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FACTORS AFFECTING AGGRESSION DURING NEST GUARDING IN THE EASTERN RED-BACKED SALAMANDER (*PLETHODON CINEREUS*)

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ABSTRACT: Defending young against intruders is a potentially risky behavior, and is energetically costly. Yet female eastern red-backed salamanders, *Plethodon cinereus*, guard their clutches for many weeks and aggressively defend eggs against predators and conspecifics. I examined the effect of clutch age, clutch size, and attendant size on the level of aggression attained during nest defense by staging conspecific invasions of nests of brooding female *P. cinereus* in the laboratory. I predicted that older and larger clutches would elicit increased aggression from the guarding females, and that larger females would be more aggressive when defending. The females were significantly more aggressive when guarding older clutches (6 wk postoviposition) than younger clutches (4 wk postoviposition). However, there was no difference in aggressive behavior when females guarded large (10-egg) or small (4-egg) clutches. There was also no relationship between body size and level of aggression; females were aggressive regardless of their size. These results suggest that females are able to evaluate the age (or developmental stage) of their eggs and adjust expenditure accordingly, but are not differentially responsive to clutch size.

Key words: Aggression; Nest guarding; *Plethodon*; Reproduction; Salamander

PARENTAL CARE THEORY predicts that an animal should invest in caring for their young when expected future benefits are substantial and outweigh costs (Clutton-Brock, 1991; Trivers, 1972). Costs of caring for offspring typically include increased risk of predation or injury, and decreased mating opportunity, fecundity, and foraging time (Fitzpatrick, 1973). Aggressively defending eggs or neonates may be a particularly costly reproductive strategy in terms of energy expenditure (Ng and Wilbur, 1995). Thus, nest-guarding behavior may be a useful gauge with which to examine investment decisions by parents. Ideally, parents should respond to proximate cues regarding costs versus benefits of defending offspring by adjusting the level of

parental care. Evaluation of clutch characteristics such as clutch size or age may enable a parent to predict the reproductive value of offspring, and may influence parental care choices. Interestingly, few studies have examined the effects of clutch properties or size of the attendant on aggression during clutch defense in species other than birds.

Although nest defense has been extensively studied in birds, less is known about aggression during nest guarding in amphibians. Approximately 20% of caudates are known to care for eggs or neonates (Crump, 1995), yet little is known about which factors may influence parental investment decisions in this group. The majority of salamander species that care for young belong to the family Plethodontidae. Studies on the eastern red-backed salamander, *Plethodon cinereus*, have demonstrated that clutch attendance by parents increases hatchling survivorship (Crespi

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and Lessig, 2004; Highton and Savage, 1961); therefore, this behavior has the potential to increase lifetime fitness of the nest attendant. I used *P. cinereus* as a model for studying parental decisions, and predicted that female salamanders should respond to proximate cues regarding costs versus benefits by adjusting the level of parental care.

The red-backed salamander is a lungless terrestrial salamander common to deciduous forests of North America. Females may reproduce only biennially, because the energy required to produce and attend a clutch is substantial (Sayler, 1966). Broods typically consist of 3–15 large eggs (Bachmann, 1984), which measure approximately 3–4 mm in diameter (Petranka, 1998; J. K. Tornick, personal observation). Females remain sequestered beneath embedded rocks (Forester and Anders, 2000) or inside decaying logs (this study) with their clutch of eggs for as long as 10 wk (Bachmann, 1984; Gibbons et al., 2003). During brooding, female *P. cinereus* curl their bodies around their eggs, occasionally vibrating them. Tactile stimulation maintains aeration and uniform distribution of egg contents, and prevents desiccation and fungal infection (Forester, 1979). After eggs hatch, females may remain with the neonates for several additional weeks (Bachmann, 1984). Females also actively defend their nests against predation and cannibalization (Highton and Savage, 1961).

