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The role of the kisspeptin system in regulation of the reproductive endocrine axis and territorial behavior in male side-blotched lizards (*Uta stansburiana*)

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ABSTRACT

The neuropeptide kisspeptin and its receptor are essential for activation of the hypothalamic-pituitary-gonadal (HPG) axis and regulating reproduction. While the role of kisspeptin in regulating the HPG axis in mammals has been well established, little is known about the functional ability of kisspeptins to activate the HPG axis and associated behavior in non-mammalian species. Here we experimentally examined the effects of kisspeptin on downstream release of testosterone and associated aggression and display behaviors in the side-blotched lizard (*Uta stansburiana*). We found that exogenous treatment with kisspeptin resulted in an increase in circulating testosterone levels, castration blocked the kisspeptin-induced increase in testosterone, and testosterone levels in kisspeptin-treated animals were positively related to frequency of aggressive behaviors. This evidence provides a clear link between kisspeptin, testosterone, and aggressive behavior in lizards. Thus, it is likely that kisspeptin plays an important role more broadly in non-mammalian systems in the regulation of reproductive physiology and related behaviors.

1. Introduction

Reproduction is controlled by a wide suite of endocrine and neural factors, which in turn heavily influence behavior (Moore and Lindzey, 1992; Rhen and Crews, 2002). The science of exploring these links between the neural, endocrine, and behavioral systems is an emerging field as we better understand how different genetic components of each system relate to each other. These links are especially poorly understood in non-mammalian organisms, despite similarities in the systems (Moore and Jessop, 2003). Understanding how these links function in organisms with different evolutionary histories can help us determine the basic underpinings of physiological systems.

One such link in this neural, endocrine, and behavioral system has recently been identified. The neuropeptide kisspeptin and its receptor are essential for activation of the hypothalamic-pituitary-gonadal (HPG) axis in mammals (Lee et al., 2009); mutations in either the gene encoding kisspeptin, *Kiss1*, or its receptor *Kiss1R* (previously identified as the orphan receptor gene GPR54) leads to reproductive deficiencies in males and females (d'Anglemont de Tassigny et al., 2007; de Roux et al., 2003; Roa and Tena-Sempere, 2007; Seminara et al., 2003). Further, exogenous administration of kisspeptin to mammals activates the HPG axis leading to significant elevations of lutenizing hormone (LH) from the pituitary gland and downstream secretion of sex steroids in animals with capable gonads (Greives et al., 2007, 2011; Irfan et al., 2014; Saito et al., 2012). Activation of the kisspeptin system appears to be possible at the hypothalamus by releasing gonadotropin releasing hormone (GnRH; Tena-Sempere et al., 2012; Roa et al., 2009) or at the pituitary gland by directly stimulating the release of luteinizing hormone (LH; Luque et al., 2011; Tena-Sempere et al., 2012; Richard et al., 2009).

While the role of kisspeptin in regulating the HPG axis in mammals has been well established, little is known about the functional ability of kisspeptins to activate the HPG axis in non-mammalian species. This is despite the fact that comparative work has shown the presence and functionality of the kisspeptin system in fish, amphibians, and reptiles (reviewed in Tena-Sempere et al., 2012; Um et al., 2010), although it seems to have been lost in birds (Pasquier et al., 2014). Recent investigations of kisspeptin in non-mammalian vertebrates have identified

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variants of the *Kiss1* and *Kiss1R*, deemed *Kiss2* and *Kiss2R* respectively, in animals including zebrafish (*Danio rerio*), African clawed frogs (*Xenopus laevis*) and grass lizards (*Takydromus tachydromoides*) (Lee et al., 2009). Interestingly, this study revealed that grass lizards possess the gene for *Kiss2*, but not *Kiss1*. Synthetic zebrafish and *Xenopus* Kiss2 peptide based on the *Kiss2* mRNA sequence binds to both Kiss1R and Kiss2R in vitro (Lee et al., 2009). An immunohistochemical examination of the lizard *Anolis carolinensis* using a human kisspeptin antiserum revealed the presence of kisspeptin-like immunoreactivity in the preoptic area (Dunham et al., 2009), an area important for activation of the HPG axis in lizards. Because the putative protein product of the *Kiss2* gene is able to activate Kiss1R, and as the c-terminus of both Kiss1 and Kiss2 are highly conserved, these neurons identified with human Kiss1 antisera likely represent cells with functional Kiss2 peptide. The observation of kisspeptin expressed in areas of the hypothalamus associated with reproduction in *Anolis* lizards suggests a likely role for the kisspeptin system in regulating reproductive function in reptiles, similar to its actions in mammals. However, these studies did not measure testosterone production or other potential down-stream effects as a result of activation of these genes.

