CHOOSY MALES IN A LITTORINID GASTROPOD: MALE *LITTORINA SUBROTUNDATA* PREFER LARGE AND VIRGIN FEMALES

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ABSTRACT

Mate choice has been considered to be a uniquely female behaviour. However, recent studies suggest that males strategically allocate their sperm among females with the greatest reproductive value. We investigated mating behaviour in the direct-developing Northern Pacific gastropod, *Littorina subrotundata*. Our field survey showed significant sexual dimorphism and the presence of size-assortative mating in our study population. Laboratory studies demonstrated that larger males may physically out-compete smaller males for access to females. Additional laboratory mate-choice experiments showed that males preferentially copulated with larger females. We also found that males were significantly more likely to copulate with virgin females than with females that had recently copulated. This suggests that males can detect the presence of sperm from rival males within a female's reproductive tract.

INTRODUCTION

The differential ability of individuals to compete for mates is known as sexual selection (Lincoln, Boxshall & Clark, 1987). Until recently, it was believed that sexual selection was primarily concerned with access to fertile females. It was thought that males had a limitless supply of sperm, and consequently their optimal strategy is to inseminate as many females as possible (Andersson, 1994; Judson, 2002). Belief in the sole existence of this system led the majority of studies on sexual selection to examine patterns of female choice (Jones, Adams & Arnold, 2002). However, Dewsbury (1982) argued that in many cases sperm, not ova, are limited. Therefore, males may increase their fitness by strategically allocating sperm among females with the highest fecundity (Preston *et al.*, 2001). Males are expected to become the selective sex in species where there is a high level of male parental care for offspring, a high risk of sperm competition, or if female fecundity shows substantial variability (Shine *et al.*, 2001).

Among littorinid gastropods, female fecundity is positively correlated with female body size and is therefore variable (Hughes & Answer, 1982). In these species, males may preferentially mate with larger females because they are physically capable of producing either a greater number of clutches or more eggs per clutch, which represents a superior sperm investment.

Littorinid snails are able to lay egg masses that are fertilized by stored sperm from multiple males. This multiple paternity can be attributed to the female's complex reproductive tract (Paterson, Partridge & Buckland-Nicks, 2001): sperm enters the female's body via the bursa copulatrix, a temporary sperm storage organ, where it may remain for up to 3 months (Buckland-Nicks *et al.*, 1999). It then migrates to the smaller seminal receptacle (Reid, 1996), where it may persist in the presence of other males' sperm (Paterson *et al.*, 2001). Baur (1994) believes sperm can viably exist in the seminal receptacle of some gastropod species for up to a year. This complex reproductive method sets up an ideal arrangement for sperm competition. Among species where females store sperm, males are often able to detect the presence of other male's ejaculates within the female's reproductive tract (Wedell, Gage & Parker, 2002). Males may then discriminate against non-virgin females either by avoiding mating with these females or by increasing ejaculate size to out-compete sperm already present. When virgin females are infrequent among a population and in species where the risk of sperm competition is high, it is advantageous for males to increase their reproductive investment with virgin females (Wedell *et al.*, 2002).

Saur (1990) studied the mating behaviour of *Littorina littorea* and *L. saxatilis* and found that male *L. littorea* demonstrate a preference for copulating with larger females. In addition, males spent significantly less time copulating with females that were heavily parasitized. This suggests that male *L. littorea* alter their reproductive effort based on the perceived quality or fecundity of the female. This trend was also observed by Erlandsson & Johannesson (1994), who found that male *L. littorea* copulated more often and for a longer period of time with larger females. They also found evidence for size-assortative mating in one of the populations they investigated.

The purpose of our study was to characterize patterns of mate choice among an Eastern Pacific species of marine snail *Littorina subrotundata* (Carpenter, 1864). *L. subrotundata* are gonochoristic snails that inhabit both wave exposed shores and salt marshes, along a geographic range from northern California to the Kurile Islands (Reid, 1996). This broad range of habitat gives rise to a correspondingly broad range of morphological variation. In this study, we examined the 'barnacle ecotype', which occurs on heavily wave-exposed shores, and seeks refuge from high wave action between barnacles and in rock grooves (Reid, 1996).

