Freshwater Biology

Freshwater Biology (2014) **59,** 2137–2149 doi:10.1111/fwb.12417

Egg fertilisation in a freshwater mussel: effects of distance, flow and male density

TYLER L. MOSLEY*, WENDELL R. HAAG† AND JAMES A. STOECKEL*

**School of Fisheries, Aquaculture, and Aquatic Science, Auburn University, Auburn, AL, U.S.A.*

†US Forest Service, Center for Bottomland Hardwoods Research, Oxford, MS, U.S.A.

SUMMARY

1. Small or sparse populations can experience Allee effects if egg fertilisation is reduced because of a shortage of sperm.

2. Freshwater mussels are spermcasters that often occur as sparse, patchy populations. Previous studies suggested that sperm shortage limits these populations unless facultative hermaphroditism and self-fertilisation occur at low density. We conducted experiments in ponds to examine fertilisation in the mussel, *Lampsilis straminea*, in response to flow, presence of and distance from males, male density, and the presence of upstream females that could compete for sperm with downstream females.

3. Self-fertilisation in the absence of males did not occur in either experiment. Female fertilisation success was uniformly high in most treatments and was not related to flow or distance from males (1–25 m). Fertilisation success was significantly lower at low male density (0.02 m^{-2}, compared with 0.16 m^{-2}) but remained relatively high even in most low male density treatments. The proportion of females that became gravid was higher in the presence of upstream females, but fecundity was significantly lower when upstream females were present; these conflicting results made it difficult to assess the role of competition among females for sperm.

4. Overall, high fertilisation success occurred at densities three orders of magnitude lower than previously proposed thresholds for mussels. Sperm dispersal and acquisition and egg fertilisation appear to be complex processes associated with adaptations for spermcasting. These adaptations are likely to facilitate persistence at low population density and buffer mussels from reproductive Allee effects.

Keywords: Allee effects, fertilisation, population density, spermcasting, unionid

Introduction

Small or sparse populations are highly vulnerable to chance fluctuations in environmental conditions or random variation in survival and reproductive success, known as environmental and demographic stochasticity, respectively (Shaffer, 1981). Declines in population size from either of these processes can create Allee effects, which accelerate the rate of population decline (Lande, 1998). An Allee effect is defined as a positive relationship between any component of individual fitness and the number or density of conspecifics (Stephens, Sutherland & Freckleton, 1999). A common type of Allee effect is when a decline in population size causes females to experience reduced fertilisation of eggs due to a shortage of sperm or difficulty finding mates. Populations that fall below a minimum threshold size for successful fertilisation may enter a vortex that leads rapidly to extinction (Courchamp, Clutton-Brock & Grenfell, 1999; Dennis, 2002).

The type of mating system employed by an organism can mediate the strength of Allee effects (Kokko & Rankin, 2006; Lee, Saether & Engen, 2011). For example, at low population density, organisms that actively search for or attract mates may be less susceptible to Allee effects than organisms that rely on passive encounters between individuals or gametes, such as broadcast spawners (Yund, 2000; Gascoigne *et al.*, 2009; Kramer *et al.*, 2009). Broadcast spawning involves release of both male and female gametes into the water

---

Correspondence: James A. Stoeckel, School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, 203 Swingle Hall, Auburn, AL 36849, U.S.A. E-mail: jimstoeckel@auburn.edu

where fertilisation occurs externally. At low population density, dilution of sperm can make egg fertilisation unlikely, resulting in strong Allee effects (Levitan, Sewell & Chia, 1992; Lundquist & Botsford, 2011). Spermcasting is a variation on broadcasting that is thought to reduce susceptibility to Allee effects. In spermcasters, males release gametes into the water, but females retain eggs in a brood chamber where eggs are fertilised by sperm obtained from the water (Bishop & Pemberton, 2006). Spermcasting typically is accompanied by release of sperm in aggregations called spermatozeugmata, which enhance sperm motility and longevity and allow fertilisation of numerous eggs simultaneously (Yund, 2000; Pemberton *et al.*, 2003; Falese, Russell & Dollahon, 2011). Gradual acquisition and concentration of sperm by females over long time periods through suspension feeding can also increase the efficiency of spermcasting. Consequently, egg fertilisation in spermcasting species may be less dependent on male density or population size than in broadcasters (Phillippi, Hamann & Yund, 2004).

Allee effects are important in conservation because populations of many endangered species are small or isolated (Groom, 1998; Boukal & Berec, 2002). In addition to influencing the viability of existing populations, Allee effects must be considered in restoration programmes involving reintroduction or augmentation. Because reintroduced or augmented populations often are small, Allee effects can lead to reproductive failure even if environmental conditions are favourable (Deredec & Courchamp, 2007; Armstrong & Wittmer, 2011). Freshwater mussels (Order Unionoida) are a highly endangered group of animals, and many remaining populations are extremely small and show little evidence of recent recruitment (Williams *et al.*, 1993; Haag, 2012). In addition to the protection of existing populations, mussel conservation efforts include aggressive reintroduction and augmentation (Neves, 2004). The extent to which Allee effects influence mussel population viability is an important issue in the conservation of these animals.

Minimum viable population size has not been estimated for any freshwater mussel species, but density-dependent sperm limitation has been invoked as a mechanism that could contribute to or hasten population declines. In a population of *Elliptio complanata* in a lake, female fertilisation success was positively correlated with local mussel density, with 100% fertilisation success observed only at densities >40 mussels m^{-2} and complete fertilisation failure at densities <10 m^{-2} (Downing *et al.*, 1993). These results suggest that small, sparse mussel populations are likely to be sperm limited, but several other observations do not support the requirement of high densities for fertilisation. In a river, fertilisation success of *Actinonaias ligamentina* was independent of mussel density and >90% fertilisation occurred even at local densities <1 m^{-2} (Moles & Layzer, 2008). A microsatellite DNA study showed that broods of female *Lampsilis cardium* were fertilised by sperm from a number of males originating as far as 16 km upstream (Ferguson *et al.*, 2013). Even in headwater streams or other habitats with very low mussel density, high fertilisation success is often observed (Barnhart, 1997; Neves, 1997; Haag & Staton, 2003), and many mussel species rarely, if ever, occur at densities >10 m^{-2} even in healthy populations with vigorous recruitment (Neves, 1997; Haag, 2012). Together, these observations suggest that stream currents facilitate fertilisation over great distances and, in general, they provide little support for a strong density-dependent relationship of egg fertilisation in mussels.

The consistently high fertilisation success seen in many species and habitats could be explained in two ways. First, facultative hermaphroditism expressed in response to low population density could allow self-fertilisation (Kat, 1983; Bauer, 1987; Neves, 1997). Hermaphroditism is common in many molluscs, but it is rare in freshwater mussels, and populations of several species that exhibited high fertilisation at low density had few or no hermaphroditic individuals (Van der Schalie, 1970; Heard, 1975; Haag & Staton, 2003). Alternatively, because mussels are spermcasters, adaptations for efficient fertilisation may release them from dependence on high male density. Like other spermcasters, male mussels release sperm into the water, and sperm are captured by females during filter feeding after which eggs are fertilised within the suprabranchial chamber (McMahon & Bogan, 2001). Mussel spermatozeugmata contain about 3000–9000 sperm, and they appear to contain a favourable osmotic environment, and potentially lipid reserves, that extend the longevity of sperm to 48–72 h (Ishibashi, Komaru & Kondo, 2000; Falese *et al.*, 2011). Spermatozeugmata also exhibit directional movement, and it is proposed that they exhibit taxis towards chemical signals produced by ovigerous females (Barnhart & Roberts, 1997; Ishibashi *et al.*, 2000; Falese *et al.*, 2011). Spermatozeugmata are reported for all five North American tribes within the family Unionidae, in the Margaritiferidae, and in two European and six Asian species, suggesting that they are a general feature of freshwater mussels worldwide (Haag, 2012 and sources therein). The prevalence of spermcasting and spermatozeugmata in freshwater mussels suggests that selection has favoured modes of fertilisation that are effective even in low-density populations.

