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Microsatellite evidence for sperm storage and multiple paternity in the marine gastropod *Crepidula coquimbensis*

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ABSTRACT

In gregarious species with copulation and internal fertilization, male–male competition and female cryptic choice may affect reproductive success of both sexes. We carried out a molecular analysis to study paternity and sperm use by females in the protandrous marine brooding gastropod *Crepidula coquimbensis*. In the field, a single female inhabits an empty hosting shell with up to six males. This gregarious behavior may promote intra-brood multiple paternity if females can store sperm from several consecutive copulations with the surrounding males. To study female sperm usage, the males sharing shelters with five different adult females were collected and preserved for paternity analysis. Females were transported alive to the laboratory and isolated for six months. After that, an additional male was offered to each of the five study females. Once the females had laid capsules, a total of 528 embryos from the five females were assigned paternity based on five microsatellite loci. Paternity analysis showed that every male sharing the empty hosting shell of a female as well as the additional male were assigned fatherhood of embryos laid by this specific female. Females can thus use sperms from multiple males including sperms stored for at least six months. In addition, in four out of the five offspring arrays, a similar contribution of each male to the brood was observed, a pattern associated with the close relationship between the number of fathers observed and the effective paternity index calculated. These results contrast with those of paternity analyses carried out in another species of the same genus, *C. fornicata* which is characterized by a stacking behavior in which the closest male to the female achieves the highest reproductive success. Male reproductive success may be largely influenced by the aggregation pattern and male mating opportunities in the *Crepidula* complex, a hypothesis to be examined further by studying other species exhibiting different grouping behavior.

1. Introduction

Polyandry (i.e. females mate with more than one male) is a common reproductive behavior observed among females in nature, across taxa and environments (e.g. Plants: Bernasconi, 2004; Mollusks: Dupont et al., 2006; Insects: review in Arnqvist and Nilsson, 2000; Reptiles: Laloi et al., 2004; Fishes: Soucy and Travis, 2003; Birds: review in Birkhead and Møller, 1995; Mammals: DeYoung et al., 2002). Multiple paternity is a likely straightforward consequence of polyandry. Recent advances in genetic techniques and statistical analyses have helped to test this hypothesis and to determine fatherhood in broods produced in the wild or in laboratory experiments. However, a large proportion of research assessing the importance of polyandry in marine invertebrates has been conducted with brooding free-spawner species in which females do not seem to exert active mate choice (e.g. Bishop and Pemberton, 1997; Bishop et al., 2000; Ayre and Miller, 2006). In such species, the occurrence of multiple paternity has often been considered as a correlated response to increased fertilization success (Yund and McCartney, 1994; Levitan, 1998). In marine invertebrate species exhibiting complex mate interactions or reproductive behavior, including species with copulatory behavior, male–male competition and female cryptic choice may affect the reproductive output of both sexes (e.g. Shaw and Sauer, 2004; Toonen, 2004; Dupont et al., 2006; Walker et al., 2007).

One of the factors that may be favoring polyandry and shaping the level of multiple paternity within marine invertebrates exhibiting copulation is aggregative behavior. This has been observed in *Crepidula fornicata*, one of the marine gastropods for which paternity patterns have been previously investigated (Gaffney and McGee,

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1992; Dupont et al., 2006; Proestou et al., 2008; Le Cam et al., 2009). This species forms perennial stacks of variable numbers of male and female individuals (Coe, 1936). Paternity analyses carried out for this species allowed for the testing some general hypotheses related to the effects of reproductive behavior and social interactions on the incidence of multiple paternity and male reproductive success. Females may store sperm for at least 1 year (Hoagland, 1978). Multiple paternity seems to be the rule with on average 1.8 to 3.3 fathers participating per brood (Dupont et al., 2006; Proestou et al., 2008; Le Cam et al., 2009). As the position of adult individuals within the stack is stable through time, the male located closest to the female has easier access to the female and achieve the highest reproductive success (Dupont et al., 2006; Proestou et al., 2008; Le Cam et al., 2009). Polyandry and multiple paternity in this species is thus favored by a gregarious behavior and the capacity of females to store sperm but is limited by the restricted social interaction of individuals within the aggregation (i.e. the reproductive success of males is strongly determined by their position within the stack). In other species with gregarious behavior characterized by more flexible interactions among individuals, all else being equal, it could be hypothesized that a higher level of multiple paternity and a more balanced participation of the surrounding males to the brood is expected given that more males have access to the female.

