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Short communication

The relationship between hatching rate and number of embryos of the brood pouch in *Littorina saxatilis*

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ABSTRACT

Littorina saxatilis is an ovoviviparous organism in which shelled embryos can be directly observed in nearly all mature females captured in the wild. This characteristic has been used a number of times as an indirect estimate for fecundity of natural populations. However, there is no experimental corroboration that the number of embryos within a female brood pouch is actually related to the rate of hatching per unit of time, a more realistic estimate of female fecundity. In order to make this corroboration we estimated the correlation between the number of embryos in the brood pouch and the hatching rate of isolated females grown in the laboratory. We also compared the hatching rates of females from two sympatric ecotypes (RB and SU) of this species, which differ significantly in the number of embryos. We found a high agreement between hatching rate and number of embryos, concluding that the latter is a good proxy for fecundity.

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1. Introduction

Littorina saxatilis (Olivi), a dioecious gastropod from intertidal North Atlantic rocky shores, has low dispersal ability because of its internal fertilization and direct development (Reid, 1996). A number of studies have used the shelled embryos from inside the female brood pouch to estimate indirectly the fecundity of the species in the wild (Cruz et al., 1998, 2001; Cruz and García, 2001, 2003; Rolán-Alvarez et al., 2004), to use them as possible bio indicators (Janson, 1985), or to infer indirectly their effect on the phenotype based on the space occupied within the mother's shell (Grahame and Mill, 1992). This latter approach has also been used in different ovoviviparous fish species (Jones and Ugland, 2001; Veríssimo et al., 2003; Chatzispyrou and Megalofonou, 2005; Abilhoa et al., 2007). The number of embryos present within the brood pouch should be related with the overall female capability for producing offspring and, therefore, it should be directly related with fecundity. This has been the assumption made in most studies, where it was concluded, for example, that fecundity was positively correlated with female size (Fish and Sharp, 1985; Cruz et al., 1998, 2001; Cruz and García, 2001; Jones and Ugland, 2001; Cruz and García, 2003; Rolán-Alvarez et al., 2004; Chatzispyrou and Megalofonou, 2005). However, so far no experimental work in *L. saxatilis* has evaluated the hatching rate, which would be a more realistic estimate of fecundity (see Tripathi and Singh, 1990; Ortega-salas and Reyes-Bustamante, 2006). Here we provide an estimate of the hatching rate of females of *L. saxatilis* grown in the laboratory and obtain estimates of its correlation with the number of embryos within the brood pouch. We also compare the hatching rate of two sympatric ecotypes of *L. saxatilis* known to differ in the mean number of embryos within the brood pouch (Johannesson et al., 1993; Cruz et al., 1998), and presumably involved in an ecological process of reproductive isolation (Quesada et al., 2007; Rolán-Alvarez, 2007).

The two ecotypes are living preferentially at different vertical levels of the exposed Galician rocky shores (NW Spain; Rolán-Alvarez, 2007), and differ in morphological characteristics associated with their respective habitats. The ridged, banded and bigger form (RB) is usually found on the upper shore dominated by barnacles, where an intense predation by crabs and heat desiccation exist, while the smooth, unbanded and smaller one (SU) is found on the lower shore dominated by blue mussels, living in a high wave-action environment. They meet and produce phenotypically intermediate hybrids in the midshore. Genetic differences have been found between the ecotypes for several fitness-related traits (including fecundity; reviewed in Rolán-Alvarez, 2007). Thus, both ecotypes are exposed to intense divergent selection favouring morphological differences in shell size and shape to cope with different environmental factors (Johannesson et al., 1993; Johannesson and Johannesson, 1996; Rolán-Alvarez et al., 1997; Rolán-Alvarez, 2007). The two ecotypes show a considerable difference in shell size, RB individuals (mean size 5.43 ± 3.95 mm) being much larger than SU ones (3.48 ± 0.61) (Pérez-Figueroa et al., 2005). The ecotypes also show different apparent fecundities, the mean number of embryos in the brood pouch being significantly

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Table 1
Analysis of variance for rate of hatching in the three experiments

