FEMALE COPULATORY STATUS AND MALE MATE CHOICE IN *NEPTUNEA ARTHRITICA* (GASTROPODA: BUCCINIDAE)

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ABSTRACT

Male mate choice in *Neptunea arthritica* was investigated in copulation trials, where female copulatory status and intermating interval were manipulated. Males preferred recently copulated to noncopulated females and did not discriminate between single- and twice-copulated females. Our results show a startling divergence from the results reported in other study models, with significant implications for our general understanding of the way sperm competition shapes male mate choice. We discuss the importance of female copulatory status, female intermating interval and sperm removal probability as factors extending competitive scenarios among males and how preference for recently copulated females might be maintained.

INTRODUCTION

Females frequently mate with more than one male, across a wide array of species (Birkhead & Møller, 1998). The phenomenon of female remating has received considerable attention because it is associated with patterns of sperm use and sexual selection (Parker, 1984). Promiscuity and sperm storage are common in gastropods and it is likely that sexual selection through sperm competition has been a strong evolutionary pressure on male traits correlated with reproductive success (Anderson, 1994; Chase, 2002).

Under sperm competition, precopulatory and postcopulatory selection mechanisms are predicted to evolve in order to optimize reproductive effort (Trivers, 1972; Parker, 1984, 1998; Wedell, Gage & Parker, 2002). In gastropods, promiscuity and sperm storage promote strong postcopulatory selection, whereas precopulatory selection (mate choice) has been traditionally regarded as having a weak effect, largely based on body size (Chase, 2002; Rogers & Chase, 2002). However, new evidence for male mate choice associated with female copulatory status in gastropod species is beginning to appear (Johannesson *et al.*, 2008; Loose & Koene, 2008; Zahradnik, Lemay & Boulding, 2008). For species in which females store sperm, males might be able to assess sperm-competition risk via sperm storage cues (Paterson, Partridge & Buckland-Nicks, 2001; Wedell *et al.*, 2002; Uhía & Cordero-Rivera, 2005) or other evidence of recent mating such as pheromones (Saur, 1990; Friberg, 2006). In some reports males prefer to mate with virgin females and can discriminate against mated females (Arnaud, 1999; Zahradnik *et al.*, 2008). There is also evidence that males might provide smaller ejaculates or terminate copula prematurely when they mate with females with higher risk of sperm competition (Yasui, 1996; Bukowski & Christenson, 1997; Loose & Koene, 2008), of different copulatory status (Sauter & Brown, 2001; Zahradnik *et al.*, 2008) or of different ecotype (Johannesson *et al.*, 2008).

The Neptune whelk, *Neptunea arthritica*, is a dioecious species with internal fertilization. Females possess a bursa copulatrix (sperm storage/digestion site) and seminal receptacle (sperm storage/fertilization site) (Martel, Larrivée & Himmelman, 1986; Miranda, 2005). Females mate several times with different males which attempt forced matings, while females resist matings by escape and biting the male foot and penis with prominent proboscis extension during approach, mounting and intromission (Miranda, Lombardo & Goshima, 2008). Females produce a single egg mass and one mating is enough to produce a fertile clutch, which is laid *c.* 7–23 days after copulation (personal observation).

When all the above is considered along with the existing conditions for sperm competition in *Neptunea arthritica*, discriminatory male behaviour in relation to female copulatory status is expected. We predict that when confronted with the choice between mated and unmated females, males should discriminate against mated females. We also expect males to discriminate among females on the basis of extended intermating intervals due to unfavourable chances of fertilization success (Parker, 1984; Wedell *et al.*, 2002). We aim to test this predicted importance of female copulatory status in relation to sperm competition by documenting male mate choice with regards to female copulatory status in *Neptunea arthritica*.

