SIGNALING AND SENSORY ADAPTATIONS IN WEAKLY ELECTRIC FISH

A Dissertation

by

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ABSTRACT

In recent decades, neuroscience research has become largely dependent on a few "model organisms" (e.g. Drosophila melanogaster, Mus musculus). A holistic understanding of nervous systems requires study of a diversity of species and should consider the ecological context under which behaviors and their neural underpinnings evolved. My dissertation used a multidisciplinary and comparative phylogenetic approach to study signaling and sensory traits in a clade of weakly electric fishes, providing a more robust foundation for a burgeoning model in neuroscience. Gymnotiformes is a speciose and ecologically diverse order of Neotropical fish. These mostly nocturnal fishes generate electric organ discharges (EODs) to communicate with other electric fishes and to navigate and detect objects in dark waters. Previous studies in three species showed that melanocortin hormones can regulate the ion channels in the electrogenic cells (electrocytes) and modify EOD waveform properties such as amplitude and duration.

In the first study, I describe variation in electric signaling behavior in response to adrenocorticotropic hormone (ACTH) in 21 species. Responses to ACTH varied greatly, suggesting there are species-specific differences in how melanocortins regulate electric signaling. Only species from the Hypopomidae and Sternopygidae consistently showed increases in EOD amplitude; however, individuals from species in all five families showed some form of waveform modulation. The second study examined the effects of ACTH at the level of electrocyte membranes, compared ion channel distributions in two

representative species, and described the kinetics of a sodium channel in a unique monophasic species, Brachyhypopomus bennetti. B. bennetti showed significantly less EOD plasticity relative to biphasic congeners. Sodium channels in this species were unexpectedly detected on both electrocyte membranes, and I determined that a second action potential reduces the EOD amplitude. The final study describes variation in the dimensions and distributions of electroreceptor pores on the heads of seven species. In some species, I found unique electroreceptor distributions associated with specialized feeding strategies, suggesting species habitat use influences electroreceptor organization. Together, these studies highlight the value of gymnotiforms as excellent models for studying the pleiotropic functions of melanocortin hormones, ion channel evolution, and the role of ecology in shaping specialized sensory systems.

DEDICATION

This dissertation is dedicated to my parents, Jorge and Jacqueline, who have supported my curiosity from the beginning. I feel overwhelmingly privileged to have spent a significant portion of my life reading, traveling, and experimenting for the simple purpose of learning more about the fascinating planet we live in and the sensory systems that allow us to experience it all.

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NOMENCLATURE

ACTH	Adrenocorticotropic hormone
α-MSH	α-melanocyte stimulating hormone
AP	Action potential
CRF	Corticotropin releasing factor
EO	Electric organ
EOD	Electric organ discharge
HPA/HPI	Hypothalamic-pituitary-adrenal/interrenal axis
I_{Na}	Sodium channel conductance

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CHAPTER I

INTRODUCTION

Animal sensory systems have been evolving for hundreds of millions of years with the primary purpose of gathering information about an organism's environment. Perhaps unsurprisingly, the process of evolution has generated an impressive variety of ways by which animals gather this information. The known diversity of sensory modalities reflects the various ecosystems and individual niches that organisms occupy. Obviously, certain methods work better in some environments over others and animals face energetic trade-offs for developing and maintaining the modalities that serve them best. For example, it makes little sense for a star-nosed mole or cave-dwelling fish to invest in maintaining elaborate eyes and associated processing centers in the cortex.

Another important role of sensory systems is mediating communication with other organisms. Animals commonly use auditory, visual, and olfactory signals to coordinate mating opportunities, to alert conspecifics to the presence of predators, or to avoid costly encounters with aggressive competitors. Sensory systems are also important for mediating mutualistic interactions between species, such as the coevolution of plant-pollinator relationships. On the other hand, sensory systems allow eavesdropping predators to take advantage of conspicuous communication signals. These biotic as well as several abiotic pressures can vary over different spatial and temporal scales. Consequently, communication signals and their respective sensory organs have evolved

dynamic mechanisms of control to ensure the use of appropriate signals depending on the environmental context.

The main goal of this dissertation was to examine how communication signals and sensory organs vary in weakly electric fishes in terms of their physiology, ecology, and phylogeny. These fish are excellent models for studying the dynamic control of sensory systems and communication signals because their primary method of sensing their environment and finding food is inextricably intertwined with their method of communication. Weakly electric fish use an organ made-up of modified muscle cells called electrocytes to generate low-voltage electric fields for a short distance around their bodies. Various types of specialized electroreceptors cover the head and body of the fish allowing the animal to perceive its surroundings by assessing the resistive and capacitive properties of objects that distort their electric fields (electrolocation). Furthermore, their electric organ discharges (EODs) can have species-, ontogenetic-, and sex-specific differences, such as the rate of discharge and waveform shape, enabling fish to identify and communicate with conspecifics (electrocommunication).

Therefore, the physiological mechanisms controlling the electrosensory system can simultaneously affect both active sensing and communicative behavior. Because their behavior uses the same currency as that of excitable tissue in all vertebrates, it allows workers to make predictions about behavioral regulatory mechanisms at the level of membrane biophysics (Stoddard et al., 2006). Additionally, these animals are highly diverse, making comparative approaches powerful tools for gaining insights toward the evolution of these systems. Electroreception is an ancient sensory modality found in

many animals, including elasmobranchs, lungfishes, coelacanths, and chondrosteans as well as some teleosts, amphibians, monotremes, and even a freshwater dolphin (Bullock et al., 2006; Czech-Damal et al., 2012). However, organisms known to combine the use of electrogeneration and electrosensation as described above are restricted to two teleost orders, Gymnotiformes and Mormyriformes. The latter are found throughout tropical regions of Africa and consist of 227 described species (Fricke et al., 2020). Despite many remarkable similarities in their ecology and the overall function of their electrosensory system, the two groups have several important differences—some of which are briefly discussed in the fourth chapter of this dissertation.

The studies included here focus on the Neotropical gymnotiform fishes, commonly referred to as knifefishes. All gymnotiforms possess a modified undulating anal fin that they use in a unique swimming style that allows for omnidirectional maneuverability (Ruiz-Torres et al., 2013). Ranging from the southern tip of Mexico to northern Argentina, there are currently 260 described gymnotiform species in five families that can be found in a variety of aquatic ecosystems, including deep river channels, floodplain channels and lakes, and permanent streams outside flooded regions frequently referred to in the literature as *terra firme* streams (Albert and Crampton, 2005; Crampton, 2011). The five families are usually categorized into two types defined by the time interval or lack thereof between EODs.

The Gymnotidae, Hypopomidae, and Rhamphichthyidae are referred to as pulse-type fish because they generate series of discrete EODs (Stoddard, 2002). They have discharge rates between 0-120 Hz and as a general rule are restricted to relatively lentic

habitats, such as floodplain lakes or slow-moving *terra-firme* streams. The Sternopygidae and Apteronotidae are referred to as wave-type fish because they generate continuous EODs. The discharge rates of wave-type species recorded in this study were between 100-1300 Hz but can be faster than 1800 Hz (Stoddard, 2002). In contrast to pulse-type fishes, wave-type fishes are most commonly found in lotic habitats, such as deep regions in river channels, sandbanks, mats of aquatic vegetation along channel margins, and even rapids (Albert and Crampton, 2005; de Santana and Vari, 2010; Lundberg et al., 1987). However, several species do not follow these habitat generalizations (especially some rhamphichthyids), and others move between habitats to exploit seasonally available resources (Winemiller, 1989).

The EOD waveforms can vary greatly both within and across families. Several studies have suggested that eavesdropping predators, primarily catfishes, have contributed to this diversity.

Over the last few decades, electric fishes have been important models for studying how hormones regulate social and sex-specific signals at the behavioral and cellular level. Hormones (in the broadest sense of the term) can play a major role in optimizing the dynamic control of communication signals and their receptive counter parts. This is easily recognizable in hormonal regulation of seasonal mating behavior, where fitness costs can be high if expensive mating signals are generated at a time when mates or resources are unavailable. On a finer time-scale, even when mating opportunities are ample, it would not be beneficial to broadcast conspicuous mating signals under imminent threat of predation. Hormonal regulation of signals on this time

scale is not as well understood, but at least in balancing predatory avoidance with other behaviors (e.g. feeding/mating), recent findings have implicated the hypothalamic-pituitary-adrenal/interrenal axis (HPA/HPI) or stress associated hormones (Burmeister et al., 2001; Coddington and Moore, 2003; Harris and Carr, 2016).

The second chapter of this work focuses on the affects adrenocorticotropic hormone (ACTH) on short-term signal plasticity in gymnotiforms, comparing 21 species with representatives from all five families. In vertebrates, ACTH has been primarily studied for its role in stress and as a signaling component of the HPA/HPI-axis. The stress response system plays a key role in optimizing energy allocation for crucial functions such as homeostasis, reproduction, feeding, and immune responses. The HPI axis is a classic example of hormonal feedback regulation. For an animal under duress, the hypothalamus produces corticotropin releasing factor (CRF) to stimulate release of adrenocorticotropic hormone (ACTH) by the pituitary gland. ACTH then stimulates the interrenal glands (in fish) to release glucocorticoids that in turn can inhibit CRF (and thus HPI activation) via classic negative feed-back loop. However, recent evidence suggests that ACTH and the other melanocortin hormones are directly involved in a much broader range of peripheral functions (Ducrest et al., 2008; Roulin and Ducrest, 2011).

For those who are not specialists in the study of electric fish biology, a brief background on the physiological mechanics of EOD function is provided, which may be especially useful for the third chapter. The mechanics of EODs in general are reviewed by Markham (2013; See Fig.1 therein), but here I focus on the biphasic discharge

commonly observed in the genus B*rachyhypopomus.* The electrocytes in the EO of *Brachyhypopomus* are large disc-shaped cells innervated on the posterior side (Bennett, 1970; Trujillo-Cenóz et al., 1984). The biphasic EOD is the result of two action potentials, one on the posterior face and one on the anterior face, whereas the edges in between are not excitable. Though both membrane faces are depolarized simultaneously, the action potential that occurs on the anterior side is slightly delayed. The summation of action potentials in a single electrocyte is referred to as the μEOD, and the whole EOD is a sum of the composite μEODs.

Electrocyte morphology and membrane properties, such as the repertoire and spatial arrangement of their ion channels, can also contribute to differences in the EOD waveforms. This is a major focus of the third chapter, wherein I compare the spatial arrangement of ion channels and their role in generating μEODs in the genus *Brachyhypopomus*. Also included in the third chapter are electrophysiological experiments describing the sodium channel kinetics in an unusual monophasic congener, *B. bennetti*. In other gymnotiforms, more complex waveforms are the result of groups of electrocytes having different innervation patterns on the anterior and posterior faces, as well as the timing of their discharges relative to that of other electrocyte groups. Interestingly, several species have non-homologous accessory electric organs that fire independently of the main organ with distinct waveforms and frequencies (Bennett, 1971, 1970; Giora and Carvalho, 2018). Lastly, the spatial organization of electrocytes, which are often in distinct rows and columns, along with adjacent tissue surrounding the

organ, can direct the flow of current to generate distinct spatiotemporal patterns in the electric field potentials around the fish's body (Caputi, 1999; Waddell et al., 2016).

Whereas the second and third chapters compare properties of electrogeneration in different species, the fourth chapter focuses on the electroreceptors. I provide descriptions of electroreceptor pore dimensions, densities, and distributions on the heads of seven species. Differences in the distributions of electroreceptors between species are discussed in the context of habitat use and feeding behavior. The fifth and final chapter draws conclusions based on findings from chapters 2-4, briefly discusses gymnotiform diversity relative to other Otophysan orders, and also explores areas for future research that would advance our understanding of the evolution of behavior and neurophysiology in this fascinating group of fishes.

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CHAPTER II

THE ROLE OF ADRENOCORTICOTROPIC HORMONE IN THE SHORT-TERM SIGNAL PLASTICITY OF WEAKLY ELECTRIC GYMNOTIFORM FISHES

Introduction

All organisms are confronted with environmental variation, including physical, chemical, and biological stressors. Animals cope with these stressors using repertoires of behavioral and physiological responses (e.g. metabolic, immune, endocrine). Accordingly, animal sensory and communication systems also show dynamic regulation, several aspects of which are under hormonal control (Sisneros, 2009). Much of what is known about hormonal regulation of animal behavior and physiology in response to the external environment has derived from the study of seasonal fluctuations, yet it is widely known that many seasonally-varying hormones have daily cycles as well. Despite this, there is surprisingly limited knowledge of how circadian hormone rhythmicity regulates physiology or behavior, and far less in regards to sensory and communication systems (Adkins-Regan, 2005; Zera, 2016). Furthermore, while short-term plasticity in communication signals has been documented (Brumm and Zollinger, 2017; Dorado-Correa et al., 2018; Ord et al., 2010), knowledge of its underlying physiology is lacking, especially at the molecular level. In this chapter, I use weakly electric fishes to study the hormonal regulation of a short-term electric signal plasticity, which has independently been shown to depend on both social context and circadian rhythm.

As described in the broader introduction, gymnotiform fishes use an organ composed of modified muscle cells called electrocytes to constantly emit weak electric signals, termed electric organ discharges (EODs), for active electrolocation and electrocommunication. Because they generate a continuous signal used for both navigation and communication with conspecifics, signal modifications are associated with ecologically significant behavior. Furthermore, changes in signaling behavioral can be hormonally regulated and directly translate into intrinsic changes in the excitability of electrocytes. Because the electrogenic system is highly amenable to physiological experiments, electric fishes are exceptional models for studying hormonally-regulated mechanisms of signaling plasticity. Finally, these speciose fishes occur in a wide range of aquatic habitats, providing an opportunity to evaluate the role of ecological differences in maintaining signal plasticity. Using a comparative phylogenetic framework, this study describes the extent of a specific type of signal plasticity regulated by melanocortin hormones in species from all five families of the order Gymnotiformes.

Gymnotiform EODs are highly diverse and vary in parameters such as discharge frequency and waveform (Albert and Crampton, 2005). The discharge frequency (also referred to as pulse rate in other studies) is regulated by a pacemaker nucleus in the gymnotiform hindbrain, whereas the EOD waveform is primarily the product of an electrocyte's innervation pattern and morphology, as well as its repertoire of ion channels and their properties (Markham, 2013). This study focuses on the plasticity of EOD waveform properties, specifically EOD amplitude and duration, as these parameters directly reflect the properties of the electrocyte membranes and ion channels.

Previous studies have shown that some species can modulate EOD waveforms in response to seasonal and circadian cycles as well as during social interactions (Hagedorn, 1995; Silva et al., 2008, 2007).

In particular, three species, *Eigenmannia virescens* (Markham et al., 2013), *Sternopygus macrurus* (Markham et al., 2009b) (both Sternopygidae) and *Brachyhypopomus gauderio* (Hypopomidae) (Markham and Stoddard, 2005), were shown to display circadian and social-dependent regulation of EOD amplitude and, in the case of the latter two species, EOD duration as well. This behavior is induced by the melanocortin hormones adrenocorticotropic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH). The changes to signaling behavior can be replicated in vivo by intramuscular injections of ACTH, as well as in vitro at the μEOD level by ACTH application to single electrocytes (α-MSH has only been tested in *B. gauderio*). Amplitude is important for determining the active space of the signal, but an increase in EOD amplitude can incur significant additional metabolic costs (Markham et al., 2009b). Additionally, decreasing the amplitude may further reduce the potential for eavesdropping electroreceptive predators to detect the signal. Therefore, the ability to regulate EOD amplitude is highly adaptive.

ACTH has primarily been studied for its role in stimulating glucocorticoid production in the vertebrate stress response system (see general introduction), but knowledge of the broader functions of ACTH and its derivatives is still lacking. The function of the stress response system has been considered to be a component of strategies to optimize energy usage. By integrating multiple systems in a context-specific

manner, it acts to balance the benefits and costs of functions as diverse as homeostasis, reproduction, feeding, and immune responses. However, other than its role in the production of glucocorticoids, the function of ACTH and other melanocortin hormones in regulating behavior and physiology at the periphery has only recently received more attention (Roulin and Ducrest, 2011). While several hormones have been shown to modulate EOD waveforms (reviewed by Dunlap et al. 2017), knowledge of which hormones can directly affect electrocyte activity and their mechanisms of action is incomplete. This is important because gymnotiforms are premier models for studying hormonal effects on behavior and provide unique advantages for studying the underlying molecular correlates. Also, it is known that the function of these hormones may vary across species. For example, the stress-associated hormone cortisol does not affect EOD amplitude in *E. virescens* (Sinnett and Markham, 2015), but cortisol decreases EOD amplitude in *B. gauderio* (Gavassa and Stoddard, 2012), an interesting finding considering cortisol is the primary end-product of ACTH release by the pituitary gland. While this chapter only discusses differences in behavioral responses to hormone treatment, comparing responses across a phylogeny is a critical first step toward understanding how these differences manifest at the molecular level.

Gymnotiform fishes are speciose and ecologically diverse, and based on studies of only a few species, their neurophysiology of electrogeneration may be equally diverse. Whether or not ACTH functions in the same manner for all 240+ gymnotiform species, or whether their EOD waveforms are even capable of similar plasticity, are unknown. Unpublished evidence suggests that other gymnotiform species may not

display circadian modulation of EOD amplitude (Harold Zakon, Univ. of Texas, personal communication). As mentioned previously (see general introduction), gymnotiforms occur in a wide variety of habitats within a geographic distribution that extends from southern Mexico to Uruguay and Argentina (Albert and Crampton, 2005). Thus, fish experience different environmental conditions, involving aspects of water quality and biotic interactions (e.g. predation, competition) that vary spatially and temporally at various scales. Water quality components, such as conductivity and dissolved oxygen concentration, generally influence how fish use habitat, but for electric fishes they can pose significant challenges. For instance, water conductivity determines the electrical resistance of the external environment through which electric signals are transmitted (Hopkins, 1999), and low dissolved oxygen levels present a significant challenge given that the electrosensory system incurs substantial metabolic costs (Crampton, 1998a; Reardon et al., 2011; Salazar et al., 2013).

Given that EOD circadian amplitude modulation may exist in some species but not others, this trait may have contributed to the notable ecological diversification and phyletic evolution observed in the five gymnotiform families (Fig. 1). I hypothesize that the presence and level of ACTH-regulated EOD waveform modulation will vary significantly among species. Also, given that EOD amplitude can be a major component of the EOD's metabolic cost (Markham et al., 2009b), I further predict that species inhabiting aquatic habitats with low levels of dissolved oxygen and/or low conductivity will show a greater degree of EOD amplitude plasticity. To test these hypotheses, I collected several gymnotiform species (Fig. 1) and recorded various environmental parameters in their respective habitats, performed in vivo injections of ACTH, and developed a new package in the R programming platform for analyzing multiple parameters of the various waveforms.

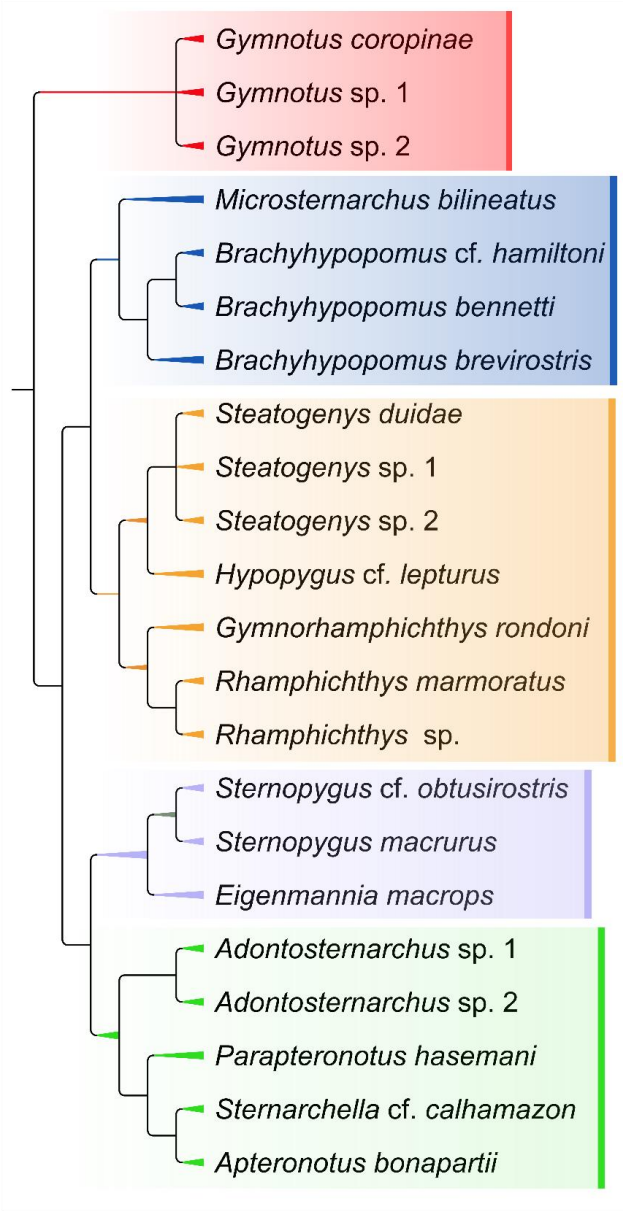

Figure 1. Gymnotiform phylogeny using the species examined in this study. Relationships are based on Tagliacollo et al. (2016).

Methods

Collection of fish and habitat data

Fish were collected from various habitat types during field trips in collaboration with Dr. Jose Alves Gomes's Laboratory for Behavior and Physiology at the Brazilian National Institute for Amazonian Research under ICMBio authorizations #14833 and #14834. Fish were first located in the field with the use of a mini audio amplifier and collected using methods that depended on the habitat type. For capturing fish in small streams, I used dipnets and seines. For sampling in lakes and rivers, seines were used to surround floating mats of aquatic macrophytes, habitats where many gymnotiforms shelter during the daytime. The lead line of the seine was pulled upward to enclose floating macrophytes, and then lifted into a boat where fishes were removed from the seine. Seine hauls were also performed in the littoral area along the banks of river channels. Fish surveys were conducted in (1) the Negro and Solimões rivers within 6 kilometers from the city of Manaus (2) Lake Catalão, a floodplain lake located at the confluence the aforementioned rivers approximately 3 kilometers from Manaus, and (3) various streams located in non-flooded regions approximately 20-30 kilometers from Manaus. One additional survey was conducted in the Tefé River within 4 kilometers of the city of Tefé in collaboration with Mr. Jonas Oliveira from the Mamiraua Research Institute.

During each survey, a YSI water quality multiprobe was used to record the following parameters: dissolved oxygen concentration, conductivity, pH, and temperature. For stream collections, I also recorded substrate composition (e.g. leaf

litter, sand, or root banks). For rivers and lakes where most fish were collected from floating aquatic macrophytes during the daytime, nocturnal habitat use may be different for some weakly electric fishes. For this study, I was not able to obtain live specimens of gymnotiforms that normally are found in the main channels of large rivers at great depths. Surveys yielded 81 individuals from 21 species that were in a condition suitable for use in laboratory experiments.

Animal care

At INPA, the fish were housed in multiple 50-L tanks with water resembling that of the streams from their natural habitats. For fish kept in black water, the pH was approximately 5.6 ± 1.1, the conductivity was approximately 21 µS/cm, and the dissolved O_2 was above 7.0 mg/L. The holding water for the few species collected from white water habitats had additional treatment of salts (calcium carbonate) to maintain the pH at approximately 7.2 with a conductivity of 90 ± 5 µS/cm. The aquarium room had large open windows with screens so that tanks were kept at ambient temperature, which in Manaus ranges from 25 – 28º C. This also exposed fish to a natural light cycle, which in Manaus is approximately 12:12 h light:dark year-round.

Injections and recordings

Injections and recordings were completed in the lab of Dr. Jose Alves-Gomes at INPA. For *in vivo* recordings, I weighed each fish and measured both the total length and the standard length (straight-line distance along body midline from the snout to the posterior margin of the anal fin). For recording EODs, fish were placed individually into a 10-L tank using water from the original holding tank to maintain consistent water

quality and to minimize stress. Measurement of EOD waveform and amplitude varies depending on the distance and orientation of the fish relative to the recording electrodes. Because the recording setup was unable to monitor fish position, it was necessary to restrict fish movement in order to measure EOD amplitude as accurately as possible. To accomplish this, fish were gently pushed by hand to induce entrance into a plastic mesh tube, after which the ends were plugged using nylon mesh or pieces of sponge. Most gymnotiforms have a natural tendency to seek shelter in confined spaces during daylight hours, and the experimental subjects were accustomed to sheltering in similar tubes placed in their housing tanks.

Some previous studies have suggested restricting fish movement alters behavior and may increase stress (Franchina and Stoddard, 1998). However, since the objective was to measure the effects of the treatment (ACTH/saline) on the EOD waveform rather than to describe natural behavior, restriction of free movement within the tank should not have significantly affected results. Furthermore, a previous study successfully employed this method for measuring natural circadian fluctuation in EOD amplitude and duration (Hagedorn, 1995), and several studies have published amplitude measurements while recording movement-restricted fish (Ardanaz et al., 2001; Dunlap et al., 2000; Silva et al., 1999).

EODs were recorded using a pair of silver electrodes that were placed 36 cm apart and equidistant from either side of the fish. EODs were amplified using a BMA 200 AC/DC Bioamplifier (CWE, Inc., USA) and digitized at 16 bits resolution using an A/D converter (CE Datatranslation USB Data Acquisition, USA) at a sampling rate of at

least 50 kHz. The digital signal was fed into a laptop running MatLab to complete the recording as well as a Tektronix (Brazil) TDS 2024 oscilloscope to monitor the condition and orientation of the fish.

To record a baseline EOD, a custom-written MatLab protocol ensured 30-sec recordings every 10 min for a minimum of 30 min (sometimes longer baselines were necessary because the fish was moving a lot or would attempt to escape from the tube). Fish were then quickly removed from the tank and given an intramuscular injection (1µl/g) of either ACTH or saline in a process that took less than 15 sec. Each fish served as its own control, so if the first injection it received was saline, the following week (~6 days later) it received ACTH, or vice versa. The order of ACTH and saline injections was randomized when more than 1 individual was available. After the injection, the subject was returned to the experimental tank, and it typically re-entered the plastic tube voluntarily or was easily induced to enter the tube. EOD recording resumed using a different MatLab protocol that recorded 5 sec of EODs every 3 min for at least 55 min. All injections occurred between 0900 h and 1500 h to avoid the influence of natural circadian fluctuations of EOD waveform/amplitude.