To test for this latter hypothesis, we examined paternity patterns in broods of *Crepidula coquimbensis* (Calyptraeidae), a congeneric species of *C. fornicata*. As with all members of the calyptraeid group, *C. coquimbensis* is a protandrous marine gastropod changing sex from male to female during its lifespan. Fertilization in this species is internal and males transfer sperm during mating. Females encapsulate and brood their offspring for a period of about 40 days. *C. coquimbensis* is a direct developer which means that juveniles hatch from the capsules at the end of the incubation time. Unlike *C. fornicata*, this species does not form stacks of individuals, although it does display gregarious behavior with one female and two to six males sharing the same micro-habitat, for instance the empty shell of another marine gastropod (e.g. genera *Tegula* and *Argobuccinum*; Brown and Olivares, 1996). Any of these males can move within the micro-habitat and reach the female. Given the gregariousness and the absence of stacking behavior of *C. coquimbensis* we specifically hypothesized that: (1) females may store sperm from different males and lay broods resulting from multiple fathers and (2) the contribution of different males to the brood is evenly distributed among them. To test for these hypotheses, we carried out paternity analyses based on previously developed microsatellite markers (Daguin et al., 2007) and examined: (i) the sperm storage ability of females of *C. coquimbensis*, (ii) the level of multiple paternity within a capsule and/or within a brood and (iii) the occurrence of skewed paternity (i.e. non-identical contribution of potential fathers to the clutch). Finally, we tested for the contribution to clutches of the last male offered to females using an experimental approach.

2. Methods

2.1. Sampling

Crepidula coquimbensis is a poorly known and studied species. The distribution range of *C. coquimbensis* seems to be very restricted with only two natural populations reported for this species (Véliz et al., 2003). We conducted our study in one of these two known populations. Empty shells of marine snails hosting females and males of *C. coquimbensis* were collected in Puerto Aldea (30°17′32″ S, 71°36′ 30″W), Chile, during January 2006. The samples were transported to the laboratory in individual plastic bags filled with sea water in order to prevent the movement of males between shells. As we were interested in conducting a fine-scale paternity analysis at the clutch level, we examined a large number of embryos per female using five females rather than few embryos in a large number of females. Five hosting shells were haphazardly chosen from the sample for the subsequent analyses. In each hosting shell, one female and one to four males of different body sizes were found (Table 1). The size of the individuals defined as the longest length between the apex and the front part of the shell was measured to the nearest 0.1 mm. The cephalic portion of the males was preserved in ethanol (95%) for subsequent genetic analyses. The five females were kept alive in the hosting shells and maintained, isolated, in running, non-filtered sea water (14 °C) to allow feeding by filtration over the period of the experiment. In order to test for sperm storage and use, after six months of isolation under laboratory conditions, one additional male (average body size of 6.3 mm, SE ± 1.5; Table 1) was offered to each of the five females to determine the genetic contribution of the last mate to the fatherhood of the brood. This male, hereafter named 'extra-male' (EM), was collected from a different hosting shell from the same population in order to reduce the probability of previous copulations with the female. We used six months of isolation because this is the half time of the maximal time for sperm storage reported for a calyptraeid species (Hoagland, 1978). Also, this time period ensured for animal acclimation in order to preclude abnormal reproductive behavior. No capsule production was observed during this period. Within one month after the addition of the extra-male the five females had laid capsules. Three capsules per brood, representing roughly 10 to 15% of the total number of capsules per brood, were haphazardly selected and removed from each female. Embryos at the very early stage of each of the three capsules were preserved in ethanol (95%) for subsequent paternity analyses. Finally, the cephalic portion of each of the five females and extra-males was individually stored in alcohol (95%) for subsequent analyses.

