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Effects of self- and cross-fertilization on parental fecundity and offspring survival in eight populations of *Lymnaea stagnalis*

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ABSTRACT

In freshwater snail *Lymnaea stagnalis* which is a self-compatible simultaneous hermaphrodite capable of reproducing by both self-fertilization and cross-fertilization, mating system evolution depends on the relative fitness of reproduction via self-fertilization and cross-fertilization. Self-fertilizing hermaphrodites and their progeny are supposed to suffer lowered fitness compared to outcrossing ones, referred as inbreeding depression and self-fertilization depression. In this study, the relative fitness of snails from eight natural populations were quantified and compared between the two reproductive modes. The experimental manipulations of mating system in the laboratory revealed that self-fertilization had no effect on parental fecundity, and only minor effects on offspring survival. Also evidence for a delayed onset of reproduction in self-fertilizing snails was found. These results indicate that self-fertilization does not result in significant (>50%) fitness reduction compared with cross-fertilization in *L. stagnalis*. The occurrence of delayed selfing indicates preferential cross-fertilization.

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TIIVISTELMÄ

Kaksineuvoisella makean veden isolimakotilolla (*Lymnaea stagnalis*) on samassa yksilössä sekä naaras- että koiraspuoliset lisääntymiselimet. Sama yksilö pystyy siis tuottamaan sekä naaras- että koiraspuolisia sukusoluja, jolloin kotiloilla on teoriassa mahdollisuus lisääntyä sekä itsesiitoksen että ristisiitoksen avulla. Näiden lisääntymistapojen esiintyvyys saattaa vaihdella sekä populaatioiden sisällä, että niiden välillä. Tämä lisääntymistapojen vaihtelu tarjoaa mahdollisuuden tutkia niitä olosuhteita, joissa itsesiitosta ja ristisiitosta esiintyy sekä niitä vaikutuksia, joita kukin lisääntymistapa tuottaa. Lisääntymistapojen evoluutio on riippuvainen itsesiitoksen ja ristisiitoksen suhteellisesta vaikutuksesta yksilöiden kelpoisuuteen. Yleisesti oletetaan, että itsesiitos johtaa alentuneeseen kelpoisuuteen. Kelpoisuuden aleneminen voi johtua mm. kotiloiden heikentyneestä hedelmällisyydestä (alentunut munien tuotto) tai jälkeläisten heikentyneistä selviytymismahdollisuuksista. Jälkimmäistä kutsutaan sisäsiitosheikkoudeksi ja molempia yhdessä itsesiitosheikkoudeksi. Tässä tutkimuksessa määritettiin kvantitatiivisesti lisääntymistavan vaikutuksia kahdeksasta eri luonnonpopulaatiosta peräisin olevien kotiloiden kelpoisuuteen. Tuloksena havaittiin, että itsesiitoksella lisääntyvien kotiloiden ja ristisiitoksella lisääntyvien kotiloiden munantuottokyky oli samanlainen. Lisäksi havaittiin, että itsesiitoksella lisääntyvien kotiloiden jälkeläiset kärsivät vain vähäisestä sisäsiitosheikkoudesta. Kotiloilla todettiin myös ns. viivästynyttä itsesiitosta, mikä saattaa olla enimmäkseen ristisiitoksella lisääntyvien kotiloiden keino pidättäytyä lisääntymästä kun olosuhteet eivät ole suotuisat (ei lisääntymiskumppaneita). Kokonaisuudessaan itsesiitoksella lisääntyvillä *L. stagnalis* -kotiloilla ei havaittu merkittävää (>50%) kelpoisuuden heikentymistä.

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1. INTRODUCTION

Mating system evolution of hermaphroditic snails depends on the relative fitness of reproduction via self-fertilization and cross-fertilization. Current theory holds that the main evolutionary force favouring the evolution of self-fertilization is the 3:2 transmission advantage for a mutant allele causing selfing in an outcrossing population (Fisher, 1941). The evolution of self-fertilization in hermaphrodites is opposed by cots that decrease the value of selfed progeny relative to that of outcross progeny. Inbreeding depression (δ) is considered the most important force opposing the evolution of self-fertilization (Charlesworth & Charlesworth, 1998). Early models with fixed inbreeding depression for the evolution of self-fertilization found that if selfing does not affect the numbers of male gametes available to outcrossing, $\delta < 0.5$ should favour the evolution of self-fertilization, whereas $\delta > 0.5$ should favour the evolution of cross-fertilization (Lloyd, 1979; Charlesworth, 1980). More sophisticated models of the evolution of selfing specify the genetic basis of inbreeding depression, and these models allow changes in inbreeding depression.

Two major explanations exist for inbreeding depression; over dominance hypothesis and partial dominance hypothesis. In both hypothesis, increased homozygosity following inbreeding leads to decline in the mean genetic value of the character, either because alleles that are complementary as heterozygotes segregate as homozygotes (overdominance hypothesis), or because deleterious (partially) recessive alleles are expressed in homozygous combinations (partial dominance hypothesis) (Waser & Williams, 2001). Most evidence supports the partial dominance hypothesis (Wright, 1977; Charlesworth & charlesworth, 1987; Lynch & Walsh, 1998; Byers & Waller, 2000).

When deleterious (partially) recessive alleles are expressed in homozygous combinations the inbreeding history of a population should influence the magnitude of inbreeding depression and the evolution of mating systems (Waser & Williams, 2001). Lande and Schemske (1985) developed a model of mating system evolution in which the history of inbreeding will purge (i.e. reduce equilibrium frequencies of unfavourable recessive and partially recessive alleles) a population of its genetic load and thereby reduce the magnitude of inbreeding depression over time. They concluded that most populations should therefore evolve either toward complete selfing or complete outcrossing.

Even if strong inbreeding depression is necessary for the maintenance of a stable outcrossing system, ecological factors, rather than levels of inbreeding depression, may be of primary importance in determining when inbreeding evolves. Such factors may include small population sizes or biparental inbreeding (outbreeding between relatives) under limited dispersal (leading respectively to fixation or purging of some portion of the mutational load) (Jarne & Charlesworth, 1993). Low density (lack of mating partners) may give a greater advantage to selfing due to reduced cross-fertilization success (reproductive assurance), so that selfing can evolve even when inbreeding depression occurs (Jarne & Charlesworth, 1993). Several works have indicated that intermediate mixed-mating systems may be common and evolutionarily stable (e.g. Uyenoyama et al. 1993; Johnston et al. 1998; Byers & Waller 1999).

Pulmonate snails like *L. stagnalis* provide a nice opportunity to compare selfing and outcrossing fitness values e.g. number of eggs produced (fecundity) and number of surviving progeny (offspring fitness). The reduced fitness of selfed or inbred offspring relative to outcrossed ones has referred as inbreeding depression. The combined effect of

self-fertilization on parental fecundity and on offspring fitness has been termed self-fertilization depression (Jarne et al., 1991).

