EFFECTS OF RISK OF SPERM COMPETITION, FEMALE SIZE, AND MALE SIZE ON NUMBER OF EJACULATED SPERM IN THE STONE CRAB *HAPALOGASTER DENTATA*

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ABSTRACT

Little is known about sperm allocation patterns in crustaceans, especially in anomuran crabs. We investigated whether male stone crabs, *Hapalogaster dentata*, change the number of ejaculated sperm depending on risk of sperm competition, and whether the pattern of sperm allocation to females of different body size varies with male body sizes in laboratory experiments where the male:female sex ratio (SR) and body size of both sexes in mating pairs were controlled. The number of ejaculated sperm differed significantly between matings under SR of 1:1 and 2:1, and males showed an increase in number of ejaculated sperm in the presence of a potential rival male. These results suggest that sperm competition may occur in the stone crab, and superiority in number of ejaculated sperm relative to rival males would be important for increasing male reproductive success in this species. Larger males showed a significant increase in the number of ejaculated sperm with increasing female size, while smaller males did not. The size of the ejaculate passed to larger females by smaller males was significantly reduced, resulting in low fertilization rates of larger females. The ability of males to provide sufficient sperm for fertilization may be one factor resulting in preference of female stone crabs for larger males.

INTRODUCTION

Sperm production is both costly and slow, and is often the limiting factor in male reproductive success (Dewsbury, 1982; Pitnick and Markow, 1994; Sato et al., 2005; Sato and Goshima, 2006). Therefore, males should partition their sperm reserves judiciously to successive matings to maximize their reproductive success (Warner et al., 1995; Parker et al., 1997; Sato et al., 2006; Sato and Goshima, in press). Sperm allocation strategies have been observed in males of many animal taxa, especially in terrestrial insects, birds, and fishes (Pitnick and Markow, 1994; Gage and Barnard, 1996; Marconato and Shapiro, 1996; Wedell and Cook, 1999; Rondeau and Sainte-Marie, 2001; Pizzari et al., 2003; Sato et al., 2006; Sato and Goshima, in press).

Allocation of sperm by males is dependent on a number of factors. For example, sperm competition and female reproductive quality have played major roles in shaping optimal sperm allocation strategies (Wedell et al., 2002). Sperm competition, i.e., when sperm from two or more males compete to fertilize a female's eggs (Parker, 1970), is an important force in shaping optimal sperm allocation, and males have evolved ways to assess the risk of sperm competition. The risk of sperm competition, e.g., the presence of potential rival males, induces an increase in ejaculate size per mating to achieve higher paternity than rival males (Gage and Barnard, 1996; Jivoff, 1997; Rondeau and Sainte-Marie, 2001; Pizzari et al., 2003). Female reproductive quality is also an important factor influencing male ejaculate size. In species in which females vary in reproductive quality, theoretical models predict that males should increase sperm investment with increasing quality of females (Galvani and Johnstone, 1998; Reinhold et al., 2002). In some species in which female reproductive potential is dependent on body size, males have been shown to provide larger ejaculates to larger females (Gage and Barnard, 1996; Marconato and Shapiro, 1996; MacDiarmid and Butler, 1999; Wedell and Cook, 1999; Sato et al., 2006; Sato and Goshima, in press).

Interest in the topic of sperm allocation in crustaceans also has increased considerably in recent years (Jivoff, 1997; MacDiarmid and Butler, 1999; Rondeau and Sainte-Marie, 2001; Gosselin et al., 2003; Brockerhoff and McLay, 2005; Rubolini et al., 2006; Sato et al., 2006; Sato and Goshima, in press). However, there have been very few attempts at such studies in anomuran crabs despite the inclusion in this group of large numbers of important fishery resources, e.g., *Paralithodes camtschaticus* (Tilesius, 1815) and *P. brevipes* (H. Milne Edwards and Lucas, 1841), (but see Sato et al., 2006; Sato and Goshima, in press). The responses of males to the risk of sperm competition through sperm allocation strategies have also not been studied in anomuran crabs. Toonen (2004) showed that multiple mating of females is widespread in the porcelain shore crab, *Petrolisthes cinctipes* (Randall, 1839), a species belonging to the anomuran crabs. There is growing interest regarding sperm competition in anomuran species (see Contreras-Gaduño and Córdoba-Aguilar, 2006) because sperm competition is generally an important feature of the reproductive biology in various animal taxa, which may explain behavioral traits of males (Parker, 1970).

