Repeateable intra-individual variation in plasma testosterone concentration and its sex-specific link to aggression in a social lizard

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ARTICLE INFO

Article history:
Received 19 November 2009
Revised 17 March 2010
Accepted 23 March 2010
Available online 31 March 2010

Keywords:
Animal personalities
Egernia whitii
Hormonal repeatability
Reptile
Sex steroids

ABSTRACT

Individual hormone profiles can be important generators of phenotypic variation. Despite this, work on the consequences of hormone profiles has traditionally ignored the large inter-individual variation within natural populations. However, recent research has advocated the need to explicitly consider this variation and address its consequences for selection. One of the key steps in this process is examining repeatability in hormone profiles and their links to behavioral traits under selection. In this study we show that individuals within a free-ranging population of the Australian lizard Egernia whitii exhibit temporal repeatability in their circulating baseline testosterone concentrations as well as their aggressive response towards conspecific intruders. Furthermore, we show significant, sex-specific links between testosterone and aggression. Specifically, testosterone and aggression is negatively linked in males, while there is no relationship in females. As conspecific aggression has significant consequences for fitness-related traits (parental care, mating strategies) in this species, inter-individual variation in testosterone concentrations, through their effects on aggression, could have important implications for individual fitness. We discuss the potential causes and consequences of hormonal repeatability as well as provide explanations for its sex-specific links with aggression. Specifically, we suggest that these patterns are the result of alternative hormonal pathways governing aggression within Egernia and may indicate a decoupling of aggression and testosterone across the sexes.

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Introduction

Individuals of the same species and sex may differ profoundly in both hormone profiles and hormone-related phenotypic traits. Although individual variation in circulating hormone concentrations has received increased attention since the postulation of the challenge hypothesis (Wingfield et al., 1990), these differences are usually studied in terms of environmental variation, especially social stimulation (Oliveira, 2004). Inter-individual variation of hormone levels characterizing individuals has so far rarely been studied, let alone in relation to inter-individual variation in behavior, despite repeated pleas for this (Zera et al., 2007; Wingfield et al., 2008; Williams, 2008) and its potential relevance to the field of animal personalities (Sih et al., 2004).

A crucial first step to understanding the consequences of inter-individual variation in hormonal profiles is to identify the extent to which individual profiles are repeatable across time and/or contexts (i.e., understanding intra-individual variation; Kempenaers et al., 2008; Williams, 2008). Identifying intra-individual repeatability in hormone profiles is crucial for two reasons. Firstly, it represents an initial step in disentangling the sources of variation in hormone profiles between individuals, i.e., the extent to which inter-individual variation reflects differences in underlying quality, as the result of heritable genetic variation or developmental programming (Schwabl, 1993; King et al., 2004), or differences in the social and/or non-social environment (Oliveira, 2004). Secondly, once repeatable individual differences in particular hormones or hormone profiles are identified, it allows one to further examine the potential links with variation in functionally significant hormone-dependent traits. For example, hormone repeatability after a response to hormone treatments in the dark-eyed junco (Junco hyemalis) is linked to variation in aggression which has significant implications for individual fitness via its effects on parental and territorial behavior (Jawor et al., 2006; McGlothlin et al., 2007). Combined, this approach allows for a better understanding of the role of the endocrine system in the organization and evolution of integrated phenotypic packages, or suites of traits (e.g., behavioral syndromes/animal personalities; Sih et al., 2004; Adkins-Regan, 2005; Kempenaers et al., 2008; Williams, 2008; Wingfield et al., 2008). Establishing a proximate basis for animal personality is crucial to our understanding of...
how these suites of traits are generated and the extent to which they can be uncoupled (Sih et al., 2004; Stamps and Groothuis, 2010).