It is usually assumed that fertility is lowered in self-fertilizing individuals and previous studies have observed significantly reduced fecundity in *Lymnaea peregra* (Coutellec-Vreto et al., 1998). The opposing results, high fecundity of self-fertilizing *L. stagnalis* have also been observed (Noland & Carriker, 1946). Much is known about the factors affecting reduced fecundity in self-fertilizing hermaphroditic snails and some of the factors may be confounding ones. First, there may be physiological constraints that limit the number of eggs that can be efficiently self-fertilized (e.g. self-sterility) (Coutellec-Vreto et al., 1998). Second, individuals in self-fertilization treatment may delay the onset of egg laying (or lay no eggs at all and delay sexual maturity) compared to individuals allowed to mate, leading to lower total number of eggs produced (Jarne et al., 1991; Wethington & Dillon, 1993). Third, reduced fecundity may also be a result from an early expression of inbreeding depression (Coutellec-Vreto et al., 1998).

It has been found factors which reduce fecundity in cross-fertilizing hermaphroditic snails e.g. multiple matings in *L. stagnalis* (van Duivenboden et al., 1985) and the 'grouping effect' (Doums et al., 1994). Some factors have shown to increase and speed up the egg production of cross-fertilizing snails (mating manipulation) (Michiels, 1998; Koene et al., 2006). What ever is the cause of reduced fecundity, it is a component of self-fertilization depression.

Estimating the magnitude of inbreeding depression (as well as self-fertilisation depression) in *L. stagnalis* requires fitness comparisons of selfed progeny and presumably outcrossed progeny (and parental fecundity). There are potential problems with this approach; outcrossings cannot be inforced in partially self-fertilizing snails (unlike in plants) and 'grouping effects' resulting lowered fecundity of grouped snails, irrespective of density (Städler & Jarne, 1997, Doums et al.,1994). Moreover an absence of 'social facilitation', rather than inbreeding depression, might be responsible for the low fecundity and poor hatching success in isolated snails of predominantly outcrossing freshwater pulmonates (Vernon, 1995). In addition Doums et al. (1996) has defined so called self-fertilization syndrome in hermaphrodite freshwater snails in which selfing populations are chacterized by high selfing rates in spite of copulations, limited deleterious effects of selfing and large heterozygote deficiencies (and fitness decrease may be limited only to fecundity).

The aim of this study was 1.) to quantify the fitness components (fecundity and offspring survival) of outcrossed and selfed *L. stagnalis* individuals derived from laboratory experiments which where designed to control for the effects of mating activity (no grouping or multiple matings, social facilitation included) and 2.) to quantify the amount of inbreeding depression (δ) and self-fertilization depression (SFD) in the study populations.

2. MATERIALS AND METHODS

2.1. Study species

Lymnaea stagnalis (Linnaeus 1758) is a holarctic freshwater snail (Gastropoda: Pulmonata: Basommatophora) inhabiting stagnant or sluggish low altitude waters (lakes, ponds, brackish waters) with luxuriant vegetation (eutrofic). *L. stagnalis* is a simultaneous hermaphrodite, which is able to reproduce by both self- and cross-fertilization. For this reason, and because the species is easy to rear in laboratory conditions, *L. stagnalis* is an

excellent study species for comparing the fitness of outcrossing and selfing individuals in controlled laboratory conditions.

2.2. Populations and laboratory rearing conditions

Egg masses of *L. stagnalis* were collected between the 12th of June and 23rd of July in 1999, from eight natural populations, situated near Jyväskylä in Central Finland (Fig.1). Half of the populations came from small lakes or ponds (A, B, C and F) which do not have streams running into them. The other half of the populations came from shallow bays of larger lakes (D, E, G and H) but each population from a different lake. Populations were situated several kilometers apart.

After the collection, all egg masses were brought to laboratory, where each egg mass was separately placed into small water filled vial and checked daily. During the whole study, temperature in the laboratory was kept at 19 °C (+/- 1°C) and artificial photoperiod of 16:8 h light:dark cycle was maintained. Hatchlings were transferred to larger (V=1l) jars and the hatchlings from the same egg mass were kept together. Before the onset of sexual activity, five snails from each egg mass were isolated to family groups and each member of the group put to a separate rearing jar (V=1l). There were 20 to 24 family groups for population A, B, D, E, F and H. Seventeen family groups were formed from population G and twelve from population C. Two of the family groups of population C had more than five family members to balance the number of snails among population. Snails in the family groups became the parental snails in the experiments.

For very young hatchlings a pinch of dried cat food was added to the jars to supplement the early diet. During the laboratory rearing and study period, snails were fed with fresh lettuce ad libitum. In rearing jars, we used groundwater to avoid possible problems caused by disinfectants and dissolved (heavy) metals in tap water. Before use, groundwater was aerated, calcified by keeping $CaCO_3$-stones in the water tank, and stabilized to laboratory air temperature. Water in rearing jars was changed once a week and snails were fed on daily bases. The positions of rearing jars were changed regularly to avoid any kind of bias due to rearing conditions.

Four weeks after the isolation of laboratory reared individuals, the first became sexually mature. Reared individuals were considered mature, when they had laid their first self-fertilized egg masses in the laboratory. At that time, snail's size was measured with a digital caliper (to exclude the possible size effects of snails in the analysis), as the distance from the apex to the spire of the shell. Size was also measured for the second time, at the onset of study treatments. Study treatments were started when at least 50 % of the snails from reared parental populations were sexually mature and had laid at least one egg mass by selfing.

2.3. Study design

From each population, there were on average 16 family groups with five members on each to be used in treatments. Two of the snails within a family group were randomly designed to self-fertilization treatment and the other two were randomly designed to cross-fertilization treatment. The fifth snail was used as a 'sperm donor' individual in the cross-fertilization treatment and as a 'partner' individual in the self-fertilization treatment.

2.3.1. Self-fertilization treatment

In self-fertilization treatment, the randomly chosen selfer snail was placed in a small jar (V=1l) with another snail ('partner' individual) from the same population but from a different family. Snails were however, separated by a plastic mesh, which made

copulations impossible but allowed the snails to have some tactile contact. The 'partner' individual in the selfing treatment was necessary in order to minimize the procedural differences between the two study treatments and control for possible social facilitation effects (Vernon, 1995; Baur & Baur, 2000). In all occasions, the 'partner' individual in the selfing treatment was later used as a sperm donor (i.e. as a 'male' individual) in outcrossing treatments. Within each population, selfing treatments were all done before outcrossing treatments. Each selfing treatment lasted about three hours, after which snails were separated and put in isolation.

2.3.2. Cross-fertilization treatment

In cross-fertilization treatment, the randomly chosen crosser snail was placed in a small (V=0.5l) transparent pairing jar with a 'sperm donor' snail from the same population but from different family. 'Sperm donor' snails were marked with nail polish at the apex of the shell for identification. The mating behavior of these pairing snails (whose sexual roles were chosen beforehand) was carefully observed and copulations were identified in accordance with detailed mating behavior work done by van Duivenboden & ter Maat, (1988).

Copulation was considered as qualified, when the 'sperm donor' individual had been seen to insert his penis (preputium) into the crosser's genital pore and had stayed in the copulatory position for at least 20 minutes (intromission). In *L. stagnalis*, the copulation is always unilateral, i.e. within a pair an individual can only mate as a male or a female, simultaneous intromission is not possible. So called sham-copulations (like a copulation but no actual intromission and sperm donation) were also distinguished from real copulations. After the copulation, the 'sperm donor' and the 'crosser' were separated and put in isolation. If the snail designed to cross-fertilization did not mate as female in the first trial, mating trial was repeated during the following days. Despite repeated mating trials and alternative sperm donors, some individuals never mated in the female role, and were excluded from further analysis. In the study of van Duivendoden & ter Maat (1988), the success of copulation was nearly 100%. As their instructions of copulation observation were carefully followed in this study, the same success accuracy can be assumed as in their study.

