Access to multiple mates increases fecundity but does not affect per-offspring maternal investment in a marine gastropod

Rachel Collin[a] and Isis Ochoa

Smithsonian Tropical Research Institute, Balboa, Panama

Abstract. Females of many organisms mate more than once and with more than one male, suggesting that polyandry confers some advantage to the female or her offspring. However, variation in maternal investment in response to mate choice and mate number can confound efforts to determine if there are benefits of polyandry. Access to multiple mates could increase maternal investment in offspring via a number of different mechanisms. Few studies have determined if investment is influenced by mate choice and number, and data are particularly lacking for marine invertebrates. This study was designed to determine if maternal investment and offspring size increase with access to increasing numbers of mates in the protandrous intertidal slipper snail *Crepidula* cf. *marginalis*. Virgin female slipper limpets were exposed to one, three, or five potential mates and their fecundity, egg size, and hatchling size were measured for multiple clutches. Treatment had a significant effect on fecundity, with fecundity increasing with the number of potential mates. Treatment did not have an effect on the size of eggs or hatchlings, on the variation in egg size or hatchling size within broods, or on the frequency of oviposition. Treatment did alter the variation in average offspring size among females, but not in the way predicted by theory. The main result, that access to multiple mates does not have an effect on per offspring maternal investment, makes *C.* cf. *marginalis* an ideal candidate to study the effects of polyandry on offspring fitness without having to take into account confounding effects of variation in maternal investment.

Additional key words: larvae, life-history, maternal effects, maternal investment, polygamy

Females usually control the ultimate success of courtship and mating attempts by males (Eberhard 1996) and often mate more than once and/or with more than one male (polyandry) (Arnqvist & Nilsson 2000; Hosken & Stockley 2003; Simmons 2003, 2005; Gowaty et al. 2010; Avise et al. 2011; Slatyer et al. 2012). It is likely that multiple mating confers some advantage to the female or her offspring (Gwynne 2008; Gowaty et al. 2010), and many theoretical and empirical studies have aimed to identify these advantages. Females also often control the amount of resources allocated to their offspring by manipulating egg size and or extra-embryonic resources (Collin & Spangler 2012; McDonald et al. 2014; Pélabon et al. 2015). Mate choice and polyandry can be linked to variation in maternal investment (Cunningham & Russell 2000; Slatyer et al. 2012; Pélabon et al. 2015). Increased maternal investment in association with increased number of

mates is relevant to our understanding of the evolution of mating strategies, but also confounds evaluation of the genetic benefits of polyandry (Simmons 1987; Cunningham & Russell 2000).

Behavioral and genetic studies demonstrate high levels of polyandry in many invertebrates (e.g., Avise et al. 2011), but little is known about how this influences female investment in offspring or offspring fitness (Slatyer et al. 2012). For example, a recent meta-analysis of genetic benefits of polyandry included only three publications on free-spawning marine invertebrates, and no papers on marine invertebrates with copulation or protected development (Slatyer et al. 2012). Effects of polyandry or access to variable numbers of potential mates on maternal investment have been examined in two marine invertebrates, the simultaneous hermaphroditic sea slug *Chelidonura sandrana* Rudman 1973 (Sprenger et al. 2008a,b) and the dioecious dumpling squid *Euprymna tasmanica* (Pfeffer 1884) (Squires et al. 2012). Only a handful of studies with

[a]Author for correspondence. E-mail: collinr@si.edu

marine invertebrates have examined other aspects of reproductive success affected by number or choice of mates. As marine invertebrates have a vast array of reproductive and life-history strategies, documenting effects of mate selection and polyandry on offspring quality and quantity in these groups will allow comparisons among more evolutionarily independent lineages and will help to extend current understanding to novel sexual systems.

There are several reasons why maternal investment and/or offspring quality are expected to increase with a female's access to increasing numbers of potential or realized mates. Females should invest more in offspring when their breeding attempt is likely to succeed (Williams 1966; Stearns 1992) and success can be linked to paternal quality. Females from diverse taxa are known to increase maternal investment in the quality and number of eggs or offspring when they mate with relatively attractive males (Cunningham & Russell 2000; Sheldon 2000; Galeotti et al. 2006; Evans et al. 2010; Horvathova et al. 2012). Many examples come from studies of birds (Ratikainen & Kokko 2010; Horvathova et al. 2012). For example, mallard ducks lay larger eggs that produce offspring with better body condition when paired with preferred males than when paired with non-preferred males (Cunningham & Russell 2000). Likewise, when crickets are allowed to choose a mate they increase the number of eggs produced, and the offspring reach maturity earlier than when the females are given no choice (Simmons 1987). Presumably the more males a female can choose from, the better able she is to select a more highly preferred partner and the greater her expected reproductive investment.

This effect has been most clearly demonstrated when females are allowed to mate with only one male. However, post-copulatory cryptic female choice could lead to a similar result when females mate with multiple males in non-resource based mating systems (Slatyer et al. 2012). Since polyandry increases the opportunity for sperm competition, as the level of polyandry increases so should the likelihood that a female has acquired and used preferred sperm to fertilize her eggs. If she adjusts egg size in response to the "quality" of the sperm she uses, then polyandrous females are more likely to invest more in their offspring on average compared to the investments made by females mated to only one male.

Finally, even if females are unable to identify or actively select for fertilization by preferred males, polyandrous females may still invest more highly in offspring than monandrous females. In an unpredictable environment the expected mean success of the genetically diverse offspring of a polyandrous female may be higher than the less genetically diverse offspring from a single sire (diversifying bet-hedging) (Fox & Rauter 2003; Garcia-Gonzalez & Simmons 2005).

