Can sexual selection and disassortative mating contribute to the maintenance of a shell color polymorphism in an intertidal marine snail?

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Abstract Littorina fabalis is an intertidal snail commonly living on the brown algae Fucus vesiculosus and showing frequent shell-color polymorphisms in the wild. The evolutionary mechanism underlying this polymorphism is currently unknown. Shell color variation was studied in mated and non-mated specimens of this species from different microareas in one locality from NW Spain, in order to estimate sexual selection and assortative mating that may (still) be operating in this population. The analyses across microareas allowed us to investigate frequency-dependent selection and assortative mating components, mechanisms that could maintain the polymorphism. The presence of shell scars caused by crab attacks, an environmental variable not related with sexual selection or assortative mating, was used as experimental control. This study provides new evidence of significant disassortative mating and some degree of sexual selection against some shell colors, supporting the results found 21 years ago in a similar study, i.e. in the same species and locality. The similarity of these estimates during the studied period suggests that this experimental approach is consistent and valid to be extended to other populations and organisms. In addition, sexual selection and assortative mating estimates did not change across microareas differing in shell color frequencies, suggesting than the polymorphism can not be maintained by a frequency-dependent (sexual selection-based) mechanism. Our main hypothesis is that negative assortative mating could contribute to the maintenance of the polymorphism, perhaps by males showing distinct female color preferences when searching for mates [Current Zoology 58 (3): 463–474, 2012].

Keywords Fitness estimate, Mate choice, Mate propensity, Negative assortative mating, Sexual selection, Frequency-dependent selection

Mating behavior, one of the key life history traits (Andersson, 1994; Futuyma, 2009), may cause two different evolutionary consequences: (1) sexual selection and (2) assortative (or disassortative) mating (Merrel, 1950; Lewontin et al., 1968; Rolán-Alvarez and Caballero, 2000). Sexual selection, referring to the component of natural selection, occurs when the genotype proportions of mature males and females are different from the actual genotype or mating proportions among successful mates (Hedrick 2005). It can be estimated by comparing mated and unmated specimens for a specific trait either in laboratory (Merrel, 1950) or natural conditions (see Rolán-Alvarez et al., 1995a; Rolán-Alvarez and Enderahl, 1996). Sexual selection, referred to the component of natural selection occurring during mating, has been considered one of the most important selective components capable of maintaining natural polymorphisms by different mechanisms (e.g. heterosis, frequency dependence; see Endler, 1977; Andersson, 1994; Hunt et al., 2008; Shuster, 2009; Futuyma, 2009). On the other hand, assortative mating is estimated for a particular trait by quantifying the deviations from random mating across mates (Merrel, 1950), but not in the same form than sexual selection does. Note that this definition does not account for unmated specimens, while the sexual selection does. The tendency for mating assortatively between similar morphs, ecotypes or incipient species (known as sexual isolation in this context) has been considered one of the most important mechanisms of reproductive isolation in Drosophila as well as in other species (Coyne and Orr, 2004; Ritchie, 2007), and is therefore a key component during speciation.

Sexual selection and assortative mating effects can be independently estimated from multiple choice experiments (Merrel, 1950; Lewontin et al., 1968; Rolán-Alvarez and Caballero, 2000), although they do
not produce identical evolutionary outcomes. Moreover, there are two main biological mechanisms that could cause both sexual selection and assortative mating effects: (1) mate propensity (the intrinsic tendency of one sex type to mate more than the other types) and (2) mate choice (any tendency of one sex type for preferentially choosing another sex type when looking for mates; see Rolán-Alvarez and Caballero, 2000). The relationship between the biological mechanisms (mate propensity and mate choice) and their evolutionary effects (sexual selection, assortative mating) is, however, rather complex. In fact, it has been shown theoretically that it is not possible to disentangle the estimation of any of these two mechanisms from any single multiple-choice experiment (Marin, 1991). Moreover, theoretical models show that these two mechanisms can produce sexual selection and assortative mating effects separately or combined (see Pérez-Figueroa et al., 2005). Therefore, as experimental strategy, it has been suggested to focus first on the evolutionary effects (sexual selection and assortative mating) and try to infer a posteriori the true biological mechanisms (mate propensity or mate choice; see Rolán-Alvarez and Caballero, 2000). These subtle relationships between mechanisms and evolutionary effects could partially explain the difficulty of developing explicit theoretical models to investigate the relationship between sexual selection and assortative mating (see as an example Ritchie, 2007; Servedio et al., 2011).

Laboratory mating experiments allow the control of some factors contributing to mating decisions, although it might be far from what is really occurring in the wild. There are few but notorious examples in which certain mating patterns observed in laboratory are apparently contradictory to those observed in the wild. Coyne et al. (2005) studied the occurrence of sexual isolation (assortative mating) in a laboratory experiment between two Drosophila species that naturally overlap in São Tomé Island, D. santomea and D. yakuba. They observed that sexual isolation was mainly caused by discrimination against D. yakuba males by D. santomea females (i.e. mate choice). However, in another experiment, most wild hybrids captured in São Tomé resulted from the D. yakuba male and D. santomea female cross (Llopart et al., 2005). These contrasting results suggest that addressing evolutionary questions about the previously mentioned mechanisms using data from the wild is absolutely necessary, although a major drawback is finding enough mating pairs to get enough statistical power to infer the evolutionary effects or even their biological mechanisms. A second related problem is knowing whether any snapshot estimate obtained from the wild (or in laboratory) is self informative or has lost its predictive value due to stochastic processes and natural biases. A strategy for dealing with these alternatives may be to replicate in time the same study in order to confirm (or not) previous patterns.