MATERIAL AND METHODS

A sample of 335 *Neptunea arthritica* individuals larger than the size at maturity (70 mm in shell length, SL; Fujinaga, 1985) were collected in March 2007 before the natural mating season (April to June, Fujinaga, 1985) at Kikonai, Hokkaido, Japan, from the accumulated bycatch of the local fishermen's cooperative. Whelks were separated by sex and immediately transported to the Hokkaido Corporation for Aquaculture Promotion (Shikabe branch), where they were kept in two 10-ton tanks with running seawater at environmental temperature, with natural seasonal daylight/dark cycle and moderate aeration. Whelks were fed weekly with a mixed diet of dead fish (*Cololabis saira*, *Paralichthys olivaceus*), crushed mussels (*Mytilisepta galloprovincialis*) and scallops (*Mizuhopecten yessoensis*). Two weeks before the experimental period (May to June 2008), the shell of whelks was measured (length) with a digital calliper (accuracy 0.1 mm) and marked with indelible ink according to sex and identity number.

Female whelk attractiveness

To control for a possible bias in male mate choice arising from female differences in attractiveness, size-matched female pairs were chosen and each presented to a set of five randomly

chosen males. Males within sets were allowed to interact with female pairs one at a time, inside 16-l acrylic plastic tanks (53 cm length × 36 cm width × 18.5 cm height). If the male did not attempt mating within 2 h from placement, it was removed and another one was introduced in the tank. Female whelk attractiveness was determined from a complete sequence of male approach, mounting and clear intromission attempts, however no copulation was allowed. Females were classified as 'attractive' if approached by at least three males in separate instances. To validate female attractiveness, the resulting non-attractive females were paired and retested after 24 h in a similar fashion against both familiar and new males.

Since whelks were allowed contact and considering the role that mucus plays in gastropod species interactions (Chase, 2002), only females approached by the same number of males were paired in the following experiments. Additionally, to control for familiarity effects, males that approached females during this phase were later presented only to novel females.

After this process, only highly motivated males and attractive females were selected to record their mating behaviour in a series of copulation trials detailed as follows.

Experiment A: effect of male and female copulatory status

To test the effect of male and female copulatory status on male mate choice, copulated females (CF) and copulated males (CM) were obtained by crossing pairs consisting of noncopulated females (NCF) and noncopulated males (NCM). Individuals were left undisturbed inside 16-l acrylic plastic tanks (53 cm length x 36 cm width x 18.5 cm height) and separated after the end of copulation. The CF and CM were then kept individually in different tanks as above, with a 3-h 'resting' period before the trials in which male mate choice was evaluated. The experiment was subdivided into two parts: (A1) to test the effect of female copulatory status on male mate choice and (A2) to analyse male copulatory status effect on male mate choice.

Experiment A1: Trials of experiment A1 consisted of NCM ($n = 10$; mean $\pm$ SD $= 92.0 \pm 3.7$ mm SL) presented to novel pairs of NCF ($n = 10$; 83.4 ± 2.1 mm SL) and CF ($n = 10$; 81.6 ± 4.3 mm SL). The binomial test was used to analyse male mate choice, and copulation duration was compared using the *t*-test.

Experiment A2: Thirteen trials (Fig. 1) were completed for experiment A2 where CM ($n = 13$; 90.0 ± 1.6 mm SL) were presented to novel female pairs with the same characteristics as above. All female pairs were size-matched (*t*-test, $t = 0.77$, df $= 36$, $P = 0.44$) and had only a single copulation previous to the trials. The binomial test was used to analyse mate choice, and copulation duration was compared using the *t*-test. The effect of male copulatory status on preference from experiment A1 and A2 was analysed with the two-proportion Z-test.

Experiment B: effect of number of female matings

In order to determine if the number of previous matings by the females affected male mate choice, a different set of size-matched female whelks was conditioned as in experiment A, where two groups of CF were obtained. One CF group had a single copulation (SCF) whereas the other copulated twice (TCF) with different NCM. During conditioning, the intermating interval of TCF never exceeded 5 h between each copulation. Both female types were kept separately with a 3-h resting period before trials. Fifteen trials were conducted in which size-matched (*t*-test, $t = 1.80$, df $= 36$, $P = 0.08$) female pairs, comprising one SCF ($n = 15$; 89.3 ± 5.5 mm SL) and
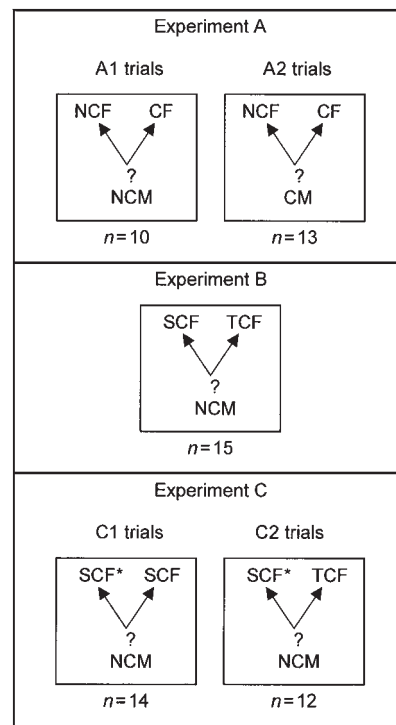

