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Inbreeding Linked to Amphibian Survival in the Wild but Not in the Laboratory

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Abstract

We examined the effects of inbreeding on the performance of wood frog (*Rana sylvatica*) larvae in the field and in the laboratory. We used microsatellite analysis to establish the parentage and degree of inbreeding of the larvae. Two different estimators of inbreeding were used. The first was based on average multilocus heterozygosity, and the second was based on a molecular relatedness estimator. The estimators were highly correlated, and both showed a significant negative relationship between inbreeding and survival in the wild. However, there was no evidence that inbreeding influenced growth or development in the wild. Neither was there any evidence that inbreeding affected survival, growth, or development in the laboratory. These results suggest that, for wood frogs, inbreeding has a bigger effect on fitness in the wild than in captivity and that measurements of survival are more sensitive than measures of growth or development.

Inbreeding depression can be defined at the individual or population level as a decline in the fitness of the offspring that is a direct consequence of increasing relatedness between mates. Inbreeding results in a reduction in the average heterozygosity of the offspring, which can reduce survival and fecundity either because decreased heterozygosity by itself decreases fitness, as has been shown for the major histocompatibility complex, or because such individuals are more likely to be homozygous for deleterious recessive alleles (Charlesworth D and Charlesworth B 1987; Flajnik and Du Pasquier 1990; Wedekind et al. 1995).

Long known from studies of captive and domestic populations, inbreeding depression may also be a threat to wild populations—a consequence of the reduced gene flow and population size that results from habitat fragmentation and degradation (e.g., Soule 1980). However, it is unclear whether inbreeding has the same consequences in the wild as it does in captive populations (Caro and Laurenson 1994). The effects of inbreeding depression may be diminished in the wild because 1) individuals avoid mating with close relatives, 2) inbred individuals can adjust for the deleterious alleles by making use of the typically more heterogeneous environments found in the wild, and 3) inbreeding may actually increase adaptation to local environments and thereby increase survival and fecundity (Bateson 1983; Crnokrak and Roff 1999). On the other hand, inbreeding depression may be more severe for individuals in the wild because the environment is often more harsh or unpredictable and the potentially beneficial effects of heterozygosity may therefore be at a premium (Ralls et al. 1988; Miller 1994).

In a review, Crnokrak and Roff (1999) found that inbreeding depression was more severe in the wild than in captivity. However, such comparisons can be confounded by the use of different estimators. In captive populations, inbreeding is often estimated based on pedigree information. Pedigree techniques can be extremely precise for the known number of generations and individuals, but they rest on the assumption that all individuals at the top of the pedigree are unrelated (Markert et al. 2004).

Because pedigrees are not typically available, estimates of inbreeding for wild populations have typically been based on molecular markers such as allozymes and, more recently, neutral markers like microsatellites. Molecular techniques like these generate a direct estimate of heterozygosity, the primary factor believed to drive inbreeding depression, and, in fact, numerous studies have shown a correlation between fitness and multilocus heterozygosity (reviewed in Crnokrak and Roff 1999). However, such techniques depend on assumptions about such things as mutation rates and mechanisms, linkage, and neutrality (Scribner and Pearce 2000). In addition, recent evidence suggests that because of its wide variance, multilocus heterozygosity may be poorly correlated with an

individual's inbreeding coefficient. Although some studies have suggested that microsatellite heterozygosity-fitness correlations are the result of generalized effects (e.g., Lesbarreres et al. 2005), others suggest that these correlations are best explained by linkage between the neutral markers and loci for which heterozygosity confers a fitness advantage (Hansson and Westerberg 2002; Balloux et al. 2004; Slate et al. 2004).

One means of minimizing the disadvantages of pedigree and microsatellite inbreeding estimates is to combine the 2 techniques (Pemberton 2004). In this study, we used a combination of these methods to estimate inbreeding in a wild population of wood frogs (*Rana sylvatica*) and evaluate its effects on growth, development, and survival. Amphibians and especially wood frogs have been commonly used in fitness studies, providing a broad context for this study (Wilbur 1980; Berven 1990). We used microsatellite analysis 1) to evaluate the relatedness of the adults in a breeding population and 2) to establish the parentage of the offspring. With this information, we estimated the level of inbreeding of the offspring with 2 techniques as follows: 1) a simple heterozygosity measure and 2) a measure based on relatedness estimator by Queller and Goodnight (1989) that incorporates information about the allele frequencies in the population as a whole. To facilitate a comparison of the effects of inbreeding in the wild and in captivity, we also collected embryos from the population under study in the wild and raised them in the laboratory.

Methods

Field Experiments

Two different ponds known as Quarry and Little Thing, hereafter called LT pond, in the Yale-Myers forest in northeast Connecticut were used in this study. Quarry is the closest wood frog breeding pond to LT pond; it lies less than 50 m away. LT pond is a smaller (pond full surface area = 79 m^2) and shallower (pond full maximum depth = 65 cm) pond than Quarry (pond full surface area = 197 m^2, pond full maximum depth = 110 cm). LT pond receives somewhat more direct sunlight than Quarry because it has less forest canopy cover, but the difference is small (Halverson et al. 2002; Halverson, unpublished data). Fewer amphibian species and fewer invertebrate wood frog predators live in LT pond than in Quarry (Halverson and Freidenburg, unpublished data).

