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# Sex-based hatching asynchrony in an oviparous lizard (*Bassiana duperreyi*, Scincidae)

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**Abstract** Sex disparities in mean dates of hatching are widespread, but are generated by a variety of mechanisms. For example, a correlation between offspring sex and hatching date is produced by temperature-dependent sex determination (TSD) in turtles and lizards, and by laying asynchrony and differential incubation behaviours in birds. Our laboratory experiments on montane lizards (*Bassiana duperreyi*, Scincidae) provide the first direct evidence of an intrinsic sex effect on the date of hatching in a reptile. All eggs within a clutch are laid on the same day but, even when incubated under identical thermal and hydric conditions, male lizards hatch about 2 days earlier than do their sisters. The effects are not due to laying asynchrony, differential incubation by parents, TSD or sex differences in mean egg size. Hormonally induced sex reversal did not eliminate the sex disparity in incubation periods, suggesting that this sex disparity is due to events occurring prior to oviposition rather than to sexual differences following gonadal differentiation. In the field, the intrinsically briefer incubation of male embryos is counterbalanced by two other trends: smaller eggs more often produce males and tend to hatch later than larger eggs within the same clutch; and cooler (and thus, later-hatching) nests overproduce male offspring via thermally induced sex reversal. Thus, somewhat ironically, the sex-specific hatching asynchrony of laboratory-incubated eggs may reflect adaptation to minimize sex differences in dates of hatching.

**Key words:** embryogenesis, incubation, life history, reproduction, sex reversal.

## INTRODUCTION

In many species, the date that an individual hatches or is born has long-term consequences for its microevolutionary fitness (Clutton-Brock *et al.* 1982; Olsson & Shine 1997). Earlier hatching can provide opportunities to acquire territories or other resources without intense competition from other individuals from the same cohort, or can facilitate success in such competition (Clark & Wilson 1981; Amundsen & Stokland 1988; Magrath 1990; Stoleson & Beissinger 1995). In environments with seasonally variable resources or with intense sibling competition for resources, even a small difference in the date of hatching can translate into significant fitness differentials. For example, hatchling lizards born earlier in the season have more time to feed and grow prior to winter, and thus overwinter at larger body sizes than their later-hatching conspecifics (Olsson & Shine 1997; Qualls & Shine 2000; Warner & Shine 2005). Similarly, earlier hatching provides a strong survival advantage in many bird species with hatching asynchrony (Drent 1975; Clark & Wilson 1981; Magrath 1992). Accordingly, early hatching may be a strong predictor of individual fitness (Magrath 1990, 1992; Stoleson & Beissinger 1995; Olsson & Shine 1997).

Life-history models suggest that the advantages of earlier birth or hatching may often differ between the sexes, because of differences in the ways in which fitness is determined in males *versus* females. Accordingly, we might expect the evolution of mechanisms that result in sex-based hatching asynchrony, whereby the sex that would benefit most from early emergence is indeed the first to hatch. Empirical data support this prediction, with frequent reports of sex-based hatching asynchrony in oviparous vertebrates (see above). However, the link between sex and hatching date might be generated by two quite distinct types of proximate mechanism: those determined by the parent and those intrinsic to the egg. Most or all cases in birds appear to fall into the former category, with sex-based asynchrony in hatching dates generated mostly by asynchrony in oviposition dates (i.e. eggs that are laid earlier, hatch earlier) or by maternal behaviour (i.e. eggs that are incubated more assiduously hatch earlier) (Drent 1975; Clark & Wilson 1981; Magrath 1992). A recent report of a sex difference in incubation periods in Eurasian kestrels (*Falco tinnunculus*) offers a possible exception to this conclusion, but (because the data were gathered on parentally incubated eggs), the possibility of sex-selective incubation by parents cannot be discounted (Blanco *et al.* 2003). In contrast, studies on reptiles and fishes suggest that the link between sex and hatching date results from temperature-dependent sex determination (TSD),

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with incubation temperature simultaneously determining both hatching date (via its effects on developmental rate) and offspring sex (Bull 1983; Bull & Charnov 1989; Shine 1999). For example, the eggs of turtles and lizards that are laid in relatively cool nests develop slowly and hatch relatively late in the season, whereas the eggs in hotter nests develop rapidly and hatch earlier (Harlow 1994). If incubation temperature also determines sex, the end result is earlier emergence of one sex than the other. A similar phenomenon occurs in some fishes, where sex-based hatching asynchrony results from seasonal shifts in water temperature that in turn generate shifts in offspring sex ratios (Conover *et al.* 1992).

