

Experimental Evidence that Nest Attendance Benefits Female Marbled Salamanders (*Ambystoma opacum*) by Reducing Egg Mortality

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ABSTRACT.—To understand the selective pressures that have influenced the evolution and maintenance of parental care, it is necessary to assess the consequences and function(s) of specific behaviors. We used field and laboratory experiments to investigate possible fitness benefits and proximate functions of female nest attendance in marbled salamanders (*Ambystoma opacum*). In the first field experiment, nests at which females remained until flooding had higher hatching success than those without attendant females, but results were somewhat equivocal. In the second field experiment we used unattended eggs in artificial clutches randomly assigned to one of four treatment groups: eggs physically agitated to possibly reduce developmental malformations and/or fungal growth, eggs protected from predators, eggs moistened by addition of water and eggs in an unmanipulated control group. Clutches that were protected from predators had significantly higher hatching success than control, agitation and supplemental water groups. In the laboratory experiment, nests in which fungal infection was controlled had higher hatching success than those without sterilized substrates. Also, physical agitation and increased moisture did not confer higher hatching success when fungi were eliminated in the laboratory. Predation and fungal infections appear to decrease hatching success in this species, and female nest attendance may reduce these risks.

INTRODUCTION

Parental behavior occurs in a wide variety of taxa, from annelids to chordates (Clutton-Brock, 1991), and has received considerable attention from theoretical and empirical evolutionary biologists (*see* Crawford and Balon, 1996; Gans, 1996; Trumbo, 1996). Specifically, researchers have been interested in the evolutionary progression of parental care from the presumed ancestral state of no care, to uniparental male or female care, to biparental care (*e.g.*, Szekely and Reynolds, 1995; Goodwin *et al.*, 1998), as well as the phylogenetic distribution of these differing life histories (Ridley, 1978; Perrone and Zaret, 1979; Gross and Shine, 1981). Central to these questions are the ecological, anatomical and behavioral factors that result in suites of costs and benefits associated with parental behavior (Trivers, 1972; Dawkins and Carlisle, 1976; Blumer, 1979).

Parental behavior is widespread among amphibians, especially those exhibiting terrestrial reproductive modes (Salthe and Mecham, 1974; Duellman and Trueb, 1986; Crump, 1995, 1996 and references therein), and may consist of nest attendance, egg transport, tadpole attendance, tadpole transport, tadpole feeding or internal gestation (*e.g.*, Crump, 1995,

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1996; Jungfer, 1996; Kaminsky *et al.*, 1999; Burrowes, 2000). Several studies have shown that removal of the parent results in high egg mortality in frogs (Townsend *et al.*, 1984; Juncá, 1996; Jungfer, 1996; Burrowes, 2000) and salamanders (Tilley, 1972; Forester, 1979a; Harris and Gill, 1980; Juterbock, 1987; Carreño and Harris, 1998), suggesting that parental behavior increases reproductive success (but *see* Jacobson, 1985). The costs of parental care have been less frequently measured in amphibians (but *see* Kaplan and Crump, 1978; Townsend, 1986; Ng and Wilbur, 1995).

In salamanders, the behavioral repertoire of caregivers primarily consists of nest attendance (Salthe and Mecham, 1974; Duellman and Trueb, 1986), which has been documented in at least eight urodele families (Nussbaum, 1969; Godley, 1983; Crump, 1995, 1996). Most care-giving salamander species are members of the plethodontid clade (Crump, 1995, 1996), which exhibits the most advanced parental behaviors among urodeles. Because their anamniotic eggs are vulnerable to desiccation, the association of terrestrial reproduction with nearly universal (in those species for which data are available) nest attendance in plethodontids lends credence to Wilson's (1975) view that especially inhospitable environments have led to the evolution of parental care (but *see* Nussbaum, 1985, for the alternative view that the trophic structure of lotic habitats gave rise to parental behavior in plethodontids).

Parental presence at the nesting site presumably increases egg survival directly (*e.g.*, Tilley, 1972; Forester, 1979a; Juterbock, 1987). The attending parent may retard desiccation of eggs (Forester, 1984), reduce egg predation by actively defending the clutch (Highton and Savage, 1961; Forester, 1978, 1979a; Bachmann, 1984), reduce egg infestation by pathogenic fungi either via antifungal skin secretions or physical disruption of fungal hyphae (Tilley, 1972; Forester, 1979a; but *see* Vial and Preib, 1966, 1967), enhance aeration of aquatic eggs (Salthe and Mecham, 1974) and/or prevent developmental malformations by jostling the eggs (Salthe and Mecham, 1974). Nest attendance may impart benefits to the hatchlings as well. Highton and Savage (1961) found that attended hatchlings were larger than their abandoned counterparts in *Plethodon cinereus*, although the cause of this result is unknown. Huheey and Brandon (1975) observed a female *Desmognathus fuscus* leading her young out of an enclosed nest site. These functions of nest attendance are reasonably well documented in plethodontid salamanders but few studies have assessed the behavior in other urodele families.