2.4. Fitness measures

As some of the snails had not started to reproduce before the mating system treatment, a Mann-Whitney test was used to determine whether the onset of egg laying of these snails was affected by the mating system treatment. The low number of snails available per population for this comparison precluded calculation of population-specific delays in self-fertilization. Therefore, data from all populations were pooled.

In isolation, both selfed and outcrossed snails laid egg masses on the sides and bottom of the isolation jars, on the lettuce leaves and sometimes even free floating egg masses were observed. Egg masses were then collected at weekly bases. From snails that had laid egg masses before the treatment, all the egg masses laid within two weeks after the treatment were collected and counted. From snails that had not laid egg masses before the treatment, all the egg masses from the first time egg masses were found and for a period of two full weeks after that were collected and counted (the very first egg masses were excluded from the fecundity analysis). All the eggs surrounded by an egg capsule within a clear gelatinous egg mass were counted under a stereomicroscope. Often there were more than one egg in egg capsule but fecundity of snails was estimated as the number of egg capsules in egg mass (approximately equal to number of eggs). From all study individuals, minimum of three egg masses per individual, were randomly chosen for

recordings of egg hatching success. However, if the total number of eggs from these three masses of eggs was less than one hundred, then additional egg masses were included until the total was at least one hundred eggs per study individual. The chosen egg masses were put on special 'hatching plates', and each egg mass was checked daily for six weeks period, and all hatched juveniles were counted and removed.

After the period of six weeks of checking for hatchlings, it was investigated what remained in the egg mass. The number of remaining intact egg capsules that had no egg inside (empty egg capsules) was recorded and the number of eggs that had not started to develop or the development was arrested at very early stage (undeveloped eggs) was recorded. The number of dead unhatched snails was also recorded, and classified as 'small larva' or 'large larva'. A 'large larva' was at the size of a typical hatchling and a 'small larva' was less than half of that. Further, the number of ruptured egg capsules from which snails had emerged was recorded. This data made it possible to double-check the estimate of hatching success. The empty egg capsules were not included in the analysis of hatching success. Of the egg capsules with more than one egg, only those that had two or three eggs were included to analysis, as eggs from these capsules often successfully developed into hatchlings.

2.5. Estimate of inbreeding depression (δ)

An estimate of inbreeding depression in hatching success (δ) in each population was calculated as

$$\delta = 1 - h_s/h_c$$

where h_s and h_c are hatching success of eggs of self-fertilizing and cross-fertilizing snails respectively.

2.6. Estimate of self-fertilization depression (SFD)

An estimate of self-fertilization depression (SFD) in each population was calculated as

$$SFD = 1 - f_s/f_c \times h_s/h_c$$

where f_s and f_c are fecundities of self-fertilizing and cross-fertilizing snails respectively, and h_s and h_c are hatching success of eggs from self-fertilizing and cross-fertilizing snails respectively.

2.7. Statistical analysis

2.7.1. Fecundity

Fecundity was analyzed with a linear mixed model (error distribution normal, checked graphically to be satisfactory) in which population, treatment (self- or cross-fertilization) and the maturation stage (matured before treatment, matured after treatment) were specified as fixed effects. Family (within population) was entered as a random effect. The final model was obtained by dropping out non-significant interactions with family or maturation stage. The estimation of the model parameters as well as their significance tests were carried out by SAS procedure MIXED using the restricted maximum likelihood (REML) approach with related Wald-type F tests and Z tests for the fixed effects and variance parameters, respectively (Brown & Prescott 1999). The data was also analysed with population as a random factor, but this did not change the conclusions.

2.7.2. Hatching success and egg mortality

The analysis of hatching success and egg mortality was complicated because mortality increased with the number of eggs per egg mass, and the number of eggs per egg mass was itself affected by maternal snail, population and treatment (see Results). Consequently, the number of eggs per egg mass should not be used as a covariate in the analysis of mortality because it obscures the effects of other factors on mortality. To solve this problem, a generalized mixed model was estimated for the number of eggs per egg mass with the same explanatory variables as in the model for mortality. The residuals of this model were used as a covariate in modeling the mortality. The model was estimated with SAS macro GLIMMIX (Littell et al. 1996), using the restricted pseudo-likelihood (REPL) estimation method (Wolfinger & O'Connell 1993), which is expected to perform well with large data sets.

Four separate logistic mixed models corresponding to the experimental design were built in order to study mortality at the three developmental stages (undeveloped egg, small and large larva), and to study the total pre-hatching mortality. Each model describes the probability that a single egg will die at the specified stage, thus the sample size in these models is the number of eggs observed. In the first modeling stage population, treatment and their interaction were specified as fixed effects; and family (nested within population), maternal snail (nested within family), and their interactions with treatment were specified as random effects. Population was entered as a fixed effect because it was desirable to obtain unbiased estimates for mortality in each population by treatment combination. Total mortality was also analyzed with population as a random factor; the results did not change. The effect of maternal snail includes the possible effects of paternal snail for the cross-fertilizing snails. Residuals of the mixed model for the number of eggs per egg mass were used in the model as a covariate (see above). The final models for mortality were obtained by dropping out those random effects whose variance components were zero or non-significant. All models were estimated with the GLIMMIX macro. For each model, the model fit was assessed by examining residual deviance with respect to degrees of freedom. The scale parameter for possible overdispersion was also calculated.

To illustrate the relative magnitude of different factors contributing to mortality, the proportion of explained deviance that could be attributed to each factor was estimated. The approximations were obtained by entering all factors in the final model as fixed effects and recording the total explained deviance. The contribution of each factor to the total explained deviance was then defined as the increase in the unexplained deviance when omitting the factor from the final model. Nested effects make an exception, since higher effects cannot be omitted if lower effects in the hierarchy are still present. For instance, the approximate contribution of the population effect must be calculated from a model in which the family effect is not present. The same applies for interaction and main effects: the contribution of main effects must be approximated from a model in which the interaction is not present. Treating random effects as fixed in the analysis does not generate serious error, as the difference between the explained deviance of the mixed model and that of the model with all factors fixed was negligible.

2.7.3 Inbreeding depression in hatching success

The estimates of inbreeding depression in hatching success in each population were calculated from the estimated model for total pre-hatching mortality (hatching success = 1 – total pre-hatching mortality). Since inbreeding depression is a nonlinear function of hatching success, approximate standard errors and confidence intervals utilizing the Taylor series linearization method and the asymptotic normality of the estimates were calculated. These calculations were carried out by self-made SAS programs.

2.7.4. Self-fertilization depression

The estimates of self-fertilization depression in each population were calculated from the estimated models for total mortality and fecundity. The approximate standard errors and confidence intervals again were based on the Taylor series linearization and the asymptotic normality of estimates.