Investigation of the pattern of sperm allocation is important not only to understand male mating strategies but also to predict female mating strategies because allocation of an insufficient number of sperm to females often limits their reproductive success, i.e., sperm limitation (Dewsbury, 1982; Nakatsuru and Kramer, 1982; Birkhead, 1991; Pitnick

and Markow, 1994; Sato et al., 2005, 2006; Sato and Goshima, 2006; Sato et al. 2007). For example, larger males adjust the ejaculate size passed to females depending on the female body size, passing larger ejaculates to larger females, although smaller males have a limited capacity to increase the ejaculate size (MacDiarmid and Butler, 1999; Sato et al., 2006). Reproductive success of larger females mated with small males can be limited by insufficient sperm supply (MacDiarmid and Butler, 1999; Sato et al., 2006). Such size-dependent fertilization ability of males should cause females to be selective in mate choice (Sato and Goshima, 2007) or to mate again with another males, i.e., it protects against sperm limitation (Gosselin et al., 2005).

Male stone crabs, *Hapalogaster dentata* (De Haan, 1844), have spermatophores in the vasa deferentia, which they attach to the abdomen of females near their gonopores during mating (Sato and Goshima, 2006, in press). Mated females fertilize their eggs externally within the brood chamber formed by a flap under the body. Males guard females after mating until the oviposition is finished (Goshima et al., 1995). In laboratory experiment, so far only a male was shown to superpose his own spermatophores on that attached previously to the pleon of a female by another male during mating without elimination of the previously attached spermatophores (Sato, personal observation). This observation represents evidence for sperm competition in this species.

Larger females spawn larger numbers of eggs (Sato and Goshima, 2006) indicating the larger females have higher reproductive quality to males. Stone crab males have limited sperm reserves and show little recovery rate of spermatophores, resulting in few possible matings (Sato and Goshima, 2006). Therefore, males judiciously adjust the number of ejaculated sperm to female reproductive quality (Sato and Goshima, in press). However, the response of sperm allocation in stone crab to risk of sperm competition remains unclear. In addition, it is also unknown whether sperm allocation pattern to females of different size varies with male size, while stone crab females show a preference for larger males as mates (Sato and Goshima, 2007).

First, we investigated whether stone crab males show a sperm allocation pattern in response to the risk of sperm competition, i.e., the presence of a potential rival male. Then, we examined whether the pattern of sperm allocation to females of different body size varies with male body size and whether stone crab males show size-dependent fertilization ability.

MATERIALS AND METHODS

Study Species

The life history and reproductive ecology of the stone crab, *Hapalogaster dentata*, are as follows (Goshima et al., 1995, 2000). Stone crabs inhabit intertidal and subtidal cobble rocky shores throughout their life. The reproductive season is from early October to late November, and females spawn once a year. During the reproductive season, the male:female sex ratio (SR) of the stone crab is 1:1. Males guard ripe females just before female molting by grasping or covering the female. Guarded females mate with males after molting, and spawn after mating. The period between mating and spawning is generally about 20 min (Sato, unpublished data). Females have no spermatheca to store sperm and, once mated, can only mate again during the short period between the first mating and spawning. In addition, when a sexually receptive female is placed with two males in an aquarium, the female will mate with only one male, and will not

subsequently remate with either male (Sato and Goshima, 2006). There may be sperm competition in this species, although the probability is low and most females would mate with only one male in a reproductive season.