Data analysis

Previous studies analyzing EOD waveforms have generally focused on single species and most labs have used custom-written scripts in software such as MATLAB. Since a primary objective of this study was to compare EODs of multiple species representing all five gymnotiform families and variety of waveforms, a new package was developed in R to automatically identify EODs within recordings and extract

measurements for parameters of interest. This package remains under development, but the version used for this study was validated for its capability to extract data on the amplitude and duration of the whole EOD and for each EOD phase. When reporting results of individual EOD phases, phase designations refer to those established in (Crampton et al., 2013), where the dominant head-positive phase is P_1 and the dominant head-negative phase is P_2 (see inset of Fig 4). This retains consistency with the primary background literature on melanocortin-regulated EOD waveform plasticity and recent studies gymnotiform ecology, but for older literature (Caputi et al., 1994), the phases, P_{-1}, P_0, P_1, P_2, P_3, P_4, and P_6 correspond to V1-V6 in order. Whole EOD amplitude refers to the peak-to-peak difference between P_1 and P_2. This was the primary metric measured and reported in this study. Duration for the whole EOD and for individual phases was measured at 10% of the total amplitude to avoid including any noise in the signal. Consequently, it was not always possible to measure duration for EOD phases with amplitudes smaller than 10% of the total amplitude. Therefore, durations for minor phases are not consistently reported here, and EOD duration for multiphasic species refers to the duration of $P_1 + P_2$.

Gymnotiform locomotion (involving undulation of the anal fin) facilitates a high degree of maneuverability, making it difficult to completely prevent fish from moving without some means of restraint. Therefore, individual EODs were averaged for each 5 sec recording to reduce any amplitude fluctuations caused by fish movement within the plastic tube. To determine the percent change in EOD amplitude and duration, average values from baseline recordings were subtracted from the average values of 4-5

recordings taken 35-50 min post-injection, and then divided by the baseline average. Occasionally, a recording was omitted when it was obvious that the fish had moved and distorted the recording. For all species with $n \geq 3$, I conducted a two-sample t-test assuming unequal variances and calculated the standard error of the mean. For the graphs provided, no error bars are shown when there were less than three individuals tested. Due to limitations on equipment availability and time constraints, I prioritized testing species for which I collected multiple specimens. Therefore, no control trial is shown for some species with n=1.

Modeling EOD plasticity and habitat associations

To test whether ACTH-regulated amplitude plasticity is related to differences in habitat, I modeled species' change in EOD amplitude as a function of associated environmental factors. In order to find the model(s) that best explain changes in EOD amplitude, I used the Akaike Information Criterium (AIC) using an all-subsets approach. This approach consists of generating multiple models that consider all potential combinations of environmental variables and then ranking the models according to their AIC. I calculated the difference in AIC between each model and the best model in the collection, and then, as suggested by Burnham and Anderson (2002), I only considered models with a ΔAIC less than 2 as potential competitors. Prior to model selection, variables were tested for collinearity, and consequently pH was excluded. I performed the analyses in the R environment using the package MuMIn (Barton and Barton, 2019).

Results

Effects of ACTH injections on EOD waveforms

Evaluation of average values for species indicated that ACTH injections had little effect on EOD waveforms in most of the species tested (Table 1). At the family level, only the Hypopomidae and Sternopygidae showed consistent increases in EOD amplitude (Fig. 3 & 5, respectively). Within the Hypopomidae, one species, *Brachyhypopomus bennetti*, showed a weak increase in EOD amplitude, a result that was explored further in the following chapter. In addition to increasing EOD amplitude, both *Brachyhypopomus brevirostris* and *Brachyhypopomus* cf. *hamiltoni* showed an increase in EOD duration (Table 1), which for both species was driven by an increase in the duration of P2, similar to what has been shown in *B. gauderio* (Markham and Stoddard, 2013, 2005).

Among the Sternopygidae, the species *Eigenmannia macrops* showed a similar pattern to that of published results for *E. virescens* (Markham et al., 2013), with an increase in EOD amplitude, but no detectable differences in EOD duration. However, the increase in EOD amplitude was not statistically significant ($p = 0.13$, $t = 4.3$), possibly because only 3 individuals were available for testing and the effect in one of them was very weak. For *Sternopygus* cf. *obtusirostris* (n=5), though there was an overall average increase in EOD amplitude, the results were highly variable. Two individuals showed large increases (~18 and 30%), one individual showed a very small increase (~6%), another showed essentially no difference (0.2%), and one individual even showed a slight decrease in EOD amplitude (-3.5%). The experimental controls were far more

consistent, with all individuals of all species showing a slight decrease in EOD amplitude. A slight decrease in EOD amplitude in response to saline injections has been observed for several other gymnotiform species (A. Goldina, personal communication).

Among the five species of Apteronotidae tested, two species, *Apteronotus bonapartii* (n=5) and *Adontosternarchus* sp.1 (n=3), showed decreases in EOD amplitude in response to ACTH that were not statistically significant relative to saline injections, while the other three species ($n \leq 2$) displayed slight increases (< 2%) that, again, were not statistically significant. Control injections consistently resulted in slight decreases in EOD amplitude (Fig. 6). EOD durations were much more consistent in response to both ACTH and saline injection, with little to no change, with the exception *Sternarchella* cf. *calhamazon*, for which one individual showed a 10% increase in EOD duration (data not shown). This is interesting because most species with wave-type EODs (Apteronotidae & Sternopygidae) showed stable EOD durations, with multiple trials showing less than a 0.01% change (Table 1).

Three species of *Gymnotus* were tested (Fig. 2). For *Gymnotus coropinae* (n=5), on average there was a slight increase in EOD amplitude (4 ± 2%) in response to ACTH. This result was primarily driven by one individual that increased its amplitude nearly 12%. However, for *G. coropinae*, the control trials showed an even higher average increase in amplitude (8 ± 2.6 %). Two individuals of *Gymnotus* sp. 1 were given ACTH injections. In both cases, the entire EOD amplitude increased slightly (7.8%). In all cases in which the amplitude increased for either *G. coropinae* or *Gymnotus* sp.1, either for ACTH and saline trials, the largest increase occurred rapidly and was evident in the first

recording post-injection. This differs from what was observed in the Hypopomidae and Sternopygidae, for which the increase was more gradual and did not reach a maximum amplitude until ~30 min post-injection. For *Gymnotus* sp.2 only one individual was tested and the amplitude decreased slightly after both ACTH and saline injection.

There were no significant changes in average EOD duration for the species of Gymnotidae tested. However, the same three *Gymnotus* individuals that showed an increase in EOD amplitude (measured as P_1-P_2) also exhibited an increase in EOD duration and a large decrease in the amplitude of P_3. A second individual of *G. coropinae* showed no increase in amplitude, but a similar increase in EOD duration and a decrease in P_3 amplitude. *Gymnotus* sp.2 also showed a minor increase in EOD duration, but its waveform is nearly biphasic to begin with, so there is no P_3 comparison.

Seven species from the family Rhamphichthyidae were tested, but again, results were largely inconclusive based on evaluation of species averages (Fig. 4). In response to ACTH injection, most species exhibited a small non-significant change in EOD amplitude relative to the saline treatment, but *Gymnorhamphichthys rondoni* was an exception. *G. rondoni* only showed a slight increase in EOD amplitude on average (1.3 ± 7%), and individual responses were highly variable, with differences as extreme as a 15% increase and a 9% decrease. To lesser degree, this sort of intraspecific variation occurred in almost all of the Rhamphichthyidae. For *Rhamphichthys marmoratus,* three individuals showed large reductions in EOD amplitude (17-37%) in response to ACTH, and this also occurred for one individual in response to a saline injection (29% decrease).

None of the rhamphichthyid species showed significant changes in average EOD duration. However, two individuals of *R. marmoratus*, one *Rhamphichthys* sp.1, and two *Steatogenys duidae* showed substantial increases in EOD duration (6-14%) in response to ACTH, but not saline. In all cases, this was almost entirely driven by an increase in the major head-negative phase P_2, for which durations increased between 22-27% (Fig. 4 inset). This increase in duration did not necessarily correlate with an increase in EOD amplitude for those individuals. Interestingly, the two individuals of *R. marmoratus* and the one *Rhamphichthys* sp.1 were later identified as males (I was unable to determine the sex of *S. duidae* specimens).

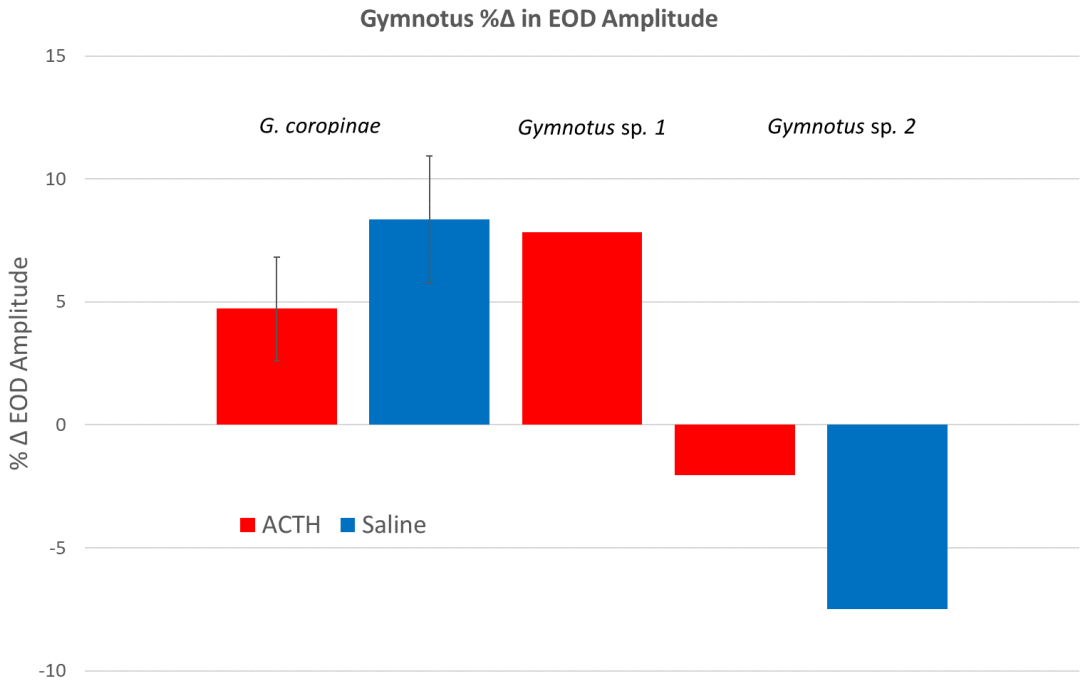

Figure 2. Bar plot showing results for 3 species of Gymnotidae. Bars indicate the species averages for percent changes in EOD amplitude in response to ACTH (red) and saline (blue) injections. Error bars indicate standard error of the mean. For species that had fewer that 3 individuals no error bars are shown. For *Gymnotus* sp. 1, no saline trial was conducted. See Table 1 for full species name and results of statistical analyses.

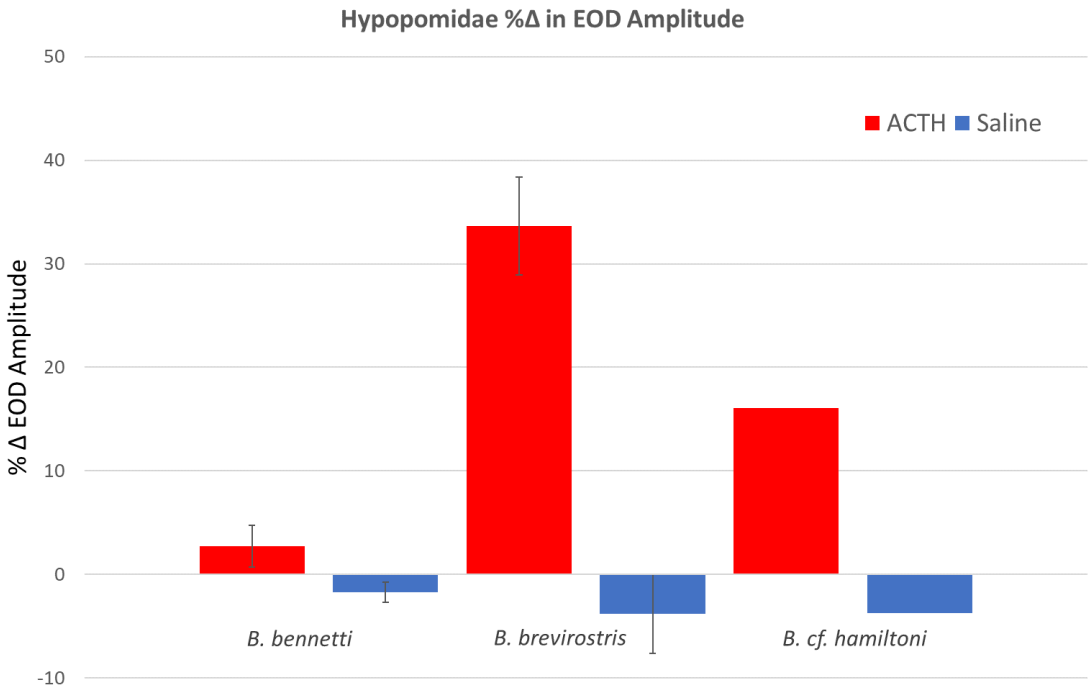

Figure 3. Bar plot showing results for 3 species of Hypopomidae. Bars indicate the species averages for percent changes in EOD amplitude in response to ACTH (red) and saline (blue) injections. Error bars indicate standard error of the mean. For species that had fewer that 3 individuals no error bars are shown. See Table 1 for full species name and results of statistical analyses.

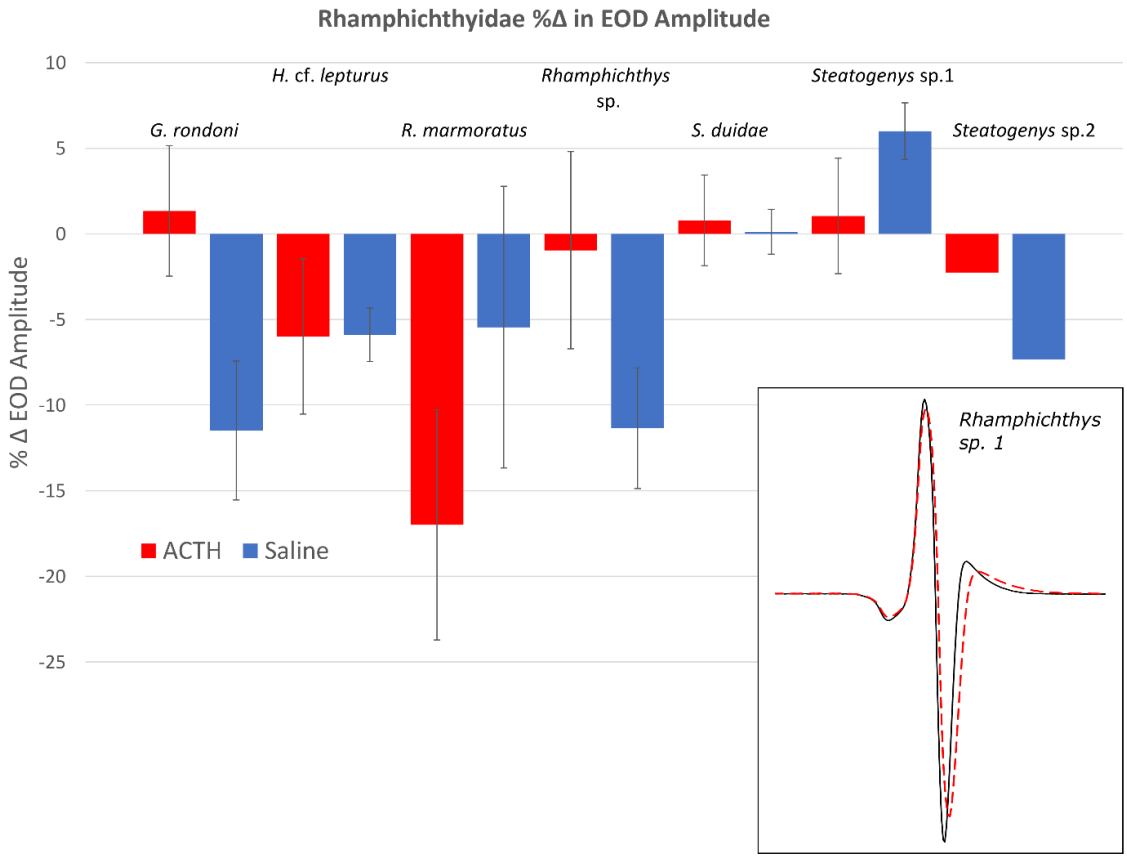

Figure 4. (version 2) Bar plot showing results for seven species of Rhamphichthyidae. Bars indicate the species averages for percent changes in EOD amplitude in response to ACTH (red) and saline (blue) injections. Error bars indicate standard error of the mean. For species that had fewer that 3 individuals no error bars are shown. The inset shows a representative waveform for one male Rhamphichthys sp. 1. Note that while EOD amplitude decreased, duration of P_2 increased. Similar waveform changes were observed in two individuals of R. marmoratus (both males) and two S. duidae (not sexed). See Table 1 for full species name and results of statistical analyses.

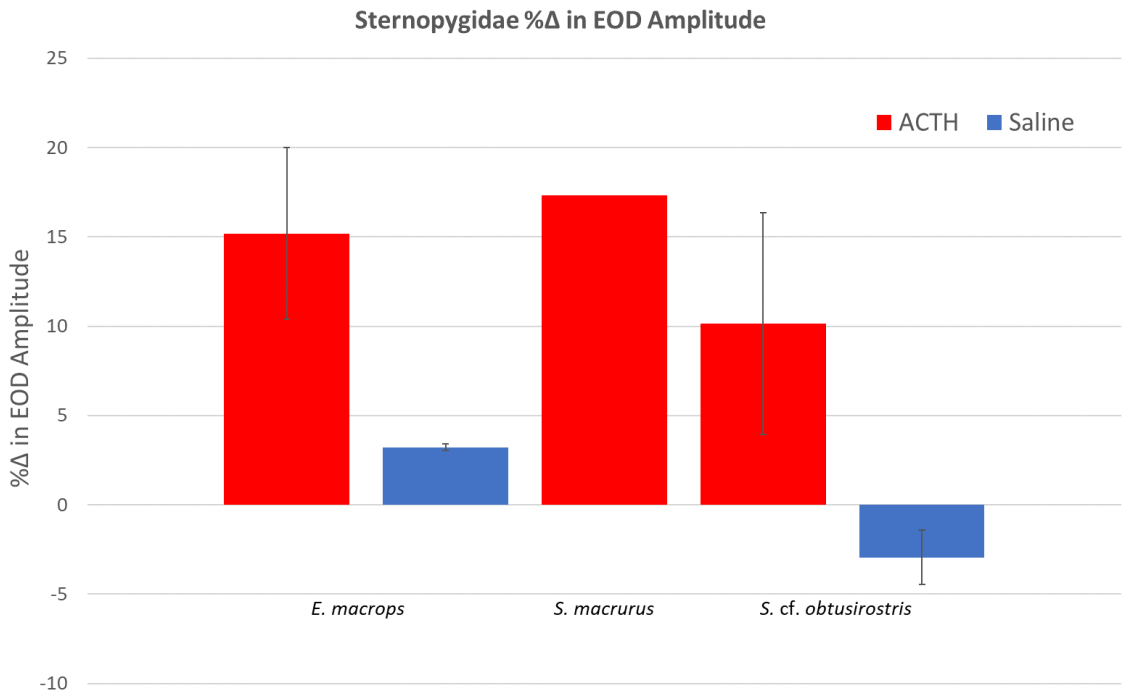

Figure 5. Bar plot showing results for 3 species of Sternopygidae. Bars indicate the species averages for percent changes in EOD amplitude in response to ACTH (red) and saline (blue) injections. Error bars indicate standard error of the mean. For species that had fewer that 3 individuals no error bars are shown. For *S. macrurus*, no saline trial was conducted, however see Markham et al. (2009b). See Table 1 for full species name and results of statistical analyses.

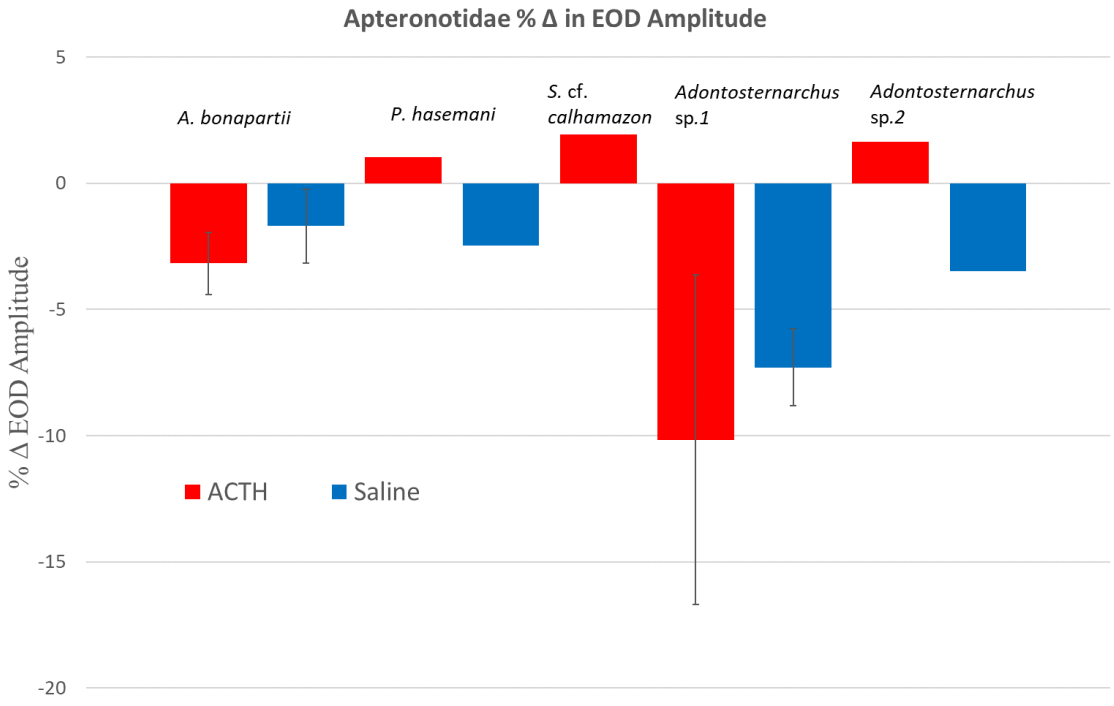

Figure 6. Bar plot showing results for 5 species of Apteronotidae. Bars indicate the species averages for percent changes in EOD amplitude in response to ACTH (red) and saline (blue) injections. Error bars indicate standard error of the mean. For species that had fewer that 3 individuals no error bars are shown. For *S.* cf. *calhamazon*, no saline trial was conducted. See Table 1 for full species name and results of statistical analyses.

	Species	EOD Parameter	Mean %Δ - ACTH	Mean %Δ - Saline	p-value / tstat
Gymnotidae	*Gymnotus coropinae*	Duration	5.35	7.61	0.521 / 2.306
	n = 5	Amplitude	3.73	6.83	0.306 / 2.306
	Gymnotus sp. 1	Duration	3.08	N/A	---
	n = 1	Amplitude	7.83	N/A	---
	Gymnotus sp.2	Duration	1.42	1.05	---
	n = 1	Amplitude	-2.05	-7.50	---
Hypopomidae	*Brachyhypopomus brevirostris*	Duration	31.53	0.73	<0.001 / 2.571
	n = 6	Amplitude	29.85	-6.57	<0.001 / 2.228
	Brachyhypopomus cf. hamiltoni	Duration	6.62	0.62	---
	n = 1	Amplitude	16.95	-4.46	---
	Brachyhypopomus bennetti	Duration	1.92	0.01	0.331 / 2.571
	n = 5	Amplitude	2.70	-1.74	0.0941 / 2.447
Rhamphichthyidae	*Gymnorhamphichthys rondoni*	Duration	4.15	1.95	0.117 / 2.306
	n = 5	Amplitude	1.34	-11.49	0.099 / 2.447
	Hypopygus cf. lepturus	Duration	3.16	-0.36	0.146 / 2.306
	n = 7	Amplitude	-5.99	-5.90	0.984 / 2.365
	Rhamphichthys marmoratus	Duration	4.27	3.88	0.862 / 2.365
	n = 5	Amplitude	-17.00	-5.45	0.319 / 2.447
	Rhamphichthys sp.	Duration	2.81	1.39	0.534 / 2.776
	n = 4	Amplitude	-0.96	-11.36	0.184 / 2.571
	Steatogenys duidae	Duration	2.97	-0.38	0.837 / 2.365
	n = 6	Amplitude	0.79	0.12	0.827 / 2.365
	Steatogenys sp. 1	Duration	1.74	1.18	0.701 / 2.447
	n = 4	Amplitude	1.05	6.00	0.258 / 2.776
	Steatogenys sp.2	Duration	1.49	1.23	---
	n = 2	Amplitude	-2.26	-7.35	---
Sternopygidae	*Eigenmannia macrops*	Duration	0.40	0.20	0.638 / 2.776
	n = 3	Amplitude	15.18	3.22	0.13 / 4.303
	Sternopygus macrurus	Duration	0.01	N/A	---
	n = 1	Amplitude	17.34	N/A	---
	Sternopygus cf. obtusirostris	Duration	0.45	0.33	0.7 / 2.365
	n = 5	Amplitude	10.14	-2.94	0.109 / 2.776
Apteronotidae	*Apteronotus bonapartii*	Duration	1.19	0.00	0.357 / 2.776
	n = 5	Amplitude	-3.17	-1.71	0.471 / 2.447
	Parapteronotus hasemani	Duration	0.00	0.00	---
	n = 1	Amplitude	1.04	-2.47	---
	Sternarchella cf. calhamazon	Duration	4.05	N/A	---
	n = 2	Amplitude	1.94	N/A	---
	Adontosternarchus sp.1	Duration	-1.36	-1.10	0.888 / 2.776
	n = 4	Amplitude	-14.10	-8.57	0.698 / 3.182
	Adontosternarchus sp.2	Duration	0.00	0.00	---
	n = 1	Amplitude	1.64	-3.48	---

Table 1. List of all species and species averages for the percent change in EOD amplitude and duration in response to ACTH and saline injections. t-tests were only conducted for species with $n \geq 3$.