In February 2002, we surrounded LT pond with a drift fence made of 1-m aluminum window screening before the wood frog breeding season began. Pit traps were located every 5 m around the inside and outside of the fence. We checked the traps and searched the pond exhaustively every day from 8 March 2002 to 7 April 2002. There was no more calling and no more egg masses appeared in the pond after this date. We weighed, measured snout-ischium length, and took a toe clip from all adult wood frogs captured on the outside of the fence during this time and released them on the inside of the fence. Toe clips were stored in 70% alcohol for later analysis. Other vertebrates captured in the traps were released on the opposite side of the fence from where they were found. We marked and documented all egg masses. To count the eggs, we gently sandwiched each mass in situ between a white background and a clear piece of plastic and took a photograph with a digital camera. We counted the eggs in the photographs by marking each one in ArcView 3.2. We also collected 20–30 embryos from at least 3 different parts of each egg mass as soon as the egg mass was discovered and raised them in the laboratory until they hatched. The hatchlings were euthanized and stored in 70% alcohol for later analysis. We opened sections of the fence after the last wood frog egg mass appeared to allow free access to the pond by other organisms. On 26 June 2002, when it appeared that wood frog larvae in the pond were approaching metamorphosis, we again closed the fence and checked the traps every day. We closed the traps and opened the fence on 27 July 2002, 14 days after the pond dried up and 13 days after the last metamorph was found in a trap. All wood frog metamorphs captured on the inside of the fence were weighed, and a toe clip was taken and stored in 70% alcohol. To avoid confusion, all wood frog metamorphs captured on the outside of the fence were released on the outside of the fence.

In 2003, we surrounded Quarry with a drift fence and a similar array of pit traps. The same sampling protocol used in LT pond in 2002 was used in Quarry in 2003. However, unlike in LT pond in 2002, we kept the drift fences up and the traps open for 48 days after the last egg masses appeared to capture adult frogs leaving the pond. We made this change to the protocol because we suspected that some frogs may have overwintered inside the fence. All unclipped adult frogs captured on the inside of the fence were measured, weighed, and toe-clipped and released on the outside.

Laboratory Experiment

Not all the embryos collected from Quarry in 2003 were euthanized immediately on hatching. Instead, some of the hatchlings from each egg mass were raised in the laboratory. The sampled embryos were stored in an incubator at 8 °C with the lights on for 12 h a day from the time they were collected until 18 April 2003. They were then moved to an incubator in which there were 2 cycles: the lights were on and the temperature was 14 °C for 12 h a day, and the lights were off and the temperature was 12 °C for 12 h a day. These temperatures approximated the temperature regimes measured in the ponds in previous years. On 28 April 2003, after all developing embryos had hatched, one hatchling from each egg mass was combined with other hatchlings from the same pond in a clear plastic container with 3.5 l of aged, dechlorinated tap water. This treatment was replicated 5 times such that there were 5 containers with 25 hatchlings representing the 25 egg masses found in Quarry in 2003. These containers were placed in an incubator that was lit and set to 14 °C for 12 h a day and dark and 12 °C for 12 h a day. As the density of the larvae in the laboratory experiment was higher than we estimate it to be in the wild, we changed the water frequently (every 3–4 days) and gave each container fresh food after every water change. To

Table 1. Summary statistics for the microsatellite loci used in this study

Locus	No. of alleles	H(O)	H(E)	Exclusion probability 1	Exclusion probability 2	HWE probability	HWE significance
C11	18	0.85	0.903	0.664	0.798	0.104	NS
C23	7	0.436	0.455	0.109	0.252	0.299	NS
C41	12	0.778	0.833	0.506	0.676	0.025	NS
C83	5	0.744	0.715	0.284	0.451	0.083	NS
D20	19	0.765	0.897	0.65	0.788	<0.001	S
D32	17	0.856	0.889	0.626	0.77	0.481	NS
D40	20	0.837	0.869	0.596	0.747	0.841	NS
D55	17	0.785	0.889	0.626	0.771	0.096	NS
D77	25	0.936	0.917	0.707	0.828	0.689	NS
D88	24	0.922	0.933	0.755	0.86	0.299	NS
All loci				0.999	0.999		

H(O) is the observed heterozygosity. H(E) is the expected heterozygosity based on the number of alleles. Exclusion probability 1 reflects the probability that a given locus will be able to exclude an individual as the parent when there is no information about the other parent. Exclusion probability 2 reflects the probability that a given locus will be able to exclude an individual as the parent when one parent is known. HWE probability is the probability that a locus at Hardy–Weinberg equilibrium would generate the observed allele combinations in the population for a given locus. HWE significance is the significance of the HWE probability values after a Bonferroni correction has been applied where S is significant and NS is not significant.

accommodate the growth of the larvae, the amount of food given to individual containers increased from 0.1 g of food at the beginning of the experiment to 0.5 g at the end. All containers received the same amount of food. The food consisted of 3 parts rabbit chow and 1 part fish food. On 19 June 2003, we euthanized all the larvae that remained in the containers and stored them in 70% alcohol. Preserved larvae were measured, and their developmental stage was assessed (Gosner 1960). Part of the tail was removed for molecular analysis.