Figure 1. *Neptunea arthritica* male mate-choice trials scheme. In experiment A1, pairs consisting of one noncopulated female (NCF) and a copulated female (CF) were presented to noncopulated males (NCM). Similar female pairs were presented to CM for experiment A2. In experiment B, pairs consisting of a single copulated female (SCF) and a twice-copulated female (TCF) were presented to noncopulated males. In experiment C1, pairs consisting of single copulated females were presented to noncopulated males. One of the single copulated females in each pair (SCF*) had a long intermating interval (mated 4–5 days before trials) while the other had a short intermating interval (mated 3 h before). In experiment C2, the single copulated female (SCF*) had a long intermating interval, whereas the TCF had a short intermating interval.

one TCF ($n = 15$; 85.7 ± 7.0 mm SL) were presented to novel NCM ($n = 15$; 89.0 ± 3.7 mm SL) (Fig. 1). The binomial test was used to analyse mate choice, and copulation duration was compared using the *t*-test.

Experiment C: effect of female intermating interval

The objective of this experiment was to assess male mate choice under different intermating intervals in females. First, SCF and TCF were obtained as in experiment B although their intermating intervals were further modified. Both female types were kept separately previous to main trials. Experiment C consisted of two parts (Fig. 1).

Experiment C1: To evaluate the effect of female extended intermating interval on male mate choice, 14 pairs of SCF ($n = 28$; 89.3 ± 5.5 mm SL) were formed where one half of the SCF had copulated 4–5 days previous to the trials, while the other half had copulated 3 h before the trial (Fig. 1). Each female pair was presented with one novel NCM ($n = 14$; 89.0 ± 3.7 mm SL). The binomial test was used to analyse male mate choice, and copulation duration was compared using the *t*-test.

Experiment C2: The objective of experiment C2 was to assess the effect of female extended intermating interval on male mate choice with reference to the number of previous matings by the

females (Fig. 1). Twelve size-matched (*t*-test, *t* = 0.50, df = 20, *P* = 0.63) female pairs consisting of one SCF (*n* = 12; 85.0 ± 4.8 mm SL) and a TCF (*n* = 12; 87.3 ± 6.6 mm SL) were formed and presented to a novel NCM (*n* = 12; 89.0 ± 3.7 mm SL). The SCF were used in the trials 4–5 days after being mated, whereas TCF were included in the trials 3 h after their last copulation. The intermating interval of TCF never exceeded 5 h. The binomial test was used to analyse male mate choice, and copulation duration was compared using the *t*-test.

Successful copulation was not considered necessary before a male could be classified as having chosen a particular female. It has been reported that females resist mating in this species (Miranda *et al.*, 2008), and therefore failed copulation attempts under female resistance were also classified as male choice. In addition, males might terminate copulation prematurely with females representing higher sperm-competition risk (Yasui, 1996; Bukowski & Christenson, 1997; Loose & Koene, 2008), thus individual copulation duration history was also analysed via one-way ANOVA along with Tukey's test.

The beginning of copulation was determined after the male achieved intromission, and duration was measured with a chronometer until penis withdrawal. Mate-choice trials were carried out within a 2-h time limit. If copulation continued over the time limit, it was allowed to carry on. When a male copulated within the time limit, observations continued until the end of the trial.

