Physiology & Behavior 179 (2017) 220–225

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/physbeh

Mammalian nonapeptides activate territorial behavior in an amphibian

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ARTICLE INFO

Keywords:
Arginine vasopressin
Oxytocin
Arginine vasotocin
Nonapeptides
Mammal
Amphibian
E. coqui

ABSTRACT

Arginine vasopressin (VP) and oxytocin (OT) are two nonapeptides present in mammals and execute a wide array of physiological and behavioral functions. In amphibians arginine vasotocin (VT) is hypothesized as a homologous nonapeptide for VP and also performs physiological and behavioral tasks. Studies have demonstrated that the structural and functional relationships between VP, OT, and VT receptor families are similar; however, little behavioral data has complimented these studies. The objective of this investigation was to determine if the mammalian nonapeptides VP and OT would activate behavioral manifestations naturally activated by VT. Frogs are particularly attractive for such an investigation because it is well documented that VT activates advertisement calling and territorial behavior. This investigation was a large sample size field study that utilized the territorial frog, *Eleutherodactylus coqui*. Fieldwork occurred on the Islands of Puerto Rico and Hawai'i and focused on territorial (calling) and non-territorial (silent) males. Frogs were administered exogenous injections of VP, OT, VT (positive control), or saline (control) in the field, placed back in their original locations, and were observed for behaviors. Exogenous injections of VP and OT significantly activated silent males to emit advertisement calls and exhibit territorial behavior. Additionally, silent males moved into new areas prior to calling whereas territorial males remained in their own territories. Control (saline) males displayed normal behaviors. This is the first study to demonstrate that mammalian nonapeptides activate calling and territorial behaviors in frogs and corroborates the close evolutionary relationships within the nonapeptide family.

1. Introduction

Nonapeptides are an archaic family of conserved peptides that have evolved for > 700 million years. Functional properties of nonapeptides and their phylogenetic dispersion within vertebrate clades are wide-ranging and diverse [1], and yet the biochemical differences among the nonapeptides are remarkably minor [2]. Vasopressin- and oxytocin-related nonapeptides are present in representatives of both protostomian and deuterostomian lineages [3–8]. This suggests that this signaling system originated very early in metazoan evolution. Due to the structural and positional similarities of the vasopressin and oxytocin genes it is hypothesized that they originated from the duplication of a common ancestral gene, likely following the radiation of the jawless fish about 500 million years ago [3,6,9–10]. This genetic event is certainly plausible since gene duplication is a common evolutionary pathway toward the adaptation of genes to new functions [11].

Arginine vasopressin (VP) and oxytocin (OT) are two nonapeptides that are present in mammals and execute a wide array of physiological and behavioral functions [12]. In other classes of vertebrates these two neuropeptide systems are not present but homologous neuropeptide systems are existent. Instead of VP, arginine vasotocin (VT) occurs in birds, reptiles, amphibians, and fish [2,3]. Co-evolving with these peptides were their respective receptors and signaling pathways that are responsible for conducting molecular and cellular functions.

Functionally, nonapeptides execute an assortment of physiological and behavioral tasks. Traditionally, VP is known as an antidiuretic hormone for its role in water retention [13], vasoconstriction [14], and water homeostasis [15]. Meanwhile OT is classically recognized for its physiological role during parturition and lactation [16–18]. Intriguingly, a large volume of literature has been generated on the behavioral outcomes of nonapeptides. While substantial variation ensues between taxa, all linages of vertebrates are characterized by having specific behaviors governed by these peptides that can include: aggression, agonistic behavior, pair-bond formation, vocalizations, gregariousness, cooperation, and paternal and/or maternal care [for reviews see: [1,3,19–22]].