2.2. DNA extraction and microsatelllite genotyping

Adult samples and embryos were washed in PBS prior to DNA extraction to remove residual ethanol content. DNA extraction from adults was performed using a Nucleospin®Multi-96 Tissue Kit (Macherey-NAGEL) following the manufacturer's protocol. We collected between 60 and 80 embryos from each capsule for DNA extraction which was performed following the protocol of Higuchi (1989): each embryo was incubated for 4 h at 60 °C with 100 µl of Higuchi buffer and 5 µl of proteinase K (10 µg/ml), followed by 15 min at 95 °C. This dilution was used to perform DNA amplification.

Samples (adults and embryos) were genotyped at five microsatellite loci (CcoqAC2F4, CcoqCT1H5, CcoqCT1F4, CcoqCT3F2 and CcoqCT1D10) previously characterized for *C. coquimbensis* by Daguin et al. (2007). The five loci used were highly polymorphic in the study population and displayed clear amplification patterns (i.e. clear banding patterns and allele size). Genotyping was carried out following the protocol detailed in Daguin et al. (2007). A minimum number of 56 embryos (from a total of three capsules) per female were genotyped for the five broods analyzed. All embryos were analyzed twice to avoid genotyping errors. Genetic analyses were carried out in the Biological Station in Roscoff (SBR), France.

2.3. Paternity and relatedness analyses

In order to assess multiple paternity within a capsule and brood, and the occurrence of skewed paternity (i.e. non-identical relative contribution of potential fathers to the clutch), paternity analyses were performed using the software CERVUS 2.0 (Marshall et al., 1998) based on the procedure proposed by Meagher (1986) using maximum likelihood calculations. Given the genotypes of the embryos, of their known mothers and of the potential fathers the paternity was assigned to the male with the highest log-likelihood ratio (LOD). To perform this analysis we compared embryo genotype with the genotype of every male sampled during the study; this means the 15 males collected from the five hosting shells, plus the 5 extra-

Table 1
Paternity assignments per capsules and broods for *C. coquimbensis*. Shell refers to the hosting empty shell in which one single female and their associated males were sampled. The percentage of embryos assigned to known males is indicated. Known males are the males sampled with the female (Mi–j; where i stands for the female/shell number and j for the male number within the shell i) or the extra-male (EMi, where i stands for the female number) used later for laboratory experiments (see Methods). For each father, the number (N) and percentage (%) of embryos assigned per capsule and brood (i.e. result over 3 capsules) is indicated. See Fig. 1 for an overview per brood.

Shell (female size, mm)	Assigned to a known male (%)	Fathers									
		Name	Size (mm)	Capsule 1		Capsule 2		Capsule 3		Brood	
				N	%	N	%	N	%	N	%
1 (24.1)	100.0										
		EM1	8.0	10	50.0	9	45.0	8	36.4	27	43.5
		M1-1	5.0	3	15.0	4	20.0	7	31.8	14	22.6
		M1-2	7.0	3	15.0	4	20.0	2	9.1	9	14.5
		M1-3	6.0	4	20.0	3	15.0	5	22.7	12	19.4
		All	–	20	100.0	20	100.0	22	100.0	62	100.0
2 (30.0)	86.0										
		EM2	5.0	21	46.7	19	42.2	23	50.0	63	46.3
		M2-1	6.0	18	40.0	19	42.2	17	37.0	54	39.7
		Unknown	–	6	13.3	7	15.6	6	13.0	19	14.0
		All	–	45	100.0	45	100	46	100.0	136	100.0
3 (22.8)	95.3										
		EM3	7.7	9	31.0	6	20.7	11	37.9	26	30.2
		M3-1	7.5	5	17.2	6	20.7	4	13.8	15	17.4
		M3-2	5.5	6	20.7	7	24.1	4	13.8	17	19.8
		M3-3	4.2	8	27.6	8	27.6	8	27.6	24	27.9
		Unknown	–	1	3.3	2	6.9	1	3.4	4	4.7
		All	–	29	100.0	29	93.1	28	96.6	86	100.0
4 (21.6)	100.0										
		EM4	6.0	5	27.8	6	33.3	5	25.0	16	28.6
		M4-1	6.2	2	11.1	3	16.7	1	5.0	6	10.7
		M4-2	3.6	4	22.2	3	16.7	6	30.0	13	23.2
		M4-3	4.4	3	16.7	3	16.7	2	10.0	8	14.3
		M4-4	5.4	4	22.2	3	16.7	6	30.0	13	23.2
		All	–	18	100.0	18	100.0	20	100.0	56	100.0
5 (24.1)	100.0										
		EM5	4.6	16	25.8	17	27.4	17	26.6	50	26.6
		M5-1	7.1	15	24.2	15	24.2	17	26.6	47	25.0
		M5-2	5.6	12	19.4	12	19.4	9	14.1	33	17.6
		M5-3	5.1	10	16.1	9	14.5	14	21.9	33	17.6
		M5-4	5.1	9	14.5	9	14.5	7	10.9	25	13.3
		All	–	62	100.0	62	100.0	64	100.0	188	100.0