Extensive studies of *P. cinereus* have demonstrated territorial aggression by both males and nonbrooding females (Jaeger et al., 1982; Jaeger and Peterson, 2002; Jaeger and Schwarz, 1991). Its agonistic repertoire has been categorized into easily recognizable threat behaviors such as Head Up, Body Up, Nudging, and Biting (Jaeger and Schwarz, 1991). These distinct postures facilitate scoring of aggressive behavior. Although a great deal is known about territorial aggression in this species, very little is known about the aggressive behavior specifically exhibited during nest guarding.

The objective of this study was to investigate parental investment during nest guarding by manipulating several variables affecting aggressive behavior. I examined clutch properties (clutch age and clutch size) and size of

the brooding female (body size). Older and larger clutches should have higher reproductive value than younger and smaller ones; therefore, I predicted that such clutches would elicit increased aggression. I also predicted that large females, which may have greater energy reserves and an increased likelihood of successfully driving off intruders, would be more aggressive than small females. To test these predictions, I staged invasions by unfamiliar conspecifics of brooding females in their nest cavities in the laboratory.

MATERIALS AND METHODS

Collection and Husbandry

Salamanders were collected from six sites within the city limits of Durham, Strafford County, New Hampshire, USA, in northern hardwood forests consisting mainly of eastern hemlock (*Tsuga canadensis*), white pine (*Pinus strobus*), red oak (*Quercus rubra*), and red maple (*Acer rubrum*), with an abundance of decaying logs used by red-backed salamanders for cover. In early spring of 2006 and 2007, I collected mature female (gravid and nongravid) salamanders as well as females with clutches of eggs, by carefully hand-searching in fallen logs and under rocks. Sex and reproductive status of each individual was determined by “candling” because their gonads and eggs are easily visible when backlit (Gillette and Peterson, 2001). Internare distance, the distance between nares at the tip of the snout, was used to verify sex (Quinn and Graves, 1999). In many salamander species, protein and fat are stored in tail tissue for energy reserves (Maiorana, 1977); therefore, only individuals with intact tails were collected.

All salamanders were housed in covered plastic containers measuring 19 cm × 13 cm × 9.5 cm. Approximately 2 cm of compressed soil covered the bottom of each container. At one end of the container, an inverted 9-cm plastic petri dish covered with a circular piece of brown paper towel served as a refugium. The towel helped to retain moisture and to keep the refugium dark. This technique also allowed observation by occasionally lifting the towel without disturbing the resident. The containers were kept at 20 °C, on a 14L:10D light cycle, and were misted occasionally to

TABLE 1.—Aggression ethogram for *Plethodon cinereus* during nest guarding. Scores are ranked from lowest (1) to highest (9) in increasing aggression. Each movement was scored as a distinct event. Most of the salamander's movements are abrupt and distinct, enabling each event to be scored discretely. Raw aggression score is the sum of all acts times the score for each act. Adjusted aggression score is the raw aggression score divided by the number of times the intruder approached the resident.

Score	Behavior	Description
1	Guard eggs	Moving to or curling around clutch
2	Head up	Lifting head above neutral
3	Body up	Lifting head and body off substrate
4	Move toward	Moving slowly toward (any distance)
5	Nudge	Pushing intruder with the nose
6	Chase	Running or darting after in pursuit
7	Snap	Bite attempt, no contact
8	Bite	Bite <1 s
9	Bite hold	Bite >1 s

maintain a high relative humidity (Bachmann, 1984). Salamanders were fed white worms (*Enchytrae*) and wingless fruit flies (*Drosophila melanogaster*) ad libitum. In 2007 brooding females lost mass over their duration in captivity (\bar{X} = 0.12 g, SD = 0.19 g, n = 17).