Littorinids are marine gastropods that commonly live in intertidal areas showing separated sexes and internal fertilization (Reid, 1996). They comprise several species with striking polymorphisms and it is often possible to observe and capture mating pairs directly in the wild. The flat periwinkle Littorina fabalis (formerly L. mariae) shows extreme shell color polymorphisms varying across countries or even from one rocky shore to another (Reid, 1996). This species lives typically on the brown seaweed Fucus vesiculosus and shows a seasonal reproductive behavior. Most matings occur during early summer, at least in certain geographic areas, facilitating the finding of many mating pairs (Rolán-Alvarez and Ekendahl, 1996). For these reasons, this species is an ideal candidate for estimating sexual selection and assortative mating components directly from wild populations.

There is a lack of consensus about how to explain the observed shell color polymorphism in this and other littorinid species, although two main mechanisms have been traditionally proposed. First, the polymorphism may be the result of neutral variation, and therefore the pattern of shell colors we currently observe is the result of a transitory phase during fixation of neutral alleles (Cook, 1992). Second, it may exist as a stable polymorphism maintained by natural selection (on the trait itself or on correlated traits), due to overdominance, frequency-dependent, spatial or even temporal heterogeneity mechanisms (Cook, 1986; O’Donald and Maje rus, 1988; Phifer-Rixey et al., 2008). The alternative hypothesis of phenotypic plasticity could be discarded on the grounds that this trait usually follows a simple mendelian inheritance in most species (Murray and Clarke, 1966; Murray, 1975; Innes and Haley, 1977). It has already been shown that one or two loci are directly involved in the shell color of L. fabalis (Reimchen, 1989), as well as in color patterns (bands, spots) in its sibling species, L. obtusata (Kozmynskii et al., 2010; Kozmynskii, 2011).

The main objective of this study was to analyze the possible effects of sexual selection and assortative mating that might be operating in a wild population of L. fabalis on the northwest coast of Spain. This population has been previously studied for the same purpose 21
years ago (Rolán-Alvarez and Ekendahl, 1996). In that study, we found evidence of strong (negative) assortative mating for shell colors and some degree of sexual disadvantage for yellow shells. In the present work we included a new sampling and analysis (dataset 2011 henceforth), as well as a re-analysis from Rolán-Alvarez and Ekendahl (1996) study (dataset 1990 henceforth). This design allowed us to investigate whether the strong negative assortative mating detected in the past is still operating nowadays and can be considered as a potential mechanism for maintaining the shell color polymorphism in this population. We also investigated if sexual selection could be frequency-dependent and discussed to what extent this mechanism could contribute to the polymorphism maintenance. We found that sexual selection and (negative) assortative mating did not change between the two studies, suggesting that our estimates are robust and representative of the species. In addition, we found that sexual selection was frequency-independent, being disassortative mating the only factor contributing to the maintenance of the polymorphism in the long term. The biological details of the mechanism are unknown, although we hypothesize that males may show particular preferences for some female colors when searching for mates.

1 Material and Methods

1.1 Sample collection and processing

Copulates of Littorina fabalis as well as unmated specimens were collected on July 2011 over Fucus vesiculosus algae in the semi-exposed rocky shore of Abelleira at the Muros-Noya Ría (NW Spain; 42° 48' 0.30"N and 9° 1' 14.87"W). The sampling was carried out at the same shore level and during the same season than the previous study, a period in which most matings occur (Rolán-Alvarez and Ekendahl, 1996). A pair was considered a mating pair when the male had inserted the penis into the female reproductive tract or was just removing it from that position (see Fig. S1 from online complementary material). In this species, mating occurs during 1–2 hours or even more, since males show mate-guarding behavior (Rolán-Alvarez et al., 1995a). For each mating pair we captured the 2–4 closest unmated specimens within a circular microarea of 25 cm diameter. Mated and unmated specimens within microareas were also used as data for analyses of color frequencies (see below). Specimens were dissected in the laboratory to exclude the sibling species L. obtusata (following Reid, 1996) and to determine the sex (male, female, immature; Rolán-Alvarez et al., 1995a), an easy task in males but more difficult in females. Immature, as well as L. obtusata specimens were discarded in males before the analyses (overall including both exclusions 2.4%), although a small proportion of wrongly assigned specimens could remain within females.

1.2 Shell color assignment and codification

We made the dissections separating soft parts from shells for species and sex classification and codifying the shells using a blind design. We then determined the shell color using a printed pattern according to Figure S2. Shell colors were primarily scored according to 8 phenotypic classes (thereafter *phenotypic nomenclature*) described in Rolán-Alvarez and Ekendahl (1996): brown (Brw), olive (Oli), yellow (Yel), orange (Ora), and their corresponding shell banded colors (showing two parallel and longitudinal bands over the former colors): Brw2, Oli2, Yel2 and Ora2.

