DIVERSITY IN THE STRUCTURE OF SIGNALS PRODUCED BY SOUTH AMERICAN WEAKLY ELECTRIC KNIFEFISH

Jacquelyn M. Petzold

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biology,
Indiana University
December 2016

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Doctoral Committee

_____

G. Troy Smith, Ph.D.

_____

Gregory Demas, Ph.D.

_____

Laura Hurley, Ph.D.

_____

Katherine Kearns, Ph.D.

_____

Dale Sengelaub, Ph.D.

November 29, 2016

Acknowledgements

Writing this dissertation was the culminating experience of my intense and rewarding immersion in the world of scientific research. My journey through graduate school was supported and enriched by many wonderful people, and I owe a debt of gratitude to you all. To my advisor Troy, thank you for your unending patience, your enthusiasm, and your eloquent explanations of nearly everything. To my committee members, thank you for your thoughtful advice, your encouragement, and your willingness to let me forge my own path. To my friends and comrades in the EEB program, thank you for enthusiastically celebrating my successes and empathetically commiserating with my failures. To Cathy, thank you for your steady support and your unshakeable belief in my potential. To my friends and family, thank you for providing love, laughter, and frequent reminders that are other things in the world besides electric fish.

And finally to my wife Hil, thank you for your boundless support and encouragement and for your occasional brilliant flashes of insight.

Jacquelyn M. Petzold

DIVERSITY IN THE STRUCTURE OF SIGNALS PRODUCED BY SOUTH AMERICAN WEAKLY ELECTRIC KNIFEISH

Natural and sexual selection shape animal communication signals according to the demands of social context and the environment, which results in enormous variation in signal properties. My dissertation uses the electrocommunication signals of South American weakly electric knifefish to compare signal structure across several closely related species, with particular emphasis on signals that are extreme or unusual. Weakly electric fish continuously generate an electric field using an electric organ discharge (EOD). During short-range social interactions, fish produce chirps by rapidly and transiently increasing EOD frequency. I used recordings with playbacks of conspecific signals and hormone manipulation to characterize the sexually dimorphic chirp duration of *Parapteronotus hasemani*, a species of electric fish with high-frequency, long-duration chirps and huge variation in male morphology. I also described signaling behavior in *Distocyclus conirostris*, a species of electric fish with a low-frequency EOD and an unusual asymmetrical behavioral response to "jamming" created when EODs of similar frequencies interact. Next, I compared across species to examine how signal properties (EODs and chirping) interact to influence each other's detection and evolution. Certain signal parameters such as chirp frequency modulation and EOD frequency difference have substantial effects on chirp conspicuousness. Contrary to expectations, there was little support for a strict co-evolution in which a species' chirps are most conspicuous on their own EOD waveforms. Thus, although EOD properties influence chirp conspicuousness, other factors such as the social or physical environment also likely

shape chirp structure. Additionally, I show that EOD waveform may differ in perceptibility based on the EOD waveform complexity of the interacting fish. I consider how chirp conspicuousness could drive the evolution of sexually dimorphic chirps (such as those produced by *P. hasemani*), and I raise questions about whether low-frequency EODs (such as those produced by *D. conirostris*) contain sufficient information for fish to detect conspecific EOD frequencies using the neural mechanisms described in fish with high-frequency EODs. Taken together, these results show how the properties of multi-component signals shape each other and impact signal detectability. Finally, my dissertation concludes with a description of an innovative approach to teaching scientific communication skills in a highly structured undergraduate introductory biology lab.

_____

G. Troy Smith, Ph.D.

_____

Gregory Demas, Ph.D.

_____

Laura Hurley, Ph.D.

_____

Katherine Kearns, Ph.D.

_____

Dale Sengelaub, Ph.D.

Table of Contents

Chapter 1

Introduction

Animal communication occurs when one organism (the sender) transmits a signal to another organism (the receiver). The information transmitted in the signal enhances the fitness of the sender, the receiver, or both. The dazzling array of signals produced by animals highlights the bountiful diversity of nature and the unyielding influences of natural and sexual selection. Animals use an arsenal of communication signals for different purposes including to facilitate mating (Endler, 1992; Gröning & Hochkirch, 2008), to prevent or ameliorate intra-specific aggression (Barnard & Burk, 1979; Lopez & Martin 2001; VanderWerf & Freed, 2003), to warn conspecifics of danger (Manser, 2001; Owings & Virginia, 1978; Sherman, 1977), to discourage predators (reviewed in Skelhorn et al., 2016; Speed, 2000; Stevens & Ruxton, 2012), and to lure prey (Lewis & Cratsley, 2008; White & Kemp, 2015; Zuk & Kolluru, 1998). Given these varied uses for signals and the different conditions under which they are transmitted, it is not surprising that the properties of animal communication signals are exceedingly diverse and designed to stimulate nearly all known animal sensory modalities. Signaling often occurs in environments teeming with biodiversity, which can favor the partitioning of the signal space (spectral content, timing, etc.) into niches in order to enhance transmission of many different signals (Amézquita et al., 2006; Greenfield, 2015). If signal production occurs in environments with predictable environmental noise, signal properties may evolve in certain directions to overcome the sensory constraints inflicted by the noise (Tobias et al., 2010; Endler, 1992). Closely related species living in sympatry sometimes evolve mechanisms whereby certain signals (such as those used in courtship) differ in

predictable and perceptible ways from each other. This diversity in signal structure, which can function in part to prevent wasteful heterospecific mating attempts, is often reinforced by preferences for conspecific signals, usually among the "choosier" sex (Kozak & Boughman, 2009; Saetre, 1997). The forces of selection shape signals to enhance their utility in challenging social and environmental contexts.

In some systems, selection acts differently on males and females, either via intersexual selection (when one sex preferentially chooses mates with certain traits) or via intrasexual selection (when certain traits give individuals of one sex a competitive advantage over same-sex conspecifics). When individuals of one sex signal to attract the attention of the opposite sex for mating purposes, the individuals of the signaling sex (often males) must compete with each other to produce the loudest or most attractive song, the brightest plumage, or the largest ornament (Jawor & Breitwisch, 2003; Meyer, 1997; Searcy & Andersson, 1986). Such traits that are advantageous for one sex but neutral or disadvantageous for the other can become more prominent via sexual selection (Tazzyman et al., 2014). Under certain circumstances, some males that may otherwise be at a tremendous disadvantage in securing mating opportunities will use alternative reproductive strategies. These strategies generally involve forgoing sexually dimorphic signaling in order to achieve mating opportunities through covert means (Gross, 1996; Taborsky, 1994). Males using alternative reproductive strategies often shift reproductive investment to enhanced gamete production. For example, the midshipman fish (*Porichthys notatus*) has two types of males: larger Type 1 males that guard nests and produce attractive songs, and smaller Type 2 males that do not guard nests, do not produce songs, and dump huge numbers of gametes during "sneak" mating attempts

(Brantley & Bass, 1994). The two types of midshipman males illustrate the trade-off between investment in signaling (advertising quality via larger body size and robust song-production) and investment in gametes (to the detriment of conspicuous traits preferred by females).

Obviously, signals must be perceptible to the receiver's sensory system and accurately decoded by the nervous system. After all, a signal that cannot be perceived by the intended recipient is a signal that represented wasted time and energy and perhaps needless exposure to predators (Magnhagen, 1991). The sensory drive hypothesis states that the sensory machinery with which the receiver captures signals is a significant force in shaping signal properties (Endler, 1992; Endler & Basolo, 1998). Signal reception determines the success of certain signal types and the failure of others, which shapes the properties of future signals. However, a particular sensory system may be used for more than one signal type. For example, many songbirds produce two signal types (calls and songs), which differ in structure and are used in different social contexts (reviewed in Marler, 2004). The songbird brain has evolved two distinct processing paths that accommodates these two signal types (reviewed in Margoliash, 1997; Maul et al., 2010). The selective processes that shape one type of signal need not necessarily affect the evolution of other type of signal; the same is true on the sensory processing side.

Sometimes animals produce two or more signals that simultaneously impinge on different receiver sensory systems. Such multimodal signaling may serve to attract the receiver's attention or to provide redundancy in the event that one signal component degrades too much to transmit information to the intended receiver effectively (Hebets & Papaj, 2005). The anti-predator signals of California ground squirrels (*Spermophilus*

beecheyi) are a particularly fascinating example of a multimodal signal that is adapted to the sensory machinery of the receiver. The rattlesnake *Crotalus oreganus* can detect prey (including California ground squirrel pups) using visual cues at close range. However, these rattlesnakes can also detect prey from further away by using specially adapted pit organs to detect thermal cues from body heat (Bullock & Diecke, 1956; Haverly & Kardong, 1996). Adult California ground squirrels produce a conspicuous tail-waving display to deter predation on their pups (Hennessy et al., 1981; Owings & Koss, 1977). When the approaching predator is a rattlesnake, the squirrel increases the temperature of its tail by 2-3° C during the tail-waving display. The combined visual and thermal signal increases the probability of eliciting rattlesnake defensive behaviors compared to the effect of the visual signal alone (Rundus et al., 2007). However, when the predator is a thermally insensitive gopher snake (*Pituophis melanoleucus*), the squirrel forgoes the temperature increase and instead produces only the visual signal (Rundus et al., 2007). Thus, the squirrel flexibly responds with the combined visual and heat display only when it is likely to induce the desired behavior in the receiver. By tailoring the signal to the sensory capabilities of the receiver, the squirrel may benefit from lower thermoregulatory and metabolic costs. This example illustrates that signals can be adapted in specific, predictable ways based on the sensory predispositions of the receiver and that the properties of signals themselves can evolve to facilitate the signaler/receiver match.

Multicomponent signals are animal communication signals composed of two or more distinct parts that simultaneously impinge upon the same sensory system. For example, chemosensory signals, which are evolutionarily quite old and likely the first communication signals to evolve, tend to contain mixtures of metabolic compounds that

simultaneously provide multiple types of information about the sender that deposited them (Hebets & Rundus, 2011). Multicomponent olfactory signals have been found to be particularly important for some eusocial insects, including ants (Denis et al., 2006; Liebig et al., 2000; Newey, 2011). Discriminating between nestmates and non-nestmates in complex eusocial societies is crucial because the cost of behaving altruistically to non-nestmates is high (Newey, 2011; Smith et al., 2009). At the same time, the social structure and dominance hierarchy may require specific individual indicators of fertility and/or caste to direct colony-supporting activities. For example, in the ant *Pachycondyla goeldii*, cuticular hydrocarbons used in signaling colony identity are synthesized in abdominal tissues and stored in the postpharyngeal gland (PPG). Ants transfer these compounds to their body surface during self-grooming and share them with other ants during allogrooming, trophallaxis, and physical contact (Soroker et al., 2003). The chemical components in the common mixture from the PPG are distinct from other cuticular hydrocarbons that vary among colony members according to fertility status (Hannonen et al., 2002; Liebig et al., 2000). The use of this chemical blend as a signal must therefore balance the mixing of common cuticular hydrocarbons with the restricted expression of specific fertility-signaling compounds. Multicomponent signals present an interesting challenge to sensory systems since the relevant sensory and neural structures must simultaneously extract, analyze, and potentially act upon two or more different types of information.

The goal of my dissertation is to make sense of signal diversity with particular emphasis on signals that are extreme or unusual relative to other signals. I use a comparative approach to sample natural signal diversity and then ask how different

features of complex multicomponent signals may have co-evolved to be detectable by nearby receivers. To do this, I studied the electrocommunication signals of South American weakly electric knifefish, a speciose group of teleost fish with a unique multi-purpose electrosensory system. Weakly electric fish produce an electric organ discharge (EOD) that generates a weak electric field. The EOD functions in object localization/identification and electrocommunication. Much like bright bird plumage, the EOD serves as a constant signal of identity by communicating varying types of information such as sex, species, or social status (Zakon & Dunlap, 1999; Smith, 2013). Modulations of the EOD called chirps are important transient, context-dependent social signals, much like bird songs and calls (Zupanc & Maler, 1997). The electrosensory system senses modulations of the EOD that are important for social communication (EODs and chirps of conspecifics) and object detection (predictable distortions of the fish's own EOD), which has led to the evolution of complex and fascinating neural strategies for simultaneously processing all this incoming information. Thus, electric fish are "champions" of sensory performance, adept at perceiving minute variations in signal properties and capable of receiving and analyzing complex multicomponent signals through a single remarkable sensory system (Heiligenberg, 1991). In my dissertation, I have harnessed the behavior of this unique animal to discover how signals vary between sexes and across species and to explore how such variation in signal properties might be encoded by animals immersed in a complex sensory milieu.

