Multifactorial Sex Determination in Chameleons

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Abstract.—Classifying a species as having either genotypic or environmental sex determination may oversimplify the processes that influence offspring sex. Using two independently gathered data sets on veiled chameleons (*Chamaeleo calyptratus*), we show that a hatchling's sex is affected by the interaction between egg mass and incubation temperature. In both studies, larger eggs produced daughters at lower temperatures and sons at higher temperatures; however, the relationships between incubation temperature, egg mass, and sex diverged markedly between the two studies. The shift in egg-size effects was seen between 25°C and 28°C in one study and between 28°C and 30°C in the other. The links between offspring size, sex, and incubation temperature are not an artifact of differential mortality; in one of the studies, egg survival rates were uniformly high. Other data on scincid and agamid lizards also do not support a simple dichotomy in sex-determining systems. Our results challenge simplistic classificatory schemes and call for a reanalysis of existing data sets to look for multifactorial as well as unifactorial effects on offspring sex.

In vertebrates, sex-determining mechanisms usually are categorized as either genotypic (an individual's sex is fixed at conception, based upon its genotype) or environmental (in which an external trigger, often nest temperature, determines the sex of the animal postconception) (Gamble and Zarkower, 2012). Both types of mechanisms can occur in closely related species and sometimes even within the same species (Conover and Kynard, 1981; Pen et al., 2010). Phylogenetic reconstruction shows multiple evolutionary switches between the two systems (Janzen and Krenz, 2004), leading to a complex distribution of sex-determining mechanisms. For example, many reptiles (all crocodilians, the tuatara, and many turtles and lizards) show temperature-dependent sex determination (TSD), whereas others (all snakes, and some turtles and lizards) exhibit genotypic sex determination (GSD) (Modi and Crews, 2005). Inferences about sex-determining mechanisms may be in error, however, if the classification scheme oversimplifies the real situation. Reports that nest temperatures can influence offspring sex ratios, even in lizards with heteromorphic sex chromosomes, challenge the validity of a dichotomy between these two mechanisms (Shine et al., 2002; Sarre et al., 2004; Quinn et al., 2007). Our data cast further doubt on the traditional dichotomous classification scheme, suggesting instead that offspring sex in veiled chameleons is affected by an interaction between incubation temperature and egg mass.

Materials and Methods

Egg Incubation.—Between May 2010 and September 2011, we collected four clutches of eggs ($N = 178$ total eggs; 53, 42, 43, 40 eggs per clutch) from female *Chamaeleo calyptratus* that had laid <12 h earlier at the University of Sydney. Eggs were weighed and then placed in individual glass jars (125 mL) containing moist vermiculite (-200 kPa). Glass jars were sealed with plastic wrap to prevent evaporation and placed into either a "cold" (25°C) or "warm" (28°C) constant-temperature incubation treatment for the entirety of incubation. Half of the eggs from each clutch went into each incubation temperature (i.e., split-clutch design). We placed data loggers (Thermochron iButtons, model DS1921: Dallas

Semiconductor, TX; resolution ±0.5°C) on each shelf holding eggs to monitor temperatures in the incubators every 2 h and rotated the shelves biweekly to minimize thermal gradients. When eggs hatched ($N = 81$ total eggs survived; 17, 13, 30, 21 eggs survived per respective clutch), we scored hatchlings as male if they possessed distinctive heel spurs (Andrews, 2005) on their back feet (Fig. 1). We also reanalyzed an existing data set on the same species, from a published study (Andrews, 2005) that had concluded these lizards have genotypic sex determination; methods for that study were very similar to our own.

Statistical Analyses.—We completed multilevel logistic regressions of offspring sex as the response variable with "Proc Glimmix" SAS 9.3 (SAS Institute, Cary, NC) using temperature treatment as a fixed factor, clutch (female) identity as a random factor, and egg mass as a covariate and including the interaction between egg mass with temperature treatment. We used residual pseudo-likelihood as the estimation technique: the link function was logit; and the degrees of freedom were calculated using Sattethwaite's algorithm. Because egg mass varied among clutches, we standardized egg mass for clutch ID (i.e., mean set to zero and SD to 1.0). We report Rao Scott *F*s from adjusted Wald Chi-squares. Two data sets were analyzed, one from the current study and one from the Andrews (2005) study.