Collection and Holding of Crabs

Crabs were collected at an intertidal rocky shore in Kattoshi on the southwest side of Hakodate Bay, southern Hokkaido, Japan (41°44′N, 140°36′E) before the reproductive season in 2002 and 2003. We sampled the stone crabs by turning over or lifting cobbles at low tide. All captured crabs were sexed according to the shape of the pleon, their CL was measured to the nearest 0.1 mm using Vernier calipers, and they were transported to Usujiri Marine Station, Aquatic Research Station, Field Science Center for Northern Biosphere, Hokkaido Unversity, where all laboratory experiments were performed. Only individuals with all their limbs present were used. Reared crabs were fed an artificial diet (diet for crayfish, Kyorin) every day. During the experiments, the light-dark regime matched the local conditions, and the seawater temperature was 9.1-19.9°C.

Experiments

Experiment 1: Effects of Risk of Sperm Competition on Number of Ejaculated Sperm.—To investigate whether males show a sperm allocation pattern in response to risk of sperm competition, we controlled the male:female sex ratio (SR) (1:1 or 2:1 SR) in laboratory experiments because the presence of a potential rival male means an increase in risk of sperm competition. We used large males (carapace length (CL) 13.0-14.0 mm, n = 26) and large females (pre-molt CL 12.0-13.0 mm, n = 18). Each male was placed into a separate aquarium (19.5 × 12 × 7 cm) containing about 1.5 l of filtered seawater, which was changed every day. Females were placed in a tank (244 × 89 × 18 cm) containing stones with flowing filtered seawater. We checked the tank containing females every day for female molts. If a female had molted, the mating trial was performed as follows.

First, to investigate the number of ejaculated sperm under 1:1 SR, a mating trial was performed with one molted female and one male together in the same aquarium. In this study, each individual was used in only one trial, and "molted females" were those that had molted within 48 h. A molted female was transferred to an aquarium containing a male for mating, and observed until they mated. Just after mating, the female was separated from the male and removed carefully from the aquarium. The attached spermatophores were removed from the abdomen by cutting out the part of the exoskeleton where the spermatophores were attached. All sperm were extracted from the spermatophores using 20% NaOH solution as follows (Sato et al., 2004; Sato and Gosihima, 2006). The parts of the female exoskeleton were put into a 15 ml tube filled with 5 ml 20% NaOH solution. After soaking in 20% NaOH solution for 60 min, the contents of the tube were mixed using a vortex-type mixer for 30 s. The mixed solution was then placed immediately into a Thoma hemocytometer and allowed to settle for 3 min. Sperm in 0.1 μl of the solution were then counted under an optical microscope at 400× magnification and under medium-high lighting conditions. The total number of extracted sperm in the tube, i.e., the number of ejaculated sperm, was calculated as: (number of sperm counted/volume of NaOH containing the counted sperm, i.e., 0.1 μl) × total volume, i.e., 5 ml. The counts were repeated four times, and the average was used as the number of ejaculated sperm. This mating trial was replicated ten times.

To determine number of ejaculated sperm under 2:1 SR, a mating trial was performed with one molted female and two males together in the same aquarium. One male was placed into the aquarium containing a male as a potential rival at least three hours before the start of each mating trial. At the end of the acclimation period, a molted female was transferred into the aquarium containing the two males, and observed until the molted female mated with either male. Just after mating with either male, the female was separated from the male and removed carefully from the aquarium. The method of collecting ejaculated spermatophores and estimating the number of ejaculated sperm was the same as described for the above experiment. This trial was replicated eight times. The effects of risk of sperm competition on number of ejaculated sperm were analyzed by *t* test after testing for homogeneity of variance using the Levene test.