A number of investigations (and reviews) on nonapeptides have concentrated on the functional aspects of nonapeptide systems [23–27]. While the majority of these studies have utilized mammals as their model organism some have employed amphibians [28,29] and fish [30,31]. For example, it was discovered replacing the fish gene for isotocin (teleost homolog of OT) with the mammalian gene for OT in

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http://dx.doi.org/10.1016/j.physbeh.2017.06.020
Received 14 October 2016; Received in revised form 26 June 2017; Accepted 26 June 2017
Available online 29 June 2017

transgenic rats did not adversely affect their physiology [31]. Further, it was demonstrated that the mammalian nonapeptides, OT and VP, can modulate social behavior in fish [32]. This could indicate that receptor mechanisms and signaling factors mediating the physiological regulation of nonapeptides are possibly conserved between mammals and fish. Investigations in both newts and frogs disclosed that both mesotocin and vasotocin 1a nonapeptide receptors are present [24,28,29]. In amphibians, the VT system activates indispensable social and reproductive behaviors [33–37] and it is firmly established that in male frogs VT activates territorial and reproductive behaviors [38]. In fact, previous research in the Puerto Rican coquí frog, *Eleutherodactylus coqui*, has demonstrated that VT will activate advertisement vocalizations [37]. These are signals used in the establishment and maintenance of territories as well as the attraction of mates. This investigation also found that exogenously activated, non-territorial frogs moved into and established new territories and commenced advertisement calling [39].

Clearly, nonapeptides perform a key role in the expression of social behaviors and that distinctive classes of nonapeptides exist in different clades of vertebrates. The objectives of this investigation were to 1) determine if the closely related non-amphibian nonapeptides, VP and OT, would activate territorial behavior in male Puerto Rican coquí frogs, *E. coqui*; 2) determine if non-territorial frogs move into new territories and commence advertisement calling, and 3) to elucidate, if VP and/or OT activate additional and/or alternative social or reproductive behaviors. Due to functional and structural components of nonapeptide receptor-ligand binding properties it is hypothesized that VP and OT will activate advertisement calling and territorial behaviors in *E. coqui*.

2. Materials and methods

2.1. Field site locations

Field work was carried out at two locations: 1) the Caribbean National Forest located in the Luquillo Mountains of northeastern Puerto Rico, approximately 1 km east of the El Verde Field Station (350–400 m) of the University of Puerto Rico at Rio Piedras; and 2) the Waiakea Field Research Area (500 m) of the University of Hawai'i at Hilo, 924 Stainback Rd., Hilo, HI. Permits were obtained from the Departmento de Recursos Naturales y Ambientales of Puerto Rico and from the Department of Land and Natural Resources, Division of Forestry and Wildlife of the State of Hawaii for work on *E. coqui*. Care of all animals was conducted in accordance with the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committees at New York University and the University of Hawaii. Experiments were conducted throughout the year that coincided with *E. coqui* continuous year-around breeding season. No differences of reproductive behavior have been noted during various times of the year [39–42]. At both locations, choruses typically begin around sunset, approximately 6–7 pm [either AST or HST] depending on season of the year, and lasted well into the night; although most males ceased calling between 2400 and 0100 h. *E. coqui* is endemic to the Island of Puerto Rico; populations of Hawaiian *E. coqui* are the result of accidental release on the Island of Hawai'i [43]. No differences in any category of results were found between Puerto Rican and Hawaiian frogs.

2.2. Male identification and individual marking

Prior to capture, territorial and silent males were observed for several minutes to ensure behavioral/hierarchical status. Males that were detected to produce advertisement calls to advertise (and defend) an area were assigned as territorial males. Males within one meter (distance explained later) of a territorial male that did not produce any vocalizations and did not physically contact the territorial male were assigned as silent ("satellite-like") males. Silent males were always captured in the territory of a calling male and observed for several minutes to ensure silent status, i.e., no calling or other territorial characteristics. After determining the social status of the male, the frog was captured and injected with a drug or saline (control). To identify and observe individual males the dorsum of the frog was marked with a non-toxic fluorescent powder (Pearl Ex Pigments; Rupert, Gibbon, Spider, Inc., Healdsburg, CA) that would produce excellent illumination when exposed to a UV flashlight. Following injections, all vocalizations, behavior, movement, and respected time were recorded. Frogs were injected shortly after sunset 7–8 pm [local AST or HST] depending on the season of the year and were observed throughout the remainder of the evening until the time when the majority of frogs ceased calling (~2400–0100 h).