3. RESULTS

3.1. Snail size

At the time of mating manipulation treatment, there were no significant differences in size of snails designed to self-fertilization and cross-fertilization (Mann-Whitney U, $P \geq 0.248$ for all populations). Populations did have significant differences in size of snails at the time of treatment (Kruskall-Wallis X^2 =368.465, df = 7, $P < 0.001$) and those differences between populations were due to differences in the size of snails at the time of maturation (also highly significant; Kruskall-Wallis X^2 = 241.876, df = 7, $P < 0.001$).

3.2. Fecundity

Population and family (nested within population) had significant effects on fecundity (Table 2). The effects of mating system treatment (self-fertilization or cross-fertilization) and the effects of maturation stage, and population by treatment interaction were all non-significant. Figure 2 shows the model estimates for fecundity in each population by treatment combination.

Thirty-five percent of snails in the cross-fertilization treatment mated in both male and female role. To exclude all the possible fecundity effects of mating in the male role the fecundities of snails that had mated as both male and female were compared to snails that had only mated as female in the cross-fertilization treatment. This revealed no differences in the fecundities of the two groups of snails (t-test: all populations combined t_{225} = -1.260, P = 0.209, each population separately $P > 0.05$ for each test).

3.3. Onset of egg-laying

Snails that had not laid eggs before the beginning of treatments started to lay eggs sooner in the cross-fertilization treatment than in the self-fertilization treatment (medians 1 and 2 weeks, respectively; Mann-Whitney U = 2061, P = 0.030).

3.4. Number of eggs per egg mass

For the number of eggs per egg mass, the family variance component was estimated to be zero and the family by treatment variance component was not significant (Z = 1.14, P = 0.128) thus they were excluded from the mixed model. According to the final estimated model, the number of eggs per egg mass was affected by the maternal snail (Z = 8.93, $P < 0.001$), by population ($F_{7, 462} = 10.35$, $P < 0.001$) and by treatment ($F_{1, 462} = 6.61$, $P < 0.001$). Overall snails in the cross-fertilization treatment laid larger egg masses than snails in the self-fertilization treatment but in two populations the egg masses laid by selfers were larger (a nearly significant treatment by population interaction ($F_{7, 462} = 1.89$, P = 0.069) (see Fig. 3). Model residuals were used as a covariate in the model for mortality.

3.5. Egg mortality

Results from the analysis of total pre-hatching mortality and mortality during the three developmental stages are presented in Table 3, together with estimates of the proportion of

explained deviance that could be attributed to each factor. The variance component of family by treatment interaction was not significant at any stage, and was excluded from all final analyses. According to standard goodness-of-fit criteria (deviance, scale parameter for overdispersion), all estimated models fit adequately to the data.

Maternal snail and population had significant effects on total pre-hatching mortality and mortality at all three developmental stages and explained a large fraction of model deviance. Family had a significant effect only on the number of undeveloped eggs. Mating system treatment (self- vs. cross-fertilization) had a significant but minor effect on the number of undeveloped eggs, mortality as a small larva, and on total mortality. Population by treatment interaction was not significant at any stage. The number of eggs per egg mass (residual) had a significant effect on the number of undeveloped eggs, on mortality as a large larva, and on total mortality. Overall mortality at large larva stage was higher in larger egg masses.

The distribution of pre-hatching mortality among the three developmental stages for all population by treatment combinations is presented in Figure 4 (calculated from the parameters of the mixed models). Most of the variation in mortality resulted from mortality at the large larva stage. Mortality at the small larva stage was relatively insignificant. The proportion of undeveloped eggs varied moderately among populations and treatments. Overall self-fertilized eggs in all populations suffered higher mortality than cross-fertilized eggs during the first two developmental stages.

3.6. Inbreeding depression in hatching success

The estimate of inbreeding depression in hatching success was positive in all eight populations i.e. self-fertilized eggs had lower hatching success than cross-fertilized eggs (Table 1). In three of the populations (A, C and H), the estimate of inbreeding depression was significantly greater than zero. The 95% confidence intervals for inbreeding depression in population C do not overlap with those of populations A, B, D, E, and F, suggesting that population C has a different level of inbreeding depression (despite the non-significant treatment by population interaction term in the analysis of egg mortality). Overall, the estimates of inbreeding depression in all populations were low to moderate, with the minimum value of 0.01 (population B) and maximum of 0.34 (population C). Importantly, in all eight populations, the confidence intervals for inbreeding depression were below the critical 0.5 value.

3.7. Self-fertilization depression

Estimates for self-fertilization depression (includes the effect of self- and cross-fertilization treatment on fecundity and hatching success) in each population are given in Table 1. The estimated values for self-fertilization depression ranged from -0.28 to 0.24, and were not significantly different from zero in any of the populations. Despite rather wide confidence intervals of the estimates, self-fertilization depression was significantly less than 0.5 in three of the populations (A, D, and G).

4. DISCUSSION

In this study with *Lymnea stagnalis* it was found that the fecundities of self-fertilized and cross-fertilized snails were similar when the experimental design controlled for the effects of mating activity. On the other hand, there were huge differences in fecundity between populations (nearly five fold amount of egg capsules between the lowest and highest value). Although there is no exact certainty that fertilizations took place in copulations

observed, there is reason to believe so, as the behavioural instructions of van Duivenboden and ter Maat (1988) were carefully obeyed and the preference of *L. stagnalis* for cross-fertilizations when mating was known (Cain, 1956). As the experimental mating system manipulation had no effects on the fecundity of snails, the evolution of self-fertilization seems not to be limited by the snails' ability to produce self-fertilized eggs in the studied populations.

Interestingly, the snails that had not been laying eggs before the beginning of the treatment started to lay eggs sooner in the cross-fertilization treatment than those in the self-fertilization treatment. Previously, mating in the female role has been reported to accelerate the onset of egg laying in *L. stagnalis* by about 2 weeks (van Duivenboden 1983). Such a delayed onset of reproduction in self-fertilizing snails can be an adaptive strategy for preferentially cross-fertilizing hermaphrodites to just 'wait and see' whether sexual partner encounters or not. And if not, in such occasions, self-fertilization can be an option favoured as a reproductive assurance (Maynard Smith 1978; Kalisz et al. 2004).

On the other hand, *L stagnalis* is known to be capable of storing and utilizing foreign sperm for about 100 days (Cain 1956). Hence possible mate encounters do not have to be very frequent, as in the case of low population densities. It has been reported that postponing the onset of reproduction when mating partners are not available together with sperm storing capability may lead to stable mixed mating and high outcrossing rates also in populations with low densities (Tsitrone et al., 2003).

There is a possibility however, that the observed earlier onset of egg laying is a consequence of mate manipulation (Michiels,1998; Koene et al., 2006) which this experimental design could not exclude.

In all but two populations, the cross- fertilizing snails laid larger egg masses than self-fertilizing snails, but this did not have effects on the fecundity of snails as a whole. It was found that the number of eggs per egg mass explained a great deal of mortality at the large larva stage. On the other hand, most of the variation in total mortality was due to mortality at the large larva stage. More stressful conditions, especially a lack of dissolved oxygen for development in larger egg masses could explain this observation (greater mortality at the final developmental stage where the demand of oxygen is highest). Chaffee & Strathmann, (1984) and Marois & Croll, (1991) among others have shown that the membranes and jelly of the egg mass affect like a barrier to the diffusion of gases, nutrients and metabolites.