Experiment 2: Effects of Male and Female Size on Number of Sperm in the Ejaculate or Fertilization Rate.—To determine whether the pattern of sperm allocation to females of different body size varies with male body size, we controlled male (small or large) and female sizes (small or large) in mating

pairs. In this experiment, we used large males (CL 13.0-14.0 mm, n = 19), small males (CL 9.0-10.0 mm, n = 21), large females (pre-molt CL 12.0-13.0 mm, n = 21), and small females (pre-molt CL 9.0-10.0 mm, n = 19). Four groups of male and female pairs were formed: small male and small female, n =10; small male and large female, n = 11; large male and small female, n = 9; large male and large female, n = 10.

Each male was placed into a separate aquarium (19.5 × 12 × 7 cm), and all females were placed in a tank (244 × 89 × 18 cm). The conditions of the aquaria containing males and the tank containing females were the same as described in Experiment 1. Similar to Experiment 1, the tank containing females was observed every morning, and then one molted female was transported to an aquarium containing a male for mating. After the male and molted female mated in the aquarium, the ejaculated sperm were collected and counted. The method of collecting ejaculated sperm and estimating the number of ejaculated sperm was the same as described in Experiment 1. The effects of male and female size on the number of ejaculated sperm were analyzed by two-way ANOVA and Bonferroni's multiple-comparison tests after testing for homogeneity of variance using the Levene test.

As the number of ejaculated sperm from small males to large females differed significantly from that from large males (see Results section), we investigated the relationship between male size and fertilization rate when males mated with large females in a field experiment. We used small males (CL 9.0-10.0 mm, n = 12), large males (CL 13.0-14.0 mm, n = 10), and large females (pre-molt CL 12.0-13.0 mm, n = 22). Two groups of male and female pairs were formed: small male and large female, n = 12; large male and large female, n = 10.

Each male was placed into a separate aquarium (19.5 × 12 × 7 cm) and all females were placed in a tank (244 × 89 × 18 cm). The conditions of the aquaria containing males and the tank containing females were the same as described in Experiment 1. The tank containing females was observed every morning for molting.

Before conducting mating trials, we made 22 isolated blocks from three cobbles to simulate the natural habitat of this crab in the intertidal cobble rocky shore zone. Each block was placed at least 5 m from the others, and all cobbles around and between the blocks were removed completely during low tide. All stone crabs within and around the blocks were removed completely, and transported to another cobble zone far from the blocks.

If a female had molted in the tank in the laboratory, the molted female and paired male were placed individually into a separate aquarium (19.5 × 12 × 7 cm), and then transported to the blocks during low tide. Before release, they were tethered as pairs (one male and one molted female) to a plummet (35 g) as mating pairs. The tethers were about 15 cm long and consisted of cotton (about 0.5 mm) attached at one end to the mid-dorsal surface of the carapace with a small drop of cyano-acrylate glue and the other end to the plummet. The day after placement, we collected the mating pairs from each block after confirming spawning of each tethered female. Twenty-two ovigerous females were transported to the laboratory from the field.

To estimate fertilization rate, after eggs spawned by the females had divided into 32-64 cells at about three days after spawning (Sato, unpublished data), more than 150 of the eggs were collected at random from three parts of the clutch using a pair of tweezers, and then cell division of a total of 150 eggs was observed under a stereomicroscope. We used the percentage of dividing eggs in the sample as the fertilization rate for the clutch. The effects of male size on fertilization rate when males mated with large females were analyzed using Mann-Whitney's U test because data sets were not homogeneous for variance.