2.3. Pharmaceutical administrations

All pharmaceutical treatments were conducted in the field and specific treatments (drugs) were blind to the observer. Frogs (calling and silent) were located with the aid of a flashlight and captured by hand with the assistance of a headlamp. Once captured all males were quickly measured for snout-vent length (SVL) and then injected intraperitoneal (IP) with a respected drug or control (randomly selected). Frogs were injected within the first hour and half after sunset. All treated frogs (experimental and controls) were at least 10 m away from other treated frogs. In addition, no territory had both territorial and silent treated individuals and no frogs were every used more than once. Following injection, experimental and control groups, all frogs were placed back in the exact location that they were captured.

2.4. Nonapeptides

Territorial and silent males were IP injected with either 50 μg VP, OT, or VT (Sigma Chemical Co., St. Louis, MO)/100 μl amphibian Ringer's saline cocktail or 100 μl of amphibian Ringer's saline (control). The dosage of administrations were determined from a previous VT study on *E. coqui* [39] and a preliminary study (10–100 μg VP and 10–100 μg OT in 100 μl of amphibian Ringer's saline) that indicated 50 μg of VP and OT was the lowest dose that resulted in the most consistent response of vocalizations and behavioral changes. Furthermore, it has been demonstrated that all three of the peptides cross the blood-brain barrier when given systemically (IP) [44–46] and that the average variance in adult male frog size was < 5% (SVL = 32.6 mm ± 0.4).

2.5. Control groups

Two groups of controls were included in the experiments. Positive controls consisted of VT-injected territorial and silent males due to the fact that it has been demonstrated that this nonapeptide activates territorial behaviors in male *E. coqui* [39] and is a closely related neuropeptide to both VP and OT [3,6,9–10]. Controls (negative) consisted of saline injections into both territorial and silent males.

2.6. Pharmaceutical and control groups

Experimental groups: Fifty territorial and 50 silent males were IP injected with 50 μg of VP/100 μl amphibian Ringer's saline cocktail. For the OT assemblages, another 50 territorial and 50 silent males comprised another set of experimental groups and were injected with 50 μg of OT/100 μl amphibian Ringer's saline cocktail. No individual frog received more than one drug treatment and all frogs were placed back in the exact location that they were captured.

Control frogs: Fifty territorial and 50 silent were injected with 100 μl of amphibian Ringer's saline solution and placed back in the exact location where they were captured. The positive control group consisted of 50 territorial and 50 silent males that were injected with

50 μg of VT/100 μl amphibian Ringer's saline cocktail.

2.7. Behavioral observations and statistical analyzes

Following injections data was recorded for each frog including movement from original placement, detection of calling, location and characteristics of calling site (vegetation, height, etc.; see below), aggressive behavior (wrestling, biting, etc.), and the time of all behaviors.

Calling locations were categorized based on a study by Narins and Hurley [47]. This study examined the relationship between call intensity and function with surrounding vegetation at the call sites. Calling sites are based on a 1–5 scale: "1 – open with no surrounding cover (e.g., on a rock, leaf, or bare tree trunk); 2 – generally open, but with sparse surrounding vegetation partially covering the frog (e.g., on a tree trunk amid small leaves or vines); 3 – closely covered by vegetation or substrate but with at least one side and the anterior uncovered (e.g., between two bamboo shoots or in a palm axil; 4 – covered closely on all sides by vegetation and substrate but open anteriorly (e.g., in a palm axil with leaves near the dorsal surface of the frog); 5 – completely surrounded by vegetation (e.g., inside a tightly curled leaf)."

