nauplii are released into the water column. The nauplii moult through six stages to the cyprid stage (e.g. Coelho, 1990; Molares et al., 2002), which is responsible for surface selection and settlement.

Due to its seasonal breeding habit, conditioning broodstock of *P. pollicipes* is essential for the provision of larvae for culture, allowing extension of the production season and reducing reliance on natural reproductive cycles. Embryos that are ready to hatch are extracted from the adults in a process whose yield depends largely upon natural

availability, seasonal larval production, the synchrony of gonad development and individual egg lamella maturation. Alternatives, such as the collection of larvae from planktonic samples, are unreliable due to the difficulty in identifying larvae to species and artificial insemination appears to have limited potential for stalked barnacles (Walley et al., 1971; Lewis, 1975a; Qui et al., 1994). Reproductive conditioning is a more plausible alternative and although the conditions required for this have not been investigated, Kugele & Yule (1996), Candeias (2005) and Cribeiro (2007), all maintained broodstock in captivity for extended periods with mating and spawning occurring naturally, although sparsely. These authors did not, however, detail the culture conditions under which this occurred, or the spawning frequency and batch fitness. Of these reports, Cribeiro (2007) provided the most detail of rearing conditions.

For *Pollicipes pollicipes* in the wild, the effect of temperature is hard to dissociate from latitude, when studies focus on the reproduction of a species across a distribution range (e.g. Cruz, 2000), or from food abundance, when variations in breeding throughout the year are considered (e.g. Molares, 1994; Cardoso & Yule, 1995; Cruz & Hawkins, 1998; Cruz & Araújo, 1999; Pavón, 2003). In fact, latitude determines the onset of reproduction in several barnacle species whose distribution covers a wide range (Crisp, 1950; Hines, 1978; Burrows et al., 1992). Studies on *P. polymerus* on the west coast of North America, indicate the presence of two distinct physiological races (Cimberg, 1981) comprising a northern stock that breeds at lower temperatures than the southern equivalent (Lewis & Chia, 1981; Page, 1983; 1984). Similarly, for *C. mitella* the length of the reproductive season is related to latitude (Leung, 2002), with barnacles at higher temperatures having a longer reproductive season than those at lower temperatures. In many species, reproduction and maturation of broodstock are known to be controlled by both endogenous and exogenous factors, among which temperature and food are often considered to be the most important (Bayne et al. 1976, Mann 1979a, 1979b; Barber & Blake 2006; Liu et al., 2008). In fact, gonadal development and maturation in *P. polymerus* in the wild has been shown to be controlled more by temperature than by food (e.g. Cimberg, 1981; Page, 1983). The importance of temperature has also been verified for *P. pollicipes* (Cardoso & Yule, 1995; Cruz & Hawkins, 1998), though from field studies it is hard to assess to what extent gonadal development is a function of temperature or food availability, as these are often correlated (e.g. Molares et al., 1994b; Cardoso & Yule, 1995; Cruz & Hawkins, 1998).

Water temperature on the Atlantic coast of Portugal and Spain varies between 10 to 24 ºC (according to location) and from 10 to 16 ºC from November to April and 14 to 24 ºC from May to October (Instituto Hidrográfico, 2012; Meteo Galicia, 2012; Euskalmet, 2012). However, most studies involving captive rearing of *P. pollicipes* did not impose strict temperature control and were not targeted at monitoring the adult reproductive condition and larval quality. Elevated rearing temperatures are often used to induce reproduction in aquaculture (e.g. bivalves; Loosanoff & Davies, 1950; Sastry, 1966), and it is hypothesized that this might also be the case in *P. pollicipes*. The conditions required for spawning in captivity have not yet been fully investigated, although some field studies have focused on the timing of larval release in natural populations (e.g. Macho et al., 2005). Macho et al. (2005) observed that *P. pollicipes* tend to release their larvae in the water column at the morning high tide, which might indicate the necessary triggering factors for spawning, such as tidal cycle and photoperiod. Observations by Lewis (1975b), when rearing *P. polymerus* in captivity, also suggest that the presence of food can stimulate the release of jets of hatching nauplii. These, together with reports from Candeias (2005) and Kugele & Yule (1996), strongly suggest that *P. pollicipes* spawning can be achieved in captivity provided that various conditions are met. On the other hand, spawning synchrony might more difficult to achieve since this species is a multiple brooder, reproducing asynchronously (Molares et al., 1994b; Cardoso & Yule, 1995; Cruz & Araújo, 1999).

This study focuses on the effect of rearing temperature on the reproductive development of *P. pollicipes* broodstock, by following fecundity index, lamella development index, release patterns, total number of larvae released and nauplius size and survival. Others factors, such as growth and survival, were also monitored due to their relevance for assessment of fitness. It further aims to establish a suitable rearing protocol for broodstock, that allows for maintenance in culture and spawning stimulation, and which can serve as a basis for future broodstock reproduction in captivity.

2.2. Materials and methods

2.2.1. Stock collection and acclimatization

Clusters of *P. pollicipes* were collected from Cabo Sardão (37°36'24.70'', -8°49'2.00'', Portugal; April, 2014) and transported (within 3 h) to the rearing facilities at the Centro de Ciências do Mar (CCMAR, Faro, Portugal), where they were acclimatized for 10 days, to assure the stabilization of mortality rates and adjustment to culture feeding regime. During acclimatization the clusters were kept in identical recirculating

conditions and at a fixed temperature of 16 ± 1 ºC. Prior to the experiment, barnacles in clusters were counted, photographically mapped within each cluster and measured for rostro-carinal distance (RC) and stalk length (SL). The clusters, which were all weighed and photographed (Olympus© E-410), were divided in groups of similar population structure (in relation to size class distribution), number of barnacles (adults and juveniles) and biomass.

2.2.2. Reproductive conditioning experiments

Barnacles were separated into similar groups (146 ± 13 barnacles; 15.34 ± 4.76 mm RC; mean ± SD), with equal population structure (16.17 ± 3.00 % under 10.0 mm RC, 24.01 ± 3.90 % between 10.0 to 12.5 mm RC, 33.40 ± 4.03 % between 12.5 and 15.0 mm RC, and 42.60 ± 3.89 % above 15.00 mm RC; mean ± SD) and distributed across 3 recirculating systems (3 aquaria of 60 L each). Over the following 4 weeks (from May 9th to June 6th, 2013), they were subjected to different temperature regimes (fig. 2. 1). Temperature treatments were as follows (*spT*) constant spring temperature of 16 °C (from day 1 to 28); (*sp-suT*) increase from spring temperatures 16 °C (on day 1) to summer temperatures 24 °C (on day 28); (*sp-suT2*) increase in temperature from 16 °C (on day 1) to 24 °C (on day 28) with diel temperature fluctuations (of ± 1 °C of the daily mean temperature). Temperature was controlled by the Aquatronic Aquarium Controller ACQ110 © and monitored in each aquarium by temperature data loggers. Each system had a total volume of 380 L, fully exchanged in 12 – 24 h (100 – 200 % renewal d^{-1}), including biological filtration.

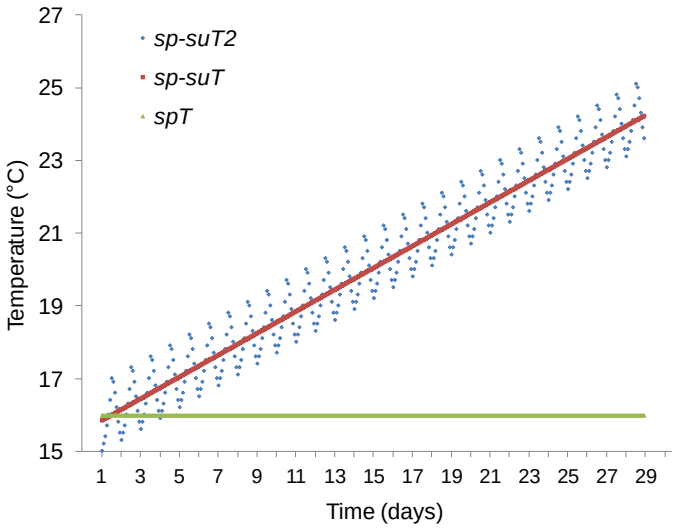


Fig. 2.1 Daily temperature, over the 28-day experimental period, according to conditioning regime. Treatments were as follows: (*spT*) constant spring temperature of 16 °C (from day 1 to 28); (*sp-suT*) increase from spring temperature 16 °C (on day 1) to summer temperatures 24 °C (on day 28); (*sp-suT2*) as previous regime but with diel temperature fluctuations (of ± 1 °C the daily mean temperature).

Each aquarium held between 100 – 120 adult barnacles attached to a net and suspended in the aquarium at approximately half the height of the water column (fig. 2.2). Aquaria contained natural filtered seawater (20 µm) at 36 ± 1 psu, natural photoperiod, dim light (100 – 200 lux), high water circulation (Hydor Koralia Pumps ©) and bottom aeration (Algarde 6'' airstone©). Each day aquaria were subjected to a tidal cycle of 3 h, during which the water level decreased to half of the aquarium, leaving the barnacles exposed to air for approximately 2.5 h. Daily feeding included *Artemia* sp. (4 % DW per day; Artemia International GSL®), for 2 h prior to the tide. *Artemia* cultures were maintained in 15-L conical aquaria with natural seawater, 36 ± 1 psu, 28 ± 1 ºC and strong bottom aeration. After 24 h, *Artemia* nauplii were separated from the cysts and samples were counted to estimate daily feeding volumes. Monitoring was done daily for dissolved oxygen, oxygen saturation, salinity and temperature.

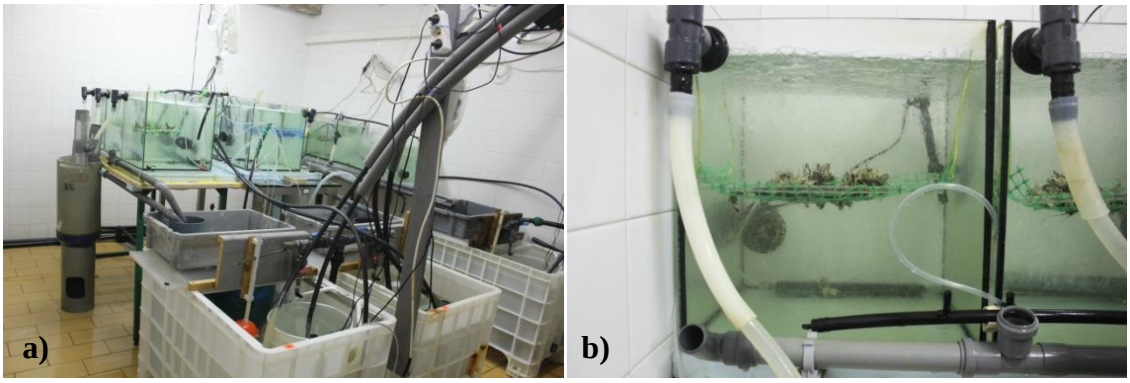


Fig. 2.2 System used for reproductive conditioning, with detail of (a) broodstock holding at mid-column height to allow for full emersion daily tidal cycles, and (b) individual system of 3 aquaria and respective nauplii collection systems.

2.2.3. Data collection and treatment

Clusters were monitored periodically for growth rate, survival rate, fecundity index, lamella development index, nauplius release rates, naupliar size, and nauplius survival over a 24-h period. At day 1 and day 28, individuals were measured with a digital calliper for RC and SL, to allow for calculation of specific growth rate according to RC distance (SGR-RC; % $28d^{-1}$; Eq. 1) and RC/SL proportion (RC/SL; Eq. 2). A sample of barnacles (n=30) was also used to extract egg lamellae for assessment of fecundity index (FI; Eq. 3) and macroscopical analysis of lamella development stage index (LDSi), according to Table 2.1. Extracted egg lamellae were left to hatch and the nauplii released (n=30) were cultured for 24 h to allow for estimation of extracted nauplius size (eGW) and 24-h nauplius survival (enS; Eq. 4). Cultures were screened daily for dead individuals, extracted and measured for RC length, to estimate daily adult mortality

b)

rates (% d^{-1}, dM; Eq. 4), total survival (% $month^{-1}$, tS) and average size of dead individuals (mm RC, dRC). Naturally released nauplii were also collected daily from each aquarium and counted, for estimation of daily (# day^{-1}, dRR), weekly (# $week^{-1}$, wRR) and total nauplius release rates (# $month^{-1}$, tRR) per aquarium (Eq. 5) and per individual. Nauplius collection was achieved using 80 µm filters placed on the outlet of each aquarium. For estimation of naturally released nauplius size in comparison to extracted nauplius size, larvae were also photographed and measured (Image J ®) for greatest width (GW). Samples of 30 nauplii were cultured for estimation of 24 h nauplii survival (24hS). Larval culture was conducted for 24 h in static conditions in Petri dishes (50x9 mm; 12 ml; BD Falcon®), in FSW, 18 ± 1 ºC, 16:8 L/D (dim light) and 35 ± 1 psu.

Eq.1 $$SGR = \frac{\ln(\frac{RC_{t+1}}{RC_t})}{(t+1)-t} \times 100$$

Eq. 2 $$RC/SL = \frac{RC}{SL}$$

Eq.3 $$FI = \frac{N_{\ lamellae}}{N} \times 100$$

Eq.4 $$dM = \frac{N_{\ dead}}{\mathrm{N}} \times 100$$

Eq.5 $$RR = \frac{n_{\ released}}{N}$$

Equations 1 to 5, where *SGR-RC* is the specific growth rate of barnacles in relation to RC distance (% $28days^{-1}$), *RC* is the rostro-carinal distance (mm) at day *t* or *t+1*, *RC/SL* index is a proportion of these two measurements, *RC*, as stated earlier, is rostro-carinal distance (µm), *SL* is stalk length (µm), *FI* is fecundity index, $N_{\ lamellae}$ is the number of individuals with lamellae (#), *N* is the total number of individuals (#), *dM* is daily mortality (% d^{-1}), $N_{\ dead}$ is the daily number of dead individuals, *RR* is the release rate or number of nauplii released per tank (*dRR*, #nauplii day^{-1} or *wRR*, #nauplii $week^{-1}$ or *tRR*, #nauplii $28days^{-1}$) and $n_{\ released}$ is the number of nauplii released (#) over the respective period (per day, week or 28 days).

Class	Development stage	Colour	Texture	Embryo development		
				Eye	Appendages	Stomach
0	Undifferentiated	Pink	Rigid	U	U	U
1	Early differentiation	Pink	Rigid	pD	U	U
2	Mid differentiation	Pink-Yellow	Semi-rigid	D	U	U
3	Late differentiation	Yellow-Brown	Flaccid	D	D	U
4	Differentiated	Yellow-Brown	Cloudy	D	D	D

Table 2.1. Criteria for the classification of lamellae according to development stage, adapted from Cruz (1993) and Molares (1994). Lamellae were analyzed macroscopically and classified according to colour and texture. Microscopical analysis was also done according to nauplii maturation stage, considering the differentiation stage of the eyes, appendages and digestive system. These were classified as *U* undifferentiated, *pD* partially differentiated and *D* differentiated.

2.2.4. Statistical analysis

All statistical analyses were performed using STATISTICA 7.0 ® and data in percentage (%) were arcsine transformed. Data were subjected to parametric tests including analysis of variance (ANOVA) or analysis of covariance (ANCOVA), with time as co-variate, when assumptions for normality and homoscedasticity were met (Shapiro-Wilk and Levene test, respectively). The significance level was set at α=0.05. Significant ANOVAs and ANCOVAs were followed by a Tukey test to identify differences among groups. Data that did not fulfil the assumptions for normality and homoscedasticity were subjected to non-parametric tests (Krushkal-Wallis test). All figures and tables shown have represented the mean ± standard error (SE).

2.3. Results

2.3.1. SGR, Survival rates and RC/SL proportion

Differences between treatments were not significant for daily mortality (ANOVA, F=1.77; p=0.17) and total survival (ANOVA, F=4.36; p=0.07; Table 2.2). Daily mortality averaged 0.38 ± 0.03 % d^{-1}, while total survival was of 93.66 ± 0.76 % after 28 days (Table 2.2). However, there were significant differences between RC of dead individuals according to treatment (ANOVA, F=6.76; p<0.01), with treatment *sp-suT2* being significantly different from the remainder (Tukey Test, p<0.01). The size of dead individuals in *spT* and *sp-suT* averaged 14.40 ± 0.34 mm RC, while in *sp-suT2* they measured 11.31 ± 0.59 mm RC.

	sp-suT2	sp-suT	spT
SGR-RC (% $28d^{-1}$)	0.83 ± 0.24 [a]	0.93 ± 0.31 [a]	0.73 ± 0.29 [a]
RC/SL (#)	1.47 ± 0.40 [b]	1.29 ± 0.29 [a]	1.37 ± 0.38 [b]
dM (% d^{-1})	0.42 ± 0.56 [a]	0.29 ± 0.53 [a]	0.43 ± 0.54 [a]
tS (% $28d^{-1}$)	91.33 ± 1.63 [a]	94.98 ± 2.27 [a]	91.68 ± 0.76 [a]
RCd (mm RC)	11.91 ± 3.44 [a]	14.54 ± 2.76 [b]	14.27 ± 3.04 [b]

Table 2.2. Growth and survival (mean ± SD) metrics for *P. pollicipes* adults grown for 4 weeks under different temperature regimes. Temperature treatments were as follows (*spT*) constant spring temperature of 16 °C (from day 1 to 28); (*sp-suT*) increase from spring temperatures 16 °C (on day 1) to summer temperatures 24 °C (on day 28); (*sp-suT2*) as previous but with diel temperature fluctuations (of ± 1 °C the daily mean temperature). Growth metrics considered included specific growth rate based on rostro-carinal distance (*SGR-RC*, % RC $28d^{-1}$) and proportion between rostro-carinal distance and stalk length (*RC/SL, #*; $RC/SL_{Initial}$ =1.28 ± 0.38). Survival metrics included daily mortality (dM, % d^{-1}), total survival (tS, %) and rostro-carinal distance of dead individuals (RC_d, mm RC). Different superscripts indicate significant differences. Temperature regime did not affect significantly SGR-RC (ANOVA; F=0.10, p=0.90), dM (ANOVA, F=1.77; p=0.17) and tS (ANOVA; F=4.36, p=0.07), but had significant effect on RC/SL (ANOVA; F=3.68, p=0.01) and RCd (ANOVA; F=6.76, p<0.01).

Specific growth rate was of 0.84 ± 0.16 % RC month^{-1} (Table 2.2), without significant differences between treatments (ANOVA, F=0.10; p=0.90). However, differences were found for RC/SL proportion (ANOVA, F=3.68; p=0.01; Table 2.2). Collected broodstock showed a RC/SL index of 1.28 ± 0.07, with individuals in *spT* and *sp-suT2* having higher RC/SL indices, *sp-suT* individuals showed lower index values, comparable to the initial broodstock. Significant differences were found between *sp-suT* and *sp-suT2* (Tukey Test; p=0.02), but not between the remaining treatments (Tukey test; p≥0.06).

2.3.2. Fecundity index and lamella development

The temperature regime significantly affected the fecundity index (fig. 2.3; ANOVA; F=11.35, p<0.01), with increasing temperatures stimulating breeding. Treatments *sp-suT2* and *spT* did not differ significantly from initial fecundity (Tukey Test; p≥0.30), unlike *sp-suT* (Tukey Test; p<0.01).

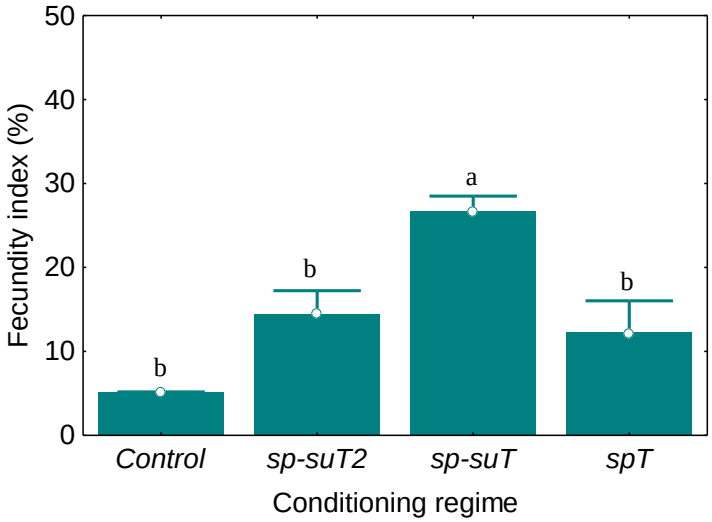


Fig. 2.3 Fecundity index (%) of broodstock cultured for 4 weeks at different temperature regimes. Treatments were as follows: (*spT*) constant spring temperature of 16 °C (from day 1 to 28); (*sp-suT*) increase from spring temperature 16 °C (on day 1) to summer temperatures 24 °C (on day 28) and (*sp-suT2*) average increase in temperature from spring 16 °C (on day 1) to summer 24 °C (on day 28) with diel temperature fluctuations of ± 1 °C. Different superscripts indicate significant differences.

The fecundity index of broodstock reared at *sp-suT* was significantly higher than *sp-suT2* (Tukey Test; p=0.05) and *sp-suT* (Tukey Test; p=0.02), with the latter not significantly different (Tukey test; p=0.93). From the initial broodstock 5.09 ± 0.08 % of individuals carried egg lamellae, while, after 4 weeks of conditioning, broodstock

achieved 12.22 ± 4.00, 26.67 ± 1.92 and 14.44 ± 5.09 % fecundity, when reared at *spT*, *sp-suT* and *sp-suT2*, respectively.

Temperature regime did not affect the size of individuals found bearing egg lamellae (fig. 2.4; ANOVA F=2.91, p=0.07), which averaged 15.94 ± 0.31 mm RC. No differences were found between the size of fecund and non-fecund individuals in any treatment (Tukey Test, p≥0.99). Nevertheless, significant differences were found between the size of fecund and non-fecund individuals in the initial broodstock (ANOVA, F=8.11, p=0.01; Tukey Test, p=0.02), which were respectively 17.71 ± 0.65 mm RC and 15.37 ± 0.15 mm RC.

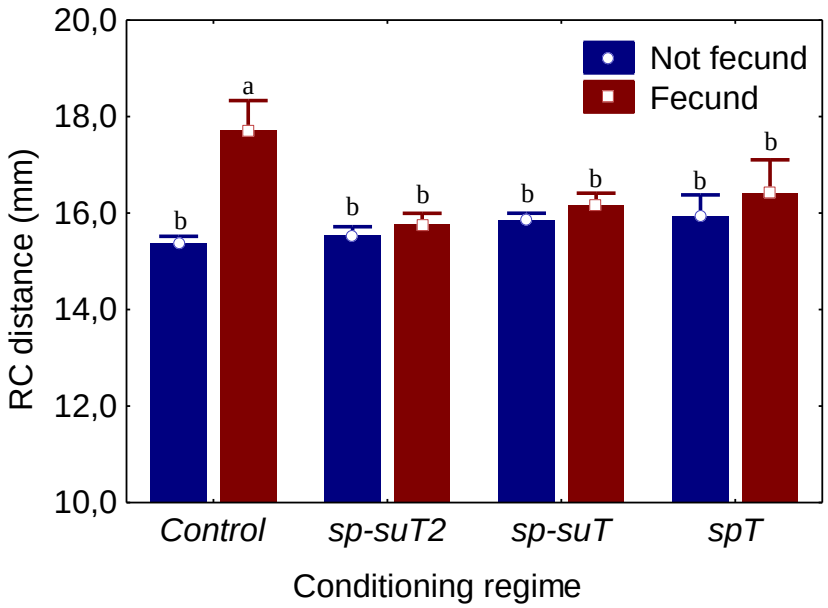


Fig. 2.4 Size (RC distance, mm) of broodstock reared at different temperature regimes, considering the rostro-carinal (RC) distance of all individuals (*n total*, blue) and individuals found bearing egg lamellae (*n lamellae, red*), i.e. fecund. Treatments were as follows: (*spT*) constant spring temperature of 16 – 18 °C, (*sp-suT*) linear temperature increase from spring 16 °C (on day 1) to summer 24 °C (on day 28) and (*sp-suT2*) average increase in temperature from spring 16 °C (on day 1) to summer 24 °C (on day 28) with diel temperature fluctuations of ± 1 °C. Different superscripts indicate significant differences.

The initial lamella development index was 0.33 ± 0.21, increasing to 0.80 ± 0.36, 2.53 ± 0.47 and 3.15 ± 0.60 after 4 weeks of conditioning at *spT*, *sp-suT* and *sp-suT2* (fig. 2.5).

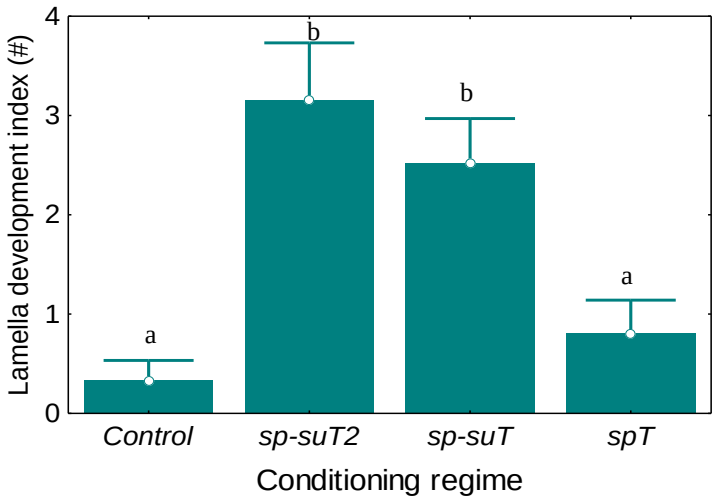


Fig. 2.5 Lamella development stage index (#) of broodstock reared under different temperature regimes. Treatments were as follows: (*spT*) constant spring temperature of 16 °C, (*sp-suT*) linear temperature increase from spring 16 °C (on day 1) to summer 24 °C (on day 28) and (*sp-suT2*) average increase in temperature from spring 16 °C (on day 1) to summer 24 °C (on day 28) with diel temperature fluctuations of ± 1 °C. Lamellae development stages were classified according to Table 1. Different superscripts indicate significant differences.

Lamella development stage index was different according to conditioning regime (ANOVA, $F=7.28$, $p<0.01$), as *sp-suT* and *sp-suT2* were higher than initial values (Tukey Test, $p\leq0.01$), while *spT* was not (Tukey Test, $p=0.93$). Final lamella development index was significantly different between *spT* and *sp-suT2* (Tukey Test, $p=0.02$), but not between *spT* and *sp-suT* (Tukey Test, $p=0.08$) and *sp-suT* and *sp-suT2* (Tukey Test, $p=0.76$). The percentage of extracted egg lamellae according to development stage is shown for the various treatments and initial broodstock in Fig. 2.6. Broodstock initially had 80 % of stage 0 lamellae, and 20 % of stage 1 and 2 lamellae. After 28 d in culture, *spT* had 60 % stage 0 and 40 % stage 1 and 2, while *sp-suT* and *sp-suT2* had only 36 % and 23 % of stage 0 lamellae, and 24 % and 39 % of stage 1 and 2 lamellae. Furthermore, despite no mature egg lamellae being observed at the beginning of the experiments, after conditioning *sp-suT* and *sp-suT2* had respectively 32 and 38 % of mature egg lamellae.

2.3.3. Nauplius release rates

Average daily release rates did not vary with treatment (ANCOVA, $F=1.14$, $P=0.32$; table 2.3), averaging 4670.90 ± 506.63 nauplii d^{-1}, although significant differences in release rates with time (ANOVA, $F=2.74$, $p>0.01$; fig. 2.7). Individuals in *spT* showed one peak release (≥10000 larvae) by week 3, while in *sp-suT* and *sp-suT2* they showed

two release peaks in weeks 2 and 4. Individuals reared at *spT* showed peak larval release rates at day 23 (15833.33 ± 2976.90 nauplii) (fig. 2.7; Tukey Test, p>0.01). Larval release rates for *sp-suT* peaked (Tukey Test, p>0.01) at day 11 (16531.20 ± 3937.31 nauplii) and day 28 (11969.00 ± 2246.22 nauplii), while for *sp-suT2* this happened at day 14 (13179.33 ± 7371.58 nauplii) and 27 (25806.67 ± 4677.13 nauplii).

	sp-su T2	sp-su T	sp T
dRR (larvae d^{-1})	4972 ± 722.15 [a]	4062.83 ± 466.15 [a]	4495.917 ± 635.55 [a]
tRR (larvae)	145674.51 ± 11292.40 [a]	113075.34 ± 10223.32 [a]	125692.33 ± 20154.10 [a]
GW (µm)	203.67 ± 2.84 [a]	202.33 ± 2.85 [a]	202.67 ± 5.90 [a]
24hS (%)	91.01 ± 1.52 [a]	92.00 ± 1.53 [a]	91.67 ± 2.91 [a]

Table 2.3. Daily release rates (dRR, # nauplii tank d^{-1}), total release rates (tRR, # nauplii tank $month^{-1}$), released nauplii I size (TL, µm) and nauplii survival after 24h (24h S; %). Average daily release rates did not vary significantly according to treatment (ANCOVA, F=1.14, P=0.32), in spite of significant differences on release rates with time (ANOVA, F=2.74, p>0.01). There were no differences in total release rates (126292.60 ± 2700.29; ANOVA, F=1.27, P=0.35), nauplii size (202.89 ± 0.69; ANOVA, F=0.03; p=0.97) and nauplii survival (91.56 ± 0.35; ANOVA, F=0.06, p=0.94). Values are presented as mean ± SD.

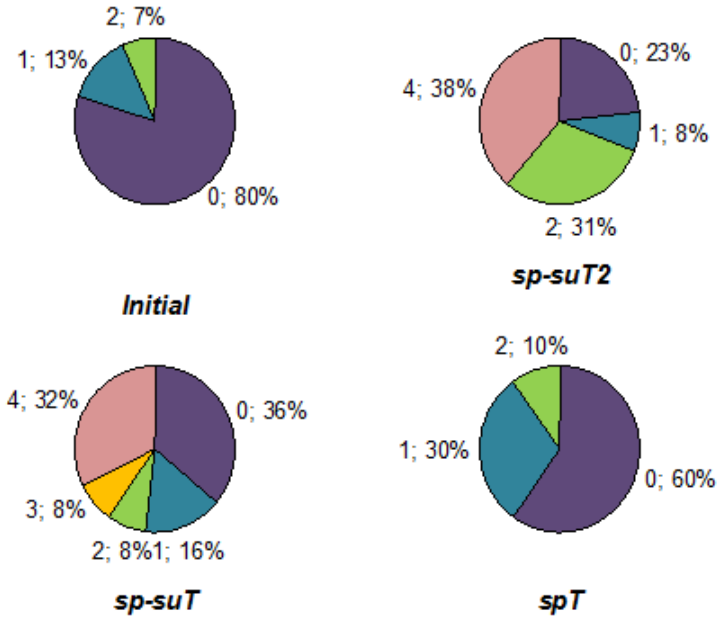


Fig. 2.6 Percentage of egg lamellae according to development stage, extracted from broodstock reared under different temperature regimes. Stages were classified as (0) undifferentiated, (1) early differentiation, (2) mid differentiation, (3) late differentiation, (4) differentiated, according to Table 1. Stage 4 lamellae were ready to hatch, and nauplii would swim freely upon egg lamella membrane rupture. Treatments were as follows: (*spT*) constant spring temperature of 16 °C, (*sp-suT*) linear temperature increase from spring 16 °C (on day 1) to summer 24 °C (on day 28) and (*sp-suT2*) average increase in temperature from spring 16 °C (on day 1) to summer 24 °C (on day 28) with diel fluctuations of ± 1 °C.

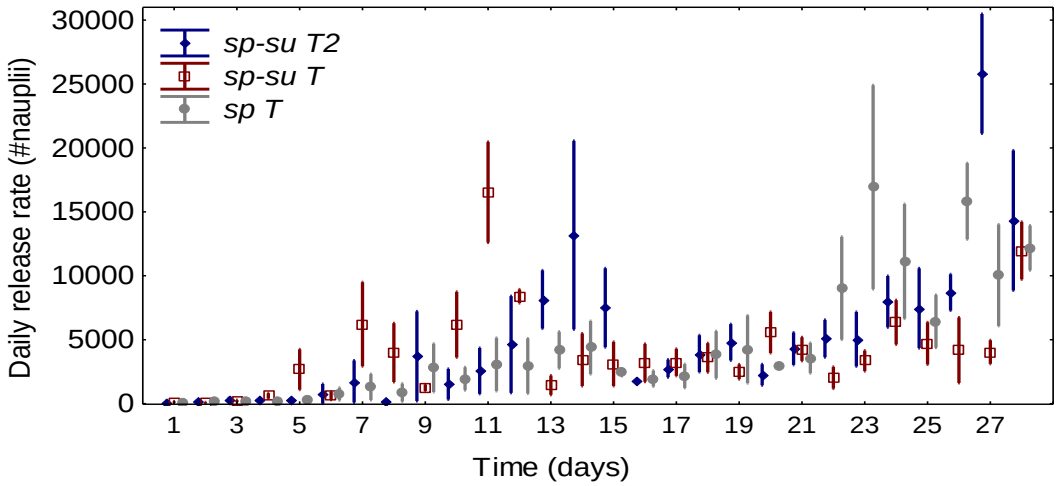


Fig. 2.7 Daily release rates, i.e. number of nauplii released per tank per day, during 4 weeks of conditioning and according to temperature regime. Treatments were as follows: (*spT*) constant spring temperature of 16 °C, (*sp-suT*) linear temperature increase from spring 16 °C (on day 1) to summer 24 °C (on day 28) and (*sp-suT2*) average increase in temperature from spring 16 °C (on day 1) to summer 24 °C (on day 28) with diel temperature fluctuations of ± 1 °C.

Average release values increased with time and weekly rates were significantly different (fig. 2.8; ANOVA, $F=35.43$; $p<0.01$). Significanlty lower numbers of larvae were released in week 1 when compared to week 4 (5310.69 ± 1891.22 to 66424.56 ± 9505.39 nauplii week^{-1}; Tukey Test, $p<0.01$). Average release rate did not differ with temperature (Tukey Test; $p\geq0.68$), except on week 4 when *sp-suT* was significantly lower than both *sp-suT2* and *sp-suT* (Tukey Test; $p=0.01$), while these two did not differ from each other (Tukey Test; $p=1.00$).

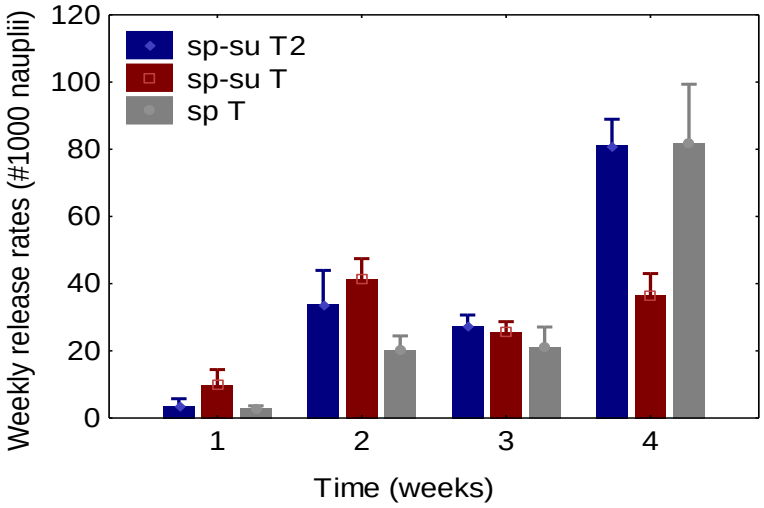


Fig. 2.8 Weekly release rates, i.e. number of nauplii released per tank per week, throughout 4 weeks of conditioning and according to temperature regime. Treatments were as follows: (*spT*) constant spring temperature of 16 °C, (*sp-suT*) linear temperature increase from spring 16 °C (on day 1) to summer 24 °C (on day 28) and (*sp-suT2*) average increase in temperature from spring 16 °C (on day 1) to summer 24 °C (on day 28) with diel temperature fluctuations of ± 1 °C.

Total release rates did not vary according to treatment (fig. 2.9; ANOVA, F=1.27, p=0.35). On average 126292.60 ± 2700.29 larvae were released per tank, although *sp-suT2* presented the highest values, followed by *spT* and *sp-suT*. Total numbers of nauplii released per tank were 125692.33 ± 20154.10, 113075.34 ± 10223.32 and 145674.51 ± 1129.40 for *spT*, *sp-suT* and *sp-suT2*, respectively (Table 2.3).

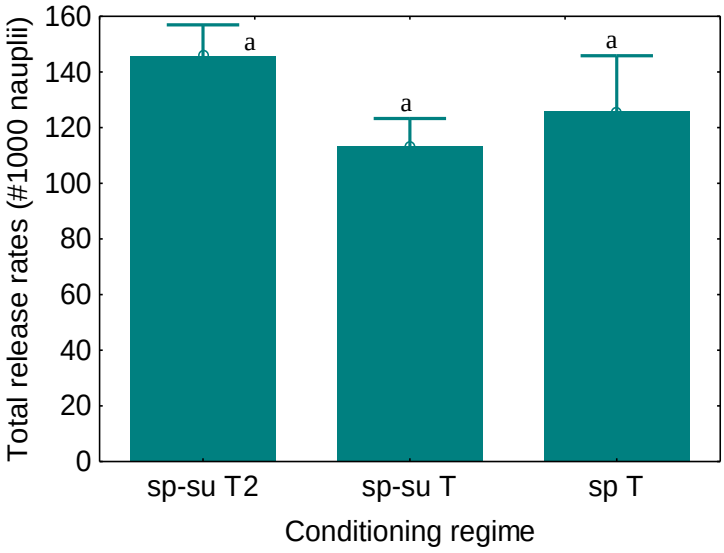


Fig. 2.9 Total release rates, i.e. number of nauplii released per tank during 4 weeks, according to temperature regime. Treatments were as follows: (*spT*) constant spring temperature of 16 °C, (*sp-suT*) linear temperature increase from spring 16 °C (on day 1) to summer 24 °C (on day 28) and (*sp-suT2*) average increase in temperature from spring 16 °C (on day 1) to summer 24 °C (on day 28) with diel fluctuations of ± 1 °C. Total release rates did not vary according to treatment (ANOVA, F=1.27, p=0.35).

2.3.4. Nauplius size and 24-h survival

Size did not vary between extracted and released nauplii (ANCOVA: F=0.01, p=0.99), which measured 202.89 ± 8.69 µm GW. Similarly, nauplius size was not significantly different among treatments (ANCOVA, F=0.03; p=0.97), as is shown in table 2.3 Nauplius 24-h survival averaged 91.56 ± 0.35 %, without differences between temperature regimes (ANOVA, F=0.06, p=0.94; Table 2.3).

2.4. Discussion

The present results show that conditioning can be achieved in captivity in a relatively short period. Essential to fast conditioning and synchronous breeding, however, is the timing of broodstock collection: early season (March/April) or late season (September/October). Individuals in the former season are more likely to show developed gonads and be in relative synchrony, as at the start of reproduction. A higher

degree of variability is expected in late season, as well as the presence of individuals with post-release gonads. Therefore, a shorter conditioning time is most likely necessary for early season stock, while post-season stock might provide a more accurate assessment of conditioning time from the point of gonad degeneration. In fact, studies of the *Capitulum mitella* showed that conditioning success can vary markedly between individuals collected in our out of the reproductive season (Leung, 2002). In the present study, broodstock were collected in the early season in April 2013 and, in spite of about 5 % of the individuals brooding eggs, no individuals were observed carrying mature egg lamellae, as 80 % of egg lamellae found were undifferentiated. Although this might not allow exact estimation of the conditioning time required for broodstock to develop from the post-reproduction state, it provides an initial basis for conditioning in this species.

Rearing temperature regime was observed to have significant effects on broodstock conditioning, affecting fecundity index, lamella development index and daily release patterns, although the lack of differences in total number of released nauplii, nauplius size and nauplius 24-h survival.

2.4.1. SGR, survival rates and RC/SL proportion

Broodstock SGR-RC showed no differences between treatments, varying between 0.73 and 0.93 % RC $month^{-1}$, within what has also been observed in the wild (e.g. 0.17 – 0.66 mm RC $month^{-1}$; Cruz, 2000). Moreover, by considering a growth period of only one month, it is difficult to accurately evaluate changes, due to high variability. Similarly, no differences in mortality rates were found, with a daily mortality rate of 0.34 % d^{-1} with a total of 6.44 % in 28 days. Mortality in the wild has been reported at 4.0 to 4.6 % $month^{-1}$ (Cruz, 2000) and 4 % $month^{-1}$ in individuals grown in offshore culture systems (Goldberg, 1984). In captive broodstock, mortality occurs mostly during the acclimatization period, as individuals can often be injured during collection, perishing within a few hours, with mortality stabilizing afterwards to negligible levels. From previous experience, *P. pollicipes* adults and juveniles take between 1 to 2 weeks to acclimatize to feeding in captivity.

Unlike SGR and mortality rates, RC/SL proportion differed between treatments, with *sp-suT2* showing higher RC/SL than *spT*, which was closer to the initial broodstock value. This measure is used as an indication of the quality of the growth of individuals rather than expressing this in terms of width or length that may not always be proportional, particularly if individuals are limited in their growth by environmental constraints. This has been observed in our facilities, where individuals subjected to

increased competition for food often showed elongated stalks in proportion to capitulum width. This phenotype has also been observed in the wild (Cruz, 2000). These individuals, with correspondingly lower RC/SL index, presented generally a more aqueous stalk. The higher RC/SL ratio for *sp-suT2* might indicate that, despite no differences in SGR, conditions in this treatment were the most suitable as individuals grew mostly by plate growth (RC), instead of relying solely on stalk extension (SL) for competitive advantage, while not showing any proportional capitular growth. Nevertheless, the fact that RC/SL did not decrease in any of the treatments suggests a lack of competitive pressure and limiting conditions for development.

2.4.2. Fecundity index and lamella development

Temperature, as well as food availability, is known to affect the reproductive cycles in many barnacle species (Patel & Crisp, 1960ab; Hines, 1978; Cimberg, 1981; Page, 1983; Cruz & Hawkins, 1998), such as *P. pollicipes*, *P. polymerus*, *Chthamalus depressus*, *C. mitella*, *C. fissus*, among others. This effect of temperature over reproductive regulation is due to effects on metabolic and physiological regulation, and can affect gonadal maturation and development rate, mating, brooding and larval release.

The three treatments were selected in order to allow a comparison between temperature regimes, from off-season temperature (*spT*), to reproductive season temperatures, with daily fluctuations (*sp-suT2*) or without (*sp-suT*). For conditioning purposes, several temperature cycles have been used for barnacles, from a broad range of stable temperature (e.g. Leung, 2002), to stable but higher (e.g. Patel, 1959; Patel & Crisp, 1960ab) or lower temperatures (e.g. Crisp, 1957) for species inhabiting temperate or cold waters. Further results from bivalves show that regimes of increasing temperature can induce breeding (e.g. Loosanoff & Davies, 1952; Chavez-Villalba et al., 2002). Notwithstanding varying results across species, breeding tends to be maximal within the natural range, with the use of higher temperatures often leading to improved reproductive performance in temperate species. Reproductive cycles are often characterized by brooding frequency and the pattern of gonad development (e.g. Cardoso & Yule, 1992; Cruz & Araújo, 1999). Although gonadal development allows a more accurate estimation of reproductive development, its application is limited in terms of day-to-day assessment of reproductive state under culture conditions. Furthermore, though mature individuals and brooding often occur contemporarily (e.g. Leung, 2002), maturity does not imply successful mating and brooding, and therefore

the presence of egg lamellae or spawned larvae are important tools for assessment of reproduction.

Fecundity index increased in all treatments, from 7 to 22 % in 4 weeks, compared to the initial broodstock fecundity. The best results were for *sp-suT* where 27 % fecundity was recorded. Successful conditioning studies with other barnacles have shown 20 – 100 % fecundity, achieved in 2 to 6 weeks, depending on species and temperature (e.g. Patel & Crisp, 1960). *Chthamalus stellatus* (found often within the range of *P. pollicipes*; Sousa et al., 2000) showed 20 to 60 % fecundity from 20 to 30 °C (maximum at 20 °C of 60 %), in spite of also being reproductive between 15 and 31 °C (10 – 12 % fecundity; Patel & Crisp, 1960). Conditioning studies with *C. mitella* (e.g. Leung, 2002) could not achieve fecundity above 5 % for in-season individuals (or 0 % for off-season individuals) even after 6 weeks of conditioning and independently of the temperatures tested (17, 22 and 28 °C). In the present case, not only did the fecundity increase significantly, but it reached values comparable to wild populations of *P. pollicipes* within 28 days of rearing. Cruz (2000) reported fecundity indices in the wild of 0 – 80 %, varying with the time of year. Higher values were recorded from May to September (18 – 80 %). Molares (1994) referred to fecundity below 10 % off-season, to 10 – 15 % between April to June, peaking from August to October to 20 – 50 %. These results were substantiated by Pavón (2003) and Cardoso and Yule (1995), with high inter-annual and site-specific variation. Other relevant factors, such as mean *P. pollicipes* population size, site and tidal level might heavily impact fecundity index (e.g. Cruz & Araújo, 1999; Cruz, 2000). Results from *C. mitella* (Leung, 2002) showed fecundity indices on the Hong Kong coast between 30 to 33 %, though in Japan and China recorded values were as high as 70 to 80 %. Cruz (2000) registered that fecundity was higher in large individuals (RC > 15 mm; 40 – 80 %) than smaller barnacles (12 < RC ≤ 15 mm; 15 – 40 %). Site and tidal variations were significant and while some site/tidal combinations showed fecundity for large animals of 60 – 100 % (e.g. Cabo de Sines, mid-tidal), others (e.g. Cabo de Sines / subtidal) were as low as 5 % during breeding season (Cruz & Araújo, 1999). Hilgard (1960) noted that for *P. polymerus* the number of fecund individuals at the height of the breeding season was never above 50 – 60 %.

Fecundity in barnacles has been further correlated to age, population density and food supply (Barnes & Barnes, 1986; Burrows et al., 1992; O'Riordan et al., 1992; Cruz & Araújo, 1999). Cruz (2000) showed that the minimal size for maturity is 10 mm RC for males and 12.5 mm RC for females, although the majority of mature individuals were

found to be over 15 mm RC, which is in accordance to what was observed here. Furthermore, despite egg-carrying individuals in the present treatments being on average of similar size to the initial broodstock, the fecund individuals of the conditioned broodstock were significantly smaller than the fecund individuals in the initial broodstock. This might indicate that in the wild there is a higher propensity for larger individuals to reproduce early on in the season or that smaller individuals might be in limiting feeding conditions at the same time. Interestingly, Cruz (2000) and Cruz & Araújo (1999) also noted that, in the wild, smaller individuals seem not to start and finish breeding as early or as late as larger individuals.