males. One advantage of using CERVUS 2.0 is the possibility to assess the statistical significance of LOD through computer simulations (Marshall et al., 1998). These computations were carried out using 10,000 iterations based on population allelic frequency estimated using the genotypes of the 25 adults studied.

Percentage of participation of different males to the brood was compared using two approaches. Assuming that males transfer to the female an equal amount of sperm and that females mix viable sperm from the different males with which copulation occurred before and after their isolation, the relative contribution of the different fathers to the total brood should not differ significantly. If sperm mixing is occurring before capsule production, the paternity profiles (number and relative contribution of each male) should not be significantly different among capsules. Thus, the contribution of each male within capsule and between capsules was computed and compared using chi-square tests for each brood. In addition, the effective paternity index (K_E) recently adapted by Johnson and Yund (2007) from Bernasconi (2004) to examine if skewed paternity was occurring in the colonial ascidian *Botryllus schlosseri* was computed. This index comes from an index used by community ecologists (Simpson's diversity index; Simpson, 1949). K_E is defined as follows:

$$K_E = \frac{1}{\sum_{i=1}^{k} p_i^2}$$

where p_i is the proportion of offspring fathered by male i ($i = 1 \ldots k$, k being the total number of fathers participating to the brood). K_E is maximum (i.e. equals to k) when the relative contribution of the different fathers is equal (i.e. absence of skewed paternity).

In all the above analyses, only embryos assigned to a known male were used as we could not ascertain with accuracy the exact number of fathers contributed to the other embryos (see discussion below). Note that these embryos fathered by an unknown male represented only a very small proportion of the study embryos.

In cases where embryos were not assigned to a known father (i.e. all the males collected from the five hosting shells, plus the 5 extra-males), an estimation of the minimum number of fathers that accounted for the genotypes of the unassigned embryos was calculated using the software GERUD 2.0 (Jones, 2005). This program was designed to analyze progeny arrays that are known to have a single mother (or a single father), but the other parent is unknown. We performed the exhaustive search option using only the information from the pattern of Mendelian segregation with the maternal genotype known. Because of computing process limitations (as pointed out in the documentation of GERUD 2.0), only the three most polymorphic microsatellite loci (CcoqCT1F4, CcoqCT3F2, and CcoqCT1D10b) were used for these analyses.

Finally, because *C. coquimbensis* is a direct developer and a sedentary gastropod, we cannot exclude the hypothesis that, after hatching, the juveniles develop as adults in the parental hosting shells. We evaluated the relatedness between all the adults (males, extra-males and females) used for the experiment, within and among hosting shells, using pairwise microsatellite-based relatedness coefficient calculated with the ML-RELATE software (Kalinowski et al., 2006).