Although existing data thus suggest that sex-based hatching asynchrony is achieved by different proximate mechanisms in endothermic *versus* ectothermic vertebrates, few species have been studied in detail and hence, any such generalizations may be premature. The present paper reports data on montane lizards that reveal sons hatch earlier than daughters, and do so via a mechanism different from that reported in previously studied reptiles. Even when all eggs within a clutch are laid on the same day and incubated at the same thermal regime, our highland skinks displayed a sex difference in hatching dates within a clutch (i.e. sons hatched before daughters).

## METHODS

### Study species and area

*Bassiana duperreyi* are medium-sized (to 79 mm snout-vent length (SVL)) oviparous scincid lizards that are widely distributed through south-eastern Australia. Extensive previous research on this species has focused on populations in the Brindabella Range 40 km west of Canberra in the Australian Capital Territory (1240 m a.s.l.; 148°501′E, 35°21′S). These cool high-elevation sites are close to the upper elevational limits for oviparous reproduction by Australian lizards (Shine & Harlow 1996) and thus, nests are concentrated in the few sites that provide sufficient sun penetration for relatively warm nesting conditions (Shine *et al.* 2002a). Female *B. duperreyi* aggregate in these sites prior to oviposition, facilitating collection of gravid animals (Shine *et al.* 2002a). We captured lizards ($n = 71$) from these sites on 24 and 29 November 2004, and took them to the University of Sydney. All gravid females were weighed, measured and individually marked. They were housed in separate cages (each 22 × 13 × 7 cm) and allowed to oviposit. Each cage contained moist vermiculite (for oviposition), a shelter site and a water dish. The lizards were fed on live crickets three times a week. The room was kept at 20°C with a 12-h light : 12-h dark photoperiod. A thermal gradient from 20 to 35°C was provided within each cage by means of an underfloor heating element that was switched on for 8 h per day; cage temperature fell to ambient room temperature (20°C) overnight (see Shine & Harlow 1996 for details). The lizards thus had ample opportunity for behavioural thermoregulation during daylight hours. All cages were visually inspected twice each day for eggs.

### Egg incubation

Oviposition occurred over a 2-week period overall, but any given clutch was always laid within a single day (and generally, within a few hours). Most females oviposited within 10 days of capture. Average clutch size was 6.95 ± 0.75 (range 2–11) eggs. On the day of oviposition, eggs were removed from the cages, weighed and then placed individually in 64 mL jars containing moist (–200 kPa) vermiculite. The jars were kept in a cycling-temperature incubator with a sinusoidal diurnal thermal cycle (20 ± 7.5°C). This thermal regime mimics conditions that eggs experience in the field in natural nests (Shine & Elphick 2001), and produces offspring sex ratios close to 50:50 (Shine 2002; Shine *et al.* 2002a). Heteromorphic sex chromosomes (male XY, female XX) determine sex in *B. duperreyi* under these conditions, although these genetic factors can be over-ridden by incubation at lower temperatures (Shine *et al.* 2002b).

### Hormone application

Any sex difference in the duration of incubation might be either a direct consequence of the genetic composition of the offspring, or induced during the cascade of embryonic differentiation that accompanies sex determination (Sarre *et al.* 2004). In *B. duperreyi*, we may be able to decouple genotypic sex from phenotypic sex if a genetically male (XY) individual can be sex-reversed by applying estradiol to the eggshell early in development, as has been reported in several other reptile species (Crews *et al.* 1991; Crews 1994; Freedberg *et al.* 2006). Thus, using a split-clutch design, we topically applied 5 μg of 17 β-estradiol in 5 μL of ethanol to eggs ($n = 53$) immediately after oviposition. Control eggs ($n = 51$) from the same clutches received only 5 μL of ethanol, or were not manipulated at all (results from the two latter groups were indistinguishable, and thus are presented together in our analyses). All eggs were then incubated separately as above, to provide data on three sets of hatchlings: ‘normal’ (control) males, ‘normal’ females, and a group of phenotypically female hatchlings from estradiol-treated eggs. Presumably, about half of this latter group were genetically male rather than female, but we cannot discriminate genetic sex within this group because all were phenotypically female.