We carried out a series of laboratory experiments to investigate whether males discriminate between different females on the basis of their reproductive quality. We tested the prediction that males will increase the number of copulations with larger, more fecund, females. We also predicted that male *L. subrotundata* can detect the presence of rival sperm in the female reproductive tract, and that males will preferentially mate with virgin females. We also tested whether larger males physically out-compete smaller males for access to high quality females. In addition, we surveyed a field population of

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L. subrotundata to test for sexual dimorphism, size-assortative mating, and for differences in the relative growth rates of males and females.

MATERIAL AND METHODS

All experiments were carried out using *Littorina subrotundata* collected from Prasiola Point (48°48′N, 125°10′W) in Barkley Sound, British Columbia, Canada, during the summer of 2002 and 2003 and winter of 2002. Sexes of all snails were determined by the presence or absence of a penis when viewed under a dissecting microscope. Shell heights were measured along the longest axis of the shell from the tip of the spire to the bottom of the operculum using digital calipers.

For all experiments, copulation was defined as the active male being mounted on the passive partner, while facing the same direction with his right shell margin in contact with the substrate. Once in this position the male will insert his penis into the mantle cavity of the female as described by Saur (1990). It has been suggested that when littorinid males copulate for a longer period of time that they can transfer more sperm (Erlandsson & Johannesson, 1999). However, controversy exists as to whether or not the number of copulations is an accurate measure of future reproductive success. Some studies have found no relationship between the two measures (Pilastro & Bisazza, 1999); whereas others show a positive relationship between mating behaviour and sperm allocation (Evans & Magurran, 1999). No direct assessment of a relationship between the number of copulations and reproductive success has yet been documented for *L. subrotundata*. However, we believed that number of copulations was the most appropriate measure, given the lack of others available at the time of the experimentation.

Sexual dimorphism

To test for the presence of sexual dimorphism, a random sample of snails ($n = 317$ snails in 2002, $n = 197$ in 2003) was collected from our study population. In the laboratory the sex and shell height of all individuals were determined. An ANOVA was used to compare the log transformed mean shell heights of males and females from the two successive years.

Size-assortative mating

The presence of size-assortative mating was tested by randomly collecting 146 copulating pairs from the study site in the summer of 2002. Each pair was placed in an individual centrifuge tube for transport back to the laboratory where the sex and species of the active and passive partner from each pair was confirmed and all shell heights measured. The regression between the shell heights of the active and passive partner was estimated using SPSS version 10.

Growth rate

To test for differences in growth rate between the two sexes, four quadrats were randomly placed on the substrate and 250 snails were collected from each, for a total of 1,000 individuals. Two quadrats were from our study population on Prasiola Point (Prasiola North); the other two quadrats were collected from another population located 20 m away (Prasiola South). All snails were brought to the lab where permanent Mar-Tech™ paint was applied around the aperture of each snail's shell. The paint was allowed to dry overnight. The following morning snails were released back to their original location and recaptured after 30 days. New shell grows from the aperture lip up; the new growth below the area where paint was applied could be measured and compared as described by Behrens Yamada (1989). Snails in different quadrats were painted different colours to allow detection of snails migrating from one quadrat to the next. New shell growth was measured using a digitizing tablet connected to a dissecting microscope with a camera lucida.

Effect of male and female size

For the following two experiments, only pairs of snails found copulating in the field were used. This was done to ensure that all experimental snails were sexually mature. Snails found copulating in the field were separated and then placed in individual centrifuge tubes for transport to the lab. All snails were then held separately to ensure that they were not sexually active again until used in the experiments. The shell height and sex of each snail was recorded as above then all snails were divided into three size classes (Male: small < 3.0 mm; medium $3.0 - 3.5$ mm; large > 3.5 mm. Female: small < 3.5 mm; medium $3.5 - 4.5$ mm; large > 4.5 mm). A small coloured dot of Mar-Tech™ paint was applied to the apex of each snail's shell to identify it to the size class in which it belonged. All snails were then placed in individual petri dishes with a mesh screen inserted in the lids (Boulding & Hay, 1993), which were kept in outdoor seawater tanks (mean water temperature = 12°C) until used in the experiments. Snails were used only once.