It was detected in this study that inbreeding depression in hatching success was low to moderate in the studied populations. However, inbreeding depression in late life-history stages (e.g. offspring fecundity) was not investigated in this study even though inbreeding depression can be expressed at any stage of the life-cycle (Ritland, 1990). In addition inbreeding depression could be underestimated by laboratory experiments as inbred genotypes may be particularly susceptible to poor environmental conditions (Jarne & Charlesworth, 1993). Hence, inbreeding depression can be much higher than what we estimated.

Even though the estimates of inbreeding depression were not near the critical 0.5 value (that opposes the competitive selfing), the estimates of inbreeding depression in hatching success were above zero in all eight populations, meaning that self-fertilized eggs had lower hatching success than cross-fertilized eggs. Moreover, in populations A, C and H, the estimate of inbreeding depression was significantly greater than zero although less than the critical 0.5 value. However, it has been shown in theory and practise that even predominantly selfing populations may show fitness reduction of selfed progeny; recurrent mutations to mildly deleterious alleles should be able to maintain substantial inbreeding

depression even in highly selfing populations (Lande et al., 1994; Johnston & Schoen, 1995).

There was a significant effect of mating system treatment on mortality during the first two developmental stages. Inbreeding depression may be especially marked in early stages of life where many important developmental functions occur, and early loss of genotypes with high inbreeding coefficients has been observed (Stevens & Bougourd, 1988; Ritland, 1991). The results from above suggest that some proportion of the genetic load of studied populations is due to deleterious (partially) recessive alleles segregating in the population. In that light the relatively low inbreeding depression estimates in all populations are not surprising given that purging is most efficient on early life-history stages, presumably because mutations affecting early survival have high selection coefficients and low dominance coefficients (Husband & Schemske, 1996; Charlesworth & Charlesworth, 1998; Byers & Waller, 1999). Such mutations are easily purged by non-random mating (Lande & Schemske 1985; Charlesworth et al., 1990; Glemin, 2003) or by an increase in homozygosity resulting from a small effective population size (Wang et al., 1999; Bataillon & Kirkpatrick, 2000; Hedrick, 2002; Glemin, 2003).

Jarne et al., (1991) have pointed out that in addition to inbreeding depression (hatching success) the fecundities of selfed and outcrossed parental snails must be taken into account when studying the evolution of mating systems (both can select for or against self-fertilization). This overall fitness reduction is referred as self-fertilization depression. There was no self-fertilization depression in this study as the estimated values were not significantly different from zero in any of the populations. This is not surprising because fecundities of outcrossed and selfed snails were similar within populations and inbreeding depression observed was only low to moderate.

In this study however, parental snail had the largest effect on total mortality and mortality at all three measured developmental stages. An explanation for this, and for the significant family effect on the number of undeveloped eggs, can be seen the work of Schultz & Willis (1995) in which they used mutation-selection recursion models to evaluate the relative contributions of mutation and inbreeding history to variation among individuals in inbreeding depression. Their models showed that Poisson (i.e. random) mutation to deleterious additive or recessive alleles generally produces far more variation among individuals in inbreeding depression than variation in history of inbreeding, regardless of selfing rate. Moreover the variation in inbreeding depression can be higher in a completely outcrossing or selfing population than in a mixed mating population (Schultz & Willis, 1995).

The strong effect of population on mortality at each developmental stage indicates that the populations differ in the amount of genetic load affecting hatching success, and some of this variation may be due to differential purging of the genetic load (as discussed above). Several authors have showed that inbreeding increases the genetic variance among populations and decreases the within –population genetic variance (Whitlock & McCauley, 1990; Maruyama & Tachida, 1992; Jarne, 1995).

The theory, together with empirical studies predict that inbreeding depression should be high in out-crossing populations or species and low in those with self-fertilization (Husband & Schemske, 1996; Lande & Schemske, 1985; Glémin, 2003). As this laboratory study shows low to moderate amount of inbreeding depression and no significant indication of self -fertilization depression, the prevailing mating system for *L. stagnalis* should be selfing in nature. Puurtinen et al. (2007) performed genetic analyses (microsatellite loci) to reveal the exact mating system of *L. stagnalis* in nature (investigated the same eight populations as this study), and found *L. stagnalis* to have predominantly cross-fertilizing mixed mating system in natural population. This contrasts

with the conclusion drawn from the theoretical work because *L. stagnalis* has been shown to predominantly cross-fertilize and yet this study showed inbreeding depression estimation values lower than the critical 0.5 value and a non significant amount of self-fertilization depression. The contrast might disappear with the assumption that the strength of inbreeding depression has been underestimated. This could be possible as offspring fecundity was not measured. Moreover, recent work made by Kalisz et al. (2004) shows that even when inbreeding depression is low (lower than the ~0.5 value that opposes competing selfing) intermediate selfing rates through mixed mating system has observed. Selfing can act as a reproductive assurance (together with delayed selfing) when cross-reproduction is constrained e.g. due to lack of mates (Kalisz et al, 2004). In that case individuals can outcross when it is possible to find mates, but resort to selfing if no mates are available. This can result in different annual/seasonal selfing rates as a functional response to lack of mates. Mating system models have shown that an intermediate level of selfing can be evolutionarily stable (Schoen et al., 1991; Johnston, 1998).

Overall the results of Schultz & Willis (1995) suggest that genetic associations between mating loci and inbreeding depression loci could be difficult to demonstrate within populations and observable only transiently during rapid evolution to a substantially new selfing rate. Selfing is by no means the only factor contributing to inbreeding depression. Several theoretical studies have shown that various factors can maintain mixed mating systems besides selfing e.g. biparental inbreeding (Uyenoyama, 1986) and population subdivision (Ronfort & Couvet, 1995).

It has also been shown that inbreeding depression is not a static phenomenon but itself coevolves with the mating system (e.g. Uyenoyama et al. 1993) and with the spatial genetic structure of the population (Campbell & Waser 1987). This dynamical interaction has important implications for equilibrium patterns (including alternative equilibria) of mating and of fitness in natural populations.

In conclusion, in this study the fecundities of outcrossed and selfed *L. stagnalis* snails were similar and thereby the evolution of selfing is not limited by the ability of snails to produce eggs by self-fertilization. It has been investigated that partial selfing occurs in the wild in these studied populations (Puurtinen et al., 2007) and this mode of reproduction has shown to have costs in terms of hatching success (this study). There was low to moderate inbreeding depression in hatching success in the study populations. In three of the populations, the estimate of inbreeding depression was significantly greater than zero but lower than the critical 0.5 value. Inbreeding depression may be greater than estimated as offspring fecundity was not measured. There was a hint of individual snails' or of populations genetic load playing a role in the amount of inbreeding depression found and populations differed in the amount of genetic load. *L. stagnalis* showed possible delayed selfing (together with the ability to store sperm), which can be adaptive strategy for self-compatible preferentially cross-fertilizing hermaphrodites to have some reproductive assurance. This result is nicely compatible with the results obtained from other studies that even if inbreeding depression is low (less than the critical 0.5 value) intermediate selfing rates through mixed mating system can be evolved. On the whole, there was no sign of significant (>50%) fitness reduction of selfing snails compared to outcrossing snails.