### Hatchling methods

Incubators were checked twice daily for hatchlings to determine hatching dates and thus, incubation periods. Any hatchling found was removed and measured and weighed, and its sex was determined by squeezing the tailbase to manually evert hemipenes (Harlow 1996). All hatchlings were then returned to the field sites and released at the location of their mother's capture, except that we retained 12 randomly selected samples in captivity until they were 8–10 weeks of age; they were then killed for histological examination of gonad morphology so that we could verify the accuracy of the hemipene-based sexing method.

### Data analysis

Sex ratio data were analysed by contingency-table (chi-square) tests. Sex differences in incubation duration were analysed by ANOVA and ANCOVA as well as with paired *t*-tests; these three different analytical approaches were adopted for the following reasons: (i) ANOVA gives a straightforward direct comparison of incubation periods of males *versus* females; (ii) ANCOVA with egg mass as the covariate allows us to examine the effect of offspring sex on incubation duration after controlling for the possible effect of sex differences in mean egg mass; and (iii) because eggs within the same clutch are not necessarily independent, we used a paired *t*-test to avoid pseudoreplication (using mean values to compare sons and daughters within each clutch). The relationship between egg mass and incubation duration was analysed by Pearson correlation coefficient analysis. For some of the parametric statistical analyses (Pearson correlation coefficient analyses and *t*-tests) we also included additional data from eggs that belonged to eight 'control' clutches incubated under similar conditions, to increase the power of our tests.

## RESULTS

Among 46 'control' eggs (i.e. that did not receive topical application of estradiol soon after laying) from 16 clutches, 26 eggs produced male hatchlings and the remaining 20 eggs produced females. Thus, the overall sex ratio (57% male) did not differ significantly from 50% ($\chi^2 = 0.78$, $P > 0.50$). In contrast, 51 of 53 hatchlings from estradiol-treated eggs hatched as females (96% *vs.* a null of 50%, $\chi^2 = 45.30$, $P < 0.0001$). Comparing the two data sets directly, estradiol application significantly increased the proportion of females among hatchlings ($\chi^2 = 20.25$, $P < 0.0001$). Thus, we examine these two sets of hatchlings separately below.

To examine sex differences in incubation periods, we first looked only at 'control' eggs. Incubation periods for female embryos ranged from 61 to 75 days, whereas male embryos required 60–68 days incubation prior to hatching. On average, males hatched 2 days earlier than did their sisters (mean 64.3 ± 0.34 *vs.* 66.5 ± 0.90 days). This sex difference was statistically significant regardless of whether each egg was treated as an independent unit (ANOVA: sex effect, $F_{1,44} = 5.96$, $P < 0.02$), or mean values were used for sons *versus* daughters in a paired comparison within each clutch (paired $t = 2.24$, d.f. = 21, $P < 0.04$).

Because hatchling sex in *B. duperreyi* is related to egg size also (smaller eggs are more likely to produce males: Shine *et al.* 2002b), we tested the hypothesis that this sex difference in incubation period might be a result of sex differences in egg size. As reported by Shine *et al.* (2002b) the mean sizes of eggs that produced sons were smaller than those of the eggs that produced their sisters (mean ± SE, 352.96 ± 14.48 mg for female-producing eggs *vs.* 342.26 ± 14.51 mg for male-producing eggs). Incubation duration was related to egg size in comparisons within each sex, with larger eggs hatching sooner than smaller eggs (for females only, Pearson's $r = -0.58$, $n = 20$, $P < 0.01$; for males only, $r = -0.55$, $n = 26$, $P < 0.005$). The same pattern was evident overall (combining both sexes, $r = -0.53$, $n = 46$, $P < 0.0002$). However, this pattern was driven largely by among-clutch differences in mean egg sizes and mean incubation periods, with no consistent correlation between egg size and incubation period within clutch-mates (seven of 14 within-clutch correlations were negative, seven were positive). The sex difference in mean egg mass, combined with the trend for egg size to influence incubation period, suggests that sex-based hatching asynchrony might be caused by the difference in mass of eggs destined to develop into males *versus* females. However, the direction of the sex difference in egg mass (sons from smaller eggs) is the opposite of that expected from this hypothesis (i.e. smaller eggs take longer to incubate, so we would expect sons to hatch *after* their sisters rather than *before*). ANCOVA with egg mass as a covariate shows that relative to egg mass, sons hatched earlier than their sisters (slopes homogeneous, $F_{1,42} = 2.18$, $P = 0.15$; intercepts sex effect, $F_{1,43} = 6.57$, $P < 0.015$). Thus, the sex disparity in incubation periods is not attributable to sex differences in mean egg mass.