The purpose of the first size-experiment was to compare the relative mating success of small and large males in order to test for patterns of dominance and subordinance. For each trial, one large male and one small male were placed simultaneously with an intermediate (medium) sized female in a petri dish with circular 1 mm^2 mesh covered openings cut into the top and bottom. There was a total of 20 petri dishes. All experimental dishes were placed in an outdoor seawater tank (mean water temperature = 12°C) and each dish was checked for the presence of copulatory behaviour five times per day for 5 days. The number of observed copulations by large and small males was compared using one way χ^2-test.

Our second size-experiment was carried out to test whether males have a preference for larger females. For this experiment, one large and one small female were placed in a meshed petri dish simultaneously with an intermediate (medium) sized male. As above, all 20 dishes were placed in an outdoor seawater tank and checked five times a day for 5 days. The total number of copulations by large and small females was compared using a one way χ^2-analysis.

Male preference for virgin females

For the following two experiments, approximately, 600 juvenile snails of shell length less than 2 mm were collected from the study site in the winter of 2002. At this size, snails are not yet sexually mature (Boulding & Van Alstyne, 1993), due to lack of penis formation. To ensure their virgin status until they reached sexual maturity, snails were raised individually in meshed petri dishes that were kept in outdoor seawater tanks (mean temperature = 12°C). These snails were shipped to the AquaLab at the University of Guelph, Ontario, where they were used to examine whether male *L. subrotundata* preferentially mate with virgin females. After the snails had reached sexual maturity, virgin females of a shell length greater than 4 mm ($n = 100$) were placed with one female of the same size (shell length no more different than ± 0.1 mm). One individual from each pair was randomly selected to become the 'experienced' female and painted white with a small dot of MarTec Paint™ for identification. This female was placed in a glass bowl containing seawater with two males of similar size. Snails were continuously observed until copulation between the female and one of the males occurred. Following copulation, the female was removed and placed in a petri dish with the virgin female from her group. This continued until 50% of females were classified as 'experienced', and were no longer virgins.

Each pair of females (one virgin and one experienced) was placed in their own glass bowl and allotted 30–45 min to manoeuvre and lay down mucus trails. Saur (1990) believed that females can be 'smelly', leaving a pheromone in their slime trail that indicates status of reproductive willingness. We hypothesized that the males in this experiment were detecting a different chemical signature from mated and from virgin females. A male of the same size (± 0.1 mm) as the two females was placed in the centre of each bowl. They were observed continuously until copulation occurred. Female type and duration of the copulatory event were recorded. Our data analysis assumed that both female types had an equal chance of first copulation; therefore, a one-way χ^2-analysis was used to compare which female type the male selected to mate with first. The mean time spent in the copulatory position with each female type was compared using a Kruskal–Wallis test.

Males were also tested for preference to four distinct female phenotypes (small virgin, large virgin, small experienced and large experienced), when presented simultaneously. Each male was presented with a group of four females. Each group contained: one large virgin female with a shell length 7.0 mm or greater; one large experienced (see above for protocol) female with a shell length 7.0 mm; one small virgin female with a shell length 4.0 mm or less; and one small experienced female with a shell length of 4.0 mm. Fifty groupings of four females were made in total.

All four females of one group were placed in a glass bowl containing seawater (as described above). Females were allotted 30–45 min to ensure they left mucus trails throughout the bowl. Snails were observed continuously until copulation occurred. The female type and the duration of the copulatory event were recorded. Our data analysis assumed that all female types had an equal chance of first copulation; therefore, a one-way χ^2-analysis was used to compare which female type the male mated with first. The mean time spent in the copulatory position with each female type was compared using a Kruskal–Wallis test.

Given the high population density of *L. subrotundata* and the propensity for males to engage in copulation, virgin females are likely to be very rare in natural populations. We therefore carried out a laboratory experiment to test whether males also demonstrate a preference for pseudo-virgin females (i.e. females that were not virgins but had not recently mated). In the laboratory, the sex and shell height of all snails was determined. Male snails were divided into two size categories; small (2.5–3.5 mm) and large (3.5–4.5 mm). For consistency, only females between 4.0 and 5.0 mm were used. Differently coloured Mar-Tech^{TM} paint was used to mark the shells of each of the three categories of snail (small male, large male and female). All snails were kept in individual meshed petri dishes for 4 days before the onset of the experiment so that any sperm present in the female reproductive system would no longer be present in the exterior portion of the reproductive tract. These females were considered pseudo-virgins.

