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Ontogenetic and mechanistic explanations of within-sex behavioral variation in a lizard with temperature- dependent sex determination

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Ontogenetic and mechanistic explanations of within-sex behavioral variation in a lizard with temperature- dependent sex determination

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Dedication

This dissertation is dedicated to my family for giving me everything.

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Ontogenic and mechanistic explanations of within-sex behavioral variation in a lizard with temperature- dependent sex determination

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The leopard gecko (*Eublepharis macularius*) is a reptile species in which embryonic temperature contributes both to sex determination and within- sex polymorphisms. Its life history makes the leopard gecko a model system for seeking ontogenic and proximate explanations for within-sex variation in sexually dimorphic behavior and neurophysiology, necessary attributes for reproductive success. For my dissertation I have incorporated the role of androgens that potentially modulate incubation temperature effects on behavioral and brain variation, which I approached using embryo and adult leopard geckos. First, I found that that the bias of same-sex clutch siblings is primarily incubation temperature- dependent and any maternal or genetic effects on same-sex clutch siblings are secondary. Second, I found that testosterone concentrations in the yolk-albumen were higher in eggs of late development than early development at 26 °C, a female-producing incubation temperature, but did not differ from eggs incubated at another female-biased temperature. This increase in testosterone concentrations during the temperature sensitive period in putative females is a finding opposite of reported trends in most other reptiles studied to date. Further, I found that the embryonic environment influences male sociosexual investigation in the absence of gonadal hormones. Lastly, in adult males of 32.5 °C, a male-biased incubation temperature, I found that the phosphoprotein DARPP-32 that is activated by the D1 dopamine receptor in limbic brain regions is correlated to this sociosexual investigatory

behavior. Neurons immunopositive for phosphorylated DARPP-32 were not only less dense in the nucleus accumbens of males who spent more time with other males, but also more dense in the preoptic area of males who spent more time with females. The use of phosphorylated DARPP-32 as marker for sociosexual exposure is novel in a lizard species. Taken together, in support of previous studies, these results show that differences in embryonic environment stem primarily from incubation temperature, can explain behavioral differences in adulthood in the absence of hormones, and, in concert with hormonal manipulation, can influence neuronal marker sensitivity to sociosexual exposure.

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CHAPTER 1: The leopard gecko as a model system for studying within-sex variation in behavioral neuroendocrinology

The leopard gecko is native to central and western Asia, including Iran, Pakistan, Afghanistan, and Turkmenistan. Generally, the topography of the region varies from valleys to foothills, with hot and dry summers and winter rains, and the sparse vegetation subject to seasonal change and anthropogenic development (Anderson 1963). Leopard geckos are terrestrial and nocturnal; they spend the day below the surface and under rocks, and feed on arthropods in the evening. Relative to other long-lived reptiles, leopard geckos are easily bred and reared in captivity. Hatchlings weigh about 3g and reach sexual maturity between 1-2 years, at about 55 and 45g for adult males and females, respectively, allowing studies to follow an embryonic manipulation with behavioral and physiological experiments in adults. The life history, sexually dimorphism, incubation temperature-based variation, and brain chemistry of the leopard gecko (*Eublepharis macularius)* makes it a feasible model system to study within-sex variation.

TEMPERATURE-DEPENDENT SEX DETERMINATION AND VARIATION

In many reptiles gonad sex is determined by the temperature of the incubating egg, a process termed temperature-dependent sex determination (TSD) (Crews 1994). In the leopard gecko extreme incubation temperatures (IncT) produce all-female (26–28 °C) or female-biased (34 °C) sex ratios (Bull, 1987; Gutzke and Crews, 1988; Viets *et al*. 1993; 1994; Crews *et al*. 1998). Intermediate IncTs result in biased sex ratios, with 30 °C producing a female-biased sex ratio (20:80) and 32.5 °C producing a male-biased (70:30) sex ratio (Figure 1). These temperatures are within the range of soil temperatures in the wild (Anderson 1963).

Leopard geckos have sexually dimorphic characteristics and stereotypical sociosexual behavior. Morphologically, males have a wider head and neck as well as pre-anal pores and hemipenes. Females are smaller, and have no detectable preanal pores, however testosterone treatment can induce hemipenile growth with females (Holmes et

al. 2005). While both males and females have translucent ventral abdomens, follicular vascularization and regression are only visible in the females.

Leopard geckos have stereotyped aggressive and sexual behaviors that differ between the sexes, but males and females both display sociosexual investigative and defensive behaviors. Both males and females detect the environment by air licks, where the tongue is flicked into the air or substrate. Leopard geckos also lick to assess the sex of conspecifics (Mason and Gutzke 1990). In captivity, females can cohabit with both sexes, whereas males can only cohabit with females and fight to the death with other males. Males will scent mark their enclosure by dragging the caudal region of the abdomen and preanal pores on the substrate, so that their hind limbs are splayed when walking forward. Females do not display territorial scent marking.

Though females are less aggressive than males, levels of aggression vary among temperature morphs (Flores et al. 1994), and both sexes display aggression by slowly undulating a raised tail in an 'S' shape. Males and high temperature females (Gutzke and Crews 1988) exhibit offensive aggression, assuming a high posture stance where their abdomen is lifted by straightening all limbs. Lunging attacks are preceded by the animal displaying a 'C' shape with the tail curved toward the head. Attacks are characterized by a gripping bite, which often results in serious tissue damage. This differs from a defensive bite, which is transient and displayed by both sexes. A defensive tail wave is not raised and flicks out to the tip instead of undulating slowly in the air.

Mating behavior is initiated and escalated in a series of progressive stereotyped male behavior. After licking a female, a male will vibrate the tip of his tail rapidly if aroused. The male will attempt to grip the female's tail in his jaws, and move his grip in a rostral direction to her flank and head, where he will align his body and mount by lifting his left or right limbs over the female. At this point the female lifts her tail and the male intromits one hemipenis. After a minute of copulation the male will loosen his head grip, dismount, and lick his hemipenes. Aroused males do not always successfully mate--unreceptive females will respond with a defensive bite and flee. Although leopard geckos have sexually dimorphic traits and stereotyped sexual behaviors, the varying degrees of

these traits within a sex are dependent on their incubation temperature. These IncT-dependent variations are addressed in subsequent chapters.

ORGANIZATIONAL AND ACTIVATIONAL ROLES OF HORMONES

In their classic study Phoenix et al. (1959) treated pregnant guinea pigs with exogenous androgen. The observation of the offspring as adults formed the basis of the Organization-Activation hypothesis. In this model hormones produced early in life (pre and postnatal adrenal and gonadal secretions) organize the brain such that these same steroid hormones produced late in adolescence and adulthood activate sex-typical behaviors. While the unified endocrine and genetic model is appropriate for mammals, it does not apply to vertebrates lacking sex chromosomes (Crews, 1993). Tinbergen (1963) framed proximate explanations for behavior as developmental (ontogenetic) and mechanistic processes. These two approaches can provide a conceptual framework when investigating androgen effects, in concert with effects of incubation temperature, on sociosexual behaviors in adulthood in TSD species. One tenet is that a window of environmental sensitivity in embryonic development allows for sex determination and, consequently, sexual differentiation. The extent to which maternal or endogenous steroid hormones interact with incubation temperature to influence sexual differentiation has yet to be described. Another point is the permanence of organizational effects. It is known IncT contributes to that variation in behavioral, morphological, and neural traits, with the results that within- sex variance can exceed the between sex variance (Sakata and Crews 2004). IncT also influences the effects of acute hormone treatment in adulthood (Rhen and Crews 2000). Finally, the leopard gecko allows investigation of the social context as a modifier of the transient, activational effects of androgens and can help explain the neurophysiological mechanisms underlying sociosexual behavior.

Considering the neuroendocrine and ethological framework established by Phoenix et al. (1959) and Tinbergen (1963), the following chapters seek ontogenetic and mechanistic explanations for within-sex variation in leopard gecko behavior (Figure 2). The studies consider environmental influence on within-clutch sex ratios at different

incubation temperatures, as well as interaction with androgen levels during embryonic development. In the context of social stimuli, studies examine adult male behaviors and neurophysiology to find causal explanations for within-in sex differences.

CHAPTER 2: Constraints on temperature-dependent sex determination in the leopard gecko (*Eublepharis macularius*)

INTRODUCTION

Sex steroid hormones, whether maternally derived or produced in situ during incubation, play a central role in gonad differentiation in reptiles with temperature-dependent sex determination (TSD) (Crews, Bull, and Wibbels 1991; P.K Elf, Lang, and Fivizzani 2002). The influence of maternally allocated hormones on sex determination may be species-specific; the sex ratio of the painted turtles (*Chrysemys picta*) is influenced by season and yolk hormones although similar correlations between yolk estrogens and within-clutch sex ratios are not seen in other turtles or lizards (R. M. Bowden, Ewert, and Nelson 2000; St. Juliana, Bowden, and Janzen 2004; R. Radder, Ali, and Shine 2007). Individual variation of clutch sex-ratio bias is found in the painted dragon (*Ctenophorus pictus*) though consistency within individuals may be genetic or hormonal (Uller et al. 2006). In the leopard gecko (*Eublepharis macularius*), temperature- sensitive steroidogenic enzymes and hormone profiles during incubation affect gonad and brain differentiation (Elf PK 2004; Sakata and Crews 2004; T. Rhen et al. 2006; Endo, Kanaho, and Park 2008). Studies of TSD in this species reveal a type II pattern with incubation temperatures (IncT) between 31 and 33°C produce male-biased sex ratios while temperatures 30°C and lower as well as 34°C and higher produce female-biased sex ratios; incubation temperatures of 26–28°C produce only females although no IncT has been identified that produces only males (Fig. 1) (Bull 1987; Gutzke and Crews 1988; Viets et al. 1993; Viets et al. 1994; Crews, Sakata, and Rhen 1998). There are two pivotal temperatures where the sex ratio of hatchlings is 1:1; the lower pivotal temperature (between 30 and 31°C) where sex ratio changes from female biased to male biased, and an upper pivotal temperature (between 33 and 34°C) where sex ratio changes from male biased to female biased (Mrosovsky and Pieau 1991; Viets et al. 1994). In the wild, these temperatures are within the range of soil temperatures (Anderson 1963). Sex

determination occurs during the early to middle stages of embryonic development, after which the bipotential gonads become irreversibly ovaries or testes (Bull 1987).

Within the family Eublepharidae, some species exhibit genotypic sex determination (GSD) while others exhibit TSD; in both sex determining systems, species have little intra-clutch variation of maternally derived yolk steroid hormones concentration (Kratochvíl, Kubička, and Landová 2006; T. Rhen et al. 2006). Maternal contributions to sex determination in the leopard gecko have only recently begun to be explored. Sex steroid hormones from the mother are deposited into the egg during follicular development, and the concentration of yolk steroids between eggs within a clutch have been found to be similar (Elf 2004; T. Rhen et al. 2006). The similarity of yolk steroid concentrations in eggs within a clutch therefore provide a good reason to study the leopard gecko in determining the extent of maternal influences in the context of temperature-dependent sex determination. Recently, Kratochvil et al. (2008) observed that unlike four GSD gecko species, in the leopard gecko siblings from the same clutch (two eggs sharing the same mother, father, and date of deposition) were always the same sex when both eggs were incubated at 30°C. The authors argued that the lack of mixed-sex clutches was attributed to the similar concentrations of yolk steroid hormones deposited during follicular development; in other words, maternally derived steroids constrain sex differentiation in geckos with TSD but not in those with GSD.

We have monitored sex ratios in our breeding colony of leopard geckos at the University of Texas at Austin and reading Kratochvil et al. (2008) motivated us to assess whether mixed-sex clutches were also absent in our large dataset. Breeding records from 1992 to 2001 were examined to: (1) compare the observed frequency of same- and mixed-sex clutches when both eggs in a clutch were incubated at 30°C (the same temperature as that reported in Kratochvil et al. (2008)) and 32.5°C, (2) reexamine the sex ratios across incubation temperatures, and (3) assess the magnitude of maternal contributions to sex ratios of clutches where eggs were incubated at different incubation temperatures.

METHODS

Generating and hatching eggs

Leopard geckos were bred in captivity at the University of Texas at Austin. Eggs were collected from moistened nest boxes within a day or two of oviposition. Clutches usually consisted of two eggs, though occasionally only of one egg. In same-IncT studies, both eggs within a clutch were incubated at either 30 or 32.5°C. In different-IncT studies, eggs within a clutch were split and incubated at different IncTs. Individual eggs were placed in plastic cups with moist vermiculite then covered with perforated plastic and maintained at a constant temperature within incubators (Precision Scientific, Chicago, IL, USA) monitored by calibrated mercury thermometers until hatching (Viets et al. 1993; Coomber et al. 1997). Across years, the moisture content ranged from 1:1 to 1.5:1 ratio of water: vermiculite. Moisture content during incubation has not been demonstrated to affect hatchling sex ratio in the laboratory under constant temperature (Viets et al. 1993); therefore, the data from 1:1 and 1:1.5 ratios of water: vermiculite were combined. The sex of the individual was determined by monitoring morphological development from hatching to sexual maturity (40–50 weeks). Individuals were assigned as males if they developed pre-anal pores and hemipenes bulges (Rhen et al. 2005).

Predicting frequencies of clutch siblings with different sexes

We used a $\chi 2$ test to examine whether the observed frequencies of same- vs. mixed-sex clutches differed from expected frequencies. We used the ratio of same- vs. mixed- sex clutches as a proxy for maternal effects because (1) females deposit sex steroids into the egg during follicular development, (2) steroid composition is comparable be- tween eggs within a clutch, and (3) because sex steroid hormones influence sex determination in TSD species (Elf 2004; Rhen et al. 2006), therefore, a maternal contribution would be manifested as an increase in the prevalence of same-sex siblings, We used a similar method to calculate the expected frequency of same- vs. mixed-sex clutches as Kratochvil et al. (2008). For the experiments in which we incubated both eggs

in a clutch at the same IncT, we used the observed frequency of males and females at each IncT to calculate the expected frequencies of same- and mixed- sex clutches. For example, at the IncT of 30°C, the expected ratio was calculated as $(30M)^2 + (30F)^2 : 2\times (30M) \times (30F)$, where 30M and 30F refer to the proportion of males and females, respectively, at 30°C, and 32.5M and 32.5F refer to the proportion of males and females, respectively, at 32.5°C. Instead of analyzing the frequency of two male siblings and of two female siblings separately as did Kratochvil et al. (2008), we simply asked whether the observed frequency of same- vs. mixed-sex clutches differed from expected. This was due to the hypothesis that maternal deposition of yolk steroids biases eggs in a clutch to be either both male or both female; there is no a priori prediction as to which scenario should dominate. Same- IncT clutches of 26 and 34°C were excluded from the analysis because the former produces only female offspring and the latter produces predominantly females (<5% males; Viets et al. 1993). We included data only from females who had a single clutch incubated at either 30 or 32.5°C to avoid pseudoreplication.

Another test of maternal contributions to sex determination is to examine whether the observed frequencies of same- vs. mixed-sex clutches differed from the expected frequencies when eggs within a clutch were separated and incubated at different IncTs. For example, if maternal deposition of yolk steroids strongly biases both embryos within a clutch to develop into females, then one should observe a higher incident of same-sex clutches even if eggs were separately incubated at 30 and 32.5°C; in other words, if maternal contributions are equally influential across various IncT, they should noticeably affect within-clutch sex ratios independently of IncT. For this analysis, we first computed the sex ratios at 30, 32.5, and 34°C from a group of hatchlings that was distinct the dataset used to analyze same-IncT and different-IncT clutches. Each of these hatchlings had a clutch sibling from a different IncT whose sex was unrecorded, so that no two individuals from the same clutch were used in the sex-ratio calculation. Eggs from the same clutch were separated and incubated in one of the following temperature pairs: 30 and 32.5°C, 30 and 34°C, or 32.5 and 34°C. Both 30 and 34°C are female- biased temperatures within a degree of the lower and upper pivotal temperatures, respectively

(Viets et al. 1993, 1994). The expected ratio of same- vs. mixed-sex clutches for clutches separated and incubated at 30 and 32.5°C, for example, was: (30M) × (32.5M) + (30F) × (32.5F):(30M) × (32.5F) + (30F) × (32.5M).