Results

A total of 81 chameleons hatched from either a cold (25°C) or warm (28°C) constant-temperature incubator during the current study. The overall sex ratio of hatchlings did not differ from 1 : 1 in either incubation group (from cold incubation, total $N = 30$; 13 females, 17 males, 2-tailed binomial test, $P = 0.58$; from warm incubation, total $N = 51$; 27 females, 24 males, $P = 0.78$), or differ between incubation temperatures (logistic regression $F_{1,23} = 0.59$, $P = 0.45$). Similarly, the mean mass of eggs (at oviposition) that produced male hatchlings was similar to that of eggs that produced females (means 1.018 g and 1.024 g, respectively; $F_{1,77} = 0.35$, $P = 0.55$). Therefore, sex of hatchling chameleons was not significantly affected by incubation temperature or by egg mass. Similarly, Andrews (2005) reported approximately equal numbers of males and females from each temperature treatment group, with hatchling sex unaffected by temperature ($F_{2,146} = 0.95$, $P = 0.39$) or egg mass ($F_{1,146} = 0.16$, $P = 0.69$).
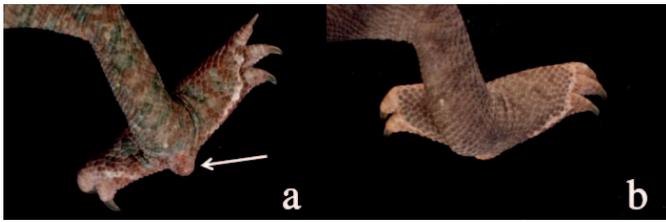
FIG. 1. In hatchling veiled chameleons, heel-spurs are present on the back feet of all males (a) but not females (b). This sexual dimorphism facilitates accurate noninvasive sexing of recently hatched offspring. Photo by Malcolm Ricketts.

FIG. 2. Average egg mass (±SE) of male and female chameleons hatching from eggs incubated at a range of temperatures. The upper graph (a) shows data from the current study, whereas the lower graph (b) shows a reanalysis of data from a study by Andrews (2005). Offspring sex is affected by the interaction between egg mass and incubation temperature.

This result changed dramatically when the analysis included interaction terms. In our study, larger eggs produced sons at high incubation temperatures, but produced daughters at low temperatures (Fig. 2a). Therefore, a multilevel logistic regression with progeny sex (male or female) as the dependent variable showed a significant interaction between egg mass and incubation temperature ($F_{1,77} = 6.84$, $P = 0.01$). Reanalysis of data from the Andrews (2005) study similarly showed that offspring sex was determined by a significant interaction between egg mass and incubation temperature ($F_{2,167} = 3.33$, $P = 0.03$). Intriguingly, the relationships between incubation temperature, egg mass, and sex differed between the two studies (Fig. 2b). For example, incubation at 28°C generated a pattern for larger eggs to produce sons in our study but to produce daughters in Andrews's study (Fig. 2b).

When we combined the two data sets, adding incubation site (United States vs. Australia) to the analysis as an additional variable, the interaction between egg mass and incubation temperature remained ($F_{2,227} = 4.99$, $P < 0.01$) but with no significant main effect of either incubation temperature ($F_{2,227} = 1.23$, $P = 0.29$) or egg mass ($F_{1,227} = 0.82$, $P = 0.37$). Hence, despite differences in the two studies (e.g., in the relationship between incubation temperature and sex), the same general pattern was seen in both and in an analysis that included data from the two studies simultaneously. Offspring sex ratios did not differ significantly between different incubation temperatures, nor was a hatchling's sex related directly to egg mass; however, a significant interaction between these two factors was evident: larger eggs produced daughters at one incubation temperature but produced sons at a different incubation temperature.

Mortality of eggs was high in our study (46% survival); hence, we cannot reject the possibility that differential mortality caused the patterns we saw. In keeping with that possibility, mortality was nonrandom with respect to egg mass (mean mass of surviving eggs 1.34 g, of nonsurviving eggs 1.21 g; one-way ANOVA $F_{1,178} = 18.54$, $P < 0.0001$). Egg survival in the Andrews (2005) study was high (92%), however, therefore, differential mortality cannot explain the sex ratio biases observed in her work. Even if all of the larger-than-average eggs (>1.17 g) in the earlier study are allocated as the "wrong" sex (males at 25 and 28°C, females at 30°C incubation), the temperature × egg mass interaction remains significant ($F_{2,167} = 3.33$, $P = 0.04$).