Molecular Analysis

We extracted DNA from the toes of the adults and portions of the tails of the larvae using the guidelines and materials in the Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN; http://www.gentra.com) and suspended the product in 50 μl of the DNA hydration solution.

We analyzed 10 microsatellite loci described by Julian and King (2003) (RsyC11, RsyC23, RsyC41, RsyC83, RsyD20, RsyD32, RsyD40, RsyD55, RsyD77, and RsyD88). Polymerase chain reactions (PCRs) were carried out in 10-μl volumes containing 1X Applied Biosystems PCR Buffer, 2 mM $MgCl_2$, 5× New England Biolabs Purified BSA, 0.4 μM of each primer, 0.2 mM of each dNTP, and 0.5 units of Applied Biosystems AmpliTaq® DNA Polymerase. The products were resolved on a 4% polyacrylamide sequencing gel on an ABI 377 sequencing machine and analyzed and scored using ABI Genescan® and Genotyper® softwares.

We used CERVUS software to analyze the allele frequencies of the sampled adults for Hardy–Weinberg equilibrium, observed and expected heterozygosity, and exclusion probabilities as described by Marshall et al. (1998). One of the loci (RsyD20) had a significant departure from Hardy–Weinberg equilibrium and was dropped from the analysis (Table 1).

Parentage and Inbreeding Analysis

For the purposes of the parentage analysis, it was necessary to determine whether we had sampled all the adults that might have bred. The presence of unclipped adults in traps on the inside of the fence in Quarry in 2003 suggests that we did not, perhaps because some individuals overwintered inside the fence. Because adult frogs were marked with toe clips and captured going in and out of the pond in 2003, it was possible to use mark-recapture analysis to estimate the number of breeding adults in a pond in a given year and the proportion that were sampled. We did not capture adults exiting LT pond in 2002, so in that case, we used the estimates of the proportion sampled from Quarry in 2003.

To maximize the power of the parentage analysis, we first assigned adult females to egg masses. We analyzed which females were compatible with each embryo based on exclusion analysis. Potential null alleles were handled in the most conservative way possible: all homozygotes were treated as heterozygotes with the detected allele and a null allele (Jones and Ardren 2003). This meant that a homozygous adult could not be excluded as the parent of a homozygous offspring at any given locus. We then examined which females' genotypes were compatible with the genotypes of all the embryos from a given egg mass, allowing for one mutation or mistyping error per embryo and assigned parentage accordingly.

To establish a confidence level in the parentage assignments, we used a Monte Carlo simulation in which pseudoadults were generated based on the allele frequencies in the population as a whole. The proportion of simulations in which a pseudoadult was more likely to be the mother of a sampled egg mass was multiplied by the estimated number of adults that were not sampled in a pond during a given year. Mutations and mistyping errors were allowed and accounted for in the Monte Carlo analysis.

Because we could not assume that all embryos in an egg mass had the same father, we established paternity of each of the embryos individually using CERVUS (Marshall et al. 1998). As the maternity of all the egg masses was established and the maternal alleles in the offspring could be removed from the analysis, the power of CERVUS to assign paternity was greatly increased. Parameters necessary for this analysis

include the estimated total number of adult males, the proportion captured, and the estimated mutation or mistyping error rate. The first 2 parameters were estimated by mark-recapture analysis. The latter was estimated from the maternity analysis of the embryos.

Finally, we assigned maternity and paternity to all the metamorphs captured in the field and to the larvae raised in the laboratory. Because of the quantity of samples and because, in most cases, they conferred sufficient discriminatory power, only the 8 most polymorphic loci were used in these analyses (RsyC11, RsyC23, RsyC41, RsyD32, RsyD40, RsyD55, RsyD77, and RsyD88) (Table 1). We first established the most likely mother using CERVUS. We then used CERVUS to assign the most likely father.

The null hypothesis in all cases was that there was no multiple paternity. Thus, in the few cases where CERVUS found multiple different most likely fathers for the offspring of a single mother, the results were examined more carefully. If, allowing for zero or one mutation or mistyping errors, the genotype of the offspring could be explained without multiple paternity, it was so done.