RESULTS

Analysis of eggs incubated at the same incubation temperature. We recorded the sexes of hatchlings from 32 clutches in which both eggs were incubated at 30°C and compared the frequency of same- vs. mixed-sex clutches against the expected frequencies. Of the 64 hatchlings, 20 developed into males and 44 developed into females (31.3% male). We observed both mixed- and same-sex clutches. Specifically, six mixed-sex and 26 same-sex clutches were observed. This observed frequency was significantly different from the predicted frequency of 13.75 mixed-sex and 18.25 same-sex clutches ($\chi 2 = 7.7$, df = 1, $p = 0.0056$; Table 1). Of the 30 hatchlings from 15 clutches in which both eggs were incubated at 32.5°C, 18 developed into males and 12 developed into females (60% male). Again, we observed both mixed- and same-sex clutches. Specifically, we observed six mixed-sex and nine same-sex clutches, and this observed frequency did not differ significantly from the expected frequencies of 7.2 mixed-sex and 7.8 same-sex clutches ($\chi 2 = 0.4$, df = 1, $p = 0.54$; Table 1).

Analysis of eggs incubated at different incubation temperatures Here, we analyzed whether the frequency of same- vs. mixed-sex clutches significantly differed from the expected frequencies of 128 clutches wherein clutches were split and the eggs placed in different IncT. The expected frequencies were calculated based on the observed sex ratios at 30, 32.5, and 34°C from a distinct set of hatchling data (514 hatchlings from 514 clutches); the sex ratios (percent male) at these IncTs were, respectively, 31.4%, 63.0%, and 7.0% (Figure 1). We analyzed instances in which eggs within a clutch were separated and incubated at 30 and 32.5°C, 30 and 34°C, and 32.5 and 34°C. In each case, the observed frequencies of same- vs. mixed-sex clutches were not significantly different from the expected frequencies (Table 2). This analysis indicates that IncT effects on sex

determination dominate potential maternal effects, and that high frequencies of same-sex clutches are not seen when eggs of one clutch are separated into different IncTs.

DISCUSSION

Based on the observation in the leopard gecko (*E. macularius*) that siblings were always either both male or both female (i.e., same-sex clutches) when both eggs within a clutch were incubated at 30°C, Kratochvil et al. (2008) hypothesized that maternal contributions (via sex steroid hormone deposition into the egg) significantly constrain sex-determining mechanisms. Here, we analyzed a larger dataset of offspring sex ratios from our colony of leopard geckos and assessed maternal contributions to sex ratios using two approaches. Maternal contributions were estimated by comparing the frequencies of same- vs. mixed-sex clutches for clutches in which both eggs were incubated at 30 or 32.5°C as well as for clutches in which eggs were separately incubated at 30, 32.5, or 34°C; therefore, we provide a more extensive test of maternal contributions to sex determination in this species.

If maternal contributions were significant, the frequency of same-sex clutches should be greater than expected for each experimental manipulation. First and foremost, in contrast to the data presented in Kratochvil et al. (2008), our analysis revealed the occurrence of mixed-sex clutches (20%) when both eggs within a clutch were incubated at 30°C (Table 1). We also observed mixed-sex clutches (40%) when both eggs in the clutch were incubated at 32.5°C (Table 1). Second, the observed frequency of same- vs. mixed-sex clutches was significantly different from expected at 30°C but not at 32.5°C (Table 1); this suggests a maternal contribution, albeit a weaker one than that observed in Kratochvil et al. (2008). This notion of relatively weak maternal contributions to sex determination was supported by the fact that the observed frequencies of same- vs. mixed-sex clutches were not different from expected when eggs within a clutch were separately incubated among 30, 32.5, and 34°C (Table 2). Taken together, these data strongly support the model proposed by Sakata and Crews (2004) that incubation temperature sets the threshold by which sex is determined via temperature-sensitive

mechanisms that interact with circulating hormones or generate new hormones. The incubation temperature-as-threshold model is supported by temperature- correlated differential decreases in yolk steroid concentrations in leopard geckos as well as turtles (Elf et al. 2002). The model is also open to the possibility of temperature- sensitive gonad differentiation in the absence of yolk hormones or other steroidogenic tissue (Jeyasuria and Place 1998; Pieau and Dorizzi 2004; Ramsey and Crews 2007; Endo, Kanaho, and Park 2008). Likewise, reptiles with GSD have sex ratios altered by certain temperatures, or even exogenous hormones; one mechanism of sex determination is not always exclusively found within a species (Shine et al. 2002; Quinn et al. 2007; R. S. Radder et al. 2008).

The difference in absence and presence of mixed-sex clutches between Kratochvil et al. (2008) and our results is possibly due to clutch effects from incubation methods or gene × environment interaction. Both studies incubated eggs within a clutch at constant temperatures, but the method of incubating eggs was slightly different; Kratochvil et al. (2008) placed eggs within the same plastic container, whereas we incubated eggs individually in covered plastic containers. It is possible that eggs placed in the same enclosed container are exposed to the same concentrations of metabolic waste such as heat and carbon dioxide, which can subsequently homogenize the external nesting environment that may not necessarily clarify the role of maternal contributions. Heat would be less uniform within a clutch where eggs are incubated individually, potentially creating differences in temperature during incubation (Ewert and Nelson 2003). Carbon dioxide concentration, which alters the activity of pH-sensitive 5-alpha-reductase, can subsequently homogenize steroidogenic activity of eggs in the same container (Jeyasuria and Place 1998). Neither our study nor that of Kratochvil et al. (2008) measured temperature or carbon dioxide concentration next to each egg. Differences in the observed sex ratios at 30°C cannot account for the presence of mixed-sex clutches, as our observed sex ratio at 30°C was comparable to that reported in Kratochvil et al. (2008), and, moreover, our reported sex ratios across incubation temperatures followed the same type II pattern as observed by Viets et al. (1993). The presence of mixed-sex clutches at

30°C, albeit at a frequency lower than expected, could result from gene× environment interactions (Janes and Wayne 2006). One clutch was represented per mother, but some fathers had more than one clutch represented. As we have aimed to follow up on Kratochvil et al. (2008) in the context of maternal contribution in TSD reptiles, we must further assess gene × environment interaction with full and half siblings with the same father.

The magnitude of maternal influences via yolk steroid hormone deposition on sex-determining mechanisms is unclear. In the leopard gecko, the concentration of yolk estradiol is positively correlated to circulating estradiol levels in gravid females, indicating that individual differences in circulating estrogens during follicular development could lead to variation in the amount of estrogens deposited into the egg (T. Rhen et al. 2006). The influence of estrogens on female development in this species as well as other TSD species is well-established, suggesting that maternal estrogen deposition into the egg could bias hatchlings to develop into females (Bull et al. 1988; Rhen et al. 1994; Tousignant and Crews 1994). We have previously documented that initial yolk steroid hormones do not differ significantly among eggs within a clutch due to synchronous follicular development and oviposition (Elf 2004; Rhen et al. 2006). Therefore, maternal contributions to gonadal differentiation should be comparable across eggs within a clutch. Despite similar initial yolk concentrations, we observed instances in which hatchlings from eggs incubated at the same incubation temperature developed into a male and female sibling (mixed-sex clutches; Table 1) and the frequency of same- vs. mixed-sex clutches was not significantly different from expected when clutches were split across IncTs (Table 2). Consequently, if maternally derived yolk steroids do influence sex determination in this TSD lizard, it is of secondary importance to the temperature of the incubating egg.

Our primary objective of the current study was to assess the extent to which maternal effects influence hatchling sex within a clutch observed by Kratochvil et al. (2008). Specifically, we set out to examine whether the frequency of same- vs. mixed-sex clutches, a proxy for maternal contributions to sex determination as used in the previous

study, deviated from expected under a variety of experimental manipulations. Our objective was not to fully quantify maternal contributions to sexual differentiation; this would require a more extensive analytical approach akin to techniques used in quantitative genetic analyses. Unlike Kratochvil et al. (2008), we observed that in the same-incubation temperature studies, 20% and 40% were mixed-sex clutches in which both eggs were incubated at either 30 or 32.5°C, respectively. Furthermore, by testing maternal contributions when eggs within a clutch were incubated at different temperatures, we failed to find consistently significant maternal effects. Taken together, we documented much stronger support for the notion that incubation temperature sets the threshold to which yolk steroid hormones influence sex determination.

CHAPTER 3: Yolk-albumen testosterone in a lizard with temperature-dependent sex determination: relation with development

INTRODUCTION

Yolk steroid hormones contribute to phenotypic plasticity by modulating development. The function of yolk steroid hormones has been described as providing information from the environment via maternal effects. In birds, environmental factors correlated with yolk steroid hormone fluctuations include proximity to nesting conspecifics, maternal condition, and egg or clutch laying order. Yolk steroid hormone differences, natural and manipulated, can contribute to differences in juvenile social rank, begging behavior, and growth rate, as well as changed secondary sexual characteristics and behavior in adults (Schwabl 1993; 1996; Adkins-Regan et al. 1995; Strasser and Schwabl 2004; Partecke and Schwabl 2008; Bonisoli-Alquati et al. 2011). In various lizard species with genotypic sex determination, sex can be correlated or uncorrelated to yolk steroid hormone concentrations (Lovern et al. 2001; Radder et al. 2007). In an Australian montane skink species (*Bassiana duperreyi*), Dihydrotestosterone is higher in smaller eggs, and that smaller eggs are more likely to produce males(Radder et al. 2009).

In reptile species with temperature-dependent sex determination (hereafter TSD), gonadal sex is determined by incubation temperature during a thermosensitive period in development. Gene expression patterns in the–undifferentiated gonad is initiated by incubation temperature at the beginning of the thermosensitive period, and is sex specific but varies across taxa (Shoemaker et al. 2007; Matsumoto and Crews 2012). After this window, the development of testes or ovaries is not affected by exogenous hormones or temperature changes (Bull 1987; Bull, Gutzke, and Crews 1988). The influence of exogenous steroid hormones on gonadal sex has been studied in various reptile species, mainly turtles. In painted turtles (*Chrysemys picta*), at a 28ºC incubation temperature, the ratio of testosterone: estradiol is correlated with the sex ratio of clutches and season (Bowden et al. 2000). It has been noted in most oviparous species regardless of the mode of sex determination that yolk steroid hormones declined during development (Conley et

al. 1997; Bowden et al. 2002; Elf et al. 2002), and the mechanism for yolk estradiol metabolism has recently been described (Paitz et al. 2012). Jeyasuria and Place (1998) proposed that testosterone acts as a substrate for the enzymes aromatase or 5a-reductase that furthers sexual differentiation in the brain, although the source, from local production in the brain, gonads, or yolk, was not specified. Aromatase activity has been found in the whole brain during the thermosensitive period of TSD reptiles, although its function has yet to be determined (Willingham et al. 2000; Endo et al. 2008).

In the leopard gecko (*Eublepharis macularius*), incubation temperature determines gonadal sex; low and high incubation temperatures (26 and 34 °C respectively) produce either only females or female-biased sex ratios, (100 vs 95% females respectively), while an intermediate temperature (32.5 °C) generates a male-biased sex ratio (approximately 75% males) (Viets et al. 1993). The embryo is at developmental stage 28 at oviposition, experiences a thermosensitive period from stages 32 to 37, and hatches at stage 42 (Bull 1987; Wise et al. 2009). Incubation time is temperature-dependent; embryos incubated at lower temperatures spend more time at each stage than those incubated at high temperatures (Bull 1987; Tousignant and Crews 1994; Valleley et al. 2001; Endo et al. 2008).

Incubation temperature also contributes significantly to adult leopard gecko intrasexual polymorphisms; males and females from each incubation temperature exhibit significant within-sex variation hereafter called temperature morphs (Sakata and Crews 2004). Animals of both sexes differ between male- vs. female- biased incubation temperatures, as well as between two female-biased incubation temperatures. Although the 26 °C and 34 °C incubation temperatures produce exclusively or predominantly females, temperature morphs from the 34 °C incubation temperature are more aggressive, reach sexual maturity later, and have higher levels of circulating corticosterone relative to females from the 26 °C incubation temperature, but they do not differ in baseline circulating sex steroid hormones (Flores et al. 1994; Tousignant et al. 1995). The two temperature morphs also differ in the metabolic capacity of hypothalamic regions associated with male-typical behavior (Coomber et al. 1997).

Studies demonstrate that steroid hormones activate sociosexual behaviors in leopard geckos (Sakata and Crews 2004), and relatively little is known about the levels of steroid hormones in the egg (Elf 2004; T. Rhen et al. 2006). Females treated with estradiol *in ovo* did not display significant growth differences at either a female-biased or male-biased incubation temperature (Tousignant and Crews 1995), though it is known that estradiol can reverse the sex in these species (Bull et al. 1988). Like some other oviparous species, maternal condition does influence yolk steroid hormone concentration; in this species dihydrotestosterone concentration has an inverse relation to female mass depending on the laying season (Rhen et al. 2006).

It is important to note that there is no distinct separation between the yolk and albumen in leopard gecko eggs, therefore the extra-embryonic material is best described as yolk-albumen (YA). The leopard gecko system provides a unique opportunity to look at YA testosterone concentration across development within a sex, at two female-biased incubation temperatures with an 8 °C difference. Because of the large difference in incubation temperatures that both produce putative females, any hormone difference detected would be due to incubation temperature and less likely sex differences.

In this experiment, we measured testosterone concentrations in the YA of eggs incubated at 26 or 34 °C collected in either early or late in development to answer (1) how YA testosterone fluctuates during embryonic development, and (2) if the YA steroid hormone concentration across development is temperature-dependent. We predicted that YA testosterone levels would decrease during development, similar to most amniotes. Finally, we wanted to examine how the hydric state of the YA varied between high and low incubation temperatures across development, as different YA water content may provide a varying microenvironment *in ovo,* and other TSD species can influence sex determination at low incubation temperatures (Gutzke and Paukstis 1983; Reed and Vleck 2001). Despite the water exchange-conducive, parchment-like structure of leopard gecko eggshells, we predicted no difference in YA water content between incubation temperatures during early development due to the same initial hydric environment *ex ovo* (Werner 1972).

METHODS

Egg collection

Eggs and samples were collected in accordance of Institutional Animal Care and Use Committee (IACUC) protocol AUP-2008-00135. Eggs were collected from Tremper Leopard Gecko (Boerne, TX, USA), a leopard gecko breeder in July 2009. Within each breeding group, eggs could not be attributed to individual geckos, but all egg-laying females were of the same incubation temperature (26 °C for 2/3 of their development time). Eggs collected and frozen within 4 days of oviposition were considered "oviposition" with embryonic stages to be determined. The eggs were incubated for 7-30 days in a commercial incubator (Precision Instruments, OH, USA) at either 26 or 34°C. After incubation, eggs were frozen at -20°C until YA collection. Eggs frozen after seven days incubating at 34 °C and 14 days at 26 °C were categorized as "Early", and after 17 days incubating at 34 °C and 30 days at 26 °C were categorized as "Late". The days of incubation that correspond with early and later developmental stages including the thermosensitive period were estimated from Bull (Bull 1987) and Endo et al. (2008). Frozen YA was separated from embryos, which were staged according to Wise et al. (Wise et al. 2009).

