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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

THE EFFECT OF MALE-MALE COMPETITION AND ITS UNDERLYING REGULATORY MECHANISMS ON THE ELECTRIC SIGNAL OF THE GYMNOTIFORM FISH *BRACHYHYPOPOMUS GAUDERIO*

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Vielka Lineth Salazar

2009

To: Dean Kenneth Furton
College of Arts and Sciences

This dissertation, written by Vielka Lineth Salazar, and entitled The Effect of male-male competition and its Underlying Regulatory Mechanisms on the Electric Signal of the Gymnotiform fish *Brachyhypopomus gauderio*, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Philip K. Stoddard, Major Professor

Date of Defense: October 30, 2009

The dissertation of Vielka Lineth Salazar is approved.

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Dean Kenneth Furton
College of Arts and Sciences

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Dean George Walker
University Graduate School

Florida International University, 2009

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DEDICATION

To my loving parents, Vielka Hayer and Juan Salazar, and to my husband, Dr. Timothy Rawlings, for their love, and for always believing in my potential.

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ABSTRACT OF THE DISSERTATION

THE EFFECT OF MALE-MALE COMPETITION AND ITS UNDERLYING REGULATORY MECHANISMS ON THE ELECTRIC SIGNAL OF THE GYMNOTIFORM FISH *BRACHYHYPOPOMUS GAUDERIO*

by

Vielka Lineth Salazar

Florida International University, 2009

Miami, Florida

Professor Philip K. Stoddard, Major Professor

Sexually-selected communication signals can be used by competing males to settle contests without incurring the costs of fighting. The ability to dynamically regulate the signal in a context-dependent manner can further minimize the costs of male aggressive interactions. Such is the case in the gymnotiform fish *Brachyhypopomus gauderio*, which, by coupling its electric organ discharge (EOD) waveform to endocrine systems with circadian, seasonal, and behavioral drivers, can regulate its signal to derive the greatest reproductive benefit. My dissertation research examined the functional role of the EOD plasticity observed in male *B. gauderio* and the physiological mechanisms that regulate the enhanced male EOD. To evaluate whether social competition drives the EOD changes observed during male-male interactions, I manipulated the number of males in breeding groups to create conditions that exemplified low and high competition and measured their EOD and steroid hormone levels. My results showed that social competition drives the enhancement of the EOD amplitude of male *B. gauderio*. In addition, changes in the EOD of males due to changes in their social environment were

paralleled by changes in the levels of androgens and cortisol. I also examined the relationship between body size asymmetry, EOD waveform parameters, and aggressive physical behaviors during male-male interactions in *B. gauderio*, in order to understand more fully the role of EOD waveforms as reliable signals. While body size was the best determinant of dominance in male *B. gauderio*, EOD amplitude reliably predicted body condition, a composite of length and weight, for fish in good body condition. To further characterize the mechanisms underlying the relationship between male-male interactions and EOD plasticity, I identified the expression of the serotonin receptor 1A, a key player in the regulation of aggressive behavior, in the brains of *B. gauderio*. I also identified putative regulatory regions in this receptor in *B. gauderio* and other teleost fish, highlighting the presence of additional plasticity. In conclusion, male-male competition seems to be a strong selective driver in the evolution of the male EOD plasticity in *B. gauderio* via the regulatory control of steroid hormones and the serotonergic system.

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LIST OF ABBREVIATIONS

8-OH-DPAT	8-hydroxy-*N,N*-dipropyl-2-aminotetralin
5-HT	5-hydroxytryptamine or serotonin
$5HT_{1A}R$	serotonin receptor type 1A
$5HT_2R$	serotonin receptor type 2
$5HT_{2A}R$	serotonin receptor type 2A
11-KT	11-ketotestosterone
α-MSH	alpha-melanocyte stimulating hormone
aa	amino acid
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ARE	androgen response element
cdk5	cyclin-dependent kinase 5
CRH	corticotropin-releasing hormone
Cys	cysteine
DHEA	dehydroepiandrosterone
DOC	deoxycorticosterone
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
EMN	electromotoneuron
EO	electric organ
EOD	electric organ discharge
F	cortisol

GC	glucocorticosteroids
GLM	general linear model
GPCR	G-protein coupled receptor
GRE	glucocorticosteroid response element
HPA	hypothalamic-pituitary adrenal axis
HPG	hypothalamic-pituitary gonadal axis
HPI	hypothalamic pituitary interrenal axis
IACUC	Institutional Animal Care and Use Committee
LSD	least significant difference
MAPK	p38 mitogen-activated protein kinase
MATLAB	matrix laboratory
NCBI	National Center of Biotechnology Information
nt	nucleotide
P1	positive phase 1 of the EOD waveform
P2	negative phase 2 of the EOD waveform
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
Pn	pacemaker nucleus
PPn	prepacemaker nucleus
PCR	polymerase chain reaction
RHP	resource-holding potential
RSAT	regulatory sequence analysis tools

S-15535	4-(benzodioxan-5-yl)1-(indan-2-yl)piperazine
SEM	standard error of the mean
Ser	serine
SPPn	sublemniscal prepacemaker nucleus
SPSS	statistical package for the social sciences
T	testosterone
TBE	Tris-Borate-EDTA buffer
Thr	threonine
TM	transmembrane
τ_{P2}	time constant of repolarization of phase 2
UniProtKB	universal protein resource knowledgebase

CHAPTER I

Introduction

Social systems and sexually-selected communication signals

In animal social systems, individuals constantly monitor their social environment and their internal motivational state, and integrate these two sources of information to make decisions on their behavioral output (Figure 1) (Bradbury & Vehrencamp 1998). Social interactions require that individuals in a social group change their motivational states over minutes to hours, and continually fine-tune their behavioral responses to other individuals' actions (Figure 1). Communication signals facilitate the exchange of information among individuals in a social group (Bradbury & Vehrencamp 1998). Information encoded in these signals, such as the songs of territorial male birds, the claw-waving in fiddler crab males, and the advertisement calls of male frogs, can help individuals to decide who to challenge and who to mate with, both of which are critical decisions to attain maximal reproductive output (Andersson 1994; Bradbury & Vehrencamp 1998). Reliable communication signals are particularly important in mating systems with skewed sex ratios, scarce resources, and limited reproductive time periods (Andersson 1994). Nevertheless, the reliability of the information encoded in signals depends on the overall benefits and costs to the sender and the receiver, and on whether they share common interests (Searcy & Nowicki 2005). When interests differ between the sender and the receiver, senders benefit by emitting unreliable signals and receivers incur costs by attending to them (Searcy & Nowicki 2005). Although through time, receivers will selectively pay attention to reliable signals, they may be tolerant to low levels of deception (Dawkins & Guilford 1991; Johnstone & Grafen 1993).

Both female mate choice and intrasexual competition prevail in polygamous mating systems, where the number of interested males exceeds the number of receptive

females at any given time, and females are more limited than males in potential reproductive output (Andersson 1994). Under these conditions, sexual selection will favor communication signals that convey reliable information about males' quality (body size, body condition), fighting ability or resource holding potential (RHP) (Andersson 1994; Parker 1974). In the context of male-male aggressive interactions, males can use these signals to avoid unnecessary physical contests that may lead to injury, depletion of energy stores, and high predation risk (Bradbury & Vehrencamp 1998). Furthermore, the outcome of the contest affects the present state of a male and his motivation in future contests, and this is mediated via steroid hormones and neuromodulators, such as biogenic amines and neuropeptides (Figure 1). In turn, changes in steroid hormone levels and neuromodulators feed back to modulate the organism's motivational state and behavioral output, thus affecting the social environment of conspecifics in the social group (Figure 1).

Aggressive interactions regulate and are regulated by steroid hormones and serotonergic neuromodulation

Male aggressive interactions regulate circulating levels of the steroid hormones glucocorticosteroids (GCs) and androgens (Figure 1) (Abbott et al. 2003; Elofsson et al. 2000; Goymann & Wingfield 2004; Oliveira et al. 2002; Overli et al. 1999; Summers & Winberg 2006; Wingfield et al. 1990; Wingfield et al. 1987). Dominant individuals, those that consistently win aggressive interactions, typically have higher levels of androgens than subordinate individuals (Elofsson et al. 2000). In contrast, the relationship between dominance status and circulating GC levels in competing males is

more complex. Although GC levels increase during the initial stages of a contest in both dominant and subordinate individuals, they quickly revert back to pre-contest levels in dominant males but stay high longer in subordinate males (Overli et al. 1999; Summers & Winberg 2006). In the context of male-male interactions, androgens typically also facilitate male signaling behavior (e.g., mating calls/displays), while elevated GCs facilitate the release of energy stores necessary to sustain signaling for high-energy signalers. In addition, increases in the density of competing males or in the number of perceived competitors in a population have been shown to increase androgen levels in competing males (Carlson et al. 2000; Oliveira et al. 2001; Pankhurst & Barnett 1993; Remage-Healey & Bass 2005). As discussed by Oliveira and colleagues (2002) and as predicted by the Challenge Hypothesis (Wingfield et al. 1990), an increase in potential competitors could intensify agonistic interactions leading to the activation of the hypothalamic-pituitary-gonadal (HPG) axis and an increase in circulating androgen levels.

Male aggressive interactions are also regulated by GCs and androgens (Figure 1) (Abbott et al. 2003; Elofsson et al. 2000; Goymann & Wingfield 2004; Oliveira et al. 2002; Overli et al. 1999; Summers & Winberg 2006; Wingfield et al. 1990; Wingfield et al. 1987). These steroid hormones typically facilitate aggressive behaviors while suppressing behavioral and physiological functions that would otherwise consume the time and nutritional resources that could be advantageously reallocated to aggression. High circulating levels of androgens and GCs can facilitate agonistic performance but incur collateral physiological and behavioral costs such as suppression of immune function, depletion of energy stores, and reduced parental care (Romero 2004; Sapolsky

et al. 2000). While high GC levels are typically related to the suppression of androgen levels (Sapolsky 1994; Sapolsky et al. 2000), in many species, both glucocorticoid and androgen levels are increased during reproduction (Beletsky et al. 1989; Creel et al. 1996; Emerson & Hess 2001; Remage-Healey & Bass 2005).

Serotonin (5-hydroxytryptamine or 5-HT), a biogenic monoamine neurotransmitter, also plays a major role in the regulation of aggressive behavior in both invertebrate and vertebrate organisms (rev. Nelson 2006). Aggressive interactions regulate and are regulated by the activity of the serotonergic system (Figure 1). Serotonin not only regulates overt aggressive physical behaviors but it also regulates production of aggressive communication signals (Albers et al. 2002; Larson & Summers 2001), including electrocommunication signals (Allee et al. 2008; Maler & Ellis 1987; Smith & Combs 2008; Stoddard et al. 2003a; Telgkamp et al. 2007). Although high chronic serotonin activity is typically related to low levels of aggressive behavior, this is not always the case (de Boer & Koolhaas 2005; Nelson & Chiavegatto 2001; Summers 2001; Veenema et al. 2005). Serotonergic activity in dominant and subordinate males matches their changes in GC levels. Similar to the trends observed for GC levels, serotonin activity increases in both dominant and subordinate individuals during an encounter but this rise in serotonin rapidly returns to baseline levels in dominant males while it stays chronically high in subordinate males (Overli et al. 1999; Summers & Winberg 2006). Associated with these changes in the serotonergic system and GC levels, subordinate individuals also display increase levels of the melanocortins adrenocorticotropic hormone (ACTH) and alpha-melanocyte stimulating hormone (α-MSH) (Hoglund et al. 2000).

The effect of serotonin on the regulation of aggressive behaviors seems to be mediated by the activation of the HPG axis and the hypothalamic-pituitary-adrenal or interrenal (HPA, or HPI in teleost fish) axis. Ultimately activation of the HPG leads to the secretion of androgens into circulation, while activation of the HPA/I axis leads to the secretion of melanocortins and glucocorticosteroids (GCs). In addition, the relationship between serotonergic activity and regulation of aggressive behavior depends on the type of serotonin receptor, the quantity and location of the receptor in brain centers that regulate aggression, and the intracellular signaling pathway activated by the receptor (de Boer & Koolhaas 2005; Schiller et al. 2006; Schiller et al. 2003; Veenema et al. 2005). For instance, the serotonin receptor type 1A ($5HT_{1A}R$) is a key player in the regulation of aggressive behavior (rev. Nelson 2006). Activation of the $5HT_{1A}R$ by 5-HT or specific agonists (e.g., 8-OH-DPAT) inhibits or reduces aggression in many invertebrate and vertebrate species (rev. Nelson 2006). Closing this regulatory loop, circulating androgens and GCs can alter the expression pattern of serotonin receptors resulting in changes in the neuronal activity of brain areas that regulate aggressive behavior.

Gymnotiform fish are a great model system to study the connection between social stimuli, motivational state, and the regulation of communication signals

The *main objective* of my dissertation research is to determine the effects of male-male competition and its underlying regulatory mechanisms on the modulation of the electric signal of male gymnotiform fish, *Brachyhypopomus gauderio*. All members of the order Gymnotiformes, electric fish from Central and South America, emit easily-quantified electric signals generated by a well-mapped neural motor network. These

electric signals, known as electric organ discharges (EODs), are regulated by social interactions, androgens, GCs, melanocortins, and the serotonergic system (Stoddard et al. 2006). Thus, changes in the electric signal of males during social interactions give a real-time broadcast of the motivational and physiological state of the fish and their effect on the electrocommunication networks of other fish with whom they interact.

The nocturnal gymnotiform fish *Brachyhypopomus gauderio* (Giora & Malabarba 2009) uses its EODs to navigate in its environment, electrolocate objects, and communicate with conspecifics in the dark. *B. gauderio* is the sister species to *B. pinnicaudatus* (Hopkins 1991). The southern species, *B. gauderio*, is found from the Pantanal of Brazil and Paraguay south to the Pampas of Argentina and Uruguay, whereas *B. pinnicaudatus* resides in the Amazon and Oronoco basins to the north (Giora & Malabarba 2009). The literature to this point has referred to both as *B. pinnicaudatus*. Given the extreme similarity of these sister species, it seems reasonable to expect that the understanding of the physiology developed for one species applies to both.

The EOD waveform of *Brachyhypopomus* species is the product of summed action potentials generated by specialized cells, known as electrocytes, found in bilateral structures that run longitudinally from behind the gills to the tip of the tail (Bennett 1970; Hopkins et al. 1990). In many *Brachyhypopomus*, including *B. gauderio*, electrocytes generate two action potentials which produce the two phases (conventionally depicted as a head-positive phase or P1, followed by a head-negative phase or P2) of the EOD waveform (Figure 2A) (Bennett 1970). The EOD can be deconstructed into: 1) peak-to-peak waveform amplitude (voltage in mV, Figure 2A), 2) the time constant of repolarization of P2 (τ_{P2}), a measure of the EOD waveform's P2 duration (Figure 2A),

and 3) repetition rate or frequency (EODs per second) (not shown). The EODs of *B. gauderio* have a distinct signature or waveform which can potentially encode information about the gender, size, and reproductive status of the fish producing it (Silva et al. 1999; Stoddard 2002).

In addition, both EOD amplitude and τ_{P2} appear to be important in the context of reproductive behaviors (Stoddard 2002). During the breeding season, sexually-mature *B. gauderio* males have longer body lengths and tails than females (Caputi et al. 1998; Silva et al. 1999). A male's body length determines the length of the EO, the number of electrocytes, and the magnitude of the EOD waveform (Caputi et al. 1998; Curtis & Stoddard 2003; Franchina & Stoddard 1998; Hopkins et al. 1990). Accordingly, *B. gauderio*'s EOD waveforms are also sexually-dimorphic (Figure 2B) (Caputi et al. 1998). When compared to female's EODs, males emit EODs with bigger amplitudes and extended τ_{P2} (Figure 2B) (Franchina & Stoddard 1998; Stoddard et al. 2007). In addition, males further enhance their electric signals following a circadian rhythm, increasing the EOD at night and decreasing it during the day (Franchina & Stoddard 1998; Stoddard et al. 2007). Thus, the enhanced male circadian rhythms in the EOD waveform and EOD repetition rate further exaggerate the EOD sexual dimorphism in the early evening hours of courtship (Figure 2B) (Stoddard et al. 2007). These enhanced traits drift back towards their baseline values to coincide with the period of daytime quiescence.

Circadian rhythmicity is not only affected by reproductive status, but also by social interactions. For instance, EOD circadian rhythm plasticity is sensitive to changes in the social environment. Social isolation decreases the EOD circadian rhythm, and addition of a social companion to the tank of an isolated male restores the reduced EOD circadian

rhythm of the isolated male to levels observed in males sampled directly from social groups. Nevertheless, social regulation of the EOD is sex-specific: a male social companion induces a bigger and faster effect than a female social companion (Franchina et al. 2001). Yet, the enhanced EOD circadian rhythms of males seem to favor reproduction success. When given a choice between a small and a big male, *B. gauderio* females prefer larger males with larger EODs (Curtis & Stoddard 2003).

The effect of male-male interactions in the magnitude of the EOD's circadian rhythm indicates that this change in the EOD could be used to communicate dominance status. Information regarding dominance status may be critical for males to secure a territory and attract females. In their natural habitat, *B. gauderio* males are spatially-distributed in a manner consistent with either an exploded-lek or a nest site polygynandry mating system, and display site-fidelity with non-overlapping spatial patterns suggesting that males may defend territories to attract females and procure spawning locations (Miranda et al. 2008). Therefore, the rises in nighttime EOD waveform (both amplitude and duration) may be adaptations to advertise social status. In other gymnotiform species, both sexes display dominance hierarchies (Black-Cleworth 1970; Hagedorn 1986; Hagedorn & Heiligenberg 1985; Hopkins & Westby 1986; Westby 1975). For instance, in *B. occidentalis* both sexes are known to be highly territorial (Hagedorn 1988; Hagedorn & Zelick 1989). Winners of these contests were bigger and enhanced their EODs more than their opponents (Hagedorn & Zelick 1989). In *B. gauderio*, dominance interactions may also be present in both sexes. Thus, individual *B. gauderio* may advertise their social status via their EOD waveform.

The electrocommunication neural network is a simple and tractable system to understand how steroid hormones and the serotonergic system regulate social behavior

Gymnotiform fish have an EOD control center composed of a hierarchical chain of nuclei located in telencephalic, midbrain, and medullary brain areas (Heiligenberg et al. 1981). The premotor areas, the sublemniscal prepacemaker nucleus (SPPn) and the diencephalic prepacemaker nucleus (PPn), send direct input to the electrogenic motor command circuit, the medullary pacemaker nucleus (Pn) (Juranek & Metzner 1998). The Pn directs the activity of the spinal cord's electromotoneurons (EMNs). Subsequently, EMNs innervate the electrocytes that compose the peripheral EO (Bennett 1970; Bennett 1971; Bennett et al. 1967). The electrocytes fire synchronously to generate the EOD (Bennett et al. 1967). In gymnotiform fish, the contributions of the central and the peripheral components of the electrocommunication neural network to the electric signal phenotype can be dissected out: the EOD repetition rate is controlled by the medullary pacemaker nucleus while the EOD waveform's amplitude and duration are determined by the intrinsic properties of the electrocytes (Zakon 1998).

Accordingly, the electric signal is modulated centrally and peripherally by steroid hormones (Bass & Zakon 2005; Zakon 2003). Androgens can alter the EOD centrally (EOD frequency) and peripherally (EOD waveform), and the effects at either level are independent from each another (Bass 1986; Bass & Volman 1987; Few & Zakon 2001). The androgen 11-ketotestosterone (11-KT) and the glucocorticosteroid cortisol are positively related with EOD rate modulations (Dunlap 2002; Dunlap et al. 2002). In addition, several studies have shown that steroid hormones modulate the ion

conductances of the electrocytes (Bass & Volman 1987; Dunlap et al. 1997; Mills & Zakon 1991; Stoddard et al. 2006; Zakon et al. 1991).

Serotonergic neurons send inputs to the electrocommunication neural network of gymnotiform fish (Johnston et al. 1990). Peripheral injections of serotonin increase the magnitude of the EOD in *B. gauderio*, mimicking the changes observed during male-male interactions (Stoddard et al. 2003b). Serotonin regulates the EOD waveform via two serotonin receptor types, the 1A and 2A (Allee et al. 2008). In *B. gauderio* males, activation of $5HT_{1A}R$ reduces the EOD waveform, while activation of $5HT_2R$ enhances the EOD waveform to levels observed after serotonin treatment and during male-male interactions (Allee et al. 2008; Stoddard et al. 2003b). In contrast to this pattern, in a different gymnotiform fish species, *Apteronotus leptorhynchus,* activation of $5HT_{1A}R$ enhances, while activation of $5HT_{2A}R$ suppresses aggressive EOD modulations (Smith and Combs 2008). Furthermore, serotonin indirectly regulates signal waveform via central 5-HT receptors (Allee et al. 2008; Markham & Stoddard 2005; Stoddard et al. 2003a), whereas the melanocortins α-MSH and ACTH augment the electric waveform directly through action at the peripheral EO (Markham et al. 2009; Markham & Stoddard 2005; Stoddard et al. 2006).

Does male-male competition drive the EOD plasticity observed in males? Are these effects paralleled by changes in the circulating levels of glucocorticosteroids and androgens and mediated by the activity of the serotonin receptor 1a?

The results of my dissertation research are presented in three chapters (Chapters II – IV). In Chapter II, I investigate the effect of social competition and social history on

the electric signal modulation and steroid hormone profiles in *B. gauderio*. In the current study, I measure the EOD and the steroid hormone levels of isolated males, males at low competition, and males at high competition, to determine whether short-term changes in the social environment of the gymnotiform fish *B. gauderio* males are accompanied by changes in both their electric signals and their steroid hormone profiles. I also explored the effects of past social experiences on the modulation of the EOD.

In Chapter III, I examine the relationship between body size asymmetry, EOD waveform parameters, and aggressive physical behaviors during male-male interactions in *B. gauderio*, in order to understand more fully the role of EOD waveforms as reliable information signals. I specifically address the following questions: 1) How reliably do EOD amplitude and τ_{P2} predict male body size (length, weight and condition) in *B. gauderio*? 2) Can resident males use this EOD information to assess body size of distant intruder males? 3) Do males respond differently to intruders based on body size? 4) How does body size relate to social dominance when males are allowed to interact physically and electrically? 5) How do mismatches between body size and EOD waveform influence social interactions between males? I address these questions through experiments using dyads of size-mismatched resident and intruder males.

In Chapter IV, I use a molecular approach to characterize the serotonin receptor 1A, one of the serotonin receptors involved in EOD regulation in *B. gauderio*. In this study, I amplify, clone and sequence the serotonin receptor 1A from mRNA isolated from the brains of female and male *B. gauderio*. I compare *B. gauderio*'s serotonin receptor 1A to sequences from other teleost fish and characterize potential regulatory mechanisms for this receptor in teleost fish.

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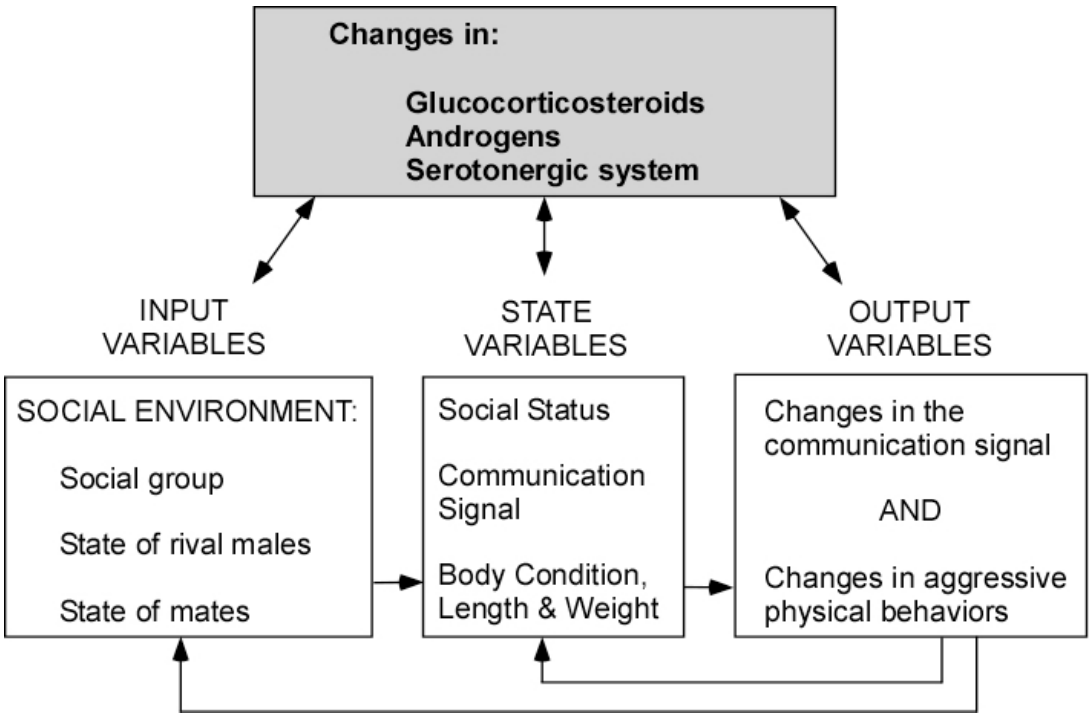

Figure 1. Conceptual framework of my dissertation research. Social individuals are constantly monitoring their social environment (input variables), comparing it against their internal state (state variables), and integrating these two sources of information to make decisions on their behavioral output (output variables). Their output behaviors feed back into their social environment, affecting the social group and the state of its members. Changes in circulating levels of steroid hormones, such as glucocorticosteroids and androgens, and the activity of the serotonergic system regulate this process at each level of this dynamic continuum.

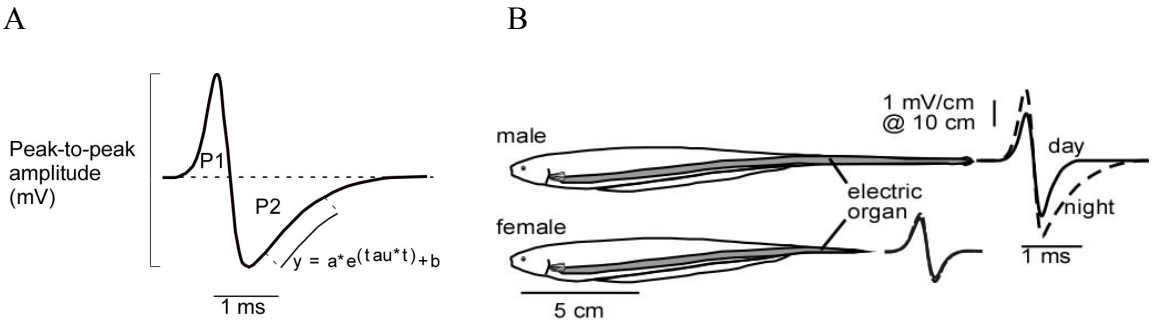

Figure 2. The electric organ discharge (EOD) waveform and its sexual dimorphism. A. The EOD waveform can be deconstructed into its peak-to-peak amplitude (mV) and its duration (ms) for phase 1 (P1) and phase 2 (P2). B. The gymnotiform fish *Brachyhypopomus gauderio* shows strong sexual dimorphism in tail size and shape, and in its EOD amplitude and the duration of the P2 measured as the time of repolarization (τ_{P2}). Males show pronounced day-night differences in the EOD. Shaded areas on each fish's body show the location of the bilateral electric organs.