For each trial, a female snail was placed with either a small or large male in a Petri dish with mesh lid. The dishes were kept in outdoor seawater tanks (as above) for 4 days. Each dish was observed five times daily and the presence or absence of copulation was recorded at each of these intervals. At the end of the 4 day period, the first male was removed and replaced by a male of the other size class. The second male with the original female was then observed five times a day for 4 days (large male first, small male second $n = 60$; small male first, large male second $n = 62$). We compared the mean number of copulations by large and small males with 'pseudo-virgin' and 'non-virgin' females using an ANOVA in SPSS version 12.

First mate precedence

To test for first male precedence, juvenile snails of shell length less than 2 mm were collected and raised in isolation until sexual maturity, as described above. For each trial, 16 glass bowls each with screen mesh attached as a lid were submerged in a large bin filled with seawater and cooled with ice. Within each bowl, a virgin female was placed with a single male. All bowls were observed four times per day for 4 days to check for the presence or absence of copulation. After 4 days, the first male was removed and replaced by a second male. Again, all bowls were checked four times per day for 4 days for the presence of copulations. The second male was then removed and replaced with a third male and observed for 4 days. This process was carried out for four temporally spaced trials. Given that copulations may be brief, this protocol did not record every copulation. However, the relative number of observed copulations from each treatment should be representative of the overall trend. An ANOVA with mean observed number of copulations, trial and male order as independent variables was carried out using SPSS version 11. Observations for Trial 1 and Trial 2 were taken at 6-h intervals. Observations for Trial 3 and Trial 4 were taken at 5-h intervals. Spreading out the time between observations reduced the chances that a single copulatory event would be recorded twice.

Fecundity of large vs. small females

All females used to test fecundity had previously been initiated in copulation at least twice before their use in this experiment. This was achieved by placing females in a glass bowl with males until a copulation event was initiated. Females were removed as soon as initiation occurred and not allowed to receive any male's full sperm investment. This was to ensure that all females were sexually receptive. Fifty females were classed as either small ($4.1 - 5$ mm shell length) or large ($5.8 - 7.1$ mm shell length). Each female was placed in a modified petri dish with two males (these males had been previously seen mating at least twice and were of intermediate size to the two female sizes: $5.1 - 5.6$ mm in shell length). Petri dishes were housed in indoor seawater tanks at AquaLab (mean temperature 10°C) for 7 days. At the end of this time, males were removed and females were allowed 45 days in which to lay eggs. Dishes were checked every $3 - 4$ days for the presence of egg masses. Once an egg mass was found it was collected and weighed and the number of eggs per mass was tabulated. Additionally, the number of egg masses laid by each female was tabulated. Initially, a fourth factor (number of offspring hatched) was to be used; however, due to indoor conditions rearing offspring was not possible.

RESULTS

Sexual dimorphism

Our study populations of *Littorina subrotundata* exhibited highly significant sexual dimorphism in both years tested (Fig. 1, Table 1). In each year, the females had a significantly greater shell height than the males ($P < 0.0001$ in 2002 and $P < 0.0001$ in 2003). However the patterns were complex, as there was also a significant difference in the mean shell heights between years.

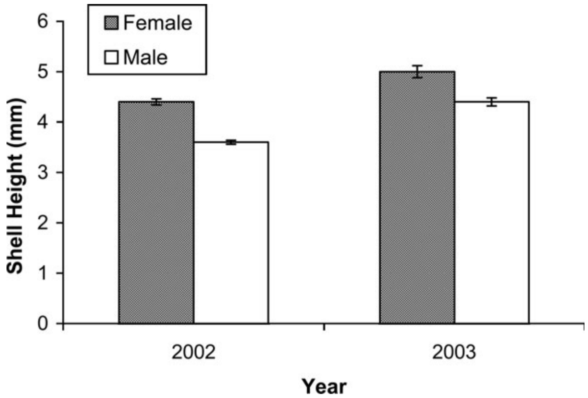

Figure 1. Mean shell height of male and female *L. subrotundata* in our study population from two successive years. Bars represent mean (±SE) shell height of males and females (In 2002 $n = 175$ males and 142 females; in 2003 $n = 91$ males and 106 females). See Table 1 for ANOVA.

Table 1. ANOVA comparing mean male and mean female shell height at Prasiola Point study site in 2002 and in 2003.