These three scenarios all predict greater investment in offspring when females have access to a greater number of potential mates. However, they make slightly different predictions about the variation in maternal investment. When females can employ pre- or post-copulatory choice, (1) variation in sire quality will decrease as the number of potential or actual mates increases, and (2) variation in genetic compatibility will decrease as the number of potential or actual mates increases. Therefore, average per offspring investment is predicted to vary less among polyandrous females or those that have been allowed to select mates than among females offered a single mate (Garcia-Gonzalez & Simmons 2005). In scenarios where females cannot select for fertilization by preferred males, within- and among-female variance in maternal investment should not be affected by number of potential sires (Sprenger et al. 2008a). Offspring quality due to paternal effects, however, should vary more within broods of multiply than singly mated females (Sprenger et al. 2008a).

The present study was designed to determine if maternal investment and/or offspring size increase with access to increasing numbers of mates in the protandrous intertidal slipper snail *Crepidula* cf. *marginalis*. We measured clutch size, egg size, and clutch frequency to determine whether access to one, three, or five potential mates altered female investment in overall reproduction and in individual eggs. We measured the size of veliger larvae at hatching to determine if there was an effect on offspring quality that was not evident in the measures of egg size. We also determined whether access to more mates increased variation in maternal investment in individual eggs within and among females.

Methods

We used *Crepidula* cf. *marginalis*, a common calyptraeid species from the intertidal of the Pacific coast of Panama, to determine if the number of potential mates contributing to a brood influences offspring size, number, and variability. These animals live under rocky rubble on sandy muddy substrate in the mid-intertidal. They often occur in dense patches with over 100 animals per square meter (Collin & Ochoa, unpubl. data). They can occur alone, in pairs with a male positioned on top

of a female, less often in triplets with two males on top of a single female, or in clusters with several males on the substrate adjacent to a female or group of females. Large females do not appear to move and often grow to match irregularities in the substrate. Smaller males are quite mobile in the laboratory, but the extent of their movement in the field is unknown. This species has been shown to alter patterns of sex allocation in response to different associations with conspecifics as well as to changes in nutrition (Mérot & Collin 2012a,b; Carrillo-Baltodano & Collin, in press). Females can adjust their maternal investment by altering egg size, brood size, and frequency of brooding in response to temperature (Collin 2012). Life-history responses to other environmental conditions have not been documented.

We collected 190 small animals at Chumical (8°30′N, 79°40′W) near the town of Veracruz, in 2010. Small males (<7 mm shell length) were assigned to pairs and placed in 350 ml cups filled almost to the top with filtered seawater. They were fed 50×10^3 cells ml^{-1} *Isochrysis galbana* daily, kept at 23°C in incubators, and the water was changed 3 times a week (following Collin 2012; Mérot & Collin 2012a,b). Because *Crepidula* are protandrous, the smaller individual in each cup remained male while the larger one became female (Mérot & Collin 2012a,b). When kept under these conditions *C.* cf. *marginalis* produce a brood of transparent capsules roughly every 3 weeks and can continue reproducing with decreasing frequency for up to a year (Collin 2012). Females brood 12–40 capsules between the walls of the container, the neck, and the propodium. At 23°C hatching occurs after 10 d, and a subsequent brood is deposited, on average, 8 d later (Collin 2012).

When the larger animals began to reach the size at which sex change can occur (~10–12 mm; Mérot & Collin 2012a,b), each cup was assigned to one of three treatments: single mate, three potential mates, or five potential mates. In the treatments with multiple potential mates, we used groups of three of five pairs and moved each male from each cup to the subsequent cup in the same group three times a week (Fig. 1). In this way, each female in the group shared the same three of five partners, but was exposed to each one at a time. Every male was continuously paired with a female. We continued the treatment until all measurements had been collected for each female in the group, or until a member of the group died, at which point data collection from females in that group ceased. In the single male treatment the male was removed and replaced in the
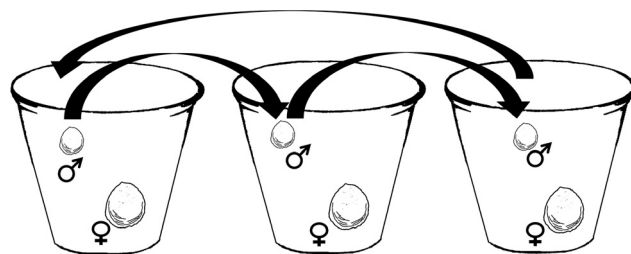

Fig. 1. Schematic of the experimental treatment with three potential mates, showing how the males were transferred between the cups. The treatment with five potential mates was organized in the same way.

same cup to act as a control for physical disruption experienced in the other two treatments.

We could not count the number of copulations with each potential mate because copulation in calyptraeids may take several hours, and it is virtually impossible to observe or to determine if sperm is transferred without disrupting the mating attempt. However, it is well documented that calyptraeids can store sperm from multiple males for several months and that broods from field-collected females are usually sired by multiple males (Dupont et al. 2006; Proestou et al. 2008; Daguin-Thiebaut et al. 2009; Brante et al. 2011). Microsatellite studies document 2–5 sires per brood for *Crepidula fornicata* (LINNAEUS 1758) and for *Crepidula coquimbensis* D.I. BROWN & OLIVARES 1996 (Dupont et al. 2006; Proestou et al. 2008; Daguin-Thiebaut et al. 2009; Le Cam et al. 2009; Brante et al. 2011), both close relatives of *C.* cf. *marginalis* (Collin 2003, 2004), and 1–4 sires (average 1.8) per brood in *Crepidula convexa* SAY 1822 (Le Cam et al. 2014). Some broods in each study included offspring that were sired by males that were not associated with the females when they were collected, or with individuals which were associated but had already become female. This shows that females can successfully use sperm that has been stored for several months and that paternity is not always attributed to the most recent copulation.

We have observed that when males are introduced to virgin females, copulation and oviposition often occur within the first several days, indicating that prolonged access to a mate is not necessary for successful copulation. We are therefore confident that these treatments allowed females ample time to choose among and to copulate with different numbers of males over the course of the experiment. We do not know, however, if they did so or to what extent copulation and fertilization are under female control. It appears that females can prevent or

curtail mating by clamping down and bringing the shell margin into contact with the substrate; however, this also prevents the water currents necessary for feeding, so is unlikely to deter a persistent partner. What level of post-copulation cryptic female choice occurs is unknown for this or any other calyptraeid.

