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Reduced survival associated with precopulatory mate guarding in male *Asellus aquaticus* (Isopoda)

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Precopulatory mate-guarding in aquatic crustaceans is known to have immediate costs for the guarding male. The extent to which guarding behavior may reduce future reproductive opportunities, however, is less established. We examined the survival and antennae length, suggested to be important in detecting receptive females, in male freshwater isopods (*Asellus aquaticus*) collected paired or unpaired. Antennae length to body size relationships suggested that large, paired males had broken antennae more commonly than large, unpaired males. Moreover, broken antennae seem to be associated with increased mortality. Males collected paired had lower survival than males collected unpaired, and this reduction in survival was greatest in larger individuals. In pairing trials, males usually attempted pair formation regardless of their size, female size, or previous pairing status. Our results suggest that some costs associated with male guarding behavior may extend beyond the actual guarding episode, though the consequences for mating decisions remain to be determined.

Introduction

Precopulatory mate guarding is a common male behavior when the time the female is receptive for fertilization is restricted. In many peracarid crustaceans (amphipods and isopods), copulation can only occur after females undergo a reproductive molt, so, prior to this molt, males engage in guarding behavior usually by positioning females ventrally and carrying them for an extended period of time (reviewed by Jormalainen 1998). Guarding is a beneficial male competitive strategy when the expected fitness gain of guarding the encountered female is higher than that of continuing searching for a receptive female (Parker 1974). Thus, the optimal guarding duration for the male is determined by the encounter rate of females and the costs of guarding relative to those of searching (Yamamura 1987).

Guarding may be costly for males owing to decreased mobility and feeding (Adams *et al*. 1985, Robinson & Doyle 1985) as well as to increased predation risk while guarding (Verrel 1985, Ward 1986). Males also incur energetic costs associated with carrying females (Sparkes *et al*. 1996, Plaistow *et al*. 2003a) and possibly fighting costs through intrasexual conflicts (Ridley & Thompson 1979). Females, on the other hand, frequently resist male-guarding behavior (Jormalainen & Merilaita 1993, 1995, Sparkes *et al*. 2000), which entails energetic and, consequently, fecundity costs for both sexes (Jormalainen *et al*. 2001). Thus, several costs manifested during guarding have been documented. Much less is known, however, about possible long-term guarding costs, i.e. the extent to which guarding decreases future reproductive opportunities (but *see* e.g. Robinson & Doyle 1985). Such costs may be particularly important for males because they do not allocate time and energy to brooding offspring, and can thus engage in many sequential pairings. For example, guarding may reduce future reproductive success of males by impairing the ability to find and pair with females or by lowering survival after completion of the current mating event. Understanding costs of mating decisions for current and future reproduction of both males and females is necessary to formulate expectations about decisions on engagement and duration of guarding behavior (Yamamura 1987, Härdling *et al*. 2004, Härdling & Kokko 2005) as well as about the extent of subsequent evolutionary conflicts that may arise between males and females (Yamamura & Jormalainen 1996).

Male freshwater isopods of the species *Asellus aquaticus* engage in precopulatory guarding behavior for several days (Ridley & Thompson 1979) during which they physically lift females off the substrate and carry them (Adams *et al*. 1985). Pairing is size assortative with large males generally found guarding larger and more fecund females than small males (Ridley & Thompson 1979, Vespoor 1982, Bertin & Cézilly 2003). This pattern may result from several mechanisms possibly acting in concert: contest competition in which large males may ‘take over’ females from smaller guarding males (Ridley & Thompson 1979), size-dependent guarding duration and male choice for large females (Elwood & Dick 1990), and female-resistance and size-dependent ability of males to start guarding (Jormalainen *et al*. 1994). Large males also tend to have longer antennae than the small ones which may give them an advantage in detecting and orienting towards receptive females, i.e. via scramble competition (Bertin & Cézilly 2003, 2005). In antagonistic interactions between males, antennae are commonly grabbed and broken (D.P.B. unpubl. data); such injuries may reduce the ability to locate females.