Marbled salamanders (*Ambystoma opacum*) are one of only two ambystomatid species that reproduce terrestrially and the only one that provides parental care (Petranka, 1998). Previous studies have provided evidence that the presence of female marbled salamanders at the nest site increases egg survivorship. Petranka (1990) noted that inviable clutches were less likely to be attended. Jackson *et al.* (1989) reported that the hatching success of a clutch was positively correlated with the number of days a female attended the nest. These observations suggest that attendance directly increases the female's reproductive success but do not eliminate the possibility that females are simply more likely to desert an unsuccessful nest (Petranka, 1990). Even without any selective advantage for nest attendance, the behavior could be maintained if its costs, in terms of lost opportunities for future reproduction (Trivers, 1972), are negligible. Kaplan and Crump (1978) detected no energetic costs of 2 to 3 wk of nest attendance and also found no evidence of feeding in males and females collected during the nesting season (*see also* Noble and Brady, 1933). Although post-reproductive food availability affects future probability of reproduction in *A. opacum* (Scott and Fore, 1995) and other species (Harris and Ludwig, 2004), there is no evidence that there is a future reproductive cost related to nest attendance. Thus, it has yet to be determined if nest attendance in marbled salamanders has a selective advantage, as suggested by correlational data (Jackson *et al.*, 1989; Petranka, 1990), or if it is selectively

neutral, in terms of egg survivorship, as has been suggested in some other amphibians (Woodruff, 1977).

The function(s) of nest attendance has not been studied rigorously in *Ambystoma opacum*. Several authors suggested that females inhibit desiccation of the eggs (*e.g.*, Noble and Brady, 1933; Petranka and Petranka, 1981a), presumably either by urinating on them or via passive moisture transfer from their permeable integuments. According to Salthe and Mecham (1974), nests are often located at the center of a small area devoid of fungal hyphae, suggesting that females inhibit fungal growth. Eggs also may benefit from agitation by the female's movements during clutch attendance (Salthe and Mecham, 1974). Although some plethodontids engage in defense of the nest against potential egg predators (*e.g.*, Forester, 1979a; Bachmann, 1984), Kaplan and Crump (1978) and D. C. Forester (pers. comm.) suggested that *A. opacum* females do not perform such behavior. Clearly, more studies of nest attendance in marbled salamanders are necessary to assess its fitness consequences and distinguish among its possible functions.

The purpose of this study was to experimentally examine whether nest attendance benefits females by increasing hatching success in *Ambystoma opacum* and, if so, to assess the potential proximate functions of nest attendance. In our first field experiment we manipulated female presence/absence on nests and compared the hatching success of attended and unattended clutches. Subsequently, we used field and laboratory experiments with unattended eggs to investigate the specific mechanism(s) by which a female salamander may impart advantages to her progeny. These experiments tested three competing, but not mutually exclusive, hypotheses concerning the function of female nest attendance: (1) agitation of eggs to reduce developmental malformations and/or disrupt fungal infection, (2) protection from egg predators and (3) inhibition of egg desiccation.

METHODS

Study species.—Marbled salamanders (*Ambystoma opacum*) breed during the late summer and fall in dry woodland ponds or floodplain pools in the eastern United States. Females construct cavities beneath logs, leaf litter and vegetation in which they oviposit and attend the clutch for variable durations, usually from 1 to 3 mo (Petranka and Petranka, 1981a, b; Jackson *et al.*, 1989; Petranka, 1998). When fall rains inundate nests, the eggs hatch due to hypoxia (Petranka *et al.*, 1982) and the larvae develop aquatically until metamorphosis, as in other *Ambystoma*. The length of the embryonic period is variable and temperature-dependent (Kaplan and Crump, 1978; Scott, in press), and may range from less than 2 wk (Petranka (1998) to 40 d (J. S. Doody, pers. comm.).

Study site.—This study was conducted at Ginger's Bay (GB), a small (0.8 ha) shallow Carolina bay (Sharitz and Gibbons, 1982) in Aiken County, South Carolina, that typically fills with water in late autumn and dries by midsummer (Scott, 1990, 1993). GB supports large populations of breeding amphibians, especially *Ambystoma opacum* and *A. talpoideum*. *Notophthalmus viridescens*, *Bufo terrestris*, *Gastrophryne carolinensis*, *Scaphiopus holbrookii* and *Rana sphenoccephala* are also commonly found at GB. The bay consists of three elevation zones that may be recognized by vegetation patterns (Jackson *et al.*, 1989). Our first field experiment was conducted with nests that were dispersed throughout the entire wetland; the second field experiment was conducted in the mid- to high-elevation zone (23–42 cm), which contains sweetgum (*Liquidambar styraciflua*), red maple (*Acer rubrum*) and loblolly pine (*Pinus taeda*) trees along with greenbriar (*Smilax* sp.).