The dual use of the EOD in electrolocation and communication makes it likely that signal evolution is mediated by the competing demands of both functions. Finding prey via electrolocation requires detecting and localizing tiny distortions in the electric

field created by invertebrate prey items. Finding mates and monitoring threats of intra-sexual aggression requires detecting global, periodic distortions in the electric field generated by electrically signaling conspecifics (Chacron, 2003). In other animals systems, these types of competing sensory inputs sometimes lead to sensory bias, which occurs when characteristics of the sensory machinery or pre-existing receiver preferences for certain types of stimuli exert selective pressure on the properties of signals used in communication (Endler, 1992). For example, water mites (*Neumania papillator*) adopt a characteristic "net stance" while hunting and remain stationary in order to hone in on the vibrations created by copepod prey. When courting male water mites detect the chemosensory cues of a nearby female, the male begins to vibrate his legs at the same frequency as the copepods. Females clutch the vibrating males, seemingly without distinguishing between the vibrations of courting males and potential prey (Proctor, 1991). Spermatophore transfer occurs immediately after the female clutches the male, which indicates that the exploitation of this female sensory bias by the males is a successful strategy for eliciting reproduction (Endler & Basolo, 1998; Proctor, 1991). In this case, foraging strategies useful for prey acquisition likely led to the adoption of behavioral strategies useful for mate acquisition. Since the electrosensory system of electric fish undoubtedly has certain adaptations necessary to enhance the salience of signal distortions created by prey, it may follow that transient social signals such as chirps have co-evolved in specific patterns that make them more easily detected or more attractive to the receiver. The use of appropriately designed signals is crucial for coordinating fitness-enhancing social behaviors because signals that are more salient to the receiver are more likely to elicit the context-appropriate behavioral response.

In my dissertation, I look broadly at sex- and species-level diversity to understand how multicomponent signals evolve and how the structure of such signals transmits meaningful information to the sensory system. The input to the electrosensory system is complex because it contains information relevant for communication (EODs, beat, and chirps) and for electrolocation (slow amplitude modulations associated with movement and distortions created by electrically non-transparent objects and organisms). The complexity of this input presents an extreme need to optimize coding of information to extract parameters of multiple signal types contained within the same sensory stream. The immensity of this task is exacerbated by the phenomenal diversity in signal properties across species that often co-exist in close proximity within the same river systems (Kramer, Kirschbaum, & Markl, 1981). The first two chapters of my dissertation focus on communication signals in two species of electric fish that produce unusual signals. In Chapter 2, I describe the sex differences in the signals produced by *Parapteronotus hasemani*, a species of electric fish with substantial variation in male jaw morphology. *P. hasemani* has the most extreme chirps (in duration and in degree of frequency modulation) among all currently characterized weakly electric knifefish. Males and females produce chirps that differ in duration, but there are no detectable differences in signaling (EODs or chirping) between long-jawed and short-jawed males. This chapter also addresses the hormonal regulation of the *P. hasemani* sex difference in chirp duration by showing that treatment with the androgen receptor blocker flutamide feminizes chirp duration in males without affecting non-sexually-dimorphic aspects of signaling. In Chapter 3, I describe the structure of signals (EODs, jamming avoidance response, and chirping) produced by *Distocyclus conirostris*, which emit EODs with a

fairly broad range of low frequencies. The jamming avoidance response is a behavior that is thought to alleviate the electrosensory interference created by nearby EODs of similar frequency. The *D. conirostris* jamming avoidance response was interesting because it was somewhat anomalous: sometimes the fish shifted EOD frequency away from the jamming stimulus (jamming avoidance) and sometimes the fish shifted EOD frequency toward the stimulus (anti-jamming avoidance). This contrasts with two other closely related species, *Sternopygus macrurus* (which has no JAR) and *Eigenmannia virescens* (which is thought to consistently shift its frequency away from the jamming stimulus). This cross-species comparison led me to hypothesize an additional (or alternative) social function for the jamming avoidance response.

In later chapters, I zoom out to look at how diversity in signal structure across many species of electric fish might affect how readily the receiver's sensory machinery can perceive signals. In Chapter 4, I quantify the conspicuousness of the chirps produced by twelve species of weakly electric fish in an effort to show how variation in chirp structure (duration, frequency modulation, etc.) affects the ability of chirps to stand out in the signal context of a typical social environment (i.e., on background EODs). In this chapter, I also use a technique to synthesize hybrid chirps that have the characteristics of one species but the EOD waveform of another species. This approach tests whether chirps are optimally conspicuous on a conspecific background. Finally, I offer insight into how EOD waveform might be perceptible/useful for some species and less important or less perceptible for others. The data I present from this signal analysis generates predictions about how diversity in signal structure might influence the sensory system. I also consider how signals that simultaneously impinge on the same sensory system might

interact to influence each other's detection and evolution. In Chapter 5, I consider how the signal diversity described in Chapters 2 and 3 can be interpreted within the framework outlined in Chapter 4. Specifically, I discuss how variation in sex differences might influence chirp conspicuousness across species. Additionally, I show that the *D. conirostris* EOD signal does not have the necessary properties to allow fish to detect each other's EODs using the neural circuitry that has been well-described in fish with high-frequency EODs. This finding challenges the conventional understanding of how weakly electric fish determine the EOD frequency conspecifics. I suggest that there are likely other, as-yet undiscovered mechanisms for fish to determine EOD frequency, at least among fish with low-frequency EODs. This could potentially have interesting implications for signal processing.

Finally, in the scientific pedagogy chapter (Chapter 6), I explore how changing the day-to-day structure of classroom activities influences students' ability to use the scientific literature and to design, interpret, and describe hypothesis-driven experiments. I show that it is possible to teach undergraduate students higher-order thinking skills without changing the laboratory exercises in a highly structured introductory biology lab course.

References

Amézquita, A., Hodl, W., Lima, A. P., Castellanos, L., Erdtmann, L., & De Araujo, M. C. (2006). Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog Allobates femoralis. *Evolution, 60*(9), 1874-1887.

Barnard, C. J., & Burk, T. (1979). Dominance hierarchies and the evolution of individual recognition. *Journal of Theoretical Biology, 81*(1), 65-73. doi:10.1016/0022-5193(79)90081-x

Brantley, R. K., & Bass, A. H. (1994). Alternative male spawning tactics and acoustic-signals in the plainfin midshipman fish Porichthys-notatus Girard (Teleostei, Batrachoididae). *Ethology, 96*(3), 213-232.

Bullock, T. H., & Diecke, F. P. J. (1956). Properties of an infra-red receptor. *Journal of Physiology-London, 134*(1), 47-87.

Chacron, M. J., Doiron, B., Maler, L., Longtin, A., & Bastian, J. (2003). Non-classical receptive field mediates switch in a sensory neuron's frequency tuning. *Nature, 423*(6935), 77-81. doi:10.1038/nature01590

Denis, D., Blatrix, R., & Fresneau, D. (2006). How an ant manages to display individual and colonial signals by using the same channel. *Journal of Chemical Ecology, 32*(8), 1647-1661. doi:10.1007/s10886-006-9099-7

Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. *American Naturalist, 139*, S125-S153. doi:10.1086/285308

Endler, J. A., & Basolo, A. L. (1998). Sensory ecology, receiver biases and sexual selection. *Trends in Ecology & Evolution, 13*(10), 415-420. doi:10.1016/s0169-5347(98)01471-2

Greenfield, M. D. (2015). Signal interactions and interference in insect choruses: singing and listening in the social environment. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology, 201*(1), 143-154. doi:10.1007/s00359-014-0938-7

Gross, M.R. (1996). Alternative reproductive strategies and tactics: Diversity within sexes. *Trends in Ecology & Evolution, 11*: 92-98.

Gröning , J., & Hochkirch, A. (2008). Reproductive interference between animal species. *Quarterly Review of Biology, 83*(3), 257-282. doi:10.1086/590510

Hannonen, M., Sledge, M. F., Turillazzi, S., & Sundstrom, L. (2002). Queen reproduction, chemical signalling and worker behaviour in polygyne colonies of

the ant Formica fusca. *Animal Behaviour, 64*, 477-485. doi:10.1006/anbe.2002.4001

Haverly, J. E., & Kardong, K. V. (1996). Sensory deprivation effects on the predatory behavior of the rattlesnake, Crotalus viridis oreganus. *Copeia*(2), 419-428.

Hebets, E.A., Papaj, D.R. (2005). Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology 57*, 197-214.

Hebets, E. A., & Rundus, A. (2011). Chemical Communication in a Multimodal Context. In T. Breithaupt & M. Thiel (Eds.), *Chemical Communication in Crustaceans* (pp. 335-354). New York: Springer.

Heiligenberg, W. (1991). The neural basis of behavior: A neuroethological view. *Annual Review of Neuroscience, 14*:247-267.

Hennessy, D. F., Owings, D. H., Rowe, M. P., Coss, R. G., & Leger, D. W. (1981). The information afforded by a variable signal: constraints on snake-elicited tail flagging by California ground squirrels. Behaviour, 78(3), 3-4.

Jawor, J. M., & Breitwisch, R. (2003). Melanin ornaments, honesty, and sexual selection. *Auk, 120*(2), 249-265. doi:10.1642/0004-8038(2003)120[0249:mohass]2.0.co;2

Kozak, G.M. & Boughman, J.W. (2009). Learned conspecific preference in a species pair of sticklebacks. *Behavioral Ecology, 20*(6): 1282-1288.

Kramer, B., Kirschbaum, F. and Markl, H. (1981). Species specificity of electric organ discharges in a sympatric group of gymnotoid fish from Manaus (Amazonas). Advances in *Physiological Sciences, 31*: 195-217.

Lewis, S. M., & Cratsley, C. K. (2008). Flash signal evolution, mate choice, and predation in fireflies *Annual Review of Entomology* (Vol. 53, pp. 293-321). Palo Alto: Annual Reviews.

Liebig, J., Peeters, C., Oldham, N. J., Markstadter, C., & Holldobler, B. (2000). Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant Harpegnathos saltator? *Proceedings of the National Academy of Sciences of the United States of America, 97*(8), 4124-4131. doi:10.1073/pnas.97.8.4124

Lopez, P., & Martin, J. (2001). Fighting rules and rival recognition reduce costs of aggression in male lizards, Podarcis hispanica. *Behavioral Ecology and Sociobiology, 49*(2-3), 111-116. doi:10.1007/s002650000288

Magnhagen, C. (1991). Predation risk as a cost of reproduction. *Trends in Ecology & Evolution, 6*(6), 183-185. doi:10.1016/0169-5347(91)90210-o

Manser, M. B. (2001). The acoustic structure of suricates' alarm calls varies with predator type and the level of response urgency. *Proceedings of the Royal Society B-Biological Sciences, 268*(1483), 2315-2324. doi:10.1098/rspb.2001.1773

Margoliash, D. (1997). Functional organization of forebrain pathways for song production and perception. *Journal of Neurobiology, 33*(5), 671-693. doi:10.1002/(sici)1097-4695(19971105)33:5<671::aid-neu12>3.0.co;2-c

Marler, P. (2004). Bird calls - Their potential for behavioral neurobiology. *Behavioral Neurobiology of Birdsong, 1016*, 31-44. doi:10.1196/annals.1298.034

Maul, K. K., Voss, H. U., Parra, L. C., Salgado-Commissariat, D., Ballon, D., Tchernichovski, O., & Helekar, S. A. (2010). The development of stimulus-specific auditory responses requires song exposure in male but not female zebra finches. *Developmental Neurobiology, 70*(1), 28-40. doi:10.1002/dneu.20751

Meyer, A. (1997). The evolution of sexually selected traits in male swordtail fishes (Xiphophorus: Poeciliidae). *Heredity, 79*, 329-337.

Newey, P. (2011). Not one odour but two: A new model for nestmate recognition. *Journal of Theoretical Biology, 270*(1), 7-12. doi:10.1016/j.jtbi.2010.10.029

Owings, D. H., & Coss, R. G. (1977). Snake mobbing by California ground squirrels - adaptive variation and ontogeny. *Behaviour, 62*, 50-69. doi:10.1163/156853977x00045

Owings, D. H., & Virginia, R. A. (1978). Alarm calls of California ground squirrels (Spermophilus-beecheyi). *Zeitschrift Fur Tierpsychologie-Journal of Comparative Ethology, 46*(1), 58-70.