Table 2
Polymorphism of the five microsatellite loci over the 25 adults (five females and 20 males) used for the paternity analysis. Locus name, number of observed alleles (N_a), unbiased expected (H_e) and observed (H_o) heterozygosity are indicated for each locus and over the 5 loci used. The probability value of an exact test for the null hypothesis of Hardy–Weinberg equilibrium (P_{HW}) and the exclusion probability (P_{excl}, at 95% of confidence level) defined as the probability of excluding a randomly chosen unrelated candidate parent from parentage given the genotype of the offspring and of the mother are indicated for each locus and over all loci.

Locus	N_a	H_e	H_o	P_{HW}	P_{excl}
CcoqAC2F4	6	0.760	0.759	0.659	0.344
CcoqCT1D10	15	0.841	0.866	0.400	0.541
CcoqCT1F4	18	0.920	0.938	0.299	0.727
CcoqCT1H5	12	0.840	0.850	0.483	0.510
CcoqCT3F2	17	0.922	0.942	0.460	0.805
Over all loci	13.6	0.857	0.871	0.620	0.990
Standard deviation	4.8	0.067	0.075		

3. Results

3.1. Paternity analyses: from capsules to the brood level

The five loci used in this study showed a very high level of genetic diversity with a mean number of alleles per locus of 13.6 ± 4.8 over the 25 adults analyzed (5 females and 20 males; Table 2). Each adult was characterized by a unique multi-locus genotype. Altogether, the five loci used were polymorphic enough to show a paternity exclusion rate of 99% with a confidence level of 95% (Table 2). Analyses performed using the software ML-RELATE showed no relatedness (i.e. relatedness level equal to zero) between adults (males, extra-males and females) within and between hosting shells.

As the embryos used for paternity analysis were at a very early stage only a small quantity of DNA was available and some of the amplifications failed or were too faint despite several genotyping attempts. These embryos were discarded from the analyses. These failures were more important in some broods as compared to others and might be due to the storage procedure. There was no evidence for a systematic bias towards bigger or smaller embryos. Altogether, between 18 and 64 embryos per capsule (32–90% of the total number of embryos per capsule) were unambiguously genotyped (i.e. clear banding patterns and allele size) at each of the five loci and used for parentage assignment.

Null alleles can be a problem for assigning paternity with high confidence, but three observations make their occurrence very unlikely in the present study: (1) Hardy–Weinberg equilibrium was observed over the putative parental pool for each locus (Table 2), (2) when DNA from embryos could be amplified, the amplification was obtained for every locus and (3) maternal alleles were found in all amplified embryos and results across loci were always congruent when assigning parentage. Altogether, from a total of 528 embryos genotyped, 505 embryos (96%) were unequivocally assigned to a male either present in our field sampling (all males inhabiting one of the five hosting shells) or to the extra-males presented to the females after six months of isolation (Fig. 1, Table 1). Unassigned embryos were observed only in broods of females inhabiting hosting shells 2 and 3. One of the most striking results was that multiple paternities were observed in every brood and every capsule. The number of fathers identified in each brood ranged from two to five (Fig. 1), with an average of four males ($SD \pm 1.2$). For the unassigned embryos, a minimum numbers of one and two fathers were estimated for the females inhabiting hosting shells 3 and 2, respectively. Thus, all females analyzed used sperm from at least three different males to produce the studied capsules. It is noteworthy that the same combination (identity and number) of fathers was observed within each capsule and over the total brood (the same males were present in all capsules within the aggregations; Table 1). Moreover, the relative contribution of each male within capsules was the same between the three capsules analyzed for the five females studied (Chi-square tests: $P > 0.05$ in all cases).