RESULTS

Effects of Risk of Sperm Competition on Number of Ejaculated Sperm

Males responded to the risk of sperm competition. The spermatophores of all pairs were correctly attached to the abdomen of the female. Under 2:1 SR, after transfer of a molted female, males scrambled to mate with the female. The number of ejaculated sperm differed significantly between mating under 1:1 and 2:1 SR (t test, $t = -3.51$, $d.f. = 16$, $P = 0.003$), and there was an increase in number of ejaculated sperm when a potential rival male was present (Fig. 1).
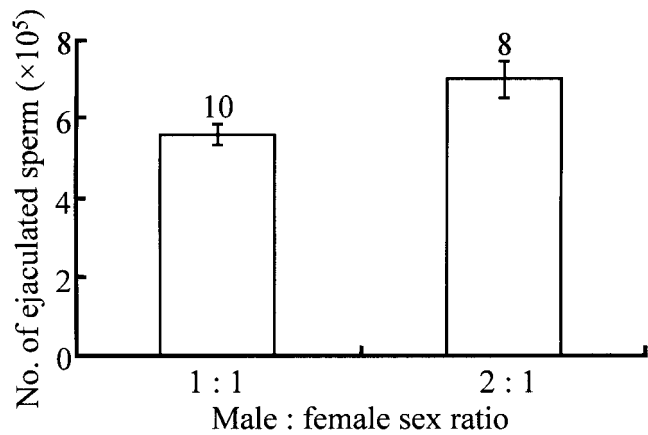

Fig. 1. Effects of the sex ratio on the number of ejaculated sperm. Error bars are SE. Numbers above the bars indicate the number of replicates.

Effects of Male and Female Size on Number of Sperm in the Ejaculate or Fertilization Rate

The pattern of sperm allocation to females of different sizes differed between small and large males. In all pairs, spermatophores were correctly attached to the abdomen of the female. Two-way ANOVA showed significant relations between male and female size and the number of ejaculated sperm (male size: $F_{1,36} = 73.27$, $P < 0.001$; female size: $F_{1,36} = 112.52$, $P < 0.001$), and indicated a two-way interaction between male and female size on the number of ejaculated sperm (male size × female size: $F_{1,36} = 86.04$, $P < 0.001$). Significant differences were observed between number of ejaculated sperm from large males to large females and that in the other pairs (Bonferroni multiple-comparisons, $P < 0.001$). In large males, the number of ejaculated sperm increased significantly with increasing female size, but small males showed no changes in the number of ejaculated sperm passed to females regardless of female size (Fig. 2). Thus, the ejaculate size passed to large females by males of different size differed significantly (Fig. 2).

Male size influenced the fertilization rate. In the field experiment, all tethered females spawned eggs. No individuals were observed within or around any blocks except for mating pairs when we collected ovigerous females, suggesting that all tethered females mated with the male tethered alongside them on each block. The fertilization rate differed significantly between females mated with small males and those mated with large males (Mann-Whitney's U test, $z = -3.33$, $P = 0.001$), and the fertilization rate of females mated with small males was lower than that of females mated with large males (Fig. 3).

DISCUSSION

Generally, the operational sex ratio and presence of potential rival males provide information about the probability of sperm competition at a particular mating in polyandrous mating systems (Wedell et al., 2002). The presence of potential rival males causes an increase in number of ejaculated sperm per mating (Gage and Barnard, 1996; Jivoff, 1997; Rondeau and Sainte-Marie, 2001; Pizzari et al.,
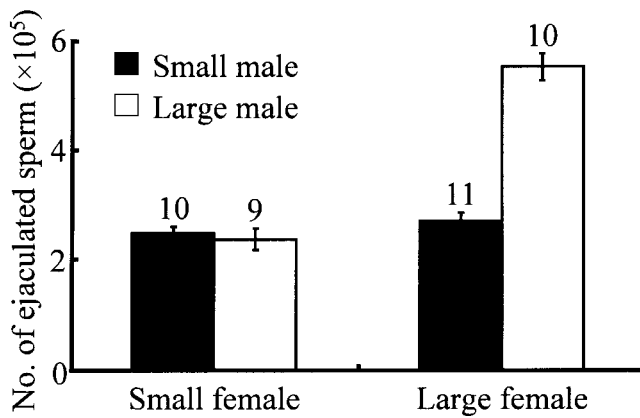
Fig. 2. Effects of male and female size on the number of ejaculated sperm (small male, CL 9.0-10.0 mm; large male, CL 13.0-14.0 mm; small female, pre-molt CL 9.0-10.0 mm; large female, pre-molt CL 12.0-13.0 mm). Error bars are SE. Numbers above the bars indicate the number of replicates.
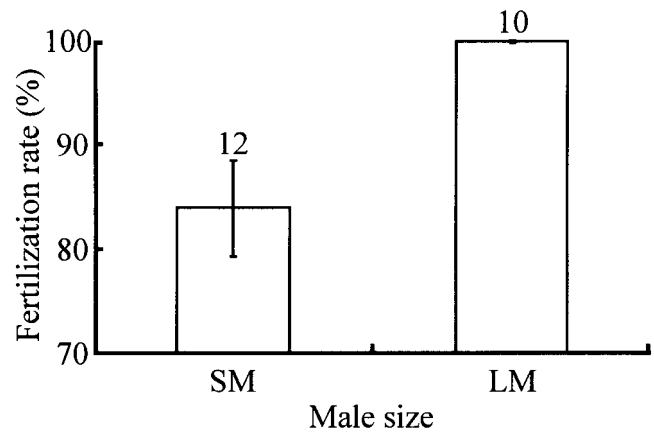