We also looked for correlations between incubation period of eggs and two other potentially relevant traits: maternal body size and date of oviposition. No significant links were evident for either maternal body size (incubation duration *vs*. maternal SVL, Pearson's $r = 0.36$, $n = 22$ clutches, $P > 0.09$ for male embryos, and $r = -0.01$, $n = 22$ clutches, $P > 0.99$ for female embryos; *vs*. maternal body mass, $r = 0.29$, $n = 22$ clutches, $P > 0.19$ for male embryos, and $r = 0.28$, $n = 22$ clutches, $P > 0.20$ for female embryos), or date of oviposition (between female incubation duration and ranked date of oviposition, $r = -0.27$, $n = 22$ clutches, $P > 0.23$; male incubation duration and ranked date of oviposition, $r = -0.33$, $n = 22$ clutches, $P > 0.13$).

Our measurements (mean ± SE) of hatchling lizards showed that females were larger than their brothers both for SVL (mean female SVL = 25.15 ± 0.18 mm, mean male SVL = 24.43 ± 0.20 mm; paired $t = 2.168$, $P < 0.02$) and for body mass (mean female mass = 268.30 ± 7.41 mg, mean male mass = 258.90 ± 6.92; paired $t = 2.40$, $P < 0.03$).

All of the analyses above were based only on 'control' eggs. Because we sex-reversed some eggs by applying estradiol to the shell, we can examine incubation periods of those eggs to see whether the shift in phenotypic (*vs*. genetic) sex modified the duration of incubation. Presumably about half of the estradiol-treated eggs were genetically male whereas the remainder were genetically as well as phenotypically female. ANOVA with all three groups included ('normal' males, 'normal' females and estradiol-produced phenotypic females) showed that incubation periods differed among the groups ($F_{2,93} = 4.30$, $P < 0.017$), and Fisher's PLSD *post hoc* tests showed that 'normal' females took longer to incubate than did either males or estradiol-produced females (in both cases, $P < 0.05$). ANOVA with only two groups (all controls *vs*. estradiol-produced females) showed no significant effect of estradiol treatment on incubation period ($F_{1,94} = 1.08$, $P = 0.30$). Inspection of these data (Fig. 1) confirms that despite their female morphology (later confirmed by histology of gonads), estradiol-treated animals tended to resemble males rather than 'normal' females in terms of incubation duration.