EOD plasticity and habitat associations

As described in the methods, I used an all-subsets approach to compare multiple models to examine the relationship between environmental variables and EOD amplitude changes post-ACTH treatment. This approach indicated that the best model included water type (i.e. black, white, and clear) as the sole explanatory variable for EOD amplitude plasticity. However, the second-best model included only the intercept, suggesting that some alternative models explained the variation in the data as well as the model solely containing the intercept. Thus, all of the models were weak and could not account for the observed variation in the data.

Discussion

As predicted, species varied greatly in their response to ACTH injections. For the majority of species tested, there was little to no effect, and in others, the results were mixed. At the family level, only the Hypopomidae and Sternopygidae showed increases in EOD amplitude consistently across species, but even then there were some peculiarities. Two species of *Brachyhypopomus* (Hypopomidae), showed responses similar to what has been observed in *B. gauderio* (Markham et al., 2009a; Markham and Stoddard, 2005), with the exception of the monophasic species, *B. bennetti* that only exhibited a minor increase in EOD amplitude and no change in duration. These results are discussed further in the following chapter, so here I only focus on the other four

gymnotiform families. Because I observed a large amount of intraspecific variation for multiple species, I first explore a few potential sources of this variation.

Sources of intraspecific variation: Biological and technical considerations

First, the EODs of many gymnotiforms are sexually dimorphic (Few and Zakon, 2007; Giora et al., 2008; Hagedorn and Carr, 1985; Ho et al., 2010; Hopkins et al., 1990; Smith, 2013). In *S. macrurus*, males discharge at a lower frequency and have EODs with longer durations. During the breeding season, *B. gauderio* males develop slightly larger caudal appendages and increase the EOD amplitude and EOD duration (especially of P2) more so than females (Franchina and Stoddard, 1998). There is even evidence that the physiological mechanism underlying the increase in EOD amplitude of *B. gauderio* in response to ACTH is sexually dimorphic (Markham and Stoddard, 2013). Therefore, it is possible that in some of the species tested, only one sex responded to ACTH injections. This could occur if expression or regulation of melanocortin receptors and/or their downstream effectors is sexually dimorphic in electrocytes.

The melanocortin system has been implicated in numerous functions of physiological and behavioral phenotypes, many of which can be sexually dimorphic, such as food intake and weight regulation, melanogenesis, and sexual behavior (Ducrest et al., 2008; Roulin and Ducrest, 2011). Moreover, sexual dimorphism in expression of the melanocortin-4 receptor has been shown in mice (Qu et al., 2014). Unfortunately, sex determination for most gymnotiform species is only possible by euthanizing the fish, and in many cases this was not possible. Several specimens were subsequently used as subjects for another project and unavailable for later dissection.

Second, while I used a consistent weight-to-volume dosage of ACTH/saline per fish, it is possible that baseline levels of this hormone differ and that different dose-response relationships or dosage-thresholds may exist for some species—a characteristic that would be difficult to determine with a small sample size. Third, as described in the methods, my recording setup had the potential to overestimate changes in EOD amplitude. While several measures were taken to reduce any potential effects of fish movement, species varied greatly in their size, and therefore not all restraining tubes limited movement equally. Species also differed in their rate of activity within the restraint. For example, most hypopomids and sternopygids would remain still within tubes, regardless of whether they were provided in their housing tank or the recording tank. Several of the apteronotids usually refuged within mats of yarn rather than the tubes provided in their housing tanks, and these species tended to move more within the tube of the recording tank. In their natural habitats and in the housing tanks, *Gymnorhamphichthys rondoni* burrows into sandy substrate during the daytime, and *Steatogenys duidae* lies flat on the substrate and mimics a leaf. These innate behavioral patterns probably account for interspecific differences in activity during confinement in tubes.

Fourth, species might differ in their response and tolerance to/of stress. ACTH is a major component of the HPI axis and helps to regulate stress and energy allocation by stimulating cortisol release. Although precautious were taken to minimize stress, fish nonetheless experienced handling and were confined for an extended period of time

(Franchina and Stoddard, 1998), and stress therefore could have influenced EOD response to injections.

Related to stress is the issue of acclimation time. Baseline recordings were taken over a period of 45-60 min before fish were injected—a conservative length of time compared to previous studies measuring EOD amplitude using a movement restriction recording method (Ardanaz et al., 2001; Dunlap et al., 2000). However, recent unpublished experiments using automated recording tanks with free-swimming fish have shown that some individuals require several hours to properly acclimate to a new tank before their EOD amplitude returns to "normal" resting values (Markham, personal communication).

Lastly, it is possible that stress from injections was inconsistent based on body size. Some species (e.g. *Microsternarchus*, *Hypopygus*) are very small as adults (< 5 cm in standard length and < 1 cm body depth), and a few individuals died the following morning. Consequently, *Microsternarchus* was excluded from analysis.

Gymnotidae

For the Gymnotidae, injections of both ACTH and saline produced variable results, suggesting that either the injection process itself, some factor discussed above (see "*Sources of intraspecific variation"*), or another unknown factor in the experiment may have caused changes in EOD waveforms. Interestingly, some individuals from all three species showed notable increases in EOD duration, occasionally in conjunction with an increase in EOD amplitude. Perhaps more interesting is the fact that these individuals also showed a decrease in P_3, a minor head-positive phase (with the

exception of *Gymnotus* sp. 2 which is nearly biphasic and therefore has no P_3). Previous studies have reported evidence of sexual dimorphism in the timing, duration, and amplitude of P_3 relative to P_2 for some species of *Gymnotus* (Crampton et al., 2013, 2011). However, because in some subjects this was also observed after saline injections it is unlikely this modulation was induced by ACTH; instead, it is possible that they occurred in response to the stress of the injection process.

A previous study that looked at the response of *Gymnotus carapo* to testosterone treatments inferred that hormone treatment increased EOD amplitude (specifically P_2) and reduced sensitivity to temperature changes (Ardanaz et al., 2001). Androgen-induced changes in EOD parameters of several species have been well documented in gymnotiforms (McAnelly and Zakon, 2007; Zakon and Dunlap, 1999) as well as the African electric fishes (Mormyridae) (Bass and Hopkins, 1983), but these changes occur over long periods of time. While further research is clearly necessary, the results presented here may be the first evidence of rapid changes in EOD waveform in Gymnotidae.

Rhamphichthyidae

Fishes of the family Rhamphichthyidae currently are the least-studied gymnotiforms, this despite the fact that this group encompasses the greatest ecological diversity. The genus *Hypopygus* contains the smallest of the gymnotiform species (min. length at maturity = 42 mm) (De Santana and Crampton, 2011), and the genus *Rhamphichthys* contains some of the largest species, with some reaching over half a meter in length (Carvalho and Albert, 2015). They are found in diverse aquatic habitats,

including “terra-firme” streams, floodplains, and deep river channels, (Albert and Crampton, 2005; De Santana and Crampton, 2011; Fernandes et al., 2004), and some species, such as *Gymnorhamphichthys rondoni,* are sand-dwelling (Zuanon et al., 2006). Therefore, it is perhaps not surprising that responses to ACTH and saline injections varied greatly, however, the amount of intraspecific variation was unexpected. Reductions in EOD amplitude post-injection (saline/ACTH) in some individuals of *G. rondoni*, *Rhamphichthys marmoratus*, and *Rhamphichthys* sp. were by far the largest observed in this study (~23-37%). Upon closer examination of recordings from those individuals, I occasionally observed low amplitude electric discharges (1-5% of full EOD amplitude) immediately following an EOD or during the inter-pulse period. These minute discharges closely resembled the EOD waveform (>80% correlation), so it is unlikely this was noise in the recording.

To my knowledge, this phenomenon has not been published previously, but anecdotal evidence suggests this occurs when fish are very stressed (Michael Markham, personal communication). Species averages for the decrease in EOD amplitude in response to ACTH were not statistically different from saline treatments for any of the Rhamphichthyidae. However, it seems likely that some stress-related mechanism may rapidly decrease EOD amplitude in some rhamphichthyids. In *Eigenmannia virescens* (Sternopygidae) cortisol does not affect the EOD waveforms, but in *Brachyhypopomus gauderio* (Hypopomidae) cortisol decreases EOD amplitude.

Furthermore, two individuals of *R. marmoratus* (both males), one *Rhamphichthys* sp.1 (male), and two *Steatogenys duidae* (unsexed) exhibited notable differences in EOD

duration in response to ACTH (but not saline) that could be due to sexual dimorphism. These individuals all exhibited elongated P_2 phases (Fig. 4 inset), a trait associated with distinct sexual dimorphism in some species of *Brachyhypopomus* (Franchina and Stoddard, 1998; Hagedorn and Carr, 1985). Increasing the duration of P_2 in relation to P_1 is interesting because this should decrease the peak power frequency (PPF) of the signal, making it more conspicuous to electroreceptive predators (Stoddard, 1999; Stoddard et al., 2019; Stoddard and Markham, 2008). For this study, PPF was not calculated, but efforts are currently underway to incorporate this metric into the new R package for EOD analyses. Sexual dimorphism has only been reported for morphological traits in *G. rondoni* (Garcia and Zuanon, 2019), so this would be the first report of sexual dimorphism in EOD waveform for any species of Rhamphichthyidae; however, the sample size is very low.

It should be noted that for *Brachyhypopomus gauderio,* the gymnotiform most studied for its waveform plasticity, an increase in P_2 duration is generally reported to coincide with an increase in EOD amplitude (Markham et al., 2009a). However, the changes in P_2 reported here in rhamphichthyid species coincided with either a slight decrease or no change in EOD amplitude. One study has reported elongation of P_2 in natural populations of *Brachyhypopomus gauderio* without any circadian changes in EOD amplitude (Silva et al., 1999). This suggests that the changes in EOD amplitude and P_2 duration could be regulated by distinct physiological mechanisms, perhaps involving multiple regulatory hormones (see also Goldina et al., 2011).

Sternopygidae

Whereas *Eigenmannia macrops* showed a similar response to ACTH as that of its previously studied congener *E. virescens*, *Sternopygus* cf. *obtusirostris* differed relative to *S. macrurus* in that only some individuals showed an increase in EOD amplitude and no change in EOD duration was observed following ACTH injection. The fact that I consistently observed no change in EOD duration in *S.* cf. *obtusirostris* (as has been shown *S. macrurus*) despite sometimes large increases in EOD amplitude, at least suggests that species-specific differences are real and not a consequence of the recording setup. Future studies could explore the possibility of sexual dimorphism in the effects of melanocortins in *S.* cf. *obtusirostris*.

Apteronotidae

With the exception of *Adontosternarchus* sp.1, no species of Apteronotidae exhibited a change in EOD amplitude, positive or negative, greater than 3.5% to either treatment (Fig. 6). In *Adontosternarchus* sp.1, the large decrease in EOD amplitude was driven by one individual. Because saline injections also caused moderate decreases EOD amplitude, it is possible this result could be stress related. During development, apteronotids lose the juvenile myogenic electric organ and the spinal neurons themselves develop into electrocytes, instead of synapsing on myogenic electrocytes as occurs during development of other gymnotiforms. Thus, the function of the melanocortin system in relation to the neurogenic electrocytes might be expected to differ for apteronotids.

Still, a recent study of a sodium channel in apteronotids showed that to generate EODs the neural-derived electrocytes express a muscle-type sodium channel similar to the myogenic electrocytes in other gymnotiforms, instead of adapting a sodium channel typically associated with spinal neurons (Thompson et al., 2018). This suggests that some critical components of the electrogenic system maybe more easily modified than others, and perhaps there also is parallel adaptation of the melanocortin system for EOD waveform regulation in the Apteronotidae that was not uncovered here. A species that deserves further study is *Sternarchella* cf. *calhamazon*, considering that one individual increased its EOD duration by 10% and in both individuals tested, a polymorphic third phase (P_3) became more apparent. Additionally, it would be interesting to see if melanocortin hormones have any effect on the EOD waveform in juvenile apteronotids that still possess a myogenic electric organ.

Circadian rhythms, hormones, and EOD waveform plasticity

This study was based on previous work that had shown ACTH modulates circadian changes in the EOD waveforms of *B. gauderio, E. virescens,* and *S. macrurus* (Markham et al., 2009a, 2009b; Markham and Stoddard, 2005). Although the present study provides evidence that ACTH modulates the EOD waveforms of several other species, this does not necessarily mean that these species also exhibit circadian rhythmicity in EOD waveform plasticity, a topic that requires further research. Nevertheless, several gymnotiform species show circadian rhythmicity in EOD frequency, increasing EOD frequency at night during periods of heightened activity (Migliaro and Silva, 2016; Perrone et al., 2010; Silva et al., 2007) and suggesting

circadian regulation of EODs is common. Several gymnotiform species are abundant at great depths in channels of large turbid rivers (Lundberg et al., 1987) where light is unlikely to be present even during the daytime, and at least one species of *Eigenmannia* is cave-dwelling (Fortune et al., 2019; Triques, 1996). It would be interesting to study whether circadian rhythmicity in signaling behavior of these fishes is maintained and how it might be entrained.

Though several species tested here did not exhibit evidence of short-term waveform plasticity in response to ACTH, this does not rule out the possibility that other hormones could regulate short-term plasticity in these and other untested species. Another melanocortin hormone, α-MSH has been shown to have the same effects as ACTH in *B. gauderio* (Markham et al., 2009a). Therefore, it is possible that α-MSH may be the predominant melanocortin responsible for regulating waveform plasticity in other species and why ACTH only produced small effects in some of the species tested here. This could occur with species-specific expression of different melanocortin receptors in the electrocytes. The amino acid sequence of ACTH is highly conserved in vertebrates (Costa et al., 2004), however, the relative affinities of the melanocortin receptors to ACTH and the other pro-opiomelanocortin peptides in teleosts apparently is unknown. The hormone leptin, also associated with the melanocortin system, has been shown to increase EOD amplitude in *E. virescens* via direct action on electrocytes (Sinnett and Markham, 2015). Serotonin has been shown to increase EOD amplitude and duration in *B. gauderio*, but does not act directly on electrocytes and instead likely stimulates the melanocortin system via the HPI axis (Stoddard, 2003).

As discussed in the section "*Gymnotidae,*" androgens have also been shown to modulate EOD waveforms, but over longer periods of time (days to months) and are typically associated with seasonal changes in sexually dimorphic signals (Bass, 1986; McAnelly and Zakon, 2007; Zakon and Dunlap, 1999). Seasonal differences in circulating androgens might also regulate the capacity for short-term waveform plasticity. In *B. gauderio*, testosterone and 11-ketotestosterone can enhance increases in P_2 when fish are faced with social challenges (Goldina et al., 2011), and females given high dosages of dihydrotestosterone show greater increases in EOD amplitude and duration when fish are faced with social challenges or treated with ACTH (Allee et al., 2009).

Waveform changes have been reported in response to temperature changes in *A. leptorhynchus, B. gauderio* and *G. carapo*, and testosterone was shown to reduce temperature sensitivity in the latter two (Ardanaz et al., 2001; Dunlap et al., 2000; Silva et al., 1999). Interestingly, when challenged with a change in temperature, *B. gauderio* can quickly compensate for this change in waveform, but *G. carapo* does not. Considering all these examples, it is possible that another reason why certain species or individuals did not show a significant change in EOD waveform in this study is because they had low levels of circulating androgens. Certain thresholds of androgens could be necessary for the expression of melanocortin receptors and other downstream effectors that regulate short-term waveform plasticity. Lastly, it is possible that there is differential expression of melanocortin receptors in different groups of electrocytes. For instance, given that the waveforms of some gymnotids result from electrocyte groups

with different innervation patterns (Caputi, 1999), it is possible that only one or two of these electrocyte groups express the melanocortin receptors and/or downstream effectors necessary to modify the ion channels regulating the waveform.

The adaptive value of EOD waveform regulation

The comparative approach taken here provides the opportunity to make inferences on the adaptive value of melanocortin regulation of waveform plasticity. Is it the same for all species that exhibit this trait or does it vary depending on their ecology? Previous studies have shown that *B. gauderio*, *E. virescens*, and *S. macrurus* all modify EOD waveforms in response to social challenges, so obviously waveform changes are important for communication. Nevertheless, consider that in this study multiple species only showed changes in either EOD amplitude or duration and similar evidence has been reported in natural populations of *B. gauderio* (Silva et al., 1999; also see discussion under "Rhamphichthyidae"). It therefore seems probable that there are different hormone-related mechanisms regulating the two parameters (Goldina et al., 2011). Indeed, different mechanisms have been proposed for *S. macrurus* and *B. gauderio* males, though *B. gauderio* females may employ the same mechanism as *S. macrurus* (Markham et al., 2009b; Markham and Stoddard, 2013). This suggests there may be different adaptive values for regulating duration and amplitude.

The electrosensory system functions in two primary capacities, electrolocation and electrocommunication. Various studies suggest that generating EODs can incur high metabolic costs (Lewis et al., 2014; Markham et al., 2016; Salazar et al., 2013). The two major components of this cost are the frequency at which the fish discharges and the

amplitude of the EOD. Increasing either of these components requires additional expenditure of ATP by increased activity of the Na^+/K^+ ATPases to repolarize the electrocytes. Previous studies had proposed that wave-type fishes might have higher metabolic costs due to their faster discharge rates; however, this does not seem to be the case (Julian et al., 2003). One proposed explanation is a trade-off in which fish with higher frequencies have lower amplitudes and vice versa.

A higher frequency provides the advantage of a higher sampling rate and thus perception of the environment with better resolution. The great majority of wave-type species with high frequencies are found in lotic habitats (Crampton, 1998b, 1998a) where higher sampling rate maybe important for quick navigation and location of prey within the water column. Conversely, amplitude determines the active space of the signal and therefore the distance at which fish can communicate and actively electrolocate. For the most part, wave-type fish do not modify their frequencies other than briefly during "chirping" behavior or minimally in a jamming avoidance response (Bullock et al., 2006). Therefore, the ability to regulate EOD amplitude would seem highly adaptive in wave-type species for reducing metabolic costs. In this manner, increasing EOD amplitude at night could enhance electrolocation abilities while foraging and also reduce the costs of active sensing during periods of inactivity.

Whether fish with greater EOD amplitudes are more effective electrolocators has not been tested. An unpublished undergraduate thesis (see Stoddard et al., 2006) suggested that male *B. gauderio*, which are typically larger and therefore have larger EOD amplitudes, are not better electrolocators than females. If this is also the case for

wave-type species such as *S. macrurus* and *E. macrops*, then an increase in EOD amplitude may primarily serve to enhance communication abilities. Interestingly, some *Eigenmannia* species are often observed in groups (Tan et al., 2005; Kirk Winemiller, personnel communication). In the current study, individuals of Gymnotidae and Rhamphichthyidae that exhibited notable changes in EOD waveform only changed EOD duration and/or EOD phases associated with sexual dimorphism. These aspects of EOD waveform are unlikely to affect a fish's ability to electrolocate and further suggests that ACTH functions to regulate communication-related behaviors, not electrolocation. The finding that serotonin can mediate EOD waveform plasticity upstream of ACTH (Stoddard, 2003) is consistent with evidence from other vertebrates that serotonin is important for mediating contextually appropriate, social behavior.

Several gymnotiform species are fractional spawners (Cognato and Fialho, 2006; Giora and Fialho, 2009; Waddell et al., 2019), and therefore, conspicuous changes in EOD signals may be used for coordinating mating behavior. This may be especially important for species of *Brachyhypopomus* that apparently exhibit a semelparous life history strategy (Waddell et al., 2019; Waddell and Crampton, 2020). Strong selection to acquire a mate during a single season of reproduction may explain why species of *Brachyhypopomus* generally exhibit both EOD duration and amplitude plasticity. In *Brachyhypopomus*, this may be further influenced by sexual selection and female preference for males with larger EOD amplitudes (Curtis and Stoddard, 2003; see also chapter III).

EOD amplitude plasticity and habitat associations

When testing whether habitat variables could explain species variation in ACTH-mediated amplitude plasticity, none of the models generated were effective. This is not totally unexpected considering the low sample size. Due to logistical limitations during my study, all individuals of a given species were caught from the same location. Therefore, I was unable to obtain replicate samples for species-habitat associations. Additionally, the tremendous intraspecific variation in individual responses to ACTH certainly limited the predictive power of the model. Future studies of short-term EOD waveform plasticity should be designed to account for both environmental as well as sex differences. My study was unable to provide any evidence of an environmental association. Fish habitat use generally is spatially and temporally dynamic, especially for species that exploit seasonally fluctuating resources, and extensive field surveys are required to reveal environmental relationships.

Evolution of melanocortin-mediated waveform plasticity

A goal of the comparative approach used here was to explore the evolution of melanocortin-mediated waveform plasticity across the gymnotiform phylogeny. Though this study has presented further evidence for species-specific differences in waveform plasticity, the findings did not reveal any clear evolutionary relationships. The gymnotiform phylogeny has been a topic of much debate (Albert and Crampton, 2005; Alda et al., 2019; Alves-Gomes et al., 1995; Arnegard et al., 2010; Elbassiouny et al., 2016; Kirschbaum and Schwassmann, 2008; Lovejoy et al., 2010; Tagliacollo et al., 2016). A recent phylogenetic study proposed Gymnotidae as the basal clade,

Hypopomidae and Rhamphichthyidae as sister taxa, and the Sternopygidae and Apteronotidae as sister taxa, with the Apteronotidae as the most derived (Tagliacollo et al., 2016). Figure 1 shows a version of that phylogeny modified to represent the species examined in this study. Others have suggested that Apteronotidae is the sister group to all other Gymnotiformes (Arcila et al., 2017; Elbassiouny et al., 2016). The most recent phylogeny proposes a new phylogeny with the pulse-type families (Gymnotidae, Hypopomidae, and Rhamphichthyidae) reciprocally monophyletic to the wave-type families (Sternopygidae and Apteronotidae) (Alda et al., 2019).

There is consensus on one thing. Radiation of the early gymnotiforms was likely very rapid (Alda et al., 2019; Crampton, 2011). Alda et al. (2019) suggest that rapid radiation and incomplete lineage sorting are the primary causes for incongruence of the evolutionary relationships at the family level. What is inferable is that the melanocortin system seems to be fairly adaptable for regulating electric signaling behavior, and downstream effectors at the electrocyte level have likely undergone many species-specific shifts. This speaks to the physiological diversity of the electrogenic system and how it has rapidly evolved with the ecologically and taxonomically diverse Gymnotiformes. Whether melanocortin-mediated waveform plasticity was present in the common ancestor of extant gymnotiforms or convergently evolved and then lost in certain lineages remains unknown.

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CHAPTER III

DERIVED LOSS OF SIGNAL PLASTICITY IN A GENUS OF WEAKLY ELECTRIC FISH

Introduction

Signal plasticity is one means by which organisms can reduce costs associated with communication. This is especially true for multi-functional signals such as the echolocation calls of bats and cetaceans or the electric organ discharges (EODs) of weakly electric fishes. Few studies have examined the evolution and maintenance of signal plasticity in a phylogenetic context. Here I compare signal plasticity in a genus of weakly electric fish and report a case in which this plasticity is significantly reduced, seemingly, at great metabolic costs.

Across the gymnotiform phylogeny, lineages have evolved a large diversity of signals that vary in parameters such as the discharge rate and waveform shape (Albert & Crampton 2005). The impressive diversity of gymnotiform EOD waveforms is due in part to differences in electrocyte morphology and innervation, as well as the diversity, kinetics, and spatial distribution of the ion channels and transporters in these excitable cells (Markham 2013; Markham & Stoddard 2013). Despite its high species diversity and wide geographical range in the Neotropics, the genus *Brachyhypopomus* (Hypopomidae) is well studied relative to other gymnotiforms, especially in terms of its phylogeny (Fig. 1) (Crampton et al. 2016a, b) and ecology (Waddell et al. 2019), making

these fishes particularly suitable for comparative evolution and neurophysiology research.

Of the 28 *Brachyhypopomus* species described, the EOD waveforms of 18 species are known, 12 of which are biphasic (Fig. 7– "*B. gauderio"*), 5 are multiphasic, and 1 is monophasic (Fig. 7– "*B. bennetti"*). Monophasy is a rare characteristic among weakly electric fishes, presumably because of its potential detection by electroreceptive predators (Stoddard 1999; Stoddard & Markham 2008). Of the larval *Brachyhypopomus* species recorded, all of them begin generating monophasic EODs and transition to more complex waveforms as they mature (Franchina 1997, Crampton et al. 2016b). This is thought to be the case for all gymnotiform fishes, and phylogenetically, the monophasic EOD is considered the ancestral trait (Stoddard 2002), though this has not been confirmed due to phylogenetic uncertainties between the families (Arnegard et al. 2010; Lovejoy et al. 2010; Tagliacollo et al. 2016; Alda et al. 2018). In some gymnotiforms, crucial signal parameters, such as phase amplitude and duration, can vary by sex and ontogeny, and are regulated in response to seasonal, circadian (Fig. 7), and social cues (Silva et al. 2002; Silva et al. 2007; Markham et al. 2009). As discussed in the previous chapter, this plasticity is regulated by steroid and peptide hormones, with peptide hormones playing a major role in regulating short-term circadian and social changes (Allee et al. 2009). Here I show how one form of short-term signaling plasticity regulated by melanocortin hormones varies between closely related species of *Brachyhypopomus*.

Previous work in *Brachyhypopomus gauderio* and *Sternopygus macrurus* has shown that these fish increase EOD amplitude in response to circadian cycles and social cues (Fig. 7), and that this amplitude plasticity is regulated by melanocortin hormones, such as adrenocorticotropic hormone (ACTH) (Markham & Stoddard 2005; Markham et al. 2009). However, as discussed in the previous chapter, the mechanism behind this increase in amplitude could differ between species and even ontogenetically or sexually within a species (Markham & Stoddard 2013). In *S. macrurus*, ACTH-mediated increases in EOD amplitude occur by trafficking vesicles containing ion channels to the electrocyte membranes (Markham et al. 2009). Higher densities of voltage-gated sodium channels in the membranes allow for a greater influx of sodium during each action potential, producing an increased amplitude in the μEOD and a greater amplitude for the EOD overall. In *B. gauderio*, increases in EOD amplitude are differentially regulated according to sex and developmental state. In males and juveniles, this occurs by increasing the delay between the overlapping posterior and anterior action potentials (AP1 and AP2, respectively). Decreasing the overlap between these action potentials allows AP1 to contribute more to the μEOD, increasing the amplitude of the EOD. Females *B. gauderio* might use a mechanism similar to that of *S. macrurus.*

To better understand the function and physiology of this EOD plasticity, I compared the effects of ACTH in three other species of *Brachyhypopomus*. I focused on one species in particular, *B. bennetti*, the only congener that possesses a monophasic EOD. This species is particularly interesting because, unlike its biphasic relative *B. gauderio* (and presumably other biphasic congeners), this species might have an EOD

driven entirely by a sodium current. I further hypothesize that reversion to a monophasic EOD means the voltage-gated sodium channels are only found on the posterior membrane, and that there is little to no excitation of the anterior membrane, similar to what has been shown in the monophasic electric eel, *Electrophorus electricus*. To study the effects of ACTH-related plasticity and test these hypotheses, I used multiple techniques, including in vivo recordings and hormonal injections, in vitro electrophysiology (current and voltage clamping), and immunolocalization. Also discussed are results from a computational model, developed by Dr. Michael Markham (Saenz et al. 2020 *in prep*), that manipulates experimentally recorded values to test what changes in electrocyte kinetics might approximate the observed effects of ACTH.