Much of the functional research into kisspeptin has focused on the neuropeptide's effects on regulating puberty (Ball and Wade, 2013). However, the HPG axis controls a suite of hormones and down-stream effects, such as territorial behavior, that warrant further analysis. Activity of the HPG axis, particularly the secretion of the sex steroid testosterone, is closely linked with territorial and aggressive behaviors in a number of species (Hau, 2007; Wingfield et al., 1990), including in lizards (Kabelik et al., 2006; Moore, 1986; Weiss and Moore, 2004). Indeed, Grone et al. (2010) determined that cichlid fish that displayed stronger territoriality (higher social status) had a higher abundance of *Kiss1r* mRNA expression in their brains. However, investigations of the role the HPG axis plays in regulating reproductive and territorial behavior has often relied on long-term release implants or multiple daily injections of the sex steroid tesosterone (e.g. Adkins and Schlesinger, 1979; Moore, 1986; Zysling et al., 2006). The link between kisspeptin as an upstream regulator of behavior has not been well documented, especially in non-mammlian species.

Determining the physiological and genetic regulation of the HPG system cannot necessarily reveal how organisms will respond in a functional context. Therefore, it is critical to examine the consequences of this regulation on endpoint metrics, such as behavior (Ketterson and Nolan, 1992; Knapp, 2003; Neuman-Lee et al., 2015). Measuring reproductive and territorial behavior is especially valuable when attempting to understand the intricacies of HPG activation and control (Knapp, 2003). Quantifying behavior can be difficult in wild organisms, so it is important to select metrics that are serialized, well-described, and linked to the physiological metric (Graham et al., 2008; Greenberg, 1978). It is well-documented that animals may display behaviors differently in the laboratory and field, so ideally behaviors in both environments should be observed (Dickens and Bentley, 2014). Finally, other endocrine signaling axes may also be involved with HPG signaling and behavior, and thus, it is impotrant to more comprehensively investigate endocrine and interactions with behavior. Especially significant is the hypothalamo-pituitary-adrenal (HPA) axis and downstream adrenal hormones (i.e., corticosterone), known to have important feedbacks and regulatory effects on reproduction and behavior in many species including reptiles (Moore and Jessop, 2003).

Utilizing the seasonally breeding and territorial side-blotched lizards (*Uta stansburiana*) as a model, we explicitly test the hypothesis that kisspeptins are able to activate the HPG axis in non-mammalian terrestrial vertebrates, by measuring secretion of the sex steroid testosterone in response to injection with kisspeptin. Additionally, we investigated whether injections of kisspeptin alters behavioral output in lizards over a very short time-scale (i.e. 30 min), and relate these behaviors with kisspeptin-induced activation of the HPG axis in both the laboratory and field. *Uta stansburiana* is similar to many lizards in that it displays robust territorial and reproductive behaviors that can be visually measured (Sinervo et al., 2000). Behavioral measures in lizards are easily quantifiable, especially in males, because male lizards have discrete territorial displays (e.g. push-ups) and defenses (e.g. charging) (Greenberg, 1978; Lovern et al., 2004; Sinervo et al., 2000). Taken together, this makes *U. stansburiana* an ideal model to investigate the effect of kisspeptin-induced HPG activation on behavior.

To determine the effects of kisspepetin on HPG axis activity in side-blotched lizards, we completed three experiments: 1) male lizards in the field were injected with either kisspeptin or a vehicle and aggressive and behavioral responses to kisspeptin injection were recorded, as well as the relationship between these behaviors and testosterone and corticosterone levels 2) males in the lab were injected with either kisspeptin or a vehicle and behavioral and hormonal responses were recorded and 3) castrated and non-castrated males were injected with kisspeptin and testosterone concentrations were measured post-injection the lab. We used these experiments together to determine whether kisspeptin-related behavioral effects were mediated via the HPG axis to alter testosterone concentrations or via other endocrine pathways, especially the HPA axis and corticosterone.