Movement of individual animals following drug delivery was measured and recorded to the nearest cm. Movement into new areas (or potential new territories) was defined as individual frogs relocating from their initial location to sites > 1 m; although somewhat arbitrary, the biological significance of this relates to the minimum distance between two territorial males, based on sound pressure thresholds and vegetative properties of call sites, which does not result in territorial disputes, i.e., aggressive calls and/or aggressive (including physical) interactions [47]. Additionally, in a previous behavioral neuropharmacological study, distances 1 m or more never resulted in any aggressive or territorial dispute [39].

Several statistical analyzes were run to determine significance. A MANOVA analysis using Tukey posthoc test was utilized to test for whether there are any statistically significant differences between means and also to examine the independent and combined effects of drug and reproductive modes. Analyses included call latency (defined as time needed for advertisement call activation following drug delivery), distance males moved following injection, calling rate after call activation, and initial and final call site preference. A *t*-test was utilized to determine if there was a significant different in size (snout-vent-length) between territorial and silent males used during the experiment. Although territorial males trended to be slightly larger there was no significant difference in mean (± SE) SVL between silent (32.2 ± 0.49) and territorial (32.9 ± 0.36) males (*t*-test: $t = 0.797$, $N = 400$, ns). We employed the Fisher's Exact Test to examine the significance of the association (contingency) between two kinds of classifications to determine significance between pharmaceutically injected and saline-injected frogs, pharmaceutically injected silent and pharmaceutically injected territorial frogs, and between males that moved or did not move into a new area. Statistical analyses were conducted using SigmaStat 3.1 and WS Excel 2016 and statistical results are reported as means ± SE.

3. Results

3.1. Advertisement call activation

Activation of advertisement calling was significantly higher in VP-injected silent males (33/50) compared to saline-injected silent males (0/50) ($p < 0.00001$, Fisher's Exact Test) (Table 1; Fig. 1). VP-injected territorial males (42/50) were not significantly more activated to call than saline-injected territorial males (37/50) ($p = 0.326$, Fisher's Exact Test) but did have significantly higher call activation than saline-injected silent males (0/50) ($p < 0.00001$, Fisher's Exact Test). There was not a significant difference in call activation between VP-injected territorial males (42/50) and VP-injected silent males (33/50) ($p = 0.063$, Fisher's Exact Test).

Following OT injections, significantly more OT-injected silent males (41/50) called compared to saline-injected silent controls (0/50) ($P < 0.00001$, Fisher's Exact Test) (Table 1; Fig. 1). There was no significant difference in call activation between OT-injected territorial males (44/50) and saline-injected territorial males (37/50) ($p = 0.12$); however, there was a significant between OT-injected territorial males (44/50) and saline-injected silent males difference (0/50) ($P < 0.00001$, Fisher's Exact Test). There was not a significant difference between OT-injected territorial males (44/50) and OT-injected silent males (41/50) ($p = 0.58$, Fisher's Exact Test).

Even though less VP-injected silent males (33/50) were activated to call compared to OT-injected silent males (41/50) there was not a significant difference between the two groups ($p = 0.11$, Fisher's Exact Test) (Table 1; Fig. 1). Likewise, there was not a significant difference in advertisement calling activation between VP-injected territorial males (42/50) and OT-injected territorial males (44/50) ($p = 0.77$, Fisher's Exact Test).

With regard to positive controls, there was no significant difference between VT-injected territorial (44/50) and VP-injected territorial males (42/50) ($p = 0.77$, Fisher's Exact Test) as well as VT-injected territorial (44/50) and OT-injected territorial males (44/50) ($p = 1.0$) (Table 1; Fig. 1). Further, there was no significant difference between VT-injected silent (40/50) and VP-injected silent males (33/50) ($p = 0.18$, Fisher's Exact Test) and VT-injected silent (40/50) and OT-injected silent males (41/50) ($p = 0.99$, fisher's Exact Test).

