Sex of incubation neighbours influences hatchling sexual phenotypes in an oviparous lizard

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PHYSIOLOGICAL ECOLOGY - ORIGINAL PAPER

Sex of incubation neighbours influences hatchling sexual phenotypes in an oviparous lizard

Florentino Braña

Abstract In many litter-bearing mammals and in a few viviparous reptiles the sex ratio of the entire brood or the sex of the adjacent fetuses induces sex-specific differences in the hatchling's phenotype. This study examines whether the sex of incubation neighbours affects hatchling characteristics in oviparous common lizards (*Lacerta vivipara*). Oviparous common lizards lay eggs with thin eggshells and, therefore, are an optimal model organism for studying the effects of hormone leakage among developing embryos since the strongest evidence for prenatal sex ratio effects on offspring development comes from viviparous populations of the same species. Groups of three eggs were incubated together and were categorised according to the sex of the resulting hatchlings as either homosex (three hatchlings of the same sex) or heterosex (one male or one female hatchling plus two siblings of the opposite sex). Hatchlings incubated adjacent to siblings of the same sex had larger body mass and body condition. Males tended to have lower ventral scale counts when incubated with other males. Conversely, females tended to have more ventral scales when incubated with other females, indicative of a more feminised phenotype. There was also a significant interaction between hatchling sex and incubation environment with respect to the length of the fourth digit of the hindlimb, likely indicative of masculinisation in heterosex females. This study suggests steroid diffusion between adjacent eggs in a minimally manipulative experiment and provides the first evidence for developmental effects of the exogenous hormonal environment in near natural conditions in an oviparous amniote. Implications of these results for the evolution of within-clutch sex ratio are discussed.

Keywords Prenatal environment · Sexual phenotype · *Lacerta vivipara* · Exogenous steroids · Hormone leakage

Introduction

Environmental variation during early life can profoundly influence developmental outcomes and can have long-term effects and fitness consequences (Crews 1996; Lindström 1999). Endogenous steroid hormones are known to play a decisive role in the configuration of sexual phenotypes, but there is growing evidence that prenatal exposure to exogenous steroids, either maternally derived or produced by sibling fetuses, can also be important in shaping newborn phenotypes and subsequent life histories in many vertebrates (Dufty et al. 2002; Rhen and Crews 2002). The effects of prenatal intrauterine position or litter sex ratio have been extensively studied in litter-bearing mammals (vom Saal 1989; Clark and Galef 1995; human twins, McFadden 1993; Lummaa et al. 2007) and more recently in a few viviparous reptiles (garter snake, *Thamnophis elegans*, Osypka and Arnold 2000; common lizard, *Lacerta vivipara*, Uller and Olsson 2003a; Uller et al. 2004, 2005). In viviparous common lizards, prenatal sex ratios produced sex-dependent allometry in head size, growing larger in males from male-biased broods (Uller and Olsson 2003a). Head size relative to abdomen size is the main dimorphic trait in lacertid lizards (Braña 1996) and larger head size may affect male reproductive success by improving both the fighting capacity in male–male contests and the ability

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to grasp females for copulation (see, for *L. vivipara,* Gvoždík and van Damme 2003). Therefore, phenotypic variation produced by prenatal exposure to exogenous hormones is potentially important in modulating reproductive success.

Oviparous amniotes deposit eggs encased in shell membranes surrounded by an external mineral layer (Packard and De Marco 1991; Board and Sparks 1991). Embryos develop separated from each other for most or all of the developmental period and, consequently, steroid leakage between sibling fetuses is considered unlikely (Uller 2003). However, in contrast to birds and crocodilians, many oviparous squamates lay eggs with a compliant, parchment-like eggshell that is highly permeable to water and solutes (Vleck 1991). The thin calcareous layer that surrounds the embryo is further reduced during the late stages of development due to mobilization of calcium during the growth phase of embryogenesis (Packard and DeMarco 1991; Shadrix et al. 1994). Thus, shell membranes of squamate eggs constitute relatively thin barriers that could allow steroid diffusion between adjacent fetuses during incubation, and the hormonal environment created by incubation neighbours could influence sexual phenotypes in oviparous squamates. To examine this possibility, the present study looked at several aspects of sexual dimorphism in hatchling lizards incubated in close contact with known-sex neighbours. The oviparous strain of the reproductively bimodal *L. vivipara,* which has both oviparous and live-bearing populations, was used in this experiment, because previous studies on the viviparous lineages of this species have found that embryos are sensitive to hormonal exposure during prenatal development (de Fraipont et al. 2000; Uller and Olsson 2003a, b, 2006).