2. Materials and methods

2.1. Animals

All procedures were approved by the Utah State University Institutional Animal Care and Use Committee (IACUC protocol #1449). Male adult side-blotched lizards (*Uta stansburiana*) were collected within Dixie National Forest, Utah (Washington County), USA, via noosing in May 2010 (Experiment 1) and May 2011 (Experiment 2 and 3). All individuals were weighed and measured (snout-vent-length; SVL) upon capture.

2.2. Experiment 1: field trials

Side-blotched lizards ($n = 20$) were caught in the field via noosing, and given a 0.02 ml injection of either 50uM solution of kisspeptin (#048-56 KiSS1, Phoenix Pharmacueticals) in PBS buffer ($n = 11$) or PBS buffer alone ($n = 9$) intra-coelomically immediately upon capture. We used kisspeptin-10, a human variant, because it was the only commercially available variant. However, this variant is likely active in side-blotched lizards because it is active in other reptiles (Lee et al., 2009; Li et al., 2009), and the endogenous variant in this species has not been determined. We restained lizards individually for 30 min in breathable, opaque cloth sacks in one location, after which they underwent a social interaction with a randomly selected stimulus male in a behavioral arena (plastic tub (55 cm × 43 cm × 32 cm) filled with "playground" sand and a single rock). The stimulus male was not injected with either kisspeptin or PBS at any time point. Encounters lasted 5 min and were scored manually and videotaped using a Canon HD Vixia HFS10 to additionally score after the trials. Video camera and researcher were positioned behind a blind such that entire arena was in video field of view. Video analysis was ultimately used to analyze all behavioral encounters, although both methods were highly related ($P < 0.01$, $r^2 = 0.92$). Behaviors measured on the treatment male included: latency to interaction with the stimulus male, pushups, full shows (i.e., lizards hold themselves up for extended duration), headbobs, charge (runs at other individual), and charge plus bite. All trials were completed between 09:10–11:59 am. Mean air temperature during trials was approximately 35 °C. After the 5 min behavioral trial we collected a blood sample from all animals retro-orbitally (within 1 min of capture). All blood was centrifuged and plasma was separated from red blood cells and stored at −20 °C until further analysis (described below). Each stimulus male was used in no more than two encounters and was rotated so as not to participate in multiple consecutive encounters. All animals were then released at their site of capture.

2.3. Experiment 2: laboratory trials

To measure these relationships in a more controlled environment, we measured both circulating sex steroid hormone and functionally related (i.e., behavioral) responses in male lizards provided either kisspeptin or vehicle injection in a laboratory setting. Male side-blotched lizards ($n = 16$, different individuals than in Experiment 1) were caught via noosing and brought back to Utah State University, Logan, UT, USA, where they were indiviudally housed for one week prior to the experiment. Animals were housed individually in 26 × 28 × 50 cm polycarbonate terraria, in a room maintained on a 14L:10D photoperiod. A 25 W heat lamp was suspended over one end of the cage providing a thermogradient within the cage (27–40 °C) and enabling the animals to behaviorally thermoregulate.

On the day of experimentation, all animals were hand captured from their individual terraria and given a 0.02 ml injection (within 2 min of capture) of either a 50 uM solution of kisspeptin (#048-56, Kiss-1, Phoenix Pharmacueticals) in Phosphate Buffered Solution (PBS) buffer ($n = 8$) or PBS buffer ($n = 7$) alone intra-coelomically. Animals were then placed back into their terraria for 30 min, after which they were re-captured and underwent a social interaction with a stimulus male in a behavioral arena, as described above. The stimulus male was not injected with either kisspeptin or PBS at any time point and all stimulus males were significantly smaller and weighed less than injected males ($P < 0.01$) to ameliorate the risk that a larger stimulus male would alter the territorial behavior of injected males. All trials were completed between 09:10–11:59 am. Mean air temperature during trials was 27 °C and mean humidity was 20%. Encounters lasted 5 min with no acclimation period and were scored manually and videotaped as described above.

In addition to the behaviors scored in the field study, the video was analyzed using Noldus EthovisionXT® Software (Leesburg VA, USA). Using Ethovision, we determined the following parameters for each treatment lizard: total distance moved within the arena, mean distance from rock, total distance from rock, total duration on rock, latency to first time on rock, and mean velocity. We chose parameters utilizing the rock as lizards will make territorial displays on perches (Greenberg, 1978; Sinervo et al., 2000). Investigations into *U. stansburiana* behavior have elucidated alternative reproductive tactics depending upon color morph (Sinervo and Lively, 1996), but our work with this population shows no evidence of physiological differences between color morphs (Lucas and French, 2012) and color morph was ignored.