3.2. Movement

Following injection frogs were place back in their original locations and typically remained motionless for a period of time. Initial movement always preceded initiation of advertisement calling (see Section 3.3). Individuals in the three silent groups (VP, OT, VT) moved into new areas, which were always > 1 m from other territorial males, significantly more than individuals from territorial groups (VP, OT, VT), respectively (all pairs were at $p < 0.00001$, Fisher's Exact Test). There was no significant ($p > 0.05$, Fisher's Exact Test) difference among injected territorial male or among the saline (control) groups (Table 1; Fig. 2). The average distance males moved following injection varied; however, there was a significant difference between the two behavioral clusters, territorial groups vs. silent groups $*(F_{(3,392)} = 13.88$, $p < 0.00001)$ (Table 1; Fig. 3).

3.3. Call latency

There was variation among groups in call latency (Table 1; Fig. 4). There was no significant distinction between treatment groups; however, all treatment groups including positive controls were significantly different from the control (saline-territorial) ($F_{(3,392)} = 8.23$, $p < 0.0001$). Examining results from the treatment groups, it is evident that VP territorial males had the longest call latency; in fact, the two VP groups (territorial and silent) recorded the two longest mean time periods, followed by the two OT groups, and then the two VT control groups.

3.4. Calling rate

The rate of advertisement calling ranged from 15 to 17 calls/min (Table 1) within the first few minutes of calling and there were no significant differences among the experimental or control groups in calling rate ($F_{(3,392)} = 0.31$, $p > 0.05$). The saline-injected silent males did not produce advertisement calls.

3.5. Calling locations

Averages for call site preferences based on Narins and Hurley [47]

Table 1
Summary of behavioral data. A) There was a significant ($P < 0.00001$, Fisher's Exact Test) difference in advertisement call activation between all treatment groups (VP, OT, VT) and the saline-injected territorial males from the control group, saline-injected silent males. There was no significant ($p > 0.05$, Fisher's Exact Test) difference among the treatment groups and saline-injected territorial males; specific statistics are in the Results section. B) There was a significant difference between all treatment groups (VP, OT, VT) and the two saline control groups in calling latency *($F_{(3,392)} = 8.23$, $p < 0.0001$). Although there was substantial variation, no significant difference among the treatment groups (VP, OT, VT) of males occurred. C) The number of males that moved into a new area (> 1 m) following pharmaceutical treatments displayed significant differences whereby exogenously injected silent males moved into new areas significantly *($p < 0.00001$, Fisher's Exact Test) more than exogenously injected territorial males and both saline control groups. D) There was a significant difference in the distance moved following IP injections between the silent male treatment groups (VP, OT, VT) and the territorial male treatment (VP, OT, VT) and saline groups ($F_{(3,392)} = 13.88$, $p < 0.00001$). E) Advertisement call rate after nonapeptide activation displayed no significant ($F_{(3,392)} = 0.31$, $p > 0.05$) differences among the groups. F) There was no differences among groups (all modes = 2) for initial call site preference (based on Narins and Hurley [47]) ($F_{(3,392)} = 1.02$, $p > 0.05$) and G) final call site preference ($F_{(3,392)} = 0.89$, $p > 0.05$).

Behavior	VP-terr	VP-silent	OT-terr	OT-silent	VT-terr	VT-silent	Saline-terr	Saline-silent
A) Advertisement calls	42/50*	33/50*	44/50*	41/50*	44/50*	40/50*	37/50	0/50
B) Call after injection (mins)	61.4 ± 9.3*	62.5 ± 7.2*	58.1 ± 12.7*	53.1 ± 7.9*	43.0 ± 8.1*	46.2 ± 8.6*	28.0 ± 4.12	0
C) Movement to new area	15/50 = 30%	45/50 = 90%*	8/50 = 16%	43/50 = 86%*	6/50 = 12%	42/50 = 84%*	3/50 = 6%	0/50 = 0%
D) Distance moved (m)	0.6 ± 0.5	2.1 ± 0.8*	0.4 ± 0.3	1.9 ± 0.4*	0.5 ± 0.4	2.2 ± 0.6*	0.4 ± 0.2	0.3 ± 0.2
E) Calling rate (ave.)	15/min	15/min	16/min	15/min	16/min	16/min	17/min	0
F) Initial call site perference (ave.)	2.2	2.3	2.1	2.2	2.3	1.9	2.2	2.1
G) Final call site perference (ave.)	2.2	2.1	2.3	2.3	2.2	2.1	2.2	2.2