Materials and methods

The common lizard, *L. vivipara,* the world's most widely distributed reptile, is live-bearing over most of its range, but some populations in the south-west limit of its distribution range are oviparous (Braña et al. 1991; Heulin et al. 2002). Oviparous females lay eggs containing well-developed embryos (most frequently at stages 31–33 according to Dufaure and Hubert's 1961 table; Braña et al. 1991). Animals for this study were obtained from an oviparous population located in the central part of the Cantabrian mountain range (Puertos de Tarna, Señales, and Isoba, northern Spain; 43°05′N, 5°15′W; elevation: 1,500–1,750 m). Sixty-nine free-living females bearing oviductal eggs were captured by hand and noose in late June 2003 and placed in terraria with moist potting ground as substrate for egg laying. Oviposition occurred in all cases within 10 days of capture. Three freshly laid eggs from each clutch were incubated in close contact, half-buried in moistened vermiculite (ratio 2:1 of distilled water to dry vermiculite by weight). Eggs were placed in 50-ml covered plastic containers and were incubated until hatching at 25°C (mean ± SD, for incubation temperature, 24.95 ± 0.29°C; for incubation time, 21.56 ± 1.44 days). Each group of three eggs incubated in the same container was categorised, according to the sex of the resulting hatchlings, as either homosex (three hatchlings of the same sex) or heterosex (one male or one female plus two hatchlings of the opposite sex). Only one individual from each trio was selected for analysis. One individual was randomly chosen from each homosex trio and the individual of the uncommon sex was selected from each heterosex trio.

Within a few hours of hatching, lizards were weighed to the nearest 0.001 g and sexed by applying gentle pressure on the tail base, which causes the eversion of the hemipenis in males (Harlow 1996). Morphological measurements (see below) and ventral scale counts were obtained from digital images of every hatchling taken within 24 h of hatching. The following measurements were taken on each neonate: mass, snout–vent length, abdomen length, head length, and ventral scale counts. These variables were selected for analysis because they are the main dimorphic traits in *L. vivipara* (Boulenger 1920; Braña 1996). Digital images were also used to measure the length of the fourth digit of the right hindlimb of hatchlings since digit lengths are dimorphic and sensitive to exposure to steroid hormones in early life in many tetrapods (e.g. in mammals, mostly in humans, Brown et al. 2002; in birds, Burley and Foster 2004; in lizards, Rubolini et al. 2006). The frequently used second-to-fourth digit ratio was disregarded because the second digit is small and difficult to stretch in hatchling lizards, resulting in unreliable measurements.

The effects of neonate sex and hormonal environment on hatchling phenotype were tested with a two-factor ANOVA [or analysis of covariance (ANCOVA), with log hatchling mass as the covariate], with the sex of the offspring and the incubation environment (as determined by the sex of the incubation partners) as the two factors. Data were log transformed when necessary to achieve normality.
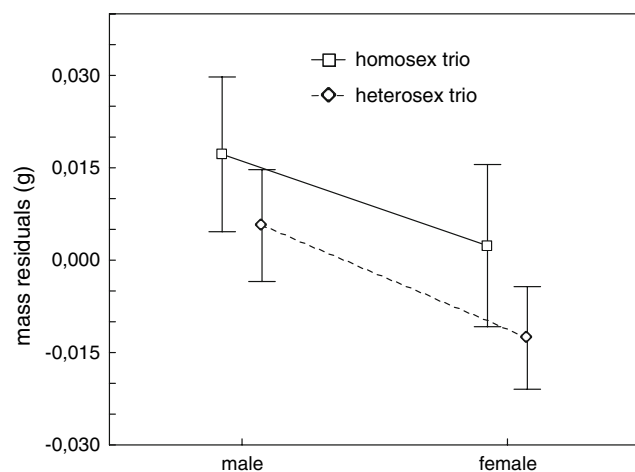
Results