The relatedness of the adults was estimated with the microsatellite data, using the method described by Queller and Goodnight (1989) and SPAGEDI software (Hardy and Vekemans 2002). This estimator weighs the number of alleles shared by individuals and the frequencies of those alleles in the population as a whole to estimate relatedness. Because the coefficient of inbreeding of an individual is, by definition, one-half the coefficient of relatedness of the individual's parents, the coefficient of inbreeding for all the embryos and metamorphs was defined as such for this analysis (Hartl and Clark 1997). Based on the parentage analysis of egg masses and metamorphs, we assumed that all individuals in an egg mass were full sibs. To facilitate comparison with previous studies, the average multilocus heterozygosity of all the offspring in an egg mass was also calculated and analyzed from the parental genotypes. We compared the relationship between average multilocus heterozygosity and the estimated inbreeding coefficient.

We calculated the survival rate for a given sibship in the field experiment as the ratio of metamorphs to embryos. Linear regression was used to compare the effects of the estimated inbreeding coefficient and heterozygosity on the survival, size, and date of capture of the metamorphs from the field experiment. The date of capture of each metamorph is probably not perfectly correlated with the actual date of metamorphosis because some metamorphs may have taken longer to exit the pond than others. However, it is the best estimator we could use in the field situation, and we refer to this as the date of metamorphosis in the rest of the manuscript.

In the laboratory experiment, we used linear regression to determine whether the proportion of the collected embryos that successfully hatched was a function of the coefficient of inbreeding or heterozygosity. We also used linear regression to determine whether the average size, developmental stage, or proportion of hatchlings that survived in the laboratory experiment was a function of the coefficient of inbreeding or heterozygosity. The power of the regression analyses for the inbreeding coefficient and heterozygosity versus survival in these experiments was estimated using the methods and software described by Dupont and Plummer (1998).

We used Monte Carlo techniques to perform sensitivity analyses on those regression analyses that showed a significant relationship. In the first test, we selected the 14 metamorphs that were of questionable parentage, randomly reassigned their parentage to other known breeding pairs, and redid the least-squares regression analyses 1000 times. In the second test, we randomly selected twice this number of metamorphs (more than 10% of the total number of metamorphs analyzed), randomly reassigned their parentage to other known breeding pairs, and redid the least-squares regression analyses 1000 times.

To determine whether the wood frog adults avoided inbreeding, an analysis of variance (ANOVA) was used to compare the average pairwise relatedness of the individuals that bred and the average pairwise relatedness of the individuals that did not breed in each pond.

Results

Molecular Analysis

The microsatellite loci used here were all polymorphic, with 5–25 alleles each (Table 1). Expected heterozygosities ranged from 0.455 to 0.933. In most loci, the observed heterozygosity was somewhat lower, suggesting that there may have been some null alleles. However, only one locus (RsyD20) showed a significant departure from Hardy–Weinberg equilibrium and was dropped from the analysis. The exclusion probabilities for a parent given no information about the other parent ranged from 0.109 to 0.755 with a combined exclusion probability of 0.999. The exclusion probabilities for a parent when the other parent is known ranged from 0.252 to 0.860 with a combined exclusion probability of 0.999.

Parentage Analysis

Five egg masses were laid in LT pond in 2002 with an average of 664 eggs apiece (SD = 172). We captured 76 males and 35 females in Quarry in 2003. Twenty-five egg masses were laid in Quarry in 2003, with an average of 523 eggs apiece (SD = 220). DNA from 6 embryos from each of the 5 egg masses in LT pond in 2002 and 5 embryos from each of the 25 egg masses Quarry in 2003 was extracted and analyzed. Three of the embryos (each from a different egg mass) from Quarry failed to amplify any products at any locus, probably because the extraction failed, and were not used in the analysis (Table 2).

With 4 exceptions, based on the exclusion analysis, we found one and only one female with a genotype that was compatible based on strict Mendelian inheritance with the genotype of all the embryos in the egg mass and assigned maternity accordingly. For 3 of the 4 unassigned egg masses, a potential mother was found that was only incompatible

Table 2. Summary of the parentage analysis for the embryos, metamorphs, and larvae raised in the laboratory

Pond	LT pond	Quarry
No. of egg masses	5	25
Average no. of embryos per egg mass	664	523
No. of embryos/egg mass analyzed	6	5
No. of embryos not analyzed	0	3[a]
No. of egg masses in which all analyzed embryos were assigned to one captured female: strict Mendelian inheritance	5	21
No. of egg masses in which all analyzed embryos were assigned to one captured female: one mutation	0	4
No. of egg masses in which all analyzed embryos were assigned to one captured male: strict Mendelian inheritance	4	22
No. of egg masses in which all analyzed embryos were assigned to one uncaptured male: strict Mendelian inheritance	0	3
No. egg masses in which all analyzed embryos were assigned to one captured male: one mutation	1	0
No. of metamorphs	43	292
No. of metamorphs assigned to a known parent pair: strict Mendelian inheritance	41	286
No. of metamorphs assigned to a known parent pair: one mutation	1	4
No. of metamorphs not analyzed	1	2
No. of laboratory larvae analyzed	NA	104
No. of larvae from laboratory matching known parent pair: strict Mendelian inheritance	NA	103
No. of larvae from laboratory matching known parent pair: one mutation	NA	1

As indicated in the table, all individuals for which the parentage was assigned were compatible with the assigned parents under "strict Mendelian inheritance," or given "one mutation." These results are described in more detail in the text.