3.2. Total individual male contribution to the brood

In every family analyzed, the extra-male was assigned as a father with a moderate to a large relative contribution (26–46%, Fig. 1). The percentage of eggs fertilized with stored sperm (i.e. from males collected in the hosting shells of the study females) ranged between 54 and 74%. Male contribution to the brood differed significantly from an even distribution only for one female (Female from shell 1: $X^2 = 12.2$; df = 3; $P = 0.007$) with most of the paternity allocated to

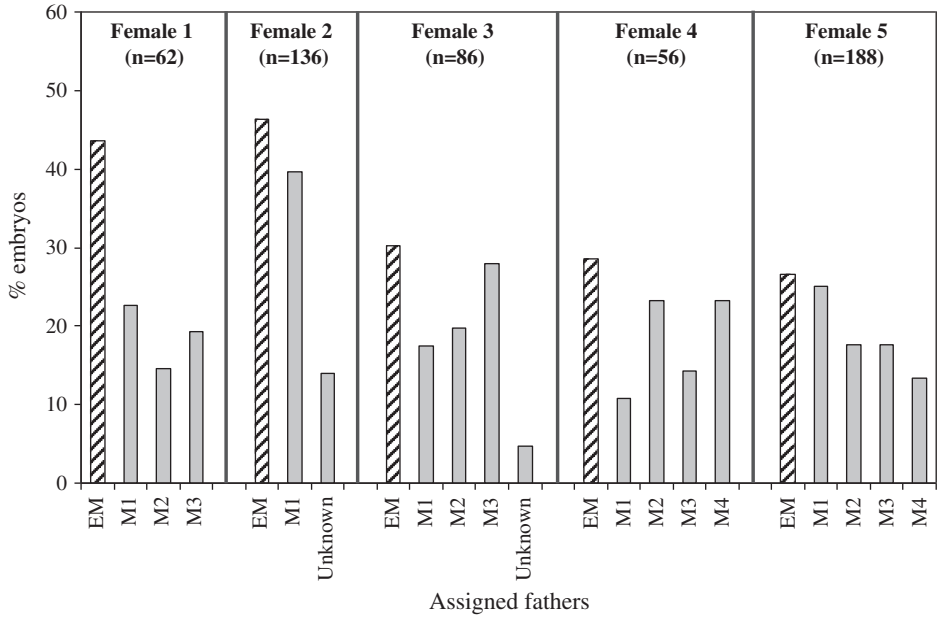

Fig. 1. *C. coquimbensis*. For each of the five broods analyzed, the total number of embryos genotyped per female is indicated within brackets. The percentage of embryos assigned to each father is given with males sampled in the hosting shell of the female indicated by grey bars and extra-males by hatched bars. Detailed information per capsule (three capsules per brood) is given in Table 1.

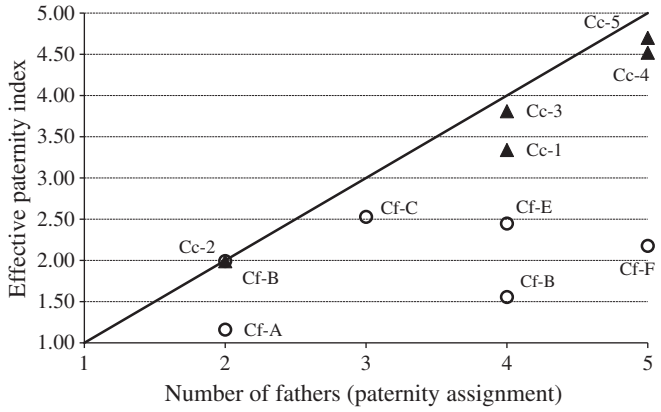

Fig. 2. Relationship between the effective paternity index K_E and the number of assigned father for *C. coquimbensis* (this study) and *C. fornicata* (computed from data provided in Le Cam et al., 2009). For each of the five broods examined in this study (black triangle) and six broods of *C. fornicata* (open circles) studied by Le Cam et al. (2009), the effective paternity index is indicated as a function of the number of assigned fathers. The black line is the hypothetical linear relationship under the assumption of identical contribution of each assigned father (slope = 1, intercept = 0). The labels refer to species and brood numbers: Cc and Cf for *C. coquimbensis* and *C. fornicata*, respectively, and numbers according to family name as indicated in Table 1 of this study and Table 1 in Le Cam et al. (2009).

the extra-male (Table 1; Fig. 1). For the remaining four females, the contribution of all males to the brood was not statistically different from an even distribution (Female from hosting shell 2: $X^2 = 0.7$; df = 1; $P = 0.41$; Female from hosting shell 3: $X^2 = 4.1$; df = 3; $P = 0.25$; Female from hosting shell 4: $X^2 = 5.8$; df = 4; $P = 0.21$; Female from hosting shell 5: $X^2 = 11.7$; df = 4; $P = 0.19$; Table 1). The effective paternity index K_E was ranging from 2.0 to 4.7 according to the brood but the K_E values were always very close or equal to the number of assigned fathers (Fig. 2). This suggests that the contribution of males to the brood is roughly identical.