After the five minute behavioral trial we collected a blood sample from all animals retro-orbitally (within 3 min of capture) for analysis of testosterone. Blood was processed and plasma was stored as described above. Each stimulus male was used in no more than two encounters and was rotated so as not to participate in multiple consecutive encounters.

2.4. Experiment 3: castrations

Male side-blotch lizards ($n = 15$) from the laboratory experiment (described above) were either castrated or underwent a sham castration surgery. One week following surgery all animals were then given a kisspeptin challenge, where they were injected with 0.02 ml of a 50 uM solution of kisspeptin and bled 35 min later. Briefly, bilateral castration of adults was performed using techniques described by Moore (1987). Surgical procedures were performed with the animals completely unconscious and unresponsive to stimuli under anesthetic deep hypothermia, which is induced by packing the animals in crushed ice for 10–30 min prior to surgery. Briefly, the testes of the hypothermically anesthetized animal were exteriorized through a small ventral incision, a ligature was tied around the testis blood vessels, the testis was cut off above the ligature, the ends of the severed blood vessels were cauterized, and the incision was closed with sutures. Sham surgeries followed a similar procedure except that no tissue was removed. Individuals with sham surgeries were returned to their site of capture after healing, while the castrated males were euthanized.

2.5. Plasma samples and radioimmunoassay

Circulating hormone levels were determined using a previously described and established radioimmunoassay (RIA) protocol (Neuman-Lee and French, 2014). Briefly, extractions were performed using a solution of 30% ethyl acetate: isooctane. Following extraction, using the same protocols as Moore (1986), samples were dried down, resuspended in PBS buffer, and assayed in duplicate for testosterone (Fitzgerald, #WLI-T3003–01916) and corticosterone (Experiment 1; Fitzgerald 20-