[a] Each of these embryos came from a different egg mass.

with one embryo at one locus, and in the latter 2 of these cases, the alleles only differed by one repeat unit. Such misfits are consistent with a mutation or mistyping error at one locus in the offspring. In the fourth case, the most compatible mother was incompatible with 4 out of 5 embryos at the same locus, a situation that might have occurred if this putative mother was mistyped or if there was a premeiotic mutation at that locus.

The Monte Carlo analysis showed that it was extremely unlikely ($P < 0.01$) that a female not sampled would have been compatible with any of the sampled egg masses under a strict exclusion analysis or even if 1–3 mutations or mistyping errors at the level of the embryos or the mother were allowed. We thus determined that a mutation or mistyping error had occurred in the case of the 4 unassigned egg masses and assigned maternity accordingly.

The paternity analysis of the embryos revealed little or no evidence for multiple paternity. With 6 exceptions, the same male was the most likely father of all the embryos in an egg mass. In the case of 3 of the remaining egg masses, 3 of the embryos within a given egg mass were found to have different most likely fathers. However, the genotypes of all the embryos from a given egg mass could also have been explained if they were fertilized by a single male that was not captured. It appears more likely that a single male that was not captured fertilized these eggs than that multiple different males with very similar genotypes all mated with a single female. In the case of another two of the egg masses, CERVUS determined that one embryo had a different more likely father than the other embryos in the mass. However, the genotype of the father of the other embryos in the mass was also compatible with the genotype of the single embryo under strict Mendelian inheritance. Again, it appears more likely that the same male fertilized all the embryos in these egg masses than that multiple males with very similar genotypes mated with the same female. Finally, one embryo in one egg mass was not compatible with the other embryos in the egg mass under strict Mendelian inheritance, but would have been so given one mutation. This was deemed the most parsimonious explanation, and paternity was assigned accordingly (Table 2).

There were 43 metamorphs captured inside the fence in LT pond in 2002 and 292 metamorphs captured inside the fence in Quarry in 2003. Though we are not able to state it with any certainty, we assume that the fence was reasonably impermeable to the metamorphs and that if any did manage to get out of the fenced area without being trapped in the buckets, they were a sufficiently small and random group that they did not bias the sample of metamorphs we were able to collect. Two of the metamorphs failed to amplify any alleles at any locus, probably due to an extraction error, and were dropped from the analysis. For 316 of the 333 metamorphs that were analyzed from both ponds, the most likely parents, according to CERVUS, were pairs that matched one of the parent pairs found in the embryo analysis. Of the remaining metamorphs, 11 had genotypes that were compatible with the genotypes from one of the known parent pairs under strict Mendelian inheritance. Five had genotypes that would be compatible with the genotypes from one of the known parent pairs under strict Mendelian inheritance given one mutation. This was deemed the most parsimonious explanation, and parentage was assigned accordingly (Table 2).

For one metamorph from LT pond in 2002, there were no mothers compatible with its genotype without mutations or mistyping errors at 3 different loci and no potential fathers without mutations or mistyping errors at 2 different loci. A more parsimonious explanation is that this metamorph came

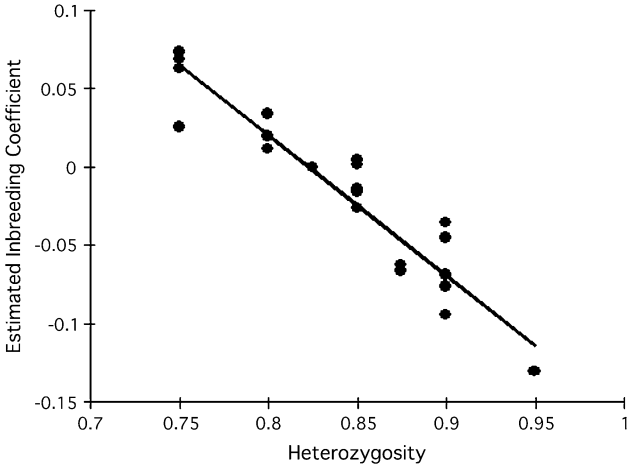

Figure 1. Estimated average inbreeding coefficient of all the embryos in each egg masses in Quarry in 2003 versus estimated average multilocus heterozygosity. The least-squares regression line shows the strong negative relationship between these 2 estimators.