Knowledge of egg fertilisation in mussels is vital for understanding population dynamics and for developing effective conservation strategies. We conducted experiments in experimental ponds to study egg fertilisation in *Lampsilis straminea* in relation to male presence and density, distance between males and females, competition for sperm between females, and the effects of flow in dispersing sperm. These experiments were designed to evaluate processes occurring at local scales, such as within mussel beds or in short stream reaches. Specifically, we tested the following hypotheses:

1. Fertilisation depends on outcrossing between males and females and does not occur as a result of facultative hermaphroditism and self-fertilisation expressed at low population density.
2. Fertilisation decreases with increasing distance from males, owing to dilution of sperm.
3. Decreases in fertilisation with increasing distance are stronger in static water environments than in flowing water, which can increase sperm dispersal.
4. Fertilisation decreases with decreasing male density, owing to sperm limitation.
5. The presence of upstream females can reduce fertilisation of downstream females owing to interception of sperm; these effects should be strongest when male density is low.

Methods

Study system and study species

We conducted the study in a series of 0.1 ha earthen ponds at the South Auburn Fisheries Research Station (SAFRS) near Auburn, Alabama. All ponds were similarly constructed and were 56 × 18 m with an average depth of about 1 m (maximum depth 2 m). We drained all ponds and allowed them to dry completely for several months prior to the experiments to eliminate existing populations of aquatic organisms, but *Lampsilis straminea* was not present in ponds prior to the experiments. Ponds were filled from a common reservoir that lacked mussels. We filled all ponds 2 months prior to conducting experiments to allow water conditions to equilibrate. We monitored pH, alkalinity and calcium hardness biweekly; these values did not differ significantly among ponds (ANOVA, $F = 0.29–1.09$, $P > 0.14$), and high water quality was maintained throughout all experiments.

In a subset of the ponds, we created a simulated stream raceway by placing a 31-m-long vinyl partition along one side of the pond *c.* 3.0 m from the bank (raceway area = 93 m^2) and installing an airlift at one end of the raceway (Fig. 1). The airlift was *c.* 2.5 m wide, stretching across nearly the entire width of the raceway, and consisted of a corrugated fibreglass baffle and a perforated airline placed on the pond bottom. Two 3 hp air blowers (Aquatic Ecosystems, Apopka, FL, U.S.A.) supplied air. Air rising from the airline was deflected by the baffle, creating water current through the raceway. We constructed

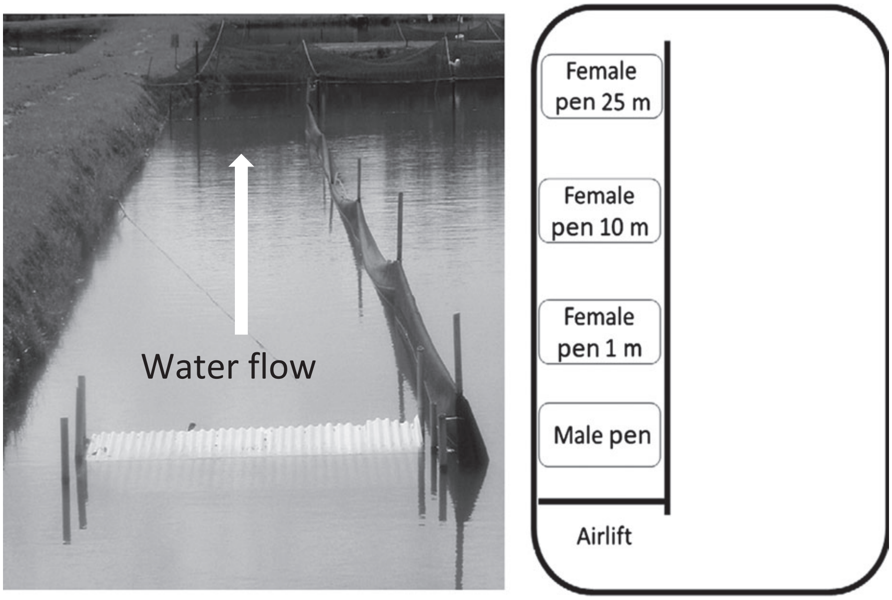

Fig. 1 (Left) Experimental pond with airlift and raceway after filling, showing direction of water flow; (Right) Schematic of mussel pen placement in pond in relation to the raceway and airlift systems (not to scale).

similar raceways in no-flow treatment ponds but did not operate the airlifts. We measured water velocity 0.4–0.7 m below the surface in all raceways (Flo-mate 2000, Flow-tronic, Welkenreadt, Belgium) at five equidistant points along an upstream-downstream transect at the approximate centre line of the raceway. The average water velocity in flow treatments was 0.03 m s^{-1} (± 0.01 SD); this velocity mimicked low flow conditions in low gradient, coastal plain streams from which study animals were collected (J. Stoeckel, unpublished data). Flow was not detectable in no-flow treatments (minimum detectable flow = 0.01 m s^{-1}). Within each raceway, we constructed 1 × 2 m rectangular pens of 1.4-cm mesh, rigid plastic netting, which was buried 0.2 m into the pond bottom and extended 0.1 m above the substrate surface. These pens were effective at containing mussels at specified locations in the raceways, and we had minimal escape (see Results). Male pens were constructed at the head of each raceway (3 m downstream of the airlift), and female pens were constructed at distances of 1, 10 and 25 m downstream of the male pens (Fig. 1).

Our study species was *Lampsilis straminea*, which is widespread in Gulf Coast rivers from the Suwannee River, Florida, west to the Amite River, Louisiana (Williams, Bogan & Garner, 2008). This species is largely restricted to streams, but it occurs typically in pools, stream margins and other lentic microhabitats (Haag, 2012). It is a long-term brooder in which eggs are fertilised in late summer or early autumn, and glochidia are brooded in the outer gills over the winter and released in spring to early summer. The shells are strongly sexually dimorphic, allowing reliable identification of males and females in the field. We collected adult *L. straminea* from Line Creek, Bullock County, Alabama, and Opintlocco Creek in Macon County, Alabama, between May and August 2010 and 2011. Collections from both creeks were mixed, and animals were randomly assigned to treatments in all experiments. At the time of collection, most females were not gravid, having released glochidia from the previous season but not yet deposited the subsequent brood. Gravid females have greatly swollen and distended outer gills, while the outer gills of non-gravid females are flaccid and similar in appearance to the inner gills. Mussels were transported to the SAFRS, identified with individually numbered tags affixed to the shell and sexed based on shell morphology. Despite the strong sexual dimorphism, we verified the sex of a subset of 20 individuals by extracting gonadal fluid with a syringe and examining it under a microscope to identify sperm or eggs (Saha & Layzer, 2008). Examination of gonadal fluid confirmed our sex determination for all individuals, but these individuals were not used in experiments because the effect that the procedure might have on subsequent egg release and fertilisation was unknown. After tagging and sex determination, we held all animals in ponds adjacent to experimental ponds until initiation of experiments. Twenty-four hours prior to beginning each experiment, we examined the gills of all test females by gently prying apart the valves; no females were gravid prior to the experiments.