RESULTS

Female whelk attractiveness

From a total of 170 male whelks, 36 were discarded because of injury or low motivation. From the remaining 134 individuals, 64 highly motivated males were selected to participate in the mate-choice trials and the remaining 70 male whelks were employed in the conditioning of females prior to the mate-choice trials. Out of 165 female whelks, 11 were excluded due to detection of imposex (masculinization of females by tributyltin pollution), and 130 normal females were classified as 'attractive'. Of these females 9% were approached by at least three different males, whereas 17% were approached by four

males and 74% by all five of the presented males. The remaining 24 females were not attractive to the presented males. No external difference between females was observed to account for attractiveness. However, males displayed intense siphon movements while orienting towards a particular female, and seemed to survey the female's aperture before to mounting and intromission.

Mating features

A total of 64 mate-choice trials were conducted, in which 59 males achieved intromissions of variable duration while five of them were rejected by the females, three on approach and two at mounting (Table 1). Copulation duration decreased in relation to the number of times the females had previously copulated (one-way ANOVA, $F_{(2,132)} = 28.98$, $P < 0.001$). A female's first copulation averaged 40.90 ± 19.76 min, second 26.48 ± 11.76 min and third 11.12 ± 5.84 min (Tukey's test, $P < 0.05$) (Fig. 2). Females displayed their reluctance to mate by trying to escape and resisted a male by biting its foot and penis before and during intromission in all trials. Of the intromissions 81% were terminated as a result of sustained female resistance (biting). Postcopulatory guarding was observed in 70% of the mate-choice trials. Male whelks retained their grip and/or flipped the female shell, restraining females from resuming normal activity beyond the trial time limit, sometimes for up to 3 h. Furthermore, white granules of a gel-like substance were observed trickling from the interior of the shell of 52 CF during intromissions that took place soon after the previous copulation (Fig. 3A). Examination under a microscope determined that the substance consisted of live sperm cells (Fig. 3B).

Mate choice

In experiment A, both NCM and CM preferred CF over NCF (Table 1). Male copulatory status had no effect on preference ($Z = 1.04$, $P = 0.29$) or on copulation duration (*t*-test, $t = 1.04$, df = 18, $P = 0.311$; NCM, 33.24 ± 9.32 min and CM, 29.47 ± 8.40 min). In experiment B, males showed no preference between single- and twice-copulated females (TCF) (Table 1), while copulation duration was significantly less for

Table 1. Summary from mate-choice experiments with *Neptunea arthritica*.

Experiment	*n*	Male mate choice		*P*	Copulation			Postcopulatory guarding	White granules 'loose sperm'
					Male overcame resistance	Female terminated (biting)	Rejected		
		NCF	CF						
A1	10	0	10	0.002	2	8	0	6	8
A2	13	2	11	0.02	4	8	1	6	11
		SCF	TCF						
B	15	9	6	0.61	3	11	1	9	13
		Long interval	Short interval						
C1	14	1 SCF	13 SCF	0.002	1	12	1	9	11
C2	12	1 SCF	11 TCF	0.006	1	9	2	11	9
Total	64	–	–	–	11 (19%)	48 (81%)	5 (8%)	41 (70%)	52 (88%) (59 matings)

