

Sequential alternation of offspring sex from successive eggs by female green anoles, *Anolis carolinensis*

Matthew B. Lovern and Kelly M. Passek

Abstract: We examined the relationships between maternal size, breeding season stage, and offspring sex at hatching for the oviparous lizard *Anolis carolinensis*. Specifically, we tested two hypotheses: (1) large females produce offspring with male-biased sex ratios and small females produce offspring with female-biased sex ratios; and (2) females, regardless of size, produce offspring with male-biased sex ratios early in the breeding season and female-biased sex ratios late in the breeding season. We found no support for either hypothesis. Rather, we found that individual females of all sizes and throughout the breeding season alternated offspring sex of sequentially laid eggs. Because female anoles also alternate the ovary that produces successive eggs, we tested a third hypothesis: females produce eggs of only one sex with each ovary. Both palpation to track which ovary produced which eggs, and unilateral ovariectomies, leaving females with only one functioning ovary, failed to support this hypothesis. Regardless of whether an ovary was removed, females were capable of producing both male and female offspring from either ovary. To account for this and potentially other unusual patterns of offspring production, we provide evidence that maternal hormone levels around the time of fertilization may affect offspring sex.

Résumé : Nous avons examiné la relation entre la taille de la mère, le moment dans la saison de reproduction et le sexe de la progéniture chez le lézard ovipare *Anolis carolinensis*. Nous avons éprouvé deux hypothèses : (1) les femelles de grande taille produisent une progéniture où les mâles prédominent et les femelles de petite taille, une progéniture où les femelles prédominent; (2) quelle que soit leur taille, les femelles produisent une progéniture à prédominance de mâles au début de la saison de la reproduction et à prédominance de femelles en fin de saison. Nous n'avons rien trouvé qui puisse corroborer ces hypothèses. Nous avons plutôt constaté que les femelles de toutes les tailles, pendant toute la saison de la reproduction, alternent les progénitures de mâles et de femelles au cours de pontes consécutives. Les femelles utilisent aussi leurs ovaires en alternance, ce qui nous a amenés à poser une troisième hypothèse qui veut que les femelles produisent seulement des rejetons de même sexe dans chacun de leurs ovaires. La palpation pour déterminer quel ovaire a produit les oeufs, combinée à des ovariectomies unilatérales laissant aux femelles un seul ovaire fonctionnel, ne supportent pas l'hypothèse. Qu'elles aient ou non subi l'ablation d'un ovaire, les femelles sont capables de produire des rejetons des deux sexes avec chaque ovaire. Pour expliquer cette modalité inusitée de reproduction, et peut-être d'autres, nous donnons ici des indices qui laissent croire à l'intervention de concentrations d'hormones maternelles à l'époque de la fécondation dans la détermination du sexe de la progéniture.

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Introduction

The extent to which sex ratios at birth or hatching deviate from parity, and under what conditions such deviations might be adaptive, has received widespread attention. Offspring sex ratios of invertebrates can deviate substantially from parity as a means of increasing parental fitness (Charnov 1982). Offspring sex ratios in some species of vertebrates, such as fishes and reptiles, are related to environmental conditions like temperature (e.g., Conover 1984; Korpelainen 1990;

Pough et al. 2001). Research on birds and mammals has generally yielded inconsistent results. Replicable data that demonstrate deviations from a 1:1 offspring sex ratio are uncommon, as are data that can be used to propose mechanisms for such deviations (reviewed in Clutton-Brock 1986; Clutton-Brock and Iason 1986; Gowaty 1991; Krackow 1995; Dittus 1998; Hewison et al. 1999; Brown 2001). Nevertheless, cases of convincing deviations from parity do exist in both birds (e.g., Heinsohn et al. 1997; Komdeur et al. 1997) and mammals, including humans (reviewed in James 1996).

The most widely tested and debated adaptive explanation for variation in offspring sex ratios involves parental investment and its effect on the future reproductive success of offspring. Trivers and Willard (1973) proposed that for species in which variance in reproductive success is greater for males than for females (e.g., species with polygynous social organization), mothers in good condition (i.e., who can invest more) should differentially invest in male offspring, and mothers in poor condition should differentially invest in female offspring. The Trivers–Willard model (TWM), as applied to

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M.B. Lovern^{1,2} and K.M. Passek. Department of Biology,
Virginia Tech, Blacksburg, VA 24061, U.S.A.

¹Corresponding author (e-mail: lovern@msu.edu).