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REFERENCES

Bataillon T. & Kirkpatrick M. 2000. Inbreeding depression due to mildly deleterious mutations in finite populations: size does matter. *Genet. Res.* 75: 75-81.

Baur B. & Baur A. 2000. Social facilitation affects longevity and lifetime reproductive success in a self-fertilizing land snail. *Oikos* 88: 612-620.

Brown H. & Prescott R. 1999. Applied mixed models in medicine. John Wiley & Sons, Chichester.

Byers D. L. & Waller D. M. 1999. Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. *Annu. Rev. Ecol. Syst.* 30: 479-513.

Cain, G. L. 1956. Studies on cross-fertilization and self-fertilization in Lymnaea stagnalis appressa Say. Biol. Bull. (Woods Hole), 111: 45-52.

Campbell D. R. & Waser N. M.1987. The evolution of plant mating system: multilocus simulations of pollen dispersal. *Am. Nat*. 129: 593-609.

Chaffee C. & Strathmann R. R. 1984. Constraints on egg masses. I. Retarded development within thick egg masses. *J. Exp. Mar. Biol. Ecol.* 84: 73-83.

Charlesworth B. 1980. The cost of sex in relation to mating system. *J. Theor. Biol.* 84: 655-671.

Charlesworth D. & Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Syst.* 18: 237-268.

Charlesworth B. & Charlesworth. D. 1998. Some evolutionary consequences of deleterious mutations. *Genetica* 102 /103: 3-19.

Charlesworth D., Morgan M. T. & Charlesworth B. 1990. Inbreeding depression, genetic load, and the evolution of outcrossing rates in a multilocus system with no linkage. *Evolution* 44: 1469-1489.

Coutellec-Vreto M. A., Jarne P. Guiller A., Madec L. & Daguzan J. 1998. Inbreeding and fitness in the freshwater snail *Lymnaea peregra*: an evaluation over two generations of self-fertilization. *Evolution* 52: 1635-1647.

Doums C., Delay B. & Jarne P. 1994. A problem with the estimate of self-fertilization depression in the hermaphrodite freshwater snail *Bulinus truncatus*: The effect of grouping. *Evolution* 48: 498-504.

Doums C., Viard F., Pernot A. F., Delay B. & Jarne P. 1996. Inbreeding depression, neutral polymorphism, and copulatory behaviour in freshwater snails: a self-fertilization syndrome. *Evolution* 50: 1908-1918.

Fisher R. A. 1941. Average excess and average effect of a gene substitution. *Ann. Eugen*. 11: 53-63.

Glémin S. 2003. How are deleterious mutations purged? Drift versus nonrandom mating. *Evolution* 57: 2678-2687.

Hedrick P. W. 2002. Lethals in finite populations. *Evolution* 56: 654-657.

Husband B. C. & Schemske D. W. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* 50: 54-70.

Jarne P. 1995. Mating system, bottlenecks and genetic polymorphism in hermaphroditic animals. *Genet. Res*. 65:193-207.

Jarne P. & Charlesworth D. 1993. The evolution of the selfing rate in functionally hermaphrodite plants and animals. *Annu. Rev. Ecol. Syst.* 24: 441-466.

Jarne P., Finot L., Delay B. and Thaler R. 1991. Self-fertilization versus cross-fertilization in the hermaphroditic freshwater snail *Bulinus globosus*. *Evolution* 45: 1136-1146.

Johnston M. O. 1998. Evolution of intermediate selfing rates in plants: pollination ecology versus deleterious mutations. *Genetica* 102/103, 267-278.

Johnston M. O. & Schoen D. J. 1995. Mutation rates and dominance levels of genes affecting total fitness in two angiosperm species. *Science* 267: 226-229

Kalisz S., Vogler D. M. & Hanley K. M. 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature*. 430: 884-887.

Koene J. M., Montagne-Wajer K., & ter Maat A.. 2006. Effects of frequent mating on sex allocation in the simultaneously hermaphroditic great pond snail (*Lymnaea stagnalis)*. *Behav. Ecol. Sociobiol.* 60: 332-338.

Lande R. & Schemske D. W. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* 39: 24-40.

Lande R., Schemske D. W. & Schultz S. T. 1994. High inbreeding depression, selective interference among loci, and the threshold selfing rate for purging recessive lethal mutations. *Evolution* 48: 965-978..

Littell R. C., Milliken G. A., Stroup W. W. & Wolfinger R. D. 1996. *SAS system for* mixed *models.* SAS Institute, Cary (N.C.).

Lloyd D. G. 1979. Some reproductive factors affecting the selection of self-fertilization in plants. *Am. Nat.* 113: 67-79.

Lynch M. & Walsh B. 1998.Genetics and analysis of quantitative traits. Sinauer, Sunderland, Mass.

Marois R. & Croll R.P. 1991. Hatching asynchrony within the egg mass of the pond snail, *Lymnaea stagnalis. Invertebr. Reprod. Dev.* 19: 139-146.

Maruyama K. & Tachida H. 1992. Genetic variability and geographical structure in partially selfing populations. *Jpn. J. Genet.* 67:39-51.

Maynard Smith J. 1978. The Evolution of Sex. Cambridge University Press, Cambridge.

Michiels N. K. 1998. *Mating conflicts and sperm competition in simultaneous hermaphrodites* in Birkhead T. R. & Møller A.P.(eds) Sperm Competition and Sexual Selection. Academic Press, San Diego.

Noland L. E. & Carriker M. R.. 1946. Obseravations on the biology of the snail Lymnaea stagnalis appressa during twenty generations in laboratory culture. *Am. Midl. Nat.* 36: 467-493.

Puurtinen M., Knott K. E., Suonpää S., Nissinen K. & Kaitala V. 2007. Predominance of outcrossing in Lymnaea stagnalis despite low apparent fitness costs of self-fertilization. 20: 901-912.

Ritland K. 1990. Inferences about inbreeding depression based upon changes in the inbreeding coefficient. *Evolution* 44: 1230-1241.

Ritland K. 1991. A genetic approach to measuring pollen discounting in natural plant populations. *Am. Nat.* 138: 1049-1057.

Ronfort J. & Couvet D. 1995. A stochastic model of selection on selfing rates in structured populations. *Genet. Res*. 65: 209-222

Schoen D. J. & Brown A. H. D. 1991. Whole- and part-flower self-pollination in *Glycine clandestina* and *G.argyrea* and the evolution of autogamy. *Evolution* 45: 1665-1674.

Schultz S. T. & Willis J. H.. 1995. Individual variation in inbreeding depression: the roles of inbreeding history and mutation. *Genetics* 141: 1209-1223.

Stevens J. P. & Bougourd S. M. 1988. Inbreeding depression and the outcrossing rate in natural populations of *Allium schoenoprasum* L. (wild chives). *Heredity* 60: 257-261

Städler T. & Jarne P. 1997. *Population biology, genetic structure, and mating system parameters in freshwater snails* in Streit, B., T. Städler and C. M. Lively (eds) Evolutionary Ecology of Freshwater Animals. Birkhäuser Verlag, Basel.