Gravid females were acclimated and given time to oviposit eggs in the refugium within their containers. Oviposition success was low; in 2006, only 4 of 23 gravid females oviposited. This is consistent with other studies that suggest that plethodontids are often reluctant to lay eggs in the laboratory (Bernardo and Arnold, 1999; Houck and Schwenk, 1984; Jockusch, 1996). To obtain enough clutches and attendants, additional females with their eggs were collected from the field. I photographed oviposited eggs under a dissecting microscope every 3–4 days to document the developmental stages. I then compared field-collected clutches to developmental photos of eggs from clutches produced in the laboratory to estimate the date of oviposition. Several females ate or pushed one or more of their eggs out of the nest cavity (n = 7). Abandoned eggs were removed; however, data were retained in the analysis if females continued to guard partial clutches (n = 5). Two clutches failed due to unknown causes (n = 2) and were abandoned, these were excluded from the analysis. In 2006 nongravid nonbrooding females, to be used as intruders and non-breeding resident (NB) controls (n = 65),

were allowed the same amount of time as the brooding females to establish territories in their refugia.

Behavioral Observations

Based on previously documented aggressive postures and behaviors (Davis, 2002; Forester, 1983; Jaeger and Schwarz, 1991; Mathis et al., 2000), as well as careful preliminary laboratory observations, an ethogram was developed for scoring the level of aggression displayed by brooding females during staged nest-site invasions (Table 1). Behavior increasing in intensity was scored ranging from nonaggressive (1 = guard eggs) to very aggressive (9 = bite hold). The rationale for this scoring system is based on the increasing effectiveness of each behavior in deterring an intruder; for example, in *Desmognathus ochrophaeus*, bites are more effective than nudges (Forester, 1983). The raw aggression score for each individual was quantified by the sum of all acts multiplied by the score for each act. To account for the behavior of the intruder, I calculated an adjusted aggression score by dividing the raw aggression score by the number of times the intruder approached the resident. Latency (time in seconds) to first contact with the intruder (any behavior scoring 5 or above) was also recorded.

Behavioral observations took place between the hours of 2000 and 2400, and were conducted under red light. Container covers and petri dishes covering the egg masses were removed. At the beginning of each trial, a nongravid female conspecific intruder was placed within one body length of a resident female. *P. cinereus* are more aggressive toward unfamiliar individuals than familiar individuals (Jaeger and Peterson, 2002); thus, to ensure that intruders were unfamiliar to the residents, individuals collected from distances greater than 1 km apart were paired. Intruders and residents were also matched by length and mass (as closely as possible) to minimize size asymmetry effects (Mathis et al., 2000). Behavior of residents and intruders was observed for 15 min, using focal sampling.

Clutch Size

In the spring of 2006, clutches of previously undisturbed brooding resident females (n =

17) were randomly manipulated into either small (4 eggs) or large clutches (10 eggs). All clutches contained at least one foreign egg. The maximum number of eggs added was four ($\bar{X} = 2.40$, $SD = 1.04$). Females were monitored for 10 d postmanipulation, to ensure that they were attending the altered clutches. If a female did not accept the foreign eggs, data were excluded from the analysis ($n = 3$). There was no bias between small and large manipulated clutches in these failures. Nonbrooding female residents (controls, $n = 24$; intruders, $n = 24$) were simultaneously allowed to acclimate to their individual refugia. Time spent in the refugia between collection and testing ranged from 40 d to 76 d.

The adjusted aggression scores were used in statistical analysis comparing females with small ($n = 7$) and large ($n = 7$) clutches with two-tailed two-sample *t*-tests, assuming unequal variance. To examine whether females base their decision to guard on initial rather than manipulated clutch size, the initial (unmanipulated) clutch size of each female ($n = 14$) was regressed against aggression scores.

Clutch Age

In late July of 2007, when clutches were approximately four weeks old, early intrusions of undisturbed brooding females ($n = 17$) by nonbrooding nongravid females ($n = 17$) were staged. Each female was tested again 2 wk later for the late encounters. The age of field-collected clutches was based on comparison to developmental photographs of eggs of known age from 2006. To further confirm egg age at test date, age was backdated from the date of the first appearance of hatchlings. Development is 51–55 d for this population at 20 °C in the lab (J. K. Tornick, personal observation), although Bachmann (1984) reported 60 d; an average of 53 d was used to calculate egg age. The early test occurred approximately 3–4 wk prehatch (4 wk after oviposition) and the late test occurred approximately 1–2 wk prehatch (6 wk after oviposition). A different female intruder was used in early and late encounters. In the previous year the nonbrooding controls had extremely low aggression scores; therefore, a control group was not included

for the clutch age experiment. Adjusted aggression scores for the early and late encounters for each female were compared using two-tailed paired *t*-tests ($\alpha = 0.05$).

