Mate search and aggregation behaviour in the Galician hybrid zone of *Littorina saxatilis*

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Abstract

In Galician rocky shores two ecotypes of the snail *L. saxatilis* can be found in sympatry. A ridged and banded ecotype (RB-morph) and a smooth and unbanded ecotype (SU-morph) overlap in midshore with the production of some hybrids. The distinct morphs mate assortatively and there is evidence of a partial reproductive barrier between them. This sexual isolation is caused by a nonrandom microdistribution and mate choice behaviour. Mucus trail-following, movement rate and aggregation behaviour were studied to determine their roles in the mating behaviour and sexual isolation of this species. Morph-specific mucus trail-following could not, in our experiments, explain either of these two processes. The reasons for the aggregation of morphs were investigated by Monte Carlo simulations of data from natural populations, which showed that size aggregation (refuge sizes fit different sized morphs differently) could explain only about 36% of the morph aggregation in adult snails. In the laboratory, morph aggregation was still present, and simulations suggested that size aggregation was the possible explanation. Thus, morph aggregation in Galician *L. saxatilis* has to be explained also by other causes in addition to size aggregation. These may be a combination of contrasting preferences for barnacle and mussel patches in the two morphs, and possibly longer copulation and pair formation time with similar sized snails of the same morph. Thus aggregation behaviour, but not trail-following, contributes to incipient reproductive isolation and perhaps sympatric speciation in Galician *L. saxatilis* populations.

Introduction

Speciation has been considered a key for understanding evolution (Coyne, 1992). Despite our poor knowledge of the biological mechanisms contributing to speciation, theoretical studies have shown that the dynamics of speciation may be importantly affected by the type of trait contributing to the reproductive isolation (Johnson *et al*., 1996; Schluter, 1996). Prezygotic reproductive isolation between populations, ecotypes or species can be directly estimated from natural populations if mating pairs can be observed, which has been done for *Littorina saxatilis* (e.g. Johannesson *et al*., 1995) and therefore can be regarded as a valuable tool to study speciation processes.

On exposed rocky shores of Galicia (NW Spain), *L. saxatilis* has two distinct ecotypes: the larger ridged and banded (RB) morph inhabits the barnacle-dominated upper shore, while the smaller smooth and unbanded (SU) morph is mainly found in the low-shore zone of blue mussels (Johannesson *et al*., 1993). The distributions of the two morphs overlap in the midshore (together with the occurrence of intermediate hybrid forms) where the distribution of morphs, as well as barnacles and mussels, is very patchy (Johannesson *et al*., 1993; Kostylev *et al*., 1997). In the midshore there is a nonrandom distribution of morphs, perhaps caused by direct choice of mussel or

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barnacle microhabitats (Otero-Schmitt *et al*., 1997) and/or choice of size-specific refuge sites created by the different architectural complexity of mussel and barnacle habitats (Kostylev *et al*., 1997). Other authors have pointed out that habitat choice may be an important prerequisite for reproductive isolation to evolve in sympatry (see Garcia-Dorado, 1986). This aggregation of similar morphs in Galician midshore populations can indirectly produce assortative mating and, coupled with behavioural mate choice, can contribute to the evolution of incipient reproductive isolation (Johannesson *et al*., 1995; Rolán-Alvarez *et al*., 1999).

Identification of chemical cues in gastropod mucous trails by other conspecifics may result in trail-following behaviour, which is frequently observed in littorinids (e.g. Gilly & Swenson, 1978; Stirling & Hamilton, 1986; Tankersley, 1989). Trail following can be important for foraging (Imrie, 1992) and aggregation behaviours (Chapman, 1998), but also for mate search behaviour (Erlandsson & Kostylev, 1995). Erlandsson & Kostylev (1995) found that males of *L. littorea* are able to discriminate between male and female mucous trails, and recognize females prior to copulation. Together with the shorter 'copulation' duration of male–male pairs compared with male–female pairs (Saur, 1990), this may explain the low frequency (compared to many littorinid species) of male–male copulating pairs (≈5%) in natural populations of *L. littorea* (Saur, 1990; Erlandsson & Johannesson, 1994). *Littorina saxatilis* has been found to be involved in both interspecific and homosexual copulations (Raffaelli, 1977; Saur, 1990) and copulations between males are very common (about 30%) in *L. saxatilis* (Saur, 1990; Johannesson *et al*., 1995; Erlandsson & Rolán-Alvarez, 1998). While *L. littorea* males are able to recognize female mucous trails, are *L. saxatilis* males unable to do so? And can microscale morph aggregation in the Galician midshore be explained by morph-specific trail-following, or are there other explanations for the aggregations of morphs?