Experiment I: Effects of flow and male distance, and evaluation of potential hermaphroditism

In this experiment, we used two ponds without airlifts as no-flow treatments and two ponds with airlifts as flow treatments (Table 1). In each pond, we stocked 15 male mussels in the upstream-most pen (hereafter, the male pen) and 10 females in each of the three pens downstream (1, 10 and 25 m downstream). We also included an additional pond without flow, in which we stocked 20 females in a single pen but did not stock males in the pond; this control treatment allowed us to evaluate the potential for self-fertilisation or fertilisation via sperm storage from previous spawning events in the wild. All mussels were placed in the ponds on 17 August 2010 and were left undisturbed until 17 November 2010, at which time they were collected from the ponds and returned to the laboratory for analysis.

Experiment II: Effects of male density and sperm competition between females

In the following year, we conducted an additional experiment in which we used eight ponds, all with airlifts, to create flow (Table 1). In four ponds, we stocked two males in each male pen as a low male density treatment, and we stocked 15 males in the male pen of each of the other four ponds as a high male density treatment. In two of the ponds in each male density treatment, we stocked 10 females in each pen (1, 10 and 25 m), but the other two ponds received 10 females only in the 25 m pen, with no females in the 1 or 10 m pens. This allowed us to test the effect of sperm interception by upstream females on long-distance fertilisation (25 m) in conditions of high and low male density. Because of the large volume of water in experimental raceways, upstream female mussel density was too low for them to intercept a substantial percentage of sperm by simple filtration of passively dispersed sperm. However, the proposal that spermatozeugmata exhibit taxis towards ovigerous females (Barnhart & Roberts, 1997) makes

Table 1 Experimental and analytical design for evaluating factors related to mussel fertilisation success

Experimental design					Number of females		
Experiment	Number of ponds	Flow (Y/N)	Pond ID	Number of males	1 m	10 m	25 m
I	2	Y	A, B	15	10	10	10
	2	N	C,D	15	10	10	10
II	2	Y	E, F	2	10	10	10
	2	Y	G, H	15	10	10	10
	2	Y	I, J	2	0	0	10
	2	Y	K, L	15	0	0	10

Analytical design				
Experiment	Factor (levels)	Ponds	Pens	N (main effects)
I	Distance (1, 10, 25 m)	A,B,C,D	All pens: 1 m vs. 10 m vs. 25 m	4
	Flow (flow, no flow)	A,B vs. C,D	All pens	6
II, model 1	Distance (1, 10, 25 m)	E,F,G,H	All pens: 1 m vs. 10 m vs. 25 m	4
	Male density (low, high)	E,F vs. G,H	All pens	6
II, model 2	Upstream females (present, absent)	E,F,G,H vs. I,J,K,L	25 m pens only	4
	Male density (low, high)	E,F,I,J vs. G,H,K,L	25 m pens only	4

Experimental design shows the number of mussels in each treatment combination. Each experiment also included a single control pond with no males (not shown, see text). Analytical design shows the level at which the influence of each factor was examined. Main effect sample sizes are for each level of the factor.

sperm interception by upstream females a plausible outcome even at low female density. As in experiment I, we also included an additional, control, pond in which we stocked 20 females in a single pen but did not stock males in the pond. This experiment began on 12 July 2011 and ran until 1 November 2011.

Laboratory analysis

At the end of each experiment, we prized open the valves of each female and examined the gills to determine the proportion of females in each pen that became gravid. We also removed the gills from five gravid females in each pen and estimated for each individual: (i) the proportion of glochidia (representing fertilised eggs) and unfertilised eggs and (ii) the total number of glochidia produced (individual fecundity). We counted glochidia and unfertilised eggs by removing and rupturing the gills and flushing the contents into a beaker. We then diluted the contents to a volume of 300–2500 mL depending on the size of the gills, mixed the sample thoroughly with a plunger and counted under a dissecting microscope the number of glochidia or eggs in three 1-mL subsamples; if subsample counts varied by more than 10%, we counted a fourth subsample. We then extrapolated the total number of glochidia based on the diluted sample volume. We also examined the gills of a subsample of 15 non-gravid females to ensure that flaccid gills contained neither glochidia nor unfertilised eggs.

Data analysis

The experiments were 2^3 factorial designs, each with two factors at either two (flow, male density, presence of upstream females) or three (distance) levels, and each factor was crossed with all other factors (Table 1). In each experiment, each full treatment combination (experiment I: flow × distance from males; experiment II, model 1: distance from males × male density; experiment II, model 2: presence of upstream females × male density) was replicated only twice, but main effects had higher replication (Table 1). Distance treatments within ponds (i.e. pens at 1, 10 and 25 m) are not strictly independent, but we considered them as such for the following reasons. First, the main confounding factor that we would expect among distance treatments is that upstream females (i.e. at 1 m) would intercept a substantial amount of sperm, and therefore, downstream females may experience reduced fertilisation due to this effect and not directly to the effect of distance and sperm dilution. However, the lack of a distance effect in either experiment and the conflicting results of the presence of upstream females in experiment II (see Results) indicate that interception of sperm by upstream females was not an important factor in explaining variation among distance treatments within a pond. Second, the grand mean of response variables (proportion of gravid females, mean proportion of fertilised eggs, mean fecundity) across distance treatments did not differ significantly among ponds in experiment I, showing that pond effects were negligible. In experiment II, the overall proportion of gravid females did not differ among ponds, but one pond had a significantly lower proportion of fertilised eggs and fecundity than the other ponds, and a blocking factor was used in these models to account for this variation (see below).

We analysed the results from both experiments with two-factor ANOVA treating all independent factors as class variables (distance, presence of flow, male density and presence of upstream females). Proportional data were arcsine-transformed, and fecundity was log-transformed. Initially, we included variation among individuals within pens (percentage of fertilised eggs and fecundity) as nested factors, but these factors were not significant in any model, and all final models were based on pen means. Fecundity in mussels is strongly related to shell length (Haag, 2013), but we observed no such relationship in either experiment, probably because of the narrow size range of individuals (56.6–90.0 mm). Furthermore, individuals were randomly assigned to treatments, and shell length of females sampled for fecundity did not differ among any treatment combination in either experiment (ANOVA; experiment I, $F = 1.18$, $P < 0.333$, 5, 50 d.f.; experiment II, $F = 0.76$, $P < 0.582$, 5, 59 d.f.). Consequently, we used mean individual fecundity (log-transformed) as a dependent variable in all ANOVA models.

In experiment II, we analysed the effects of distance from males and presence of upstream females separately in two, two-factor ANOVA models (Table 1). The first model (model 1) included as independent variables distance and male density, but it excluded females at 25 m without upstream females. The second model (model 2) included as independent variables male density and presence of upstream females, but it excluded females at 1 and 10 m. In both experiments, all ANOVA models were run initially with interaction terms between independent variables, but if interaction terms were not significant (using a conservative threshold of $P > 0.20$) they were omitted and final models included only main effects. In experiment II, models evaluating the response variables proportion of fertilised eggs and fecundity included an additional term, pond identity, as a blocking factor to account for differences among ponds in these variables. Models evaluating the effect of upstream females did not include a blocking factor because we used observations for only one pen per pond.