Lamella development index increased significantly in *sp-suT* and *sp-suT2*. The egg lamellae transitioned from average stage 0 to stage 3, while for *spT* this increase was less marked, from stage 0 to stage 1, in 4 weeks. Furthermore, the initial 0 % mature lamellae found, increased to between 23 and 36 % in *sp-suT* and *sp-suT2*, respectively, though in *spT* mature lamellae remained at 0 %. This, together with fecundity results, indicate that within 4 weeks lamellae can not only develop from fully immature to ready to hatch, but also non-fecund individuals can mate and develop lamellae in that period if conditions are favourable. It is interesting to note that although *spT* had an increase in fecundity, it did not have an increase in lamella maturity, which suggests that not only brooding time might be significantly reduced in the treatments of higher temperatures, but that there might be a limiting temperature that triggers gonadal development. At the end of the reproductive season in the wild, the female gonad takes longer to regenerate (Molares et al., 1994a). This coincides with an abrupt decrease in upwelling, but no major temperature change (after maxima from July to September; Fiuza et al., 1982), supporting the importance of food for regeneration and temperature for triggering reproduction. Patel & Crisp (1960) further confirmed in several barnacle species that starved individuals were unable to reproduce. In a situation such as that in the present study, where food is non-limiting, temperature is most likely the triggering factor for reproduction and, specifically, lamella development. Lending support to this theory, one of the treatments in the present study used stable spring temperatures (*spT*) and showed the smallest increase in fecundity, although still above that of the initial broodstock. This result indicates that even when temperature does not change reproductive development still occurs, but at a slower rate. Nevertheless, the number of nauplii released throughout the experimental period suggests that some lamellae did mature, supporting such a larval production. However, the lack of mature lamella by the

end would signify a most likely unpredictable and decaying subsequent spawning period. This echoes the observations of Kugele & Yule (1996) and Candeias (2005), where continuous larval release was observed, but was unreliable. It is likely, therefore, that stock kept under suitable conditions will always produce nauplii, but reliability on a continuously high productivity might not be guaranteed if conditions are not ideal.

Broodstock reared at *sp-suT* showed a significantly higher fecundity index when compared to the initial broodstock and, though *sp-suT2* and *spT* also showed increased fecundity, this was not significantly higher than the control. Fecundity increased by 6 % in these treatments (*sp-suT2* and *spT*) and about 21 % in the previous treatment (*sp-suT*), in the space of 28 days. However, when lamella development stage at day 28 is considered, *sp-suT* and *sp-suT2* showed a significantly higher number ready to hatch; between 32 and 38 % compared to *spT*. When total release rates are compared between treatments, *sp-suT2* released in total 14 % more nauplii then *spT* and 23 % more than *sp-suT*, which produced the lowest number of nauplii. Under these conditions, the treatment with highest fecundity was *sp-suT*, while the treatment with highest total release rate was *sp-suT2*. This raises the question, as to whether it is better to rear apparently highly fecund individuals that release fewer larvae, or less fecund individuals that release more larvae. These observations may be explained by the fact that the sampling frequency for assessment of fecundity occurred only at day 0 and day 28, while releases of larvae occurred throughout and were monitored daily. Therefore, while dRR provides a pattern in time, FI and LDS provide a snapshot of reproductive fitness pre- and post-conditioning. One could argue either that the higher fecundity of *sp-suT* could indicate a higher propensity for nauplius release, or that the lowest fecundity of *sp-suT2* could be due to a previously high release rate. Therefore, the percentage of mature lamellae found at day 28 could also be dependent on the previous release events, and a period of intense release could lead to lower percentages of mature lamellae, especially considering that egg incubation is thought to take between 15 to 24 days (Molares et al., 1994a; Cruz, 2000). If nauplii had been released within that conditioning period it is unlikely that new lamella development would have had time to occur. However, this could only be confirmed by sampling the treatments weekly throughout the experimental period, by dissecting a significant number of adults that would deplete the experimental population beyond use.

2.4.3. Nauplii release rates

Similar daily release rates were recorded across treatments, averaging approximately 4500 nauplii day^{-1}, although peak values were frequently above 10000 nauplii day^{-1}. These values might seem low, especially when compared to the number of embryos produced per barnacle (25429 ±1024 on average; 16229 ± 1094 for smaller individuals [14 ≤ RC ≤ 16 mm RC] and 34172 ± 1807 for large individuals [18.5 ≥ RC ≥ 21 mm RC]) (Cruz & Araújo, 1999; Cruz, 2000). Results on the related *C. mitella* indicate similar brood sizes, varying from 12600 to 87000 embryos (Leung, 2002). In the present study, lamellae were found to contain around 15547 ± 2589 embryos, although when lamellae were left to hatch over a 24-h period, hatching rate was about 15 % increasing to 25 % by 48 h. Embryos at the periphery of the egg lamella were often observed to be several development stages ahead of the central embryos, and hatched between 2 – 10 days earlier than the central embryos. This is probably due to differences in oxygenation between central and peripheral embryos (Crisp, 1959). Although these observations relate to isolated egg lamellae hatched in vitro, lamellae brooded inside the mantle cavity of the adults might be subject to similar conditions with partial hatching occurring over time. Therefore, it is hypothesized that adults carrying mature egg lamellae might release their newly hatched nauplii over a period of days, instead of in a single event. This would explain the low daily release rate associated with comparatively high fecundity index and high percentage of ready to hatch lamellae in some treatments.

Given the experimental design, it was not possible to estimate the number of adults releasing larvae. This would have provided valuable insight into the proportion between mature adults and actively releasing individuals, and consequently the number of adults needed to assure a suitable volume of production and maintain a diverse larval genetic background. This could be confirmed by studies following single individuals and not cohorts which, despite being more applicable to aquaculture, have inherent limitations. It should also be considered that the adults might have consumed a percentage of nauplii before these were collected by the filters. *Pollicipes* spp. are known to be cannibalistic and, in spite of being selective in terms of prey (e.g. *P. polymerus,* Lewis, 1981), the conspecific nauplii are within the size range of *P. pollicipes* consumption (see Norton, 1996). Loss of nauplii is also possible given the daily tidal cycle system in place. Nevertheless, such a continuous system allows for the identification of release peaks, as peak days are normally preceded and followed by days of above average

release, allowing for the adjustment of collection strategies during these periods. This might be as simple as moving the stock to more controlled systems, where nauplii can be frequently collected then and transferred back to culture. Considering that these peaks were above 10000 nauplii collected per tank, the use of multiple aquaria, as in the current experiment, could lead to the collection of considerable number of nauplii, especially in heavy release events (e.g. *sp-suT2* at day 26, on average ≥ 25000 nauplii).

Temperatures increased progressively in two treatments, oscillating (*sp-suT2*) or not (*sp-suT*), while remaining stable in the other (*spT*). As a consequence, at any one time, all treatments could be subjected to a different instantaneous temperature (though identical on average for *sp-suT* and *sp-suT2*) and to a previously distinct temperature pattern. In the case of *spT,* dRR increased progressively until peaking at day 23 and then slowly decreased until the end of the experiment. As temperatures remained stable, this increase could be due to the natural maturation of lamellae, following the natural timing and wild cycle. As the individuals were collected in late April 2013, by the time the experiment was terminated it was early June 2013. Judging by previous years, the reproductive season in the wild could have begun already. For *sp-suT*, release rates seemed to increase and peak at day 11 (19.6 °C), followed by a decrease until almost day 28 (23.0 °C) when they increased again. On the other hand, for *sp-suT2*, the same behaviour was observed, but peaks occurred at days 14 and 27, where temperatures averaged 20.1 °C and 22.6 °C. In both treatments, peak releases started as they approached 20 °C, indicating that this temperature might trigger reproduction. These patterns differed between groups in terms of peak frequency, between stable and increasing temperatures, but also the timing and intensity of the peaks in treatments kept at increasing temperature.

It should be noted that *spT* took considerably longer to reach peak larval release than the other treatments and only had one peak. This may be due to an effect of lower temperature on gonadal and embryonic development, or a decrease in reproductive activity. Leung (2002) suggested for *C. mitella* that the effect of temperature on brooding might be related to the change in length of the embryonic brooding time. This might also be the case for *P. pollicipes* and would further explain the observation of only one breeding peak. Field results show that *P. pollicipes* is a multiple brooder, (Molares et al., 1994b; Cardoso & Yule, 1995; Cruz & Araújo, 1999), spawning apparently asynchronously in spite of two broad breeding peaks at the beginning and the end of the reproductive season (Molares et al., 1994b). The two peaks observed in

the increasing temperatures might also be supported by the effect of higher temperature and non-limiting food; conditions under which gonad recovery is likely to be faster. The hypothesis that individuals had two broods cannot be excluded, though unlikely. The timing between releases could also be due to a decrease in fecundity as temperature rose above 20 °C, as seen by Patel & Crisp (1960) in *C. stelattus*.

The second noteworthy pattern was observed between the two treatments of increasing temperatures, which peak at similar times and at similar intensities. However, *sp-suT2* shows a higher average dRR and less time between dRR peaks compared to *sp-suT*. The similarities in timing are compatible with the similar response temperatures and are therefore not surprising. However, the effects of temperature are still poorly understood, as in different species the relative importance of temperature has been suggested to be related to temperature intensity, critical values or even the effect on development rates (Muranaka & Lannan, 1984; Cardoso & Yule, 1995; Spirley et al., 2000). The reduced time between dRR peaks in *sp-suT2* might suggest that daily changes of temperature, as would be experienced in the wild, induce faster development. Temperature cycles are reproduction regulators, being advantageous for allowing the synchronization of reproduction between individuals, particularly essential when female and male development does not occur contemporaneously. In general, for *P. pollicipes*, it is accepted that the increase in temperature triggers reproduction, and this is supported by current results, although in the wild this occurs in synchrony with the increase in food availability. For *Semibalanus balanoides*, the final gonadal maturation is triggered by both decreased temperature and photoperiod (Barnes, 1963, 1989; Crisp, 1986), while for *C. stellatus*, *Elminius modestus*, *B. perforatus* and *B. amphitrite*, high temperature and food affect gonad development (Patel & Crisp, 1960). The case might be different however, for *P. pollicipes*, as the maturation of female and male gonads is distinct throughout the year. Cruz (2000) observed that during the breeding season, the percentage of mature female gonads ranged between 20 – 60 %, while most of the population had the seminal vesicles mature all year round. Molares (1994) suggested that the maturation of the female gonad would be what restricts reproduction to the period between March and September. In the absence of limiting conditions, it is likely that gonad recovery occurs at a faster pace, allowing for shorter brooding periods and therefore multiple release peaks. Under this scenario, temperature variations might effectively act as release triggers, leading to closer release peaks and to higher intensity spawning, as seen in *sp-suT2* in comparison to *sp-suT*. The effect of temperature

changes on larval and gamete release has been observed in bivalves (Helm & Bourne, 2004), and is often used as an artificial inducer of spawning in these species.

It is also important to compare the current results with the only study that has investigated the timing of larval release of *P. pollicipes* in the wild (Macho et al., 2005) and its relation with tidal and lunar cyles. The previous author reported peaks of larval abundance in synchrony with the waxing moon, and full moon (to a lesser extent), in spite of having a low larval representation of *P. pollicipes* during sampling. Though the animals in the current study were maintained without being subjected to the natural lunar cycle, it is possible that a synchrony might have still been kept with natural cycles. During the period of the current study, the corresponding lunar cycle progressed from a new moon at day 1 of the experiment to first quarter at day 9, followed by a full moon at day 16, last quarter moon at day 22 and new moon at day 30 (post-finishing of the study). Considering this, it is clear that the periods of higher release coincide with the waxing and waning moon (with the highest values across treatments), with lower periods during new and full moon. Nevertheless, the current study was only developed at early breeding season, covering the period of less than a month, and therefore care must be taken regarding the extrapolation of the relation between current results and lunar cycles. In spite of this, it cannot be excluded the possible influence of lunar cycles on larval release cycles, as reported by Macho et al. (2005), and further studies should further focus on confirming or refuting this hypothesis.

If the dates of the last release peak are evaluated considering the observed fecundity across treatments at the end of the conditioning period, it is interesting to note that although *spT* had an early peak (day 23), there were no mature egg lamellae by the end, while *sp-suT* and *sp-suT2* showed the last peak considerably later (days 26 and 28), but presented respectively 32 and 38 % of mature egg lamellae. However, although it could be argued that a recent release peak would lead to fewer mature lamellae, this does not seem to be the case when both increased temperature treatments are considered comparatively, though it is impossible to assess if these values have decreased post-peak due to the low frequency of lamellae sampling. In fact, although *sp-suT2* had more egg lamellae by day 28, the intensity of the last peak was significantly higher than *sp-suT*, which suggests an overall higher fecundity in that treatment.

A trend of increased larval release from week 1 to 4 was observed in all treatments, raising the question of whether the values recorded on the last week could still be increased by extending the conditioning period. Total release rates did not differ

significantly between treatments, although *sp-suT2* had the highest values, followed by *spT* and *sp-suT*. However, care must be taken when drawing conclusions, as total values would vary significantly according to when the experiment is stopped, occurring before or after a release peak. The lower values for the *sp-suT* treatment might in fact, reflect the impact of not continuing conditioning for a longer period, since values seemed to start peaking by day 28. Although total numbers of nauplii released provide a valuable insight into the productivity of each treatment; if these are not considerably different, the frequency and intensity of release events can provide a more useful comparative tool for aquaculture.

2.4.4. Nauplii size and 24h survival

Extracted and collected nauplii were further analysed for size and 24-h survival rate, with no differences found between treatments and between extracted and collected nauplii. These are promising results for culture-produced nauplii, since nauplius quality does not seem to be affected by source or adult rearing conditions. Nevertheless, it cannot be excluded that wild-raised and naturally-released larvae might be healthier in the long run. Further studies should evaluate effects, if any, on cyprid metamorphosis, survival and larval settlement. To our knowledge, no report of such effects has been observed in barnacle species, although the larval period of other species (e.g. *B. improvisus*) can be significantly extended by dietary deficiency, yet still results in ostensibly healthy larvae. Observations from experiments developed in our laboratory with *P. pollicipes*, show that between batches of extracted larvae, reared under identical conditions, survival and moulting success to the cyprid can vary markedly (Franco, pers. obs.).

2.5. Conclusions

Conditioning of *P. pollicipes* can be achieved in culture in less than a month, provided that individuals are previously acclimatized. Better results for conditioning were achieved for broodstock reared at increasing temperature, in terms of fecundity, percentage of mature lamellae and frequency of larval release peaks. It is suggested that increasing temperature might decrease development time and accelerate egg lamella maturation, as food availability was not limiting, allowing for multiple release peaks. Furthermore, broodstock kept with daily temperature variations showed slightly better results in terms of lamella development index, lamella maturation and time between release peaks. However, no significant differences were noted between the total number of larvae produced, suggesting that the conditioning effect mostly relates to timing and

concentration of release events, rather than total number of nauplii produced. Interestingly, the daily average of nauplii released was below that expected, given the number of embryos known to be produced by each adult. Therefore it is proposed that individuals might be releasing larvae of the same brood over a period of days, as the embryos hatch within the mantle cavity. Furthermore, analysis of nauplius size and 24h survival did not reveal differences between extracted and released larvae, validating both protocols. The results from the present study support the proposal that *P. pollicipes* conditioning can be a valuable tool for larval collection in captivity and production of larvae under culture conditions, not limited to the breeding season, and in significant numbers for scaling up cultures.

Chapter 3. Larval culture of stalked barnacles (*Pollicipes pollicipes*): Effects of temperature, food quality, photoperiod and salinity on larval growth and survival

Abstract

Pollicipes pollicipes (Crustacea: Pedunculata) is highly prized for food in Portugal and Spain and a species of significant interest to aquaculture. Culture conditions have, however, never been optimized. Efficient larval production is an essential step towards sustainable aquaculture production of stalked barnacles. The present study investigated the effects of temperature, food quality, photoperiod and salinity on the growth and survival of *P. pollicipes* larvae. Results showed that higher temperatures significantly increased specific growth rate, from 2.60 ± 0.08 d^{-1} at 11 ºC to 5.93 ± 0.35 d^{-1} at 24 ºC, decreasing development time from 24.83 ± 0.29 days at 11 ºC to 9.81 ± 0.25 days at 24 ºC. However, mid-range temperatures (15 – 20 ºC) maximized total survival to the cyprid (19.08 ± 2.83 to 31.11 ± 5.26 %, respectively). Higher temperatures reduced development time but this was accompanied by a significant increase in mortality, with temperatures above 22 ºC having mortalities over 90 %. With regard to food quality, monodiets and mixed diets of *Tetraselmis chuii*, *Isochrysis galbana* and *Skeletonema costatum* resulted in no significant differences in specific growth rate, which on average varied between 3.26 - 3.78 d^{-1}. Mixed diets of *T. chuii*, *T. chuii/S. costatum* and *S. costatum/I. galbana*, led to significantly higher total survival (on average 39.22 ± 0.23 % in 15 days), as well as a higher rate of metamorphosis to the cyprid for the latter two diets (90.33 ± 6.94 %). A monodiet of *I. galbana* led to the poorest survival of 3.03 ± 3.03 %. Photoperiod did not significantly affect survival, although short-day photoperiods (8/16 L/D) led to the highest survival, averaging 37.33 ± 4.42 %. Specific growth rate, on the other hand, was significantly higher at 24/0 L/D, followed by long- and short-day photoperiods, with the lowest values recorded for 0/24 L/D. With respect to salinity (20 – 40 psu range), no significant effects on growth and survival were recorded. Considering the results obtained, optimization of growth and survival could be accomplished using rearing temperatures of 15 – 20 ºC, daily feeding with *T. chuii/S. costatum or I. galbana/S. costatum* and a photoperiod of 24/0 L/D. Future studies should investigate the use of recirculating systems for larviculture as well as optimal rearing density and feeding protocol.

3.1. Introduction

Pollicipes pollicipes (Gmelin, 1970) is one of the most commercially important barnacle species for human consumption (Lopez et al., 2010). As concerns have grown over stock management, interest has risen over aquaculture of the species. The few published investigations into culture conditions of *P. pollicipes* (e.g. Goldberg, 1984; Coelho, 1990, 1991; Molares, 1994; Candeias, 2005; Cribeiro, 2007) have often identified larval production and settlement as the main bottlenecks to production. Hatchery production is essential to ensure a regulated supply of larvae throughout the year with controlled timing and yield, as well as to guarantee a high standard of larval quality. Furthermore, larval collection from the wild is economically unsustainable and besides providing an unreliable solution for aquaculture, presents conservation issues and conflicts with stock management programmes. Larval culture and settlement have long been investigated in various barnacle species (e.g. Knight-Hones & Stevenson, 1950; Knight-Jones, 1953; Keough & Downes, 1982; Brown & Roughgarden, 1985; Gabbott & Larman, 1987; Maki et al., 1988). Although protocols for species such as *Elminius modestus*, *Balanus improvisus* and *Balanus amphitrite* are well established (after Moyse, 1963; Tighe-Ford, 1970), protocols for others such as *P. pollicipes* are in their infancy.

In the case of *P. pollicipes*, hatchery production of larvae encompasses two key phases: larval development to the cyprid, as the nauplius moults successively through six stages, and cyprid settlement, when the cyprid locates a suitable surface on which to settle. For *P. pollicipes*, larval development has been described (e.g Coelho, 1990; Molares et al., 1994b) and nauplii have been cultured to the cyprid stage by various authors (e.g. Coelho, 1990; Molares, 1994; Molares et al., 1994a; Kugele & Yule, 1996). Nevertheless, knowledge of the effects of environmental factors and feeding on larval growth and survival remains poor and these essential steps are rarely elaborated upon in the literature (e.g. Coelho, 1990; Candeias, 2005). Similarly, studies on the remaining Pollicipipedidae are scarce and limited to works with *Capitulum mitella* (e.g. Lin et al., 1994; Qiu et al., 1994a, 1994b; Zhang et al., 2009; Lin et al., 2002; Rao et al., 2010) and *P. polymerus* (e.g. Lewis, 1975b). In the absence of an integrated investigation of optimal larviculture for *P. pollicipes*, the preliminary works of several authors on larval production systems (Molares, 1994), rearing diets (Coelho, 1990, Candeias, 2005) and the effects of temperature and culture density (Coelho, 1990) have provided a basis from which to build.

One of the major factors known to affect larval culture performance is temperature. Studies on *P. polymerus* have suggested that higher temperatures can lead to increased growth rate, despite decreasing fitness and survival (Lewis, 1975). From the few studies on *P. pollicipes* development in the laboratory (Coelho, 1990; Molares, 1994; Molares et al., 1994a; Kugele & Yule, 1996; Candeias, 2005), only Coelho (1990) specifically investigated larval growth at different temperatures. Coelho measured larval growth at 15 °C and 22 °C, concluding that although development time to the stage VI nauplius decreased from 20 days to 9 days, mortality was significantly higher at elevated temperatures, supporting the observations of Lewis (1975). However, mortality rate was not presented clearly by Coelho (1990). The question therefore remains, under the conditions of hatchery production, would this decrease in development time compensate for the reported increase in mortality? And at what temperature would this ratio be optimal? Furthermore, both Rao et al. (2010) and Zhang et al. (2009) reported the same decrease in development time with increasing temperature (24 – 31 ºC) for *C. mitella*, with Rao et al. recording survival above 90 % for mid-range temperatures (27 ºC). These remarkable results for larval survival support the suggestion that protocols in place for *P. pollicipes* could benefit from significant optimization. Despite the lack of detailed mortality data in most studies that describe larval rearing of *P. pollicipes*, several authors (Molares, 1994; Candeias, 2005) have reported massive mortality (≥ 85 %) during the first two weeks of rearing.

Feed quality and quantity are also of importance and can directly influence fitness and growth, with inadequate feeding causing mechanical interference with the swimming, the accumulation of lethal metabolites, deficient development and precocious death (e.g. Lewis 1975b; Moyse, 1963; Franco, pers. obs.). The only studies to investigate larval food quality for *Pollicipes* sp. were conducted by Lewis (1975b) for *P. polymerus*, and by Coelho (1990) and Candeias (2005) for *P. pollicipes*. Candeias (2005) performed the first systematic comparison of how ingestion rates varied with larval size and algal species provided as food, although the focus was mainly on food acceptability rather than growth and survival. Candeias (ibid.) concluded that there was a widespread preference for ingesting *Isochrysis galbana* and *Skeletonema costatum* and, to a lesser extent, *Tetraselmis suecica*. In terms of carbon content, however, *T. suecica* was superior to the former species. Additionally, mixed diets and flagellates (e.g. *Rhinomonas reticulata*) were responsible for a higher survival than monodiets of diatoms (e.g. *S. costatum*). Coelho (1990), on the other hand, concluded that from

monodiets of *S. costatum*, *T. suecica*, *Thalassiosira pseudonana*, *I. galbana*, *Chaetoceros gracilis* and *Chlorella* sp., only *T. suecica* and *I. galbana* assured larval development to the last naupliar stage, with the remaining monodiets being insufficient to sustain adequate development beyond nauplius stages II and III. However, no mention was made of total survival and growth rates, simply the time of appearance of each stage, which provides limited information about culture performance.

Other potentially important factors such as larval density, photoperiod, light intensity, salinity and water quality also remain poorly investigated. In fact, the results of the few preliminary studies conducted provide limited basis to culture, having focused only on density (Coelho, 1990) and response to light (Molares, 1994). Concerning salinity, no studies have so far specifically investigated the optimal range for culture, and most studies mention only the use of natural salinities (Coelho, 1990; Molares, 1994; Molares et al., 1994). This is similar to the usual choice of photoperiod, with natural photoperiods prevailing (e.g. Coelho, 1990) or full day photoperiod (e.g. Molares 1994; Molares et al., 1994a), due to the lack of supporting evidence for any other photoperiod being preferable.

This chapter aims to address these gaps, establishing which culture conditions would be most suitable for the larval rearing of *P. pollicipes* and overcoming the need for empirical guidelines. It is hypothesized that there is a range of environmental and dietary conditions that optimize culture performance. Larvae of *P. pollicipes* were reared at different temperatures (11, 15, 20, 22, 24 ºC), diets (monodiets and mixed diets of *I. galbana*, *T. chuii* and *S. costatum*), photoperiod (0/24, 8/16, 16/8 and 24/0 L/D) and salinity (20, 30 and 40 psu) and their survival and growth rates were followed to the cyprid stage.

3.2. Materials and methods

3.2.1. Broodstock collection, egg lamellae extraction and culture conditions

Clusters of barnacles were collected from the SW coast of Portugal (Cabo Sardão, Portugal, 37°36'24.70", -8°49'2.00") and transported to the rearing facilities at the School of Marine Science of Technology (Newcastle University, UK), where they were dissected ($n \geq 60$ ind) for the extraction of egg lamellae. Lamellae that were ready to hatch were separated (class 4, as according to Table 2.1, see previous chapter; adapted from Cruz, 1993; Molares, 1994) and cut into pieces to assist naupliar release. Healthy

nauplii were separated from dead nauplii, unhatched embryos and lamella membranes (which were discarded) by phototatic behaviour and placed into culture.

The newly hatched nauplii were sampled and distributed into experimental groups, with three replicates per treatment. Experiments were run under a standard protocol adjusted accordingly for the environmental factor under investigation. Cultures were done in 500 ml conical aquaria (JBL Artemio ®), with weak bottom aeration of 0.22 µm filtered natural seawater (NSW). Standard conditions included temperature of 20 ± 1 ºC (except in Exp. 1), photoperiod of 16:8 L/D (except in Exp.3), salinity of 33 ± 1 psu (except in Exp. 4), antibiotics (0.0232 g l^{-1} Penicillin and 0.0369 g l^{-1} Streptomycin) and an initial larval density of 5 larvae ml^{-1}. Feeding was done every 2 days with a mixed diet of *T. chuii* and *I. galbana* (100000 cells mL^{-1} 1:1) (except in Exp. 2), after the changes in seawater.

3.2.2. Experiments

3.2.2.1. TEMPERATURE

Experiment 1 tested the effect of temperature (11, 15, 20, 22, 24 ºC) on larval growth and survival. This experiment was divided in two parts due to limited larval numbers. The first part tested temperatures of 11 ± 1 °C, 15 ± 1 °C and 20 ± 1 °C, while the second part tested temperatures of 20 ± 1 °C, 22 ± 1 °C and 24 ± 1 °C. Each treatment was kept in a separate controlled-temperature incubator (LabHeat© and LabCold© RLCH0400 Incubator Units). Cultures were sampled daily until over 50 % of individuals reached the cyprid stage, at which point the treatment was terminated.

3.2.2.2. DIET

Experiment 2 tested the effect of food quality, by using a multifactorial design to test different monodiets and mixed diets (*T. chuii*, *I. galbana* and *S. costatum*). Diets were as follows: (a) *Isochrysis galbana*, (b) *Tetraselmis chuii*, (c) *Skeletonema costatum*, (d) *I. galbana* and *S. costatum*, (e) *I. galbana* and *T. chuii*, (f) *T. chuii* and *S. costatum*. Cultures were provided with 100000 cells ml^{-1} in a 1:1 ratio for mixed diets (50000 cells ml^{-1} of each food item). Algae were cultured in 10-L carboys in autoclaved natural seawater (NSW), 20 ± 1 °C, 37 ± 1 psu, 16/8 L/D photoperiod, 2022 ± 58 lux (RS-01 Light Meter©), grown with F/2 medium (Guillard & Ryther, 1962, Guillard, 1975).

3.2.2.3. PHOTOPERIOD

Experiment 3 tested the effect of photoperiod on larval growth and survival. The cultures were reared under the following photoperiods: (a) 24:0 L/D, (b) 16:8 L/D, (c)

8:16 L/D and (d) 0:24 L/D. Treatments were kept in a temperature and photoperiod-controlled incubator (LabHeat© RLCH0400 Incubator Unit), with light intensities of 2245 ± 256 lux (lamps Osram Fluora, L 36W/77).

3.2.2.4. SALINITY

Experiment 4 evaluated the effects of salinity (20, 30 and 40 psu) on larval growth and survival. Salinity was checked daily (Hand Held Refractometer B+S©) and adjusted accordingly, to account for variations caused by feeding and water evaporation.

3.2.3. Data collection and analysis

Cultures were sampled daily (n ≥ 30 nauplii per sample), pre-feeding, and the larvae were fixed with Lugol's iodine solution until observation. Samples were analyzed using light microscopy and counted for total number of larvae. Each larva was classified according to developmental stage (following Molares, 1994). Further samples were also taken when relevant for estimation of larval quality, according to Table 3.1. Samples were then photographed (Olympus© E-410) and larvae were measured for size estimations, using the software Image J©. Size measurements were also made for total length (TL) and total width (TW) and, for cyprids, carapace length (CL) and carapace width (CW) (see Cruz, 2000). TW and CL were used for estimations of growth in nauplii and cyprids respectively, due to the decreased variability when compared to TL and CW. Larval specific growth rate to nauplii VI (SGR, %; Eq.1) was estimated from the data collected, as were average cyprid length (CL, μm; Eq.2), percentage of high-quality larvae (%HQL, %; Eq.3) and cyprids (%C, %; Eq.4), stage progression index (SPI, #; Eq.5), median development time to cyprid from nauplii I (MDT, d; Eq. 6), total survival (TS, %; Eq.7) and measured daily survival rate (S, % d^{-1}). Each culture was followed until cyprids became dominant (≥ 50 %), at which time it was filtered, with final values reflecting directly population data and not samples.

Class	Criteria for classification of larval quality			
	Lipidic drops	Activity	Appendages	Fouling
L-Q larva	Absent	Static	Protruded	Fouled
H-Q larva	Present	Motile	Healthy	Unfouled

Table 3.1. Criteria for the classification of high-quality *P. pollicipes* larvae. Larvae were observed and compared for presence of lipid drops, motility/activity level, position of appendages and fouling of the larvae. The larvae were classified as high-quality larvae (H-Q larvae) if they satisfied 3 out of 4 of the assigned criteria for high-quality larvae, or of low-quality (L-Q larvae) if they scored 2 or more criteria for low quality.

Eq.1 $$SGR_N = \frac{lnTW_{t_f} - lnTW_{t_i}}{t_f - t_i} \times 100$$

Eq.2 $$CL = \frac{\sum_{i=1}^{n}(CL_i)}{n}$$

Eq.3 $$\%HQL = \frac{nHQL}{n} \times 100$$

Eq.4 $$\%C = \frac{nC}{n} \times 100$$

Eq.5 $$SPI = \frac{\sum_{i=1}^{n}(n_i \times s_i)}{n}$$

Eq.6 $$MDT = t_f - t_i$$

Eq.7 $$TS = \frac{n_{t_f}}{n_{t_i}} \times 100$$

Equations 1 to 7, where *SGR* is the specific growth rate of nauplii from nauplii I to VI (SGR_N), *TW* is nauplii total width (µm), *CL* cyprid carapace length (µm), CL_i cyprid carapace length of individual *i* (with *i*=1,…,30; µm), t_f final time (d), t_i initial time (d), %*HQL* percentage of high quality larvae (%), *nHQL* number of high quality larvae (#), *n* total number of larvae (#), %*C* percentage of cyprids (%), *nC* number of cyprids (#), *SPI* stage progression index, n_i number of individuals at development stage *i* (with *i*=1,2,3,4,5 or 6), s_i development stage *i* (with *i*=1, 2, 3, 4, 5, 6 and 7, respectively for nauplii I, II, III, IV, V, VI and cyprids), *MDT* median development time to cyprid, *TS* total survival (%), nt_i initial number of larvae (#), nt_f final number of larvae (#).

Results were analyzed using Statistica ® at a significance level of 0.05. Analyses were carried out to determine homogeneity of variance (Levene's test) and normality (Kolmogorov-Smirnof test), while significant differences were detected using one-way ANOVA or ANCOVA with time as co-variate. Post-hoc investigation used Tukey's HSD test when relevant. Data in percentage format were arcsine transformed pre-analysis. All results are presented as mean ± SE, unless stated otherwise.

3.3. Results

3.3.1 Temperature

The effect of temperature was tested in two separate experiments. Specific growth rate (ANOVA, F=0.25; p=0.77) and total survival (ANOVA, F=0.45, p=0.61) data for the overlapping temperature (20 °C) did not differ between experiments, allowing for further comparisons.

Specific growth rate was significantly influenced by both low (ANOVA, F=57.50; p<0.01) and high (ANOVA, F= 48.47, p<0.01) temperatures. Higher temperatures led to significantly higher growth rates, varying from 5.93 ± 0.35 d^{-1} at 24 ºC to 2.60 ± 0.08 d^{-1} at 11 ºC (Table 3.2.). Differences were significant between all temperatures (Tukey

Test, p<0.01), with the exception of both values recorded at 20 ºC (Tukey Test, p=0.89). Consequently, median development time to the cyprid varied significantly (ANOVA, F=86.03, p <0.01) from 24.83 ± 0.29 days at 11 °C to 9.81 ± 0.25 days at 24 °C.

Experiment	T (ºC)	SGR (d^{-1})	Survival (%)	MDT (d)	CL (µm)
Low temp.	11	2.60 ± 0.08 [a]	10.81 ± 5.26 [a]	24.83 ± 0.29 [a]	200.00 ± 7.96 [a]
	15	2.97 ± 0.10 [b]	19.08 ± 2.83 [b]	17.67 ± 0.58 [b]	209.65 ± 3.52 [a]
	20	4.57 ± 0.15 [c]	22.87 ± 3.86 [c]	15.23 ± 0.68 [c]	211.78 ± 2.34 [a]
High temp.	20	4.50 ± 0.14 [c]	31.11 ± 5.26 [c]	16.06 ± 0.39 [c]	205.12 ± 4.52 [a]
	22	5.31 ± 0.47 [d]	8.69 ± 5.38 [d]	13.45 ± 0.51 [d]	208.24 ± 3.83 [a]
	24	5.93 ± 0.35 [e]	7.67 ± 2.2.9 [e]	9.81 ± 0.25 [e]	203.03 ± 2.54 [a]

Table 3.2. Specific growth rate (SGR, #), total survival to cyprid (Survival, %), median development time to cyprids (MDT, days) and cyprid width (CW, µm), for larvae grown at low temperatures (11, 15, 20 °C) and high temperatures (20, 22 and 24 °C). Temperature significantly affected specific growth rate (ANOVA, F=57.50; p<0.01), survival rates (ANOVA, F=1.59; p=0.03) and median development time to cyprids (ANOVA, F=86.03; p<0.01), but not cyprid length (ANOVA, F=0.27; p=0.79).

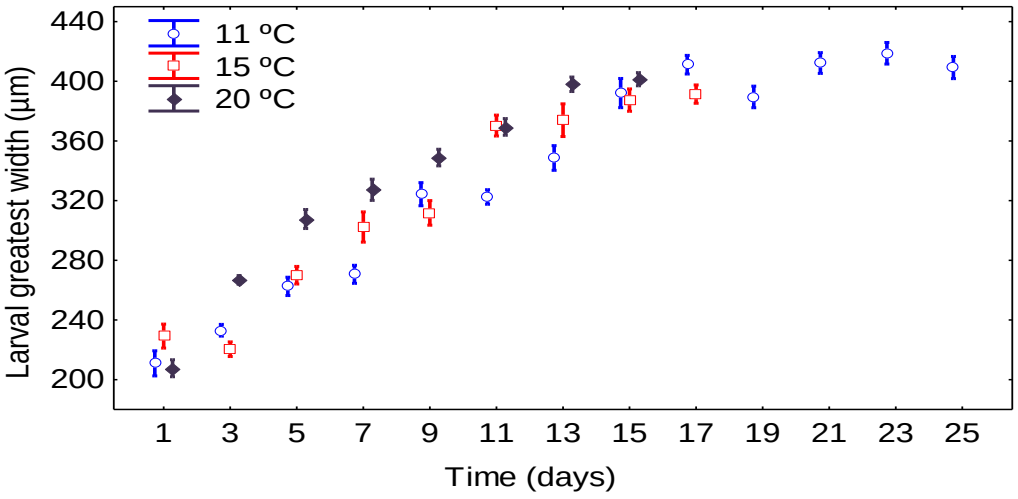
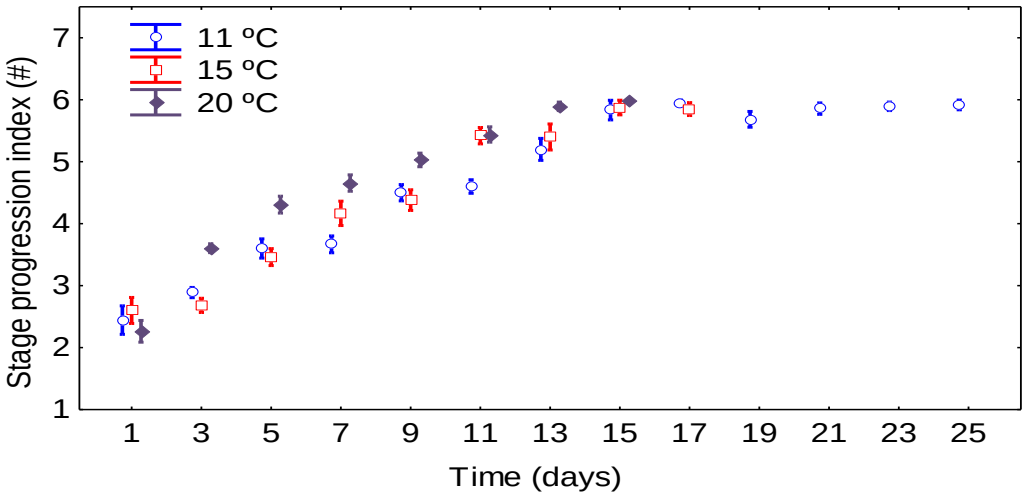
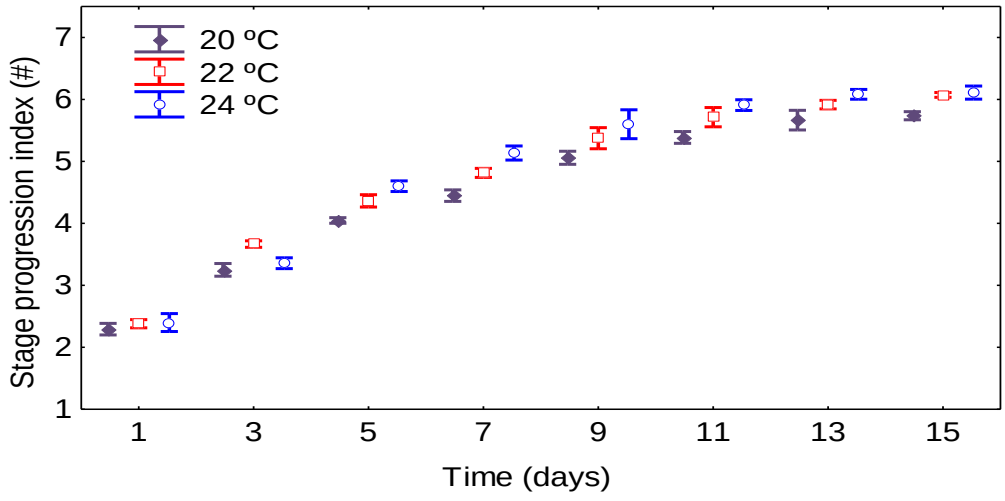


Fig. 3.1 Average larval greatest width (µm) according to culture time (days), for larvae grown at a) low temperatures (11, 15, 20 °C; Experiment 1.1) and b) high temperatures (20, 22 and 24 °C; Experiment 1.2).

In Fig. 3.1 the increase in larval size with time is clear, despite different growth rates among temperatures, to a stabilization point that coincides with cyprid appearance. After there were >50% cyprids in the culture, average size decreased slightly due to the smaller size of cyprids compared to nauplius VI. No significant differences were detected in cyprid size data for cultures reared at different temperatures (low temperatures: ANOVA, F=0.31, p=0.73; high temperatures; ANOVA, F=0.25; p=0.81) and averaged 209.03 ± 1.92 µm of CL. Larval stage progression gives a clearer view of stage dominance (fig. 3.2), showing clearly when the cultures became cyprid-dominated. At this point, high mortality was observed if no appropriate settlement substrata were provided. The mixing of different stages in a single culture (data not shown) was higher in cultures grown at lower temperatures (e.g. 11 – 15 ºC).



a)



b)

Fig. 3.2 Larval stage (#) progression according to culture time (days), for larvae grown at a) low temperatures (11, 15, 20 °C; Experiment 1.1) and b) high temperatures (20, 22 and 24 °C; Experiment 1.2). Stages were assigned as follows 1) nauplii I, 2) nauplii II, 3) nauplii III, 4) nauplii IV, 5) nauplii V, 6) nauplii VI, 7) cyprids.

Larval survival decreased steadily with time at all temperatures (fig. 3.3). At low temperatures, daily survival rates did not vary significantly according to temperature (ANOVA, $F=2.01$, $p=0.14$), though total mortality was significantly different (ANOVA, $F=59.10$, $p<0.01$) due to differences in median development time. However, temperatures changes in the higher range (≥ 20 ºC) had a significant effect on survival rates (ANOVA, $F=5.43$, $p<0.01$) in addition to the expected effect on total mortality (ANOVA, $F=64.55$, $p<0.01$).

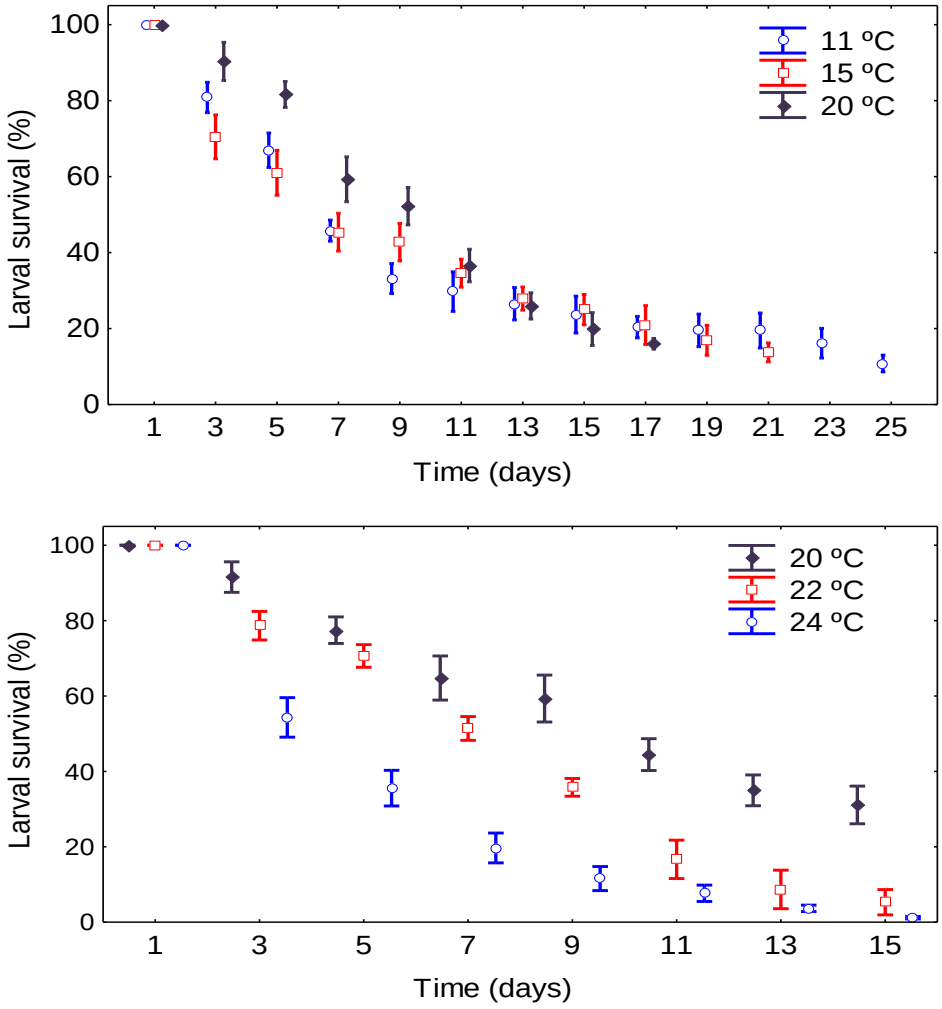


Fig. 3.3 Survival rate (%) according to culture time (days), for larvae grown at a) low temperatures (11, 15, 20 °C; Experiment 1.1) and b) high temperatures (20, 22 and 24 °C; Experiment 1.2).

Total survival increased significantly with increasing temperatures until optimal conditions were achieved, from 10.81 ± 5.26 % at 11 °C to 26.99 ± 4.56 % at 20 °C (average), decreasing subsequently. Temperatures of 22 and 24 °C led to mortalities above 90 % (Table 3.2).

3.3.2 Diet

Contrary to what was observed in differing temperature regimes, there were no significant differences in larval specific growth rate (ANOVA, F=1.87; p=0.17) according to food type. Similarly, cyprid length was the same irrespective of larval diet (ANOVA, F=179.36; p<0.01). Average growth rates varied between 3.17 and 3.78 d^{-1} (Table 3.4). The differences observed had only a minor influence on median development time, which varied between 14.99 and 16.05 days (Table 3.3).

Diet	SGR (d^{-1})	Survival (%)	MDT (d)	CL (µm)
T. chuii	3.78 ± 0.28 [a]	38.30 ± 6.65 [a]	14.99 ± 2.54 [a]	212.35 ± 3.62
I. galbana	3.17 ± 0.38 [a]	13.03 ± 3.03 [b]	n.a.	n.a.
S. costatum	3.40 ± 0.30 [a]	16.89 ± 2.26 [b]	15.30 ± 2.88 [a]	209.45 ± 2.99
T. chuii /I. galbana	3.39 ± 0.28 [a]	14.60 ± 3.93 [a]	15.32 ± 3.55v	210.98 ± 3.17
I. galbana /S. costatum	3.26 ± 0.24 [a]	38.72 ± 8.60 [a]	16.05 ± 3.18 [a]	207.15 ± 4.03
T. chuii /S. costatum	3.43 ± 0.17 [a]	40.82 ± 2.37 [b]	15.27 ± 2.54 [a]	202.86 ± 3.88

Table 3.3. Specific growth rate (SGR, #), total survival to cyprid (Survival, %), median development time to cyprids (MDT, days) and cyprid width (CW, µm), for larvae cultured using various diets, namely monodiets of *Tetraselmis chuii* (*T. chuii*), *Isochrysis galbana* (*I. galbana*), *Skeletonema constatum* (*S. costatum*), and mixed diets of *T. chuii* and *I. galbana* (*T. chuii/I. galbana*), *I. galbana* and *S. costatum* (*I. galbana*/ *S. costatum*) and *T. chuii* and *S. costatum* (*T. chuii*/ *S. costatum*). Diet significantly affected total survival to cyprid (ANOVA, F=9.93; p<0.01), but not specific growth rate (ANOVA, F=24.39; p<0.01), cyprid length (ANOVA, F=0.41; p=0.65) or median development time (ANOVA, F=0.23; p=0.88). In treatments where cyprids were not observed, median development time was classified as *n.a.* - not-applicable.

Total survival varied significantly with diet (ANOVA, F=9.93, p<0.01) (Table 3.4). Larvae grown on diets of *T. chuii* (Tukey, p≤0.01), *T. chuii/S. costatum* and *I. galbana/S. costatum* led to significantly higher total survival (the mixed diets did not differ significantly from each other; Tukey, p≥0.99). The lowest survival (Tukey, p≤0.01) was recorded with diets of *I. galbana,*followed by *T. chuii/ I.galbana* and *S. costatum*, being statistically similar (Table 3.4, Fig. 3.4). These differences were visible, in the case of *I. galbana*, from the first day of culture when immediate mortality was observed, as opposed to more progressive mortality in the other diets. Independently, none of the diets tested were toxic to the larvae and all were capable of sustaining development beyond nauplius stage II, although with different degrees of success.