Proctor, H. C. (1991). Courtship in the water mite Neumania-papillator - males capitalize on female adaptations for predation. *Animal Behaviour, 42*, 589-598. doi:10.1016/s0003-3472(05)80242-8

Rundus, A. S., Owings, D. H., Joshi, S. S., Chinn, E., & Giannini, N. (2007). Ground squirrels use an infrared signal to deter rattlesnake predation. *Proceedings of the National Academy of Sciences of the United States of America, 104*(36), 14372-14376. doi:10.1073/pnas.0702599104

Saetre, G.P. (1997). Differential species recognition abilities of males and females in a flycatcher hybrid zone. *Journal of Avian Biology, 28*(3): 259-263.

Searcy, W. A., & Andersson, M. (1986). Sexual selection and the evolution of song. *Annual Review of Ecology and Systematics, 17*, 507-533. doi:10.1146/annurev.es.17.110186.002451

Sherman, P. W. (1977). Nepotism and evolution of alarm calls. *Science, 197*(4310), 1246-1253. doi:10.1126/science.197.4310.1246

Skelhorn, J., Halpin, C. G., & Rowe, C. (2016). Learning about aposematic prey. *Behavioral Ecology, 27*(4), 955-964. doi:10.1093/beheco/arw009

Smith, A. A., Holldober, B., & Liebig, J. (2009). Cuticular hydrocarbons reliably identify cheaters and allow enforcement of altruism in a social insect. *Current Biology, 19*(1), 78-81. doi:10.1016/j.cub.2008.11.059

Smith, G. T. (2013). Evolution and hormonal regulation of sex differences in the electrocommunication behavior of ghost knifefishes (Apteronotidae). *Journal of Experimental Biology, 216*(13), 2421-2433. doi:10.1242/jeb.082933

Soroker, V., Lucas, C., Simon, T., Fresneau, D., Durand, J. L., & Hefetz, A. (2003). Hydrocarbon distribution and colony odour homogenisation in Pachycondyla apicalis. *Insectes Sociaux, 50*(3), 212-217. doi:10.1007/s00040-003-0669-1

Speed, M. P. (2000). Warning signals, receiver psychology and predator memory. *Animal Behaviour, 60,* 269-278. doi:10.1006/anbe.2000.1430

Stevens, M., & Ruxton, G. D. (2012). Linking the evolution and form of warning coloration in nature. *Proceedings of the Royal Society B-Biological Sciences, 279*(1728), 417-426. doi:10.1098/rspb.2011.1932

Taborsky, M. (1994). Sneakers, satellites, and helpers: Parasitic and cooperative behavior in fish reproduction. *Advances in the Study of Animal Behavior, 23*: 1-100.

Tazzyman, S. J., Iwasa, Y., & Pomiankowski, A. (2014). Signaling efficacy drives the evolution of larger sexual ornaments by sexual selection. *Evolution, 68*(1), 216-229. doi:10.1111/evo.12255

Tobias, J. A., Aben, J., Brumfield, R. T., Derryberry, E. P., Halfwerk, W., Slabbekoorn, H., & Seddon, N. (2010). Song divergence by sensory drive in Amazonian birds. *Evolution, 64*(10), 2820-2839. doi:10.1111/j.1558-5646.2010.01067.x

VanderWerf, E. A., & Freed, L. A. (2003). 'Elepaio subadult plumages reduce aggression through graded status-signaling, not mimicry. *Journal of Field Ornithology, 74*(4), 406-415.

White, T. E., & Kemp, D. J. (2015). Technicolour deceit: a sensory basis for the study of colour-based lures. *Animal Behaviour, 105*, 231-243. doi:10.1016/j.anbehav.2015.04.025

Zakon, H.H. & Dunlap, K.D. (1999). Sex steroids and communication signals in electric fish: A tale of two species. *Brain, Behavior, and Evolution 54*(1): 61-69.

Zuk, M., & Kolluru, G. R. (1998). Exploitation of sexual signals by predators and parasitoids. *Quarterly Review of Biology, 73*(4), 415-438. doi:10.1086/420412

Zupanc, G.K.H. & Maler, L. (1997). Neuronal control of behavioral plasticity: the prepacemaker nucleus of weakly electric gymnotiform fish. *Journal of Comparative Physiology A, 18*0: 99-111.

Chapter 2.

Androgens regulate sex differences in signaling but are not associated with male variation in morphology in the weakly electric fish *Parapteronotus hasemani*

Jacquelyn M. Petzold[a,b] & G. Troy Smith[a,b]

Affiliations:
[a] Department of Biology, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405, USA
[b] Center for the Integrative Study of Animal Behavior, Indiana University, 409 N. Park Ave., Bloomington, IN 47405, USA

This chapter follows the format of the journal *Hormones & Behavior*

Petzold, J.M. & Smith, G.T. (2016). Androgens regulate sex differences in signaling but not inter-male differences in morphology in the weakly electric fish *Parapteronotus hasemani*. *Hormones & Behavior*, *78*: 67-71.

ABSTRACT

Sexually dimorphic signaling is widespread among animals and can act as an honest indicator of mate quality. Additionally, differences in signaling and morphology within a sex can be associated with different strategies for acquiring mates. Weakly electric fish communicate via self-generated electrical fields that transmit information about sex, reproductive state, and social status. The weakly electric knifefish *Parapteronotus hasemani* exhibits sexual dimorphism in body size as well as substantial within-male variation in body size and jaw length. We asked whether *P. hasemani* exhibits hormonally mediated sexual dimorphism in electrocommunication behavior. We also asked whether males with short versus long jaws differed significantly from each other in morphology, behavior, hormone levels, or reproductive maturity. Males produced longer chirps than females, but other signal parameters (electric organ discharge frequency; chirp rate and frequency modulation) were sexually monomorphic. Pharmacologically blocking androgen receptors in males reduced chirp duration, suggesting that this sexually dimorphic trait is regulated at least in part by the activational effects of androgens. Males sorted into two distinct morphological categories but did not differ in circulating 11-ketotestosterone or testosterone. Short-jawed males and long-jawed males also did not differ in any aspects of signaling. Thus, chirping and high levels of 11-ketotestosterone were reliably associated with reproductively active males but do not necessarily indicate male type or quality. This contrasts with other alternative male morph systems in which males that differ in morphology also differ in androgen profiles and signaling behavior.

INTRODUCTION

Sexual selection can act upon numerous traits to produce sexual dimorphism in morphology and behavior. Sexually dimorphic traits that exhibit variation within one sex can serve as honest indicators of quality and may differentially attract potential mates (Badyaev & Hill, 2000; Owens & Hartley, 1998; Zahavi, 1975). Variation within a sex can either be continuous or discrete. Discrete within-sex variation is often associated with the presence of alternate reproductive tactics, whereby individuals of one sex (typically males) use one of two or more mutually exclusive strategies to secure mating opportunities (Godwin, 2010; Gross, 1996; Oliveira et al., 2008). Distinct male reproductive phenotypes may result from either fixed developmental trajectories or plastic responses to internal or external cues including age, size, or energy reserves (Godwin, 2010; Humfeld, 2013). Fish species with alternative reproductive tactics often have two behaviorally distinct male morphs that differ substantially in size. One subset of males (*courting males*) make a relatively large investment in growth and development of secondary sexual characteristics while other males (*noncourting males*) invest more heavily in gonad development and sperm production at the expense of growth. The larger courting males reproduce by attracting females and defending a territory, while the smaller noncourting males rely on "sneak-spawning" or "streak-spawning" to surreptitiously fertilize eggs (Taborsky, 1994). The mechanisms that regulate the development and maintenance of alternative reproductive tactics among fish vary, but there is strong evidence that androgens play a role, often with higher levels of 11-ketotestostone relative to testosterone in courting males and the reverse in non-courting males (Brantley et al., 1993).

South American weakly electric knifefish are an excellent system in which to study sexually dimorphic morphology and behavior. Weakly electric fish communicate via a specialized electric organ that continuously emits a weak electrical field. The electric organ discharge (EOD) frequency is a distinguishing characteristic that can indicate sex, species, dominance, and/or individual identity (Zakon & Dunlap, 1999). During social interactions, fish can rapidly increase EOD frequency on short timescales to produce context-specific chirps (Hagedorn & Heiligenberg, 1985; Zakon & Dunlap, 1999). Sexual dimorphism in signaling appears ubiquitous, although there is a great diversity across species in the signal parameters that differ between the sexes. Sex differences in EODs and chirps are regulated by sex steroid hormones (reviewed in Smith, 2013).

Parapteronotus hasemani is a species of electric fish in which reproductively mature males vary dramatically in morphology. Short-jawed males have relatively small body sizes with short jaws that make them visually indistinguishable from females. Long-jawed males have longer, heavier bodies and disproportionately larger jaws with pronounced upward curvature (Fig. 1A&B). Indeed, long-jawed males have such a drastically different appearance compared to females and short-jawed males that they were previously misclassified as a separate species (Cox Fernandes et al., 2002). Here, we assess whether the communication signals of *P. hasemani* are hormonally mediated indicators of sex. Additionally, we test whether the two male morphs show differences in signaling or androgen concentration that could be correlated with male quality or differential attractiveness to females.

METHODS

Animals

Sexually mature male (n=18) and female (n=6) *P. hasemani* were collected from the Amazon and Nanay Rivers in Peru in August 2006 and January 2008 by commercial suppliers and transported to Indiana University. Fish were housed separately in 35-L tanks within a 2000-L recirculating aquarium on a 12:12 light/dark cycle. Temperature was maintained at 25-27° C, conductivity at approximately 150 μS/cm, and pH 5.5-6.5. These housing conditions have been shown to stimulate gonadal recrudescence in captivity (Kirschbaum, 1975; Kirschbaum, 1979). Animal care and experimental protocols were approved by the Indiana University Bloomington Institutional Animal Care and Use Committee.

Morphology

To quantify morphology, we measured body mass, body length, and jaw size. Two measures of jaw size were used: the distance from the anterior tip of the mandible to the meeting point of the mouth (gape length) and the distance from the most anterior point of the premaxilla to the posterior edge of the operculum (jaw length). Males were divided into two groups (short-jawed males and long-jawed males) based on the bimodal distribution of jaw measurements (Fig 1A). Jaw length was not measured for four males. Data from those males were included in analyses of sex differences but not within-male differences. Sex was assessed by measuring plasma 11-ketotestosterone (11-KT) and later confirmed by gonadal inspection (19 out of 24 fish). Sex was always determined by

gonadal inspection for any fish that did not provide sufficient samples for 11-KT analysis.

EOD and Chirping Behavior

To test for the presence of sexually dimorphic signaling behavior, we recorded EOD frequency and chirping behavior of male and female *P. hasemani* using a chirp chamber paradigm that has been described elsewhere (Kolodziejski et al., 2005). Briefly, the fish was enclosed in a shelter tube inside a darkened tank. Two carbon electrodes placed parallel to the fish's body were used to record the EOD and chirping. Stimulus signals were generated in audio software (CoolEdit Pro, Syntrillium; Phoenix, AZ) and presented via carbon electrodes placed perpendicular to the fish's body. The stimulus was calibrated midway between the playback electrodes to a root-mean-square (RMS) amplitude of approximately 1.5 mV/cm. After a thirty-minute acclimation period and a four-minute baseline recording, each fish was randomly presented with five sinusoidal stimuli simulating EODs of other fish +5 Hz, -20 Hz, +20 Hz, -150 Hz, and +150 Hz relative to the fish's EOD frequency. Each recording consisted of a one-minute pre-stimulus period, a two-minute playback period, and a one-minute post-stimulus period. Recordings were interspersed with ten-minute rest periods. These recordings were conducted in February-March of 2011.

EOD and Chirp Analysis

EOD frequency was measured by performing a fast Fourier transform (Blackman-Harris window, size 65536) on the baseline recording and was adjusted to the frequency

expected at 26° C using a $Q_{10°C}$ of 1.6 (Dunlap et al., 2000). Chirps were analyzed offline in Igor Pro (Wavemetrics; Lake Oswego, OR) using the custom procedure eFish (efish23e, Brian Nelson, University of Oregon, Eugene, OR; http://nelsonbs.com/eFish/efish.html). For details of the algorithm and processing, see Turner and colleagues (2007). For each fish, we combined data from the six recordings to determine mean chirp rate, duration, and frequency modulation.