Here we studied some potential pairing costs that may decrease future mating success of male *A. aquaticus*. First, we studied how antennae length varied with the pairing status of males. Second, we assessed whether guarding behavior influences male survival after the guarding period. Finally, we determined whether prior pairing status affected the willingness and/or ability of males to enter into precopulatory guarding.

Materials and methods

Animal collection and maintenance

Isopods were collected at the beginning of June 2005 with a dipnet from Niemijärvi, a small pond in central Finland (62°12´N, 25°45´E) in which the only fish species is crucian carp (*Carassius carassius*). Isopods were transported to the laboratory and precopulatory pairs as well as unpaired male isopods were sorted out. Pairs and unpaired males were placed in separate tanks (5-l volume) and left overnight. The following day, individual males from the paired tank no longer guarding females ($n = 216$) were isolated individually in plastic containers (10 × 15 × 5 cm) with 400 ml of lake water. Likewise, unpaired males of similar size from the other tank were placed singly in the containers ($n = 361$). It is possible that the isopods collected unpaired had engaged in precopulatory guarding before being brought to the laboratory. Thus, the distinction between paired and unpaired isopods is that paired isopods were engaged in precopulatory guarding immediately prior to the beginning of the experiment. Copulations in the paired group were not directly observed, but many males in this group probably mated successfully before being isolated. This distinction could also contribute to differences between paired and unpaired males. All isopods were fed *ad libitum* on a diet of conditioned leaves, primarily alder (*Alnus glutinosa*). Leaves were conditioned in aerated lake water for at least two weeks to allow microbial colonization prior to being offered to isopods (Hasu *et al*. 2006). Animals were maintained at a temperature of approximately 18 °C under constant illumination. Every five days, half the water volume in each container was replaced with fresh, aerated lake water.

Measuring survival and antennal injuries

Isopod survival was monitored over the course of 60 days. After an isopod died, its length (from the tip of the cephalon to the end of the telson) was measured to the nearest 0.5 mm. Antennae length, a secondary sexual characteristic in male isopods (Bertin & Cézilly 2003), was also measured for each individual to the nearest 0.5 mm. Right and left first antennal lengths were averaged. The relationship between average antennae length and isopod size was compared among males collected paired and unpaired using ANCOVA.

To assess whether broken antennae also affect isopod viability, it was necessary to establish which isopods likely had antennal injuries. A baseline allometric relationship between antennae length and isopod size was constructed using male isopods collected from Niemijärvi in September 2005 ($n = 122$). Reproductive behavior is rarely observed at this time of the year, and individuals commonly have intact antennae. For these isopods, the relationship between isopod size and average antennae length was linear ($y = 0.796x + 1.289$, $r = 0.91$, $P < 0.001$). The negative boundary of the 95% CI for the slope and intercept of this line were 0.730 and 0.702, respectively. Using these lower boundary parameters, a regression line was plotted and used as a conservative cutoff to establish which isopods in the June sample likely had broken antennae, i.e. individuals falling below this line had antennae shorter than expected on the basis of body size ($n = 332$) while those above it had antennal lengths relatively similar to expectations ($n = 245$). In central Finland, *A. aquaticus* populations exhibit considerable turnover in July (D.P.B. unpubl. data), so the isopods collected in September are likely younger than those collected in June. However, the size ranges of these two groups overlapped completely (June: 6–11.5 mm; September: 6–12 mm), so the inferred antennae length–body size relationship should be applicable to the isopods collected in June.