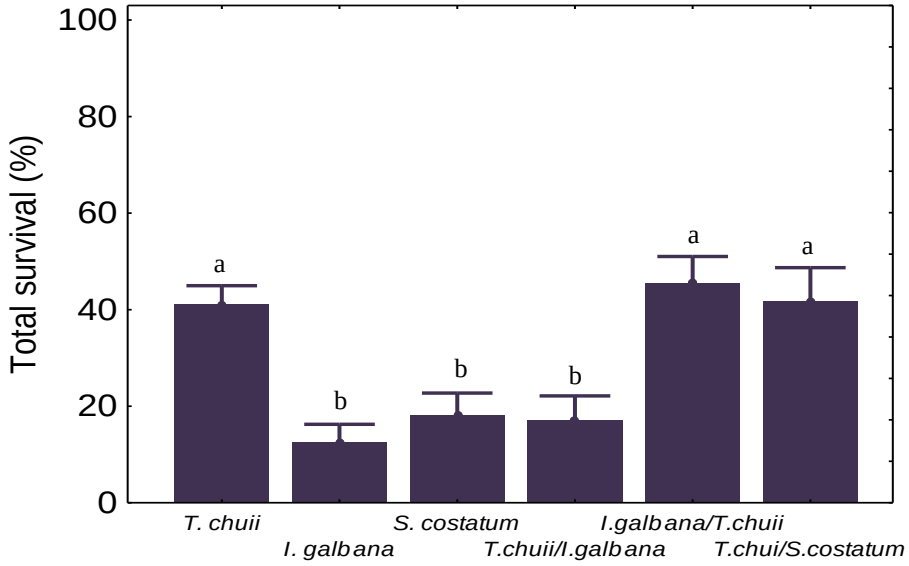


Fig. 3.4 Survival rate (%) according to culture time (days), for larvae cultured using various diets, namely monodiet of *Tetraselmis chuii* (*T. chuii*), monodiet of *Isochrysis galbana* (*I. galbana*), monodiet of *Skeletonema constatum* (*S. costatum*), mixed diet of *T. chuii* and *I. galbana* (*T. chuii/I. galbana*), mixed diet of *I. galbana* and *S. costatum* (*I. galbana*/ *S. costatum*) and mixed diet of *T. chuii* and *S. costatum* (*T. chuii*/ *S. costatum*).

Interestingly when larvae were assessed for quality (Table 3.5), there were significant differences between treatments (ANOVA; $F=89.92$; $p\leq0.01$). Diets of *S. costatum* and *I. galbana/ S. costatum* led to higher quality larvae (69.67 ± 7.09 and 63.67 ± 6.51 %, respectively; statistically not different; Tukey, $p=0.74$), followed by *T. chuii/ S. costatum* (not different from *I. galbana/S. costatum*; Tukey, $p=0.17$), and *T. chuii* and *T. chuii/ I. galbana* (not different between themselves; Tukey, $p=0.95$), with *I. galbana* leading to the lowest larval quality (Tukey, $p<0.01$), with no high quality larvae observed. Similarly, the percentage of cyprids varied significantly with diet (ANOVA; $F=179.36$; $p\leq0.01$). This was higher for mixed diets with *S. costatum* (90.33 ± 4.00 %; statistically non different between themselves; Tukey, $p=1.00$), followed by monodiets *S. costatum* (49.67 ± 1.45 %; Tukey, $p<0.01$) and *T. chuii/ I. galbana* (32.67 ± 3.48 %; Tukey, $p<0.01$). The lower percentage of cyprids was observed in cultures reared on *T. chuii* and *I. galbana* (not statistically different between themselves; Tukey, $p=0.24$), where cyprid percentage was 9.67 ± 2.03 and 0.00 ± 0.00 % respectively (Table 3.4).

Diet	H-Q larvae (%)	Cyprids (% larvae)	%H-Q cyprids (% n)
T. chuii	14.33 ± 4.04 [c]	9.67 ± 2.03 [c]	0.21 ± 018 [b]
I. galbana	0.00 ± 0.00 [d]	0.00 ± 0.00 [c]	0.00 ± 0.00 [b]
S. costatum	69.67 ± 7.09 [a]	49.67 ± 1.45 [b]	1.27 ± 2.19 [b]
T. chuii /I. galbana	18.00 ± 5.57 [c]	32.67 ± 3.48 [b]	0.20 ± 0.35 [b]
I. galbana /S. costatum	63.67 ± 6.51 [ab]	90.33 ± 3.76 [a]	15.15 ± 2.62 [a]
T. chuii /S. costatum	52.33 ± 5.51 [b]	90.33 ± 4.26 [a]	11.32 ± 4.66 [a]

Table 3.4. High quality larvae (%) and cyprids (%) obtained after rearing and larvae collection, for larvae cultured using various diets. High quality larvae were identified following the criteria outlined in table 1. Diets tested included monodiets of *Tetraselmis chuii* (*T. chuii*), *Isochrysis galbana* (*I. galbana*), *Skeletonema constatum* (*S. costatum*), and mixed diets of *T. chuii* and *I. galbana* (*T. chuii/I. galbana*), *I. galbana* and *S. costatum* (*I. galbana*/ *S. costatum*) and *T. chuii* and *S. costatum* (*T. chuii*/ *S. costatum*). Diet significantly affected larval quality (ANOVA, $F=89.92$; $p<0.01$), number of cyprids (ANOVA, $F=179.36$; $p<0.01$) and the percentage of high-quality cyprids (ANOVA, $F=24.39$; $p<0.01$).

3.3.3 Photoperiod

Photoperiod was observed to affect larval specific growth rate significantly (ANOVA, $F=223.47$; $p<0.01$) and therefore median development time to cyprid (ANOVA, $F=17.18$; $p<0.01$).

Growth rates varied on average between 4.03 and 5.27 d^{-1} (Table 3.6) Larvae grown at 24:0 L/D had the highest specific growth rate, not significantly different from 16:8 L/D (Tukey, $p=0.79$), followed by 8:16 L/D, and 0:24 had the lowest growth rate (all different statistically; Tukey, $p<0.01$). This is noticeable in Fig. 3.6 and is also reflected in the different median development times to cyprids (Table 3.6).

Photoperiod	SGR (d^{-1})	Survival (%)	MDT (d)	CL (µm)
0:24 L/D	4.03 ± 0.05 [c]	21.94 ± 5.45 [a]	16.67 ± 0.33 [c]	206.76 ± 2.76 [a]
8:16 L/D	4.87 ± 0.07 [b]	37.33 ± 4.42 [a]	15.33 ± 0.31 [b]	208.87 ± 3.55 [a]
16:8 L/D	5.22 ± 0.08 [a]	24.44 ± 4.12 [a]	15.13 ± 0.11 [b]	204.45 ± 3.71 [a]
24:0 L/D	5.27 ± 0.07 [a]	27.00 ± 3.01 [a]	13.67 ± 0.29 [a]	211.27 ± 3.82 [a]

Table 3.5. Specific growth rate (SGR, #), total survival to cyprid (Survival, %), median development time to cyprids (MDT, days) and cyprid width (CW, µm), for larvae cultured at various photoperiods, namely full dark photoperiod (0:24 L/D), 16 h of light and 8 h of darkness (16:8 L/D), 8 h of light and 16 h of darkness (8:16 L/D), and full light photoperiod (24:0 L/D). Photoperiod significantly affected specific growth rate (ANOVA, $F=223.47$; $p<0.01$) and development time (ANOVA, $F=17.18$; $p<0.01$), but not survival (ANOVA, $F=2.39$; $p=0.08$) and length (ANOVA, $F=0.35$; $p=0.71$).

Larvae grown in full-day photoperiod developed into cyprids in 13.67 ± 0.29 days, significantly faster than other photoperiods (Tukey Test; $p\leq0.03$), with mixed photoperiods showing no significant differences (Tukey Test; $p=0.96$) and the longest

development time in full darkness, 16.67 ± 0.33 d^{-1}. The effect was also noted in the early appearance of nauplius VI and cyprids in the culture exposed to 24:0 L/D. In this culture, stage VI nauplii and cyprids were clearly visible after day 11, and later in the other photoperiods, around day 15 (fig. 3.7). However, although total survival varied between treatments, these differences were not significant (ANOVA, F=2.39; p=0.08), in spite of the markedly different development times. Cultures grown at 0:24 L/D, 16:8 L/D, 24:0 L/D and 8:16 L/D showed total survival rates of 21.94 ± 5.45 %, 24.44 ± 4.12 %, 27.00 ± 3.01 % and 37.33 ± 4.42 % respectively (Table 3.5).

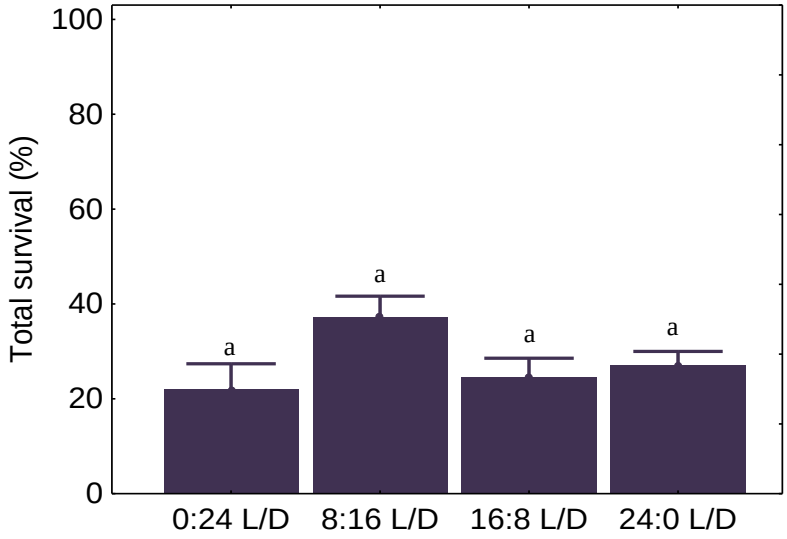


Fig. 3.5 Survival rate (%) according to culture time (days), for larvae grown at various photoperiods, namely full dark photoperiod (0:24 L/D), 16 h of light and 8 h of darkness (16:8 L/D), 8 h of light and 16 h of darkness (8:16 L/D), and full light photoperiod (24:0 L/D).

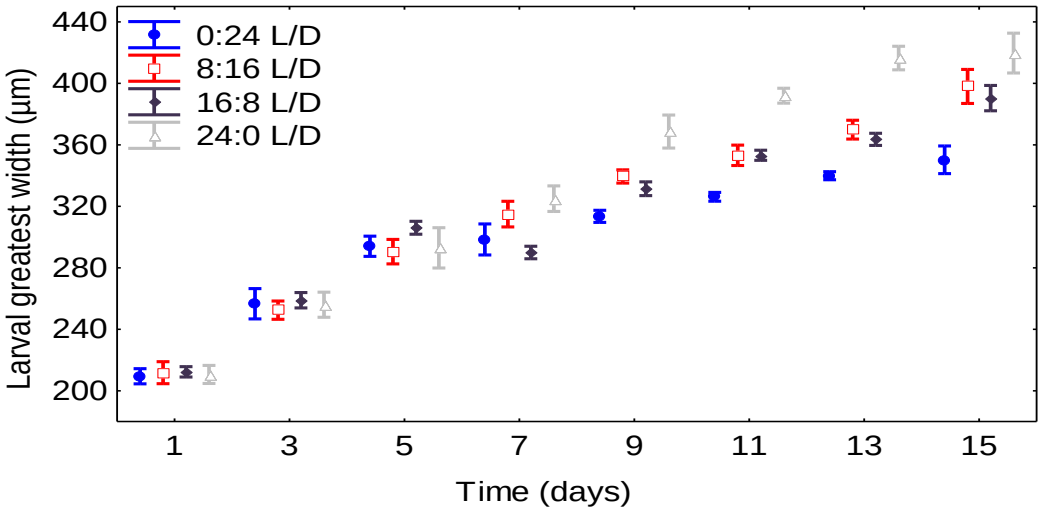


Fig. 3.6 Larval greatest width (µm) according to culture time (days), for larvae grown at various photoperiods, namely full dark photoperiod (0:24 L/D), 16 h of light and 8 h of darkness (16:8 L/D), 8 h of light and 16 h of darkness (8:16 L/D), and full light photoperiod (24:0 L/D).

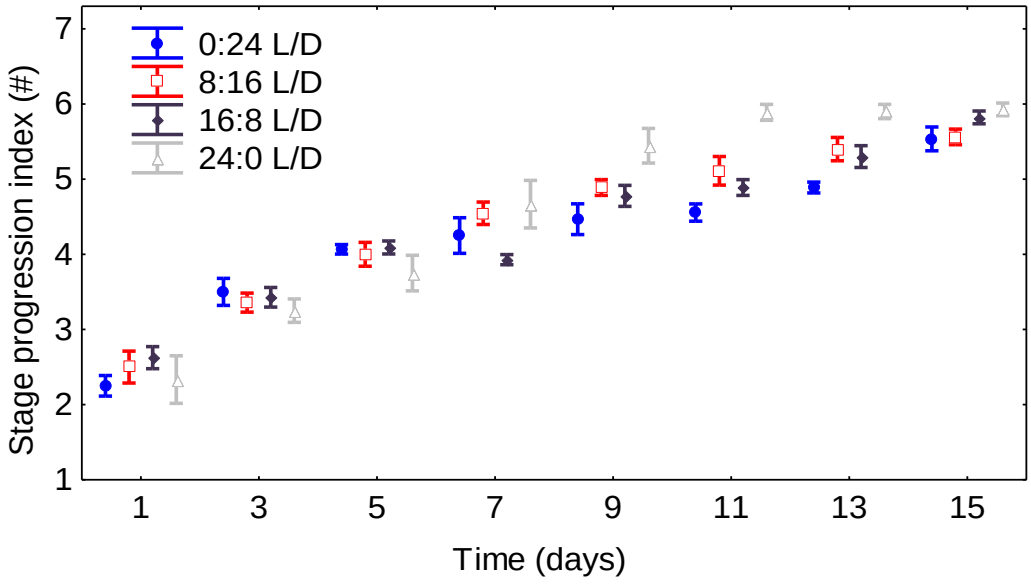


Fig. 3.7 Larval stage (#) progression according to culture time (days), for larvae grown at various photoperiods, namely full dark photoperiod (0:24 L/D), 16 h of light and 8 h of darkness (16:8 L/D), 8 h of light and 16 h of darkness (8:16 L/D), and full light photoperiod (24:0 L/D).

3.3.4 Salinity

No effects of salinity were observed (20, 30 and 40 psu) on larval specific growth rate (ANOVA, F=0.66; p=0.52), total survival (ANOVA, F=0.87; p=0.42; fig. 3.8) or median development time to cyprid (ANOVA, F=13; p=0.98). Results are summarized in Table 3.6. Specific growth rate, total survival and median development time averaged 4.18 ± 0.05 d^{-1}, 24.26 ± 3.89 %, and 14.52 ± 2.22 days respectively.

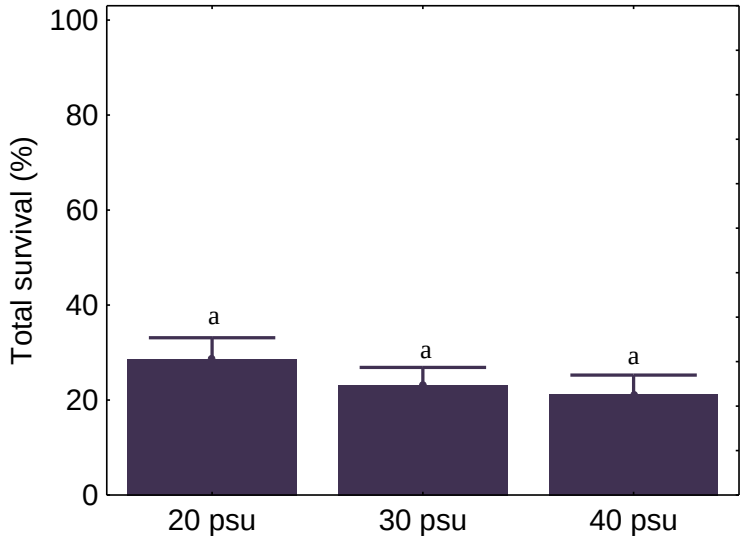


Fig. 3.8 Survival (%) according to culture time (days), for larvae grown at various salinities (20, 30 and 40 psu).

Salinity	SGR (d^{-1})	Survival (%)	MDT (d)	CL (µm)
20 psu	4.20 ± 0.07 [a]	28.61 ± 4.51 [a]	14.35 ± 1.99 [a]	210.33 ± 2.59 [a]
30 psu	4.18 ± 0.06 [a]	23.06 ± 3.81 [a]	14.45 ± 2.08 [a]	212.18 ± 3.62 [a]
40 psu	4.14 ± 0.07 [a]	21.11 ± 4.15 [a]	14.78 ± 1.87 [a]	205.88 ± 4.11 [a]

Table 3.6. Specific growth rate (SGR, #), total survival to cyprid (Survival, %), median development time to cyprid (MDT, days) and cyprid width (CW, µm), for larvae cultured at various salinities (20, 30 and 40 psu). There were no significant differences between salinities, in terms of total survival to cyprid (ANOVA, F=0.87; p=0.42), specific growth rate (ANOVA, F=0.66; p=0.52), median development time (ANOVA, F=0.13; p=0.98) and cyprid length (ANOVA, F=0.22; p=0.91).

3.4. Discussion

3.4.1 Temperature

Unsurprisingly, temperature had a significant effect on specific growth rate, as had been reported previously for *P. polymerus* (Lewis, 1975), *C. mitella* (Rao et al., 2010; Zhang et al., 2009) and *P. pollicipes* (Coelho, 1990). The studies of Coelho (1990) were, however, highly preliminary, mentioning median development time but failing to detail the observed specific growth rate and only following growth to the last naupliar stage. Development time from nauplius stage I to nauplius VI decreased from 20 days at 15 ºC to 9 days at 22 ºC. In the present study, SGR to cyprid increased with temperature from 2.60 to 5.93 d^{-1}, with median development time decreasing from 24.85 d at 11 ºC to 9.81 d at 24 ºC. Coelho's (1990) results are in accordance with the timeframe observed in this study, though it is hard to make an accurate comparison given the differences in protocol. The literature suggests a minimum development time of *P. pollicipes* to the cyprid of 23 – 28 d at 20 ºC (Molares et al., 1994a), 14 d at 17.5 ºC (to nauplii VI; Candeias, 2005) and 21 to 28 d at 15 – 24 ºC (Kugele & Yule, 1996). In light of current results, development time can be decreased by 14 days if culture temperature is increased by 11 ºC. These differences in SGR can be explained by the effects of increasing temperature on metabolism, also heavily influenced by body mass. Similarly in *C. mitella*, which has a longer larval phase (Qiu et al., 1994b), higher temperatures decreased development time from 11 d (at 24 ºC) to 7 d (at 30 – 31 ºC) (Rao et al., 2010; Zhang et al., 2009).

In the current study, we observed a direct relation between SGR and total survival at low temperatures, as the higher the SGR, the lower the mortality. However, at higher temperatures (> 20 ºC) an effect was also observed in daily mortality, which increased with temperature, creating an opposing trend. This is in accordance with Coelho (1990) who reported mass mortality at 22 ºC, as well as with the conclusions of Lewis (1975).

In fact, at cultures above 20 ºC, survival to cyprid was less than 9 %, and at 24 ºC, barely any cyprids were found. This could also be ascribed to a putative decrease in water quality at these temperatures or eventual proliferation of contaminating species, as reported by others (e.g. Candeias, 2005). Candeias (2005) reported 30 % mortality in the first 3 days of culture and overall mortality upon nauplius stage VI stage of 86.8 %. Coelho (1990) simply referred to "high mortality", but did not present supporting data in relation to temperature. From our experience, most of the culture problems observed result directly from the long development time to the cyprid (a minimum of 15 days at 20 ºC), which maximizes the chance for abrupt mortalities to occur. In comparison to other species, such as *B. amphitrite* and *B. improvisus*, whose cyprids can be obtained in 4 to 5 days (28 °C), a rearing period of 15 days is extensive and harder to control. Common problems encountered include the increased probability for system failure (failure in aeration, temperature control, etc.), culture contamination, proliferation of contaminant species (e.g. ciliates/bacteria), clogging of larval appendages and failure to feed/swim properly. Total survival varied between 19 – 31 % at an optimal range of 15 – 20 ºC, to values below 11 % at 11 ºC and above 22 ºC. Although a clear indication of optimal rearing temperature, these values certainly remain sub-optimal, as seen by comparison with studies on *C. mitella*, which at the optimal temperature of 27 °C, total survival ranges from 90 – 99 % and metamorphosis from 73 – 81 % (Rao et al. 2010).

In light of these results, the use of culture temperatures at or below 20 ºC would be advisable to maximize both growth and survival, although higher temperatures are recommended to reduce the chances of abrupt mortality due to a long development time. However, a note should be made regarding when to extract the cyprids from culture. Due to the fast succession of larval stages in culture, close attention should be given to the appearance of the nauplius VI stage, as a prelude to cyprid appearance, since it is advisable to filter the cultures when over 50 % of the individuals have reached the cyprid stage. If cyprids remain in culture too long, due to the high surface selectivity of this species (Kugele & Yule, 1996), high mortality can occur and cyprid quality can decrease abruptly. The cyprid is the stage responsible for surface selection and unlike nauplius VI is non-feeding, relying entirely on lipid and protein reserves for survival during the exploratory and attachment phase (Holland & Walker, 1975; Satuito et al., 1996; Thiyagarajan et al., 2002b). Results from other species also suggest increased mortality prior to the cyprid stage (e.g. 49.2 % for *B. amphitrite*; Costlow & Bookhout, 1959).

3.4.2 Diet

Feeding and nutritional requirements are of paramount importance in larval culture of *Pollicipes* spp. (Lewis, 1975; Moyse, 1963). From the diets tested, no significant differences were recorded on growth rate and development time, although monodiets of *T. chuii* led to higher growth rates, followed by *S. costatum* monodiet and mixed diets *T. chuii*/*I. galbana* and *T. chuii*/*S. costatum*. The lowest growth rates were attained for *I. galbana* and *I. galbana*/*S. costatum*. Notwithstanding the lack of statistical significance, this trend could be predicted on the basis of carbon content, with diets such as *T. chuii* having a higher carbon content with *I. galbana* considerably inferior. In fact, in these algal species, organic weight averages 200, 20 and 29 µg C 10^{-6}cell^{-1}, respectively for *T. chuii*, *S. costatum* and *I. galbana*, which in great part is proportional to cell volume, averaging 300, 45 and 85 µm^3 cell^{-1} (Helm & Bourne, 2004). Though algal density is not an adequate measure to compare algal volume available to the larva, cell density does give an accurate balancing of encounter rates. Though carbon content could have been balanced between diets, rather than cell density, balancing diets via carbon content is often impractical in day-to-day feeding. Rather, density is often used as a replacement measure since it directly relates to pre-predator encounter rates. Furthermore, it allows for easier comparison between studies with most referring to diets in terms of cell density (e.g. Candeias, 2005; Kugele & Yule, 1996; Molares et al., 2002). Feed quantity used in the literature varies, with Coelho (1990) using water turbidity as a feeding measure (sufficient to make the water turbid the water but not to obscure the larvae). Protocols have ranged from 50 – 100 cells µl^{-1} fed daily or every 2 – 3 days (Kugele & Yule, 1996; Molares, 1994; Molares et al., 1994; Candeias, 2005). Candeias (2005) recommended the use of algal densities ≤ 500 cells µl^{-1}, when antibiotics are not used, to avoid the deterioration of water quality. Nevertheless, with the marked differences between studies and the differences in larval density from 1 to 5 larvae ml^{-1} (Coelho, 1990; Molares, 1994; Molares et al., 1994; Molares et al., 2002), care must be taken when making comparisons across studies. Furthermore, consumption rate has been shown to vary according to algal species provided as food (Candeias, 2005). Lewis (1975b) concluded that, from the variety of algal species used, only *Procentrum micans* and *Platymonas* sp. could sustain larval development in *P. polymerus* to the cyprid stage. Moreover, nutritional requirements might change with larval development, as suggested by Kugele & Yule (1996).

However, mixed diets should not, as a rule, be considered superior to monodiets, as demonstrated by the positive survival results attained with *T. chuii*. Molares et al. (2002) investigated the use of *Isochrysis*, *Tetraselmis* and *Skeletonema* spp., but with unsatisfactory results and high mortality. Although mixed diets are considered to result in better development and survival for larvae (e.g. Kuban et al., 1985), these parameters are also known to depend on the nutritional quality of the algae that constitute the diet (e.g. Stone, 1989; Vanderploeg et al., 1996). Furthermore, when it comes to nutritional profile, these species can show marked differences, varying further with culture conditions. *T. chuii* is known to be rich in polyunsaturated fatty acids (PUFA), namely eicosapentaenoic acid (EPA) and arachidonic acid (ARA) (Dunstan et al., 1992), while *I. galbana* is richer in docosahexaenoic acid (DHA) than EPA (Renaud et al, 1991; Liu et al., 2013), making them good complementary diets. On the other hand, *S. costatum* is rich in palmitic, myristic and ARA (Winaryo, 2009), though its wide use in crustacean and bivalve aquaculture is principally due to its calcium carbonate content, essential to the development of these species. Independent of diet, development time ranged between 14.99 to 16.05 d, without significant differences.

Although diet did not significantly influence growth and development time, it did significantly affect survival. This study indicates that the algal diets tested are not necessarily unsuitable as food sources, allowing development through the naupliar stages, but their bioavailability and interaction with the larvae might impact survival. These species will be represented differently in the water column, as *Tetraselmis* sp. and *Isochrysis* sp. are flagellated species, moving freely and dispersing more evenly, unlike *Skeletonema* sp. which is known to form long chains that tend to flocculate and settle, in particular if aeration is insufficient to keep the algae in solution. Furthermore, these long chains of cells can often become entangled in the limbs of the larvae and hinder feeding efficiency, leading to increased mortality. Nevertheless, it is interesting to note that the larvae obtained with the *S. costatum* monodiet and mixed diets containing that species had the greatest larval quality, and the largest percentage of cyprids. These data indicate that although *S. costatum* alone might be insufficient as a food source, or not sufficiently bioavailable, when used in conjunction with *T. chuii* or *I. galbana* it constitutes a suitable diet that maximizes growth, survival and number of cyprids, over monodiets of the other species.

Coelho (1990) also concluded that from monodiets of *S. costatum*, *Tetraselmis suecica*, *Thalasiosira pseudonana*, *I. galbana*, *Chaetoceros gracilis* and *Chorella* sp., only *T.*

suecica and *I. galbana* facilitated larval development to cyprid stage. This contradicts the results reported here, where monodiets of *S. costatum* also sustained development to the cyprid stage. However, the three monodiets diets of *I. galbana*, *T. chuii* and *S. costatum* produced an overall percentage of high quality cyprids respectively of 0.00 ± 0.00, 0.21 ± 0.18 and 1.27 ± 2.19 % of initial larvae added to the cultures; considerably less than that obtained with mixed diets. Diets of *I. galbana/S. costatum* and *T. chuii/S. costatum* produced 15.15 ± 2.62 and 11.32 ± 4.66 % high-quality cyprids respectively from the initial larval numbers. In spite of representing the best results, these values are by themselves indicative of the need of optimization of culture conditions. Although mixed diets ranked better overall, *S. costatum* did support development to the cyprid, especially when associated other species, while *I. galbana* led to the poorest overall results. Candeias (2005) concluded that all *P. pollicipes* larval stages show a preference for ingesting *I. galbana* and *S. costatum* and, to a lesser extent, *T. suecica*. Additionally, mixed diets and flagellate-based diets (e.g. *Rhinomonas reticulata*) produced higher survival rates than monodiets of diatoms (e.g. *S. costatum*; Candeias, 2005), as observed here also. Kugele & Yule (1996) used a mixed diet of *S. costatum* and *R. reticulata* (1:2), Stone (1988) *R. reticulata* until nauplius III and *T. chuii* afterwards, while Molares (1994) and Molares et al. (1994a, 2002) used *I. galbana*. Though temperate species tend to prefer flagellates (Stone, 1983; Moyse, 1963), Candeias (2005) suggested that as *P. pollicipes* is subjected to seasonal upwelling there may be a propensity for feeding on diatoms. Here, however, the nutritional profile of this species probably plays a more significant role than ecological preference *per se*.

Although in terms of survival mixed diets were comparable to *T. chuii*, when larval quality and number of cyprids are considered, the latter diet proved to be insufficient to attain satisfactory survival and is not recommended. The best overall results for high-quality larvae and successful development to the cyprid were obtained with mixed diets including *S. costatum*. In the case of *I. galbana*, the results attained were poor overall, with minimal survival associated with null larval quality and cyprid percentage. Furthermore, other non-diet specific studies (Molares, 1994; Molares et al., 1994a) have used *I. galbana* and, although growth and survival were not monitored, the authors report massive mortality and insufficient numbers of cyprids for use in further experiments. Nevertheless, observations from several authors (e.g. Coelho, 1990; Candeias, 2005) indicate that monodiets of flagellate species such as *Tetraselmis* sp. and *I. galbana* would be sufficient to sustain larval development and survival, although

values are not presented by these authors and this assertion is not supported by the current results. It is interesting to note, however, that when these species are provided together, larval quality and survival to the cyprid were superior to the pooled individual results, suggesting that the results for survival might have been underestimated in this study. In fact, this seems to be supported by previous experiments undertaken, where with diets of *T. chuii*/*I. galbana* resulted in average survival of 31.05 ± 5.14 % (unpublished results) similar to what was obtained in the experiments testing temperature and photoperiod.

From the current results, mixed diets, including *S. costatum*, produced maximal survival, quality and percentage of cyprids. Optimal rearing of larvae should be a priority as apparently healthy larvae, raised on sub-optimal diets may not be competent to settle, as the case of with *Dunaliella* sp. used for *B. amphitrite* larvae (Aldred per. com.). Therefore, future studies should further investigate settlement effects, extending the current work, providing that suitable settlement surfaces are identified. *P. pollicipes* has reportedly poor settlement on artificial surfaces in captivity (e.g. Kugele & Yule, 1996; Coelho, 1991).

3.4.3 Photoperiod

Tough there have been no studies on the effects of light exposure on *P. pollicipes* larval growth and survival, there have been studies of how behaviour is affected by light (Molares et al., 2002). *P. pollicipes* larvae are known to be phototactic and Molares (1994) established that most of the time this will manifest as a positive swimming response to light. This characteristic has long been used for selection of the fittest larvae, by attraction to light. In the present study, it was observed that photoperiod can affect both larval growth and survival, though the differences in survival were not a statistically significant result. In fact, larvae grown in 24:0 L/D and short/long day photoperiod had the highest specific growth rate (SGR) and survival, respectively. Although this might seem contradictory, SGR and survival can often be inversely related, as faster growth can translate into decreased fitness and increased mortality. Longer-day photoperiods can directly promote algal proliferation, allowing for greater food availability for an extended period of time, which may contribute to faster growth in the full-light photoperiod. This may also negatively impact larval fitness, however, as increased algal concentration can lead to increased fouling of limbs and consequently decreased survival (e.g. Candeias, 2005). Additionally, excessive feeding can accelerate the deterioration of water quality, due not only to high growth rates, but also to the

accumulation of metabolites and proliferation of secondary species (e.g. ciliates), which are difficult to remove from culture, even with frequent cleaning. In the Crustacea, there is high variability in response to photoperiod during larval rearing (e.g. *Strombus pugilis*, *Portunus pelagicus*, *Jasus edwaedsii*; Manzano et al., 1998; Andres et al., 2010; Bermudes & Ritar, 2008) and therefore species-specific protocols are mandatory. In larvae that are visual predators, light can play a significant role in prey capture, as well as the contrast between environment and prey. The simple eye structure is highly reliant on absolute illumination, making it more dependent on not only the amount of light provided, but also on light dispersion in the tanks (Naas & Huse, 1996), in particular in phototactic species as *P. pollicipes* (Molares et al., 2002). Nevertheless, no studies to date have investigate this hypothesis.

Highest survival was recorded at 8:16 and 24:0 L/D, while the lowest was in full-darkness, which also had the lowest growth rate, although differences were not significant. This is not surprising, since full-darkness will negatively affect algal growth. Another possible factor is the effect of light on larval activity, as observed by Molares (1994). As light leads to higher swimming activity, this might possibly induce higher consumption of food reserves in larvae subjected to longer day photoperiods or even increase larva-larva or larva-container encounters. Nevertheless, larvae tend to accumulate in the side of the tank that is closer to the light source (Franco, pers. obs.) despite in-tank turbulence. In this sense, tanks kept in darker conditions create a more even larval distribution, though using different tank design (e.g. Plankton kreisel; Greeve, 1968) might be also of interest. Independently of this, the present results support the use of full-day photoperiod to mixed photoperiods, which is in accordance to what has been used to date by previous authors.

3.4.4 Salinity

Salinity is known to affect growth and survival during larval development of several species. However, unnatural regimes have proven beneficial in other species of bivalves, crustaceans and fish (e.g. Innes & Haley, 1977; Fonds, 1973; Kumlu et al., 2000; Boeuf & Payan, 2001) and several authors working with larvae (e.g. *Penaeus semisulcatus*, *Mytilus edulis*, *Solea solea*, *Sparus sarba*) have shown that higher salinities, within a tolerance range, tend to enhance growth, while lower salinities often improve survival. This may be due to osmotic stress and the need to increase metabolic expenditure to compensate for sub-optimal salinities. In the case of *P. pollicipes* larval culture, salinities are most frequently within the natural range of salinities 33 – 34 psu

(Coelho, 1990; Molares, 1994; Molares et al., 1994a). Nevertheless, many species are known to be wide-range osmoregulators, being considered euryhaline. The effects of various salinities in these species will depend largely on their ability to osmoregulate at early stages (e.g. Charmantier, 1998), and can strongly affect larval lipid reserves (e.g. Torres et al., 2002). In the present study, no effects of salinity, ranging from 20 to 40 psu, were observed in terms of larval survival, growth rate, development time and cyprid length, indicating that *P. pollicipes* is highly tolerant within this range. In the wild, *P. pollicipes* is normally found at salinities of 33 to 37, although salinity in the intertidal environment can vary significantly due to evaporation in the hours of air exposure and rainfall. Results suggest that *P. pollicipes* can be grown anywhere in this range, although further studies should be done to investigate what the exact tolerance limits for this species are.

3.5. Conclusions

Based on the present results, optimization of growth and survival can be accomplished using rearing temperatures of 15 – 20 ºC, daily feeding with *T. chuii/S. costatum or I. galbana/S. costatum* and a photoperiod of 24/0 L/D. The use of higher temperatures significantly increases mortality and provides unsatisfactory numbers of cyprids, while lower temperatures lead to the extension of the growth period, which allows for higher risk of acute mortality. The decision of which temperature to use within this range (15 – 20 ºC) should rely upon the relative benefits of having a longer/shorter growth period in any given situation. With regard to diet, despite the better growth rates seen using *T. chuii*, the negative impact of this diet on larval quality and number of cyprids make it less suitable than *T. chuii/S. costatum or I. galbana/S. costatum*, which in spite of lower growth rates lead to higher quality and number of cyprids, therefore maximizing rearing efficiency. Photoperiod, on the other hand, had marked effects on survival, with better results in full-day photoperiods and long/short-day photoperiods, as full-dark photoperiods should be avoided. Regarding salinity, no effects were observed, suggesting that there is a wide range of acceptability. Future studies should investigate the use of recirculating systems for larviculture, as a way of improving water quality and decreasing maintenance costs, as well as investigating the effects of rearing density and feeding protocol on rearing efficiency.

Chapter 4. Larval settlement and recruitment of stalked barnacles (*Pollicipes pollicipes*) on natural and artificial substrata

Abstract

The stalked barnacle, Pollicipes pollicipes, is in high demand in Portugal and Spain, where it is considered a delicacy with high market prices. In recent years, interest relating to the sustainable aquaculture of this species has increased, although the conditions necessary to promote larval settlement remain unknown. The cyprid of *P. pollicipes* is known to be highly selective, with settlement associated almost exclusively with the adults. In the present paper, the settlement of *P. pollicipes* was investigated with a focus on the environmental conditions that promote cyprid settlement on the adult, as well as settlement and recruitment to artificial and natural substrata in the laboratory and field. Maximum settlement on adults in culture was about 30 – 35 %, with a one-week metamorphosis rate of 70 – 80 %. The conditions necessary for such behaviour included the use of natural salinities (30 – 40 psu), temperature of 20 ºC, 24:0 L/D, circulating water conditions or the presence of a water meniscus and cyprids no older than 3 days. Settlement in the laboratory occurred preferentially on the capitulum of adult conspecifics, while in the wild most recruits were found on the stalk, possibly due to a higher post-settlement survival on the stalk in the wild or advantageous larval transport. Very low settlement was also recorded on artificial substrata in the laboratory and, in much larger numbers, on marine epoxy in the field where settlement was comparable to that on the adults. Settlement, in both the laboratory and field, was also recorded in association with *Chthamalus* sp., *Corallina* sp., rocks (uncolonized or cleared) and a variety of other substrata. In the laboratory, however, settlement to non-natural substrata only occurred in the absence of adult conspecifics. Although the adult conspecific remains the principal driver of larval attraction in this species, for those that settle on artificial substrata, the nature of the surface also appears to determine both surface selection and post-settlement survival. On the basis of these data, there may be significant value in studying surface-related parameters such as roughness and micro-topography, and their interactions with environmental factors including hydrodynamics, predation, competition and desiccation, as well as the use of chemical settlement inducers.

4.1. Introduction

Harvesting *Pollicipes pollicipes* is an important economic activity in Portugal and Spain, where the species is subject to an intensive fishery (Freire & García-Allut, 2000; Molares & Freire, 2003; Bald et al., 2006; Borja et al., 2006a, 2006b). Their market value and increasing conservation concerns have continued to provoke interest in the aquaculture production of this species, although very few studies have investigated the subject in any depth (Goldberg, 1984; Coelho, 1990, 1991; Molares, 1994; Norton, 1996; Cribeiro, 2007; Candeias, 2005). Larval production and settlement remain unresolved phases in the synthetic culture of *P. pollicipes* and represent the most significant bottleneck in their adoption to aquaculture. The development of protocols for larval settlement in captivity and larval recruitment in the wild would thus constitute major steps towards commercial culture and wild recolonization programmes, bridging fisheries and conservation.

P. pollicipes is a pedunculate barnacle, locally abundant in the very exposed low-intertidal rocky shore (Barnes, 1996; Cruz & Araújo, 1999; Cruz et al., 2010) and normally found in clusters of sessile adults, attached to each other and to the primary rock substratum. These simultaneous hermaphrodites and obligate cross-fertilisers require gregariousness in order to mate with conspecifics (Molares et al., 1994b; Cruz, 2000; Pavón, 2003). After mating, embryonic development occurs in the mantle cavity of the adults (Cruz & Araújo, 1999) until hatching and release of first stage nauplii. Adults of this species produce 1 to 5 batches of larvae per year during the breeding season, from March to September (Cardoso & Yule, 1995; Molares, 1998; Cruz & Hawkins, 1998; Cruz & Araújo, 1999; Cruz, 2000; Molares et al., 2002; Pavón, 2003; Macho et al., 2005; Cruz et al., 2010). The first stage nauplius moults through a further five naupliar stages to a non-feeding cypris stage, responsible for surface selection and settlement (e.g. Coelho, 1990; Molares et al., 2002). The cyprid of *P. pollicipes* is highly discriminating and tends to select as settlement substrata the peduncle of adults of the same species, or substrata that have been in contact with conspecific adults (e.g. Kugele & Yule, 1996; Cruz et al., 2010). The barnacle cyprid is known to explore surfaces prior to settlement using a mechanism of temporary adhesion, which allows the cyprid to survey a surface's physical and chemical characteristics before attaching permanently (Aldred et al., 2008; Aldred & Clare, 2009). Barnacle cyprids can postpone their settlement (Wilson, 1932; Krug, 2006) in order to select a substratum that is suitable, possibly exploring surfaces and returning to the water column repeatedly

before committing to permanent attachment. Once the settlement site has been selected, the barnacle cyprid secretes a second adhesive that permanently fixes it to the substratum (Nott & Foster, 1969; Walker, 1972; Ödling et al., 2006; Phang et al., 2006). Immediately after settlement, the cyprids metamorphose into juvenile barnacles of *P. pollicipes*, which can be found in the natural environment from September to January (Cardoso & Yule, 1995; Cruz & Hawkins, 1998; Molares, 1998; Cruz & Hawkins, 1998; Cruz & Araújo, 1999; Cruz, 2000; Molares et al., 2002; Pavón, 2003; Macho et al., 2005; Cruz et al., 2010). Recruits are commonly referred to as spat after successful settlement and a period of post-settlement survival, and are early juveniles in the adult form.

Settlement of barnacles is influenced by many factors, from larval culture (e.g. Harder, et al., 2001; Thiyagarajan et al., 2002) to settlement conditions, such as hydrodynamics (e.g. Crisp, 1955; Knight-Jones, 1955), temperature (e.g. Marechal et al., 2004), salinity (e.g. Dineen & Hines, 1992) and light (e.g. Crisp & Ritz, 1973; Pawlik, 1992). Cyprid-related factors, such as origin (e.g. Holm et al., 2000; Holm et al., 2012), condition (e.g. Thiyagarajan et al., 2002b) and age (e.g. Holm et al., 2000; Marechal et al., 2012) are also determining factors affecting barnacle settlement. It is generally considered that, with age, barnacle larvae become less selective about their settlement site (Rittschof et al., 1984; Satuito et al., 1996, 1997; Holm et al., 2000). Cyprids of some species can delay metamorphosis for up to one month and still subsequently settle (e.g. Lucas et al., 1979). Unsurprisingly, gregariousness plays a pivotal role in barnacle settlement, as cyprids are positively stimulated to settle by the presence of settled conspecific cyprids and adults (e.g. Clare et al., 1994; Kugele & Yule, 1996). Besides conspecific effects, surface characteristics, chemical cues and biofilms are also significant (Aldred & Clare 2008).

The importance of chemical cues to barnacle settlement is well studied (e.g. Clare & Matsumura, 2000; Thyagarajan, 2010), and cyprids are known to respond to surface-bound chemicals while settling, as well as waterborne cues (e.g. Clare & Yamazaki, 2000; Elbourne et al., 2010). Natural surface-bound chemical cues are produced by adults, as the settlement-inducing protein complex (SIPC) (e.g. Matsumura et al., 2000), or left on surfaces by conspecific cyprids (Matsumura et al., 1998b; Dreanno et al., 2006b). Artificial chemical cues can also modulate settlement in barnacles (Clare et al., 1995; Holm et al., 2000; Yu et al., 2008), as well as the presence of biofilms (e.g. Maki et al., 1992, 1998). Many authors have devoted significant effort towards the

identification of surface characteristics that influence barnacle settlement (e.g. Crisp & Barnes, 1954; Le Tourneux & Bourget, 1988), mainly due to the importance of barnacles as fouling organisms. Although barnacle settlement has long been of interest, with extensive literature on ecology, research has historically been directed towards repelling settlement of cosmopolitan barnacles, rather than inducing the settlement of those with very specific requirements. Cyprids of *Pollicipes* sp. reportedly have incredibly high cyprid selectivity (e.g. Lewis, 1975a; Hoffman, 1989), settling only on or around adult conspecifics, or perishing in the water column. However studies are extremely limited for *P. pollicipes* specifically (e.g. Coelho, 1991; Kugele & Yule, 1996). Kugele & Yule (1996) investigated settlement of *P. pollicipes* in culture but could not achieve consistent settlement on the variety of surfaces used, with rates below 1 % of which most (93 %) of cyprids settled on the adults. In addition, several authors (e.g. Coelho, 1990; Molares et al., 1994) have reported difficulty in rearing significant numbers of healthy larvae to the cyprid stage.

It is essential to make a distinction between settlement studies in the laboratory and in the wild, and the terms settlement and recruitment. The former refers to the number of larvae that successfully attach and metamorphose to the juvenile, while the second refers to the number of larvae that, having settled, survive for a period of time. An alternative to larval rearing and settlement in culture and thus circumventing the problem of securing settlement to artificial substrata, is collection of larvae from the wild using artificial collectors. Lopez et al. (2005, 2010 and 2012) and Pham et al. (2008, 2011) have explored this option for commercial recruitment and growth of respectively *Austromegabalanus psitaccus* and *Megabalanus azoricus* with positive results. Their procedure simply involved settlement panels placed into the natural habitat. Notwithstanding obvious advantages in terms of practicality and costs, cyprid recruitment in the field is a product of larval availability, influenced by factors such as seasonality and variability of larval production during the reproductive season, as well as the impact of environmental factors on larval survival, transport, and selectivity (e.g. Shanks, 1986; Farrell et al., 1991; Pineda, 1991; Bertness et al., 1996; Shkedy & Roughgarden, 1997). Additionally, in the case of *P. pollicipes*, the collector systems and substrata to be used remain unexplored. The only studies to investigate recruitment on artificial structures were by Coelho (1991) and Cruz (2000), who used a variety of panels (e.g. tufnol, PVC, plastic, rubber, sisal ropes, etc.) but recorded no settlement, even when used in conjunction with crude barnacle extract. Additional complications

emerged in the form of loss and damage to infrastructure (e.g. Coelho 1991) due to the highly energetic environments that these species occupy. The loss of structures is common between studies (e.g. Goldberg, 1984) and makes it difficult to draw conclusions from the materials tested.

The present study investigates the factors affecting *P. pollicipes* larval settlement in the laboratory and the field. Specifically, 1) the conditions required to promote settlement on adult conspecifics in the laboratory, 2) settlement on artificial and natural substrata in the laboratory, both in conditions of choice and no-choice, and 3) recruitment to natural and artificial structures placed in the field.

4.2. Material and methods

4.2.1. Larval culture

Clusters of barnacles were collected from the SW coast of Portugal (Cabo Sardão, Portugal, 37°36'24.70", -8°49'2.00") and transported to the School of Marine Science of Technology (Newcastle University, UK), where the barnacles were dissected ($n \geq 60$ individuals) to extract egg lamellae. Lamellae were separated according to development stage and mature lamellae were cut to assist naupliar release. Healthy nauplii were selected by phototatic behaviour and transferred into culture. Standard culture was achieved using static culture conditions, in 10L plastic carboys, 0.2 µm natural filtered seawater (FSW), kept at 20 ± 1ºC, 16:8 L/D (dim light, 200-1500 lux), 33 ± 1 psu, antibiotics (0.0232 g l^{-1} Penicillin and 0.0369 g l^{-1} Streptomycin), with 1 larva ml^{-1} and weak, bottom aeration. Cultures were fed after water changes, every 2 days, with a mixed diet of *Tetraselmis chuii* and *Isochrysis galbana* (100000 cells mL^{-1} 1:1). Larval cultures were monitored daily for larval development in order to allow the identification of the peak in cyprid abundance (≥ 50 % cyprids), at which point cultures were filtered through a 60 µm mesh.

4.2.2. Experimental design

4.2.2.1. Environmental and cyprid-related factors affecting settlement on conspecifics in culture

Preliminary experiments (not presented here) tested a multitude of substrata and indicated the importance of cyprid quality, also revealing the well-documented tendency of cyprids of this species to reject artificial structures and to settle preferentially on adults. This initial study directed subsequent experiments towards the identification of the conditions that maximize settlement on the adult and also to reference values for

larval attachment, metamorphosis and survival in culture. A series of six experiments (Exp. 1 to 6) were then conducted to test the factors that mediate cyprid settlement on the adults. This included the testing of various environmental conditions, of hydrodynamics (Exp. 1), temperature (Exp. 2), salinity (Exp. 3) and light (Exp. 4), as well as cyprid related factors, such as cyprid age (Exp. 5) and batch (Exp. 6). Observations on cyprids (behaviour and morphology) and adults (behaviour, characteristics of the stalk and capitulum) were noted when relevant. The differences in settlement rates over time and those relating to location of settlement on the adult body, were also recorded, as well as cyprid survival in culture in a series of three further experiments (Exp. 7 to 9). A list of the experiments conducted is given in Table 4.1 for clarity.