Additionally, we used two different color-associated categories based on published information about the color trait inheritance in this and related species (Reimchen, 1989; Kozminskii et al., 2010; Kozminskii, 2011; thereafter *genotypic nomenclature*): color background (Brw, Oli, and Yel; Ora was excluded due its low frequency) and color pattern (Unbanded and Banded). Note that for each new category we pooled previous color classes according to the phenotypic nomenclature (see above), e.g. background Brown is the class formed by Brw and Brw2 phenotypes and so on (see Figure S2).

Finally, we made specific analyses using the two main background color frequencies (Brown and Yellow; *genotypic nomenclature*), grouping microareas depending on their color frequencies: Yellow microareas (yellow frequency higher than 60%), Brown microareas (brown frequency higher than 60%) and mixed microareas (intermediate frequencies for both background colors).

Intertidal populations of L. fabalis from NW Spain are heavily predated by crabs (e.g. by Carcinus maenas; but see Reid, 1996; Ekendahl, 1998). Marine snails may survive crab attacks, but a characteristic shell scar is often produced (see Fig. S2 from online complementary material). We included a second variable (qualitative) in our study, the presence/absence of a shell scar, which was used as a control. The rationale for including such control variable is that we do not expect any *a priori* contribution of shell scars (accidental) to mating decisions. Therefore, we only considered significant results regarding sexual selection and assortative mating on shell colors as far as this effect was not simultaneously found in shell scars. These analyses could only be ap-
plied on the 2011 dataset, as this trait had not been recorded in the previous study.

1.3 Sexual selection and assortative mating analyses

Analysis of sexual selection and assortative mating were done according to Rolán-Alvarez et al. (1995a) and Rolán-Alvarez and Ekendahl (1996), but also including the new estimators described in Rolán-Alvarez and Caballero (2000). These analyses assume that we only have access to a snapshot of both processes and therefore the estimates are representative of what is happening in the species during the mating season. The estimators and the statistical tests have been explained and discussed in detail in former studies (see Carvajal-Rodríguez and Rolán-Alvarez, 2006). Consequently, here we will just mention them briefly.

The sexual selection effects on colors and shell scars were estimated by the cross product estimator (values range from 0 to 1, with 1 being the class with the highest sexual advantage), which compares mated and unmated classes. This estimator was developed for frequency data and is unbiased for differences in data-frequency itself, being therefore especially useful for estimating frequency-dependent selection components (Knoppien, 1985). Sexual selection effects for the whole dataset (including all values of the trait; i.e. all colors) were evaluated by a likelihood G test (Gsel), while their significance on each trait (i.e. each color separately) was estimated by bootstrapping their cross product estimator values (bootstrapping both pre-mating and mating frequencies).

Assortative mating was estimated by the 1pti coefficient (values range from -1 to 1; -1 corresponding to the maximum negative assortative mating, 0 to random mating and 1 to maximum positive assortative mating; see Rolán-Alvarez and Caballero, 2000).

Finally, the total mating preferences (the tendency of each pair to be more or less represented than expected by chance during mating, compared to unmated frequencies) were estimated by the pair total index (PTI coefficient; values ranging between zero and infinite, with 1 being the absence of mating preferences and values below or above 1 being cases in which the mating pair was less or more frequently observed than expected by chance), as described in Rolán-Alvarez and Caballero (2000). These PTI coefficients account for both sexual selection and assortative mating effects (as well as all their putative causes: mate propensity and mate choice) detected in the population, but for each pair combination separately.

All estimators and corresponding tests were obtained implementing the software JMATING (Carvajal-Rodríguez and Rolán-Alvarez, 2006) and Poptools (Hood, 2010).

2 Results

Phenotypic and genotypic color frequencies in the mated and unmated classes from the 1996 and 2011 datasets as well as shell scars occurrence for the 2011 dataset are recorded in the Supplementary Material section. In the 1990 dataset, phenotypic color frequencies differed significantly between mated and non-mated males (G = 14.2, df = 8, P = 0.028) and females (G = 35.3, df = 4, P < 0.001), although in the 2011 dataset, mated and non-mated males phenotypic color frequencies did not differ significantly in any sex (males G = 11.4, df = 6, P = 0.076; females G = 9.4, df = 7, P = 0.222).

A clearer trend was detected when using the background color (genotypic nomenclature; pooling for each color banded and unbanded specimens; see Table 1). However, in this case sexual selection primarily acted against yellow snails of both sexes and datasets. The fact that most sexual selection estimates were not significant in the 2011 dataset could be due to a lower number of mating pairs sampled. The results also showed a stronger effect in females than males, suggesting the action of female-female competition or male choice mechanisms for explaining the observed patterns (see below). On the other hand, there were no significant differences in sexual selection between banded and unbanded specimens for any dataset or sex. All these results pointed to individuals with certain shell colors (yellow and brown) having some sexual disadvantage when competing for mates, a process that, acting alone, would deplete the population diversity for color genes rather quickly.

Additionally in the 2011 dataset, a control using shell scars was performed. Phenotypic shell color frequencies did not differ between snails with and without shell scars (G = 6.6, df = 7, P = 0.475). Presence or absence of shell scars seemed to be not influenced by sexual selection too (Gsel = 0.3, df = 2, P = 0.839), both suggesting that sexual selection estimates might not be biased.

