High Levels of Multiple Paternity in Littorina saxatilis: Hedging the Bets?

TUULI MÄKINEN, MARINA PANOVA, AND CARL ANDRÉ

From the Department of Marine Ecology, Tjärnö Marine Biological Laboratory, Göteborg University, S-452 96 Strömstad, Sweden (Mäkinen, Panova, and André).

Address correspondence to T. Mäkinen at the address above, or e-mail: tuuli.makinen@tmbl.gu.se.

Abstract

The mating system of a species can have great effects on its genetic structure and evolution. We studied the extent of multiple paternity in a gastropod with internal fertilization, the intertidal snail Littorina saxatilis. Paternal genotype reconstruction based on microsatellite markers was performed on the offspring of wild, naturally fertilized females from 2 populations. The numbers of males contributing to the offspring per female were among the highest detected in invertebrates so far, with the exception of social insects. No reproductive skew in favor of males that were genetically more distant from the females was detected, and the pattern of fertilization appeared random. The result fits a hypothesis of indiscriminate mating, with genetic bet hedging as the most likely explanation. Bet hedging may have evolved as a form of inbreeding avoidance, if the snails are not able to recognize relatives. However, nutritional benefits from sperm or sexual conflict with males are additional possibilities that remain to be assessed in this species. Whatever the causes, such high levels of multiple paternity are remarkable and are likely to have a large impact on population structure and dynamics in a species in which migration between populations is spurious.

Observations of multiple paternity in single clutches or broods have been commonplace since Darwin's time (Birkhead 2000). However, a classic paradigm in evolutionary biology stated that females benefit little from mating with more than one male (Bateman 1948). This view changed when modern genotyping methods revealed polyandry to be common in a wide variety of animal groups (Birkhead 2000; Jennions and Petrie 2000). Mating with different males usually entails costs but does not always bring obvious benefits for females. Various models have therefore been proposed to explain the occurrence of multiple mating, including “trading up” to a more desirable partner, or “bet hedging” to avoid committing all resources to reproduction with a possibly inferior partner (Jennions and Petrie 2000). Factors that may contribute to some males being genetically more desirable than others include sexually selected “good genes” (Pai and Yan 2002), inbreeding avoidance (Stockley et al. 1993), and genetic incompatibility from the interaction of selfish genetic elements (Zeh JA and Zeh DW 1996). On the other hand, evidence of the ubiquity of sexual conflict, often leading to increased costs of mating, is also emerging (Arnvist and Rowe 2005). Fertility assurance may be another reason why females in many species need to mate with multiple males. In theory, a male can contribute enough sperm in a single mating to last a female’s lifetime in most species; but in practice, sperm use is inefficient, and sperm stores may degrade over time. Sperm can be energetically costly to produce, so males may choose to invest less sperm in females of lower reproductive quality, or when there is a low chance of fertilization per mating (Bonduriansky 2001; Wedell et al. 2002).

The evidence for multiple mating is extensive but comes largely from vertebrates and insects (Birkhead 2000). Here, we present genetic evidence for high levels of multiple mating in the rough periwinkle (Littorina saxatilis), a marine prosobranch gastropod with internal fertilization. In this intertidal, rocky shore snail, reproduction is ovoviviparous, and dispersal capability is therefore expected to be low. Multiple paternity in the wild has previously been suggested in a related species, Littorina obtusata (Paterson et al. 2001), which is oviparous. In that study, females were mated in the laboratory to known males, but a genetic analysis revealed that some offspring derived from previous matings in the wild. Littorina saxatilis has been observed to be highly promiscuous: when a few males and females are placed in a dish with some moisture, several matings per hour can often be observed. In this study, we investigated the effect of promiscuity by estimating multiple paternity rates exclusively from natural matings. Males of L. saxatilis actively seek out mating partners by following the mucus trails of other snails (Johannesson K, Sundin A, Lindegård M, Jonsson P, Havenhand J, and Hollander J, in preparation). Matings between snails of different size or shell shape are interrupted more frequently,
possibly indicating assortative mating and partial reproductive isolation between ecotypes (Hollander et al. 2005). Such findings have been interpreted in terms of male mate choice (Johannesson K, Sundin A, Lindegarth M, Jonsson P, Havenhand J and Hollander J, in preparation). Although much more ubiquitous in nature, mate choice by females has not been studied in this species. Here, we examine one aspect of female choice: whether the pattern of sperm use by females indicates postcopulatory sexual selection. Such mechanisms are termed sperm competition if male driven and cryptic female choice if female driven (Birkhead and Pizzari 2002). Sometimes the distinction between them is artificial: providing opportunities for sperm competition within the female can be a form of cryptic female choice (Birkhead and Pizzari 2002). On the other hand, sperm competition can also have negative consequences for the female, such as the well-known example of seminal fluid in Drosophila having toxic effects on the female (Chapman et al. 1995).