	Exp.	Variable	Assay (C/nC)	Factors
A	1	Hydrodynamics	nC	Static, moving, aerated, interface
	2	Temperature	nC	14, 17, 20 ºC
	3	Salinity	nC	20, 30, 40 psu
	4	Light	nC+mC	Dark, light, dark-light choice
	5	Cyprid age	n.a.	0, 3, 6 days
	6	Larval batch	n.a.	2011, 2012, 2013
	7	Time	n.a.	0, 24, 48, 72, 96h ; 0, 3, 6, 9, 12 days
	8	Body zone	mC	Base stalk, lower stalk, upper stalk, stalk/capitulum, capitulm
	9	Survival	n.a.	0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 days
B	1	Artificial substrata	nC+mC	Clay, slate, nylon mesh, PVC, epoxy resin 1, epoxy resin 2, tufnol, glass epoxy, carbon epoxy, PMMA, nylon, calcium silicate, glass vinyl ester
	2	Natural substrata	nC+mC	Adults, rocks ucolonized, rocks cleared, *Chthamalus* sp., *Corallina* sp., incrusting algae, stalk cuticle
C	1	Artificial substrata	n.a.	(as B1)
	2	Natural substrata	n.a.	(as B2)

Table 4.1. List of experiments developed on (a) environmental and cyprid related factors affecting settlement, (b) settlement on natural and artificial substrata, and (c) recruitment in the wild, according to assay type (*C*, choice or *nC*, no-choice), variable and factor levels investigated.

Cyprid settlement assays were conducted using no-choice assays (except Exp. 4 and 8) in 500 ml conical flasks, 0.2 µm natural FSW, kept at 20 ± 1ºC, 16:8 L/D (dim light, 500-1000 lux), 33 ± 1 psu, antibiotics (as previously) and low, bottom aeration. To exclude the possibility of cannibalism, adults were placed in 50 mL Falcon tubes ® (whose lid had been cut to allow the stalk through and sealed with parafilm), with the stalk towards the inside of the tube and the capitulum on the outside. The tubes were filled with FSW and the larvae were added (≈ 50 larvae per tube; 1 larvae ml^{-1}). The

tubes were then fixed and placed inside the conical bottles, to allow the adult to be submerged and extend the cirri. The larvae could swim inside the tube, having as available substrata the adult stalk and the inner surface of the polypropylene tube, with the adults being unable to predate the larva. For each treatment, 9 replicate adults were used (except when stated otherwise), each in a tube system with a distinct set of larvae from the same batch. This assay and sampling was used for all experiments, but adjusted according to the factor being tested, as detailed below. The number of larvae temporarily and permanently attached was then counted after 48h and expressed as a % of the total. Metamorphosis was monitored after one week and the respective rate calculated.

Experiment 1 tested the effects of hydrodynamics on larval settlement. To simulate different hydrodynamic conditions, different setups were tested. Hereafter these are referred to as static, moving, aerated and interface. The static treatment (*static*) was achieved by leaving the systems (flask and tube) fixed and undisturbed, while for the moving treatment (*moving*) all replicate systems were placed on an orbital shaker (SSL1 Stuart®), set for constant 30 rpm. The aerated treatment (*aerated*) was achieved by placing weak point aeration inside the adult tube, allowing the larvae to move by force of air bubbling inside, and the air to escape by means of a secondary tube. The treatments with air-water interface (*interface*) had the tubes containing the adults fully filled with FSW until 0.5 cm from the top, which allowed a liquid meniscus and air-water interface within each tube. Experiment 2 tested the effects of temperature (14, 17, 20 ºC) on settlement. Each treatment was kept in a separate controlled-temperature incubator (LabHeat© and LabCold© RLCH0400 Incubator Units) at either 11 ± 1 °C, 15 ± 1 °C or 20 ± 1 °C and monitored as before. Experiment 3 tested the effect of salinity (20, 30 and 40 psu) on larval settlement. Larvae were kept at 20 ± 1 psu, 30 ± 1 psu or 40 ± 1psu. Salinity was checked daily (Hand Held Refractometer B+S©) and adjusted accordingly, to account for variations. Experiment 4 tested the effect of light (dark, light and light-dark choice) on larval settlement. A total of 27 replicate adults were used, from which 9 were left in full dark, 9 were left with full light and the remaining 9 were subjected to incident light from one direction, being consequently shaded on the other side. In the assay tubes in the light-dark choice treatment, adults were placed in fixed positions inside each conical flask and marked to highlight areas of light and shade. Treatments were kept in photoperiod-controlled incubators (LabHeat©

RLCH0400 Incubator Unit), with light intensities of 1233 ± 145 lux (lamps Osram Fluora, L 36W/77).

Experiment 5 evaluated the effects of cyprid age (0-, 3- and 6-days-old) on settlement. Newly metamorphosed larvae were filtered from culture and separated into replicate groups, aged at culture temperature for 0, 3 or 6 days. Replicate groups were kept in 0.2 µm natural FSW, at 20 ± 1ºC, 0:24 L/D, 33 ± 1 psu until use in the experiments. Experiment 6 tested the effects of larval origin on settlement, namely inter-annual variability on larval settlement rate, given the same culture conditions. This aimed at investigating the variability in settlement and metamorphosis according to larval batch (named A, B and C). Larvae were extracted from wild-collected adults (n=60), as previously described, over 3 consecutive years and cultured to cyprids.

Experiment 7 investigated cyprid settlement rates on adult conspecifics over time (18 replicates). Temporary and permanent attachment were monitored at 24, 48, 72 and 96 hours after substrata were provided (9 replicates of each). On the remaining replicates, the 48 h permanently attached larvae were monitored for metamorphosis rate after 3, 6, 9 and 12 days post-attachment. Experiment 8 investigated the preferential settlement to different areas of the adult body (9 replicates). The adults were divided into five parts (A, stalk base to E, capitulum), adapted from Cruz (2000) (fig. 4.1). These included (A) stalk base, (B) lower stalk, (C) higher stalk, (D) stalk-capitulum transition and (E) capitular plates. The adults were placed in culture, without restriction of cannibalistic behaviour, and the cyprids were allowed to settle on the preferred region, being subsequently described as either attached or metamorphosed. Experiment 9 tested how long cyprids survive in culture, in the absence of settlement substrata, using 9 replicate sets of larvae from the same batch. Survival was monitored every 48h over a period of 20 days, after which the experiment was terminated.

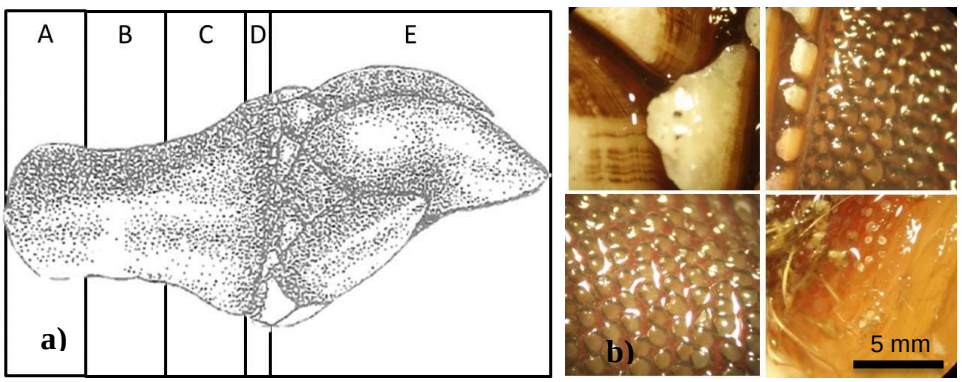


Fig. 4.1 (a) Adult body of *P. pollicipes* divided according to settlement zones, adapted from Cruz (2000). The adult body was divided in 5 zones, A to E, where A, B and C

correspond equally size areas of the stalk, D corresponds to the transition between the stalk and capitulum, and E to the area occupied by the capitular plates. The respective areas were classified as follows: (A) stalk base, (B) lower stalk, (C) upper stalk, (D) stalk-capitulum transition, and (E) capitulum. (b) Details of *P. pollicipes* body for the various zones considered (b1) capitulum, (b2) stalk, (b3) stalk-capitulum and (b) base.

4.2.2.2. SETTLEMENT ON NATURAL AND ARTIFICIAL SUBSTRATA IN CULTURE

A variety of different no-choice (nC) and multiple choice (mC) settlement assays were conducted in the laboratory, testing separately a range of artificial (A) and natural (N) substrata (fig. 4.2.). Settlement assays were done sequentially, due to insufficient numbers of larvae at a synchronous cyprid stage to conduct all the assays in parallel. Artificial and natural substrata were evaluated separately, in nC and mC settlement assays, always using the adults and a blank (untreated polystyrene IWAKI® plates) as control to reveal underlying interactions and larval preferences. Natural substrata tested included conspecific adults, uncolonized natural rocks, natural rocks cleared, but not completely cleaned of previous *P. pollicipes*, allospecific species such as *Chthamalus* sp., *Corallina* sp. and encrusting algae, and adult stalk cuticle, clean of all other tissues. All natural substrata (excluding stalk cuticle) were attached to natural rocks, sectioned to identical area (2.25 cm^2). To minimize differences in surface area on substrata not fully flat, surfaces such as *Chthamalus* sp., *Corallina* sp and incrusting algae were selected for testing with full surface coverage, while for *P. pollicipes*, individuals were measured to calculate surface area and selected accordingly. Artificial substrata included clay, slate, nylon mesh, PVC, epoxy biomimetic 1, epoxy biomimetic 2 (described below), Tufnol, glass epoxy, carbon epoxy, PMMA, nylon, calcium silicate and glass vinyl ester. All artificial surfaces were 2.25 cm^2 (1.5 per 1.5 cm). Biomimetics were cast in two types of epoxy resin (1) epoxy 199-1468 RS® and (2) epoxy E45TZ ENT (Terrarientechnik®) with moulds taken with material for dental impression (Extrude Medium-28416 Kerr®) from *P. pollicipes* stalk cuticle, dissected from the adults and cleaned.

Preliminary experiments testing many substrata indicated the importance of using larger working volumes than the standard 24-well plate assays (e.g. Elbourne et al., 2008), and the necessity for water movement to stimulate cyprid activity. Consequently, no-choice assays were conducted in 50 mL Falcon tubes®, 0.2 µm natural FSW, with one surface per tube and 50 cyprids (1 larvae ml^{-1}). The assays were run under the same conditions as described before for larval settlement testing, with the tubes (9 replicates per substrata) placed on an orbital shaker (as above), to ensure water movement and

monitored for temporarily and permanently attached larvae after 48h, and metamorphosis after 1 week. Multiple-choice assays, were conducted in 500 ml conical flasks (as standard). The settlement surfaces were fixed to 60 µm plankton mesh (1.5 x 12 cm; 4 substrata per mesh) and hung vertically from the walls of the conical flasks. To each flask, holding in total 8 or 15 test substrata for natural and artificial substrata, 500 cyprids were added (1 larvae ml^{-1}). Cyprids were monitored for temporary and permanent attachment, and metamorphosis as described before.

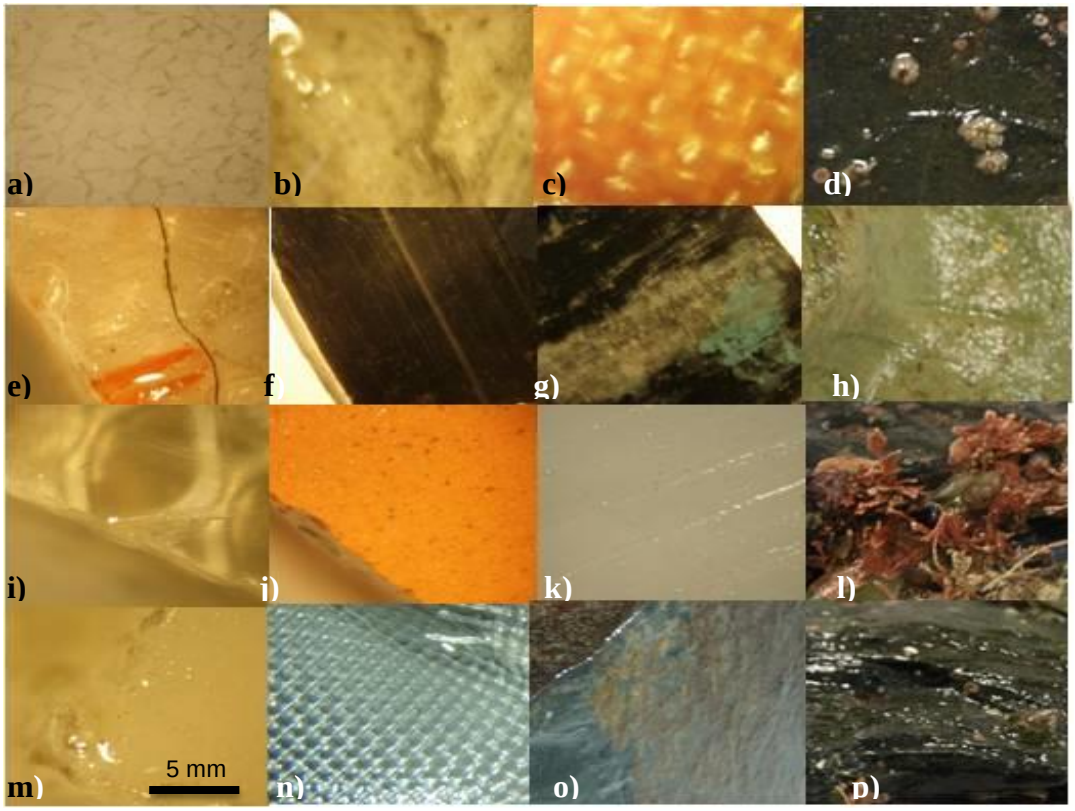


Fig. 4.2 Detail of artificial and natural substrata tested. Artificial substrata tested were as follows: (a) epoxy biomimics, (b) calcium silicate, (c) Tufnol, (d) *Chthamalus* sp., (e) glass epoxy, (f) carbon epoxy, (g) PMMA, (h) marine epoxy, (i) glass vinyl ester, (j) clay, (k) nylon, (l) *Corallina* sp., (m) PVC, (n) nylon mesh, (o) slate, (p) natural rocks.

4.2.2.3. RECRUITMENT TO NATURAL AND ARTIFICIAL SUBSTRATA IN THE FIELD

Artificial substrata were deployed in the intertidal zone (Cabo Sardão, Portugal, 37°36'24.70", -8°49'2.00"), in mid-April, at the start of the breeding season. Substrata (mentioned above) were distributed and deployed (7 replicate structures of each type) within an area of 10 m^2 in various orientations and tidal heights, within the natural limits of *P. pollicipes* distribution. Control groups of previously colonized natural rocks were also established within each location. No frames were used to mount the substrata, due to the characteristics of the terrain and the difficulty of safely attaching large panels for prolonged periods without significant ecological impact. Instead, the structures were

attached to the rocks with marine epoxy (Z-Spar Marine Epoxy®), placed under each substrate and at the edges to reduce drag/pull forces. After 6 months, in mid-October, the substrata were retrieved and the *P. pollicipes* recruits were counted and mapped.

Concurrently, a second set of natural rocks (7 replicates) presenting barnacles grown in culture was transferred to the shore in order to assess relative growth and survival in the field, as well as the tolerance of captively-reared juveniles for a 6-month grow-out period. These rocks, holding only the primary rock substrata and the juveniles (≥ 0.9 mm RC) of *P. pollicipes*, were attached to the natural rocks with marine epoxy, as previously described. However, during the 6-month period, heavy settlement was observed on these natural substrata which became colonized not only by new *P. pollicipes* recruits, but also *Chthamalus* sp. and *Corallina* sp., which were found attached to the rock, epoxy and around the juveniles of *P. pollicipes*. This created, within each rock, distinct settlement areas for *P. pollicipes* larvae, with different biological and physico-chemical characteristics, as recruits were found settled in association with (1) the primary rock, (2) the marine epoxy, (3) *P. pollicipes* juveniles, (4) *Chthamalus* sp. and (5) *Corallina* sp. aggregates. Therefore, although not being the primary experimental goal of the adopted set-up, resulting settlement data per area were recorded and analysed. Each rock was considered to have five areas (bare rock, marine epoxy, *P. pollicipes*, *Chthamalus* sp. and *Corallina* sp.) and counts were made for the number of recruits within each area. The rocks were photographed and the area occupied by each type of surface was measured using Image J®.

4.2.3 Data analysis

Results were analysed using Statistica ®, with a significance level of 0.05. Data analysis was carried out on the basis of homogeneity of variances (Levene's test) and normality (Kolmogorov-Smirnof test). Significant differences were detected by using one-way ANOVA, followed by a post-hoc Tukey HSD test. Data as percentages were arcsine transformed pre-analysis. All results are presented as mean ± SE.

4.3. Results

4.3.1. Environmental and cyprid-related factors affecting settlement on conspecifics in the laboratory

Temporary, permanent attachment, and metamorphosis of larvae settled on the adults, according to the different environmental conditions of hydrodynamics, temperature, salinity, light and cyprid-related factors, as ageing and batch, are shown in Figs. 4.3, 4.4 and 4.5.

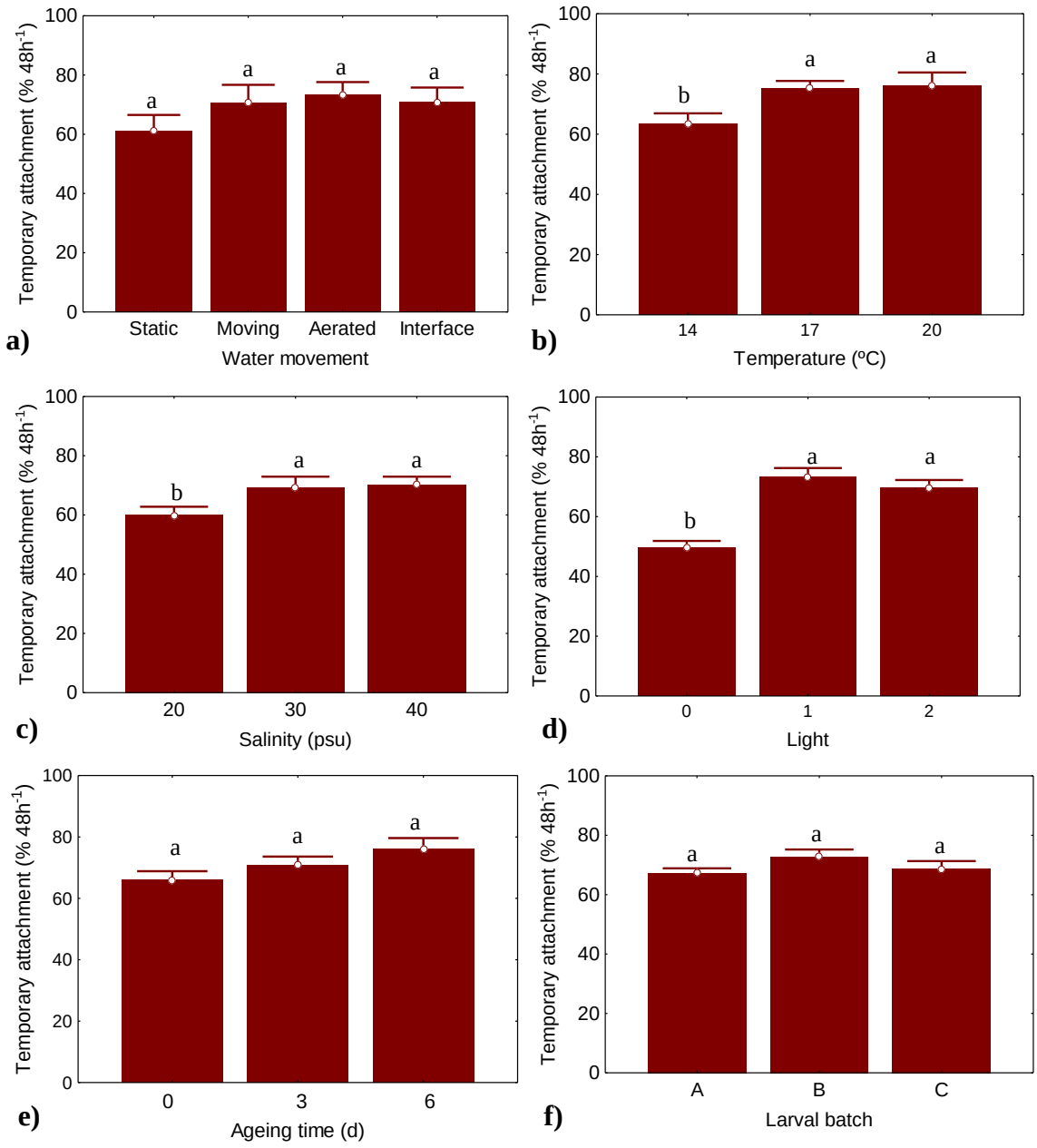


Fig. 4.3 Temporary attachment (% $48h^{-1}$) of *P. pollicipes* larva, allowed to settle on the adults in culture, under various environmental conditions, of hydrodynamics (*a*), temperature (*b*), salinity (*c*) and light (*d*), as well as according to various cyprid related factors, such as cyprid age (*e*) and batch orygin (*f*). Hydrodynamic conditions tested (a) were as follows: (static) no water movement, (moving) circular water movement, (aerated) water kept under constant aeration and (interface) surface not fully submerse, but with interface between air and water. Temperature tested (b) included 14, 17 and 20 ºC, and salinities (c) were of 20, 30 and 40 psu. Light treatments (d) were as follows: (0) 0:24 L/D photoperiod on a no-choice assay; (1) 24/0 L/D photoperiod, created by all surrounding light, on a no-choice assay; (2) 24:0 L/D photoperiod, created with a single light source, on a double choice assay between the side directly exposed to light (*light*) or shaded from light (*shade*). Cyprid ageing (e) was done for 0, 3 and 6 days and batch tested (f) were collected form adults during the breeding season on the years of 2011 (*A*), 2012 (*B*), and 2013 (*C*).

Hydrodynamic conditions did not affect temporary attachment significantly (ANOVA; $F=1.10$, $p=0.36$), with 69.03 ± 2.59 % of larva found temporarily attached to the adult, although significant differences were found in relation to permanent attachment (ANOVA; $F=12.23$, $p<0.05$) between treatments (fig. 4.3a, 4.4a and 4.5a). Permanent attachment was significantly lower for larvae in static conditions (12.67 ± 1.62 %) compared to the other treatments (29.93 ± 2.98 %; Tukey Test; $p\leq0.01$), which were not significantly different from each other (Tukey Test; $p\geq0.17$). Larvae maintained in static conditions were less active than those kept in hydrodynamic conditions. Interestingly, larvae in the interface treatment generally swam predominantly close to the water surface and settled at the meniscus. The percentage of larvae metamorphosing within a week, was not affected by hydrodynamic conditions (ANOVA; $F=0.27$, $p=0.84$), averaging 73.81 ± 1.69 %.

Temperature (fig. 4.3b, 4.4b and 4.5b) significantly affected temporary attachment (ANOVA, $F=4.16$, $p=0.03$). Larvae kept at 14 ºC showed significantly lower percentage of temporary attachment (63.33 ± 3.59 %; Tukey Test, $p=0.04$) compared to larvae at 20 ºC (76.22 ± 4.26 %), although not different to larvae at 17 ºC (75.22 ± 2.45 %; Tukey Test, $p=0.97$). Larvae kept at higher temperatures were considerably more active than at lower temperatures. Nevertheless, no significant differences were observed for permanent attachment (ANOVA; $F=0.32$, $p=0.73$), which averaged 32.30 ± 1.26 % across treatments. However, total metamorphosis over the first week varied significantly according to treatment (ANOVA; $F=9.00$; $p<0.01$), increasing in line with temperature from 59.78 ± 3.81 % at 14 ºC to 79.22 ± 2.44 % at 20ºC (Tukey Test; $p<0.01$).

Salinity affected temporary attachment (ANOVA; $F=3.41$, $p<0.05$), permanent attachment (ANOVA; $F= 5.69$, $p<0.01$) and metamorphosis rate (ANOVA; $F=13.45$, $p<0.01$) (fig. 4.3c, 4.4c and 4.5c). Larvae allowed to settle at 20 psu showed significantly lower percentage of permanent settlement (23.78 ± 1.98 %; Tukey Test; $p<0.04$) when compared to larvae at 30 and 40 psu (on average 31.56 ± 1.91 %). The same pattern was observed for metamorphosis with 52.11 ± 2.84 % at 20 psu and averaging 69.11 ±1.97 % at higher salinities (30 and 40 psu; Tukey Test; $p<0.01$).

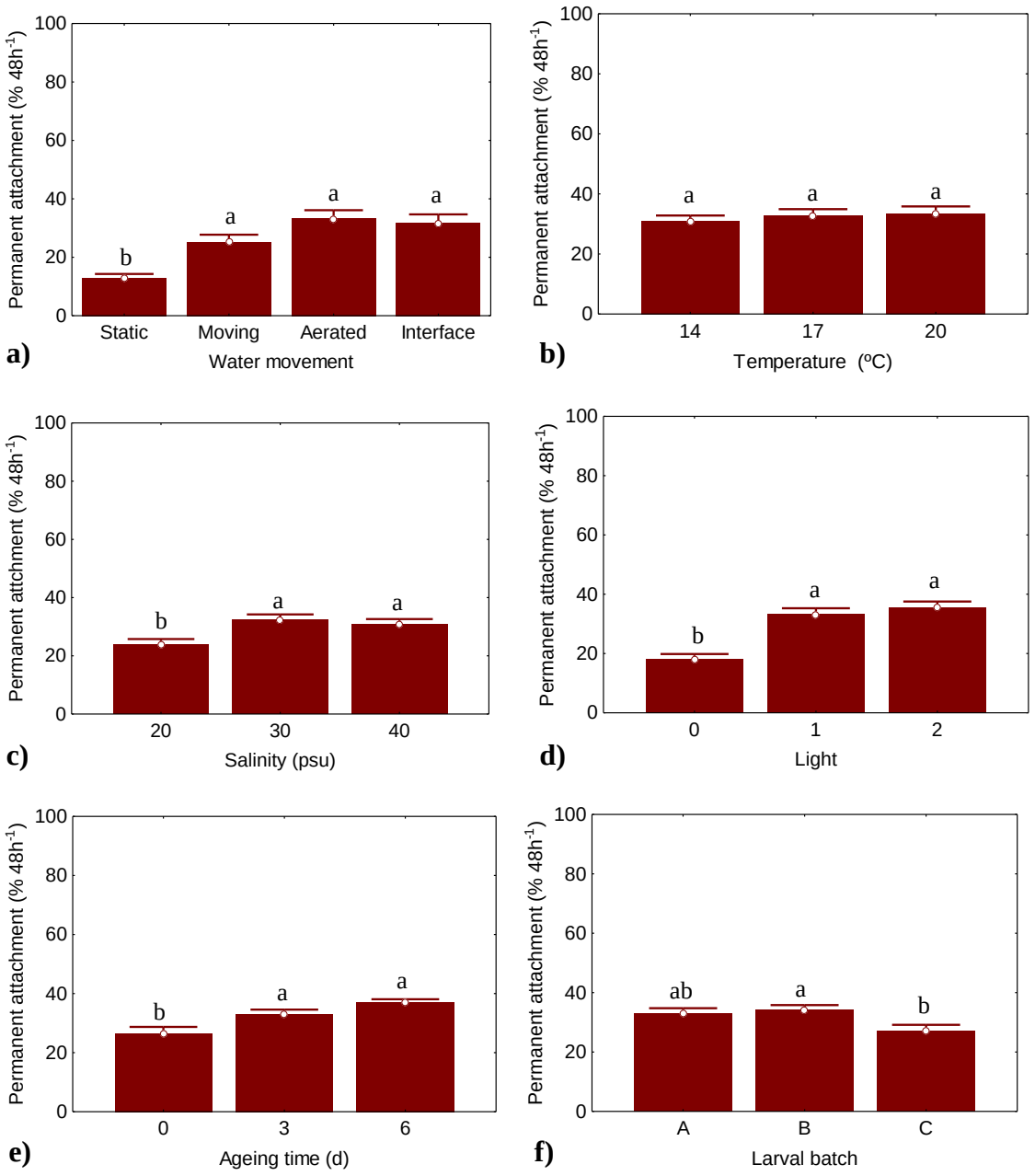


Fig. 4.4 Permanent attachment (% $48h^{-1}$) of *P. pollicipes* larva, allowed to settle on the adults in culture, under various environmental conditions, of hydrodynamics (*a*), temperature (*b*), salinity (*c*) and light (*d*), as well as according to various cyprid related factors, such as cyprid age (*e*) and batch orygin (*f*). Hydrodynamic conditions tested (a) were as follows: (static) no water movement, (moving) circular water movement, (aerated) water kept under constant aeration and (interface) surface not fully submerse, but with interface between air and water. Temperature tested (b) included 14, 17 and 20 ºC, and salinities (c) were of 20, 30 and 40 psu. Light treatments (d) were as follows: (0) 0:24 L/D photoperiod on a no-choice assay; (1) 24/0 L/D photoperiod, created by all surrounding light, on a no-choice assay; (2) 24:0 L/D photoperiod, created with a single light source, on a double choice assay between the side directly exposed to light (*light*) or shaded from light (*shade*). Cyprid ageing (e) was done for 0, 3 and 6 days and batch tested (f) were collected form adults during the breeding season on the years of 2011 (*A*), 2012 (*B*), and 2013 (*C*).

Light significantly affected larval temporary attachment (fig. 4.3d; ANOVA; F=63.46, p<0.01), with settlement assays conducted in the dark leading to lower temporary attachment (49.67 ± 2.20 %; Tukey Test; p<0.01) in comparison to full light (69.67 ± 2.58 %) and light-dark choice assay (73.33 ± 2.92 %). Likewise permanent attachment (fig. 4.4d; ANOVA; F=39.89, p<0.01; Tukey Test; p<0.01) was 16 % higher in light treatments (Table 4.2). This preference for settlement in light conditions was confirmed in the choice assay where, from the 33.11 ± 2.17 % of settled larvae, 26.11 ± 2.04 % settled in the light and 7.00 ± 0.64 % settled in the shade (Tukey Test; p<0.01), in spite of the lack of differences in terms of temporary attachment (Tukey Test; p=0.07). No differences were observed in metamorphosis within the first week (ANOVA; F=0.34, p=0.84), which averaged 72.82 ± 1.03 % (fig. 4.5d).

Light	Temporary attachment (%)	Permanent attachment (%)	Metamorphosis (%)
Dark – no choice	48.67 ± 2.20	18.00 ± 1.83	73.89 ± 3.06
Light – no choice	69.67 ± 2.58	35.44 ±2.04	71.56 ± 2.01
Light or shade – choice	73.33 ± 2.92	33.11 ±2.17	72.89 ± 1.18
Light exposed	40.89 ± 1.72	26.11 ± 1.83	71.33 ± 2.03
Shaded	32.44 ± 1.43	7.00 ± 0.65	74.44 ± 3.06

Table 4.2. Temporary attachment (% $48h^{-1}$), permanent attachment (% $48h^{-1}$) and metamorphosis (% w^{-1}) of larva allowed to settle on the adults, in culture, at different light conditions. Treatments were as follows: (*dark – no choice*) 0:24 L/D photoperiod on a no-choice assay; *(light – no choice*) 24/0 L/D photoperiod, created by multiple surrounding light sources, on a no-choice assay; (*light or shade – choice*) 24:0 L/D photoperiod, created y a single light source, on a double choice assay between the side directly exposed to light (*light*) or shaded from light (shade). Cyprid numbers for the choice assay were counted for both *light* and *shade* sides separately and added for calculation of the total values for the *light or shade* vales. Differences between treatments were significant for temporary attachment (ANOVA; F=63.46, p<0.01) permanent attachment (ANOVA; F=39.89, p<0.01), but not metamorphosis (ANOVA; F=0.34, p=0.84). Values are presented as mean ± SE.

Cyprid ageing (fig. 4.3e, 4.4e and 4.5e) did not affect temporary attachment (ANOVA; F=2.57, p=0.10), which averaged 80.89 ± 1.93 %, but had significant effects on permanent attachment (ANOVA; F=8.77, p<0.01) and metamorphosis (ANOVA; F=5.71, p<0.01). Permanent attachment was higher with aged larvae (32.89 ± 1.69 for 3-days and 36.89 ± 1.19 for 6-days larvae; not significantly different; Tukey Test; p=0.27), than non-aged larvae (26.44 ± 2.28 %; Tukey Test; p≤0.04). However, successful metamorphosis within the first week decreased with ageing and was significantly lower for larvae aged for 6 days (60.89 ± 2.61; Tukey Test, p<0.05). Larvae in all groups presented visible numbers of lipid droplets, and although the total

volume of these was not calculated, no clear differences between treatments were observed.

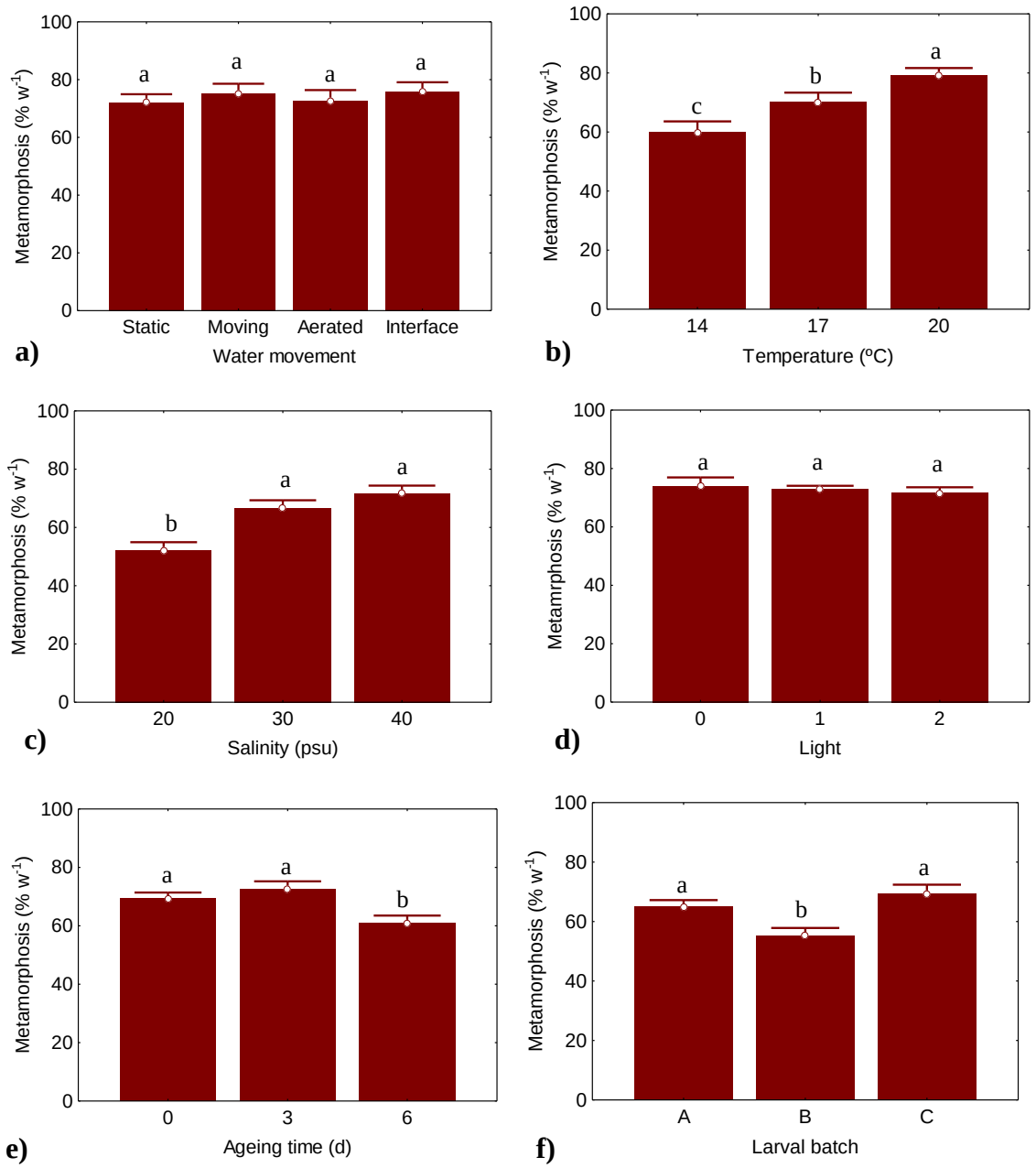


Fig. 4.5 Metamorphosis (% w^{-1}) of *P. pollicipes* larva, allowed to settle on the adults in culture, under various environmental conditions, of hydrodynamics (*a*), temperature (*b*), salinity (*c*) and light (*d*), as well as according to various cyprid related factors, such as cyprid age (*e*) and batch orygin (*f*). Hydrodynamic conditions tested (a) were as follows: (static) no water movement, (moving) circular water movement, (aerated) water kept under constant aeration and (interface) surface not fully submerse, but with interface between air and water. Temperature tested (b) included 14, 17 and 20 ºC, and salinities (c) were of 20, 30 and 40 psu. Light treatments (d) were as follows: (0) 0:24 L/D photoperiod on a no-choice assay; (1) 24/0 L/D photoperiod, created by all surrounding light, on a no-choice assay; (2) 24:0 L/D photoperiod, created with a single light source, on a double choice assay between the side directly exposed to light (*light*) or shaded from light (*shade*). Cyprid ageing (e) was done for 0, 3 and 6 days and batch tested (f) were collected form adults during the breeding season on the years of 2011 (*A*), 2012 (*B*), and 2013 (*C*).

Permanent attachment (ANOVA; F= 4.13, p=0.03) and metamorphosis rate (ANOVA; F=7.45, p<0.01) varied with larval batch (fig. 4.3f, 4.4f and 4.5f). These differences were significant between batches B and C, for permanent attachment (Tukey Test; p=0.03) and B and A as well as C, for metamorphosis (Tukey Test; p=0.04 and p<0.01, respectivelly), with all other comparisons not significantly different (Tukey Test; p≥0.08). Permanent attachment varied between 27.00 ± 2.16 and 34.11 ± 1.69 %, while metamorphosis ranged between 55.22 ± 2.61 and 69.33 ± 3.06 %. Batch differences therefore accounted for variations of the order of 7 and 15 % respectively for permanent attachment and metamorphosis for groups of larvae reared under identical conditions, but collected from different groups of adults in consecutive years. Larval exploratory behaviour, however, was not different between batches, with no significant differences in temporary attachment (ANOVA; F=1.60, p=0.22).

With regard to larval preferences over settlement areas on the adult body (previous fig. 4.1), significant differences were observed between zones for temporary attachment (ANOVA; F=7.21, p<0.01) and permanent attachment (ANOVA; F=675.32, p<0.01), but not for metamorphosis (ANOVA; F=0.14, p=0.97; Table 4.3).

	Body zone	Temporary attachment (%)	Permanent attachment (%)	Metamorphosis (%)
A	Stalk base	12.33 ± 1.97 ^{a}	5.00 ± 0.45 ^{a}	71.22 ± 3.97 ^{a}
B	Lower stalk	21.44 ± 2.01 ^{b}	7.67 ± 0.53 ^{ab}	73.11 ± 3.53 ^{a}
C	Upper stalk	24.89 ± 1.98 ^{b}	9.69 ± 0.71 ^{b}	72.22 ± 3.43 ^{a}
D	Stalk-capitulum	22.44 ± 1.60 ^{b}	16.68 ± 0.65 ^{c}	72.67 ± 2.56 ^{a}
E	Capitulum	18.89 ± 1.24 ^{b}	61.00 ± 1.62 ^{d}	70.11 ± 2.27 ^{a}

Table 4.3. Temporary attachment (% $48h^{-1}$), permanent attachment (% $48h^{-1}$) and metamorphosis (% w^{-1}) of larva allowed to settle on the adults, in culture, and monitored according to settlement zone in the body (A, B, C, D, E; according to Fig. 4.2). The adult body was divided in zone (A) stalk base, (B) lower stalk, (C) upper stalk, (D) stalk-capitulum interface, and (E) capitulum. Differences between zones were significant for temporary attachment (ANOVA; F=7.21, p<0.01), permanent attachment (ANOVA; F= 675.31, p<0.01), but not metamorphosis rate (ANOVA; F=0.141, p=0.97). Values are presented as mean ± SE.

Temporary attachment from the lower stalk upwards ranged between 18 and 25 % (Tukey Test, p≥0.65), with the base of the peduncle recording the lowest values (Tukey Test, p<0.01) of 12.33 ± 1.97 %. Permanent attachment increased towards the capitulum, from 5 – 8 % at the base and lower stalk (not different; Tukey Test, p=0.24), to significantly higher values above the upper stalk (Tukey Test, p<0.01), different between the upper stalk (9.67 ± 0.71 %), interface (16.67 ± 0.65 %) and on the capitular

plates, which recorded the highest values (61.00 ± 1.62 %; fig. 4.6). Larvae settled between the scales on the stalk and between capitular plates (fig. 4.7). Variation in the stalk cuticle of different barnacles is shown in Fig.4.8.

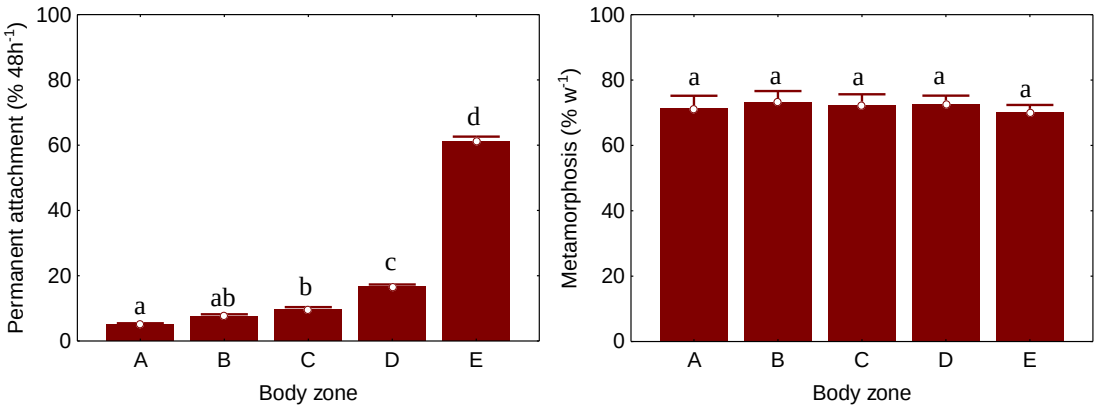


Fig. 4.6 Permanent attachment (a - % $48h^{-1}$) and metamorphosis (b - % w^{-1}) of larva allowed to settle on the adults, in culture, and monitored according to settlement zone in the body (A, B, C, D, E). The adult body was divided in zones (A) stalk base, (B) lower stalk, (C) upper stalk, (D) stalk-capitulum interface, and (E) capitulum.

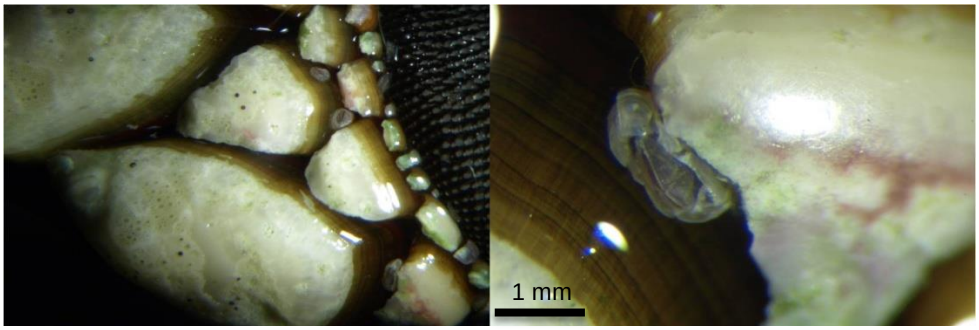


Fig. 4.7 Early spats of *P. pollicipes* after settlement on the capitulum of the conspecific adult. Cyprid were often found exploring the ridges between the capitular plates, followed by permanent attachment and metamorphosis to early spats. Even when observed out of water, the cyprid or spat were always covered with a water cuticle.

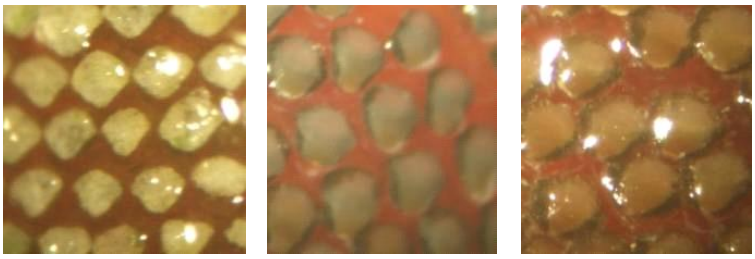


Fig. 4.8 Examples of stalk cuticle of various adults of *P. pollicipes*.

Significant differences were recorded for temporary (ANOVA; $F=338.17$, $p<0.01$) and permanent attachment (ANOVA; $F=102.04$, $p<0.01$; fig. 4.9a) with time. Attachment was observed to increase over the first 48h, after which it stabilized, with no difference in the attachment rates after 48h (Tukey Test, $p\geq0.14$). Similarly, metamorphosis also

varied with time (ANOVA; F=467.34, p<0.01), increasing for the first 6 days (Tukey Test, p<0.01), to 65.33 ± 2.19 %, after which the number of larvae metamorphosing decreased drastically, reaching 80.67 ± 1.66 % by the 12th day (fig. 4.9b). About 10 % of cyprids were observed to have failed to metamorphosed even after 18 days.

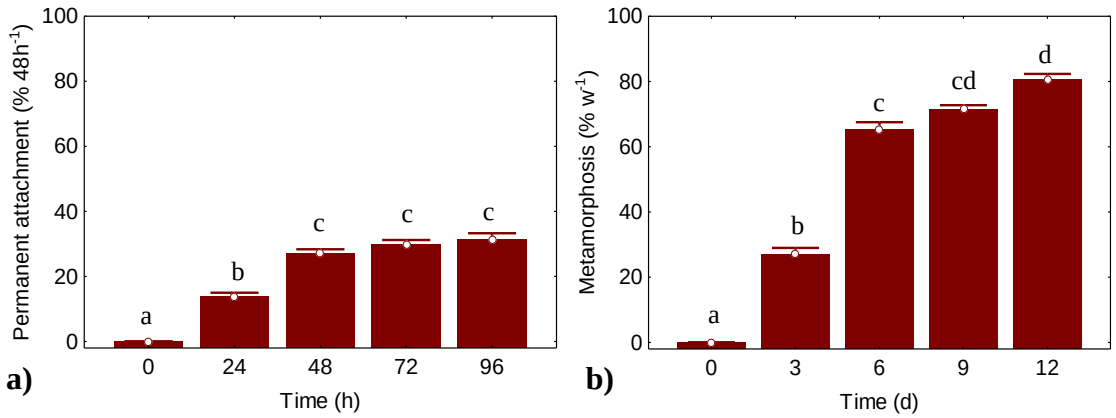


Fig. 4.9 Permanent attachment (a - %) and metamorphosis (b - %) of larva allowed to settle on the adults, in culture, according to time. Permanent attachment was observed in 24h intervals for a period of 96h, while metamorphosis was monitored every 3 days for a period of 12 days post-settlement.

When larval survival in culture was evaluated in the absence of substrata, cyprids were observed to live for extended periods, with mortality after 20 days at around 50 %. Survival for the first 6 days post-metamorphosis to the cyprid was of over 95 %, slowly declining until day 16 (to ≈70 %), at which point a steeper decline commenced (fig. 4.10). Cyprid activity declined with time, as well as visible lipid reserves (fig. 4.11). Live cyprids were observed even after 30 days in culture, although the amount of available lipid was virtually non-existent and cyprid activity was minimal.