Field experiment 1.—In late October 1988 we searched for nests and measured nest success at GB similar to the methods of Jackson *et al.* (1989). Seventy nests with recently oviposited eggs were discovered from 1 November through 14 November 1988. Upon discovery of a

TABLE 1.—Nest success and snout-vent length (SVL) for female *Ambystoma opacum* in field experiment 1 at Ginger's Bay. E = nests enclosed with aluminum cylinder, U = nests unenclosed until 1–2 d prior to nest flooding (*see* Methods)

Treatment group	# Nests	% Nest success (mean \pm 1 SE)	SVL (mm) (mean \pm 1 SE)
Female abandoned (E)	9	9.7 \pm 6.3	58.3 \pm 2.8
Female abandoned (U)	12	7.4 \pm 5.6	57.2 \pm 1.1
Female removed (E)	8	8.9 \pm 7.9	59.4 \pm 1.3
Female removed (U)	14	8.0 \pm 3.8	57.9 \pm 0.6
Female present (E)	14	26.1 \pm 7.9	57.5 \pm 1.1
Female present (U)	13	14.3 \pm 4.9	58.8 \pm 1.2

nest, the attending female was briefly removed while her eggs were counted and then returned to the nest and covered with the original nest structure material. Many females are sensitive to nest disturbances and prone to abandon their nests (J.W. Petranka, pers. comm.). The day following the initial egg counts we rechecked each nest to determine female presence/absence. Nests that females had abandoned were non-randomly assigned to a “female abandoned” treatment group. Remaining nests with females were randomly assigned to either a “female removed” treatment, where we physically removed the attending female, or a “female present” treatment, where a female was allowed to remain on her nest.

We crossed the three female presence/absence treatments with a nest enclosure treatment. Within each group (*i.e.*, female abandoned, removed or present) we randomly assigned nests to be enclosed by a 25 cm diameter by 51 cm high aluminum cylinder (Jackson *et al.*, 1989) or not enclosed (*see* Table 1 for sample sizes). The cylinder enclosures were buried 8 to 10 cm below the ground surface. The purpose of the enclosures was to exclude any large predators from the nests and reduce numbers of invertebrate predators. In the “female present” treatment the enclosures probably also served to keep females from abandoning nests. These enclosures may have reduced, but did not eliminate, colonization of nests by millipedes, which at the time were not known to be potential nest predators.

Regardless of treatment, all nests were enclosed 1–2 d prior to predicted nest flooding (Jackson *et al.*, 1989). Nest flooding ranged from 26 November 1988 to 10 March 1989. Two to 3 d after submersion we sampled nests for hatchlings, dead larvae and unhatched eggs. Nest success was calculated as the number of hatchlings divided by the original number of eggs.

Field experiment 2.—Given evidence from Field Experiment 1 that nest attendance enhances egg survival, we investigated some potential causes in autumn 2000. We used four experimental treatments to explore the mechanism by which an attending female salamander may enhance the survivorship of her eggs. Each treatment consisted of 30 artificial clutches of 20 eggs in the field, without attending females. The eggs we used were chosen from a pool of approximately 40 clutches to randomize both the developmental stage of the embryos and individual maternal effects. We chose 20 eggs to control for clutch size and simultaneously keep the egg sample size manageable and sufficient for detecting differences among treatment groups.

In the first treatment, each clutch received daily five-second periods of physical agitation by gently lifting eggs with a spoon before allowing them to roll back into the nest. We used a separate plastic spoon for each replicate throughout the experiment. This treatment was designed to test the hypothesis that eggs which are moved regularly experience higher survival as a consequence of reduced susceptibility to developmental malformations and/or fungal infection by disruption of hyphae.

The second treatment tested the hypothesis that predation is an important source of mortality in the absence of the female. We enclosed this group of artificial clutches in small rectangular boxes (5 cm × 5 cm × 1 cm) of stainless steel mesh (<2 mm) to deny potential predators, including small invertebrates, access to eggs. Soil was added to each box so that eggs contacted it throughout the experiment.

The third treatment tested the hypothesis that additional moisture increases survival of unattended embryos. We regularly added water to this group of clutches. On most days, we applied distilled water to each clutch for five seconds with a small water sprayer. We chose 5 s because it was the time required to sufficiently fill the spaces between the eggs without completely inundating them. On days when the high temperature did not surpass 5 C (six total during the 30 d of the experiment), we did not add water because of the possibility that the experimental manipulation could increase egg mortality due to freezing.