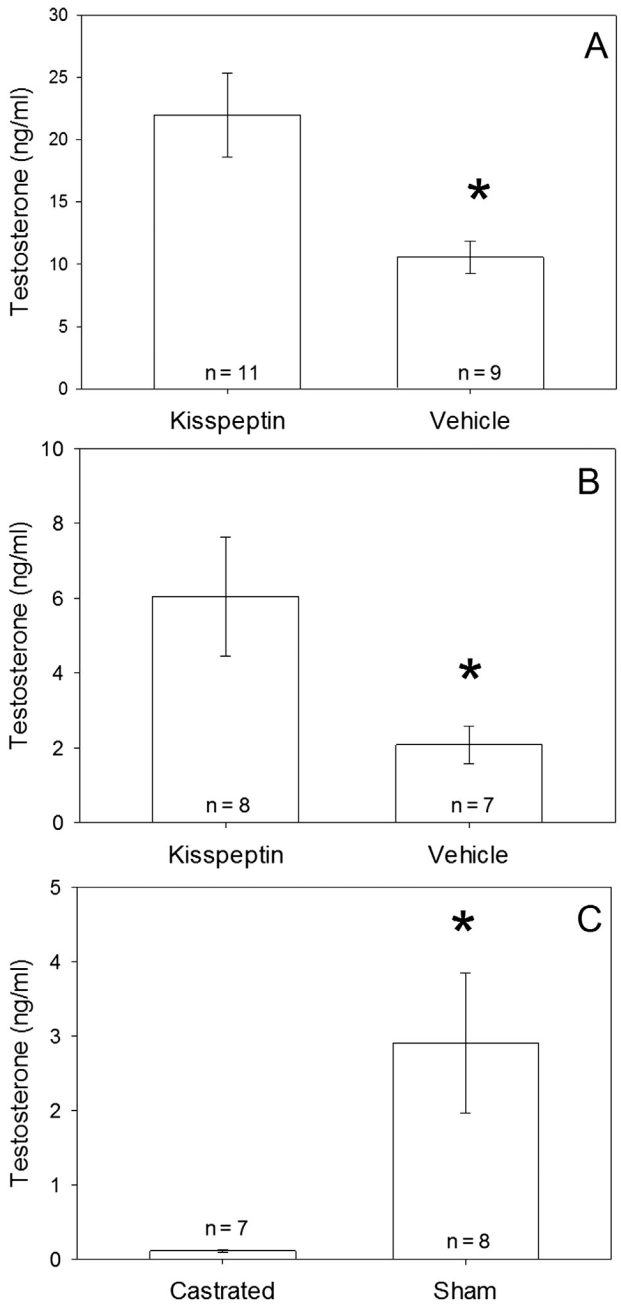

Fig. 1. Testosterone concentrations in male side-blotched lizards (*Uta stansburiana*) given either kisspeptin or a vehicle challenge in the field (A) or laboratory (B), and given kisspeptin after castration (C). Data are shown non-transformed. Error bars represent ± 1 standard error of the mean. Asterisks represent significant differences between treatment groups.

CR45, Lot # P0012502). For each sample, we used an aliquot of the resuspended fractions to measure individual recoveries following extraction and chromatography. Final sample concentration values were adjusted based on individual recoveries to account for any losses during extraction and chromatography procedures (intra-assay coefficient of variation = Testosterone 2010 = 4.9%, 2011 = 9.11%; Corticosterone 2010 = 10.35%).

2.6. Statistical analyses

Because the animals receiving the castrations had previously been used for the laboratory kisspeptin trials, we also performed an analysis of covariance (ANCOVA) on the testosterone concentrations after castration using the testosterone concentrations after the behavioral trial as a covariate to the castration/sham treatment. Because treatment was significant while the previous levels of testosterone or the interaction between treatment and testosterone were not ($P > 0.05$), we analyzed testosterone levels from the lizards using a *t*-test. For the other two experiments, we also compared the two treatment groups using a *t*-test. To compare treatment effects on behavior, additional *t*-tests were conducted. To examine the effect of testosterone levels on the behavioral parameters, we employed linear regressions. Testosterone, pushups, time to first interaction, total distance moved, and latency to first time on rock were $\log_{10}$-transformed to satisfy the assumption of normality and equal variances in all of the tests. One male was removed from the mean velocity analyses because his velocity could not be reliably calculated by the EthoVision software. There was no effect of time of bleeding on the metrics, so it was removed from all models ($P > 0.2$). All statistical analyses were performed using JMP·IN version 8.1 analyses software (SAS Institute Inc., Cary, NC) or R version 3.3.0 (R Development Core Team, 2016).

3. Results

3.1. Experiment 1: field trials

Kisspeptin treatment significantly affected circulating testosterone concentrations in the field ($F = 7.67$, $df = 1, 19$, $P = 0.01$); kisspeptin treated animals had significantly higher testosterone levels than did vehicle treated controls (Fig. 1A). However, there was no significant effect of kisspeptin treatment on any of the aggressive behaviors measured in the field (Table 1; all $F < 1.30$, all $P > 0.27$), nor was there a relationship between testosterone and any of the behavioral metrics (all $P > 0.10$).

There was also no significant effect of kisspeptin treatment on circulating corticosterone concentrations ($F_{(1,19)} = 1.64$, $P = 0.22$). Males did not significantly differ in snout vent length (SVL, 51.8 ± 0.4 mm) or body mass (3.8 ± 0.2 g) across treatment groups (all $F < 0.46$, all $P > 0.51$).

3.2. Experiment 2: laboratory trials

Kisspeptin treatment significantly affected circulating testosterone levels in laboratory housed-males ($F_{(1,15)} = 5.02$, $P = 0.04$); kisspeptin treated males had higher testosterone levels than did vehicle injected controls (Fig. 1B). There was no significant effect of kisspeptin treatment on aggressive behaviors (Table 1; all $F < 2.50$, all $P > 0.14$) or movement behaviors (Table 1; $F < 3.1$, all $P > 0.10$) directly. When examining animals in both treatment groups, testosterone levels were positively correlated to the number of headbobs ($F_{(1,13)} = 5.35$, $P = 0.04$, $r^2 = 0.29$). No other correlation was found in any of the variables ($F < 2.88$, all $P > 0.11$). When treatment groups were analyzed separately, testosterone was positively correlated to intensity of some aggressive behaviors in kisspeptin animals (head-bobs: $F_{(1,7)} = 5.52$, $P = 0.06$, $r^2 = 0.49$, Fig. 2A; push-ups: $F_{(1,7)} = 6.77$, $P = 0.04$, $r^2 = 0.53$, Fig. 2B; time to first interaction: $F_{(1,6)} = 5.27$, $P = 0.07$, $r^2 = -0.51$, Fig. 2C). However, all other parameters were not related to testosterone in kisspeptin-treated animals ($F < 3.0$, $P > 0.14$; full-show displays = insufficient data). There was no correlation between testosterone and aggressive behaviors in vehicle treated control animals (all $F < 0.85$, all $P > 0.40$).