²Present address: Department of Psychology, Psychology
Research Building, Michigan State University, East Lansing,
MI 48824, U.S.A.

polygynous species, relies on three assumptions: (1) the condition of the mother is correlated with that of her offspring; (2) offspring condition carries over into adulthood and is correlated with fitness; and (3) maternal condition has a greater effect on the eventual reproductive success of male offspring than on that of female offspring (Trivers and Willard 1973).

In the present study, we examined offspring sex ratios at hatching in green anoles (*Anolis carolinensis*). Unlike many of the reptiles in which variation in offspring sex ratios has been studied, this lizard does not have temperature-dependent sex determination (TSD), but its genetic sex-determining mechanism (i.e., male or female heterogamety) is not known (Gorman 1973; Viets et al. 1994). Whatever that underlying mechanism may be, the life history of *A. carolinensis* suggests that the TWM could apply. *Anolis carolinensis* has a polygynous mating system in which territorial males compete for exclusive access to multiple females (Ruby 1984; Jenssen et al. 1995). Females set the stage for male–male competition with spatially clumped distributions and single-egg clutches laid approximately weekly over the breeding season (late April – early August) (Andrews 1985; Nunez et al. 1997; Jenssen and Nunez 1998). After oviposition, there is no further parental care in this species. Thus, males put reproductive effort primarily into securing mating opportunities, and females put it primarily into egg production. For males, the major determinant of successful acquisition and maintenance of a breeding territory is body size: larger adult males hold larger territories, encompassing more females, than do smaller adult males, which may not acquire a territory (Ruby 1984; Jenssen et al. 1995; Jenssen and Nunez 1998). From available data, the assumptions of the TWM listed above appear to be met by *A. carolinensis*. First, larger females (and, we assume, females in better condition) produce larger offspring (Michaud and Echternacht 1995). Second, juvenile males grow faster than juvenile females, and larger juveniles grow disproportionately faster than smaller juveniles (Michaud 1990). Third, although it is probable that all females which are physiologically capable of reproduction do successfully reproduce, there is considerable variance in the reproductive success of adult males that is positively related to body size (Ruby 1984; Jenssen et al. 1995; Jenssen and Nunez 1998). Thus, body size may be important to females, but appears to be more important to males; small females still reproduce, but small males, who are physiologically capable of reproduction, may be excluded from doing so if they are not large enough to acquire a territory (Tokarz 1998).

Specifically, we tested two hypotheses: (1) large female *A. carolinensis* produce offspring with male-biased sex ratios and small females produce offspring with female-biased sex ratios; and (2) females, regardless of size, produce offspring with male-biased sex ratios early in the breeding season and offspring with female-biased sex ratios late in the breeding season. Our first hypothesis explicitly tested the TWM for *A. carolinensis* and our second hypothesis tested a logical extension of the TWM that incorporates a seasonal component (e.g., Daan et al. 1996). Such a pattern of offspring sex ratio manipulation could be adaptive because juvenile males would benefit more than juvenile females because of the extended growth period (growth slows or stops during

the winter months), owing to the greater importance of body size to males than to females and to the fact that neither sex can reproduce in the summer in which they hatch (Michaud 1990).

Methods

We collected data from a population of *A. carolinensis* located along the Augusta Canal in Columbia County, Georgia, U.S.A. (33°N, 82°W), during the 1998 and 1999 breeding seasons. Between April and August we captured 70 adult females by hand or noose from their territories within the riparian vegetation along the canal ($n = 42$ in 1998, $n = 28$ in 1999). Snout–vent length (SVL) was determined to the nearest millimetre for each lizard. Lizards were housed singly in the laboratory in cages measuring 30 × 60 × 70 cm under environmental conditions approximating those in the field. Lights (Durotest Vitalite plus) were on a 14 h light : 10 h dark cycle and temperatures ranged from 23°C during the night to 27–34°C during the day, depending on location within the cage. We misted cages with water daily and fed lizards vitamin-dusted crickets or mealworms at least every other day. In addition to perches and artificial vegetation, each cage contained a dish of moist soil into which females could oviposit. These eggs were fertilized from copulations in the field; females have the ability to store sperm for later fertilizations (Conner and Crews 1980). We checked all dishes daily for eggs, and the date of oviposition was recorded for each egg as a measure of breeding-season stage.

We incubated eggs in plastic cups in a vermiculite:water mixture (1:1 mass). Cups were covered with plastic wrap, secured with a rubber band, and incubated at 28°C until hatching (approximately 38 d). Upon hatching, sex was determined by noting the presence (males) or absence (females) of enlarged post-anal scales (Greenberg and Hake 1990). Females were kept 3–6 weeks under the laboratory conditions described above, and produced 1–6 eggs that hatched successfully (89% hatching success; 154/173 eggs).