Mortality and escape from pens was low (see Results), and in most cases, mortality was confirmed by recovery of dead shells at the end of the experiment. Only a few individuals were unaccounted for at the end of the experiment. For unrecovered or dead males, we assumed that these individuals participated in reproduction prior to death. This assumption cannot be tested, but the number of males that died was low and loss of males even prior to spawning probably had a minimal effect on our results.

Results

Across both experiments and all treatments, 73% of surviving females became gravid (Fig. 2; not including control ponds with no males). Among gravid females, the proportion of eggs that became fertilised was uniformly high and averaged 94%. The lowest percentage of fertilised eggs in any individual was 48%, but few individuals exhibited fertilisation <90% (Fig. 2).

Experiment I: Effects of male distance and flow, and evaluation of potential hermaphroditism

Only one mussel, a control female, escaped from its pen during this experiment, but it was found along the outside edge of the pen at the end. Mortality during the experiment was low (1.5%, $n = 200$ individuals), including two males and one female, and no pens lost more

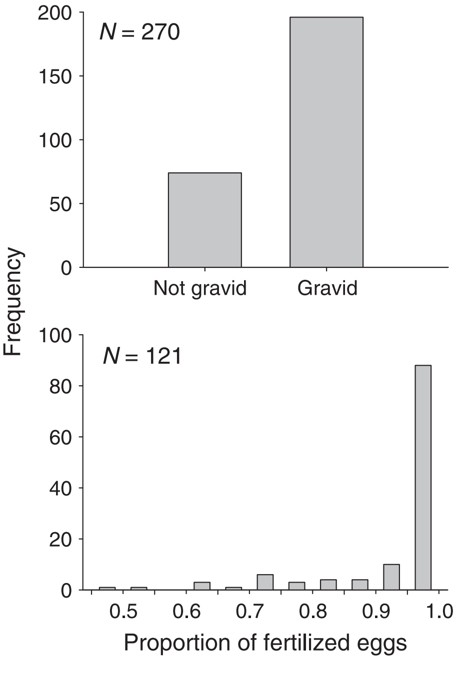

Fig. 2 Frequency distributions for (a) female gravidity and (b) proportion of fertilised eggs across both experiments and all treatments. Numbers of non-gravid females do not include females in control ponds with no male mussels.

than a single individual. None of the females in the control pond became fertilised in the absence of males, and all had flaccid gills. Dissection and examination of the gills from five control females revealed no unfertilised eggs or glochidia.

Female fertilisation success was not related to the presence of flow and was only minimally affected by distance from males (Table 2; Fig. 3). The proportion of females that became gravid was not related to distance, flow or the interaction between these factors. At 1 and 10 m, ≥90% of females became gravid in all ponds, regardless of the presence of flow. The only apparent effect of distance was that the percentage of gravid females was much more variable at 25 m (20–100%) than at 1 or 10 m. Among females that did become gravid, an average of >95% of eggs were fertilised in all treatment combinations, and only two of 56 total individuals had fertilisation <90% (minimum = 77.3%). The percentage of eggs that were fertilised and the number of glochidia produced by females were not related to flow, distance or the interaction between these factors.

Table 2 ANOVA results for factors influencing fertilisation success in freshwater mussels.

Response variable	Factor	d.f.	SS	*F*	*P*
Experiment I					
Proportion of females gravid	Model	3,8	1029.149	0.99	<0.4465
	Distance	2	980.33	1.41	<0.299
	Flow	1	48.82	0.14	<0.718
Proportion of fertilised eggs	Model	3,8	47.978	2.00	<0.192
	Distance	2	45.691	2.86	<0.1155
	Flow	1	2.287	0.29	<0.6071
Fecundity	Model	3,8	0.008	0.23	<0.874
	Distance	2	0.003	0.29	<0.759
	Flow	1	0.001	0.11	<0.745
Experiment II – model 1					
Proportion of females gravid	Model	3,8	1694.089	3.92	<0.054
	Distance	2	939.320	3.26	<0.092
	Male density	1	754.770	5.24	<0.051
Proportion of fertilised eggs	Model	5,6	962.057	6.26	<0.023
	Distance	2	48.066	1.56	<0.284
	Male density	1	431.711	14.04	<0.010
	Pond	2	434.212	7.06	<0.027
Fecundity	Model	5,6	0.450	4.36	<0.051
	Distance	2	0.042	1.02	<0.416
	Male density	1	0.188	9.09	<0.024
	Pond	2	0.221	5.35	<0.046
Experiment II – model 2					
Proportion of females gravid	Model	3,7	3679.931	6.59	<0.050
	Upstream females	1	1178.647	6.33	<0.066
	Male density	1	1642.315	8.83	<0.041
	Upstream females* male density	1	858.969	4.62	<0.098
Proportion of fertilised eggs*	Model	2,6	444.137	5.08	<0.080
	Upstream females	1	9.117	0.21	<0.672
	Male density	1	443.888	10.15	<0.033
Fecundity*	Model	2, 6	0.072	29.97	<0.004
	Upstream females	1	0.013	10.94	<0.030
	Male density	1	0.049	40.33	<0.003

For models without interaction terms, interaction was non-significant and these terms were omitted from the final model (see text). Results are based on Type I sums of squares (SS) for all models except those indicated by an asterisk, which are based on Type III; these were unbalanced models because of an absence of fertilised females in one pond. For experiment II, model 1 excludes treatments with no upstream females and model 2 excludes females at 1 and 10 m (see text).

Experiment II: Effects of male density and sperm competition between females

Two male mussels escaped from pens during the experiment, one from a low male density pond containing females only at 25 m and another from a high male density pond containing females at all distances. Neither of these males was recovered after the experiment but, because we did not find dead shells of either individual, we assumed that they had simply buried deeply in the substratum after the reproductive period. No females escaped from pens. Mortality was low (4.4%, $n = 248$) and included two males and nine females. One pen lost three females, but no other pens lost more than one individual.

None of the females in the control pond became fertilised in the absence of males, and all had flaccid gills. Dissection and examination of the gills from five control females revealed no unfertilised eggs or glochidia.