CHAPTER II

"Social competition affects electric signal plasticity and steroid levels in the gymnotiform fish *Brachyhypopomus gauderio*"

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Abstract

Sexually-selected communication signals can be used by competing males to settle contests without incurring the costs of fighting. Steroid regulation of these signals can render them as reliable indicators of a male's physiological state. We investigated how plasticity in electrocommunication signals is driven by social competition for mates, mediated by steroid hormones, and subject to the effects of past social experience. We measured the electric waveform's amplitude and duration and steroid hormone levels of male gymnotiform electric fish (*Brachyhypopomus gauderio*) following week-long periods of social isolation, and low or high social competition. To quantify the effect of social history on the modulation of the electric signal, six groups of six males experienced all the above three social conditions but in different order. We found that males differentially modulate their electric signals depending on the order they experienced these conditions. Thus, past social interactions affect both present and future social electric signals. Cortisol levels and the amplitude of the electric signal appeared to track the intensity of competition, while androgen levels and the duration of the electric signal only responded to the presence (low and high competition) or absence (isolation) of a social environment (low and high androgens respectively). In addition, cortisol levels were related to the body size of the males at high social competition. Taken together, these findings suggest that the capacity of males to modulate their signals in response to social competition is regulated by steroids.

Keywords: male competition; communication signal; steroid; androgen; cortisol; social history; gymnotiform; electric fish; electric organ discharge.

Social experiences influence an animal's motivational state during present and future social interactions. Males compete fiercely or adopt alternative mating strategies when fewer mates are available, resources are limiting, and the reproductive period is short (Andersson 1994). But contests between males consume time and energy in the best case, or can lead to injury in the worst case (Neat et al. 1998). Ritualized behaviors and reliable signals facilitate the resolution of contests while minimizing their costs (Grafen 1990; Parker 1974; Smith 1973; Zahavi 1975). Furthermore, male aggressive interactions regulate and are regulated by steroid hormones such as glucocorticosteroids and androgens (Abbott et al. 2003; Elofsson et al. 2000; Goymann & Wingfield 2004; Oliveira et al. 2002; Overli et al. 1999; Summers & Winberg 2006; Wingfield et al. 1987; Wingfield et al. 1990). Yet, this bidirectional relationship can result in collateral costs such as suppression of immune function, depletion of energy stores, and reduced parental care (Romero 2004; Sapolsky et al. 2000; Wingfield et al. 1990).

Some organisms have evolved innovative adaptations to balance the benefits and costs of energetically-demanding signals and displays. Such is the case of the gymnotiform fish *Brachyhypopomus gauderio*[1] (Giora & Malabarba 2009), which, by coupling its electric signal waveform to endocrine systems with circadian, seasonal, and behavioral drivers, can direct its expensive signal displays to the times when it might derive the greatest benefit (Salazar & Stoddard 2008). Four features of the life history of the nocturnal gymnotiform fish *B. gauderio* make it an excellent candidate to understand the adaptive role of the circadian regulation of communication signals. First, these fish

[1] Recently, *B. pinnicaudatus* was divided into two species. Specimens found in the northern range of its distribution remain as *B. pinnicaudatus*, while those found in the southern range are now classified as *B. gauderio*. Drs. William Crampton and David de Santana have confirmed that our laboratory colony originated from the southern species *B. gauderio*.

generate an electric organ discharge (EOD) to navigate, locate prey, and communicate with conspecifics in the dark. During the breeding season, not only do males have larger amplitude and longer duration EODs, but they further enhance their EODs by increasing these sex differences at night while decreasing them during the day (Franchina & Stoddard 1998; Stoddard et al. 2007b). Second, these sexually dimorphic characters are associated with reproductive success. Gravid female *B. gauderio* preferentially associate with bigger males with larger amplitude and longer duration EODs (Curtis & Stoddard 2003). Third, EOD circadian rhythm plasticity is sensitive to changes in the social environment. Social isolation decreases the EOD circadian rhythm, and addition of a social companion to the tank of an isolated male restores the reduced EOD circadian rhythm of the isolated male to levels observed in males sampled from social groups. Nevertheless, this effect is sex-specific: a male social companion induces a bigger and faster effect than a female social companion (Franchina et al. 2001). Fourth, the EOD is modulated by melanocortins (Markham et al. 2009; Markham & Stoddard 2005) and by steroid hormones (Mills & Zakon 1991; Stoddard et al. 2006).

The EOD operates in the same modality as the nervous system's action potentials, making it a tractable system to investigate the role of hormones in the regulation of signal production mechanisms in the context of sexually-selected communication. The EOD can be deconstructed into its waveform amplitude (voltage in mV, Figure 1A), its waveform duration (time in ms, Figure 1A), and its repetition rate or frequency (EODs per second). The contributions of the central and the peripheral components of the electrocommunication neural network can be dissected out (Zakon 1998): the EOD repetition rate is controlled by the medullary pacemaker nucleus (Dye & Meyer 1986;

Kawasaki & Heiligenberg 1989; Kawasaki & Heiligenberg 1990; Keller et al. 1991), while the EOD waveform's amplitude and duration are determined by the intrinsic properties of the peripheral electric organ (EO)'s electrocytes (Bennett 1970; Bennett et al. 1967). Androgens can alter the EOD centrally (EOD repetition rate) and peripherally (EOD waveform) (Bass & Volman 1987; Mills & Zakon 1991; Mills et al. 1992; Stoddard et al. 2006; Zakon et al. 1991). The effects at either level are independent from each other (Few & Zakon 2001). Furthermore, 11-ketotestosterone (11-KT) and cortisol are positively related to modulations of the EOD repetition rate in taxa with sex differences in this parameter (Dunlap 2002; Dunlap et al. 2002).

Increasing the density of males in a population has been shown to increase the incidence of aggressive encounters and the levels of androgens in teleost fish (Oliveira et al. 2002; Pankhurst & Barnett 1993). Nevertheless, the effect of changing social group dynamics on steroid-regulated, condition-dependent communication signals is not well understood. In this study, we measured the EOD and the steroid hormone levels of isolated males, and males at low and high competition. Males experienced all three conditions but in different order which allowed us to quantify the effect of social history on the modulation of the EOD.

Methods

Animals

Our subjects were male *B. gauderio*, a gymnotiform pulse-type weakly electric fish native to marshes and slow waters of South America. Fish were selected randomly from a captive-reared, 11th generation breeding colony located at Florida International

University, Miami, Florida. Males' body length ranged from 13.0 to 24.6 cm and females' body length ranged from 14.1 to 17.9 cm. We categorized juveniles by length (7 cm or smaller) and by the absence of sexually-mature characters (e.g., long and thick tails indicative of breeding males or swollen abdomens indicative of gravid females). Fish were reared and housed in 450-liter (185 x 95 x 26 cm) outdoor pools with water conductivity at 90±10 μS cm^{-1} and mean ambient temperature at 27±2 °C. The water surface of each pool was covered 80-100% with water hyacinths (*Eichhornia crassipes*). Each breeding pool contained 10-20 fish. All fish were fed live oligochaete blackworms (Gulfstream Tropical Aquarium, Dania, Florida) three times per week. Experiments took place during the reproductive months, typically from May to September.

Before the beginning of the experiment, we tagged male subjects with fluorescent visible implant elastomer (VIE, Northwest Marine Technology, Inc.) for individual identification. For individual tagging, we anesthetized each fish using 0.075% 2-phenoxyethanol for 2-3 min and injected the elastomer tags on the same side of each fish caudal to the pectoral fin. The elastomer tags were injected subcutaneously following a numerical code consisting of a combination of orange, yellow and green vertical and horizontal lines approx. 2-3 mm in length (supplementary materials 1). Experiments complied with NIH 'Principles of Animal Care' publication no. 86-23, rev. 1985, and were approved by the FIU IACUC (protocol approval no. 07-004).

The EOD machine

This method has been described in detail by (Stoddard et al. 2003). In brief, this automated system allowed us to record and perform online analysis of calibrated EODs in

free-swimming fish. We placed male fish into one of the outer compartments of the recording tank (Figure 1C) and recorded the fish's EOD only when it was positioned in the center of the tank. Every 60 s, the peak-to-peak amplitude and τ_{P2} (time constant of repolarization of the 2nd phase, a measure of EOD duration) of nine consecutive EODs were recorded provided that the fish was in the center of the tank (Stoddard et al., 2003) (Figure 1A). During the night, the EOD was sampled at irregular intervals since the fish were more active and did not necessarily swim through the center of the tank during all sampling intervals. Therefore, we fitted a smoothing cubic spline function using the MATLAB Spline Toolbox (Mathworks, Natick MA) to the selected 48 h data block of each parameter to interpolate for any gaps in the data collection (Stoddard et al. 2007b). We used the fitted data to calculate the peak-to-peak amplitude and τ_{P2} values at the day minimum and the night maximum (Figure 1B). The night-day changes were calculated by subtracting the day minimum from the night maximum (Figure 1B).

Design of social treatment groups

From May to August, thirty-six male fish were randomly sampled from our outdoor colony for inclusion in the experiment. **Baseline** social conditions in our outdoor pools during the experiment period consisted of groups of 10-20 fish with 2-4 males per group. Each group experienced each of the following three social conditions (Figure 2A) but in different order: **Isolation** (1 male, 0 juveniles, and 0 females), **Social 2** (low male competition: 2 males, 4 juveniles, and 6 females), and **Social 6** (high male competition: 6 males, 0 juveniles, and 6 females). In the two competition treatments, juveniles were included to keep the total number of fish constant.

Before and after placement in each of the three social conditions, we weighed and measured each male, and recorded the circadian oscillation of his EOD for 48 h in the EOD machine. Therefore, each group of fish alternated between being housed in outdoor pools under their respective social condition (isolation, Social 2, or Social 6) for seven days and being individually housed in tanks in the EOD machine for 48 h (Figure 2B). Since one group of six males went through one of the six possible combinations, at any time, males in the Social 6 group were together in one pool, males in the Social 2 group were housed in pairs in three pools, and socially isolated males were housed individually in six pools. At the end of the experiment, each social condition had two replicates (Figure 2B). In all, the design had six groups of six males, counterbalanced by assigning subject males to six possible permutations of the three social treatments (Figure 2B).

Blood collection

At the end of the experiment, fish were returned to their most recent social condition housing pools for another week (Figure 2B). Then, six males from each social condition were quickly netted from their respective social pools in the late afternoon (15:00-16:00), lightly anesthetized by immersion for 2-3 min in a solution of 0.075% 2-phenoxyethanol, quickly bled from the ventral vertebral sinus, and returned back to their pools. We collected 50-200 μl of blood with a 10% EDTA-treated needle and syringe. The blood was transferred to a 10% EDTA-treated 0.5 ml polypropylene tube and kept on ice until centrifugation. Blood samples were centrifuged for 15 min at 7000 rpm using an Eppendorf MiniSpin centrifuge at 2-4 °C and the plasma was removed and stored at -80°C for later analysis. We also sampled blood from 12 females following the same

protocol as with the males to quantify sex-specific differences in steroid hormone plasma levels. Females were sampled randomly from either Social 2 or Social 6 pools.

Steroid hormones analyses

For males in each of the three social treatments, circulating levels of unbound cortisol (F), testosterone (T), and 11-ketotestosterone (11-KT) were quantified in plasma using enzyme immunoassays (EIAs) specific for each hormone (Cayman Chemical Co.). The detection limits for these immunoassays were 1.2×10^{-2} ng/ml for cortisol, 1.3×10^{-3} ng/ml for testosterone and 6×10^{-3} ng/ml for 11-ketotestosterone. The Cayman EIA kits have sufficiently high sensitivity that after proper dilution (1:100 for isolated males and 1:500 for social males) one male plasma sample of 10 µl is sufficient for 3 triplicate immunoassays of three steroids.

We collected sufficient plasma from six females to assay 11-KT and T, but not cortisol, so we sampled six additional females to assay cortisol levels. From these additional plasma samples, four of the samples were sufficient to assay a second hormone so we measured circulating T levels a second time. To ensure that the hormone concentrations fell within the immunoassay detection range, at least two dilutions were tested in duplicate in pilot immunoassays. Because of their small body size, fish could not be bled more than once within a 2-3 weeks period, therefore all the fish in this experiment were only bled once. Fish were bled within the first 3 min after capture; therefore, we assumed that the plasma cortisol levels do not reflect the effects of handling during blood sampling (Fox et al. 1997; Pottinger & Moran 1993), an assertion reinforced by uniformly low cortisol levels in our social isolates (see Results).

A pilot immunoassay using plasma aliquots from untreated fish detected assay interference. Therefore, we triple-extracted steroid hormones from the plasma using 4x sample volume of hexane:ethyl acetate (90:10 for samples tested for 11-KT and 70:30 for samples tested for T and F). Systematic pilot tests showed these solvent combinations and ratios yielded the best recoveries for all the hormone standards across the entire detection ranges of the kits. Extracted samples were evaporated in a vacuum centrifuge (Eppendorf Vacufuge, using the organic mode at 30°C) and reconstituted using the immunoassay kit's EIA buffer provided with Cayman's EIA kits. Standards of known concentrations were processed using the same extraction protocol applied to fish plasma samples to calculate percent recovery. Steroid hormones concentration values were adjusted to account for the percent recovery calculated for each assay.

Plasma samples were assayed in triplicate using two 96-well assay kits on the same day. The non-extracted and extracted standards were assayed in triplicate in both of the two 96-well plates. We used the extracted standard triplicates from the two plates for each hormone to calculate the intra-assay and inter-assay coefficients of variation. For the two cortisol plates, intra-assay variation was 2.6% and 1.9% and the inter-assay variation was 2.3%. For the two testosterone plates the intra-assay variation was 2.6% and 2.0% and the inter-assay variation was 2.3%. For the two 11-KT plates the intra-assay variation was 2.1% and 2.3% and the inter-assay variation was 2.2%. Cross-reactivities of the cortisol antiserum reported by the manufacturer were 100% for cortisol, 22% for prednisolone, 6.1% for cortexolone, 1.3% for corticosterone, 0.2% for DOC and 17-hydroxy-progesterone, and less than 0.01% for 18-hydroxy-DOC, progesterone, pregnenolone and 17-hydroxy-pregnenolone. The assays were highly specific for the

steroids tested. Cross-reactivity of the testosterone antiserum was 100% for testosterone, 27.4% for 5α-dihydrotestosterone, 18.9% for 5β-dihydrotestosterone, 4.7% for methyltestosterone, 3.7% for androstenedione, 2.2% for 11-KT, 0.51% for 5-androstenediol, 0.2% for *Epi*-testosterone, 0.14% for progesterone, 0.11% for testosterone enanthane, 0.05% for androsterone, 0.04% for androsterone sulfate, 0.03% for testosterone sulfate, 0.02% for DHEA sulfate, and less than 0.01% for estradiol. The cross-reactivity for the 11-KT antiserum was 100% for 11-KT, 0.01% for 4-androsten-11β,17b-diol-3-one, and less than 0.01% for testosterone, 5α-androstan-17β-ol-3-one and 5α-androsten-3β,17β-diol. We plated, incubated, and developed the samples following the kit manufacturer's instructions specific for each hormone tested. All developed plates were read at 405 nm with the ELx808 Ultramicroplate Reader (Biotek Instruments, Inc.) using the software interface KCJunior.

Data analyses

Plasma steroid levels were calculated against the standard curve (8 standards in triplicate) and the extraction recovery values using the Cayman Chemicals Analysis Tools (EIA tools available at http://www.caymanchem.com). We analyzed the effect of condition order versus the differences in EOD τ_{P2} and amplitude across the social conditions using a two-way mixed ANOVA with repeated measures [GLM repeated measures procedure in SPSS v. 14.0, Model I – Fixed factors] with two factors: (1) social conditions [within-subjects factor with 4 levels = Baseline, Isolation, Social 2 and Social 6] and (2) condition order [between-subjects factor with 6 levels]. We $\log_{10}$ transformed all plasma steroid levels to fulfill the normality assumption. Whenever significant

interactions were found between these 2 factors, we used one-way ANOVA for each social condition level and evaluated that social condition for each condition order. For non-significant interactions, we calculated the main effects' p-level across each variable. Fisher's LSD multiple comparison tests were calculated to determine significant pairwise differences. We used the GLM multivariate procedure in SPSS v. 14.0 with social group as a fixed factor and steroid hormone as a covariate to evaluate the relationship between each steroid hormone with the dependent variables: day minima and night maxima for EOD τ_{P2} and amplitude. We used multiple linear regression to evaluate the relationship between each steroid hormone with body length and mass. All statistical analyses were performed with MATLAB or SPSS v.14.0, α=0.05 two-tailed.

Results

Order of social experiences influences their effects on the EOD

The magnitude of the males' EOD τ_{P2} in any particular social treatment condition depended on the order in which they experienced the three social treatments. Social condition treatment showed a significant order effect in daytime EOD τ_{P2} (social condition x condition order interaction: $F(15, 75) = 2.58$, $p = 0.004$). Males that experienced isolation as their first treatment (Figure 3, orders 3 and 6) depressed their daytime EOD τ_{P2} significantly below baseline levels (LSD post-hoc tests, $p < 0.001$ and $p = 0.011$, respectively). Furthermore, the males in these orders did not recover their previous daytime EOD τ_{P2} upon experiencing either the low or high competition conditions (Figure 3). By comparison, males in all other orders produced mean daytime EOD τ_{P2} values during the low or high competition conditions that matched or exceeded

their baseline levels (Figure 3). Although we found a significant social condition x condition order interaction (GLM repeated measures: $F(15, 66) = 2.12$, $p = 0.02$) in daytime EOD amplitude, post hoc tests revealed no significant differences between social conditions at each condition order.

Differences in EOD circadian rhythm magnitudes across the social conditions

Mean EOD amplitude tracked the differences in level of competition (Social 6 > Social 2 > Isolation), while mean EOD τ_{P2} only tracked presence or absence of a social environment (Social > Isolation). Overall, social competition increased the magnitudes of minimum daytime values when the fish were at rest (amplitude: $F = 11.07$, $p < 0.001$; τ_{P2} : $F = 8.18$, $p < 0.001$), maximum nighttime values when the fish were active (amplitude: $F = 10.55$, $p < 0.001$; τ_{P2} : $F = 8.17$, $p < 0.001$) , and day-night differences reflecting the magnitudes of circadian rhythms (amplitude: $F = 5.06$, $p < 0.001$; τ_{P2} : $F = 13.08$, $p < 0.001$) (Figure 4).

Differences in plasma steroid levels across the social conditions

Plasma concentrations of both androgens varied significantly with social condition and sex (T: $F(3, 20) = 39.53$, $p < 0.001$; 11-KT: $F(3, 20) = 3.84$, $p = 0.025$) (Figure 5). Overall, males' androgen levels were higher in both social conditions than in social isolation, but did not differ significantly between high and low social competition conditions. Females sampled from Social 2 and Social 6 pools had plasma 11-KT levels comparable to those of isolated males, but surprisingly, had testosterone levels significantly higher than males in any of the three social treatments (Figure 5).

The cortisol pattern followed the overall pattern of the androgens with one key difference – cortisol appeared to track the intensity of competition (number of males in the pool), whereas androgens tracked only the presence or absence of competition. Mean levels and variability of cortisol differed significantly between social conditions and sexes ($F = 7.587$; $p = 0.001$; Bartlett's test of variance = 8.95, $p = 0.03$) (Figure 5). Cortisol levels among males in the high competition group, Social 6, were significantly higher than for males in the low competition group, Social 2, ($p = 0.019$, Fisher's LSD post-hoc test). While mean cortisol levels were comparable between isolation and Social 2, the latter was far more variable. Cortisol levels in females were high, comparable to those of males in the Social 6 treatment, and significantly higher than in the isolated and low competition males ($p < 0.001$ for both) (Figure 5). Recall that females were sampled directly from Social 2 and Social 6 pools, a sex-specific competitive environment comparable to the Social 6 treatment that males received.

When looking at the variance in the steroid levels of males across the three social conditions, we found no significant differences in T levels (Levene statistic$_{1,10}$: Isolation-Social2 = 2.09, $p = 0.18$, Isolation-Social6 = 0.15, $p = 0.70$, and Social2-Social6 = 1.76, $p = 0.21$). We found a significant difference in 11-KT levels for the Isolation-Social6 comparison (Levene statistic$_{1,10} = 11.83$, $p = 0.006$), but not for the Isolation-Social2 (Levene statistic$_{1,10} = 4.58$, $p = 0.06$) or Social2-Social6 (Levene statistic$_{1,10} = 3.43$, $p = 0.09$) comparisons. We also found a significant difference in cortisol levels for the Isolation-Social6 (Levene statistic$_{1,10} = 8.53$, $p = 0.02$) and Isolation-Social2 (Levene statistic$_{1,10} = 11.97$, $p = 0.006$) comparisons, but not for the Social2-Social6 (Levene statistic$_{1,10} = 1.38$, $p = 0.27$) comparison. In addition, we found no significant

relationships between T, 11-KT and cortisol among the males at each social condition. For instance, T levels did not predict 11-KT levels (Isolation: F (1,5) = 1.93, R^2 = 0.32, p (2-tailed) = 0.24, Social 2: F (1,5) = 1.13, R^2 = 0.22, p (2-tailed) = 0.35, and Social 6: F (1,5) = 3.38, R^2 = 0.46, p (2-tailed) = 0.14). Also, T levels did not predict cortisol levels (Isolation: F (1,5) = 0.26, R^2 = 0.06, p (2-tailed) = 0.64, Social 2: F (1,5) = 0.40, R^2 = 0.09, p (2-tailed) = 0.56, and Social 6: F (1,5) = 0.005, R^2 = 0.001, p (2-tailed) = 0.94). In addition, 11-KT levels did not predict cortisol levels in males at each social condition (Isolation: F (1,5) = 0.54, R^2 = 0.12, p (2-tailed) = 0.50, Social 2: F (1,5) = 0.34, R^2 = 0.08, p (2-tailed) = 0.59, and Social 6: F (1,5) = 0.27, R^2 = 0.06, p (2-tailed) = 0.63).

Relationship between steroid hormone levels and the EOD circadian rhythm across the different social conditions

Cortisol plasma levels covaried significantly and strongly with the EOD circadian rhythm across the different social conditions (Wilks' λ = 7.94, p=0.003, eta-squared = 0.74, observed power = 0.97). Plasma levels of cortisol were significantly and strongly related to daytime and nighttime EOD amplitude (univariate between-subjects tests; day amplitude: p = 0.001, partial eta-squared = 0.57, observed power = 0.98 and night amplitude: p < 0.001, partial eta-squared = 0.60, observed power = 0.99). In contrast, plasma levels of cortisol were not significantly related to daytime or nighttime EOD τ_{P2} (univariate between-subjects tests; day τ_{P2}: p = 0.874, partial eta-squared = 0.002, observed power = 0.05 and night τ_{P2}: p = 0.25, partial eta-squared = 0.09, observed power = 0.20). When evaluating these relationships across each social group, we found that plasma cortisol levels positively predicted EOD amplitude in males under both low

and high competition treatments but not in the isolated males, which showed almost no variance in cortisol (Isolates: day $R^2 = 0.24$, $p = 0.32$, night $R^2 = 0.26$, $p = 0.30$; Social 2: day $R^2 = 0.71$, $p = 0.035$, night $R^2 = 0.70$, $p = 0.038$; Social 6: day $R^2 = 0.90$, $p = 0.004$, night $R^2 = 0.90$, $p = 0.004$) (Figure 6). Plasma cortisol levels and EOD τ_{P2} showed no apparent relationship (Figure 6).

Neither T nor 11-KT plasma levels covaried with the EOD circadian rhythm across the different social conditions (11-KT: Wilks' $\lambda = 1.68$, $p = 0.22$, partial eta-squared = 0.38, observed power = 0.36, and T: Wilks' $\lambda = 0.173$, $p = 0.95$, partial eta-squared = 0.06, observed power = 0.08). Even though we saw no significant relationship between either androgen and EOD amplitude or τ_{P2} at each social treatment (Figure 7 and 8), when the three social treatments were pooled, T levels positively predicted EOD τ_{P2} at night ($R^2 = 0.22$, $p = 0.05$) (supplementary materials 2). We found no significant relationship between plasma T levels and the EOD amplitude when all social treatments were pooled. We also found no significant relationship between 11-KT and the EOD amplitude or EOD τ_{P2} on pooled analysis (Figure 8).

We found significant relationships between the two key EOD parameters amplitude and τ_{P2}. Day-night change in EOD amplitude positively predicted EOD τ_{P2} ($R^2 = 0.23$, $p = 0.046$), whereas the day minima and night maxima were not associated in either parameter (day: $R^2 = 0.02$, $p = 0.63$; night: $R^2 = 0.09$, $p = 0.24$) (data not shown).

Relationship between steroid hormone levels and body size

Both body length and mass positively predicted plasma cortisol levels in the high competition condition only (length: $R^2 = 0.82$, $p = 0.045$; mass: $R^2 = 0.65$, $p = 0.05$)

(Figure 9). Body length was associated with mass (Figure 9) although the effect was weakest and thus not significant in isolated males (isolates: $R^2 = 0.39$, $p = 0.19$; Social 2: $R^2 = 0.79$, $p = 0.018$; Social 6: $R^2 = 0.97$, $p < 0.001$). In addition, although the mean mass of all the males was similar across all three social conditions, a high proportion of Social 2 males lost weight (66%) ($\chi^2 = 19$; $df = 2$; $p < 0.001$).

Discussion

Our results demonstrate that changes in the social environment of the gymnotiform fish *B. gauderio* males are accompanied by changes in both their electric signals and their steroid hormone profiles. By increasing the number of male competitors in a social group, we show that the EOD amplitude, but not the EOD τ_{P2}, is responsive to these changes. In addition, we also show an increase in the plasma cortisol levels, but not androgens, of these males as a function of the increase in the number of male competitors in the social group. In contrast, the EOD τ_{P2} and the plasma androgen levels only increased significantly when the males transition from social isolation to social conditions (either low or high competition). Our findings also suggest that altering the order in which males experienced isolation versus social competition had dramatic effects on their ability to fully enhance their electric signals during periods of intense competition for mates.

Etho-ecological validity of our study

Although both male competition groups included six females, the sex ratio between these two social conditions varied (low competition: 1 male: 3 females versus

high competition: 1 male: 1 female). Three weeks into the breeding season, a field study of *B. gauderio* populations in Uruguay found a 1:4 female-biased operational sex ratio across the multiple sites (Miranda et al. 2008). Males were spaced out in their habitat and displayed non-overlapping home ranges, while females occupied much larger ranges overlapping other females and the home ranges of multiple males (Miranda et al. 2008). *B. gauderio* spatially aggregated by day and night, keeping a meter or less of distance between each other in an uniform habitat (Miranda et al. 2008). Based on these findings, we suggest that of the three pool conditions, the low competition condition (Social 2) is closest to the densities and sex ratios observed in wild *B. gauderio* populations partway through the breeding season (initial conditions have not been observed). It is important to note that although the males in the high competition group were potentially subjected to fewer reproductive opportunities, we collected comparable numbers of eggs from both the low and high competition pools throughout the experiment indicating that our experimental design and manipulations did not disrupt breeding success in our test fish.

Social environment affects the EOD plasticity and steroid levels

Our experimental design revealed an effect of past social environment on the outcome of present and future social interactions. The males that experienced social competition as their first treatment were more resilient to reduction of their basal (daytime) EOD τ_{P2} when subsequently isolated (orders 1, 2, 4 and 5; Figure 3). It is important to note that although all the fish in this experiment were randomly sampled from social groups of 10-20 fish with 2-4 males per group (baseline social conditions), and that these conditions are equivalent to what they experienced in the experimental

social conditions, the baseline social conditions were sustained, stable conditions which adult fish had experienced since they were juveniles. We believe that it is the combined effect of initial absence of non-specific social stimuli followed by a continually-changing social environment (fish were moved every 7 days to a different social condition for almost a month) that drives the differences observed in the isolation treatment for those condition orders where isolation was experienced first (orders 3 & 6; Figure 3).