Factor	df	Mean square	F	P	Eta^2
Sex	1	3.08	99.04	<0.0001	0.163
Year	1	2.94	99.11	<0.0001	0.163
Sex*Year	1	0.18	1.40	0.237	0.003

$r^2 = 0.312$ (adjusted $r^2 = 0.308$).

Size-assortative mating

The majority of mating pairs collected in the field were heterosexual. However, there were a small number of cases where the passive partner was male (6.8%) and a small number of cases where the passive partner was the sympatric species *L. scutulata* (6.2%). These pairs were excluded from the analysis as they would confound statistical testing for size-assortative mating. Among the remaining snails we found a significant positive correlation ($r^2 = 0.033$, df = 145, $P = 0.029$) between the shell heights of the active partners and passive partners.

Growth rate

There was a low recapture rate of the snails in our growth rate experiment and a noticeably large amount of the paint flaking off over the course of the experiment. In general, we found that mean growth was highly variable between the quadrats in each site. Given vastly unequal sample sizes recaptured from each quadrat and unequal variance in growth rate, we had an unbalanced experimental design with little remaining statistical power. What we present here are analyses within each quadrat that suggest an increased growth rate for females relative to the males (Fig. 2). Ninety-five percent confidence interval limits suggested a significantly slower growth rate for males in two of our quadrats, and indicate a similar but non-significant trend in the other two quadrats.

Effect of male and female size

When both large and small males were simultaneously introduced to a female, there were a significantly higher number of copulations by large males compared to small males (Table 2; $\chi^2 = 8.3$, df = 1, $P = 0.004$).

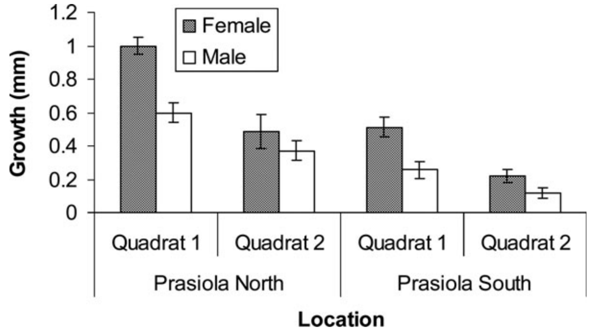

Figure 2. Mean (±95% confidence interval limits) growth observed in *L. subrotundata* among the four quadrats. From Prasiola North, $n =$ 49 females and 47 males in quadrat 1; $n = 18$ females and 20 males in quadrat 2. From Prasiola South, $n = 23$ females and 22 males in quadrat 1; $n = 12$ females and four males in quadrat 2.

Table 2. Single classification χ^2-test of the number of observed copulations by large and small males that were held in petri dishes containing an intermediate-sized female.

Male size	Observed copulations	P
Large (>3.5 mm)	34	0.004
Small (<3.0 mm)	14	

Table 3. Single classification χ^2-analysis of the total number of observed copulations by intermediate-sized males that were held in petri dishes containing a large and a small female.

Female size	Observed copulations	P
Large (>4.5 mm)	59	<0.0001
Small (<3.5 mm)	16	

When we introduced a large and a small female simultaneously to an intermediate size of male, we found that the male spent a significantly more time copulating with the larger female (Table 3; $\chi^2 = 24.7$, df = 1, $P < 0.0001$).

Male preference for virgin females

When a male was introduced simultaneously to a virgin and experienced female of the same body size, the male preferentially mated with the virgin female (Fig. 3; $\chi^2 = 5.12$, df = 1, $P = 0.024$). However, the mean time spent in copulation with a virgin (V) or experienced female (E) did not differ ($\mu_V =$ 15.0 min; $\mu_E = 21.5$ min; $n = 33$; t-test, df = 48, $P = 0.53$).

When given access to four females the order of preference was: large virgin, large experienced, small virgin and small experienced (Fig. 4). When virgins and experienced females were grouped together for data analysis, large females were selected more often than small females ($\chi^2 = 15.7$, df = 1, $P <$ 0.001). The mean time spent in copulation with a large virgin (LV), large experienced (LE), small virgin (SV) or small experienced (SE) female did not significantly differ for an intermediate sized male ($\mu_{LV} = 21.1$ min ($n = 14$), $\mu_{SV} =$ 16 min ($n = 2$); $\mu_{LE} = 8.3$ min ($n = 14$); $\mu_{SE} = 34$ ($n = 4$); $Z = -0.616$, $P = 0.54$).