In experiment II, model 1 (excluding treatments with no upstream females), female fertilisation success was not related to distance from males, but it was affected by male density (Table 2; Fig. 4). There was no strong effect of male distance on the proportion of females that became gravid, but the mean proportion was lower and more variable at 1 m, contrary to our expectations, and there was weak evidence for a significant difference among means ($P < 0.092$). The proportion of gravid females was significantly lower in low male density treatments (mean across distance treatments = 0.57) than in high-density treatments (mean = 0.79). In low male density treatments, the proportion of gravid females was highly variable (0.2–0.9), but it exceeded 0.70 in only one pond; in contrast, fertilisation was consistently high in high male density treatments (0.67–1.0). Among gravid females, the proportion of fertilised eggs was not related to distance. When differences among ponds were accounted for, male density had a significant effect on

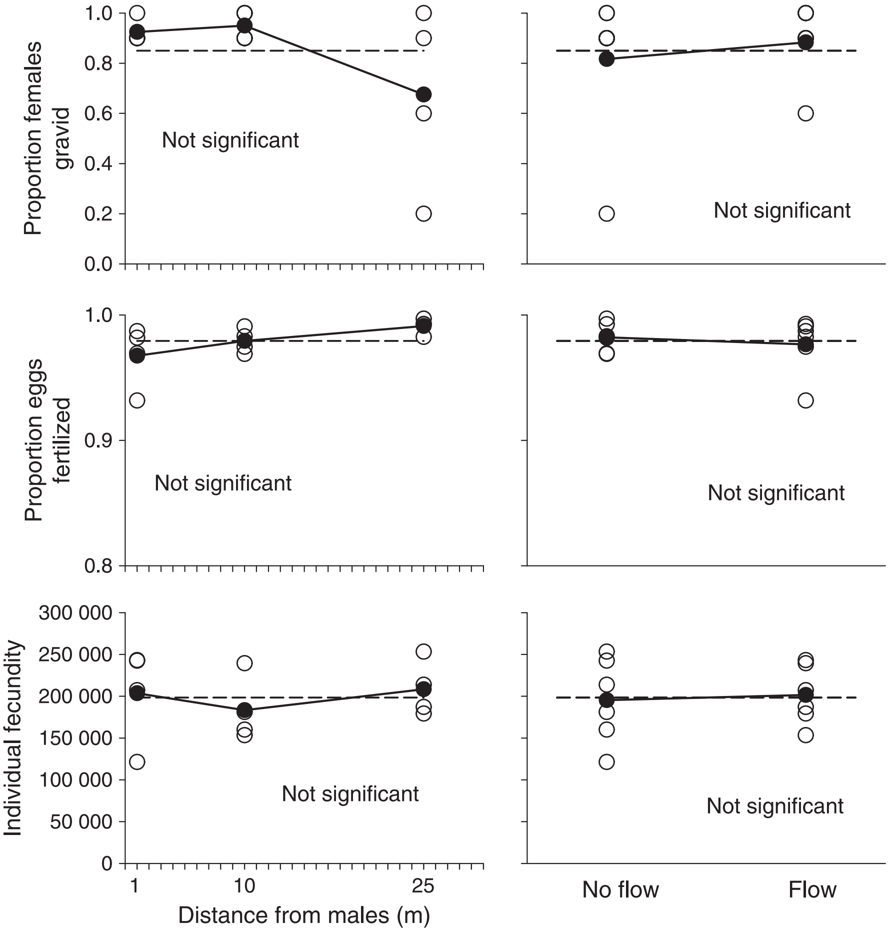

Fig. 3 Main effects plots depicting fertilisation success in experiment I in relation to flow and male distance. Open circles are results from individual pens and, in the lower two panels, are based on mean values for female mussels in each pen. Solid circles are means across pens in each treatment combination. Horizontal dashed lines are grand means for each response variable across all treatments.

the proportion of fertilised eggs, reflecting a slightly, but consistently lower proportion in females at low male density (means across distance treatments: low = 0.82, high = 0.95). Similarly, the number of glochidia produced by females was not related to distance, but male density had a significant effect. Overall, fecundity in low male density ponds was 64% of fecundity at high male density (means across distance treatments: low = 75 009, high = 117 990).

In experiment II, model 2 (excluding females at 1 and 10 m), the presence of upstream females had variable effects on female fertilisation success (Table 2; Fig. 4) but, as in model 1, male density had a strong, consistent effect (Table 2). Male density had a significant effect on the proportion of females that became gravid, reflecting a higher proportion in high male density ponds (means across upstream female treatments: low = 0.45, high = 0.77; comparison not shown on Fig. 4). The proportion of gravid females was highly variable in low male density ponds (range = 0.00–0.90) but uniformly high at high male density (0.78–0.89). The effect of upstream females on the proportion of gravid females was marginally insignificant ($P < 0.066$), but any effect was opposite to that expected. The mean proportion of gravid females was higher when upstream females were present (0.77) than when upstream females were absent (0.45). However, the interaction term in the model also was marginally insignificant ($P < 0.098$), suggesting that the effect of upstream females was dependent on male density. Within gravid females, male density had a significant effect on the proportion of fertilised eggs, reflecting slightly higher fertilisation success at high male density (means across upstream female treatments: low = 0.83, high = 0.98; comparison not shown on Fig. 4); the presence of upstream females and the interaction term were not significant factors. Male density and presence of upstream females both had significant effects on fecundity (interaction term not significant). Mean fecundity was higher at high male density (means across upstream female treatments: low = 87 069; high = 132 825; comparison not shown on Fig. 4) and higher in treatments without upstream females (means across male density treatments: upstream females present = 100 564; upstream females absent = 130 083).