Sixty-seven trios hatched successfully and were used in later analyses. There were 21 homosex trios (11 all-male and ten all-female) and 46 heterosex trios (21 prevailing-female and 25 prevailing-male). The number of clutches of the four group types did not differ from the expected number under the hypothesis of equal and independent probability for ocurrence of both sexes (i.e. $0.125 \times n$ for each homosex group and $0.375 \times n$ for each heterosex group; $n = 67$, $\chi^2 = 1.816$, $P = 0.612$). Overall sex ratio of the
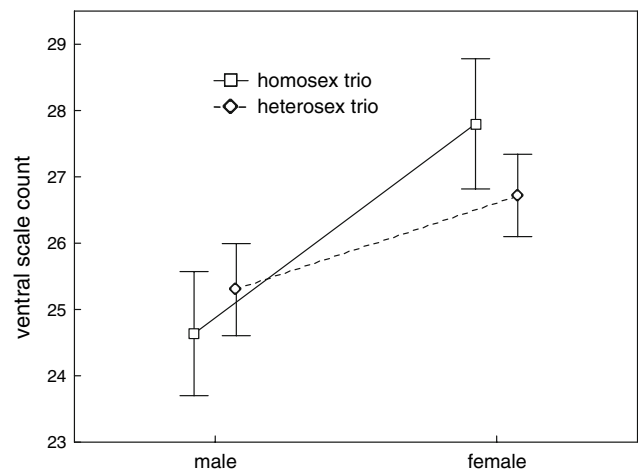
hatchlings did not significantly depart from equality (male/female: 102/99; $\chi^2 = 0.045$, $P = 0.832$).

Head length was similar for male and female hatchlings ($F_{1,63} = 0.07$, $P = 0.788$), whereas the abdomen was much longer in females ($F_{1,63} = 32.05$, $P < 0.0001$) even when controlled for differences in body size (ANCOVA, with log hatchling mass as covariate; $F_{1,62} = 58.21$, $P < 0.0001$). Head length and abdomen length did not differ between incubation environments and there was no significant sex by environment interaction, whether considering direct measurements or values controlled for body size (ANOVA or ANCOVA, with log hatchling mass as covariate; $P > 0.1$ in all cases). Hatchling mass varied depending upon the sex of the incubation partners, heavier neonates resulting when incubated with two siblings of their same sex ($F_{1,63} = 6.69$, $P = 0.012$, for the effect of incubation environment; there were no significant sex or sex by environment interaction effects: $P > 0.5$ in both cases). Given that females are larger than males (snout–vent length; $F_{1,63} = 18.04$, $P < 0.0001$) and both sexes had similar body mass, I further analysed body condition as assessed by the residuals from the regression of hatchling mass on snout–vent length; mass residuals were affected by incubation environment (homosex hatchlings had larger residuals; $F_{1,63} = 5.83$, $P = 0.018$; Fig. 1) and exhibited a clear sex effect ($F_{1,63} = 9.04$, $P = 0.004$; males had larger relative mass).

Females had more ventral scales than males ($F_{1,63} = 31.99$, $P < 0.0001$). The incubation environment did not influence scale counts by itself ($F_{1,63} = 0.28$, $P = 0.598$), but there was a significant interaction effect of sex and environment ($F_{1,63} = 5.53$, $P = 0.037$; Fig. 2), so that males tended to have lower ventral scale counts when

Fig. 2 Ventral scale counts for male and female hatchling common lizard incubated in homosex or heterosex environments (see Fig. 1). Mean values and 95% confidence limits are represented

incubated with other males, while females tended to have more ventral scales when incubated with other females ($F_{1,33} = 3.99$, $P = 0.055$).

The length of the fourth digit of the hindlimb did not show significant differences according to the sex of the hatchlings (slightly larger in males; ANCOVA; $F_{1,62} = 1.20$, $P = 0.277$) or to incubation environments ($F_{1,62} = 0.54$, $P = 0.466$) but there was a significant interaction effect of both factors ($F_{1,62} = 4.36$, $P = 0.040$; Fig. 3) with two main underlying trends: within homosex trios males tended to have longer digits than females ($F_{1,18} = 3.87$, $P = 0.063$), and among females, the heterosex incubation group tended to have longer digits than the homosex group ($F_{1,32} = 3.26$, $P = 0.080$).
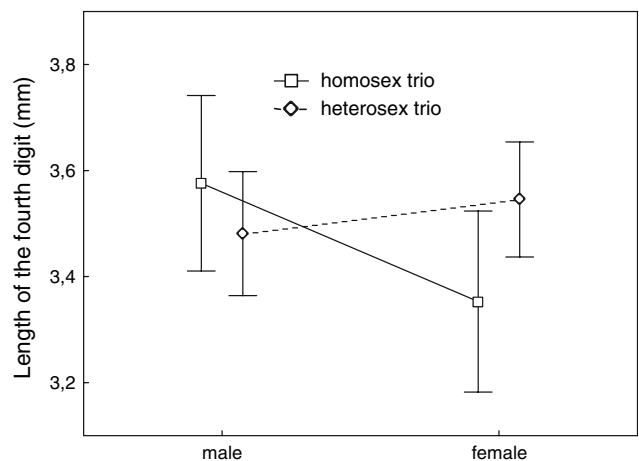