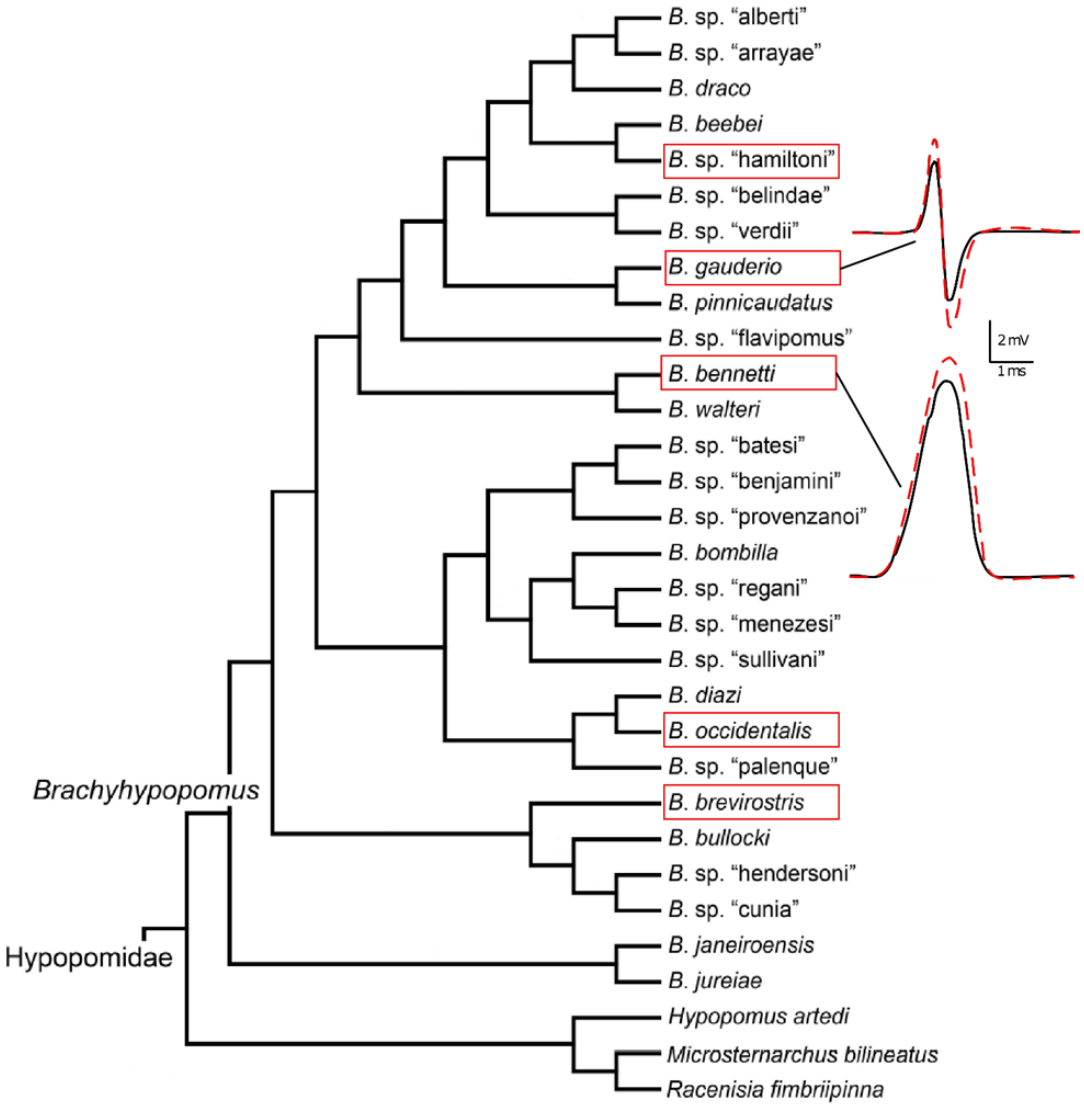

Figure 7. Current *Brachyhypopomus* phylogeny adapted with permission from Crampton et al. 2016a. Species in red boxes have been shown to display circadian changes in EOD waveform. *B. occidentalis* was recorded by Hagedorn (1995). Insets show the EOD waveforms of two species, the biphasic *B. gauderio* and monophasic *B. bennetti*. Red dashed lines show nightly waveform changes. Note the total magnitude of EOD amplitude is much larger in *B. bennetti*, however the magnitude of the change is much smaller.

Materials and Methods

Animals

Fish were wild caught from Brazil and obtained from Rehoboth Aquatics with the exception of *B. gauderio,* which were captive-bred from colonies maintained at the University of Oklahoma. All methods were approved in advance by the Institutional Animal Care and Use Committees of Texas A&M University and the University of Oklahoma.

Immunohistochemistry

Sections of electric organ were embedded in OCT compound, flash frozen in isopentane (MilliporeSigma) chilled with liquid nitrogen, and kept at -80 ºC until further use. Tissue was sliced in longitudinal sections (15-20 µm thick) at -25ºC using a cabinet cryostat (Leica CM 1900) and mounted on gelatin-subbed slides. The slides were dried overnight at RT and then fixed in PBS containing 4% paraformaldehyde (Electron Microscopy Services) for 20 minutes in an incubator at RT. Tissue sections were then washed three times in PBS containing 0.05% Tween-20 (MilliporeSigma) (PBST) for 10 min per wash. Next, they were blocked in an incubator with PBST containing 2% bovine serum albumin (BSA) and 5% goat normal serum (Jackson ImmunoResearch) for 1 hour at RT, and then incubated overnight with primary antibodies diluted in PBST at 4ºC. Control slides were incubated in PBST without primary antibodies. Following primary antibody incubation, tissue sections were washed three times for 10 min each in PBST, then incubated for 1 hour at RT in PBST containing 1:200 Alexa Fluor®488 or 594-conjugated secondary antibodies (Jackson ImmunoResearch). The slides were then

washed in PBST again as above, air dried, and mounted using VectaShield® with DAPI (Vector Laboratories). Slides were kept in the dark at 4 ºC until imaged on a Zeiss ApoTime.2 microscope with 5x/0.16NA, 10x/0.45NA, and 20x/0.80NA dry objectives.

Images were acquired using a Zeiss AxioCam MRm and then processed by Zeiss AxioVision Rel.4.8. I created optical sections of the fluorescent samples using structured illumination. Images were further processed using ImageJ-win64 version 1.52 (NIH). To label voltage-gated Na^+ channels, I used rabbit polyclonal antibody (1:100) against an intracellular epitope of $Na_v1.x$ channels (Anti-Pan Na_v, obtained from Alomone Labs). I labeled Na^+/K^+ ATPase using a mouse monoclonal antibody (1:100) against the α subunit of Na^+/K^+ ATPase (a5, developed by D.M. Fambrough (Lebovitz et al., 1989), and obtained from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa). I labeled acetylcholine receptors using a rat antibody (1:10) against the muscle-type acetylcholine nicotinic receptor (mAb 35, developed by J. Lindstrom (Tzartos et al., 1981), and obtained from DSHB). Lastly, I labeled axon terminals using a mouse monoclonal antibody (1:100) against neurofilament-associated antigen (3A10, developed by T. Jessel and J. Dodd, and obtained from DSHB).

Injections and recordings

EODs were recorded using a pair of silver electrodes on either side of the fish. EODs were amplified using a BMA 200 AC/DC Bioamplifier (CWE, Inc., USA) and digitized at 16 bits resolution using an A/D converter (CE Datatranslation USB Data Acquisition, USA) at a sampling rate of at least 50 kHz. Baseline EOD recordings were made for a minimum of 30 minutes. Fish were then quickly removed from the tank and

given an intramuscular injection (1µl/g) of either ACTH or saline in a process that took less than 15 seconds. For more details, see supplemental material.

Circadian recordings

Circadian recordings were conducted in a lab at the University of Oklahoma using an automated system for recording calibrated EODs from freely swimming fish described in full detail by Stoddard et al. (2003). Briefly, fish were placed in the automated measurement tank (300L) with nichrome recording electrodes on either side. The tank was partitioned into three sections by mesh dividers and an unglazed ceramic tube in the center for fish to swim through. Fish passing through the tube or resting inside it were detected using custom-developed circuitry. Detection of the fish elicited EOD amplification and digitization. The automated measuring tank was located in a light and temperature-controlled room on a 12:12D light cycle. EODs were recorded at intervals of approximately 1 min for multiple days to assess circadian variation in EOD waveform.

Solutions

The normal saline contained 114 NaCl, 2 KCl, 4 $CaCl_2 \cdot 2H_20$, 2 $MgCl_2 \cdot 6H20$, 5 HEPES, 6 glucose; pH to 7.2 with NaOH. However, for voltage clamp experiments with *B. bennetti* electrocytes, the NaCl was substituted with choline to achieve a sodium concentration of ~ 14 mM. Adrenocorticotropic hormone (ACTH from porcine pituitary) was purchased from Sigma Aldrich (St. Louis, MO). The collagenase was prepared in a saline solution and was obtained from Worthington Biochemical, Lakewood, NJ.

Electrophysiology

To record discharges from single electrocytes (μEODs), I removed a small piece of the tail (~ 1cm) and carefully dissected the skin off to expose electric organ. This step was done without using anesthesia, which causes more harm to a gymnotiform fish than does a tail clip. Gymnotiform fishes regenerate their tails and specimens captured from natural habitats frequently have their tails missing or have tails in various stages of regeneration (Winemiller, personal communication and Saenz, personal observation). The tissue was pinned into a Sylgard-coated recording dish with a saline solution containing 2% collagenase for 45 minutes to weaken the tissue surrounding the electrocytes. The preparation was then flushed several times with normal saline at RT (23 ± 1 ºC) over a period of at least 15 minutes before recording.

Current clamp: Intracellular stimulation and recordings were completed using an Axoclamp 900A amplifier (Molecular Devices, Union City, CA) and extracellular recordings were completed using a Dagan TEV200A amplifier (Dagan Corp, USA) in current clamp mode. A Digidata 1440 interface and PClamp 10.0 software (Molecular Devices) were used to control all amplifiers. Data were sampled and digitized at a rate of 100 kHz. Extracellular pipettes had resistances between 400-600 KΩ when filled with normal saline, and intracellular pipettes had resistances between 0.8-1.2 MΩ when filled with 3 M KCl.

I used a multi-electrode current clamp to record action potentials from single electrocyte membranes in a procedure described in more detail elsewhere (Bennett 1961; Markham & Stoddard 2005, Markham & Zakon 2014). One intracellular pipette

delivered a depolarizing current step to elicit the μEOD. A second intracellular pipette recorded the intracellular potential, and two extracellular pipettes placed within 50 μm of the posterior and anterior membranes recorded extracellular potentials, one from each membrane. Off-line subtraction of the posterior extracellular record and the anterior extracellular record from the intracellular record result in AP1 and AP2, respectively. Subtraction of the posterior extracellular record from the anterior extracellular record returns the μEOD.

Only electrocytes with stable resting potentials and input resistances were recorded. Once all the electrodes were in place, I delivered depolarizing current steps while manually adjusting the current magnitude until a μEOD was dependably elicited. For one preparation of *B. bennetti* tissue, this was as high was 25,000 nA. A baseline recording was made of μEODs every 60 seconds for at least 15 minutes, after which I perfused normal saline for control cells or normal saline containing 100 nM of ACTH. Solutions were changed during the interstimulus interval with a quick perfusion of 5 mL followed by slow continuous perfusion at 5 mL/h. I recorded μEODs at 60 second intervals for the remainder of the experiment.

Two-electrode voltage clamp: From a holding voltage of -90 mV, the voltage clamp protocol to assess Na^+ current (I_{Na}) activation and inactivation consisted of a 50 ms conditioning step to -120 mV, followed by 20 ms voltage steps from -120 to 25 mV in 5 mV increments, and then a 20 ms step to 0 mV. Recovery of I_{Na} from inactivation was assessed with a protocol that consisted of a 50 ms conditioning step to -120 mV

followed by an activation step to 0 mv and a recovery step to -120 mV for 0.5 ms to 12.5 ms in 0.5 ms steps and then a step to a test potential of 0 mV for 20 ms.

Data analysis

For in vivo experiments, paired t-tests were used to compare percent changes in EOD parameters (relative to baseline) between in ACTH and saline. The same was done for the current clamp data. Unfortunately, I was unable to conduct statistical analyses for the injection data on *B.* cf. *hamiltoni* or for current clamp data on *B. brevirostris* because I only had one individual for each experiment.

Results

Immunohistochemistry confirmed the site of innervation in *B. bennetti* and *B. gauderio*. Figure 8 A&D shows the single innervation of the caudal stalks in both species. As expected in *B. gauderio*, the Na^+ channels and the Na^+-K^+-ATPases were found on both the posterior and anterior membranes (Fig. 8 E-F). Contrary to my expectations, both the Na^+ channels and the Na^+-K^+-ATPases were also found on the posterior and anterior membranes of *B. bennetti* (Fig. 8 B-C). Whereas the density of Na^+ channels is far greater on the posterior membrane, the Na^+-K^+-ATPases are present in large quantities at both membranes.

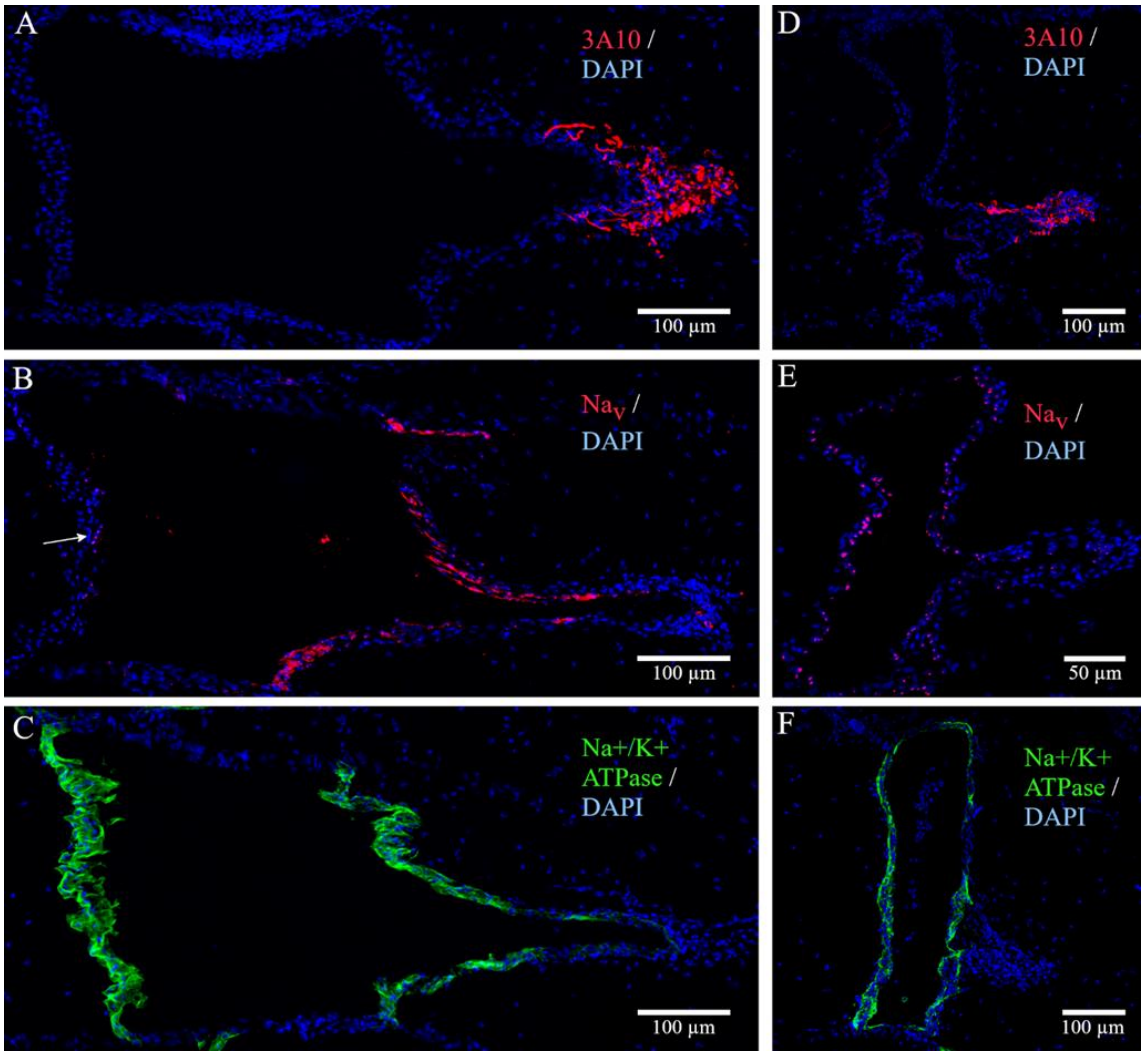

Figure 8. Immunohistochemistry staining of Na^+ channels and Na^+-K^+-ATPases in *Brachyhypopomus* electrocytes. DAPI (blue) labels the electrocyte nuclei and provides a relative outline of the cells shown anterior (left) to posterior (right) with the caudal stalk prominently visible. A-C: electrocytes from the monophasic species, *B. bennetti*. D-F: electrocytes from the biphasic species, *B. gauderio*. **A, D**: in both species, the axons of the spinal electromotor neurons, labeled with 3A10 (red), only innervate the electrocytes on the posterior side on the caudal stalk. **B, E**: expression patterns of voltage-gated sodium channels. The arrow in B indicates the presence of Na^+ channels on the anterior membrane, contrary to our expectations. **C, F**: in both species, Na^+-K^+-ATPases are abundant on both the anterior and posterior membranes.

Effects of ACTH in vivo

I found that intramuscular injections of ACTH significantly increased the overall EOD amplitude (P_1-P_2) in the biphasic species, *B. brevirostris*, and *B.* cf. *hamiltoni* (33.7 ± 4.7% and 16.1%), as has been shown for *B. gauderio* (21.9 ± 4.2% Markham & Stoddard 2005). Separate evaluation of the phases in *B. brevirostris* showed that both P_1 and P_2 increased, and P_1 always increased more than P_2 ($t=2.23$, $p = 0.056$). On average, ACTH also increased EOD amplitude in *B. bennetti*, however, the effect was considerably weaker (Fig. 9; $t = 2.45$, $p = 0.094$). Saline controls slightly decreased the EOD amplitude in all four species. The duration of both P_1 and P_2 (measured at 2% of the peak amplitude) increased in the biphasic species *B. brevirostris* ($p < 0.001$, $t = 2.57$) and *B.* cf. *hamiltoni.* The increase in duration was significantly larger for P_2 than for P_1 in *B. brevirostris* (Fig. 10) and still noticeably so for the single individual of *B.* cf. *hamiltoni*. In *B. bennetti*, the duration of the EOD did not change significantly relative to the saline control.

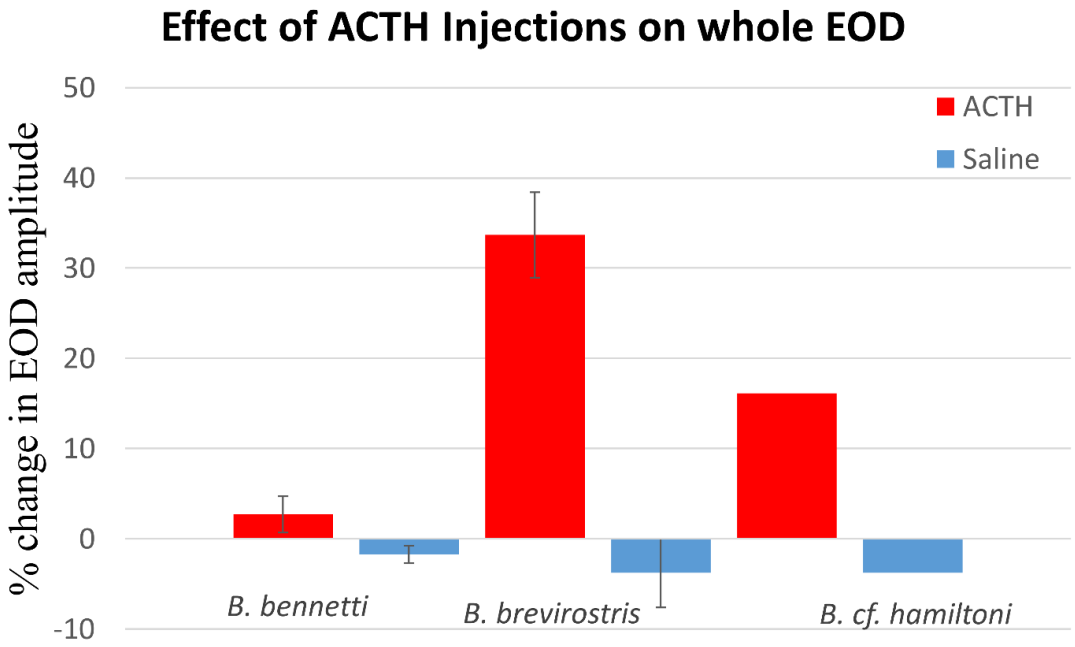

Figure 9. *In vivo* intramuscular injections of ACTH or saline control in 3 species of *Brachyhypopomus* (n=6, 6, & 1, respectively). Bars show the percent change in whole EOD amplitude.

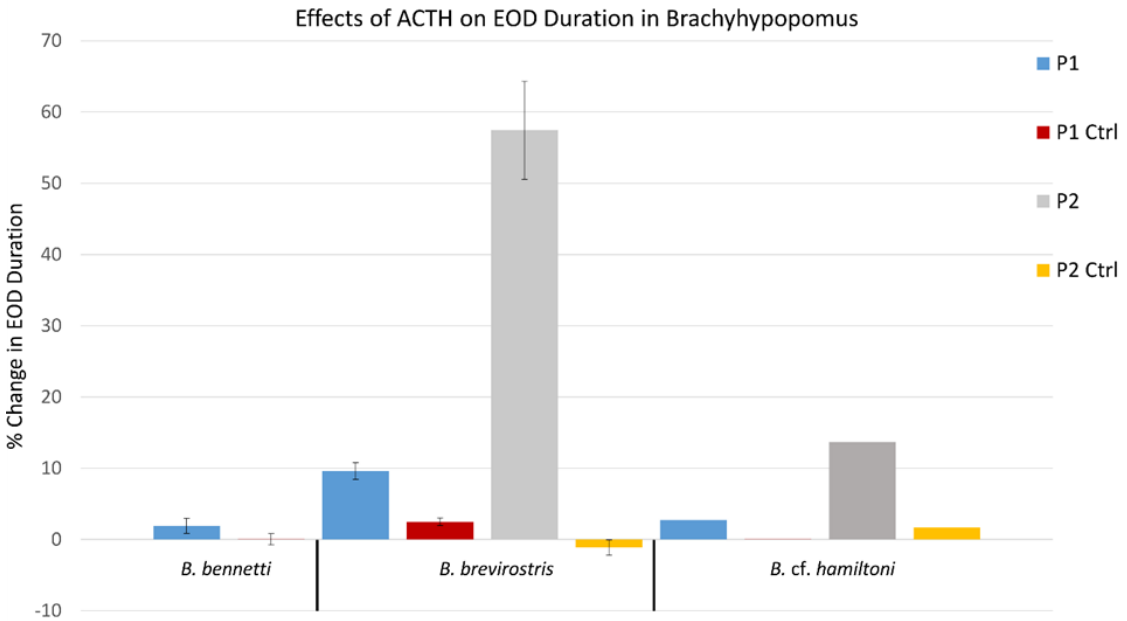

Figure 10. In vivo intramuscular injections in 3 species of *Brachyhypopomus* (n=6, 6, & 1, respectively). Bars show the percent change in the duration of individual EOD phases after application of either ACTH or saline control.

Effects of ACTH in vitro: Current clamp

Using the multi-electrode current clamp, I measured µEOD parameters from individual electrocytes of *B. brevirostris* and *B. bennetti*. Like previous studies have shown (Markham & Stoddard 2005; Markham et al. 2009; Markham et al. 2013) in *B. gauderio,* ACTH increased amplitudes of both P_1 and P_2 of the µEOD (primarily P_1) and concurrently the total µEOD amplitude as well as the half-width of P_2 (Fig. 11). Because only one individual was available for testing, I attempted to wash out the ACTH after 30 minutes with saline (60 min of the total recording). Although the effect of ACTH is typically strong and long lasting, during the washout I observed a slow decrease in the

amplitude of mainly P_1 over the course of the next hour. At minute 120, I reapplied ACTH and saw a small increase again in the amplitudes of both P_1 and P_2. I did not, however, observe the return of this small increase in the AP1-AP2 delay. The recording was stopped after 145 minutes because the cell was showing signs of degradation.

In *B. bennetti*, ACTH also increased the amplitude of the μEOD (Fig. 12). Similar to the in vivo experiments, the effect was much smaller relative to *B. gauderio* and *B. brevirostris*, but here it was more consistent. There was considerable variation among *B. bennetti* individuals, possibly due to sex differences, however we were unable to confirm the sex. Again, counter to my expectation, I observed action potentials on both the posterior and the anterior membrane. Even more surprising was the magnitude of AP2 which was approximately 40% of AP1 (Fig. 13). Perfusion of ACTH slightly increased both AP1 and AP2 (Fig. 13). No other parameters showed consistent changes in response to ACTH, but this could be due to the low sample size (n=4).