We applied the assortative mating 1pti coefficient to check deviations from random mating in both the 1990 and 2011 datasets (Table 2). The coefficients were negative but not significant using the phenotypic nomenclature, but they were negative and moderate to highly significant using the background color definition in the
Table 1  Sexual selection estimates for each sex for the different background shell colors and banded patterns (genotypic nomenclature) in the 1990 and 2011 datasets, as well as in both datasets pooled

| Year | Group | N | Brw | Yel | Oli | G_{sel} | Brw | Yel | Oli | G_{sel} |
|------|-------|---|-----|-----|-----|--------|-----|-----|-----|--------|
|      |       |   |     |     |     |        |     |     |     |        |
| 1990*| Pairs | 237| 0.76*| 0.78**| 1 | 58.6***| 0.88| 1 | 0.7 |
|      | Males | 174 | 0.08***| 0.06***| 1 |        | 0.86| 1 |        |
|      | Females | 258 | 0.08***| 0.06***| 1 |        | 0.86| 1 |        |
| 2011 | Males | 155 | 0.67 | 0.68 | 1 | 11.9* | 0.52| 1 | 2.7 |
|      | Females | 206 | 0.45 | 0.24* | 1 | 0.92 | 1 |
|      | Pairs | 96 |     |     |     |        |     |     |     |        |
|      | Pooled | 333 | 0.55* | 0.52* | 1 | 49.7***| 0.71| 1 | 3.3 |
|      | Males | 329 |     |     |     |        |     |     |     |        |
|      | Females | 464 | 0.18***| 0.13***| 1 |        | 0.87| 1 |        |

* Data re-interpreted (using a different color nomenclature) from Rolán-Alvarez and Ekendahl (1996).  
\* P < 0.10; \* P < 0.05; \* P < 0.01; \* P < 0.001.

Note that significant indices indicate that sexual selection is acting for some shell colors in the population (values of 1 means the reference color with maximum sexual advantage and values below 1 means colors with some sexual disadvantage). G_{sel} represents a maximum likelihood G test comparing mated and unmated color frequencies in each sex and data, and including male and female data simultaneously.

Table 2  Assortative mating statistic (I_{PSI}) estimated for different datasets and shell color nomenclatures (both phenotypic and genotypic nomenclatures) and presence/absence of shell scars

| Year | Shell color | Phenotypic | Genotypic | Scars |
|------|-------------|------------|-----------|-------|
|      |             | Brw/Yel/Oli| Brw/Yel   |       |
| 1990*|              |            |           |       |
|      |              | -0.36      | -0.33***  | -0.15*|
|      |              | (0.256)    | (0.047)   | (0.070)|
| 2011 |              | -0.32      | -0.26**   | -0.06 |
|      |              | (0.564)    | (0.084)   | (0.121)|
| Pooled |            | -0.31      | -0.30***  | -0.13*|
|      |              | (0.489)    | (0.041)   | (0.060)|

* Data re-interpreted (using a different color nomenclature) from Rolán-Alvarez and Ekendahl (1996).  
\* P < 0.10; \* P < 0.05; \* P < 0.01; \* P < 0.001.

Negative values are expected for negative assortative (disassortative) mating and zero values for random mating. The bootstrapped standard errors of the statistics are shown in parentheses.

1990 and 2011 datasets and both pooled (genotypic nomenclature, Table 2). The absence of significance in the I_{PSI} coefficients for the phenotypic classes is explained because several pair combinations present at rather low sample sizes, increasing the estimated variance during bootstrapping (see standard deviations of I_{PSI} from Table 2). To eliminate the possibility that all former results were mainly caused by a rare color class (olive specimens), we repeated the estimation using exclusively the two more frequent background color classes (Brown and Yellow), which produced a similar tendency, with weaker disassortative mating significant in the 1990 and pooled datasets. This would allow us to use the PTI estimates in a simpler context (see below). Color pattern (banded and unbanded) showed random mating (data not shown).

We accomplished some further analysis on the dataset from 2011 using exclusively the two main back-
ground color classes (Table 3). This included the whole sample as well as the sample subdivided in three classes depending on the color frequencies within microareas. Note that sexual selection estimates for all microareas pooled are identical to those obtained in Table 1 (after transforming to relative values the fitness from Table 3). The sexual selection or the disassortative mating did not change with the microareas defined on color frequency, thus suggesting the estimates are frequency-independent.

In order to understand the mechanism causing both sexual selection and negative assortative mating, the mating preferences were estimated from the PTI statistics for the two main background colors in both datasets (Table 4; genotypic nomenclature). For background colors, the mating pairs were homogeneous across years (see Table S2; G test = 3.8; df = 4, P = 0.434), allowing pooling of the data (Table 4). The clearest (negative) effect was observed for Yellow/Yellow pairs in all cases (only significant in the pooled sample). Furthermore, the total mating preferences were apparently more variable across columns (PTI variances = 0.170) than across rows (PTI variance = 0.069) using both datasets, explaining why sexual selection effects should be stronger in females (see Table 1 and 3). Therefore, we can use these estimates to infer the mating rules in this species. For example in the pooled dataset, yellow males mate preferentially with brown females (PTI = 1.2) rather than with yellow females (PTI = 0.64; see Table 4). Notice, however, that these results could be similarly interpreted in terms of female competition (Brown females might show certain advantage when competing for mates compared to yellow ones).