Radioimmunoassay

The yolk-albumen fraction collected from eggs was used to measure testosterone concentrations using a competitive-binding radioimmunoassay (RIA) with testosterone-specific antibody (Wien Laboratories T3003). The samples were divided into two assays, with each assay containing an equivalent number of samples from each treatment group. The RIA protocol followed that of Schwabl (1993). For each egg, 50 mg YA was weighed and homogenized in 1ml water. To calculate recoveries, 2000 cpm of tritiated testosterone (Perkins Elmer NET553, Boston, MA USA) was added per sample.

After equilibrating over night, samples were extracted with 6ml diethyl ether/petroleum ether (70:30, v:v), dried with nitrogen and reconstituted in 1ml ethanol in

-20 °C overnight. To pellet neutral lipids, samples were spun at 2000 rpm for 5 minutes, supernatants were dried with nitrogen at 37 °C. Samples were then re-suspended in 500 ml 10% ethyl acetate in isooctane (2,2,4-trimethylpentane) before being transferred to celite columns consisting of water phase and a propylene glycol/ethylene glycol (1:1, v:v) phase. After increasing concentrations of ethyl acetate in isooctane, testosterone samples were collected with 4.5 ml of 20 % ethyl acetate in isooctane. The average recovery rate of testosterone for the samples was 51%. The intra-assay coefficients of variation were 26.6 % and 13 % respectively. The interassay coefficient of variation was 26.6 %.

Yolk-Albumen dehydration

Because YA water content can vary among eggs, with the possibility of dried YA mass increasing during development (Reed and Vleck 2001), dried weights of the samples were measured. To determine how YA water content was related to embryonic development or incubation temperature, YA was aliquoted in a pre-weighed 1.5 ml Eppendorf centrifuge tubes (Eppendorf, Hamburg, Germany), and dried overnight at 45 °C. The YA aliquot and tube were collectively weighed before and after drying. Water content was calculated as the difference between the before and after drying YA weight. The dry YA weight was the difference between the after drying weight and the tube weight. The dry YA weight divided by the aliquot weight gave the proportion of YA as dry weight. This fraction multiplied by 100 gave the percent dry weight. As these aliquots were not taken at the same time as the radioimmunoassay aliquots, some samples for RIA were not available for dehydration.

Statistics

R: A Language and Environment for Statistical Computing was used for data analyses (R Foundation for Statistical Computing, Vienna, Austria 2012). A Pearson product correlation test was performed on samples of all developmental stages to detect correlation between developmental stage and YA testosterone concentration. Considering YA taken at the thermosensitive period, two factors were considered as factors influential

to YA testosterone concentration: development (Early vs. Late) and incubation temperature (26 ºC vs. 34 ºC). The combination of factors generated four treatment groups: Early26, Early34, Late26 and Late34.

The combinations made four groups and each was normally distributed (Shapiro-Wilk $p > 0.05$). However, sample sizes were taken to account and the non-parametric analysis was used. Kruskal-Wallis with *post hoc* pairwise Wilcoxon rank-sum test with Benjamini-Hochberg corrections was used to detect the effects of development and incubation temperature on YA testosterone and water content(Benjamini and Hochberg 1995). To detect an influence of development on dry YA weight, a Pearson product correlation test was performed.

RESULTS

Yolk-Albumen testosterone concentration

Analysis of our radioimmunoassay data detected a significant difference in YA testosterone concentration between early and late development but no difference between low and high female-biased incubation temperatures (Figure 3). One egg frozen in the "oviposition" category had a developmental stage in the thermosensitive period, and was excluded from analysis of thermosensitive period because it did not undergo an incubation treatment. Some eggs frozen at the "Late" interval (see Methods) had embryos that had developed beyond the thermosensitive period; these eggs were excluded when considering effects of thermosensitive period.

Including YA testosterone measurements from eggs at all recorded developmental stages (stage 28 to 41), there was a significant positive correlation between yolk-albumen testosterone concentration and developmental stage ($df = 51$, $p = 4.2e^{-7}$, $r^2 = 0.40$). During the thermosensitive period, YA testosterone concentrations differed among the four treatment groups (Kruskal-Wallis $X^2 = 14.801$, $p = 0.002$). The ranges of stages in Early and Late development were 33 to 35 and 36 to 37, respectively. A *post hoc* pairwise Wilcoxon rank-sum test with Benjamini-Hochberg corrections

indicated that the Early vs. Late development difference in YA testosterone concentration was detected at 26 ºC but not at 34 ºC incubation temperature ($p = 0.003$, and $p = 0.056$, respectively).

Dried Yolk-Albumen weight

There was a significant positive correlation between the dry YA weight and developmental stage ($df= 50$, $p = 3.4e^{-4}$, $r^2 = 0.23$), but there was no significant difference between 26 or 34 ºC incubation temperature at either early or late thermosensitive period (Kruskal-Wallis $X^2 = 0.8487$, $df = 3$, $p= 0.8378$). Nor was there significant correlation during the thermosensitive period between the dry YA weight and YA testosterone concentration at either 26 or 34 ºC ($p = 0.085$, $p = 0.16$, respectively).

DISCUSSION

In order to describe changes in testosterone across development in the leopard gecko, we measured egg YA testosterone concentrations Early and Late during the thermosensitive period. We had predicted that, according to most other studies of oviparous animals with genetic and temperature-dependent sex determination, testosterone concentration in the YA would decrease during embryonic development, possibly as a consequence of steroid metabolism. However, we detected the opposite, where concentration of testosterone in the YA was greater in eggs at the Late temperature-sensitive period than at the Early temperature-sensitive period, specifically at the 26 °C incubation temperature. There was a similar trend in YA testosterone concentration at 34 °C but the difference between Early vs. Late was not significant. Our data suggest that females of this Eublepharid species with temperature- dependent sex determination experience an increase in the concentration of testosterone in YA during development. In addition, although we detected a loss of YA water content across development, the YA water content remained similar during the thermosensitive period, and did not differ between incubation temperatures.

Testosterone concentration in the YA of eggs did not differ between 26 and 34 °C incubation temperatures during development. These findings complemented other studies of steroid hormone exposure, such as hormone synthesis and sensitivity during development. In leopard gecko embryos from 26 and 34 °C incubation temperatures, steroidogenic enzyme p450scc and androgen receptor expressions are detected in both the brain and gonad-adrenal-mesonephric complex at all stages of the thermosensitive period, but aromatase expression patterns across development are different (Endo et al. 2008). Steroidogenic enzyme aromatase expression is not detected in the gonad-adrenal-mesonephric complex of embryos at 26 ºC incubation temperature until the late thermosensitive period, unlike embryos of 34 ºC incubation temperature. However, in other TSD species gonadal activity separate from the adrenal and mesonephros have shown a different gene expression timeline (Ramsey and Crews 2007). Alternative explanations are that behavioral differences in 26 and 34 ºC incubation temperature females do not originate during the thermosensitive period, or that the behavioral differences are not organized by testosterone in the YA.

The increase over time in testosterone concentrations in YA suggests an increase in gonad steroidogenesis occurring in Early vs. Late temperature-sensitive period, across five developmental stages. Although YA water content decreased during development, the decrease was not detected during the temperature- sensitive period (stages 33-37) at either 26 or 34ºC. It is worth noting that the aliquots were taken from the same stock sample but not taken at the same time, but each egg YA was homogenized before collecting the stock sample. Another possible shortcoming is that in Late thermosensitive period samples, the YA becomes vascularized and hormones in circulating blood vessels could potentially contribute to an increase in testosterone concentration. However with frozen samples, it is easy to identify and remove the vessels from YA, so there was minimal input in our samples from circulating steroids present in the vasculature.

In the context of other species, the pattern of increasing YA testosterone is in the minority. In contrast to species with decreasing yolk steroid hormone, it is suggested that developing leopard gecko embryos may be generating testosterone during the

thermosensitive period, in addition to metabolizing YA testosterone. A few studies in species with genetic sex determination have reported an increase in steroid hormones in later development, possibly attributing the increase to gonad and adrenal steroidogenesis or decrease in absolute yolk volume (Lovern and Wade 2001; Pamela K. Elf and Fivizzani 2002). In other TSD species, estrogen metabolites move between the yolk and embryo, suggesting that other steroid hormones, if conjugated and polar, can also move between yolk and embryo during development (Paitz, Sawa, and Bowden 2012). Neither the absolute YA volume nor gonadal steroidogenesis for these leopard gecko samples were quantified, so it is possible that both factors can contribute to the measured increase in testosterone concentration across development. Recently, the yolk testosterone level of another gecko species with TSD (*Gekko japonicus*) has been described across development among different incubation temperatures, with similar results; the two female-biased incubation temperatures did not differ in yolk testosterone levels, but both experienced an increase in testosterone from 1/3 to 2/3 of their incubation time (Ding et al. 2012). It was suggested that the increased yolk testosterone concentration followed a proposed mechanism in the GSD green anole (*Anolis carolinensis*), that nonpolar steroids generated by the embryo enter the lipophilic yolk (Lovern and Wade 2003), but steroidal movement is also possible in a conjugated state (Paitz et al. 2012). Further work is needed to determine whether the observed increase in YA testosterone is the product of embryonic steroidogenesis, and to compare the mechanisms between opposing changes in YA steroid hormones.

In summary, the most important finding was that the YA testosterone concentration increased during development in eggs, specifically at the female-biased incubation temperature of 26 ºC. This is an uncommon examination of within-sex variation of YA hormones in a species with TSD where a developmentally dependent increase was found, that there was no incubation temperature difference in YA testosterone concentration, even considering a decrease in YA water content during development. Because it is possible to separate sex and temperature effects in this

system, future studies focused on determining the source of YA testosterone may be particularly interesting.

CHAPTER 4: Differences induced by incubation temperature, versus androgen manipulation, in male leopard geckos (*Eublepharis macularius*)

INTRODUCTION

Organizational Contributions to Intra-sexual Variation

The embryonic environment is the prologue to life experiences, influencing morphological and physiological development and priming the individual to behave in a certain way when exposed to future stimuli. This early organization of adult morphological and behavioral characteristics generally are related to dimorphic traits and stereotypical behavior (Arnold and Breedlove 1985). The variation within a sex for any given trait, however, is greater than the average difference between the sexes. This variance is often thought of as the spectrum of masculinity and femininity within a trait among males and females. One of the earliest descriptions of this within-sex variation is the 'freemartin', a condition in cattle in which there is anastomosis of the placentae, resulting in the female twin being both masculinized and defeminized while the male twin is normal (Lillie 1916). Similarly, Vom Saal and Bronson (1978; 1980) described the intrauterine position effect of one female between two males to become a less attractive and more aggressive adult than a female that developed next to females in utero. Conversely, estradiol exposure from neighboring sisters *in utero* demasculinizes male behavior (vom Saal et al. 1983).

Long-term behavioral variation can also originate from maternal condition in both oviparous and viviparous species. Stressing pregnant rats can prevent a normal testosterone surge in fetal males, producing sons that as adults have demasculinized play behavior(Ward and Stehm 1991). Both sexes of juvenile viviparous common lizards (*Lacerta vivipara*) alter their basking behavior depending on the corticosterone levels of their mothers during embryonic development, but only the female sex experience change in escape behavior after prenatal hormone manipulation (Belliure et al. 2004). In ring-

necked pheasants (*Phasianus colchicus*), males with testosterone treatment *in ovo* have greater copulation frequency as well as wattle hue (Bonisoli-Alquati et al. 2011a; 2011b). The sex that is affected by yolk testosterone depends on the species, reflecting the diversity of maternal effects among avian taxa (reviewed in Groothuis, et al. 2005; Gil 2008).

While prenatal environments have a resonating effect on individuals in adulthood, circulating hormones are important to activate reproductive behavior. Androgens, both testosterone and the non-aromatizable dihydrotestosterone, as well as estradiol, are important in permitting male-typical behavior in a social context (Arnold and Breedlove 1985; Moore and Lindzey 1992). In females, estrogens, in combination with progesterone and testosterone, facilitate female-typical reproductive behavior (reviewed in Beach 1976).

Temperature Polymorphism in Leopard Geckos

In species with temperature-dependent sex determination (**TSD**), it is the temperature experienced during the mid-trimester of development that determines the sex of the offspring. In the leopard gecko (*Eublepharis macularius*) an incubation temperature (**IncT**) of 26 °C produces only female offspring, while IncT of 30, 32.5 and 34 °C produce approximately 25%, 75%, and 5% males, respectively (Viets et al. 1993). In both males and females individuals from different incubation temperatures (hereafter Temperature Morph), vary predictably in brain, behavior, and physiology.

As in other vertebrates, the preoptic area (POA) and ventromedial nucleus of the hypothalamus (VMH), are associated with male-typical and female-typical reproductive behaviors (Dörner et al. 1968; Crews and Silver1985; Moore and Lindzey 1992), respectively. In leopard geckos the POA is not sexually dimorphic in size, but in metabolism (Crews et al. 1996; Coomber et al. 1997). Rather, within each sex the size of the POA depends upon IncT such that it is larger in individuals from a male-vs. female-biased IncT; the opposite finding is evident in the VMH (Coomber et al. 1997). Testosterone treatment in one year-old adults increases POA and decreases VMH

volumes in both males and females at a male-biased IncT, but not in 26 °C IncT females, demonstrating an intersection of temperature polymorphism with sexual dimorphism in hormone sensitivity (Crews et al. 1996).

Males of different Temperature Morphs also differ in the time spent with females according to the female's IncT (Putz and Crews 2006). Males exhibit both stereotyped copulatory and aggressive behaviors, and within males there is temperature dimorphism in sexual activity and aggression among morphs (Sakata and Crews 2003). Females from a 30 °C IncT are more attractive to 32.5 °C IncT males and less aggressive than their counterparts from a 32.5 °C IncT (Flores et al. 1994; Sakata and Crews 2003). Consistent with these behavioral differences, both males and females from 30 °C IncT have higher circulating estradiol levels than individuals from 32.5 °C IncT (Flores et al. 1994; Sakata and Crews 2003). Behavioral and neural studies primarily address the two aforementioned Temperature Morphs, but 34 °C males, like 30 °C males differ from male-biased 32.5 °C IncT males in lower circulating androgen:estradiol ratio (Coomber et al. 1997).

This is the first thorough study of the behavior of the few males that are produced at the extreme, female-biased incubation temperature of 34 °C. We sought to determine if (1) IncT-dependent behavioral polymorphisms in male sociability extend to include a male temperature morph at a higher IncT, and also an extreme, lower male sex ratio, (2) whether exogenous androgen affects this behavior in the social context of both male and female conspecific stimuli. Based on previous aggressive and sexual behavioral studies of hormonally-manipulated 30 °C IncT and 32.5 °C IncT males, we predicted that differences in sociability behavior would originate between geckos of a different female-biased IncT, 34 °C vs. 32.5 °C IncT, particularly that the differences would still be detected in Temperature Morphs with the same hormonal manipulation. We also predicted that androgens would modulate sociosexual behaviors in 32.5 °C IncT males.