We measured the diameters of uncleaved eggs for three broods from each female. Once these measures were obtained, we counted the number of capsules and eggs per capsule for three capsules from each of the subsequent three broods. Finally, we collected hatchlings from two additional broods to measure the shell length of the veliger larvae the day they hatched. Embryos do not generally develop normally away from the mother; therefore, it was not possible to obtain these data all from the same brood. In some cases, cleavage had already begun when we collected eggs from the first broods, so the egg diameter measures come from three of the first four, or in some cases five, broods produced by each female. The hatchlings come from the 6th through 10th broods produced by each female. Eggs and hatchlings were photographed for each brood and measured with Image J (Abràmoff et al. 2004). The shape-descriptor plug-in was used to measure the major and minor axes of the eggs. The diameter was calculated as the average of these two measures for eggs with a roundness (measured area/π (measured radius)^2) greater than 94 to ensure cleavage had not begun. Shell length of veligers was measured using the Feret (i.e., longest) length of the shape, which corresponds to the traditional shell length measurement used for larval gastropods. Whenever possible, at least 20 uncleaved eggs or hatchlings were measured.

Significant effects of mating treatment on egg diameter and hatchling length were tested for using restricted maximum likelihood (REML) and expected mean squares (EMS) ANCOVA implemented in JMP (version 9.0.2). To test exclusively for the effects of treatment, we used treatment as a fixed effect, maternal identity (hereafter "dam") as a nested random effect within treatment, and brood number as a nested random effect within dam. Maternal length at the time the eggs were produced or hatchlings collected was included as a covariate. The interaction between treatment and maternal length was also included. Because previous studies have found effects of polyandry change with brood number, we conducted a similar analysis using average egg diameter or hatchling length for each brood as the dependent variable, and with treatment and brood number both as fixed effects and female

length as a covariate. All interaction effects were included, and the removal of non-significant interaction terms from the analyses did not alter the results. Sample sizes for these analyses were somewhat smaller as the same broods could not always be measured for each female, and the analysis was limited to broods 1–4 for egg diameter and 6–9 for hatchling length.

To test the hypothesis that access to more potential mates increases the within-brood variation in egg diameter or hatchling length the coefficient of variation (CV) was calculated for each brood and ANCOVA was used to test for the fixed effects of treatment and brood number and the covariate of maternal length on within-brood CV. To test the hypothesis that access to more potential mates decreases variation among females, the CV of the mean egg diameter or hatchling length was calculated for each female in each treatment. An exhaustive set of n − 1 jackknifed datasets of female means were created for each treatment and the 95% confidence interval of the CV was calculated from these sets. In addition, 100 replicate datasets of female means randomized across the three treatments were used to generate the 95% confidence intervals of the random expectation.

One-way ANOVA with treatment as a fixed effect was used to determine if the number of potential mates affects female size at first oviposition, and the average rate of brood production over the first four broods. Brood size was calculated as the number of capsules times the average number of eggs per capsule. ANOVA was used to test for the fixed effect of treatment and brood number as fixed effects and maternal size as a covariate.

Residuals from all models were checked for deviations from a normal distribution using either the Shapiro–Wilk W-test when sample sizes were less than 2000, or the Kolmogorov–Smirnov test for sample sizes larger than 2000. Brood size and CV in hatchling length were log-transformed to comply with the normality requirement. However, transformations failed to bring CV in egg diameter and brood frequency into compliance with normality and therefore the results of 1-way non-parametric tests are reported for these variables.

Results

Egg diameter

We measured 6019 eggs from 265 broods from 91 females. In the first model, 42% of the variation was explained by the random effect of dam nested

Table 1. Analysis of covariance table showing the effect of multiple potential mates on egg size in *C.* cf. *marginalis*. Bold p-values are statistically significant (p≤0.05). Model 1 was analyzed with restricted maximum likelihood (REML) and Model 2 with Expected Mean Squares (EMS) estimations.

	df	df Den	F ratio	p	% var.
Egg size—model 1					
Treatment	2	85.2	0.3	>0.7	
Dam [treatment] random	—	—	—	—	42.1
Brood number [dam] random	—	—	—	—	40.0
Maternal length	1	391.6	31.3	**< 0.0001**	
Treatment×maternal length	2	134.8	0.8	>0.4	
Model r^2=0.85					
	df	SS	F ratio	p	
Egg size—model 2 (brood means)					
Treatment	2	111.5	1.0	>0.3	
Maternal length	1	2756.3	50.2	**< 0.0001***	
Brood number	3	99.2	0.6	>0.6	
Treatment×brood number	6	327.1	1.0	>0.4	
Treatment×maternal length	2	44.4	0.4	>0.6	
Brood number×maternal length	3	44.5	0.3	>0.8	
Brood number×maternal length×treatment	6	293.6	0.9	>0.5	
Model r^2=0.23					

*Stepwise removal of the non-significant interaction terms does not increase significance of non-significant factors.

within treatment, 40% was explained by the effect of brood nested within dam, and 85% of the overall variation was explained by the model. Treatment did not have a significant direct effect on egg diameter (p>0.5), nor did the interaction between treatment and maternal size. However, egg diameter did increase significantly with maternal shell length (p=0.002) (Table 1; Fig. 2). Likewise, the second model showed a significant effect of maternal size but no significant effect of any of the other variables or interactions. Average egg diameters were 175.49 μm (s.e.=1.14) for single males, 176.81 μm (s.e.=1.16) for three males, and 176.42 μm (s.e.=1.15) for five males. Power analysis of the ANCOVA shows a 95% probability of detecting a 1.8-μm effect and a 50% chance of detecting a 1-μm effect in both treatment and brood number. Effects smaller than this may not be biologically meaningful.