The fourth treatment was the unmanipulated control group. For all groups, when the predicted nightly low temperature was below 0 C we covered the clutches with plastic tarps to minimize frost damage. Tarps were removed early on the following morning.

Experimental clutches were placed in artificial nest sites constructed by excavating small basins (ca. 5 cm diameter, 3 cm deep). It was sometimes necessary to remove root mats and other litter. We used small pieces of pine (*Pinus*) bark to cover the nest basins. Surrounding litter was used to cover each piece of bark. The replicates were arranged in two blocks that were each four nests wide. To ensure that the experimental clutches were placed near one another in the bay, we fit them among the trees in one block of 10 rows and another of 20. All treatment groups were present within a four-clutch row, but their order was randomized to control for position effects. Each nest was 14 cm away from its nearest neighbor within a row, and rows were separated by 14 cm.

The experiment lasted for 30 d, from 7–8 December 2000 to 6–7 January 2001. We performed no experimental manipulations on 25 December 2000. At the end of the experiment, clutches were brought into the laboratory and placed immediately in plastic or styrofoam cups of distilled water for hatching. During this phase of the experiment, the embryos were kept in a windowless room at 16 C. Each cup was checked one to three times per day for hatchlings, which were removed and counted.

Laboratory experiment.—Female salamanders may retard fungal growth on their eggs by physically disrupting fungal hyphae or by passively applying antifungal skin secretions. The unattended artificial clutches in the field experiment experienced visible fungal infections, which may cause significant mortality (Salthe and Mecham, 1974). Therefore, the presence of fungi in all treatment groups was a possible confounding factor. To remove this confounding effect and experimentally test the hypothesis that fungi cause egg mortality, we performed a laboratory experiment with four groups of 10 artificial clutches of 20 eggs each, chosen randomly from the same group of eggs used in the second field experiment. For three of the four groups, we sterilized the substrate and container. One of the sterile substrate groups received daily water addition to test the hypothesis that hydration increases egg survival in the absence of fungal infestation in the laboratory. A second sterile substrate group was spooned daily for five seconds to test the hypothesis that agitation is beneficial to embryo survival. The third group of eggs was a sterilized substrate control, whereas the fourth group consisted of unmanipulated eggs in unsterilized substrate. Thus, this laboratory experiment assessed the possibility that fungi cause significant mortality in unattended eggs as well as the importance of hydration and mechanical agitation as functions of nest attendance in the absence of fungal infestation. We did not add attendant females to the experimental clutches because previous observations showed that they do not exhibit normal nest attendance behavior under laboratory conditions or after being disturbed from their original nest sites.

Plastic freezer containers (8.5 cm × 8.5 cm × 6 cm) with a depth of 2 cm of soil were used to house the clutches. As in Field Experiment 2, a circular depression was constructed and covered with a small piece of *Pinus* bark. We covered all containers containing the egg-free artificial nests and bark with aluminum foil and sterilized them at 115 C for 30 min in an autoclave. We rinsed the eggs repeatedly (five to seven times) with distilled water to remove as many fungal spores and bacteria as possible and then added the eggs to the sterilized containers. This procedure was successful at controlling fungal infestation. We did not perform the sterilization or rinsing procedures for the unsterilized treatment that remained susceptible to infection.

The physical agitation treatment was identical to that used in the second field experiment. All clutches received autoclaved water (115 C for 30 min) at least every other day. The moisture treatment consisted of daily water additions. We used a small plastic spray bottle to apply water without agitating the eggs. A bleached spoon was used to lift the bark cover, except for the unprotected treatment. For each water addition, we sprayed the nest depression until the spaces between the eggs were inundated but before the eggs were entirely covered with water. Using this procedure, we were able to prevent catastrophic desiccation while allowing for the possible effects of stress due to limited moisture. No clutches entirely succumbed to moisture stress, yet, at each moisture addition, the soil substrate in the low-water groups was completely dry and eggs were noticeably smaller than those in the high moisture treatment. As in Field Experiment 2, no manipulations were performed on 25 December 2000.

The experimental clutches were housed in an environmental chamber with controlled day and night temperatures (21 C and 10 C, respectively) and a 13.75:10.25 h (light:dark) photoperiod. Light was provided by three fluorescent bulbs (116 W). The experiment lasted for 30 d, from 10 December 2000 to 9 January 2001. At the end of the experiment, we followed the same protocol as in the second field experiment to determine hatching success.