3.3. Experiment 3: castration

Castration surgery effectively blocked testosterone release to kisspeptin challenge, whereby castrated animals had significantly lower testosterone levels than did animals that underwent sham castration surgeries ($F_{(1,14)} = 13.53$, $P < 0.01$; Fig. 1C). For castrations and laboratory trials, males did not significantly differ in snout vent length (SVL, 51.7 ± 0.4 mm) or body mass (3.9 ± 0.1 g) across treatment groups (all $F < 0.68$, all $P > 0.42$).

4. Discussion

The data presented here demonstrate a functional kisspeptin system capable of activating the HPG axis in a non-mammalian vertebrate, the side-blotched lizard; injections of kisspeptin elevated levels of circulating testosterone. Further, we demonstrate that exogenous administration of kisspeptin alone did not significantly alter behaviors, when compared with control-injected animals. However, the amount of pushups was correlated with the elevated testosterone concentrations induced by kisspeptin in treated males.

Table 1
Behavioral measurements of male side-blotched lizards (*Uta stansburiana*) in the field and laboratory in response to a stimulus male after a single injection of either kisspeptin or a vehicle. There are no significant differences between control and kisspeptin injected individuals in all behaviors analyzed (all $P > 0.05$). All values are presented as mean ± 1 standard error.

Parameter	Field		Laboratory	
	Control $N = 9$	Control $N = 7$	Control $N = 7$	Kisspeptin $N = 11$
Latency to interaction (s)	175.0 ± 37.8 $N = 4$	163.83 ± 37.38 $N = 6$	59.00 ± 35.25 $N = 6$	64.43 ± 16.80 $N = 7$
Pushups (#)	9.89 ± 5.27	13.55 ± 5.85	26.86 ± 9.74	19.63 ± 5.46
Full shows (#)	0.11 ± 0.11	0.45 ± 0.28	0	0
Charges (#)	0	0.64 ± 0.36	0.86 ± 0.40	0.25 ± 0.25
Bites (#)	0	0.36 ± 0.28	1 ± 0.58	0.13 ± 0.13
Headbobs (#)	2.78 ± 1.84	2.27 ± 1.07	1.57 ± 0.84	1.88 ± 0.74
Total distance moved within the arena (cm)	–	–	884.17 ± 210.93	2011.59 ± 809
Total distance from rock (cm)	–	–	89,402.7 ± 16,947	55,204.7 ± 10,790
Mean distance from rock (cm)	–	–	9.36 ± 1.95	6.16 ± 1.38
Total duration on rock (s)	–	–	76.63 ± 39.29 $N = 6$	160.10 ± 38.34 $N = 7$
Latency to first time on rock (s)	–	–	36.00 ± 15.85 $N = 6$	28.44 ± 17.37 $N = 7$
Mean velocity (cm/s)	–	–	2.67 ± 0.61	4.03 ± 0.75 $N = 7$