Littorinid snails commonly store sperm for long periods. Sperm is received in the bursa copulatrix and later transferred to the seminal receptacle for storage (Buckland-Nicks et al. 1999). Storage longer than 1 year has been observed in L. saxatilis (Johannesson K, personal communication). This provides ample opportunity for multiple paternity and sperm competition. In this study, wild L. saxatilis females were maintained in the laboratory, where they produced offspring from stored sperm. The mothers and a sample of their offspring were genotyped for microsatellite DNA markers at 5 loci, and the pattern of paternity was reconstructed from their genotypes.

Materials and Methods

Collection and Incubation of Snails

In Sweden, 2 ecotypes of L. saxatilis occur: a larger and thicker shelled morph (δ) on sheltered shores and a smaller, thinner shelled morph (Ε) on wave-exposed shores (Janson 1982). To control for ecotype differences, L. saxatilis individuals were collected in July 2004 from one sheltered and one exposed shore separated by 100 m on the island Saltö (N58°53′, E11°10′) on the Swedish west coast. The snails were sexed and measured, and 9 females of each ecotype, with shell lengths of approximately 9 mm for the δ ecotype and 12 mm for the Ε ecotype (over the minimum size for sexual maturity; cf. Erlandsson and Rolán-Alvarez 1998), were chosen for incubation. They were placed individually in 11 jars with a constant flowthrough of seawater. The jars were covered with fine mesh to prevent escape and kept illuminated during daytime to promote the growth of microalgae for the snails to feed on. The families were incubated for a total of 2 and a half months, and at the end of the experiment, all snails were collected on the same date. One δ female died and 2 did not produce any offspring most likely owing to sterility caused by trematode infection, which is not uncommon in Swedish populations, particularly in the δ ecotype (Granovitch and Johannesson 2000). The remaining 15 females produced 23–302 juveniles, which corresponded well to the number of embryos recorded earlier in uninfected females of L. saxatilis, mean (range) δ ecotype 163 (1–526), Ε ecotype 64 (8–200); data from Janson (1985). Out of these females, we randomly chose 4 families from each population for parental analysis. These families contained 23, 24, 34, and 87 offspring for Ε ecotype and 34, 40, 69, and 71 offspring for δ ecotype, respectively. From each family, we sampled 23 offspring randomly. The largest diameter of each juvenile was measured to the nearest millimeter, and the snails were frozen and kept at −70 °C until DNA extraction.

Genetic Analysis

DNA was extracted with the cetyltrimethylammonium bromide protocol (Murray and Thompson 1980). For the juveniles, the whole animal was used for extraction, and about 1 mm³ of muscle tissue was taken from the females. The snails were genotyped at 5 microsatellite loci: Lsub62, Lsub32, Lsub8 (Tie et al. 2000), Lsx6, and Lx23 (Sokolov et al. 2002). The polymerase chain reaction (PCR) protocol was as follows: 2 μl DNA, 0.6 U Taq polymerase (TaKaRa Inc., Otsu, Japan), 1× supplied buffer, 1.5 mM MgCl2, 0.2 mM of each diethylnitrophenyl thiophosphate, 0.5 μM of fluorescence-labeled forward primer (Sigma Proligo AB, The Woodlands, TX) and 0.5 μM of reverse primer, and ddH2O to a total volume of 12 μl. The amplification was done in Eppendorf thermal cyclers in conditions described by the primers’ authors, with the exception of different annealing temperatures for Lsub62 (54 °C) and Lsub32 (55 °C). The DNA from some juveniles failed to amplify at all loci probably due to poor extraction quality, resulting in final sample sizes of 20–23 offspring per mother. The PCR products were run on a Beckman Coulter CEQ 8000 automatic sequencer using standard chemical kits (Beckman Coulter GmbH, Krefeld, Germany), and the results were analyzed with the CEQ Fragment Analysis software.