Experiment	Variable	Source	df	MS	F
1 (1998)	Rate of hatching	Ecotype	1	4108.2	9.2^{**}
		Distribution	1	294.0	0.7^{ns}
		Interaction	1	840.2	1.9^{ns}
		Error	20	444.2	
	Rate of hatching/volume[a]	Ecotype	1	0	0.133^{ns}
		Distribution	1	0.025	7.56^{*}
		Interaction	1	0.012	3.66^{ns}
		Error	20	0.003	
2 (1999)	Rate of hatching	Ecotype	1	8816.7	30.4^{***}
		Distribution	1	130.7	0.4^{ns}
		Interaction	1	620.2	2.1^{ns}
		Error	20	289.9	
	Rate of hatching/volume[a]	Ecotype	1	0.002	0.201^{ns}
		Distribution	1	0.002	0.207^{ns}
		Interaction	1	0.008	0.863^{ns}
		Error	20	0.009	
3 (2007)	Rate of hatching	Ecotype	1	27960.1	33.8^{***}
		Error	15	827.1	
	Rate of hatching/volume[a]	Ecotype	1	0.013	0.96^{ns}
		Error	15	0.013	
		Error	15	827.1	
	Rate of hatching/volume[b]	Ecotype	1	0.015	1.33^{ns}
		Error	15	0.011	

In experiments 1 and 2 the fixed factors are Ecotype (RB and SU) and Distribution (allopatric and sympatric) in a two-way ANOVA. In experiment 3, the fixed factor is Ecotype (RB and SU) in a one-way ANOVA.
$^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$; ns is non-significant.
[a] Volume estimated as shell height to the third power.
[b] Volume of a sphere whose diameter is the female shell width.

larger in the RB ecotype (119.4 ± 9.23) than in the SU one (38.1 ± 3.05) (Cruz et al., 1998), possibly due to their differences in shell size.

2. Materials and methods

We evaluated the hatching rate of recently caught females representative of the two ecotypes in three laboratory experiments (1–3). Laboratory conditions were similar in all experiments. The specimens were maintained in transparent metacrilate tubes (15 cm long and 10 cm in diameter) closed on both sides by a net of 300 μm. The containers were aerated and a 14/10 h cycle of day/night respectively was disposed. However, experiments 1 and 2 were carried out on a closed circulating system at a laboratory of the Biology Faculty, where aeration was provided by air pumps and seawater needed to be hand-replaced every 4 days, whereas experiment 3 was done on the new marine station ECIMAT, having a circulating seawater flow for the breeding system and a central system for aeration. In both cases the food was naturally supplied by the seawater in the form of diatoms and other microalgae. However, in experiments 1 and 2, a supplementary food (tetramin) was added every 15 days, and in experiment 3, *Patella* spp. dissected from the same shore and habitat

were supplied as an extra diatoms source (deposited on their surface). Those from specimens 1 and 2 were maintained for only two months because mortality rates were relatively high (about 10% per month).

Specimens were randomly chosen from the locality of Silleiro at different shore levels and years. Specimens from experiments 1 and 2 were captured at three different shore levels (lower, mid and upper shore), and in two consecutive years (June, 1998 for experiment 1 and June, 1999 for experiment 2). At lower and upper shores only SU or RB specimens are available, respectively, whereas at the mid shore both types and a variable percentage of hybrids are present. RB and SU specimens were sexed following Johannesson et al. (1993), and 6 females of each ecotype, shore level and year (24 in each experiment) were isolated. Specimens from experiment 3 were randomly chosen on November 2007 exclusively from upper (11 RB) and lower shore (6 SU) habitats. Each isolated female was inspected fortnightly (experiments 1 and 2) or weekly (experiment 3) to detect recently hatched juveniles over a period of two months, and those remaining alive were counted under a binocular microscope LEICA MZ12. At the end of the period, the shell size of every female was measured with a caliper following De Wolf et al. (2001), and the number of embryos in the brood pouch was counted.

The rate of hatched juveniles (either raw or corrected for female shell volume) was analysed by a two-way ANOVA with the fixed factors *ecotype* (RB and SU) and *distribution* (Upper/Lower and Mid shore) in experiments 1–2, and by a one-way ANOVA with the fixed factor *ecotype* (RB and SU) in experiment 3. Correction for an approximate estimate of shell volume was carried out by dividing the number of hatched juveniles by the (female) shell height to the third power. For experiment 3 (where estimates of shell width were available) an additional correction was made by using the volume of a sphere whose diameter is the female shell width. Heteroscedasticity among treatments was checked by the Levene test (Nie et al., 1975), and it was not detected. The normality of the dependent variable was not checked but the *F* test in ANOVA is extremely robust about normality violations in the dependent variable (Scheffe, 1959). All the analyses were accomplished by the SPSS/PC ver 12.0 software (Nie et al., 1975).