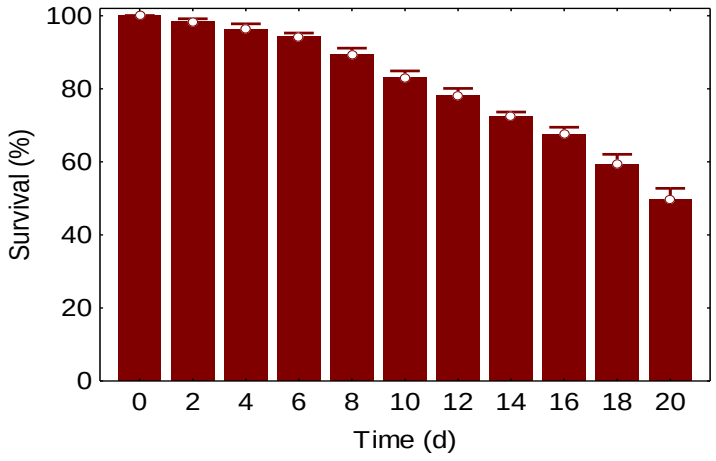


Fig. 4.10 Cyprid survival (%) over time under culture. Monitoring was done at 2 day intervals for a period of 20 days.

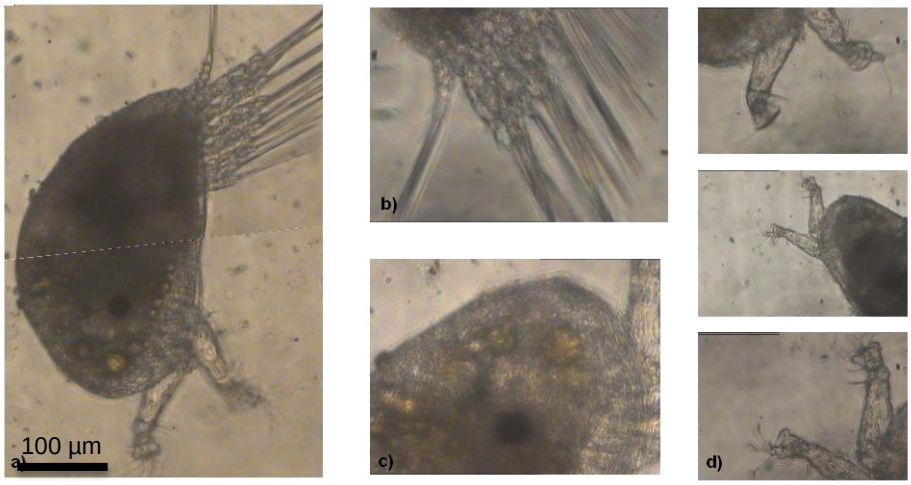


Fig. 4.11 Cyprid of *P. pollicipes* on optical microscopy, where (a) cyprid lateral view, (b) detail of swimming appendages, (c) detail of lipidic drops, and (e) detail of feet.

4.3.2. Settlement on natural and artificial substrata in culture

The results of settlement on natural and artificial substrata are shown in Tables 4.4 and 4.5, respectively. The adults were the preferred substrata for attachment, both in no-choice and multiple choice assays and independently of the other substrata tested.

Substrata	nC assay			mC assay
	Temporary attachment (%)	Permanent attachment (%)	Survival (%)	Permanent attachment (%)
Adults	72.44 ± 2.19	29.00 ± 1.66	97.56 ± 0.63	41.11 ± 10.03
Blank	9.00 ± 0.90	0.78 ± 0.28	96.11 ± 1.97	0.00 ± 0.00
Rocks uncol.	17.78 ± 2.29	5.44 ± 1.17	89.67 ± 1.71	1.00 ± 0.37
Rocks cleared	31.89 ± 2.61	16.67 ± 2.03	89.11 ± 2.16	4.61 ± 1.22
Chthamalus sp.	25.44 ± 1.45	12.11 ± 0.98	91.56 ± 1.67	2.17 ± 0.76
Corallina sp.	15.67 ± 1.29	8.56 ± 1.26	91.89 ± 2.21	1.06 ± 0.41
Incrusting algae	13.22 ± 1.40	4.11 ± 0.79	88.11 ± 1.90	0.44 ± 0.20
Stalk cuticle	3.00 ± 1.00	0.00 ± 0.00	18.11 ± 10.10	0.00 ± 0.00

Table 4.4. Temporary attachment (% $48h^{-1}$), permanent attachment (% $48h^{-1}$) and survival (% $48h^{-1}$) of larva allowed to settle on a variety of natural substrata, including the adults, on a no-choice assay (nC assay), as well as under multiple-choice assay (mC assay) for permanent attachment (% $48h^{-1}$).. The substrata provided were as follows (1) adults, (2) blank, (3) rocks ucolonized, (4) rocks cleared, (5) *Chthamalus* sp., (6) *Corallina* sp., (7) incrusting algae, and (8) stalk cuticle. For the non-choice assay, no statistical analysis was preformed due to the high variability effects on the power of parametric statistical tests. For the multiple-choice assay, the same was also true, while data on temporary attachment showed an identical trend, being therefore not represented, and larval survival was of 96 % $48h^{-1}$. Values are presented as mean ± SE.

Substrata	nC assay			mC assay
	Temporary attachment (%)	Permanent attachment (%)	Survival (%)	Permanent attachment (%)
Adults	71.66 ± 2.25	27.89 ± 1.37	98.67 ± 0.58	31.22 ± 1.50
Blank	9.00 ± 0.90	0.56 ± 0.24	99.22 ± 0.47	0.56 ± 0.24
Clay	18.44 ± 1.92	0.33 ± 0.17	88.78 ± 2.27	0.11 ± 0.11
Slate	21.22 ± 1.43	1.56 ± 0.50	95.78 ± 0.93	0.00 ± 0.00
Nylon mesh	16.56 ± 1.72	0.22 ± 0.15	95.89 ± 0.59	0.11 ± 0.11
PVC	15.11 ± 1.17	0.67 ± 0.23	92.89 ± 0.75	0.00 ± 0.00
Epoxy resin 1	3.67 ± 1.00	2.44 ± 0.64	46.44 ± 3.84	0.00 ± 0.00
Epoxy resin 2	17.56 ± 2.47	1.00 ± 0.44	94.11 ± 0.63	0.11 ± 0.11
Tufnol	10.67 ± 0.87	0.78 ± 0.28	94.33 ± 0.83	0.00 ± 0.00
Glass epoxy	9.44 ± 1.09	0.88 ± 0.26	95.00 ± 0.97	0.11 ± 0.11
Carbon epoxy	13.00 ± 0.96	0.56 ± 0.24	94.55 ± 0.56	0.11 ± 0.11
PMMA	8.00 ± 1.71	0.33 ± 0.17	94.78 ± 1.01	0.00 ± 0.00
Nylon	10.44 ± 1.52	0.34 ± 0.24	90.00 ± 0.97	0.11 ± 0.11
Calcium silicate	8.67 ± 1.15	0.22 ± 0.15	93.78 ± 1.64	0.00 ± 0.00
Glass vinyl ester	11.33 ± 1.83	0.23 ± 0.14	94.33 ± 1.00	0.00 ± 0.00

Table 4.5. Temporary attachment (% $48h^{-1}$), permanent attachment (% $48h^{-1}$) and survival (% $48h^{-1}$) of larva allowed to settle on a variety of artificial substrata, on a no-choice assay (nC assay), using the adults as control group, as well as under multiple-choice assay (mC assay) for permanent attachment (% $48h^{-1}$). The substrata provided were as follows (1) adults, (2) blank, (3) clay, (4) slate, (5) nylon mesh, (6) PVC, (7) epoxy resin 1, (8) epoxy resin 2, (9) tufnol, (10) glass epoxy, (11) carbon epoxy, (12) PMMA, (13) nylon, (14) calcium silicate, and (15) glass vinyl ester. For the non-choice assay, no statistical analysis was preformed due to the high number of zeros and inherent variability effects on the power of statistical tests. For the multiple-choice assay, the same was valid, while data on temporary attachment showed an identical trend, being therefore not represented, and larval survival was of 95 % $48h^{-1}$. Values are presented as mean ± SE.

When a choice was given between adults and other substrata, the majority of cyprids chose to settle on the adults. Settlement on the adult, of cyprids given a choice between settling on the adult and on artificial substrata, was 31.22 ± 1.50 %, while against natural substrata settlement on conspecifics was 41.11 ± 10.30 %. This corresponds respectively to 97 % and 82 % of settled larvae, tested against artificial and natural substrata. In situations of choice, the percentage of larvae settled on the various artificial substrata was below 1 %. On natural substrata, settlement to surfaces other than the adult varied up to 5 %, with highest settlement on cleared rocks (previously colonized by *P. pollicipes*) and *Chthamalus* sp.. In conditions of no-choice, permanent settlement on substrata other than the adult increased. Settlement on artificial structures was never above 3 %, while on natural structures it reached 16.67 ± 2.03 % and 12.11 ± 0.98 % on cleared rocks and *Chthamalus* sp., respectively. Other surfaces such as *Corallina* sp., encrusting algae and uncolonized rocks had settlement rates between 4 and 9 %. Interestingly, temporary attachment varied widely according to substrata,

ranging between 3 and 21 % on artificial substrata and between 9 and 31 % for natural substrata, and about 70 % on the adults in both cases. Survival was generally high (> 88 %) on all the substrata tested, with the exception of the epoxy resin 1 and the stalk cuticle.

4.3.3. Recruitment on natural and artificial substrata in the wild

Recruitment on artificial structures placed in the wild was zero, regardless of the type of substrata. Nevertheless, there was visible colonization by other species, such as *Corallina* sp., especially on the stalk biomimics cast in epoxy resin (fig. 4.12).

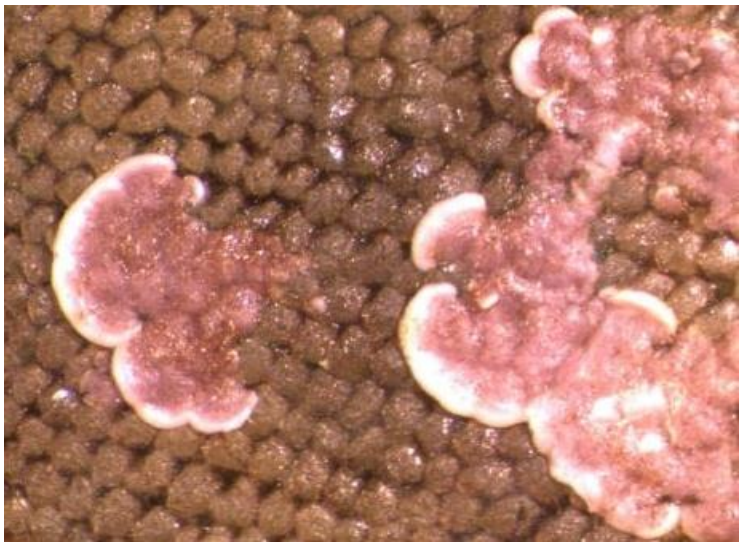


Fig. 4.12 Epoxy biomimics form stalk cuticle, placed in the wild during *P. pollicipes* breeding season, covered in *Corallina* sp.

On the contrary, recruitment was observed on all of the natural substrata, as well as on the marine epoxy used to fix the substrata to the rocks, with the percentage of recruits varying with substrata (ANOVA; $F=15.39$, $p<0.01$). An example of this is shown in Fig. 4.13. Most recruits settled on epoxy (49.37 ± 5.81 %), on and around conspecific adults (22.31 ± 6.01 %) and in association with *Corallina* sp. (15.54 ± 2.79 %; fig. 4.14a). However, the surface occupied by each substrata also varied significantly (ANOVA; $F=7.71$, $p<0.01$), affecting the surface area available for colonization.

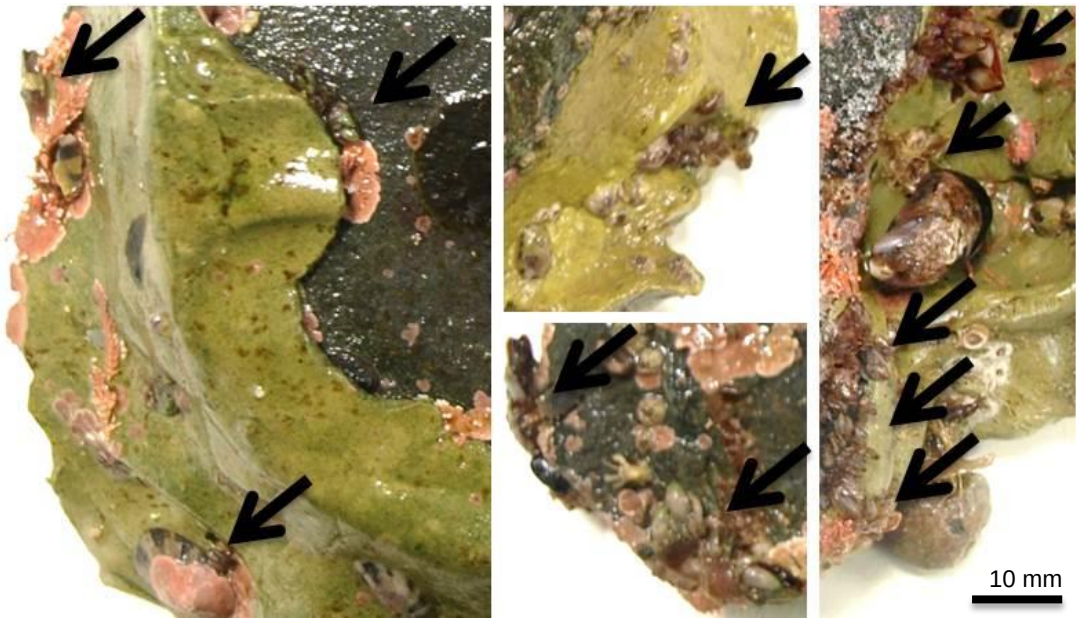


Fig. 4.13 Recruits of *P. pollicipes* found settled on the epoxy area of the natural structures placed in the wild. Arrows indicate where recruits were found settled.

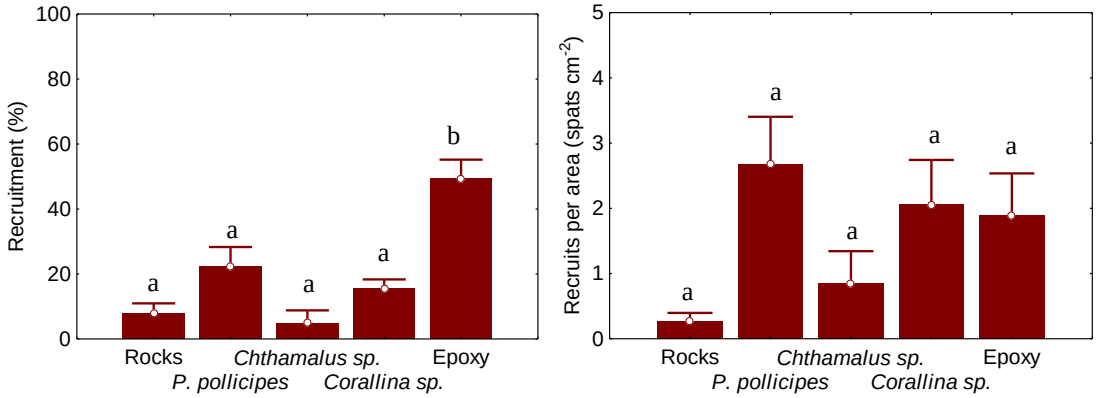


Fig. 4.14 Recruitment in natural rocks with captivity grown *P. pollicipes* adults, placed in the wild for a period of 6 months during breeding season, according to a) total number of spat and b) number of recruits per area. At the time of transfer the rocks were cleaned of species other than *P. pollcipes*. In each rock, after 5 months in the wild, distinct areas were identified, as follows: (rock) base rock, without settled species; (*P. pollicipes*) conspecifc adults transplanted from culture and with no recruits by the time of surface transferring; (*Chthamalus* sp.) colonizing adults of *Chthamalus* sp.; (*Corallina* sp.) colonizing *Corallina* sp.; and (epoxy) Z-Spar Marine Epoxy ® used for gluing the transplanted rocks to the rock in the wild. The area occupied by each surface varied (ANOVA; $F=7.71$, $p<0.01$), being of 49.08 ± 11.24, 32.89 ± 7.02, 16.50 ± 6.47, 8.31 ± 2.6 and 3.67 ± 1.94 cm^2 respectively for rock, epoxy, *P. pollicipes*, *Corallina* sp. and *Chthamalus* sp..The percentage of recruits varied with substrata (ANOVA; $F=15.39$, $p<0.01$), with the percentage on the epoxy being significantly higher than the on others (Tukey Test; $p<0.01$), but not the number of recruits per area (ANOVA; $F=1.84$, $p=0.05$), due to high variability and uneven area representation.

Rock, epoxy and *P. pollicipes* adults were the best represented surfaces (respectively 49.08 ± 11.24, 32.89 ± 7.02 and 16.50 ± 6.47 cm^2), with both covered by *Corallina* sp. and *Chthamalus* sp. receiving lower settlement. Consequently, the number of recruits per area (fig. 4.14b) did not vary significantly according to substrata (ANOVA; F=1.84, p=0.05). More recruits per area were found settled on the adults (2.68 ± 0.76 spats cm^{-2}), *Corallina* sp. (2.06 ± 0.69 spats cm^{-2}) and epoxy (1.89 ± 0.64 spats cm^{-2}), with the lowest numbers occurring in association with *Chthamalus* sp. (0.84 ± 0.49 spats cm^{-2}) and rock (0.26 ± 0.13 spats cm^{-2}). Over 90 % of spat found on the adults were settled on the adult stalk, which was not observed in culture. Due to the unplanned nature of this experiment it is impossible to state when colonization by *Corallina* sp. and *Chthamalus* sp. occurred and therefore how this may have impacted results.

4.4. Discussion

P. pollicipes cyprids were highly discerning in their choice of settlement substratum, with selectivity and metamorphosis influenced by cyprid and substratum-related factors, as well as the environmental conditions at the time of settlement.

4.4.1. Environmental and cyprid-related factors affecting settlement on conspecifics in the laboratory

Of the cyprid-related factors that might affect settlement on conspecifics in culture, only ageing and larval batch were investigated. Cyprids of *Balanus amphitrite* become less selective for substrata as they age (Rittschof et al., 1984); a phenomenon known as the desperate larva hypothesis (Toonen & Pawlik, 1994). Although temporary attachment did not differ between treatments in the present experiments, settlement increased as larvae aged, consistent with the aforementioned hypothesis. Rittschof et al. (1984) further reported that age-dependent responses of cyprids decrease in the presence of settlement inducers, which may suggest that if settlement was being tested on substrata other than the adult, these settlement differences could be in fact be higher. The temperature at which cyprids are aged is also crucial to this effect. Satuito et al. (1996) reported that storage protein in *B. amphitrite* decreases with time when ageing is carried out at 15, 20 and 25 ºC, but not at 5 ºC. This indicates that at low temperatures the effect could be minimal. Nevertheless, longer ageing implies decreased storage reserves, which signify a reduced competence, or ability to metamorphose, in aged cyprids (Holland and Walker 1975; Lucas et al. 1979; Satuito et al., 1996). This seems to be the case for *P. pollicipes* where, after a week, fewer metamorphosed cyprids were observed in stocks aged for 6 days. This does not imply an absolute decrease in metamorphosis,

rather a possible decrease in rate. A settled cyprid will always attempt to moult to a juvenile barnacle (West & Costlow, 2005), although a minimal oil cell volume is required to ensure successful metamorphosis. As cyprids age, therefore, the number of cyprids incapable of metamorphosis will increase. Furthermore, it should be noted that post-settlement growth can decrease in aged cyprids, even if metamorphosis rate was not affected (Pechenik et al., 1993). Although cyprid ageing (3 days) might be used to increase settlement of *P. pollicipes* cyprids in culture, care should be taken when extending the ageing period to 6 days or more since this may have a detrimental effect on the rate of metamorphosis. There are also interesting implications as regards to what might be happening in the wild. Barnacle cyprids are known to postpone their settlement for varying periods that differ between species (Krug, 2006; Wilson, 1932; Knight-Jones, 1953). However, although cyprids of *P. pollicipes* might be able to survive in the wild for long periods, the ability to successfully settle will likely decrease steadily with time, selecting for only the fittest individuals. In the laboratory, *S. balanoides* lasted 28 days (10 ºC) on energy reserves, after which the capacity to metamorphose was compromised due to a drop in reserves below the critical minimum for moulting (Lucas et al., 1979). As the quality of the storage reserves will determine the possible survival time until a settlement site is identified, the timing of larval release and development rate is of paramount importance.

When larval batch is considered, significant variability was noted for temporary and permanent attachment, as well as the rate of metamorphosis. Although permanent attachment and metamorphosis varied significantly, no clear pattern was observed between batches and their respective rates of metamorphosis. This highlights the fact that all results are batch specific and that, depending on the stock used, settlement can vary significantly. This should be considered when making extrapolations from the present data or comparing between different studies. Differences from 10 to 40 % in settlement can be found between larvae reared under the same conditions in different batches for *B. amphitrite* (Holm et al., 2000). In the present study the maximum differences were approximately 10 %. These are likely to be due to parental as well as environmental effects as discussed by Holm (2000).

Environmental conditions (hydrodynamics, light, salinity and temperature) and their effects on settlement were also investigated. The effects of water movement on barnacle settlement have been widely investigated (e.g. Crisp, 1955; Knight-Jones, 1955; Mullineaux & Butman, 1991). Settlement systems in the laboratory have been shown to

benefit from conditions of flow, which maximize larval movement and larva-surface encounters. Temporary attachment and metamorphosis rate did not vary according to hydrodynamic conditions in this study, although permanent attachment was lower in static conditions. Permanent attachment was higher for larvae kept in aerated or interface conditions, although these were not significantly different from dynamic conditions. According to the type of movement, larval circulation was facilitated (e.g. aeration) or hindered (e.g. orbital shaker). Furthermore, larvae with an air-water interface were observed in the water meniscus and out of water stuck to the walls of the container. This settling behaviour has been observed in other species (e.g. *B. amphitrite*) that when allowed to settle in culture containers, are often found settled at the water line. When observed under the stereomicroscope, the *P. pollicipes* larvae that settled out of water were always covered by a thin water layer that kept the cyprids completely covered, apparently reducing desiccation. The same was observed for the cyprids attached to adults, which were mostly found settled in the ridges between the stalk scales. This can possibly be an adaptive characteristic, which in nature can serve as a way of targeting upper water column levels, by avoiding transportation by bottom currents and likely placement in the intertidal habitat where conspecifics are found. Cyprids of *B. improvisus* and *Elminius modestus* reared in our laboratory often become trapped in the meniscus during assays. Whether this represents an adaptation for habitat selection, or an artefact of rearing under culture (see Di Fino, 2014), remains unconfirmed. Furthermore, the driving forces for this behaviour, either the transport by the currents or bubbles generated, or the larvae actively swimming to light, remain unclear. Other studies have observed that barnacle larvae respond positively to directional flow, within a certain range, accepting or rejecting surfaces accordingly. In fact, Larsson & Jonsson (2006) suggested that larvae of *B. improvisus* that rejected surfaces at flows above 15 cm s^{-1} did so as a way to prevent sub-optimal growth and survival post-settlement. On the other hand, Mullineaux & Butman (1991) reported that although certain flows can promote surface rejection, this does not necessarily lead to lower settlement due to differences in contact frequency between flows. Water movement seems to invariably impact settlement outcome in barnacle assays, with *P. pollicipes* being no exception.

The effects of salinity on settlement have been investigated in several barnacle species (e.g. *B. improvisus*, *B. eburneus*, *B. subalbidus*; Dineen & Hines 1992, 1994a, 1994b). For *P. pollicipes*, the present results suggest that this variable has an effect across

temporary and permanent settlement, with exploration and settlement decreasing (≈ 10 % at 20 psu) at salinities below natural (30 – 40 psu) in a similar way to metamorphosis (≈ 20 %). *P. pollicipes* inhabit coastal environments were salinity ranges between 33 – 37 psu and it is therefore not surprising that larvae will be better adapted to survive and develop within this range. The regulatory effort needed to cope with sub-natural salinities might lead to a decrease in surface exploration, but also a reduced capacity to metamorphose. Other authors (e.g. Dineen & Hines 1994ab) have observed that the response to salinity varies significantly with species selectivity, correlating with their distribution range, but can also be heavily influenced by other factors such as age and the use of barnacle extracts. Barnacles are often maintained at salinities between 30 – 40 psu, although estuarine species, such as *B. improvisus* can cope with salinities in a lower but broader range.

Studies with *P. pollicipes* larvae have shown that all larval stages show positive phototaxis (with the exception of nauplii II, whose results were inconsistent), although cyprid behaviour was not evaluated (Molares et al., 2002). It is here hypothesized that this phototactic behaviour has ecological advantages, allowing the placement of the larvae higher in the water column, which is beneficial for on-shore transport to the intertidal environment. Furthermore, throughout development, the larvae are planktotrophic (except for stage I larvae) and are therefore dependent on phytoplankton abundance for feeding. As cyprids are lecithotrophic, however, transport back to the coast becomes the essential factor. It is therefore not surprising that both temporary and permanent attachment are affected by light, with higher attachment responses when subjected to light in comparison to darkness (≥ 15 % higher attachment). Furthermore, when larvae are given a choice, the overall preference is for permanent settlement under full day photoperiod, with rates almost 4 times higher than those in the shade. No differences were observed in temporary attachment, indicating that the larvae will explore both sides equally and make a choice to settle in light for *P. pollicipes*. The lack of differences in metamorphosis rate suggests that this is independent of light. Results from other authors indicate that settlement can vary with light, with many species showing positive phototaxis and with species-specific preferences for settlement under direct or diffused light (e.g. Daniel, 1957; Crisp & Ritz, 1973; Pawlik, 1992), which have been suggested to be adaptive responses.

Temperature has well documented effects on barnacle larval development and survival (Satuito et al., 1996; Marechal et al., 2004). Results from Coelho (1990) indicate that

the use of higher temperatures significantly decreases larval development time in *P. pollicipes*, as well as survival. This also applies to cyprids, as higher temperatures increase metabolic rate and oxygen consumption, with effects on the depletion of reserves and a reduction in competence (Satuito et al., 1996). In the present study, permanent settlement was not affected by temperature, but temporary attachment was reduced at lower (14 ºC) temperatures. This might be related to the effect of temperature on larval activity, as larvae kept at higher temperatures were often observed to move faster and show more active behaviour. The temperatures tested were within the range observed in the wild during reproductive season. Only three temperatures were considered, but studies on other species (e.g. Thiyagarajan et al., 2003) have shown that settlement can vary according to temperature, with species exhibiting preferential settlement at certain temperatures. Although permanent attachment was not influenced by temperature, metamorphosis rate was higher (≥ 20 %) at higher temperatures (17 and 20 ºC). This can be related to temperature accelerating development in the first week and not necessarily implying a higher rate of metamorphosis at these temperatures. However, results from Anil et al. (2001) on *B. amphitrite* suggest that larvae reared at 20 °C could successfully metamorphose over a shorter period than those reared at 30°C, and therefore temperature effects might also be absolute. The use of higher temperatures with *P. pollicipes* lead to faster cyprid metamorphosis, though the effects on juvenile survival and development were not followed. Pechenik et al. (1993) reported that cyprids that were forced to delay metamorphosis showed slowed post-settlement growth, despite no effects on metamorphosis success and post-settlement survival, highlighting the need for further investigation. In nature, temperatures over the breeding season vary 14 to 24 ºC from May to October, which is within the range tolerated by the larva while in laboratory conditions. Accelerating metamorphosis in response to higher temperatures might represent the added advantage of shortening a transition phase when the larva is highly fragile and dependent on reserves and adequate environmental conditions to successfully moult.

Patterns of *P. pollicipes* larval settlement over time are in accordance with what has already been reported for other species (e.g. Rittschof et al., 1994), with settlement increasing over the first 48h and then slowly stabilising over time. However, the present study has also focused on the acquisition of reference values for *P. pollicipes*. Metamorphosis showed a similar pattern, with most larvae moulting within 6 days (≈ 65 %), increasing up to ≈80 % after 12 days with about 10 % larvae failing to

metamorphose. Kugele & Yule (1996) reported a similar pattern, with 64 % of cyprids metamorphosing in 6 days and 18 % failing to metamorphose. The percentage of cyprids failing to metamorphose in the laboratory, together with the observations of settlement on non-conspecific substrata in the field, suggest that health of laboratory reared larva might be an issue to consider. Survival, on the other hand, showed a steady rate of decline to ≈50 % after 20 days in culture. These values reflect the larval capacity to survive over long periods in the water column. Results from other barnacle larvae indicate particularly long cyprid phases for larvae reared in culture (Batham, 1946). In fact, larvae not provided with settlement substrata were observed, in the present study, to become increasingly lethargic and reduce their activity periods to the minimum, with increasing activity depending upon stimulation (e.g. light, heat).

When settlement on the adult was evaluated according to body region in culture, results indicated a significant preference for temporary attachment in all areas except the base of the peduncle and permanent attachment preferentially in between the capitular plates. Larvae were always found in protected ridges, either between the scales of the stalk or the plates of the capitulum. This might be explained by the exposure of the integument in these locations, acting as a chemical attractant, or associated protection value. Metamorphosis rate did not change according to settlement region, indicating that this is not a factor in site selection. These settlement results, of preferential settlement on the capitulum, contradict our field results and what Cruz (2000) also observed for larval recruitment in the field. Instead, most recruits were found attached to the stalk, similar to the observations by Macho (2006). Interestingly Kugele & Yule (1996) reported that two thirds of the larvae observed to settle on adults in the laboratory did so on the captitulum, in accordance to what was observed herein. These differences between results from laboratory and field suggest that settlement areas on the adult hold distinct survival value, in spite of larval preferences in culture. Settlement on the capitulum, as observed in the laboratory, although preferential, might lead to higher mortality in nature. Whether this is due to biotic factors, such as predation or intraspecific competition, as seen in mussels (e.g. Navarette & Castilla, 1988; Guinez, 1996), or abiotic factors (e.g. temperature stress), remains unknown. However, it also implies that the number of settled larvae in nature could be much higher than the values noted to date. Probably, more likely though, is the explanation that larvae in nature might find higher protection value (e.g. from drag, currents, predators) in the smaller space that exists between the stalk's scales, than the larger ridges in between capitular plates.

Fouling of the capitular plates in the wild could be another reason for low settlement in this region in the wild, as suggested by Hoffman (1989) who also noted that spat were rarely found on the capitular plates except when under constant immersion. Furthermore, it might also be a fact of differences in larval transport across body regions, both to the aggregate effect or to emmersion cycles. Other factors, such as the effect of water speed on larval transport, might also be important since, within complex aggregates such as those observed in barnacle and mussels clusters, water speed can vary between areas of the aggregate that are more exposed (e.g. capitulum) or protected (e.g. stalk). Alvarado & Castilla (1996) explained the greater number of juveniles in the lower layer as a higher retention of recruits in these areas due to the reduced turbulence and higher-retention of suspended matter. This also raises the question of how predictive laboratory settlement assays are of settlement patterns in the wild.

4.4.2. Settlement and recruitment on natural and artificial substrata

Studies carried out in the field often refer to recruitment, and studies done under laboratory conditions often refer to settlement. In the laboratory, post-settlement survival is intrinsically high and the differences between settlement and recruitment may be negligible. In the wild, however, the presence of predators and stress factors (e.g. desiccation, tides, etc.) can significantly impact the number of larvae that survive the period immediately after settlement.

Research on *P. pollicipes* larval settlement in the laboratory is restricted to Kugele & Yule (1996), who investigated settlement on various substrata, and Molares et al. (2002), who assessed the importance of chemical cues to settlement. Results from these studies confirmed the apparently high cyprid selectivity in this species. Kugele & Yule (1996) reported no consistent settlement on substrata such as slate in various colours (either with pits, smooth or slotted), as well as Perspex plates roughened on one side, black plastic, sandstone, plastic mesh of 80 µm and epoxy models of adults, even when combined with crude extracts and concentrated protein extracts from conspecifics. Although the present study tested a wider range of surfaces, results remained inconclusive, even when the same structures were tested in association with barnacle extract (unpublished results). However, it should be noted that the quality of laboratory-raised cyprids might be an issue, since rearing temperature and feeding have been observed to affect barnacle cyprid competence (Harder et al., 2001; Thiyagarajan et al., 2002a; West & Costlow, 2005; Emlet & Sadro, 2006). Although settlement in the laboratory occurred also on artificial substrata, this was rare and no pattern was

observed that supported the induction of settlement by any of the tested artificial substrata. In particular, when a choice was given, the adults were invariably the preferred substratum. This was supported by results in the wild that returned zero settlement on the artificial structures tested, including textured adult mimics, in spite of strong settlement on conspecifics. An exception was the recruitment to the marine epoxy used in the field. This event indicated that, despite larvae rejecting most substrata tested, settlement on artificial substrata could be accomplished in numbers comparable to recruitment on adults, under certain conditions that have not yet been adequately explained. Though the marine epoxy was not tested in culture, future works on this might provide valuable insight into differences between culture and wild studies and the impact of post-settlement survival on these differences, using a substrata other than the adult. The marine epoxy used was not flat like the other structures tested, but was moulded around the rocks, having therefore a series of depressions and concave/convex areas that could have impacted on the micro-hydrodynamics as well as protection given to spat. Results from Aldred et al. (2010) demonstrated texture effects on the rate of removal of cyprids of *B. amphitrite*, with preferential settlement on textures to which the adults subsequently adhered to best. Results from barnacle settlement in the field show that post–settlement survival is critical and studies of *Balanus glandula* showed that mortality in the first day post settlement could be up to 38 %, almost equivalent to the 40 % mortality recorded in the subsequent 44 days as a result of of predation, desiccation, wave exposure or size-specific vulnerability (Gosselin & Qian, 1996).

Previous field studies (Cruz, 2000; Coelho, 1991) tested an array of substrata with and without barnacle extract, including smooth and rough PVC, tufnol, plastic, rubber and ropes, all with negative results. These studies, however, faced limitations such as the loss of structures and had very different periods of field exposure. It is important to note that, beside the present results, other observations on *P. pollicipes* recruitment suggest that the conspecific adults are not mandatory settlement substrata, as *P. pollicipes* settle on various materials (e.g. epoxy, metal nuts and screws) placed in the field for ecological studies (Cruz, pers. com.). Interestingly, early work on *P. polymerus* reflected similar concerns over settlement selectivity, with Hoffman (1989) suggesting that pre-colonization of substrata by conspecifics could be essential for *P. polymerus*. However, the accumulation of current results and previous literature maintain that recruitment is more dependent on the survival value of a surface compared to its

physicochemical properties, which might also explain the lack of settlement response in the laboratory.

Gregariousness in barnacles, is often attributed to the chemical interactions between the adult and cyprids, and the fitness value associated with settling in proximity to conspecifics. The basics of this interaction have been studied extensively in other barnacle species and it is known that the adults produce surface-bound settlement cues (e.g. settlement-inducing protein complex or SIPC; Dreanno 2006a, 2006b) as well as waterborne cues (e.g. Elbourne & Clare, 2010), that initiate gregariousness (see Aldred & Clare, 2008; Clare, 2011). Although barnacle settlement, adhesion and surface selection have been widely investigated (Lewis, 1975a, 1978; Crisp, 1984; Bourget, 1988; Aldred & Clare, 2008; Clare & Aldre, 2009), very little is known about the settlement of *Pollicipes* sp. cyprids, with results limited to Lewis (1975a), Hoffman (1989), Kugele & Yule (1996) and Molares et al. (2002). A wide range of studies has been carried out in the laboratory to confirm the function of the SIPC as a pheromone promoting barnacle settlement (Clare & Yamazaki, 2000; Matsumura et al., 2000; Clare 2011). Studies of *P. pollicipes* settlement in vitro are limited to Kugele & Yule (1996) and Molares et al. (2002). Moreover, evidence for the presence of the SIPC in *P. pollicipes* has been presented (Clare et al., 1997; Molares et al., 2002) . The current study showed that there were clear differences in settlement on previously uncolonized and cleared structures, with the latter accumulating 2 – 3 times more spat, in accord with Cruz (2000).

Whether chemical attraction is the dominant cue for *P. pollicipes* settlement is unclear. Physical characteristics of the substratum have long been known to affect barnacle cyprid settlement (Crisp & Barnes, 1954; Knight-Jones, 1951; Barnes, 1956; Le Tourneux & Bourget, 1988). The preference of *P. pollicipes* cyprids for the rough cuticle of adult conspecifics is not surprising, as some barnacle cyprids tend to settle preferentially in cracks and pits (e.g. Bergeron & Bourget 1986; Le Tourneux & Bourget, 1988), with SIPC being likely associated with the cuticle. Even on surfaces that would naturally attract cyprid settlement, settlement rates in the laboratory have never been high; e.g. 20 % for *P. polymerus* (Lewis, 1975a) and 1 % for *P. pollicipes* (Kugele & Yule, 1996). Such low settlement may, however, be a consequence of using unhealthy cyprids resulting from laboratory culture. When no choice of substrata is given, the percentage of settlement in the laboratory increases for natural surfaces other than conspecifics, with most substrata attracting 2 or 3 times the colonisation by

percentage. This might indicate that although the adults present the strongest settlement cue, in the wild cyprids have a limited choice over which surface to settle on provided that protection is assured. This appears to be supported by the results of settlement on artificial structures, marine epoxy and also by the positioning of the settled barnacles within the structures of natural substrata. Larvae were mostly found to inhabit pits, edges and ridges. When settlement was on allospecific species, it was mostly on their edge and between substrata, reflecting the preference for settlement on pits and ridges. Though not necessarily reflected in recruitment, settlement is apparently higher in association with other barnacle species, such as *Chthamalus* sp., compared to non-barnacle species such as *Corallina* sp.. Thus in spite of the preference for settling on barnacles, the survival value of this behaviour may indeed be less relevant in the wild, if protection and proximity to conspecifics is assured. However, laboratory settlement assays with other barnacle species have noted a relation between settlement, settlement cues and systematic affinity (e.g. Molares et al. 2002; Dreanno et al., 2007), i.e. the more closely related the species the greater is the inductive effect.

4.5. Conclusions

When evaluating the overall results of settlement on adults in the laboratory, maximum temporary and permanent attachment were of the order of 70 – 75 % and 30 – 35 %, respectively. Furthermore, the rate of metamorphosis over a period of 1-week reached of 70 – 80 % of settled larvae. These results raise the question of whether optimal larval quality is being obtained in the laboratory, as settlement rates are still below 50 % even when using the adults as settlement inducers. A comparison with larvae settled in the field is impossible as these represent recruitment rather than settlement and therefore subject to other forces.

Preferential conditions, maximizing both permanent settlement and metamorphosis rate include natural salinities (30 – 40 psu), a temperature of 20 ºC, illumination, water circulation and the presence of an interface area, and cyprid age not exceeding 3 days. These conditions have evident implications for laboratory settlement assays, as besides the poor settlement observed on non-adult substrata, they limit the use of common settlement protocols used for other barnacle species (e.g. *B. amphitrite*, *B. improvisus*). If this inductive effect of the adults on settlement is due to a response by the larvae to proteins produced by the adults (e.g. SIPC) or due to gaining a refuge by settling in between the scales or capitular plates, remains unresolved. The benefits of settling on the adult range from immediate protection (e.g. avoidance of desiccation, predation and

refuge from hydrodynamic forces) to suitable habitat selection (e.g. feeding, reproduction, etc.), though competition for food and space is also increased. However, this pressure can be reduced, as *P. pollicipes* have been reported to move on the adult stalk (Kugele & Yule, 1993, 2000), and might potentially be able to relocate towards the primary substrata. Larger juveniles are frequently found towards the base of the stalk instead of the upper stalk, though it remains unclear what mechanism(s) mediates this behaviour.

Importantly, settlement does not occur exclusively in association with conspecific adults; it also occurs on both artificial and natural substrata., An effective induction of settlement was not achieved with the artificial substrata tested. The only artificial substratum where recruitment was consistent was on marine epoxy placed in the field, possibly due to the higher protection afforded by the irregularity of the surface. However, when using natural substrata, settlement was observed on other species and natural rocks. Settlement was higher when larvae were not offered a choice of substrata (in the laboratory), and was comparable to recruitment on the adults, when the observations from the wild are considered. Though the conspecific adult constitutes the main factor in larval attraction, being essential to gregariousness, habitat selection in nature is likely mediated by other factors, such as transport to the coast, larval choice and post-settlement survival, as spat are found on a variety of natural substrata besides the adult. This is in accordance to the natural formation of new clusters not being ultimately dependent on previous colonization or presence of conspecifics. It further highlights that current beliefs over apparently high larval selectivity in this species might be a matter of overlooking other relevant settlement factors (besides type of material and induction by chemical cues), such as surface associated survival value, and explicitly the interaction of surface related parameters (e.g. roughness, micro-topography) with environmental factors (e.g. hydrodynamics, predation, intraspecific-competition, desiccation), as further research into these hypothesis is essential.

Chapter 5. Feeding behaviour of stalked barnacles (*Pollicipes pollicipes*) in response to hydrodynamics, temperature and food

Abstract

Pollicipes pollicipes is an edible barnacle of considerable economic importance in Portugal and Spain. However, few studies relating to the rearing of *P. pollicipes* in captivity exist and the specifics of feeding behaviour and feeding preferences are largely unknown. In this study, captive cultures of *P. pollicipes* were subjected to varying hydrodynamic conditions, temperature, food quality and quantity in order to evaluate the effects of these parameters on feeding behaviour, thus informing efforts to develop this species for commercial aquaculture. Furthermore, individuals maintained in the laboratory for different lengths of time were investigated separately in order to better understand the consequences of long-term captive rearing.

Laboratory-conditioned *P. pollicipes* respond to slower water speeds (≥ 6 cm s^{-1}; 77.83 ± 9.52 % of indindividuals showing captorial feeding) than unconditioned individuals (≥23 cm s^{-1}; 84.46 ± 8.73 % of indindividuals showing captorial feeding). Capture rates of conditioned individuals averaged 75.00 ± 2.41 captures min^{-1} ind^{-1}, significantly above unconditioned individuals (42.52 ± 1.88 captures min^{-1} ind^{-1}). From the diets tested, live nauplii of *Artemia* sp. promoted the highest feeding rate (15.67 ± 0.47 J ind^{-1} $3h^{-1}$). *P. pollicipes* also responded to other prey including: frozen *Artemia* sp. nauplii, *Brachionus plicatillis* and *Tisbe battagliai* and microalgae (*Tetraselmis chuii*), but did not respond to other 'inert' foods (freeze-dried *Daphnia* sp., pellet food) or *Isochrysis galbana*. There were no significant differences in response to diet between conditioned and unconditioned individuals, other than the more prevalent cirral beating shown by unconditioned animals. *Artemia* sp. concentration (7, 12 and 25 $nauplii^{-1}$ ml^{-1}) did not affect ingestion rates, suggesting that the conditions of food availability tested were not limiting. However, temperatures of 20 ºC increased captorial behaviour as well as ingestion rates (of 3.63 ± 0.50 J ind^{-1} h^{-1}), in comparison to 15 ºC and 11 ºC (1.77 ± 1.63 and 1.26 ± 0.21 J ind^{-1} h^{-1}). Cumulative *Artemia* sp. consumption increased steadily until 27.20 ± 2.17 06 J ind^{-1}, at which time consumption began to decrease. The present results advise on which food quantity/quality to use and flow rates that promote active feeding response, as well as temperature effects on feeding. Further studies are essential to establish how the present results compare to growth and survival rates in culture.

5.1. Introduction

Historically, research focussing on the stalked barnacle *Pollicipes pollicipes* has related to its biology and ecology in the wild, with a view to establishing solid conservation and stock management practices (Molares, 1994; Cruz, 2000; Cruz et al., 2010). Few studies have investigated *Pollicipes* sp. juvenile feeding behaviour in the wild (Barnes & Reese, 1959; Lewis, 1981) or in captivity (Norton, 1996; Cribeiro, 2007). Norton (1996) described the feeding apparatus and feeding patterns of *P. pollicipes*, while Cribeiro (2007) made some important observations on culture conditions and feeding behaviour for this species. *Pollicipes* sp. cirral activity is related to both feeding and respiration, and can vary between retracted cirri with an open capitulum, rhythmic cirral extension, to prolonged cirral extension accompanied by the movement of individual in-curling cirri that transport captured food to the mouthparts (Norton, 1996). Food quality and water flow are known to influence the feeding response in *P. pollicipes* (Barnes & Reese, 1959; Norton, 1996; Cribeiro, 2007), although the effects of flow rate, temperature, quantity and quality on ingestion rates and feeding behaviour remain poorly investigated.

The effects of water flow on feeding activity have been studied in other barnacle species (e.g. Southward, 1955a; Anderson, 1981; Trager et al., 1990, 1994) and adequate hydrodynamics are considered to be crucial for feeding in many species (Barnes & Reese, 1959, 1960; Barnes, 1996; Lauzier, 1999). It is believed that, for some species, there is a critical water flow below which the cirral response is not stimulated (Southward, 1955b; Barnes & Reese, 1959, 1960). In the case of *P. pollicipes*, distribution is limited to sites with high wave exposure (Borja et al., 2006a, 2006b; Cribeiro, 2007; Cruz et al., 2010), which might support the existence of critical flows. Furthermore, individuals in the wild have been observed to re-orient themselves according to wave exposure and microtopographical features, to face incoming flow so as to benefit from food transport (Barnes & Reese, 1959; Barnes & Reese, 1960; Barnes, 1996; Lauzier, 1999). The perception of flow is thought to be facilitated by the stalk and the opercular flaps, with individuals showing decreased cirral beating with increased flow, ceasing at about 15 cm s^{-1} (Norton, 1996). A differential response to water flow has also been described for juveniles and adults of *Pollicipes* sp.; juveniles maintain cirral beating under static conditions unlike the adults that remain apparently unresponsive. In turbulent conditions cirral extension occurs in both adults and juveniles (Barnes & Reese, 1960; Lewis, 1981; Norton, 1996; Cribeiro, 2007). As early

juveniles are generally found attached to the stalks of adult conspecifics, and often in areas of lower water speed, this behaviour may reduce their dependence on currents for feeding and respiration. In fact, both Cribeiro (2007) and Barnes (1996) suggested that turbulent conditions with continuous streams of running water are essential to stimulate *Pollicipes* sp. feeding in captivity. This hypothesis has not been verified experimentally, however, despite the significant impact that such a requirement would have on running costs for any facility designed to support a culture of this species. Neither have the effects of flow on feeding behaviour and capture rate been adequately investigated.

To date, the natural diet of *P. pollicipes* requires further studies, but works on the related *P. polymerus* suggest prey items ranging from diatoms, detritus and exuviae to pelagic crustaceans for younger juveniles and various phytoplankton, microalgae, copepods and polychaetes for older juveniles (Barnes & Reese, 1959; Howard & Scott, 1959; Lewis, 1981). The shift in diet during development may either hold evolutionary significance (Lewis, 1981), or else be a consequence of the changes in size and efficiency of the cirral net as individual barnacles grow (Norton, 1996). When rearing *P. pollicipes* in captivity, Cribeiro (2007) observed that the acceptability of prey was higher for live foods, with some inert food items being rejected even post-capture. Norton (1996) also established a relation between ingestion rates of *P. pollicipes* and food quality and quantity for some prey. When juveniles of *P. pollicipes* were fed with diets of *Rhinomonas reticultata*, *Skeletonema costatum* or *Brachionus plicatillis*, ingestion rate increased with food density until an asymptote was reached, with maximal ingestion rates of 0.679 J ind^{-1} h^{-1} of *S. costatum* (1.16 x 10^6 cells ind^{-1} h^{-1}), 0.23 J ind^{-1} h^{-1} of *R. reticulata* (1 x 10^5 cells ind^{-1} h^{-1}), 0.53 J ind^{-1} h^{-1} of *B. plicatillis* (81 ind^{-1} h^{-1}) and 2.14 J ind^{-1} h^{-1} (58 ind^{-1} h^{-1}) for *Artemia* sp. nauplii. Nevertheless, the energetic requirements calculated by Norton (1996) contrast with Page (1983) who recorded maximal ingestion rates of 8.9 - 9.2 *Artemia* sp. adults ind^{-1} d^{-1} and daily maintenance requirements of 28.3 J ind^{-1} d^{-1}, depending on temperature, for the related *P. polymerus*. These values were, considerably higher than those in earlier reports for *P. pollicipes*. Furthermore, none of these studies used test conditions similar to those found in aquaculture, which could affect the applicability of the results significantly. The inconsistency, both in experimental data and methods, raises the question of the exact energetic requirements of *P. pollicipes*, and how different diets can support this. Moreover, differences between the behaviour of laboratory conditioned and recently collected (unconditioned) individuals may be significant.