Social isolation suppressed both EOD parameters and androgen levels of male *B. gauderio*. Isolated males had lower EOD circadian rhythm magnitudes than males in either the low or the high competition conditions (Figure 4). These results agree with the findings reported by Franchina and her colleagues (2001), wherein males isolated for 3-5 days displayed a significant reduction in the circadian modulation of their EOD amplitude and duration. In our study, isolated males reduced their day and night EOD waveforms (Figure 4) along with circulating plasma levels of the androgens T and 11-KT (Figure 5, 7 and 8). Our isolation treatment not only removed all potential male competitors but it also removed all potential mates and non-sexual social stimuli (i.e., juveniles). Therefore, we cannot rule out the possibility that the reductions observed in the EOD and androgens are due to the lack of complete non-specific social stimuli rather than specifically due to the lack of male competitors. Cortisol levels were significantly higher under high competition than low competition, but were not higher in low competition than isolation, though isolates had significantly less variance (Figure 5). Nevertheless, gymnotiform *Apteronotus leptorhynchus* size-matched male pairs displayed significantly higher circulating levels of cortisol and more EOD chirps, an agonistic EOD frequency modulation, than isolated males (Dunlap et al. 2002). Even though males

were tested under isolation for 7 days in both studies, Dunlap and his colleagues (2002) kept their males isolated for 2-weeks prior to their experiments, while our fish were under either baseline social conditions or the experimental social conditions (Social 2 and Social 6) before they were isolated. In addition, when compared to *Apteronotus leptorhynchus*, *B. gauderio*'s baseline cortisol levels are much higher (Dunlap et al. 2002) (Figure 5 and supplementary material 3). Elevated cortisol levels during the breeding season is a pervasive adaptive strategy among vertebrates (Landys et al. 2006), a mechanism to deal with high metabolic demands associated with reproduction (Wingfield & Kitaysky 2002). Either of these factors can account for the lack of difference in cortisol levels between isolated and social males in our study.

The power of the EOD (i.e., voltage squared or the voltage area under both waveform's phases at a constant resistance) perfectly matches the energy expended on electrogenesis (Salazar & Stoddard 2008). Thus for isolated males to reduce both basal EOD parameters and their circadian augmentation appears to be an adaptive strategy to minimize energetic costs when social benefits due to the enhanced signal parameters are absent. The EOD of *B. gauderio* males seems to be a condition-dependent trait (Salazar & Stoddard 2008). Male *B. gauderio*, like male orthopterans, frogs, and some birds, invest a considerable fraction of their metabolic energy into signal production (Bucher et al. 1982; Eberhardt 1994; Hoback & Wagner 1997; Kavanagh 1987; Prestwich et al. 1989; Prestwich & Walker 1981; Taigen & Wells 1985). While androgens typically facilitate male signaling behavior, elevated glucocorticoids would be expected to release energy stores necessary to sustain signaling for high-energy signalers. Although high glucocorticoid levels are typically related to the suppression of androgen levels (Sapolsky

1994; Sapolsky et al. 2000) in many species, both glucocorticoid and androgen levels are increased during reproduction (Beletsky et al. 1989; Creel et al. 1996; Emerson & Hess 2001; Remage-Healey & Bass 2005).

Social competition affects EOD amplitude's circadian plasticity and cortisol levels

Cortisol and EOD amplitude covary positively with density of males in the social group (Figures 4-6), suggesting that cortisol may be a modulator of the EOD amplitude. We do not know if this potential modulatory effect is direct or indirect. Activating the hypothalamic-pituitary-interrenal (HPI) axis at various levels enhances the EOD amplitude and τ_{P2} in *B. gauderio* males (Markham et al. 2009; Markham & Stoddard 2005). But only the melanocortins, adrenocorticotropic hormone (ACTH) and alpha-melanocyte-stimulating hormone (α-MSH), exert rapid effects directly at the electrocytes (Markham et al. 2009; Markham & Stoddard 2005). In this study, we measured cortisol levels several days after the males had been housed at their specific social conditions. Although cortisol could directly initiate slow transcriptional effects that can drive the enhancement of the EOD amplitude within this period of time, ACTH and/or α-MSH can increase both the EOD waveform and cortisol levels independently within the same period of time. Therefore, we cannot rule out the possibility that the relationship that we observed between cortisol and EOD amplitude could be mediated by the effect of social experience on melanocortins. Beyond that, cortisol may support the enhanced EOD by increasing availability of glucose and lipids to support energetically costly signaling and swimming behaviors associated with territoriality and courtship (Landys et al. 2006; Sapolsky et al. 2000).

Increases in the density of competing males or in the number of perceived competitors in a population have been shown to increase androgen levels in other teleost fish (Carlson et al. 2000; Oliveira et al. 2001; Pankhurst & Barnett 1993; Remage-Healey & Bass 2005). As discussed by Oliveira and his colleagues (2002) and as predicted by the Challenge Hypothesis (Wingfield et al. 1990), an increase in potential competitors could intensify agonistic interactions leading to the activation of the hypothalamic-pituitary-gonadal (HPG) axis and an increase in circulating androgen levels.

Unexpectedly, circulating androgen levels did not differ for males in the low and high competition groups (Figure 5). However, if only one male dominates in the low competition pool and one or two males dominate in the high competition pools, they alone might show elevated androgens while suppressing androgen levels in the subordinates. Thus variance would increase but not group mean, a hypothesis consistent with the obtained distribution of 11-KT measurements (Figure 5). Nevertheless, we only found a significant difference in the variance of plasma levels of 11-KT between the isolated males and the males in the low competition group. Carlson and his colleagues (2000) found in the mormyrid fish *Brienomyrus brachyistius*, an African electric fish, that the dominance status of males housed in social groups determined the levels of 11-KT and the changes on their EOD total duration. We did not assess the dominance status of the males in our study. Body size is usually a good predictor of dominance status in size-mismatched male-male contests (Maynard Smith 1982; Parker 1974). We randomly paired the males in the low competition (Social 2) condition rather than matching them for size. Eleven of the eighteen Social 2 pairs were size-mismatched, and all three Social 2 pairs assayed for steroid levels were also size-mismatched. Therefore, it is possible that

by looking at the males as a group in each social condition rather than as subgroups (i.e., alpha males, beta males and omega males) based on their dominance status (Carlson et al. 2000), we were not able to identify the link between 11-KT levels and male-male competition as we increased the number of competing males.

In both gymnotiform and mormyrid electric fish species, the electric organ (EO) that generates the EOD has been shown to be a direct androgen-target tissue (Few & Zakon 2001), and androgen receptors have been identified in the nuclei of the electrocytes that composed the EO (Bass et al. 1986; Dunlap & Zakon 1998). Furthermore, androgens directly change the ion conductances of the electrocytes leading to changes in the EOD waveform (Zakon et al. 1999). Androgen implants in male and female *B. gauderio* (Allee et al. 2009; Silva et al. 2002; Stoddard et al. 2007a) and its congener *B. occidentalis* (Hagedorn & Carr 1985) enhance the duration of the 2nd phase of the EOD but have little or no effect on EOD amplitude. Androgen implants increase the effect of melanocortins on the EOD of *B. gauderio* (Allee et al. 2009; Stoddard et al. 2007a). Therefore, we speculate that during the breeding season, in *B. gauderio*, interaction with competing males and prospective mates stimulates the HPG axis leading to an increase in baseline circulating androgen levels. Higher level of circulating androgens would enhance the EOD τ_{P2}. The additional metabolic cost associated with the enhancement of the EOD via androgens and melanocortins would be sustained by increasing cortisol to release energy.

Sex differences in steroid hormone levels across gymnotiform species

In teleost fish, 11-KT and T are the major androgens in circulation in breeding

males (Kime 1993). In many species, females have similar or higher levels of the aromatizable androgen, testosterone (Borg 1994). In our study, *B. gauderio* females had significantly higher plasma levels of T than the males irrespective of their experimental condition (Figure 5). Females tend to have much lower levels of 11-KT than breeding males (Borg 1994). Accordingly, the plasma 11-KT levels of *B. gauderio* females were very similar to those observed in isolated males and were in the lower end of the range of plasma 11-KT levels observed in the males under social conditions (Figure 5). The androgen profiles of *B. gauderio* males and females follow a similar trend as in other gymnotiform species tested, where females have lower 11-KT levels than males but similar or higher levels of T (supplementary materials 3).

Although females had similar plasma cortisol levels than the males in the high competition group, they displayed higher levels of cortisol when compared to isolated males and the males in the low competition group (Figure 5). To our knowledge, this is the first time that circulating plasma cortisol levels for female gymnotiform fish have been reported. As mentioned earlier, *B. gauderio* baseline cortisol levels are much higher than the cortisol levels reported for one other gymnotiform species (Dunlap et al. 2002).

In conclusion, social environment regulates the enhanced male EOD potentially via 11-ketotestosterone and cortisol. Social competition further enhances the day to night changes in male EOD suggesting that circadian regulation of the EOD plays a role in male-male interactions. Cortisol levels increase in males as the level of competition increases, and cortisol is related to the social males' body size. Since the male EOD is an energetically expensive trait, we speculate that cortisol may be regulating EOD amplitude directly as well as indirectly by providing the fuel to sustain it.

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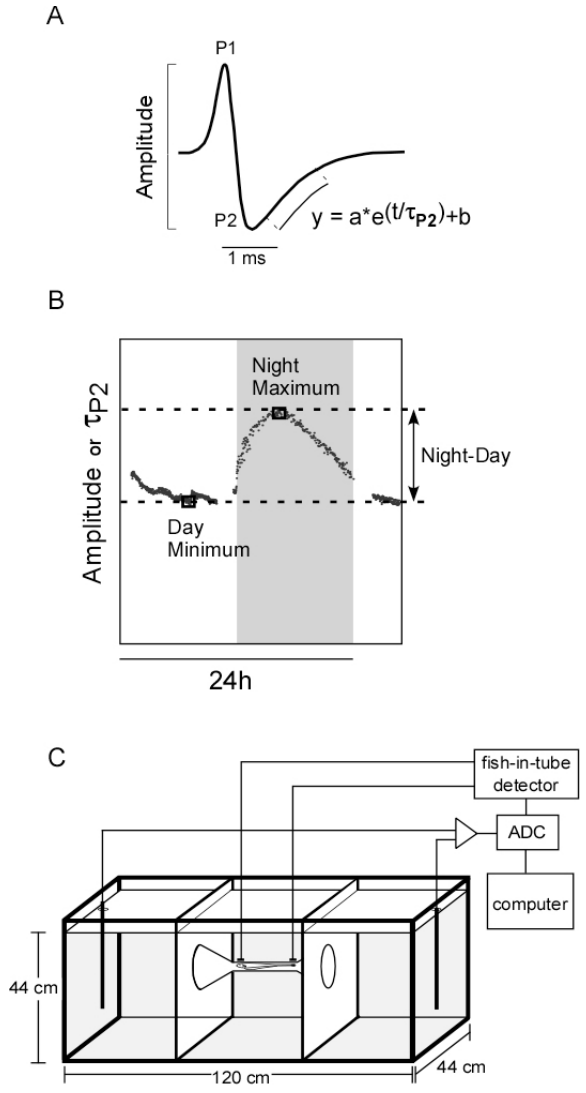


Figure 1. EOD data acquisition. A. The EOD of *B. gauderio* is a biphasic waveform composed of a positive phase (P1) and a negative phase (P2). We measured the peak-to-peak amplitude and the time constant of P2 repolarization (τ_{P2}). B. To determine changes in the EOD circadian rhythm, we measured daytime low, nighttime high, and the night-to-day difference for the amplitude and τ_{P2}. C. The tank set-up in the EOD machine automated system (Stoddard et al., 2003) allowed us to record the EOD of free-swimming fish continuously and accurately.

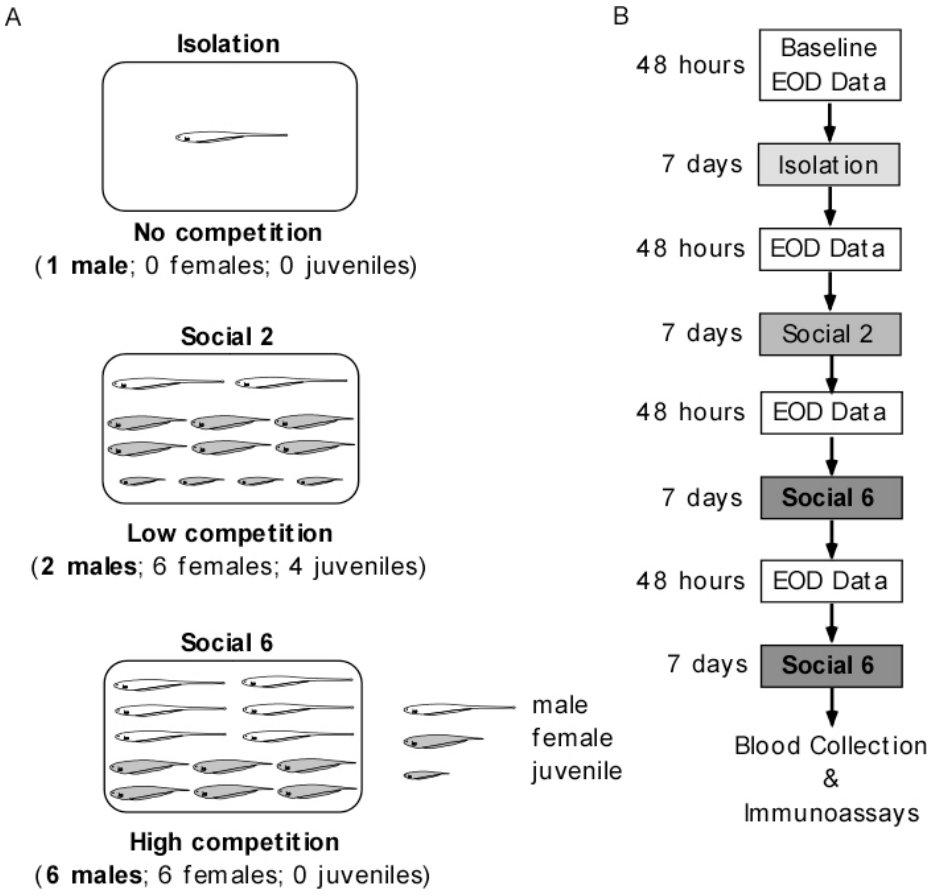


Figure 2. Social conditions and experimental design. A. We used three social conditions: Isolation, Social 2 and Social 6. For the Isolation condition, we housed males singly in a pool to deprive them of any social stimuli. For the Social 2 condition, we housed two males with six females and 4 juveniles in a pool to create a low competition social environment. For the Social 6 condition, we housed six males with six females in a pool to create a high competition social environment. B. We used a counterbalanced design where six groups of six males experienced all three social conditions but in different order. We chose this approach to evaluate the effect of social experience on the EOD changes displayed by the males at each social condition. For each group of six

males, we recorded their EODs for 48 hours continuously at the beginning of each experimental order (baseline values) and after each social condition. Males remained at each social condition for a week. At the end of an experimental order, we chose one replicate for each condition, kept those males under this condition for another week and then bled them to assay their plasma for circulating levels of steroid hormones.

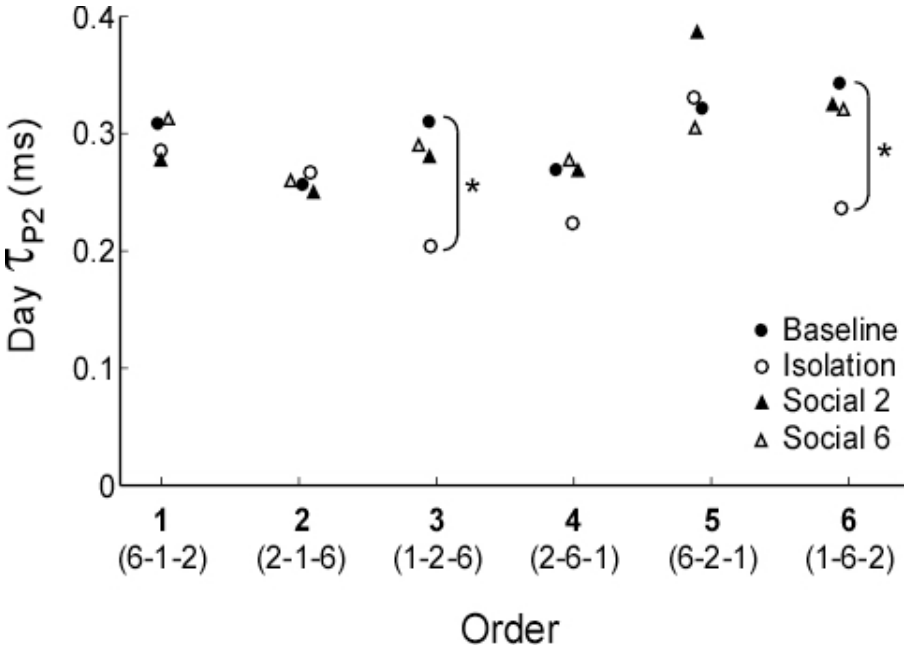


Figure 3. Males displayed differences in their EOD circadian rhythm plasticity according to the order they experienced the different social conditions. The magnitude of the day EOD τ_{P2} was affected by social experience. Particularly, males that experienced the Isolation condition first in the order displayed significantly lower day EOD τ_{P2} values than their prior baseline values. In addition, when compared to the day EOD τ_{P2} of the males on the other orders, the day EOD τ_{P2} of these males (orders 3 and 6) was lower than baseline levels during both the Social 2 and the Social 6 conditions.

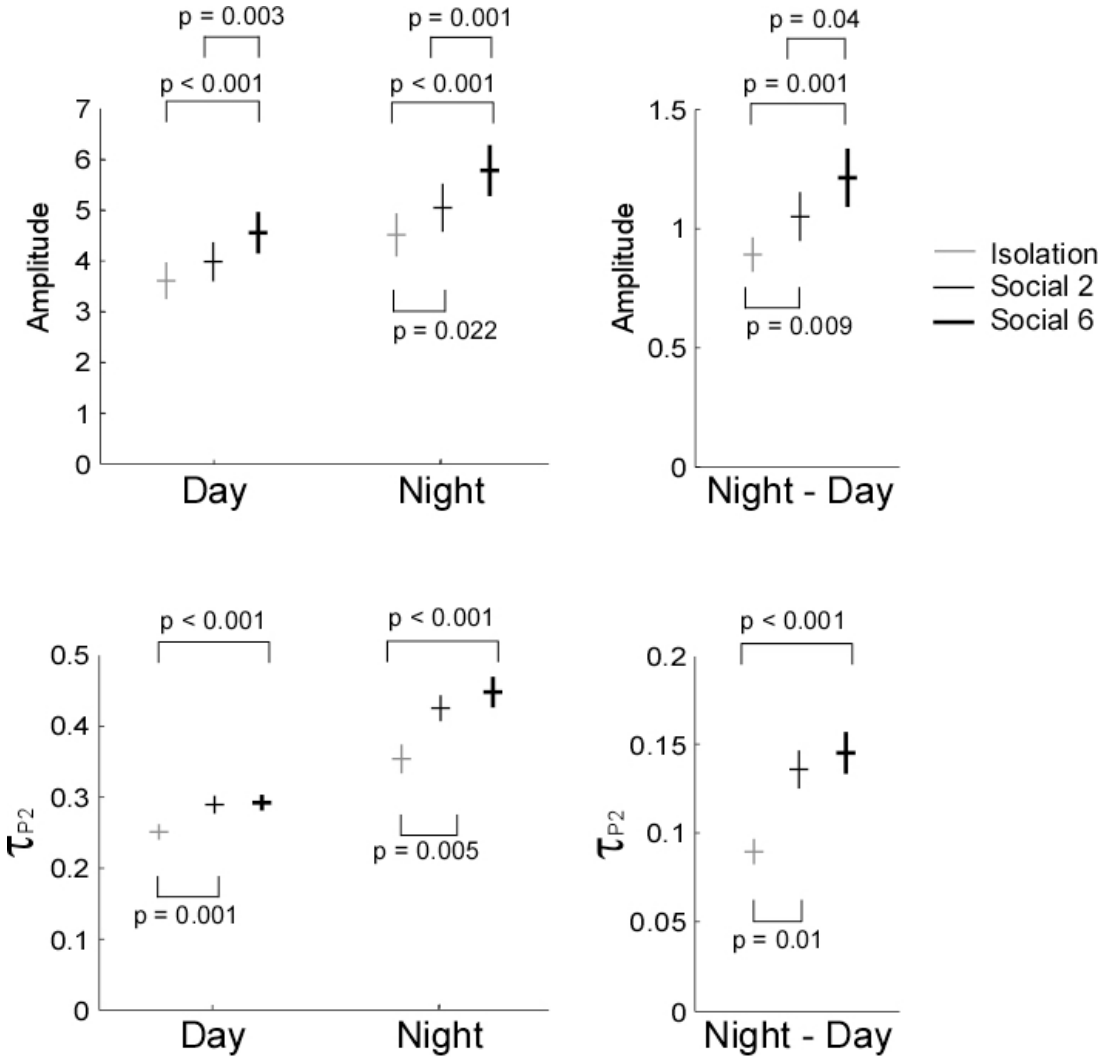


Figure 4. Means±SEM are shown for the EOD parameters amplitude and τ_{P2} across the social conditions. Daily minima, nightly maxima, and day-night differences follow the same trends wherein the highest competition (Social 6) promoted the highest values and social isolation promoted the smallest. P values are derived from post-hoc LSD pairwise tests following repeated-measures ANOVA.

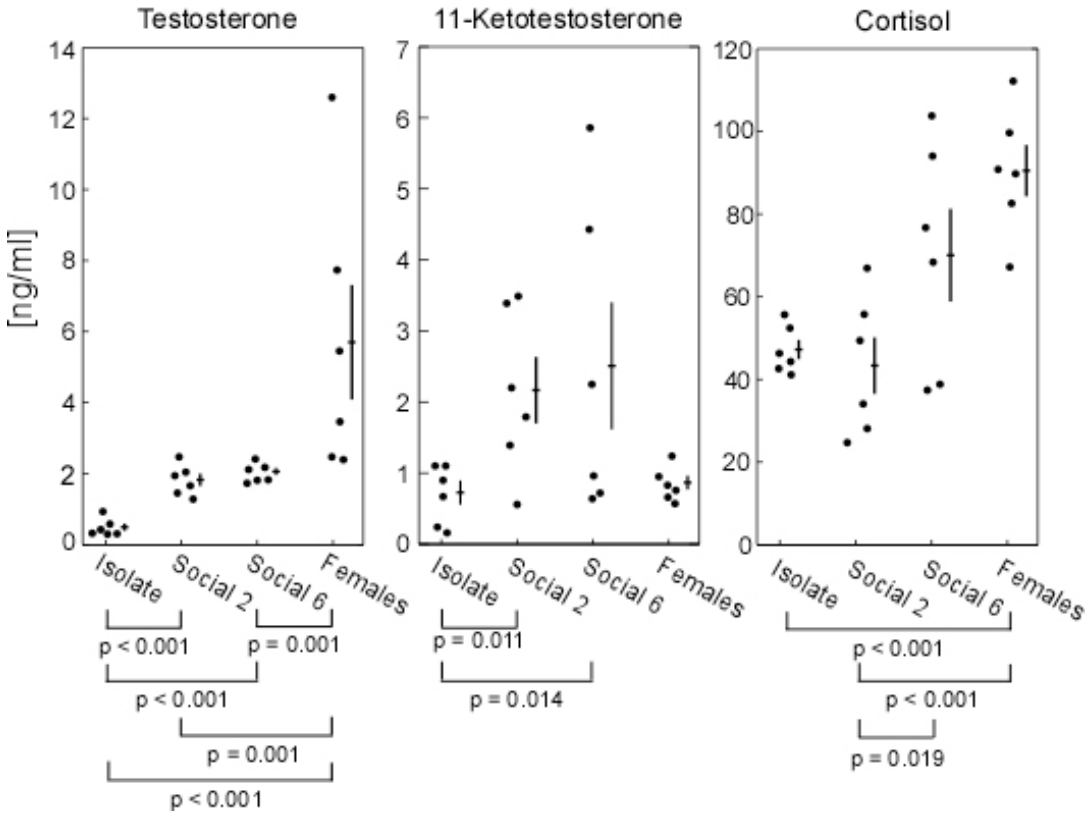


Figure 5. Plasma levels of T, 11-KT and cortisol (ng/ml) varied significantly with social condition and sex. Both T and 11-KT were lower in isolated males than in social males. Although females' T levels were higher than males', their 11-KT levels were similar to those of isolated males. High competition males' cortisol levels were higher than low competition males'. Females displayed higher cortisol levels than those of isolated and low competition males. Filled circles depict raw values while crosses depict mean ± SEM. Significant p-values from Fisher's LSD post-hoc pairwise comparisons are also shown.

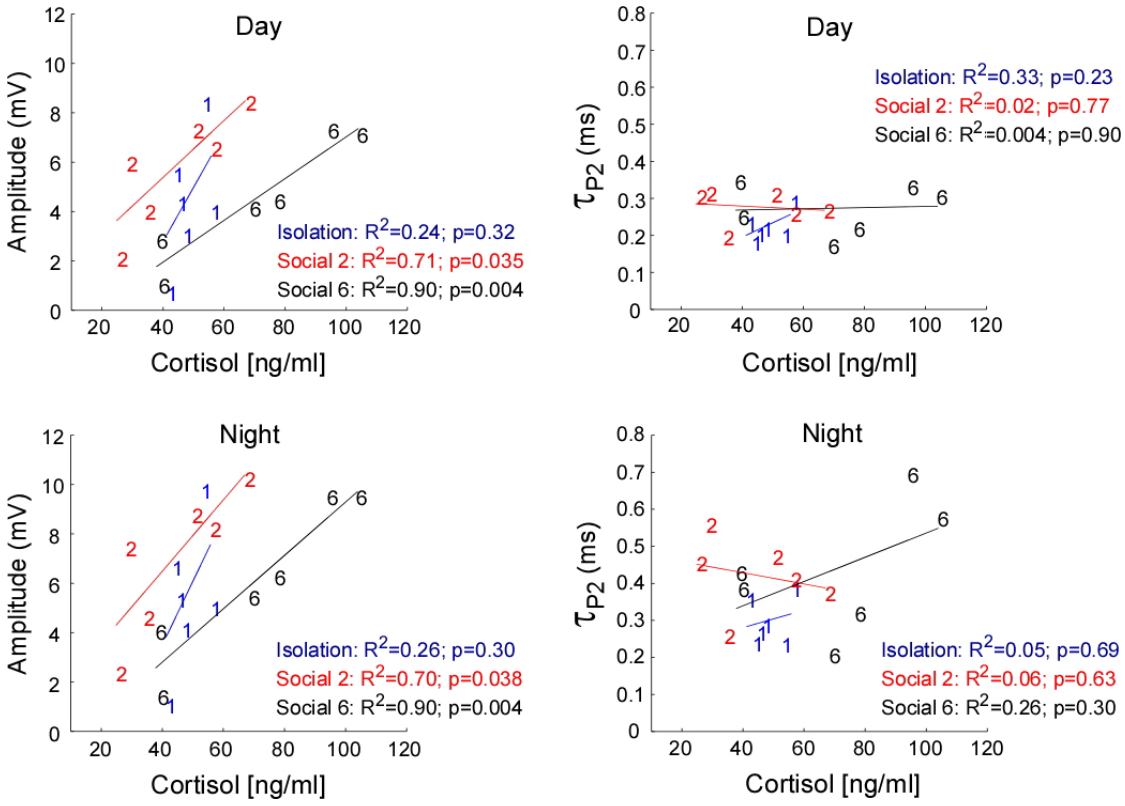


Figure 6. As plasma cortisol levels (ng/ml) increase, EOD amplitude (mV) increases for the low and high competition males but not for the isolated males (low competition; day: $y = 0.12x + 0.77$ & night: $y = 0.15x + 0.70$ and high competition; day: $y = 0.09x - 1.44$ & night: $y = 0.11x - 1.52$). EOD τ_{P2} and cortisol were not related in our dataset.