Therefore, we first tested the hypothesis that each morph follows mucous trails of its own ecotype more than trails of the contrasting morph, and whether this can explain some parts of the morph aggregation in midshore, and to some degree the assortative mating. We also tested the hypothesis that the distinct ecotypes move with different rates, which may perhaps suggest different strategies during mate or food searching. Finally we tried to understand the biological mechanisms causing the observed morph aggregation in polymorphic Galician populations.

Materials and methods

Movement and mucous trail-following

The methodology of these experiments, which were performed during summer 1997, was similar to trail-following experiments in *L. littorea* (see Erlandsson & Kostylev, 1995) except as noted below.

We used only the female marker and male tracker combination, because it showed the highest trail-following in pilot experiments (unpublished results) and because of its evolutionary relevance. Snails were picked 2–4 times from the midshore of three different localities (Senín, Silleiro 1 and 2), and we used them for experimental treatment not later than 2 days after collection during summer 1997. We constructed an experimental arena in the following way: a circle of plastic pipe was glued to a glass plate creating a circular basin with a diameter of 80 cm, and we used 88 females (44 of each morph) individaully as markers which were each left to move from the centre of the arena to the edge or until 30 min had passed. Males (44 of each morph; 22 for each female morph) were put individually on the same point facing the same direction as the female. Before each experimental treatment (between each pair of marker and tracker) we cleaned the glass plate with tissue paper and added about 2 cm of filtered sea water to remove the effect of 'old' trails on the behaviour of the next pair, and to be sure that no pheromones could have been left in the water. A randomization table was used to decide in what order to use snails from different localities in the experiment.

The movement of snails was recorded with a video camera and an observer plotted the movement trail on a transparent film attached to the TV screen. After this the trails were scanned into a computer and analysed with 'NIH Image' software. The movement time did not exceed 30 min for each snail. We measured the degree of trail-following (i.e. coincidence of trails) by using an index of coincidence, where coincidence was defined as a complete overlap between the marker's and the tracker's trails (see Townsend, 1974; M and M in Erlandsson & Kostylev, 1995). The range of the coincidence index lies between 0 and 1, where 0 indicates no coincidence and 1 complete coincidence. We also recorded the movement rate (tortuosity) of male and female movement and the number of times males of the two ecotypes encountered their own trail or the female marker trail, and we also estimated the mean movement rate.

Analyses of variances were applied to test the differences in trail-following and movement rate between morphs and sexes, following parametric inference. However, if the variances were heterogeneous we applied randomization ANOVA (4999 randomizations) programmed in BASIC.

Aggregation experiments

We made additional analyses of data from Rolán-Alvarez *et al*. (1999), where midshore microareas were sampled for mating pairs and, from a few square centimetres around the pairs, noncopulating snails. The different microareas (which included mating pairs and surrounding noncopulating snails) can be considered as a

representative sample of possible morph and size aggregations during matings. Localities from summer 1994 (Barcelos, Portocelo, Caneda, Escuma, Senín and Silleiro 4) were rather homogeneous, which makes it possible to pool samples. We performed a Monte Carlo simulation to test the null hypothesis of absence of morph aggregation. We resampled 5000 times a random allocation of morphs, following the number of observed microareas present and their known sample sizes, from the pooled samples. This allows us to test if the observed degree of morph aggregation (variance of morph frequencies across microareas) deviates significantly from random aggregation. Since size is a morph-specific character, and thus mean sizes and morph frequency are highly correlated among microareas, the nonrandom aggregation of morphs can be just due to the size differences between morphs. We therefore performed a second Monte Carlo test using the morph and size of each snail, and independently the observed size distribution in the microareas. Then, snails of a certain size (and indirectly a morph) for a particular microarea were resampled (5000 times), following the known size distribution among microareas, from the pooled sample. The process is completed by obtaining a new resampling of the snail sizes (not necessary with the same morph distribution) across microareas. Three different statistics were calculated: (i) the variance of the morph frequency among patches, (ii) difference in mean size between morphs within patches (averaged across patches) and (iii) the correlation between mean size and morph frequency across patches. Thus, if the observed statistic is outside the 95% confidence intervals of the Monte Carlo resampling distribution, then a direct morph or size aggregation mechanism can be suggested.