In our experiment that tested male behaviour towards pseudo-virgin females, we found that the sexual activity of the males varied with their size (Fig. 5, Table 4). Small males copulated significantly more often than large males when introduced in the absence of competing males to pseudo-virgin

Figure 3. Number of times a male copulated first with either a virgin or 'experienced' female when presented simultaneously with both female types of identical body size.

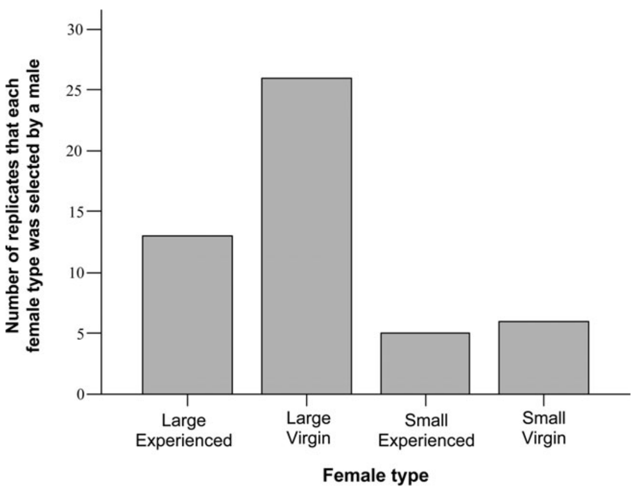

Figure 4. Number of times a male copulated first with either large virgin, large 'experienced', small virgin and small 'experienced' female when presented simultaneously with all four female types.

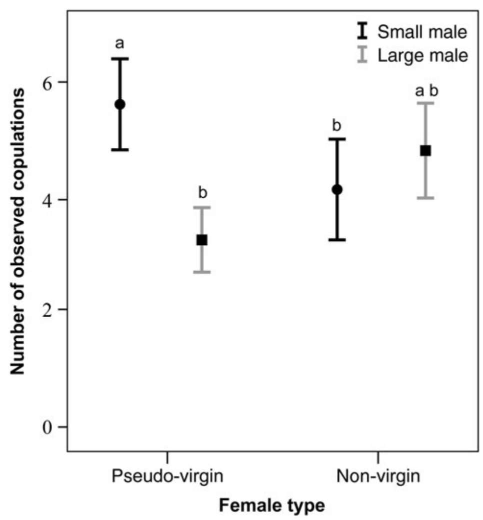

Figure 5. Mean number of observed copulations by large and small males when individually introduced to either a 'pseudo-virgin' or non-virgin female. Error bars indicate 95% confidence intervals around the mean.

Table 4. ANOVA comparing the mean number of observed copulations by large and small males when individually introduced to either a 'pseudo-virgin' or non-virgin female.

Source	Type III sum of squares	df	Mean square	*F*	*P*	*Eta*^{2}
Total	552.0	114	4.8			
Intercept	2,273.3	1	2,273.3	469.5	<0.001	0.805
Male size	23.7	1	23.7	4.9	0.029	0.041
Female type	0.3	1	0.3	0.05	0.817	0.000
Male size* Female type	67.7	1	67.7	14.0	<0.001	0.109
Error	2,938.0	118				

Table 5. ANOVA: dependent variable was number of copulations observed for each male that was sequentially introduced to an initially virgin female (Fig. 7).

Source	Type III sum of squares	df	Mean square	*F*	*P*	*Eta*^{2}
Total	2,824	187				
ORDER	38.12	2	19.06	3.994	0.02	0.044
TRIAL	12.94	3	4.31	0.904	0.441	0.015
ORDER*TRIAL	78.08	6	13.01	2.726	0.015	0.085
Error	835.31	175	4.77			

females. However, when introduced to females that had very recently copulated, there was no difference in the number of copulations by large and small males.

First mate precedence

When we sequentially introduced three different males to a single female, there was a slight trend for an increased number of copulations with the initial virgin female (Table 5, Fig. 6), followed by a decreased number of copulations when the second male was introduced, and then an increased number of copuations by the third male.