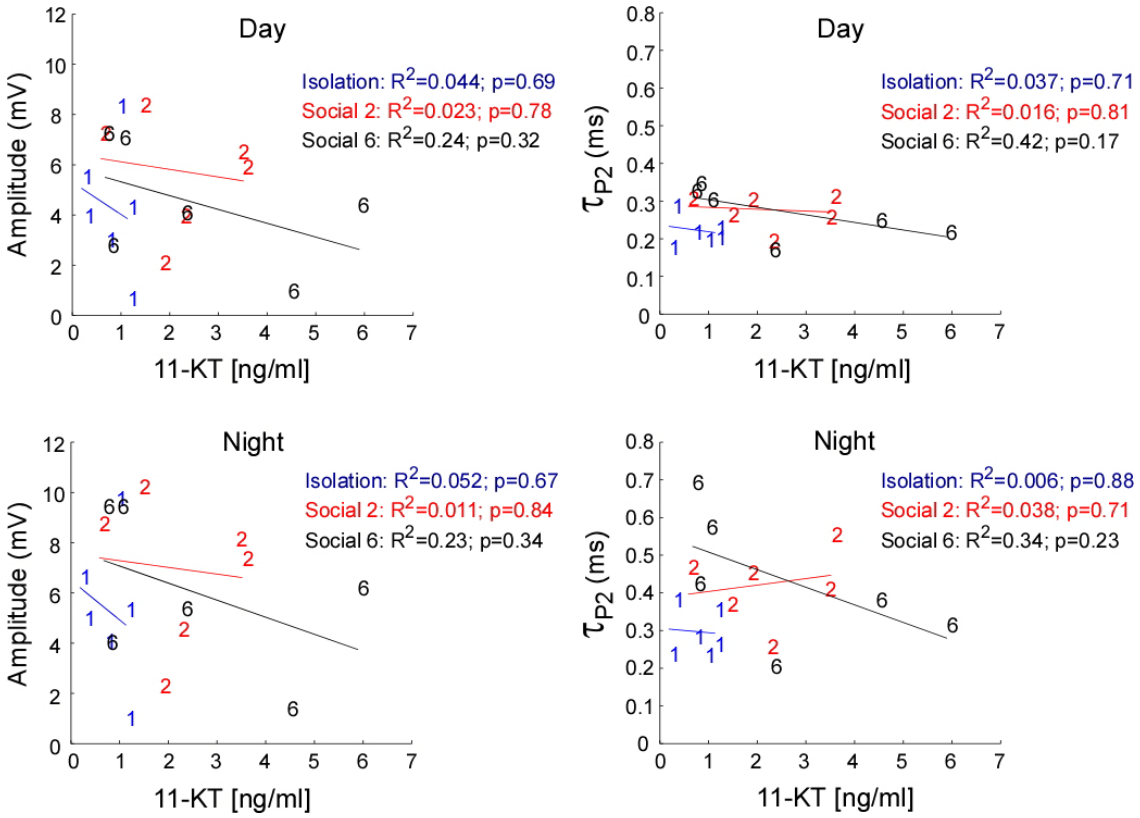


Figure 7. Testosterone plasma levels were not significantly related to EOD amplitude or τ_{P2} at any of the three conditions, isolation, Social 2 or Social 6.

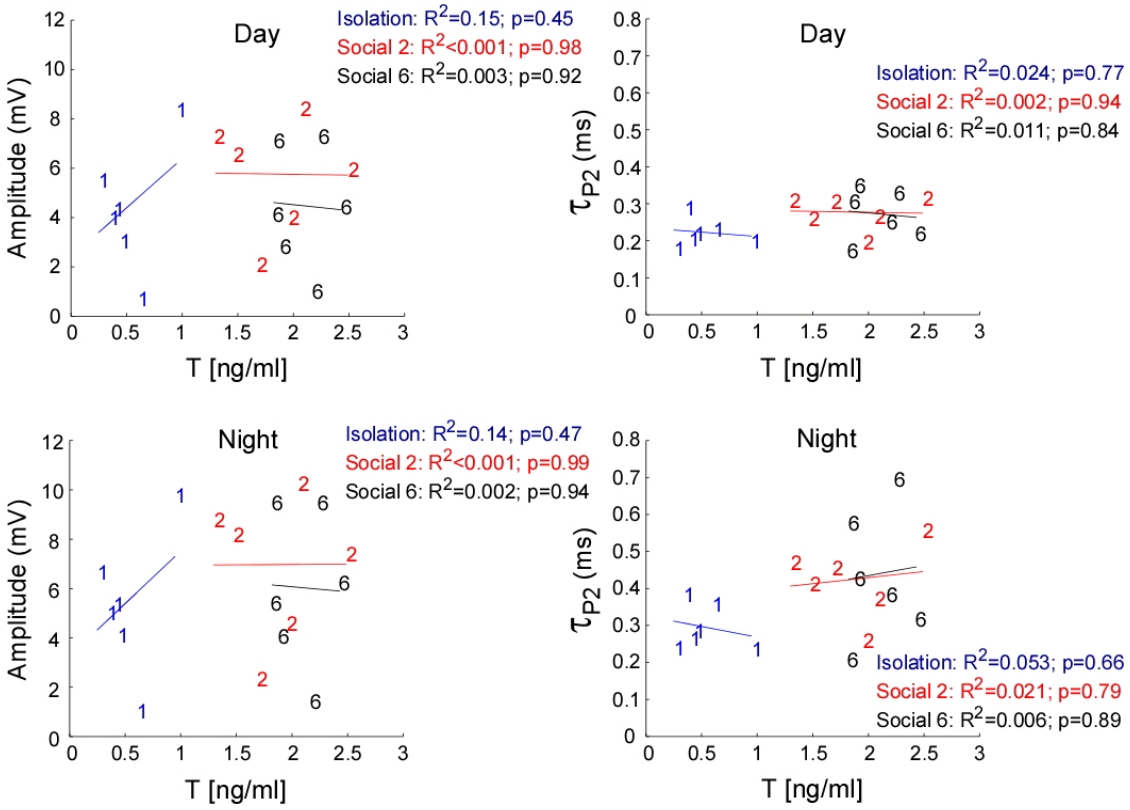


Figure 8. 11-ketotestosterone plasma levels were not significantly related to EOD amplitude or τ_{P2} at any of the three conditions, isolation, Social 2 or Social 6.

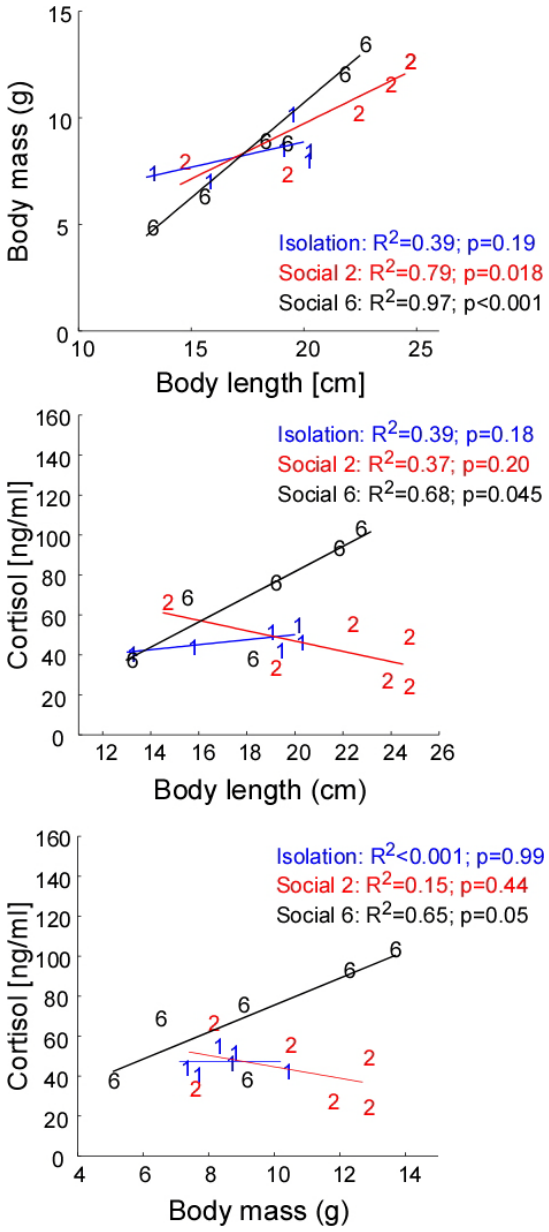
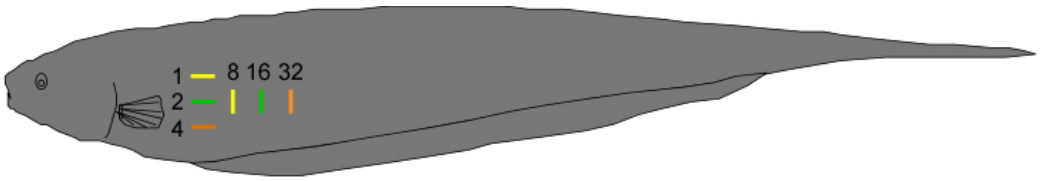
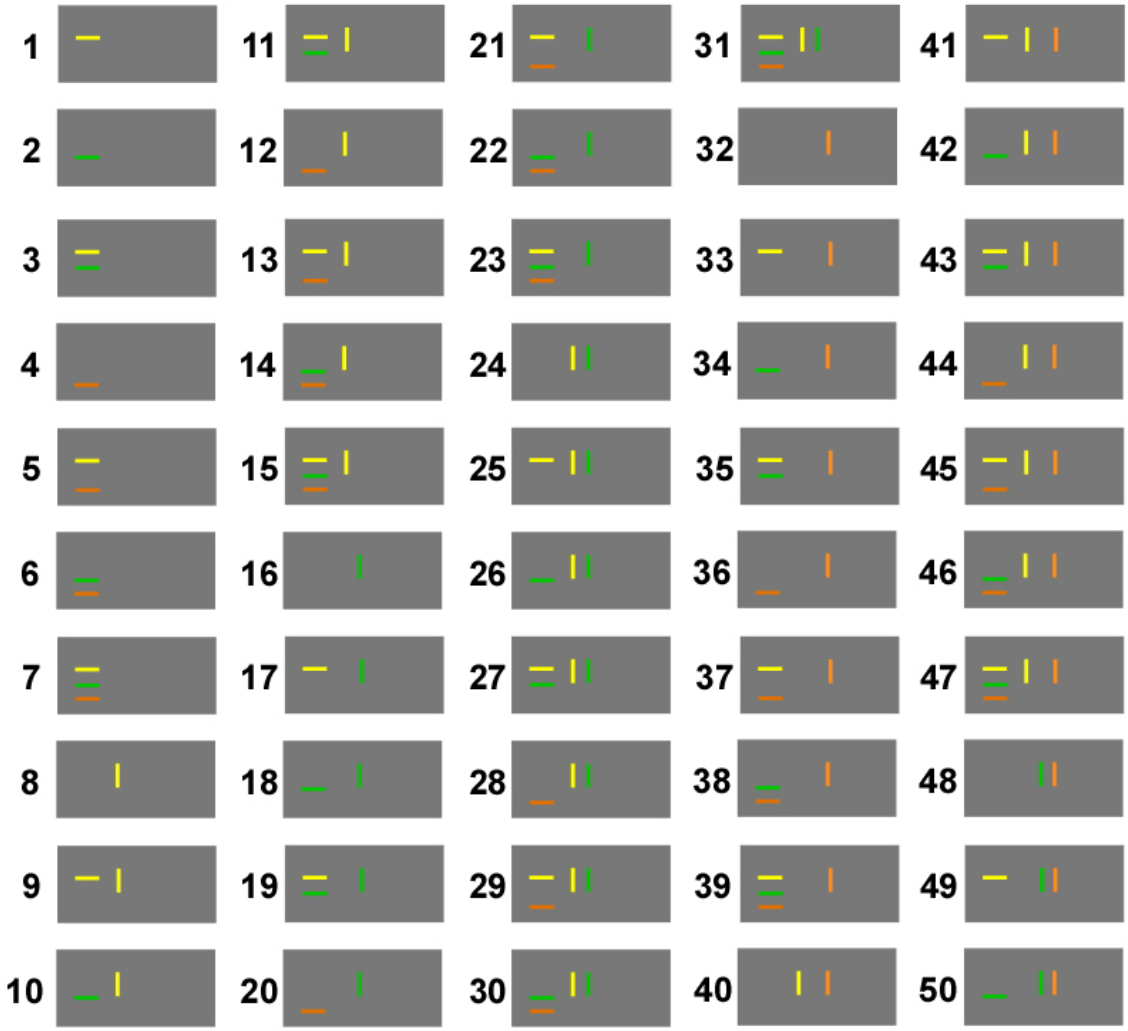


Figure 9. Body length (cm) predicts body mass (g) in low and high competition males but not in isolated males (low competition; y = 0.52x – 0.67 and high competition; y = 0.89x – 7.15). Only under high competition (Social 6), the body length (cm) and body mass (g) of the males predicts their circulating plasma cortisol levels (ng/ml). The regression models are y = 6.21x – 43.24 for body length vs. cortisol and y = 6.75x + 8.40 for body mass vs. cortisol.

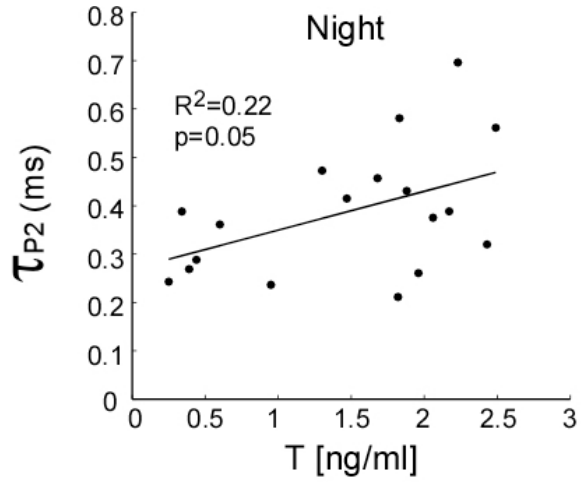
Supplementary material 1

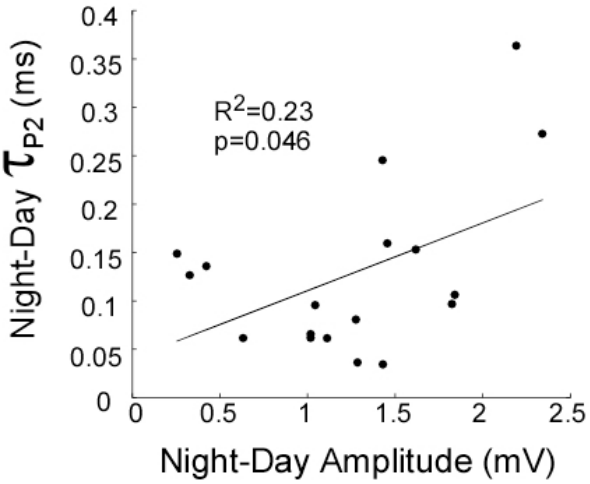




All the males in this study were individually-tagged using fluorescent elastomer of three colors, yellow, green and orange. We devised a binary code using these three colors and a combination of horizontal and vertical lines. Tags were placed on the right side of each male fish immediately caudal to the pectoral fin. Here we displayed the code in a cartoon fish and the tag combinations from 1 to 50.

Supplementary material 2





A pooled sample of all males tested for steroid hormones irrespective of the social condition showed that high testosterone levels (ng/ml) predict high EOD τ_{P2} (ms) following the regression model: y = 0.07x + 0.04.

We did not observe this relationship within each social condition or for the other androgen tested, 11-KT. When all the males tested for steroid hormones are combined irrespective of the social condition they experienced, we found that the night-day change in EOD amplitude (mV) predicts the night-day change in τ_{P2} (ms).

Supplementary material 3

Table 1. Comparison of plasma steroid levels across different gymnotiform species.

Species	Sex	11-KT (ng/ml)	T (ng/ml)	Cortisol (ng/ml)
Sternopygus macrurus	male	0.48 ± 0.33 (n=8)[5] 1.45 ± 0.80 (n=15; 1986)[6] 1.48 ± 1.56 (n=20; 1987)[6] 2.61 ± 1.41 (n=10; 1989)[6]	0.66 ± 0.44 (n=8)[5] 0.90 ± 0.32 (n=15; 1986)[6] 0.80 ± 0.99 (n=20; 1987)[6] 1.69 ± 1.47 (n=10; 1989)[6]	NM
	female	ND[6]	0.58 ± 0.49 (n=7)[5] 0.245 ± 0.13 (n=7; 1986)[6] 1.42 ± 2.52 (n=11; 1987)[6] 0.91 ± 0.48 (n=10; 1989)[6]	NM
Eigenmannia virescens	both	0.97 ± 0.26 (n=2)[3]	1.27 ± 0.41 (n=3)[3]	NM
Apteronotus albifrons	male	6.12 ± 1.22 (n=9)[2]	8.45 ± 3.2 (n=9)[2]	NM
	female	0.82 ± 0.12 (n=7)[2]	14.21 ± 4.1 (n=7)[2]	NM
Apteronotus leptorhynchus	male	5.33 ± 1.74 (n=10)[2] 0.31 ± 0.06 (n=10, Expt1)[4] 0.63 ± 0.22 (n=8)[4] (Expt3, paired individuals)	10.6 ± 2.41 (n=10)[2]	10.6 ± 2.6 (n=10; Expt 1)[4] 14.6 ± 5.4 (n=8)[4] (Expt3, paired individuals)
	female	0.89 ± 0.25 (n=6)[2] 1.3 ± 0.05[1]	12.2 ± 3.11 (n=6)[2]	NM
Brachyhypopomus gauderio	male	0.73±0.42 (isolation; n=6) 2.17±1.15 (Social 2; n=6) 2.51±2.19 (Social 6; n=6)	0.50±0.25 (isolation; n=6) 1.83±0.43 (Social 2; n=6) 2.06±0.25 (Social 6; n=6)	47.26±5.72 (isolation; n=6) 43.34±16.81 (Social 2; n=6) 83.13±41.64 (Social 6; n=6)
	female	0.87±0.24 (n=6)	5.74±3.46 (n=10)	90.50±15.23 (n=6)

All reported studies were conducted under laboratory conditions. Except for our study, all reported studies used radioimmunoassays to quantify steroid levels. ND = not detectable levels; NM = not measured. References: 1. Dunlap, 2002; 2. Dunlap *et al.*, 1998; 3. Dunlap & Zakon, 1998; 4. Dunlap *et al.*, 2002; 5. Zakon *et al.*, 1990; 6. Zakon *et al.*, 1991.

CHAPTER III

"The effect of body size asymmetry on electric signal waveform plasticity in male-male dyads of the gymnotiform fish *Brachyhypopomus gauderio*"

Abstract

Sexually-selected communication signals can convey both reliable as well as dishonest information about an organism's status or resource-holding potential (RHP). Here I examine the information encoded in the electric organ discharge (EOD) of the South American weakly electric fish, *Brachyhypopomus gauderio*, with reference to male-male interactions, to determine if signal characteristics act as reliable indicators of a male's body size and dominance status. My results suggest that while body size is the best determinant of dominance in this fish, EOD amplitude can reliably predict body condition, a composite of length and weight, for fish with good body condition (positive residuals). As such EOD amplitude (but not EOD duration) can act as an honest signal of RHP under some circumstances. Interestingly, I also observed evidence of bluffing (increased EOD amplitude) in small resident male fish when they encountered a larger male intruder, and diminished responses of resident males upon encountering a smaller intruder. Such responses suggest a more complex suite of physiological and behavioral processes at work in these agonistic interactions between males when size mismatches occur between males. The fact that size/dominance can be encoded in some elements of the EOD (EOD amplitude) but not others (EOD duration) suggests that these two signal properties can be regulated independently and may encode different information content.

Keywords: body size; body condition; *Brachyhypopomus gauderio***;** communication signal; dominance; electric fish; electric organ discharge; Gymnotiformes; knifefish.

Introduction

Sexually-selected signals can convey information about an organism's resource-holding potential (RHP), such as body size, fighting ability and ownership of a territory (rev. Andersson, 1994; Maynard-Smith and Harper, 2003). For instance, complex signals such as birdsongs, and the calls of frogs and insects can act as both indices of RHP and handicaps of a condition-dependent trait (Maynard-Smith and Harper, 2003). Females can use this information to make decisions about who to mate with, while males can make decisions about whether or not to challenge a specific rival. Failure to make the correct decision can lead to ineffective investment of energy in contests and/or poor reproductive output. Although signals are, on average, honest because they are constrained by physical characteristics of the organism (indices) or incur high costs (handicaps) (Maynard-Smith, 1982), low frequency cheating using unreliable signals may be tolerated under certain conditions (rev. Searcy and Nowicki, 2005).

Many studies have established how receivers use visual, acoustic and chemical signals to obtain information about a sender's RHP (Andersson, 1994). Whether signals in other sensory modalities, such as the electric signals of weakly electric fish, operate as reliable indices and/or handicaps in the context of male competition for mates and mate attraction is less clear. The gymnotiform fish *Brachyhypopomus gauderio* (Giora and Malabarba, 2009), a nocturnal species found in various freshwater habitats in South America, provides a good model system for exploring the information content encoded in electrical communication signals and its reliability as a measure of RHP. This gymnotiform fish continuously generates discrete electrical pulses (approx. 1mV), known as electric organ discharges (EODs) which are used to navigate in their environment,

electrolocate objects, and communicate with conspecifics in the dark. The EODs of *B. gauderio* have a distinct signature or waveform which can potentially encode information about the gender, size, and reproductive status of the fish producing it (Silva et al., 1999; Stoddard, 2002).

Body size and EOD waveforms as indices

The EOD waveform of *B. gauderio* is the product of summed action potentials generated by specialized cells, known as electrocytes. These cells are organized in rows and columns within the electric organ (EO), a bilateral structure that runs longitudinally along approximately two-thirds of the length of the fish, (Bennett, 1970; Hopkins et al., 1990). In *B. gauderio*, electrocytes generate two action potentials which produce the two phases (conventionally depicted as a head-positive phase or P1, followed by a head-negative phase or P2) of the EOD waveform (Figure 1A) (Bennett, 1970). The biphasic EOD waveform has two behaviorally-relevant measurable parameters: 1) amplitude and 2) the time constant of repolarization of P2 (τ_{P2}), a measure of EOD P2 duration (Figure 1A).

Both EOD amplitude and τ_{P2} appear to be important in the context of reproductive behaviors (Stoddard, 2002). During the breeding season, males emit EODs with bigger amplitudes and extended τ_{P2} when compared to female's EODs. Males further enhance these traits during the night hours of courtship (Franchina and Stoddard, 1998; Stoddard et al., 2006). Males' EOD amplitude and τ_{P2} are also sensitive to changes in the social structure of the species (Salazar and Stoddard, 2009; Silva et al., 1999). For instance, males enhance their EOD amplitude and τ_{P2} even more by the presence of other males,

and suppress these enhancements if they are isolated artificially (Franchina et al., 2001; Franchina and Stoddard, 1998; Stoddard et al., 2006).

When assessing potential mates or rivals, *Brachyhypopomus gauderio* have the potential to use either relative body size differences or information encoded in their EOD waveform to decide when to challenge a competitor or select a mate. Although body size appears to play an important role in mate attraction in *B. gauderio,* less is known about its role in male-male competition. For instance, sexually-mature *B. gauderio* males have longer body lengths and tails than females (Caputi et al., 1998; Silva et al., 1999), enabling gender to be distinguished based on morphology alone. Female *B. gauderio* also prefer large males over small males in a two-choice test, suggesting that large males have access to more reproductive opportunities (Curtis and Stoddard, 2003). How can weakly electric fish assess the size and condition of conspecifics? Weakly electric fish can determine the size and volume of objects in the dark via active electrolocation within approximately one body length distance (von der Emde and Fetz, 2007). Consequently, *B. gauderio* males can determine the length of other conspecifics without receiving EOD waveform cues or physically interacting with them, as long as both fish are within a few centimeters of each other. Physical interactions between fish, such as orienting antiparallel to one another, may also provide more precise information relating to differences in relative size (Aguilera et al., 2001; Terleph and Moller, 2003).

Because body length is also related to the length of the EO, body size information may also be encoded in the electrical signal. Several studies have shown that a male's total body length determines the length of the EO, the number of electrocytes, and the EOD amplitude (Caputi et al., 1998; Curtis and Stoddard, 2003; Franchina and Stoddard,

1998; Hopkins et al., 1990). A fish can determine the magnitude of a conspecific's EOD amplitude at a distance of several body lengths, via passive electrolocation (by comparing distance to electric field strength) and scan sampling (Hopkins, 1986; Hopkins et al., 1997; Hopkins and Westby, 1986). Therefore, gymnotiform fish may use EOD amplitude to assess a composite of length, weight, and body condition of conspecifics, particularly when these fish are positioned further apart. Although, based on this evidence, EOD amplitude could be used as an RHP index, the relationship between length and EOD amplitude can vary depending on the recent social experience of the males (Franchina et al., 2001; Salazar and Stoddard, 2009). Consequently, EOD amplitude can vary independently of size and thus may not always be an honest indicator of RHP.

EOD waveforms as handicaps

The EOD is also a condition-dependent signal. The power of the EOD is significantly more energetically expensive in males than in females and strongly related to body condition in males (Salazar and Stoddard, 2008). Furthermore, the enhanced asymmetric male EOD is more attractive and readily detected by electroreceptive predators (Stoddard 1999). As such, the enhanced male EOD operates as a handicap signal and presumably conveys honest information on a male's condition and survival ability (Zahavi, 1975). Interestingly, males can boost their EOD amplitude within minutes by increasing the time between the firing of the two action potentials within each electrocyte, a process under the control of melanocortins (Markham and Stoddard, 2005), that renders the EOD as an unreliable signal if only assessed for a short time interval.

Therefore, males with low body condition could take advantage of this melanocortin-driven EOD dishonest enhancement to deter competitors of superior body condition.

Objectives

In this study, I investigated the relationship between male size, behavior, and EOD waveform parameters in male-male agonistic interactions to understand the role of EOD waveforms as reliable information signals. I specifically addressed the following questions: 1) How reliably can EOD amplitude and τ_{P2} predict male body size (length, weight and condition) in *B. gauderio*? 2) Can resident males gain body size information from an intruder male at a distance, using only electrical cues, and do they respond differently to these fish based on size? 3) How does body size relate to social dominance when males are allowed to interact physically and electrically? And 4) How do body size and EOD waveform mismatches influence these social interactions between males? I addressed these questions by undertaking experiments using dyads composed of size-mismatched resident males and intruder males. In the first of these sets of experiments, I physically isolated resident males from intruder males to explore the information content passed between males based on EOD waveforms alone. The second experiment explored the effect of both waveform and physical contact in male-male behavioral interactions.