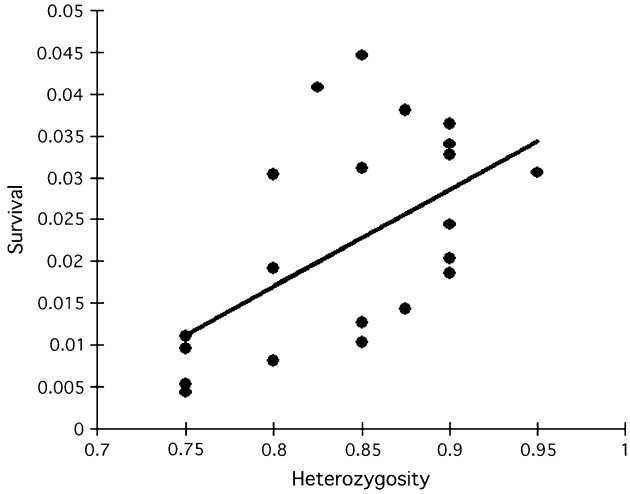

Figure 2. Proportion of individuals from a given egg mass that survived to metamorphosis as a function of the average multilocus heterozygosity of the individuals in the egg mass in Quarry in 2003. The line represents the least-squares regression line.

from another pond and somehow slipped through the fence into LT pond before getting caught in one of the inside buckets. It was therefore dropped from further analysis. Every sibship was represented in the metamorphs by a minimum of 1 and a maximum of 18 individuals in LT pond in 2002 and by a minimum of 2 and a maximum of 37 individuals in Quarry in 2003.

According to CERVUS, for 91 of the 104 larvae that survived the laboratory experiment and were analyzed, the most likely parents were pairs that matched one of the parent pairs found in the embryo analysis. Of the remaining 13 larvae, 12 had genotypes that were compatible with the genotypes from one of the known parent pairs under strict Mendelian inheritance and 1 would have been strictly compatible given one mutation. Parentage was assigned accordingly.

On an experiment-wide basis, the probability that there were instances of multiple paternity that we failed to detect depends on the number of eggs in the egg mass and the percentage of eggs that were fertilized by another male. On average, across all ponds, in all pond years, there were 547 eggs in an egg mass, and we examined paternity in no less than 5 eggs from every egg mass. If we assume that another male fertilized 10% of the eggs in an average egg mass, then there is only a 41% chance that we would have detected multiple paternity in the 5 embryos examined. However, there were, on average, 10 metamorphs from every egg mass, which increases the sample size to 15 and the detection probability to 79%. On a study-wide basis, the probability of detection increases even more. Even if multiple paternity occurred in only 10% of the 30 egg masses analyzed, using the parameters described in the last instance, there is less than a 1% chance that we would have failed to detect multiple paternity in at least one egg mass. Thus, we assume that multiple paternity did not occur in these ponds in these years.

Inbreeding Analysis

Linear regression showed that there was a strong relationship between the estimated inbreeding coefficient and the average multilocus heterozygosity ($F_{1,20} = 156.50$, $P < 0.01$, $R^2 = 0.89$; Figure 1).

Because we did not know the genotype of the father for 3 of the egg masses in Quarry in 2003, we could not define the coefficient of inbreeding or average heterozygosity for these sibships. We thus dropped these sibships from the inbreeding analysis.

For individuals in Quarry in 2003, linear regression with the 22 analyzed sibships showed a significant negative relationship between the estimated inbreeding coefficient and survival ($F_{1,20} = 8.09$, $P = 0.01$, $R^2 = 0.29$) and a significant positive relationship between heterozygosity and survival ($F_{1,20} = 8.50$, $P < 0.01$, $R^2 = 0.30$; Figure 2). For those individuals that reached metamorphosis in Quarry in 2003, linear regression showed no significant relationship between the estimated inbreeding coefficient and the average weight ($F_{1,20} = 0.44$, $P = 0.52$, $R^2 = 0.021$), average date of metamorphosis ($F_{1,20} = 2.45$, $P = 0.13$, $R^2 = 0.11$), or average number of days to metamorphosis ($F_{1,20} = 2.73$, $P = 0.12$, $R^2 = 0.056$). Results for heterozygosity were similar (weight: $F_{1,20} = 0.06$, $P = 0.81$, $R^2 < 0.01$; date: $F_{1,20} = 2.57$, $P = 0.13$, $R^2 = 0.11$; days: $F_{1,20} = 2.46$, $P = 0.13$, $R^2 = 0.11$).

For the embryos raised in the laboratory, linear regression showed no significant relationship between the estimated inbreeding coefficient and hatching success ($F_{1,20} = 2.85$, $P = 0.11$, $R^2 = 0.11$) or between heterozygosity and hatching success ($F_{1,20} = 3.26$, $P = 0.085$, $R^2 = 0.13$). For the hatchlings from Quarry in 2003 that were raised in the laboratory, linear regression showed no significant relationship between the estimated inbreeding coefficient and survival ($F_{1,20} = 0.078$,

$P = 0.79$, $R^2 < 0.01$) or between heterozygosity and survival ($F_{1,20} = 0.38$, 0.54, $R^2 = 0.02$). The power of each of these linear regression analyses, assuming the regression coefficients were the same as in the pond, was 0.73 and 0.76, respectively (Dupont and Plummer 1998). Nor was there a significant relationship between the estimated inbreeding coefficient and the average size of the larvae ($F_{1,20} = 0.97$, $P = 0.34$, $R^2 = 0.05$) or average developmental stage ($F_{1,20} = 0.93$, $P = 0.35$, $R^2 = 0.04$). Results for heterozygosity were similar (weight: $F_{1,20} = 1.11$, $P = 0.30$, $R^2 = 0.05$; developmental stage: $F_{1,20} = 0.56$, $P = 0.46$, $R^2 = 0.03$).