MATERIALS AND METHODS

Animals

All animals were hatched from eggs of controlled matings in the laboratory. Eggs were incubated individually in covered plastic cups containing a water:vermiculite ratio of 1:1 (w:v) in an environmental incubation chamber (Precision, Illinois) monitored by HOBO temperature logger (Onset Computer Corp, Pocasset MA). Hatchlings were fed live crickets every other day for 10 weeks and received water *ad libitum*. As juveniles and adults their diet changed to vitamin-dusted mealworms. Animals were housed individually and maintained on a 14:10 day: night cycle. Animals were sexed early in adulthood using prominent secondary sexual characteristics (presence of hemipenes and preanal pores) and their sex was confirmed by presence of testes during castration. Adult male geckos between 4-6 years old from 32.5 °C IncT or 34 °C IncT were used. All work was approved by the University of Texas at Austin Institutional Animal Care and Use Committee protocol number 07022603.

Hormonal Implants

All animals were castrated under hypothermia anesthesia in accordance with IACUC protocol. Males from 32.5 °C IncT were given a 20 mm Silastic capsule containing dihydrotestosterone (**DHT**) or no hormone (blank or **BL**) (from (Dias et al. 2007)). Males from 34 °C IncT were given a BL capsule only, as there were not enough individuals for a DHT group. Three groups were generated for testing: 11 individuals were **34 °C-BL**, 18 were **32.5 °C-BL**, and 18 were **32.5 °C-DHT**.

Behavior testing

All animals were given one social association test in a Y-maze (Putz and Crews 2006). The test male was placed in a sanitized Y-maze for a total of 15 minutes, with each stimulus animal at the left and right ends of the short arm for 7:30 minutes to prevent side bias of the test male. Stimulus conspecifics were a castrated, DHT-implanted

male and an intact female with vitellogenic follicles, and hence receptive (Rhen et al. 2000). Both stimulus conspecifics were of 30°C IncT, different than the experimental male Temperature Morphs. We measured two types of behavior: 1) time spent associating with stimulus conspecifics at the short arms of the Y-maze and 2) presence of scent marking, a type of territorial behavior.

Statistics

To compare group differences in Y-maze scent marking a Chi square test was used between Temperature Morphs and different hormone manipulations. Temperature Morph comparisons received the same hormone manipulation. Conversely, implant comparisons were made within 32.5 °C males. Using R (R, a Language and Environment for Statistical Computing, Vienna, Austria 2010), Wilcoxon rank-sum tests were conducted to 1) compare behavioral differences between castrated males of two Temperature Morphs, and 2) compare differences between hormonally manipulated 32.5 °C males.

RESULTS

Time Spent with Conspecifics

We detected a Temperature Morph difference in social association behavior (Figure 4). On average, 34 °C-BL males spent significantly more time than 32.5 °C-BL males associating with conspecifics (Wilcoxon rank-sum, W=50, p=0.03). When responses to male and female stimulus animals were analyzed separately, 34 °C castrates spent more time than 32.5 °C castrates associating with the stimulus female (W=45, p = 0.02) and stimulus male (W= 55.5, p = 0.05). Males in groups 32.5 °C-BL and 32.5 °C-DHT did not differ in social association behaviors. There was no difference of time spent with stimulus males vs. females in any of the treatment groups.

Scent Marking

We did not detect a Temperature Morph difference in the proportion of individuals that scent marked in the Y-maze. We did detect a hormone implant difference in scent marking (figure 5); more than two-thirds of 32.5 °C-DHT males scent marked compared to one-third 32.5 °C-BL males ($c^2 = 5.51$, $p = 0.02$).

DISCUSSION

In this study, castrated leopard geckos from different incubation temperatures (34 °C, an extreme, female-biased, IncT versus 32.5 °C, a male-biased IncT) were tested in a Y-maze with male and female stimulus animals. The use of castrates allowed us to examine Temperature Morph differences in the absence of circulating concentrations of androgen. In addition, the effects of DHT were further examined in the male-biased IncT morph; the lack of adequate number of female-biased IncT morph prevented similar treatment. We demonstrated that time spent with stimulus conspecifics was predicted by Temperature Morph while scent marking was increased by exogenous DHT.

Temperature polymorphism

While there are extensive behavioral neuroendocrinology profiles for males of 30° and 32.5 °C IncTs (see 1.2), this finding of behavioral differences between 34 °C and 32.5 °C males contributed to the behavioral profile of 34 °C males. Though 34 °C and 32.5 °C males differ in circulating androgens and estradiol, as well as POA and VMH both volume and metabolic capacity (Coomber et al. 1997), we were able to detect behavioral differences in the absence of gonadal differences. This was consistent with the idea that the embryonic environment had an organizational effect on conspecific visits, because the behavioral difference was not activated by gonadal steroids. As these animals were castrated as adults, we must also consider the possibility of postnatal organizational effects that are Temperature Morph- specific. Juvenile gonadectomy effect on adult female size depends on the Temperature Morph, but its effect on sociosexual behavior has not been tested (Tousignant and Crews 1995). Sexual behaviors in non-TSD animals

such as red-sided garter snakes and mammals are organized by gonadal hormones prior to sexual maturity (Crews 1985; Schulz and Sisk 2006). Sex discrimination was not detected in any of the treated groups, so our suggestion was limited to the possibility that conspecific association was a type of social investigation. This lesser social "intolerance" shown in 34 °C males vs. 32.5 °C males can be interpreted as another temperature polymorphic behavioral trait.

Androgen-sensitive characteristics

The hormone implant difference in proportion of scent markers between 32.5 °C-BL and 32.5 °C-DHT males is consistent with previous studies that androgen-treated 32.5 °C males spent more time scent marking than castrated 32.5 °C males in the presence of females (Rhen and Crews 1999). Castrated males of 32.5 °C and 34 °C IncT did not differ in proportion of scent markers. Previous studies show no difference in scent marking time between 32.5 °C and 30 °C castrated males (Rhen and Crews 1999). Interestingly, 32.5 °C male scent marking is independent of sexual experience, where sexually naïve and experienced 32.5 °C males have the same low proportion of scent markers (Sakata and Crews 2003). These observations of scent marking are in different social contexts.

While some behavioral and brain characteristics are androgen dependent, others are not. The observation that social association was DHT-independent in 32.5 °C males was previously undocumented, but not surprising, since more stereotyped behavior, such as aggressive high posturing, in the presence of stimulus males, is androgen-independent(Rhen and Crews 2000). However, high posturing is androgen-dependent in the presence of stimulus females, suggesting a role of social context in hormone manipulation (Rhen and Crews 1999; 2000). Likewise, 32.5 °C males demonstrate androgen insensitivity in sexually- and reward-relevant regions of the brain (Crews et al. 1996; Dias et al. 2007). It is worth noting is that the relationship of androgens to the two measured behaviors is limited to the observation of 32.5 °C males, as these

aforementioned behaviors and brain characteristics of other temperature morphs are sensitive to hormonal manipulation (Sakata and Crews 2003).

Conclusion

Consistent with previous research, we have demonstrated differences in conspecific association between gonadectomized Temperature Morphs, indicating that in a species lacking sex chromosomes, social behavior is organized directly by incubation temperature. The finding that exogenous androgens stimulate scent-marking behavior is also consistent with previous work and suggests that gonadal hormones modulate the latent effects of incubation temperature on the brain.

CHAPTER 5: Sociosexual investigation in sexually experienced, hormonally manipulated male leopard geckos: relation with phosphorylated DARPP-32 in dopaminergic pathways

INTRODUCTION

Behaviors that lead to the individual repeating the action, such as sexual behavior, and can activate dopaminergic pathways. Dopamine is generated and released during sexual exposure (Hull et al. 1995), and its metabolism affected by sexual experience (Mitchell and Stewart 1989). The mesolimbic pathway includes the basolateral amygdala, nucleus accumbens, ventral pallidum, hippocampus, septum, striatum, and ventral tegmental area (VTA) (O'Connell and Hofmann 2011). The projection of dopamine from the VTA to the nucleus accumbens is conserved across taxa and is involved in appetitive male-typical sexual behaviors and influenced by sociosexual exposure (Gonzalez et al. 1990; O'Connell and Hofmann 2011). In the nigrostriatal pathway, dopamine generated in the substantia nigra projects to the striatum and is involved in motor coordination including copulation in male rats (Hull et al. 2004).

Dopaminergic activity in the preoptic area of the hypothalamus (POA) is associated with both appetitive and consummatory male-typical copulatory behavior in mammals (Dominguez and Hull 2005). In male rats dopamine levels increase in the POA during exposure to females and prior to copulation (Hull et al. 1995) and ejaculations during copulation decreased when dopamine antagonists are administered in the POA (Pehek et al. 1987). In the Japanese quail, the POA and dopamine are associated with both appetitive and consummatory behavior, with dopamine potentially modulating POA steroid hormone metabolism (Balthazart et al. 1997; 1998; Balthazart et al. 2002; Cornil et al. 2002).

Dopaminergic pathways have been established by studying the ligand and receptor distributions in brain nuclei, as well as afferent and efferent connections of the nuclei. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine synthesis, and its immunoreactive neurons are found in dopaminergic nuclei such as the VTA and putative dopaminergic organon periventricular nucleus (OPN) in the

hypothalamus of the Tokay gecko (*Gekko gecko*) (Smeets et al. 1986). In mammalian, avian, and fish taxa, TH immunoreactivity can be colocalized with Fos, the product of the immediate early gene *cFos* (Dragunow and Faull 1989), as a proxy to indicate general activity in neurons that also express TH. In these studies, immunohistochemical colocalization is greater among individuals that experience sexual behavior compared to those that do not (Asmus and Newman 1994; Dragunow and Faull 1989; Goodson et al. 2009; Petersen et al. 2013). Dopamine D1 receptors, when activated, can enhance cellular activity through the dopamine-and cAMP-regulated phosphoprotein (DARPP-32) via phosphorylation at the threonine-34 residue (pDARPP-32) (Hemmings et al. 1984; Ouimet et al. 1984; Fienberg et al. 1998). Immunoreactive studies involving pDARPP-32 in the mesolimbic nuclei focus on dopamine activity under drug and alcohol manipulation (Maldve et al. 2002; Nairn et al. 2004; Zachariou et al. 2005). A handful of studies related pDARPP-32 in the hypothalamus of male and female rodents to steroid hormone treatment and sexual behavior (Mani et al. 2000; Auger et al. 2001; McHenry et al. 2012); of particular interest is that D1 dopamine antagonist treatment reduced copulatory behavior in male rats (McHenry et al. 2012).

In lizards, like mammals, the nucleus accumbens receives dopaminergic input from the VTA. The lizard striatum, however, has less subnuclei distinction than in mammals and receives input mainly from the substantial nigra and some input from the VTA (Smeets and Medina 1995; Gonzalez et al. 1990; Smeets et al. 1986; Smeets et al. 2001), and is associated with male sexual vigor in the whiptail lizard (*Cnemidophorus inornatus*) (Woolley et al. 2004). The POA has DARPP-32 and TH immunopositive cells in the Tokay gecko and leopard gecko, respectively (Smeets et al. 2001; Dias et al. 2007).

Although the POA is sexually dimorphic in size and metabolic activity in mammals (McCarthy et al. 2010), in the leopard gecko such differences are dependent upon the temperature of the incubating egg (hereafter temperature morphs) (Coomber et al. 1997); hence, within each sex the POA is larger and has greater metabolic activity in individuals from a male-biased incubation temperature (IncT) compared to female-biased IncT. Incubation temperature variation in the leopard gecko brain also exist in the

mesolimbic pathway, where males of 30 ˚C IncT have greater dopamine levels in the nucleus accumbens than males of 32.5 ˚C IncT, as well as androgen-sensitive TH neurons in the VTA (Dias et al. 2007). In addition to IncT effects, it is possible that androgen manipulation can influence dopaminergic activity in the mesolimbic and nigrostriatal pathways and the hypothalamus, as seen in rats (Alderson and Baum 1981; Mitchell and Stewart 1989; Du and Hull 1999). The involvement of the POA in sexual behavior is influenced by steroid hormones across vertebrate taxa (Crews and Silver 1985; Balthazart et al. 1998; Ball and Balthazart 2004; Woolley et al. 2004). In the leopard gecko, males of 32.5 ˚C IncT have POA size and metabolic activity that are androgen sensitive (Crews et al. 1996).

Studying intrasexual variation of neural protein expression that arise from different incubation temperatures may explain developmental influences on adult sociosexual behaviors. The focus of this study is to understand how the dopaminergic system is influenced by embryonic experience in the leopard gecko, a lizard species with temperature-dependent sex determination. In the dopaminergic and dopaminoceptive nuclei, I quantified tyrosine hydroxylase, Fos, and pDARPP-32 immunoreactivity in males of different treatment groups to look for IncT and hormone manipulation effects in a sociosexual context. I predicted that TH colocalization with cFos would not differ among temperature morphs but would differ according to hormone manipulation, as there was no difference between TH neuronal number between 32.5 and 30 ˚C males but within 30 ˚C males there was a difference dependent treatment. If dopaminoceptive neuronal activity was similar to male rats, then pDARPP-32 expression in the POA would be correlated to exposure to females. If dopaminoceptive neuronal activity was proportional to dopamine concentration in leopard geckos, I would expect an IncT difference in pDARPP-32 expression in the nucleus accumbens, but not in the POA or the VTA. Behaviorally, I would expect temperature morphs to differ in the time spent investigating conspecifics, with males of 32.5 ˚C IncT spending less time than other temperature morphs as is seen in sexually inexperienced males (Huang and Crews 2012). However, I would not expect differences in non-social investigative behavior.

METHODS

Animals

Animals were hatched from eggs obtained from controlled matings in the laboratory. Eggs were incubated individually in covered plastic cops containing a water: vermiculite ratio of 1:1 in a monitored incubation chamber (Precision, Illinois, USA) with a HOBO temperature logger (Onset Computer Corp, Pocasset MA, USA). Hatchlings were fed live crickets every other day for 10 weeks and received water *ad libitum*. As juveniles and adults their diet changed to vitamin-dusted mealworms in a 14:10 day: night cycle. Animals (n = 37) were cared and treated under the guidance of IACUC protocol number AUP-2011-00135.

Castration and hormone treatment

Virgin adult males from different IncTs (3-8 years of age) were placed in a tank with 2-4 adult females to gain sexual experience. This lasted for 4 to 12 weeks and ended 1 to 2 weeks before males were castrated and given one 20mm Silastic implant of dihydrotestosterone (DHT) (Steraloids, Rhode Island, USA) or empty or blank capsule (BL). Castration was performed using hypothermia anesthesia in accordance with IACUC protocol. Experimental males were from two female-biased IncTs (30 and 34 ˚C) and one male-biased IncT (32.5 ˚C) to generate temperature morphs with either a blank or DHT implant. There were not enough animals to generate a 34 ˚C-blank group, as the percentage of males is < 5% at this IncT.

Behavioral testing

Four weeks following castration and hormonal manipulation, experimental males were given a series of behavioral tests in a Plexiglas Y maze (Putz and Crews 2006; Huang and Crews 2012). The first test was a food odor discrimination test using a cricket frass-embedded filter paper vs. control filter paper in the short arms of the Y maze. The second and third tests were a sex discrimination test, the initial test using an unfamiliar

male and female from the 32.5 ˚C IncT as stimulus animals, followed by a test with a stimulus male and female from the 30 ˚C IncT. For the social Y maze tests, focal males were exposed to the same stimulus male. However, the stimulus females varied to ensure that all stimulus females were receptive and had vitellogenic follicles, which was verified by vascularized follicles seen through their translucent abdomens. Time spent at each end of the short arms was recorded, as well as time spent scent marking, a territorial behavior. The first two tests were conducted 2-8 days apart, and the third test was conducted 12 weeks later to reduce any effects of social exposure experience from the different temperature morph. Each test lasted 15 minutes; where halfway through the test the stimuli were switched in between the short arms to prevent side bias.