Flutamide Treatment

Male *P. hasemani* (n=8; 3 long-jawed, 5 short-jawed) were treated with the androgen receptor blocker flutamide to test for activational effects of androgens on EOD frequency and chirping. Before treatment, we recorded chirps and took a baseline blood sample from each fish. Then fish were divided into two groups, and treatment was assigned using a balanced within-subjects repeated measures design. One group received flutamide treatment followed by a ten-day washout period and then the control (ethanol vehicle) treatment. The other group received the control treatment, then the flutamide treatment, and then a ten-day washout followed by another control treatment. Thus, all fish received both control and flutamide treatments, but the order was balanced, and all fish received a vehicle washout treatment after the flutamide treatment. This design allowed us to control for possible time effects. Each treatment period (control or flutamide) lasted for three weeks. During flutamide treatments, flutamide (Sigma-Aldrich; St. Louis, MO) dissolved in ethanol was added to tank water (0.5 mg flutamide/L of tank water) in a recirculating aquarium system. In the vehicle control treatments, ethanol without flutamide was added to yield the same tank water ethanol

concentration (0.0025%) as in the flutamide treatment. Aquarium water was periodically replenished by adding water treated with the appropriate dose of flutamide or ethanol. We recorded chirps and took a blood sample after each treatment. This experiment was conducted in March-July of 2012.

Hormone Assays

We collected blood samples 1-3 days after each recording in order to measure plasma concentrations of T and 11-KT. Each fish was lightly anesthetized with 0.075% 2-phenoxyethanol (Sigma-Aldrich; St. Louis, MO) in deionized water before 10-15 µL of blood was collected from the caudal vein using a 1 mL syringe and a heparinized needle. Blood samples were centrifuged for five minutes at 10,000 rpm to extract plasma. Plasma samples were initially frozen at -20° C and then stored at -80° C. T and 11-KT enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI; T kit catalog #582701; 11-KT kit catalog #582751) were performed according to the manufacturer's specifications. Plasma samples were diluted in assay buffer at a range of concentrations to ensure at least one sample fell within the range of sensitivity (T: 1:25 and 1:50; 11-KT: 1:25, 1:50, 1:100, and 1:300). Samples from putative males were diluted more than samples from putative females. Each sample was assayed in duplicate for each dilution. The assay detection limit was 1.6 pg/mL for 11-KT and 3.9 pg/mL for T. Intra-assay variability was calculated using the coefficient of variation of the four replicate wells distributed across the plate and containing the 12.4 pg/mL standard (11-KT) or the 62.5 pg/mL standard (T). The intra-assay variability was 12% for 11-KT and 25% for T. Insufficient plasma

was available to conduct 11-KT assays on 3 females and 3 males or to conduct T assays on 3 females and 4 males.

Statistics

Statistical analyses were performed with JMP 11 (SAS Institute, Inc.; Cary, NC) and Statistica 7 (StatSoft Inc.; Tulsa, OK). Comparisons of 11-KT and T concentrations, EOD frequency, and chirp parameters between the sexes and between the two male types were accomplished with two-tailed Welch's t-tests assuming unequal variances. A 2-factor repeated measures ANOVA was used to compare changes in chirp parameters between fish that received flutamide vs. vehicle as their first treatment. Within-subjects comparisons of chirp parameters in baseline vs. flutamide vs. vehicle washout treatments were made with a single-factor repeated measures ANOVA. Effect size was estimated using Cohen's d for pairwise comparisons and eta squared for the ANOVAs.

RESULTS

Between Sex Comparisons

As expected, jaw morphology was sexually dimorphic and varied between the two male morphs. Because gape length and jaw length were tightly correlated (R^2=0.95, $p<0.001$), only comparisons with jaw length are reported here. Males as a group had longer jaws than females (Fig. 1A & 1B; $t(16.9)=4.78$, $p<0.001$, $d=2.31$). Males also had greater total body lengths than females (Table 1; $t(8.8)=3.22$, $p=0.01$, $d=1.52$). Chirp duration was the only sexually dimorphic signal parameter. Chirps produced by males lasted significantly longer than chirps produced by females (Fig. 1C; $t(14.5)=2.63$,

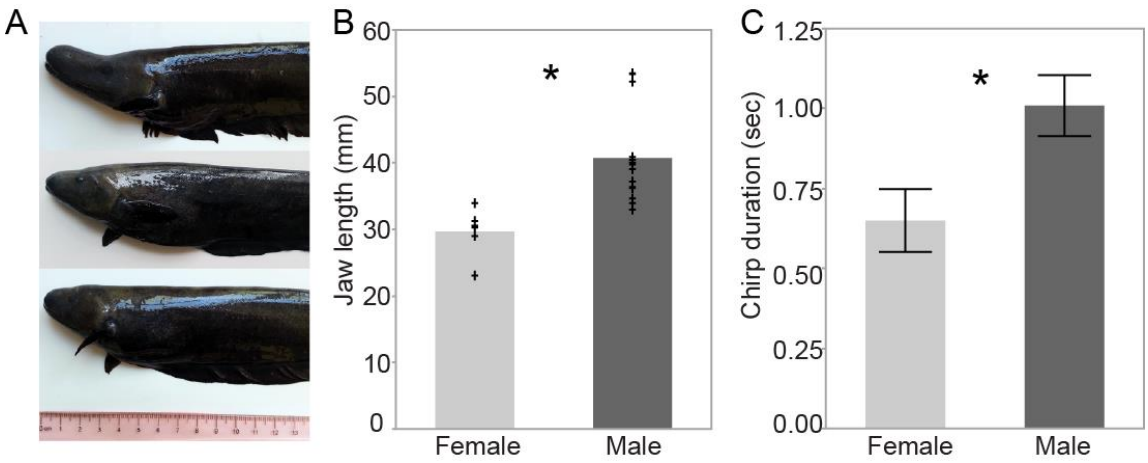


Fig. 1. Comparison of female and male morphology and chirping behavior in *Parapteronotus hasemani*. A) Long-jawed males (top) have larger, more curved jaws than short-jawed males (middle) and females (bottom). B) Mean jaw length is greater for males than females. Males fell into two categories: long-jawed males (>50 mm) and short jawed (< 45 mm). Individual data points shown with crosses. C) Chirps produced by males were longer in duration (mean ± S.E.M.) than chirps produced by females. Asterisks indicate a statistically significant difference (t-test, $p<0.05$)

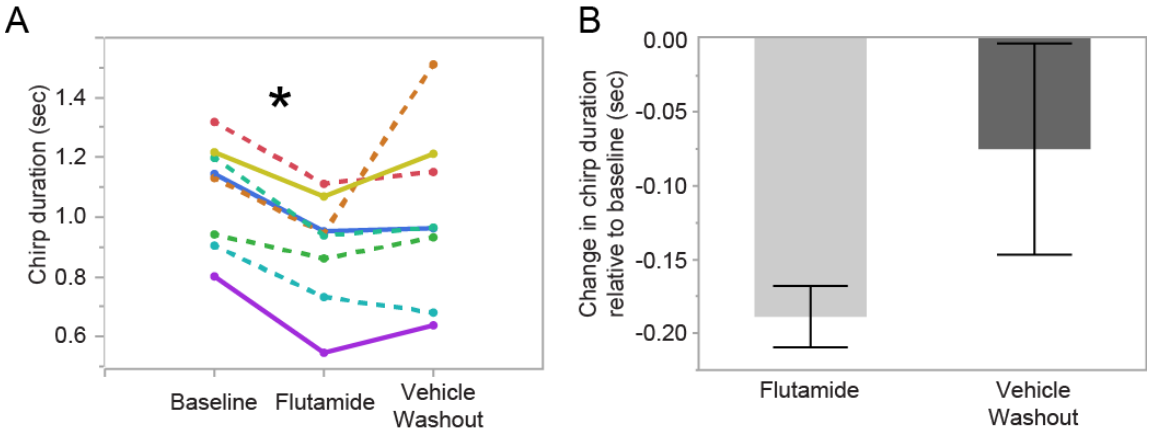


Fig. 2. Male chirp duration before, immediately after, and three weeks after flutamide treatment. A) Males treated with flutamide showed decreased chirp duration relative to baseline. Short-jawed males are indicated with dashed lines; long-jawed males are indicated with solid lines. B) Mean (± S.E.M) change in chirp duration relative to baseline. Chirp duration after a post-flutamide three-week vehicle/washout treatment was marginally greater than chirp duration immediately following flutamide treatment ($F(2,14)=5.2$, $p=0.07$).

Table 1

	Between Sexes		Within Males	
	Females	All males	Short-jawed males	Long-jawed males
Body length (mm)	228.0±14.0[a] *(6)*	280.4±8.3 *(18)*	272.6±6.1[b] *(12)*	317.3±19.1 *(3)*
Jaw length (mm)	29.7±1.5[a] *(6)*	40.7±1.8 *(15)*	37.7±.08[b] *(12)*	53.0±0.4 *(3)*
Electric organ discharge frequency (Hz)	877.5±23.7 *(6)*	875.0±15.8 *(18)*	887.4±19.1 *(12)*	842.5±34.9 *(3)*
Chirp duration (sec)	0.65±0.10[a] *(6)*	1.01±0.10 *(16)*	1.08±0.13 *(11)*	0.96±0.13 (*3)*
Chirp rate (chirps/min)	3.7±1.4 *(6)*	6.7±1.1 *(16)*	7.4±1.5 *(11)*	4.0±0.13 *(3)*
Chirp FM (Hz)	549.8±37.9 *(6)*	541.2±15.7 *(16)*	554.9±18.5 *(11)*	494.7±40.3 *(3)*
11-KT (ng/mL)	0.14±0.05[a] *(3)*	2.56±0.42 *(15)*	2.69±0.55 *(11)*	2.34±0.61 *(3)*
T (ng/mL)	0.76±0.19 *(3)*	0.35±0.09 *(14)*	0.35±0.10 *(11)*	0.36±0.26 *(3)*

[a] significant difference between sexes, $p<0.05$, t-test
[b] significant difference between long vs. short jawed males, $p<0.05$, t-test
Some data points were not available for all fish; see Methods for details. Sample sizes are shown in parentheses. All values are mean ± S.E.M.

p=0.02, d=1.26). EOD frequency, chirp rate, and chirp frequency modulation did not differ between the sexes (Table 1; t-tests, p>0.10). Males did not differ from females in T concentration (t(3.0)=1.89, p=0.15, d=1.20), but males did have higher 11-KT concentrations than females (Table 1; t(14.3)=5.79, p<0.0001, d=3.66).

Within-Male Comparisons

Long-jawed males had significantly longer jaws than short-jawed males, which is consistent with our visual morphological categorizations (t(12.7)=16.86, p<0.0001, d=10.98). Long-jawed males and short-jawed males did not differ in 11-KT concentration (t(5.8)=0.68, p=0.43, d=0.28), T concentration (t(2.7)=0.05, p=0.97, d=0.03) or 11-KT/T ratio (t(3.2)=0.51, p=0.64, d=0.33). The two male types also did not differ in EOD frequency, chirp rate, chirp duration, or degree of chirp frequency modulation (t<1.9; p>0.08).

Flutamide Treatment

Chirp duration decreased significantly in fish that received flutamide as their first treatment, whereas vehicle treatment did not significantly affect chirp duration relative to baseline (baseline chirp duration for flutamide group: 1.16±0.03 sec; post-flutamide: 0.96±0.03 sec; baseline chirp duration for vehicle group: 0.96±0.06 sec; post-vehicle: 0.90±0.05 sec; treatment x time interaction, F(1,6)=20, p<0.01, η_p^2=0.77; PLSD, baseline vs. post-treatment in the flutamide group, p<0.001). Fish in the initial vehicle control group subsequently received flutamide, and we also used within-subjects comparisons to examine the effects of flutamide and subsequent washout treatments across all males in

the experiment. Among all males, flutamide treatment decreased chirp duration relative to pre-treatment baseline recordings (Fig. 2; $F(2,14)=5.2$, $p=0.02$, $\eta_p^2=0.43$; PLSD, baseline vs. flutamide, $p<0.05$). This effect was reversible, as chirp duration in post-flutamide washout/vehicle recordings was not significantly different from baseline (PLSD, baseline vs. washout, $p=0.21$) and was marginally greater than chirp duration post-flutamide treatment (PLSD, flutamide vs. washout, $p=0.07$). This effect was specific to the sexually dimorphic chirp duration parameter; flutamide treatment did not affect EOD frequency, chirp rate, or chirp frequency modulation ($F(2,14)<2.1$, $p>0.15$).