Paternity Inference and Statistical Analyses

Initially, we used the GERUD1.0 program (Jones 2001) for paternity inference, but because our data sets exceeded the maximum limit of fathers in this program, we repeated the analyses with the likelihood-based COLONY (Wang 2004). COLONY has no maximum limit of contributing parents and estimates the most probable number of fathers instead of the minimum. The number of alleles in a family at the most polymorphic locus, divided by 2, was calculated as another measure of the minimum number of fathers. All analyses other than estimating the number of fathers were done exclusively in COLONY. COLONY provided the most probable configuration of paternity, including assignments of every offspring to one of the estimated paternal genotypes. If the program provided 2 options, the most probable genotype was always chosen.

The genetic data were checked for occurrence of null alleles by mother–offspring comparison. Two of the loci (Lsx6 and Lsub8) were found to contain possible null alleles (when half of the offspring of an apparently
homozygous mother appeared to be homozygous for the other allele). These loci were excluded for the analyses on GERUD1.0. The results from COLONY were controlled by doing 2 separate estimations: first, by allowing the program to treat the null alleles as genotyping errors, and second, manually assigning a value for the suspected null alleles where a mother and her offspring did not match. The latter is a more conservative approach according to Jones and Ardren (2003). The subfamilies reconstructed with the 2 methods were identical, except for a few offspring in one family that were assigned to different fathers by the 2 methods. In those cases, the analysis with unspecified genotyping errors suggested several alternative paternal genotypes with approximately equal posterior probabilities, whereas the analysis with indicated null alleles still produced one paternal genotype with much higher posterior probabilities than others (data not shown). The latter estimate was chosen for the analysis. Therefore, it can be assumed that null alleles were dealt with adequately in our analysis.

The average numbers of contributing males in the 2 populations (7 and 8 for S and E, respectively) were not significantly different (t-test, \( P = 0.56 \)), and the data for the 2 ecotypes were pooled, resulting in a sample size of 8 families. After the families had been divided into subfamilies by likelihood, a truncated Poisson distribution (Cohen 1960) was fit to the distribution of offspring numbers per father. The expected distribution was then compared with the observed one by a chi-squared test. This tested the null hypothesis that the sampling of sperm at fertilization was random from a relatively large pool. Genetic similarity between the maternal and estimated paternal genotypes, expressed as the proportion of shared alleles (Bowcock et al. 1994), was calculated with Microsatellite analyzer (Dieringer and Schlötterer 2003) and correlated to the estimated fertilization success of the male (i.e., number of offspring). To determine if the sperm from different males was used sequentially or simultaneously (indicating sperm mixing), we used offspring size at the end of the experiment as a proxy for age, with the assumption that the time from fertilization to emergence and the growth rate of juveniles were relatively constant. Nested analysis of variance (ANOVA) was used to test whether the offspring of different males by the same female were significantly different in size. The males that had less than 4 offspring assigned to them were excluded from this test.