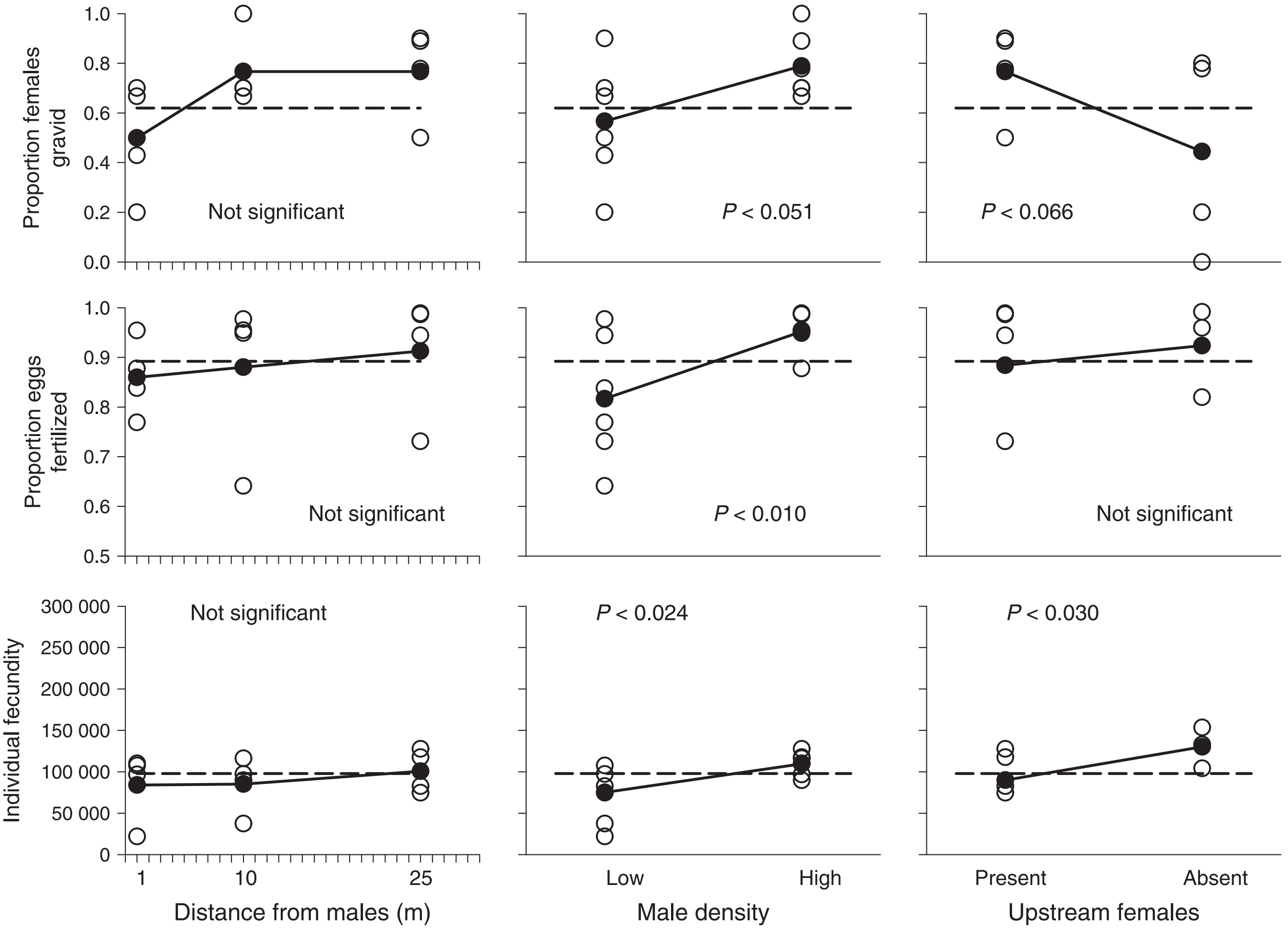

Fig. 4 Main effects plots depicting fertilisation success in experiment II in relation to male distance, male density and the presence of upstream females. Open circles are results from individual pens and, in the lower two panels, are based on mean values for female mussels in each pen. Solid circles are means across pens in each treatment combination. Horizontal dashed lines are grand means for each response variable across all treatments. Note that the left and middle columns (distance from males and male density) exclude ponds with no upstream females (model 1), and the right column (upstream females) includes results only from 25 m distance treatments (model 2). Fertilisation success in relation to male density is not shown for model 2, but these results were similar to those from model 1 and are reported on Table 2.

Discussion

Like other spermcasters, *Lampsilis straminea* has highly effective sperm dispersal and acquisition that allows complete fertilisation of female broods even at a considerable distance from males and in the absence of flow. Furthermore, we observed no fertilisation in the absence of males in two consecutive reproductive seasons, which indicates that *L. straminea* did not exhibit short-term facultative hermaphroditism in response to low population density. There exists a potential for a change to hermaphroditism over longer time periods, and this issue requires additional study. Nevertheless, the traits we observed for *L. straminea* stand in sharp contrast to previous depictions of mussel fertilisation as strongly dependent on high population density or proximity among males and females or dependent on self-fertilisation at low density (Bauer, 1987; Downing *et al.*, 1993; Neves, 1997).

The mechanisms that allow efficient fertilisation in mussels remain poorly known, but they are probably similar to those of other spermcasters. Male spawning is loosely synchronous in some mussel populations, occurring over 1–3 weeks, but can continue for up to 4 months in others (Haag, 2012 and sources therein). Through filter feeding, females exposed to even very low sperm densities for protracted periods may concentrate sufficient sperm for fertilisation of complete broods. Release of sperm in spermatozeugmata is also likely to be important in facilitating efficient fertilisation. Even at the low flows in our experiments (0.03–0.04 m s^{-1}), viable sperm could be transported about 5 km in 48 h and nearly 8 km in 72 h, corresponding to the range of extended sperm longevity provided by spermatozeugmata (see Ishibashi *et al.*, 2000; Falese *et al.*, 2011). The directional movement exhibited by spermatozeugmata could further extend dispersal distance and may be especially important in lentic environments without flow. Finally, the large number of sperm contained in a single spermatozeugmata facilitates fertilisation of many eggs even if the number of spermatozeugmata encountered by females is low (Waller & Lasee, 1997). Spermatozeugmata have not been documented in *L. straminea*, but they occur in all major unionid groups, including other *Lampsilis* (Utterback, 1931; Waller & Lasee, 1997).

Sperm dispersal and acquisition and egg fertilisation appear to be complex processes that result in an unusual 'all-or-none' pattern of fertilisation within individual females. We rarely observed females that were brooding a large number of unfertilised eggs or appeared to have only partial broods and sharply reduced fecundity. Even in treatments having a low percentage of gravid females, females that did become gravid had a high percentage of fertilised eggs, and total fecundity was usually similar to individuals in treatments with a high percentage of gravid females. This pattern appears to be characteristic of many mussel species. It was reported in another experimental study of mussel fertilisation in *Ligumia subrostrata* (Landis, Haag & Stoeckel, 2013), and with the exception of species for which unfertilised eggs impart structure to conglutinates (e.g. *Cyprogenia, Dromas, Fusconaia, Pleurobema*), the percentage of fertilised eggs in gravid females is typically high in the wild in most mussel species (Downing *et al.*, 1993; Neves, 1997; Haag & Staton, 2003; Barnhart, Haag & Roston, 2008; Moles & Layzer, 2008).

Details of mussel egg fertilisation are poorly known, but the prevalence of this pattern suggests some mechanisms. The infrequent observation of females brooding a large percentage of unfertilised eggs suggests that either females do not retain largely unfertilised broods (likely to avoid reduced gill function; see Richard, Dietz & Silverman, 1991; Tankersley & Dimock, 1993), or egg deposition into the suprabranchial chamber occurs only after sufficient sperm are encountered during filter feeding. The high fertilisation success observed in gravid females even at low male density supports the idea that relatively few spermatozeugmata are required to fertilise an entire brood. An average brood of *L. straminea* (about 150 000 ova) could be fertilised completely by about 25 spermatozeugmata (assuming 6000 sperm/spermatozeugmata; see Barnhart & Roberts, 1997; Waller & Lasee, 1997).

Despite the high fertilisation success we observed in most treatments, our results support the possibility of sperm limitation and resulting Allee effects in some situations. Although there were no significant differences in the proportion of gravid females related to distance or flow in experiment I, the more variable fertilisation in the 25 m/no-flow treatment suggests that flow plays some role in facilitating fertilisation at greater distances. Experiment II suggests that low male density can result in sperm limitation leading to reduced fertilisation and fecundity. The mechanism by which sperm limitation results in reduced fecundity is unclear. The proportion of fertilised eggs in a brood was on average only 15% lower in low male treatments, but fecundity was reduced by nearly half; consequently, fertilisation failure alone cannot fully explain the sharp reduction in fecundity. One possible explanation for this result is that the number of ova released from the ovary is controlled by the number of sperm encountered during filter feeding. Regardless, this interesting result offers a glimpse of the complexity of mussel fertilisation and highlights the need for additional research.

Other results of experiment II are similarly difficult to interpret. We observed almost complete fertilisation failure in the 25 m/low male density treatments in the absence of competing females, but high fertilisation occurred in this treatment when upstream females were present. The latter result shows that a single male produces sufficient sperm to fertilise several females and, indeed, multiple paternity has been documented in mussels (Christian *et al.*, 2007; Ferguson *et al.*, 2013). The lower overall fertilisation in the absence of upstream females is perplexing, but this result should be viewed with caution because fertilisation probably has a large random component, especially at low density. For example, in low male density treatments, poor sperm production or viability in a single male could have had a large effect on the results. The potential for such random effects underscores the threat that demographic stochasticity poses in extremely low-density populations in the wild.