Table 3  A re-analysis of the 2011 data set, after grouping for the relative frequency of the two main colors within microareas (using genotypic nomenclature; see text)

| Microarea | N Pairs | Sex | Brw | Yel | Gsel | $I_{re}$ |
|-----------|--------|-----|-----|-----|------|---------|
|           |        | Male| 0.96| 1   | 6.5$^*$ | -0.06 |
| All       | 78     | Female | 1   | 0.55$^*$ | (0.119) |
|           |        | Male| 1   | 0.91 | 4.8$^*$ | -0.43 |
|           | Yellow | Female | 1   | 0.18$^*$ | (0.396) |
|           | Mix    | Female | 1   | 0.62 | 1.9 | -0.44$^{**}$ |
|           | Brown  | Female | 1   | 0.35$^*$ | 6.3$^*$ | (0.294) |

$P < 0.10$; $^* P < 0.05$; $^{**} P < 0.01$, $^{***} P < 0.001$

Table 4  Total mating preferences estimated by the PTI statistics in the two main shell color classes in both datasets (using genotypic nomenclature)

|          | 1990$^a$ | 2011 | Pooled |
|----------|---------|------|--------|
| Brw      | 1.02    | 1.20 | 1.12   |
| Yel      | 1.04    | 0.67$^*$ | 0.76 |

$^a$Data re-interpreted (using a different color nomenclature) from Rolán-Alvarez and Ekendahl (1996).

Values below 1 represent pairs mating less frequent than expected under random mating, while values above 1 represent those more frequent than expected. The significance of the coefficients was obtained by bootstrapping the observed frequencies (see text).

3 Discussion

The Abelleira population of L. fabalis, as well as other local populations of this species, shows a striking shell color polymorphism that could be maintained by genetic drift or a balancing selection mechanism (see...
Clarke et al., 1988; O’Donald and Majorus, 1988; Cook, 1992; Ekendahl, 1995, 1998; Phifer-Rixey et al., 2008). The shell color frequencies in the same population 21 years later have remained remarkably similar, suggesting that the observed polymorphism might be maintained by balancing selection rather than genetic drift. In addition, we found direct evidence to support the hypothesis of balancing selection mechanisms acting on background shell colors. The fact that we obtained the same result in the same population 21 years later strongly suggests that a snapshot estimate of these processes may be valuable (repeatable) in similar cases. Certainly, our sexual selection inference is based on samples collected only during a few days in early summer, but this season is the time of the year with higher sexual activity in this species and geographical area and a high number of specimens and mating pairs were sampled. In addition, our estimates seem to be valid and meaningful due to the absence of any significant result from the experimental control (shell scars) used, a trait considered a priori as environmentally determined and unrelated to mating decisions. Therefore, these results might serve to encourage other researchers to try a similar approach in their studies, i.e. capturing mating pairs in the wild in order to better obtain direct estimates of mating behavior in vivo.

Sexual selection may be a powerful mechanism to reinforce adaptation in sympatry (Cruz et al., 2001) and even to promote speciation during mollusk taxa radiation (Sauer and Hausdorf, 2009). In this study we have the possibility of investigating the relationship between sexual selection and disassortative mating, a mechanism that therefore can help to maintain the population genetic diversity. First, we will focus on discussion about patterns of sexual selection, then on disassortative mating and finally how both patterns can be similarly produced.

It was observed that sexual selection may act against the most frequent shell colors (brown and yellow), irrespective of the color nomenclature (phenotypic and genotypic) used to classify individuals samples before the analysis (Rolán-Alvarez and Ekendahl, 1996; this study), even though the results were clearer and simpler to interpret when using the (genotypic nomenclature) background color and the banded pattern traits. However, the observed sexual selection effects cannot explain the observed shell color polymorphism in L. fabalis at Abelleira, because it would produce a dramatic increase of the olive specimens in the population, therefore decreasing the overall variability. The existence of particular fitness differences among shell colors does not necessarily imply that a polymorphism can be maintained, unless a frequency-dependent or similar mechanism is operating. Despite this, we did not find any evidence supporting that this mechanism was occurring. On the contrary, we found clear evidence that the shell color frequencies have remained stable in Abelleira from 1990 to 2011 (see table S1 from appendix). The apparent contradiction between our sexual selection estimates and the observation of the polymorphism maintenance could be resolved by estimating other fitness components (viability, fecundity, etc.). In fact, we have observed some significant differences in shell colors between age cohorts in this population and species (Rolán-Alvarez and Ekendahl, 1996 and unpublished material), perhaps suggesting differences in viability for some colors (e.g. higher in yellow ones). Nevertheless, it remains to be quantified and shown whether or not the interaction between both selection components (viability and sexual) may contribute to the maintenance of this polymorphism (for example producing a frequency-dependent mechanism). The distribution of shell colors has been claimed to be related to distinct selective mechanisms in different studies. For instance, shell color was found to be associated with distinct microhabitats in some Swedish L. fabalis populations (Ekendahl, 1995). A similar result was obtained by a manipulative laboratory experiment made by Ekendahl (1994), while in other populations certain shell colors may show a mimetic function regarding blend predation (Reimchen, 1989). The red shell ecotype of L. fabalis, living exclusively on the red algae Mastocarpus stellatus on some exposed rocky shores from Galicia (Rolán and Templado, 1987), seems to be adapted to that particular microhabitat (Rolán-Alvarez et al., 1995b). In L. saxatilis, it was also suggested that the observed shell color polymorphism might be maintained by visual selective crab predation, although laboratory manipulative experiments did not find support for such a hypothesis (Ekendahl, 1998), or be correlated with some physiological differences involved in adaptation to changes in salinity (Sokolova and Berger, 2000).