Fig. 1 Mass residuals from the regression on snout–vent length for male and female hatchling common lizard incubated together with two embryos of the same sex (*homosex trio*) or with two embryos of the opposite sex (*heterosex trio*). Mean values and 95% confidence limits are represented

Fig. 3 Length of the fourth digit of the right hindlimb of male and female hatchling common lizard incubated in homosex or heterosex environments (see Fig. 1). Mean values and 95% confidence limits are represented

Discussion

This study presents evidence for effects of the sex of incubation neighbours on hatchling phenotype in oviparous common lizards (*L. vivipara*), a species for which previous studies conducted on viviparous populations showed that prenatal sex ratio influences sexual dimorphism (Uller and Olsson 2003a; Uller et al. 2004, 2005). In the studied population females oviposit eggs containing embryos at Dufaure and Hubert's (1961) stages 31–33 (Braña et al. 1991, and additional unpublished data), at which time endogenous production of steroid hormones is unlikely. Detailed studies in the lizard *Calotes versicolor* indicated that gonadal differentiation begins at stage 34 and there was no appreciable steroidogenesis until stage 36 in both male and female embryos (Doddamani 2005, 2006). Similar results were reported in the lizard *Sceloporus undulatus* (Austin 1988) and in green anoles (*Anolis carolinensis*; Lovern and Wade 2003b). In addition, some traits that exhibited treatment effects are not developed (scales) or are hardly outlined (digits; at stage 33 only three digits begin to differentiate; Dufaure and Hubert 1961) at egg laying. Therefore, the effects on offspring development are most likely attributable to the direct influence of the sex of the neighbouring fetuses during the experimental incubation. Because the experiment involved minimal manipulation of eggs and incubation environment, these results suggest that the influence of hormonal environment created by sibling embryos during development could be an important source of phenotypic variation in natural populations.

Oviparous *L. vivipara* have thin eggshells (6–7 μm at oviposition; Heulin et al. 2002), and lay clutches with eggs closely attached, glued by oviductal secretions. The mineral crust of the eggshell is further reduced during embryogenesis because of the removal of calcium for embryonic development, as generally occurs in lizards (Shadrix et al. 1994; Ji and Braña 1999). In oviparous *L. vivipara* of northern Spain, a 22% reduction was recorded in eggshell dry weight from oviposition to hatching (ratio of mean dry weight loss of eggshells from a sample of ten clutches; F. Braña, unpublished data). These characteristics may favour the transport of steroids between adjacent eggs by diffusion through the amniotic fluid and across the fetal membranes, as has been demonstrated between adjacent mammalian fetuses (Even et al. 1992; vom Saal and Dhar 1992). In contrast, rigid shell structure appears to insulate avian eggs from steroids produced by other embryos of the same clutch (Burley and Foster 2004).

Results of the current study strongly suggest steroid leakage among eggs not mediated by maternal production during development or by diffusion through the mother's oviduct. However, although seemingly unlikely, we cannot rule out, as an alternative or complementary explanation, that offspring sex ratio could be influenced by levels of maternally derived yolk steroids present at fertilization, which could differentially affect male- and female-inducing sperm, as suggested by Lovern and Wade (2001, 2003a) for *Anolis carolinensis*. However, other studies have failed to demonstrate any influence of maternally derived yolk steroids in sex determination in lizards (see Kratochvíl et al. 2006 for the geckonid *Paroedura picta;* Radder et al. 2007 for the scincid *Bassiana duperrey*). If common lizards follow the pattern suggested for *A. carolinensis*, high yolk testosterone level at the time of fertilization could have produced a male-biased offspring sex ratio and, consequently, higher probability of three-male trios. If so, further masculinisation of hatchlings from all-male trios could be, at least partially, a maternal effect. Uller and Olsson (2003a) stress the difficulty in separating the effects of fetal hormonal interactions from the maternal steroid allocation. In this study, the knowledge of the sex of the siblings adjacent to the focal hatchling, instead of the sex ratio of the entire clutch or litter, greatly improved the reliability of causal attribution for the observed effects on hatchling phenotypes. Furthermore, both the overall sex ratio of the hatchlings and the number of clutches of the four group types agreed with expectations under the hypothesis of equal and independent probability for successive recruitment of hatchlings of both sexes. This is the expected outcome from the primary chromosomal sex determination mechanism and discredits the possibility of hormonal interference.