Egerinia whitii is a social lizard species in which aggression, an ecologically important behavioral trait, exhibits strong repeatability within and between breeding seasons (Sinn et al., 2008; While et al., 2009a). Furthermore, an individual’s aggressive phenotype is linked to a number of fitness-related traits, including the proportion of extra-pair offspring (While et al., 2009a) and offspring survival (Sinn et al., 2008). Crucial to our understanding of the causes and consequences of this behavioral repeatability is knowledge of its proximate mechanisms (Sih et al., 2004). Therefore, the aim of this study is (i) to document repeatability of variation in baseline testosterone concentrations, a key mediating hormone of aggression within many vertebrate systems (e.g., Wingfield et al., 1987) and (ii) to assess the extent to which aggression and testosterone are linked in both males and females.

Methods

Study species

E. whitii is a medium sized (up to 100 mm snout–vent length (SVL)) viviparous lizard found throughout a broad altitudinal (0–1600 m) and habitat (coastal heaths, grasslands, and forests) range in south-eastern Australia. We studied E. whitii from the East coast of Tasmania, Australia (42°57’S, 147°88’E). Morphology and life history traits vary geographically in Egerinia (Chapple, 2005), but in Tasmania, males and females are sexually monomorphic, become reproductively mature at approximately 3 years, and display an overall lifespan of 9–10 years. Reproduction occurs annually, with mating occurring during the Austral spring (September–October) and gestation spanning 3–4 months (While et al., 2007). Birth of offspring occurs in the Austral summer (January–February), and each female usually completes parturition over several days (While et al., 2007; While and Wapstra, 2008). Egerinia populations are typically highly saturated, characterized by intense competition over limited permanent shelter sites (Chapple, 2003; O’Connor and Shine, 2004; Langkilde et al., 2005), which has led to the formation of small family groups (Chapple, 2003; O’Connor and Shine, 2003; While et al., 2009c).

Field protocol

All subject lizards were part of a larger life history study which has taken place across five subsequent reproductive seasons, 2004/2005 to 2008/2009 (see While et al., 2007; While et al., 2009a, b, c, d). All individuals in the population were therefore toe-clipped to allow for unique identification previous to the present study. At the beginning of each field season, all individuals in the population were captured using a mealworm ‘fishing’ technique. This was achieved by attaching a mealworm to the end of a fishing line and placing the mealworm in front of the lizard and lifting the lizard into a collection bucket once it had grabbed the mealworm. Once captured, individuals were measured for weight (± 0.1 mg) and length (snout–vent and total length, ± 0.5 mm using digital calipers), and sex was determined via visual inspection (the corner of the mouth). There was no effect of latency to bleed on an individual’s plasma testosterone concentration (Pearson’s correlation; $r = -0.18, N = 81, P = 0.11$). Subsequent to sampling, blood was immediately placed on ice until centrifugation (6000 rpm for 5 min) at the end of each field day. Plasma was stored at −25 °C until assayed.

Testosterone extractions and radioimmunoassay (RIA)

For extraction, we followed Kingma et al. (2009), with minor modifications. Briefly, samples were extracted twice by using diethyl-ether (70:30 v/v), dried under nitrogen, and then reconstituted in 70% methanol and stored over night. The following day samples were spun down, decanted, and dried. Competitive-binding radioimmunoassay (RIA) was used to determine the testosterone concentrations with anti-testosterone immunoglobulin coated tubes, with the antibody having 5.8 and 2.3 cross-reactivity with dihydrotestosterone and androstenedione, respectively (Diagnostic Systems Laboratories, Texas, USA). The average recovery rate was 78%. In order to validate the assay for the current species, we made dilution curves for four samples and confirmed parallelism. All samples were analyzed within one assay and the intra-coefficient of variation was 3.8%.