### Results

The total numbers of alleles detected per locus were 8 in Lsub62, 11 in Lsub32, 18 in Lsub8, 15 in Lsax6, and 27 in Lx23 (see also Mäkinen et al. forthcoming). The paternal allele count, divided by 2, in the most polymorphic locus (Lx23) is a conservative estimate of the minimum number of fathers and resulted in estimates of 3–5 sires for our samples of up to 23 offspring. Minimum numbers of fathers, estimated from all loci in GERUD1.0, ranged from 3 to 8 fathers, 8 being the maximum limit of the program. The estimates of most likely numbers of fathers from COLONY ranged from 4 to 10 contributing males per female with an average of 7.6 (Figure 1). The estimates of contributing fathers by different methods matched relatively well, considering that a single locus will result in lower estimates than multiple loci, and the first 2 methods estimate only the minimum number of fathers, whereas the most probable number of sires given by COLONY can be higher than the minimum (Figure 1). Further results refer only to those from COLONY, and males or sires refer to male genotypes reconstructed by the same program.

Female fertility (total offspring number) was not correlated with the number of sires (Figure 1; \( R^2 = 0.24, P = 0.22 \)).

The division of offspring among different males is presented in Figure 2. The mean size of offspring of different males within the same females was not significantly different (nested ANOVA; between subgroups comparison: degrees of freedom [df] = [16, 139], \( F = 1.04, P = 0.42 \)), suggesting either that the sperm from different fathers was mixed during fertilization or that the size of juvenile snails is not a good indicator of their age and/or fertilization time. The distribution of paternal offspring numbers was not significantly different from an expected distribution resulting from a random fertilization process, approximated by a truncated Poisson distribution (Figure 3; \( df = 5, \chi^2 = 11.1, P = 0.82 \)), indicating that there was no apparent reproductive skew. Only in one family had a single male sired more than half of the offspring (F1; 57%). Genetic similarity between the estimated male genotypes and females, measured by the proportion of shared alleles between them, had no effect on male fertilization success (Figure 4; \( P > 0.05 \)).

---

**Figure 1.** The numbers of sires in 8 half-sib families of *Littorina saxatilis* estimated by 3 different methods: allele count: an estimation of minimum number of sires as the number of paternal alleles at the most polymorphic locus (Lx23) divided by 2, GERUD1.0 software: estimation of minimum number of sires based on multilocus genotypes (maximum limit 8), and COLONY software: estimation of the most probable number of sires based on multilocus genotypes (no maximum limit). N: number of genotyped offspring/total number of offspring per female.
Discussion

The number of sires per brood was among the highest detected in invertebrate species so far, with the exception of social insects: the army ant and the honeybee (Table 1; species that to our knowledge have relatively high levels of multiple paternity in their respective groups were chosen for this comparison). Moreover, our estimate is likely to be a conservative one because only a subset of offspring was sampled, and many sires were estimated to have only one offspring in the sample. Such a high level of multiple paternity suggests a high rate of multiple mating in the species and is in need of evolutionary explanation because mating rates are usually assumed to be the result of balancing costs and benefits of mating (Arnqvist and Rowe 2005).

Mating can be costly for females in many ways, for example, by taking time and energy or creating a higher risk of predation (Watson et al. 1998). In *L. saxatilis*, the male mounts the shell of the female during mating, which makes the pair more susceptible to being dislodged by waves (Erlandsson and Rolán-Alvarez 1998; Johannesson K, Sundin A, Lindegarth M, Jonsson P, Havenhand J and Hollander J, in preparation). Multiple mating may also deplete energy resources through reduced time for feeding, which has been suggested to be the most generally occurring cost of mating (Daly 1978). *Littorina saxatilis* males follow the slime tracks of other snails but unlike *L. littorea*, a species with pelagic larvae, seem unable to use chemical cues from the tracks to differentiate between males and females (Johannesson K, Sundin A, Lindegarth M, Jonsson P, Havenhand J and Hollander J, in preparation). It has been suggested that the loss of sexual identification in *L. saxatilis* is a female adaptation: whereas females of a species with a shorter reproductive season, like *L. littorea*, would advertise their identity by pheromones, in a species with continuous reproduction like *L. saxatilis*, it pays to avoid excess matings (Erlandsson and Rolán-Alvarez 1998). In many species, suboptimal matings result from male coercion, for example, in marine turtles (Lee and Hays 2004), damselflies (van Gossum et al. 2001), and dung flies (Mühlhäuser and Blanckenhorn 2002). Such behavior, where multiple mating may not be beneficial to the female, but the cost of resistance is higher than the cost of mating, has been termed convenience polyandry (Cordero and Andres 2002). In the face of intense sexual conflict, females more often than not evolve some countermeasures (Arnqvist and Rowe 2005). Sometimes *L. saxatilis* females seem to be “wrestling” with the males sitting on top of their shells, with the male possibly using its shell as a lever to lift the female’s shell to enable copulation (Hollander J, personal communication). Similar mating behavior is seen in the hermaphrodite snail *Physa*: a recipient snail sometimes tries to reject a sperm donor by swinging the shell (DeWitt 1996). This behavior usually makes the sperm donor dismount and does not involve a risk of injury or death for the partners (DeWitt
is unlikely to explain multiple mating in Littorina saxatilis, compared with other studied invertebrate species with internal fertilization.