Data from 1998 unexpectedly indicated that females alternated the sex of their offspring with successive single-egg clutches, far above what would be expected by chance. Because *A. carolinensis* typically alternates the ovary from which successive eggs originate (Smith et al. 1973), the coincident pattern of alternating offspring sex suggested to us that each ovary might produce only one sex. Such a pattern could result from nonrandom ovulation of female gametes (if females are the heterogametic sex), nonrandom fertilization of ova by male gametes (if males are the heterogametic sex), or sex-specific embryonic mortality. Therefore, as a simple and direct first step, in 1999 we tested a third hypothesis: in *A. carolinensis* each ovary produces only one sex. We tested this hypothesis in two ways. It was indirectly tested by externally palpating females to determine from which ovary each egg originated and then noting the sex of that egg at hatching. The hypothesis was also directly tested by removing an ovary from females, then determining the sex of the subsequently laid eggs originating from the remaining ovary. We expected that these unilaterally ovariectomized females would only produce either male or female offspring. Nine lizards were palpated prior to each oviposition to determine the ovary from which each egg originated (either left or

right, based on whether the egg was in the left or right oviduct). We performed unilateral ovariectomies (henceforth ovariectomies) on 17 lizards after they laid 2 or 3 eggs in the laboratory. To determine which ovary to remove, we palpated each lizard to identify the side containing the most mature follicle or egg. Then, we removed the contralateral ovary. Females were anesthetized and placed on ice during surgery to reduce bleeding. Then we removed the ovary and its associated follicles through a 0.5 cm long ventrolateral incision, after which the incision was sealed. Following surgery, lizards were housed in plastic containers (19 × 32 × 11 cm) for 24 h to facilitate monitoring their recovery, after which they were returned to their home cages. Eggs laid by ovariectomized females were incubated as described above and sex was determined at hatching. Ovariectomized lizards were palpated prior to each oviposition to confirm that only one ovary was producing eggs (i.e., to ensure that we did not miss any ovarian tissue or developing follicles during surgery). Hatching success of eggs laid by ovariectomized lizards was 70% (16/23 eggs hatched).

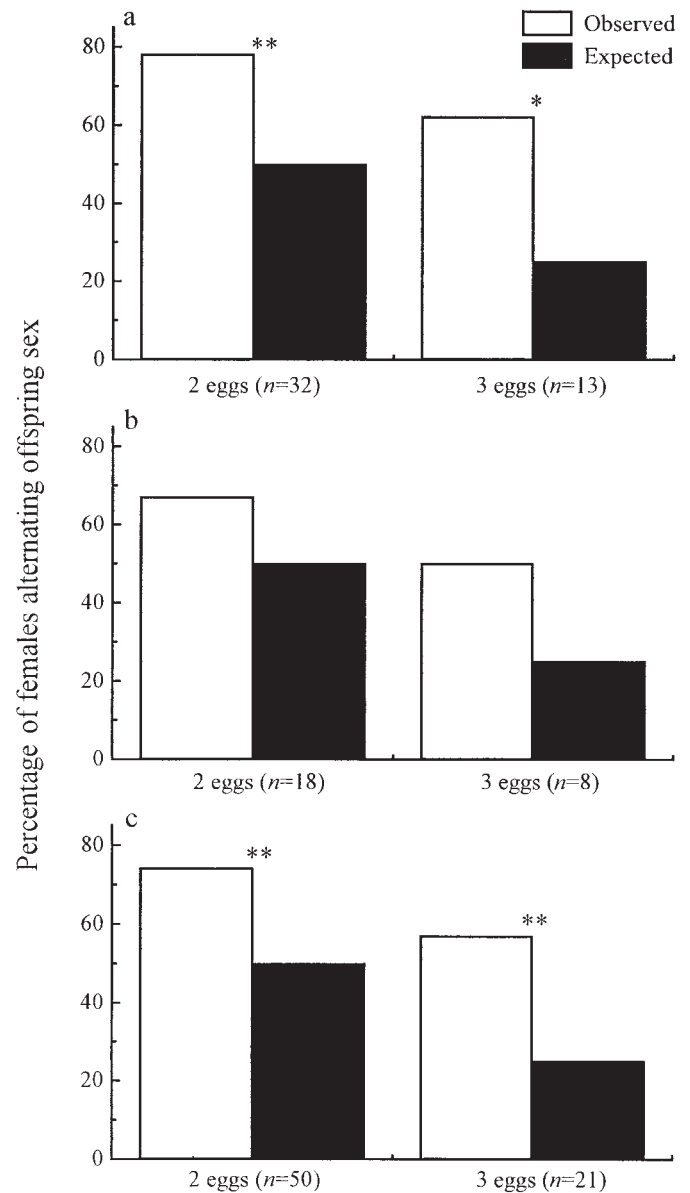
Logistic regression was used to determine if year, female size, or season affected offspring sex. We also performed χ^2 analyses to determine if patterns of offspring sex and egg sequence differed from chance. Finally, Wilcoxon's signed-ranks test was used to determine if females had different offspring sex ratios before and after ovariectomy. All tests were two-tailed, and the level of significance required to reject the null hypothesis was $P \leq 0.05$.