Water temperature is well known to affect the metabolism of test organisms and, presumably therefore, cirral behaviour with its direct links to feeding and respiration. Norton (1996) evaluated beating frequency at temperatures between 16 and 24 °C and identified an increase from 1.25 ± 0.25 to 4.03 ± 2.85 as temperature was raised. This response was presumably respiration-related since no food was provided. Accordingly, no studies have been conducted into the potential effects of water temperature on the cirral movement exhibited by *P. pollicipes* juveniles, and the consequent impact on ingestion rates. Although passive food intake may increase in line with respiration rate, feeding is, by and large, active in *P. pollicipes*, dependent upon captorial behaviour by the curling of individual cirri. So the question remains, would higher temperatures stimulate active feeding and, if so, to what extent?

In the case of *Artemia* sp. nauplii used as prey, ingestion rate has been shown to be independent of food density (Norton, 1996), as hypothesized by Crisp (1961) for large particles. This suggests that individuals have a maximal capture capacity per unit time, which may therefore limit the effects of temperature. Additionally, the fact that beating might be more common at high temperatures could lead to reduced energy allocation to captorial feeding, causing a decrease in active capture rate. In fact, it has been observed that in the presence of food, beating is lower than in its absence (Norton, 1996).

The present work investigated the response of juvenile *P. pollicipes* to different hydrodynamics, temperature, food quality and quantity, and how the responses varied between conditioned and unconditioned individuals. The aim was to provide a basis for understanding *P. pollicipes* feeding behaviour in response to environmental conditions, which could be used as a guideline for establishing rearing protocols in captivity and analysis of ecological preferences in the wild.

5.2. Material and methods

5.2.1. Collection and culture of P. pollicipes

Clusters of *P. pollicipes* were collected from Cabo Sardão (37°36'24.70", -8°49'2.00"; Portugal) and transported to the facilities at Newcastle University, where the stocks were maintained in non-recirculating conditions. Recently collected, unconditioned stocks were maintained in captivity for 2-4 days prior to experiments to allow for acclimatisation, while conditioned stocks were maintained for a minimal period of 6 months, to adjust to feeding regimes. All experiments and acclimatization periods were run under a standard protocol (except where stated otherwise), adjusted as needed

according to the experiments and, using 5L plastic transparent aquariums, maintained in static conditions. Standard culture conditions comprised UV filtered natural seawater (NSW), 20 ± 1 ºC, photoperiod of 16:8 L/D (dim light, 300-400 lux), salinity of 33 ± 1 psu and turbulent hydrodynamic conditions maintained by high-flow water pumps (Rio® +1000 Aqua Pump) and multiple point source aeration (Blagdon® Koi Air 50 and Interpet© Aquarium 2.5cm air stones). *Artemia* sp. nauplii (GSL-INT Artemia LCC©) were provided daily in excess quantities (25 nauplii mL^{-1} day^{-1}), sporadically supplemented with *Tetraselmis chuii* and *Isochrysis galbana* (≥100.000 cells mL^{-1} 1:1). Culture water quality, temperature (±1 ºC) and salinity (±1 psu) were monitored regularly to control conditions of culture. *Artemia* sp. were cultured in 5L carboys with UV-filtered NSW, 33 ± 1 psu, 24:0 L/D photoperiod (1800-2200 lux), 28 ± 1 ºC and strong aeration. After 24h, instar I were separated from the cysts and samples were counted to estimate daily feeding volumes. Microalgae cultures were conducted in 10L carboys with autoclaved NSW, 33 ± 1 psu, 16:8 L/D photoperiod (1900-3500 lux), 20 ± 1 ºC and strong bottom aeration, supplemented with F/2 medium (Guillard & Ryther 1962; Guillard 1975).

5.2.2. Experimental design

Prior to the experiment, all barnacles were measured for rostro-carinal distance (RC) and stalk length (SL) (see Cruz, 2000). Clusters of barnacles were divided into similar sized groups ($p= 0.751$; Kruskal-Wallis test), according to number of barnacles, mean size and equivalent population structure. Population structure was analysed by assigning the barnacles into size classes according to RC ([0-5mm], [5-10mm], [10-15mm], [15-20mm] and [20-25mm]) and analysing the size distribution of each cluster according to the proportion of each size class. The feeding response was always evaluated using barnacles in clusters, to approximate natural conditions. Feeding behaviour of individuals was also analysed, however this was done by observing each clustered individual singularly. Due to technical limitations and number of barnacles, the experimental design was separated into four experiments testing the effects of hydrodynamics (Exp.1), food quality (Exp.2), food quantity with temperature (Exp.3) and food saturation (Exp. 4) respectively. Three replicate tanks per treatment were used, as well as a control group. Feeding behaviour was analysed through assessment of (1) capture rate, (2) clearance and ingestion rates and (3) behavioural classes. Capture rate (CR) was calculated by counting the number of times in a minute that the individual cirri of the barnacle curled to the mouthparts opening. Clearance rate (*F*; Eq. 1) was

calculated following Candeias (2005) and ingestion rate (*IR*; Eq. 2) following Frost (1982). Individual barnacle feeding behaviour was observed within the clusters and barnacles were classified according to behavioural classes listed in Table 5.1.

Eq.1 $F = \frac{v}{nt} ln\left(\frac{C_1 \times C_f}{C_2 \times C_i}\right)$

Eq.2 $IR = \bar{C} \times F$, with $\bar{C} = \frac{C_2(e^{(k-g)t}-1)}{(k-g)t}$, being $k = \frac{1}{t} ln \frac{C_f}{C_i}$ and $g = \frac{1}{tT} ln \frac{C_f}{C_1}$

Equations 1 and 2, where *IR* is ingestion rate, $\bar{C}$ is average food concentration, *F* is clearance rate, *k* is growth constant, *g* is grazing constant, t is time, c_1 is initial food concentration, c_2 is final food concentration, c_i is initial food concentration in control, c_f is final food concentration in control, *v* is volume, and *n* is number of barnacles.

Class	Behavioural class	Capitulum	Cirral net	Cirral movement
1	Non-feeding closed	Closed	Retracted	Absent
2	Non-feeding retracted	Partially open	Retracted	Absent
3	Non-feeding extended	Partially open	Extended	Absent
4	Passive feeding extended	Open	Extended	Absent
5	Active feeding beating	Open	Extended	Rythmic
6	Active feeding captorial	Open	Extended	Captorial

Table. 5.1 Description of *P. pollicipes* juvenile behaviour according to capitulum opening and cirral net extension and movement. Behaviour was evaluated according to the following categories: (1) capitulum closed, (2) capitulum partially open with retracted cirral net, (3) capitulum partially open with extended cirral net, (4) capitulum open with open cirral net but without individual cirri movement (passive feeding activity), (5) capitulum open with open cirral net with beating movement (beating active feeding activity), (6) capitulum open with open cirral net and individual movement of cirri (captorial active feeding activity).

Due to differences in the feeding behaviour of conditioned and unconditioned animals previously observed by the authors, experiments were done with both groups (results shown when relevant) in order to demonstrate the extent to which the measured behaviours vary with duration in captivity. Conditioned animals were kept in the aquarium for approximately 6 months, while unconditioned individuals were used 24-48h post-collection.

5.2.2.1. EXP.1 HYDRODYNAMICS

The effects of hydrodynamics (water speed of 3, 6, 12, 23, 32, 64 cm s^{-1}) on the feeding behaviour of *P. pollicipes* were investigated by recording individual capture rates and observing the feeding behaviour of barnacles in clusters. Each cluster of *P. pollicipes* (62 ± 5 ind; 8.55 ± 3.23 mm RC), was placed in a separate 10 L glass tank (artificial SW, 15 °C, non-recirculating, 300 – 400 lux) at identical distances from the water pump (Rio® +30, 60, 1500 and 3000 Aqua Pumps). Each treatment was assigned a water

pump of different power, and water speed at the point of contact with the barnacles was quantified using a Novonic® streamflow impeller. Water flows were as follows: (3) 3.22 ± 0.12 cm s^{-1}; (6) 6.45 ± 0.42 cm s^{-1}; (12) 11.89 ± 0.19 cm s^{-1}; (23) 23.38 ± 1.32 cm s^{-1}; (32) 32.05 ± 1.85 cm $s^{-1;}$ and (64) 64.45 ± 1.89 cm s^{-1}. The animals were left to acclimatise to the hydrodynamic conditions for 5 min, after which they were fed *Artemia* sp. nauplii at 25 ml^{-1} and left feeding for 20 min. Observations were made during this period. From each group, individual capture rate was estimated from 6 mid-cluster barnacles selected at random. The cluster's feeding behaviour was also observed and the number of individuals in each behavioural class (as previously described) was counted. The clusters were then left resting for 10 min and then assigned to a treatment, at random, so that all clusters were tested with all treatments.

5.2.2.2 EXP.2 FOOD QUALITY

The effects of food quality on feeding behaviour, clearance and ingestion rates of *P. pollicipes* were investigated. Treatments included (a) *Artemia* sp. live nauplii (25 ind ml^{-1}: GSL-INT Artemia LCC©), (b) *Artemia* sp. frozen nauplii (25 ind ml^{-1}), (c) *Brachionus plicatillis* (25 ind ml^{-1}), (d) *Tisbe battagliai* copepodites (25 ind ml^{-1}), (e) *Tetraselmis chuii* (≥ 1000 µgC l^{-1}), (f) *Isochrysis galbana* (≥1000 µgC l^{-1}), (g) freeze dried *Daphnia* sp. (Interpet Freeze Dried Daphnia©) and (h) pellet food (King British Turtle and Terrapin Complete Food©). Each replicate (33 ± 2 ind; 7.96 ± 2.76 mm RC), was initially fed with a single diet and monitored every 30 min for a period of 3 hours. During that time, observations were made of feeding behaviour within each cluster. *Artemia* sp. and microalgae cultures were done as previously described. Rotifer and copepod cultures were done in 500 mL conical bottles with UV filtered NSW, 33 ± 1 psu, 16:8 L/D photoperiod (300 – 550 lux), 20 ± 1 ºC and weak aeration. Feeding was done with microalgae, in excess, namely *T. chui*. Early larval stages were collected daily by filtering the cultures and were counted to estimate daily feeding volumes.

5.2.2.3 EXP.3 WATER TEMPERATURE AND FOOD QUANTITY

The effect of water temperature (11, 15 and 20 ºC) and food quantity (7, 12, 25 nauplii ml^{-1} of *Artemia* sp.) on the feeding behaviour of *P. pollicipes* was investigated by means of a full factorial design. Feeding treatments were as follows: (a) 7 nauplii ml^{-1} at 11 ºC; (b) 12 nauplii ml^{-1} at 11 ºC; (c) 25 nauplii ml^{-1} at 11 ºC; (d) 7 nauplii ml^{-1} at 15 ºC; (e) 12 nauplii ml^{-1} at 15 ºC; (f) 25 nauplii ml^{-1} at 15 ºC; (g) 7 nauplii ml^{-1} at 20 ºC; (h) 12 nauplii ml^{-1} at 20 ºC; and (i) 25 nauplii ml^{-1} at 20 ºC. Each replicate (42 ± 4 ind; 8.12 ±

1.97 mm RC) was initially fed with the referred rations and monitored every 30 min, for a period of 4 hours as described in the previous section.

5.2.2.4. EXP.4 FOOD SATURATION

The effect of increasing food quantity on the feeding behaviour of individuals was investigated by feeding the groups with increasing quantities of *Artemia* sp. nauplii, until saturation was reached. Each tank replicate (36 ± 4 ind; 9.15 ± 2.87 mm RC) was initially fed with *Artemia* sp. nauplii and re-fed every 20 min, for a period of 80 min, at which time cultures were left feeding without further food addition. Food quantity was therefore topped-up every 20 min to the following food concentration levels: 5 nauplii ml^{-1} at 0 min; 10 nauplii ml^{-1} at 20 min; 15 nauplii ml^{-1} at 40 min; 25 nauplii ml^{-1} at 60 min; and 35 nauplii ml^{-1} at 80 min, after which no more food as added. Each replicate was monitored every 20 min for the first 80 min, before and after each feeding event, and afterwards every 20 min for further 160 min, for a total period of 4 hours, as previously described, for clearance and ingestion rates.

5.2.3. Data treatment and statistical analysis

All statistical analyses were performed using STATISTICA 7.0® and data in percentage (%) were arcsine transformed. Data were subjected to parametric tests as analysis of variance (ANOVA) or analysis of covariance (ANCOVA), with time as a co-variate, when assumptions for normality and homocedasticity of variance were met (Shapiro-Wilk and Levene test, respectively). Tests were conducted at $\alpha = 0.05$. Significant ANOVAs and ANCOVAs were followed by Tukey's test to identify differences among groups. Data that did not obey the assumptions for normality and homocedasticity were subjected to non-parametric tests (Kruskal-Wallis test). All figures and tables report the mean ± standard error (SE).

5.3. Results

5.3.1. Hydrodynamics

Water speed significantly affected the feeding behaviour and capture rate of *P. pollicipes*. Conditioned and unconditioned animals showed significantly different responses to water speed, both in terms of capture rate (ANOVA, $F=21.25$; $p\leq0.01$) and percentage of captorially feeding individuals (ANOVA, $F=17.74$; $p\leq0.01$) (figs. 5.1 and 5.2ab). Conditioned animals showed a significantly higher average capture rate (75.00 ± 2.41 captures min^{-1} ind^{-1}) and responded to a broader range of water speeds in comparison to unconditioned animals (42.52 ± 1.88 captures min^{-1} ind^{-1}) (ANOVA;

F=112.84; p≤0.01; fig.5.1). The former also responded promptly to slower water speeds, having increased captorial behaviour above 6 cm s^{-1}, while unconditioned animals responded only to flows above 12 cm s^{-1}. Feeding behaviour was in accord with these observations, since for conditioned animals maximal rates were observed above 6 cm s^{-1}, with an average of 77.83 ± 9.52 % of the feeding done captorially, while for unconditioned individuals similar values were only recorded above 23 cm s^{-1} (84.46 ± 8.73 %; fig. 2). Maximum capture rates (fig. 5.1) were observed between 6 – 31 cm s^{-1} (from 86.33 ± 3.35 captures min^{-1} ind^{-1} to 97.56 ± 3.73 captures min^{-1} ind^{-1}; statistically indistinguishable among treatments; Tukey's test, $0.08 \leq p \leq 0.98$) and at 23 cm s^{-1} (68.50 ± 2.45 captures min^{-1} ind^{-1}; statistically different from all other flows; Tukey's test, $p \leq 0.01$), for conditioned and unconditioned animals respectively. Capture rate decreased slightly at maximum water speed (62 cm s^{-1}) in both groups, as individuals struggled to keep the cirral net open and move the cirri. Conditioned individuals were observed to close in the extreme flow regimes (12.50 ± 8.80 % closed; 68.17 ± 2.50 captures min^{-1} ind^{-1}; fig. 5.2a) unlike unconditioned individuals (0.00 ± 0.00 % closed; 42.23 ± 2.17 captures min^{-1} ind^{-1} fig. 5.2b). At 6 – 64 cm s^{-1} only a few conditioned animals were not actively capturing prey, and the majority of these were observed to have a partially open capitulum with retracted cirri (on average 6.17 ± 3.95 %) or extended cirral net (on average 2.57 ± 2.92 %). This was in contrast to the unconditioned individuals that at stimulatory water speeds (23 – 64 cm s^{-1}) had a much higher percentage of non-captorial individuals (Tukey's test, $p \leq 0.01$), mostly with partially open capitulum with retracted cirri (14.37 ± 7.54 %) or open cirral net (20.00 ± 7.22 %). No beating was observed, regardless of water flow and conditioning state.

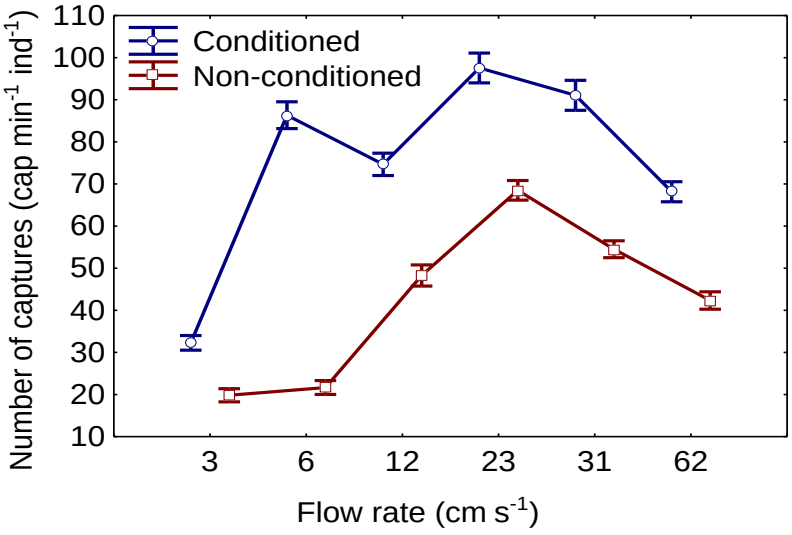


Fig. 5.1 Capture rate (captures min-1 ind-1) of conditioned (blue) and unconditioned (red) *P. pollicipes* when exposed to various water speeds (3, 6, 12, 23, 32, 64 cm s^{-1}).

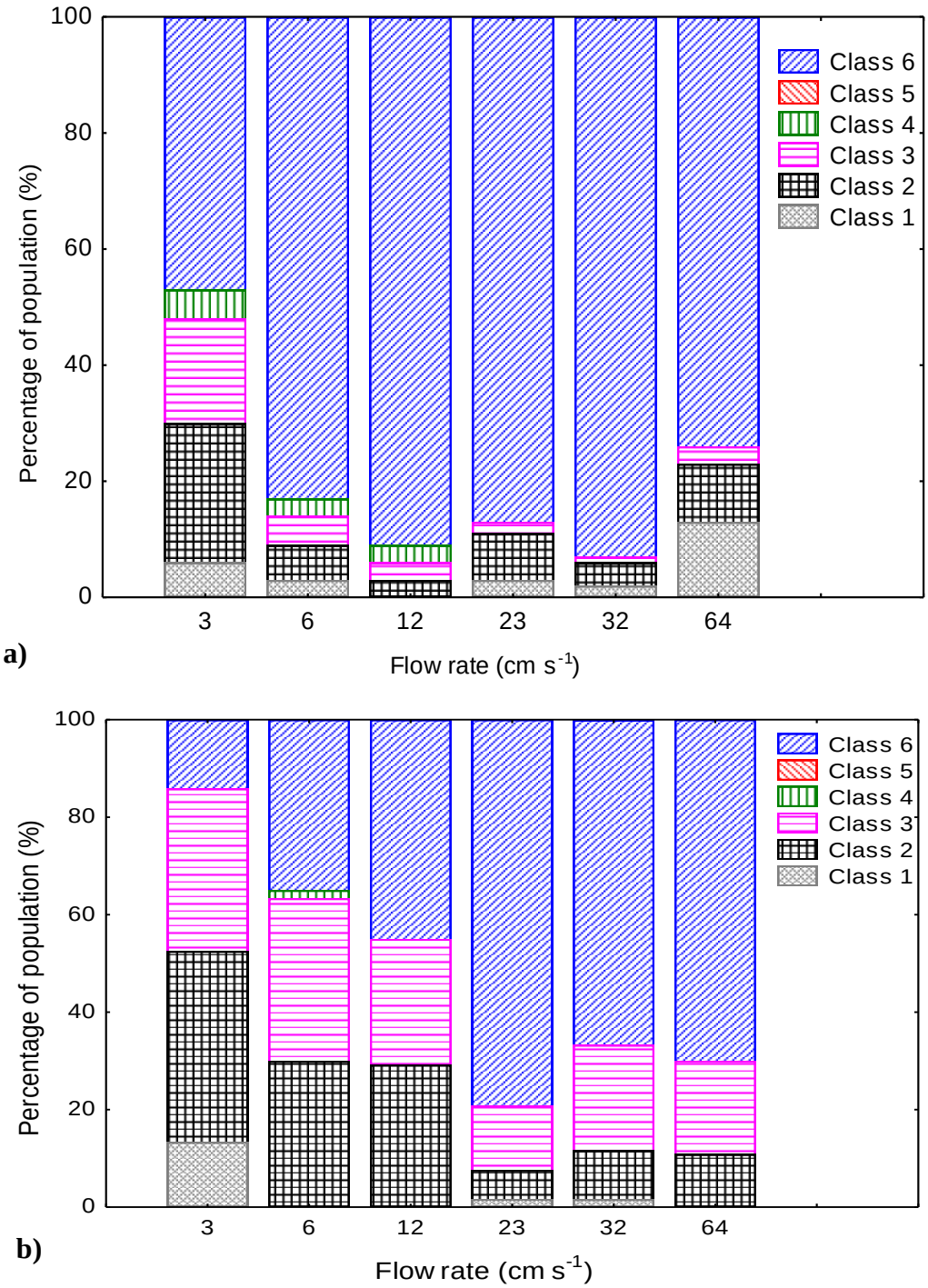


Fig. 5.2 Behavioural response of (a) conditioned and (b) unconditioned individuals of *P. pollicipes* (% of population), according to behavioural classes (1 to 6, from closed capitulum to fully captorial behaviour), when exposed to various water speeds (3, 6, 12, 23, 32 and 64 cm s^{-1}). Behaviour classes were as follows (1) closed capitulum and fully retracted cirri, (2) partially open capitulum with retracted cirri, (3) partially open capitulum with extended cirri, (4) open capitulum with fully extended cirri, (5) open capitulum with beating cirri, (6) open captilum with cirri individually capturing prey.

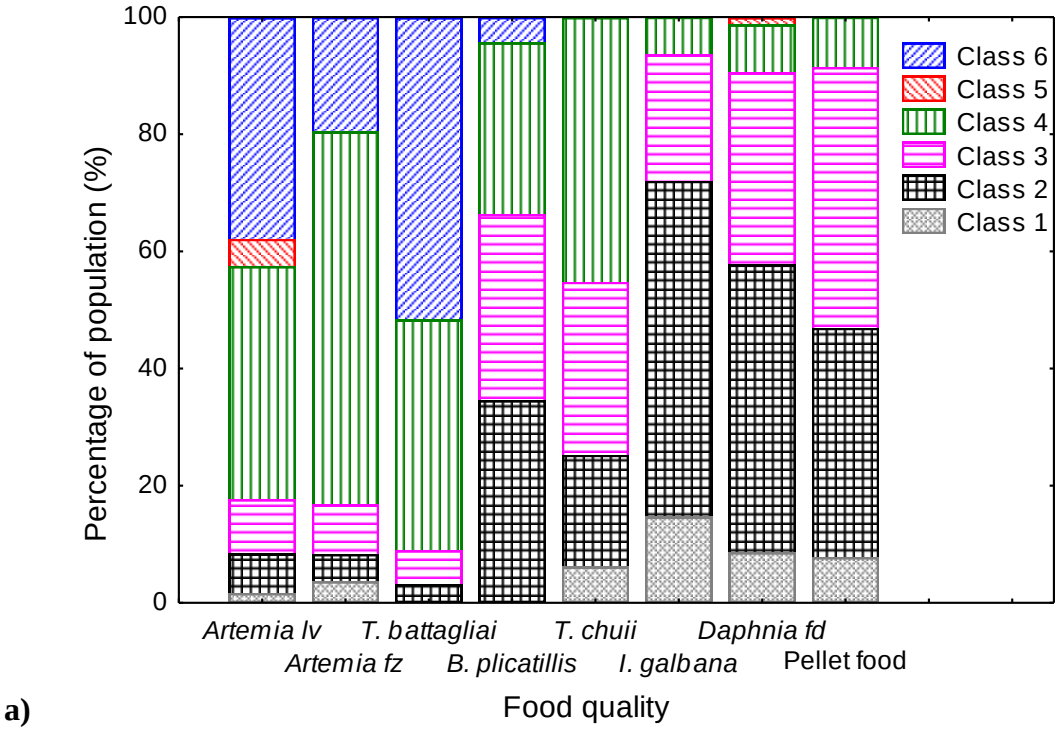
5.3.2. Food quality

Behavioural responses, and in particular, captorial feeding behaviour, varied significantly with conditioning stage (ANOVA, $F=58.39$, $p<0.01$) and diet (ANOVA, $F=65.54$, $p<0.01$) (fig. 5.3.). Active predation was observed in conditioned and unconditioned animals only when *P. pollicipes* were fed *Artemia* sp. nauplii (live and frozen), *B. plicatillis*, *T. battagliai* and *T. chuii*. From these species, *T. chuii* invoked a distinct behaviour, with a high percentage of the population exhibiting capitulum opening and cirral extension. *I. galbana*, *Daphnia* sp. and pellet food, in comparison, led to individuals keeping the capitulum either open or closed, or just partially open. Interestingly, beating behaviour was only observed in unconditioned animals feeding on *Artemia* sp., *B. plicatillis*, *T. battagliai* and *T. chuii*, averaging between 9.33 to 13.17 % of those groups, as well as conditioned individuals given *Artemia* sp. (although at a reduced rate; 4.66 ± 0.90 %). Relative to unconditioned individuals, conditioned animals showed significantly higher feeding response to live *Artemia* sp. (37.8 ± 2.88 % to 28.95 ± 3.25%, Tukey's test, $p<0.01$), lower feeding response to *B. plicatillis* (51.63 ± 5.24 to 7.29 ± 1.17; Tukey Test, $p<0.01$), and no differences for frozen *Artemia* sp. (19.57 ± 2.23 % to 10.43 ± 1.51 %; Tukey Test, $p=0.35$) and *T. battagliai* (4.32 ± 0.80 % to 9.65 ± 4.76 %; Tukey Test, $p=0.98$). Nevertheless, the highest response in terms of captorial behaviour was recorded with live *Artemia* sp. nauplii at 37.83 ± 2.98 % and 28.95 ± 3.25 % for conditioned and unconditioned individuals, as well as *B. plicatillis* in conditioned individuals, (51.63 ± 5.24 %). Ingestion rates decreased with time for all diets (fig. 5.4). Ingestion and clearance rates (fig. 5.5a and 5.5b) varied significantly with food quality (ANOVA, $F=368.62$, $p<0.01$, and ANOVA, $F=17.80$, $p<0.01$, respectively). Total ingestion, over the 3h period, also varied significantly among live feeds (ANOVA, $F=524.29$, $p<0.01$; Table 5.2), being significantly higher for live *Artemia* sp. nauplii (15.67 ± 0.47 J ind^{-1}; Tukey Test, $p<0.01$), followed by frozen *Artemia* sp. (10.76 ± 0.46 J ind^{-1}; Tukey Test, $p<0.01$). The lower values for *B. plicatillis* (0.26 ± 0.01 J ind^{-1}) and *T. battagliai* (0.27 ± 0.03 J ind^{-1}) were not different from each other (Tukey Test, $p=0.99$).

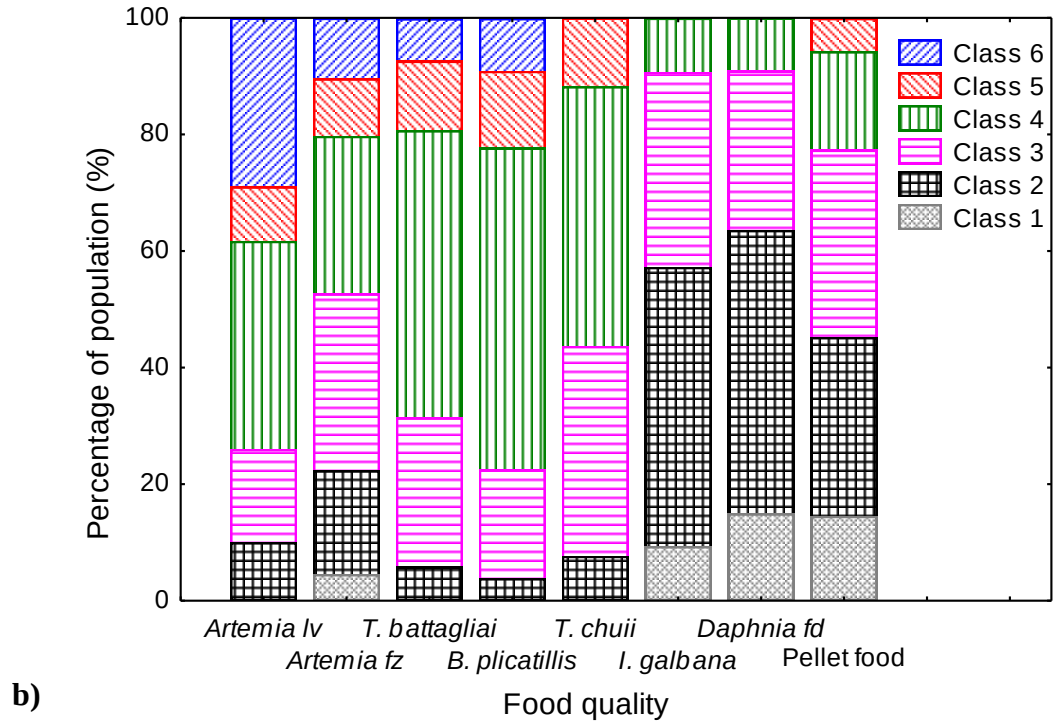
Diet	Ingestion rate (J ind^{-1} h^{-1})	Capture rate (# ind^{-1} h^{-1})	Total ingestion (J ind^{-1})
Artemia lv	4.88 ± 0.18 [a]	137.01 ± 5.15 [a]	15.16 ± 0.47 [a]
Artemia fz	1.74 ± 0.15 [a]	48.83 ± 4.17 [b]	10.76 ± 0.46 [a]
T. battagliai	0.07 ± 0.01 [b]	133.76 ± 3.81 [a]	0.26 ± 0.01 [b]
B. plicatillis	0.07 ± 0.01 [b]	131.05 ± 3.29 [a]	0.27 ± 0.03 [b]

Table 5.2 Average ingestion rates (J ind^{-1} h^{-1}), capture rate (prey ind^{-1} h^{-1}) and total ingestion over 3h (J ind^{-1}) of unconditioned *P. pollicipes* when fed live *Artemia* sp.

nauplii, frozen *Artemia* sp. nauplii, live *Tisbe battagliai* copepodites and live *Brachionus plicatillis* nauplii.



a)



b)

Fig. 5.3 Behavioural response of (a) conditioned and (b) unconditioned individuals of *P. pollicipes* (% of population), according to behavioural classes (1 to 6, from closed capitulum to fully captorial behaviour), when fed with different foods. Treatments were as follows: *Artemia* sp. nauplii (*Artemia* sp.), *Artemia* sp. nauplii frozen (*Artemia* sp. frozen), *Tisbe battagliai* copepodites (*T. battagliai*), *Brachionus plicatillis* nauplii (*B. plicatillis*), *Tetraselmis chuii* (*T. chuii*), *Isochrysis galbana* (*I. galbana*), freeze-dried *Daphnia* sp. (Interpet Freeze Dried Daphnia©) and pellet food (King British Turtle and Terrapin Complete Food©). Behaviour classes were as follows (1) closed capitulum and

fully retracted cirri, (2) partially open capitulum with retracted cirri, (3) partially open capitulum with extended cirri, (4) open capitulum with fully extended cirri, (5) open capitulum with beating cirri, (6) open captilum with cirri individually capturing prey.

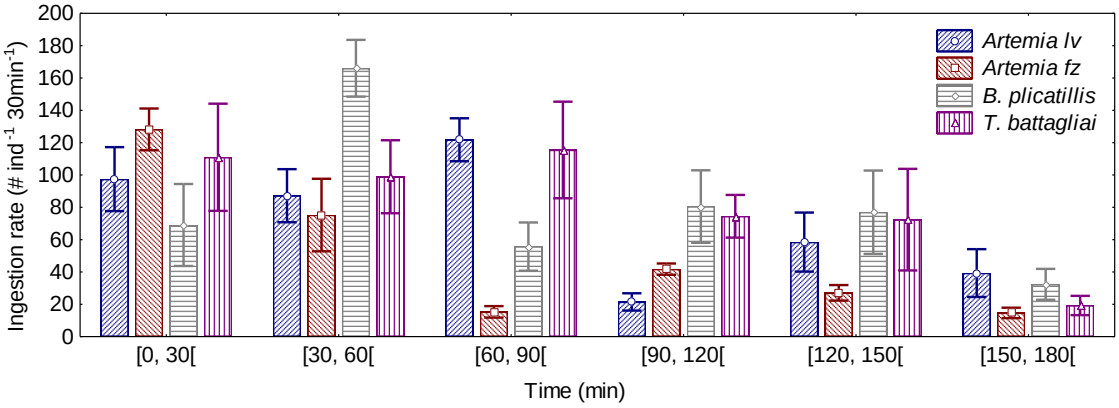


Fig. 5.4 Ingestion rate (# ind^{-1} 30min^{-1}) of unconditioned *P. pollicipes* when fed live *Artemia* sp. nauplii, frozen *Artemia* sp. nauplii, live *Tisbe battagliai* copepodites and live *Brachionus plicatillis* nauplii.

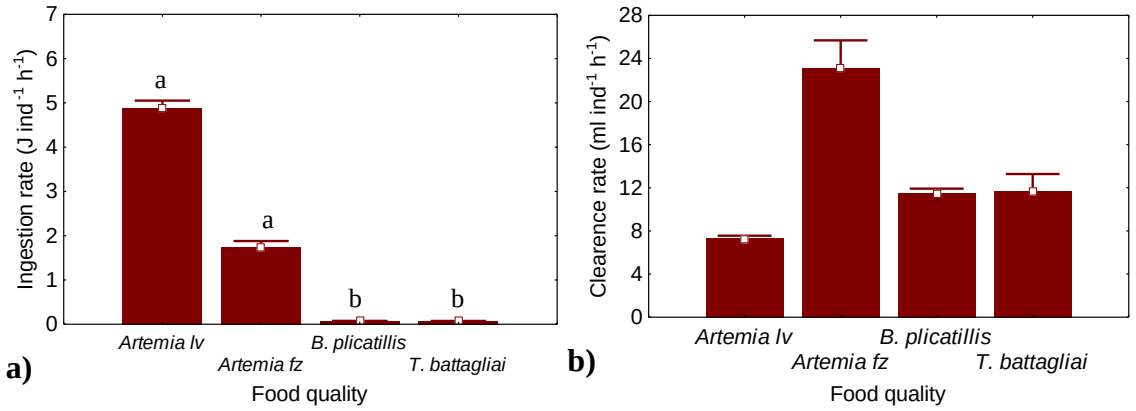


Fig. 5.5 (a) Ingestion rate (J ind^{-1} h^{-1}) and (b) clearance rate (ml ind^{-1} h^{-1}) of unconditioned *P. pollicipes* when fed live *Artemia* sp. nauplii, frozen *Artemia* sp. nauplii, live *Tisbe battagliai* copepodites and live *Brachionus plicatillis* nauplii.

5.3.3. Water temperature and food quantity

Ingestion rate was significantly different between temperatures (ANOVA, F=18.47, p<0.01), but not between food ration (ANOVA, F=1.15, p=0.34) for unconditioned individuals (fig. 5.6a). At 20 ºC ingestion rates were relatively high (Tukey Test, p<0.01) compared to the lower temperatures of 11 and 15 ºC, which were not significantly different (Tukey Test, p=0.45) at 3.63 ± 0.50 J ind^{-1} h^{-1} compared to 1.77 ± 1.63 and 1.26 ± 0.21 J ind^{-1} h^{-1}. Although ingestion rate did not vary significantly with prey density, it was higher at 15 and 20 °C, but not at 11 °C, with maximal ingestion being recorded at 25 nauplii ml^{-1} at 20 ºC, equating to 162.07 ± 30.65 nauplii ind^{-1}.

Food was fully consumed at 7 nauplii ml^{-1} after 2 h, at 12 nauplii ml^{-1} after 4 h and at 25 nauplii ml^{-1} food depletion was not observed during the experimental period.

Clearance rates were also significantly different between temperatures (ANOVA, F=26.10, p<0.01), but not feed quantity (ANOVA, F=0.75, p=0.49) (fig. 5.6b). Over 80 % of the experimental population showed active feeding behaviour, in spite of temperature or food quantity differences.

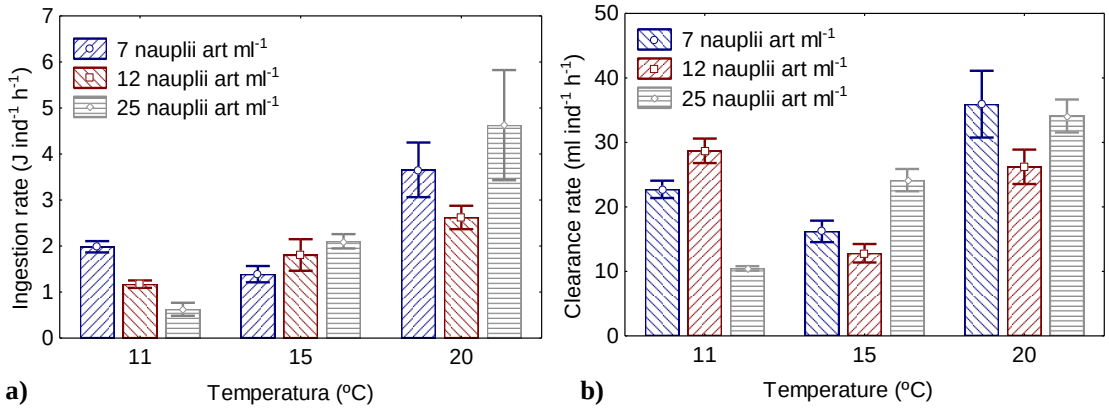


Fig. 5.6 (a) Ingestion rate (J ind^{-1} h^{-1}) and (b) clearance rate (ml ind^{-1} h^{-1}) of conditioned *P. pollicipes* fed live *Artemia* sp., at 7 nauplii ml^{-1} (blue), 12 nauplii ml^{-1}(red), 25 nauplii ml^{-1}(grey), and kept at the rearing temperatures of 11, 15 and 20 ºC.

5.3.4. Food saturation

Cumulative food consumption increased significantly with time (ANOVA, F=71.20, p<0.01; fig. 5.7), until 120 min (Tukey test, p<0.01), to 27.20 ± 2.17 06 J ind^{-1}, at which time food saturation was achieved and food consumption plateaued to a maximum of 33.60 ± 1.06 J ind^{-1} after 4h (Table 5.3). Cumulative consumption increased significantly No significant differences between values recorded from 120 to 240 min (Tukey test, p≥0.07) were observed. Consumption rates averaged 5.08 – 7.70 nauplii min^{-1} ind^{-1} at non-saturating food conditions. But as saturation approached, food ingestion decreased to 0.80 – 2.86 nauplii min^{-1} ind^{-1}.

Diet	Max ingestion (J ind^{-1})	Capture rate (artemia min^{-1})	Max ingestion (artemia ind^{-1})
Artemia sp.	33.60 ± 1.06	3.93 ± 0.41	942.86 ± 29.74

Table 5.3 Maximal ingestion rate (J ind^{-1} and artemia ind^{-1}) and average capture rate (artemia ind^{-1} min^{-1}) of *P. pollicipes* fed increasing quantities of live *Artemia* sp. nauplii for a total period of 4h.

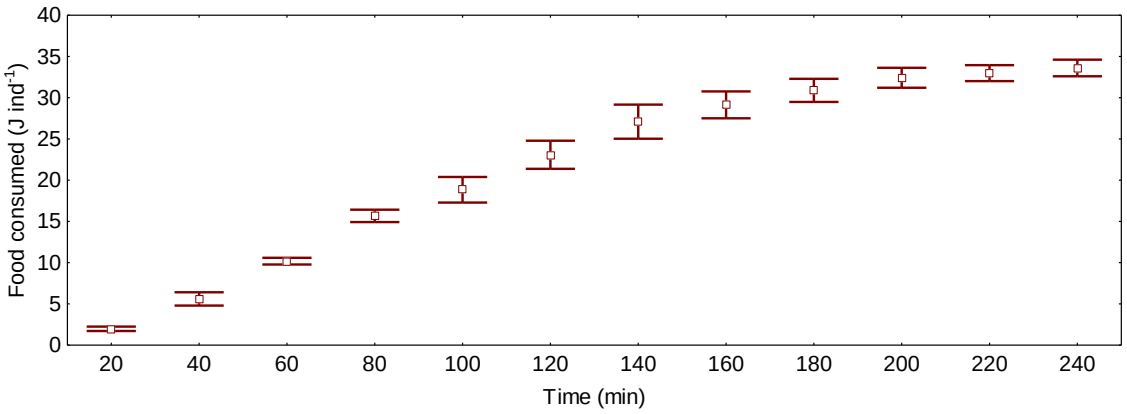


Fig. 5.7 Cumulative food consumption (J ind-1) with time (min) of *P. pollicipes* fed increasing quantities of live Artemia sp. nauplii.

5.4. Discussion

5.4.1. Hydrodynamics

Pollicipes pollicipes are intertidal barnacles adapted to life in high-energy environments. According to Denny (1988), water velocity can vary from 5 to 1000 cm s^{-1} in a coastal environment of high wave exposure. Flow is essential for obtaining adequate oxygen and food as in other species (Okamura, 1985; Trager et al., 1990), but also for spawning and waste removal as well as reduced risks of desiccation and predation. *P. pollicipes* depends upon adaptations such as the strong muscular stalk, thick stalk cuticle, capitular plates and strong adhesion to the substratum not only to withstand the strong hydrodynamic forces, but also to protect against desiccation during periods of air exposure.

Present results indicate that unconditioned individuals (in culture for 1 to 2 days) do not cope well with slower water speeds, feeding less actively, unlike conditioned individuals (in culture for over 6 months) that actively feed under these conditions. This suggests that individuals alter their response to hydrodynamics during conditioning to captivity, presumably in order to cope with a less turbulent environment than that found in the wild. This effect is also supported by the differences in capture rate, with similarities to the feeding differences observed between juveniles and adults (Lewis, 1981). As individuals get older, decreasing their dependency on the adults and no longer being subjected to sub-optimal flows, they can reduce their energy expenditure on feeding and rely more on the naturally high-speed flows. This capacity to adjust to the environment might justify the varying response to hydrodynamics of conditioned and unconditioned individuals, since when unconditioned individuals, used to high flow conditions, are put in weaker flows they show a reduced cirral response (e.g. closed

capitulum, retracted ciri) which will impact food intake. Therefore, with conditioning time, individuals will compensate by increasing active feeding and showing higher responses at reduced flow rates. A minimal flow is still required to stimulate feeding, although it is possible that at even lower flows individuals would have exhibited cirral beating (see Norton, 1996). These results further support the suggestion that although *P. pollicipes* distribution is limited to the high energy intertidal, these animals are not strictly dependent upon the highly hydrodynamic conditions to survive and are able to adjust to feeding in slower water flows. Therefore, the limitations on the distribution of this species may be found elsewhere (e.g. predation, food resources, currents, temperature).

The implications for rearing are that newly collected individuals should be exposed to faster, but not excessive, water speeds of 23 – 31 cm s^{-1}, which can be progressively decreased to standard levels (e.g. 6 – 12 cm s^{-1}). Conditioned individuals also showed higher capture rates than unconditioned animals and this measure may be used as a tool to determine the length of the conditioning period. It is also interesting to note that exaggerated flow rate (e.g. 62 cm s^{-1}) has an inhibitory effect on feeding behaviour, as individuals have difficulty in keeping the cirral net open and curling the cirri when prey are encountered. Furthermore, at excessive water currents, food was frequently observed to be lost from both the cirri or the inside of the oral cone, hindering feeding efficiency. These observations, support Cribeiro (2007), who observed that highly turbulent conditions were essential for adequate feeding by *P. pollicipes*, as well as sustained growth and survival. They are also in accordance with Southward (1955b) and Barnes & Reese (1959, 1960), who speculated that there should be an optimal flow above and below which feeding is limited. However, observations by Thomason et al. (1998) suggest the notion that the density of barnacle aggregations can affect the local flow characteristics. In fact, these authors report that flow velocity alone only accounts for part of the hydrodynamic regime to which an individual is subjected, as upstream conspecifics will play a role in flow interference (e.g. flow reduction, creation of vortexes, etc.). This might not necessarily reduce particle capture, however, due to changes in particle deposition, although it may affect prey size. In cases such as this, where mid-cluster barnacles were selected to evaluate capture rates, there can be a biasing factor since differential prey capture has been reported in other species (Crisp & Bourget, 1985; Pullen & LaBarbera 1991). Nevertheless, results indicate that *P. pollicipes* respond to flow rates similar to what would be experienced in the wild, as

seen in other species (e.g. Hunt & Alexander, 1991), but after a period in captivity they also respond to lower flows. It is our experience (unpublished observations) that conditioning time can vary greatly according to stock, but most individuals will have adjusted to feeding in captivity within a month of collection.

5.4.2. Food quality

With regard to food quality, individuals were observed to use captorial behaviour on live prey, such as *Artemia* sp., *B. plicatillis* and *T. battagliai*. Live instar I of *Artemia* sp. (508 ± 22 µm length) promoted a higher feeding response in *P. pollicipes*, being heavily consumed, probably due to their relatively large size compared to nauplii of *B. plicatillis* (265 ± 20 µm length) and *T. battagliai*. Norton (1996) showed that the cirral net of *P. pollicipes* is responsible for food selectivity, with the prey changing as individuals grow. When using smaller juveniles (5.8 mm RC) in experiments (Norton, ibid.), estimated that ingestion rates of *B. plicatillis* (0.53 J ind^{-1} h^{-1} at densities of 35 rots ml^{-1} or 0.23 J ml^{-1}) were considerably higher than those reported here. Assuming that densities were non-limiting, the prey size in comparison to the cirral net size of these smaller individuals can possibly explain the differences in ingestion to this study. Furthermore, it is suggested that there is a maximal number of prey captured per hour that juveniles are able to ingest, as no differences were seen using different prey densities. Nevertheless, the high values of stimulation observed for conditioned animals in the presence of *B. plicatillis* are also of relevance and might indicate that despite its size, this would still be a suitable feed for use in captivity and has the advantage of improved nutritional profile in comparison to *Artemia* sp..

Maximum consumption of nauplii of *Artemia* sp. was recorded to be considerably above that for frozen nauplii of *Artemia* sp., *T. battagliai* and *B. plicatillis*. This is within range presented by Page (1983) who reported 598 – 611 cal ingested of adult *Artemia* sp. in 40 d^{-1} during the experiment, which would be equivalent to maximal daily ingestion of 62.29 – 63.65 J ind^{-1} d^{-1}. Norton (1996) however, reported an average ingestion rate for *Artemia* sp. nauplii of 2.14 J ind^{-1} h^{-1}, significantly below what was observed in this study. This, again, might be related to the fact that this author estimated ingestion rates not only from smaller isolated barnacles, but they were kept in rotating 20 mL glass vials for a 1-2 h period at 16 °C. In the present experiment, clusters of larger volume were used and the temperature was 20 ºC. The higher rates recorded herein support Barnes (1996), who suggested that individuals would only exhibit full feeding activity for a few hours each day. Nevertheless, the fact that the feeding

behaviour of *P. pollicipes* was observed to vary markedly within a single cluster, with part of the cluster being permanently closed while others fed actively, also has implications for estimating food quantity.