The factors affecting isopod survival were assessed with the Cox regression, a method commonly used for survival analyses (Andersen 1991). An assumption of Cox regression models is that the ratio of the hazard function for any two individuals is dependent on their covariate values and the baseline hazard function, but not time (proportional hazards assumption). This assumption was tested by entering time as a covariate into the regression model and checking whether it significantly interacted with any of the predictor variables. No significant interactions were observed, suggesting the proportional hazards assumption was met. Thus, a time-dependent covariate was not included in the final Cox regression model. Pairing status and antennae status (i.e., presumably broken or intact) were incorporated into the model as categorical covariates and isopod size was treated as a continuous covariate. All main effects and interactions were included in the initial model, and the terms included in the final model were assessed with likelihood ratio tests. Forwards and backwards selection algorithms produced the same final model.

Isopod molting was followed throughout the course of the experiment. When a shed exoskeleton was observed, it was removed from the container and the date was recorded. The majority of isopods molted once or not at all; only a small proportion molted multiple times during the experiment. Thus, the occurrence, as opposed to the frequency, of molting was compared between paired and unpaired isopods. The chances of recording a molt depended on how long an individual was observed (i.e. how long it survived), so the data were divided into comparable subsets. Each subset was defined by a discrete length of observation (i.e. isopods living at least 10, 20, 30, 40, 50, or 60 days), and, therefore, every individual in a given subset was observed the same minimum amount of time. For example, all isopods in the first subset survived at least 10 days and therefore had equal “opportunity” to molt during the initial 10 days of the experiment. For each subset, the propor-

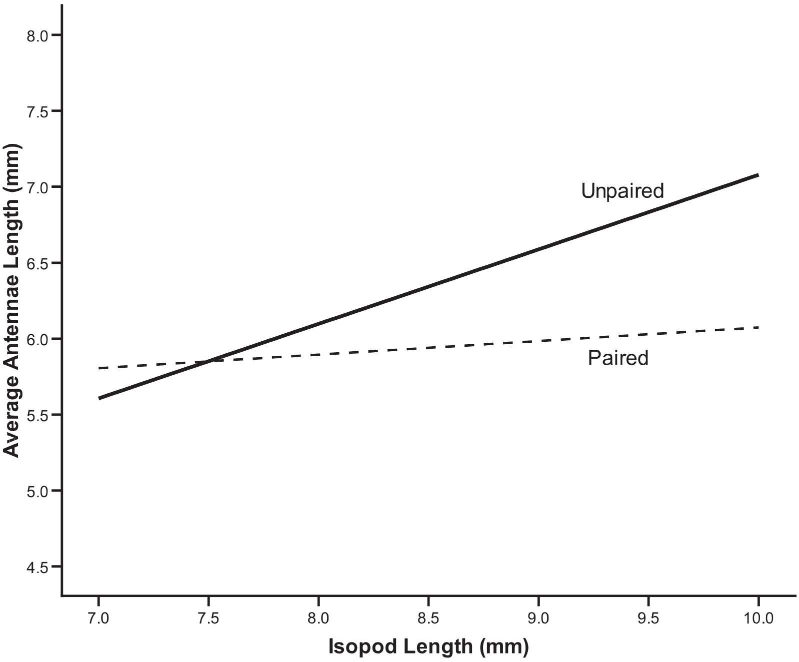

Fig. 1. The relationship between the average length of the first antennae and isopod length for paired (dashed line; $n = 216$, $y = 0.89x + 5.18$; $r = 0.05$) and unpaired (solid line; $n = 361$, $y = 2.17x + 0.49$; $r = 0.275$) male isopods. The length of males ranged from 6 to 11.5 mm, but 95% of both the paired and unpaired males were between 7 and 10 mm. Thus, the regression lines are only shown for that range.

tion of isopods which molted during this time span was compared between paired and unpaired isopods with a χ^2-test.