DISCUSSION

The prevailing paradigm—that offspring sex in vertebrates is determined either by genotype or by nest temperature—has encouraged researchers to anal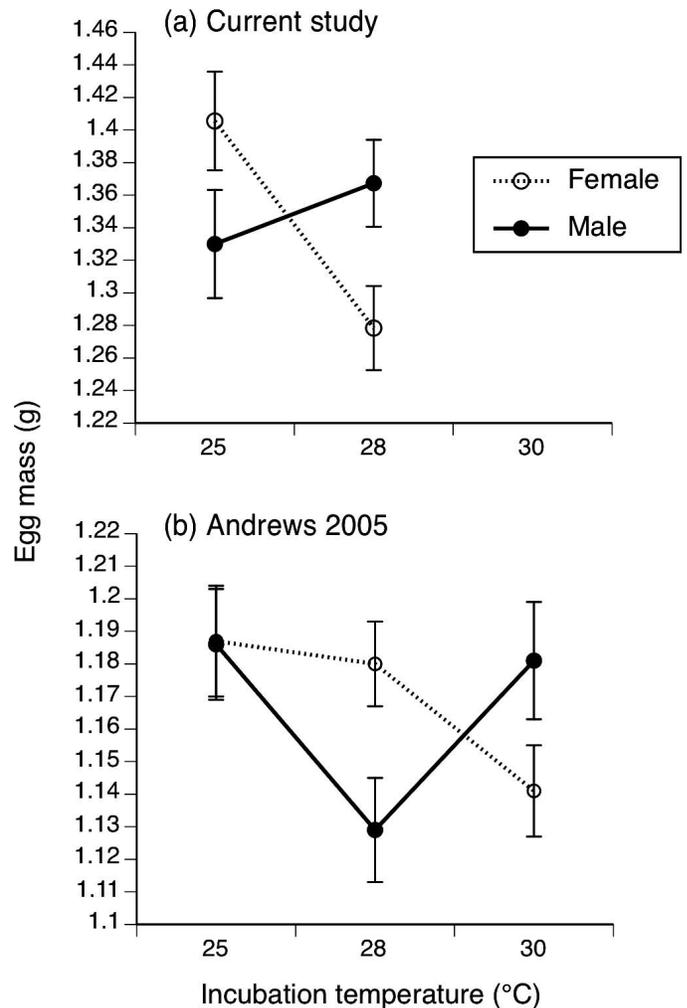yze their data using unifactorial approaches. If offspring sex ratios are 1 : 1 at a range of incubation temperatures, researchers tend to conclude that incubation temperature does not influence offspring sex (Sarre et al., 2004). With these types of unifactorial analyses, previous studies of chameleons have inferred GSD (Andrews, 2005; Long, 2008). One anecdotal report hints at TSD: eggs of *Chamaeleo chamaeleon* incubated at 27–29°C produced only female progeny (Schmidt et al., 1994). Our results on *C. calyptratus* suggest that effects of incubation temperature and egg mass on offspring sex may be evident only when analyses specifically look for interactive effects. Our reanalysis of the earlier study on this species (Andrews, 2005) illustrates exactly this reversal of conclusions. Despite the popularity of chameleons among reptile breeders and in the pet trade, sex-determining mechanisms in these animals are largely unstudied, with a few exceptions. Chameleons within the genus *Furcifer* exhibit female heterogamety (Rovatsos et al., 2015), and unpublished reports suggest this same mechanism may determine sex in *Bradypodion ventrale* also (reported in ref. Olmo and Signorino, 2005).