4. Discussion

Paternity analyses carried out in this study showed the occurrence of sperm storage and multiple paternity in the marine gastropod *Crepidula coquimbensis*. In addition, the different fathers did not segregate among the capsules of a given brood but contribute to the three study capsules. Finally, for four of the five offspring arrays analyzed, the relative contribution of fathers to the brood did not deviate significantly from an even distribution. Computation of an effective paternity index also pointed out such a similar contribution of different males to the brood. These findings may have several implications in light of the reproductive system of *C. coquimbensis*.

We showed that females of *C. coquimbensis* may store viable sperm for at least six months and on average more than 60% of eggs in a brood may be fertilized by that sperm. Sperm storage has been widely reported for marine invertebrate species (e.g. Hoagland, 1978; Emery et al., 2001; Shaw and Sauer, 2004; Bishop and Ryland, 2005; Walker et al., 2007) including for other species of the genus *Crepidula*. Females of the congeneric species *C. fornicata*, may store viable sperm for long periods, more than one year in some cases (Hoagland, 1978; Gaffney and McGee, 1992; Dupont et al., 2006; Le Cam et al., 2009). The mechanisms by which females of *C. coquimbensis* store the sperm and use it for fertilization inside the reproductive tract are unknown. However, the fact that multiple paternity occurred in every capsules and that each assigned father at the brood level contributed to the three study capsules offers two possible explanations: (1) that sperm from different males are first stored separately then mixed before egg fertilization, or (2) that sperm received from different males is stored in only one compartment (and thus mixed with previous stored sperm). For *Busicon carica*, the paternity assignment of eggs within and between egg case-strings suggests that successive fertilization events normally occur as near random draws from a well, but not perfectly, mixed pool of gametes (Walker et al., 2007). More studies on the reproductive anatomy and the internal process of egg fertilization in *C. coquimbensis* are needed to determine these mechanisms and the role played by mate precedence on the reproductive output of males.

We observed here an average of at least four assigned fathers per brood for *C. coquimbensis*. This value is a minimum number of fathers at least for two of the five study broods for which we could not always assign fatherhood to the sampled males. These unknown fathers however only represented a small proportion of the total paternity assignment (i.e. 14% and 4.7% of the embryos were not assigned to a known male for female 2 and 3 respectively, see Table 1). In addition, for all the study broods, our estimates may be conservative given that in some capsules less than 50% of the embryos produced by the female were genotyped. When considering the fifth brood for which a large number of embryos was studied (i.e. 188), patterns observed were similar to the other broods: in particular, all known males contributed to the brood. This paternity assignment study in *C. coquimbensis* thus reinforces previous findings in marine invertebrates: intra-brood multiple paternity is commonly observed regardless of mating behaviors (Bishop et al., 2000; Toonen, 2004; Ayre and Miller, 2006; Johnson and Yund, 2007; Mäkinen et al., 2007).