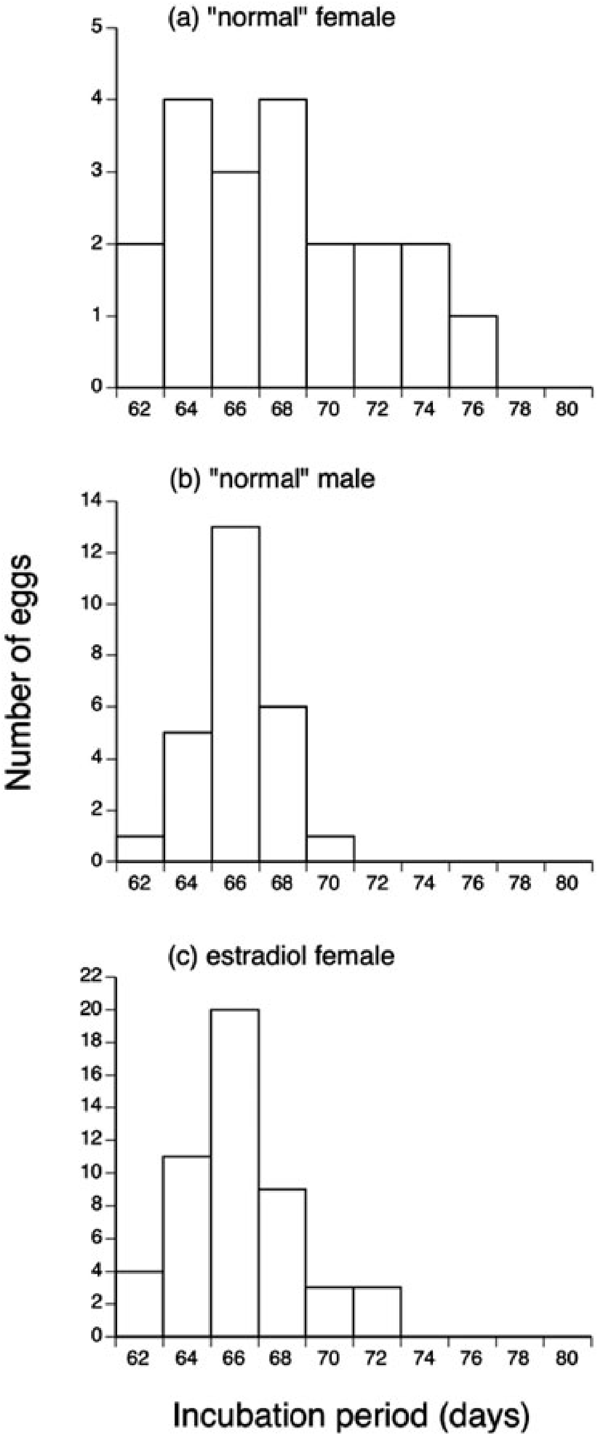

**Fig. 1.** Incubation periods of montane scincid lizards (*Bassiana duperreyi*), as a function of hatchling sex and experimental treatment. The graphs show (a) 'normal' (control) males, (b) 'normal' (control) females, and (c) a group of phenotypically female hatchlings from estradiol-treated eggs; presumably about half of this third group were actually genetically male.

## DISCUSSION

In our laboratory trials, male montane lizards (*B. duperreyi*) hatched out earlier (on average, about 2 days earlier from a 10-week incubation) than did their sisters. This difference was not attributable to any disparity in incubation regimes (thermal and hydric regimes were identical), nor to sex differences in egg mass, nor was it related to maternal body size or date of oviposition. Because each egg was in a separate container, post-laying interactions among eggs cannot have affected the results either. Thus, at least under the conditions used in the present study (which were designed to mimic natural-nest regimes as closely as possible: Shine & Harlow 1996), there is an intrinsic sex-based difference in incubation periods within *B. duperreyi*. To our knowledge, this is the first documented clear-cut case of this phenomenon in any species with genotypic sex determination. The closest parallels to our results come from Burke's (1992) study on broiler chicks and Blanco *et al.*'s (2003) work on Eurasian kestrels, but the former study was based on long-term captive stock and revealed only a very minor sex effect, whereas the design of the latter study did not eliminate the possibility of parental favouritism: that is, males may have hatched earlier because male-containing eggs were incubated more consistently (Blanco *et al.* 2003).

Two major questions arise from our results: (i) what is the proximate mechanism by which incubation is shortened in sons relative to daughters? and (ii) what is the adaptive significance of this pattern? We address these issues below.