Methods

Study subjects

Males of the pulse-type weakly electric fish *Brachyhypopomus gauderio* (Giora & Malabarba, 2009) were sampled randomly from a captive-reared, 11th generation

breeding colony located at Florida International University, Miami, Florida. Fish were housed in 450-liter (185 x 95 x 26 cm) outdoor pools with water conductivity at 90±10 $\mu S\ cm^{-1}$ and mean ambient temperature at 27±3 °C. The water surface of each pool was covered 80-100% with water hyacinths (*Eichhornia crassipes*). Each breeding pool contained 10-20 fish. All fish were fed live oligochaete blackworms (Gulfstream Tropical Aquarium, Dania, FL, U.S.A.) three times per week. I weighed and measured the length of all the males at the beginning of the experiment. Experiments complied with NIH 'Principles of Animal Care' publication no. 86-23, revised 1985, and were approved by the F.I.U. IACUC (protocol approval no. 07-004).

EOD machine recording

The EOD machine consists of 12 recording tanks and an automated real-time data acquisition system designed to record the EOD of one fish per tank. Each recording tank (120 x 44 x 44 cm) was kept in a light- and temperature-controlled room on a 12:12 light-dark cycle and was divided with two screen-mesh partitions into three compartments of equal size. The two outer compartments were connected via a ceramic tube (Stoddard et al., 2003). Resident fish typically hid inside the ceramic tube during the day, and swam back and forth between the outer compartments of the tank during the night. An electrode pair monitored the position of the resident fish, while another electrode pair sampled the EOD when the fish was centered in the tube (for more details, see Stoddard et al. 2003). This system was not capable of measuring the EOD of a second stimulus fish while the resident fish was being recorded. The EOD data were analyzed in real-time with MATLAB (The MathWorks, Inc, Natick, MA, U.S.A.) to yield the peak-to-

peak amplitude and τ_{P2} (Figure 1A) (Stoddard et al., 2003). I measured the EOD amplitude and τ_{P2} at the following points in the 24h period before and after the addition of the stimulus male: (1) daytime low, (2) nighttime peak, and (3) time of peak (Figure 1B).

Test of body size effects - dyads with no physical contact

To determine the relationship between body length, weight, condition and EOD change that resulted from male-male interaction, I randomly sampled 27 males from their outdoor breeding pools (hereafter deemed the "resident" male) and paired each one with a second male fish (the "stimulus") (Figure 1C). Prior to the experiment, I placed each resident male in a tank in the EOD machine and recorded its electric signal for 24h. During the early hours of the morning of the second day, the stimulus male was placed in the middle compartment of the EOD machine tank of the resident male (Figure 1C), allowing the two males to interact electrically without direct body contact. I measured the EOD waveform of the resident male continuously for 24h throughout the social interaction. At the end of this social interaction, I returned the stimulus male to his previous tank. This design allowed me to measure the EOD waveform modulation experienced by the resident male before and during the social interaction. I calculated differences in daytime low, nighttime peak, and peak time for amplitude and τ_{P2} between the 24h prior to the addition of the second male and during the 24h of interaction between the two males.

I calculated the percent difference in body length (cm) and weight (g) between the resident male and the stimulus male. Then, I calculated the percent change (from the 24h

prior to addition of the stimulus male and 24h of interaction) in the daytime low and nighttime peak EOD amplitude and τ_{P2} of the resident male (Figure 1B). I used linear regression to evaluate the relationship between body size (length and weight) differences and EOD changes. Previous studies have shown that male *B. gauderio*'s EOD amplitude highly correlates with body length (Curtis and Stoddard, 2003; Franchina and Stoddard, 1998; Salazar and Stoddard, 2008). To determine whether the relation that I observed between body size difference and EOD change was the result of the intrinsic relationship between these variables in individual males, I looked at this relationship in resident males at their daytime low and nighttime peak before and during the intrusion. I regressed resident males' total length against their weight and regressed mass residuals against both EOD amplitude and τ_{P2}. I partitioned the males into two analysis groups, those that were lighter than predicted for their specific lengths (negative residual mass) and those that were heavier (positive residual mass). All statistical analyses were performed with MATLAB or SPSS v.15.0 (SPSS Inc., Chicago, IL, U.S.A.), α=0.05 two-tailed.

Test of body size effects - dyads with physical contact

To determine the relationship between body size and EOD with physical behaviors during male-male interactions, I selected seven dyads of size-mismatched males and individually isolated these fourteen males for 5 days in EOD machine tanks to reset their waveforms following their previous social encounters (Franchina et al., 2001) (Figure 1C). I recorded the EODs of each male during the last 24h of isolation (Figure 1C). At the end of this isolation period, just before dark, one dyad at a time was reunited in a behavioral observation tank (122 cm x 45 cm x 52cm) containing only one plant

refuge. The plant refuge contained the only food dish, provisioned daily with live oligochaete food ("blackworms"). The tank was kept on a 12:12 light cycle, at 27°C (±1), and water conductivity100 μS (±5).

I videotaped social interactions starting at the onset of darkness using a digital camcorder (Sony DCR-TRV310) and an infrared LED array for illumination. The changes in the EOD rate were detected with two carbon electrodes, amplified, and recorded on the digital audio track of the camcorder. I used pilot videos to generate an ethogram listing all behaviors observed during male-male interactions (supplementary materials 1). Analysis of pilot videos showed that the first 30-min of interaction were sufficient to determine the dominance status of each male in a dyad. Accordingly, I converted the first 30-min recording of each interacting pair to Quicktime video using iMovie (Apple Computer, Cupertino, CA, U.S.A.) and continuously scored behaviors using the behavioral analysis program JWatcher (v. 1.0, Animal Behaviour Laboratory, Macquarie University, Sydney, Australia). I assigned to each behavior a unique computer keyboard code (supplementary materials 1) for data acquisition in JWatcher, and a general description (supplementary materials 1) based on a previously described ethogram for another gymnotiform species, *Gymnotus carapo* (Black-Cleworth, 1970).

I took the 30-min continuous sequence of scored behaviors for each of the 10 dyads and analyzed them in two ways. First, I compiled them into one dataset, tallied repeated key codes of behaviors performed by the big male and the small male in the dyad, and computed the transitional probabilities (for lag 0 to lag 1) matrices using JWatcher's Sequential Analysis tool. I used transition probabilities that were greater than 0.15 and had a p-value smaller than 0.05 to build first-order Markov chains of offensive-

defensive behavioral sequences classified by behaviors performed by the big male and the small male in a dyad. In addition, in the context of agonistic interactions, *B. gauderio* males modulate their EOD rate to generate stereotypic EOD patterns such as accelerations (Perrone et al., 2009). Therefore, I scored the occurrence of EOD accelerations in relation to physical offensive behaviors. Second, I took the first 10 min of each 30 min dyad sequence, and counted the number of offensive behaviors (e.g., bites), and mutual assessment behaviors (e.g., parallel and antiparallel lateral body orientations) to assess if differences in the body size and the EOD of the two males predicted the incidence of these behaviors.

Results

Resident males increased EOD amplitude when paired with a larger stimulus male

Despite the lack of physical contact with stimulus males, resident males responded to the presence of an intruder through changes in the amplitude of their EOD. The direction and magnitude of the change in EOD amplitude of resident males in the 24h of electrical interaction with a stimulus male depended on the extent of mismatch in their relative sizes (length and weight). Residents increased amplitude when the intruders were larger, and decreased amplitude when the intruders were smaller (Figures 2A and 2B). The percent difference in length or weight between the two males predicted the percent change in the resident male's EOD amplitude during the intrusion (Figure 2). I did not observe a similar effect for EOD τ_{P2}. Neither difference in length nor in weight predicted the change in the resident males' τ_{P2} (Figures 2C and 2D).

In addition, neither the body length nor the weight of the resident males

significantly predicted EOD amplitude or τ_{P2} (Figure 3). Therefore, the relationship between percent size (length and weight) difference and EOD amplitude change was not confounded by an intrinsic relationship between the body size and the EOD amplitude of resident males (Figures 2 and 3).

The body lengths of resident males were strongly related to body weight (Figure 4). I found two different EOD responses depending on whether the male had a positive residual mass (heavier than predicted for length) or negative residual mass (lighter than predicted). Residual mass of heavy resident males was positively associated with day and night EOD amplitude both before and during the intrusion (Figure 4B), and negatively associated with τ_{P2} before and during the intrusion during the day (Figure 4C). Night values of τ_{P2} showed the same trend as day but the p value was marginal (Figure 4C). In contrast, the residual mass of light males predicted neither EOD amplitude nor τ_{P2} (Figures 4D and 4E).

Body size predicted execution of offensive behaviors and dominance in male dyads

In physical encounters between size-mismatched males, larger males consistently dominated the interactions despite the fact that these males did not consistently have higher EOD amplitudes. During the first 30 min of observation within dyads, large males performed all offensive behaviors, such as bites-nudges, chases, nose rubs and head butts, while small males consistently swam away after each confrontation (Figure 5). Of those behaviors expressed during the first 10 min of the session, the first male to perform a bite, a forward chase or a reverse chase proceeded to display all offensive behaviors during the entire 30 min observation period. I classified these males as dominant or winners of the

interaction. Losers or subordinate males were identified as those who first withdrew or swam away from an approaching or chasing male within the first 10 min of the interaction.

To determine the behavioral responses of small males to offensive behaviors by larger dominant males, I evaluated the sequence of behaviors with significant transition probabilities. I identified the following chain of events as predictive patterns of behaviors leading to the establishment of dominance relationships in size-mismatched male dyads. After a large male bit-nudged or chased (forward or reverse) a smaller male, the small male swam away from the large male (Figure 5). In some instances, the big male's bite-nudge was so forceful that the small male's body jerked before swimming away (Figure 5). Forward chases by the larger male were typically associated with an increase in its EOD rate followed by a sharp EOD acceleration after nose-rubbing the smaller fish (Figure 5). In addition, EOD accelerations had a significant probability of occurring in the context of 'a big male's bite followed by small male retreat' sequence (Figure 5). Both a head butt and a nose rub led to the antiparallel lateral body orientation (Figure 5). Optimal electrolocation range is attained when the fish are within few centimeters of each other. In addition, the majority of the electroreceptors are located at the fish's head. Therefore, running the head along the full length of another fish's body at close proximity provides accurate information on that fish's body characteristics. As such, males may obtain more precise information of an opponent's body length and weight by engaging in these behaviors. I also found that forward chases were followed by a rise in EOD rate presumably to increase electrosensory sampling rate and to accurately track the position of the opponent fish (Figure 5). In addition, nose rubs were

always followed by EOD accelerations and were only performed by the big male (Figure 5). The nose rub-EOD accelerations sequence could serve two purposes, as a mechanism to obtained refined information of another fish's position, size, and electric field, and as an aggressive signal of close-range electrical interference.

Relative EOD amplitude between the two males predicted number of bites

While size differences explained many aspects of the interaction between dominant and subordinate fish, EOD differences between the fish also were important in explaining some behavioral responses. Aggressiveness, interpreted as the number of times the large male bit the small male within the first 10 min of the interaction, was reduced when the dominant male's EOD amplitude was smaller than the small male's EOD amplitude (Figure 6). Conversely, aggressiveness increased when the dominant males displayed larger EOD amplitudes when compared to the subordinate fish.

Discussion

EOD amplitude as an honest signal of body condition?

Body size and condition are typically good indicators of fighting ability because they are related to physical strength and availability of energy stores (Beaugrand et al., 1996; Breitburg, 1987; Lindstrom, 1988). Although I found no relationship between the EOD parameters of resident males and their body length and weight (Figure 3), body condition, measured as residual mass adjusted for length, showed more complex effects on the EOD than previously reported for this species (Figure 4) (Salazar and Stoddard, 2008). In males of above average condition, body condition was associated with a larger

EOD amplitude and smaller τ_{p2} (Figures 4B and 4C), whereas males of below average condition showed no relationship between body condition and EOD parameters (Figures 4D and 4E). Thus for males of average to high body condition the signal tracks body condition (Figure 4). As males lose condition, some continue to put out an EOD signal that is not honest (Figure 4D). Therefore, the EOD is not a reliable indicator of body condition, though it may still honestly indicate instantaneous energy expenditure given the energetic expense of signal production (Salazar and Stoddard, 2008).

If males are using EOD amplitude to assess each other's body condition when they are several body lengths away from each other, they may not be receiving reliable information. Such constraints may influence the behavioral interactions displayed by competing males, including the stereotypical displays observed in dyads here (Figures 5 and 6). For instance, males may approach each other to be within their active electrolocation range and in doing so gain more precise information on their relative lengths. Nevertheless, even at close range, they may not be able to determine accurately relative volume differences, a potential indicator of an opponent's body condition. Although weakly electric fish can discriminate objects based on size and volume, their ability to perform this task accurately depends on the shape and conductance properties of the objects (von der Emde and Fetz, 2007). Therefore, a fish may not be able to determine the overall size (length and volume) of another fish accurately using active electrolocation because a fish's body is not composed of a uniform material and has a complex geometry lacking sharp edges (Kelly et al., 2008; von der Emde and Fetz, 2007). Thus, even though EOD amplitude may not be a reliable indicator of body condition, it may be a useful proxy because 1) it is reliable for males with above average condition

who can put up a good fight, and 2) information on an opponent's body condition cannot be obtained consistently via active electrolocation.

EOD responses to body size mismatches between males

While resident male fish were expected to detect and respond to male intruders, size asymmetries had unexpected effects on enhancement of EOD parameters previously associated with body length and condition (Curtis and Stoddard, 2003; Hopkins et al., 1990; Salazar and Stoddard, 2008). During simulated intrusions with no physical interaction, resident males that were smaller than their intruders increased their EOD amplitude, whereas resident males that were larger than their intruders decreased theirs (Figures 2A & 2B). No effects were seen in other components of the signal. These results were opposite to my predictions. I was expecting to see an EOD increase from the resident males that were larger than their intruders, and an EOD decrease from the resident males that were smaller than their intruders. I based these predictions on the assumption that unlike the smaller males, larger males had the energy stores to sustain the energetic cost and the predation risk associated with enhanced EODs (Salazar and Stoddard, 2008; Stoddard, 1999; Stoddard, 2002). Despite this expected response, perceived ownership of a territory can reverse a size asymmetry effect if the value of the territory is high (Turner, 1994). Similar interactions between size asymmetry and perceived value of a resource have been documented in pumpkinseed sunfish *Lepomis gibbosus* (Dugatkin and Biederman, 1991; Dugatkin and Ohlsen, 1990). I speculate therefore that perceived ownership of the tank acts as a motivational driver for resident males.

There is some supporting evidence for this in field studies of *B. gauderio*. During the breeding season, *B. gauderio* males are spatially-distributed in a manner consistent with either an exploded-lek or a nest site polygynandry mating system (Miranda et al., 2008). In addition, Miranda and her colleagues (2008) found that *B. gauderio* males displayed site-fidelity and non-overlapping spatial patterns suggesting that males may defend territories to attract females and procure spawning locations. Given this site fidelity, it is possible that large resident males perceive their size and tank ownership advantage, and adjust the energy allocated to their EODs by decreasing their EOD amplitude, in preparation for energetically-expensive behaviors such as courtship and physical confrontation with other males. In contrast, smaller resident males may perceive their size disadvantage, but have a high motivational drive because of their perceived tank ownership, thus increasing their EOD amplitude. In addition, the mesh dividers provided an artificial condition that allow for small resident males to have a higher motivational drive to enhance their EOD amplitudes in the presence of a large challenger since the threat of a potential physical challenge was absent. Therefore, a male's perceived ownership of a tank seems to provide him with the motivational drive to enhance his EOD amplitude upon the presence of a challenger, regardless of the presence of a size disadvantage.

Empirical evidence has also shown that when hawk-dove models are put to the test, small males do not necessarily act as predicted by the model (i.e., as doves) but instead display a 'Napoleon complex' (Dugatkin and Ohlsen, 1990; Jenssen et al., 2005). As such, small males may adopt a hawk strategy when in the presence of a larger challenger, presumably because they have nothing to lose, and if their bluff is not put to

the test by the large male challenger, they have much to gain (Just and Morris, 2003). Although the melanocortin-activated spike time-shifting mechanism may provide the means for a small male to bluff his EOD amplitude in a particular night, predation risk may prevent the enhanced EOD from becoming pervasively unreliable in the population. Enhancing the EOD can increase predation risk (Stoddard, 2002). Electroreceptive predators readily detect the enhanced EODs of males over the symmetrical EODs of females (Hanika and Kramer, 1999; Hanika and Kramer, 2000; Stoddard, 1999; Stoddard, 2002).

Body size: the honest signal of dominance?

The inability of the small male to withstand a physical challenge by a larger male may ultimately prevent the relationship between body condition and EOD amplitude from being unreliable (Searcy and Nowicki, 2005). In males, body size typically determines the likelihood of winning a physical contest (Andersson, 1994). Likewise, when I placed two males in a tank simultaneously and allowed them to interact physically, the larger male consistently bit and chased the smaller male, causing the smaller male to retreat by swimming away from his attacker (Figure 6). In fact, body size predicted dominance in male *B. gauderio* in physically interacting dyads irrespective of differences in the signal properties between these two fish (Figure 6).

The EOD signals still are important, however. Although I could assess dominance within the first 10 min of physical interactions by identifying which fish performed the first bite or chase, the aggressiveness of the dominant fish depended on whether there was a mismatch between length and EOD amplitude asymmetry (Figure 5).

Accordingly, the large male bit the small male more often when length and EOD asymmetry matched, but bit him less if there was a mismatch (Figure 5). This observation suggests that, despite size advantages, males pay attention to the EOD amplitudes of their rivals and compare this information with the length information obtained via active electrolocation, adjusting his level of aggressiveness accordingly. Despite potential ambiguities between length and EOD amplitude information, however, length differences seem to be what ultimately determines which male dominates a physical interaction.

EOD signals: is different information encoded in EOD amplitude and duration?

EOD power in male *B. gauderio* is energetically-expensive (Salazar and Stoddard, 2008). Males may be able to generate high amplitude EODs at low cost for a short time interval by changing the timing between their electrocytes' action potentials under the regulation of melanocortins (Markham and Stoddard, 2005), however, concordant increases in τ_{P2} will necessarily use extra energy. Curiously, most studies to date on EOD modulation in *B. gauderio* have reported co-modulation of EOD amplitude and τ_{P2}. For instance, social stimulation of isolated males causes both amplitude and duration to increase (Franchina et al., 2001). Serotonin and melanocortins mediate both effects (Allee et al., 2008; Markham et al., 2009; Stoddard et al., 2003). The present study finds males regulating amplitude and τ_{P2} in opposite directions depending on their relative body condition (Figure 4). Although the mechanism underlying this division is not known, androgens have been shown to regulate EOD τ_{P2} but not amplitude, while cortisol

regulates EOD amplitude but not τ_{P2}, suggesting a possible mechanism for independent regulation of the two parameters (Allee et al., 2009; Salazar and Stoddard, 2009).

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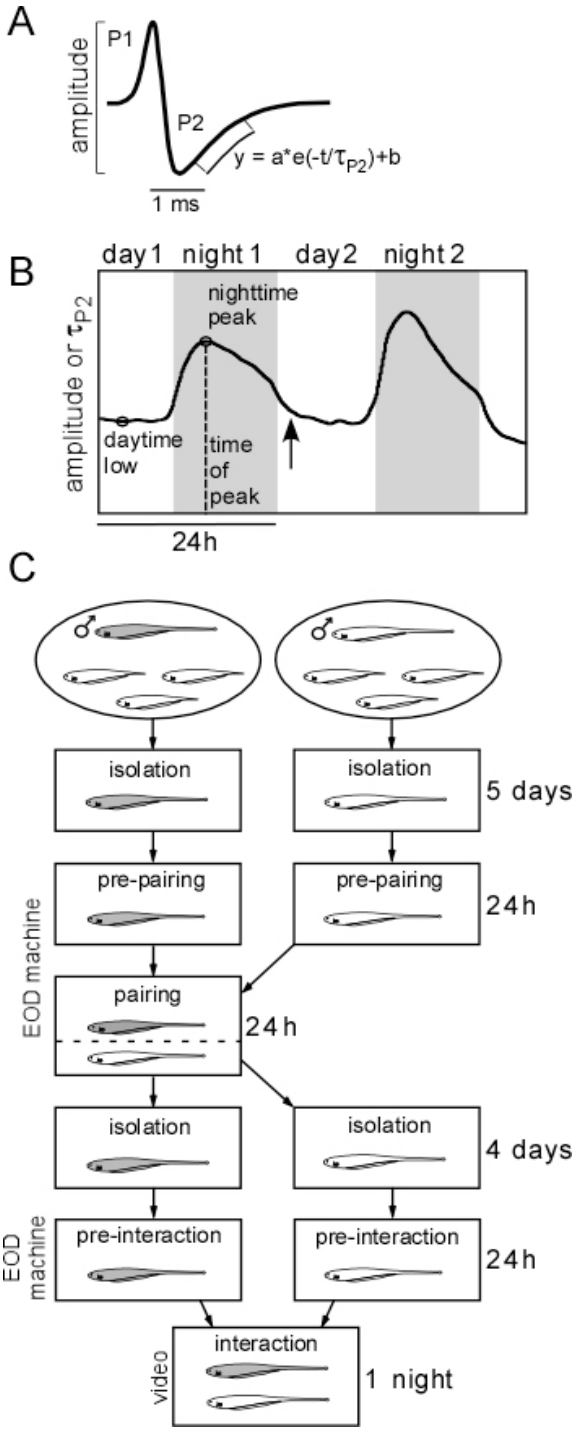

Figure 1. EOD metrics and experimental design using male dyads. A. *B. gauderio*'s biphasic EOD waveform is composed of a positive phase (P1) and a negative phase (P2).

The amplitude of the waveform is measured peak-to-peak for P1 and P2 and τ_{P2} is measured as the slope of the repolarizing segment of P2. B. For both amplitude and τ_{P2}, I sampled a 10-sec train of EODs every min constantly for the 24h before and after a stimulus male was added to the resident male's tank (indicated by arrow). For night-day comparisons of the EOD amplitude and τ_{P2}, I focused on the daytime low, nighttime peak, and the time of the nighttime peak within a 24h cycle. C. Males were randomly sampled from different outdoor breeding pools. The EOD of resident males was recorded 24h before and 24h after pairing. Resident and stimulus males were physically separated by a mesh divider (dashed lines). A subset of resident and stimulus males were subsequently isolated in EOD machine tanks. Their EODs were recorded for the last 24h of isolation. Then, I re-paired them in an observation tank where they could interact physically for one night. I videotaped and recorded EOD modulations for the first 30min of the interaction.

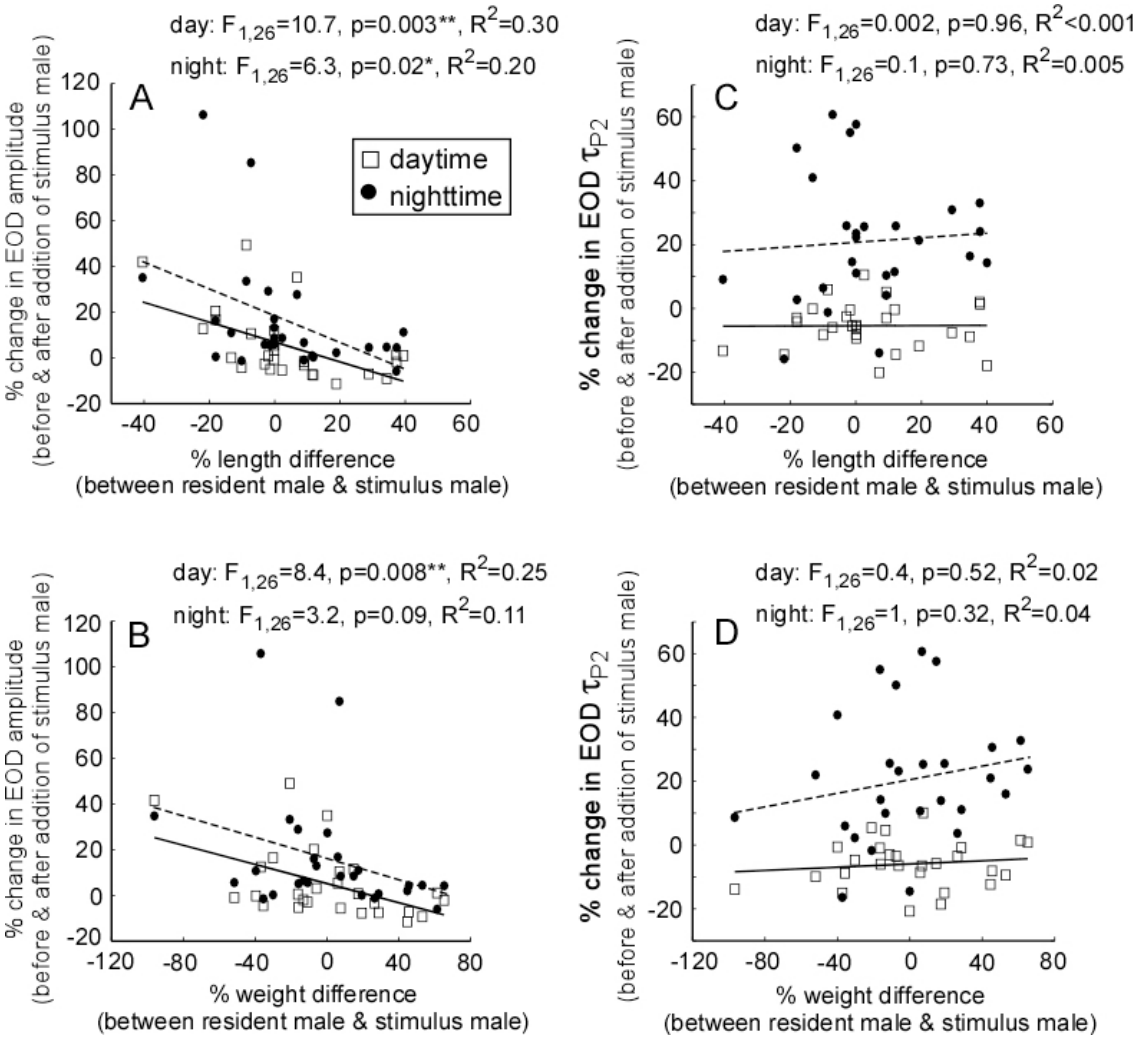

Figure 2. The percent length and weight difference between the resident and stimulus males predicted the percent change (before and after the addition of stimulus male) in EOD amplitude, but not in τ_{P2}. A. Increasing percent difference in length between resident and stimulus males predicted a smaller change in the resident male's EOD amplitude upon addition of the stimulus male, while decreasing percent length difference predicted a bigger change in the resident male's EOD amplitude. B. Although I observed the same trends for the percent difference in weight between resident and stimulus males and the change in the resident male's EOD amplitude, this negative relationship was only

significant for the change in day EOD amplitude, and not for the change in night EOD amplitude. C. The percent difference in length between resident and stimulus males did not significantly predict the change in the resident male's EOD τ_{P2} from before to after the addition of the stimulus male. D. The percent difference in weight between resident and stimulus males also did not significantly predict the change in the resident male's EOD τ_{P2} from before to after the addition of the stimulus male. Open squares and solid lines = daytime values and filled circles and dashed lines = nighttime values.