In the present study we observed the existence of negative assortative mating between some phenotypic shell colors. Such patterns were also detected in the 1990 dataset (Rolán-Alvarez and Ekendahl, 1996), and in a re-analysis of both datasets using background colors (Table 2 and 3). Negative assortative mating has been rarely reported in the literature (see for example; Castrezana and Markow, 2008; Handcox et al., 2010). A
During trail following, males are usually looking for ing (Gilly and Swenson, 1978; Erlandsson et al., 1999). Littorinid snails show a behavior of mucus trail follow- arguments suggesting that the underlyi ng mechanism or extreme temperatures (Phifer-Rixey et al., 2008) in city of individuals to resist salinity (Sergievsky, 1992) colors have been commonly associated with the capa-

questions. 

Before going a discussion about the mechanism behind the patterns observed, we will focus on the biological meaning of shell color change in this species. Whether this organism has the ability to visually distinguish different colors is presently unknown, but the shell color trait may show other pleiotropic effects. Background colors are produced by expressing different pigments in both soft and hard components of the organism, perhaps also including chemical substances within the mucus trail (see below). In fact, shell colors in littorinid have shown apparently pleiotropic effects on behavior and fitness in several cases. Certain shell colors have been commonly associated with the capacity of individuals to resist salinity (Sergievsky, 1992) or extreme temperatures (Phifer-Rixey et al., 2008) in L. obtusata.

In summary, we may preliminarily assume that the genes determining a particular color may imply some other pleiotropic effects on other traits.

The observed pattern of mating preferences suggests that both negative assortative mating and sexual selection effects can be caused by either female-female competition or by male choice preferences for distinct colors (Table 4). Although unlikely in this case, we can not reject the scenario of female-female competition, in which brown females could have some general advantage compared to yellow ones. However, we have some arguments suggesting that the underlying mechanism might be instead mediated by a male choice mechanism. Littorinid snails show a behavior of mucus trail following (Gilly and Swenson, 1978; Erlandsson et al., 1999). During trail following, males are usually looking for mates (Erlandsson and Kostylev, 1995). Some snails are known to deliver certain information in the mucus trail, as some species can distinguish between male and female mucus (Erlandsson and Kostylev, 1995) or tracker size (Conde-Padín et al., 2008). Male trail following has been suggested as the mechanism responsible for the known positive assortative (prezygotic sexual isolation) mating existing between two sympatric ecotypes of the related species Littorina saxatilis (Conde-Padín et al., 2008). The involvement of this trail following mechanism in L. fabalis, which would explain the mating preferences, could be experimentally tested in the future. In addition, it would be interesting to confirm the observed patterns by using laboratory mate choice experiments with different male and female shell colors.

Finally, we can briefly speculate about the reason why such a mechanism could exist in this organism. In this sense, we would like to highlight the fact that this species usually lives in low density populations, suffering common bottlenecks during winter time leading to a considerable level of population genetic subdivision (Rolán-Alvarez et al., 1995b). The mechanism underlying the polymorphism could have been selected in the past as a potential mechanism for reducing the rate of inbreeding, the different pigments being either equivalent to incompatibility alleles in plants (Futuyma, 2009), or being physically linked or phenotypically associated with the true alleles driving the mating preferences. Interestingly, in the related species L. saxatilis, typically living in high density populations, there is positive assortative mating for distinct ecotypes (having indirectly different shell colors; reviewed in Rolán-Alvarez, 2007; Conde-Padín et al., 2008). Obviously, we do not expect any simplistic relationship between population density and type of assortative mating, but the life history characteristics of a particular species certainly limits/ conditions its potential for speciation in one way or another.

Shell color polymorphisms still demand a consistent evolutionary explanation in this and other species. We know that sexual selection and assortative mating potentially affects the population dynamics of shell colors in L. fabalis from Abelleira. Future studies in this model system could be decisive for a complete understanding of the reasons and the mechanisms maintaining the polymorphism as well as for elucidating its biological causes.

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References

Andersson M, 1994. Sexual Selection. Princeton: Princeton University Press.

Carvajal-Rodríguez A, Rolán-Alvarez E, 2006. JMATING: A software for the analysis of sexual selection and sexual isolation effects from mating frequency data. BMC Evol. Biol. 6: 40.

Castrezana SJ, Markow TA, 2008. Frequency-dependent selection, metrical characters and molecular evolution. Phil. Trans. R. Soc. B. 319: 631–640.

Conde-Padin P, Cruz R, Hollander J, Rolán-Alvarez E, 2008. Revealing the mechanisms of sexual isolation in a case of sympatric and parallel ecological divergence. Biol. J. Linn. Soc. 94: 513–526.

Cook LM, 1986. Polymorphic snails on varied backgrounds. Biol. J. Linn. Soc. 29: 89–99.

Cook LM, 1992. The neutral assumption and maintenance of color morph frequency in mangrove snails. Heredity 69: 184–189.

Coyne JA, Orr HA, 2004. Speciation. Massachusetts: Sinauer Associates.

Coyne JA, Elwyn S, Rolán-Alvarez E, 2005. Impact of experimental design on Drosophila sexual isolation studies: Direct effects and comparison to field hybridization data. Evolution 59: 2588–2601.