Tsitrone A., Duperron S. & David P. 2003. Delayed selfing as an optimal mating strategy in preferentially outcrossing species: theoretical analysis of the optimal age at first reproduction in relation to mate availability. *Am. Nat.* 162: 318-331.

Uyenoyama M. K. 1986. Inbreeding and the cost of meiosis: the evolution of selfing in populations practicing biparental inbreeding. *Evolution* 40: 388-404.

Uyenoyama M. K., Holsinger K.E. & Waller D.M. 1993. Ecological and genetic factors directing the evolution of self-fertilization. *Oxf. Surv. Evol. Biol.* 9: 327-381.

van Duivenboden Y.A. 1983. Transfer of semen accelerates the onset of egg-laying in female copulants of the hermaphrodite freshwater snail *Lymnaea stagnalis*. *Int. J. Invertebr. Reprod.* 6: 249-257.

van Duivenboden Y.A., Pieneman W. & ter Maat A.. 1985. Multiple mating suppresses fecundity in the hermaphrodite freshwater snail *Lymnaea stagnalis*: a laboratory study. *Anim. Behav.* 33: 1184-1191.

van Duivenboden Y.A. & ter Maat A. 1988. Mating behaviour of *Lymnaea stagnalis*. Malacologia, 28: 53-64.

Vernon J. G. 1995. Low reproductive output of isolated, self-fertilizing snails: inbreeding depression or absence of social facilitation? *Proc. R. Soc. Biol. Sci*. Ser. B., 259: 131-136.

Wang J., Hill W. G., Charlesworth D. & B. Charlesworth B. 1999. Dynamics of inbreeding depression due to deleterious mutations in small populations: mutation parameters and inbreeding rate. *Genet. Res*. 74: 165-178.

Waser N.M. & Williams C.F. 2001. *Inbreeding and Outbreeding* in Fox C.W., Roff D.A. & Fairbairn D.J. (eds) Evolutionary Ecology Concepts and Case Studies. Oxford University Press, Oxford.

Wethington A. R. & R. J. Dillon R.J. 1997. Selfing, outcrossing, and mixed mating in the freshwater snail *Physa heterostropha*: Lifetime fitness and inbreeding depression. *Invertebr. Biol*. 116: 192-199.

Whitlock M.C. & McCauley D. E. 1990. Some population genetic consequences of colony formation and extinction: Genetic correlations within founding groups. *Evolution* 44: 1717-1724.

Wolfinger R. D. & O'Connell M. 1993. Generalized linear mixed models: a pseudo-likelihood approach. *J. Stat. Comput. Simul*. 48: 233-243.

Wright S. 1977. *Evolution and the genetics of populations*. University of Chicago Press, Chicago.

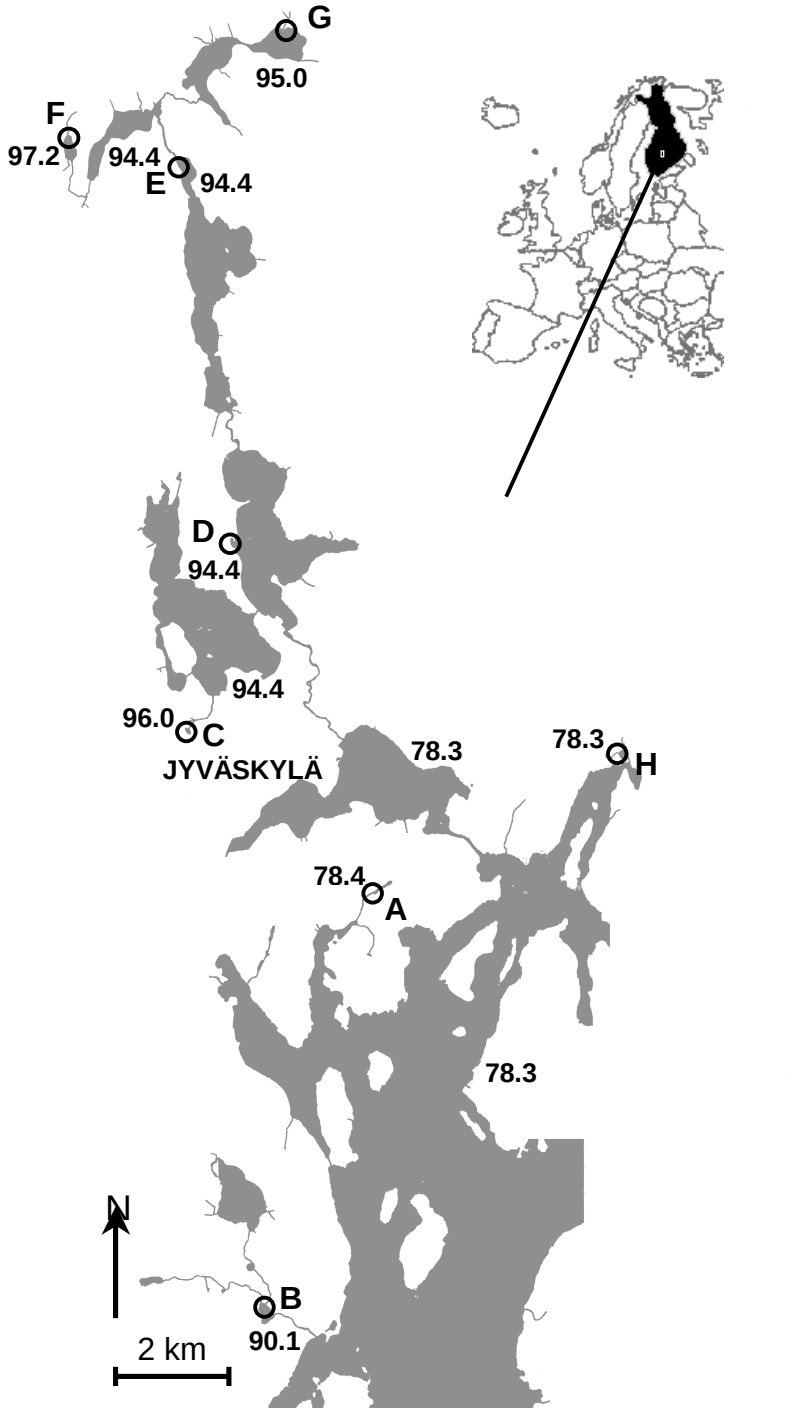

Figure 1. Locations of the study populations. Numbers denote the level of the lake/pond (meters above sea level).

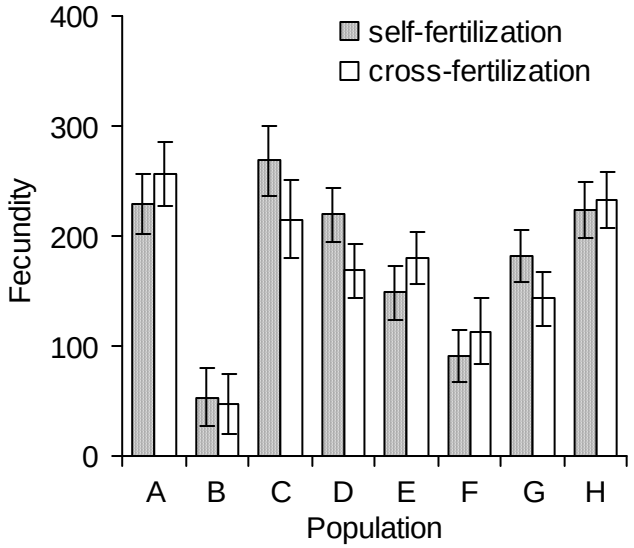

Figure 2. Mixed model estimates of fecundity (number of egg capsules / two weeks) in each population by treatment combination. Error bars denote 1 S.E.