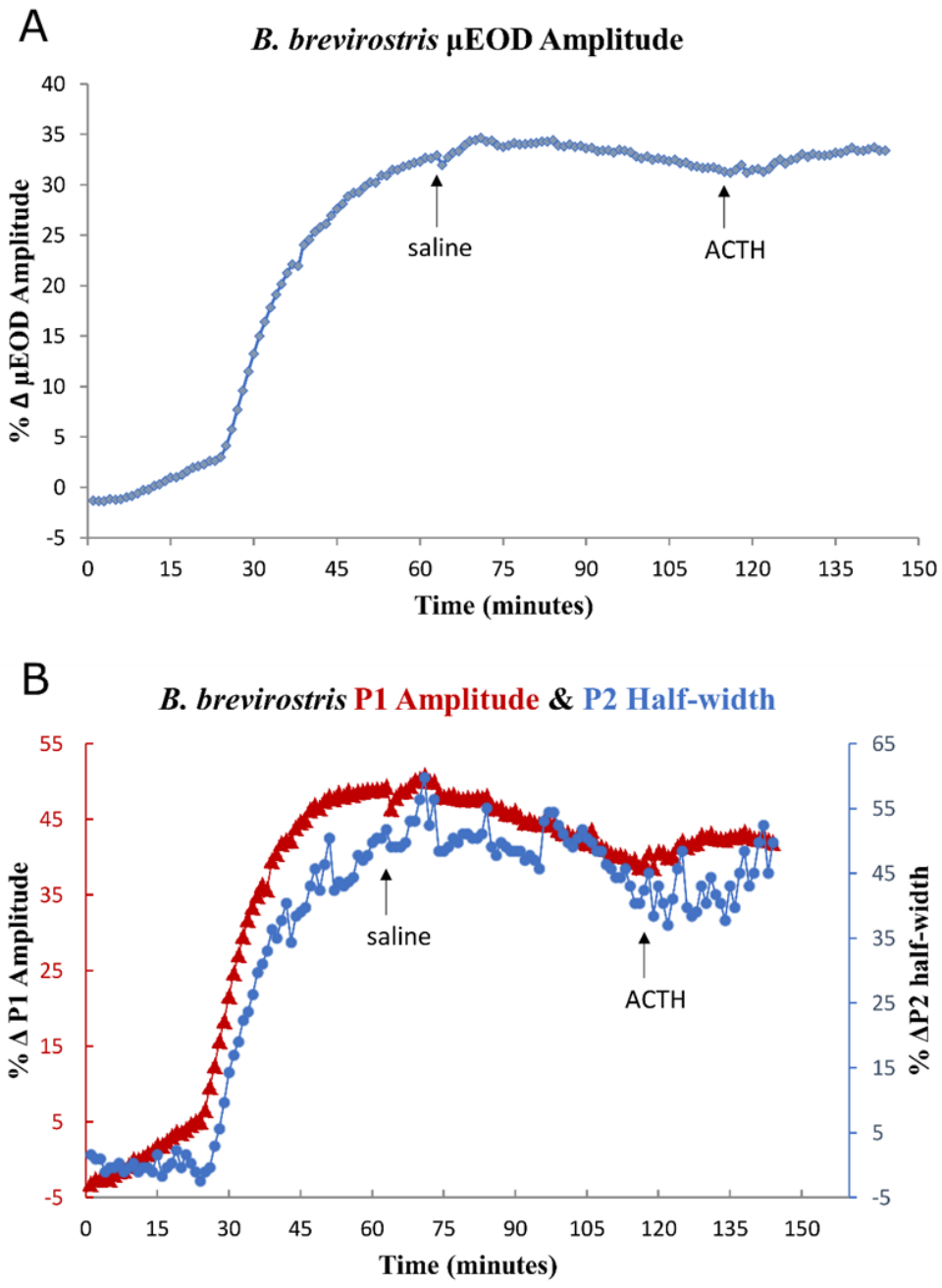

Figure 11. Current clamp data of *B. brevirostris*. ACTH was first applied after a 20-minute baseline recording. Because only one individual was available, we washed out the ACTH after 40 minutes (min 60) with saline. After another hour (min 115), we reapplied ACTH. **A,** Percent change in µEOD amplitude. **B,** Left vertical axis shows percent change in amplitude of P1 (parameter that changed the most). Right vertical axis shows percent change the half-width of P2. This has also been shown to occur in males of *B. gauderio*.

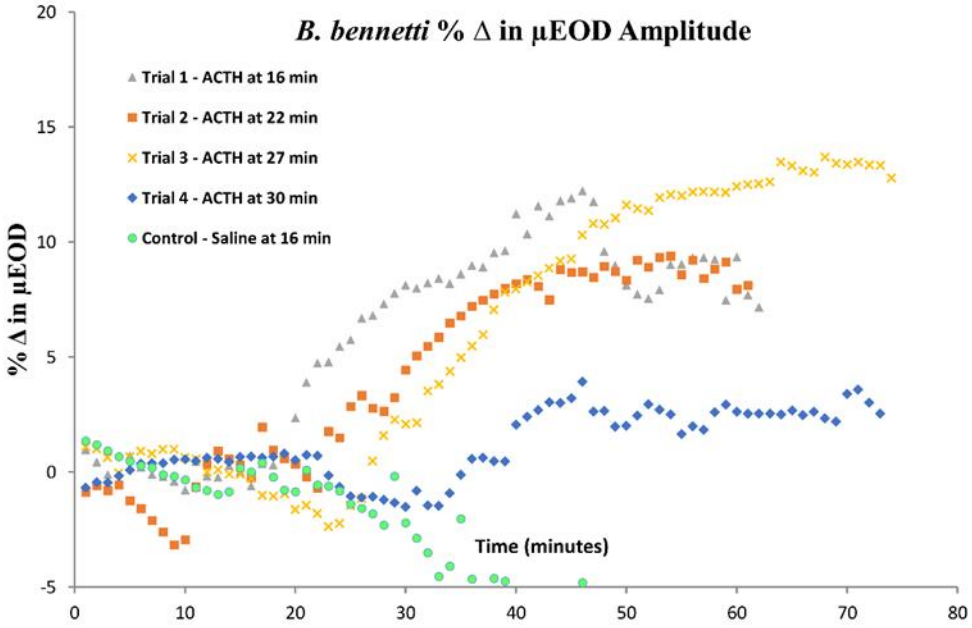

Figure 12. Current clamp data of *B. bennetti*, n=4. Each individual is represented by a different color and shape. ACTH was applied after recording a baseline amplitude. Because so few individuals were available, the time of application was staggered to further highlight any effects of the treatment. The control is shown in green circles. The decay below zero is due to natural degradation of the cell membrane during the recording process. While, ACTH does increase µEOD amplitude, the effect is very small relative to other *Brachyhypopomus*.

Ionic currents in Brachyhypopomus bennetti

Because only two individuals were available for testing, I focused on describing the sodium channels in *B. bennetti* electrocytes since these have not yet been described for any species of *Brachyhypopomus*. At normal extracellular Na^+ concentrations (114 mM), inward Na^+ currents were so large that voltage control could not be maintained during depolarizing voltage steps, even at the amplifier's maximum TEVC gain of 50,000 V/V. I therefore used a reduced-Na+ extracellular saline solution to reduce the

driving force on I_{Na} and allow stable voltage clamp control during I_{Na} activation. The reduced-Na^+ saline contained 14.25 mM Na^+ (choline substitution for 100 mM Na^+), 50 mM of TEA, and 5 mM BaCl to record isolated transient inward Na^+ currents (Fig. 14A). Inactivation and activation curves and time constants were consistent with previously described sodium currents in *Steatogenys elegans* (Rhamphichthyidae) (Fig. 14B&C) (Markham & Zakon 2014). Sodium currents showed typical voltage dependence of activation and inactivation as well as fast inactivation component (Fig. 14D). The recovery tau (τ = 3.80 ms) is the slowest for any gymnotiform species thus far reported. This is not unexpected considering *B. bennetti* has a very slow EOD frequency (<10 Hz).

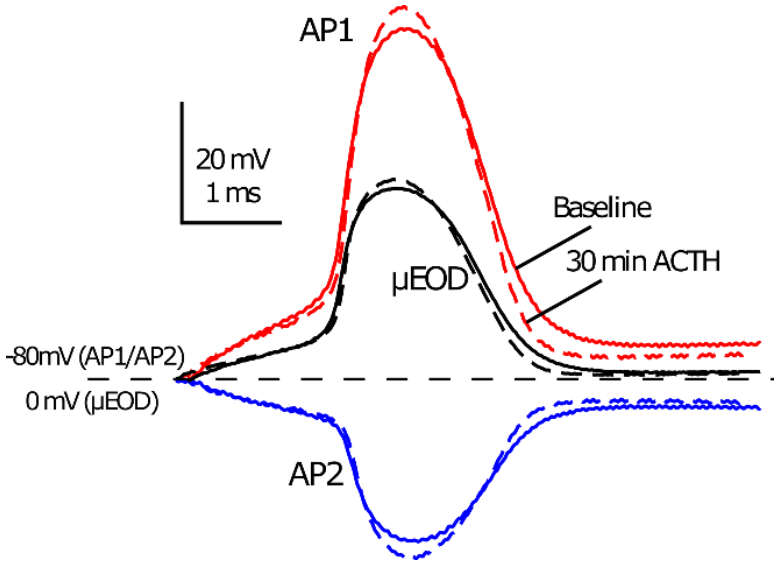

Figure 13. Multi-electrode current clamp recording of *B. bennetti* showing superimposed individual action potentials from each membrane. AP1 (red) is the potential difference from the extracellular posterior membrane voltage recording and the intracellular voltage recording. AP2 (blue) is the potential difference from the extracellular anterior membrane voltage recording and the intracellular voltage recording. The difference between the two extracellular potentials provides the whole-cell voltage and the µEOD (black). Dashed lines represent the same potentials 30 minutes after perfusion of ACTH.

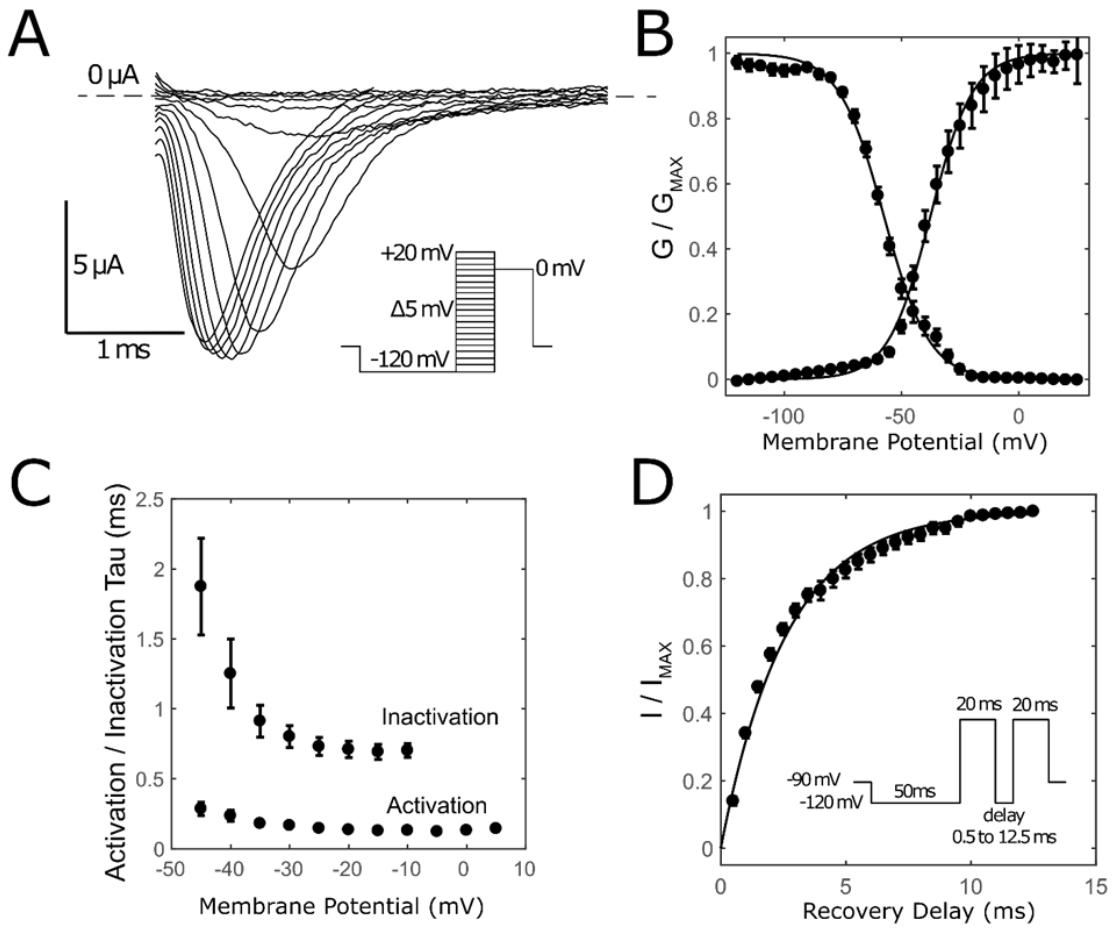

Figure 14. Voltage clamp recordings of *B. bennetti* from a holding potential of -90 mV. **A**, Currents induced by voltage steps from -120 to 20 mV in 5 mV increments show a transient inward current (protocol shown in inset also used for B & C). **B**, Inactivation and activation IV plots. **C**, Activation and inactivation time constants. **D**, Recovery from inactivation.

Electrocyte simulation

Using experimental data from the voltage clamp recordings, *B. bennetti* electrocytes were modeled using the Hodgkin-Huxley formalism to explore how ACTH

might induce the effects observed during the current clamp recordings (Fig. 15)[1]. Briefly, the electrocyte was modeled as a three-compartment cell with a passive central compartment coupled to two flanking active compartments (anterior and posterior). The central compartment contained only a passive leak conductance while the anterior compartment contained passive leak and a voltage-gated Na+ conductance constrained to experimentally-derived parameters with a maximum conductance of 150 mS/cm^2. The posterior compartment contained passive leak and the same voltage-gated Na+ conductance with a baseline maximum conductance of 400 mS/cm^2. External stimulation current was delivered in the central compartment, consistent with experimental procedure. The capacitance for the central compartment was 100 nA, and it was 50 nA for the anterior and posterior compartments, giving a total membrane capacitance of 200 nF. Differential equations were coded and integrated with Matlab (Natick, MA). While maintaining all other parameters equal to my experimentally recorded values, increasing the Na^+ conductance by 10% in both anterior and posterior compartments slightly increased AP1, AP2, and μEOD amplitude similar to the results observed during my experiments. Under these conditions, the simulated μEOD waveform was consistent with the experimentally recorded waveform, suggesting that increasing voltage-gated Na^+ conductance is sufficient to produce my results.

[1]Figure 15 was generously contributed by Michael Markham for a manuscript that is currently in prep (Saenz et al. 2020) and reproduced with his permission here. For details on the methods, see Saenz et al. (2020) and Markham & Zakon (2014).

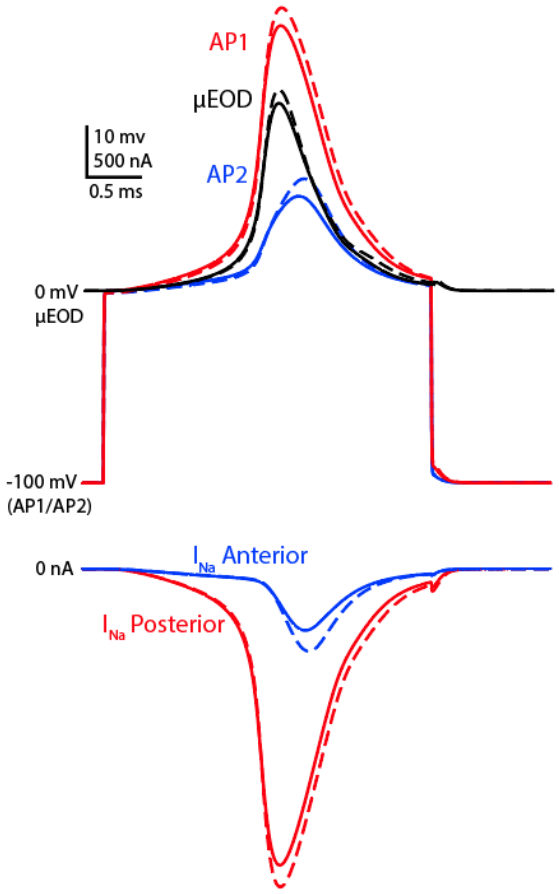

Figure 15. Simulated action potentials and µEOD of *B. bennetti*. For comparison, see experimental data from Figure 13. Top: AP1 (red), AP2 (blue), µEOD (black) are elicited by 0.5 ms depolarizing current steps. Bottom: conductance of voltage-gated sodium channels (I_{Na}) on the posterior (red) and anterior (blue) membranes. Dashed lines show the result of increasing I_{Na} on both membranes by 10%. AP1, AP2, and µEOD amplitudes increase similar to experimentally recorded results, suggesting these conditions are sufficient to reproduce the effects of ACTH in *B. bennetti* electrocytes. This figure was generously contributed by committee member Dr. Michael Markham.

Discussion

EOD plasticity in Brachyhypopomus

Compared to congeneric species that have been tested, *B. bennetti* revealed low EOD plasticity. Previous studies have shown how *B. gauderio* can significantly modify its EOD waveform in response to circadian and social cues via direct action of melanocortin peptide hormones on the electrocytes (Markham & Stoddard 2005; Allee et al. 2009; Markham et al. 2013). Circadian plasticity of the EOD waveform has also been reported in *B. occidentalis* (Hagedorn 1995). Here we report similar ACTH-mediated EOD plasticity in two additional species of *Brachyhypopomus (B. brevirostris* and *B.* cf. *hamiltoni)* and a reduction in waveform plasticity in *B. bennetti*.

The extent of this EOD plasticity and its regulation varies by sex and ontogeny in *B. gauderio* (e.g. ΔP2 amplitude is greater in males and P2 half-width only increases in males; Markham et al. 2013). Although my sample size is insufficient to confirm demographic variation for the species tested here, it is interesting that the magnitude of this plasticity remains consistent throughout the genus (Fig. 7), with *B. bennetti* as an exception. While fishes from some other gymnotiform genera exhibit EOD amplitude plasticity (McAnelly & Zakon 1996; Markham et al. 2009), others do not (see chapter 1). The function of this plasticity is not yet fully understood, but two probable roles are social and energetic. The active electrosensory system can be metabolically costly. Estimates of EOD production range from 4% to 22% of the daily metabolic budget in species with relatively slow EOD repetition rates, such as *B. gauderio* (Salazar & Stoddard 2008), and energetic cost may exceed 30% in species with higher repetition

rates, such as *Eigenmannia virescens* (Sternopygidae) (Lewis et al. 2014). Previous studies have reasonably argued that circadian regulation of EOD amplitude is adaptive for conserving energy. So why might *B. bennetti* lose such a trait, especially considering that the amplitude of *B. bennetti* EODs is 3 to 8 times larger than those of sympatric congeners (see Fig. 7) (Crampton & Albert 2006)?

The curious case of the monophasic fish

Another surprising finding was the magnitude of the discharge from the anterior, un-innervated face of *B. bennetti* tail electrocytes and the presence of voltage-gated sodium channels on the same membrane. Here, I specify "tail electrocytes", because in some multiphasic gymnotiforms it has been suggested that only the tail electrocytes are biphasic and that electrocytes closer to the head are monophasic. This also could be the case for *B. bennetti*. I showed that while the EOD of *B. bennetti* is monophasic, the μEOD is still composed of two action potentials (Fig. 7). Because they completely overlap, the discharge from the anterior face (AP2) cancels out up to 40% of the initial posterior action potential (AP1), seemingly wasting considerable energy. Bennett (1971) recorded electrocytes from a monophasic hypopomid, likely *B. bennetti,* but argues that the anterior membrane is electrically inexcitable. Instead, it is suggested that a smaller spike from the un-innervated membrane is a discharge of the membrane's capacitance, similar to what has been shown in the un-innervated face of electrocytes in *Gymnarchus niloticus,* an African weakly electric fish. My finding that voltage-gated sodium channels are indeed present on the anterior face (Fig. 8B) suggests the membrane is excitable and that the spike is the action of voltage-gated sodium channels. This is further supported

by the results of the computational simulation, which showed that two overlapping APs with similar kinetics can produce the µEOD waveform observed in *B. bennetti*.

In some biphasic gymnotiforms, multiple action potentials are achieved via the innervation of both the anterior and posterior membrane. In species of *Brachyhypopomus* studied thus far, the electromotor neurons only innervate a caudal stalk and the second action potential on the anterior side is achieved via auto-excitation. Immunolabeling results also reaffirm Bennett's (1971) suggestion that the stalk is more important for spike generation than the innervated face. I found generally high densities of Na^+ channels on the stalks relative to the posterior face. This highlights another yet-unanswered question about the physiological purpose of these stalks, which are not found in all gymnotiforms. Furthermore, the magnitude of AP2 and the presence of Na^+ channels on the anterior membrane provide evidence against the long-standing assumption that *B. bennetti*'s EOD is a retention of the paedomorphic monophasic condition. Is this a costly vestige of *B. bennetti*'s biphasic ancestry, or does AP2 serve some unknown function? With regard to biphasic species of *Brachyhypopomus*, Stoddard & Markham (2008) hypothesized that the outward flowing K^+ ions from AP1 and the inward flowing Na^+ ions from AP2 sum to form a larger current for P2 (see Box 1 in Stoddard & Markham 2008), potentially saving energy in the overlap of APs. This strategy would clearly not benefit the monophasic *B. bennetti.*

In *B. gauderio* males (but not females), ACTH increases EOD amplitude by increasing the AP1-AP2 delay via a cAMP/protein-kinase A-regulated pathway (Markham & Stoddard 2005, 2013). Increasing the delay between APs decreases their

overlap, thereby increasing the μEOD amplitude. The mechanism for maintaining this highly precise delay has only been studied in *S. elegans*, in which a ~30 μs delay between APs is probably maintained by a difference in the activation voltage for Na^+ currents between the two membranes. This could occur by having different sodium channel isoforms on each membrane, but this has not been studied further (Markham & Zakon 2014). Interestingly, the EOD of *S. elegans* does not seem to be greatly affected by ACTH (see chapter 1). In *B. bennetti*, the lack of the AP1-AP2 delay is what creates the monophasic waveform. The computational model shows that increasing Na^+ conductance on both membranes is sufficient to reproduce the effects of ACTH without modifying the timing between the two APs in a way that impacts the μEOD waveform. If the AP1-AP2 delay is maintained by different Na^+ channel isoforms in other species, this suggests that *B. bennetti* may only have one sodium channel isoform. So how does ACTH elicit the small increase in μEOD amplitude in *B. bennetti* electrocytes if not by increasing the AP1-AP2 delay? It is possible that ACTH triggers a second messenger pathway that traffics vesicles of sodium channels to the electrocyte membranes in a manner similar to what has been demonstrated in *S. macrurus.* Further study of *B. bennetti* could help elucidate the full range of mechanisms involved in electrocyte physiology.

Predator avoidance

The lack of EOD plasticity exhibited by *B. bennetti* stimulates further debate concerning the adaptive vs. maladaptive value of the monophasic EOD. Evidence from models and controlled experiments suggest the biphasic EOD is an adaptation in

response to predation pressure from eavesdropping electroreceptive predators (i.e. catfishes Hanika & Kramer 1999, 2000; Stoddard 1999), and this trait was subsequently modified by other evolutionary forces, such as genetic drift, sexual selection, and reproductive character displacement (Arnegard et al. 2010; Crampton et al. 2011; Picq et al. 2016). The second phase of the biphasic EOD is slightly delayed and therefore sums with the first phase at a short distance away from the fish. This attenuates the low-frequency DC component of the EOD detectible by the ampullary receptors of catfishes. Consequently, at greater distances, DC-balanced EODs are theoretically not detectible by electroreceptive predators (Stoddard 1999; Stoddard & Markham 2008; Stoddard et al. 2019). The monophasic EOD represents the asymmetrical extreme, with a large DC component that potentially carries significant costs due to its detectability by electroreceptive predators.

Previous studies have suggested that *B. bennetti*'s EOD functions as a Batesian mimic of the Electric Eel's monophasic EOD (Stoddard 1999). This fish is the only gymnotiform capable of producing a strong electric discharge to incapacitate predators and prey alike. Crampton & Albert (2006) argue that this mimicry is not a likely evolutionarily stable strategy due to a large discrepancy in the relative abundance of *B. bennetti* and *E. electricus*, the former being three orders of magnitude more abundant than the latter. In fact, *B. bennetti* is purportedly one of the most common gymnotiforms in the Amazon basin (Crampton et al. 2013, 2016b). Instead, Crampton & Albert (2006) suggested that the monophasic EOD of *B. bennetti* is associated with species recognition. Acknowledging that the mimicry hypothesis is feasible, Sullivan et al.

(2013) noted that specimens of *B. bennetti* are often caught with damaged or regenerated tails at frequencies similar to those of a sympatric biphasic congener *B. walteri*. This suggests that monophasic EOD mimicry is not particularly effective at preventing "tail-grazing." The identity of these tail-grazers is poorly documented; however, most of these predators probably are non-electroreceptive. Major predators of *Brachyhypopomus* include piranhas (*Pygocentrus* and *Serrasalmus* spp,) and traíras (*Hoplias* spp.) (Winemiller unpublished data). Furthermore, the large electroreceptive catfishes that reportedly feed on gymnotiforms use suction to ingest prey whole, and typically do not tear off pieces of flesh when feeding (Westneat 2004). During aggressive encounters in captivity, several species including *B. brevirostris*, will bite caudal appendages off of adversaries (Kirschbaum & Schugardt 2002 and personal observations) so tail-grazing could also be evidence of intraspecific competition.

Sullivan et al. (2013) point out that damaging the tail of a biphasic species removes a portion of P2, eliminating the "cloaking" feature by increasing the low frequency component of the EOD and potentially impeding communication and identification by other conspecifics. Removing the tail of a monophasic fish does not alter the EOD waveform, preserving the species-specific signal, thus supporting the species recognition hypothesis (Sullivan et al. 2013). Interestingly however, Tran (2014) showed that *B. occidentalis* captured in the field with damaged or regenerating tails did not have EODs that differed significantly from intact conspecifics. Though experimentally removing the tails shows an immediate effect on the EOD waveform, fish were able to produce normal EODs just a few days later, before the tail even began

regenerating (Tran 2014). It's possible that this mechanism of plasticity is similar to that of the other hypopomids described here and by Markham et al. (2009), involving ion channel trafficking.