The sensitivity analyses showed that the significant linear regressions of inbreeding coefficient versus survival and heterozygosity versus survival were very robust. When the 14 metamorphs of questionable parentage in Quarry in 2003 were randomly reassigned to other known breeding pairs, the inbreeding coefficient was significantly negatively related to survival in 97.9% and heterozygosity was significantly positively related to survival in 99.8% of the 1000 tests performed. The R^2 value was as high or higher than the value found for the original data in 49.9% of the 1000 tests performed for inbreeding coefficient versus survival and in 24.6% of the 1000 tests performed for heterozygosity versus survival. When 28 metamorphs were randomly selected and reassigned to other known breeding pairs, the inbreeding coefficient was significantly negatively related to survival in 88.6% and heterozygosity was significantly positively related to survival in 92.0% of the 1000 tests performed. The R^2 value was as high or higher than the value found for the original data in 55.2% of the 1000 tests performed for inbreeding coefficient versus survival and in 32.4% of the 1000 tests performed for heterozygosity versus survival.

For individuals in LT pond in 2002, linear regression with the 5 analyzed sibships showed no significant relationship between the estimated inbreeding coefficient and survival ($F_{1,3} = 0.29$, $P = 0.62$, $R^2 = 0.09$) or between heterozygosity and survival ($F_{1,3} = 0.31$, $P = 0.61$, $R^2 = 0.09$). However, the trends for these regressions were in the same direction as for the Quarry metamorphs in 2003. Linear regression also did not show any significant relationship between the estimated inbreeding coefficient and the average weight ($F_{1,3} = 0.36$, $P = 0.59$, $R^2 = 0.11$), average date of metamorphosis ($F_{1,3} < 0.01$, $P = 0.94$, $R^2 < 0.01$), or average number of days until metamorphosis ($F_{1,3} = 0.068$, $P = 0.81$, $R^2 = 0.02$) of those individuals that reached metamorphosis in LT pond in 2002. Results for heterozygosity were similar (weight: $F_{1,3} = 0.69$, $P = 0.47$, $R^2 = 0.19$; date: $F_{1,3} < 0.01$, $P = 0.96$, $R^2 < 0.01$; days: $F_{1,3} = 0.28$, $P = 0.63$, $R^2 = 0.09$).

Pairwise relatedness of those individuals that mated with each other was not any different than the average pairwise relatedness of the population as a whole (ANOVA—Quarry 2003: $F_{1,2772} = 0.89$, $P = 0.35$; LT pond 2002: $F_{1,194} = 0.16$, $P = 0.69$).

Discussion

In this study, inbreeding (estimated either through multilocus heterozygosity or relatedness estimator of Queller and Goodnight [1989]) negatively affected the survival of wood frog larvae in the wild (Figure 2). The effects were large; there was more than a 7-fold difference in the survival of the least and most heterozygous individuals in Quarry in 2003. However, in the laboratory, inbreeding had no effect on the hatching success of the embryos or the survival of the larvae. Neither did it have any significant effect on size or development of the larvae in the laboratory or the field. Although interesting, it is difficult to interpret the latter results because it is possible that the smallest or least-developed individuals in the field did not survive as well and thus were not measured. The results from the field experiments in LT pond in 2002 yielded no significant results for any comparisons; however, the sample size and thus the power were low.

Determining parentage and then estimating average multilocus heterozygosity and the Queller and Goodnight (1989) inbreeding coefficient for an entire sibship as we did here has several advantages over measuring the heterozygosity of the individuals alone. First, it utilizes more information; it makes use of the 4 alleles at a locus in the parental generation instead of the 2 alleles present in individual offspring. This combination of pedigree and microsatellite techniques provides a better estimate of inbreeding at the whole-genome level and lessens the effects of linkage disequilibrium (Pemberton 2004). Second, the Queller and Goodnight (1989) inbreeding coefficient makes use of information about the allele frequencies in the population as a whole to determine relatedness. This information could not be generated reliably solely from the offspring generation because the presence of sibships would skew estimates. It is interesting to note, however, that heterozygosity and the Queller and Goodnight (1989) inbreeding coefficient were highly correlated (Figure 1). Third, it minimizes the number of microsatellites that must be analyzed because not all loci must be analyzed in the offspring generation—only those necessary to determine parentage.