Fig. 3. Fertilization rate of large females mates with small (SM) or large (LM) males (small male, CL 9.0-10.0 mm; large male, CL 13.0-14.0 mm; large female, pre-molt CL 12.0-13.0 mm). Error bars are SE. Numbers above the bars indicate the number of replicates.

2003). Stone crab males also passed larger numbers of ejaculated sperm under 2:1 SR than under 1:1 SR, suggesting that sperm competition would occur in this species. This is surprising, because we thought that sperm competition is rare in such species in which females have no sperm storage organ and spawn immediately after mating.

In species in which females have sperm storage organs, males do not always show an increase in number of ejaculated sperm in response to the risk of sperm competition because the winner in sperm competition can be determined through several mechanisms acting within the sperm storage organ, e.g., sperm stratification (Urbani et al., 1998; Sainte-Marie et al., 2000) or sperm removal (Waage, 1979; Rubenstein, 1989). In contrast, in species that conduct external fertilization, the advantage in sperm competition is determined mainly through the raffle principle (lottery-based competition; Parker, 1970, 1990). The probability of fertilizing a female's eggs increases with a male's proportion of sperm in competition. In laboratory experiment, stone crab male superposed his spermatophores on previously attached spermatophores without eliminating them and then the mated female spawned and fertilized her eggs with these spermatophores without eliminating either (Sato, personal observation), i.e., there would be no possibility that polyandrous female exercise cryptic female choice as suggested by Thiel and Hinojosa (2003) in this species. Therefore, the simple numerical superiority by increasing the sperm investment may be important for stone crab males to attain higher reproductive success. Further studies are required to determine how widespread sperm competition is in this species, to examine whether males that pass larger numbers of ejaculated sperm achieve higher paternity than rival males, and to determine the importance of mate order for males.

In species in which females differ in reproductive quality, males attain higher reproductive success by strategically allocating their sperm reserves to females that provide larger fertilization returns (Wedell et al., 2002). In various species, including the stone crab, in which female reproductive potential is dependent on body size, there is a great deal of

evidence that males provide larger ejaculates to larger females (Marconato and Shapiro, 1996; MacDiarmid and Butler, 1999; Wedell and Cook, 1999; Sato et al., 2006; Sato and Goshima, in press), which is an adaptive strategy by which stone crab males maximize their reproductive success with limited sperm reserves (Sato and Goshima, in press). However, only large male stone crabs applied this mating strategy. In this study, the pattern of sperm allocation in response to female size differed with male size and the number of sperm ejaculated by small males did not vary with female size. It is likely that smaller stone crab males would also have limited reproductive potential and would be unable to increase the number of ejaculated sperm above a certain number (approximately 2.7×10^5, Fig. 2). The fertilization rate of large females mated with small males was significantly lower than that of large females mated with large males, which would result from insufficient sperm supplies from small males to fertilize all the eggs spawned by larger females. Such reduction of reproductive success of females mated with smaller males has also been reported in the spiny king crab (Sato et al., 2006) and spiny lobster, *Panulirus argus* (MacDiarmid and Butler, 1999) as well as several other species (McLain et al., 1990; Bissoondath and Wiklund, 1996). The limited capacity to increase the number of ejaculated sperm of small males influences male-male competition. If the advantage in sperm competition is determined mainly through the raffle principle in this species, small males are disadvantaged in sperm competition. Furthermore, larger male stone crabs are the dominant males in male-male competition for females (Goshima et al., 2000). Thus, reproductive success measured as the number of progeny in likely to be much less in smaller than in larger males.