Other cases of monophasy

Given its apparent waste of energy and potential advertisement to electroreceptive predators, the monophasic signal of *B. bennetti* is puzzling. There are five other species of gymnotiform with intermittent or pulse-type monophasic EODs, all from the family Gymnotidae, (as well as the sister taxon *Electrophorus* which need not worry about electroreceptive predators). Though now it seems plausible to suggest these species may not have monophasic μEODs, and could retain a second AP like *B. bennetti*. At least two *Gymnotus* species occur in seasonally hypoxic habitats without major electroreceptive predators. Therefore, predator release leading to relaxed selection has been suggested as one explanation for the reversion to monophasic EODs in *Gymnotus* (Brochu 2011; Crampton et al. 2013). In combination with predator release, sensory bias and a female preference for signals with low frequency energy could further explain this reversion toward a monophasic signal. Sexual dimorphism has been documented in a few species of *Gymnotus* as well as several species of *Brachyhypopommus.*

According to Crampton & Albert (2006), *B. bennetti* males produce EODs with amplitudes 2-3 times larger than females of comparable sizes, likely because they possess relatively larger sized electrocytes compared to juveniles and females (Crampton et al. 2016b). Building on the hypothesis of species recognition, I suggest that the monophasic EOD of *B. bennetti* could be a result of sexual selection. Consistent with the

handicap hypothesis (Zahavi 1975), I propose that female preference for a large amplitude has selected for a costly signal both in terms of energetics and susceptibility to predation. A mate choice study on *B. gauderio* suggests that EOD amplitude is the most salient EOD feature used by females to assess potential mates (Curtis and Stoddard 2003). In *B. occidentalis*, males with the largest EOD amplitude and lowest peak-power frequency maintain dominant status in paired male interactions (Hagedorn & Zelick 1989). A fish's maximum EOD amplitude is largely a function of size (within a species), with larger fish having larger EO's with more electrocytes that produce larger EODs. Larger amplitude also implies greater metabolic costs (i.e., larger APs require more ATP to restore electrocyte membrane potential per EOD), meaning a large amplitude could serve as an honest indicator of male condition. Additionally, larger males of sexually dimorphic species may be able to obtain more food and defend higher-quality territories (Johnsson et al. 2005; Serrano-Meneses et al. 2007). In populations with higher densities, *B. gauderio* maintain higher EOD amplitudes (Gavassa et al. 2012), suggesting competition influences basal EOD amplitude. Even though *B. gauderio* can enhance their EOD amplitudes, the extent to which they are able to do so depends on their basal amplitude rather than social context. Gavassa et al. (2012) proposed that this EOD plasticity could be a form of handicap disposal, allowing individuals in low density populations to expend less energy.

Perhaps in combination with a female preference for monophasic signals with large amplitudes, high population densities and high levels of intraspecific competition in *B. bennetti* have been sufficient to render extreme EOD plasticity impractical,

reinforcing both the handicap and signal reliability. Despite that *B. bennetti* is abundant in the Amazon basin (Crampton et al. 2016b), little is known about its population structure. Mate choice studies are needed to test whether or not female preference is based on EOD amplitude. Nonetheless, since amplitude is actively regulated and seems to play an important role in other species of *Brachyhypopomus*, it seems possible that female preference for larger amplitude could be a phylogenetically conserved trait in the genus. When placed in a phylogenetic context, mate choice can reveal strong evolutionary conservatism in contemporary preferences (Ryan & Rand 1995).

Conclusion

This chapter investigated the physiological underpinnings of variation in signal plasticity within a genus of weakly electric fish. Even though a major mechanism that regulates EOD amplitude seems to be strongly conserved throughout the genus, I found that there is at least one exception. I showed that *B. bennetti* demonstrates reduced EOD plasticity, the ion channel distribution that has been presumed for its electrocytes is incorrect, and the actual distribution implies its signal is energetically inefficient.

This is not the first instance in which major assumptions of ion channel distributions have been wrong. Until recently, it was assumed that AP generating ion channels were restricted to the posterior innervated face of electrocytes in *Eigenmannia virescens* (Sternopygidae), however this has been shown not to be the case. A recent study revealed that sodium-activated potassium channels on the anterior membrane can be activated by Na^+ influx from over a millimeter away (Ban et al. 2015). Clearly, our understanding of electrocyte physiology is incomplete. Since the early studies on the

Electric Eel (Ellisman & Levinson 1982; Fritz et al. 1983), researchers have recognized the importance of the heterogenous distribution of ion channels in electrocytes, and yet little is known about the regulatory mechanisms contributing to their localization. In many cases, the density and distribution of specific ion channels is critical for the function of nerve cells, such as in the nodes of Ranvier (Schultz et al. 2008) or the axon initial segment (AIS) (Kole et al. 2008), and abnormalities in channel localization can lead to neural pathologies. For example, irregular distributions of sodium channels in axons are associated with painful neuromas (England et al. 1996). Further study of electrocyte ion channels in weakly electric fishes could improve our understanding of the mechanisms regulating ion channel localization as well as their evolution.

Findings from this chapter reveal the need for a better understanding of the ecology of gymnotiform fishes. Given the broad geographical distribution and abundance of *B. bennetti* (see Fig. 14 of Crampton et al 2016b.), it seems the energetic and predation handicaps considered here have not hindered its ecological success and suggest influences from other aspects of its ecology, such as mate choice and intraspecific competition. Clearly, a great deal of ecological research is needed, but we could be running out of time. Due to their unique sensory system, weakly electric fishes are particularly vulnerable to environmental degradation, including effects of pollution, climate change and watershed alteration, all of which are currently occurring in the Amazon (Markham et al. 2016).

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CHAPTER IV

DOES ELECTRORECEPTOR DISTRIBUTION IN GYMNOTIFORM FISHES CORRESPOND WITH THEIR ECOLOGY?

Introduction

Sensory systems undergo strong selection to optimize the means by which organisms obtain information about their environments. Organisms rely on sensory systems for orientation, detecting food, finding shelter, locating potential mates and rivals, and avoiding danger. Environmental conditions strongly influence the evolution of sensory systems. A clear example of this is provided by cave or subterranean habitats where organisms largely depend on auditory, olfactory, and mechanosensory systems, accompanied by reduction or even complete loss of vision. For most species in most habitats, the effects are more nuanced than this example. Studies manipulating the light environment have demonstrated how critical aspects of vision, such as lens properties (Kröger et al., 2001), opsin expression (Fuller et al., 2005) and the relative abundance of photoreceptor cells (Shand et al., 2008) and their neural connectivity (Wagner and Kröger, 2005), can vary. Whereas some sensory modalities such as vision are fairly well studied, the influence of environmental variation on electroreception is not as well understood.

Electroreception is an ancient sensory modality that has been lost and re-evolved in multiple lineages. One class of electroreceptors, known as ampullary receptors, are highly sensitive to DC and low-frequency AC electric fields. They can be found in

elasmobranchs, lungfishes, and coelacanths as well as some non-teleost actinopterygians, teleosts, amphibians, monotremes, and even a freshwater dolphin (Bullock et al., 2006; Czech-Damal et al., 2012). Comparative studies of elasmobranchs have shown that the organization of electroreceptors are strongly associated with marine habitat types. In a survey of 40 different species of skates, Raschi (1986) found that the number of electrosensory pores and their distribution differed among species from different habitats. Species found at greater depths had fewer electrosensory pores and a greater proportion of these pores were distributed along the dorsal surface. Furthermore, sharks occupying different habitats (e.g. pelagic, coastal, reef) show patterns in brain morphology and electrosensory pore count/distribution that coincide with their respective habitats (Kajiura et al., 2010; Kempster et al., 2012; Lisney et al., 2008). Kajiura et al. (2010) proposed that these patterns reflect specializations in foraging strategies and diet for each habitat type. Elasmobranchs only exhibit ampullary receptors, and although these can have multiple roles (e.g. orientating to Earth's magnetic field, predator avoidance), their primary function is to locate prey (Kempster et al., 2012).

In addition to ampullary receptors, freshwater teleosts of the orders Gymnotiformes and Mormyriformes also possess tuberous receptors. These specialized electroreceptors are tuned to the frequency of the fish's own electric organ discharge (EOD) and are used for active electrolocation and electrocommunication (see Chapter 1). Although the organization of electroreceptors largely influences the spatial resolution of the electric image (Carr et al., 1982; Kempster et al., 2012), it is unknown whether

their spatial distributions exhibit patterns associated with specific habitat types or diets. As in elasmobranchs, gymnotiforms use electroreception to locate prey, but it also does much more. Electrocommunication has been shown to play a critical role in the evolution of the electrosensory system and the rapid diversification of both gymnotiforms and mormyriforms (Albert and Crampton, 2005; Arnegard et al., 2010; Carlson et al., 2011; Crampton et al., 2013; Hopkins, 1981; Nagel et al., 2017), and therefore may influence the spatial distribution of electroreceptors (Carlson et al., 2011; Xu-Friedman and Hopkins, 1999). Though these studies have documented large variation in the waveforms of EOD signals, few studies have explored interspecific variation in the electroreceptors in gymnotiforms, and to my knowledge none have considered species habitat differences.

Here I compare the distribution and density of both electroreceptor types (ampullary and tuberous) on the heads of seven species of gymnotiforms from two different habitats (i.e. deep river channels vs. floodplain lakes). In addition to describing previously unknown patterns of electroreceptor pore distributions, I explore whether organization of electroreceptors differs non-randomly among these taxa and whether differences are consistent with ecological factors such as habitat type or trophic strategy. Fish in lotic habitats often face directly into the flow of water and thereby may encounter drifting particles (e.g. debris, food); fish in lentic habitats are unconstrained by water current. Therefore, I hypothesize that fish from lotic environments should have higher densities in the rostral region, and species from lentic environments will have more uniform electroreceptor distributions.

Additionally, it is possible that electroreceptor organization is constrained by phylogenetic or developmental constraints (as defined by McGhee, 2007). While studies in other taxa often highlight how habitat use influences trait evolution, evidence from electric fishes suggest that preexisting traits related to the electrosensory system may constrain species movement between habitats or into new habitats (Stoddard, 2002). Crampton (2011) used gymnotiforms to illustrate the importance of phylogenetic niche conservatism in structuring neotropical fish communities. Species from the same family frequently occupy similar habitats and have similar trophic niches, potentially as a consequence of traits related to the electrosensory system. Therefore, I hypothesize that closely related species will have similar pore distributions. My analysis was focused on the head, because electrosensory capability in this region is most likely to influence food detection and feeding success. Also, the few studies that have described electroreceptor distribution in gymnotiforms report a general trend of decreasing density in the rostral-caudal direction, and the rostral region has been referred to as an electroreceptive fovea (Castelló et al., 2000), so differences in habitat use may be more apparent in this sensitive region.

Materials and Methods

Formalin-fixed, ethanol-preserved specimens were borrowed from the Biodiversity Research and Teaching Collections (TCWC) at Texas A&M University, the Biodiversity Center (TNHC) at the University of Texas at Austin, and the Academy of

Natural Sciences (ANSP) of Drexel University. Specimens were first inspected under a light microscope, and the least-damaged specimens were selected for analysis. Measurements were made on each specimen using calipers, with resolution to the nearest 0.1 mm. Straight-line measurements were total length, the length from the snout to the end of the anal fin, and head length. Surface area of the head and head+body was estimated by wrapping paper around the fish and then trimming the paper to cover only the head or the entire body. A picture was taken of the paper with a ruler for scale and the area of the paper was then measured using the open-source software FIJI (Schindelin, 2012).

The electroreceptors of gymnotiform fishes lie deep in the corium beneath a layer of epidermis that can be further divided into four sublayers (Szabo, 1974). Ampullary receptors are found at the base of a jelly-filled canal with a pore that opens up to the surface of the epidermis. Tuberous receptors are found encapsulated at the base of a canal. The canal is filled with a plug formed by elongated epithelial cells, so the receptors are not in contact with the surface. The naming convention used here follows Zakon (1987) and Wachtel and Szamier (1966), where the group of encapsulated sensory *receptor cells* within a single canal / pore opening are collectively referred to as a *receptor organ*, (though some receptor organs may share a pore opening, likely, during receptor organ division; Zakon, 1987). In general, each receptor organ is innervated by a single axon (but see section on *Hypopomus* sp. in Zakon, 1987 and Vischer, 1995). Receptor organs may divide over the life of a fish but are innervated by the same neuron.

Collectively these are referred to as a *receptor unit*. For this study, I did not examine receptor innervation patterns and only quantified receptor pores (i.e organs, not units).

Previous studies that have looked at lateral line and electroreceptor distribution in gymnotiforms have estimated receptor types simply by the characterizing the pore overlying the receptor organ (Bennett, 1967; Carr et al., 1982; Szamier and Wachtel, 1969; Wachtel and Szamier, 1966). Ampullary organs generally have the smallest canal openings (25 µm) and occur in recognizable clusters called rosettes. Here I use the term "cluster" over rosettes because, although rosettes were observed, they were not standard. Tuberous organs reportedly have intermediate sized pores (50 µm) and may or may not cluster. Clusters are formed by tuberous organs innervated by a single nerve fiber, forming receptor units (Harold H. Zakon, 1984). Both electroreceptor organ pores can be differentiated from canal neuromast pores which have large openings (100 µm) (Bennett, 1967; Carr et al., 1982). However, I found that accurate identification and quantification of tuberous organs by their pores is exceptionally difficult to do in species with little pigmentation, because there is little to no contrast between the underlying pores and the other epidermal tissue. Moreover, fish that have been fixed in 10% formalin and preserved in 70% ethanol, as in the case of the museum specimens used here, lose pigmentation over time.

I attempted to differentiate the pores without removing skin by using stains traditionally used for electroreceptor studies (e.g. toluidine); however, this yielded little success. Removal of skin was a delicate procedure, and large intact pieces of skin could rarely be removed. Ease of skin removal could have been influenced by specimen age.

Because the goal of this study was to quantify the density and distribution of pores on the head, using many small pieces of skin would make it difficult to accurately assign the orientation of pores. Therefore, I opted to use scanning electron microscopy to image the surface of the head. This required removal of the epidermal layer, but it enabled much more accurate measurements of pore sizes, counts of pores, and estimates of total surface area.

The thin layer of epidermis was removed using a pair of fine forceps in a large dissecting dish with 70% ethanol under a light microscope (though in some preserved specimens this layer is already partially absent). The head was then removed by dissection along a vertical line passing through the proximal margin of the pectoral fins, and the head was then placed in 90% ethanol for 12 hours. The following day, the head was chemically dried using a protocol based on Jusman et al. (2014). This consisted of a graded series of solutions containing 100% ethanol and hexamethyldisilazane (HMDS), for a total of thirteen 30-min wash steps. The full desiccation protocol is listed in Appendix A. HMDS was obtained from Fisher Scientific. After each specimen was dried, it was mounted on a custom-made aluminum stub using Ted Pella colloidal silver paint, coated with gold using a Cressington 108 sputter coater, and imaged using a TESCAN Vega 3 Scanning Electron Microscope (SEM) at the Texas A&M Microscopy & Imaging Center.

An average of 20 images were taken per specimen on the dorsal, ventral, lateral, rostral, and caudal surfaces of the left side of each fish. An effort was made to maintain the same working distance and magnification for each image. For a few cases, a change

in working distance was necessary due to the rotating mechanism of the Vega 3, and appropriate adjustments were made later to scale images by the magnification used. FIJI was used to process the images and count the pores using a cell counter plug-in. In some regions of the head, all pores could not be counted because sections of epidermis remained. In these cases, a grid (mesh size: 0.1 mm^2) was superimposed on the image, and the average pore density of the immediately surrounding squares was used to estimate the pore density in the missing area. FIJI also was used to measure the surface area in image sections where pores were counted. Image distortion associated with variance in the z-plane may have yielded a small degree of error in estimates.

Positions of neuromast canal pores in gymnotiforms match those found in most teleost fishes. Neuromast canal pores were used by Carr et al. (1982) as landmarks to compare receptor distribution in specimens of *Apteronotus albifrons* of varying sizes and by (Vischer, 1989) to compare lateral-line development in *Eigenmannia*. Therefore, in addition to the eye, neuromast canal pores were used to delineate six zones on the head of each fish (Fig.16), three pre-orbital and 3 post-orbital. Using abbreviated versions of nomenclature for lateral-line systems proposed by Coombs et al. (1988), the zones were designated as follows. The region dorsal to the supraorbital (SO) line, with pores along the line inclusive, was divided into two zones: SO-pre = anterior to and including the eye; SO-post = posterior to the eye. The region ventral to the mandibular-preopercular (MP) line, with pores along the line inclusive, was divided into two zones: MP-Pre = anterior to and including the eye; MP-post = posterior to the eye. Lastly, the regions in between the SO and MP lines were designated as the infraorbital (IO) zones and were

again divided into: IO-pre = anterior to and including the eye; IO-post = posterior to the eye.

Pores were counted for each zone and divided by the surface area to determine zone pore density. Due to time limitations, only 1 specimen per species and 7 species are included in this chapter; however, 3 additional species and at least 1 replicate of *Eigenmannia* are currently being processed and will be included in a forthcoming manuscript.[2] Due to the limited sample size, formal statistical analysis comparing the same zones across different species would be inappropriate. Instead, pore densities and distributions within and across species were compared graphically using the package 'fmsb' in the open-source software R (Nakazawa & Nakazawa, 2019). Notwithstanding the small sample size, a linear model was used to generally evaluate whether the number of receptors were a function of fish total length or head length. Using four different models, total fish length and fish head length were each used as an independent variable, and total pore counts and total pore densities were each used as a dependent variable.

[2] A specimen of *Distocyclus conirostris* was also prepared and imaged, but a comparable analysis was not possible because much of the epidermis was either not fully removed (this process can be challenging) or the underlying surface was damaged. Interestingly, this specimen contained several spines scattered along the snout that, after some discussion with Kevin Conway, I now believe are sponge spicules. A similar peculiarity has been observed in specimens of *Sternarchorhynchus goeldii* by Mark Sabaj. Gymnotiform and freshwater sponge associations have not been previously documented, so efforts are underway to publish a note on this material.

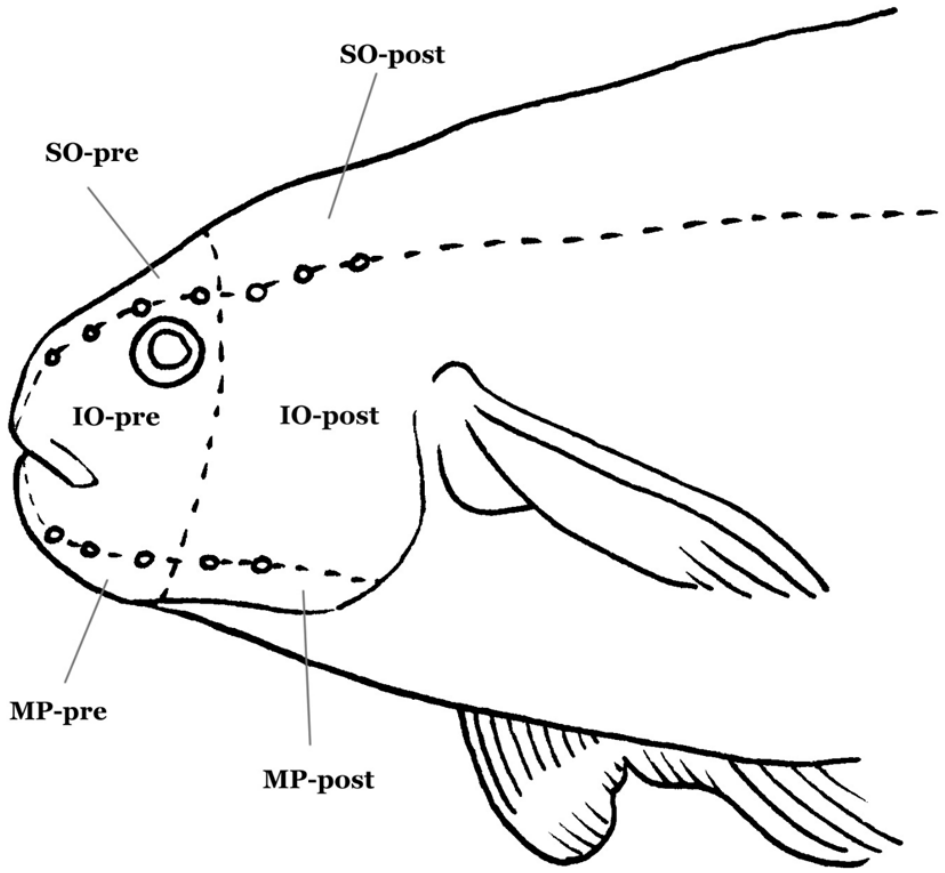

Figure 16. Illustration of *Adontosternarchus* with dotted lines demarcating zones used for pore density comparisons. Circles represent canal neuromasts that were used to as landmarks to delineate zones across species. The dorsal and ventral lines separate the supraorbital (SO) zone and the mandibular-preopercular (MP) zone from the infraorbital zone (IO). These were further divided into -pre and -post orbital zones. Pores were only counted on the head, which was cut at the anterior base of the pectoral fin, just passed the operculum.

Results

All species exhibited a general decline in pore density in the rostral to caudal direction on the head. Densities among the six zones of the head varied considerably across species, with most exhibiting either a dorsal or ventral specialization in pore distribution (Fig. 17&18). A linear model (not shown) suggested that total pore counts were not a function of fish total length or head length (p-values = 0.83 and 0.86, respectively; similar p-values were calculated when pore density was compared). Pore densities are compared by type across species in Figure 17 and their distributions within each species are illustrated in Figure 18. Familial relationships and broad habitat categories are also compared in Figure 18. Overall, *Rhabdolichops eastwardi* (Sternopygidae) had the highest total pore density (tuberous + ampullary) and the highest pore counts (Table 2 in Appendix A). *Steatogenys elegans* had the lowest total pore density, and *Adontosternarchus clarkae* had the lowest total number of pores. In general, the center of the operculum had the lowest density of pores, with *R. eastwardi* and *B. diazi* (Hypopomidae) having the highest densities in IO post.

Nearly all species had tuberous and ampullary pores with dimensions that did not always match previously published descriptions (see Methods). In some species, tuberous organs appeared to have pores with a large range of sizes. The receptor organs associated with each pore size class could not be confirmed by histology. Pore diameter was not necessarily equal to the size of the receptor organ, because canals sometimes were narrower than the organ below. Therefore, the first two columns of bar graphs in Figure 17 contrast interspecific densities of the two receptor types (ampullary and

tuberous), and the third column contrasts the total electroreceptor pore densities. Mucous cells are irregularly interspersed on the epidermis, and in *Eigenmannia* sp. pores are reported to be approximately 8-9 μm in diameter (Vischer, 1995). Therefore, it is possible that mucous cells were occasionally mistaken for ampullary organ pores for species in which those were small (i.e. *R. marmoratus*, *R. eastwardi*, and *B. diazi*). Details of pore densities, distributions, and dimensions, as well as any distinct patterns observed for each species are summarized below.

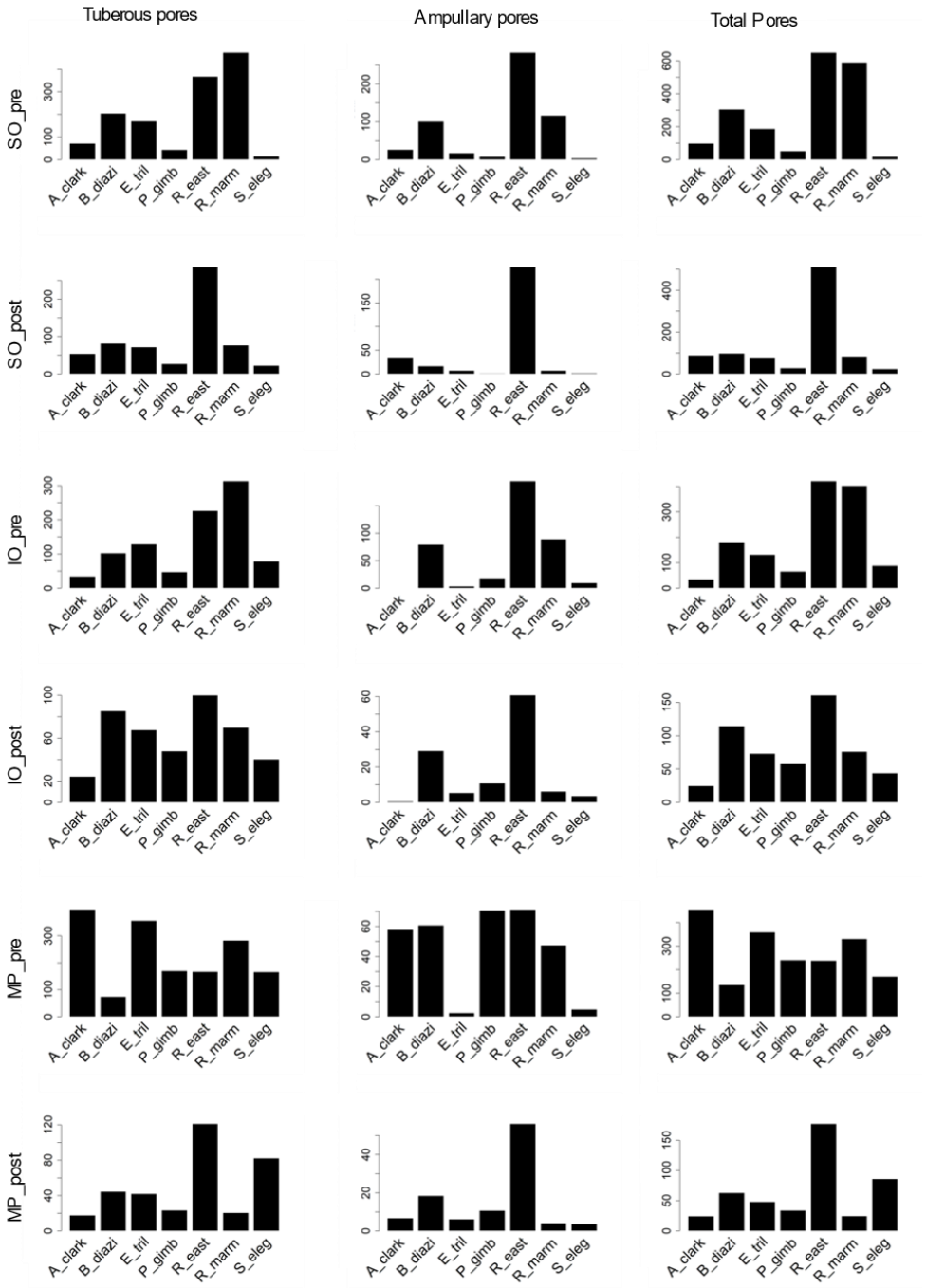

Figure 17. Bar graphs contrasting the densities of electroreceptor pores in each zone. The first column contrasts tuberous organs densities, the second contrasts ampullary organ densities, and the third contrasts densities of all pores (tuberous and ampullary combined). Each row shows the density for a different zone.