Experiment A1 tested female copulatory status effect on male mate choice. Pairs consisting of one noncopulated female (NCF) and a copulated female (CF) were presented to noncopulated males (NCM). Experiment A2 analysed male copulatory status effect on male mate choice. Female pairs were presented to copulated males (CM). Experiment B determined if the number of previous matings by the females affected male mate choice. Pairs consisting of a single copulated female (SCF) and a twice-copulated female (TCF) were presented to NCM. Experiment C1 evaluated female extended intermating interval effect on male mate choice. Pairs consisting of single copulated females (SCF) were presented to NCM. One of the single copulated females (SCF) in each pair had a long intermating interval (copulated 4–5 days before trials) while the other had a short intermating interval (copulated 3 h before). Experiment C2 assessed female extended intermating interval effect on male mate choice with reference to the number of previous mating by the females. Pairs consisting of a single copulated female (SCF) and a TCF were presented to NCM. The single copulated female (SCF) had a long intermating interval whereas the TCF had a short intermating interval. The binomial test was used to analyse mate choice.
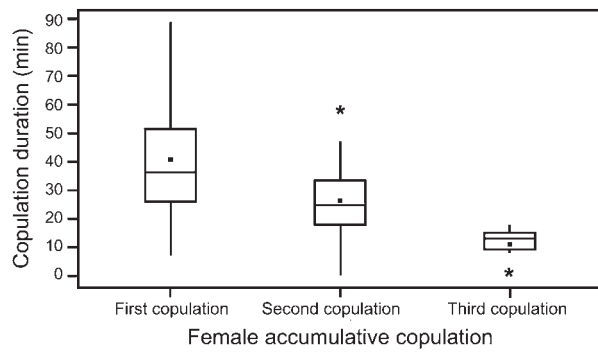
Figure 2. *Neptunea arthritica*. Copulation duration as a function of accumulated number of female matings. The solid dot is the mean, and horizontal lines across the box represent the median. Asterisks correspond to outlier points.
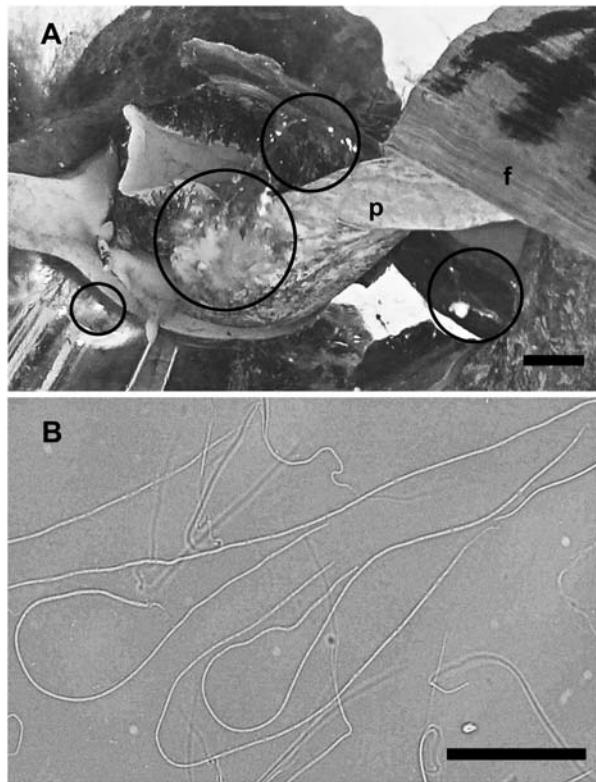

Figure 3. *Neptunea arthritica*. **A.** 'Loose sperm' coagulated into white granules (black circles) trickling from the female aperture during copulation. **B.** Sperm cells from white granules. Abbreviations: f, female; p, penis. Scale bars: **A** = 10 mm; **B** = 100 μm.

the latter (*t*-test, $t = 6.07$, df = 10, $P < 0.05$; SCF, 24.47 ± 5.82 min and TCF, 11.54 ± 2.17 min). When the intermating interval was manipulated in experiment C, males showed a marked preference for recently mated females over those with longer intermating interval (Table 1). Copulation duration was significantly less for TCF (*t*-test, $t = 11.29$, df = 20, $P < 0.05$; SCF, 26.76 ± 3.93 min and TCF, 11.83 ± 2.48 min).

DISCUSSION

In experiment A, males appeared to discriminate between NCF and CF, mating consistently with CF. This male mate choice is unusual, since in view of much work in the field of sperm competition (Parker, 1984, 1998; Birkhead & Møller,

1998; Wedell *et al.*, 2002) a bias towards NCF was expected. Furthermore, there was no significant preference between once-copulated females (SCF) and TCF in experiment B, indicating that males did not adjust their preference in relation to female copulatory status or, alternatively, they could not detect the female's previous matings. Moreover, in experiment C, when males were presented with the choice between females of short and long intermating interval (3 h and 4–5 days before trials, respectively), males chose females with a shorter intermating interval regardless of the female's number of previous matings, suggesting that males are sensitive to cues indicative of recent mating in females. Interestingly, the CF that were preferred in experiments A and B also had a short intermating interval (the 3-h resting period) before interaction in male mate-choice trials. To our knowledge, this is the first time, at least in gastropods, that males have been shown to prefer recently mated females, implying a variant in male mate-choice mechanism among gastropods.