DISCUSSION

Sexually dimorphic signaling can be critical for identifying and attracting a high-quality mate (Zahavi, 1975). Chirping in electric fish is an important social signal used during courtship and aggressive interactions (Hagedorn & Heiligenberg, 1985). The sexual dimorphism observed here suggests that chirp duration could serve as an indicator of sex in *P. hasemani*. Blocking androgen receptors with flutamide partially de-masculinized male chirp duration in the direction and approximate magnitude predicted by sex differences, suggesting that this feature of the EOD is at least partially controlled by the activational effects of androgens. This effect was specific to chirp duration and did not alter other aspects of signaling. Further work should examine whether other gonadal steroids such as estradiol affect this sexually dimorphic signaling parameter.

Animals that employ alternative reproductive tactics show within-sex differences in morphology that are associated with different tactics for acquiring mates (Taborsky, 1994). Interestingly, the striking morphological variation among male *P. hasemani* was

not correlated with any detectable differences in EOD frequency or chirping that could indicate functional differences in mate quality or social status. Likewise, the similar levels of 11-KT and T among long-jawed and short-jawed male *P. hasemani* are in stark contrast with the hormonal variation between male morph types exhibited by fish species with alternative reproductive tactics such as bluegill sunfish and midshipman fish (Brantley et al., 1993; Kindler et al., 1989). Short-jawed and long-jawed *P. hasemani* males have androgen levels that are similar to each other and dissimilar from those of females. The hormone levels reported here are comparable to those seen among reproductively mature individuals in breeding condition in other species of weakly electric fish within the family Apteronotidae (Cox Fernandes et al., 2010; Dunlap et al., 1998; Ho et al., 2010). This hormonal evidence combined with previous descriptions of gonadal state among males of varying sizes indicates that both male types are reproductively competent and that short-jawed males are not simply immature long-jawed males (Cox Fernandes et al., 2002). Nevertheless, over a three-year period we saw one short-jawed male develop the distinct curvature and jaw length of a long-jawed male while other similar-sized males did not. Thus it remains unclear how the development of long jaws is initiated and maintained and why some males develop this striking morphology while others do not.

Further behavioral experiments in more naturalistic environments are needed to observe courtship behaviors, mate preference, and intrasexual aggression. These observations could provide insight into whether males that vary in jaw morphology have different strategies for attracting mates or defending limited resources. For example, exaggerated male body mass and jaw size could correlate with differential levels of

aggression that allow long-jawed males to limit short-jawed males' access to territories and females. There may also be contextual differences in how chirping is used during social interactions that were not apparent in our recordings of chirps elicited by playbacks. These observations of social behaviors would help link the observed morphological variation among *P. hasemani* with its functional and evolutionary contexts.

ACKNOWLEDGMENTS

Thanks to Karen Heindl, Rose Stewart, and the Center for the Integrative Study of Animal Behavior at Indiana University for expert technical support. This research was supported by NSF IOS 0950721 (GTS) and NIH T32HD049336 (Common Themes in Reproductive Diversity training grant, JMP).

REFERENCES

Badyaev, A.V., Hill, G.E., 2000. Evolution of sexual dichromatism: contribution of carotenoid- versus melanin-based coloration. Biol. J. Linn. Soc. 69, 153-172.

Brantley, R.K., Wingfield, J.C., Bass, A.H., 1993. Sex steroid levels in Porichthys notatus, a fish with alternative reproductive tactics, and a review of the hormonal bases for male dimorphism among teleost fishes. Horm. Behav 27, 332-347.

Cox Fernandes, C., Lundberg, J.G., Riginos, C., 2002. Largest of all electric-fish snouts: Hypermorphic facial growth in male Apteronotus hasemani and the identity of Apteronotus anas (Gymnotiformes : Apteronotidae). Copeia, 52-61.

Cox Fernandes, C., Smith, G.T., Podos, J., Nogueira, A., Inoue, L., Akama, A., Ho, W.W., Alves-Gomes, J., 2010. Hormonal and behavioral correlates of morphological variation in an Amazonian electric fish (Sternarchogiton nattereri: Apteronotidae). Horm. Behav 58, 660-668.

Dunlap, K.D., Smith, G.T., Yekta, A., 2000. Temperature dependence of electrocommunication signals and their underlying neural rhythms in the weakly electric fish, Apteronotus leptorhynchus. Brain Behav. Evol. 55, 152-162.

Dunlap, K.D., Thomas, P., Zakon, H.H., 1998. Diversity of sexual dimorphism in electrocommunication signals and its androgen regulation in a genus of electric fish, Apteronotus. J Comp Physiol A 183, 77-86.

Godwin, J., 2010. Neuroendocrinology of sexual plasticity in teleost fishes. Front. Neuroendocrin. 31, 203-216.

Gross, M.R., 1996. Alternative reproductive strategies and tactics: Diversity within sexes. Trends Ecol. Evol 11, 92-98.

Hagedorn, M., Heiligenberg, W., 1985. Court and spark: Electric signals in the courtship and mating of gymnotoid fish. Anim. Behav. 33, 254-265.

Ho, W.W., Fernandes, C.C., Alves-Gomes, J.A., Smith, G.T., 2010. Sex differences in the electrocommunication signals of the electric fish Apteronotus bonapartii. Ethology 116, 1050-1064.

Humfeld, S.C., 2013. Condition-dependent signaling and adoption of mating tactics in an amphibian with energetic displays. Behav. Ecol. 24, 859-870.

Kindler, P.M., Philipp, D.P., Gross, M., Bahr, J.M., 1989. Serum 11 ketotestosterone and testosterone concentrations associated with reproduction in male bluegill (Lepomis macrochirus: Centrarchidae). Gen. Comp. Endocrinol 76, 446-453.

Kirschbaum, F., 1975. Environmental factors control periodical reproduction of tropical electric fish. Experientia 31, 1159-1160.

Kirschbaum, F., 1979. Reproduction of the weakly electric fish Eigenmannia-virescens (Rhamphichtyidae, Teleostei) in captivity .1. Control of gonadal recrudescence and regression by environmental-factors. Behav. Ecol. Sociobiol. 4, 331-355.

Kolodziejski, J.A., Nelson, B.S., Smith, G.T., 2005. Sex and species differences in neuromodulatory input to a premotor nucleus: A comparative study of substance P and communication behavior in weakly electric fish. J. Neurobiol. 62, 299-315.

Oliveira, R.F., Taborsky, M., Brockmann, H.J., 2008. Alternative Reproductive Tactics: An Integrative Approach, first ed. Cambridge University Press, New York.

Owens, I.P.F., Hartley, I.R., 1998. Sexual dimorphism in birds: why are there so many different forms of dimorphism? Proc. R. Soc. B 265, 397-407.

Smith, G.T., 2013. Evolution and hormonal regulation of sex differences in the electrocommunication behavior of ghost knifefishes (Apteronotidae). J. Exp. Biol. 216, 2421-2433.

Taborsky, M., 1994. Sneakers, satellites, and helpers: Parasitic and cooperative behavior in fish reproduction. Adv. Stud. Behav. 23, 1-100.

Turner, C.R., Derylo, M., de Santana, C.D., Alves-Gomes, J.A., Smith, G.T., 2007. Phylogenetic comparative analysis of electric communication signals in ghost knifefishes (Gymnotiformes : Apteronotidae). J. Exp. Biol. 210, 4104-4122.

Zahavi, A., 1975. Mate selection - selection for a handicap. J. Theor. Biol. 53, 205-214.

Zakon, H.H., Dunlap, K.D., 1999. Sex steroids and communication signals in electric fish: A tale of two species. Brain Behav. Evol. 54, 61-69.

Chapter 3.

Jamming avoidance and chirping in the weakly electric fish *Distocyclus conirostris*

Jacquelyn M. Petzold [a,b], José A. Alves-Gomes[c], G. Troy Smith [a,b]

Affiliations:

[a] Department of Biology, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405, USA

[b] Center for the Integrative Study of Animal Behavior, Indiana University, 409 N. Park Ave., Bloomington, IN 47405, USA

[c] Laboratório de Fisiologia Comportamental e Evolução (LFCE), Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM 69083-000, Brazil

This chapter is an expanded version of a manuscript
that will be submitted to the *Journal of Experimental Biology*
and therefore follows that journal's format.

ABSTRACT

The electrosensory system of weakly electric fish must accommodate the competing demands of sensing the environment (electrolocation) and receiving social information (electrocommunication). The jamming avoidance response (JAR) is a behavioral strategy thought to reduce electrosensory interference from conspecific signals close in frequency. We used playback experiments to characterize the electric organ discharge frequency (EODf), chirping behavior, and JAR of *Distocyclus conirostris*, a gregarious species of weakly electric fish. *D. conirostris* produced low-frequency EODs (~80-200 Hz) and chirps of modest durations (~90 ms) that sometimes interrupted the EOD. These low EOD frequencies likely prevent fish from determining the EODf of conspecifics using currently described mechanisms since the carrier (EOD) frequency overlaps substantially with the frequency of beats created by social interactions. Like other electric fish, *D. conirostris* responded to playbacks by shifting EOD frequency. Fish consistently lowered EODf in response to higher frequency stimuli but inconsistently raised/lowered EODf in response to lower frequency stimuli. This led to jamming avoidance or anti-jamming avoidance, respectively. We compare these signaling behaviors to those of other sternopygid electric fish (*Eigenmannia* and *Sternopygus*) and comparatively describe the circuitry that controls the JAR and chirping. Based on these data, we conclude that the JAR may have some additional social function and may not solely minimize the deleterious effects of jamming, as its name suggests.

INTRODUCTION

Active sensory systems such as echolocation and electrolocation rely on accurate detection of self-generated signals and are thus sensitive to interference from signals produced by nearby conspecifics (Bullock et al., 1975; Nelson and MacIver, 2006). The jamming avoidance response (JAR) of South American weakly electric knifefish is one example of a behavioral strategy that minimizes deleterious interference by increasing spectral differences between co-occurring signalers (Watanabe and Takeda, 1963; Rose, 2004). Weakly electric fish generate weak electric fields by emitting an electric organ discharge (EOD) from a specialized electric organ. Social interactions with other electric fish create global distortions in the EOD. Simultaneously, fish can detect the position and properties of biotic and abiotic environmental features via localized distortions of the EOD (Chacron et al., 2003). For wave-type electric fish, the EOD is produced continuously at a particular frequency (Scheich, 1977; Zakon et al., 2002). When two fish are in close proximity, each fish perceives the other by the interference created when that fish's EOD interacts with its own. The regular constructive and destructive interference of two of more EODs creates a periodic amplitude and phase modulation (beat). Beat frequency is equal to the difference between the EOD frequencies of the two interacting fish (difference frequency; DF). Each fish uses the beat and the relative geometry of the interacting signals to estimate conspecific EOD frequencies, which carry important social information (Bastian and Heiligenberg, 1980; Benda et al., 2006; Heiligenberg et al., 1978; Smith, 2013). However, slow beats (approximately 4-10 Hz) created by interactions between fish with similar EOD frequencies can impair the electrolocation function of the EOD by masking localized EOD distortions (Matsubara and Heiligenberg,

1978). The JAR is a stereotyped behavioral response produced by electric fish that involves increasing or decreasing EOD frequency in order to increase beat frequency and thereby reduce or eliminate low-frequency interference (Bullock et al., 1972; Heiligenberg et al., 1978). In addition to the JAR, electric fish rapidly increase EOD frequency on millisecond timescales to produce context-specific social signals called chirps, which indicate motivational state during courtship and aggressive interactions (Hagedorn and Heiligenberg, 1985; Larimer and MacDonald, 1968; Zakon et al., 2002). The frequency modulation caused by a chirp is perceived by the receiving fish as a rapid disruption of the beat (Walz et al., 2013).

The JAR has been extensively studied in *Eigenmannia*, a genus of electric fish within the family Sternopygidae. *Eigenmannia* are gregarious and have EOD frequencies in the range of 300-600 Hz (Hopkins, 1974b; Tan et al., 2005). Using primarily frequency-clamping experiments, several investigators have shown that when *Eigenmannia* are presented with lower- or higher-frequency stimuli near their own EOD frequency, they shift their EOD frequency up or down respectively (Watanabe & Takeda, 1963; Heiligenberg et al., 1978). *Sternopygus*, a genus of territorial species in the same family, has low-frequency EODs in the range of 50-150 Hz (Hopkins, 1974a). Interestingly, *Sternopygus* does not produce a JAR but can still behaviorally discriminate between higher and lower frequency signals (Bullock et al., 1975; Matsubara & Heiligenberg, 1978; Rose & Canfield, 1991). This discrimination ability without a JAR has been postulated to be a pre-adaptation in *Sternopygus* that allowed the evolution of the JAR in *Eigenmannia* (Rose & Canfield, 1991).