Pair formation with regard to prior pairing status

Whether prior precopulatory pairing affects the tendency of males to form future pairs was tested. As for the survival experiment, males found in pairs were placed in a tank and allowed to complete the guarding period. The following day, all males no longer guarding a female were collected, isolated individually, and allowed to recover for two weeks ($n = 34$). Males collected unpaired were maintained in a similar manner ($n = 101$). After two weeks, precopulatory pairs were taken from the field, and receptive females were separated from the guarding males. Single, receptive females were randomly placed into Petri dishes filled with 100 ml lake water. A single male, either previously paired or unpaired, was randomly introduced into each dish and the mating behavior was observed for 30 min. Two aspects of male behavior were recorded, pairing attempts and pairing success. If the male tried to grab the female and position her ventrally, the normal guarding position, at least once during the 30 min, then the male was scored as attempting to engage in precopulatory guarding. If any of the attempts resulted in a pair being formed, then the male was considered successful. After the experiment both males and females were measured to the nearest 0.5 mm. The effect of prior pairing on male attempts and success was assessed with two separate logistic regressions. Only males that attempted to pair ($n = 107$) were used in the analysis of pairing success. In the logistic regressions, both male and female size were employed as covariates.

Results

Costs of guarding

The antennal length of isopods increased with size, but the increase was stronger in the unpaired males (Fig. 1). The effect of pairing status and isopod length, as well as their interaction, on antennae length were statistically significant (ANCOVA: $F_{1,573} = 6.10$, $P = 0.014$, $F_{1,573} = 15.28$, $P < 0.001$, and $F_{1,573} = 7.32$, $P = 0.007$ respectively).

Individuals inferred to have broken antennae

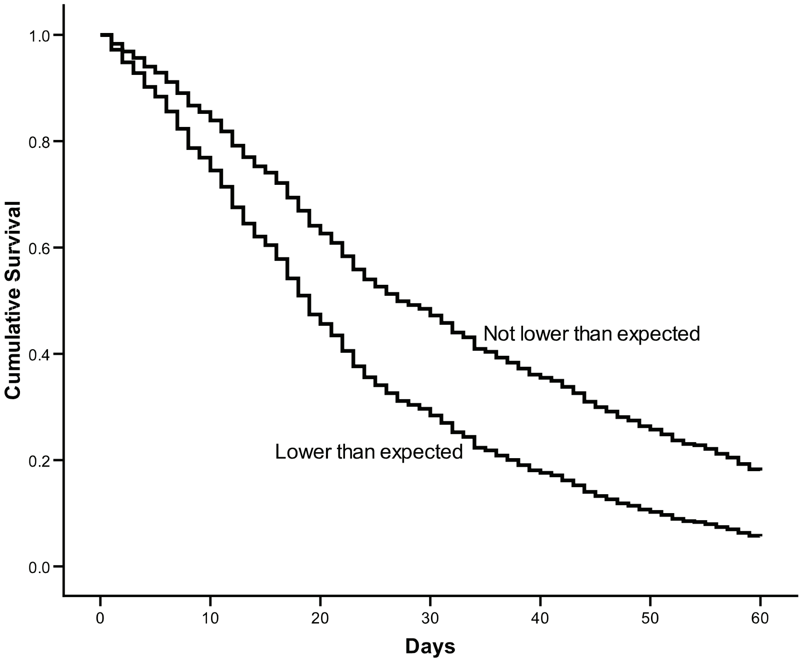

Fig. 2. Cumulative survival of male isopods with antennae lengths either similar to or lower than expected values based on an allometric relationship between antennal length and body size.