The morph and size aggregation factors were also studied in laboratory-controlled conditions. For this experiment the same experimental arena was used as in the trail-following experiments. We collected two independent samples (1 and 2) of fresh snails from Silleiro 1 during summer 1997. Fifty snails each of the RB- and SU-morphs with different sizes were allocated in a chessboard pattern so that they regularly covered most of the arena surface (replicated two times; 1–2). This was done in order to give every snail the same probability to encounter any morph. The arena was then covered with a second glass plate to prevent escape of the snails that reached the edge. Animals were left to move for 1 h and after this time most of them were aggregated in separate distinct patches (they were aggregated if they touched each other). We recorded the number of snails within each patch and the number of patches. Each patch was collected in a plastic bag and the morph and size of each snail in a patch was recorded. In a second experiment the same set-ups were used, but this time only juvenile snails of similar sizes were put into the arena. The same Monte Carlo approach was applied to the laboratory-sampled data to check the former models.

Results

Movement rate and mucous trail-following

Tracker snails (males) encountered their own trails as often as the marker snail trails (females; *t*-test: d.f. = 89, $t = 0.58$, $P = 0.56$), but trackers followed the encountered marker trails for much larger distance than their own trails (*t*-test: d.f. = 87, $t = 2.89$, $P = 0.0049$). Apart from that, markers and trackers followed their own trails to the same extent (*t*-test: d.f. = 89, $t = 0.49$, $P = 0.62$). The tracker snails (males) also moved more tortuously than marker snails (females) as indicated by the measure of the ratio of trail length to displacement between start- and end-points of moving snails (ANOVA: $F = 4.69$, $P = 0.03$). These results may suggest the presence of a search behaviour in males of Galician *L. saxatilis*.

However, degrees of trail-following were not significantly different for snails from different localities ($F = 0.35$, $\text{d.f.}_1 = 2$, $\text{d.f.}_2 = 76$, $P = 0.72$), nor different morphs ($F = 1.34$, $\text{d.f.}_1 = 3$, $\text{d.f.}_2 = 76$, $P = 0.35$), i.e. snails followed individuals of the contrasting morph as far as they followed their own. The rate of movement, however, differed between the morphs (Table 1). Both markers (RB, average = 5.9 cm min^{-1} SD = ± 1.4, $n = 46$; SU, average = 4.1 cm min^{-1}, SD = ± 1.1, $n = 44$) and trackers (RB, average = 6.1 cm min^{-1}, SD = ± 1.8, $n = 45$; SU, average = 3.8 cm min^{-1}, SD = ± 0.8, $n = 43$) of the RB-morph moved significantly faster than snails of the SU-morph (Tables 1a and 1b).

Differences in movement rates between morphs may to some degree depend on the size differences between them. When movement rates of snails of both morphs were pooled there was a positive correlation both between marker movement rate and marker size ($r = 0.47$, d.f. = 88, $P = 0.0001$) and between tracker movement rate and tracker size ($r = 0.70$, d.f. = 86, $P = 0.0001$). Also, within each morph there was a positive correlation between marker size and movement rate ($r = 0.47$, d.f. = 44, $P = 0.0001$) and between tracker size and movement rate ($r = 0.60$, d.f. = 43, $P = 0.0001$). An ANCOVA of marker movement rate, with morph as the factor and size as the covariate,

Table 1 One-factor analysis of variance of movement rate of the RB- and SU-morphs among female marker (a) and tracker (b) snails in Galician *L. saxatilis*.

Source	d.f.	MS	*F*	*P*
(a) Marker morph	1	7410	47.2	0.0001*
Residual	88	157.2		
(b) Marker morph.	1	12406	64.0	0.0001*
Residual	86	193.9		

* RB-snails move at higher rates than SU-snails.

Table 2 Microdistribution of the RB- and SU-morphs in natural populations (a) and laboratory samples (b) of Galician *L. saxatilis*. Monte Carlo simulations of natural data (resampling distribution of morphs among microareas; see text) were done to test the probability that aggregations of morphs arise by chance. Observed and resampling (i.e. random allocation of morphs) variances of morph frequency are shown. *n* is the number of microareas sampled.