Length of larvae at hatching

A similar result was obtained for the length of larvae at hatching. We measured 3162 larvae from 164 broods from 81 females. In the first model, 43% of the variation in hatchling length was explained by the random effect of dam nested within treatment, and 31% of the variation was explained by the random effect of brood nested within dam.
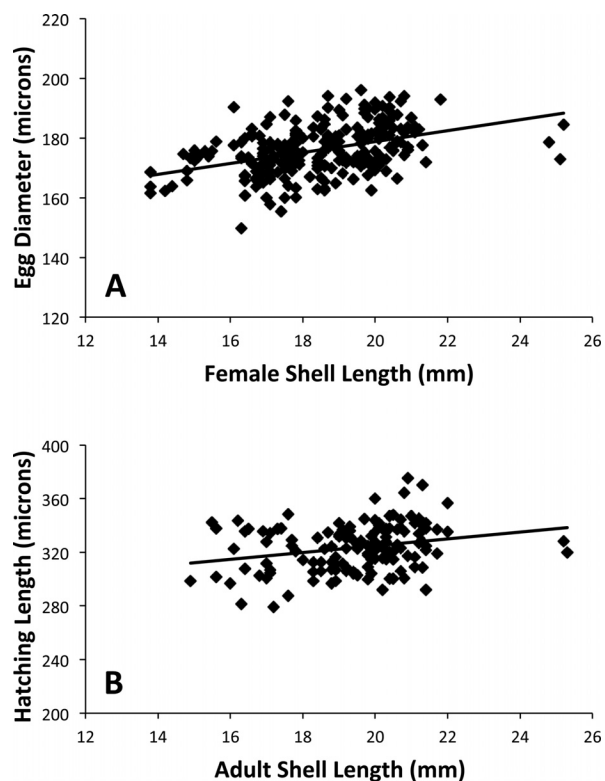

Fig. 2. The relationship between maternal length and (**A**) average egg diameter (r^2=0.17; n=248; p<0.0001; egg diameter=1.83*maternal length+142) and (**B**) average hatchling length (r^2=0.06; n=140; p=0.002; hatchling length=3.03*maternal length+264) per brood.

Table 2. Analysis of covariance table showing the effect of multiple potential mates on egg size in *Crepidula* cf. *marginalis*. Bold p-values are statistically significant (p≤0.05). Model 1 was analyzed with restricted maximum likelihood (REML) and Model 2 with Expected Mean Squares (EMS) estimations.

	df	df Den	F ratio	p	% var.
Hatchling size—model 1					
Treatment	2	80.0	1.5	>0.2	
Dam [treatment] random	—	—	—	—	42.8
Brood number [dam] random	—	—	—	—	30.7
Maternal length	1	82.7	9.3	**0.003**	
Treatment×maternal length	2	82.7	0.7	0.5	
Model r^2=0.76					
	df	SS	F ratio	p	
Hatchling size—model 2 (brood means)					
Treatment	2	382.9	0.7	>0.5	
Maternal length	1	1613.1	5.7	**0.018***	
Brood number	3	238.9	0.3	>0.8	
Treatment×brood number	6	1171.7	0.7	>0.6	
Treatment×maternal length	2	203.2	0.4	>0.6	
Brood number×maternal length	3	1461.7	1.7	>0.1	
Brood number×maternal length×treatment	6	1279.3	0.76	>0.6	
Model r^2=0.20					

*Stepwise removal of the non-significant interaction terms does not increase significance of non-significant factors.

The model explained 76% of the overall variation. There was no significant effect of treatment or interaction between treatment and maternal length on hatchling size (Table 2). However, hatchlling length did increase with maternal length (p<0.001) (Table 2). Likewise, the second model detected a significant effect of maternal length on hatchling size, but no effect of treatment, brood number, or interactions between any of the variables (Table 2). Average hatchling lengths were 320.88 μm (s.e.=2.93) for females mated to one male, 323.68 μm (s.e.=2.91) for females with three males, and 326.76 μm (s.e.=2.81) for females with five potential mates. Power analysis showed that with the observed data distribution there is a 95% probability of detecting a difference of 5 μm and a 50% probability of detecting a difference of 3 μm for treatment, and 50% probability of detected a difference of 3.4 μm and 95% probability of detecting a difference of 6 μm for brood number.

Variation

Number of potential mates did not alter the within-brood variation in either egg size or hatchling size. Within-brood CV of egg diameter could not be transformed to conform to the normality assumptions of ANOVA. Non-parametric Kruskal–Wallis tests failed to detect effects of treatment, brood number, or maternal length (Table 3). Within-brood CV ranged across the treatments from 1.76 (s.e.=0.08) for three males to 1.89 (s.e.=0.10) for one male, with an intermediate CV of 1.85 (s.e.=0.08) for five potential mates. Power analysis for the Kruskal–Wallis test showed there is a 95% probability to detect an effect size of 0.22. Likewise, ANCOVA failed to detect a significant effect of treatment on the within-brood CV of hatchling shell length (Table 4). Within-brood CVs of hatchling size were: 2.96 (s.e.=0.19) for one male, 2.53 (s.e=0.11) for three males, and 2.74 (s.e.=0.13) for five males. There was no significant effect of brood number (Table 4). Maternal length did have a significant effect on within-brood CV of hatchling shell length; however, this only accounted for 0.6% of the variation. The effect was due entirely to the

Table 3. Non-parametric Kruskal–Wallis analyses to determine the effects of treatment and brood size on egg diameter, and correlation analysis to determine the effect of maternal length on egg diameter.

	df	Chi-square	p
CV egg diameter			
Treatment	2	0.4	0.42
Brood number	3	4.6	0.20
Maternal length	1		0.38

Table 4. Analysis of covariance of the coefficient of variation of hatchling length after stepwise removal of non-significant interactions. Bold p-values are statistically significant (p≤0.05).

	df	F ratio	p
log(CV Hatchling length)			
Treatment	2	1.3	0.26
Brood number	3	0.36	0.78
Maternal length	1	5.3	**0.02***

*r^2=0.06, but effect due entirely to one particularly large female.