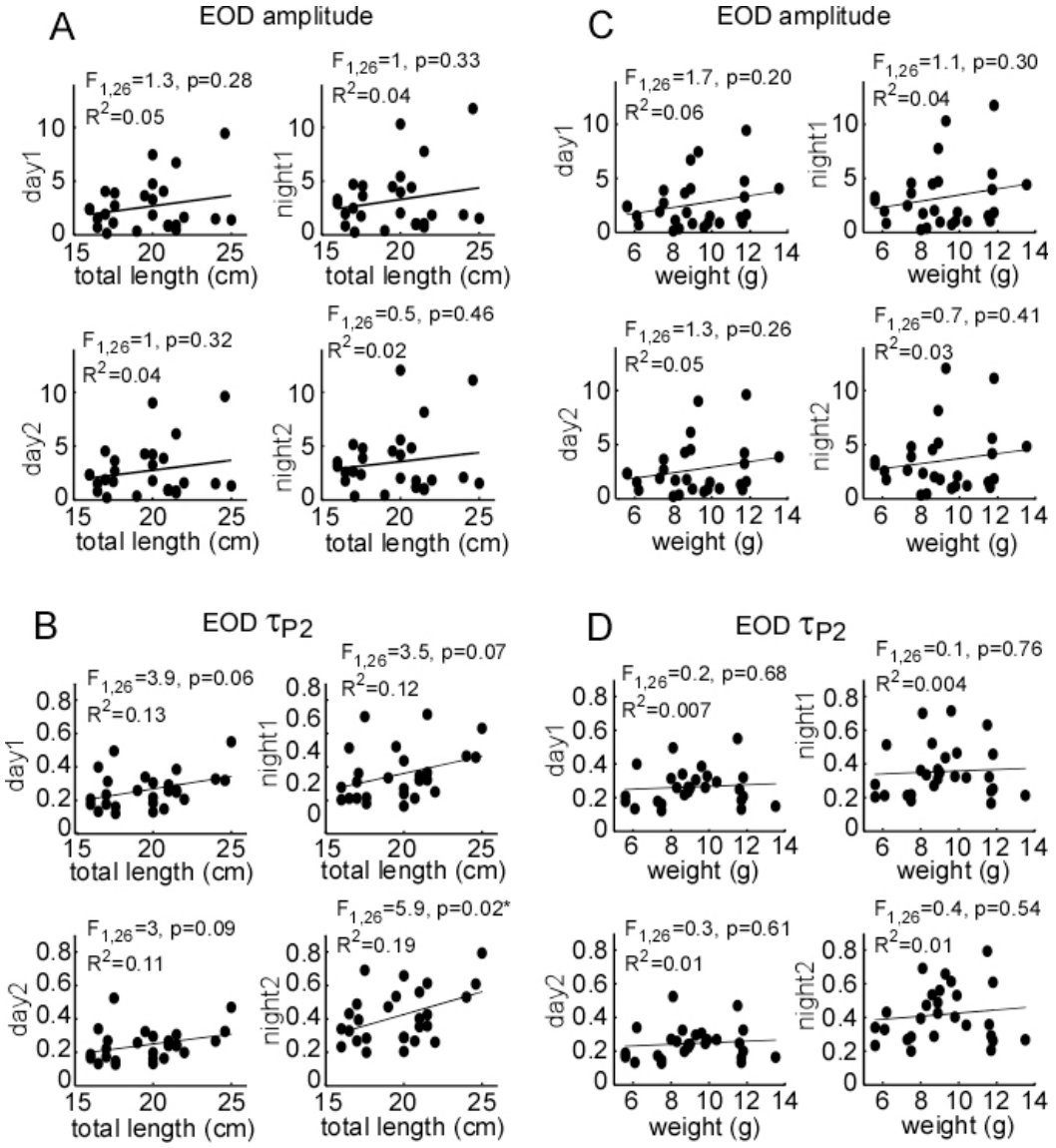

Figure 3. For the most part, the body length and weight of a resident male did not predict EOD amplitude and τ_{P2} during either the 24h before or 24h after a stimulus male was added to his tank. A. Resident males' body length did not predict the magnitude of their EOD amplitude during the day (day1) and night (night1) before or the day (day2) and night (night2) after pairing with stimulus male. B. Although resident males' length did not predict their τ_{P2} on the day and night before pairing and the day after pairing, this

relationship was significant for the night after pairing. C. Resident males' weight did not predict their EOD amplitude on the day and night before or after pairing. D. Weight did not predict EOD τ_{P2} on the day and night before or after pairing with a stimulus male.

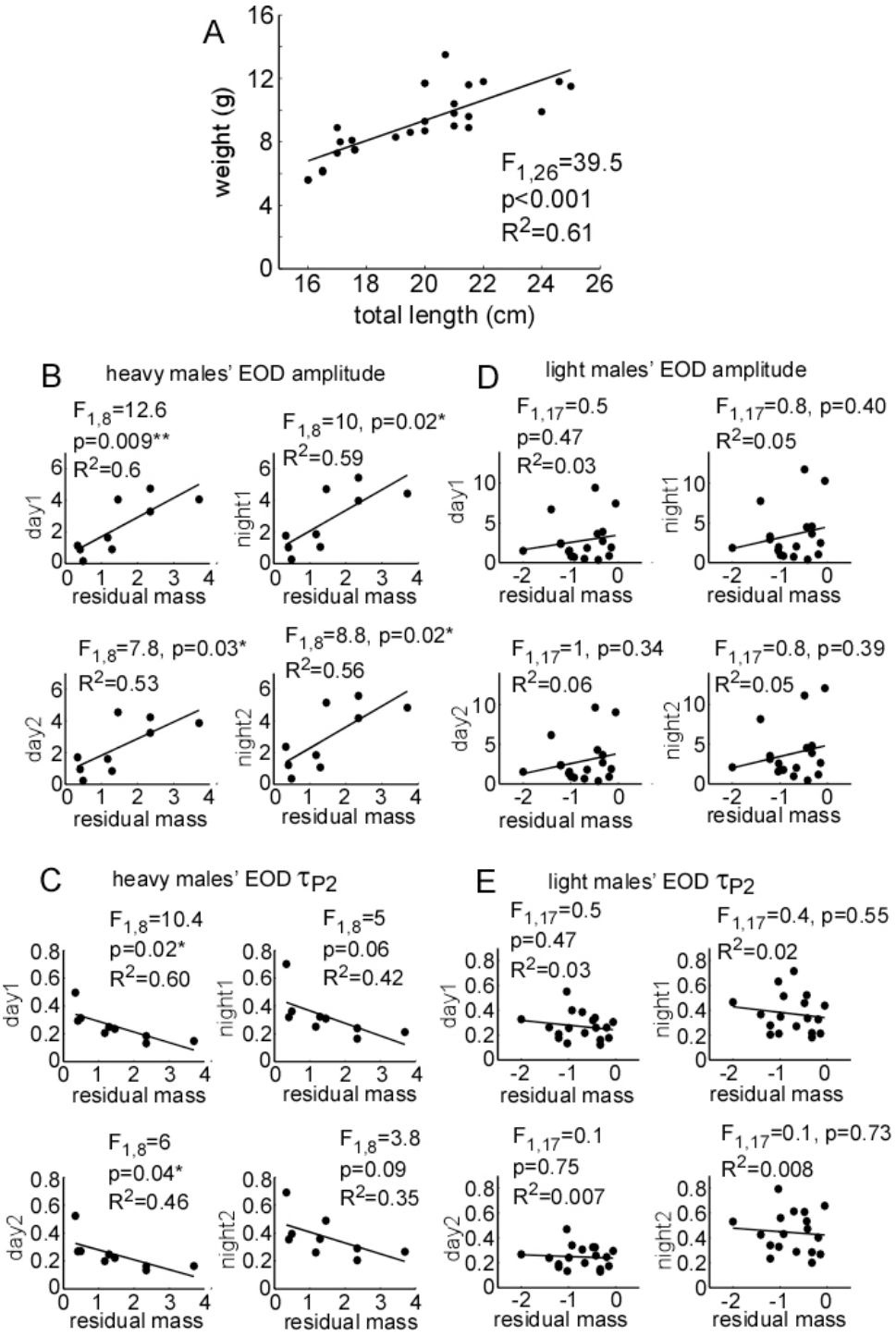

Figure 4. Residual body mass predicts EOD amplitude and τ_{P2} only in heavier males. A. The body length (cm) of a resident male positively predicts its body weight. B. Once

adjusted for length, the residual mass of the resident males that were heavier than predicted for their length (i.e., positive residuals) significantly predicted the magnitude of their EOD amplitudes before and after the stimulus males were added to the resident males' tanks. C. Heavier resident males' residual mass also significantly predicted day τ_{P2}, but not night τ_{P2}, before and after pairing with the stimulus male. D. Once adjusted for length, the residual mass for the resident males that were lighter than predicted for their lengths (i.e., negative residuals) did not predict the magnitude of the EOD amplitude either before or after the addition of the stimulus male. E. Lighter resident males' residual mass did not predict the magnitude of the τ_{P2} either before or after the addition of the stimulus male.

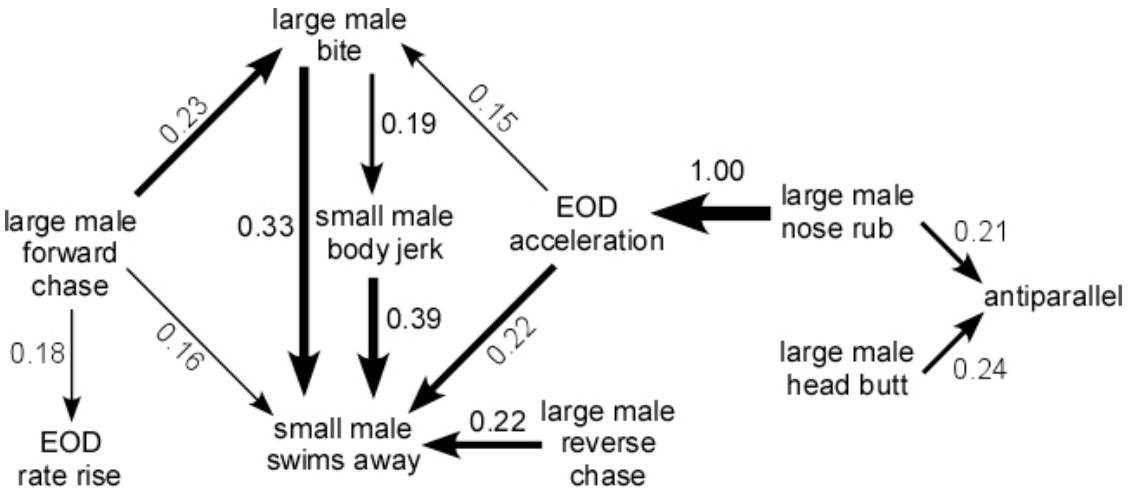

Figure 5. When the two males in a dyad were allowed to interact physically, the large male directed offensive behaviors, such as forward and reverse chases and bites or nudges, toward the small male. The small male responded by swimming away, and in few instances, his body jumped or jerked after a bite attack before he swam away from his aggressor. Large males also performed head butts and nose rubs towards the small males. These presumably offensive behaviors led to mutual inspection via antiparallel lateral orientation, rather than an attack. EOD accelerations were significantly related to the behavioral sequence of a large male biting a small male followed by the retreat of the small male, and EOD rises were significantly performed in relation to forward chases. I only included in this first-order Markov chain transition probabilities for sequences involving behaviors from the large male that significantly led to behaviors from the small male (probabilities bigger than 0.15 with a significance level smaller than 0.05). Descriptions of the behaviors are available in the supplementary materials 1.

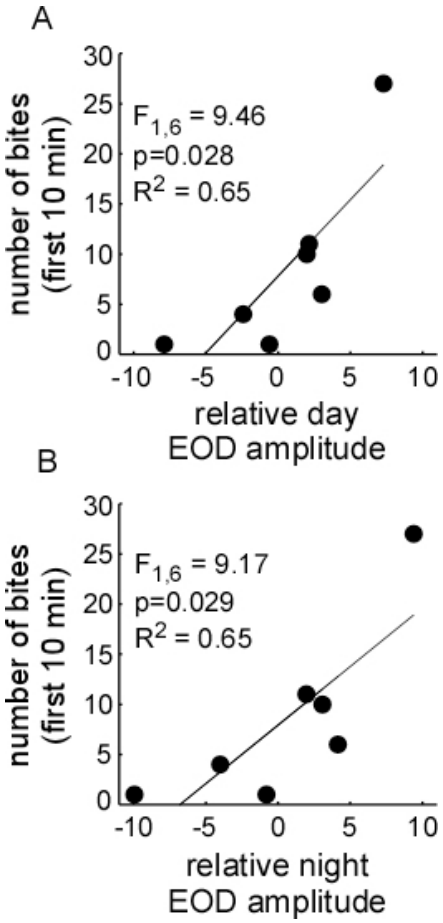

Figure 6. The relative difference in EOD amplitude between the large male and the small male in a dyad predicted the number of times the large male bit the small male within the first 10 min of physical interaction. A. When the day EOD amplitude of the large male in the dyad was smaller than the day EOD amplitude of the small male, the large male bit the small male fewer times during the first 10 min of physical interaction. The opposite pattern was observed when the large male's day EOD amplitude was higher than that of the small male. In this case, the large male bit the small male more during the first 10 min of physical interaction. B. The same trends were observed for the night EOD amplitude.

Supplementary material 1. Ethogram of physical and electrical behaviors observed during *B. gauderio* male-male interactions

Behaviour	Key Code	Description
antiparallel	a	fish oriented facing opposite directions
bite/nudge	b	one fish bites or nudges other fish's torso or tail
EOD chirp	c	Short EOD rate increase with an EOD amplitude decrease
EOD constant rate	d	EOD rates becomes regular
feeding	e	one fish digs its snout into feeding tray
forward chase	f	one fish swims forward at high speed towards other fish
EOD rate rise	g	prolonged and sustained increase in EOD rate
approach	h	one fish swims slowly towards other fish
EOD interruption	i	fish stops emitting EODs for a short period
turn	l	fish turns around and orients itself towards opposite direction
head butt	n	fish connect with each other head-to-head with force
EOD acceleration	o	short and sharp increase in EOD rate
parallel	p	fish oriented facing the same direction
EOD up-down rate change	q	continuous sequence of EOD rate increases and decreases
reverse chase	r	one fish swims in reverse at high speed towards other fish
nose rub	s	one fish runs its snout along other fish from head to tail
circling swim	t	fish swim circling each other
swim by plant	v	one fish swims by plant or within plant roots
swim away	w	one fish quickly swims away from the other fish
tail crossing	x	fish orient or rub their tails forming a cross
body jerk/jump	y	fish's body jerks or jumps after a bite or direct body contact with other fish
fish in plant	z	fish is stationary within plant roots

This ethogram was used as the Focal Master File in JWatcher (v.1.0) for analyses. Behaviors were scored for the large male and small male in each dyad using the following modifiers: "1" = large male and "2" = small male. Behaviors and descriptions are modified from Black-Cleworth, 1970.

CHAPTER IV

"Molecular characterization of serotonin receptor 1A from brains of the gymnotiform fish *Brachyhypopomus gauderio*"

Abstract

Serotonin, a neurotransmitter with a wide range of effects in the central nervous system, plays a major role in regulating aggressive behavior. Although higher serotonin levels are typically related to low levels of aggressive behavior, this is not always the case. The relationship between serotonergic activity and regulation of aggressive behavior depends on the type of serotonin receptor, the quantity and location of the receptors, and the intracellular signaling pathway activated by the receptor. The serotonin receptor 1A, $5HT_{1A}R$, is a key player in the regulation of aggressive behavior. This receptor occurs both as a somatodendritic autoreceptor in serotonergic neurons and as a postsynaptic receptor in serotonin target neurons. Its expression is regulated by glucocorticosteroids and androgens and its function is regulated by posttranslational modifications via phosphorylation. Although the $5HT_{1A}R$ is conserved across all vertebrates, two isoforms of this receptor have been found in at least two species of teleost fish.

The nocturnal gymnotiform fish *Brachyhypopomus gauderio* generates dual-function electric signals for electrolocation and communication. The serotonergic system, steroid hormones, and melanocortins augment the electric signal waveform during social interactions. Pharmacological activation of the $5HT_{1A}R$ reduces the magnitude and duration of the electric waveform in male *B. gauderio*. To further characterize the role of the serotonergic system in the regulation of gymnotiform male electric signals, I analyzed the sequence of one $5HT_{1A}R$ receptor in *B. gauderio* that is homologous to and phylogenetically more-closely related to the b/β isoform found in other teleost fish. I confirmed the presence of previously reported and identified putative novel phosphorylation sites in the $5HT_{1A}R$ of *B. gauderio* and other teleost fish. I also

identified a putative glucocorticoid response element in the promoter region of zebrafish 1Ab gene. Taken together, these findings highlight additional plasticity at $5HT_{1A}R$ in teleost fish.

Keywords: *Brachyhypopomus gauderio*; gymnotiform fish; serotonin; serotonin receptor 1A; phosphorylation

Abbreviations: aa, amino acid; ACTH, adrenocorticotropic hormone; α-MSH, alpha-melanocyte-stimulating hormone; ARE, androgen response element; cdk5, cyclin-dependent kinase 5; CRH, corticotropin-releasing hormone; EDTA, ethylenediaminetetraacetic acid; GC, glucocorticosteroids; GPCR, G-protein coupled receptor; nt, nucleotide; GRE – glucocorticosteroid response element; 5-HT, 5-hydroxytryptamine or serotonin; HPG, hypothalamic-pituitary-gonadal axis; HPA, hypothalamic-pituitary-adrenal axis; HPI, hypothalamic-pituitary-interrenal axis; MAPK, p38 mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; 5HT1A, serotonin receptor 1A; TBE, Tris-Borate-EDTA buffer; TM, transmembrane.

Introduction

Among its many functions in the central nervous system, serotonin (5-hydroxytryptamine or 5-HT), a biogenic monoamine neurotransmitter, plays a major role in the regulation of aggressive behavior in both invertebrate and vertebrate organisms (rev. Nelson, 2006). Although higher serotonin levels are typically related to low levels of aggressive behavior, this is not always the case (de Boer and Koolhaas, 2005; Nelson and Chiavegatto, 2001; Summers, 2001; Veenema et al., 2005). The relationship between serotonergic activity and regulation of aggressive behavior depends on the type of serotonin receptor, the quantity and location of the receptor in brain centers that regulate aggression, and the intracellular signaling pathway activated by the receptor (de Boer and Koolhaas, 2005; rev.Nelson, 2006; Schiller et al., 2006; Schiller et al., 2003; Veenema et al., 2005). All serotonin receptors, with the exception of the type 3, are members of the G-protein coupled receptor (GPCR) superfamily of proteins, characterized by seven transmembrane regions, an extracellular N-terminus, a short intracellular C-terminus, and three intracellular loops (Kroeze and Roth, 1998). The second and third intracellular loops (i2 and i3) play a major role in G-protein coupling, which consequently dictates the specificity of the signaling pathway that is activated or inhibited (Albert and Tiberi, 2001).

The serotonin receptor 1A ($5HT_{1A}R$) is a key player in the regulation of aggressive behavior (rev. Nelson, 2006). Activation of the $5HT_{1A}R$ by 5-HT or specific agonists (e.g., 8-OH-DPAT) inhibits or reduces aggression in many invertebrate and vertebrate species (rev.Nelson, 2006). Furthermore, the $5HT_{1A}R$ is localized in brain regions, such as the raphe nuclei, cortical and limbic areas, that control or regulate

aggressive behaviors (Hensler, 2003). An interesting feature of the $5HT_{1A}R$ is that it functions both as a somatodendritic autoreceptor in serotonergic neurons of the raphe nuclei where it inhibits serotonin release, and as a postsynaptic receptor in corticolimbic brain areas, modulating the response to serotonin of postsynaptic target neurons (Barnes and Sharp, 1999; Hensler, 2003). Furthermore, the brain area in which $5HT_{1A}R$ is expressed seems to dictate what G protein it interacts with, thus affecting the intracellular signaling pathway it regulates, and whether it is desensitized via phosphorylation (Hensler, 2003). Therefore, to understand how $5HT_{1A}R$ regulates aggressive behavior in any organism, it is important to fully characterize the receptor including its potential transcriptional and post-translational regulatory mechanisms.

The effect of serotonin on the regulation of aggressive behaviors seems to be mediated by the activation of the hypothalamic-pituitary-gonadal (HPG) axis and the hypothalamic-pituitary-adrenal or interrenal (HPA, or HPI in teleost fish) axis. Ultimately activation of the HPG leads to the secretion of androgens into circulation, while activation of the HPA/I axis leads to the secretion of melanocortins and glucocorticosteroids (GCs). Closing this regulatory loop, circulating androgens and GCs can alter the expression pattern of serotonin receptors resulting in changes in the neuronal activity of brain areas that regulate aggressive behavior. For instance, in rodents, either gonadectomy or adrenalectomy increases the expression (mRNA levels) of the $5HT_{1A}R$ (Kroeze and Roth, 1998; Zhang et al., 1999), suggesting that androgen and glucocorticoid response elements (ARE and GRE respectively) may be present in the promoter region of the $5HT_{1A}R$. This steroids-serotonergic pathway may explain why activation of the $5HT_{1A}R$ in fish that experience handling-stress (high circulating GC levels) inhibits the

HPI, while activation of this receptor in non-handled fish (low circulating GC levels) has the opposite effect (Hoglund et al., 2002). In addition, the $5HT_{1A}R$ acts directly at the level of the anterior pituitary to regulate the release of the melanocortin adrenocorticotropic hormone (ACTH) (Dinan, 1996). Furthermore, increased serotonergic activity also increases plasma levels of another melanocortin, alpha-melanocyte-stimulating hormone (α-MSH) (Olivereau et al., 1980). Thus, GCs can potentially activate $5HT_{1A}R$ transcription and exert negative feedback at the molecular level on the serotonergic regulation of the HPA/HPI axis. Less is known about the bidirectional regulation between the HPG axis and the serotonergic system.

Serotonin not only regulates overt aggressive physical behaviors but it also regulates production of aggressive communication signals (Albers et al., 2002; Larson and Summers, 2001), including electrocommunication signals (Allee et al., 2008; Maler and Ellis, 1987; Smith and Combs, 2008; Stoddard et al., 2003; Telgkamp et al., 2007). Gymnotiforms, a group of nocturnal fish from Central and South America, continuously generate electric signals to navigate and communicate with conspecifics in the dark. These electric signals, known as electric organ discharges (EODs), are regulated by aggressive interactions, androgens, GCs, melanocortins, and the serotonergic system (Stoddard et al., 2006). This group of fishes provides an excellent opportunity to explore how androgens, GCs and the serotonergic system interact to modify aggressive communication behavior, since electric signals are already in the same currency as the nervous system, action potentials.

The electrocommunication neural network of gymnotiform fishes is extensively innervated by serotonergic neuron terminals (Johnston et al., 1990). In the gymnotiform

fish, *Brachyhypopomus gauderio* (Giora and Malabarba, 2009), males injected peripherally with serotonin enhanced their EOD, mimicking the EOD enhancement displayed by both sexes during aggressive interactions (Allee et al., 2009; Stoddard et al., 2003). These effects were mediated via the serotonin receptors 1A and 2A (Allee et al., 2008). In *B. gauderio* males, activation of $5HT_{1A}R$ reduced the EOD waveform, while activation of $5HT_2R$ enhanced the EOD waveform to levels observed after serotonin treatment (Allee et al., 2008). Opposite to this pattern, in a different gymnotiform fish species, *Apteronotus leptorhynchus,* activation of $5HT_{1A}R$ enhanced, while activation of $5HT_{2A}R$ suppressed aggressive EOD modulations (Smith and Combs 2008).

In *B. gauderio*, serotonin indirectly regulates signal waveform via central 5-HT receptors (Allee et al., 2008; Markham and Stoddard, 2005; Stoddard et al., 2003), whereas the melanocortins α-MSH and ACTH augment the electric waveform directly through action at the peripheral electric organ, which generates the EOD, enhancing the EOD (Markham et al., 2009; Markham and Stoddard, 2005; Stoddard et al., 2006). I have not determined which brain regions regulate the waveform through the activation of the $5HT_{1A}R$, or whether these modulations are regulated by somatodendritic autoreceptors, postsynaptic receptors, or both. In *B. gauderio*, males treated with a $5HT_{1A}R$ specific agonist, 8-OH-DPAT, displayed split responses, some enhanced their EODs and some reduced it (Allee et al., 2008). These findings suggest the presence of $5HT_{1A}R$ both as a somatodendritic autoreceptor and as a postsynaptic receptor. Treatment of males with S-15535, a selective agonist at $5HT_{1A}R$ autoreceptors and an antagonist at postsynaptic $5HT_{1A}R$, enhanced their EOD waveforms, suggesting that the effects of $5HT_{1A}R$ on the EOD are mediated postsynaptically (P. Stoddard & A. Silva,

unpublished data). Here, I characterize the $5HT_{1A}R$ in the brains of male and female *B. gauderio* using a molecular approach. I amplified, cloned and sequenced the $5HT_{1A}R$ from mRNA isolated from the brains of female and male *B. gauderio*. I compared *B. gauderio*'s $5HT_{1A}R$ to homologous sequences from other teleosts available in GenBank, and identified putative phosphorylation sites and a GRE in the promoter region of zebrafish's $5HT_{1A}R$, to further characterize potential regulatory mechanisms for this receptor in teleost fish. My study represents the first stage in a series of studies examining the bidirectional regulation between androgens, GCs, and the $5HT_{1A}R$ in the context of aggressive behaviors.

Materials and methods

Animals

I sampled sexually mature males and females of the pulse-type weakly electric fish *B. gauderio* from a captive-reared, 13th generation breeding colony, located at Florida International University, Miami, Florida. I housed the fish in 450-liter (185 x 95 x 26 cm) outdoor pools with water conductivity at 90±10 μS cm^{-1} and mean ambient temperature at 27±2 °C. The water surface of each pool was covered 80-100% with water hyacinths (*Eichhornia crassipes*). Each breeding pool contained 10-20 fish. I fed all fish live oligochaete blackworms (Gulfstream Tropical Aquarium, Dania, FL, USA) three times per week.

Brain extraction, RNA isolation and cDNA synthesis

I netted fish from their housing pool, deeply anesthetized them with a 10 μl

injection of 10% pentobarbital, and perfused them intracardially with ice-cold Hickman's Ringer solution, pH 6.8-7.0, containing 1 mg/ml of EDTA to reduce blood clotting, 1 mg/ml of lidocaine to sustain analgesia, and 0.1 mg/ml of flaxedil (gallamine triethiodide) to minimize involuntary muscle contractions. I surgically removed the brains and placed them in RNA*later*®. All surgical materials were treated with RNase AWAY®. Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless noted otherwise.

I isolated total RNA from homogenized brain tissue using the TRIzol® reagent (Invitrogen Co., Carlsbad, CA, USA), followed by chloroform extraction (1µl/5µl of TRIzol) and purification using the RNeasy mini kit (Qiagen Inc., Valencia, CA, USA) as per manufacturers' instructions. Following RNA isolation, samples were treated with RQ1 RNase-free DNase (Promega Co., Madison, WI, USA) to further minimize DNA contamination. I synthesized the first-strand cDNA from the isolated RNA using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions.

Design of degenerate primers and product PCR amplification

I obtained both the nucleotide (nt) sequences and the deduced amino acid (aa) sequences from mouse, rat, human, gorilla, chimpanzee, silver fox, dog, horse, African clawed frog, Mozambique tilapia, fugu (japanese puffer fish), and goldfish from the NCBI database (http://www.ncbi.nlm.nih.gov) (Table 1), and aligned the nt and aa sequences as two separate groups using the multiple sequence alignment tool ClustalX (Higgins et al., 2003). From the aa alignment, I selected consensus regions from

conserved functional domains, TM segments I through VII (Barnes and Sharp, 1999), and seven functional motifs (PRINTS, protein motif fingerprint database, http://www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/, PR00512, 5HT1ARECEPTR). I located these regions in my nt alignment and chose regions for primer design that minimized the degeneracy of my primers while maintaining conserved functional relevance specific to the $5HT_{1A}R$. I avoided regions that were conserved for all the serotonin receptors because of concerns over amplifying other serotonin receptor genes (Table 2).

I used the first-strand cDNA and several combinations of the forward and reverse degenerate primers to amplify via polymerase chain reaction (PCR) products corresponding to the partial sequence of the $5HT_{1A}R$. The cycling conditions were 3 min incubation at 94°C, followed by 35 cycles of 45 s denaturing at 94°C, 30 s annealing at 58°C and 1.5 min extension at 72°C. I incubated the samples for another 10 min at 72°C and immediately stored them at 4°C.