Results

Offspring sex was not affected by the year of data collection ($t = 1.7, P = 0.09$), female size ($t = 1.5, P = 0.14$), or breeding-season date ($t = 0.4, P = 0.66$). In 1998, 44 eggs produced males and 47 produced females (12 did not hatch and sex could not be determined). In 1999, 39 eggs produced males and 24 produced females (7 did not hatch and sex could not be determined). Overall, 83 eggs produced males and 71 produced females (54% male).

Interestingly, we found that females for which we had at least 2 consecutive hatchlings alternated male and female offspring significantly more often than expected by chance in 1998 ($n = 32, \chi^2_{[2]} = 10.2, P = 0.006$). This trend also held for 3 consecutive hatchlings ($n = 13, \chi^2_{[3]} = 9.46, P = 0.02$). Females with 2 consecutive hatchlings alternated the sex of their offspring 78% of the time versus 50% expected, and those with 3 consecutive hatchlings alternated 62% of the time versus 25% expected (Fig. 1a). The same pattern occurred in 1999 (Fig. 1b), as 67% alternated the sex for 2 hatchlings and 50% alternated for 3 hatchlings (2 hatchlings: $n = 18, \chi^2_{[2]} = 2.44, P = 0.29$; 3 hatchlings: $n = 8, \chi^2_{[3]} = 5.0, P = 0.17$). For the 1999 data, comparatively small sample sizes lessened our ability to detect significant deviations from chance, if in fact they existed. However, the combined 1998 and 1999 datasets strongly indicated that females alternated the sex of their offspring significantly more than was expected by chance. This was true for both 2 consecutive hatchlings ($n = 50, \chi^2_{[2]} = 11.56, P = 0.003$) and 3 consecutive hatchlings ($n = 21, \chi^2_{[3]} = 11.95, P = 0.008$). Overall, 74% of the females alternated the sex of 2 consecutive hatchlings and 57% alternated the sex of 3 consecutive hatchlings (Fig. 1c).

Fig. 1. Percentage of female *Anolis carolinensis* with 2 and 3 sequential eggs that alternated offspring sex versus the proportion expected by chance in 1998 (a), 1999 (b), and combined (c) (*, $P \leq 0.05$; **, $P \leq 0.01$ (χ^2 test)).



Of the nine females that we palpated to determine the relationship between the ovary from which eggs originated and offspring sex, five produced offspring of only one sex from each ovary and four produced offspring of both sexes from each ovary (after 2 or 3 eggs). Ovariectomized females produced both male and female offspring, and the sex ratios did not differ from chance (1 egg: $n = 10, \chi^2_{[1]} = 0.0, P = 1.0$; 2 eggs: $n = 6, \chi^2_{[2]} = 0.6, P = 0.74$). Finally, when we examined only those females for which >1 egg hatched before and after ovariectomy, we found that ovariectomy had no effect on sex ratio (Wilcoxon's signed-ranks test, $n = 4, W = 1, P = 1.0$). Although the sample size for this test was small, the result was unambiguous: females with only one ovary could still produce both male and female offspring.

Discussion

Our data do not support the TWM for *A. carolinensis* (hypothesis 1). Larger females were not more likely to produce offspring with male-biased sex ratios than were smaller females. Furthermore, a possible seasonal component of the TWM was not supported (hypothesis 2). Females were not more likely to produce offspring with male-biased sex ratios early in the breeding season and female-biased ratios late in the breeding season. In contrast, Viets (1993) found support for the TWM, in that larger female *A. carolinensis* produced a higher proportion of male offspring than did smaller females. The effect appears to be comparatively weak, however, and may be difficult to detect consistently, as significant relationships between female size and offspring sex depended on the type of analysis used and on which eggs were included in that analysis (e.g., first and (or) second and third eggs collected; Viets 1993, p. 174). As with studies on other species (e.g., Hewison et al. 1999), if the TWM applies to *A. carolinensis* it is likely subtle at best.