Both *Eigenmannia* and *Sternopygus* produce at least two types of short-term EOD modulations, although the terminology and categorization for such modulations varies somewhat among authors. *Eigenmannia* produces chirps (or rises) during which the frequency of the EOD rapidly increases and then decreases, sometimes with complex frequency modulations in between (Hopkins 1974b; Stöckl et al., 2014). *Eigenmannia* also produces interruptions, which are temporary cessations of the EOD that last 50-100 ms (Hagedorn & Heiligenberg, 1985; Hopkins 1974b). Similarly, *Sternopygus* produces chirp/rises that increase and decrease in frequency (sometimes with multiple frequency peaks) as well as interruptions during which the EOD is mostly silenced. *Sternopygus* interruptions vary in duration and appear to be produced during agonistic encounters (short interruptions, 20-70 ms) and courtship (long interruptions, 70-100 ms; Hopkins, 1974a). In this paper, we describe and quantify the EOD, JAR, and chirping behavior of wild-caught Amazonian *Distocyclus conirostris*, a species of electric fish that is in a sister genus to *Eigenmannia* and is similarly gregarious but has a low-frequency EOD like *Sternopygus* (Kramer et al., 1981; Tagliacollo et al., 2015). We also make comparisons among *D. conirostris*, *Eigenmannia*, and *Sternopygus* that may provide insight into how EOD frequency, jamming avoidance, and chirping co-evolve.

MATERIALS AND METHODS

Four *D. conirostris* were collected with nets from floating vegetation mats in the Solimoes River just east of the Xiborena Channel on the south edge of Catalão near Manaus, Brazil, in March 2014. After collection, fish were transported to the laboratory and temporarily housed in aerated river water. Within two days of capture, we recorded

electrocommunication behavior using a “chirp chamber” paradigm that was modified from a procedure that has been described previously (Kolodziejski et al., 2005). Briefly, the fish was placed in a loose mesh hammock within a temperature-controlled tank of river water and allowed to acclimate for thirty minutes. The fish’s EOD was recorded via a pair of carbon electrodes placed parallel with the long axis of its body. The signal from the electrodes was amplified 100x and recorded on the sound card of a laptop computer using audio editing software (Cool Edit Pro, Syntrillium; Phoenix, AZ). After acclimation, we first performed a three-minute baseline recording. Then we performed playbacks in a random order with sinusoidal stimuli that spanned a range of frequencies simulating conspecific EODs (±3 Hz, ±5Hz, ±10 Hz, ±20Hz, ±40Hz relative to the fish’s own EOD). Stimuli were generated with audio editing software and presented via carbon electrodes placed perpendicular to the long axis of the fish’s body. Stimulus amplitude was calibrated between the playback electrodes to a root-mean-square (RMS) amplitude of 0.6 mV/cm measured parallel to the electrodes and halfway between them. Each 60-second playback was preceded by 45 seconds of silence and followed by 75 seconds of silence, for a total recording length of three minutes. Playback sessions were interspersed with four-minute rest periods to prevent habituation. Immediately after recording, we measured total length and body mass (Table 1). Sex was confirmed by post-mortem examination of the gonads (3 out of 4 fish).

EOD frequency was measured by generating a power spectrum (fast Fourier transform, Blackman-Harris window, size 65536) in Cool Edit Pro from a segment of the baseline recording. To account for slight variations in water temperature among recording sessions, the EOD was standardized to the frequency expected at 26° C using a $Q_{10°C}$ of

Table 1.

Fish ID	Sex	Mass (g)	Length (cm)	EOD Frequency (Hz)	Chirp Rate (chirps/min)	Chirp Duration (ms)[1]
D3164A07A	M	15.8	23.6	89.4	2.35	73.8 ± 4.3
D3164A07B	?	17.4	21.0	114.6	0.48	99.2 ± 1.4
D3164A07C	F	28.1	23.2	151.6	1.82	96.5 ± 1.8
D3164A07D	F	38.9	25.5	198.6	0.67	93.5 ± 11.0

[1] Values are mean ± 1 S.E.M.

1.6 (Dunlap et al., 2000). Chirp recordings were analyzed offline with Igor Pro (Wavemetrics; Lake Oswego, OR) using the custom procedure eFish (efish23e, Brian Nelson, University of Oregon, Eugene, OR; http://nelsonbs.com/eFish/efish.html) that has been described previously (Kolodziejski et al., 2005). However, the EOD frequency could not be reliably tracked when interruptions occurred during chirps. Instead, EOD frequency during chirps was calculated by using zero-crossings with custom code in Matlab (Mathworks; Natick, MA, USA; code available by contacting GTS at getsmith@indiana.edu). The direction and magnitude of the jamming avoidance response was measured by comparing the mean EOD frequency in the five seconds immediately preceding onset of the playback with the most extreme (minimum or maximum) EOD frequency exhibited during the playback, not including chirps. The relationship between playback difference frequency and chirp rate was examined using one-way repeated measures analysis of variance (ANOVA). The relationship between playback difference frequency and JAR frequency shift was analyzed using linear correlations.

RESULTS AND DISCUSSION

EOD Frequency and Chirping

EOD frequency was 138.6 ± 23.7 Hz (mean ± S.E.M; range 89.4-198.6 Hz; Table 1). Fish often responded to the playback by rapidly modulating their EOD, which is characteristic of electric fish responding to a social stimulus. We use the term “chirp” here to describe these modulations, based on their relatively short durations and associated abrupt increases and decreases in EOD frequency (after Hagedorn & Heiligenberg, 1985). Across individuals, chirp rate ranged from 0.48 to 2.35 chirps/min.

There was no relationship between chirp rate and the difference frequency of the playback ($F(10, 30)=1.24$, $p=0.31$). A typical *D. conirostris* chirp began with a small (approximately 10-30 Hz) increase in EOD frequency followed by a brief cessation of the EOD that lasted approximately 20-25 ms. The EOD resumed at a slightly lower frequency but then increased quickly to baseline (Fig. 1 A&B). However, some chirps consisted of only an increase or decrease in frequency, not both. A small subset of chirps (<10%) had short durations (~25 ms) that created a phase shift of the EOD rather than an interruption across one or more EOD cycles. These shorter chirps tended to occur in brief bursts of 3-7 chirps (Fig. 1 C&D). Across all fish and all chirps, chirp duration averaged 90.8 ± 5.8 ms.

In our recordings, we saw evidence of a pronounced DC offset during certain chirps, particularly those chirps with relatively larger FM and longer durations (e.g., Fig. 1B). This observation is consistent with the perceptible chirp-induced DC offset that has been observed in *Eigenmannia virescens* signals (Naruse & Kawasaki, 1998; Stöckl et al., 2014). Typically the voltage trace of an electric fish EOD is centered around zero (i.e., has no DC offset). The electrocytes of gymnotiformes with myogenic electric organs such as *Eigenmannia* and *D. conirostris* are innervated on the posterior face, which by itself would cause a substantial head-positive DC offset. However, most fish with myogenic electric organs have evolved a mechanism by which the electrocytes continuously generate a tail-positive current that counteracts the DC offset (Stoddard & Markham, 2008). This is thought to make the fish less detectable by electroreceptive predators such as catfish, since these predators have only ampullary (low-frequency-detecting) electroreceptors (Peters & Buwalda, 1972; Stoddard, 1999). When the EOD is

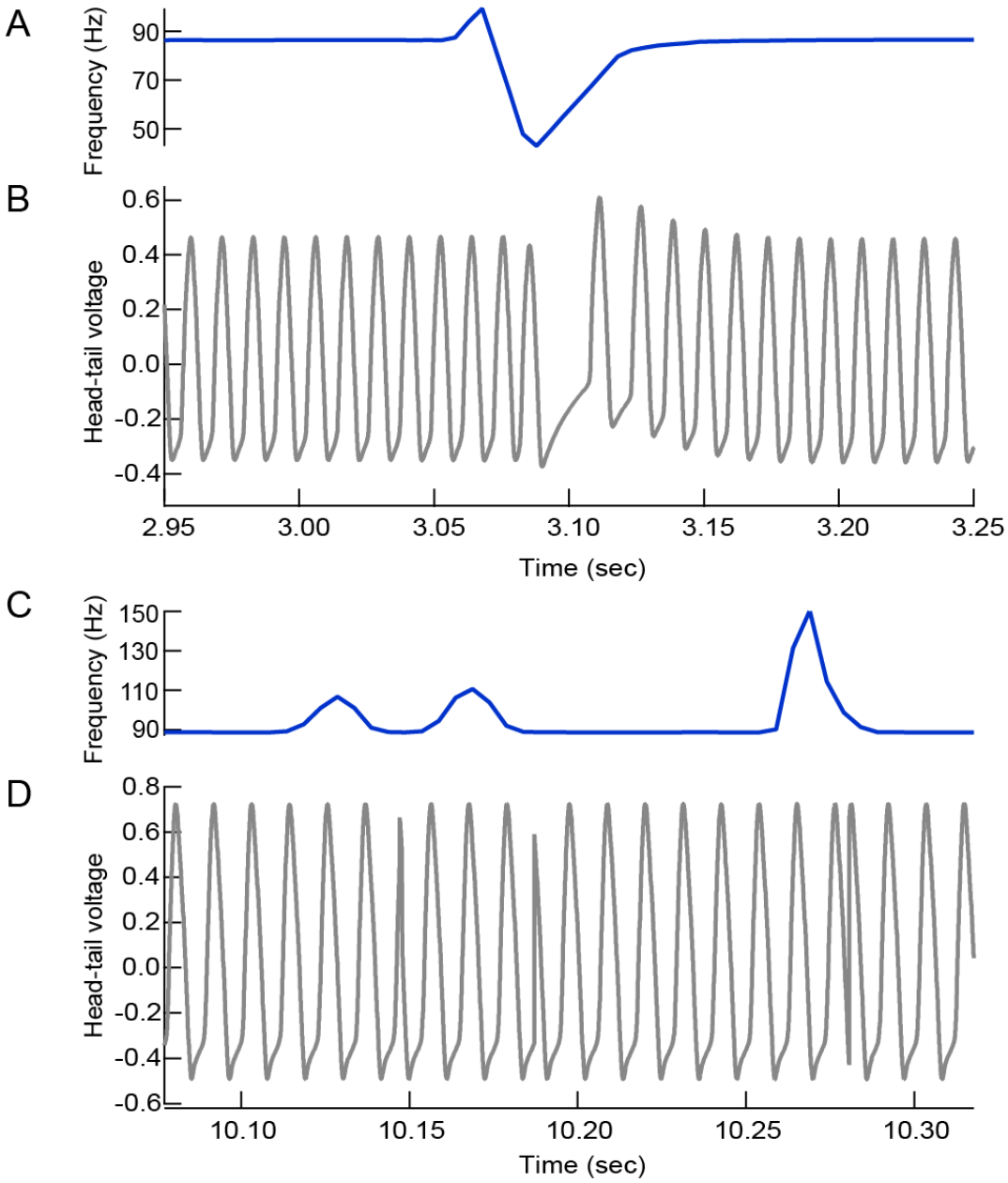


Fig. 1. Representative *Distocyclus conirostris* chirps. A) During a typical chirp, the EOD frequency transiently increased and then decreased before returning to baseline. B) These chirps temporarily interrupted the EOD, depicted here as the change in voltage of the head of the fish relative to the tail over time. Note that the undershoot lasts longer than several EOD cycles, which indicates that this frequency increase is not simply an artifact of the missed EOD. C) A small subset of chirps were substantially shorter in duration than other chirps. These chirps tended to occur in small clusters. D) The shorter duration chirps were not accompanied by an interrupted of the EOD but instead caused an EOD phase shift.

interrupted during a chirp, the head-positive current from the electrocytes is interrupted, unmasking the constant tail-positive current. This leads to the pronounced head-negative DC offset during the chirp. Although this may make the fish more conspicuous to electroreceptive predators, there is a potential benefit in that the DC offset may make chirps more detectable by other weakly electric fish. This is because weakly electric fish also possess ampullary electroreceptors, in addition to tuberous (high-frequency-detecting) electroreceptors (Carr & Friedman, 1999; Moller, 1995; Stöckl et al., 2014). Given that *E. virescens* chirps have a DC offset, it is likely that the DC offset we observed is a real property of the *D. conirostris* signal. However, because we used an AC-coupled amplifier and the sound card of a laptop computer during our recordings, we are unable to confirm that the DC offset we see in our recordings was not an artifact of the equipment we used. Thus, we cannot say unequivocally that *D. conirostris* chirps have a DC offset.