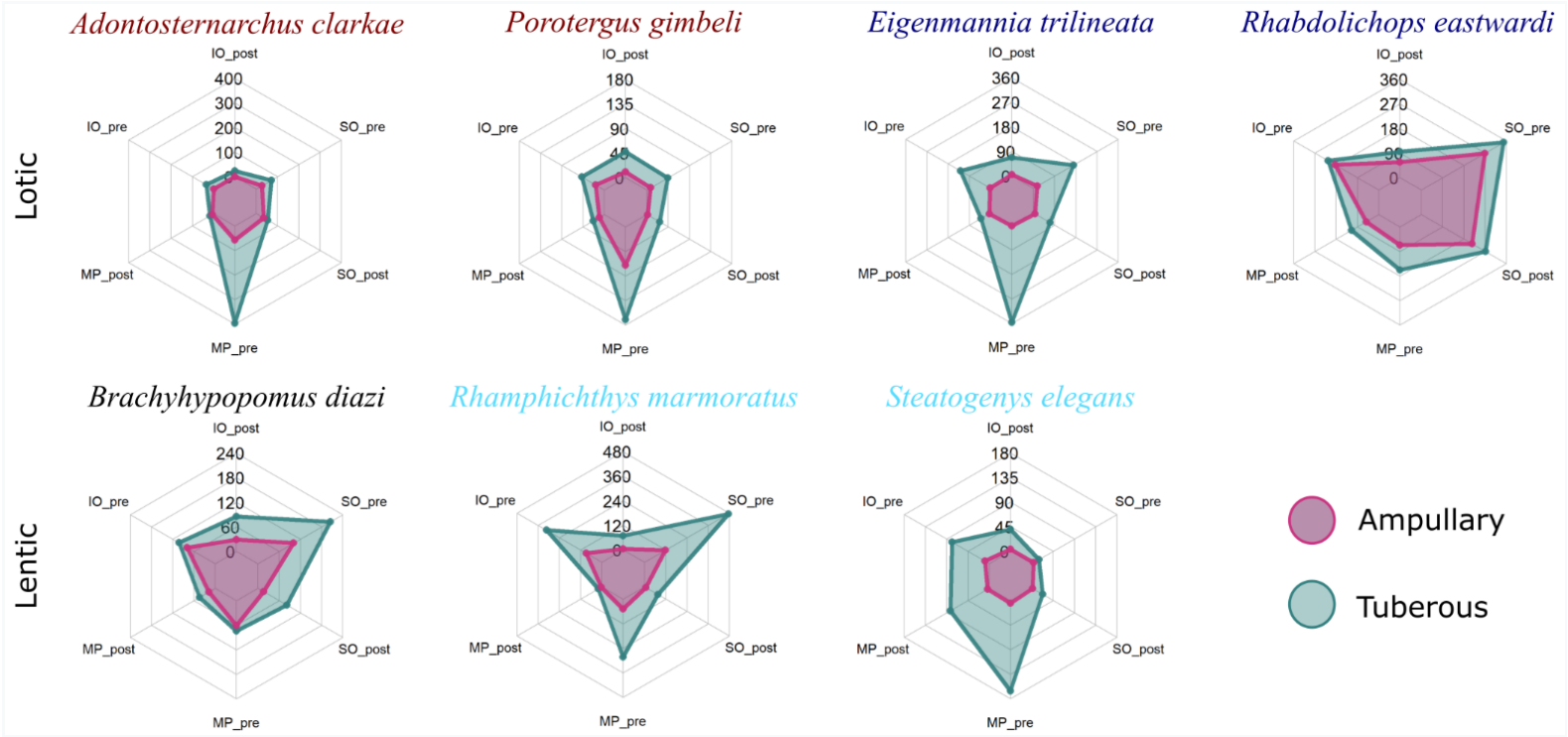

Figure 18. Radar plots showing the relative distribution of electroreceptor pore densities across the six designated zones. Note that the scale is different for each species (for interspecific comparison of zone densities see Figure 2). Species collected in lotic habitats are shown on the top row and those collected in lentic habitats are on the bottom row. Species names in the same color are in the same family.

Steatogenys elegans (Rhamphichthyidae)

The pores of ampullary organs (16-25 µm) in *S. elegans* displayed the classic rosette clustering (Fig. 19) described in *Apteronotus albifrons* (Apteronotidae) (Carr et al., 1982). The pores of tuberous organs appeared to have two size classes. Smaller pores (30-40 µm) were mostly found in the preorbital zones, especially in MP_pre. Mean pore size increased in the rostral to caudal direction and larger pores (55-63 µm) were predominantly found in the postorbital zones. Interestingly, the diameter of neuromast pores sometimes was similar and therefore they were difficult to differentiate from tuberous receptors. The highest pore density was found in the ventral zones, with the highest in MP_pre and the second highest in MP_post. Interestingly, *S. elegans* and *R. eastwardi* were the only species in which a postorbital zone had a higher density than preorbital zones. In contrast to *S. elegans, R. eastwardi* densities were higher in the dorsal zones. In *S. elegans* there was also a notable concentration of pores in the zone IO_pre. The eye is densely surrounded by pores, and it appears as if only the lens is devoid of electroreceptor pores (Fig. 20).

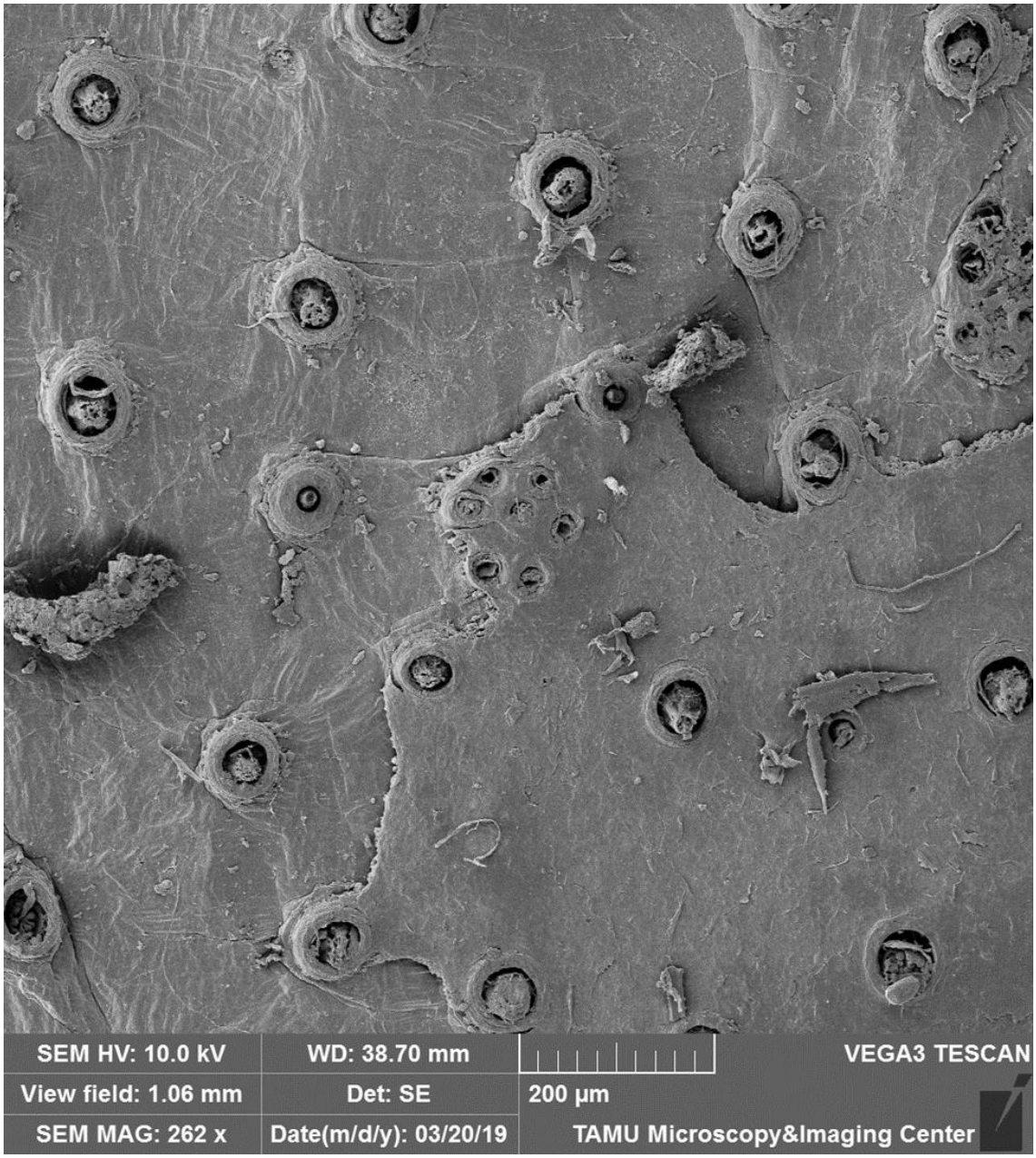

Figure 19. Electroreceptor pores of *Steatogenys elegans.* Tuberous pores and 2 rosettes of ampullary pores can be seen (center and top right). In some cases, the plugs and covering cells are still in the tuberous pores.

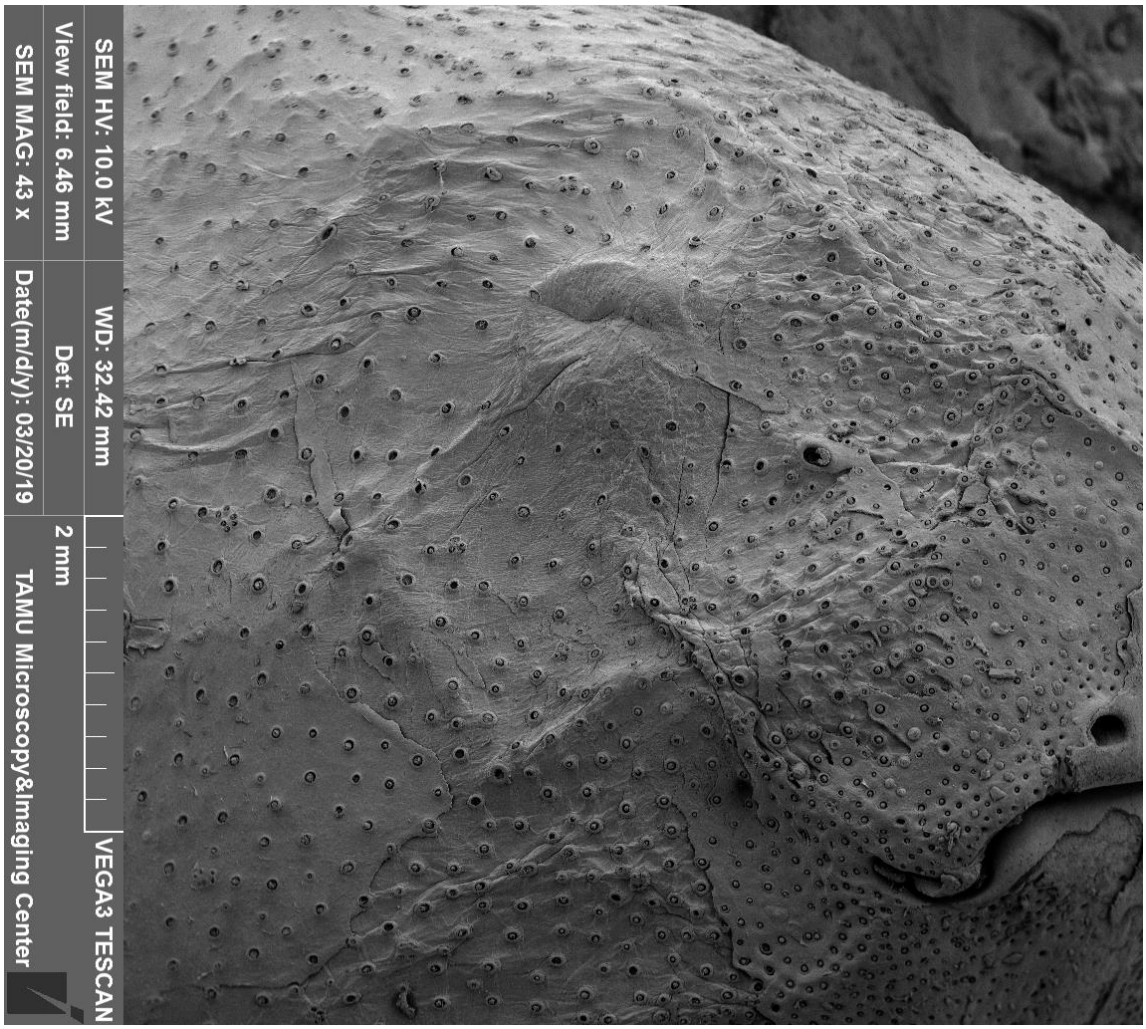

Figure 20. Eye of *Steatogenys elegans.* Note that pores are almost encroaching over the lens and neuromast pores are difficult to differentiate by size from other pores.

Rhamphichthys marmoratus (Rhamphichthyidae)

In *R. marmoratus*, none of the pore types revealed obvious clusters. There was significant variation in pore sizes with overlapping ranges, and in addition to the potential bias associated with the z-plane, it was difficult to estimate densities of different-sized pores with precision. Similar to *S. elegans*, pore diameter tended to increase in the rostral to caudal direction. The smallest pores (15-20 µm - labeled as ampullary) and an intermediate class (25-30 µm - labeled as tuberous) were predominant on the snout, whereas in the caudal zones, larger pores with (40-50 µm - also labeled as tuberous) were most common. A 33 mm^2 sample from the snout suggests the ratio between the two intermediate and the smallest size classes was approximately 9 : 4.5 : 1. The highest pore density was found on SO_pre, and this was highest pore density for a single zone among all species. Nevertheless, all 3 pre-orbital zones had high densities. Ampullary pores showed a similar pattern to tuberous pores.

Porotergus gimbeli (Apteronotidae)

Porotergus gimbeli revealed strong clustering of ampullary receptors. Tuberous receptors appeared to have two different size classes, ~40-50 µm and ~70-90 µm. The highest density was found in MP_pre, just under the chin. Both pore types were found on the snout, but the larger pores were predominantly found in the caudal zones (although at a lower relative density) and in a fairly uniform distribution.

Adontosternarchus clarkae (Apteronotidae)

Adontosternarchus clarkae was similar to *P. gimbeli* in that the highest pore density was found at MP_pre, though the density of this zone was two times larger in *A.*

clarkae. In *A. clarkae*, MP_pre density differed greatly relative to the other zones (10:1). In contrast, MP_pre density in *P. gimbeli* compared to other zones was only 4:1. Ampullary pores (15-20 µm) also occurred in clusters. Tuberous pores were generally large and uniform in size (50-60 µm); however, the chin region had pores of varying sizes (27-35 µm and 50-60 µm) (Fig 21).

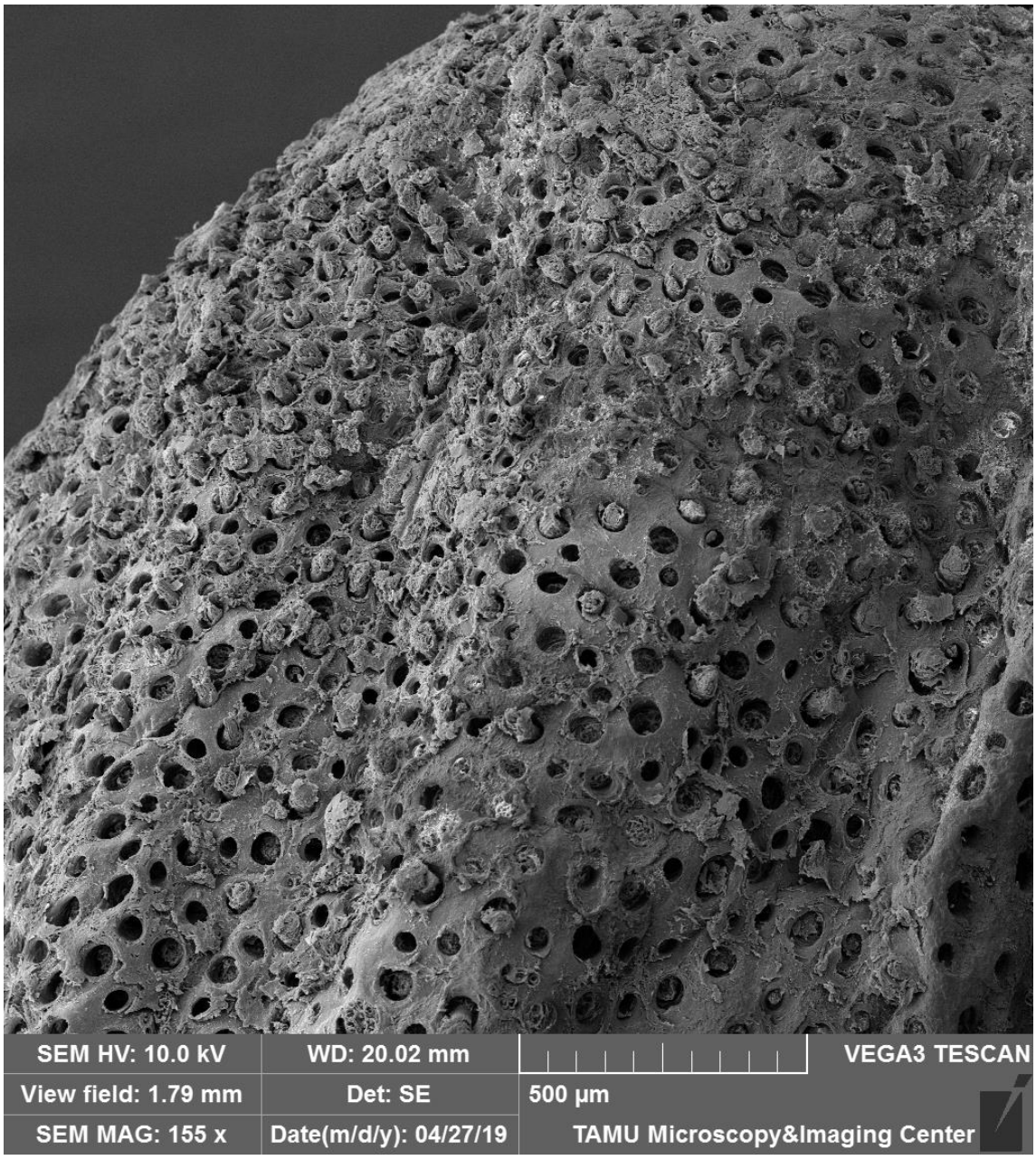

Figure 21. Close up on the chin of *Adontosternarchus clarkae*. Note the high density of pores and the variation in pore sizes.

Eigenmannia trilineata (Sternopygidae)

In *E. trilineata*, both ampullary and tuberous pores occurred in clusters. Tuberous pores that clustered were generally smaller (~30 µm) in diameter than non-clustering pores (39-43 µm) and primarily found in the preorbital zones. The entire snout is well covered, with IO_pre and SO_pre having relatively high densities, although the highest concentration was observed in MP_pre. Few ampullary pores (15-25 µm) were identified in *E. trilineata*, and those that were identified tended be fairly uniform in distribution, a pattern similar to that observed in *S. elegans*. Neuromast pores were larger (140 µm) than other pores.

Rhabdolichops eastwardi (Sternopygidae)

The highest total pore count and the highest overall pore density among all the species examined was found in *R. eastwardi*. This species also appears to have the broadest distribution of pores (Fig. 18). The entire dorsal region (both SO_pre and SO_post) had high pore densities, with highest density in SO_pre. Along with *S. elegans*, *R. eastwardi* was the only other species for which at least one post-orbital zone had a higher density than the pre-orbital zones, although IO_pre and MP_pre nonetheless had high pore densities.

Rhabdolichop eastwardi had a range of a pore sizes that did not easily separate into discrete size classifications (Fig. 22), even for the larger tuberous pores (45-68 µm). Pores were often observed in clusters, but within clusters pores were not always uniform in size. Instead, I often observed a range of sizes, with both small (10-15 µm) and medium size pores (20-36 µm) clustering together. In combination with potential bias

associated with the image z-plane, high pore density made it difficult to consistently differentiate between the different size classes in lower magnification images (measurements were taken in high magnification images), and there likely is overlap between pores classified as ampullary and tuberous. This may account for the large density of pores classified as ampullary organs in this species. Another pattern that was frequently observed in *R. eastwardi* was that clusters would form a ring around a single large tuberous organ (Fig. 22).

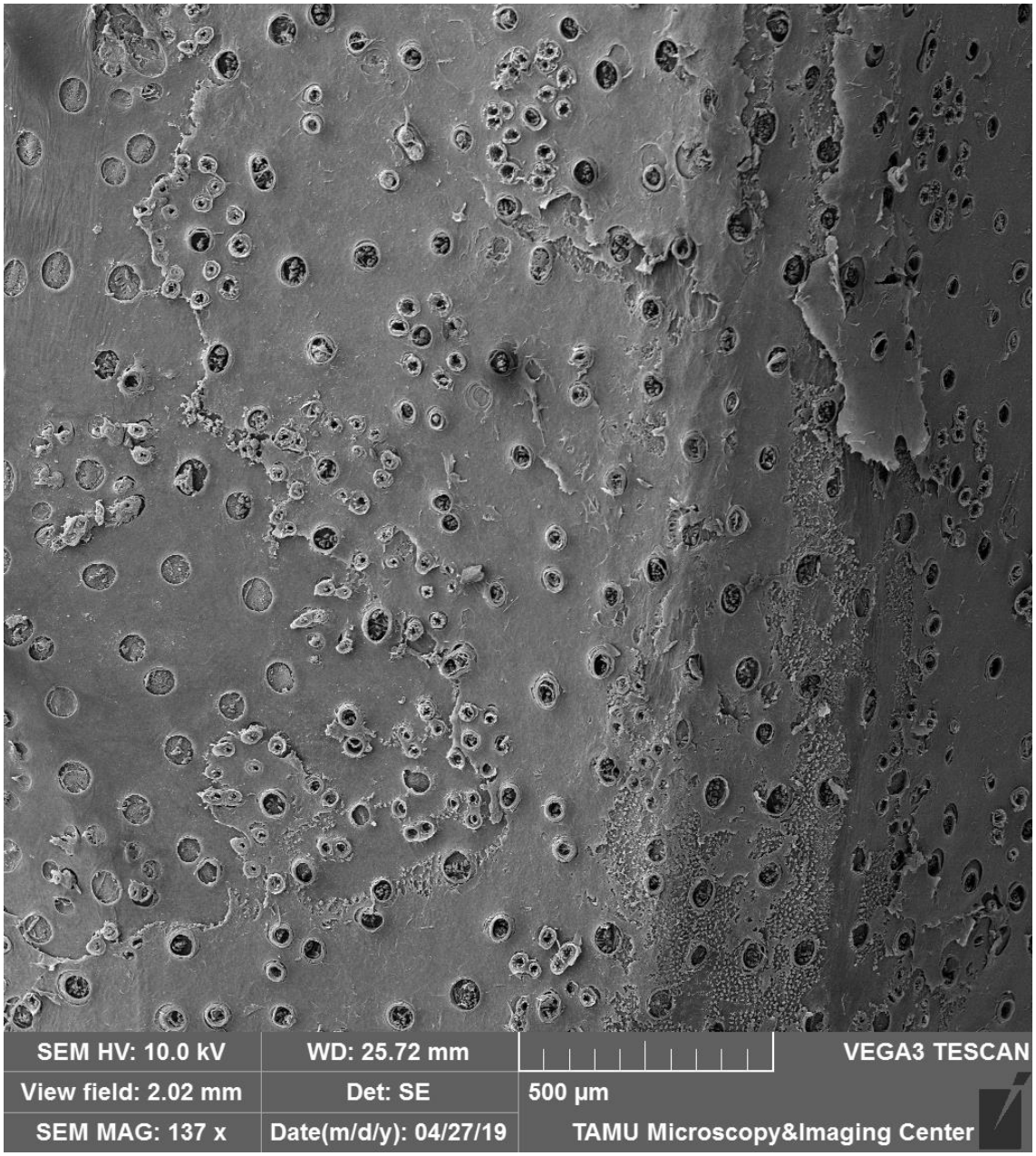

Figure 22. Section of the dorsal region in *Rhabdolichops eastwardi*. Note the variation in pore sizes. On the left, center of the image there is an obvious ring of what were labeled as ampullary pores surrounding a large tuberous pore.

Brachyhypopomus diazi

In *B. diazi,* some pores were miniscule (15 µm) and occasionally occurred in clusters. These were labeled as ampullary pores. Two intermediate size classes (20-30 µm and 45-55 µm), and one large class (70-76 µm) of pores were counted as tuberous organs. The preorbital dorsal zone, SO_pre, had the highest pore density. Other zones had similar pore densities, with the exception of MP_pre with lowest pore density.

Discussion

This study demonstrates that the distribution of electroreceptor organs on the heads of gymnotiforms varies widely between species, primarily differing in densities on ventral and dorsal preorbital surfaces. Several studies have described subtypes of tuberous organs by their morphology, innervation pattern, and physiology (Szabo & Fessard, 1974; Zakon, 1987) and are most recently reviewed by Bullock et al. (2006). Physiologically, there are two main types of electroreceptors: (1) those that code amplitude information—termed probability coders (P units) in wave type species (Sternopygidae and Apteronotidae) and burst coders (B type) in pulse type species (Gymnotidae, Hypopomidae, and Rhamphichthyidae) and (2) those that code timing or phase information—termed timing coders (T units) in wave type species and pulse markers (M type) in pulse type species. Some studies have also reported patterns in the spatial distribution of morphological and physiological subtypes (Bastian, 1977; Szabo, 1974, 1965; Yager and Hopkins, 1993). Therefore, the variation in pore sizes and

distribution observed here is probably related to these electroreceptor subtypes; however, additional information is needed with regards to receptor organ morphology as well as the number of receptor cells per organ and their innervation patterns. Variation in pore size also may be associated with fish age, because some receptor organs grow by adding more receptor cells and eventually divide, increasing the number of organs per receptor unit (Zakon, 1984, 1987). Nonetheless, physiological subtypes have not been studied extensively for more than a few species.