The selected species showed, to our knowledge, relatively high levels of multiple paternity in natural or nearly natural mating situations in their respective groups. Number of males contributing to a single female’s offspring in Littorina saxatilis, compared with other studied invertebrate species with internal fertilization.

<table>
<thead>
<tr>
<th>Species</th>
<th>N(off)</th>
<th>N(fem)</th>
<th>Males</th>
<th>Offspr./male</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eciton burchelli (army ant)</td>
<td>20–60</td>
<td>6</td>
<td>15.5 ± 5.8</td>
<td>1.7</td>
<td>Allele count</td>
<td>Denny et al. (2004)</td>
</tr>
<tr>
<td>Apis dorsata (honeybee)</td>
<td>111–288</td>
<td>13</td>
<td>80.1 ± 3.6</td>
<td>2.6</td>
<td>Allele count</td>
<td>Wattanachaiycharoen et al. (2003)</td>
</tr>
<tr>
<td>Drosophila mojavensis (fruit fly)</td>
<td>15–146</td>
<td>20</td>
<td>3.1 ± 0.3</td>
<td>12.5</td>
<td>KINSHIP</td>
<td>Good et al. (2006)</td>
</tr>
<tr>
<td>Drosophila melanogaster (fruit fly)</td>
<td>89</td>
<td>4</td>
<td>5.0 ± 0.8</td>
<td>17.8</td>
<td>Allele count</td>
<td>Imhof et al. (1998)</td>
</tr>
<tr>
<td>Gryllus binaculatus (cricket)</td>
<td>16</td>
<td>7</td>
<td>3.8 ± 0.9</td>
<td>4.2</td>
<td>PARENTAGE</td>
<td>Breman and Tregenza (2005)</td>
</tr>
<tr>
<td>Orconectes placidus (crayfish)</td>
<td>20–236</td>
<td>15</td>
<td>2 ± 1.1</td>
<td>30</td>
<td>HAPLOTYPES</td>
<td>Walker et al. (2002)</td>
</tr>
<tr>
<td>Loligo pealeii (squid)</td>
<td>34–58</td>
<td>5</td>
<td>3 ± 1.0</td>
<td>14.3</td>
<td>Allele count</td>
<td>Buresch et al. (2001)</td>
</tr>
<tr>
<td>Littorina obtusata (marine snail)</td>
<td>40–46</td>
<td>3</td>
<td>5 ± 1.0</td>
<td>8.6</td>
<td>CERVUS</td>
<td>Paterson et al. (2001)</td>
</tr>
<tr>
<td>L. saxatilis (marine snail)</td>
<td>20–23</td>
<td>8</td>
<td>7.6 ± 2.1</td>
<td>2.9</td>
<td>COLONY</td>
<td>Present study</td>
</tr>
</tbody>
</table>