D. conirostris produce a moderately broad range of low-frequency EODs, which has interesting implications for our understanding of how the electrosensory system interprets the amplitude modulations used for extracting social information. Consistent with Hopkins' (1974a) observation of male and female *Sternopygus* in reproductive condition, some individual *D. conirostris* have EOD frequencies that are approximately twice that of nearby conspecifics. Since electroreceptors are typically tuned to a fish's own EOD frequency, this means that the fish with higher frequency EOD will be more sensitive to the frequency range of the second harmonic of a neighbor's low-frequency signal, not its fundamental frequency. Furthermore, the frequency of the beat created by interacting fish will often be near that of the carrier (EOD) signals. This overlap creates

aliasing in the combined signal that impinges on the electroreceptors. Consequently, the fish cannot differentiate the properties of the beat signal from the properties of the carrier signal in order to determine the EOD frequency of the other fish. A further discussion of the signal-processing implications of these low-frequency EODs can be found in Chapter 5.

The *D. conirostris* chirps we describe here are similar in structure to *E. virescens* chirps, which have a rapid increase and decrease in EOD frequency, last 20-100 ms, and vary in duration based on social context (Hagedorn and Heiligenberg, 1985; Hopkins, 1974b). However, we did not see evidence of long interruptions that have previously been observed among *E. virescens* during live courtship interactions (Hagedorn & Heiligenberg, 1985; Hopkins, 1974b). It is possible that *D. conirostris* produces longer interruptions but that our experimental paradigm (i.e., chirp chamber recordings) was not sufficient to elicit them. That is, since *Eigenmannia* and *D. conirostris* are social, they might not produce their full repertoire of signals in the absence of live conspecifics (Stöckl et al., 2014). *D. conirostris* chirps were similar to – although somewhat shorter than – the *Sternopygus* short interruptions (average duration 800 ms) described by Hopkins (1974a). Moreover, like those interruptions, *D. conirostris* chirps often began with a frequency increase and ended with a frequency undershoot below baseline. Thus, is appears that the general patterns in EOD modulations are fairly conserved across these three groups.

Jamming Avoidance Response

D. conirostris consistently shifted EOD frequency at the onset of a conspecific signal mimic (Fig. 2). When fish were presented with a stimulus frequency higher than their own EOD frequency, they reliably responded by decreasing EOD frequency for the duration of the playback and then increasing EOD frequency back to baseline after playback cessation. However, when fish were presented with a stimulus frequency lower than their own EOD frequency, the direction of the JAR was less predictable, increasing in 40% of trails (jamming avoidance) and decreasing in 60% of trials (anti-jamming avoidance). More specifically, all four fish raised EOD frequency in response to the -3 Hz playback, two fish raised in response to the -5 Hz playback, and two fish raised in response to -40 Hz (Fig. 2). None of the fish raised EOD frequency in response to -10 Hz or -20 Hz. In two cases the fish first raised and then lowered its EOD frequency in response to higher frequency stimuli. This asymmetrical response was reflected in the statistical relationship between the difference frequency of the playback and the EOD frequency shift performed by the fish. These two variables were not linearly correlated across the entire range of playback difference frequencies (R^2=0.02, p=0.40). However, examining the positive and negative playback differences separately shows a significant linear correlation for positive playback difference frequencies (R^2=0.31, p=0.01) but no correlation for negative playback difference frequencies (R^2=0.05, p=0.32).

Like *Eigenmannia* and unlike *Sternopygus*, *D. conirostris* shifts EOD frequency at the onset of a playback stimulus. *Sternopygus* is largely solitary and territorial, but *Eigenmannia* forms large social aggregations (Hopkins, 1974a; Stamper et al., 2010; Tan et al., 2005). We strongly suspect that *D. conirostris* is gregarious like *Eigenmannia* since *D. conirostris* are typically found in groups clustered around floating

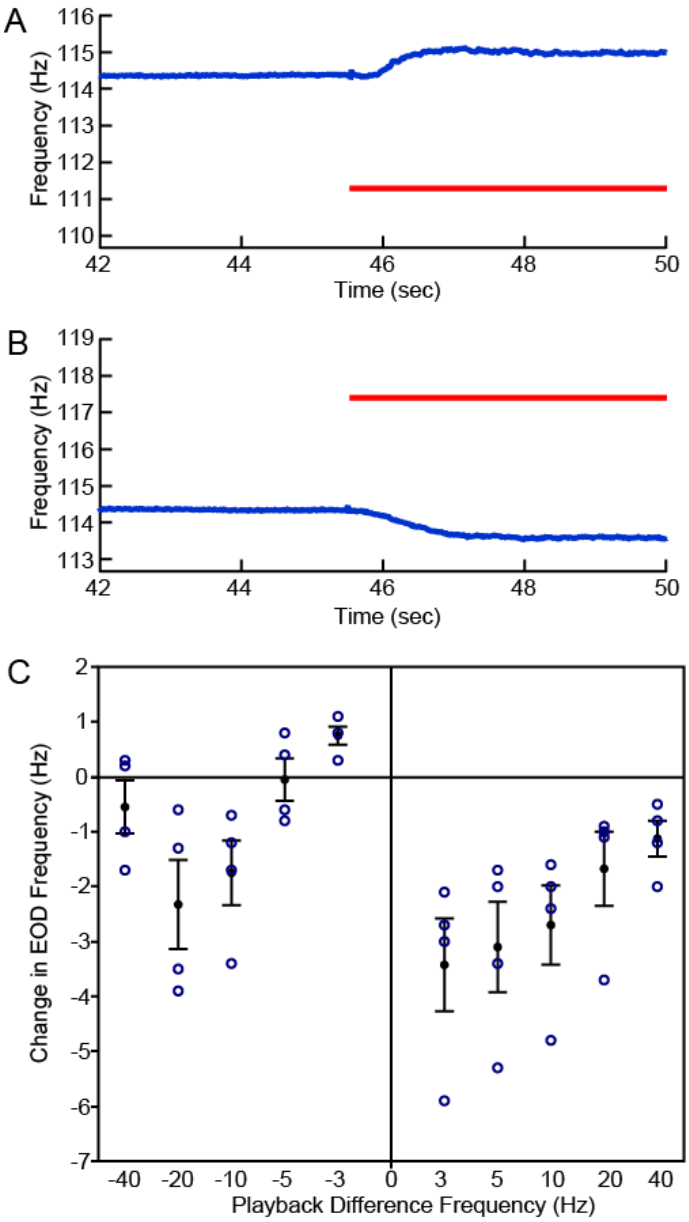


Fig. 2. Fish shifted EOD frequency (blue line) when presented with a stimulus of a similar frequency (red line). Two examples illustrate that *D. conirostris* is capable of (A) increasing or (B) decreasing EOD frequency in response to a playback. (C) Fish responded differently to higher- and lower-frequency playbacks. For stimuli higher in frequency compared to the fish's EOD (i.e., a positive playback difference frequency), all fish responded by lowering their EOD frequency, with the most robust responses to the difference frequencies closest to zero. For stimuli lower in frequency than the fish's EOD, the direction of the frequency shift was less consistent. Individual fish are shown with open circles; the black dots represent the mean for each playback difference frequency. Error bars show one standard error from the mean.

vegetation when they are collected (Alves-Gomes, personal observation). Both *Eigenmannia* and *D. conirostris* demonstrate the physiological capability for a bi-directional JAR, but *Sternopygus* does not. This could suggest that the JAR is critical for species that regularly co-exist with nearby conspecifics producing interfering signals but less important for species that live solitary lifestyles. However, the situation may be more complicated for several reason. First, *Sternopygus* does not show impaired electrolocation from signals with similar frequencies presented at ecologically relevant amplitudes, which obviates the need for a behavioral strategy to avoid jamming (Matsubara, 1981; Matsubara & Heiligenberg, 1978). Thus, *Sternopygus* is more unaffected by jamming stimuli from conspecifics and simultaneously less likely to encounter it. It is possible that *Steronpygus*' lack of a JAR is linked to its very low EOD frequency. However, we have shown that *D. conirostris* has similarly low EOD frequencies and produces a JAR. Additionally, there may be other neural mechanisms for fish to avoid jamming from nearby EODs with similar frequencies, such as comparing local and global distortions of the EOD (Chacron et al., 2003).

The neural mechanisms that control EOD modulations are evolutionarily labile. In all gymnotids, the timing of electric organ firing is controlled by neurons in the medullary pacemaker nucleus (Bennett et al., 1967). The pacemaker receives input from other brain areas to speed up or slow down the firing rate of the electric organ. The JAR is a relatively slow modulation of the EOD; chirping occurs on a much faster timescale. For weakly electric fish in the genus *Apteronotus*, the neurons in the sublemniscal pre-pacemaker (SPPn) that synapse with the pacemaker nucleus are tonically inhibited by a GABA-ergic input. The removal of this inhibition causes the pacemaker to increase firing

during the JAR and thus increase the EOD frequency (Heiligenberg et al., 1996). To generate the bidirectional JAR, *Eigenmannia* uses two largely independent pathways originating with the nucleus electrosensorius (nE), depending on whether the fish is raising or lowering its EOD frequency. To raise the EOD frequency, the nE↑ excites cells in the PPnG, which increases the firing rate of the pacemaker via glutamate acting on AMPA receptors (Dye & Heiligenberg, 1987; Metzner, 1993). The neurons in the SPPn of *Eigenmannia* are tonically active, which creates an NMDA-ergic excitation on the pacemaker nucleus. To lower EOD frequency, the nE↓ inhibits the SPPn, which removes the tonic excitation, decreases pacemaker firing, and thereby lowers EOD frequency (Metzner, 1993). Chirping in *Eigenmannia* is controlled by cells in the PPnC that act via AMPA receptors on relay cells responsible for carrying action potentials from the pacemaker to the electrocytes in the electric organ (Heiligenberg et al., 1981). *Sternopygus* does not perform a JAR and thus the SPPn is not responsible for jamming avoidance as it is in *Eigemannia* (Bullock et al., 1975). Instead, the SPPn of *Sternopygus* controls chirping by sending excitatory input to relay cells via NMDA inputs to the pacemaker nucleus. The coordinated depolarization of the relay cells blocks the action of the pacemaker and interrupts the EOD, creating a chirp (Keller et al., 1991). These varying uses of the same sub-nuclei in the pre-pacemaker nucleus to control different modulations of the EOD between *Eigenmannia* and *Sternopygus* could indicate that the inconsistent JAR we saw in *D. conirostris* represents some type of transition in the JAR circuitry. *D. conirostris* may not have the well-developed dual JAR pathways that *Eigenmannia* does but instead may use an alternative or intermediate pathway to regulate the JAR.

Still, it is perplexing that *D. conirostris* sometimes produced a jamming avoidance response to lower-frequency playbacks and sometimes produced an anti-jamming avoidance response (or a jamming seeking response) to lower-frequency playbacks. One possible explanation is that the JAR might have functions other than avoiding the deleterious effects of other EODs on electrolocation. Kramer (1987) observed anomalous JARs in *Eigenmannia* that somewhat mirror the asymmetrical response we saw in *D. conirostris*. That is, some female *Eigenmannia* responded consistently to higher-frequency stimuli by lowering EOD frequency but responded weakly or not at all to lower-frequency stimuli. Juvenile *Eigenmannia* showed somewhat more robust JARs, but males made no or only minor changes in EOD frequency when presented with stimuli at frequencies near their EOD frequency. Based on these observations, Kramer (1987) proposed a potential social function for the JAR in addition to (or in place of) its purported function of minimizing deleterious interference. Thus, if the JAR were used in preventing intra-specific aggression or mediating and maintaining dominance hierarchies within social groups, for example, it would follow that the JAR might be more highly developed in gregarious species, relative to territorial species. A communication function would also explain why the fish we recorded here showed small but consistent responses to playback stimuli that were 20 Hz and 40 Hz above their own EOD frequencies, well outside the usual range of frequencies shown to impair electrolocation (Behrend, 1977; Matsubara & Heiligenberg, 1978). A similar pattern of EOD frequency shifts produced to in response to large difference frequency playbacks has also been observed in at least one apteronotid species (Ho et al., 2010). We suggest that a comparative characterization of the JAR among other species of wave-type electric

fish could provide more insight into how the JAR might have been shaped both by social context and by the costs of impairment to electrolocation.

ACKNOWLEDGMENTS

Thanks to Cristina Cox Fernandes for assistance with fish collection and identification.

FUNDING

This research was supported by National Science Foundation IOS 0950721 (GTS) and IOS 1557935 (GTS) and by National Institutes of Health T32HD049336 (Common Themes in Reproductive Diversity training grant, JMP). Travel support #457339/2013-6 from CNPq to JAG helped GTS to visit LFCE for the experiments.