Our study is limited in that we know very little about the nature of the signal or signals that mediate reproductive interactions in *Neptunea arthritica*. The use of chemical cues plays an important role in molluscan behavioural responses (Chase, 2002). Pheromone-mediated attractiveness associated with gonad maturation and the onset of mating has been documented in a number of gastropod species (Saur, 1990; Chase, 2002; Johannesson *et al.*, 2008). Furthermore, at least three pheromones and one kariomone have been linked to aggregation of sexually active individuals in *Ilyanassa obsoleta* (Moomjian, Nystrom & Rittschof, 2003) and *Pomacea canaliculata* (Takeichi, Hirai & Yusa, 2007). Attractiveness in other buccinid species such as *Buccinum undatum* (Martel *et al.*, 1986), *Neptunea antiqua* (Power & Keegan, 2001) and *B. isaotakii* (Ilano, Fujinaga & Nakao, 2004) is thought to be regulated similarly, however the origin and nature of the compounds involved is not known. In the present study, males targeted individual females and seemed to use their siphons to examine the aperture of females before attempting to mate, suggesting the potential use of distinctive cues. Therefore, it is likely that a component of male mate choice in this species is mediated by pheromones indicative of female maturity and/or copulatory status.

In our experiments, male whelks might have perceived females only as mature, and therefore as potential targets. However, when facing sperm-competition risk and when information about the females' intermating interval was available in the form of chemical cues (e.g. impregnated mucus from other males, sperm or seminal fluid residues, female fluids from the bursa copulatrix or changes in hormone profiles), males preferred recently copulated females as mates. The consistent preference for females of short intermating interval suggests that this interval (timing of copulation) is highly important for success in sperm competition (Parker, 1984). Female *N. arthritica* produce a single egg mass, which is laid *c*. 7–23 days after copulation (personal observation). Within this time window, male fertilization success may be deeply affected by multiple mating (Parker, 1984, 1998; Birkhead & Møller, 1998; Wedell *et al.*, 2002). Opportunistically, male whelks could use recent mating cues to identify mature females that are about to ovulate and attempt positioning of their ejaculate in order to fertilize as many eggs as possible. Thus, the short intermating interval in CF (reliable information about sperm removal potential and fertilization success chances) *vs* the absence of recent mating cues or presence of an odour indicating the opposite (sperm removal may be impossible, seminal receptacle already full and/or ovulation occurred long ago), might explain why NCF and SCF of long intermating interval were not preferred.

Multiple mating generates potential for sperm competition (Parker, 1998; Wedell *et al.*, 2002) and is probably related to

the observed postcopulatory guarding behaviour (Dewsbury, 1982; Wedell *et al.*, 2002) in *N. arthritica*. Furthermore, instances of 'loose sperm' (Fig. 3A) in our experiments might have been the result of spillage by accident, full capacity of the bursa copulatrix, active ejection by females or removal by males (Buckland-Nicks *et al.*, 1999). Male genitalia in *N. arthritica* might be able to disrupt the contents of the bursa copulatrix by expanding in volume soon after intromission (Fig. 4A). First, the penis might be secured in place by a hook on its distal portion (Fig. 4B), then engorged with haemolymph to occupy most of the female's mantle cavity, producing an increase in pressure, tamping down rival sperm and allowing the male's own to be transferred first into the seminal receptacle. As a result, rival sperm might spill out from the bursa. Although there is no conclusive evidence of males physically displacing rival sperm from the bursa copulatrix and the mechanism involved, in our observations 'loose sperm' was seen only during intromissions after a short intermating interval, which suggests that it was the result of subsequent mating. In most neogastropods, sperm is first received in the bursa copulatrix and then transferred over a 3-day period to the seminal receptacle where storage and fertilization are thought to occur (Martel *et al.*, 1986). It is unknown how long ejaculates take to reach the seminal receptacle in *N. arthritica*; nevertheless, sperm removal in subsequent copulation with a short intermating interval (3–5 h in this study) is possible if ejaculates remain in the bursa copulatrix. This is likely the case since 'loose sperm' was not observed in matings (two females) after a long intermating interval (4–5 days before trials), implying that perhaps the sperm had been digested or transferred to the seminal receptacle where removal is impossible (Martel *et al.*, 1986; Buckland-Nicks *et al.*, 1999). Male ability to detect mature females near egg laying and incidentally (or deliberately) to remove sperm from previous matings results in the maintenance of a preference for recently copulated females. Such an opportunistic male strategy might be particularly useful under sperm competition as the mating season advances, when the abundance of unmated females decreases and female intermating intervals become more variable.