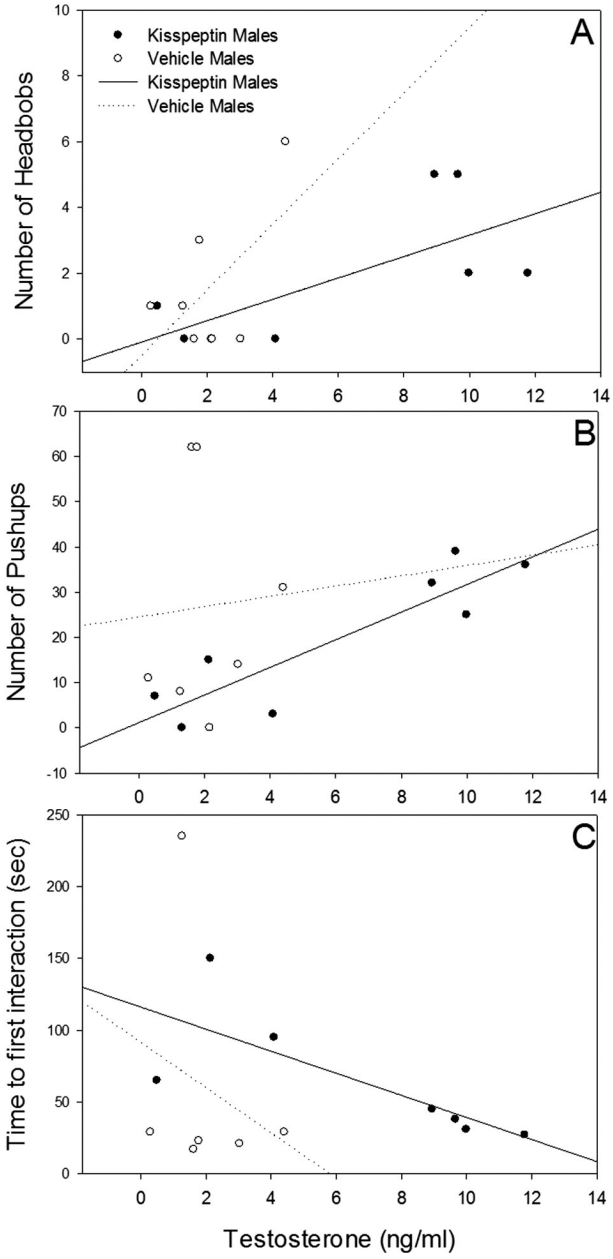

Fig. 2. Correlation between testosterone and behavioral metrics in kisspeptin-treated laboratory-housed side-blotched lizard (*Uta stansburiana*) males when presented with a stimulus male. Behavioral metrics presented include A) Number of headbobs (Kisspeptin males: $R^2 = 0.49$, Vehicle males: $R^2 = 0.07$), B) Number of pushups (Kisspeptin males: $R^2 = 0.53$, Vehicle males: $R^2 = 0.01$), C) Time to first interactions (Kisspeptin males: $R^2 = -0.51$, Vehicle males: $R^2 = -0.05$) All values are presented as non-transformed.

In the current study, side-blotched lizards displayed significant activation of the HPG axis in response to injections of commercially available kisspeptin, human kisspeptin-10, a product of the *KISS1* gene. Activation of the HPG axis was confirmed via a significant elevation in circulating levels of the sex steroid testosterone. Castration abolished this kisspeptin-induced elevation in testosterone, confirming that the measured elevation in testosterone was due to the activation of the HPG axis and was not elevated in response to release or conversion by non-gonadal tissues (e.g. adrenals; Weiss and Moore, 2004).

While numerous studies in mammals have documented the effects of the kisspeptin system on activation of the HPG axis, culminating in testosterone secretion, few studies have investigated the functional actions of this peptide system on the reproductive axis in non-mammalian vertebrates. Two studies of fishes have identified the ability of kisspeptin to elevate LH levels (Chang et al., 2012; Yang et al., 2010), however, neither of these studies investigated the downstream effects by measuring sex steroid secretion, the final hormonal product following activation of the HPG axis.

Our research reveals that a reptilian model does respond to exogenous kisspeptin by activation of the HPG axis. Not only did we see a significant increase in testosterone in kisspeptin-treated males in both the field and the laboratory, but castrating males ameliorated the increase in testosterone. While testosterone response to kisspeptin injection was dampened in laboratory compared with field caught animals, which has been documented in other lizard species (Weiss et al., 2002), the pattern of a significant kisspeptin-induced elevation in testosterone levels compared with vehicle injected animals was still maintained. Interestingly, we found that there was a high level of variation in testosterone response to kisspeptin injections, which is unsurprising given the high amount of individual variation in testosterone levels in this species (Sinervo et al., 2000). This could indicate at least two non-mutually exclusive factors: 1) baseline levels of testosterone (prior to injection or behavioral trials) were highly variable and/or 2) these individuals have varying sensitivity to kisspeptin. Regardless of the reason, this variation in testosterone after kisspeptin injections revealed that these elevations were correlated with the social behavior of pushups, while there was no relationship observed between circulating testosterone levels and behaviors in the vehicle-treated individuals.