Statistical analyses.—We counted the number of embryos that successfully hatched in each replicate from all experiments and expressed these data as a proportion of the initial total egg number. We used a square-root arcsine transformation and visually inspected frequency histograms to ensure that our data met the normality assumption of parametric statistical techniques. For each experiment, we used *a priori* contrasts in a single-factor analysis of variance (ANOVA; SAS Institute, 2003) to determine if treatment groups of interest differed. In Field Experiment 1, we tested for differences between nests with females vs. nests without females (whether abandoned or removed), nests with females vs. nests where females were removed and enclosed nests with females vs. unenclosed nests with females and enclosed nests without females. For the second field experiment, we tested for differences between the control group and each of the three treatment groups separately. In the laboratory experiment, we tested for differences between the control group and the two sterilized treatment groups, agitation and water. We also compared the unsterilized group of clutches to the three sterilized groups.

RESULTS

Field experiment 1.—Some nests at lower elevation in the bay were flooded as early as 26 November, while some nests at higher elevation were flooded much later (*e.g.*, 13 January and 10 March 1989) or not at all. The broad range of timing of nest submersion contributed to high variance in nest success in all treatment groups (Table 1). Despite high variance, nests with no females (combined “female abandoned” and “female removed” treatments) had significantly reduced hatching success compared to nests with females (Fig. 1; ANOVA contrast: $F_{1,64} = 4.98$, $P < 0.05$). Comparing only the nests that were randomly assigned to treatments, there was no significant difference between “female removed” and “female

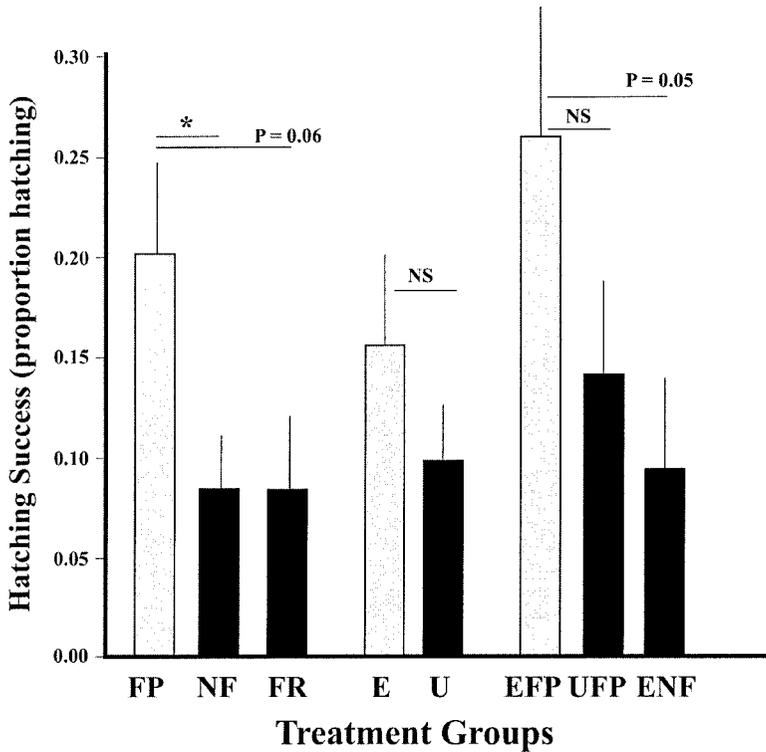


FIG. 1.—Mean (± 1 SE) proportional hatching success of *Ambystoma opacum* nest treatments in 1988–1989 at Ginger's Bay. Bars represent the treatment groups used in five ANOVA contrasts comparing: (1) nests with females present (FP; enclosed and unenclosed) to nests with no females (NF; combined female removed and female abandoned treatments, enclosed and unenclosed nests) and nests from which females were removed (FR; enclosed and unenclosed nests), (2) enclosed nests (E; with and without females) to unenclosed nests (U; with and without females) and (3) enclosed nests with females (EFP) to unenclosed nests with females (UFP) and enclosed nests without females (ENF; combined female removed and female abandoned treatments). Horizontal lines represent each statistical test and result; * indicates $P < 0.05$, NS = non-significant at $P > 0.10$

present" nests (ANOVA contrast: $F_{1,64} = 3.49$, $P = 0.06$), although the trend was consistent with a benefit from female nest attendance. In general, there was no difference between enclosed and unenclosed nests (ANOVA contrast: $F_{1,64} = 0.37$, $P = 0.54$), or in particular between enclosed and unenclosed nests with females (ANOVA contrast: $F_{1,64} = 0.81$, $P = 0.37$), although enclosed nests with females fared better than enclosed nests without females (ANOVA contrast: $F_{1,64} = 3.78$, $P = 0.05$).

Field experiment 2.—The group that received protection from predators had significantly higher hatching success than the control group (Fig. 2, ANOVA contrast, $F_{1,116} = 25.3$, $P < 0.0001$). The hatching success of the other groups was not significantly different than the control (agitation: ANOVA contrast, $F_{1,116} = 0.1$, $P = 0.80$; water: ANOVA contrast, $F_{1,116} = 0.54$, $P = 0.46$).