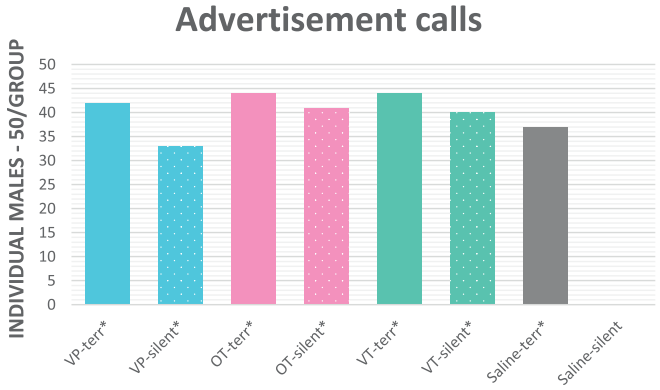

Fig. 1. The number of males activated to emit advertisement calls. All groups are significantly *($p < 0.00001$) different from the control group, saline-injected silent males. There is no significant ($p > 0.05$) difference among any of the treatment and saline-injected territorial groups.

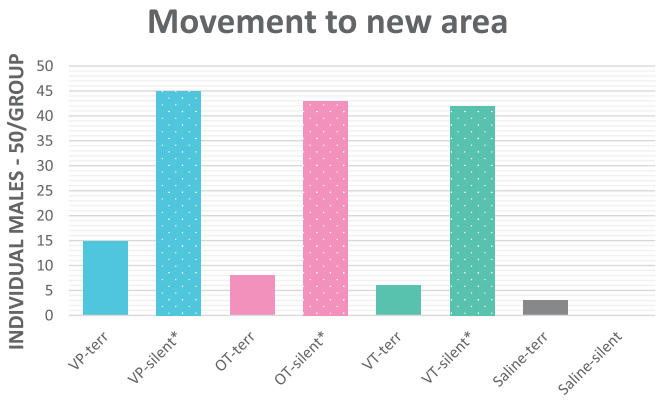

Fig. 2. Number of males that moved into a new area following injections of nonapeptide or saline. Silent males injected with VP, OT, and VT moved significantly *($p < 0.00001$) more than territorial males injected with VP, OT, and VT as well as both control groups. There was no significant ($p > 0.05$) difference among injected territorial male groups or among the silent male groups.

for territorial males ranged from 1.9 to 2.3; however, the mode for all groups was 2. There was no significant difference with regard to initial call site preference among all groups including controls ($F_{(3,392)} = 1.02$, $p > 0.05$). Likewise, there was no significant differences among the all groups (controls included) in the final call site preference ($F_{(3,392)} = 0.89$, $p > 0.05$) (Table 1).

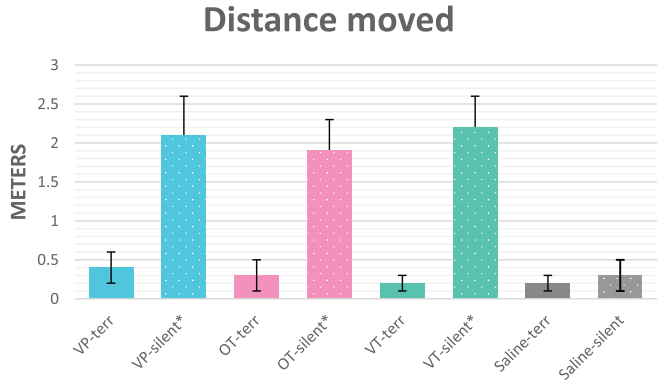

Fig. 3. Distance moved following injections. Silent males in all treatment groups moved significantly *($F_{(3,392)} = 13.88$, $p < 0.00001$) further than territorial males and both control groups. There was no significant ($p > 0.05$) difference among nonapeptide injected territorial male or among nonapeptide injected silent male groups.