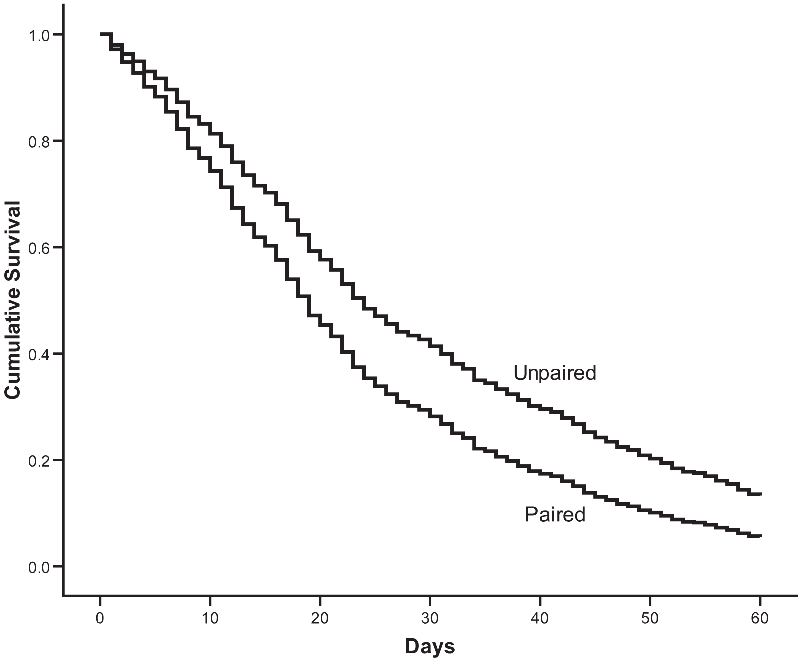

Fig. 3. Cumulative survival of male isopods collected as a precopulatory pair or unpaired over the course of 60 days.

had reduced survival compared to those with an average antennal length similar to expectations based on body size (Fig. 2 and Table 1). Paired males had slightly lower survival than unpaired males (Fig. 3 and Table 1). However, the effect of pairing on survival was dependent on isopod length (Table 1). Specifically, the negative relationship between survival and isopod length was more pronounced for paired males (Fig. 4).

Only 33% of 577 isopods molted during the experiment, and the majority of these individuals only molted once (*n* = 161). The maximum number of observed molts was three (*n* = 7). Regardless of the observation length, the pro-

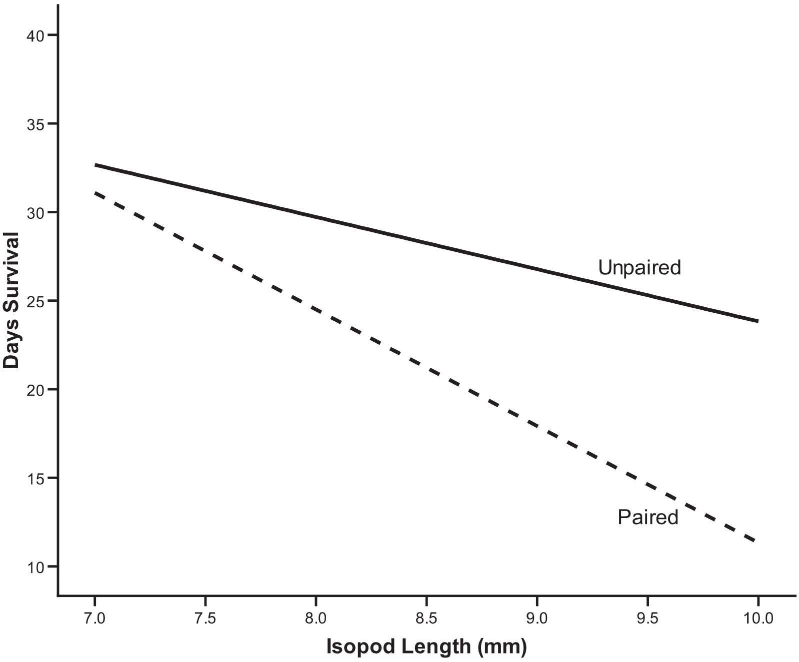

Fig. 4. The relationship between survival and isopod length for paired (dashed line; $n = 216$, $y = –6.58x + 77.2$; $r = 0.301$) and unpaired (solid line; $n = 361$, $y = –2.95x + 53.3$; $r = 0.123$) male isopods. The length of males ranged from 6 to 11.5 mm, but 95% of both the paired and unpaired males were between 7 and 10 mm. Thus, the regression lines are only shown for that range.

portion of paired and unpaired isopods which molted at least once did not differ significantly (Table 2).