The unusual results of experiment II and other aspects of our study also should be considered in the light of the low replication of our experiments. We had little ability to assess the role of random factors or to overcome the influence of this source of variation, and our power to detect small differences among treatments was low. However, sample sizes for evaluating main effects were modest, and because few models had significant interaction terms, they were informative tests of the importance of these effects. Moreover, it should be emphasised that these are large-scale experiments that combine the benefits of repeatability and replication with an environmentally relevant setting, and they provide insights that could not be obtained by either field or laboratory studies. As in other large-scale experiments, logistical constraints precluded the level of replication possible in the laboratory. Despite low replication, our study clearly shows that mussels can achieve high fertilisation success across a broad range of distances and densities, and this finding is concordant with an emerging view of the adaptive benefits of spermcasting.

Like other *Lampsilis*, *L. straminea* typically does not occur in high-density aggregations (Haag, 2012), and mechanisms allowing efficient fertilisation in sparse populations may be of particular selective advantage. In species such as *Elliptio complanata* and *Margaritifera margaritifera*, that regularly occur in dense aggregations ($\gg$10 m^{-2}), fertilisation may be more strongly dependent on high density (e.g. Bauer, 1987; Downing *et al*., 1993). However, a number of observations suggest that adaptations for high fertilisation efficiency at low density are pervasive among freshwater mussels. The routine occurrence of fully fertilised females in low-density populations across a wide range of species and habitats, and the lack of a density-dependent relationship for fertilisation in the wild for a species that commonly occurs in high-density aggregations (*Actinonaias ligamentina*) provide the most direct support for the generality of this phenomenon (Barnhart, 1997; Neves, 1997; Haag & Staton, 2003; Moles & Layzer, 2008). Furthermore, the occurrence of spermatozeugmata in all mussel groups, including species that occur at high density (e.g. *Elliptio complanata*, *Margaritifera laevis*; Okada & Ishikawa, 1959; Bringolf *et al*., 2010), shows the breadth of adaptations for efficient fertilisation.

Nevertheless, some degree of sperm limitation is to be expected in the sparsest of populations. The critically endangered species, *Quadrula sparsa*, occurs in the Powell River, Tennessee and Virginia, at densities <0.03 m^{-2}, and the difficulty of finding gravid females suggests that they are sperm limited (Johnson, 2011). However, evidence of recent recruitment in this population shows that fertilisation can occur at least occasionally even at these very low densities. In our low male treatments in experiment II, male *L. straminea* occurred at comparably low density (0.02 m^{-2} within the total area of the raceways) but, despite reduced fertilisation success, an average of 45% of females were fertilised, including one pond with 90% fertilisation at 25 m, and >80% of eggs were fertilised in these individuals. Even in other treatments, males occurred at a density of only 0.16 m^{-2}, yet fertilisation success was uniformly high. Together, these results show that substantial fertilisation can occur at densities three orders of magnitude lower than previously proposed reproductive thresholds for mussels (e.g. 10 m^{-2}; see Downing *et al*., 1993).

A patchy and often sparse distribution is a characteristic of most freshwater mussel populations (Strayer *et al*., 2004), and adaptations for efficient fertilisation under these conditions appear to be widespread. Consequently, like other spermcasters, mussels are probably buffered from Allee effects related to fertilisation. This is good news for mussel conservation and can inform efforts to re-establish or augment populations of imperiled species. Reintroduction or translocation programmes often place individuals within a small area to maximise chances of fertilisation, but this technique may render these individuals vulnerable to localised disturbance. If long-distance fertilisation is common among mussel species, reintroduced individuals could be placed at wider intervals to lessen chances of mass mortality from a single event and to maximise the chances that some individuals are placed in suitable microhabitats. The potential for long-distance and low-density fertilisation also has implications for population augmentation programmes designed to overcome potential sperm limitation, but more research is needed to determine optimal mussel densities in the wild. Furthermore, it is important that the generality of our experimental results be tested with other species and in other habitats. Sperm dispersal and acquisition and egg fertilisation in mussels appear to be complex processes, and a thorough understanding of these mechanisms is necessary to evaluate other factors that influence reproductive success. Even if fertilisation is largely density independent, other types of Allee effects, such as loss of facilitative feeding interactions, may affect mussel populations negatively, and small populations will remain highly vulnerable to environmental and demographic stochasticity (Spooner & Vaughn, 2009; Haag, 2012).

Acknowledgments

This study was supported by the US Environmental Protection Agency and US Forest Service, Center for Bottomland Hardwoods Research. We thank Catlin Ames, Mickey Bland, Daniel Foree, Michael Hart, Andrew Gascho Landis and Brad Staton for field and laboratory assistance. Two anonymous reviewers provided comments that improved the manuscript.

References

Armstrong D.P. & Wittmer H.U. (2011) Incorporating Allee effects into reintroduction strategies. *Ecological Research*, **26**, 687–695.

Barnhart M.C. (1997) Fertilization success in freshwater mussels. Report to Missouri Department of Conservation, Columbia, USA. Available at: http://biology.missouristate.edu/faculty_pages/Barnhart%20pubs/barnhart_pubs.htm.

Barnhart M.C., Haag W.R. & Roston W.N. (2008) Adaptations to host infection and larval parasitism in Unionoida. *Journal of the North American Benthological Society*, **27**, 370–394.

Barnhart M.C. & Roberts A. (1997) Reproduction and fish hosts of unionids from the Ozark uplifts. In *Conservation and Management of Freshwater Mussels II: Initiatives for the Future*. (Eds K.S. Cummings, A.C. Buchanan, C.A. Mayer & T.J. Naimo), pp. 16–20. Upper Mississippi River Conservation Committee, Rock Island, IL

Bauer G. (1987) Reproductive strategy of the fresh-water pearl mussel Margaritifera-Margaritifera. *Journal of Animal Ecology*, **56**, 691–704.

Bishop J.D.D. & Pemberton A.J. (2006) The third way: spermcast mating in sessile marine invertebrates. *Integrative and Comparative Biology*, **46**, 398–406.

Boukal D.S. & Berec L. (2002) Single-species models of the Allee effect: extinction boundaries, sex ratios and mate encounters. *Journal of Theoretical Biology*, **218**, 375–394.

Bringolf R.B., Heltsley R.M., Newton T.J., Eads C.B., Fraley S.J., Shea D. *et al.* (2010) Environmental occurrence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. *Environmental Toxicology and Chemistry*, **29**, 1311–1318.

Christian A.D., Monroe E.M., Asher A.M., Loutsch J.M. & Berg D.J. (2007) Methods of DNA extraction and PCR amplification for individual freshwater mussel (Bivalvia: Unionidae) glochidia, with the first report of multiple paternity in these organisms. *Molecular Ecology Notes*, **7**, 570–573.

Courchamp F., Clutton-Brock T. & Grenfell B. (1999) Inverse density dependence and the Allee effect. *Trends in Ecology & Evolution*, **14**, 405–410.

Dennis B. (2002) Allee effects in stochastic populations. *Oikos*, **96**, 389–401.

Deredec A. & Courchamp F. (2007) Importance of the Allee effect for reintroductions. *Ecoscience*, **14**, 440–451.

Downing J.A., Rochon Y., Perusse M. & Harvey H. (1993) Spatial aggregation, body-size, and reproductive success in the fresh-water mussel Elliptio-Complanata. *Journal of the North American Benthological Society*, **12**, 148–156.

Falese L.E., Russell M.P. & Dollahon N.R. (2011) Spermcasting of spermatozeugmata by the bivalves Nutricola confusa and N. tantilla. *Invertebrate Biology*, **130**, 334–343.