Body Size

To assess the effect of body size of the resident, data from the clutch-size experiment were used. Snout–vent length (Petranka, 1998), snout–leg length (Szuba et al., 2002), mass (g), and total length of each female ($n = 14$) were measured at time of collection in spring and again at release in late summer (for brooding females this was after all eggs hatched). Spring and late-summer measurements were averaged for use in statistical analyses. The ratio of log10 mass to total length was used to calculate each female's relative body size. Relative body size and the residuals were regressed against raw aggression score.

Additional comparisons.—Adjusted aggression scores from the clutch-size experiment were compared between brooding residents and controls (nonbrooding residents), brooding residents and intruders, and intruders and controls using analysis of variance with Bonferroni correction. Latency to first contact with the intruder was also analyzed using *t*-tests assuming unequal variance and with paired *t*-tests.

RESULTS

Most brooding females showed high tenacity in guarding their eggs, and were highly aggressive when an intruder was introduced. To emphasize the frequency of aggressive behaviors observed in the staged encounters, raw aggression scores (Table 2) are presented in addition to the adjusted aggression scores. The initial behavior of most brooding residents was to guard (curl tightly around) eggs. Next, the brooding females typically responded sequentially with head up, nudge, chase, and snap. In many encounters biting and even bite/holding occurred. In several encounters the brooding resident bit the intruder more than 10 times, and in one encounter the brooding resident bit the intruder more than 21 times in the 15-min test. Most of these bites were directed at the tail of the intruder, although a few bites to the head and body were observed. In one instance, the resident bit and physically

TABLE 2.—Summary of raw and adjusted aggression scores and latency to first contact (includes score of 5 or above) from behavioral observations for experimental groups of *Plethodon cinereus* during nest guarding. An * indicates a significant difference between groups.

	Clutch age		Clutch size	
	Early (~4 wk) vs. late (~6 wk)		Small (4 eggs) vs. large (10 eggs)	
<i>n</i> =	17		7	
Raw mean score	117.8	270.4	83.9	79.6
Adjusted mean score	46	105.4	26.1	25.4
<i>t</i> =	2.4		0.07	
<i>P</i> =	0.01*		0.47	
Mean latency (s)	380	192	213	205
<i>t</i> =	2.12		0.09	
<i>P</i> =	0.02*		0.46	

overturned the intruder. In several cases (*n* = 4), after 15 min of observations the brooding resident was still engaged in defensive behavior. In almost all encounters the nonbrooding residents were stationary and were nonaggressive, and intruders usually attempted to escape or hide. In only one encounter did an intruder attack a resident.

There was a large variance in aggression scores for females guarding clutches. Females guarding broods were significantly more aggressive when guarding older clutches (6 wk old) than they were when guarding younger clutches (4 wk old; *t* = 2.4, *df* = 16, *P* = 0.01; Table 2). Latency to first aggressive act was significantly lower in females guarding the older clutches (Table 2).

Females were not significantly more aggressive when guarding small (4 eggs) or large clutches (10 eggs), using scores adjusted for

the number of approaches by the intruder (*t* = 0.09, *df* = 13, *P* = 0.46; Table 2). Due to a small sample size and high variance, statistical power for these tests were low (0.22); however, females exhibited high aggression when guarding either clutch size. The analysis of latency to the first aggressive act also showed no significant difference by clutch size (Table 2). The smallest initial clutch size was four. We detected no relationship between raw aggression scores and female's initial clutch size (*r*² < 0.001, *F* = 0.06, *P* = 0.94).