3. Results

Estimates of hatching rate were significantly correlated with the number of embryos of the brood pouch after the dissection of the females in experiment 2 ($r=0.42$, $df=22$; $p=0.040$) and 3 ($r=0.72$, $df=15$; $p=0.001$), although not significant in experiment 1 ($p=0.874$). The rate of hatching (uncorrected for female volume) was significantly different between ecotypes (greater for RB females than SU ones) in the three experiments (Table 1; Fig. 1), in agreement with previous fecundity estimates obtained through the number of embryos in the brood pouch. In experiments 1 and 2 neither the distribution nor the interaction factors were significant. A positive correlation was found between the size of the mothers and her rate of hatching in the three
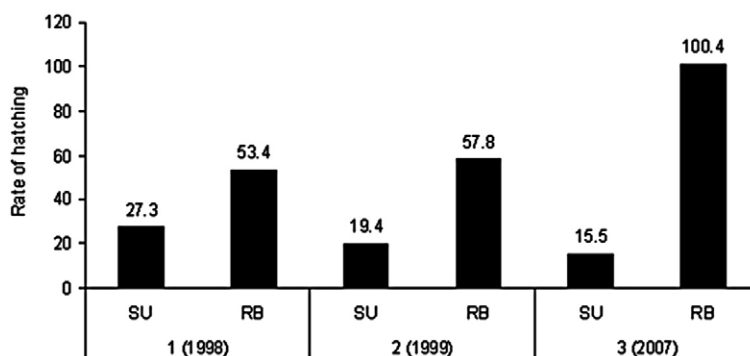

Fig. 1. Rate of hatching after two months for the two ecotypes in the three experiments carried out (allopatric and sympatric samples are averaged for experiments 1 and 2).

experiments ($r=0.64$, $p=0.001$; $r=0.57$, $p=0.004$; $r=0.83$, $p<0.001$, respectively). In fact, differences in hatching rate between the ecotypes disappeared when this was corrected by female volume (Table 1).

4. Discussion

Our results confirm that the estimates of fecundity based on the number of embryos within the brood pouch and those based on the rate of hatching per unit of time during the first two months of isolation were highly positively correlated in at least two of the three experiments. The highest correlation observed ($r=0.72$, $df=15$; $p=0.001$) was achieved in experiment 3, performed in a the new marine station ECIMAT, where a circulating seawater flow for the breeding system and a central system for aeration was available. Experimental conditions for this experiment were thus considerably improved relative to those for experiments 1 and 2, carried out on a closed circulating system, where aeration was provided by air pumps and seawater needed to be hand-replaced. Additionally, the biological patterns observed for the number of embryos within the brood pouch, i.e. a positive fecundity–size relationship and significant differences between ecotypes (Cruz et al., 1998, 2001; Cruz and García, 2001, 2003; Rolán-Alvarez et al., 2004) were analogously observed for the hatching rate. These results suggest that both approaches can be used as an estimate of fecundity, although counting the number of embryos within the brood pouch is much simpler to obtain. A similar result found with sharks revealed that fecundity estimates based on ova counts from the ovary or on embryos from the uterus were equally valid (Chatzispyrou and Megalofonou, 2005). We did not evaluate the hatching rate for more than two months as the relatively high mortality rates observed in laboratory conditions would make them little representative of the actual values. However, we think that the period used is a realistic one, as it should be rare that females could be isolated from other males for longer periods in a species which usually lives at mid to high densities in the wild (Reid, 1996).

The fecundity differences observed between ecotypes (based both on hatching rates or counted embryos) can be explained just by the existing size differences between ecotypes, at least if the relationship between fecundity and size is cubic. This is interesting since these two ecotypes show a strong pre-mating sexual isolation when they meet at the mid shore (Rolán-Alvarez, 2007), and it has been postulated that such phenomena could be a side effect of the size differences existing between them (Conde-Padín et al., in 2008). In fact, these size differences are assumed to be a consequence of adaptation to distinct habitats and conditions, the small size of the SU being necessary to protect them from the strong wave impacts, whereas the largest size in the RB is characteristic of those populations being heavily predated by crabs (Johannesson and Johannesson, 1996; Rolán-Alvarez, 2007). It seems that disruptive selection for size in the sympatric populations of this species may have a deep impact on many other life history characteristics (fecundity and sexual isolation). The specimen typical/ maximum size should be a key trait to predict the success of a particular population to a particular habitat, and this could explain the high heritability observed in this species (Conde-Padín et al., 2007).

Acknowledgements

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