Pair formation

In 79% of the trials, the male attempted to form a pair, and just over half (56%) of these trials resulted in pair formation. Previous pairing status did not predict attempts ($P = 0.643$) or pairing success ($P = 0.785$). Trials involving large females were less likely to form a pair (Fig. 5; $\beta = –1.11$, $\chi^2_1 = 15.3$, $P < 0.001$), but female size did not affect male attempts ($P = 0.612$). Male size did not significantly affect pairing attempts or success ($P = 0.098$ and 0.572, respectively). For both pairing attempts and success, the interactions between previous pairing, male size, and female size were not significant ($P > 0.1$).

Discussion

As expected, the antennae length of isopods increased with body length, but this relation-

Table 1. Summary of Cox regression model used to analyze the survival of 216 paired and 361 unpaired male isopods. The antennae of isopods were defined as either intact or broken based on an allometric relationship between antennae length and isopod size. The odds ratio indicates the ratio in the hazard function (survival probability) for individuals in contrasted classes, e.g. the survival probability of unpaired isopods was approximately 1.5 times higher than that of paired isopods.

Terms in the final model	β (SE)	df	*P*	Odds ratio
Pairing status	0.43 (0.09)	1	< 0.001	1.53
Antennae status	0.52 (0.09)	1	< 0.001	1.68
Pairing status × isopod length		1	< 0.001	
Terms excluded from the final model	Score	df	*P*	
Isopod length	0.55	1	0.46	
Pairing status × antennae status	0.11	1	0.74	
Isopod length × antennae status	0.23	1	0.63	
Pairing status × isopod length × antennae status	0.11	1	0.74	

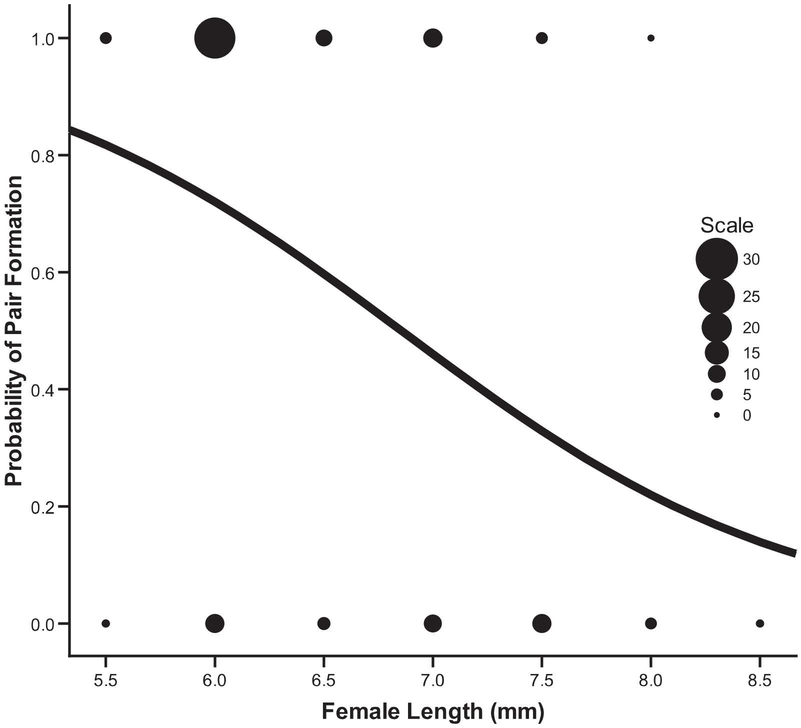

Fig. 5. The relationship predicted by logistic regression between female length and the probability of pair formation in behavioral trials in which a male was presented with a single receptive female. The actual data points (cases where the pair formation took place (1) or did not (0) occur) are shown with filled circles; circle size scales with the frequency of observations at that point.