Table 5. Coefficient of variation of female means in egg diameter and hatchling length, and the 95% jackknife confidence intervals.

Treatment	CV of female means	95% confidence interval from (n − 1) Jackknife samples	95% confidence interval from randomized female means
Egg diameter			
Single	3.86	3.82–3.89	3.61–3.85[a]
Triple	3.68	3.65–3.72	3.68–3.83
Quintuple	3.82	3.79–3.85	3.68–3.81[a]
Hatchling length			
Single	4.91	4.85–4.98	4.62–5.30
Triple	4.85	4.81–4.89	4.95–5.19[b]
Quintuple	5.32	5.26–5.38	4.77–5.00[a]

[a]Observed CV is larger than estimated 95% confidence interval of randomized data.
[b]Observed CV is smaller than estimated 95% confidence interval of randomized data.
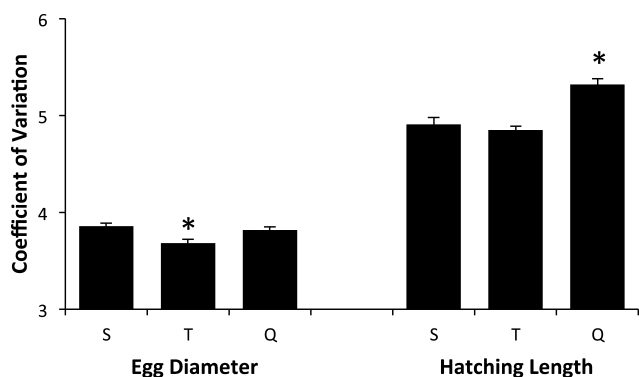

Fig. 3. The relationship between mating treatment (S=one mate; T=three mates; Q=five mates) and coefficient of variation in egg size and hatchling size. Error bars indicate 95% confidence interval calculated from jackknifing. Asterisks (*) indicate significant difference from the other treatments.

effect of one particularly large outlying female. When she was removed from the analysis the effect of maternal size on variation in hatchling size was no longer significant.

The hypothesis that multiple mates could reduce the among-female variation in egg size and in offspring size was not supported by our data (Table 5; Fig. 3). The observed CV of egg diameter for females with three potential mates was smaller than for females with one or five potential mates (Fig. 3). These values were also marginally different from the random expectation. In addition, females with five potential mates showed significantly higher CV in mean hatchling size than those with one or three potential mates. Finally, the CV of mean hatchling size was significantly larger than expected if female means were distributed at random (Table 5).

Fecundity

Treatment had some effect on reproductive output of females. The frequency of brood production was not affected by treatment (Kruskal–Wallis test; Chi-square=0.10; df=2; p=95; n=88), with females in each treatment waiting an average of 18 d between each of their first four broods. Treatment had no significant effect on the size at first oviposition (one-way ANOVA; df=2; F=0.75; p=0.47; n=158), with females in all three treatments laying their first eggs at an average shell length of 17.4–17.9 mm. Treatment did have a statistically significant effect on brood size (Table 6; Fig. 4). Females exposed to only a single mate averaged 1365 (s.e.=63.6) eggs per brood while those exposed to three or five potential mates averaged 1530 (s.e.=65.6) and 1576 (s.e.=68.4), respectively. Maternal length and brood number had no effect on brood size (Table 6), nor did their interactions. The test for significant effects of brood number had 1.0 power to detect an effect of size 0.5 in log (brood size).

Table 6. Analysis of covariance of brood size after stepwise removal of non-significant interactions. Bold p-values are statistically significant (p≤0.05).

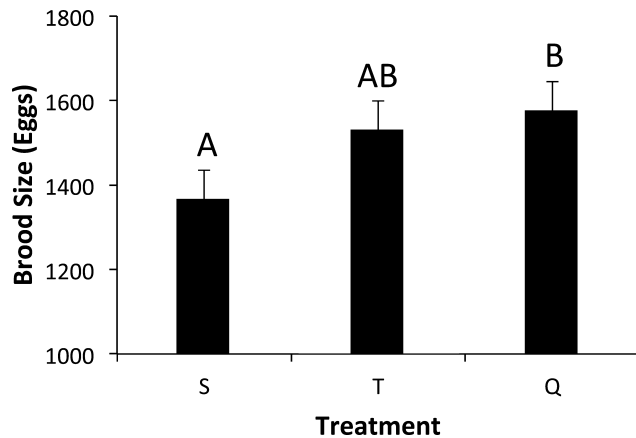|	df	F ratio	p
log (brood size)			
Treatment	2	0.53	**0.05**
Maternal length	1	0.02	0.61
Brood number	3	0.34	0.25

Fig. 4. The relationship between brood size and mating treatment (S=one mate; T=three mates; Q=five mates). Bars sharing the same letter do not differ significantly (p<0.05) in post-hoc Tukey HSD tests. Error bars indicate standard error.

Discussion

The number of potential mates to which females of *Crepidula* cf. *marginalis* were exposed had a significant effect on brood size, but no discernable effect on female investment in individual offspring. Exposure to one, three, or five mates did not alter the frequency with which clutches were produced or the maternal size at which clutches were first produced. Nor did it have a statistically significant effect on egg size or hatchling size. However, females exposed to five mates produced larger clutches than did those exposed only to a single mate, and those exposed to three mates produced intermediate-sized clutches.

Maternal investment in individual offspring

We found no discernable effect of mating treatment or brood number on female reproductive investment in the size of individual eggs or hatchlings in *C.* cf. *marginalis*. However, there was a significant positive correlation between maternal length and both egg size and hatchling size (Fig. 2). This was not unexpected: such a relationship is common among many organisms (Rollinson & Rowe 2015), and previous work with *C.* cf. *marginalis* also detected an increase in egg size with maternal shell length (Collin 2012). The previous study did not, however, detect a relationship between maternal size and hatchling length.