We observed that individuals that displayed greater elevations in testosterone in response to kisspeptin also displayed higher amounts of the pushups territorial behavior. A higher concentration of testosterone correlated with an increased level of territorial behavior similarly occurs in tree lizards (*Urosaurus ornatus*), where circulating testosterone levels were higher in individuals exhibiting higher frequency and intensity of aggressive behaviors (Kabelik et al., 2006). Artificial elevations in testosterone also incresaed some, but not all, territorial aggressive behaviors in non-breeding male mountain spiny lizards (*Sceloporus jarrovi*) (Moore and Marler, 1987). Still other studies show that other reproductive hormones, including progesterone might also be important for modulating aggressive display in lizard species (Weiss and Moore, 2004). Interestingly, we did not find any relationship between corticosterone and kisspeptin or any of the behavioral metrics. The relationship between corticosterone and behavior is clearly complicated and sometimes contradictory: studies have shown a relationship between behavior and corticosterone levels (Moore and Mason, 2001; Reedy et al., 2014; Thaker et al., 2009), no relationship between the two metrics (Bliley and Woodley, 2012; Wack et al., 2013), or complicated, context-dependent relationships (Breuner and Wingfield, 2000; Neuman-Lee et al., 2015; Robert et al., 2009).

Previous unpublished work using a rodent model revealed that Siberian hamsters (*Phodopus sungorus*) injected with kisspeptin were no more likely than vehicle injected controls to display aggressive behavior using a resident-intruder paradigm (M. Scotti, T. Greives, G. Demas, unpublished data). If kisspeptin acted directly on neural substrates without the need for elevated testosterone levels, it would be expected that a rapid and significant change in behaviors would have been observed. However, in our study, no significant differences in behavior between kisspeptin- and vehicle-injected lizards were observed 35 min following injection. This may indicate that a longer time period, with prolonged exposure to elevated levels of testosterone, would be needed to observe behavioral changes. This observation strongly suggests that kisspeptin indirectly influences behavior via activation of the HPG axis. To fully rule out the possibility of direct regulation of behavior by central actions of kisspeptin would require behavioral testing following castration and kisspeptin injection.

The current investigation utilized a commercially available kisspeptin peptide derived from the mammalian *Kiss1* gene product (human kisspeptin-10). Recent analyses suggest that the ancestral state of vertebrates is to have two genes that encode for kisspeptin,

Kiss1 and *Kiss2* and that placental mammals have lost the more potent *Kiss2* (Felip et al., 2009). It appears that other species, such as the grass lizard, have lost both *Kiss1* and *Kiss1R* (receptor), but have retained *Kiss2* and *Kiss2R* (Lee et al., 2009). In fish, products of both the *Kiss1* and *Kiss2* variants are able to potently activate the Kiss2R in vitro, as indicated by reporter gene assays (Lee et al., 2009; Li et al., 2009), suggesting that the conserved c-terminal sequence present in both Kiss1 and Kiss2 are capable of activating kisspeptin receptors present in lizards (Lee et al., 2009; Li et al., 2009). Further, immunohistochemical investigations in *Anolis* lizards using antibody against the Kiss1 peptide revealed peptide presence in areas of the hypothalamus known to be important for the regulation of the HPG axis (Dunham et al., 2009), further suggesting the results observed in the current investigation likely reflect a functional kisspeptin system in reptiles. However, it should be noted that the genome of our model species has not been investigated for the presence of either the *Kiss1* or *Kiss2* variant. Thus, although we do not yet know for certain which peptide variant may be acting in this species, the commonality of the c-terminus across taxa, immunohistochemical localization of kisspeptin in the hypothalamus, combined with evidence of bioactivity of both variants on the Kiss2R in fish indicates that our data, demonstrating a significant elevation of testosterone, reveals a role for the kisspeptin system in the regulation of the HPG axis in lizards.

In mammals, the kisspeptin system has been demonstrated to play a key role in regulating seasonal changes in reproductive physiology (Greives et al., 2008), metabolic control of reproduction (Castellano et al., 2009), and the development of reproductive capability (Ball and Wade, 2013). This study provides evidence that it plays a direct role in regulating reptilian reproductive physiology, and an indirect role in regulating territorial and reproductive behaviors. The current investigation, which clearly demonstrates a functional role for the kisspeptin system in regulation of the HPG axis, suggests that the kisspeptin system may also play a key role in regulating changes in seasonal breeding in non-mammals and reproductive development as well. Future experiments investigating seasonal and ontogonetic changes in expression of kisspeptin as well as sensitivity of the HPG axis in response to kisspeptin will help to further resolve the role of kisspeptin as a key regulator of reproduction in vertebrates.

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