Cruz R, García C, Rolán-Alvarez E, 2001. Sexual selection on phenotypic traits in a hybrid zone of Littorina saxatilis. J. Evolution. Biol. 14: 773–785.

Ekendahl A, 1994. Factors important to the distribution of color morphs of Littorina mariae Sacchi and Rastelli in a non-tidal area. Ophelia 40: 1–12.

Ekendahl A, 1995. Microdistribution in the field and habitat choice in aquariums by color morphs of Littorina mariae Sacchi and Rastelli. J. Mollus. Stud. 61: 249–256.

Ekendahl A, 1998. Colour polymorphic prey (Littorina saxatilis Olivi) and predatory effects of a crab population (Carcinus maenas L.). J. Exp. Mar. Biol. Ecol. 222: 239–246.

Endler JA, 1977. Geographic Variation, Speciation, and Cines. Princeton: Princeton University Press.

Erlandsson J, Kostylev V, 1995. Trail following, speed and fractal dimension of movement in a marine prosobranch Littorina littorea during a mating and a non-mating season. Mar. Biol. 122: 87–94.

Erlandsson J, Kostylev V, Rolán-Alvarez E, 1999. Mate search and aggregation behaviour in the Galician hybrid zone of Littorina saxatilis. J. Evolution. Biol. 12: 891–896.

Gilly WF, Swenson RP, 1978. Trail following by Littorina: Wash-out of polarized information and the point of paradox test. Biol. Bull. 155: 439.

Handcox D, Hoskin CJ, Wilson RS, 2010. Evening up the score: Sexual selection favours both alternatives in the colour-polymorphic ornate rainbowfish. Anim. Behav. 80: 845–851.

Hedrick PW, 2005. Genetics of Populations. Boston: Jones and Bartlett Publishers.

Hood GM, 2010. PopTools version 3.2.5. Available on the internet. URL http://www.poptools.org

Hunt J, Breuker CJ, Sadowski JA, Moore AJ, 2008. Male-male competition, female mate choice and their interaction: Determining total sexual selection. J. Evolution. Biol., 22: 13–26.

Futuyrma DJ, 2009. Evolution. 2nd edn. Massachusetts: Sinauer Associates.

Innes DJ, Haley LH, 1977. Inheritance of a shell-color polymorphism in the mussel. J. Hered. 68: 203–204.

Knoppen P, 1985. Rare male mating advantage: A review. Biol. Rev. 60: 81–117.

Kozminkii EV, 2011. Inheritance of longitudinal shell bands in the snail Littorina obtusata and L. saxatilis (gastropoda: Prosobranchia). Russ. J. Genet. 47: 1112–1119.

Kozminkii EV, Lezin PA, Fokin MV, 2010. A study of inheritance of white spots on the Littorina obtusata (Gastropoda, Prosobranchia). Russ. J. Genet. 46: 1455–1461.

Lewontin R, Kirk D, Crow J, 1968. Selective mating, assortative mating, and inbreeding: Definitions and implications. Eugen. Q. 15: 141–143.

Llopart A, Elwyn S, Lachaise D, Coyne JA, 2005. An anomalous hybrid zone in Drosophila. Evolution 59: 2602–2607.

Marín I, 1991. Sexual isolation in Drosophila I. Theoretical models for multiple-choice experiments. J. Theor. Biol. 152: 271–284.

Merrel DJ, 1950. Measurement of sexual isolation and selective mating. Evolution 4: 326–331.

Murray J, 1975. The genetics of Mollusca. In: King RC ed. Handbook of Genetics 3. London: Plenum Press, 3–31.

Murray J, Clarke B, 1966. The inheritance of polymorphic shell characters in Partula (Gastropoda). Genetics 55: 1161–1877.

O’Donnell P, Majerus M, 1988. Frequency-dependent sexual selection. Philos. T. Roy. Soc. B 319: 571–586.

Pérez-Figueroa A, Caballero A, Rolán-Alvarez E, 2005. Comparing the estimation properties of different statistics for measur-
izing sexual isolation from mating frequencies. Biol. J. Linn. Soc. 85: 307–318.

Phifer-Rixey M, Heckman M, Trussell GC, Schmidt PS, 2008. Maintenance of clinal variation for shell color phenotype in the flat periwinkle *Littorina obtusata*. J. Evolution. Biol. 21: 966–978.

Reid DG, 1996. Systematics and evolution of *Littorina*. London: Ray Society.

Reimchen TE, 1989. Shell colour ontogeny and tubeworm mimicry in a marine gastropod *Littorina mariae*. Biol. J. Linn. Soc. 36: 97–109.

Ritchie MG, 2007. Sexual selection and speciation. Annu. Rev. Ecol. Syst. 38: 79–102.

Rolán E, Templado J, 1987. Consideraciones sobre el complejo *Littorina obtusata-mariae* (Mollusca, Gastropoda, Littorinidae) en el Noroeste de la península Ibérica. Thalassas 5: 71–85.

Rolán-Alvarez E, Caballero A, 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution 54: 30–36.

Rolán-Alvarez E, Ekendahl A, 1996. Sexual selection and non-random mating for shell colour in a natural population of the marine snail *Littorina mariae* (Gastropoda: Prosobranchia). Genetica 97: 39–46.