The PCR products were visualized in a 1% agarose gel prepared with SYBR® Safe DNA gel stain (Invitrogen) in 0.5X TBE buffer. Because I obtained multiple fragments from the PCR reaction (Figure 1), I gel-extracted each fragment. The DNA from each gel fragment was isolated with 3 gel volumes of Buffer QG (solubilization and binding buffer, Qiagen) followed by 1 gel volume of isopropyl alcohol, and purified using the QIAquick PCR purification kit (Qiagen).

Cloning and sequencing

I cloned purified PCR products using the TOPO TA Cloning kit (Invitrogen), and

verified successful transformation by colony-PCR using M13 forward and reverse primers. I extracted plasmids from successfully transformed bacterial colonies using the QIAprep spin miniprep kit (Qiagen). Cloned inserts were sequenced in the DNA Core Facility at Florida International University (Department of Biological Sciences) using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with BigDye-Terminators and M13 primers. I assembled and edited DNA sequences using ABI AutoAssembler to generate a consensus sequence. I translated the nt consensus sequence to aa using the ExPasy Translate Tool (Swiss Institute of Bioinformatics; http://us.expasy.org/tools/dna.html). I searched the deduced aa sequence against the NCBI GenBank database using blastx and scanned with ExPasy's PROSITE (http://www.expasy.ch/prosite/) to confirm $5HT_{1A}R$ identity. Three additional piscine $5HT_{1A}R$ sequences were published in GenBank after I began this study, allowing me to align *B. gauderio* $5HT_{1A}R$ partial sequence with $5HT_{1A}R$ deduced aa sequences for the six teleost fishes (Table 1): zebrafish, goldfish, barramundi, mozambique tilapia, fugu (japanese puffer fish), and gulf toadfish. Amino acid alignments were done using ClustalW (Higgins et al., 2003). I annotated the alignment with functional regions identified from human and fugu $5HT_{1A}R$ genes (Yamaguchi and Brenner 1997) and from UniProtKB (Universal Protein Resource Knowledgebase, http://www.uniprot.org, Query=P08908, 5HT1A_HUMAN). Since Yamaguchi and Brenner (1997) identified phosphorylation sites in their fugu $5HT_{1A}R$ sequences that were not present in the human $5HT_{1A}R$ sequence, I performed an *in silico* identification of all amino acid residues in the *B. gauderio* $5HT_{1A}R$ partial sequence that showed phosphorylation probability scores greater than 0.50 (Blom et al., 1999) using the NetPhos 2.0 server

(http://www.cds.dtu.dk/services/NetPhos/). To further characterize these phosphorylation sites, I identified their putative target kinases (sites with scores > 0.75) by entering all the teleost sequences from my alignment into the NetPhosK 1.0 server (http://www.cbs.dtu.dk/services/NetPhosK/) (Blom et al., 2004).

Phylogenetic tree analysis

Using MacClade (Maddison and Maddison, 1992), I did multiple alignments on deduced aa sequences of $5HT_{1A}R$ for all fish listed in the previous section along with homologous sequences for human, rhesus macaque, gorilla, chimpanzee, bornean orangutan, european rabbit, long-tailed ground squirrel, horse, silver fox, domestic dog, house mouse, brown rat, desert grassland whiptail lizard, little striped whiptail lizard, and african clawed frog (Table 1). This alignment was imported into PAUP (Swofford et al., 1996) for phylogenetic analysis using the neighbor-joining method (Saitou and Nei, 1987). I rooted the tree at midpoint and calculated the strength of each branch via bootstrapping with 1000 replicates (Felsenstein, 1985). Only bootstrap values of 50% or more are presented.

***In silico* identification of putative GRE and ARE sequences**

To characterize the regulatory mechanism underlying $5HT_{1A}R$ expression in teleost fish, I identified putative GRE and ARE regions upstream the $5HT_{1A}R$ gene in the zebrafish genome. Using the toolkit RSAtools (Regulatory sequence analysis tools; http://rsat.ulb.ac.be/rsat/), I retrieved the upstream sequences for the two zebrafish $5HT_{1A}R$ genes (gene ID: htr1a, corresponding to $5HT_{1A}Ra$ located in chromosome 8

(DNA contig: BX571721, location: 32,697,755 to 32,699,540) and gene ID: htr1ab, corresponding to 5HT1Ab located in chromosome 21 (DNA contig: BX927332, location: 22,237,194 to 22,238,348). For comparison, I imported the consensus frequency matrices generated for GRE and ARE of human, mouse, and rat sequences (Nelson et al., 1999) and searched upstream from -2000 to -1, using pattern-matching (patser) matrices, for the presence of GRE and ARE in the promoter region of the $5HT_{1A}R$ in zebrafish (van Helden et al., 1998).

Results

Cloning of 5HT1AR

I amplified two fragments close to the expected $5HT_{1A}R$ amplicon size of 645 bp for one of my degenerate primers (Table 2 and Figure 1A). I successfully gel-extracted the two fragments (Figure 1B), cloned, and sequenced them. Although the deduced aa sequence of the $5HT_{1A}R$-like band 1 amplicon does not display any similarity to a specific gene, my band 2 amplicon displayed 82% identity with zebrafish1Ab (*E* value $1e^{-93}$), 77% identity with barramundi 1A (*E* value $2e^{-84}$), 75% identity with Mozambique tilapia 1A (*E* value $9e^{-83}$), 72% identity with fugu 1Aβ (*E* value $6e^{-80}$), and 70% identity with goldfish 1A (*E* value $5e^{-35}$).

Putative phosphorylation sites

The partial sequence of *B. gauderio* $5HT_{1A}R$ extends downstream from TM III to a little upstream from TM VI. In *B. gauderio* $5HT_{1A}R$, the Ser_{68} and Thr_{69} binding sites in TM V are conserved (for reference, these sites correspond to Ser_{208} and Thr_{209} in fugu

1Aα and Ser_{198} and Thr_{199} in human 1A). In fact, these sites are conserved for all teleost fish with sequence data for this region of the protein (Figure 2). Within *B. gauderio* $5HT_{1A}R$'s partial sequence, there are a conserved disulfide bond site at Cys_{56} (for reference, Cys_{196} in fugu 1α) and twelve putative phosphorylation sites with high probability (score > 0.53: Thr_{13}, Ser_{45}, Tyr_{65}, Thr_{98}, Thr_{102}, Thr_{127}, Ser_{130}, Ser_{131}, Ser_{152}, Thr_{171} and Ser_{186}) (Table 3 & Figure 2).

From these twelve phosphorylation sites, four (i.e., Thr_{13}, Thr_{98}, Thr_{102} and Thr_{127}) correspond to previously identified phosphorylation sites in the fugu's $5HT_{1A}R$ genes (Figure 2) (Yamaguchi and Brenner, 1997). The partial sequence for *B. gauderio* $5HT_{1A}R$ does not include the last phosphorylation site found in the fugu (Thr_{344} for fugu 1Aα and Thr_{337} for fugu 1Aβ, Figure 2). Of the four phosphorylation sites shared by *B. gauderio* and fugu $5HT_{1A}R$ sequences, one corresponds to a different aa residue. Instead of a serine found at position 264 in fugu 1Aβ, *B. gauderio* has a threonine (Thr_{102}) and the fugu 1Aα lacks a phosphorylation site at this position in the alignment (Table 3 & Figure 2). Although Yamaguchi and Brenner (1997) identified phosphorylation sites at Thr_{332} in fugu 1Aα and Thr_{325} in fugu 1Aβ, these phosphorylation sites are not supported by NetPhos. Instead, NetPhos identifies possible phosphorylation sites at Thr_{330} in fugu 1Aα and Thr_{323} in fugu 1Aβ (Figure 2). This threonine is conserved across all teleost 1A sequences with the exception of the gulf toadfish 1A and *B. gauderio* 1A, which have serine residues instead. Interestingly, these serine residues, but not the threonine residues, are predicted to be PKC phosphorylation target sites by NetPhos (Table 3).

Using an *in silico* analysis to identify putative target kinases for phosphorylation sites, I predicted with a high probability (score > 0.75) that the Thr_{13}, Thr_{98}, Thr_{102}, Thr_{127}

and Ser_{186} sites in the *B. gauderio* 1A are targeted by protein kinase C (PKC), while the Ser_{131} site is targeted by protein kinase A (PKA) and the Thr_{171} site is targeted by cyclin-dependent kinase 5 (cdk5) (Table 3). In the *B. gauderio* 1A only Thr_{98} remains consistent across teleost fish as a PKC target site (scores > 0.75). Although NetPhosK predicts that PKC targets Thr_{13} in *B. gauderio* 1A and the corresponding Thr_{148} in zebrafish 1Ab, all other teleost 1A sequences had p38 mitogen-activated protein kinase (MAPK) as the predicted kinase targeting their corresponding threonine (Table 3). Targeting of PKC at Thr_{102} in *B. gauderio* 1A is also supported for the corresponding threonine in fugu 1Aβ, barramundi 1A and tilapia 1A (Table 3 and Figure 2). Targeting of PKA at Ser_{131} in *B. gauderio* 1A is likewise supported for the corresponding serine in fugu 1Aβ, barramundi 1A and zebrafish 1Aa (Table 3 and Figure 2). Finally, targeting of cdk5 at Thr_{171} in *B. gauderio* 1A is supported for the corresponding threonine in barramundi 1A, tilapia 1A and zebrafish 1Ab (Table 3 and Figure 2).

Sequence homology and transcriptional regulation

My partial sequence for *B. gauderio* $5HT_{1A}R$ is more closely related to the zebrafish 1Ab, barramundi 1A, tilapia 1A, and fugu 1Aβ; all branches are highly supported, with bootstrap values ranging from 95-100% (Figure 3). The zebrafish 1Aa sequence is more closely related to the goldfish 1A (bootstrap value 93%, Figure 3). Although the cladogram clusters the gulf toadfish $5HT_{1A}R$ and the fugu 1Aα, the bootstrap value is too low to support this clade (Figure 3). As expected, all the mammalian 1A sequences cluster together as a sister clade to the lizards 1A clade, and both clades descend from the amphibian 1A clade (Figure 4).

I found one putative GRE site located -115 to -89 upstream (score = 7.76, ln(P) = -10, Figure 4) from the coding region for zebrafish $5HT_{1Ab}R$ in the D strand of zebrafish chromosome 21. I found no matches for ARE sites within the 2.0 kb upstream sequence region of either zebrafish $5HT_{1A}R$.

Discussion

Yagamuchi and Brenner (1997) found two $5HT_{1A}R$ genes, α and β, in fugu, suggesting that a duplication event for the $5HT_{1A}R$ took place at least within the superclass Ostheichthyes. Recently, two $5HT_{1A}R$ sequences, a and b, were also identified in zebrafish (Norton et al., 2008). These two $5HT_{1A}R$ genes are found in two different chromosomes in the zebrafish genome, 1Aa in chromosome 8 and 1Ab in chromosome 21. Although it is possible that all teleosts have two $5HT_{1A}R$ genes, my degenerate primers pulled out only one $5HT_{1A}R$ gene from *B. gauderio*. The partial sequence of *B. gauderio* $5HT_{1A}R$ has the highest aa identity and is phylogenetically most closely related to zebrafish 1Ab. The fact that *B. gauderio* 1A, zebrafish 1Ab, barramundi 1A, tilapia 1A, and fugu 1Aβ form a robust clade (bootstrap value = 100%) at the exclusion of zebrafish 1Aa and fugu 1Aα, suggests that most of the identified teleost $5HT_{1A}R$ genes are structurally and functionally like the b/β gene. As suggested by Yamaguchi and Brenner (1997), the two teleost $5HT_{1A}R$ homologs might have different functions. In mammals, the $5HT_{1A}R$ is localized as a somatodendritic autoreceptor in serotonergic neurons of the raphe nuclei, and as a post-synaptic receptor in neurons receiving serotonergic input in cortical and limbic areas (Hensler 2003). Their diverging pharmacological and regulatory characteristics suggest that the autoreceptor and

postsynaptic receptor may represent distinct subtypes of the $5HT_{1A}R$ (Clarke et al., 1996; Newman-Tancredi et al., 2009). In zebrafish, although both the 1Aa and 1Ab receptors co-localized in several brain regions, 1Ab, but not 1Aa, was present as an autoreceptor in the caudal zone of the periventricular hypothalamus (Hc), and as both autoreceptor and postsynaptic receptor in the ventral nucleus of the ventral telencephalic area (Vv) and the ventral zone of the periventricular hypothalamus (Hv) (Norton et al., 2008). The anatomical segregation of 1Aa and 1Ab distribution in the zebrafish brain could reflect functional segregation for $5HT_{1A}R$ in teleost brains.

In addition to anatomical and functional divergence of the the autoreceptor and postsynaptic receptor classes, the $5HT_{1A}R$ is coupled to different G proteins depending on where in the brain it is expressed (Albert and Tiberi, 2001; Hensler, 2003). This regional specificity confers additional plasticity to the $5HT_{1A}R$ because the type of G protein determines the intracellular signaling pathway activated or inhibited by the ligand. In addition, the receptor is susceptible to desensitization via phosphorylation by various kinases (Nebigil et al., 1995; Raymond, 1991; Raymond and Olsen, 1994). Several phosphorylation sites at the receptor's i2 and i3 have been shown to interfere with $5HT_{1A}R$ coupling to specific G proteins (Albert and Tiberi, 2001). Protein kinase C inhibits $5HT_{1A}R$ activation of phospholipase C (PLC), but has no effect on the receptor's inhibition of adenylyl cyclase, whereas PKA enhances the effects of PKC phosphorylation (Albert and Tiberi, 2001). I found PKC target residues in the i2 and i3 and a PKA target residue in the i3 of *B. gauderio* $5HT_{1A}R$. Furthermore, one of the PKC sites, Thr_{127}, is only found in *B. gauderio* 5HT1A. Therefore, *B. gauderio* $5HT_{1A}R$ may

display more plasticity on its regulatory mechanisms than observed from mammalian $5HT_{1A}R$.

Human $5HT_{1A}R$ has two putative calmodulin (CaM) binding sites on the i3 (Turner et al., 2004) a 1-8-14 motif located at aa 185-199 in the i3C region and a 1-12 motif located at aa 84-107 in the i3N region, also present in *B. gauderio*. In humans, the CaM binding sites overlap various PKC target sites (Turner et al., 2004). In *B. gauderio*, putative CaM binding sites likewise overlap putative PKC target sites located at Thr_{98}, Thr_{102}, Thr_{127} and Ser_{186} in the i3 region (Table 3 and Figure 2). CaM binding antagonizes PKC phosphorylation at the receptor's i3, and is an essential step for receptor internalization and activation of the extracellular-signal regulated kinase (ERK)/MAPK pathway (Turner et al., 2004). Therefore, CaM binding prevents PKC-driven receptor desensitization and commits the receptor to the endocytic-ERK/MAPK activation pathway (Turner et al., 2004). PKC phosphorylation prevents CaM binding and receptor endocytosis (Turner et al., 2004) and inhibits $5HT_{1A}R$ activation of phospholipase C (PLC), but does not prevent $5HT_{1A}R$ inhibition of adenylyl cyclase (Lembo and Albert, 1995). Presumably, *B. gauderio*'s $5HT_{1A}R$ is also subjected to the antagonistic regulatory effects of CaM and PKC.

Activation of the $5HT_{1A}R$ in the hypothalamus activates release of the melanocortin ACTH (Serres et al., 2000). In *B. gauderio*, the enhanced EODs observed during social interactions can be replicated by activation of the central serotonergic system and by the direct application of melanocortins to the electric organ (Markham et al., 2009; Markham and Stoddard, 2005). In addition, during intense social competition conditions, *B. gauderio* males display higher levels of circulating GCs (Salazar and

Stoddard, 2009). Increasing levels of GCs decrease $5HT_{1A}R$ mRNA (Kroeze and Roth, 1998), via the repressive action at GRE sites in the promoter region of $5HT_{1A}R$ (Ou et al., 2001; Wissink et al., 2000). I found a putative GRE site on the promoter region of zebrafish 1Ab gene in chromosome 21 but not in the promoter region of the zebrafish 1Aa gene in chromosome 8. As discussed earlier, the zebrafish 1Ab in the hypothalamus displayed two patterns, it localized as an autoreceptor in the Hc and as a heteroreceptor in the Hv (Norton et al., 2008). *B. gauderio*'s $5HT_{1A}R$ is more closely related and has the highest identity to zebrafish 1Ab. Therefore, it is possible for *B. gauderio* $5HT_{1A}R$'s transcription to be regulated by GCs. Thus GCs could exert negative feedback on $5HT_{1A}R$ expression by downregulating transcription, thereby suppressing aggression through action at $5HT_{1A}$ autoreceptors (de Boer et al., 2000), and reducing electric signal magnitude through action at $5HT_{1A}$ post-synaptic receptors (Allee et al., 2008). I currently do not know whether $5HT_{1A}R$ is found as an autoreceptor and postsynaptic receptor in the hypothalamus of *B. gauderio*. Pharmacological study of electric signal control in *B. gauderio* indicated that the EOD waveform is under tonic negative control by a postsynaptic 1A receptor (Allee et al., 2008). I did not find ARE in the promoter region of $5HT_{1A}R$. Nevertheless, in *B. gauderio* females, androgen implants enhanced the effect of 5-HT on their EODs (Allee et al., 2009). Therefore, androgens' regulation of the EOD could take place at downstream targets.

In several mammals, the location of $5HT_{1A}R$ mRNA matches the location of its binding sites (Barnes and Sharp, 1999). A study is in progress to localize $5HT_{1A}R$ receptor transcripts in brains of *B. gauderio* following behavioral interactions to identify

the relationship between serotonin receptor expression, social dominance, androgen and GC levels.

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Table 1. List of sequences from the NCBI database used for primer design, multiple alignment, and phylogenetic analysis

Common name	Scientific name		nt AC	aa AC
house mouse	*Mus musculus*		NM_008308	NP_032334
brown rat	*Rattus norvegicus*		NM_012585	NP_036717
human	*Homo sapiens*		NM_000524	NP_000515
gorilla	*Gorilla gorilla*		AB041405	BAA94490
chimpanzee	*Pan troglodytes*		AB041404	BAA94489
silver fox	*Vulpes vulpes*		Y204569	AAP12466
domestic dog	*Canis familiaris*		AY134454	AAN08044
horse	*Equus caballus*		AB264325	BAF32952
african clawed frog	*Xenopus laevis*		NM_001085830	NP_001079299
mozambique tilapia	*Oreochromis mossambicus*		AY219038	AAP83427
japanese pufferfish (or fugu)	*Fugu rubripes*	1Aα	X95936	CAA65175
		1Aβ	X95937	CAA65176
goldfish	*Carassius auratus*		EF493019	ABP88964
zebrafish	*Danio rerio*	1Aa		NP_001116793
		1Ab		NP_001139238
barramundi	*Lates calcarifer*			ABV66072
gulf toadfish	*Opsanus beta*			ACF39936
rhesus macaque	*Macaca mulatta*			AAL96692
bornean orangutan	*Pongo pygmaeus*			BAA94491
european rabbit	*Oryctolagus cuniculus*			AAF76184
long-tailed ground squirrel	*Spermophilus undulatus*			ABG81775
desert grassland whiptail lizard	*Aspidoscelis uniparens*			ACE88682
little striped whiptail lizard	*Aspidoscelis inornata*			ABY65236

nt = nucleotide, aa = amino acid, AC = accession number

Table 2. Degenerate primers

Gene	Sequence (5'-3')	Functional region[1]
5ht1a	Forward	
	AGYTAYCAARRTBRTYACMTC	motif 2
	TACTGGGCCATMACDGASCC[2]	between TM III and IV
	Reverse	
	GTTTTRCGYTCKCKKGCCA[2]	after motif 7
	CATGATGATGCCSAGVGTYTTCAC	after motif 7

[1] Consisting on the seven TM (transmembrane) segments and seven motifs characterized in PRINTS (PR00512 for 5ht1a)

[2] Degenerate primers pair that yielded a 5ht1a-like PCR product

Table 3. Putative phosphorylation sites identified by NetPhosK in the partial sequence for *B. gauderio*'s $5HT_{1A}R$.

B. gauderio	T_{13}^{PKC}	S_{45}	Y_{64}	T_{65}	T_{98}^{PKC}	T_{102}^{PKC}	T_{127}^{PKC}	S_{130}	S_{131}^{PKA}	S_{152}	T_{171}^{cdk5}	S_{186}^{PKC}
Fugu α	$T_{158}^{p38MAPK}$	NP	Y_{204}	T_{205}	T_{238}^{PKC}	T_{242}^{PKC}	E_{271}	R_{274}	G_{275}	S_{297}	T_{316}^{cdk5}	T_{330}^{PKC}
Fugu β	$T_{146}^{p38MAPK}$	S_{178}	Y_{197}	T_{198}	T_{231}^{PKC}	T_{235}^{PKC}	S_{264}	R_{267}	S_{268}^{PKA}	S_{289}	T_{308}	T_{323}^{PKC}
Goldfish	NS	NP	Y_{46}	T_{47}	T_{80}^{PKC}	T_{84}^{PKC}	NS	NS	NS	NS	NS	NS
Seabass	$T_{75}^{p38MAPK}$	S_{107}	Y_{126}	T_{127}	T_{160}^{PKC}	T_{164}^{PKC}	S_{193}	R_{196}	S_{197}^{PKA}	S_{218}	T_{237}^{cdk5}	T_{252}^{PKC}
Tilapia	$T_{66}^{p38MAPK}$	S_{98}	Y_{117}	T_{118}	T_{151}^{PKC}	T_{155}^{PKC}	S_{184}	R_{187}	S_{188}	S_{209}	T_{228}^{cdk5}	T_{243}^{PKC}
Toadfish	$T_{54}^{p38MAPK}$	NP	Y_{100}	T_{101}	T_{134}^{PKC}	T_{138}	G_{164}	R_{167}	R_{168}	S_{190}	T_{209}	S_{225}^{PKC}
Zebrafish-a	$T_{141}^{p38MAPK}$	NP	Y_{187}	T_{188}	T_{221}^{PKC}	T_{225}^{PKC}	T_{253}	R_{256}	S_{257}^{PKA}	S_{274}	N_{293}	T_{306}^{PKC}
Zebrafish-b	T_{148}^{PKC}	NP	Y_{189}	T_{190}	T_{223}^{PKC}	P_{227}	N_{252}	S_{255}	A_{256}	S_{277}	T_{296}^{cdk5}	T_{311}^{PKC}

NP = not present in alignment, alignment gap; NS = no sequence available for this region; grey squares = aa does not correspond to a phosphorylation site; squares with diagonal line = scores < 0.4. Only putative kinases with scores > 0.75 are shown.

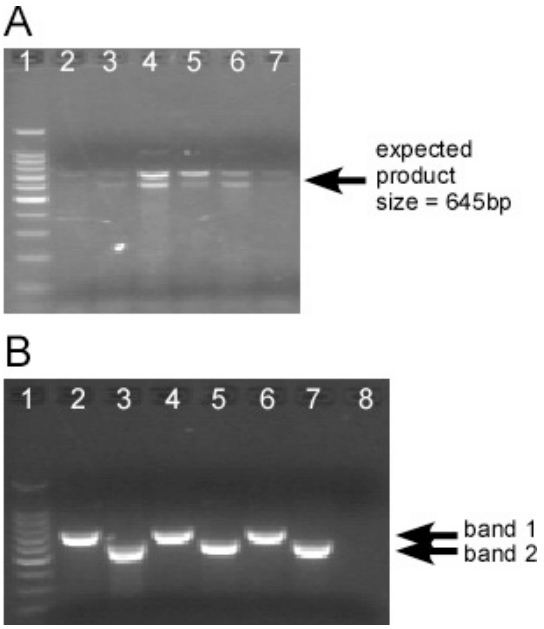

Figure 1. Putative $5HT_{1A}$R-like PCR products from *B. gauderio*. Double bands represent two potential homologous receptors. **A**. PCR products were visualized in 1% agarose gel. Lanes: 1. 100bp ladder, 2. Female-1 brain cDNA, 3. Female-1 brain cDNA from DNase-treated RNA, 4. Female-2 brain cDNA, 5. Female-2 brain cDNA from DNase-treated RNA, 6. Male-1 brain cDNA, 7. Male-1 brain cDNA from DNase-treated RNA.Table 2. See table 2 for information on degenerate primers used for the amplification of target genes. **B**. Gel-extracted bands 1 and 2. Lanes: 1. 100bp ladder, 2. Female-2 brain cDNA band 1, 3. Female-2 brain cDNA band 2, 4. Female-2 brain cDNA band 1 from DNase-treated RNA, 5. Female-2 brain cDNA band 2 from DNase-treated RNA, 6. Male-1 brain cDNA band 1, 7. Male-1 brain cDNA band 2, 8. (-) PCR control.

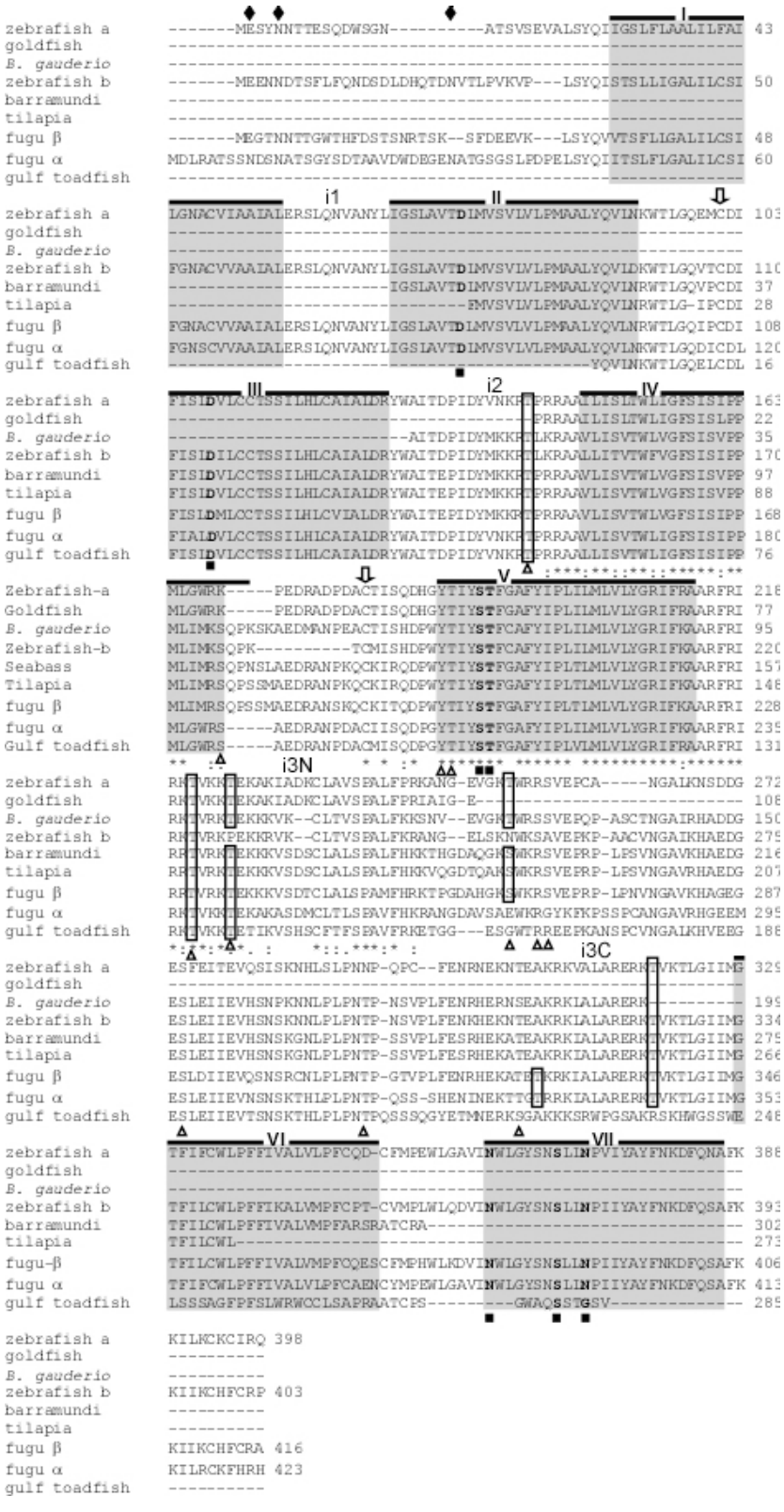
I
i1
II
III
i2
IV
V
i3N
i3C
VI
VII

Figure 2. Unlike most vertebrates, the teleost fish zebrafish and fugu have two $5HT_{1A}R$ isoforms. Yet, in the majority of teleost fish, including *B. gauderio*, sequenced for $5HT_{1A}R$, only the b/β isoform has been isolated. Shown is the alignment of $5HT_{1A}R$ deduced aa sequences for *B. gauderio*, zebrafish, goldfish, barramundi, mozambique tilapia, japanese puffer fish, and gulf toadfish (Table 1) using ClustalW. Grey shaded areas = 7TMs, black diamonds = glycosylation sites, arrows = disulfide bond sites, black squares = important residues for the receptor's binding specificity, open rectangles = phosphorylation sites identified in the fugu, open triangles = identified phosphorylation sites in *B. gauderio*.