Conspecific aggression tests

To examine aggression levels, a total of 64 E. whitii were assayed for conspecific aggression at a single time period in 2008 (February 18–March 6), comprising 25 males and 39 females. Of these, 48 individuals had been assayed for conspecific aggression at two time periods during the previous field season (September/October 2006 and December/January 2006/2007; see Sinn et al., 2008; While et al., 2009a). To assay aggression levels, individuals were captured in the field and then transported to the University of Tasmania where they were housed individually in rectangular plastic terraria (30 x 60 x 40 cm) in temperature- and light-controlled rooms with lights set to ambient day lengths (Hobart, Tasmania, Australia). Housing terraria contained a basking rock and basking light at one end and a shelter at the opposite end. This provided a thermal gradient of 17 °C to 40 °C in the terraria, allowing females to bask to their preferred body temperature of ~34 °C (Bennett and John-Adler, 1986). Food (Tenebrio larvae, crushed fruit) and water were available ad libitum. Basking lights were set on a timer to come on 1 hour after room lights were turned on and to turn off 1 hour before room lights went off. The sampling period for testosterone and aggression assays in 2007/2008 were separated temporally (1 to 2 months) as blood samples collected during the time of aggression sampling during this field season were used for metabolic analyses.

A detailed description of the methodology for our conspecific aggression tests is contained elsewhere (Sinn et al., 2008; While et al., 2009a) and only relevant details are reported below. During each of the three tests periods, all individuals were subjected to two identical conspecific aggression tests given by a single experimenter (DLS or JMcE) on two testing days, 24 and 48 hours after capture. Behavioral tests were conducted between 14:00 and 17:00, allowing lizards to obtain preferred body temperatures before tests, and the test order for individual lizards was randomized on each test day. Behavioral tests consisted of the experimenter touching the lizard with a realistic conspecific clay model attached at the end of a fishing rod (model dimensions, head width: 15.7 mm; head depth: 12.3 mm; head length: 17.6 mm; SVL: 87 mm; for similar approaches, see Sinn and Moltschaniwskyj, 2005; Lopez et al., 2005). Models were scented with male and female Egerinia urine and faeces collected from unrelated laboratory animals. Subject lizards were presented with the conspecific model only if they were found, and remained on, the basking rock for a 60-s acclimation period at the start of tests. Lizards were touched on the center of the snout by the model up to 10 times and each touch was of similar duration (ca. 0.5 s), or until they fled into or on top of the shelter. For each test, four behaviors were measured: the number of touches required before the lizard fled, the number of back arches (a display
whereby the spine of the lizard is bent in a concave manner), the number of times the lizard displayed with an open mouth, and the number of times the subject actively bit the model. These behaviors closely resemble those recorded in antagonistic interactions within this and other species of *Egernia* (e.g., Langkilde and Shine, 2004; O’Connor and Shine, 2004; Langkilde and Shine, 2005; Langkilde et al., 2005; Langkilde and Shine, 2007; J. McEvoy, 2008, personal observation). Behaviors in tests were recorded for the duration of stimulus presentation (i.e., the number of touches). Multiple frequencies of each behavior were possible since lizards could perform behaviors anew after each touch with the model.

**Statistical analyses**

The four behaviors were highly inter-correlated and loaded strongly on a single common component in PCA analyses (Table 1). Therefore, to reduce the number of variables used in subsequent analyses and to facilitate use of a reliable single score (e.g., Buss and Craik, 1983), we computed aggregate scale scores (Tabachnick and Fidell, 1996; see Sinn et al., 2008; While et al., 2009a). A unique scale score for each lizard for each collection period was computed by summing the standardized frequencies of the observed variables in the two tests given within each collection period. Standardization was according to the grand mean of behaviors to mathematically allow for mean-level changes in aggregate scale scores. Higher scale scores represented more aggressive overall responses (see Sinn et al., 2008; While et al., 2009a).