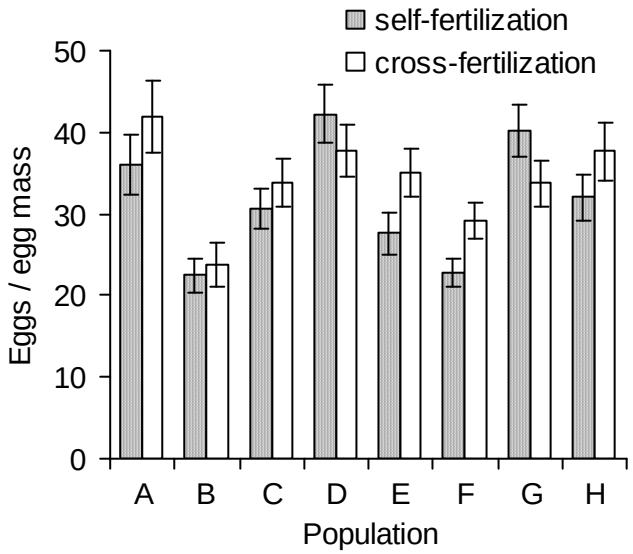

Figure 3. Mean number of eggs per egg mass in each population by treatment combination. Error bars denote 95% confidence intervals.

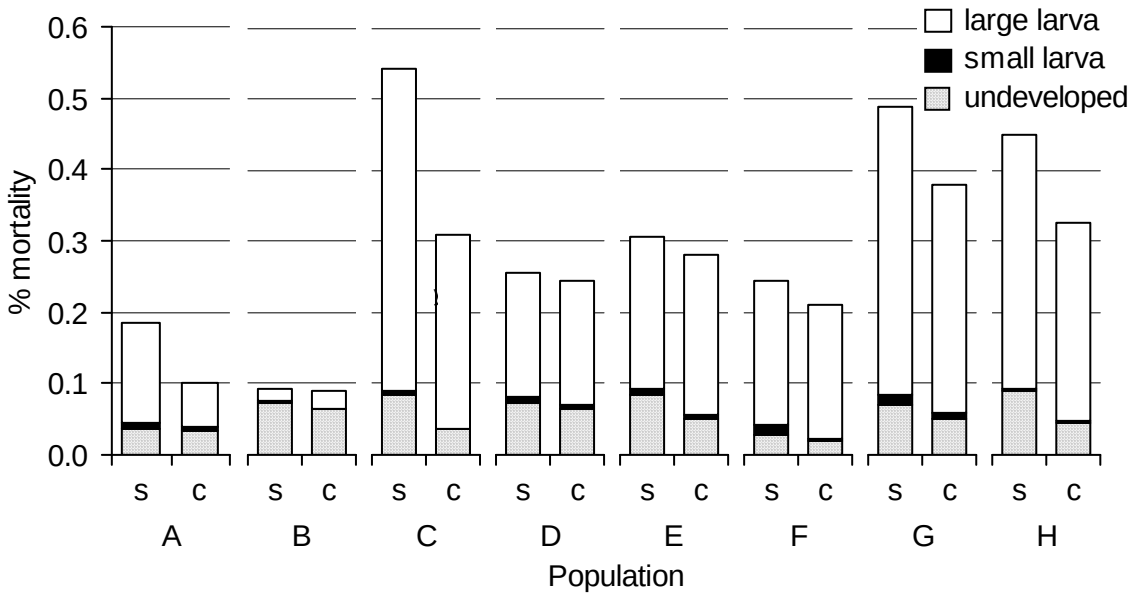

Figure 4. Mortality in different developmental stages, calculated from the mixed model for mortality. s = self-fertilization treatment; c = cross-fertilization treatment.

Table 1. The estimate of inbreeding depression in hatching success (δ) and self-fertilization depression (SFD)

Population	*δ* (95% CI)	*SFD (95% CI)*
A	0.09 (0.03 0.16)	0.20 (-0.06 0.47)
B	0.01 (-0.05 0.06)	-0.12 (-1.82 1.57)
C	0.34 (0.20 0.48)	0.15 (-0.27 0.56)
D	0.01 (-0.13 0.15)	-0.28 (-0.77 0.21)
E	0.03 (-0.12 0.19)	0.24 (-0.10 0.57)
F	0.04 (-0.08 0.16)	0.24 (-0.32 0.81)
G	0.18 (-0.01 0.36)	-0.05 (-0.55 0.46)
H	0.19 (0.00 0.37)	0.23 (-0.12 0.57)

Table 2. Mixed model results for fecundity. Non-significant interactions with family and maturation stage have been dropped from the final model.

	Test statistic	P
Family (population)	$Z = 2.40$	0,0083
Population	$F_{7,111} = 10.41$	<.0001
Treatment	$F_{1,364} = 0.36$	0,5473
Population*treatment	$F_{7,363} = 1.19$	0,3103
Maturation stage	$F_{1,453} = 0.32$	0,5745

Table 3. Analysis of factors affecting mortality during three developmental stages and total pre-hatching mortality, with estimates of the proportion of explained deviance that could be attributed to each factor.

	undeveloped egg		small larva		large larva		total mortality	
Mixed model scaled residual deviance, degrees of freedom, and extra-dispersion scale	dev = 28201; d.f. = 54721; extra-disp = 0.8418		dev = 17322; d.f. = 50227; extra-disp = 0.4223		dev = 41766; d.f. = 49045; extra-disp = 0.9312		dev = 55416; d.f. = 54830; extra-disp = 0.9778	
Source of variation	*P*	approximate % of total explained deviance (24.0%)	*P*	approximate % of total explained deviance (35.8%)	*P*	approximate % of total explained deviance (29.6%)	*P*	approximate % of total explained deviance (22.2%)
maternal snail	<0.0001	51.2	<0.0001	83.5	<0.0001	61.5	<0.0001	66.3
family	0.0024	38.8	* n.s.		† n.s.		* n.s.	
population	0.0109	6.1	<0.0001	10.8	<0.0001	22.0	<0.0001	19.0
treatment	0.0036	2.6	0.0002	4.0	0.1072	0.5	0.0005	1.8
population*treatment	0.5982	1.2	0.4841	1.5	0.4005	1.2	0.3717	1.2
# of egg capsules / egg mass (residual)	0.0002	0.1	0.1351	0.2	<0.0001	14.8	<0.0001	11.8

The family*treatment interaction was non-significant for mortality at all life-stages and was excluded from final analyses.

*) The effect was removed from final analysis. The *P*-values and explained deviances of other factors are calculated excluding this effect.

†) The estimated variance component of this factor was zero. The *P*-values and explained deviances of other factors are calculated excluding this effect.