Sex of incubation neighbours affected hatchling mass and condition. Male and female offspring developing adjacent to two siblings of the same sex were heavier than those who shared the incubation environment with two individuals of the opposite sex. Effects of steroid hormone exposure during pregnancy on neonate weight have been repeatedly reported in mammals (Kinsley et al. 1986; Petridou et al. 1990; Kaijser et al. 2000). Among squamate reptiles, manipulative experiments conducted at different postembryonic stages have frequently shown that testosterone inhibits mass gain and skeletal growth (review in Cox et al. 2005), although Uller and Olsson (2003b) reported increased growth of hatchling *L. vivipara* (live-bearing strain) exposed to exogenous testosterone as embryos. Interestingly, some studies conducted on *Sceloporus* lizards have shown growth effects of sex steroids consistent with the direction of size dimorphism. For example, experimental manipulation using testosterone implants in young males indicated that testosterone inhibits male growth in female-larger species (*S. undulatus* and *Sceloporus virgatus*) but stimulates growth in the male-larger *Sceloporus jarrovii* (Cox and John-Alder 2005; Cox et al. 2005). In *L. vivipara*, large females have larger relative abdomen size that allows production of more or bigger eggs (Braña 1996), but body

size, particularly head size, may also positively affect male reproductive success (Gvoždík and van Damme 2003). In this context, female mass increase in the homosex environment is not unexpected in *L. vivipara*, a species where females are larger. This does not necessarily imply a negative effect of androgens on male growth, as size dimorphism arises from sex differences in growth rates and growth trajectories in particular body segments (Braña 1996).

Female *L. vivipara* have more ventral scales than males (Boulenger 1920; this study) and the interaction effect of sex by incubation environment reported in the present study indicates that females incubated close to two females have higher ventral scale counts. Thus, these females are further feminised compared to those incubated adjacent to two males. Similarly, male and female hatchlings of viviparous common lizards (*L. vivipara*) tended to have an increased number of ventral scales in female-biased clutches (Uller et al. 2005). In contrast, *Thamnophis elegans* females have fewer subcaudal scales (thus are feminised) in litters in which males predominate, suggesting that female embryos are feminised by aromatizing testosterone (Osypka and Arnold 2000).

The interaction in the length of the fourth digit in *L. vivipara* largely consists of an increase in digit length in females incubated with two males compared to those incubated with two females. Knowledge of patterns of digit development with respect to symmetry and relative size in non-mammalian tetrapods is very preliminary, but it is well known that prenatal androgen exposure can differentially affect finger length in a number of mammals (mostly in humans; Brown et al. 2002; Putz et al. 2004) and birds (Burley and Foster 2004; Romano et al. 2005). To my knowledge, the only study conducted on reptiles examined the lizards *Podarcis muralis* and *Mabuya planifrons* and showed that digit ratios are sexually dimorphic in both species. However, direction of between-sex differences varied in some ratios, suggesting a relationship with sex-specific growth trajectories associated with the specific direction of sexual size dimorphism (Rubolini et al. 2006). Unfortunately, Rubolini et al. (2006) did not report measurements of particular digits. Only length ratios between digit pairs were reported, and these are confusing with respect to the source of variation, which precluded a comparison with the common lizard patterns described here.

Studies of many viviparous mammals (vom Saal 1989; Clark and Galef 1995) and a few viviparous reptiles (Osypka and Arnold 2000; Uller and Olsson 2003a) have shown that embryonic exposure to exogenous steroids can be an important factor in generating phenotypic variation. The present study demonstrates developmental effects of the exogenous hormonal environment in an oviparous lizard and, because the experiment was conducted in near natural conditions, these effects can be expected to also occur in the field. Further studies should address the persistence

of phenotypic effects produced by exogenous steroids during development and evaluate the extent to which these effects have long-term consequences for offspring fitness (see Uller et al. 2004). One particularly interesting issue in this respect concerns the potential fitness consequences of variation of within-clutch sex ratios. Although sex chromosomes favour a sex ratio near equality in most vertebrates (Charnov 1982), when the relative fitness of male and female offspring varies with environmental conditions, evolutionary theory predicts that parents should adjust the sex of their offspring accordingly (Frank 1990). Larger body size and enhanced sexual phenotypes should favour reproductive success, thus improving the fitness return on maternal investment. Thus, when considering a population-level equilibrated sex ratio, within-clutch skewed sex allocation is expected to evolve since skewed sex ratios would benefit individuals of the over-represented sex by increasing body mass and reinforcing sex-specific characteristics through steroid leakage among siblings. Furthermore, it is worth noting that one potential advantage of temperature-dependent sex determination systems in reptiles (Bull 1980; Janzen and Paukstis 1991) could be related to the benefits of producing highly skewed within-clutch sex ratios.

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