Impacts of polyandry or access to different numbers of potential mates on maternal investment in individual offspring have been examined in two other marine invertebrates (Sprenger et al. 2008a,b; Squires

et al. 2012). The relationship between hatchling size, egg size, and polyandry is complicated in the dumpling squid *Euprymna tasmanica* (Squires et al. 2012). Eggs of singly mated squid were significantly larger than eggs of squid mated twice to the same male, but eggs from squid mated once each to two different males did not differ from either of these other treatments. Hatchlings showed no effect of treatment. Therefore, no impact of the number of individual mates on maternal investment in individual offspring was detected in this species. As only one or two mates were tested, trials with larger numbers of mates could alter this pattern. In the sea slug *Chelidonura sandrana,* mate number does affect maternal investment (Sprenger et al. 2008a,b). Slugs assigned one, two, four, and eight mates over a 48 h period produced egg masses with the largest egg capsule volume, a proxy for hatchling size, at the intermediate mating rate (four mates) for the first egg mass produced in the laboratory. No significant effect was detected for subsequent masses (Sprenger et al. 2008b). In a similar study comparing females that were singly mated, mated four times with a single male, and mated once each by four males, the first egg mass showed an increase in both egg capsule volume and larval length for slugs with multiple mates (Sprenger et al. 2008a). Again, this effect was not evident for subsequent masses (Sprenger et al. 2008a).

In the studies with both the squid and the sea slug matings were actually observed, so these experiments manipulated both the identity of potential mates and the actual occurrence of matings. Due to the nature of *C.* cf. *marginalis* we were not able to observe copulations and therefore could only manipulate access to potential mates. However, the number and duration of copulations may be misleading proxies for sperm transfer and realized paternity. For example, in *Chelidonura sandrana* the duration of copulation does not correlate with the amount of sperm transferred (Lange et al. 2012a). In addition, it is well known that post-copulatory processes can influence realized paternity (Eberhard 1996). It is therefore important to interpret the results from *C.* cf. *marginalis* as indicating only a lack of impact of access to different numbers of mates and not different numbers of copulations on per offspring investment, while the two previous studies examined the impact of different numbers of actual copulations and copulatory partners on per offspring investment.

Offspring number

The effects of multiple mates on female fecundity in aquatic invertebrates are somewhat better

documented than the effects on per offspring investment, but published data are still sparse. Contrary to the results of this study, the few studies available suggest that there usually is either no relationship or a decreasing relationship between fecundity and the number of male partners or the rate of copulation. No relationship between fecundity and number of potential mating partners was found in the leech *Helobdella papillornata* Govedich & Davies 1998, where total egg volume in a brood was not affected by group size (Tan et al. 2004), and in the flatworm *Macrostomum lignano* Ladurner, Schärer, Salvenmoser, & Rieger 2005, where group size did not affect the number of offspring produced over 20 d following isolation from the group (Janicke et al. 2011). A reduction in fecundity with polyandry has been demonstrated in several heterobranch gastropods where hypodermic insemination is thought to damage the female partner. Lange et al. (2012b) found no effect of mating rate on capsule size in the sea slug *Siphopteron quadrispinosum* Gosliner 1989, but the number of eggs per egg mass was highest at the intermediate mating rate of one opportunity per day, compared to 0.3 and three chances per day. Egg production in the sacoglossan sea slug *Alderia willowi* Krug, Ellingson, Burton & Valdés 2007 increases for field-caught animals kept separately compared to slugs kept in pairs (Smolensky et al. 2009). Higher mating rates, independent of the number of individual mates, resulted in lower fecundity but higher investment per egg in the pond snail *Lymnaea stagnalis* (Linnaeus 1758) (Hoffer et al. 2012). In *Chelidonura sandrana*, fecundity decreased with mating rate, suggesting that although females may sometimes allocate more to individual offspring at intermediate mating rates, multiple matings reduce overall reproductive investment (Sprenger et al. 2008a,b). In some taxa, more complex relationships exist. For example, in the nematode *Caenorhabditis remanei* (Sudhaus 1974), female fecundity increased but survival decreased with the number of males in the group (Diaz et al. 2010). In none of these cases was there a clear monotonic increase in fecundity with increased mating or mate availability. Our data from *C.* cf. *marginalis* demonstrate an increase in fecundity with access to more potential mates (up to five). However, we cannot rule out a subsequent decrease at even higher numbers of potential mates, as observed in *S. quadrispinosum*.

Offspring quality

In contrast to egg size and egg number, which are expected to primarily reflect maternal investment,

hatchling size and variation in hatchling size could also reflect effects of maternal and paternal genomes and the genetic compatibility between the two. Because the number of potential mates had no significant effect on egg size in *C.* cf. *marginalis*, a significant effect of mating treatment on hatchling size would have given unambiguous support for some kind of genetic effect of polyandry on offspring size. However, we did not detect any effect of treatment on the shell length of hatchling larvae. Our study was primarily designed to test for differences in maternal investment rather than genetic effects on offspring fitness, and therefore we did not measure realized paternity or any additional components of offspring fitness.

Although offspring size did not increase in the females exposed to multiple males, it is possible that multiple paternity could contribute to increased larval growth, or to more subtle measures of fitness or performance that are not dependent on maternal investment. Such effects can be complicated and difficult to demonstrate. For example, microsatellite paternity analysis of larvae of *C. fornicata* showed that the difference in growth rates between the fastest and slowest growing larvae from a single brood was significantly correlated with the level of multiple paternity in the brood, and negatively correlated with the degree of relatedness among larvae in the brood (Le Cam et al. 2009). However, no particular sires were associated with either fast- or slow-growing larvae (Le Cam et al. 2009). It is also possible, but unlikely based on what we know about related species, that access to multiple partners did not result in multiple paternity in *C.* cf. *marginalis*. Such an outcome could also account for a failure to detect differences in larval size at hatching.