Rolán-Alvarez E, Zapata C, Alvarez G, 1995a. Multilocus heterozygosity and sexual selection in a natural population of the marine snail *Littorina mariae* (Gastropoda: Prosobranchia). Heredity 75: 17–25.

Rolán-Alvarez E, Zapata C, Alvarez G, 1995b. Distinct genetic subdivision in sympatric and sibling species of the genus *Littorina* (Gastropoda: Littorinidae). Heredity 74: 1–9.

Sauer J, Hausdorf B, 2009. Sexual selection is involved in speciation in a land snail radiation on crete. Evolution 63: 2535–2546.

Sergievsky SO, 1992. A review of the ecophysiological studies of the colour polymorphism of *Littorina obtusata* (L.) and *L. saxatilis* (Olivi) in the White Sea. In: Grahame J, Mill PJ, Reid DG ed. Proceedings of the third International Symposium on Littorinid Biology. London: The Malacological Society of London, 235–245.

Servedio MR, Van Doorn GS, Kopp M, Frame AM, Nosil P, 2011. Magic traits in speciation: ‘magic’ but not rare? Trends Ecol. Evol. 26: 389–397.

Shuster SM, 2009. Sexual selection and mating systems. Proceedings of the National Academy of Science 106: 10009–10016.

Sokolova IM, Berger VJA, 2000. Physiological variation related to shell colour polymorphism in White Sea *Littorina saxatilis*. J. Exp. Mar. Biol. Ecol. 245: 1–23.
## Appendix

### Table S1  Observed frequency of shell colors using the 8-classes (phenotypic nomenclature) in both mated and unmated subsamples from 1990 and 2011 datasets

| Year | State | Brw | Yel | Ora | Oli | Brw2 | Yel2 | Ora2 | Oli2 |
|------|-------|-----|-----|-----|-----|------|------|------|------|
| 1990 | Non-Mated | 201 | 185 | 0   | 16  | 29   | 0    | 1    | 0    |
|      | Mated   | 226 | 192 | 0   | 19  | 36   | 0    | 0    | 1    |
| 2011 | Non-Mated | 159 | 170 | 11  | 2   | 6    | 4    | 0    | 9    |
|      | Mated   | 94  | 77  | 6   | 2   | 3    | 1    | 1    | 9    |

*a Data from Rolán-Alvarez and Ekendahl (1996).

### Table S2  Frequency of shell colors (*genotypic nomenclature*) in mated and unmated specimens from both datasets

| Year | Brw | Yel | Ora | Oli |
|------|-----|-----|-----|-----|
| 1990 | 95  | 75  | 4   | 0   |
|      | 135 | 68  | 63  | 0   |
|      | 111 | 57  | 29  | 0   |
|      | 12  | 0   | 0   | 0   |
|      | 0   | 2   | 11  | 0   |
| 2011 | 68  | 76  | 4   | 7   |
|      | 97  | 21  | 14  | 4   |
|      | 98  | 31  | 17  | 2   |
|      | 7   | 4   | 1   | 0   |
|      | 4   | 0   | 0   | 1   |

*b Data re-interpreted (using a different color nomenclature) from Rolán-Alvarez and Ekendahl (1996). Males in columns and female in rows. Data within the squares represent the distribution of mating pairs for a particular male and female color combination (for example we observed 57 pairs from female yellow and male brown combinations in the 1990 dataset). Numbers on the first column represent the frequencies of unmated females, while in the first row the frequencies of unmated males.

### Table S3  Frequency of shell bands (*genotypic nomenclature*) in mated and unmated specimens from both datasets

| Year | Unbanded | Banded |
|------|----------|--------|
| 1990 |          |        |
|      | 242      | 200    |
|      | 16       | 17     |
| 2011 |          |        |
|      | 194      | 83     |
|      | 12       | 5      |

*c Data re-interpreted (using a different color nomenclature) from Rolán-Alvarez and Ekendahl (1996). Males in columns and female in rows. Data within the squares represent the distribution of mating pairs for a particular male and female color combination. Numbers on the first column represent the frequencies of unmated females, while in the first row the frequencies of unmated males.
Table S4  Frequency of shell scars in mated and unmated specimens from 2011 dataset

|       | ♂  | ♀  |          |
|-------|----|----|----------|
|       | No scars | Scars |          |
| 2011  | 140 | 16  |          |
| No scars | 180 | 76  | 9        |
| Scars  | 28  | 10  | 1        |

Males in columns and female in rows. Data within the squares represent the distribution of mating pairs for a particular male and female combination. Numbers on the first column represent the frequencies of unmated females while in the first row the frequencies of unmated males.

Fig. S1  Left side: photography of a mating pair (male yellow – female brown). Right side: specimens with shell scars presumably produced by crab attacks.

Fig. S2  Color scheme used as reference for the 8 shell color classes (phenotypic nomenclature)
Brown (Brw), olive (Oli), orange (Ora) and yellow (Yel) morphs on the first row, and the corresponding banded colors (Brw2, Oli2, Ora2 and Yel2) on the second row. Note that we used also two other color traits under the genotypic nomenclature (see text): background colors, by pooling banded and unbanded similar phenotypes (Brw + Brw2, Oli + Oli2, Ora + Ora2 and Yel + Yel2) and color pattern (unbanded = Brw + Oli + Ora + Yel, or banded = Brw2 + Oli2 + Ora2 + Yel2).