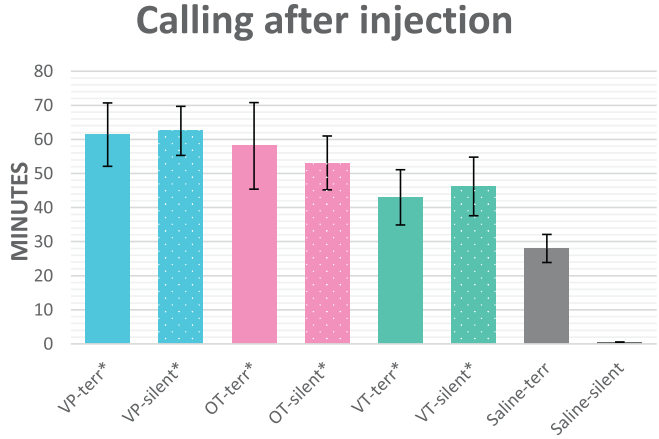

Fig. 4. Call latency varied among groups. All treatment groups displayed significantly *($F_{(3,392)} = 8.23$, $p < 0.0001$) longer call latency than the saline-injected control groups.

4. Discussion

Results indicate that the mammalian nonapeptides VP and OT significantly activated advertisement calling and territorial behavior in the anuran amphibian, *E. coqui*. Further, the VP- and OT-injected males were as significantly activated to emit advertisement frogs as VT activated males. Interestingly, a major difference between exogenously activated males was that silent males relocated into a new area prior to initiating advertisement calling significantly more often than territorial

males in both VP and OT treatment groups, as well as in the VT positive control group. Furthermore, the distance moved after the exogenous injection was significantly farther in silent males in all treatment classes (and the positive control) than in territorial males. These results are similar to another study that examined the effects of VT exogenously injected into territorial and silent (satellite) male *E. coqui* [39]. In this study, silent (termed satellite) males injected with VT also redeployed into new territories prior to initiating advertisement calling. This study hypothesized that this observed behavior may be the result of one or more of the following circumstances. Territorial males that possessed high quality territories, may want to retain them, due to the fact that moving into another area could be costly and/or maladaptive. The relatively low-density level of males compared to the high number of preferred calling sites may encourage silent male to move into these new calling sites. Furthermore, the stage of maturation in their reproductive status (young vs. old) and the behavioral experience level of the male may play a factor in initiating movement and/or advertisement calling. Lastly, the energetic level or energy reserve of the male, if low, may prohibit him from calling.

Generally, activation of the VT/VP family of neuropeptides plays an important role in territorial behavior and aggression. Studies have also indicated that VP and VT do not necessarily stimulate aggressive behavior per se but instead initiate an aggressive disposition [22]. This activation may be manifested in silent males whereby they are motivated to call but not aggressively galvanized to challenge the territorial male. For exogenously activated silent males it could be economically and evolutionarily better to move into a new area and initiate advertisement calling as oppose to challenging the resident male, expending energy, and risking injury for a calling site that could be available a meter or two away. As for territorial males, they have already established territories and can immediately call within it; thus, not moving to another territory and risking injury via aggressive interactions with conspecific males. Furthermore, territorial males have already invested time and energy into establishing and maintaining their territory so it may be beneficial to remain. These territories may have been chosen due to their superior acoustical parameters (resulting acoustical signature) for the attraction of mates and defense from other males, and/or containing or in close proximity of oviposition sites [48–49].