Euthanasia and brain removal

Twenty minutes after the last Y maze, experimental males were given an overdose of sodium pentobarbital (Euthasol, Virbac Animal Health, Fort Worth, TX, USA). After expressing no reaction from a hard digit-pinch, males were then perfused with a saline solution and then with cold 4% paraformaldehyde (PFA). After perfusion, animals were decapitated, heads placed in 4% PFA overnight, and the extracted brains were then cryoprotected in 20% sucrose before frozen in -80 ˚C. Frozen brains were then cryosectioned at 40μm on Superfrost Plus Gold microscope slides in a 1:6 series, where sections on one slide were six sections apart, and each series was used for different protein detection.

Immunohistochemistry

To detect the colocalization of proteins TH and Fos, slides from series 2 were used for 20 animals and slides from series 4 were used for 17 animals, and slides from series 3 were used to detect pDARPP-32. Slides were first placed in 4% PFA for 10 minutes, washed twice in PBS for 10 minutes and placed in 3% hydrogen peroxide to quench endogenous peroxidase activity for 20 minutes. After 10 minutes of PBS washing, the slides were placed in 1% $NaBH_4$ for 20 minutes for antigen retrieval and

washed for 10 minutes in PBS. Slides were then blocked with 4% normal goat serum for 60 minutes before being incubated with a primary antibody overnight with a dilution ratio of 1:250 dilution ratio for rabbit anti-Fos or 1:300 mouse anti-pDARPP-32 (sc253; sc21601, Santa Cruz, Dallas, USA). The second day slides were washed for 20 minutes in PBS, incubated with 1:200 biotinylated secondary antibody, anti-rabbit in goat for 120 minutes for cFos or anti-mouse in goat for pDARPP-32. Slides were washed in PBS for 20 minutes before incubating with Avidin Biotin complex (Vector Laboratories Inc., Burlingame, CA, USA) for 60 minutes. After washing for 20 minutes the protein was detected with diaminobenzidine (DAB) or DAB with nickel for double immunohistochemistry. Slides were washed with water, dried, and coverslipped for cell quantification. For double immunohistochemistry, directly after washing with water, the slides were re-immersed in PBS and then blocked with 8% normal horse serum for one hour before being incubated in with the 1:1000 dilution of second primary antibody monoclonal mouse anti-TH (MAB318, Millipore, Temecula, CA, USA) overnight. On the third day slides were washed for 20 minutes in PBS, incubated with 1:200 biotinylated secondary antibody, anti-mouse in horse for 120 minutes. Slides were washed in PBS for 20 minutes before incubating with Avidin Biotin complex for 60 minutes. After washing for 20 minutes the protein was detected with a red chromogen (Vector Red, Laboratories Inc., Burlingame, CA, USA). Slides were washed with water, dried, and coverslipped for cell quantification. Sections incubated without primary antibodies served as negative controls, and did not show immunopositive staining (Figures 6, 7). An additional negative control for pDARPP-32 included the antibody preadsorbed with 5X peptide concentration, and also did not show immunopositive staining.

Immunoreactive cell quantification

For an unbiased estimation of immunopositive cell density and cell numbers of TH, Fos, and pDARPP, the optical fractionator module of StereoInvestigator software was used (Microbrightfield, USA). To quantify TH and Fos immunoreactivity, one series

of immunohistochemistry staining, the VTA and OPN were outlined in 3 sections according to Smeets et al. (1986; 1989), and the POA was outlined in two sections, however the regional volume was not the same across individuals. Within each outline the optical disector of 50 X50 X 10 μm^3 of x-y-z dimensions was placed in a 100 X 100 μm^2 grid to ensure standard and random sampling. To quantify pDARPP-32 immunoreactivity in a different series of immunohistochemistry staining, the nucleus accumbens and striatum were outlined in three sections and the POA was outlined in two sections. Cells were counted in sample frames placed equally distanced within the outlined region using the 40X objective on a Zeiss microscope. Immunopositive neuronal densities were estimated in the aforementioned nuclei using the formula derived from Mayhew and Gundersen (1996). Total cell number was calculated with a formula from West et al. (1991). To detect TH neurons that had cellular activity, the proportion of TH colocalization was calculated by the number of neurons with both nuclear Fos-ir and cytoplasmic TH-ir divided by the total number of TH-ir cells.

Statistics

The detection for IncT or hormone implant effects on behavior and brain immunocytochemistry employed the statistical program R (R, a Language and Environment for Statistical Computing, Vienna, Austria 2010). To detect a difference between time spent with male and female conspecifics and cricket odor versus control, Wilcoxon signed rank test was conducted. ANOVA was conducted to detect differences among treatment group in time spent with stimuli in the Y maze, and Pearson product correlation was used to detect a relation between the times spent with different stimuli in the Y maze. Further, the proportion of time spent with female conspecifics or cricket odor was calculated from the total investigation time per maze. A generalized linear mixed model analysis with Poisson distribution was used to compare conspecific and nonsocial investigation time in the Y maze among treatment groups. A generalized linear mixed model analysis with binomial distribution was used to compare the proportion investigation time with cricket odor or female conspecifics in the Y maze among treatment groups. Considering only the social investigators, paired t-tests were used to

compare differences within each treatment group in proportion of investigation time with the two female temperature morphs of the two social Y maze tests. ANOVA or Kruskal Wallis tests were conducted to compare 1) TH– and Fos–ir neuronal densities and cell number and TH/cFos colocalization in the VTA, OPN and POA, and 2) pDARPP-32–ir neuronal density and cell number in the nucleus accumbens, striatum, and the POA among treatment groups. To relate brain immunohistochemistry to behavior in the last Y maze test, Pearson product correlation tests were conducted to relate protein –ir neuronal density and colocalization in the aforementioned nuclei to total time spent in the last Y maze investigating conspecifics of 30 °C IncT, as well as proportion of investigation time with females of 30 °C IncT.

RESULTS

Behavior

To detect any changes in behavior during the duration of the Y maze, the time visiting cricket odor stimulus females, males, cricket odor, and time spent scent marking during the male's time in the Y maze was first divided into the first and second 450 seconds. After no behavior differences were detected between the first and second halves, and no significant relationship was detected between the behaviors and the treatment groups, the Y maze time per stimulus was used for analysis of behavior (Table 3).

The total investigation time spent visiting stimulus conspecifics in each Y maze test were compared across treatment groups (Figure 8). No differences in time spent with non-social stimulus or 32.5 °C conspecifics were detected among treatment groups (Table 4). When exposed to conspecifics of 30 °C IncT, there was an implant and IncT × implant interaction effect on total time spent with conspecifics ($p= 0.021$ and 0.024, respectively). No IncT or implant effects were detected among treatment groups for proportion of time spent with one stimulus for each Y maze (Table 5) (Figure 9). However, males of the treatment group 34 ˚C-DHT spent a significantly greater proportion of time with female vs male in the Y maze with 32.5 °C conspecifics than in

the Y maze with 30 °C conspecifics ($t = -2.801$, $df = 6$, $p = 0.031$). No differences in the Y maze total investigation time were detected within any treatment groups (Table 6).

Immunoreactive cell number, density, and colocalization

No IncT or hormone implant effects were detected among treatment groups for either TH– or Fos–ir neuronal density or cell number (Tables 7, 8), nor were there treatment group differences detected for the colocalization of TH and Fos from immunohistochemistry (Table 9). No IncT or hormone implant effects were detected among treatment groups for pDARPP-32–ir neuronal density at the nucleus accumbens, striatum, or the POA (Tables 10, 11).

Cell immunoreactivity in relation to behavior in last Y maze test

In the VTA of 32.5 ˚C-blank males there was a positive correlation between Fos–ir neurons and time spent with 30 ˚C stimulus females ($t = 0.796$, $p = 0.027$, $R^2 = 0.527$) (Figure 10). In the POA of 32.5 ˚C-blank males pDARPP-32–ir neuronal density in the POA was positively correlated to time spent visiting 30 °C stimulus females ($t = 2.426$, $p = 0.046$, $R^2 = 0.457$), as was pDARPP-32–ir cell number ($t = 3.201$, $p = 0.015$, $R^2 = 0.594$) (Figures 10 and 11, respectively). Males in the 32.5 ˚C-blank treatment group also exhibited a negative correlation between pDARPP-32 neuronal density in the nucleus accumbens and time spent visiting a 30 °C stimulus male ($t = -2.927$, $p = 0.022$, $R^2 = 0.550$) (Figure 11). No other significant correlation between neuronal densities, number or colocalization, and time spent with conspecifics was detected within treatment groups in the POA, OPN, or VTA (Tables 12-16). No other significant correlation between pDARPP-32 neuronal density or number and time spent with conspecifics was detected within treatment groups in the nucleus accumbens, striatum, or POA (Tables 17 and 18).

DISCUSSION

The purpose of the study was to determine if sociosexual behavior differed among sexually experienced animals due to temperature morph or hormone implant, and if social exposure or manipulated factors affected the dopaminergic pathway. After measuring

time spent with cricket odor and conspecifics of different IncTs and in a Y maze behavioral design, I detected proteins TH, Fos and pDARPP-32 proteins as proxies for neurons expressing dopamine ligand and receptor activity in brain nuclei involved in dopaminergic pathways. I found that total investigation time with stimulus conspecifics of 30 °C was dependent on IncT and hormone implant, but these factors did not significantly affect total investigation time in the Y mazes with cricket odor or 32.5 °C conspecifics. Interestingly, when looking within each treatment group to see behavioral differences in the two social Y maze tests, I found in 34 °C-DHT males a significantly greater proportion of investigation time spent with female's vs males of 32.5 °C, than in the Y maze with conspecifics of 30 °C (Table 6) (Figure 9). This is consistent with previous findings that male temperature morphs differ in the proportion of time spent with different female temperature morphs in social investigation time, albeit the sexual experience and stimulus temperature morphs differed from previous experiments (Putz and Crews 2006; Huang and Crews 2012). I also found that neurons immunoreactive for phosphorylated DARPP-32 (at threonine 34) in the nucleus accumbens was negatively correlated to the time males spent in the Y maze arm with 30 °C males. Conversely in the POA, pDARPP-32–ir neuronal density and number were positively correlated to the time males spent in the Y maze arm with 30 °C females.

In the dopaminergic VTA, I found that Fos–ir neurons were positively correlated to time spent with 30 °C females, although there was no relation to TH immunoreactivity, suggesting only general activity of the VTA. Neuronal density, however, may be a more unbiased quantification, as the number of samples differed per individuals, and not all sections for TH and cFos immunohistochemistry were of the same series. Even if the entire region was traced and counted, temperature polymorphism in nucleus sizes could account for neuron number differences (Coomber et al. 1997). Three possibilities explain the correlations found in the 32.5 °C-BL treatment group: First, the neural chemistry was affected by time spent with conspecifics. Second, the neural chemistry could lead to time spent with conspecifics. It is also likely that non-social motor activity in the 32.5 °C -BL treatment group positively affected the pDARPP-32 and Fos expression in the POA and

the VTA, respectively, while negatively affecting pDARPP-32 expression in the nucleus accumbens.

While test males did not exhibit sex discrimination in the Y maze tests with stimulus conspecifics of 32.5 and 30 °C IncT, a disproportionate number of test males spent less investigation time with female versus male conspecifics. This observation was not unique to any treatment group, and the range of investigatory time was broad (4 to 838 seconds), and implant or IncT did not explain this variation. No differences in cricket odor investigation time were detected among treatment groups. The cricket olfactory test was the first non-social behavioral test conducted in a Y maze for the leopard gecko.

Relating these behaviors in the last Y maze test to brain immunochemistry in the treatment groups, only the 32.5 °C-BL treatment group had pDARPP-32–ir neuronal density correlated to time spent investigating conspecifics, which is of interest as Putz et al. (2006) found that intact 32.5 °C males prefer females of this particular IncT of 30° C, while males of 30 °C did not. It is not known whether males of 34 °C have a preference for specific female temperature morphs.

It was expected that dopamine in the nucleus accumbens and POA would increase in relation to female exposure, if the mesolimbic pathway was similar to other taxa (Damsma et al. 1992; Hull et al. 1995), thereby activating D1 dopamine receptors and phosphorylating DARPP-32 at threonine 34. However the present results suggest that any influence of female exposure on neuronal pDARPP-32 in the nucleus accumbens could have been overridden by time spent with stimulus males. No difference in pDARPP-32–ir in the striatum was found among treatment groups. A possible explanation is that even though the males were sexually experienced, non-tactile conspecific exposure was not enough interaction to elicit a change in dopamine activity in the striatum that is related to motor coordination in sexual behavior, or that IncT and hormone manipulation did not affect striatum dopamine activity. Alternatively, as the striatum is GABA-ergic and receives glutamate input, the phosphorylation of DARPP-32 at threonine 34 can be indirectly modulated by excitatory neurotransmitters that are not dopamine (Nagy et al. 1978; Nishi et al. 2011; Yger and Girault 2011). These results are comparable to sexually

experienced male rats that, combined with recent sexual behavior, experienced greater D1 dopamine receptor activity in the POA than others with sexual experience but no recent sexual behavior (McHenry et al. 2012). The relation of pDARPP-32–ir to sociosexual exposure in castrated male leopard geckos is of particular interest, as previous studies in female rodents demonstrate that hormones modulate pDARPP-32 protein expression in the POA (Mani et al. 2000; Auger et al. 2001). However, pDARPP-32–ir in the POA of female rats can also be affected by vaginal stimulation in the absence of progesterone (Meredith et al. 1998). TH and Fos immunohistochemistry studies yielded negative results in the POA, OPN, and VTA among treatment groups and were not correlated to scent marking behavior or time spent visiting conspecifics. This was unexpected, as there is a known TH–ir neuronal difference between hormonally manipulated, sexually naïve males of 30 °C IncT (Dias et al. 2007). Likewise, there was no IncT or hormone implant effect on pDARPP-32–ir neuronal density in the nucleus accumbens, POA, or striatum.

Because the Y maze included both male and female stimulus conspecifics that would elicit different behaviors following contact, this mixed sex exposure may potentially yield different reactions in the dopaminergic system. While pDARPP-32 has been primarily used in studies of drug addiction and rodent sexual behavior, immunoreactive quantification of this protein activated by D1 dopamine receptor can be extended study the mesolimbic pathway in reptiles in the context of sociosexual behavior. While the Y maze may be appropriate for behavioral tests, it may not be the optimal design for relating brain to behavior as each individual controls its exposure to the stimulus. In future experiments relating behavior to pDARPP-32, a single sex exposure design would be appropriate to delineate sex-specific exposure effects in these nuclei. Alternatively, a human-controlled exposure time to a conspecific would generate a more distinct relationship between neural protein expression and conspecific exposure duration. While DARPP-32 distribution in reptile brains have been described, and phosphorylated DARPP-32 in sexual contexts have been quantified in rats, it is worth noting that the use of pDARPP-32 as potential immunoreactive marker is novel for

studying D1 dopamine receptor activity in the limbic system of a reptile species in a sociosexual context.

CHAPTER 6: Conclusion

Variation in sexually dimorphic traits can have ontogenetic and mechanistic explanations. In leopard geckos, the embryonic environment not only determines sex, but also contributes to behavioral, morphological and physiological variation within each sex. In addition, this ontogenetic effect is modulated by steroid hormones in adulthood. The embryonic environment includes the incubation temperature, as well as steroid hormones that are maternally allocated and endogenously generated. How these factors interact with each other, and how they contribute to within- sex behavioral variation is a central question addressed in Chapter 2 and 3 of the dissertation. In Chapters 4 and 5, I ask how the incubation temperature interacts with adult hormone manipulation to influence behavior.