Additionally, the ingestion rates of live and frozen nauplii differed markedly. This is hypothesized to be a consequence of the lower bio-availability of frozen *Artemia* sp. since it does not disperse evenly in the water column and has a tendency to sediment, in spite of water movement. Nevertheless, frozen *Artemia* sp. was clearly perceived by the barnacles as indicated by changes in their behaviour, although these behavioural changes did not translate into capture rates. The inert diets did not to stimulate feeding in *P. pollicipes* and were left unconsumed. This is in accordance to Cribeiro (2007) who reported that *P. pollicipes* did not feed on inert diets, mostly rejecting fish meat, cephalopods, mussels, seabass pellet food and frozen *Artemia* sp.. The previous author further observed that some were rejected after prey capture (e.g. mussel meat). Nevertheless, evidence is insufficient to reject the hypothesis that in the presence of extreme hydrodynamic conditions these would not be bio-available and consumed.

P. pollicipes exhibited a distinct response to the microalgal diets compared to zooplankton, which were ingested via captorial feeding. *T. chuii* did not stimulate captorial behaviour, but did lead to a marked extension response, which was higher in unconditioned individuals. As noted by Norton (1996), microalgae tend to be eaten non-selectively; they are not captured by the cirri and are insufficient to sustain growth by themselves. Therefore, although feeding on phytoplankton is passive, *T. chuii* was still perceived by the barnacles. Interestingly, *P. pollicipes* seemed unresponsive to *I. galbana*. This is likely due to the small size of *I. galbana* (5 – 7 μm length) when compared to *T. chuii* (10 – 20 μm length) and that they are below the selective limit of the cirral net. No measurements of microalgae consumption were made due to the short experimental period, which did not allow for significant decrease in algae concentration.

Interestingly, mostly unconditioned individuals were observed to undertake cirral beating. Based on previous findings, this could have possibly been due to the low hydrodynamics in the tanks that in the case of unconditioned individuals would force individuals into cirral beating, both for breathing and feeding. However, in this case, beating appears to be closely related to feeding, as in conditioned individuals it was mostly observed in the diets that did not stimulate captorial behaviour and cirral extension. This seems to suggest that for high-impact diets (e.g. *Artemia* sp., *Brachionus* sp.) this behaviour is not necessary, being replaced by active capture.

5.4.3. Water temperature and food quantity

Artemia sp. food quantity had no effect on the ingestion rate of *P. pollicipes*, supporting previous observations by Norton (1996). Norton (*ibid.*) observed that *Artemia* sp. density did not affect clearance rates in contrast to results for microalgae. This is possibly due to the large prey size and also supports the theory that the densities tested here were initially non-limiting to feeding behaviour. However, the food provided was consumed rapidly and depletion occurred in the lower concentration diets within the experimental period. On the other hand, increasing the temperature significantly increased ingestion rates. This might be due to an increase in activity and metabolism, although higher consumption does not necessarily indicate higher growth and survival rates. Nevertheless, the recorded ingestion rates can be used as a guide to consumption across a temperature range. Results from other species show that higher temperatures can lead to increased oxygen consumption and higher metabolism, so care must be taken when extrapolating to rearing protocols.

5.4.4. Food saturation

Notwithstanding previous studies on the effect of various food densities on ingestion rates of *P. pollicipes* (e.g. Norton, 1996) providing a working basis for consideration of ingestion rates and feeding energetics, further experiments were necessary to assess whether individual studies represented cluster behaviour throughout a standard feeding period. Although these experiments were designed with a view to evaluating maximal ingestion rates and the effects of food density on ingestion rate, the results were not consistent with those of other authors (e.g. Page, 1983) leaving room for doubt regarding likely behaviour under culture conditions.

Maximal food consumption reached 33.60 J ind^{-1}, over 240 min, although an asymptote was reached at 120 min at 27 J ind^{-1}, indicating the start of saturation. This suggests that approximately 6 *Artemia* were consumed per minute; a value significantly below that observed during the monitoring of cirral capture movements and, perhaps, indicating a low capture feeding efficiency despite high effort. Nevertheless, when monitoring cirral activity, this was done over considerably shorter periods and without the effect of saturation, which will account for the great difference in values. However, the ingestion rate of *Artemia* sp. nauplii should, of course, be taken into account when choosing a feed density. It should also be considered that these results were obtained over 4 hours of continuous monitoring, which allows for the possibility that feeding would be higher in individuals allowed a resting period followed by re-feeding. This would also account

for the higher values observed by Page (1983) in *P. polymerus*. Norton (1996) observed that the ingestion rate of *Artemia* sp. does not relate to prey density, averaging 57.6 ± 4.2 nauplii ind^{-1} h^{-1} across densities, again, significantly less than observed here where consumption rates averaged 304.80 – 462.00 nauplii ind^{-1} h^{-1}. Norton further tested ingestion rates across a range of juvenile sizes to evaluate the magnitude of these differences observing that all barnacles were able to feed at maximal rates, provided that rostrum-carina (RC) distance was above 1.5 mm. The average rate recorded by Norton was 2.14 J ind^{-1} h^{-1}. This is considerably lower than the values presented here, which raises questions over the consistency of responses at higher temperatures or with higher feeding, but especially with the increase in capture capacity as individuals grow; in particular with the large difference in size between barnacles of RC of 1.5 mm and 10 mm, as tested by Norton (1996).

5.5. Conclusion

Based on the present results, it is advisable to use higher flow rates (23 – 32 m s^{-1}) for rearing *P. pollicipes* in captivity, which can be decreased slightly when animals become conditioned to the culture (to speeds no less than 6 m s^{-1}) without compromising the active feeding response. It should be noted, however, that as individuals compensate for decreased flow, energy expenditure might increase and this should be further studied for effects on growth. Of the prey tested, *Artemia* sp. nauplii provided live led to the best results, although *B. plicatillis* and *T. battagliai* also stimulated feeding in spite of their lower energetic value. However, further studies are required to verify the putative relationship between food acceptability and growth/survival rates. Food quantity had a negligible effect on ingestion rate, provided that food supply is not limiting, and values above 4 % of body mass would be advisable. The present results can be used to decide which feeding quantities may be used and the duration of feeding, as well as feeding frequency. Higher ingestion rate, stimulated by higher temperatures and active feeding cannot be directly translated into higher growth and survival without support from further studies, given that under these conditions metabolism is also likely to be elevated. Further studies are essential to establish how the present results compare to growth and survival rates, allowing the establishment of a basis for rearing of *P. pollicipes* juveniles in captivity.

Chapter 6. Culture of juveniles and grow-out in the wild of stalked barnacles (*Pollicipes pollicipes*)

Abstract

In the present study, stalked barnacles (*Pollicipes pollicipes*) were reared in culture under different conditions of feeding, temperature, tidal cycle and photoperiod in order to evaluate the effects on growth, survival and external morphology. Cultured juveniles were also transferred to the field for 6 months to evaluate the effect of a grow-on period. Increased growth and survival in culture can be accomplished using a rearing temperature of 15 °C (over 10 and 20°C). Food quantity (7, 12 and 25 nauplii of *Artemia* sp. ml^{-1}) did not affect growth indicating that this was not limiting. Rostro-carinal distance and stalk length (RC/SL ratio) did not change in culture except at 20 °C with high feeding, for which RC/SL increased significantly. Diets of *Artemia* sp. (provided daily) lead to higher growth and survival rates, while diets of *Tisbe battagliai* and *Tetraselmis chuii* (every 3 days) did not stimulate feeding and led to lower growth. Higher growth was observed in individuals kept without a tidal cycle, while photoperiod had no effect. No differences in survival were observed according to the feeding regimes tested, temperature and food quantity. Smaller individuals (between 0 and 5 mm RC) grew more (1.14 ± 0.20 mm RC $month^{-1}$) than larger individuals (between 5 – 20 mm RC; 0.34 to 0.61 mm RC $month^{-1}$) across all experiments. Maximum average growth and mortality rates in culture were 0.81 ± 0.11 mm RC $month^{-1}$ and 1.37 % $month^{-1}$, for individuals grown in the dark, without tidal influence and fed daily on *Artemia* sp. nauplii. However, laboratory-reared cultures exhibited alterations in morphology in comparison to wild individuals (e.g. pink capitular plates, plate decalcification and plate deformation), though these were mostly reversible after finishing growth in the wild. In the wild, no differences in growth were observed, despite the considerable lower survival of transferred animals (20.70 ± 2.40 %, in comparison to 73.70 ± 4.05 % of wild individuals), indicating that the use of protective cages might be necessary. The estimated growth period of early juveniles to commercial size is 18 to 24 months in culture, followed by a grow-on period in the wild of ≤ 6 months. Future studies should investigate the use of on-shore or suspended culture systems to grow early juveniles, either transferred from culture or recruited directly from the wild.

6.1. Introduction

Pollicipes pollicipes (Gmelin, 1970) is an edible stalked barnacle found in clusters in the highly exposed intertidal. Harvesting is a dangerous manual activity as collectors can be exposed to extreme weather and sea conditions (Cribeiro, 2007). The low product availability, associated with high demand, has maintained initial market prices (Molares & Freire, 2003; Borja et al. 2006a; Jacinto et al., 2010). Due to the economic importance of *P. pollicipes* in Portugal and Spain, it has been a subject of intensive fisheries (Bernard, 1988; Borja et al., 2006ab) and recent interest has risen over the possibility of introducing this species to aquaculture. However, in spite of being considered the most important barnacle species for consumption, other species, such as *Megabalanus azoricus* and *Austromegabalanus psitaccus*, are much better established in terms of aquaculture, due to long-term culture efforts (Lopez et al., 2010).

Very few studies have investigated juvenile rearing in culture (Cribeiro, 2007) and juvenile culture in the wild (Goldberg, 1984). As a result, there are currently no protocols for production, though for research purposes laboratory rearing seems to be well established (Molares et al., 1994a; Cardoso & Yule, 1995; Macho, 2006). Juvenile growth and survival in the wild varies greatly with environmental conditions, being on average between 0.11 to 0.66 mm per month, as measured by the distance between the tops of the rostral and carinal plates; a measure that is considered more stable than others (Cruz, 1993; Cruz, 2000), and hereafter referred to as RC. Growth to commercial size is estimated to take about 12 months (Cruz et al., 2010).

P. pollicipes juveniles have been used extensively for research, and kept for several months in closed and semi-closed production units (Molares, 1994; Norton, 1996, Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007). These were small-scale cultures, flow-through or recirculation systems, with high water renewal and strong circulating flow. Studies were often conducted using the natural range of environmental conditions, including photoperiod, light, hydrodynamics, salinity and temperature. Though no acute mortalities have been registered, the limited reports of the conditions used are insufficient to extrapolate optimum culture conditions and thus leave room for further investigation.

Hydrodynamics is known to significantly affect feeding and consequently growth. In this regard, Cribeiro (2007) subjected *P. pollicipes* cultures to an artificially-produced tidal cycle, which increased barnacle response to food significantly. However, higher growth rates have been observed for *P. polymerus* under permanent submersion

(Hoffman, 1989), presumably due to longer feeding time. Hydrodynamics and light have been suggested not only to impact feeding behaviour and growth, but also external morphology. According to Barnes & Reese (1960) light and photoperiod affect barnacle's physiology and morphology. Besides alterations in appearance, changes in proportion between RC distance and stalk length have been reported. Field observations on *Pollicipes* sp. morphology in populations subjected to decreased hydrodynamics and higher competition for food, commonly known as "percebe mijão", indicate that these tend to show severe stalk elongation, higher water content and fewer scales (Barnes & Reese, 1960; Cruz, 1993, Cribeiro, 2007). Similarly, Cribeiro (2007) observed that juveniles reared in culture have a stalk that is disproportionately long compared to the RC distance (Cribeiro, 2007). This effect on the stalk can also result from inadequate feeding. A way of monitoring *P. pollicipes* proportions is to consider the ratio between RC and stalk length (SL), or the RC/SL ratio. Alterations to external morphology can have effects on marketable value and further data is needed to assess under what conditions these changes occur.

Diet in culture has been mostly restricted to the use of *Artemia* sp. nauplii, due to high acceptability and ease of use when compared to alternate diets (Norton, 1996; Kugele & Yule, 1996; Candeias, 2005; Cribeiro, 2007). However no studies have assessed whether a monodiet of *Artemia* sp. nauplii is sufficient to meet the nutritive needs of *P. pollicipes* juveniles. In fact, there have been no investigations on *P. pollicipes* growth and survival under different diets and feeding regimes, despite important foundation provided by Cribeiro (2007) and Norton (1996) on rearing conditions and feeding, respectively. Furthermore, studies of the quantity of food needed to optimise barnacle growth are still to be developed. Pior research has usually referred to feeding as *ad libitum*, providing no reference for production scenarios. Additionally, the use of adequate diets is essential for development and pigmentation (e.g. Barnes & Reese, 1960; Cruz, 2000; Cribeiro, 2007), as food quality can significantly affect coloration in other barnacle species (Patel & Crisp, 1960).

Besides diet, water temperature has a major impact on growth and survival, and, as suggested by Page (1983), temperature is known to condition resource allocation, for growth or reproduction. There is a seasonal correlation of food availability and temperature in the wild. Furthermore, temperature influences cirral activity in barnacles and therefore feeding (Southward, 1955; Barnes, 1996). Optimal temperatures are related to species distribution and are often the basis for species-specific conditioning

temperatures (Patel & Crisp, 1960). Water temperature off the Atlantic coast of Portugal and Spain varies between 10 to 25 ºC, averaging between 9 to 16 ºC from November to April and 14 to 24 ºC from May to October.

Though several other barnacle species are currently cultured in open systems in the wild (Lopez et al., 2010), in systems based on recruitment substrata, racks or suspended cultures, very little is known about *P. pollicipes* juvenile growth and survival in such culture systems (Goldberg, 1984; Cunha & Webber, 2000). Goldberg (1984) showed that the growth of transplanted stalked barnacles on floating offshore systems was higher than on the coast. However, several systems were lost, which can be a significant problem and have a considerable impact on production. Site selection and system design should therefore be considered, as factors such as tidal level, light, productivity, temperature, predation, and hydrodynamics can have impact on growth and survival of *Pollicipes* sp. (Hoffman, 1988). Seasonal variability also exists, with stalked barnacles showing faster growth in spring and summer, when temperature conditions and food availability are more advantageous (Cruz, 1993). For *P. pollicipes*, on-shore growing culture trials have not been done and most growth and survival data are a result of ecological studies on the natural habitat, where it has been estimated that individuals of commercial size could be produced in about 1 to 2 years after settlement (e.g. Cunha & Webber, 2000; Cruz et al., 2010).

Further extension of the works developed for rearing in captivity and in the wild (Goldberg, 1984; Cribeiro, 2007) is required to broaden knowledge on juvenile *P. pollicipes* culture time and survival as well as to provide basic information on the viability of its culture. This study aimed to investigate the conditions for optimising juvenile culture, focusing on the effects of tidal cycle, photoperiod, temperature, food quantity and quality on the growth, survival and external morphology of *P. pollicipes* juveniles reared in culture. It also evaluated the effects of a follow up growth period in the wild.

6.2. Material and methods

6.2.1. Collection and cultures of P. pollicipes

Clusters of barnacles were collected from the SW coast of Portugal (Cabo Sardão, Portugal, 37°36'24.70", -8°49'2.00") and transported to the facilities at Newcastle University (Newcastle Upon Tyne, UK), where the stocks were maintained in semi-static conditions for a period of four weeks prior to experiments, to acclimatize and

adjust to feeding regimes. All experiments and acclimatization period were run under a standard protocol, adjusted as needed. Standard culture conditions comprised a water temperature of 20 ± 1ºC, photoperiod of 16:8 L/D (dim light, 300 – 400 lux), salinity of 33 ± 1 psu, no tidal cycle and turbulent hydrodynamic conditions maintained by water pumps (Rio® +1000 Aqua Pump) and multiple point-source aeration (Blagdon® Koi Air 50 and Interpet© Aquarium 2.5cm air stones). *Artemia* sp. nauplii (GSL-INT *Artemia* LCC©) were provided daily in excess quantities (25 nauplii mL^{-1} day^{-1}), sporadically supplemented with *Tetraselmis chuii* and *Isochrysis galbana* (≥100.000 cells mL^{-1} 1:1). Water quality, temperature (±1 ºC) and salinity (±1 psu) were monitored regularly to ensure controlled conditions of culture. *Artemia* sp. were cultured with UV-filtered natural seawater (NSW), 33 ± 1 psu, 24:0 L/D photoperiod (1800 – 2200 lux), 28 ± 1 ºC and strong aeration. After 24h, instar I were separated from the cysts and samples were counted to estimate daily feeding volumes. Microalgae cultures were conducted with autoclaved NSW, 33 ± 1 psu, 16:8 L/D photoperiod (1900 – 3500 lux), 20 ± 1 ºC and strong aeration, in F/2 medium (Guillard & Ryther 1962; Guillard 1975).

6.2.2. Experimental design

Prior to the experiment, all barnacles in clusters were measured for rostro-carinal distance (RC) and stalk length (SL) (see Cruz, 2000). Clusters of barnacles were divided into similar sized groups (p= 0.843; Kruskal-Wallis test), according to number of barnacles, mean size and equivalent population structure. Population structure was analysed by assigning the barnacles into size classes according to RC ([0-5mm], [5-10mm], [10-15mm], [15-20mm] and [20-25mm]) and analysing the size distribution of each cluster according to the proportion of each size class. Recruits below 1 mm RC were not considered due to the large associated error with these measurements. Barnacles were used in clusters to approximate natural conditions. The clusters of barnacles were photographed (Olympus© E-410) and each individual was mapped in relation to its cluster, to allow for individual follow-up throughout the experiment. All individuals were also observed for external morphology, with a focus on plate deformities and abnormal colouration.

The clusters were distributed in 5l recirculating aquaria (water renewal of 1 volume per hour), with artificial seawater at standard culture conditions, except when mentioned otherwise. The experimental design tested the effect of photoperiod and tidal cycle (Exp. 1), food quality and feeding regime (Exp. 2), and food quantity and temperature (Exp. 3) on production traits of *P. pollicipes* juveniles reared in culture. Three replicate

tanks per treatment were used. Each replicate was monitored regularly for growth, survival and external condition. The clusters were routinely photographed to allow the re-identification of individuals. Barnacles in clusters are observed to readjust their position and limited mobility can be observed (Kugele & Yule, 1993; Kule & Yule, 2000; Crisp, 1960), making the task of individual identification more difficult if close monitoring is not maintained.

Periodically, the clusters were taken out of water, allowed to rest for 30 min, and each individual was identified, measured for RC distance and stalk length (SL) (see Cruz, 2000), using a digital calliper, and external morphology was analyzed. Survival (% month^{-1} or % year^{-1}; *S*; Eq. 1) was estimated by considering individual identification of the barnacles present in the cluster, and whether alive, over time. Size measurements were used to calculate RC increment (mm RC month^{-1} or mm RC year^{-1}; *RCi;* Eq. 2), specific growth rate (month^{-1} or year^{-1}; *SGR*; Eq.3) and the proportion *RC/SL* (Eq.4), used as a measure for the percentage of the population that had an elongated stalk. External morphology was evaluated qualitatively by the presence of abnormal capitular plate shape, calcification and colouration, as well as abnormal stalk shape, and calculated as the percentage of abnormal individuals (%; *Ab*; Eq.5). The presence of epibionts (e.g. *Corallina* sp., *Ulva lactuca*, *Mytilus edulis*, *Chthamalus montagui*) attached to *P. pollicipes* was also monitored and quantified (%; *Fl*; Eq.6) when relevant.

Eq.1 $$S = \frac{n_{t+1}}{n_t} \times 100$$

Eq. 2 $$RCi = RC_{t+1} - RC_t$$

Eq.3 $$SGR = \frac{\ln(\frac{RC_{t+1}}{RC_t})}{(t+1)-t} \times 100$$

Eq. 4 $$RC/SL = \frac{RC}{SL}$$

Eq.5 $$Ab = \frac{nAb}{n} \times 100$$

Eq. 6 $$Fl = \frac{nFl}{n} \times 100$$

Equations 1 to 6, where *S* is survival (% month^{-1} or % year^{-1}) , n_t or n_{t+1} is number of individuals at time *t* (n_t, #) or *t+1* (n_{t+1}, #), RCi is rostrum-carina distance increment (µm), *RC* is rostrum-carina distance (µm) at time *t* or *t+1*, *SGR* is specific growth rate of barnacles (month^{-1} or year^{-1}), *ln* is nepereian logarithm, *RC/SL* is index of proportion, *SL* is stalk length distance (µm), *Ab* is percentage of externally abnormal individuals (%), *nAb* is number of individuals with external abnormalities (#), *n* is number of individuals (#), *Fl* is percentage of fouled individuals (%), *nFl* is number of fouled individuals (#).

6.2.3.1. EXP.1 EFFECTS OF TIDAL CYCLE AND PHOTOPERIOD

The effects of tidal cycle (present or absent) and photoperiod (0:24 L/D or 16:8 L/D) were tested simultaneously by means of a full factorial design (4 treatments), on the production traits of *P. pollicipes* juveniles reared in captivity for a month. Treatments were as follows 16:8 L/D and no tidal cycle (*LnT*); 16:8 L/D and tidal cycle (*LT*); 0:24 L/D and no tidal cycle (*DnT*); 0:24 L/D and tidal cycle (*DT*). The replicated groups (52.38 ± 11.82 ind; 8.17 ± 3.53 mm RC; mean ± SD) were distributed in the 12 aquaria. The tidal cycle was accomplished by controlling the water level in the aquaria, allowing for the level to decrease/increase in 30 min and with a daily emersion time of 4 h. The aquaria were held in a photoperiod-controlled room set for a 16:8 L/D cycle. Treatments on 0:24 L/D were kept dark covered to assure full darkness, while the treatments on 16:8 L/D were enclosed with transparent covers to allow for maintenance of the long-day photoperiod cycle. Monitoring was done over a period of 1 month.

6.2.3.2. EXP.2 EFFECTS OF FEEDING REGIME AND FOOD QUALITY

The effect of feeding regime (e.g. daily, every 2 days, every 3 days) and food quality (*Artemia* sp., *Tetraselmis chuii* and *Tisbe battagliai*), was tested by means of a partial factorial design (7 treatments), on the growth and survival of *P. pollicipes* juveniles over a period of 1 month. The replicate groups (40.02 ± 5.93; ind; 7.70 ± 3.48 mm RC; mean ± SD) were distributed between the 21 aquaria. Treatments were as follows: daily feeding with *Artemia* sp. nauplii (*Art-1d*); every other day feeding with *Artemia* sp. nauplii (*Art-2d*); every 3 days feeding with *Artemia* sp. nauplii (*Art-3d*); daily feeding with microalgae *Tetraselmis chuii* (*Alg-1d*); daily feeding with a mixed diet of *Artemia* sp. and *T. chuii* (*Art/Alg-1d*); and every 3 days feeding with a mixed diet of copepodites of *Tisbe battagliai* and the microalga *T. chuii* (*Cop/Alg-3d*). Feeding was done to excess at 1000 µgC l^{-1}, balanced on mixed diets at a 1:1 ratio of carbon content. Monitoring was done over a period of 1 month.

6.2.3.3. EXP. 3 EFFECTS OF WATER TEMPERATURE AND FOOD QUANTITY

The effects of water temperature (10, 15 and 20 ºC) and food quantity (7, 12, 25 nauplii ml^{-1} of *Artemia* sp.) were tested by means of a full factorial design (9 treatments), on the growth and survival of *P. pollicipes* juveniles over the 12-month period. The aquaria were distributed in three temperature controlled rooms set for 10, 15 and 20 ºC, with culture temperature averaging 10.5 ± 0.9 ºC, 14.6 ± 1.0 ºC and 19.8 ± 1.2 ºC. The replicate groups (83.70 ± 12.47 ind; 7.83 ± 4.58 mm RC; mean ± SD) were distributed between the 27 aquaria. Treatments were as follows: feeding with 7 nauplii ml^{-1} at 10 ºC

(*l0/low*); feeding with 12 nauplii ml^{-1} at 10 ºC (*l0/medium*); feeding with 25 nauplii ml^{-1} at 10 ºC (*l0/high*); feeding with 7 nauplii ml^{-1} at 15 ºC (*l5/low*); feeding with 12 nauplii ml^{-1} at 15 ºC (*l5/medium*); feeding with 25 nauplii ml^{-1} at 15 ºC (*l5/high*); feeding with 7 nauplii ml^{-1} at 20 ºC (*20/low*); feeding with 12 nauplii ml^{-1} at 20 ºC (*20/medium*); and feeding with 25 nauplii ml^{-1} at 20 ºC (*20/high*). Monitoring was done over a period of 12 months.

6.2.3.4 EXP. 4 EFFECT OF FINISHING GROW-OUT IN THE WILD

P. pollicipes reared in culture for 12 months (Exp. 3) were transferred to the natural environment for a period of 6 months (from May to November 2012) and monitored for growth, survival and external morphology. Clusters in the natural environment, exclusively grown in the wild, were also monitored for comparison. Nine clusters of cultured barnacles (*cPp*; cultured *P. pollicipes;* 57.11 ± 11.30 ind; 14.21 ± 4.06 mm RC; mean ± SD) at 15 ºC (see Exp. 3) were transferred from the rearing facilities at the School of Marine Science of Technology (Newcastle University, UK) to the SW coast of Portugal (Cabo Sardão, Portugal, 37°36'24.70", -8°49'2.00"). The clusters, together with the rocks to which they were attached, were randomly distributed and glued (Z-Spar Marine Epoxy ©) to rocks in the natural habitat. Within the same area, three native clusters (*wPp*; wild *P. pollicipes*) were randomly selected and marked for identification purposes, and were subjected to the same monitoring and sampling as the transferred groups. Within these clusters only barnacles over 10 mm RC were followed (42.24 ± 8.54 ind; 13.26 ± 2.24 mm RC; mean ± SD) to avoid a mismatch in size between cultured and wild individuals. At the beginning of the experiment the clusters were photographed and individuals were mapped, measured and observed for external morphology, as previously detailed. The previous procedures were repeated after 6 months. Due to difficulties in developing such measurements during the time constrained field work, mapping and measuring on *wPp* was done over two consecutive days of field work. Mapping and measurements on *cPp* were made in the laboratory within 24 hours of transfer (deployment or collection) to the wild. At the start of the experiment, mapping of *wPp* occurred on day 1, simultaneously to *cPp* deployment, and measurements of *wPp* were done on day 2. At the end of the experiment, the opposite was done, i.e., *wPp* were measured on day 1 and collection of *cPp* was done on day 2, leaving the same number of days between measurements.

6.2.3. Data treatment and statistical analysis

All statistical analyses were performed using STATISTICA 7.0 ® and data as percentages (%) were arcsine transformed. Data were subjected to parametric tests, namely Student's t-test (T-Test), analysis of variance (ANOVA) and analysis of covariance (ANCOVA), according to relevance, when assumptions for normality and homocedasticity were met (Shapiro-Wilk and Levene's test, respectively), with a significance level of α=0.05. Analyses of growth were conducted through an ANCOVA, using time as a covariate. Significant ANOVAs and ANCOVAs were followed by a Tukey-test to identify differences among groups. Data that did not obey the assumptions for normality and homocedasticity were subjected to non-parametric tests, namely the Kruskal-Wallis test. All figures and tables show the mean ± standard error (SE), except when mentioned otherwise.

6.3. Results

6.3.1 Tidal cycle and photoperiod

Tidal cycle significantly affected juvenile growth rate (ANOVA, $F=11.05$, $p<0.01$; Tukey Test, $p<0.01$), associated with a lower RC increment, of 0.43 ± 0.07 mm RC month^{-1} in comparison to individuals kept without a tidal cycle, which grew 0.78 ± 0.07 mm RC in the same period (fig. 1a). On the other hand, neither photoperiod (ANOVA; $F=0.85$, $p=0.36$) nor the interaction between both variables (ANOVA, $F=0.04$, $p=0.84$) significantly affected RC increment. An identical pattern was observed for SGR, which was 0.05 ± 0.01 and 0.08 ± 0.01 month^{-1} for individuals grown with or without a tidal cycle respectively. Smaller individuals (between 0 and 5 mm RC) grew more (1.14 ± 0.20 mm RC month^{-1}; ANOVA, $F=8.49$, $p<0.0.1$; Tukey Test, $p<0.01$) than their larger counterparts (between 5 – 20 mm RC). Nevertheless, they did not show significant differences in RC increment (Tukey Test, $p\geq0.17$), which ranged on average between 0.34 to 0.61 mm RC month^{-1} (Table 6.1).

Juvenile survival varied on average between 90.45 and 98.63 % (fig. 6.1b). There was a significant interaction between tide and photoperiod (ANOVA, $F=13.22$, $p<0.01$), as individuals grown in the dark under a tidal cycle showed lower survival than the ones not subjected to tidal cycles (Tukey Test, $p=0.03$).

Treatment	Size class (mm RC)			
	]0,5]	]5,10]	]10,15]	]15,20]
DnT	1.61 ± 0.70	0.96 ± 0.15	0.66 ± 0.13	0.57 ± 0.16
LnT	1.51 ± 0.30	0.72 ± 0.12	0.59 ± 0.11	0.48 ± 0.12
DT	0.93 ± 0.20	0.63 ± 0.17	0.30 ± 0.07	0.15 ± 0.15
LT	0.92 ± 0.50	0.44 ± 0.11	0.29 ± 0.07	0.21 ± 0.09

Table 6.1. Rostrum-carina increment (RCi; mm month^{-1}), according to size class (mm RC), of *P. pollicipes* juveniles cultured for a month, under different environmental conditions of tide and light. Treatment were as follows, (*DnT*) 0:24 L/D photoperiod and no-tidal cycle, (*LnT*) 16:8 L/D photoperiod and no-tidal cycle, (*DT*) 0:24 L/D photoperiod and tidal cycle, and (*LT*) 16:8 L/D photoperiod and tidal cycle. Juveniles were divided into to the following size class:]0, 5] from 0 to 5 mm RC,]5, 10] from 5 to 10 mm RC,]10, 15] from 10 to 15 mm RC, and]15, 20] from 15 to 20 mm RC.

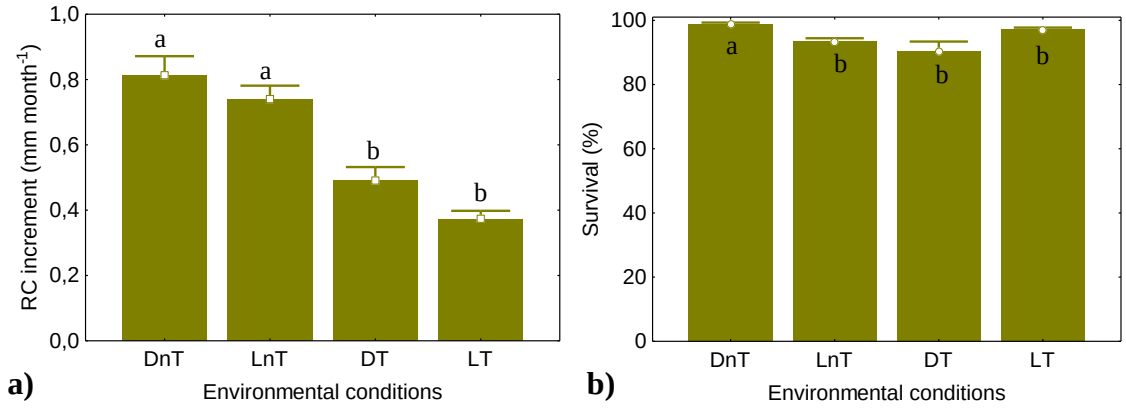


Fig. 6.1 (a) Rostrum-carina increment (RCi, mm) and (b) survival (% month-1), for juveniles grown at different tidal cycle and photoperiod. Tidal cycle was either absent or present, with a daily tide of 4h. Photoperiod was either considered (*D*) full darkness with 0:24 L/D or (*L*) light dominated with 16:8 L/D. Treatments were as follows (*DnT*) 0:24 L/D photoperiod and no tidal cycle, (*LnT*) 16:8 L/D photoperiod and no tidal cycle, (*DT*) 0:24 L/D photoperiod and tidal cycle and (*LT*) 16:8 L/D photoperiod andtidal cycle. For RC increment, significant differences were found with tidal cycle (ANOVA, $F=11.05$, $p<0.01$), but neither with photoperiod (ANOVA; $F=0.85$, $p=0.36$) or the interaction between both variables (ANOVA, $F=0.04$, $p=0.84$). For survival, there were significant differences for the interaction between tide and photoperiod (ANOVA, $F=13.22$, $p<0.01$), but neither tide (ANOVA, $F=1.74$, $p=0.22$) nor photoperiod (ANOVA, $F=0.15$, $p=0.71$).

6.3.2 Feeding regime

Growth (as RCi) varied according to feeding regime (fig. 6.2a; ANOVA, $F=9.15$, $p<0.01$). Individuals fed with diets containing *Artemia* sp. had a significantly highest growth rate (Tukey, $p\geq0.08$), with the lowest growth with *T. chuii* monodiets and every 3 day feeding with mixed diets of *T. battagliai* and *T. chuii* (Tukey, $p<0.01$). Feeding frequency and algal supplementation in *Artemia* sp. based diets did not lead to significant differences in growth, with animals fed daily with brine shrimp growing 0.81 ± 0.06 mm RC month^{-1}. Individuals on *Art-2d, Art/Alg-1d and Art-3d* grew respectively

0.72 ± 0.07 mm RC month^{-1}, 0.66 ± 0.07 mm RC month^{-1} and 0.59 ± 0.07 mm RC month^{-1}. Similarly, SGR ranged from 0.04 to 0.11 month^{-1} according to feeding regime. When growth was analyzed according to size class (Table 6.2), growth was significantly different according to RC distance (ANOVA, F=16.69, p=0.28), with higher growth observed in smaller individuals. The interaction between size class and feeding regime was not significant (ANOVA, F=1.18, p=0.29). Juvenile survival (fig. 6.2b) was 95.28 ± 0.32 % month^{-1} and did not differ between feeding (ANOVA, F=0.25; p=0.93).

Treatment	Size class (mm RC)			
	]0,5]	]5,10]	]10,15]	]15,20]
Art-1d	1.54 ± 0.12	0.85 ± 0.09	0.57 ± 0.09	0.25 ± 0.07
Art-2d	1.20 ± 0.13	0.81 ± 0.10	0.54 ± 0.09	0.32 ± 0.12
Art-3d	0.96 ± 0.19	0.72 ± 1.00	0.38 ± 0.08	0.29 ± 0.08
Alg-1d	0.57 ±0.12	0.47 ± 0.06	0.32 ± 0.08	0.28 ± 0.11
Art/Alg-1d	1.42 ± 0.74	0.88 ± 0.11	0.49 ± 0.09	0.24 ± 0.09
Cop/Alg-3d	0.53 ± 0.08	0.39 ± 0.07	0.38 ± 0.07	0.25 ± 0.15

Table 6.2. Rostrum-carina increment (RCi; mm month^{-1}), according to size class (mm RC), of *P. pollicipes* juveniles cultured for a month, under different feeding regimes. These were as follows (*Art-1d*) daily feeding with *Artemia* sp. nauplii, (*Art-1d*) daily feeding with *Artemia* sp. nauplii, (*Art-2d*) every other day feeding with *Artemia* sp. nauplii, (*Art-3d*) every 3 days feeding with *Artemia* sp. nauplii, (*Alg-1d*) daily feeding with microalgae *Tetraselmis chuii*, (*Art/Alg-1d*) daily feeding with a mixed diet of *Artemia* sp. and *T. chuii*, and (Cop/Alg-3d) every 3 days feeding with a mixed diet of copepods *Tisbe battagliai* and microalgae *T. chuii*. Juveniles were divided into to the following size class:]0, 5] from 0 to 5 mm RC,]5, 10] from 5 to 10 mm RC,]10, 15] from 10 to 15 mm RC, and]15, 20] from 15 to 20 mm RC.

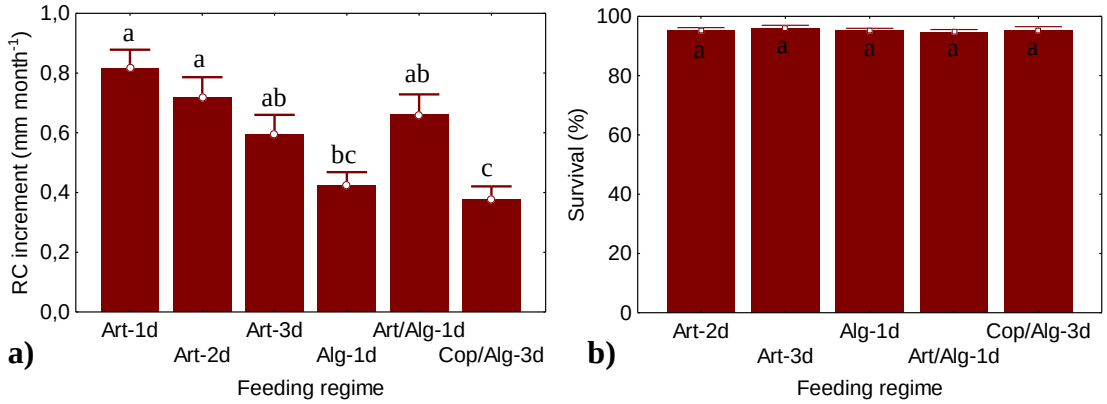


Fig. 6.2 (a) Rostrum-carina increment (RCi, mm) and (b) survival (% month^{-1}), for juveniles grown at different feeding regimes. These were as follows (*Art-1d*) daily feeding with *Artemia* sp. nauplii, (*Art-1d*) daily feeding with *Artemia* sp. nauplii, (*Art-2d*) every other day feeding with *Artemia* sp. nauplii. (*Art-3d*) every 3 days feeding with *Artemia* sp. nauplii, (*Alg-1d*) daily feeding with microalgae *Tetraselmis chuii*, (*Art/Alg-1d*) daily feeding with a mixed diet of *Artemia* sp. and *T. chuii*, and (Cop/Alg-3d) every 3 days feeding with a mixed diet of copepods *Tisbe battagliai* and microalgae *T. chuii*. Significant differences were found for RC increment (ANOVA, F=9.15, p<0.01), but not survival (ANOVA, F=0.25; p=0.93).

6.3.2 Water temperature and food quantity

When the long-term effects of temperature and food quantity on RC increment (fig. 6.3a) were evaluated, significant differences were found for temperature (ANOVA, F=89.71, p<0.01), but not food quantity (ANOVA, F=0.95, p=0.05). Temperature seemed to exert the stronger effect on growth, as individuals maintained at 10 °C, 15 °C and 20 °C showed RC increments of 3.90 ± 0.14, 6.63 ± 0.14 and 5.17 ± 0.12 month^{-1}, respectively. The RC increment values with regard to food rations of 7, 12 and 25 nauplii ml^{-1} were 5.54 ± 0.15, 5.28 ± 0.09 and 5.49 ± 0.15 mm month^{-1} respectively. Barnacles kept at 15 °C and fed 7 or 25 nauplii ml^{-1} had the highest growth rates (Tukey Test, p≥0.07), with increments between 6.5 and 7.3 mm RC (Tukey Test p<0.01). The lowest growth rates were recorded at 10 °C with a ration of 7 or 12 nauplii ml^{-1} and at 20 °C for 25 nauplii ml^{-1} (Tukey Test, p=0.09). There were otherwise no differences with treatments (Tukey Test, p≥0.05).

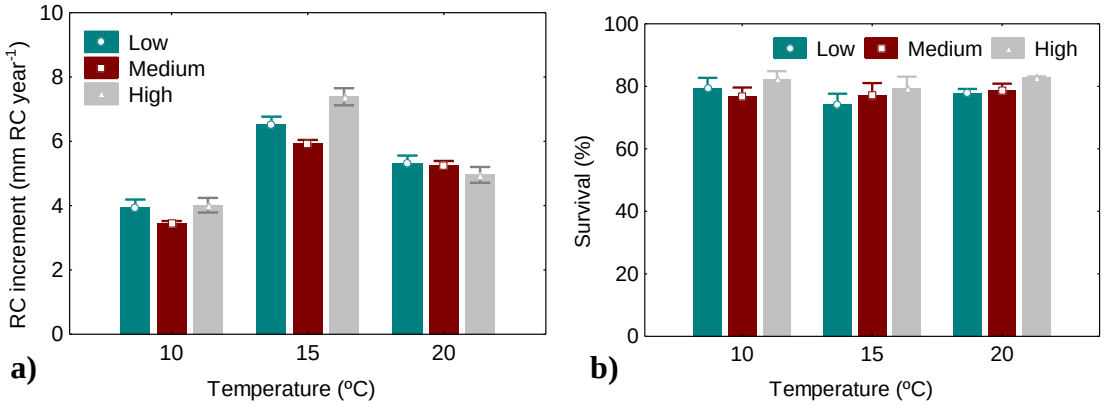


Fig. 6.3 (a) Rostrum-carina increment (RCi, mm) and (b) total survival (% 12months^{-1}), for juveniles grown at different temperatures (10, 15 and 20 °C) and feeding quantity (low, medium, high). Significant differences on RCi were found for temperature (ANOVA, F=89.71, p<0.01) and the interaction of both variables (ANOVA, F=4.37, p=0.01), but not food quantity alone (ANOVA, F=0.95, p=0.05). No significant differences on survival were observed with temperature (ANOVA, F=0.92, p=0.41), food quantity (ANOVA, F=2.08, p=0.15) and the interaction of both (ANOVA, F=0.24, p=0.91).

Neither temperature (ANOVA, F=0.92, p=0.41) nor food quantity (ANOVA, F=2.08, p=0.15), affected survival significantly (fig. 6.3b). There was also no significant interaction between both factors (ANOVA, F=0.24, p=0.91). Total survival by the end of the 12-month period ranged between 74.06 ± 3.59 and 82.72 ± 0.44 %. Differences observed according to size class supported the results of previous experiments and are shown in Fig.6. 4.

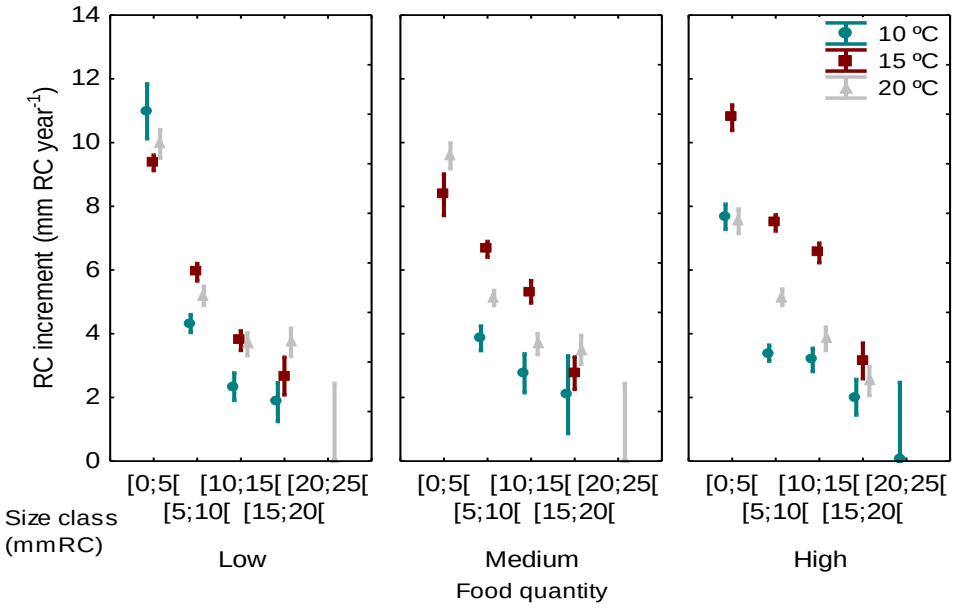


Fig. 6.4 Rostrum-carina increment (RCi; mm month^{-1}), according to size class (mm RC), for *P. pollicipes* juveniles cultured for 12 months, under different temperatures (10, 15 and 20 °C) and feeding quantity (low, medium, high). Juveniles were divided into to the following size classes:]0, 5] from 0 to 5 mm RC,]5, 10] from 5 to 10 mm RC,]10, 15] from 10 to 15 mm RC, and]15, 20] from 15 to 20 mm RC.

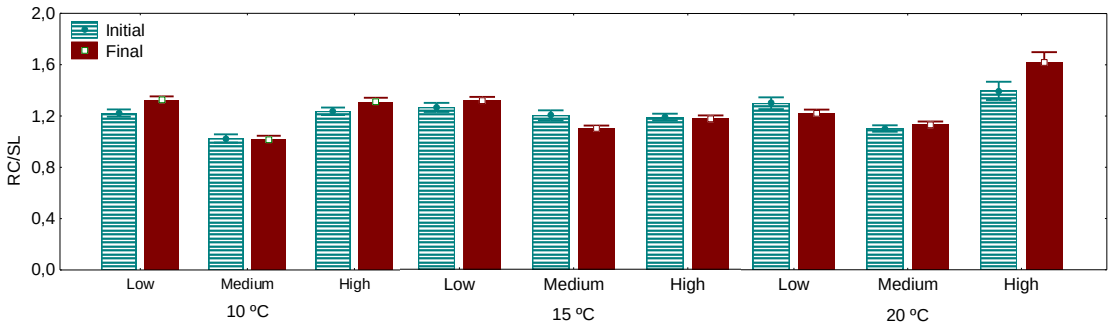


Fig. 6.5 Proportion between rostrum-carina distance and stalk length (RC/SL) for juveniles grown at different temperatures (10, 15 and 20 °C) and feeding quantity (low, medium, high), compared across time, at the beginning (initial) and end (final) of the experiment. RC/SL proportion varied significantly with time, food quality and temperature (ANOVA, F=3.34, p≤0.01).

The proportion RC/SL changed with time as shown in Fig. 6.5. Initial RC/SL averaged 1.24 ± 0.01, while the final value averaged 1.27 ± 0.01 mm, though this difference was not significant (ANOVA, F=1.07, p=0.30). The differences in this proportion were also not significantly different with time across temperature and food quantity, except at 20 °C with high feeding (ANOVA, F=3.34, p≤0.01; Tukey Test, p=0.01), where RC/SL increased from 1.39 ± 0.07 to 1.62 ± 0.08. The morphology of individuals changed

considerably over time, with different types of morphological alterations noted (fig. 6.6). These included pink capitular plates, plate decalcification and plate deformation. Over 85.55 ± 5.76 % of individuals showed at least one form of malformation, although there was no difference in prevalence across treatments (ANOVA; F=0.24, p=0.76).

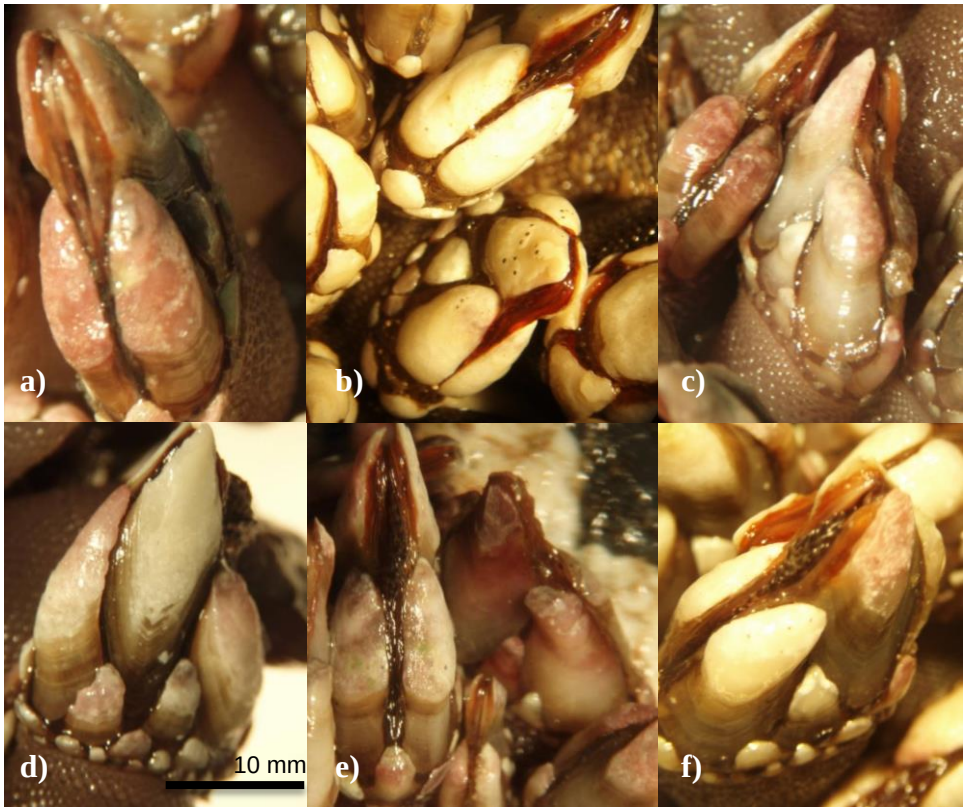


Fig. 6.6 Alterations on *P. pollicipes* juvenile morphology during growth in culture (a, c, d, e, f), in comparison to normal individuals (b). These ranged from alterations to (a) capitular plate coloration (mostly to a pink coloration), to (c-e) capitular plate deformation and decalcification.