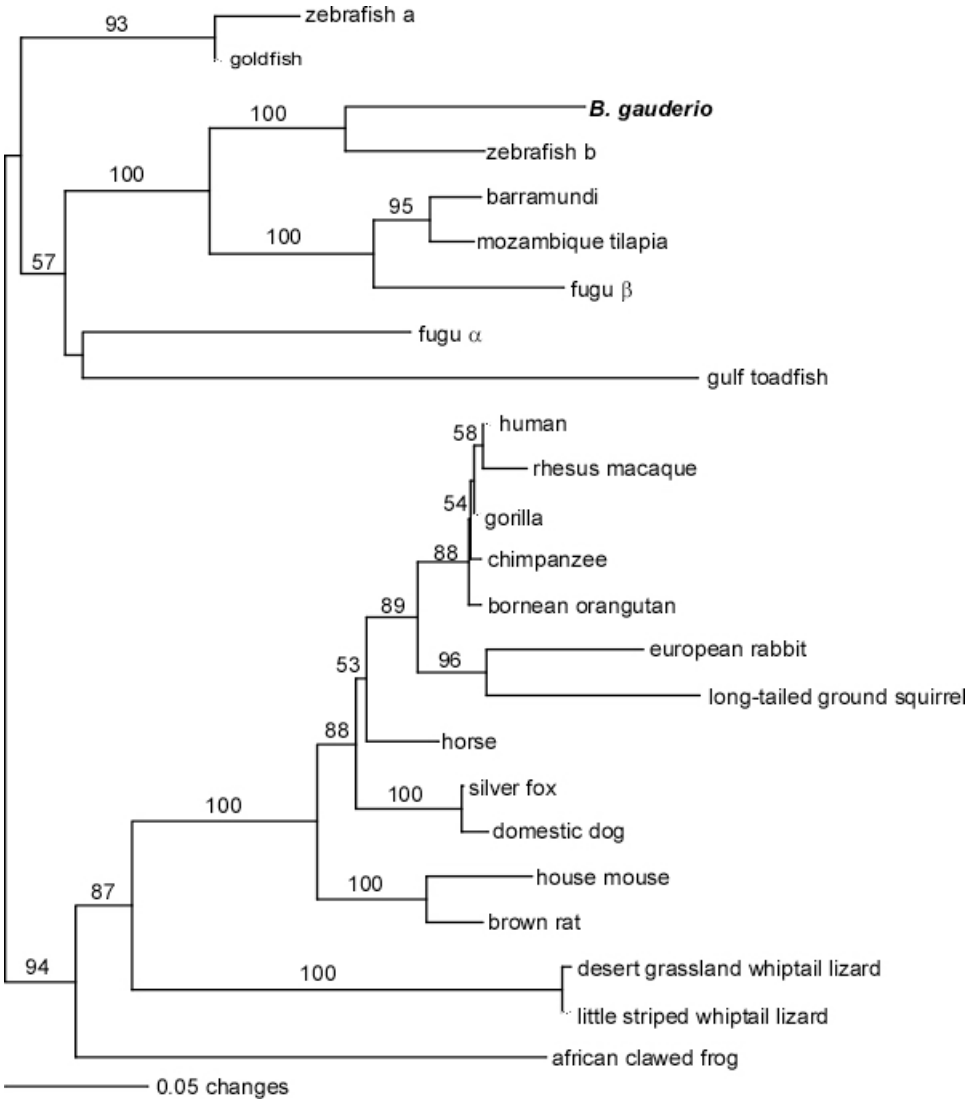

Figure 3. *B. gauderio* $5HT_{1A}R$ forms a highly-supported clade with zebrafish 1Ab, barramundi 1A, mozambique tilapia 1A, and fugu 1Aβ. Shown is a phylogenetic analysis using the Neighbor Joining method of piscine $5HT_{1A}Rs$, using the same aa sequences (Table 1) from the alignment in Figure 2. Only bootstrap values higher than 50% are reported.

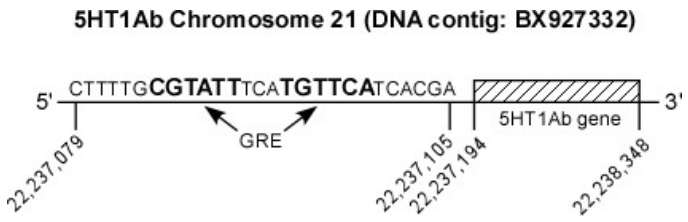

Figure 4. Glucocorticosteroids can regulate $5HT_{1A}R$ expression in teleost fish. Shown is a putative GRE upstream of $5HT_{1A}R$ sequence in zebrafish's chromosome 21. Nucleotides in bold text are regions for zinc finger binding of dimerized GR.

Chapter V

Conclusions

Ontogeny of my dissertation research: the functional and mechanistic underpinnings of electrocommunication signals

Before I started my dissertation research, it was known that the EOD waveforms of *B. gauderio* were tremendously plastic. During the breeding season, *B. gauderio*'s EOD waveforms become sexually-dimorphic, with males having longer and larger EODs. In both sexes, the EOD also changes from day to night following a circadian rhythm (Franchina & Stoddard 1998; Stoddard et al. 2007). These circadian modulations are also sexually-dimorphic, with male EODs undergoing larger circadian changes (Stoddard et al. 2007). Investigating this level of plasticity has been the basis of many studies, particularly understanding the adaptive significance of these changes and the physiological mechanisms underlying the ability to rapidly modulate the EOD.

Franchina *et al.* (2001) also discovered that the EOD waveform of *B. gauderio* could be affected by social stimulation or social deprivation. Specifically, isolated males displayed smaller EOD amplitudes and durations and their EOD circadian change was suppressed when compared to the EODs of males sampled directly from the breeding pools. The introduction of a social companion into the tank of an isolated male restored the reduced EOD waveform to normal levels, and male companions typically had a stronger and faster restorative effect (Franchina et al. 2001). These results were the first to suggest that the sexual dimorphic EOD waveform and the enhanced EOD circadian rhythm of male *B. gauderio* play a role in male-male social interactions. Alternative explanations of these results are also possible, however. An isolated male could be enhancing its EOD waveform more when presented with another male simply because

the male EOD is larger than a female and thus a stronger stimulus, or to make his EOD more conspicuous than that of a competing male to attract potential mates.

My dissertation research has attempted to advance our knowledge on the functional role of the EOD plasticity observed in male *B. gauderio* and the physiological mechanisms that regulate the enhanced male EOD. To evaluate whether social competition drives the EOD changes observed after an isolated male was paired with a social male, in Chapter II, I manipulated the number of males in a breeding group that also contained females and juveniles to create conditions that exemplified low (fewer males in a group) and high (more males in a group) levels of competition. Not only were these conditions more natural than simply pairing two males in a tank, the design of this study allowed me to show that EOD changes observed after an isolated male was paired with a social companion were part of a continuum of social responsiveness. Isolated males enhanced their EODs once they were moved into social groups with low competition, but enhanced their EODs even more if placed in social groups with high competition. As such, Chapter II of my dissertation research has revealed that social competition drives some of the plasticity observed in the electrocommunication signals of male *B. gauderio*, specifically the enhancement of the EOD amplitude.

In this study, I measured circulating plasma levels of androgens and cortisol in male and female *B. gauderio* for the first time, and demonstrated that changes in the EOD of males due to changes in their social environment were paralleled by changes in the levels of androgens and cortisol. This study complements other studies in weakly electric fish showing that steroid hormones can act directly act the electrocytes, thus changing the EOD waveform (Bass & Volman 1987; Bass & Zakon 2005). While I was conducting

this dissertation research, additional studies from Dr. Stoddard's research group demonstrated that androgens masculinize the EODs of *B. gauderio* females (Allee et al. 2009), and that activation of the hypothalamic-pituitary-interrenal (HPI) axis at various levels mimicked the EOD enhancement that males displayed when paired with another male (Markham et al. 2009). Of particular relevance, melanocortins were found to act directly on the electrocytes to mediate the enhanced male EOD (Markham & Stoddard 2005). Although the connection between the EOD enhancement and androgens is now much clearer, considerable work needs to be done to determine the connection between cortisol and EOD changes observed in males. In addition, the results from my study also revealed that EOD changes observed during these social manipulations were sensitive to a male's past social experience. This is a fascinating finding that begs for further investigation. It will be interesting to determine if this result is consistent under other social conditions and to investigate the adaptive significance of social history in this species.

In Chapter III, I evaluated the role of the male's EOD enhancement from the perspective of its information content. If indeed, as my previous study showed, the enhanced male EOD was responsive to changes in the number of male competitors, I hypothesized that information about a male's attributes as a competitor could be encoded in the EOD waveform parameters that change as a function of the changing social environment. Body size seemed the obvious candidate since is the best predictor of strength and fighting ability. Males might minimize expensive physical confrontation if they could use the size of another male's EOD to decide whether or not to challenge him or engage in direct physical aggression.

The results of this chapter demonstrated that while body size is the best determinant of dominance in male *B. gauderio*, EOD amplitude can only reliably predict body condition, a composite of length and weight, for fish in good body condition. As such, EOD amplitude can act as an honest signal of a male's resource holding potential (RHP) under some circumstances. I also examined what behaviors and sequences of behaviors predicted dominance, as defined by the ability of a fish to dominate physical interactions by exclusively performing offensive behaviors. My results showed that larger males always won contests with smaller males. When I placed two males in a tank simultaneously and allowed them to interact physically, the larger male consistently bit and chased the smaller male, causing the smaller male to retreat by swimming away from his attacker. In addition, I determined that the dominant fish could be identified within the first 10 minutes of the interaction as the first male to perform a bite, a forward chase, or a reverse chase. This information is valuable for examining the relationship between social dominance and levels of steroid hormones and the activity of the serotonergic system. For instance, future studies could use this experimental approach to design studies to test for differences in circulating levels of androgens and cortisol between dominant and subordinate *B. gauderio* males or to test for the effects of inhibiting the activity of these steroid hormones and serotonin on dominance status (i.e., display of offensive behaviors within the first 10 min of male-male interactions).

One of the limitations of my current experimental system, when investigating the relationship between social dominance and the electrocommunication system of *B.gauderio*, is my inability to record the EOD waveform changes of both the resident and intruder males simultaneously. Overcoming the methodological hurdles of measuring

two fish simultaneously while also knowing which signal comes from which fish, is not trivial. To determine whether being a resident or an intruder had an effect on the EOD changes observed after two males of different body sizes are paired, I conducted a small experiment where I recorded the EOD waveforms of six pairs of males 24h before and 24h after they were paired, with no physical contact, in separate EOD machine tanks. Then, I re-paired them and once again recorded their EOD waveforms before and after the re-pairing, but I reversed their prior roles as resident and intruder (Figure 1). I was particularly interested to see if perceived ownership of a territory (the recording tank) affected day-night changes in EOD parameters. I used the nonparametric Wilcoxon signed-rank test to compare the percent differences in the EOD between individual residents and their corresponding intruders and within individuals as they changed roles from being residents to being intruders. Interestingly, resident males tended, albeit non-significantly, to either increase their EOD amplitude or keep it unchanged and decrease their EOD τ_{P2}, while intruder males followed the opposite trend, thus decreasing their EOD amplitude and increasing their EOD τ_{P2} (Figure 1A). I also compared individual males as they went from being residents in their tank to being intruders in the other male's tank. Although I found no significant differences between the resident role versus the intruder role across day and night EOD amplitude and night EOD τ_{P2} (Figure 1B), I did find a significant difference between the resident role and the intruder role in their relative change in day τ_{P2} (Figure 1B). As residents, males tended to increase EOD amplitude and decrease τ_{P2}, but as intruders they reversed this pattern, decreasing amplitude and increasing τ_{P2} (Figure 1B). Although these findings suggest that residency affects changes in male *B. gauderio*'s EOD waveforms due to social and territorial

asymmetries in male-male interaction, the sample size was very small and the trends are not statistically significant. Future studies should evaluate this relationship in more detail with a larger sample size.

In *B. gauderio* males, the enhanced EODs observed during social interactions can be replicated by activation of the central serotonergic system and by the direct application of melanocortins to the electric organ (Allee et al. 2008; Markham et al. 2009; Markham & Stoddard 2005; Stoddard et al. 2003). Specifically, activation of the serotonin receptor 1A ($5HT_{1A}R$) reduces the EOD waveform, while activation of the serotonin receptor 2A ($5HT_{2A}R$) enhances the EOD waveform to levels observed after male-interaction or serotonin treatment (Allee et al. 2008). These findings suggest that the $5HT_{1A}R$ and $5HT_{2A}R$ are present in the brain of *B. gauderio* and that these receptors play a role in the regulation of the enhanced EOD displayed by males during male-male interactions. To further characterize the mechanisms underlying the relationship between male-male interactions and EOD plasticity, I undertook the molecular characterization of the serotonin receptors that had been pharmacologically linked to the regulation of the EOD waveform. In chapter IV, I successfully identified the expression of the $5HT_{1A}R$, a key player in the regulation of aggressive behavior, in the brains of *B. gauderio*. The sequence of *B. gauderio*'s $5HT_{1A}R$ is homologous to the b/β isoform found in other teleost fish. I also identified putative target regulatory regions in the $5HT_{1A}R$ of *B. gauderio* and other teleost fish, highlighting the presence of additional plasticity at $5HT_{1A}R$ in teleost fish.

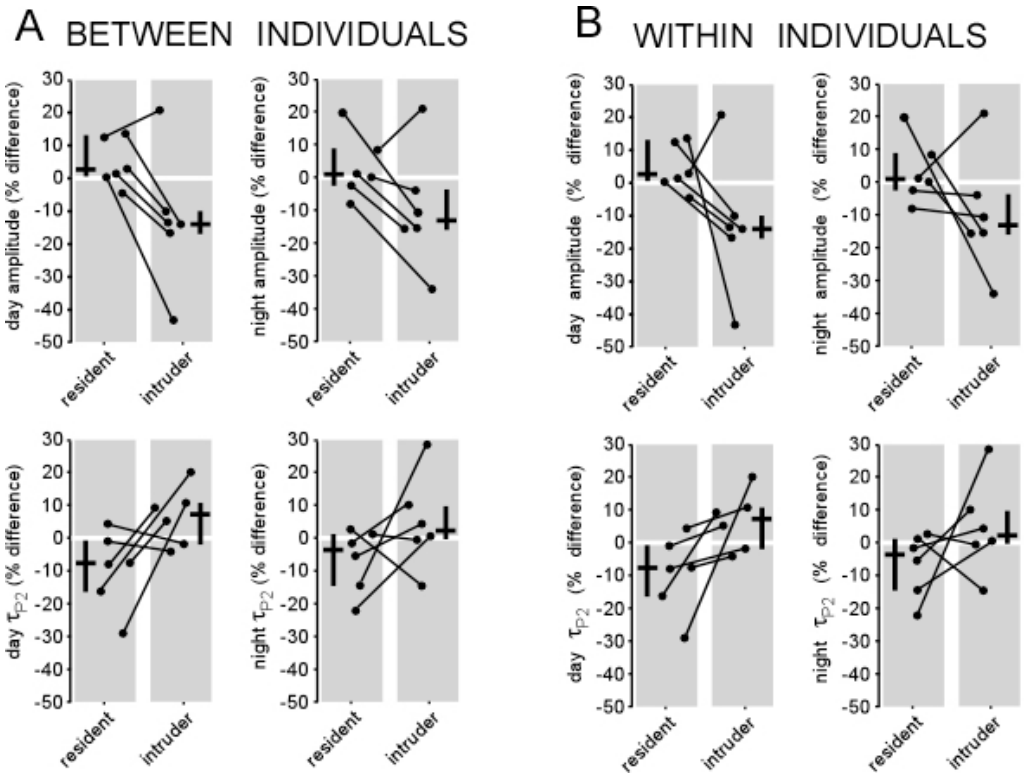

Figure 1. EOD changes varied with role as resident or intruder. **A.** Resident males usually increased EOD amplitude and decreased τ_{P2} while intruder males generally did the opposite, decreasing amplitude and increasing τ_{P2} (Wilcoxon signed-ranks test: day amplitude z=-1.99, *P*=0.06, night amplitude z=-1.57, *P*=0.16, day τ_{P2} z=-1.78, *P*=0.09, night τ_{P2} z=-1.55, *P*=0.31). **B.** A comparison of individual males as they changed roles from being residents in their own tanks to being intruders in the other males' tanks. Overall, resident males tended to increase their amplitude and decrease their τ_{P2}, but did just the reverse when they became intruders (Wilcoxon signed-ranks test: day amplitude z=-1.36, *P*=0.22, night amplitude z=-1.36, *P*=0.22, day τ_{P2} z=-2.20, *P*=0.03, night τ_{P2} z=-0.94, *P*=0.44). Resident-intruder pairs are connected with a solid line. Outside of the cluster of data points, we depict the medians (horizontal lines) and the 25th and 75th percentiles (low and high boundaries of the vertical lines).

Proposed model for the regulation of male EOD plasticity

Here I propose a model for the regulation of the male EOD plasticity observed in the context of social interactions of *B.gauderio* that I base on my experimental results and other related studies. The model is based on a modified version of the Energetics-Hormone Vocalization (EHV) model first proposed by Emerson (2001). Under the EHV model, an extension of the Challenge Hypothesis (Wingfield et al. 1990), the energetic costs of communication are included to highlight the connection between mating success, body condition and steroid hormone regulation (Emerson 2001). In insects, frogs, birds and fish, males invest a tremendous amount of their metabolic energy in the production of signals to attract females (Bucher et al. 1982; Eberhardt 1994; Hoback & Wagner 1997; Kavanagh 1987; Prestwich et al. 1989; Prestwich & Walker 1981; Taigen & Wells 1985). In male frogs, for instance, the EHV model predicts changes in corticosterone and androgen levels observed during the transition from calling to noncalling. Increasing levels of androgens in noncalling males presumably facilitate vocalization. These calling males are also predicted to display an increase in corticosterone levels to match the increase in vocalization output. Presumably, this increase in corticosterone levels releases energy stores that can allow these calling males to sustain the energetic demands associated with this behavior. Accordingly, this surge in glucocorticoids has a negative effect on circulating levels of androgens leading to their reduction and the associated decline in vocalization, which switches the males back to a noncalling state.

With the EHV model in mind, I propose that the serotonergic system might act as the neuronal connection between androgens and glucocorticoids and their dual-role in regulating communication signals. The Energetics-Steroids-Serotonergic Signaling

(ESSS) model draws from previous studies showing that the EOD of male *B. gauderio* is energetically expensive and a condition-dependent trait (Salazar & Stoddard 2008) and that the serotonergic system is involved in the regulation of the EOD via the opposing actions of a $5HT_{1A}$-like receptor (which reduces the EOD) and a $5HT_{2A}$-like receptor (which enhances the EOD) (Allee et al. 2008). These findings together with the results from Chapter II suggest that if the three manipulations that I applied (isolation, Social 2 and Social 6) are seen as a continuum (from social suppression to maximum social stimulation), I can predict that an isolated male will decrease its EOD circadian modulation and androgen levels since the enhanced social EOD is energetically expensive to generate and there is no reproductive gain (Figure 2). Once an isolated male transitions to a social environment with plenty of mating opportunities, androgen levels and the EOD circadian modulation should increase accordingly.

Cortisol and EOD amplitude covary positively as the number of competing males increases, suggesting that cortisol may be a modulator of the EOD amplitude. Cortisol may support the enhanced EOD by increasing availability of glucose and lipids to support energetically costly signaling and swimming behaviors associated with territoriality and courtship (Landys et al. 2006; Sapolsky et al. 2000). As previously mentioned, activating the HPI axis enhances the EOD amplitude and τ_{P2} in *B. gauderio* males (Markham et al. 2009; Markham & Stoddard 2005). Although cortisol could directly drive the enhancement of the EOD amplitude, the melanocortins adrenocorticotropic hormone (ACTH) and/or alpha-melanocyte-stimulating hormone (α-MSH) can increase both the EOD waveform and cortisol levels independently within the same period of time.

Therefore, the relationship that I observed between cortisol and EOD amplitude could be mediated by the effect of social experience on melanocortins.

This model also predicts that expression of the $5HT_{2A}$-like receptors should be suppressed during social isolation while the expression of $5HT_{1A}$-like receptors should be enhanced. Increasing androgen levels increases the expression of $5HT_2$ receptors (Birger et al. 2003; Fink et al. 1999; Sumner & Fink 1998; Zhang et al. 1999) and increasing glucocorticosteroid levels decreases the expression of $5HT_{1A}$ receptors but has no effect on the expression of $5HT_2$ receptors (Chalmers et al. 1993; Kuroda et al. 1994). Therefore, expression of the $5HT_2$-like receptors should increase under social competition conditions, while the expression of post-synaptic $5HT_{1A}$-like receptors should decrease (Figure 2). Finally, when these males transition to a high competition environment with relative fewer available mates, androgen levels remain the same but cortisol levels increase to support the energetic demands associated with a further enhancement of the EOD circadian modulation. Accordingly, the expression of the $5HT_2$-like receptors should further increase while the expression of the $5HT_{1A}$-like autoreceptors should decrease (Figure 2).

In this model, I suggest that androgen levels need to increase to a breeding baseline and remain there for cortisol to have an effect on EOD amplitude. Therefore, both an increase in androgen and cortisol levels is necessary to enhance the EOD amplitude. Future studies will characterize the expression pattern of the serotonin receptors $5HT_{1A}R$ and $5HT_2R$ in the brains of male and female *B. gauderio* as they relate to plasma steroid levels and social status. I have already started a study evaluating the distribution of the $5HT_{1A}R$ in the brains of male and female *B. gauderio*. I have

generated a working riboprobe using the $5HT_{1A}R$ partial sequence that I cloned (Chapter IV). I also measured the circulating levels of androgens and cortisol and videotaped staged dyadic agonistic interactions of all the males in this study. This project has three objectives: 1) to generate a brain atlas for this gymnotiform fish species, 2) to describe sex-specific serotonin receptors distribution, and 3) to determine the relationship between levels of steroid hormones, social status and serotonin receptor distribution. My preliminary results show positive binding for my $5HT_{1A}R$ riboprobe, and so far, I have identified expression of $5HT_{1A}R$ in few brain regions involved in the regulation of aggressive behaviors, such as amygdala-like nuclei (i.e., central and ventral subdivisions of the ventral telencephalon) and the dorsal raphe nucleus.

My dissertation research sets the stage for many interesting and challenging future research questions to further our understanding on the function and the regulation of electrocommunication signal plasticity. For instance, the proposed ESSS model suggests a synergy between androgens and cortisol on the regulation of the EOD amplitude during changes in social competition. In addition, EOD amplitude and τ_{P2} can change independently from each other, yet the mechanisms underlying the regulation of one parameter versus the other are not known. From a technical perspective, the development of a two-fish EOD Machine recording system and signal playbacks will provide the tools to further investigate the function of the EOD amplitude and τ_{P2} during male-male interactions as well as during male-female courtship. It is my goal that my contribution to this field will inspire many other graduate students to complete this puzzle.

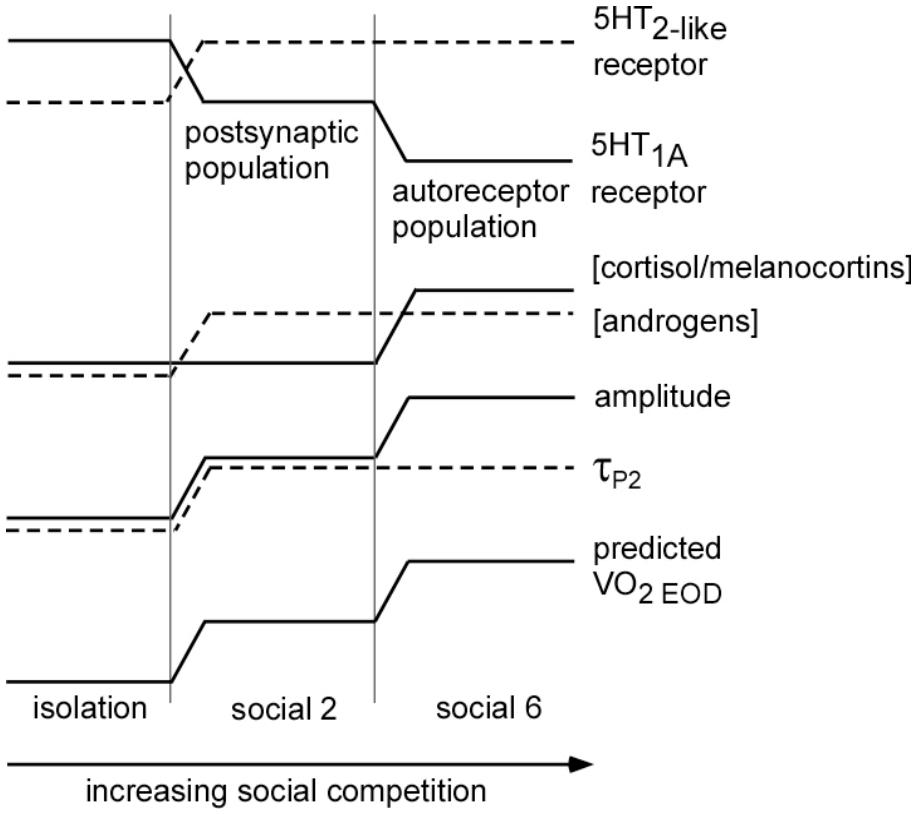

Figure 2. The Energetics-Steroids-Serotonergic Signaling (ESSS) model based on the Challenge Hypothesis (Wingfield et al. 1990) and modified from the Energetics-Hormone Vocalization (EHV) model (Emerson 2001; Leary et al. 2004). The ESSS model superimposes results from previous studies on the energetic costs of the EOD and the EOD regulatory function of the serotonergic system onto the data from my dissertation research. I propose that the opposing actions of two types of serotonin receptors explained the directionality of the modulation on the EOD by steroid hormones as a function of the organism's body condition. $VO_{2\ EOD}$ stands for the oxygen consumption associated with the generation of the EOD.

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