I tested the hypothesis that the phenomenon of same-sex bias in clutch siblings was influenced by maternal effects (Kratochvíl et al. 2008), by separating pairs of eggs of the same clutch into different incubation temperatures and comparing the sibling sex ratios to that expected of each temperature. I showed that the phenomenon of same-sex bias in clutch siblings was dependent on the incubation temperature, and that any maternal influences via yolk-albumen (YA) steroid hormone deposition were less influential. Though the influence of estrogen on female development in reptiles with temperature-dependent sex determination is well established (Bull et al. 1988; Rhen and Lang 1994; Tousignant and Crews 1994), it would not explain the mixed- sex clutches that were incubated at the same temperature. Further, a subsequent study has shown a lineage bias toward a certain sex, and that this bias is also incubation temperature dependent (Rhen et al. 2011). Despite having a lesser influence on sex determination, YA hormones could act in concert with incubation temperature to influence behavior during development by organizing brain regions that are sociosexually relevant. In avian species, yolk hormones, both natural and manipulated, can contribute to differences in juvenile behavior and adult secondary sexual characteristics (Schwabl 1993; 1996; Adkins-Regan

et al. 1995; Strasser and Schwabl 2004; Partecke and Schwabl 2008; Bonisoli-Alquati et al. 2011a), To test for incubation temperature differences in YA hormone concentration, I measured YA testosterone concentration at different developmental stages from pairs of eggs of the same clutch incubated at two female-biased temperatures with a difference of 8 °C. Though there was no YA testosterone concentration difference between incubation temperatures over time, a concentration increase was correlated to developmental stage at both incubation temperatures; this was detected during the temperature-sensitive period in eggs incubated at the lower temperature of 26 °C. The YA testosterone concentration difference during development suggests an increase in gonad steroidogenesis or decrease in absolute yolk volume, as the source of YA testosterone is undetermined. The lack of temperature influence on testosterone could be attributed to all eggs being incubated at female-biased temperatures, or to siblings having similar hormone metabolism at different incubation temperatures. The finding of higher testosterone levels in later development, while not novel for a reptile with temperature-dependent sex determination, is in the minority (Ding et al. 2012). Although testosterone from local production in the brain, gonads, or yolk could vary in their influence, testosterone can be a substrate for aromatase or 5a-reductase that furthers sexual differentiation in the brain (Jeyasuria and Place 1998).

While the embryonic environment influences individuals in adulthood, it is the social context and circulating hormones that are involved in activating behavior. One way to investigate these effects is to examine the putative mechanisms that lead to behavioral differences within a sex. Previous studies have established that aggression and attractiveness varied among males and females from different incubation temperatures, and temperature morphs were differentially sensitive to hormone manipulation (Flores et al. 1994; Crews et al. 1996; Dias et al. 2007). Using a castration and hormone replacement design, I examined sociosexual behaviors in a social context, without physical contact, in an attempt to isolate investigative behavior. In this experiment I included males ~~of~~ from 34 °C, an incubation temperature that generates extreme female bias and from which male behavior has not been previously tested. To find effects of

incubation temperature, I compared adult males from the same hormonal treatment. To find effects of hormonal status, I compared males from the same incubation temperature with castrated or dihydrotestosterone (DHT)-implant hormonal manipulations, Consistent with previous research, I found differences in conspecific association between gonadectomized temperature morphs, indicating that in a species lacking sex chromosomes, social behavior is organized directly by incubation temperature. However, as these animals were castrated as adults, it is important to consider the possibility of postnatal or peripubertal organizational effects that are incubation temperature- specific. The finding that exogenous androgens stimulate scent-marking behavior was also consistent with previous work and suggests that gonadal hormones modulate the latent effects of incubation temperature on the brain.

As investigative behavior of conspecifics potentially leads to aggressive or sexual behavior in males, sociosexual investigation could also be correlated to dopaminergic activity in brain regions associated with appetitive behavior. I modified the previous experimental design to determine if sexually experienced males from different treatments of incubation temperature and hormone manipulation exhibited different reactions in the limbic system during social exposure. I found that castrated males from 32.5°C had phosphorylated DARPP-32 immunoreactive neuronal number and density related to time spent investigating conspecifics. Specifically, pDARPP-32 neuronal density in the nucleus accumbens was negatively correlated to time spent with males, and in the preoptic area was positively correlated to time spent with females. Using pDARPP-32 as a proxy to measure D1 dopamine receptor activity, the results suggest that castrated males from 32.5°C were sensitive to conspecific investigation, though their investigatory behavior did not differ from dihydrotestosterone-implant counterparts, nor did their behavior differ from other castrated temperature morphs. In addition, because conspecifics of different sex elicited different stereotyped behaviors, they may also elicit different reactions in the dopaminergic system, rendering a sex-specific reaction in the dopaminergic system difficult to delineate if males spent time with both sexes. As this was a novel use of pDARPP-32 in a lizard species, comparisons of D1 dopamine receptor

activity were drawn to rodent studies in the context of sociosexual exposure or hormone manipulation. Both male and female D1 dopamine receptor activity affect and are affected by sexual activity (Meredith et al. 1998; McHenry et al. 2012).

Taken together, the results from these studies are consistent with previous work by re-emphasizing the primary influence of incubation temperature on sex determination and the contribution of the embryonic environment to behavioral variation within a sex. Examining incubation temperature and hormone interactions in yolk-albumen during development and dopamine receptor activity during social exposure are methods established in other species that provide further insight into temperature-mediated behavioral variation in the leopard gecko.

Table 1: Frequencies and sex ratios of same-clutch eggs at same incubation temperature (IncT).

Data from Kratochvil et al. (2008) of same-clutch, same incubation temperature (30 °C) siblings presented as reference on first row. Each clutch has two eggs sharing the same deposition date, father and mother. $\chi 2$ analysis for sibling same-sex vs. mixed sex clutches (df=1, two ♂or two♀: one♀ and one ♂). Data of the same-clutch, same-IncT hatchlings were obtained from the University of Texas breeding colony from 1995 to 2001 and 1993-1999 for 30 and 32.5°C, respectively.

Incubation Temperature (°C)	Number clutches	Number Hatchlings	Sex Ratio (male: female)	Possible Sibling Sex outcomes	Expected Sibling Sex Frequency	Observed Sibling Sex Frequency	X^2	P
Kratochvil et al. 2008 Same clutch Same IncT 30°C	26	52	20:32	Two ♂or two♀: one♀ and one ♂	12.75 12.25	26 0	23.4	<0.00001
Univ. Texas, Same Clutch Same IncT30°C	32	64	20:44	Two ♂or two♀: one♀ and one ♂	18.25 13.75	26 6	7.7	0.0056
Univ. Texas, Same Clutch Same IncT 32.5°C	15	30	18:12	Two ♂or two♀: one♀ and one ♂	7.8 7.2	9 6	0.4	0.54

Table 2: Frequencies and sex ratios of same-clutch eggs separated and incubated at a different incubation temperature (IncT).

Each clutch has two eggs sharing the same deposition date, father, and mother. analysis for same-sex vs. mixed-sex clutches (df=1; two ♂or two♀: one♀ and one ♂). Data of same-clutch, different-IncT hatchlings were obtained from the University of Texas breeding colony from 1992 to 2001.

Incubation Temperature Pairs (°C)		Number clutches	Number Hatchlings	Possible Sibling Sex outcomes	Expected Sibling Sex Frequency	Observed Sibling Sex Frequency	X^2	P
30°C	32.5°C	56	112	Two ♂or two ♀:	25.3	32	3.244	0.071
				one ♀ and one ♂	30.7	24		
30°C	34°C	35	70	Two ♂or two ♀:	23.1	23	0.001	0.971
				one ♀ and one ♂	12.9	12		
32.5°C	34°C	37	74	Two ♂or two ♀:	14.4	14	0.015	0.902
				one ♀ and one ♂	22.6	23		

Table 3. Time spent associating with respective stimuli in short arms (mean seconds ± SEM) in three Y maze tests.

Significance of comparison (ANOVA or Kruskal-Wallis p-values) among treatment groups is indicated in last row. Significance of comparisons between stimulus (cricket odor, male and female from different incubation temperatures) per treatment group is indicated in the same column (paired t-test or Wilcoxon signed- rank p- values).

Gecko group	Cricket odor Control odor	32.5 °C female 32.5 °C male	30 °C female 30 °C male
30 °C-blank	174 ± 58	149 ± 19	61 ± 27
	117 ± 82 p=0.584	179 ± 79 p=0.687	324 ± 139 p=0.170
30 °C-DHT	123 ± 58	90 ± 47	99 ± 91
	239 ± 98 p=0.208	227 ± 84 p=0.144	162 ± 75 p=0.789
32.5 °C-blank	88 ± 19	54 ± 39	35 ± 17
	170 ± 64 p=0.164	134 ± 30 p=0.205	96 ± 44 p=0.352
32.5 °C-DHT	131 ± 29	157 ± 48	146 ± 58
	120 ± 41 p=0.636	117 ± 49 p=0.834	140 ± 61 p=1.000
34 °C-DHT	121 ± 50	173 ± 55	114 ± 27
	148 ± 69 p=0.457	174 ± 63 p=0.988	268 ± 70 p=0.052
significance	0.588 0.926	0.115 0.680	0.302 0.163

Table 4. Generalized linear mixed model with Poisson distribution was performed on the total time spent investigating for three Y maze tests.

For the Y maze measuring total time spent with conspecifics, an implant and an incubation * implant effects were detected (see asterisk), but an incubation temperature effect was not detected. P values are given in each cell.

	Cricket odor and control	32.5 °C conspecifics	30 °C conspecifics
IncT	0.883	0.234	0.165
implant	0.656	0.185	0.022 *
IncT:implant	0.644	0.185	0.024 *

Table 5. Generalized linear mixed model with binomial distribution was performed on the proportion of total time spent investigating cricket odor of female conspecifics for three Y maze tests.

No incubation temperature (IncT), implant, or incubation * implant effect was detected. P values are given in each cell.

	Cricket odor and control	32.5 °C conspecifics	30 °C conspecifics
IncT	0.540	0.161	0.858
implant	0.481	0.071	0.851
IncT:implant	0.483	0.068	0.860

Table 6. Comparison of sociality and proportion of time spent with female conspecifics between Y maze tests of 32.5 and 30 °C conspecifics, within each treatment groups.

For the treatment group 34 ˚C-DHT, a difference in percent or proportion of time spent with female was detected between the two Y maze tests. No differences between the two social Y maze tests were detected in sociality or proportion of time spent with females for the other treatment groups. Significance indicated in p-values from paired t-tests or Wilcoxon signed-rank test.

	30 ˚C-blank	30˚C-DHT	32.5 ˚C-blank	32.5 ˚C-DHT	34˚C-DHT
Sociality (Total time)	0.378	0.737	0.374	0.636	0.726
Proportion of time with female	0.092	0.875	0.584	0.408	0.031 *

Table 7. Density of TH-ir followed by Fos-ir cells (mean #/ mm^3 ± SEM) in dopaminergic cell groups (preoptic area: POA, organon periventricular nucleus: OPN, ventral tegmental area: VTA) of male leopard geckos of different incubation temperatures (30, 32.5 or 34 °C) and hormone treatment (blank or dihydrotestosterone: DHT).

Significance of comparison (ANOVA or Kruskal-Wallis p-values) among treatment groups is indicated in last row.

Gecko group	POA	OPN	VTA
30-blank	1962 ± 358	1540 ± 268	1399 ± 288
	3277 ± 838	2869 ± 646	2291 ± 293
30-DHT	1751 ± 301	1563 ± 309	2100 ± 334
	2793 ± 427	2278 ± 487	2725 ± 381
32.5-blank	1674 ± 386	1072 ± 113	1278 ± 97
	2710 ± 401	1916 ± 386	1772 ± 174
32.5-DHT	1567 ± 275	1485 ± 135	1521 ± 177
	2905 ± 425	2409 ± 280	2649 ± 291
34-DHT	1249 ± 246	1544 ± 142	1516 ± 239
	2431 ± 397	2306 ± 281	1996 ± 191
Significance	0.305	0.230	0.133
	0.791	0.434	0.501

Table 8. Number of TH-ir followed by Fos-ir cells (mean # derived from formula in West et al., 1991) in dopaminergic cell groups (preoptic area:POA, organon periventricular nucleus: OPN, ventral tegmental area: VTA) of male leopard geckos of different incubation temperatures (30, 32.5 or 34 °C) and hormone treatment (blank or dihydrotestosterone:DHT).

Significance of comparison (ANOVA or Kruskal-Wallis p-values) among treatment groups is indicated in last row.

Gecko group	POA	OPN	VTA
30-blank	2682± 497	2599 ± 610	1548 ± 234
	4392 ± 947	4968 ± 1193	2750 ± 614
30-DHT	1602± 374	2022 ± 475	2580 ± 421
	2994 ± 947	3132 ± 921	3804 ± 791
32.5-blank	1692± 352	1556 ±160	1844 ± 249
	2984 ± 445	2828 ± 507	2492 ± 304
32.5-DHT	1973± 476	2664 ± 394	2254 ± 457
	3337 ± 446	4288 ± 598	3661 ± 561
34-DHT	1738± 549	2499 ± 459	2129 ± 470
	3374 ± 929	3821 ± 748	2695 ± 402
significance	0.555	0.588	0.668
	0.702	0.882	0.820

Table 9. Percent of TH-ir cells colocalized with Fos-ir staining (means ± SEM) in dopaminergic cell groups (preoptic area:POA, organon periventricular nucleus: OPN, ventral tegmental area: VTA) of male leopard geckos of different incubation temperatures (30, 32.5 or 34 °C) and hormone treatment (blank or dihydrotestosterone:DHT).

Significance of comparison (ANOVA p-values) among treatment groups is indicated in last row.

Gecko group	POA	OPN	VTA
30-blank	45.3 ± 6.5	46.3 ± 4.8	45.4 ± 7.4
30-DHT	43.3 ± 3.7	29.1 ± 4.2	38.6 ± 4.3
32.5-blank	40.7 ± 4.6	43.2 ± 5.4	35.9 ± 4.0
32.5-DHT	42.8 ± 5.8	45.3 ± 3.7	41.7 ± 4.2
34-DHT	29.7 ± 6.8	40.5 ± 4.2	31.3 ± 4.9
Significance	0.396	0.580	0.195

Table 10. Density (#/ mm^3) of pDARPP-32 cells (means ± SEM) in dopaminergic cell groups (nucleus accumbens, striatum, preoptic area:POA) of male leopard geckos of different incubation temperatures (30, 32.5 or 34 °C) and hormone treatment (blank or dihydrotestosterone:DHT).

Significance of comparison (ANOVA or Kruskal-Wallis p-values) among treatment groups is indicated in last row.

Gecko group	Nucleus accumbens	Striatum	POA
30-blank	1765 ± 69	1356 ± 439	1726 ± 272
30-DHT	1171 ± 259	676 ± 119	1423 ± 371
32.5-blank	1431 ± 195	864 ± 143	1516 ± 211
32.5-DHT	1608 ± 139	863 ± 115	1707 ± 312
34-DHT	1580 ± 184	803 ± 85	1329 ± 146
Significance	0.685	0.652	0.248

Table 11. Cell number of pDARPP-32 cells (mean # derived from formula in West et al., 1991) in cell groups with D1 dopamine receptors (nucleus accumbens, striatum, preoptic area: POA) of male leopard geckos of different incubation temperatures (30, 32.5 or 34 °C) and hormone treatment (blank or dihydrotestosterone:DHT).

Significance of comparison (ANOVA or Kruskal-Wallis p-values) among treatment groups is indicated in last row.