Mate choice by stone crab females may also respond to the limited capacity of small males to increase the number of ejaculated sperm. Female mate choice should evolve whenever males differ in ability to fertilize eggs (Trivers, 1972) and great male fecundity is honestly signaled by traits that females can recognize although little is known about female choice in crustacean species (Snedden, 1990;

Jormalainen et al., 2000; Díaz and Thiel, 2003; Sato and Goshima, 2007). Stone crab females should avoid mating with smaller males that pass an insufficient sperm supply to achieve successful fertilization. Especially, larger females should be more selective in choosing mates compared with smaller females because larger females spawn many eggs (Sato and Goshima, 2006) and require abundant sperm to fertilize all of their eggs. This prediction is supported by the observation that stone crab females prefer larger males and males capable of transferring sufficient sperm for successful fertilization as their mates (Sato and Goshima, 2007). The ability of males to ejaculate an ample number of sperm may be one factor responsible for the preference of female stone crabs for larger males. There are two alternative explanations for mate preference in the female stone crab. Females generally gain indirect benefits for their offspring, e.g., good genes, or direct benefits, e.g., resources, or both by mate choice (Andersson, 1994). Female stone crabs mated with larger males would also receive indirect benefits from the male's good genes and some direct benefits. Larger male stone crabs are dominant in male-male competition for females (Goshima et al., 2000), representing indirect benefits from the male's good genes. Furthermore, larger male stone crabs performed pre- and post-copulatory mate guarding behavior (Goshima et al., 1995), meaning that guarded females are protected from predation during the vulnerable period (Jivoff, 1997).

The limited capacity to increase the number of ejaculated sperm of small males as shown in this species is also reported in some commercially important decapod crustaceans (MacDiarmid and Butler, 1999; Sato et al., 2006). Only large males are selectively harvested in many large decapod crustacean fisheries (Ennis et al., 1990). Fishing only large males decreases the mean male size (Ennis et al., 1990; Sato et al., 2005). In fished populations, small mature males replace large males in reproduction than in non-fished populations (Ennis et al., 1990; Sainte-Marie, 1993). Therefore, the probability that female fertilization success is constrained by an insufficient sperm supply from small males as shown in this study may be high in fished populations. The present fishery regulations for large decapod crustaceans, particularly for minimum legal size, may be based on an oversimplified concept of their reproductive system. To maintain the resources of commercially important decapod crustaceans, the minimum legal size may be reconsidered based on the reproductive potential of male size. We should investigate the details of the reproductive ecology of each species to maintain resources.

Although sperm allocation strategies are now known to be common features in various animal taxa, there have been only a few studies focusing on the sperm allocation strategies in anomuran crabs (Sato et al., 2006; Sato and Goshima, in press). To our knowledge, this is the first report that addresses the effect of presence of rival male (the risk of sperm competition) on sperm allocation pattern in anomuran crabs. Our results also suggest that the limited capacity of smaller males to increase the number of ejaculated sperm may be a factor influencing intra- and inter-sex interactions, i.e., sperm competition and female choice. The details of the reproductive ecology, such as sperm allocation strategy, should be investigated to elucidate the mutual interactions between sexes in crustacean species.

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