In contrast to offspring sex ratios biased by features such as maternal size or breeding season stage, our data suggest that regardless of the conditions, females tend to alternate the sex of their offspring. To our knowledge, no other study has reported such a pattern. However, shifts in offspring sex ratio with egg sequence or season (e.g., starting male-biased and finishing female-biased, or vice-versa) occur in at least one turtle species with TSD (Bowden et al. 2000) and in some bird species as well (e.g., Daan et al. 1996; Kilner 1998). In a particularly extreme example, the parrot *Eclectus roratus* tends to produce offspring of the same sex across long runs of sequential eggs (often 10 or more eggs of the same sex in a row) before switching to the other sex (Heinsohn et al. 1997).

How might female *A. carolinensis* alternate the sex of their offspring? One possible mechanism directly involves the ovaries. This idea seemed plausible because female anoles produce single-egg clutches sequentially throughout the breeding season, and because the ovaries tend to alternate egg production (Smith et al. 1973). However, females that we palpated to determine the ovary from which eggs originated and females with one of their two ovaries removed were capable of producing male and female offspring from the same ovary. Thus, we can rule out the possibility that individual ovaries in female *A. carolinensis* exclusively produce only males or only females, but we cannot rule out the possibility that under normal circumstances one ovary is more likely than the other to produce offspring of a particular sex. Perhaps the situation is similar to that seen in gerbils, in which male embryos tend to be found in the right uterine horn and female embryos in the left (Clark and Galef 1990; Clark et al. 1991). This asymmetry is regulated by the ovaries themselves rather than by the uterus, although the proximate mechanism of regulation is not known (Clark et al. 1994).

The ovaries do not appear to control alternation of offspring sex in a strict sense (i.e., producing only males or only females) in *A. carolinensis*. However, they may indirectly influence offspring sex via hormonal secretions. Indeed, there is accumulating evidence for such an influence in numerous taxa (reviewed in James 1986, 1996; Krackow 1995; Crews 1996). For example, hormones and temperature interact to

determine the sex of reptiles with TSD (Wibbels et al. 1994; Crews 1996). The hormonal environment experienced by females during their own prenatal development affects subsequent offspring sex ratios in some rodent species (Clark et al. 1993; Vandenberg and Huggett 1994). The timing of insemination with respect to ovulation can influence offspring sex ratios in numerous mammals, and this is likely related to differences between X- and Y-sperm motility and viability, as well as to physiological changes in the female reproductive tract brought about by hormonal changes around ovulation (e.g., Harlap 1979; Hedricks and McClintock 1990; Huck et al. 1990). From these studies, gonadotrophins, testosterone, and estrogen appear to be the most likely candidates for influencing sex ratios.

Integrating our data with the results reviewed above, we suggest that features of maternal physiology, such as hormone profiles, may somehow play a role in the alternation of offspring sex in *A. carolinensis*. This claim is supported by the fact that females which were part of a study involving blood sampling prior to egg collection (M.B. Lovern, unpublished data) had higher incidences of male-biased offspring sex ratios than did the females in the present study (2 eggs: $n = 13$, $\chi^2_{[2]} = 5.91$, $P = 0.05$; 3 eggs: $n = 8$, $\chi^2_{[3]} = 10.32$, $P = 0.02$). We suggest that these manipulated females likely had disrupted hormone profiles through the actual removal (in the blood) of circulating hormones, and possibly through associated stress effects of blood sampling (e.g., Moore et al. 1991).

If the pattern of alternating offspring sex that we found holds true, at least three related issues need to be addressed. First, how is sex determined in *A. carolinensis*? The heterogametic sex is unknown (Viets et al. 1994), and determining this key fact would restrict the range of relevant hypotheses to be tested in elucidating a mechanism for alternating offspring sex. Second, can hormone levels influence the sex ratio? The potential means of hormonal influence are numerous, including effects on differences in gamete production by the heterogametic sex or differences in the likelihood of particular zygotes forming, possibly mediated by hormonal effects on sperm motility or vaginal pH and mucosity (Krackow 1995). A general hormonal influence on offspring sex ratio could be crudely tested by manipulating maternal (or paternal) hormone profiles and examining subsequent offspring sex ratios. Third, is the alternation of offspring sex adaptive? It is difficult to imagine how alternating offspring sex is an adaptive response for all females during all stages of the breeding season. However, if the pattern is an adaptive response, it is possible that it could be altered under different social conditions. For example, females housed in groups or with males may show a different pattern of offspring production from that of ours in the present study, which were housed singly. Alternatively, perhaps the pattern is actually a constraint resulting from phylogeny or selection on some other feature of reproductive function regulated by hormones (e.g., Krackow 1995). Further research will be necessary to distinguish among these alternatives.

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