Gecko group	Nucleus accumbens	Striatum	POA
30-blank	4529 ± 591	2693 ± 1041	2016 ± 338
30-DHT	2304 ± 381	1224 ± 282	1458 ± 132
32.5-blank	2836 ± 545	1228 ± 228	1836 ± 208
32.5-DHT	2966 ± 368	1552 ± 231	1886 ± 280
34-DHT	2977 ± 438	1049 ± 100	1872 ± 469
Significance	0.094	0.358	0.768

Table 12. Correlation test of total time focal male spent with 30 °C stimulus conspecifics to tyrosine hydroxylase–ir cell number in three different brain regions.

In 34-DHT males, the tyrosine hydroxylase cell number was positively correlated to the total time sent with 30 °C conspecifics (t = 2.6901, df = 5, p=0.043, R=0.769, see asterisk).

Gecko group	Preoptic area	Organon periventricular nucleus	Ventral tegmental area
30-blank	0.495	0.494	0.840
30-DHT	0.630	0.604	0.780
32.5-blank	0.961	0.197	0.272
32.5-DHT	0.130	0.587	0.219
34-DHT	0.043 *	0.456	0.244

Table 13. Correlation test of total time focal male spent with 30 °C stimulus conspecifics to tyrosine hydroxylase–ir cell density in three different brain regions.

In 30-DHT males, tyrosine hydroxlase density in the organon periventricular nucleus was positively correlated to the total time spent with conspecifics ($t = 2.9432$, $df = 4$, $p=0.042$, $R=0.827$ see asterisk). After removal of an outlier, this correlation was no longer significant ($p= 0.572$).

Gecko group	Preoptic area	Organon periventricular nucleus	Ventral tegmental area
30-blank	0.287	0.605	0.450
30-DHT	0.765	0.042 *	0.392
32.5-blank	0.131	0.071	0.145
32.5-DHT	0.274	0.948	0.586
34-DHT	0.055	0.866	0.169

Table 14. Correlation test of total time focal male spent with 30 °C stimulus conspecifics to Fos –ir cell number in three different brain regions.

P value of the correlation is given per nucleus for each treatment group.

Gecko group	Preoptic area	Organon periventricular nucleus	Ventral tegmental area
30-blank	0.455	0.385	0.446
30-DHT	0.877	0.478	0.982
32.5-blank	0.414	0.449	0.103
32.5-DHT	0.921	0.944	0.532
34-DHT	0.183	0.671	0.560

Table 15. Correlation tests of total time focal male spent with 30 °C stimulus conspecifics to Fos –ir cell density in three different brain regions.

P value of the correlation is given per nucleus for each treatment group.

Gecko group	Preoptic area	Organon periventricular nucleus	Ventral tegmental area
30-blank	0.381	0.646	0.969
30-DHT	0.562	0.385	0.311
32.5-blank	0.518	0.852	0.112
32.5-DHT	0.328	0.383	0.824
34-DHT	0.525	0.457	0.567

Table 16. Correlation test of total time focal male spent with 30 °C stimulus conspecifics to percent of tyrosine hydroxylase colocalized with Fos in three different brain regions.

In 34-DHT males, colocalization of tyrosine hydroxlase with Fos in the organon periventricular nucleus was negatively correlated to the total time spent with conspecifics ($t = -3.8939$, $df = 5$, $p = 0.011$, $R = -0.867$, see asterisk). After removal of an outlier, this correlation was no longer statistically significant ($p = 0.080$).

Gecko group	Preoptic area	Organon periventricular nucleus	Ventral tegmental area
30-blank	0.663	0.432	0.799
30-DHT	0.198	0.857	0.683
32.5-blank	0.414	0.163	0.859
32.5-DHT	0.091	0.386	0.620
34-DHT	0.817	0.011 *	0.150

Table 17. Correlation tests of total time focal male spent with 30 °C stimulus conspecifics to pDARPP-32 –ir cell number in three different brain regions.

P-value of the correlation is given per nucleus for each treatment group.

Gecko group	Nucleus accumbens	Preoptic area	Striatum
30-blank	0.203	0.724	0.536
30-DHT	0.243	0.420	0.317
32.5-blank	0.106	0.708	0.250
32.5-DHT	0.969	0.159	0.918
34-DHT	0.807	0.582	0.146

Table 18. Correlation test of total time focal male spent with 30 C stimulus conspecifics to pDARPP32 –ir cell density in three different brain regions.

In 32.5-blank males, pDARPP-32 density in the nucleus accumbens was negatively correlated to the total time spent with conspecifics ($t = -3.112$, $df = 7$, $p= 0.017$, $R= - 0.762$, see asterisk). After removal of an outlier, this correlation was no longer significant ($p= 0.138$).

Gecko group	Nucleus accumbens	Preoptic area	Striatum
30-blank	0.401	0.537	0.713
30-DHT	0.216	0.265	0.323
32.5-blank	0.017*	0.768	0.575
32.5-DHT	0.809	0.585	0.508
34-DHT	0.882	0.265	0.875

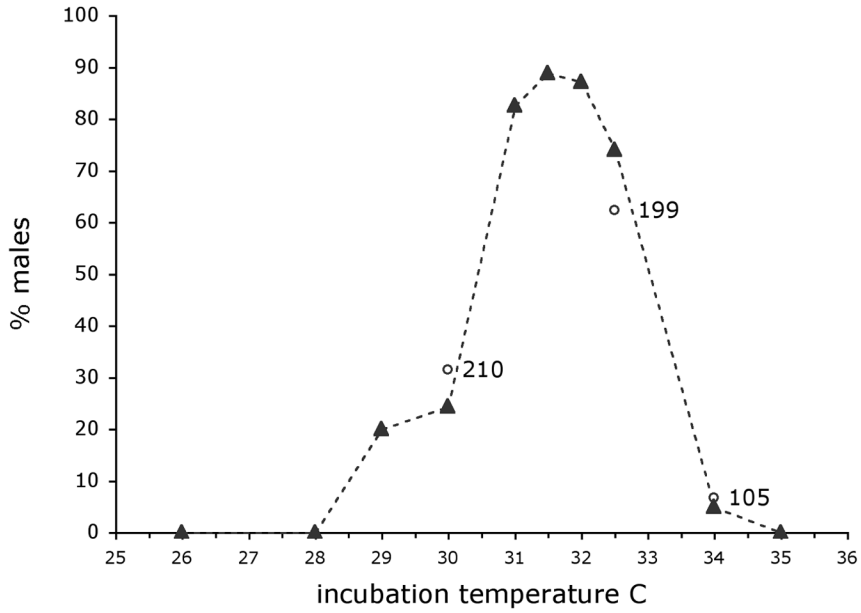

Figure 1: Sex ratios (percent male hatchlings) across different incubation temperatures (IncTs) from Viets et al. (1993) (filled triangles connected by dashed lines) and from the University of Texas breeding colony from 1993 to 2001 (open circles).

Presented are percentages of male hatchlings with different IncT with total number of hatchlings at the respective IncT. Clutch siblings were of different IncT that did not hatch or were not sexed.

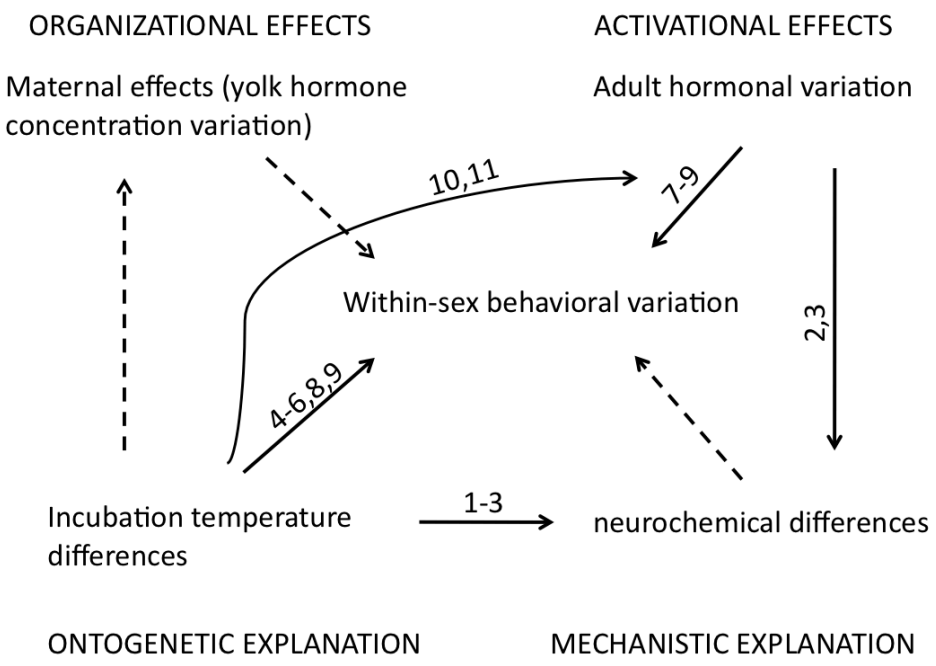

Figure 2: Framework for studying factors contributing to within-sex variation in behavior of leopard geckos.

Organizational and activational effects of hormones from a mammalian neuroendocrine framework are part of ontogenetic and mechanistic explanations of behaviors in leopard geckos. Solid arrows indicate embryonic and adult manipulation studies that affect within-sex behavioral variation. Dotted arrows are untested hypotheses. References for the studies: 1. Coomber et al.1997; 2. Crews et al. 1996; 3. Dias et al. 2007; 4. Flores et al. 1994; 5. Putz and Crews 2006; 6. Rhen and Crews 1999; 7. Rhen et al. 1999; 8. Rhen and Crews 2000; 9. Sakata and Crews 2003; 10. Tousignant and Crews 1995; 11. Tousignant et al. 1995.

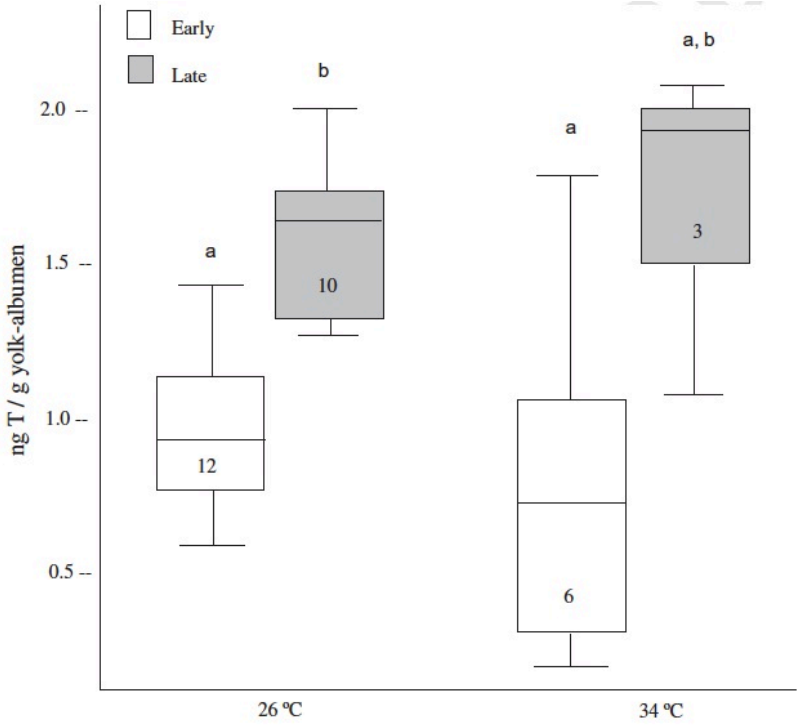

Figure 3: In the leopard gecko, testosterone concentration in the yolk-albumen is higher in Late vs. Early thermosensitive period at 26 ºC incubation temperature.

Medians of four treatment groups ± 1.5 interquartile ranges are represented; numbers in boxes indicate sample size. Groups not sharing the same letters indicate significant difference ($p<0.05$).

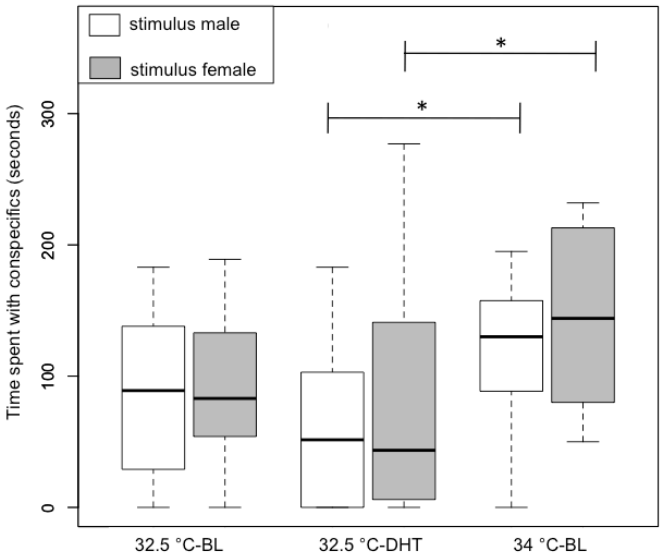

Figure 4: Box-and-whisker plots (median, 1st and 3rd quartile ± 1.58 inter-quartile range) showing association time with stimulus conspecifics in Y-maze.

32.5 °C-DHT: males from male-biased IncT received a dihydrotestosterone (DHT) implant. 32.5 °C-BL: males from male-biased IncT receiving a blank implant. 34 °C-BL: males from female-biased incubation temperature IncT receiving a blank implant. Asterisk indicates significant difference ($p \leq 0.05$).

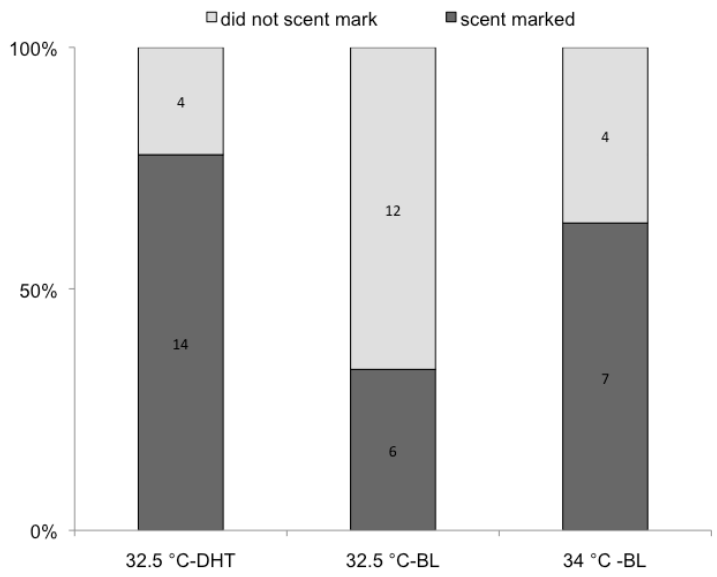

Figure 5: Proportion of animals that scent-marked in Y-maze.

32.5 °C-DHT: males from male-biased IncT received a dihydrotestosterone (DHT) implant. 32.5 °C-BL: males from male-biased IncT receiving a blank implant. 34 °C-BL: males from female-biased incubation temperature IncT receiving a blank implant. Number of individuals are indicated in bar.