In free-spawners, eggs produced by females are commonly fertilized by sperm from more than one male. Low to moderate multiple paternity levels have been documented in the brooding coral *Acropora palifera* (two to three males; Ayre and Miller, 2006) while the ascidian species *Diplosoma listeranium* and *Botryllus schlosseri* and the bryozoan, *Celleporella hyalina* provide evidence of high levels of multiple paternities (four to seven males; Yund and McCartney, 1994; Bishop et al., 2000; Pemberton et al., 2003). Similar levels of multiple paternities have been observed in marine invertebrate species exhibiting copulation and complex mating behaviors, in which females may potentially exert a mate choice (e.g. Shaw and Sauer, 2004; Toonen, 2004; Walker et al., 2007). For instance, 7.6 fathers per brood have been reported for the marine snail *Littorina saxatilis* (Mäkinen et al., 2007), exhibiting one of the highest levels of multiple paternity reported for a marine gastropod. At the genus level, our results for *C. coquimbensis* (four fathers on average) revealed a higher number of fathers as compared to the congeneric species *C. fornicata*: 1.8 fathers were reported by Proestou et al. (2008) in one American population and 3.1 fathers on average were reported by Dupont et al. (2006) in three European introduced populations. These differences in the level of multiple paternity observed between the two species might be first explained by a sample size effect. The number of larvae of *C. fornicata* analyzed was lower (on average per brood, 26 larvae in Proestou et al., 2008 and 11 in Dupont et al., 2006) than in our work (56 to 188 embryos per brood). The differences of the paternity level between the two species is however unlikely to be explained only by sampling size effects. A recent paternity study of *C. fornicata* by Le Cam et al. (2009) carried out with *C. fornicata* and using a mean number of 76 larvae per brood reported a mean number of 3.3 fathers per brood, a value close to the one previously reported by Dupont et al. (2006) in populations of *C. fornicata* from the same geographical area, but still slightly lower than the values observed in our study.

The differences in the male–female aggregation patterns may explain the higher level of multiple paternity observed in *C. coquimbensis* than in *C. fornicata*. In *C. coquimbensis*, the effective paternity index (K_E, see Cc in Fig. 2) was identical or very close to the number of assigned fathers. For *C. fornicata*, we estimated K_E from data available in Le Cam et al. (2009). As shown in Fig. 2 (see Cf data points), K_E was always lower than the number of assigned fathers, especially at higher number of fathers, indicating a differential male contribution to the brood. Adults of *C. fornicata* form perennial stacks in which the position of males with respect to females is usually

permanent over the life-time of the individuals (ca. 8 years). The male closest to the female has a highest probability of copulating with the female, increasing its individual reproductive success and skewing paternity. In contrast, *C. coquimbensis* does not form stacks and laboratory observations suggest mobility of the males (pers. obs.). All the males present with a female within a hosting shell have thus the same likelihood to get involved in a copulation event. This behavior may limit skewed paternity.

Male reproductive success may be largely influenced by other mechanisms in this protandrous species. Sex allocation theory indeed predicts that protandry will evolve if the reproductive value of females increases with their size and the reproductive value of males is independent of size (a proxy for the individual age) (Ghiselin, 1969; Charnov, 1982; Wright, 1988; Collin, 1995, 2006). As in other calyptraeid species (e.g. Chaparro et al., 1999), female fecundity of *C. coquimbensis* increases with body size (A. Brante, unpublished data). Paternity analyses are helpful to analyze male reproductive success. However, we could not properly test for the second prediction (i.e. no male size effect on their reproductive success) because of the low number of participant males, the narrow male size range in some families and a possible 'last-mate' effect due to the mating experiments (i.e. additional male effect). The absence of skewed paternity nevertheless suggests that male body size does not influence male reproductive success (see for instance brood 5 in Table 1). A dedicated study based on a different sampling procedure, with fewer embryos per female but more females and males, is needed to ascertain this hypothesis.

The life history, behavior and ecology of *C. coquimbensis* are still poorly documented and deserve further studies. Our study shed light on mating patterns occurring in one of the two reported populations of this species showing that every male sampled with a female was a successful father. None of the sampled males was found to mate with more than one female although male movements were observed in the laboratory. That 96% of the embryos analyzed were unambiguously assigned suggests that male mobility might be limited in the field over one reproductive season. Characterizing male mobility within and among successive reproductive seasons in *C. coquimbensis*, by experimental ecology or field observation, is an important objective to understand the ecology of this species. By moving among different hosting shells and fertilizing more than one female during a reproductive season, males could increase their reproductive success, compensate for the lack of pelagic larvae and reduce risks of inbreeding.

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