Although the experimental blocks and the individual clutches in the agitation and water treatments were observed daily, we never noticed extensive physical disruptions that would

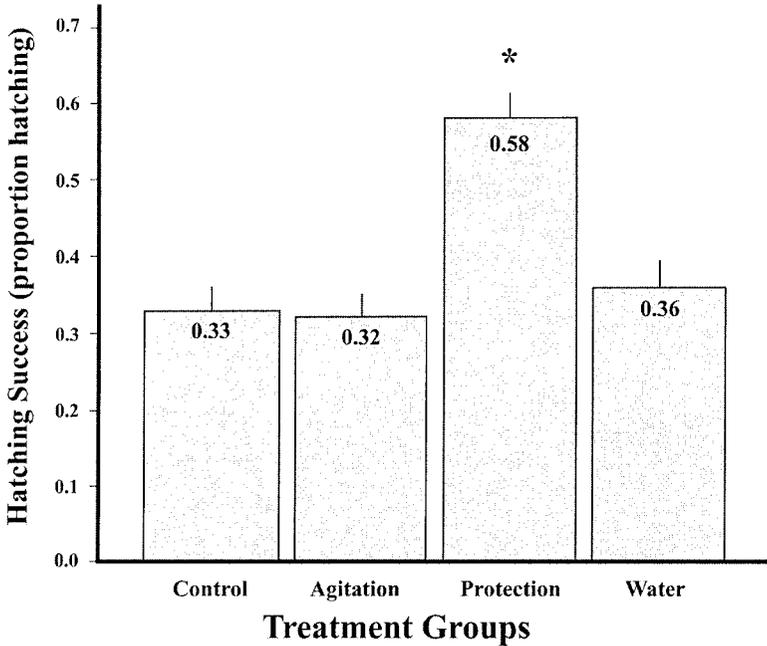


FIG. 2.—Mean (± 1 SE) proportional hatching success in the second field experiment (2000). See text for an explanation of the treatments. The asterisk (*) indicates a significant difference from the control treatment ($p < 0.017$)

indicate predation by large vertebrates (*e.g.*, frogs, snakes, shrews). However, small millipedes (generally about 20 mm long), *Oxidus gracilis* (Polydesmida: Paradoxosomatidae), invaded the clutches in large numbers and slowly destroyed many of the eggs. Through their partially translucent cuticles, we observed their digestive tracts fill as the experiment progressed and affected eggs degenerated to empty sacs. Millipedes maintained prolonged close association with eggs that eventually succumbed. *Oxidus gracilis* is a globally widespread, introduced species (Shelley, 1988) that produces strong defensive allomones (*e.g.*, phenol and guaiacol; Blum, 1996). It is unclear whether they feed on living *Ambystoma opacum* eggs or kill them with biocidal chemicals before consumption. There was considerable variation in the number of millipedes present among nest sites. Many nests remained free of millipedes, whereas others had more millipedes than eggs (clutch size = 20). Fungi infected eggs in all treatment groups and appeared to be distributed randomly among them.

Laboratory experiment.—The unsterilized group had lower hatching success than the sterilized groups (Fig. 3, ANOVA contrast, $F_{1,36} = 21.6$, $P < 0.0001$). Hatching success in the agitation and water groups was not significantly different from that of the control (agitation: ANOVA contrast, $F_{1,36} = 0.0$, $P = 0.95$; water: ANOVA contrast, $F_{1,36} = 1.1$, $P = 0.30$).

DISCUSSION

Fitness benefits of nest attendance.—Results from the 1988–89 nest manipulation experiment suggest that nest attendance by female *Ambystoma opacum* increases nest success. Nests from which females were removed had low hatching rates similar to nests that females