Ferguson C.D., Blum M.J., Raymer M.L., Eackles M.S. & Krane D.E. (2013) Population structure, multiple paternity, and long-distance transport of spermatozoa in the freshwater mussel Lampsilis cardium (Bivalvia:Unionidae). *Freshwater Science*, **32**, 267–282.

Gascoigne J., Berec L., Gregory S. & Courchamp F. (2009) Dangerously few liaisons: a review of mate-finding Allee effects. *Population Ecology*, **51**, 355–372.

Groom M.J. (1998) Allee effects limit population viability of an annual plant. *American Naturalist*, **151**, 487–496.

Haag W.R. (2012) *North American Freshwater Mussels: Natural History, Ecology, and Conservation*. Cambridge University Press, New York, NY.

Haag W.R. (2013) The role of fecundity and reproductive effort in defining life history strategies of North American freshwater mussels. *Biological Reviews*, **88**, 745–766.

Haag W.R. & Staton J.L. (2003) Variation in fecundity and other reproductive traits in freshwater mussels. *Freshwater Biology*, **48**, 2118–2130.

Heard W.H. (1975) Sexuality and other aspects of reproduction in Anodonta (Pelecypoda: Unionidae). *Malacologia*, **15**, 81–103.

Ishibashi R., Komaru A. & Kondo T. (2000) Sperm sphere in unionid mussels (Bivalvia: Unionidae). *Zoological Science*, **17**, 947–950.

Johnson M.S. (2011) *A Quantitative Survey of the Freshwater Mussel Fauna in the Powell River of Virginia and Tennessee, and Life History Study of Two Endangered Species, Quadrula Sparsa and Quadrula Intermedia*. MS Thesis, Virginia Polytechnic Institute and State University, Blacksburg, USA.

Kat P.W. (1983) Sexual selection and simultaneous Hermaphroditism among the Unionidae (Bivalvia, Mollusca). *Journal of Zoology*, **201**, 395–416.

Kokko H. & Rankin D.J. (2006) Lonely hearts or sex in the city? Density-dependent effects in mating systems. *Philosophical Transactions of the Royal Society B-Biological Sciences*, **361**, 319–334.

Kramer A.M., Dennis B., Liebhold A.M. & Drake J.M. (2009) The evidence for Allee effects. *Population Ecology*, **51**, 341–354.

Lande R. (1998) Demographic stochasticity and Allee effect on a scale with isotropic noise. *Oikos*, **83**, 353–358.

Landis A.M.G., Haag W.R. & Stoeckel J.A. (2013) High suspended solids as a factor in reproductive failure of a freshwater mussel. *Freshwater Science*, **32**, 70–81.

Lee A.M., Saether B.E. & Engen S. (2011) Demographic stochasticity, allee effects, and extinction: the influence of mating system and sex ratio. *American Naturalist*, **177**, 301–313.

Levitan D.R., Sewell M.A. & Chia F.S. (1992) How distribution and abundance influence fertilization success in the sea-urchin strongylocentrotus-franciscanus. *Ecology*, **73**, 248–254.

Lundquist C.J. & Botsford L.W. (2011) Estimating larval production of a broadcast spawner: the influence of density, aggregation, and the fertilization Allee effect. *Canadian Journal of Fisheries and Aquatic Sciences*, **68**, 30–42.

McMahon R.F. & Bogan A.E. (2001) Mollusca: Bivalvia. In *Ecology and Classification of North American Freshwater Invertebrates*. (EdsJ.H. Thorpe & A.P. Covich), pp. 315–399. Academic Press Inc, New York

Moles K.R. & Layzer J.B. (2008) Reproductive ecology of Actinonaias ligamentina (Bivalvia: Unionidae) in a regulated river. *Journal of the North American Benthological Society*, **27**, 212–222.

Neves R.J. (1997) A national strategy for the conservation of native freshwater mussels. In *Conservation and Management of Freshwater Mussels II: Initiatives for the Future*. (Eds K.S. Cummings, A.C. Buchanan, C.A. Mayer & T.J. Naimo), pp. 1–10. Upper Mississippi River Conservation Committee, Rock Island, IL

Neves R.J. (2004) Propagation of endangered freshwater mussels in North America. *Journal of Conchology Special Publication*, **3**, 69–80.

Okada S. & Ishikawa Y. (1959) The biological study of freshwater pearl mussel, *Margaritifera margaritifera* (L.). *Scientific reports of the Hokkaido Fish Hatchery*, **14**, 73–81. (in Japanese).

Pemberton A.J., Hughes R.N., Manriquez P.H. & Bishop J.D.D. (2003) Efficient utilization of very dilute aquatic sperm: sperm competition may be more likely than sperm limitation when eggs are retained. *Proceedings of the Royal Society B-Biological Sciences*, **270**, S223–S226.

Phillippi A., Hamann E. & Yund P.O. (2004) Fertilization in an egg-brooding colonial ascidian does not vary with population density. *Biological Bulletin*, **206**, 152–160.

Richard P.E., Dietz T.H. & Silverman H. (1991) Structure of the gill during reproduction in the unionids anodonta-grandis, ligumia-subrostrata, and carunculina-parva texasensis. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **69**, 1744–1754.

Saha S. & Layzer J.B. (2008) Evaluation of a nonlethal technique for determining sex of freshwater mussels. *Journal of the North American Benthological Society*, **27**, 84–89.

Shaffer M.L. (1981) Minimum population sizes for species conservation. *BioScience*, **31**, 131–134.

Spooner D.E. & Vaughn C.C. (2009) Species richness and temperature influence mussel biomass: a partitioning approach applied to natural communities. *Ecology*, **90**, 781–790.

Stephens P.A., Sutherland W.J. & Freckleton R.P. (1999) What is the Allee effect? *Oikos*, **87**, 185–190.

Strayer D.L., Downing J.A., Haag W.R., King T.L., Layzer J.B., Newton T.J. *et al.* (2004) Changing perspectives on pearly mussels, North America's most imperiled animals. *BioScience*, **54**, 429–439.

Tankersley R.A. & Dimock R.V. (1993) Endoscopic visualization of the functional-morphology of the ctenidia of the unionid mussel pyganodon-cataracta. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **71**, 811–819.

Utterback W.I. (1931) Sex behavior among naiades. *Proceedings of the West Virginia Academy of Science*, **5**, 43–45.

Van der Schalie H. (1970) Hermaphroditism among North American freshwater mussels. *Malacologia*, **10**, 93–112.

Waller D.L. & Lasee B.A. (1997) External morphology of spermatozoa and spermatozeugmata of the freshwater mussel Truncilla truncata (Mollusca: Bivalvia: Unionidae). *American Midland Naturalist*, **138**, 220–223.

Williams J.D., Bogan A.E. & Garner J.T. (2008) *Conservation Status of Freshwater Mussels of Alabama and the Mobile Basin in Georgia*. The University of Alabama Press, Tuscaloosa, Mississippi and Tennessee.

Williams J.D., Warren M.L., Cummings K.S., Harris J.L. & Neves R.J. (1993) Conservation status of fresh-water mussels of the United-States and Canada. *Fisheries*, **18**, 6–22.

Yund P.O. (2000) How severe is sperm limitation in natural populations of marine free-spawners? *Trends in Ecology & Evolution*, **15**, 10–13.

(Manuscript accepted 28 June 2014)