We used the two-way random effects intra-class correlation coefficient (hereafter referred to as ‘repeatability’: Boake, 1989; McGraw and Wong, 1996) to establish whether individuals showed sex-specific repeatability in their between-individual variation in aggression and circulating testosterone concentrations. Repeatability of aggression has already been demonstrated over a 3-month period within this population of *E. whitii* (Sinn et al., 2008; While et al., 2009a). In this study, we calculated long-term repeatability, i.e., over an 18-month period (representing 3 sampling periods). Repeatability in the level of circulating testosterone was examined over a 3-month period (representing 2 sampling periods) (see above). We also examined population-level changes in testosterone in relation to both sex and season using a repeated-measures ANOVA, with sex as the between-individual factor and season as a within-individual factor (two levels: post-mating and at the end of gestation). We used the PROC GLM procedure in SAS to assess the influence of testosterone on aggression both at the end of the mating season and at the end of female gestation. Given the strong differences in testosterone concentration between the sexes (see Results), these models were run for each sex separately, resulting in four models (one for each sex for each season). In these models, aggression was entered as the dependent variable, with testosterone and snout–vent length entered as predictor variables. Two outliers were identified in female-only linear models during the post-mating season (>3 standard deviations outside the mean), so female models at this time were re-estimated excluding them. This did not change the interpretation of our results. As these outlier females had measures from a single time only (October/November post-mating sample), they were not included in repeatability or repeated-measures models.

All data were checked for violations of assumptions. The data for testosterone concentrations were non-normal and were subsequently log-transformed for all analyses. Sample sizes differ slightly between tests as not all target traits could be measured for all individuals at all times. Since our analyses was exploratory, GLMs started with the full model and we subsequently eliminated the interaction term when $p > 0.25$ (Quinn and Keogh, 2002). As a consequence, we here report results from models containing only main effects as the interaction terms were removed in each of the models following backward elimination ($p$ values = 0.40–0.65).

**Ethical note**

Collection of lizards and experimental methods were approved by the University of Tasmania Animal Ethics Committee and the Tasmanian Department of Primary Industries and Water. Toes were removed with surgical grade iris scissors that were disinfected before and after each foot/lizard. Full toes were removed, since partial natural digit loss is common in this species, similar to other skinks (Hudson 1996). Toe clipping of lizards involved clipping only one toe per foot and no visual signs of distress were observed during the procedure. Blood loss during toe clipping was either nonexistent or minimal and ceased within 2–3 s. Physiological stress responses (i.e., corticosterone) to toe clipping are several degrees of magnitude lower than stress responses to microchip implants in lizards and are no different from those induced by handling during size measurements (Langkilde and Shine, 2006). We did not observe any adverse effects on the lizards from biting the model lizard, which was soft and ‘gave way’ when bitten.

**Results**

Both male and female aggression scores were highly repeatable across the three sampling periods (September/October 2006, December/January 2006/2007, February/March 2008). Male aggression repeatability was $\rho = 0.77$ ($F_{10, 4} = 4.49$, $P = 0.002$, 95% CI = 0.38–0.93), and for females, repeatability was $\rho = 0.62$ ($F_{18, 36} = 2.63$, $P = 0.007$; 95% CI = 0.18–0.84), indicating a high level of intra-individual repeatability in aggressiveness across the 18-month sampling period (see Fig. 1). Males exhibited high levels of repeatability in circulating testosterone ($\rho = 0.98$, $F_{6, 8} = 46.61$, $P = 0.001$), with males with high circulating concentrations of testosterone in November also having high concentrations in January (and vice versa for males with low circulating

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Component one</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of touches</td>
<td>0.874</td>
</tr>
<tr>
<td>Number of back arches</td>
<td>0.875</td>
</tr>
<tr>
<td>Number of mouth open displays</td>
<td>0.854</td>
</tr>
<tr>
<td>Number of bites/attacks</td>
<td>0.781</td>
</tr>
<tr>
<td>% variance explained</td>
<td>72.597</td>
</tr>
</tbody>
</table>