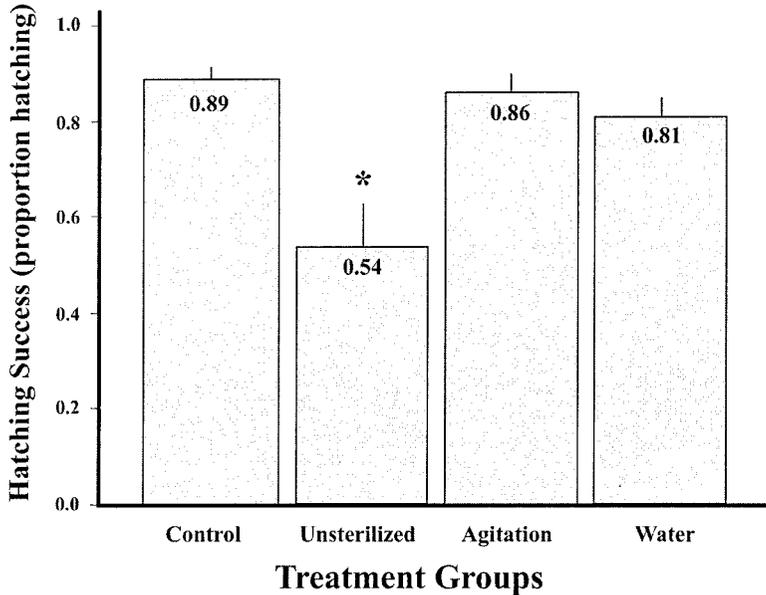


FIG. 3.—Mean (± 1 SE) proportional hatching success in the laboratory experiment. See text for an explanation of the treatments. The asterisk (*) indicates a significant difference from the sterilized substrate control group ($p < 0.01$)

abandoned, and these nests with no females (combined abandoned and removed groups) had lower success than those with females present. In addition, enclosed nests with attending females had higher success than enclosed nests without females (also combined abandoned and removed groups). We cannot rule out the possibility that females were more likely to abandon nests that either already were unsuccessful upon discovery or were likely to become unsuccessful later in the embryonic period. This bias, if present, could have resulted in the comparatively high hatching success of nests with attending females. However, we believe this to be unlikely because nests were discovered soon after oviposition, probably before egg mortality had occurred. Additionally, the similarity in overall nest success between nests in which we controlled female presence (the randomly removed female group, 8.4%) and nests in which females chose to be absent (the female abandoned group, 8.4%) does not support the idea that females abandon nests of unusually poor quality early in the developmental period.

When abandoned clutches are eliminated from the analysis, there is only a marginally significant difference ($P = 0.06$) in hatching success between attended and unattended nests. Because GB filled gradually in 1988–89 (*i.e.*, pond-filling began 3–4 wk after oviposition), low-lying nests were likely to experience high success regardless of treatment, and similarly the high elevation nests that flooded much later or not at all were likely to fail regardless of treatment. This hydrologic pattern resulted in high variance within treatment groups and low statistical power. But there remains convincing evidence that female nest attendance is beneficial to the clutch. First, all unattended nests combined had significantly lower hatching success than attended nests. Second, nests that experienced female removal had much lower hatching success (albeit non-significant) than those attended until

flooding. Thus, our data provide the strongest experimental evidence to date that female nest attendance directly increases hatching success in *Ambystoma opacum*.

Functions of nest attendance.—In the second field experiment we observed that unattended *Ambystoma opacum* eggs protected from all predators had higher hatching success than unprotected controls. Clutches that received additional water and physical agitation did not have higher survival than controls in the field or the laboratory. This result suggests that nest attendance by female *A. opacum* functions to deter predation and does not support the hypotheses that nest attendance serves to agitate or hydrate eggs. In addition, in Field Experiment 1 we observed a trend that enclosed nests with females had greater success (26% hatching) than unenclosed nests with females (14%) and enclosed nests without females (9.3%), which is consistent with the idea that nest predation by millipedes can be a source of egg mortality (because millipedes were not excluded by the aluminum cylinders, although numbers may have been reduced). In the laboratory, unattended clutches on sterilized soil had higher survival than those that were on unsterilized soil, indicating that fungi can also cause egg death. In addition to protection from predators, female nest attendance may inhibit fungal infection of eggs in the field.

It is possible that the stainless steel mesh boxes used to exclude predators served an unrecognized function (*e.g.*, prevention of fungal access to the eggs) to increase egg survival. However, fungal infestation was observed in all treatment groups, including controls. We purposely added soil to surround the eggs within the boxes. Therefore, the exclusion of predatory millipedes (*Oxidus gracilis*), which were abundant in the clutches of the other three treatment groups, probably resulted in the observed increase in hatching success.

Previous experiments with attending female *Ambystoma opacum* showed that they do not actively defend their nests from intruders (D. C. Forester, unpubl. data) as do some plethodontids (*e.g.*, Highton and Savage, 1961; Forester, 1979a; Bachmann, 1984). Females may remove small millipede predators by consuming those that venture into the nest cavity. However, Kaplan and Crump (1978) and Noble and Brady (1933) found no prey in the stomachs of attending females, nor have we in samples of females leaving the wetland (DES, unpubl. data). It is more likely that millipedes are either unaffected by the presence of the female or that they avoid adult salamanders to lessen the chance of being consumed, although the sensory mechanism that could allow such a behavior remains unknown. The possibility that female marbled salamanders repel millipedes and other potential predators from their nests warrants further investigation. Other predator species (*e.g.*, frogs, heterospecific salamanders, beetles, etc.), as well as another millipede (Mitchell *et al.*, 1996), reportedly prey on the eggs of *A. opacum* (Noble and Brady, 1933). We only observed millipedes in the nest cavities, and it is unlikely that larger vertebrate predators (*e.g.*, shrews, snakes) took any eggs because no full clutches disappeared or were visibly disturbed.