ship was rather flat for isopods collected in a precopulatory pair. This pattern may suggest that among large isopods, paired individuals had broken antennae more commonly than unpaired individuals. When breeding is widespread in early summer, *A. aquaticus* populations can be male-biased (Pashkova & Korotneva 2000), and the operational sex ratio is male-biased anyway as the females disengage from the mating population due to the long incubation period following egg-laying. Thus, the incidence of male-male contests and the risk of antennae injuries is likely to be high. This risk could be particularly high for large males which are more likely to pair with large, fecund females (Ridley & Thompson 1979). These males may experience more numerous and more aggressive take-over attempts and, perhaps, more frequently damaged antennae. Alternatively, because animals were collected from nature, it is possible, though we find it unlikely, that the causal chain goes in the other direction, i.e. large males with broken antennae

Table 2. The occurrence of molting by male isopods collected paired or unpaired during discrete lengths of observation. All isopods which survived at least 10 days, for example, had equal "opportunity" to molt during the initial 10 days of the experiment. For each minimal observation length/survival time, the proportion of individuals which molted was compared between paired and unpaired isopods with a χ^2-test.

Days of observation	Number of individuals molting at least once/ number of individuals still alive		χ^2_1	*P*
	Unpaired	Paired		
10	92/300	44/150	0.06	0.81
20	88/215	41/92	0.20	0.65
30	75/149	35/67	0.03	0.86
40	60/106	28/46	0.10	0.75
50	50/75	16/27	0.17	0.68
60	43/53	17/24	0.22	0.64

were more likely to form pairs than large males with intact antennae. Also, limb regeneration in isopods is dependent on molting (Needham 1949, Madhavan & Madhavan 1981). Given that molting rate in the observed isopods was low, reproductive males would regenerate lost antennae slowly. Slow limb regeneration would tend to decrease the distinction between paired and unpaired isopods, because some of the isopods collected unpaired may have lost antennae during previous guarding episodes. Nonetheless, large males collected paired had significantly shorter antennae than those collected unpaired, presumably as a consequence of recent guarding behavior.

Males with broken antennae may be less able to detect female presence or receptivity and would thus be less successful in scramble competition (Lefebrve *et al.* 2000, Bertin & Cézilly 2003). The importance of scramble competition in *A. aquaticus* for male fitness, however, probably depends on population density (Bertin & Cézilly 2005). At high densities, contest competition may primarily determine male success, but scramble competition may predominate at low densities (Andersson 1994). Sexual selection on antennae lengths varies between *A. aquaticus* populations (Bertin & Cézilly 2003), though it is not known if this variation is related to density. The population studied here experiences little fish predation, so, compared with nearby populations subjected to predation, the population density is high (D.P.B. unpubl. data). Under these circumstances, contest competition may be more relevant for male fitness than scramble competition and, thus, reduced antennae length could have little impact on pairing success. Nonetheless, individuals with seemingly broken antennae also exhibited higher mortality, suggesting antennal injuries may still be costly even if they do not affect mate-finding ability in the prevailing high densities. The mechanism underlying the relationship between mortality and broken antennae is not clear. It might reflect the number of antagonistic conflicts experienced by a male, it may derive from energetic costs of appendage regeneration, or it may be a consequence of infections and immunological costs associated with wounding (Plaistow *et al.* 2003b). Alternatively, broken antennae could be a surrogate measure for some other cause of isopod mortality and thus may only be indirectly related to survival. For example, broken antennae could simply be a marker for ill health in isopods.