Few studies have examined the effects of polyandry on aquatic invertebrate hatchling size and larval performance. In *Chelidonura sandrana* the significant effect of polyandry on larval size appears to be due to altered maternal investment. The increase in capsule size in polyandrous females is reflected in a significant increase in hatchling size for the first brood only (Sprenger et al. 2008a), but mating treatment had no effect on time to hatching or larval survival (Sprenger et al. 2008a,b). The relationship between hatchling size, egg size, and polyandry is more complicated (see above). It seems, in this case, that the mating treatment decouples the relationship between egg size and hatchling size (Squires et al. 2012), and the level of polyandry did not ultimately affect offspring quality. In free-spawning invertebrates, where levels of polyandry cannot affect maternal invest-

ment and where frequency of copulation is not a potential confounding factor, exposure of eggs to sperm of multiple males commonly results in higher fertilization rates than similar concentrations of sperm from a single male (Evans & Marshall 2005; Marshall & Evans 2007; McLeod & Marshall 2009). The only case where exposure of eggs to sperm from multiple males has been demonstrated to result in improved offspring quality is the higher hatching success and larval survival in the serpulid polychaete *Galeolaria caespitosa* Lamarck 1818 (McLeod & Marshall 2009). Higher hatching success in polygamous spawns of the tunicate *Pyura stolonifera* (Heller 1878) was accounted for entirely by the differences in fertilization success, and there is no evidence of improved offspring quality in this species (Marshall & Evans 2007).

Conclusions

Access to multiple mates can result in increased maternal investment in offspring via a number of different mechanisms. Offspring quality is also expected to improve when females have access to multiple mates, over and above the effect of increased maternal investment. However, *Crepidula* cf. *marginalis* showed no evidence of increased maternal investment in individual eggs or evidence of increased offspring quality, despite a clear increase in fecundity with multiple mates. The results for *C.* cf. *marginalis* are not unusual among marine invertebrates. A lack of obvious benefits of access to multiple mates to offspring might explain the lower than expected levels of realized polyandry uncovered by genetic studies of aquatic invertebrates (Avise et al. 2011). It does, however, make *C.* cf. *marginalis* an ideal candidate to study the effects of polyandry on offspring fitness without having to take into account confounding effects of variation in maternal investment.

References

Abràmoff MD, Magalhães PJ, & Ram SJ 2004. Image processing with ImageJ. Biophotonics Int. 11: 36–43.

Arnqvist G & Nilsson T 2000. The evolution of polyandry: multiple mating and female fitness in insects. Anim. Behav. 60: 145–164.

Avise JC, Tatarenkov A, & Liu J-X 2011. Multiple mating and clutch size in invertebrate brooders versus pregnant vertebrates. Proc. Natl Acad. Sci. USA 108: 11512–11517.

Brante A, Fernández M, & Viard F 2011. Microsatellite evidence for sperm storage and multiple paternity in the marine gastropod *Crepidula coquimbensis*. J. Exp. Mar. Biol. Ecol. 396: 83–88.

Carrillo-Baltodano A & Collin R in press. Sex-change in response to physical contact with conspecifics in the slipper limpet *Crepidula* cf. *marginalis*. Biol. Bull.

Collin R 2003. Phylogenetic relationships among calyptraeid gastropods and their implications for the biogeography of speciation. Syst. Biol. 52: 618–640.

——— 2004. The loss of complex characters, phylogenetic effects, and the evolution of development in a family of marine gastropods. Evolution 58: 1488–1502.

——— 2012. Temperature-mediated trade-offs in the life histories of two slipper limpets (Gastropoda: Calyptraeidae) with planktotrophic development. Biol. J. Linn. Soc. 106: 763–775.

Collin R & Spangler A 2012. Impacts of adelphophagic development on variation in offspring size, duration of development, and temperature-mediated plasticity. Biol. Bull. 223: 268–277.

Cunningham EJA & Russell AF 2000. Egg investment is influenced by male attractiveness in the mallard. Nature 404: 74–77.

Daguin-Thiebaut C, Le Cam S, & Viard F 2009. Isolation of 11 microsatellite markers in *Crepidula convexa* (Gastropoda, Calyptraeideae) for parentage analyses. Mol. Ecol. Res. 9: 917–920.

Diaz SA, Haydon DT, & Lindstrom J 2010. Sperm-limited fecundity and polyandry-induced mortality in female nematodes *Caenorhabditis remanei*. Biol. J. Linn. Soc. 99: 362–369.

Dupont L, Richard J, Paulet YM, Thouzeau G, & Viard F 2006. Gregariousness and protandry promote reproductive insurance in the invasive gastropod *Crepidula fornicata*: evidence from assignment of larval paternity. Mol. Ecol. 15: 3009–3021.

Eberhard WG 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, NJ.

Evans JP & Marshall DJ 2005. Male-by-female interactions influence fertilization success and mediate the benefits of polyandry in the sea urchin *Heliocidaris erythrogramma*. Evolution 59: 106–112.

Evans JP, Box TM, Brooshooft P, Tatter JR, & Fitzpatrick JL 2010. Females increase egg deposition in favor of large males in the rainbowfish, *Melanotaenia australis*. Behav. Ecol. 21: 465–469.

Fox CW & Rauter CM 2003. Bet-hedging and the evolution of multiple mating. Evol. Ecol. Res. 5: 273–286.

Galeotti P, Rubolini D, Fea G, Ghia D, Nardi PA, Gherardi F, & Fasola M 2006. Female freshwater crayfish adjust egg and clutch size in relation to multiple male traits. Proc. R. Soc. Lond., Ser. B: Biol. Sci. 273: 1105–1110.

Garcia-Gonzalez F & Simmons LW 2005. The evolution of polyandry: intrinsic sire effects contribute to embryo viability. J. Evol. Biol. 18: 1097–1103.