Though here I present data on interspecific differences in electroreceptor pore densities and distributions, it is unknown whether these are consistent within species with regards to sex and ontogeny. Zakon (1987) demonstrated in various species that as fish age, receptor organs contain greater numbers of receptor cells and that receptor units (clusters of receptor organs innervated by the same neuron) decrease in density. In contrast to receptor unit density, the density of receptor organs appears to remain the same in fish of different lengths (Carr et al., 1982; Castelló et al., 2000; Zakon, 1987), likely maintaining resolution of the electric sense relative to the fish's size. In future studies, it would be interesting to study species that have sexually dimorphic head morphologies, such as *Compsaraia samueli* and *Apteronotus rostratus* (Evans et al., 2019), to isolate the influence of head morphology and development on electroreceptor distribution. During the development of *Eigenmannia* sp., electroreceptor organs (ampullary and tuberous) in the trunk show a clear spatial gradient of development, with new organs developing caudal and dorsal to older ones, away from the lateral-line. However, the opposite is observed on the head where electroreceptors begin to develop

randomly without any particular spatial gradient on the head, suggesting receptor distribution in the head may be more plastic than along the trunk. As observed in previous studies (Carr et al., 1982; Castelló et al., 2000; Szabo, 1974; Zakon, 1984), the species studied here all exhibited a general decline in pore density in the rostral to caudal direction.

One caveat of this study was the problem of measuring distances in 2-dimensional images of 3-dimensional fish heads. At the magnification used, some images differed in the z-plane more than others, so that error due to cursor placement and/or pore orientation was comparable to the few microns in actual pore size differences. The main consequence of this is that the distribution of different size classes is not precisely represented here; however, size differences between pore classes were confirmed in images taken at higher magnification with no effect of the z-plane. To my knowledge, this is the first study to contrast electroreceptor pore densities in gymnotiforms at this scale.

Ecological and phylogenetic perspective on electroreceptor distributions

A primary goal of this study was to explore whether receptor pore distributions exhibited associations with habitat or diet and whether phylogeny could predict pore distributions. Gymnotiform fishes are not herbivorous and lack long guts or other adaptations for consuming aquatic vegetation or detritus (Winemiller and Adite, 1997). Their diets primarily consist of macroinvertebrates and invertebrate larvae, especially chironomids (Crampton et al., 2005, 2016a; Giora et al., 2005; Virgilio and Gomes, 2019; Zuanon et al., 2006), though some gymnotiform fishes are piscivorous (Lundberg

and Mago-Leccia, 1986; Winemiller, 1989a) and others have developed specialized head morphologies for consuming benthic larval invertebrates (Marrero and Winemiller, 1993). Though no statistical test for phylogenetic signal was included in this chapter, the results observed here, in combination with qualitative information obtained from the literature (discussed below), suggest that head morphology and feeding strategy may play a more important role than phylogeny in determining electroreceptor pore distribution.

Apteronotidae

The large majority apteronotids are rheophilic (Albert and Crampton, 2005; Crampton, 1998), and some may seasonally occupy floodplain and small stream habitats (Winemiller, 1989b), and a few can be found in lentic habitats year-round (Arantes, personal communication). According to Albert (2001) and reviewed by (Santana and Crampton, 2010), the two genera of apteronotids examined here, *Porotergus* and *Adontosternarchus,* are sister taxa, which may explain the general similarity in electroreceptor pore distributions. However, there is a large interspecific difference in total density. Species of the genus *Adontosternarchus* possess a unique V-shaped mouth as well as an accessory electric organ derived from the fibers of electrosensory nerves in the chin (Bennett, 1970). This chin organ discharges independently from the main electric organ and likely only activates receptors in the head/chin region (Bennett, 1971).

Adontosternarchus species are captured almost exclusively using bottom trawl nets, and gut content analyses have revealed their gut contents contain benthic invertebrates, such as cladocerans, nematodes, and rotiferans, and even include sand

grains (Lundberg and Fernandes, 2007; Mago-Leccia et al., 1985). Whereas sand grains are likely consumed incidentally, this information suggests that the impressive density of electroreceptors in the chin region reflect a benthic feeding habit (Lundberg et al., 1987). *Porotergus gimbeli* is also known to consume benthic invertebrates, which also may explain the relatively high density in the MP_pre zone. Interestingly, this species exhibits polymorphic swelling of the chin region (Santana and Crampton, 2010), but the specimen examined here lacked this feature.

Sternopygidae

Similar to the apteronotids, the specimen of *E. trilineata* examined here also exhibited a high total pore density in the MP_pre zone, but with relatively higher densities in the other preorbital zones. This corroborates findings from a diet study on *E. trilineata* that suggested this species feeds throughout the water column, but with a preference toward feeding on benthic invertebrates (Giora et al., 2005). Species in the genus *Eigenmannia* can be found in many different aquatic habitats, including deep river channels, floodplain lakes, terra firme streams, and even caves (Peixoto and Ohara, 2019), so it is unlikely the results presented here are representative for the genus. *Eigenmannia* are notoriously cryptic, and several species groups have recently been proposed (Waltz and Albert, 2018, 2017). One study found that adjacent populations of *Eigenmannia macrops* show significant microgeographic variation in the size and shape of specimens collected from small streams versus open water habitats such as floodplain lagoons (Lundberg and Stager, 1985). It would be interesting to examine whether

electroreceptor pore distributions also vary with these small changes in size and morphology.

Because associated receptor organs were not confirmed with histology, it is possible that the smaller (25-30 μm) clustered pores observed in *E. trilineata* labeled as tuberous were actually ampullary organ pores. A previous study quantified ampullary organ pores in *A. ablifrons* by using similar dimensions to those described here (25 μm) (Carr et al., 1982). However, in *E. trilineata* these smaller pores comprised a significant proportion of the total pores on the snout, so that ampullary organs would be highly abundant relative to tuberous organs—a pattern that has not been described before in gymnotiforms (but see results for *R. eastwardi*).

Unexpectedly, *R. eastwardi* was found to have the highest density of receptor pores, but aspects of their ecology may explain this result. *Rhabdolichops* is found in great abundance within flowing, deep channel habitats in rivers, but may occasionally be found in adjacent floodplain channels near the outflow from tributaries (Correa et al., 2006; Lundberg et al., 1987; Lundberg and Mago-Leccia, 1986). Most species possess numerous, long gill rakers that normally are associated with a zooplanktivorous feeding strategy. Some *Rhabdolichops* also eat small insects and even small fish (Lundberg and Mago-Leccia, 1986), but the only information on *R. eastwardi* suggests it feeds on planktonic copepods. Additionally, several species have distinctly upturned mouths, although the mouth of *R. eastward* is more terminal and not as upwardly oriented. This evidence induces speculation that high density of electroreceptors, especially on the dorsal (MP_pre & MP_post) and lateral (IO_pre) zones, is important for locating and

capturing prey in the water columns of turbid, swift waters (Lundberg et al., 1987). The unique method of locomotion found in all gymnotiforms (see Chapter 1) may be especially useful for *Rhabdolichops* to move forward and backward to capture prey drifting overhead in flowing water.

Rhamphichthyidae

Most rhamphichthyids are found in terra-firme streams and floodplain habitats (Albert and Crampton, 2005; Crampton, 1998). Although the two species studied here were captured in a small stream and a floodplain channel, they sometimes are collected from deep river channels (Albert and Crampton, 2005; Carvalho and Albert, 2015). The two rhamphichthyid species had different electroreceptor pore distributions. This was not unexpected considering the different head morphology and feeding strategies. *Rhamphichthys* and *Gymnorhamphichthys* species possess long tube-like snouts that are used to probe for aquatic invertebrates in undercut river banks, leaf packs, and holes and crevices in benthic substrates (Marrero, 1987; Marrero et al., 1987; Marrero and Winemiller, 1993). Once located, prey are ingested via suction through the tube-like snout. Unsurprisingly, the snout was densely covered in electroreceptor pores relative to the post-orbital zones, with an especially high density on the dorsal surface of the snout (SO_pre).

There are no obvious patterns inferable from the electroreceptor pore distribution of *S. elegans* relative to its ecology, perhaps because it is an ecological generalist. Museum records of *S. elegans* indicate this species can be found at depths of 40 m, but is most commonly associated with leaf-litter and structured habitats with woody debris

(Sazima et al., 2006). A description of a closely related species *S. ocellatus* suggests they feed on a variety of autochthonous and allochthonous invertebrates.

Interestingly, all rhamphichthyids possess accessory electric organs (Giora and Carvalho, 2018). *Steatogenys elegans* and *R. marmoratus* both have a submental accessory organ that runs along the ventral region of the jaw and is thought to be non-homologous in these species. Additionally, *R. marmoratus* has a subpectoral accessory organ, and *S. elegans* has a humeral accessory organ. Unlike the chin organ in *Adontosternarchus*, no obvious clustering of electroreceptor pores was identified in association with these organs on the head. Perhaps these organs assist in concentrating electric currents near the head and improve the fish's ability to locate prey.

Hypopomidae and Gymnotidae

The hypopomids are primarily found in lentic habitats such as floodplain lakes and in the slow-moving waters of terra firme streams (Albert and Crampton, 2005; Crampton et al., 2016a). *Brachyhypopomus diazi*, which is commonly found in floating aquatic macrophytes or emergent vegetation, exhibited a fairly even distribution of electroreceptor pores (Fig. 18), but with highest density on the dorsal surface. One may speculate that this is associated with where this species feeds within the water column. This species presumably feeds on small insects and larval invertebrates like most *Brachyhypopomus* species (Crampton et al., 2016b; Giora et al., 2014). Interestingly, *B. diazi* is reported to have an extensive network of unpigmented canals (superficially described by Crampton et al. 2016b). Crampton et al. (2016b) cite a dissertation by

Sullivan (1997) which cites unpublished data that suggest these canals lead to tuberous receptors. Likely because I removed the epidermis, I did not observe these canals.

Species of the family Gymnotidae are restricted to habitats similar to those inhabited by *Brachyhypopomus*. Though none were examined here, pore distributions for *Gymnotus carapo* have been described by Castelló et al. (2000). The authors found the highest density of electroreceptor pores in the ventral preorbital region and a high density in the dorsal preorbital region relative to postorbital surfaces. A notable characteristic in gymnotids is the protruding lower jaw. This species reportedly feeds on insects and crustaceans (Albert and Crampton, 2003) and small fish (Winemiller, 1989a). *Gymnotus carapo* males exhibit mouthbrooding of eggs and larvae (Kirschbaum and Schugardt, 2002), and it would be interesting to investigate the role of eletroreception in this form of parental care.

Electrocommunication, sensory drive, and comparison with mormyrid electric fishes

My initial hypothesis that patterns in the distribution of electroreceptor pores may reflect feeding strategies and habitat type was based on evidence for such patterns in elasmobranchs. However, most elasmobranchs do not generate electric discharges, with the obvious exception of the torpedo rays (Torpediniformes), nor do they use their electric sense for communication (with the potential exception of some skates in the family Rajidae, see Bratton and Ayers, 1987; Morson and Morrissey, 2007). In any case, these exceptions are not sufficiently documented to warrant further comment. The electrosensory system of both the gymnotiforms and the mormyriforms has been well studied for its role in electrocommunication and has been implicated in the rapid

diversification of species in both clades (Albert and Crampton, 2005; Arnegard et al., 2010; Carlson et al., 2011; Crampton et al., 2013; Hopkins, 1981; Nagel et al., 2017). Therefore, it is interesting to consider whether the communicative role of electroreceptors interferes or otherwise interacts with the electrolocating role with regard to electroreceptor distribution.

The sensory drive hypothesis (Cummings and Endler, 2018; Endler, 1992) suggests that aspects of a species environment will influence the evolutionary trajectory of communication signals and their corresponding sensory systems. Although a general relationship between EOD discharge rate and habitat type is well-known in gymnotiforms (wave type species are typically found in lotic habitats and pulse type species are generally found in lentic habitats) (Albert and Crampton, 2005; Crampton, 1998), I am unaware of any experimental evidence indicating that a particular EOD discharge frequency or waveform is more effective for communication a given habitat. Additionally, differences in the physical properties of electric signals relative to acoustic or visual signals suggest that signal refraction or reflection by the animal's environment have little effect on the electric sense (Crampton et al., 2013; Brenowitz, 1986; Hopkins, 2003, 1999). Therefore, it is unclear how communication may "override" environmental influences on the evolution of electroreceptor distribution for electrolocation.

Mormyrids have specialized electroreceptors termed knollenorgans. These receptors and their associated central processing pathways are exclusively responsible for mediating electrocommunication (Bullock et al., 2006). Equivalent receptors are not found in gymnotiforms. There is evidence that the spatial arrangement of knollenorgans

is important for determining the directionality of conspecific signals (Carlson et al., 2011; Xu-Friedman and Hopkins, 1999). Furthermore, a recent study suggests that mormyrid species that rely on the electric sense over species that use a combination of visual and electric senses differ with regard to the spatial distribution of the knollenorgans and habitat usage (Velez et al., 2018).

The tuberous receptors responsible for orientation and electrolocation in mormyrids are termed mormyromasts, but to my knowledge their distributions have not been well studied. One recent study demonstrated that morphological differences in the tube-snouts of the mormyrid genus *Campylomormyrus* are associated with preferences for feeding on different substrates (Amen et al., 2020). It would be interesting to examine whether mormyromasts in these species show distribution patterns associated with feeding adaptations in specific microhabitats.

Conclusion

This study found that electroreceptor pore distributions differed across species of gymnotiforms. I did not find conclusive evidence that broad habitat categories predict pore distributions; however, three of the species studied (i.e. *R. marmoratus, S. elegans,* and *E. trilineata*) inhabit multiple habitat types. Nonetheless, the ecological information reviewed here suggest that microhabitat, head morphology, and feeding strategy may play a role in the evolution of electroreceptor spatial distribution. Data from additional species may provide further evidence and I am examining additional specimens and species to test this idea further.

Another interesting comparison that merits further investigation is the relationship of electroreceptor distribution to species' EOD waveforms. Bastian (1977) showed that the frequency response of electroreceptors varies with their position on the body of the fish. Castelló et al. (2000) provided evidence of how the passive properties of the fish (e.g. body geometry and tissue organization) can funnel electric currents toward the head. It is well known that electric field potentials vary spatiotemporally along a fish's body as a function of the EOD waveform (Caputi, 1999). Some recent studies (Aguilera et al., 2001; Crampton et al., 2013) have claimed that the specific phases of the EOD waveform can be broken down into two functional components, one for electrolocation and one electrocommunication—a concept originally proposed by Trujillo-Cenóz et al. (1984). However, there is also compelling evidence that this is not the case (Schuster and Otto, 2002). It would be interesting to expand the species comparisons made here to the rest of the body and with additional species that have restricted use of habitats. In addition to more detailed ecological information (e.g., habitat conditions, diet), comparisons of electroreceptor tuning properties and pore distributions for additional species (Castello et al., 2000) would further elucidate the drivers of electroreceptor evolution.

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CHAPTER V

CONCLUSIONS

Historically, the field of physiology has progressed by testing hypotheses using experimental approaches and has been largely motivated by concerns for human health and medicine. Conversely, due to the enormous variation in natural and evolutionary histories as well as the inherent complexity of controlling for myriad environmental variables, the relatively young field of ecology has relied on observation and comparison, supplemented by field and lab experiments of relatively short duration and small spatial scales. Due in part to the rapid anthropogenic changes to Earth's climate, recent decades have seen a change in the approaches taken by ecologists to make testable predictions about multi-scale patterns in ecology. The eminent ecologist Robert McArthur is famously quoted for saying that the goal of all science should be to search for general patterns or rules (MacArthur, 1984). Sensory physiology and the plasticity of signaling behavior are complex traits with multiple functions. Therefore, it is difficult to make inferences about the role species ecology play in their evolution. My dissertation adopted a comparative phylogenetic approach to study aspects of sensory physiology and signaling behavior while considering the potential role of ecological factors with the goal of finding general patterns that may influence the evolution of these traits.

The second chapter examined the effects of ACTH on the short-term signaling plasticity in the EODs of twenty-one species of gymnotiform fishes. The main finding at the family level was that, when considering only species averages, only hypopomids and

sternopygids showed consistent increases in overall EOD amplitude in response to ACTH. However, slight changes the in duration and amplitudes of minor phases were observed in at least one species from every family. This was unexpected for the apteronotid *Sternarchella* cf. *calhamazon*, since the adult apteronotid electric organ develops from neural tissue in contrast to the muscle-derived electric organs of other gymnotiforms. A model using environmental factors, such as habitat type, temperature, dissolved oxygen, and conductivity—factors known to influence the function of the electrosensory system (Ardanaz et al., 2001; Crampton, 1998; Hopkins, 1999), failed to predict the degree of interspecific variation in EOD waveform plasticity in response to ACTH. A potential reason for this is that several species exhibited significant variation between individuals in their response to ACTH.

Short-term changes in EOD waveform were first described in response to circadian rhythms (Franchina and Stoddard, 1998; Hagedorn, 1995), but now many factors are known to contribute to EOD waveform plasticity, including sex, social environment, and nutrient availability (Gavasa et al., 2012; Gavassa et al., 2013; Markham and Stoddard, 2013; Salazar and Stoddard, 2008; Sinnett and Markham, 2015). Recent studies suggest that ACTH and other melanocortin hormones maybe be directly responsible for regulating a plethora of central and peripheral functions, some of which may be sexually dimorphic (Ducrest et al., 2008; Qu et al., 2014; Roulin and Ducrest, 2011). Though many unknowns remain, gymnotiforms stand as excellent models for further exploring the pleiotropic functions of the melanocortin system in the behavior, physiology, and ecology of vertebrates.

The third chapter examined cellular and molecular differences in electrocyte physiology in the taxonomically diverse genus *Brachyhypopomus*. Coupled with evidence from other studies, I suggest that *Brachyhypopomus* are specialists in rapid regulation of EOD waveforms, potentially as result of the semelparous life history strategy that apparently is predominant in this genus (Waddell et al., 2019). This chapter also highlights a unique exception to the rule in the case of the monophasic species *B. bennetti*. I provide evidence suggesting that the monophasic phenotype observed in this species is not a retention of the genus' assumed paedomorphic condition and instead appears to persist as a transitional evolutionary state. Completely overlapping action potentials from electrocyte anterior and posterior membranes significantly reduce the overall amplitude of the EOD. Despite this, *B. bennetti*'s EOD amplitude is 3-8 times larger than that those of sympatric congeners (Crampton and Albert, 2006). While multiple non-mutually exclusive hypotheses have been proposed to explain the persistence of the monophasic phenotype, I propose that mate choice may be a contributing factor (Curtis and Stoddard, 2003) and that the larger EOD amplitude observed in males (Crampton and Albert, 2006) functions as an electric ornament.

The fourth chapter quantified and compared the distribution of electroreceptor pores on the heads of seven gymnotiform species. I reported interspecific differences in pore dimensions, densities, and distributions that may correlate with physiological subtypes of electroreceptors. Additionally, I made inferences based on species ecology and suggested that observed differences in electroreceptor distributions reflect unique trophic strategies used by some of these species. Unlike what has been shown in

ecological studies of electroreception in elasmobranchs, I did not find evidence that broad aquatic habitat categories (i.e. lentic versus lotic) are robust predictors of electrocyte distribution in freshwater teleosts; however, only a limited number of species were examined and few of them are strictly found in lentic habitats. It is possible that electroreceptor distribution is more developmentally plastic than previously believed. It would be interesting to compare intraspecific variation in electroreceptor distribution in species, such as *Compsaraia samueli* and *Apteronotus rostratus*, that have sexually dimorphic head morphology (Evans et al., 2019). Additional information on the distribution of physiologically distinct electroreceptor types (e.g. amplitude versus phase/timing coders), their specific tuning frequencies, and more ecological data are needed for a better understanding of how a species environment, diet, and social interactions contribute to evolution of the electrosensory system in gymnotiforms.

Lastly, a major take-away from this research is the incredible physiological diversity observed in gymnotiforms. In many respects, these fishes are highly diverse, from the habitats they occupy (Albert and Crampton, 2005) to the various social and reproductive strategies employed (Kirschbaum and Schugardt, 2002). Gymnotiforms also are among the most taxonomically diverse groups of freshwater fishes. There are currently 260 described species, many of which are thought to encompass cryptic diversity (Waltz and Albert, 2017), and newly discovered species are described every year (Albert and Crampton, 2009, 2003; Campos-da-Paz and Santana, 2019; Carvalho and Albert, 2015; de Santana et al., 2019; Dutra et al., 2018, 2017; Meunier et al., 2011).

Gymnotiforms are members of the Ostariophysi, a superorder that contains approximately 28% of the world's known fish species, and may account for up to 68% of freshwater species (Nelson et al., 2016). At the time of this writing (and considering solely the Otophysan orders within this superorder), Cypriniformes contains ~4,639 species, Characiformes contains ~2,251 species, Suliriformes contains ~3,976 species and Gymnotiformes contains ~260 species. These Otophysan orders are well-known for their morphological and ecological diversity. With fewer species, the gymnotiforms seem to encompass a disproportionately high degree of ecological diversity. Why? It is easy to imagine that the electrosensory system has played a major role in ecological diversification. Basic anatomy and morphology are highly conserved throughout the gymnotiform phylogeny; however, the head is the exception (Evans et al., 2017, 2019), which may reflect the importance of the trunk morphology to electrosensory system. The visceral and reproductive organs are all contained in a highly compact region just posterior to the head, and the rest of their long, eel-like bodies are reserved for muscle, the electric organ, and supporting tissues.

Because the electrosensory system is intimately associated with ecological performance, factors such as temperature, conductivity, and dissolved oxygen—which can greatly impact the function of the electrosensory system—may limit their capacity to expand over larger ranges like their Otophysan sister clades. These theories have been discussed at greater length by other authors (Albert and Crampton, 2005; Crampton, 1998, 2011). I highlight these discrepancies in diversity because gymnotiform physiological diversity is often overlooked in ecological studies. My dissertation

highlights the fact that recent research has only uncovered the proverbial tip of the iceberg with regards to physiological diversity in gymnotiforms. Comparative studies, such as those included in this dissertation, could yield new insights into the evolutionary diversification of electric fishes, including trophic ecology, mating systems, reproductive strategies, habitat use, and drift (Arnegard et al., 2010; Curtis and Stoddard, 2003; Picq et al., 2016).

Experimental research on electric fishes has greatly contributed to our understanding of neurophysiology, especially the diversity of ion channel properties and their mechanisms of regulation. Electrocytes themselves are highly diverse, and the few studies that have characterized them in just a handful of species have uncovered surprising adaptations that could have significant biomedical importance (Ban et al., 2015; Markham et al., 2013; Markham and Zakon, 2014; Stoddard et al., 2006; Thompson et al., 2018). The accessibility of electrocytes and their inextricable role in in sensing the environment and communication provide a window into fundamental endocrine-regulatory mechanisms of excitable cells and behavior. There likely is considerable interspecific variation in how the melanocortin system regulates EOD waveform plasticity in gymnotiform fishes that may be related to patterns of receptor expression, circulating hormone levels, and differences in the secondary effector pathways. Many unknowns remain, and we obviously need more information on ecology and behavior to aid interpretation of physiological differences. We have scarcely begun to understand the pleiotropic functions of the melanocortin system, and electric fishes provide an excellent model system for future research.

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APPENDIX A

Species	Zone	Tube_scaled	Amp_scaled	Total Tube	Total Amp
A_clark	IO_post	24.26	0.57	155	8
A_clark	IO_pre	34.34	0.60	91	3
A_clark	MP_post	17.77	6.72	116	49
A_clark	MP_pre	397.30	57.82	663	141
A_clark	SO_post	54.01	35.12	187	94
A_clark	SO_pre	71.60	26.76	101	38
B_diazi	IO_post	85.35	29.22	621	205
B_diazi	IO_pre	102.47	79.36	382	316
B_diazi	MP_post	44.63	18.50	204	84
B_diazi	MP_pre	74.18	60.73	333	283
B_diazi	SO_post	81.44	16.71	435	93
B_diazi	SO_pre	204.69	101.01	440	244
E_tril	IO_post	67.71	5.43	567	38
E_tril	IO_pre	128.53	3.33	1311	34
E_tril	MP_post	42.08	6.23	223	33
E_tril	MP_pre	356.06	2.57	1077	9
E_tril	SO_post	71.93	7.13	414	60
E_tril	SO_pre	170.00	17.86	886	25
P_gimb	IO_post	47.84	10.83	467	119
P_gimb	IO_pre	47.50	18.34	290	116
P_gimb	MP_post	23.42	10.73	148	78
P_gimb	MP_pre	169.74	70.63	604	371
P_gimb	SO_post	26.87	1.25	167	15
P_gimb	SO_pre	44.22	8.06	193	79
R_east	IO_post	100.05	60.76	807	414
R_east	IO_pre	226.43	195.23	637	305
R_east	MP_post	121.24	56.23	295	130
R_east	MP_pre	167.07	71.22	767	306
R_east	SO_post	286.74	226.25	716	634
R_east	SO_pre	367.50	283.20	273	364
R_marm	IO_post	70.02	6.25	402	49
R_marm	IO_pre	313.43	89.39	1225	325
R_marm	MP_post	20.56	4.15	79	18
R_marm	MP_pre	282.89	47.59	365	59

Table 2. Summary of electroreceptor pore counts by type.

Species	Zone	Tube_scaled	Amp_scaled	Total Tube	Total Amp
R_marm	SO_post	76.67	7.18	362	32
R_marm	SO_pre	473.57	117.02	389	70
S_eleg	IO_post	40.32	3.66	337	33
S_eleg	IO_pre	78.86	9.68	790	99
S_eleg	MP_post	82.25	3.89	346	22
S_eleg	MP_pre	165.70	4.88	877	24
S_eleg	SO_post	22.52	1.63	151	8
S_eleg	SO_pre	15.25	3.50	61	14

Table 2. Continued

Desiccation Protocol

Protocol for chemically drying heads of preserved fish specimens using high-percentage ethanol and hexamethyldisilazane (HMDS). Steps 2-11 sum to a total of thirteen 30-minute washes each with the specimen placed in a glass vial on an orbital shaker mixing table. The solution should completely cover the specimen. HMDS should only be handled under a fume hood with adequate ventilation.

1. Leave desired piece of tissue in 90% ethanol overnight.
2. Two 30-min washes of 95% EtOH, replacing ethanol between washes.
3. Two 30-min washes of 100% EtOH, replacing ethanol between washes.
4. 90:10 EtOH:HMDS
5. 80:20 EtOH:HMDS
6. 65:35 EtOH:HMDS
7. 50:50 EtOH:HMDS
8. 35:65 EtOH:HMDS

9. 20:80 EtOH:HMDS

10. 10:90 EtOH:HMDS

11. Two 30-min washes of 100% HMDS, replacing HMDS between washes.

12. Remove all remaining liquid and leave the vial in the fume hood overnight.