Energetic costs associated with mate guarding may have contributed to the higher mortality of paired isopods. Sparkes *et al.* (1996) found guarding to reduce the glycogen reserves of male isopods, but recovery was quick; within 36 hrs after the end of guarding glycogen stores were replenished. Studies on amphipods, however, suggest that the energetic demands and reduced feeding associated with guarding may also have more long-term costs, e.g. reduced growth (Robinson & Doyle 1985, Plaistow *et al.* 2003a). Reduced somatic maintenance and survival could also be a consequence of these energetic expenditures (Kirkwood & Austad 2000). Because isopods were collected from nature, we can not exclude the possibility that pre-existing differences between paired and unpaired isopods were responsible for their disparate mortality rates. For example, the paired males may simply have initially been older than the unpaired males. Paired and unpaired isopods did not differ in body lengths measured at death (*t*-test: $P = 0.19$), so, if size is roughly representative of isopod age, this may imply they die at approximately the same age. Because the unpaired isopods lived longer and, thus, had slightly more time to grow, they were perhaps younger at the start of the experiment. However, molting was infrequent as compared with isopods from the same population collected during the autumn (Benesh & Valtonen 2007), suggesting growth was limited. Moreover, the occurrence of molts was similar in paired and unpaired isopods, providing no support for the existence of age-differences between these groups. The possibility of age dissimilarities between paired and unpaired isopods can not be ruled out, but there was also little to indicate they were of different ages.

Even if unpaired isopods were younger, their pattern of size-dependent mortality would be expected to be similar to that of paired isopods, albeit at a different level. This was not the case, however, as large, paired males experienced greater mortality than large, unpaired males. Given that pairing is size-assortative (Ridley & Thompson 1979), the decreased survival of large, paired males could indicate big females are more costly to guard. Males guarding large, fecund females may be subjected to more frequent take-over attempts or large females may be more energetically-demanding to carry (Plaistow *et al*. 2003a). Guarding large females could also be costly if they vigorously resist guarding attempts, but this seems improbable because, unlike some other isopod species, female *A. aquaticus* do not exhibit considerable resistance behavior (Jormalainen & Merilaita 1995). Additionally, large males may engage in precopula for longer periods than smaller males (*A. aquaticus*: Jormalainen & Merilaita 1995; amphipods: Dick & Elwood 1996, Hume *et al*. 2002). Whatever the causal mechanism, the size-dependent mortality of paired males raises the possibility that the fecundity benefits of guarding large females are traded off against decreased survival.

Previous pairing did not predict male pairing attempts, suggesting energetic costs associated with guarding do not deter future attempts at pair formation, given the male has had time to recover. The two-week recovery time was relatively long, so, if it were shorter, it is possible that previously paired males would have been less willing or less capable to enter precopula. However, male amphipods are also still motivated and able to pair with females shortly after ending earlier guarding episodes (Hume *et al*. 2002). Therefore, males may be generally inclined to enter precopula, regardless of the reproductive costs already incurred. Less discriminating males may be favored when the probability of meeting a receptive female is low (Härdling & Kokko 2005), which seems realistic for mate-guarding crustaceans given the limited time females are available for fertilization.

Though most males attempted to form pairs, the success rate was just over 50%. Success in establishing a precopulatory pair was particularly low when females were large. An enhanced ability of large females to resist guarding is unlikely because female *A. aquaticus* do not seem to show pronounced resistance to guarding attempts (Jormalainen & Merilaita 1995). Instead, loading constraints, i.e. males can only carry females of certain sizes, may be responsible for the observed reduction in pairing success of large females (Adams *et al*. 1985). This large-female disadvantage seems to occur in some gammarids (Hatcher & Dunn 1997).

Precopulatory mate-guarding is known to have costs (Jormalainen 1998), and the data presented here suggest the costs incurred by males extend beyond the guarding phase. These costs were not apparently reflected in male behavior, however. The various costs associated with guarding, together with encounter rates between the sexes and female behavior, dictate the relative fitness benefits associated with male guarding decisions. Determining the factors most important in shaping the cost/benefit ratio for male strategies will undoubtedly provide insight into the mechanisms of sexual selection and arousal of sexual conflicts.

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