In addition, no relationship was detected between relative body size (or any other measurement of size) of females and raw aggression scores (*r*² = 0.03, *F* = 0.41, *P* = 0.53). Brooding residents were significantly more aggressive than controls (nonbrooding residents; *F* = 16.94, *df* = 2, *P* < 0.001; Table 3). Latency to first aggressive act was significantly lower in brooding females as compared to controls (*t* = 2.6, *df* = 37, *P* = 0.02). Brooding residents were also significantly more aggressive than intruders (*F* = 16.94, *df* = 2, *P* < 0.001; Table 3). Latency to first aggressive act was significantly lower in brooding females than intruders (*t* = 1.79, *df* = 27, *P* = 0.05). Controls and intruders did not differ significantly in either aggression score (*F* = 16.94, *df* = 2, *P* = 0.92) or latency to first contact (*t* = 1.76, *df* = 47, *P* = 0.43; Table 3).

DISCUSSION

Brooding female *P. cinereus* of all sizes aggressively defended both large and small clutches, and exhibited a significant increase

TABLE 3.—Comparison of raw and adjusted aggression scores and latency to first contact (score of 5 or above) for experimental versus control groups of *Plethodon cinereus*. B = Brooding residents, NB = controls (nonbrooding residents), I = Intruders. An * indicates a significant difference between groups.

	B vs. NB		B vs. I		I vs. NB	
<i>n</i> =	14	24	14	14	24	24
Raw mean score	84.7	3.5	84.7	10	3.6	2.6
Adjusted mean score	25.7	3.5	17.6	2.7	2.4	1.6
<i>F</i> =	16.94		16.94		16.94	
<i>P</i> =	0.001*		0.001*		0.916	
Mean latency (s)	193	438	193	390	438	459
SE =	38.2	87.3	38.2	102.7	87.3	79.3
<i>t</i> =	2.6		1.79		1.76	
<i>P</i> =	0.02*		0.05*		0.43	

in aggression when defending late-stage eggs against intruding conspecifics. These findings are consistent with parental investment theory, which predicts that an older brood is more likely to survive to hatching than a newly deposited brood and, therefore, has greater reproductive value (Montgomerie and Weatherhead, 1988; Winkler and Wilkinson, 1988). The effect of clutch age on aggression is poorly understood in amphibians, and results from the few existing studies vary. In Bachmann's (1984) study, brooding *P. cinereus* did not increase aggression toward conspecifics late in the season, although neither size-matching nor familiarity of intruders was considered. On the other hand, my results are consistent with the findings of Forester's (1983) study in which *D. ocoee* was significantly more aggressive later in the brooding season. If parents increase aggression with age of the clutch, as Forester's (1983) study suggests, there may be a mechanism for recognizing egg development stage. When given a choice between thermally manipulated early and late stage eggs, another plethodontid salamander, *D. fuscus*, preferred chronologically advanced eggs (Forester et al., 2005). The mechanism underlying this ability is yet unknown. These data suggest that *P. cinereus* females either have the ability to recognize the developmental stage of eggs, or at least have a mechanism for calculating the amount of time passed since they oviposited their eggs. An alternate explanation is that brooding females simply increased aggression due to increased time spent in their refugia (Nunes and Jaeger, 1989). However this explanation is less likely, because nonbrooding females (controls) in the clutch-size experiment had very low aggression scores even though they had been in their refugia for as long as 76 d. It is possible that brooding females increase aggression with time spent in a refugium and nonbrooding females do not; but this explanation is less parsimonious. To answer this question, the clutch age experiment could be repeated with nonbrooding controls.

This is the first study to directly compare clutch size with level of aggression in a nest-guarding amphibian. Although aggression was high in many females, aggression scores during the defense of small (4-egg) versus large (10-

egg) clutches were not significantly different. Parental investment theory predicts that a large brood has greater reproductive value and should elicit greater parental efforts than a smaller brood (Montgomerie and Weatherhead, 1988; Williams, 1966; Winkler, 1987; Winkler and Wilkinson, 1988); therefore, parents should increase aggression when guarding a large number of eggs. One plausible explanation for the lack of difference in aggression when defending large (10-egg) or small (4-egg) clutches is that a 4-egg clutch is still a substantial energetic investment. Each plethodontid egg is sizable in comparison to the mother's body size. Even a small number of eggs represents a considerable energetic investment; therefore, this behavior may still be consistent with parental investment theory. These data suggest that for *P. cinereus*, the benefits of defending a clutch consisting of as few as four eggs outweigh the costs/risks associated with this behavior. Initial (unmanipulated) clutch size also did not appear to affect the level of aggression exhibited by a female, supporting my contention that attendants do not base decisions to defend on clutch size.