Copulation duration decreased as the number of previous matings by the female increased. Such a trend might be explained by male adjustment to female copulatory status (Yasui, 1996; Bukowski & Christenson, 1997; Loose & Koene, 2008) or female resistance stemming from sexual conflict (Arnqvist & Rowe, 2005). In *N. arthritica* female reluctance to copulate suggests intense sexual conflict (Lombardo & Goshima, in press). Lombardo & Goshima (in press) compared male whelk copulation duration in 'natural' trials to that of males in trials where female resistance ability had been manipulated, allowing males to end copulation at their timing and thus make adjustments depending on the perceived risk of sperm competition. Copulation duration in natural trials decreased progressively due to female resistance, while male copulation duration with manipulated females increased significantly even though these females had previously mated three times with different males, indicating that males invest heavily to win sperm-competition contests (Parker, 1984, 1998; Birkhead & Møller, 1998; Wedell *et al.*, 2002). In the present study it seems plausible that copulation duration might have been mediated by females, since 81% of intromissions were terminated as a result of sustained female resistance. Given that males are able to detect that females have recently copulated at least once, extended copulation might be in their best interest (e.g. to remove rival sperm, transfer a large ejaculate and/or stimulate the female to transport or use his sperm), provided that copulation duration is correlated with reproductive success under sperm competition (Parker, 1984, 1998; Anderson, 1994; Birkhead & Møller, 1998; Wedell *et al.*, 2002). Thus, in line with Lombardo & Goshima (in press), it seems unlikely that copulation duration was reduced by the males according to the number of previous matings by the females.

In conclusion, male mate-choice criteria in *N. arthritica* depends greatly on mating opportunities, but most importantly on fertilization chances as dictated by the interplay between female copulatory status, female intermating interval and probability of sperm removal. Further studies of paternity from controlled copulation trials will contribute to improve our understanding of the evolution of mating strategies in this species.

ACKNOWLEDGEMENTS

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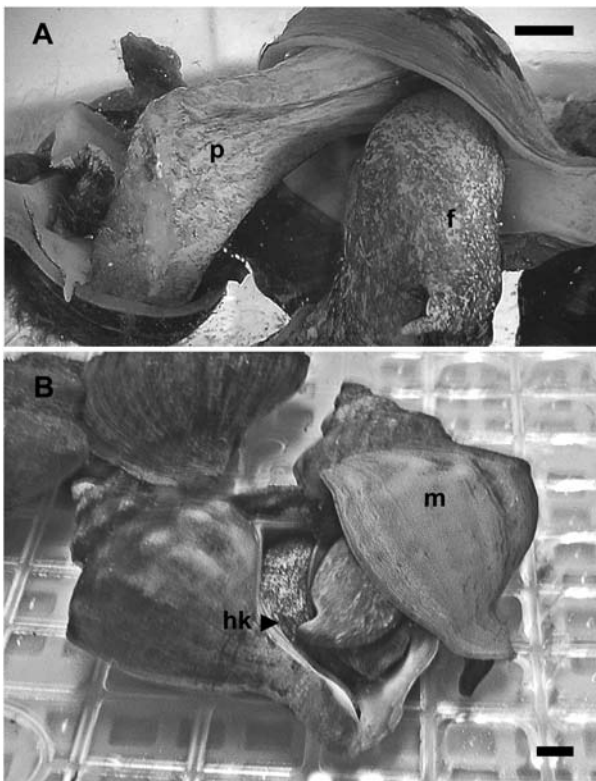

Figure 4. *Neptunea arthritica*. **A.** Engorged penis occupying most of the female mantle cavity. **B.** Tip of penis. Abbreviations: f, female; hk, hook, m, male; p, penis. Scale bars: **A, B** = 10 mm.

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