Gowaty PA, Kim Y-K, Rawlings J, & Anderson WW 2010. Polyandry increases offspring viability and mother productivity but does not decrease mother survival in Drosophila pseudoobscura. Proc. Natl Acad. Sci. USA 107: 13771–13776.

Gwynne DT 2008. Sexual conflict over nuptial gifts in insects. Ann. Rev. Entomol. 53: 83–101.

Hoffer JNA, Schwegler D, Ellers J, & Koene JM 2012. Mating rate influences female reproductive investment in a simultaneous hermaphrodite, Lymnaea stagnalis. Anim. Behav. 84: 523–529.

Horvathova T, Nakagawa S, & Uller T 2012. Strategic female reproductive investment in response to male attractiveness in birds. Proc. R. Soc. Lond., Ser. B: Biol. Sci. 279: 163–170.

Hosken DJ & Stockley P 2003. Benefits of polyandry: a life history perspective. Evol. Biol. 33: 173–194.

Janicke T, Sandner P, & Schaerer L 2011. Determinants of female fecundity in a simultaneous hermaphrodite: the role of polyandry and food availability. Evol. Ecol. 25: 203–218.

Lange R, Beninde J, Reichel V, Werminghausen J, Gerlach T, & Anthes N 2012a. Copulation duration does not predict sperm transfer in a marine hermaphrodite. Anim. Behav. 83: 469–472.

Lange R, Gerlach T, Beninde J, Werminghausen J, Reichel V, & Anthes N 2012b. Female fitness optimum at intermediate mating rates under traumatic mating. PLoS ONE 22: 7.

Le Cam S, Pechenik JA, Cagnon M, & Viard F 2009. Fast versus slow larval growth in an invasive marine mollusc: does paternity matter? J. Hered. 100: 455–464.

Le Cam S, Riquet F, Pechenik JA, & Viard F 2014. Paternity and gregariousness in the sex-changing sessile marine gastropod Crepidula convexa: comparison with other protandrous Crepidula species. J. Hered. 105: 397–406.

Marshall DJ & Evans JP 2007. Context-dependent genetic benefits of polyandry in a marine hermaphrodite. Biol. Lett. 3: 685–688.

McDonald KA, Collin R, & Lesoway MP 2014. Poecilogony in the caenogastropod Calyptraea lichen (Mollusca: Gastropoda). Invertebr. Biol. 133: 213–220.

McLeod L & Marshall DJ 2009. Do genetic diversity effects drive the benefits associated with multiple mating? A test in a marine invertebrate. PLoS ONE 4: e6347.

Mérot C & Collin R 2012a. Effects of food availability on sex change in two species of Crepidula (Gastropoda: Calyptraeidae). Mar. Ecol. Prog. Ser. 449: 173–181.

———— 2012b. Effects of stress on sex change in Crepidula cf. marginalis (Gastropoda: Calyptraeidae). J. Exp. Mar. Biol. Ecol. 416–417: 68–71.

Pélabon C, Albertsen E, Falahati-Anbaran M, Wright J, & Armbruster WS 2015. Does multiple paternity affect seed mass in angiosperms? An experimental test in Dalechampia scandens. J. Evol. Biol. 28: 1719–1733.

Proestou DA, Goldsmith MR, & Twombly S 2008. Patterns of male reproductive success in Crepidula fornicata provide new insight for sex allocation and optimal sex change. Biol. Bull. 214: 194–202.

Ratikainen II & Kokko H 2010. Differential allocation and compensation: who deserves the silver spoon? Behav. Ecol. 21: 195–200.

Rollinson N & Rowe L 2015. The positive correlation between maternal size and offspring size: fitting pieces of a life-history puzzle. Biol. Rev. doi:10.1111/brv.12214.

Sheldon BC 2000. Differential allocation: tests, mechanisms and implications. Trends Ecol. Evol. 15: 397–402.

Simmons LW 1987. Female choice contributes to offspring fitness in the field cricket Gryllus bimaculatus (de Geer). Behav. Ecol. Sociobiol. 21: 313–321.

———— 2003. The evolution of polyandry: patterns of genotypic variation in female mating frequency, male fertilization success and a test of the sexy-sperm hypothesis. J. Evol. Biol. 16: 624–634.

———— 2005. The evolution of polyandry: sperm competition, sperm selection, and offspring viability. Ann. Rev. Ecol. Evol. Syst. 36: 125–146.

Slatyer RA, Mautz BS, Backwell PRY, & Jennions MD 2012. Estimating genetic benefits of polyandry from experimental studies: a meta-analysis. Biol. Rev. 87: 1–33.

Smolensky N, Romero MR, & Krug PJ 2009. Evidence for costs of mating and self-fertilization in a simultaneous hermaphrodite with hypodermic insemination, the opisthobranch Alderia willowi. Biol. Bull. 216: 188–199.

Sprenger D, Anthes N, & Michiels NK 2008a. Multiple mating affects offspring size in the opisthobranch Chelidonura sandrana. Mar. Biol. 153: 891–897.

Sprenger D, Faber J, Michiels NK, & Anthes N 2008b. Natural female mating rate maximizes hatchling size in a marine invertebrate. J. Anim. Ecol. 77: 696–701.

Squires ZE, Wong BBM, Norman MD, & Stuart-Fox D 2012. Multiple fitness benefits of polyandry in a cephalopod. PLoS ONE 7: e37074.

Stearns SC 1992. The Evolution of Life Histories. Oxford University Press, Oxford, UK.

Tan GN, Govedich FR, & Burd M 2004. Social group size, potential sperm competition and reproductive investment in a hermaphroditic leech, Helobdella papillomata (Euhirudinea: Glossiphoniidae). J. Evol. Biol. 17: 574–580.

Williams GC 1966. Natural selection, the cost of reproduction, and a refinement of Lack's principle. Am. Nat. 100: 687–690.