Parameters such as call rate and calling site preference were not significantly different. Vasotocin affects male advertisement calling characteristics [50,51] and increases frequency of aggressive calling in paternal males [37] but it appears that in territorial and silent males, VP, OT, and VP does not significantly affect the actual calling rate. Likewise, call site preference based on Narins and Hurley [47] was not significantly affected by VT or OT injections. It appears that nonapeptide receptor activation has little direct impact on calling site preference. On average, males whether exogenously treated or not are choosing calling sites that are generally open but with some surrounding vegetation (2; Narins and Hurley [47]) or sites that are covered by vegetation on one side (3; Narins and Hurley [47]). Call site preference appears to be controlled by different variables other than nonapeptide receptor activation per se. Calling latency did vary among the groups but not significantly except to controls (saline injected territorial males). Variance in call latency was also observed in *E. coqui* when given exogenous (IP) injections of VT [39] and may be a consequence of an IP injection in general. In anurans, VT has multiple impacts on vocalizations with a varied spectrum. While it does facilitate advertisement calling [39,50,52–54], it also can promote call characteristic [50–53], or changes in calling behavior [55], or changes in calling latency [39]. At this point it is reasonable to say that all of the behavioral biology and vocalizations involving VT, or even other nonapeptides in general, has yet to be full understood.

With regard to the receptor/ligand relationship, the objective of this study was not to specifically determine which nonapeptide bound to a particular receptor, or how precisely the neuropeptide bound to that receptor; however, it was evident that the mammalian nonapeptides VP and OT did significantly activate calling and territorial behaviors in the amphibian *E. coqui*. It was also found that VP and OT activated social behaviors in fish and in transgenic rats [31]. Further, Do-Rego et al. [32] determined that VP and OT mimicked the stimulatory effect of VT on neurosteroid biosynthesis suggesting that mammalian hormones may activate non-mammalian behavioral manifestations. Several investigations have indicated that neuroactive steroids regulate reproductive behaviors in amphibians and that androgens modulate vocalizations in frogs [56–58]. It appears that multiple classes of nonapeptides can activate social behavior in various clades of vertebrates.

5. Conclusions

The mammalian neuropeptides VP and OT significantly activated advertisement calling and territorial behavior in male *E. coqui*. This activation was as robust as activation accomplished with the amphibian nonapeptide VT. Interestingly, nonapeptide activation also affected males differently. Following exogenous injections of VP and OT territorial males remained in their current territory but silent males moved into new areas and only then began to emit advertisement calls; this difference in male behavior also occurs when *E. coqui* are injected with VT. This phenomenon may reflect differential selective pressures on the two different male modes and is likely coordinated by additional neuroendocrine mechanisms.

Acknowledgments

We are grateful to the Departmento de Recursos Naturales y Ambientales of Puerto Rico for issuing collecting permits to work on *E. coqui*. Very special thanks goes to the staff at the El Verde Field Station (EVFS), Puerto Rico for providing support and use of their facilities, especially to Dr. Alonso Ramírez (Director of EVFS), Dr. Jill Thompson, and Ms. Hilda Lugo. The field station is part of the Long-Term Ecological Research project of the Institute for Tropical Ecosystem Studies, University of Puerto Rico at Rio Piedras, funded by the National Science Foundation (DEB-9411973, DEB-0620910) . We would also like to thank the staff of the Waiakea Research Station, Hilo, Hawai'i for support and use of their facilities; special thanks goes to Dr. Robert Borris, Associate Dean of Research. A special mahalo goes to the Division of Forestry and Wildlife, Department of land and Natural Resources for issuing permits to work on *E. coqui*. We would like to express our gratitude to Drs. Richard Elinson and William Mautz for collection and advice on *E. coqui* biology.

Financial support of this research was made possible partially by several sources throughout the years including a Faculty Research Grant (8062) from the Office of the Vice President for Research and the Department of Psychology at the University of Michigan, the Department of Basic Science and Craniofacial Biology in the College of Dentistry at New York University, and the Department of Pharmaceutical Sciences at the University of Hawai'i College of Pharmacy. Finally, we are exceptionally grateful to Drs. E. Dianne Rekow, Robert P. Borris, and Anthony D. Wright for unending encouragement as well as for scientific and logistical support of this project.

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