Figure 2 reveals a complex pattern. Both studies show that an interaction between incubation and temperature affects off-

spring sex, but the details of that interactive effect differ, as do mean egg masses. For example, large eggs produced males at 28°C incubation in our study but produced females in Andrews's original (2005) study. In both studies, however, larger eggs produced females at lower incubation temperatures and produced males at higher incubation temperatures (Fig. 2). Thus, the pattern is similar but with a displacement of the reaction norms. We do not understand why different populations of the same lizard species would diverge in mean egg masses and in patterns of sex determination in this way, but note that geographic variation in egg sizes and pivotal temperatures for sex determination occurs in turtles (Ewert et al., 2006). We do not know the geographic origin of the chameleons used in either study; presumably, the veiled chameleons currently held in captivity came from a limited number of founders originally collected in the native range (perhaps from multiple locations) many years ago. Even though these two studies were conducted on different populations of the species, by investigators in different continents, a decade apart, the results reveal a divergence in traits such as egg masses and norms of reaction, and both exhibit strong evidence of multifactorial sex determination.

Increasing evidence challenges the long-held (and still popular) paradigm that vertebrate sex-determining mechanisms comprise a simple dichotomy between genotypic factors on the one hand and environmental influences on the other. Offspring sex in many taxa may indeed be dictated exclusively by one or other of these two mechanisms, but multiple interactive processes can affect sex determination in other species (Sarre et al., 2004; Cassey et al., 2006). In the lizard *Bassiana duperreyi*, an individual's sex is affected not only by genes (heteromorphic sex chromosomes) and incubation temperature but also by yolk allocation (Radder et al., 2009). Similarly, in the Japanese gecko *Gekko japonicus*, heavier eggs produce females at 28°C (Ding et al., 2012). Therefore, egg mass is linked to offspring sex in at least three lizard species from phylogenetically divergent lineages (separated 250 million years ago: Kumazawa, 2007). Maternal control of offspring sex, via modification of egg size, may be widespread among lizards.

Complex multifactorial sex-determining systems are well known among invertebrates. Environmental factors involved in invertebrate sex determination include temperature, photoperiod, salinity, nutrition level, and mate availability (Cook, 2002). Also, invertebrate species that use superficially identical sex-determining systems may do so through different underlying molecular mechanisms (Cook, 2002). Vertebrate sex determination may be more similar to that of invertebrates than is currently envisaged. Even in vertebrate lineages thought to determine sex via a single system, mothers may be able to manipulate progeny sex ratio using additional mechanisms once employed (perhaps more overtly) by their progenitors (Grigg, 1992; Swain and Jones, 2000). Interactive effects of genes, incubation conditions, and maternal allocation decisions in determining offspring sex may be only the tip of the iceberg. For example, offspring sex in some lizards and turtles is affected by an interaction between mean nest temperatures and the degree of variation in nest temperatures (Bull, 1985; Valenzuela, 2001; Warner and Shine, 2011). In snowfinches (*Montifringilla tacza-nowkii*), maternal and paternal body size jointly interact to affect sex allocation (Lu et al., 2013).

An ability for mothers to manipulate the sex ratios of their progeny, based on maternal allocation decisions, also fits well with the ability of reproducing birds and mammals to facultatively adjust the sex ratio of their offspring. For example, prenatal or nutritional stress may induce mothers to overproduce offspring of the less energetically costly sex (Wiebe and Bortolotti, 1992; Catalano et al., 2006; Song, 2012; Torche and Kleinhaus, 2012). Other factors, such as birth date (Barclay, 2012), mating season duration (Elmberg, 1990), operational sex ratios, and mating behavior (Werken and Charnov, 1978; West and Godfray, 1997), also can stimulate shifts in offspring sex ratios. Such facultative adjustments may be facilitated by an ancestral suite of mechanisms that enabled maternal control over offspring sex.

The adaptive significance of multifactorial sex determination in veiled chameleons remains unclear, because we know almost nothing about the ecology of this species in nature (Hillenius and Gasperetti, 1984). Nonetheless, our study adds to a growing body of research that reveals unexpected diversity and complexity within the sex-determining systems of reptiles. Current classification schemes for sex-determining systems need to be reexamined. Information on a greater diversity of study species would be useful; but a simple way to begin that re-evaluation would be for researchers to reanalyze their existing data in a multifactorial fashion, looking for interactive effects rather than simply main effects of putative causal factors. If this reevaluation shows the sex of hatchling lizards to be influenced by maternal allocation of yolk to the egg, then these animals may provide to be an ideal model system with which to explore how reproducing animals can manipulate the phenotypic traits of their offspring.

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