	Observed values		Monte Carlo distribution under the model		
Age class	*n*	Average	Average	Variance	Probability
(a) Adults	142	0.0676	0.0286	0.00001	0.0004
Juveniles	51	0.0441	0.0245	0.00002	0.0000
(b) Adults	12	0.0394	0.0195	0.00005	0.0152
Juveniles	20	0.0289	0.0282	0.00007	0.8960

showed no morph/size interaction ($P = 0.189$), but significant effects of morph (0.00001) and size (0.028), while an ANCOVA of tracker movement rate showed no significant effects of interaction ($P = 0.66$) and morph ($P = 0.115$), but a significant effect of size ($P = 0.0005$).

Analyses of aggregation and microdistribution

We randomly resampled snails among microareas (patches) in order to check the existence of morph aggregation (Table 2). The results from natural data showed that random allocation of morphs in patches could not explain the observed morph aggregation in natural populations (Table 2a), possibly meaning that there are behavioural- or microhabitat-related factors affecting the observed pattern. An allocation of sizes (and their respective morphs) following the known size distribution among microareas was also estimated. The resampling distribution of the three statistics (variance of RB frequency, difference in mean size and correlation of sizes and frequencies among patches) is used to compare the probability of including the observed statistics of aggregation in the resampling distribution (Table 3). While the effect of morph aggregation was found to be significant in natural data, the effects of size aggregation and of correlation between size and morph frequency were not (Table 3a). Thus, despite the size difference between morphs, the results indicated that size cannot completely explain the aggregation of morphs in nature.

In a similar analysis, using the above Monte Carlo simulations, but on data collected from laboratory experiments (to remove the possibility that specific snails choose specific microhabitats in the field), we found that adult snails of similar morphs and sizes aggregate in laboratory conditions as well, but not juveniles (Table 2b). The Monte Carlo simulation on laboratory data showed that size aggregation could account for the morph aggregation in adults, since the size aggregation model could not be rejected (Table 3b).

Discussion

Reproductive isolation may evolve by the same forces that cause phenotypic evolution. Natural selection may be the most important force promoting phenotypic evolution, and thus directly or indirectly promoting speciation, but still we need clear examples of this (see Schluter, 1996). Snails of the genus *Littorina* are valuable for studies of biological mechanisms contributing to prezygotic reproductive isolation, because mating pairs can be directly observed on the rocky shore (Raffaelli, 1977; Saur, 1990; Johannesson *et al*., 1995). In particular, exposed vs. sheltered ecotypes of *L. saxatilis* are interesting because they have developed partial reproductive isolation in some localities (in exposed Galician populations) but not in others (Swedish populations), in spite of the similar environmental shifts between exposed and sheltered habitats. One of the most relevant differences between Swedish and Galician populations is that only the latter show a striking morph aggregation in hybrid populations, which contributes to partial reproductive isolation between different ecotypes and also contributes to morph cohesion.

At least two classes of processes contribute to the incipient reproductive isolation observed in Galician *L. saxatilis* populations (Johannesson *et al*., 1995; Rolán-Alvarez *et al*., 1999): morph aggregation and behavioural sexual isolation. Here, we focus on those mechanisms responsible for the morph aggregation, as further discussion on the mechanisms of reproductive isolation is presented elsewhere (Rolán-Alvarez *et al*., 1999). In principle, there are two different kind of hypotheses to account for the midshore morph aggregation: microhabitat choice and trail-following. However, snails from different localities followed mucous trails of the contrasting morph for as long a distance as they followed trails of their own morph. Furthermore, there was no morph aggregation in equally sized juveniles in laboratory experiments. These results suggest that snails are not able to detect differences between morphs in their mucous trails. However, trail-following may be important for mate or food search in other mollusc species (Townsend, 1974; Erlandsson & Kostylev, 1995). On the other hand, the search behaviour (increasing movement tortuosity) by males of Galician *L. saxatilis* indicated by the present study may function as a way to increase the probability of finding any mucous trails and individuals (by following detected trails) of potential mates. Besides, the RB-morph showed a higher rate of movement compared with the SU-morph, perhaps as a consequence of their larger size. Nevertheless, it does not seem probable that these two latter processes are important for the prezygotic reproductive isolation observed. On the contrary, they seem general strategies for finding

Table 3 Microdistribution of the RB- and SU-morphs in natural populations (a) and laboratory samples (b) of Galician *L. saxatilis*. Monte Carlo simulations of natural and laboratory data (resampling distribution of sizes, and indirectly morphs, among microareas; see text) were done to test the probability that aggregations of morphs are caused by size aggregation. Three different statistics were estimated for each resampling distribution (see text for more explanations): variance of morph frequency among patches, size difference between morphs within patches and correlation between mean size and morph frequency within patches.