**Table 1** Component matrix obtained from the principal components analysis for the *Egernia whitii* aggression tests. Matrix comprised of four aggressive behaviors (number of touches from model before lizard fled, number of times lizard arched its back, number of times lizard did a mouth open display, and number of times it bit/attacked the model) taken over three separate periods (twice in the 2006/2007 field season and once in the 2007/2008 field season, $n = 227$).
between males and females, with males having significantly lower testosterone concentrations (ng/ml plasma) at the end of gestation ($E. whitii$) for both sexes compared to the mating season (Fig. 3). In addition, there was no relationship between a female's circulating testosterone concentration and her aggressive phenotype both at the end of the mating season ($F_{1,14}=2.68$, $P=0.12$, Fig. 4b) and at the end of gestation ($F_{1,19}=3.13$, $P=0.09$), although the latter exhibited a weak positive trend. Body size (svl) did not predict an individual's aggressive phenotype for either males or females ($F_{1,14}=1.11$, $P=0.31$ and $F_{1,16}=0.11$, $P=0.74$, respectively) nor was body size correlated with an individual's concentration of circulating testosterone (males: $n=18$, $r=-0.11$, $P=0.57$; females: $n=23$, $r=0.21$, $P=0.39$).

Discussion

Despite the recent interest in the causes and consequences of repeatable behavioral differences among individuals across time and contexts (e.g., animal personalities), documentation of long-term repeatable variation in behavior is still relatively rare within wild vertebrate populations (but see Boon et al., 2008; Reale et al., 2009). Here we show that $E. whitii$, a viviparous social skink species, exhibit temporal repeatability in their aggressive response over an 18-month period. That is, both males and females were consistently more or less aggressive than others over a relatively long time span. This result supports our previous work documenting repeatability in aggression over a single reproductive season (Sinn et al., 2008; While et al., 2009a) and indicates a high degree of intra-individual stability in aggression for both males and females.

Crucial to our understanding of how this behavioral consistency is generated is knowledge of its proximate mechanisms (Sih et al., 2004). To address this, we also examined repeatability in circulating baseline testosterone concentrations, a key mediating hormone of aggression within many vertebrate systems (e.g., Wingfield et al., 1987). We found high intra-individual repeatability in hormone concentrations between the mating season and the end of (female) gestation for both males and females. These results indicate that, despite a mean-level decrease with season, individuals within this population maintained their circulating testosterone concentrations relative to all other individuals. While, there is some evidence for intra-individual repeatability in hormone production or sensitivity along the hypothalamic–pituitary–adrenal (HPA) axis (e.g., Cockrem and Silverin, 2002; Kralj-Fisher et al., 2007), this is the first time it has been shown along the hypothalamic–pituitary–gonadal (HPG) axis. Indeed, this is the first time that repeatability in un-manipulated baseline testosterone concentrations has been identified within a free-living vertebrate population, supporting recent work identifying repeatability in responses to testosterone treatments (e.g., GnRH challenges) found within the dark-eyed junco (Jawor et al., 2006).

The presence of such strong intra-individual repeatability in hormone profiles is of great potential importance to our understanding of how natural selection acts on endocrine traits as it represents an initial step in disentangling the sources of variation in hormone profiles between individuals (Adkins-Regan, 2005). For example, repeatability estimates can provide insights into the heritable nature of traits (Boake, 1989), thus repeatable intra-individual variation in circulating hormone concentrations could be the result of genetic or epigenetic (e.g., environmental influences on gene expression during ontogeny) differences among individuals (Kempenaars et al., 2008). Heritability estimates of endocrinological traits have rarely been documented, particularly for free-living populations where long-term endocrinological monitoring of populations with adequate sample sizes and pedigrees can make accurate estimates of heritability difficult to obtain (McGlothlin and Ketterson, 2008). However, repeatability could alternatively be the result of a stable environment. Due to high across-year stability of the social environment (e.g., home range size and habitat quality), factors that would act to maintain stable hormone profiles. However, further work is needed to determine the proximate mechanisms underlying such repeatable patterns in circulating hormone concentrations.
While identifying the presence of repeatable intra-individual variation is important, it is also crucial to examine co-variation between hormonal and phenotypic traits and the extent to which those traits relate to fitness (Williams, 2008). Testosterone has traditionally been assumed to activate aggression (e.g., Lincoln et al., 1972; Wingfield et al., 1987), including in reptiles (Marler and Moore, 1988; Weiss and Moore, 2004; Kabelik et al., 2008). However, recent work has challenged this view, suggesting this relationship is highly context dependent (e.g., dependent on the social context) and is often not observed outside the mating season (reviewed in Adkins-Regan, 2005). In line with this, we found a significant negative relationship between testosterone and aggression for males at the end of the mating season (see also Rubenstein and Wikelski 2005) whereas females showed no such relationship. Although relatively weak, these patterns were evident despite the temporal separation between aggression and testosterone assays (2 and 4 months) and the fact that baseline testosterone concentrations are known to be susceptible to small scale fluctuations, both of which could obscure the links between testosterone and behavior in the absence of a strong underlying relationship. One potential explanation for the negative pattern in males is that they are the result of alternative hormonal mediators of aggression, such as progesterone (Weiss and Moore, 2004) or estrogen (Schlinger and Callard, 1990; Silverin et al., 2004), both of which are precursors for testosterone. Therefore, the negative relationship between testosterone and aggression observed in this study may be an indirect effect of the relationship between testosterone and progesterone (or estrogen). The potential for interactions between multiple hormone traits to mediate behavior emphasizes the complex pathways by which hormones interact with the environment to influence phenotypic expression (DeNardo and Sinervo 1994; Zera et al., 2004).