Fecundity of large vs. small females

When given equal opportunity to mate and lay eggs, large females were 16% more likely to lay eggs than small females however this difference was not significant (Fig. 7; $\chi^2 = 1.14$, df = 1, $P = 0.28$). No female laid more than one egg mass in this short-term experiment and there was no significant difference between the weight of the egg mass or the number of embryos per egg clutch and the size class of the female ($P = 0.33$).

DISCUSSION

Among littorinid gastropods, sexual dimorphism is extremely common (Reid, 1996). In addition, several studies (Chow, 1987; Erlandsson & Johannesson, 1994) have demonstrated differential growth rates between male and female littorinid snails. In all littorinid species where sexual dimorphism is

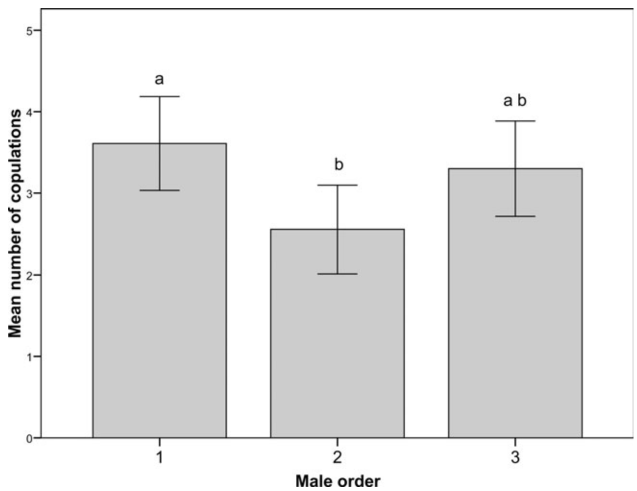

Figure 6. Mean number of copulations by each of three males sequentially introduced to a virgin female. Error bars indicate 95% confidence interval limits.

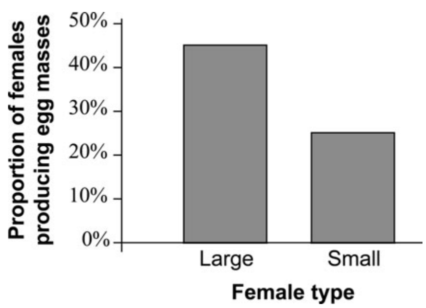

Figure 7. The proportion of large and small females producing egg masses after copulating for 7 days with three males simultaneously. Note: All females had previously mated twice before the 7 day period. Large females have a shell length as measured from the spire of shell to apex from 5.8 to 7.1 mm, small females a shell length of 4.1 − 5 mm.

present, it is females who attain greater shell heights. This is consistent with our results, which demonstrate a pronounced sexual size dimorphism in *Littorina subrotundata*, with females being significantly larger than males. That females are larger is not surprising, given that we found evidence that adult female *L. subrotundata* had a faster growth rate than adult males.

Littorina subrotundata exists in extremely wave-exposed habitats (Boulding & Van Alstyne, 1993; Reid, 1996). Egg allocation strategies of such marine species tend to differ from those of close relatives that reside in calmer waters. In high-wave areas, a smaller egg clutch is less likely to be dislodged from between the barnacles than a larger one. Consequently, female *L. subrotundata* lay small egg masses containing only 60 − 80 eggs throughout the breeding season (Boulding *et al.*, 2007). Smaller females may tend to produce only one mass per season, whereas larger females may produce multiple clutches using the same stored batch of sperm (E.G. Boulding, personal communication). If a male's ejaculate will be saved and used to fertilize more than one clutch, there will be selection for him to mate with a larger female as opposed to a smaller female that will lay only a single clutch.

We found that male *L. subrotundata* significantly increased their number of copulations with larger females. Other studies have shown that increased fecundity among larger females may cause them to be favoured by males (Reinhold, Kurtz & Engqvist, 2002). Our results are consistent with Erlandsson & Johannesson (1994) who observed male preference for larger females among *Littorina littorea*. This suggests that male *Littorina* do not indiscriminately distribute their sperm among as many females as possible; rather they strategically allocate sperm to females that will produce the greatest number of offspring. However, we were unable to quantify differences in female fecundity in this study because we had little success at maintaining egg masses in our indoor facilities.