The results from Quarry in 2003 are consistent with results from studies with other amphibian species (e.g., Rowe et al. 1999; Lesbarreres et al. 2005), with the predictions of Ralls et al. (1988), and with the results of other studies that have shown inbreeding to have a stronger effect on survival in the wild than in the laboratory (Chen 1993; Jimenez et al. 1994; reviewed in Crnokrak and Roff 1999). There are several possible explanations for such contrasting results. First, the decreased heterozygosity that accompanies inbreeding might lead to individuals that are more subject to predation—a factor that was only present in the wild. Second, such results could be generated if competition was more severe in the wild, and inbreeding had a negative effect on competitive abilities. Although growth and development—the traits that are most likely to affect predation and competition—were not correlated with inbreeding in either experiment, such an affect might have been hidden by the fact that the slower growing and developing individuals were less likely to survive to metamorphosis and thus be sampled (e.g., Rowe and Beebee 2005). Third, many abiotic factors may be more harsh in the wild than in the laboratory, and heterozygosity may be at a premium in harsher environments. Chemical differences between the water in the pond and the treated tap water used

in the laboratory may have made the pond a harsher environment. Likewise, hydroperiod may have been important; the ponds in which these studies were conducted are temporary, and thus, individuals that did not metamorphose quickly enough could have died. However, no premetamorphic larvae were found in these ponds for more than a week before the ponds dried in the years in which these experiments were conducted. Combined with the lack of any correlation between inbreeding and growth and development, these observations suggest that chemistry, hydroperiod, and other abiotic factors were not strong factors driving the different results in the wild and the laboratory.

A fourth possibility that could explain the different effects of inbreeding on survival in the field and the laboratory is that inbreeding exerts its most powerful influence during the few days that precede metamorphosis. Although the individuals in the wild were not collected until they reached this stage, the individuals in the laboratory were collected before any had begun this process. It is likely that metamorphosis is a stressful time for wood frogs and a period of high mortality. For example, during this time, the larval digestive system undergoes profound changes to make it suitable for terrestrial food sources and the larvae stop eating (Duellman and Trueb 1986). The larvae must also begin obtaining oxygen from lungs instead of gills during this time. And, as the larvae resorb their tails and begin to rely on their feet for locomotion, often in crowded conditions from imminent pond drying, they are extremely vulnerable to predation.

Fifth, and finally, it is possible that disease was more prevalent in the wild than in the laboratory and that inbreeding was correlated with immune response. Although data are lacking, the first part of this hypothesis seems likely. Because the water used in the laboratory experiment was tap water that had initially been filtered and treated for human consumption, it likely had a lesser load of parasites. In contrast, both observations in these ponds and studies conducted elsewhere suggest that water in natural ponds can have numerous different parasites that can inflict widespread mortality on amphibians. The second part of the hypothesis also has strong theoretical support and empirical support. Heterozygosity has been shown to be very important for the functioning of the immune system and, specifically, the major histocompatibility complex (Flajnik and Du Pasquier 1990; Wedekind et al. 1995).

It is often suggested that inbreeding may be correlated with fitness because inbred individuals are more likely to have 2 copies of a lethal recessive allele (Ralls et al. 1988). In extreme cases, inbred embryos that are homozygous for lethal recessive alleles may not survive past the first few cell divisions. The lack of any significant effect of inbreeding on survival of embryos or larvae in the laboratory experiment suggests that such lethal alleles played little role here. And indeed, in theory, there should be fewer such alleles in populations of this species. Wood frogs have been shown to be highly philopatric (Berven 1990) and thus should experience higher levels of inbreeding than less philopatric species. High levels of inbreeding across multiple generations have been shown to expose lethal recessive alleles to selection and thereby purge them from the gene pool (Crnokrak and Barrett 2002). In addition, wood frog populations are likely to undergo frequent bottlenecks when environmental factors such as droughts inflict extremely high mortality on a cohort. Such bottlenecks should eliminate many rare alleles such as lethal recessives. In combination, these 2 factors should limit the number of lethal recessive alleles present in wood frog populations and thus reduce any effects they may cause through inbreeding, as appears to be the case here.

It has also been suggested that inbreeding may be mitigated in wild populations because individuals can avoid breeding with their close relatives (Waldman et al. 1992; Crnokrak and Roff 1999). Although wood frog larvae have been shown to be capable of recognizing their siblings and half-siblings (e.g., Waldman 1984; Cornell et al. 1989; Halverson et al. 2006), there was no evidence that wood frog adults avoided inbreeding. The average pairwise relatedness of those individuals that mated with each other in each pond was not significantly different than the average pairwise relatedness of the population of adults as a whole. Such results should not be surprising, given the wood frog breeding system. Observation suggests that the female has little choice in her mate and the males have little reason to avoid inbreeding so long as it does not prevent them from breeding with less-related individuals.

In conclusion, it should be noted that this is not the first study of inbreeding effects on wood frog larvae. As was found here with microsatellites, Wright and Guttman (1995) found no correlation between size and allozyme heterozygosity in wood frog larvae in the wild. However, in that study, the effects of allozyme heterozygosity on survival and development were not assessed. The results of this study demonstrate the importance of measuring various components of fitness, especially survival, when assessing the effects of inbreeding. They also demonstrate the importance of studying inbreeding under natural conditions.

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