The pattern of more males gaining paternity than can reasonably be explained by females looking for superior males was also reported in Lake Malawi cichlids (Kellogg et al. 1995), tree swallows (Lifjeld et al. 1993), and insects, (Zeh et al. 1997) among others. One plausible explanation for this is bet hedging to avoid inbreeding (Stockley et al. 1993; Yasui 2001). Littorina saxatilis, unlike many marine invertebrates, lacks free-swimming pelagic larvae, and both juveniles and adults have limited mobility (Janson 1982). Although the species frequently occurs at high population densities, they are often concentrated in cracks and crevices on rocky shores, with limited movement between them. This increases the risk of inbreeding, and limited population size is also a requirement for the evolution of bet hedging (Yasui 2001). Although purging of deleterious alleles may reduce inbreeding depression in populations with continuous inbreeding, the occurrence of deformed embryos at high rates in some populations, even in unpolluted areas (Janson 1985), suggests that inbreeding depression is common in L. saxatilis. Several intriguing observations of benefits of outbreeding through mixed paternity have recently been made in many animal groups including pythons (Madsen et al. 2005), crickets (Tregenza and Wedell 2002), live-bearing pseudoscorpions (Zeh JA and Zeh DW 2006), and salmon (Garant et al. 2005).

Another suggested benefit of bet hedging is that higher genetic diversity in the offspring increases the possibility that some of them will survive in a heterogeneous or fluctuating environment (Watson 1991; Yasui 1998). However, simulations show that multiple mating solely for genetic diversity can only evolve in very limited conditions, for example, when full sibs compete more intensely than half sibs and the environment is fine grained so that individuals may choose between microhabitats (Yasui 1998), or when the environment fluctuates unpredictably (Yasui 2001). The intertidal zone may be considered such a fine-grained environment for L. saxatilis juveniles, but there is no evidence for differential resource use between half sibs.

A high level of multiple paternity combined with sperm storage may be beneficial in animals with low dispersal.
ability and a fluctuating population size because it provides a reservoir of genetic variability. Populations of *L. saxatilis* on the Swedish west coast went through a severe bottleneck in 1988, when most of the snail populations were wiped out by a toxic algal bloom. During the following few years, small islands were rapidly repopulated, probably by snails occasionally rafting with algae (Johannesson K and Johannesson B 1995). These reestablished populations did not exhibit much loss of genetic variation either in allozymes (Janson 1987) or in microsatellite loci (Panova M, unpublished data). Moreover, the species has managed to colonize remote locations, such as the island of Rockall in the Atlantic, despite its limited mobility and lack of pelagic larvae (Johannesson 1988). Colonizing success of this species is not surprising, though, taking into account the high fertility of females (Janson 1985), their ability to store sperm for a year or more (Johannesson K, Sundin A, Lindegarth M, Jonsson P, Havenhand J and Hollander J, in preparation), and that a mated female might carry genetic material from up to 10 males (present data). The genetic effects of an introduction of a single multiply mated female may be compared with a single queen establishing a colony in some ant species. Interestingly, ant species where a single queen starts a new colony are polyandrous, whereas in other ants, having several foundresses per colony, queens are usually monogamous (Keller and Reeve 1994). Multiple mating and sperm storage were also suggested to have evolved as adaptations against genetic deterioration in the land snails *Cepaea nemoralis* and *Helix aspersa*. As *L. saxatilis*, these species have limited dispersal abilities and live in environments with frequent stochastic disturbances (Murray 1964; Arnaud and Laval 2004).

In conclusion, multiple paternity is likely to be of great importance for recolonization and population expansion in *L. saxatilis*. Genetic bet hedging may evolve in response to a fluctuating environment or a high risk of inbreeding in small populations; both are plausible explanations for multiple mating with lack of preference for a particular male genotype in *L. saxatilis* females. However, the roles of direct benefits of sperm, precopulatory female choice, and sexual conflict remain to be experimentally assessed in this species.

### Funding

European commission project, European Marine Genetic Biodiversity (EVK3-CT-2001-00048; www.eumar.tmbld.gu.se); Collanders stiftelse.

### Acknowledgments

Kerstin Johannesson’s and Johan Hollander’s knowledge on the mating behavior and other peculiarities of *Littorina saxatilis* was invaluable to the planning of this study. Christer Wiklund, Kerstin Johannesson, Elizabeth Boulding, and 3 anonymous reviewers provided comments on earlier drafts.

### References


Received January 30, 2007

Accepted August 30, 2007

Corresponding Editor: Rob DeSalle