Furthermore, *P. cinereus* may not be able to distinguish the difference in quantity between a 4-egg clutch and a 10-egg clutch. Forced food-choice discrimination tests suggest that these salamanders can distinguish the difference between one and two prey items, and between two and three prey items; they preferentially selected the larger of the two numerosities. *P. cinereus*, however, failed to discriminate between three versus four prey items (Uller et al., 2003). Yurewicz and Wilbur (2004) examined brood tenacity (the tendency to continue care rather than abandon a clutch) and found that female *P. cinereus* are more likely to abandon small clutches than larger ones. This seems to indicate that *P. cinereus* can distinguish differences in clutch size (or quantity of egg material) when deciding whether to attend or abandon clutches. If clutch size had been manipulated to fewer than four eggs, females may have been more likely to abandon rather than defend them. Also, it should be noted that if the intruder is a large predator (for example a snake), selection may favor abandonment rather than defense.

The size of the attendant was also not predictive of the level of aggression attained during nest guarding. Relative size (a function of both mass and length) may be an indication of the physical condition or resource-holding potential of an individual. Increased body condition (larger reserve of energy) has a positive effect on hatchling survivorship (Horne et al., 1990). Contrary to my prediction, no correlation was found between any measurement of size (mass, total length, snout–leg length or mass/length ratio) and aggressive behavior. Power to detect an effect of body size on aggression might have been enhanced by increased sample size. It seems that the relationship between body size and level of nest defense varies greatly by species (Williams, 1975). However, my results are consistent with the Jaeger et al. (1982) study in which large territorial *P. cinereus* were not more likely to initiate attacks than small ones.

Most females in this study were aggressive, suggesting that both large and small individuals are willing to incur the costs of protecting their genetic investment. For *P. cinereus*, age or experience may play a larger role than a parent's size in explaining variations in nest defense. An older or more experienced mother may simply curl tightly around the clutch to protect it, yet an inexperienced mother might actively pursue and attack an intruder. Perhaps past maternal experience may explain some of the variation. For instance, if a mother previously lost some or all of her clutch to another salamander, she may defend her present nest more aggressively. In addition, defensive behavior may vary depending on how close a female is to the end of her lifespan. To address these questions, future experiments could examine the effects of age and experience on aggression during nest defense. To my knowledge these factors have not yet been examined in amphibians.

The elevated aggression scores in brooding residents as compared to nonbrooding residents suggest that females guard offspring more vigorously than resources such as food or refugia. Researchers have not previously compared aggression between brooding (nest guarding) and nonbrooding (territorial) female *P. cinereus*, although many studies have clearly demonstrated territorial aggression in

males and nonbrooding females of this species (Jaeger et al., 1982; Jaeger and Peterson, 2002; Jaeger and Schwarz, 1991). Data from my study may be interpreted two ways, either: (1) females are more aggressive when guarding eggs than they are when simply guarding a territory; or (2) nonbrooding females kept in captivity do not recognize their housing as their own territory. The second explanation is less likely, because territories of males and nonbrooding females are thought to be established in the laboratory after only 5 d (Nunes and Jaeger, 1989). Results from these and other studies seem to suggest that females guarding eggs are more aggressive than those simply guarding a territory.

Few studies have previously investigated factors that might contribute to the level of aggression demonstrated during nest guarding in an amphibian. Many female *P. cinereus* were quite aggressive under multiple circumstances, suggesting that each clutch of eggs (even those with as few as four eggs) represents a large investment in time and energy and is, therefore, worth vigorously defending. Importantly, these data indicate that females are able to ascertain the value of offspring based on age and adjust parental care accordingly.

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