6.3.4 Finishing grow-out in the wild

The growth rate of individuals transferred to the wild averaged 0.26 ± 0.10 mm RC month^{-1}, and ranged from 0 to 0.93 mm RC month^{-1}. These values were comparable to those of native incumbents (T-Test; t=0.19; p=0.85). Overall growth after 6 months averaged 1.66 mm RC, with individuals averaging 15.26 ± 0.15 mm RC in size. No growth differences in the field were observed in transplants originating from distinct rearing protocols (ANOVA, F=0.07, p=0.99).

Transfer to the wild impacted survival to 6 months, which averaged 20.70 ± 2.40 %, compared to 73.70 ± 4.05 % for wild barnacles. There were also significant differences in monthly mortality between previously cultured and wild barnacles (T-Test, t=9.80, p<0.01). Monthly mortality was 4.38 ± 0.68 % month^{-1} for wild barnacles and 13.33 ± 0.47 % month^{-1} for previously cultured barnacles.

No differences were observed between initial and final RC/SL index for transferred groups (T-Test; t=0.14; p=0.88). The incidence of malformations decreased significantly after rearing in the wild (ANOVA, F=125.71, p<0.01; Tukey Test, p<0.01), from 83.33 ± 0.10 % to 18.33 ± 0.29%, compared to 15.15 ± 0.16 % for clusters of wild barnacles. The incidence of malformations did not change in time in natural clusters (Tukey Test, p=0.99), being comparable to cultured groups after finishing grow-out (Tukey Test, p≥0.77). Capitular plates no longer presented the pink coloration acquired in culture or signs of decalcification, and the only malformations still observed were of plate deformity. Epibiosis of the capitular plates, mostly by green macroalgae, was observed in 60.65 ± 16.08 % of the individuals.

6.4 Discussion

Although there have been a few previous studies on the culture of *P. pollicipes* (e.g. Goldberg, 1984; Cribeiro, 2007) and maintenance of this species under culture (Molares et al., 1994a; Cardoso & Yule, 1995; Macho, 2006), little was known about the response of this species to different rearing conditions. From the present data, tidal cycle, temperature and feeding regime appear to have the highest repercussions for success of culture.

6.4.1. Tidal cycle

Tidal cycle reportedly stimulates feeding of *P. pollicipes* (Cribeiro, 2007), but current results support the observations of Hoffman (1989) on *P. polymerus* in the wild, where higher growth rates were found for animals under permanent immersion. Earlier studies on barnacles also noted that growth rate was proportional to submergence time (e.g. Barnes & Powell, 1953; Barnes & Barnes, 1962). Hoffman (1989) suggests that that results for *P. polymerus* might be due to longer feeding time. However, observations by Bourget & Crisp (1975) might also be of relevance, as these authors reported that shell growth of *Semibalanus balanoides* decreases markedly upon emersion. The higher growth rate resumed upon immersion was consistent with a need for external calcium ions for shell growth. In the present study, growth was higher in animals that did not experience a tidal cycle. Whether this result reflected more time for feeding or renewed access to external calcium, or a combination of the two, remains unknown. According to Barnes & Reese (1960), even in the most suitable sites full feeding activity is limited to certain periods of the day. In the natural habitat, *Pollicipes* sp. are naturally exposed to air for periods of up to 9 h per day, depending on intertidal height, with growth rates herein (with exposure of 4 h) matching those recorded in the wild. This suggests that

limits to feeding imposed by tidal exposure may be less significant in culture and would be more accentuated in the field. Furthermore, food was not provided continuously in culture, which is potentially the case in the wild, as *P. pollicipes* are observed to become saturated after 2h of feeding in culture, with cirral activity and ingestion declining drastically (Franco, per. obs). Nevertheless, Cribeiro's (2007) observations remain valid and though a tidal cycle might not be beneficial for growth, it might be an added advantage to reproduction. Whereas on highly exposed rocky shores flow velocities range between 0.5 – 20 m s^{-1} (Denny, 1988), when in culture, stimulation of feeding might often be insufficient to sustain continuous feeding, and turbulent tidal cycles can present confounding effects due to increased hydrodynamics. Furthermore, hydrodynamics can affect competition and therefore impact barnacle RC/SL proportion. Field observations on *Pollicipes* sp. populations of "percebe mijão" indicate that these tend to show severe stalk elongation (Barnes & Reese, 1960; Cruz, 1993, Cribeiro, 2007), possibly due to insufficient feeding. Though in the present experiment RC/SL was not followed, due to time constraints, no apparent changes were observed, supporting the absence of major hydrodynamic difference between tide exposed groups.

6.4.2. Photoperiod

Several studies have investigated the effects of light and photoperiod on barnacle development and reproduction (e.g. Barnes, 1953; Costlow & Bookhout, 1956; Barnes & Stone, 1974; Bourget & Crisp, 1975; Crisp & Bourget, 1985), but only a few have focused on *P. pollicipes* (e.g. Macho et al., 2005). Costlow & Bookhout (1953, 1956) reported no effect of photoperiod on the moulting rate of *B. amphitrite niveus* and *B. improvisus*. Likewise, the growth of *S. balanoides* is unaffected by photoperiod (Crisp & Patel, 1960; Bourget & Crisp, 1975; Crisp & Bourget, 1985). The only contradicting results are those of Klugh & Newcombe (1935) who reported decreased growth under direct sunlight, though this has received little support from subsequent studies. Barnes & Stone (1974) refer to the same lack of effects in moulting frequency, except when during and after reproduction, possibly due to hormonal control effects or due to interaction between reproduction and photoperiod. In fact, of the physiological responses studied, reproduction in barnacles seems to the only affected by photoperiod (Davenport et al., 2005). As regards *P. pollicipes*, Macho et al. (2005) found that the timing of larval release in *P. pollicipes* is affected by light. The present study has shown that there is no effect of light or photoperiod on the growth and survival of this species. According to Barnes & Reese (1960), however, light and photoperiod affect *P.*

polymerus physiology and morphology. They found that development of the stalk colour is in part dependent on light exposure, as stalk colour appeared when barnacles were transferred from the dark to light. This effect was also observed in this study when recruits, that were previously attached (deep in the clusters), were removed and kept isolated in culture, acquiring a deeper coloration, passing from a pale greyish stalk to a pigmentation similar to the deep-purple of the adults (Franco, pers. obs.). In the current experiment, no such alterations in pigmentation where observed with light regime. The only difference observed was on the proliferation of epiphyte algal species on the capitulum of *P. pollicipes* grown under long-day photoperiod, but absent on individuals grown in the dark. The presence of epiphytes is common in the natural environment, with *Ulv*a sp. and other microalgae often found covering the capitular plates of *P. pollicipes*. Whether the algae affect feeding efficiency and growth, remains unknown, though Hoffman (1989) suggested that this epibiosis on *P. polymerus* can decrease recruitment. Moreover, studies on mussels and epiphyte algae have shown that these can negatively affect abundance of mussels (Albrecht & Reise, 1994).

6.4.3. Temperature

Periods of increased reproductive effort in invertebrates are often correlated with periods of decreased growth (Page, 1983). As species distribution is restricted to specific temperature ranges, stock-specific differences in reproductive cycles have also been observed for various Pollicipedidae inhabiting over broad distributions. Distinct physiological races have been reported along the latitude of distribution of *P. polymerus* and *C. mitella*, in association with temperature (Hines, 1978; Cimberg, 1981; O'Riordan et al., 2004; Chan, 2006). In the wild, no brooding individuals of *P. pollicipes* are found below 14 ºC in the south of Portugal (Cardoso & Yule, 1995) and 11 ºC in the north of Spain (Macho, 2006). In the case of *P. pollicipes*, reduced growth has been observed in summer months, during the peak of the reproductive season (Cruz, 2000). This is the period of higher food availability, with temperatures ranging from 14 °C to 24 ºC from May to October. The results from the current study suggest that growth of *P. pollicipes* is best around 15 °C to 20 °C, whereas growth is compromised at 11 °C. Since no significant differences in growth were observed with food quantity, it is assumed that food was not limiting and might been considered in excess in some cases. In order to analyze these results, two things need to be considered: a) barnacle age and maturity and b) ecological significance of the temperatures tested. The barnacles used herein were mostly juveniles (≥ 75 %) at the start of the experiment. Age is difficult to

estimate in barnacles when studies do not start with newly settled juveniles. Estimations based on size provide the next best option for analysis, with RC distance considered the most reliable measure of growth (Cruz, 2000). In the field, mature females are found above 12.5 mm RC and mature males above 10 mm RC (Cruz, *ibid*.). According to size resource allocation will be distinct; with early recruits allocating resources exclusively to growth while juveniles closer to maturity and mature adults will focus resources on gonad development. Therefore, under identical conditions of temperature, early juveniles will be expected to show a higher growth than later juveniles and adults, though this difference may be higher according to the ecological and metabolic impact of the temperature in question.

It is hypothesized that in the wild, as temperatures are higher during the breeding season, when recruitment occurs, early juveniles benefit from a period of very high growth by the end of Summer, which would then decrease on Winter months (due to temperature, food and age). On the other hand, adults would see their growth stagnate in the Summer (despite higher temperatures), followed by an increase in growth as the reproductive season ends, when gonad recovery occurs and temperatures range from 9 ºC to 16 ºC, from November to April. As in the current study, temperatures remained stable throughout and were only compared at different levels; several scenarios were expected in accordance to the previous hypothesis, depending on whether early or late juveniles were being considered and their absolute growth in effect of temperatures. Cruz (2000) reported highly variable growth of adults and juveniles in the wild throughout the year, with, no significant differences between the two, except from mid-June to late-October, where juveniles grew on average 0.47 mm RC month^{-1} in comparison to the 0.11 mm RC month^{-1} in adults. This supports the previous hypothesis, being consistent with a shift in resource allocation towards reproduction, instead of growth.

The lack of differences, during the rest of the year, between juveniles and adults, is explained given the correlation between juveniles age and temperature variation throughout the year, making it difficult to make an accurate analysis in field studies. In the wild, younger juveniles will grow more and be the dominant cohort by the end of Autumn/Winter, just after reproductive season. However, food availability is limited and temperature is lower at that time of year, constraining growth to average values. On the other hand, by the following summer, most juveniles from the previous season will be older and their growth rate will be reduced, in spite of better growth conditions of

food and temperature, which will increase growth to average values. If this confounding effect is removed, most likely the expected pattern of growth would be based solely on seasonal variations of food/temperature, excluding the influence of age. If food ration is normalized and temperature is stable, the effect of temperature on growth alone can be evaluated. Therefore, the best temperature for growth should become evident, which may or may not coincide with the highest temperature experienced in the wild.

This is also patent in the current results, were 15 ºC seems to be the temperature that favours growth for this species, while breeding often occurs above this value in the wild. The likely implications of these observations are that, given unlimited food, the energy "trade-off" between reproduction and growth will be regulated by water temperature. In fact, for *Mytillus edulis* kept under culture, the energy available for growth when above 20^{o} C decreases as a result of increased metabolic rate and reduced suspension feeding (Bayne, 1976), so it is not always the highest temperature that is optimal. Various studies have shown that higher temperatures within the distribution range do not necessarily lead to the highest growth (e.g. Southward, 1957; Patel & Crip, 1960; Barnes & Barnes, 1969).

6.4.4. Food quantity and quality

From the current results, growth was not affected by food quantity in the long term (1-year growth), though at the temperature at which the highest growth was recorded (15 °C), higher rations led to the highest growth rates. The lack of effect of quantity is not surprising, since for large particles, such as *Artemia* sp., filtration rates of actively feeding *P. pollicipes* are independent of prey density above a minimum requirement (Norton, 1996; see also Crisp, 1961). When diets were compared over shorter growth periods (1 month), *Artemia* sp. (given daily) lead to higher growth and survival rates, with growth decreasing with feeding frequency. On the other hand, diets of *T. battagliai* and *T. chuii* (every 3 days) and mono-diets of *T. chuii* did not stimulate feeding and led to lower growth. These prey are considerably smaller than *Artemia* sp. nauplii and therefore stimulation might not be equivalent, decreasing captorial behaviour. *P. pollicipes* can exhibit various types of cirral activity, from testing, pumping and beating to active captorial behaviour. The in-curling of cirri transporting the captured food items (Norton, 1996) has only been observed in the presence of larger prey such as *Artemia* sp. and *T. battagliai*. The cultures fed on *T. chuii* alternated between the different types of cirral extension, but did not show active captorial behaviour. *Artemia* sp. has long been used as feed in aquaculture due to its availability and wide acceptability (Bengtson

et al., 1991, Légger et al. 1986; Lavens & Sorgeloos, 2000). Studies on barnacles are no exception, and Norton (1996) when maintaining *P. pollicipes* in culture on *Artemia* sp., observed maximal growth rates of 0.930 mm RC month^{-1} for older juveniles and 0.509 mm RC month^{-1} for adults in culture. This is comparable to the average values reported here of 0.81 ± 0.06 mm RC month^{-1}. Nevertheless, the nutritional profile of *Artemia* sp. varies considerably biogeographically, within strains and in time, being often surpassed nutritionally by other live feeds or through enrichment (Sorgeloos et al., 1998; Støttrup, 2000).

It is not known whether a monodiet of *Artemia* sp. nauplii is sufficient to meet the nutritive needs of *P. pollicipes* juveniles. Despite the positive results attained in the long term for growth and survival, the recorded alterations in morphology suggest serious nutritional deficiencies. On the other hand, other diets, such as copepods, rotifers and natural plankton have been presented as better alternatives for aquaculture in terms of nutritional profile and by being natural prey of many cultured species (Shields et al., 1993; McEvoy et al., 1998). In the case of *P. pollicipes*, Norton (1996) observed that younger juveniles of 5.5 to 6.6 mm RC ingested *Artemia* sp. until 3.9 mm in length, while older juveniles (11.9 mm RC) consumed prey up to 6.64 mm in length, with the change in selectivity related to the suspension-feeding efficiency of the cirral net as individuals grow. This raises the question of whether very small *P. pollicipes* may be unable to feed on a large prey, such as first stage *Artemia* sp. nauplii, whose size normally ranges between 428 - 517 nm (Léger et al., 1987). Although no differences were observed between monodiets of *Artemia* sp. and mixed diets of *Artemia* sp. and microalgae (of ≤ 20 μm) in this study, this might be due to the fact that during the 1-month experimental period, only few of the individuals most likely be impacted were followed (i.e. juveniles measuring 1 - 5 mm RC). Mixed diets are recommended for *P. pollicipes*, especially given growth-related changes in the cirral net. The diets should also be changed as the individuals grow (Norton, 1996). However, Norton (*ibid.*) stressed that algal cells on their own are insufficient to maintain body mass, and therefore feeding of younger juveniles should be adjusted by using mixed diets of suitable prey-sizes. Cribeiro (2007), working with mixed cultures, concluded that live nauplii of *Artemia salina* were more acceptable and led to higher growth rates than inert diets (e.g. fish meat, cephalopods, mussels, seabass pellet food and frozen *Artemia* sp.).

Norton (1996) tested other live diets and concluded that ingestion rates vary with prey density and diet, with *Tetraselmis chuii* and *Pavlova lutheri* ingestion being negligible.

In the case of *Rhinomonas reticultata*, *Skeletonema costatutum* and *Brachionus plicatilis*, ingestion varied with prey density. For comparatively large prey, such as *Artemia* sp. nauplii, Norton (*ibid.*) did not find a strong relationship between ingestion rate (above a minimum requirement) and prey abundance, averaging 2.14 J ind^{-1} h^{-1} (or 58 *Artemia* ind^{-1} h^{-1}). However, the question has arisen whether this diet would have an effect on the palatability of barnacles and other factors that might affect consumer's product perception. Further studies of growth in the wild and with other prey could provide further clarification on this point.

6.4.5. Morphological alterations

An interesting effect observed in laboratory-reared broodstock, was the alteration in morphology compared to wild individuals (e.g. pink capitular plates, plate decalcification and plate deformation). The most evident change in morphology was plate colouration from a base white to a light pink. This effect may be caused by diet. In some cirripede larvae, when feeding is done with particular algal species, the nauplii develop deformities (Stone, 1989). In the wild, early spat of *P. polymerus* (1 – 6 mm RC distance) have a diet based solely on diatoms, detritus, shells, sand, sponge spicules, barnacle exuviae and crustaceans, while larger juveniles (7 – 14 mm RC distance) feed also on other unicellular phytoplankton, blue-green algae, copepods, polychaetes and to a lesser extent on eggs, hydroids, molluscs and large algae (Lewis, 1981). In *Lepas anatifera* the ovaries become pink when feeding on *Artemia* sp., as opposed to the natural yellow coloration of wild individuals (Patel & Crisp, 1960). The use of adequate diets is essential for development and pigmentation *in P. pollicipes* (e.g. Barnes & Reese, 1960; Cruz, 2000; Cribeiro, 2007). Mostly due to the lack of essential fatty acids, the use of *Artemia* sp. in culture has been associated with malformations and deformities in aquaculture species (e.g. Sorgeloos et al. 2001). In the case of barnacles, this might be a significant issue as fatty acids in barnacles result not only from de novo synthesis, but also from diet. Adequate lipid provision is essential to metabolism and indispensable from growth to reproduction (Holland, 1987). Dietary deficiencies may therefore have a significant impact on development. Furthermore, barnacle shells are formed of protein, chitin, calcium, and magnesium (Barnes et al., 1976), and these components need to be available in the diet. Other potential explanations for the incidence of shell malformations include the removal of protein by endolytic algae (e.g. *C. depressus*; Klepal & Barnes, 1975; Barnes et al., 1976) and the lack of hydrodynamism (e.g. *P. polymerus*; Barnes & Reese, 1960) under culture conditions.

Shell malformations and deformities are not exclusive to barnacles, being common in bivalve culture, decreasing growth and hindering market possibilities (e.g. Lake, 1992; Wheaton and Hall, 2007). Interesting results from Dunham & Marshall (2012) on this, indicate that the incidence of shell deformities in *Clinocardium nuttallii* in the wild might be related to structural support.

6.4.6. Grow-out in the wild

The results of barnacle transfer to the wild showed that the morphological alterations observed in captivity can be mostly reversed by a period of 6 months of finishing grow-out in nature, and therefore these are likely to be associated with culture-related factors (e.g. diet, density, hydrodynamics). The incidence of malformations decreased 65 %, to 18.33 ± 0.29%, comparable to the 15.15 ± 0.16 % of wild individuals. Furthermore, there was a complete disappearance of the pink plate coloration. These are promising results and the use of shorter periods for grow-out should be investigated in terms of product finishing characteristics (e.g. appearance, palatability).

In the field, growth rates were lower than those observed in culture, most likely due to the fact that the individuals transferred were older. Furthermore a percentage of the individuals transferred was most likely mature (≥ 12.5 mm RC), with breeding being also propitiated by the transplant occurring during the breeding season, on the period of higher temperature and food availability. Growth rates were comparable to those reported by Cruz (2000), but below those of Goldberg (1984). Cruz (2000) estimated yearly adult growth rates of 3.54 mm RC respectively, with values ranging from 0.3 to 9.1 mm RC per year for adults. On the other hand, the average growth rate reported by Goldberg (1984) was 1.12 mm RC month^{-1} for juveniles above 10 mm RC, which might relate to the fact that this study was done with smaller juveniles and just over summer months.

Nevertheless, though growth was comparable to exclusively wild-grown barnacles, survival was significantly lower for transferred animals, indicating that the use of protective cages might be mandatory. Wooton (1994) showed that the use of protective cages over *P. polymerus* can significantly decrease predation and increase coverage by barnacles from < 20 % to > 60 %. Predation has been poorly studied for Pollicipedidae, with reports of predation on *P. polymerus* by crabs, seagulls and polychaetes (Bernard, 1988; Hoffman, 1989; Wooton, 1994; Meese, 1993), and by gastropods on *C. mitella* (Taylor & Morton, 1996). Mortality averaged 13.33 ± 0.47 % month^{-1} in transplanted animals and 4.38 ± 0.68 % month^{-1} in wild barnacles, suggesting that such factors as

decreased fitness in culture individuals, preferential predation or decreased protection (e.g. due to cluster size, density, attachment strength), might be key to these differences. Mortality rates in the wild, have been reported to average 4 % mortality month^{-1} for barnacles transplanted from the coast to off-shore grow-out structures (Goldberg, 1984) and 4.0 to 4.6 % month^{-1} for wild barnacles exclusively grown in the intertidal (Cruz, 2000). Furthermore, the macroalgal epibiosis observed on the capitular plates of over 60 % of the individuals, might have impacted growth and survival, though it can be related to the time of the year the study was conducted. Epibiotic pressure varies throughout the year, being generally higher during summer months (Dunham & Marshall, 2012). Studies on bivalves (e.g. Lodeiros & Himmelman, 1996) suggest that epibiosis might restrict water flow around bivalves and increase shell weight, interfering with the movement of the valves and hindering feeding.

6.5 Conclusions

Maximum average growth and survival rates in captivity were 0.81 ± 0.11 mm RC month^{-1} and 98.63 % month^{-1}, for individuals grown in the dark, with no tidal influence and fed daily with *Artemia* sp. nauplii. When only juveniles ≤ 10 mm RC are considered, these values increase considerably. A temperature of 15 °C is optimal for growth compared to 10 and 20°C, with no effect on survival. Higher temperatures do not necessarily favour growth, therefore, as energy may be transferred to reproduction and these can also impact metabolism. There were no identified effects of food quantity (7, 12 and 25 nauplii *Artemia* sp. ml^{-1}), as diets were not limiting and prey density had no effect on ingestion, as reported by other authors (e.g. Crisp, 1961; Norton, 1996). Diets of *Artemia* sp. nauplii (given daily) lead to higher growth and survival rates, with growth improving with feeding frequency but with no improvement when using mixed diets with microalgae. The use of alternative mixed diets should be investigated further to improve the nutritional profile and to ensure suitable particle size availability whatever the size of barnacle. Diets of microalgae alone led to lower growth and thus support Norton's (1996) observations. The presence of tidal cycle decreased growth, presumably through reduced opportunities to feed and grow, but did not impact on survival. The suggestion of Cribeiro (2007) that tidal cycles should be used in culture needs careful consideration, therefore, if the purpose is to optimize growth. Hoffman's (1989) observation of higher growth under submersion was supported by the present study. No effects of photoperiod were noted for morphology, growth or survival, though effects on reproduction were not studied. Laboratory-reared cultures further exhibited

alterations in morphology in comparison to wild individuals (e.g. pink capitular plates, plate decalcification and plate deformation) and though the causes for these remain unknown, they were mostly reversible after finishing growth in the wild.

Growth variation according to size classes occurred as expected, as smaller individuals (between 0 and 5 mm RC) grew more than larger individuals (between 5 – 20 mm RC), in accordance to other authors (e.g. Cruz, 2000). The previous author estimated yearly juvenile growth rates of 5.46 mm RC, with values ranging from 0.5 to 13.3 mm RC per year, and monthly growth of 0.17 – 0.66 mm RC (Cruz, 1993). Cruz et al. (2010) also reported that during the first year of life growth was 1.3 mm RC per month, averaging 15.7 mm RC in the first year. Growth in culture has matched values recorded in the wild, as did values of subsequent grow-out of adults on the coast, which being considerably lower, are consistent with growth rates for older barnacles. The extremely poor survival of transplanted barnacles is a cause of concern and should be addressed in future studies.

According to Cruz (2000), commercial size occurs above 15 mm RC, with individuals above 19 mm RC having added value. Based on current results, the estimated growth period of early juveniles to commercial size is of about 12 months in culture, followed by a finishing period in the wild. A grow-out period of 6 months proved sufficient, but shorter periods should be investigated, as well as methods for improving survival. Considering an embryonic, larval development and post-settlement period of no less than 1.5 months in culture, by current culture conditions (Franco, pers. obs.), growth from embryos to commercial size is expected to take between 18 to 24 months, including finishing period. Estimations by previous authors, from field studies, suggest *P. pollicipes* growth to commercial size within 12 – 48 months after settlement (Cunha & Webber, 2000; Cruz et al., 2010). This is within the range of other commercially exploited barnacles (*A. psittacus* and *M. azoricus*), which grow to commercial size in 17 – 24 months (Lopez, 2008; Lopez et al., 2010, 2012; Pham et al. 2011). Future studies should investigate the use of on-shore or suspended culture systems to grow early juveniles, either transferred from culture or recruited from the wild.

Chapter 7. Conclusions and recommendations

7.2. General discussion and conclusions

7.2.1. Performance of P. pollicipes under culture

The present study aimed to identify the conditions required to establish the culture of this species, over the various production phases. A phase-by-phase approach was taken, as research focused sequentially on broodstock reproductive conditioning, larval culture, larval settlement and juvenile behaviour and culture (see Table 1.17).

7.2.1.1. BROODSTOCK REPRODUCTIVE CONDITIONING

The present work has established the initial basis for adult reproduction in culture and provided the first reference values on broodstock performance in captivity. An overview of the most significant results on adult reproduction, for the treatments that optimized breeding, is shown in Table 7.1.

Parameter	Best regime	Post-conditioning results
Specific growth rate	No differences	0.84 ± 0.16 month^{-1}
RC/SL	*sp-suT2*	1.47 ± 0.40
Total survival	No differences	93.66 ± 0.76 %
Size of mature adults	No differences	15.94 ± 0.23 mm RC
Fecundity	*sp-suT*	26.67 ± 1.92 %
Lamella development	*sp-suT and sp-suT2*	3.15 ± 0.60 (*sp-suT2*) and 2.53 ± 0.47 (*sp-suT*)
Matured lamella	*sp-suT and sp-suT2*	38 % (*sp-suT2*) and 32% (*sp-suT)*
Average released larvae	No differences	4670.90 ± 506.63 nauplii day^{-1}
Total released larvae	No differences	128147.39 ± 13548.26 nauplii
GW (µm)	No differences	202.89 ± 8.69 µm GW
24hS (%)	No differences	91.56 ± 0.35 %

Table 7.1. Summary of most significant results on broodstock reproductive conditioning, indicating the regime that provided the best results and respective reference values. Temperature regimes were as follows (*spT*) constant spring temperature of 16 °C (from day 1 to 28); (*sp-suT*) increase from spring temperature 16 °C (on day 1) to summer temperatures 24 °C (on day 28); (*sp-suT2*) as previous regime but with diel temperature fluctuations (of ± 1 °C the daily mean temperature). Metrics considered included specific growth rate based on rostro-carinal distance (*SGR-RC*, month^{-1}), ratio of rostro-carinal distance and stalk length (*RC/SL*, *#*), total survival (%), size at maturity (mm RC), fecundity (%), development stage index (#), fully mature lamella (%), average release rates (# d^{-1}), total release rates (# month^{-1}), larval greatest width (GW; µm GW) and 24 h nauplii survival (24hS, %). Initial broodstock showed RC/SL of 1.28 ± 0.38, average size of mature individuals of 17.71 ± 0.65 mm RC, fecundity of 5.09 ± 0.08 %, lamella development index of 0.80 ± 0.36 and 0 % with mature lamellae.

The results indicated that conditioning of *P. pollicipes* can be achieved in culture in less than a month after acclimatisation, when subjected to increasing temperatures (from 16 °C to 24 °C), as found during the breeding season. This could be achieved either by steady increase, or an increase with daily temperature oscillations. Larvae were released daily (averaging 4670 ± 506 nauplii day^{-1}) and peak releases (≥ 10000 – 30000 nauplii per tank; ≈122 adults per tank) occurred 1 – 2 times during the conditioning period. The daily release rates were below expectations, which suggests that individuals were releasing larvae of the same brood over a period of days, as the embryos hatched within the mantle cavity. Regimes of increasing temperatures led to more frequent spawning peaks, possibly due to faster development rates, and temperature oscillations seemed to favour a shorter interval between peaks of release. Higher fecundity was recorded for broodstock reared under steady temperature increase, while lamella maturation and larval production were higher at oscillating temperatures. The total number of larvae released did not vary between treatments. No differences in larval quality (for nauplii size and 24-h survival) were recorded among temperature regimes. In spite of the need for further optimization, broodstock reproductive conditioning can be accomplished and a continuous supply of larvae obtained using the protocols described herein.

7.2.1.2. LARVAL CULTURE

Larval culture was developed under different conditions of temperature, food quality, photoperiod and salinity, tested independently. Best growth and survival was achieved using temperatures of 15 – 20 °C, daily feeding with *T. chuii/ S. costatum or I. galbana/ S. costatum* and a photoperiod of 24/0 L/D. Under these conditions, development to the cyprid occurred within 14 to 17 days, with 20 – 40 % survival. An overview of the most significant results on larval culture is presented in Table 7. 2.

Culture condition	Best regime	Total survival (%)	SGR (d^{-1})	MDT (d)
Temperature	15 °C	19.08 ± 2.83	2.97 ± 0.10	17.67 ± 0.58
	20 °C	31.11 ± 5.26	4.50 ± 0.14	16.06 ± 0.39
Food quality	*I. galbana /S. costatum*	38.72 ± 8.60	No differences	No differences
	T. chuii /S. costatum	40.82 ± 2.37	No differences	No differences
Photoperiod	24:0 L/D	27.00 ± 3.01	No differences	No differences
Salinity	30 psu	No differences	No differences	No differences

Table 7.2. Summary of larval culture conditions (temperature, diet, photoperiod and salinity) that improved growth and respective values of total survival to cyprid (%), specific growth rate (SGR, d^{-1}) and median development time (MDT, d).

Higher temperatures (> 20 ºC) significantly increased total mortality, though lower temperatures (< 15 ºC) extended the growth period allowing for higher risk of acute mortality. At low temperatures, the higher the SGR, the lower the mortality, whilst at higher temperatures (> 20 ºC) daily mortality increased with temperature despite SGR also increasing, creating an opposing trend. Mid-range temperatures (15 – 20 ºC) provided the best compromise, maximizing total survival to the cyprid (between 19 to 31 % survival). Higher temperatures significantly increased specific growth rate, from 2.60 ± 0.08 d^{-1} at 11 ºC to 5.93 ± 0.35 d^{-1} at 24 ºC, explained by the effects of increasing temperature on metabolism, decreasing development time from 25 days at 11 ºC to 10 days at 24 ºC, though temperatures above 22 ºC had over 90 % mortality. Common problems encountered ranged from system failure, to culture contamination, clogging of larval appendages and failure to feed/swim properly. Close attention should be given to the appearance of the nauplius VI stage, as a prelude to cyprid appearance. It is advisable to filter the cultures when over 50 % of the individuals have reached the cyprid stage.

Diets tested (monodiets and mixed diets of *T. chuii*, *I. galbana* and *S. costatum*) did not affect growth rates, but higher survival was recorded with mixed diets of *T. chui/ S. costatum or I. galbana/ S. costatum*. Microalgae bioavailability and interaction with the larvae should be considered, besides nutritional profile and prey size. Furthermore, different diets considerably impacted cyprid quality, which should be considered in future studies.

No differences in survival were noted according to photoperiod (0/24, 8/16, 16/08 and 24/0 L/D), but with growth rate higher at full-day photoperiod. These results can be explained by effects on algal growth, prey contrast and swimming activity. With respect to salinity (20, 30 and 40 psu), no significant effects on growth and survival were recorded, but the highest values were noted at 30 psu.

7.2.1.3. LARVAL SETTLEMENT

Settlement was observed on a range of substrata (artificial and natural), both in culture and in the wild, though the adults remain as the preferential substrata. Maximum settlement on adults in culture was about 30 – 35 %, with a one-week metamorphosis rate of 70 – 80 %. Larval settlement on the adult was higher for older cyprids (6 days), at 20 ºC, with water circulation, light and salinity of 30 – 40 psu to a maximum of 30 – 35 % settlement. Metamorphosis was affected by temperature and cyprid age.

Settlement in the laboratory occurred preferentially on the capitulum of adult conspecifics (≈60 % of settled larvae), while in the wild most recruits were found on the stalk, possibly due to a higher post-settlement survival on the stalk in the wild or advantageous larval transport. The cause for the effect of preferential settlement on the adults, is possibly due to a response by the larvae to chemical inducers, such as the settlement inducing protin complex, and/or due to gaining a refuge by settling in between the scales or capitular plates.

Settlement was greatly reduced on artificial substrata tested in the laboratory (< 3 %) and, in this experiment, was not recorded in the wild. In the laboratory, settlement to non-natural substrata mostly occurred in the absence of adult conspecifics. Settlement on natural substrata (e.g. *Chthamalus* sp., *Corallina* sp., rocks) was higher (< 17 % per surface). An effective induction of settlement was not achieved with the artificial substrata tested. The only artificial substratum where recruitment was consistent was on marine epoxy placed in the field, being comparable to that on adults, possibly due to the higher protection afforded by the irregularity of the surface. The most significant results on settlement and metamorphosis are highlighted in Table 7.3.

Substrata	Parameter	Results
Adult	Settlement	30 – 35 % (in culture) Higher on 6-d cyprids, water circulation, illumination, 30-40 psu Largely preferential over all other tested substrata (culture and wild)* Preferentially on the capitulum in culture and on the stalk in the wild Increasing over time until 48h, stabilizing afterwards
	Metamorphosis	70 – 80 % within a week Higher for younger cyprids (≤3d) and higher temperatures (20 °C) Increasing over time, though some larvae fail to metamorphose
Natural	Settlement	Higher in conditions of no-choice between the adults (maximum 17 %) Higher in *Chthamalus* sp. and *Corallina* sp.
	Metamorphosis	Comparable to the metamorphosis of cyprid settled on the adult
Artificial	Settlement	Overall residual across substrata (< 3 %), but higher in conditions of no-choice High recruitment in the wild in marine epoxy (only exception to low values)
	Metamorphosis	Comparable to the metamorphosis of cyprid settled on the adult
		* only exception is marine epoxy tested in the wild

Table 7.3. Summary of relevant results on cyprid settlement and metamorphosis, according to type of substrata (conspecific adults, natural substrata and artificial substrata).

Although the adult conspecific remains the principal driver of larval attraction in this species, for those that settle on artificial substrata, the nature of the surface also appears to determine both surface selection and post-settlement survival.

7.2.1.4. JUVENILE FEEDING AND CULTURE

Juvenile barnacles were investigated both for their feeding behaviour and performance under culture. Juvenile feeding was observed to vary with the degree of laboratory conditioning, as unconditioned individuals only responded to water speeds above 23 cm s^{-1} in comparison to above 6 cm s^{-1} for conditioned animals. This suggests the response to hydrodynamics can be altered during conditioning, presumably in order to cope with a less turbulent environment than that found in the wild. Higher flow rates (23– 32 ms^{-1}) are recommended for rearing *P. pollicipes* in captivity, which can be decreased as animals become conditioned (to speeds above 6 m s^{-1}). Conditioned individuals also showed more frequent prey captures. Higher temperatures (above 20 ºC) increased captorial behaviour as well as ingestion rates, in comparison to lower temperatures (15 ºC and 11 ºC). Live nauplii of *Artemia* sp. promoted the highest feeding rates for both groups, with ingestion rate independent of food quantity, and feeding at above 4 % of body mass is advisable. Nevertheless, *P. pollicipes* also responded to other prey, such as frozen *Artemia* sp. nauplii, *Brachionus plicatillis* and *Tisbe battagliai* and microalgae (*Tetraselmis chuii*), but did not respond to other 'inert' foods (freeze-dried *Daphnia* sp., pellet food) or *Isochrysis galbana*.

Growth and survival were optimized in cultures not subjected to daily tides, at 0:24 L/D photoperiod, 15 °C and fed daily with excess *Artemia* sp.. A summary of the most relevant results is shown in Table 7.4. Higher growth was observed in individuals kept without a tidal cycle, probably related both to prolonged feeding time and the increase in shell growth under submergence, although the lack of effect on survival. Photoperiod did not affect growth or survival either, and it is hypothesized that its impact might be mostly at the level of reproductive regulation. Current results show higher growth at 15 °C, compared to higher temperatures such as 20 °C, which supports the suggestion that an energy "trade-off" between reproduction and growth is regulated by water temperature, with higher temperatures stimulating resource allocation towards reproduction over growth. Smaller individuals (between 0 and 5 mm RC) grew more (1.14 ± 0.20 mm $month^{-1}$) than larger individuals (between 5 – 20 mm RC; 0.34 to 0.61 mm $month^{-1}$). Maximum average growth in the laboratory was 0.81 ± 0.11 mm $month^{-1}$ for RC and survival rates of 98.63 % $month^{-1}$. Alterations to juvenile morphology were observed in culture (e.g. pink capitular plates, plate decalcification and plate deformation), likely to be associated with culture-related factors (e.g. diet, density,

hydrodynamics), though reversible after growth in the wild for 6 months. In the wild, no differences in growth were observed between natural and transferred individuals, despite the considerable lower survival of transferred animals (20.70 ± 2.40 %), in comparison wild individuals (73.70 ± 4.05 %). The estimated growth period of early juveniles to commercial size is between 24 to 36 months in culture.

Rearing method	Culture condition	Best regime	Mortality (% month^{-1})	RC increment (mm RC month^{-1})
Culture	Tidal cycle	No tidal cycle	No differences	0.78 ± 0.07
	Photoperiod	No differences	No differences	No differences
	Temperature	15 °C	No differences	0.55 ± 0.02
	Feeding regime	*Artemia* sp. daily	No differences	0.81 ± 0.06
Wild	Na	Na	13.33 ± 0.47	0.26 ± 0.10

Table 7.4. Summary of the performance of juveniles reared in culture and in the wild, in terms of mortality (% month^{-1}) and RC increment (mm RC month^{-1}). The culture conditions (tidal cycle, photoperiod, temperature and feeding regime) that improved growth and survival are also shown. Na – not applicable

7.2.2. Implications of new findings for production

In the present research, the feasibility of culturing *P. pollicipes* throughout its life cycle was examined. It is concluded that this species can be reared throughout its life cycle in culture, though the culture conditions are still to be improved. The effect of environmental conditions on the behaviour and development of *P. pollicipes* was evaluated from reproduction to larval settlement and juvenile growth and initial rearing protocols can now be established with knowledge of preferred culture conditions.

An initial literature review was conducted to concentrate and evaluate the current knowledge with reference to *P. pollicipes* culture and identify potential culture bottlenecks that required further study. This review was conducted as a reference for future work. It provides the first integrative study that considered concomitantly results from ecological and applied aquaculture studies on stalked barnacles, analysed in view of their application towards the culture of this species.

Broodstock reproductive conditioning can be developed through the protocol developed in the present study, over short periods, and with significant larval production. The results herein allow production to be factored according to number of individuals and maturity, in order to design breeding groups. The fact that larval quality was not lower in conditioned animals, compared to wild animals, further supports the viability of conditioning, though additional studies should investigate potential effects on the cyprids and juvenile stages. The positive outcome of broodstock conditioning

experiments presents promising prospects for aquaculture, especially given that this is the first study targeting *P. pollicipes* reproduction in culture. The present results further support the importance of temperature on the reproductive cycle of *P. pollicipes* and breeding frequency, which is useful for further development of culture protocols and supports results from ecological studies.

The present results on larval culture outline a range of conditions that lead to improved larval growth and survival, which can be further used as reference for future studies. Larval culture can be developed in captivity to give high growth rates and production of high-quality cyprids. The conditions still need to be optimized, however, as shown by the low survival values. Observations of common problems encountered in culture, and the importance of diet and temperature as key variables, provide a framework for future research. The low survival in culture-produced larvae presents the highest challenge during this phase and raises concerns over the health of produced larva, as not all larvae were of high-quality. The present results also shed some light on the time required for larval development in the wild and the importance of release time for larval survival and growth, in terms of impact of environmental conditions on development.

Larval settlement did not occur exclusively in association with conspecific adults, oin spite of the high preference, but also on artificial and other natural substrata. Though most settlement results on non-conspecifics were low, some substrata, such as marine epoxy, *Corallina* sp. and *Chthamalus* sp. attained higher settlement, encouraging further studies. Current understanding of apparently high larval selectivity in this species may overlook other relevant factors, such as the survival value of different surfaces, and explicitly the interaction of surface-related parameters (e.g. roughness, micro-topography) with environmental factors (e.g. hydrodynamics, predation, intraspecific-competition, desiccation). Further research into these aspects is essential, as well as on the induction by chemical cues. The environmental conditions here shown to promote settlement on the adult have further implications for laboratory settlement assays, as they limit the applicability of common settlement protocols used for other species, demanding a more tailored approach for *P. pollicipes*.

The study on juvenile behaviour and rearing in culture provides an integrated approach to some of the main factors affecting development and how differential behaviours related to effective growth/survival performance. The results from testing the feeding behaviour of conditioned and unconditioned individuals support the suggestion that, although *P. pollicipes* distribution is limited to the high energy intertidal, these animals

are not strictly dependent upon the highly hydrodynamic conditions to survive and are able to adjust to feeding in slower water flows. This implies that the limitations on the distribution of this species may be found elsewhere (e.g. predation, food resources, currents, temperature). Also, for aquaculture, the current results suggest that hydrodynamics can be adjusted as conditioning occurs and provide a framework to investigate the growth response to different water speeds. In terms of food quality, the value of *Artemia* sp. nauplii has been confirmed, but the use of other prey such as *B. plicatillis* and *T. battagliai* that also stimulated feeding should be further investigated. Present data on behaviour can be used to decide what feeding quantities to use and the duration of feeding. The effects of different temperatures on feeding, growth and survival have also been considered and provide further insight into how these factors interact and how resource allocation might occur. A rearing protocol for juveniles is presented herein, as well as a comparison of culture conditions. The importance of adequate tidal regimes and temperature to stimulate growth is explored. A trend of morphological changes under culture and their reversibility in the wild was also established, indicating the need to investigate further the best rearing practices as well as growth options in the field. The relevance of mortality of individuals after transfer to the wild was also noted as an important factor to be addressed. The values presented here for juvenile growth and survival allow a better estimation of culture periods under different rearing conditions and main factors impacting development. Growth results, in particular, are promising when compared to wild studies. The acquired knowledge on juvenile performance under culture can be used to direct future work on *P. pollicipes* production and tackle expected pitfalls (e.g. changes in morphology).

Overall, the current results strongly suggest that reproduction under culture and larval rearing can be successfully undertaken in captivity, in terms of reliability and sufficient yield, though there is a need for optimization. Larval settlement on non-conspecifics in culture would be the major setback to production. Though current results of settlement on the adult are promising, under the current knowledge, larval settlement under culture using artificial substrata would not be reliable and knowledge of potential substrata is still in its infancy (e.g. marine epoxy). This contrasts with juvenile rearing, which is comparatively easy (followed by a finishing grow-out in the wild) and the production cycle closed in captivity. Considering this, a production cycle in the wild, from larval recruitment on artificial structures to juvenile grow-out might present better prospects, in spite of the need to solve the issues relating to survival.

7.3. Future research and recommendations for aquaculture

Notwithstanding the research efforts of the present study, *P. pollicipes* culture is at its earliest stages and knowledge remains limited. Future research is of paramount importance, both at basic and applied levels. Knowledge gaps range from surface-specific characteristics that affect cyprid settlement to the economical viability of juvenile growht in the wild, and in integrated approach is needed towards the culture of *P. pollicipes* culture. This study has highlighted a number of research gaps.

In regard to broodstock reproduction, further optimization of the proposed protocol should investigate the effect of other variables, such as food quality and photoperiod, on reproduction in culture. The importance of diet should not be overlooked and alternative diets (e.g. copepods) should be considered as a priority. Additionally, it would be interesting to follow larval release at the individual level, to assess how larval release occurs over a period of days. The follow-up of mating and fecundation in clusters, by direct observation of mating followed by histological analysis gonad condition, might also provide valuable insight. This can elucidate under controlled conditions how mating occurs, size of breeding groups, selection of breeding partner, distances between breeding pairs, among other factors which are useful for selecting broodstock.

Future studies on larval culture should examine the use of recirculating systems for larviculture as a way to decrease husbandry concerns and optimize larval quality and in particular larval survival. Optimal rearing density should also be a focus of further studies, to attempt to optimize yield without compromising growth and survival. A deeper understanding of feeding requirements of larvae as they age would also be valuable, given the large difference in size and most likely nutritional requirements between first and last stage nauplii. As diet is known to affect cyprid settlement and early spat quality in other species, future research should build on current results of settlement assays and extend the evaluation of larval quality until post-settlement survival, in particular using potential artificial substrata.

P. pollicipes selectivity at settlement is less than initially believed and this should encourage future studies towards assessing the use of artificial substrata (such as the marine epoxy). It further raises the question of whether the inductive effect of the adults on settlement is due to a response by the larvae to proteins produced by the adults or others such as protection value, and future studies should pursue these possibilities. Establishing the relative importance of the SIPC of *P. pollicipes* on settlement should be taken as a pressing matter. There may be significant value in studying surface-related

parameters such as roughness and micro-topography, and their interactions with environmental factors including hydrodynamics, predation, competition and desiccation. Accordingly, studies on post-settlement survival in the wild, on various substrata, are key. Early spat relocation might also have merit for aquaculture purposes, as the hypothesis of juvenile relocation towards the base of the adult's stalk remains unverified, and if confirmed could prove of use to aquaculture, using the adults as inducers and the stalk base as secondary substrata.

Future studies on juvenile rearing should investigate the use of on-shore or suspended culture systems to grow early juveniles, either transferred from culture or recruited directly from the wild. There is an urgent need for information on mortality factors in the wild, due to the impact of these on the survival of transplanted barnacles and most likely also recruitment. The scaling up of cultures in captivity is also essential, as are the optimization of culture conditions and feeding protocols. The use of alternative live prey should be considered, especially in view of the possibility of the effect of current diets in morphology. Studies on juvenile growth should be directed at assessing the economical viability of commercial-scale culture, considering both culture options in captivity and in the natural environment.

Larger-scale studies on broodstock reproduction and larval culture and settlement should be developed, in order to approximate commercial culture conditions and provide applied data. Though follow-up studies on the optimization of protocols are essential, integrated research should be made a priority, with a focus on bridging the gap between captivity and the wild.

The present study addressed several culture bottlenecks and has provided the basis for a better understanding of *P. pollicipes* culture conditions. It further produced a framework for future research and raised new questions over the culture of this species. Research wise, priority should be given to further investigating the factors that mediate *P. pollicipes* settlement and optimize larval quality in culture. Field studies should prioritise the investigation of the economic viability of recruitment on artificial substrata and juvenile growth in the field, as these are the more immediate production steps. A consideration of the socio-economic impact of these possibilities is indispensable, and should not be neglected when deciding over future directions. Furthermore, the application of knowledge of culture of *P. pollicipes* towards the conservation of this species should be considered as important as working directly towards its aquaculture, as these options might provide a solid support for future resource management policies.

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