		Observed estimate	Monte Carlo distribution under size aggregation		
Age class	Variables	Average	Average	Variance	Probability
(a) Adults	Variance (RB freq)	0.0676	0.0431	0.0018	0.0000
	Size diff (RB-SU)	1.11	1.1498	0.0026	0.4137
	Correl. (Size-Freq)	0.6726	0.7037	0.0015	0.4212
Juveniles	Variance (RB freq)	0.0441	0.0243	0.0006	0.0000
	Size diff (RB-SU)	0.82	0.8064	0.0048	0.8651
	Correl. (Size-Freq)	0.3823	0.3342	0.0134	0.7523
(b) Adults	Variance (RB freq)	0.0394	0.029	0.0009	0.1841
	Size diff (RB-SU)	1.72	1.646	0.0158	0.4059
	Correl. (Size-Freq)	0.77	0.724	0.0069	0.5643

proper habitats or mates but without any apparent discriminating ability.

The morph aggregation in midshore Galician populations is a somewhat difficult evolutionary problem. Although it has been detected previously (Johannesson *et al*., 1995; Kostylev *et al*., 1997; Otero-Schmitt *et al*., 1997; Rolán-Alvarez *et al*., 1999), the biological mechanisms promoting it were not clear. The morph aggregation can be quantified by the variance in morph frequencies, which ranges between 0.04 and 0.07 in wild populations and between 0.03 and 0.04 in the laboratory (Table 2). Moreover, in all cases aggregation could not be explained just by chance. Interestingly, morph aggregation was considerably greater in natural populations than in controlled laboratory conditions. This suggests that different mechanisms are contributing to morph aggregation in each case. One possibility is that morph aggregation may be caused exclusively by size aggregation. There is indeed significant size difference between the two parental ecotypes on the Galician coast (Johannesson *et al*., 1993, 1995), and size aggregation could be caused by size-specific barnacle and mussel refuges (Kostylev *et al*., 1997). Our simulations imply that size can fully explain the aggregation of juvenile and adult snails of the same morph in laboratory conditions (Table 3). In this case, the mechanism of size aggregation may depend on the snails' preference for being attached to other snails (not necessarily searching for mates), and thus they could perhaps recognize the 'surface' or size contours of a similar sized group of snails. This ability would be related to the searching for refuges similar to their own size in natural conditions. On the other hand, the size aggregation model could not explain the morph aggregation observed in natural populations, although the size aggregation may still explain a considerable amount of the morph aggregation in nature, i.e. about 36% in adults and 3% in juveniles.

Thus, the aggregation behaviour in wild populations may depend on other factors besides size-dependent microhabitat choice. One possibility would be if the different morphs show particular preferences for the different microhabitats (barnacles and mussels) in midshore areas (see Kostylev *et al*., 1997; Otero-Schmitt *et al*., 1997). Otero-Schmitt *et al*. (1997) found that each microhabitat supports a particular microflora, but the morphs seem to feed randomly within each microhabitat. In fact, the two distinct morphs from Galicia do seem to have different preferences for the different microhabitats, although the pattern of this microhabitat choice may vary as a consequence of complex interactions of causative factors (see Kostylev *et al*., 1997; Otero-Schmitt *et al*., 1997). Another possibility would be that snails show particular preferences during matings, for example mating longer with the same ecotype or size, and perhaps staying in the vicinity of similarly sized snails of the same morph after copulation. However, there is no experimental support for this latter alternative.

In summary, we found support for two different mechanisms contributing to the morph aggregation in midshore: size aggregation for suitable refuges and microhabitat choice. The joint simulation of these mechanisms with real data allowed us to explain successfully the observed microdistribution of morphs in wild and laboratory data. We still need, however, a fully predictive theory for the morph aggregation in wild populations. Furthermore, although previous work suggests that these mechanisms have probably evolved indirectly by natural selection adapting upper and lower shore populations to their respective habitats (see Rolán-Alvarez *et al*., 1997, 1999), a formal demonstration is still required.

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