If these results hold under further empirical scrutiny, they offer exciting prospects for future research. Firstly, they suggest that the effect of testosterone on aggression may be decoupled across the sexes (see also Clotfelter et al., 2004). This could be the result of asymmetries between the sexes in the costs and benefits of testosterone or their effects on aggression (Sandell, 2007). For example, maintaining high testosterone concentrations at key phases during reproduction can be detrimental to fitness-related traits such as female fecundity (Rutkowska et al., 2005, Veiga and Polo, 2008), immune function (Zysling et al., 2006), parental care (Clotfelter et al., 2004), mating decisions (McGlothlin et al., 2004), and the timing of egg laying (Clotfelter et al., 2004). Such costs may lead to sex-specific differences in the hormonal mechanisms underpinning aggression (e.g., Rhen et al., 1999; Elekonich and Wingfield, 2000; Davis and Marler, 2003; Jawor et al., 2006) or in the factors that influence phenotypic responses to hormone concentrations, such as the strength of the hormonal signal or the number of receptors (Godwin and Crews, 1997; Canoine et al., 2007; Sandell, 2007). Secondly, as aggression is a functionally important behavioral trait within Egeria (Sinn et al., 2008; While et al., 2009a), repeatable inter-individual variation in testosterone concentrations, through its effects on aggression, could have important implications for fitness. However, in order to fully understand the causes and consequences of the observed sex-specific relationship between hormones and behavior, further work exploring intra-individual variation of additional hormone traits and the up- and down regulation of steroids directly after aggressive interactions is required. In the long-term, such an approach will allow us to gain a greater understanding of the evolution and maintenance of suites of correlated traits, an outstanding issue in evolutionary biology (Sih et al., 2004; Gil and Faure, 2007; Williams, 2008).

Acknowledgments

We thank B. de Vries for laboratory assistance and guidance and T. Uller, S. M. Jones and two anonymous reviewers for valuable comments on earlier versions of the MS. This work was partially funded by the Environmental Futures Network, the Joyce Vickery Research Fund (to GW), the Holsworth Wildlife Research Fund, the Ecological Society of Australia (to GW and JMcE), the Winifred Scott

Fig. 4. The links between aggressive phenotype and circulating testosterone for male and female Egeria whitii at the end of the mating season [a (n = 15) and b (n = 17), respectively] and at the end of female gestation [c (n = 10) and d (n = 20), respectively].
Estate (to JMcE), and the Australian Research Council (to EW). G. White was funded by the Australian Research Council and C. Isaksen was funded by an NWO Rubicon fellowship (project number 825.07.004). All work complied with wildlife regulations imposed by the Tasmanian Department of Primary Industries and Water and the Animal Ethics Committee (permit numbers A0008055 and A0009737) at the University of Tasmania.

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