First, what mechanism generates this sex divergence? Two possibilities seem plausible. The first is that embryonic development occurs more rapidly in male-producing eggs rather than female-producing eggs, either because of maleness *per se* (i.e. the embryo somehow influences its own rate of differentiation and growth) or because of differential maternal investment into male-producing *versus* female-producing eggs. For example, a reproducing female might allocate more growth-stimulating hormones to eggs destined to produce one sex *versus* the other. In keeping with this speculation, mothers invest sex hormones differentially into male *versus* female eggs in the lizard *Anolis carolinensis* (Lovern & Wade 2003). Although our measurements of yolk hormone levels in *B. duperreyi* reveal no sex difference in oestrogen, testosterone or dihydrotestosterone (R. Radder *et al.*, unpubl. data, 2005), the sex-reversal experiment shows that changing the embryo's sexual phenotype to female did not prolong incubation to the degree that would have been expected for 'normal' female eggs (Fig. 1). That is, genetically female (control) eggs had longer incubation periods than did phenotypically female (hormonally manipulated) eggs. These data suggest that there is something about a female-producing egg, not simply the phenotypic gender of its gonads, that results in a longer incubation period. That 'something' might relate to differential maternal investment, or to embryogenesis completed prior to the time that we applied estradiol (immediately postoviposition). As in most other squamate species, *B. duperreyi* complete about one-quarter of embryogenesis prior to oviposition (Shine 1983).

An alternative mechanism for sex disparity in incubation period might be that sons grow and differentiate at the same rate as daughters, but hatch earlier because they initiate hatching (escape from the egg) at an earlier developmental stage. That is, sons may be more precocial (in avian terms) than are daughters. Shine and Olsson (2003) have argued that this altricial-precocial continuum occurs in lizards as well as birds, albeit to a much less extreme degree. However, the results of the sex-reversal experiment argue against the idea that females simply delay hatching until they are more completely developed; were this to be the case, we would expect that sex-reversal would have influenced incubation periods, whereas it did not do so.

Although the mechanism by which sex influences the duration of incubation remains unknown for *B. duperreyi*, it clearly differs from that described for other reptile species with sex-based hatching asynchrony. In all the cases known so far, the sex divergence in hatching dates is a consequence of TSD combined with a strong thermal effect on incubation period. That is, offspring from cooler nests hatch later, and with a different sex ratio, than do offspring from hotter nests (Harlow 1994, 2004). In these TSD species, the primary level of covariation between offspring sex and hatching date involves among-clutch effects: that is, entire clutches tend to be of the same sex and hatch at the same time. The system in *B. duperreyi*, in contrast, involves more subtle within-clutch modification of hatching times relative to offspring sex.

We now turn to the issue of adaptive significance. Although a pattern for males to emerge earlier than females (protandry) is very widespread among animals, its adaptive basis may be diverse (Cook & Monaghan 2004; Lezalova *et al.* 2005). More generally, the way in which the seasonal timing of hatching affects individual fitness often may differ between the sexes (Lloyd & Martin 2003; Laaksonen 2004; Langkilde & Shine 2005), and any such difference may impose selection for mechanisms that modify hatching time relative to offspring sex (Olsson & Shine 1997, 1998; Olsson *et al.* 1999; Qualls & Shine 2000; Warner & Shine 2005). Indeed, such a divergence may have been the primary selective force for TSD in squamate reptiles (Harlow 2004; Warner & Shine 2005), including viviparous species (Wapstra *et al.* 2004).

We know too little about the determinants of individual reproductive success in free-ranging *B. duperreyi* to identify links between offspring phenotype and success, let alone sex differences in such a relationship. Indeed, such data are available for relatively few reptile species (Sinervo & Huey 1990; Brown & Shine 2004). Extrapolating our results to the natural population thus remains difficult. Natural nests of *B. duperreyi* vary substantially in mean temperatures both among and within years (Shine 2002), and some communal nests display significant within-nest thermal stratification (R. Radder & R. Shine, unpubl. data, 2005). Because developmental rates are very sensitive to temperature (Andrews 2004), this thermal variation may override the relatively modest sex-based differential induced by sex divergences in incubation periods relative to temperature. This will be especially true if cooler nests overproduce males – as happens with *B. duperreyi* for at least two reasons. First, smaller eggs usually have longer incubation periods (this paper), and these smaller eggs typically produce male hatchlings (Shine *et al*. 2002b). Second, at least in some years and in some populations, very cool nests mostly produce sons (i.e. incubation temperature overrides genotypic sex determination: Shine *et al*. 2002a). Thus, the adaptive significance of the phenomenon that we have documented in the current paper may be to minimize rather than to amplify a sex disparity in mean hatching dates. That is, somewhat ironically, the intrinsic sex-based differences in incubation periods seen in the laboratory may reduce rather than increase the mean difference in hatching dates between males and females under field conditions.

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