Unattended eggs that received supplemental moisture did not show higher hatching success in the field or the laboratory. Some amphibian species can use specialized behaviors (*e.g.*, coiling around the brood, seeking moisture before returning to urinate on eggs) to inhibit clutch desiccation (Wells, 1978; Weygoldt, 1980; Forester, 1984). But *Ambystoma opacum* females show little recognition of their eggs and appear to only passively remain near or on them (Noble and Brady, 1933). However, it is possible that differential water potential between the eggs and the integument of attending females allows the passage of moisture as in *Eleutherodactylus coqui* (Taigen *et al.*, 1984). Field Experiment 2 occurred during exceptionally cool weather in December 2000 (Savannah River Technology Center, 2000). More data are needed to test the idea that nest attendance serves a hydration function in *A. opacum* during typical years when most of the nesting season occurs in October and

November, which on average are the two driest months of the year (Savannah River Technology Center, 2000).

Daily egg jostling did not result in increased hatching success in the field or the laboratory, failing to support the hypothesis that nest attendance functions to prevent lethal malformations or fungal infections by agitating the eggs. Because we did not quantify abnormalities directly, it remains possible that agitation decreased malformations but had no effect on hatching success. This result also contrasts with that of Forester (1979a), who found that gentle mechanical vibrations increased the survivorship of *Desmognathus ocoee* eggs (formerly known as *D. ochrophaeus*). Although some female salamanders may accomplish egg agitation through their respiratory gular motions (Forester, 1979a), marbled salamanders cannot touch each of their numerous single eggs continually. Normal stirring by attendant females probably jostles and displaces a high proportion of the brood regularly, apparently resulting in increased survival of terrestrial eggs in the frog, *Cophixalus parkeri* (Simon, 1983). Further work, with quantification of developmental malformations and mortality, is needed to investigate this possible function of nest attendance. Daily agitation failed to inhibit fungal infection in the field and laboratory; hyphae visibly affected eggs in these treatment groups.

The laboratory experiment suggested that infection by pathogens can cause significant egg mortality. Fungal hyphae were prominent in all groups of clutches that were left unsterilized and probably caused the observed decrease in hatching success. Females of some species may prevent egg mortality by applying antifungal dermal secretions (e.g., Stebbins, 1954; Salthe and Mecham, 1974) or physically disrupting the growth of fungal hyphae (but see Daniel and Simpson, 1954; Vial and Preib, 1966, 1967). In the field, many eggs were infested but it was unclear whether fungi were the primary cause of death or only invaded after eggs died for other reasons. If the eggs are somehow protected from fungi, the embryos themselves may produce the antibiotic compounds, although the results of the laboratory experiment indicate that this ability is limited, if it exists at all. Further data are needed to test how *Ambystoma opacum* females might protect their embryos from fungi in the field.

Evolution of parental care.—Parental behaviors are unlikely to evolve unless their fitness benefits exceed the costs of decreased future reproduction incurred by the caregiver. The higher success in nests with attending females in the first field experiment, the increased survival of protected eggs in the second field experiment and the positive effect of nest attendance documented by Jackson *et al.* (1989) may indicate that parental behavior should be favored because the energetic costs of nest attendance are reportedly negligible (Kaplan and Crump, 1978). However, other studies suggest that nest attendance may be costly, either because of reduced foraging opportunities or increased susceptibility to predation in *Ambystoma opacum* (DES, unpubl. data), *Plethodon cinereus* (Ng and Wilbur, 1995) and *Desmognathus ocoee* (Forester, 1979b). More quantitative studies are needed to assess the costs and benefits of nest attendance in *A. opacum* and other species before we can understand the evolution of parental care in salamanders.

This study addressed two proposed situations that could favor the evolution of parental care (Wilson, 1975): (1) harsh environmental conditions during development and (2) high predation on the eggs or young. The harsh dry nest site could select for parental attendance to assist in hydration of eggs. Our data do not support this hypothesis, however, as experimental addition of water did not increase hatching success in the field or the laboratory. The results of this study do suggest that predation and fungal infestation of eggs have been important selective pressures in the evolution and maintenance of nest attendance in *Ambystoma opacum*, a conclusion that may apply to other salamanders as well

because the terrestrial environment likely poses a substantially different community of predators and pathogens than do aquatic habitats. In oviparous species we should expect a variety of parental care behaviors to accompany the extreme shift in the site of egg development, *i.e.*, the transition from egg deposition in water to land in amphibians. Indeed, parental behavior is conspicuously associated with terrestrial reproduction in all extant amphibian groups (Crump, 1995, 1996).

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