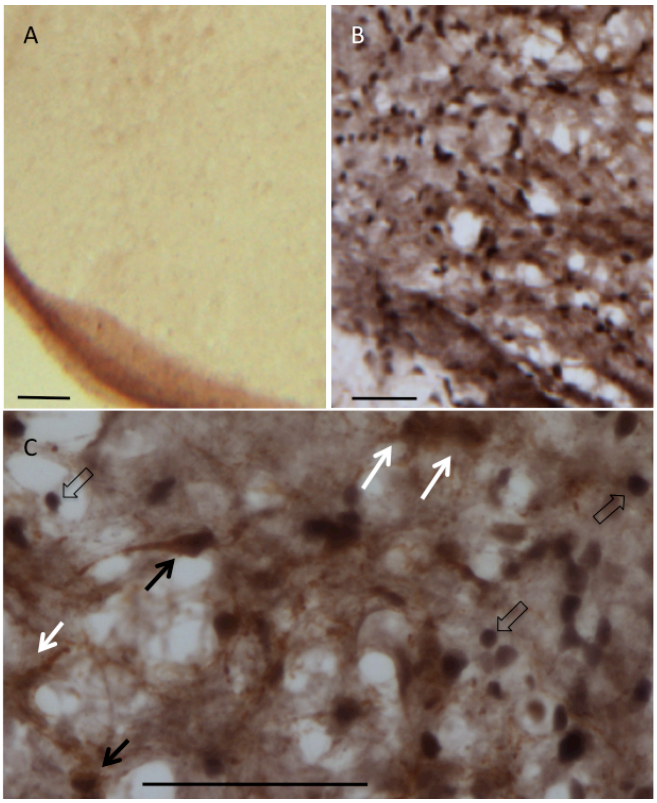

Figure 6. Representative photomicrographs of tyrosine hydroxylase (TH) and Fos immunoreactive cells in sectioned brain tissue.

Immunohistochemistry was conducted on cryosectioned gecko brain tissue using a rabbit polyclonal antibody against Fos and mouse monoclonal antibody against TH. DAB with nickel staining and Vector Red were used to visualize Fos and TH immunoreactive cells, respectively. Bars indicate 50 μm A) Without TH or Fos antibodies, no neurons were detected. B) Neurons were stained when tissue was incubated with TH and Fos antibodies. The lower left corner of A and B is the ventral edge of the tissue. When focusing though the tissue on the microscope C) TH, indicated with a white arrow, was a red, cytoplasmic stain, and was distinct from the small nuclear dark Fos stain (indicated with clear arrow), and also distinct in the (D) large colocalized stain with dark centered stain, indicated with black arrow.

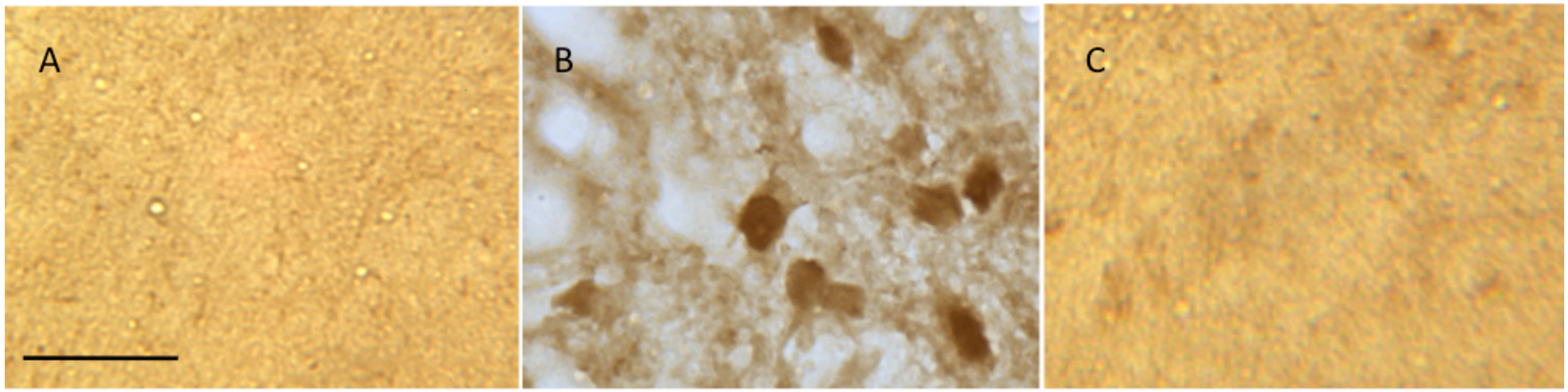

Figure 7. Antibody validation of phosphorylated DARPP-32 at threonine 34 residue (pDARPP-32) on sectioned brain tissue.

Immunohistochemistry was conducted on cryosectioned gecko brain tissue using a rabbit polyclonoclonal antibody against pDARPP-32, and DAB staining was used to visualize pDARPP-32 immunoreactive cells. Bars indicate 50μm. A) Without pDARPP-32 antibody, no cells were detected. B) pDARPP-32 neuronal staining was detected when tissue was incubated with antibody. C) pDARPP-32 neuronal stainging was not detected when antibody was preadsorbed with 5X pDARPP-32 peptide prior to incubation.

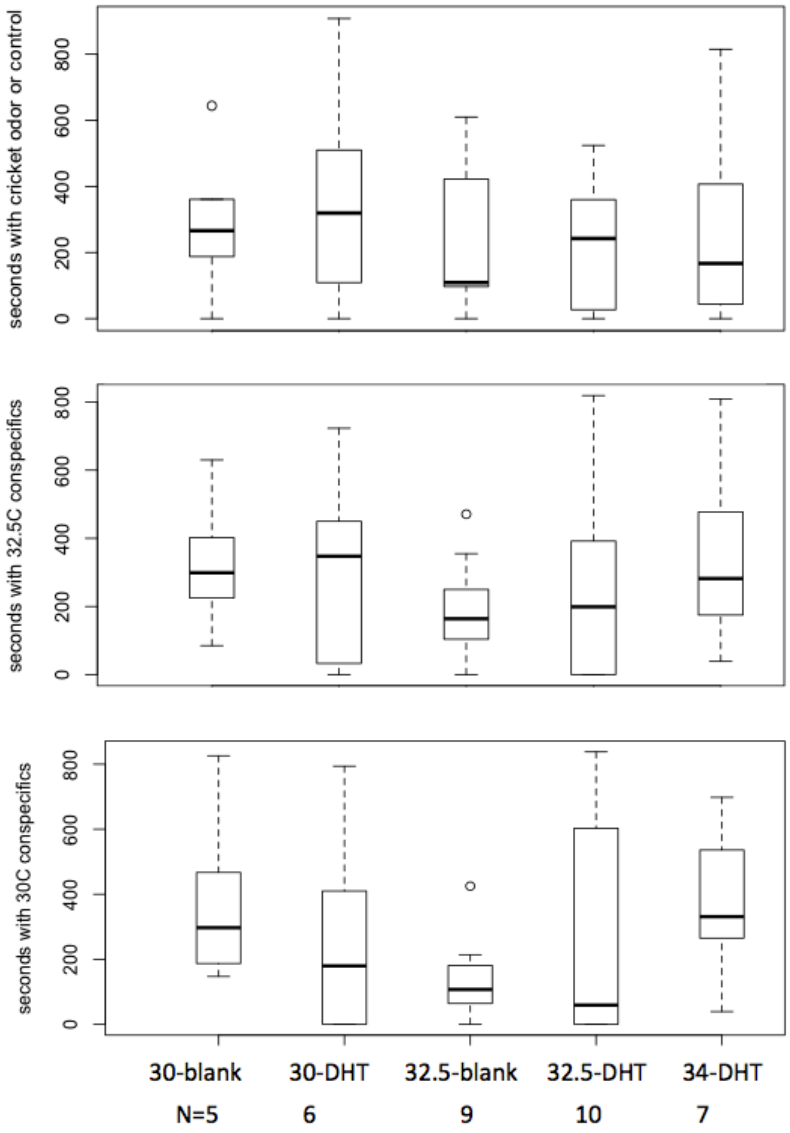

Figure 8. Total time spent in short arms of Y maze were interpreted as investigation time.

Box-and-whisker plots (median, 1st and 3rd quartile ± 1.5 inter-quartile range) per treatment group for the following Ymaze tests (top to bottom): cricket odor and control, 32.5 °C conspecifics, and 30 °C conspecifics.

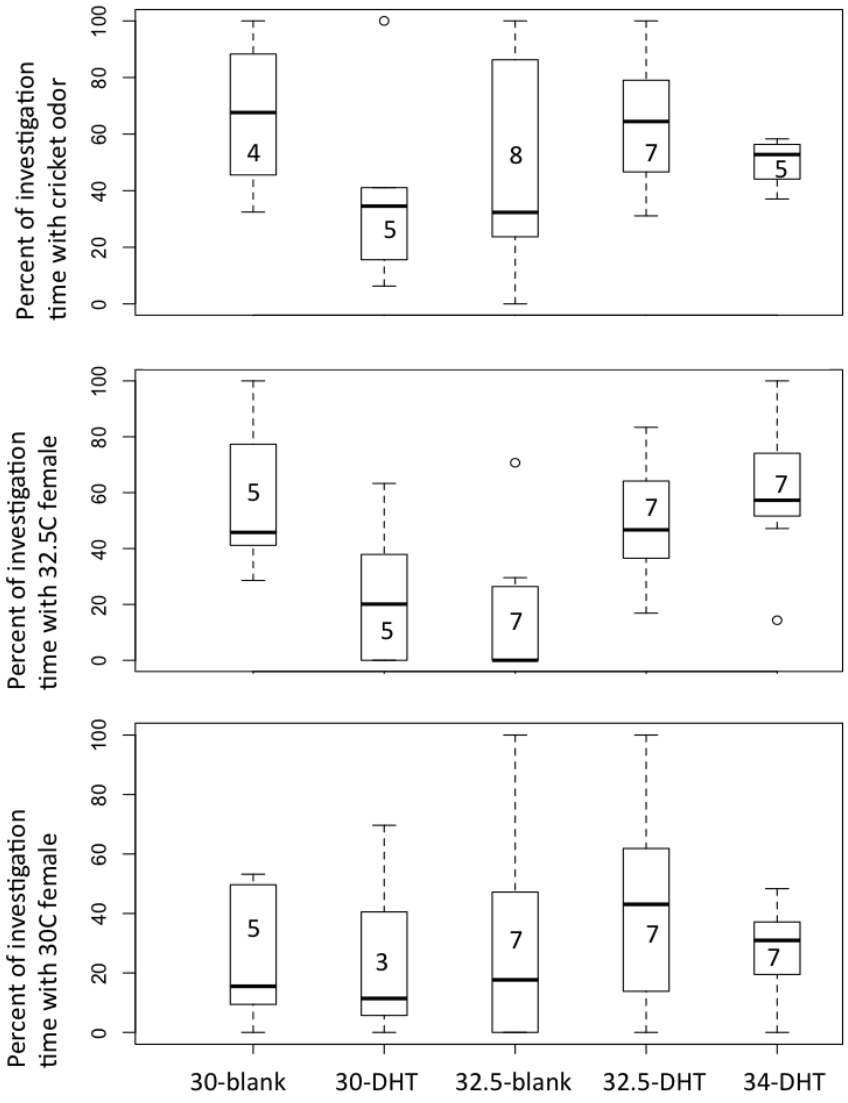

Figure 9. For each treatment group, percent of total investigation time spent with cricket odor, stimulus female of 32.5 °C and stimulus female of 30 °C in three different Y maze tests

Box-and-whisker plots (median, 1st and 3rd quartile ± 1.5 inter-quartile range). Individuals that did not investigate either short arm in the Y maze were excluded from the respective test.

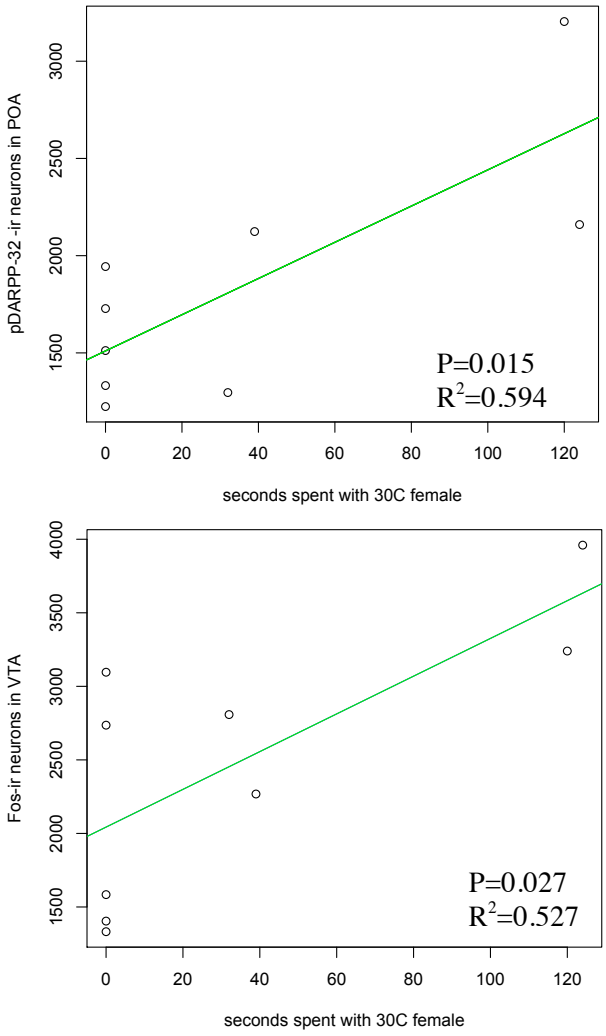

Figure 10. Protein immunoreactive cell number was positively correlated to time spent with 30 °C stimulus female.

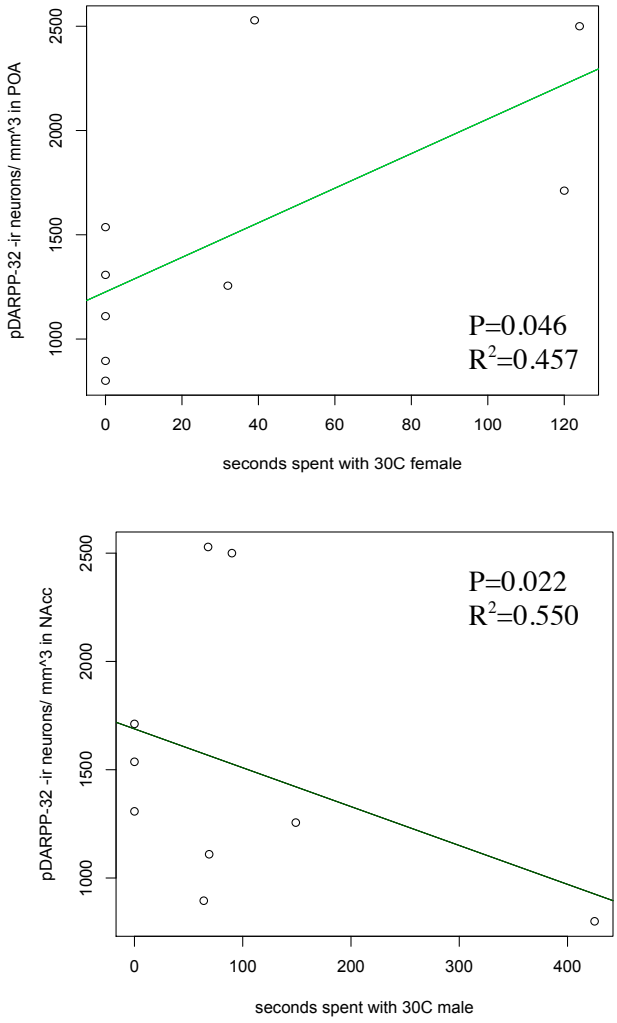

Figure 11. phosphorylated DARPP-32 at threonine 34 (pDARPP-32) immunoreactive cell density in relation to time spent with 30 °C stimulus conspecifics.

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