Our study also found significant evidence that male *L. subrotundata* preferentially mate with virgin females. This is common among species where females store sperm; in these species, males are often able to detect the presence of rival male's ejaculates within the female's reproductive tract (Wedell *et al.*, 2002). Preferentially mating with virgin females becomes advantageous in systems where virgin females are infrequent and in species where the risk of sperm competition is high (Wedell *et al.*, 2002). Natural populations of *L. subrotundata* occur at high density, and locating an abundance of mating pairs is not difficult (Zahradnik, 2005). Therefore the frequency of virgins is likely to be very low in natural populations, which contributes to their desirability among males.

We found weak evidence for the presence of first-male precedence in this system. However, we did observe a slight trend for the second male introduced to a female to have a reduced number of copulations compared with the first male. This is consistent with the 'topping-off' strategy (Jones *et al.*, 2002), where the first male fills the female reproductive tract to near capacity. Topping-off is often found in combination with first-male precedence behaviour, wherein females become less receptive to additional male copulations when sperm is already present in their reproductive tract (Yasui, 1995). We also found a slight trend for the third male to increase copulations relative to the second male. We speculate that the increase in copulation of the third male may be related to the amount of time required for sperm migration within the female reproductive tract; in a number of trials, viable eggs masses were found 7 days into trials, during the second male's copulation. For the third male it is possible that the sperm had moved too far into the female's reproductive tract to be detected. This suggests that after the sperm has moved from the bursa copulatrix, a temporary sperm storage organ to the smaller seminal receptacle, the female may be preferred equally to a true virgin female (Zahradnik, 2005). Given that the frequency of true virgin females is low in natural populations, the presence of 'pseudo-virgin' females may help to maintain male choice for virgin females.

Indeed, our experiment that tested male behaviour when introduced to pseudo-virgin females suggests that these females are favoured over those that have recently copulated. Interestingly, this trend was only true among small males. We hypothesize that this may indicate different mating strategies depending on male size: large males produce more sperm and therefore have a lower risk of sperm competition than a smaller male whose sperm is more limited. It may be that small males then opt to increase their copulation effort with virgin (or pseudo-virgin) females in which the risk of sperm competition is less. More work is needed to test this hypothesis and determine whether males employ different mating strategies based on their size.

Previous studies on sexual selection for increased male size within *Littorina* species have come to differing conclusions. Erlandsson & Johannesson (1994) found large and small males had equal success at attaining mates among *L. littorea*. However, Gibson (1965) observed mating battles between males of *L. planaxis* (now *L. keenae*). Within our own trials, we observed aggressive encounters between conspecific males during copulation. In one of these events, a larger male physically displaced a smaller male and then proceeded to copulate with the receptive female. This suggests that there may be a competitive advantage to being a large male in this species. Experimentally, we found that large males engaged in a significantly higher number of copulations than smaller males when both were placed simultaneously with a female.

Size-assortative mating among *Littorina* has been observed in *L. planaxis* (Gibson, 1965), in some populations of *L. littorea* (Saur, 1990; Erlandsson & Johannesson, 1994) and in *L. saxatilis* (Rolan-Alvarez *et al*., 1999). We found slight but significant size-assortative mating among our study population of *L. subrotundata*. In other studies where assortative mating has been observed, there has also been only a small correlation between male and female shell heights.

The exception to the generally weak evidence for size assortative mating is among Spanish populations of *L. saxatilis*. In this species, strong size-assortative mating is responsible for maintaining two distinct ecotypes that exist at different elevations in the rocky intertidal zone. Rolan-Alvarez *et al*. (1999) demonstrated that among *L. saxatilis* assortative mating acts as a prezygotic mechanism that reinforces the two distinct morphs. Though our examination of *L. subrotundata* found a significant deviation from random mating and suggests that size-assortative mating is present, it is unclear whether this represents 'true' assortative mating, in that it does not appear to be acting as a mechanism to facilitate speciation. Likely the perceived assortative mating in this study results from male preference for large females combined with the fact that larger males can physically out-compete smaller males for access to large females; smaller males are then left with the less desirable smaller females.

In conclusion, we found significant sexual dimorphism in our wild study population. Laboratory studies showed that large males may have a competitive advantage over smaller males with respect to access to mates. Male *L. subrotundata* discriminated between females of different reproductive quality when distributing their sperm. They preferred to copulate with larger females and with virgin females rather than with those that had recently mated. This suggests that males can detect the presence of rival males' sperm in the female reproductive tract.

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