REFERENCES

Bastian, J. and Heiligenberg, W. (1980). Phase-sensitive midbrain neurons in Eigenmannia - neural correlates of the jamming avoidance-response. *Science* **209**, 828-830.

Behrend, K. (1977). Processing information carried in a high-frequency wave - properties of cerebellar units in a high-frequency electric fish. *Journal of Comparative Physiology* **118**, 357-371.

Benda, J., Longtin, A. and Maler, L. (2006). A synchronization-desynchronization code for natural communication signals. *Neuron* **52**, 347-358.

Bennett, M. V. L., Pappas, G. D., Gimenez, M. and Nakajima, Y. (1967). Physiology and ultrastructure of electrotonic junctions .4. Medullary electromotor nuclei in gymnotid fish. *Journal of Neurophysiology* **30**, 236-300.

Bullock, T. H., Behrend, K. and Heiligenberg, W. (1975). Comparison of jamming avoidance responses in gymnotid and gymnarchid electric fish - case of convergent evolution of behavior and its sensory basis. *Journal of Comparative Physiology* **103**, 97-121.

Bullock, T. H., Hamstra, R. H. and Scheich, H. (1972). Jamming avoidance response of high-frequency electric fish .1. General features. *Journal of Comparative Physiology* **77**, 1-22.

Carr, C. & Friedman, M. (1999). Evolution of time coding systems. *Neural Computation* **11**, 1-20.

Chacron, M. J., Doiron, B., Maler, L., Longtin, A. and Bastian, J. (2003). Non-classical receptive field mediates switch in a sensory neuron's frequency tuning. *Nature* **423**, 77-81.

Dunlap, K. D., Smith, G. T. and Yekta, A. (2000). Temperature dependence of electrocommunication signals and their underlying neural rhythms in the weakly electric fish, Apteronotus leptorhynchus. *Brain Behavior and Evolution* **55**, 152-162.

Dye, J. and Heiligenberg, W. (1987). Intracellular-recording in the medullary pacemaker nucleus of the weakly electric fish, *Apteronotus*, during modulatory behaviors. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **161**, 187-200.

Hagedorn, M. and Heiligenberg, W. (1985). Court and spark - electric signals in the courtship and mating of gymnotid fish. *Animal Behaviour* **33**, 254-265.

Heiligenberg, W., Baker, C. and Matsubara, J. (1978). Jamming avoidance-response in Eigenmania revisited - structure of a neuronal democracy. *Journal of Comparative Physiology* **127**, 267-286.

Heiligenberg, W., Finger, T., Matsubara, J. and Carr, C. (1981). Input to the medullary pacemaker nucleus in the weakly electric fish, Eigenmannia (Sternopygidae, Gymnotiformes). *Brain Research* **211**, 418-423.

Heiligenberg, W., Metzner, W., Wong, C. J. H. and Keller, C. H. (1996a). Motor control of the jamming avoidance response of Apteronotus leptorhynchus: Evolutionary changes of a behavior and its neuronal substrates. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **179**, 653-674.

Ho, W. W., Fernandes, C. C., Alves-Gomes, J. A. and Smith, G. T. (2010). Sex Differences in the Electrocommunication Signals of the Electric Fish Apteronotus bonapartii. *Ethology* **116**, 1050-1064.

Hopkins, C.D. (1974a). Electric communication in the reproductive behavior of *Sternopygus macrurus* (Gymnotoidei). *Zeitschrift fur Tierpsychologie*, **35**, 518-535.

Hopkins, C.D. (1974b). Electric communication - functions in social-behavior of Eigenmannia-virescens. *Behaviour*, **50**, 270-305.

Keller, C. H., Kawasaki, M. and Heiligenberg, W. (1991). The control of pacemaker modulations for social communication in the weakly electric fish Sternopygus. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **169**, 441-450.

Kolodziejski, J. A., Nelson, B. S. and Smith, G. T. (2005). Sex and species differences in neuromodulatory input to a premotor nucleus: A comparative study of substance P and communication behavior in weakly electric fish. *Journal of Neurobiology* **62**, 299-315.

Kramer, B. (1987). The sexually dimorphic jamming avoidance-response in the electric fish Eigenmannia (Teleostei, Gymnotiformes). *Journal of Experimental Biology* **130**, 39-62.

Kramer, B., Kirschbaum, F. and Markl, H. (1981). Species specificity of electric organ discharges in a sympatric group of gymnotoid fish from Manaus (Amazonas). *Advances in Physiological Sciences* **31**, 195-217.

Larimer, J. L. and Macdonald, J. A. (1968). Sensory feedback from electroreceptors to electromotor pacemaker centers in gymnotids. *American Journal of Physiology* **214**, 1253-1261.

Matsubara, J. and Heiligenberg, W. (1978). How well do electric fish electro-locate under jamming. *Journal of Comparative Physiology* **125**, 285-290.

Matsubara, J. A. (1981). Neural correlates of a non-jammable electrolocation system. *Science* **211**, 722-725.

Metzner, W. (1993). The jamming avoidance-response in Eigenmannia is controlled by 2 separate motor pathways. *Journal of Neuroscience* **13**, 1862-1878.

Moller, P. (1995). *Electric fishes: History and behavior*. London: Chapman & Hall.

Naruse M, Kawasaki M. 1998. Possible involvement of the ampullary electroreceptor system in detection of frequency-modulated electrocommunication signals in Eigenmannia. *Journal of Comparative Physiology A* **183**, 543–552.

Nelson, M. E. and MacIver, M. A. (2006). Sensory acquisition in active sensing systems. *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **192**, 573-586.

Peters, R. C. and Buwalda, R. J. A. (1972). Frequency-response of electroreceptors (small pit organs) of catfish, Ictalurus-nebulosus LeS. *Journal of Comparative Physiology* **79**, 29-38.

Rose, G. J. (2004). Insights into neural mechanisms and evolution of behaviour from electric fish. *Nature Reviews Neuroscience* **5**, 943-951.

Rose, G. J. and Canfield, J. G. (1991). Discrimination of the sign of frequency differences by Sternopyguas, an electric fish without a jamming avoidance response. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **168**, 461-467.

Scheich, H. (1977). Neural basis of communication in high-frequency electric fish, Eigenmannia-virescens (jamming avoidance-response) .1. Open loop experiments and time domain concept of signal analysis. *Journal of Comparative Physiology* **113**, 181-206.

Smith, G. T. (2013). Evolution and hormonal regulation of sex differences in the electrocommunication behavior of ghost knifefishes (Apteronotidae). *Journal of Experimental Biology* **216**, 2421-2433.

Stamper, S. A., Carrera, E., Tan, E. W., Fugere, V., Krahe, R. and Fortune, E. S. (2010). Species differences in group size and electrosensory interference in weakly electric fishes: Implications for electrosensory processing. *Behavioural Brain Research***207**, 368-376.

Stöckl, A., Sinz, F., Benda, J. and Grewe, J. (2014). Encoding of social signals in all three electrosensory pathways of Eigenmannia virescens. *Journal of Neurophysiology* **112**, 2076-2091.

Stoddard, P. K. (1999). Predation enhances complexity in the evolution of electric fish signals. *Nature* **400**, 254-256.

Stoddard, P. K. and Markham, M. R. (2008). Signal cloaking by electric fish. *Bioscience* **58**, 415-425.

Tagliacollo, V. A., Bernt, M. J., Craig, J. M., Oliveira, C. and Albert, J. S. (2016). Model-based total evidence phylogeny of Neotropical electric knifefishes (Teleostei, Gymnotiformes). *Molecular Phylogenetics and Evolution* **95**, 20-33.

Tan, E. W., Nizar, J. M., Carrera-G, E. and Fortune, E. S. (2005). Electrosensory interference in naturally occurring aggregates of a species of weakly electric fish, Eigenmannia virescens. *Behavioural Brain Research* **164**, 83-92.

Walz, H., Hupe, G. J., Benda, J. and Lewis, J. E. (2013). The neuroethology of electrocommunication: How signal background influences sensory encoding and behaviour in Apteronotus leptorhynchus. *Journal of Physiology-Paris* **107**, 13-25.

Watanabe, A. and Takeda, K. (1963). The change of discharge frequency by A.C. stimulus in a weak electric fish. *Journal of Experimental Biology* **40**, 57-66.

Zakon, H., Oestreich, J., Tallarovic, S. and Triefenbach, F. (2002). EOD modulations of brown ghost electric fish: JARs, chirps, rises, and dips. *Journal of Physiology-Paris* **96**, 451-458.

Chapter 4.

Co-adaptation of Electric Organ Discharges and Chirps in South American Ghost Knifefishes (Apteronotidae)

Jacquelyn M. Petzold[a,b] , Gary Marsat[c], & G. Troy Smith[a,b]

Affiliations:

[a] Department of Biology, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405, USA

[b] Center for the Integrative Study of Animal Behavior, Indiana University, 409 N. Park Ave., Bloomington, IN 47405, USA

[c] Department of Biology, West Virginia University, 53 Campus Dr., Morgantown, WV 26506, USA

This chapter follows the format of *Journal of Physiology – Paris*

Petzold, J.M., Marsat, G., Smith, G.T. (in press). Co-adaptation of electric organ discharges and chirps in South American ghost knifefishes (Apteronotidae). *Journal of Physiology – Paris*, http://dx.doi.org/10.1016/j.jphysparis.2016.10.005

Note: Main figures appear in the text; supplementary figures can be found at the end of the chapter.

1. INTRODUCTION

Complex signals are animal communication displays that use multiple signal components (Hebets & Papaj, 2005; Partan & Marler, 2005). The function of complex signals varies across species and contexts, with complex signals used to transmit multiple messages simultaneously, to provide redundancy as a means of improving reliability, to counteract varied sources of environmental noise, or to overcome sensory constraints (Hebets & Papaj, 2005). Multimodal signals, which are complex signals that exert influence on the receiver by stimulating two or more sensory modalities, have been well-characterized, particularly in courtship displays and warning signals (reviewed in Higham & Hebets, 2013 and Rowe & Guilford, 1999). However, less is known about how the components of complex signals that share the same sensory channel interact and influence each other's detection and evolution. Some of the most intriguing examples of unimodal complex signals come from the study of animal olfactory communication. For example, some species of ants use complex blends of pheromones to simultaneously signal fertility, caste, and/or colony identity (Denis et al., 2006; Moore & Liebig, 2010; Smith et al., 2013). In this paper we take advantage of a uniquely suited model system – the communication signals of weakly electric fishes – to examine how two functionally distinct signals impinging on the same sensory modality (electroreception) interact to influence signal detection across species.

The electrosensory system of South American weakly electric knifefish is a multi-purpose sensory modality used for sampling several important types of environmental information. Weakly electric knifefish detect self-generated electric fields that are distorted in predictable ways by objects and organisms in the environment and by the

signals of other electrogenic animals. With each electric organ discharge (EOD), the fish experiences a transient increase in the voltage of its head relative to its tail followed by a concomitant decrease in the voltage of its head relative to its tail (Assad et al., 1999). For weakly electric fish in the family Apteronotidae, the EOD creates a continuously alternating high-frequency electric field. The frequency, amplitude, and waveform of this wave-type EOD can communicate information about size, sex, species, and/or social status (Hopkins, 1988; Kramer & Otto, 1991; Turner et al., 2007; Zakon & Dunlap, 1999). Fish also modulate the frequency and amplitude of the EOD on short timescales (milliseconds to seconds) to produce context-specific communication signals called chirps (Fig. 1; Hagedorn & Heiligenberg, 1985; Larimer & MacDonald, 1968). Thus, the EOD is a continuous badge of identity, whereas chirps are transient indicators of motivational state (Smith, 2013). EODs and chirps have relatively simple structures that can be easily recorded, analyzed, manipulated, synthesized, and played back. This makes them ideal candidates for examining how the properties of animal communication signals convey information.

Unlike many communication signals, EODs are not detected directly by other fish. Because the EOD is produced continuously, each fish detects a social partner's EOD as the interaction of the signaler's EOD with the fish's own EOD. Since socially interacting fish usually fire their electric organs at different frequencies, the EODs of interacting fish produce a relatively slow amplitude modulation (called a beat) that forms as the two EODs come in and out of phase with each other and thereby constructively and destructively interfere (Fig. 2; Fortune et al., 2006; Rose, 2004; Scheich, 1977a). Fish are able to use the frequency of the beat to determine the relative EOD frequency of nearby

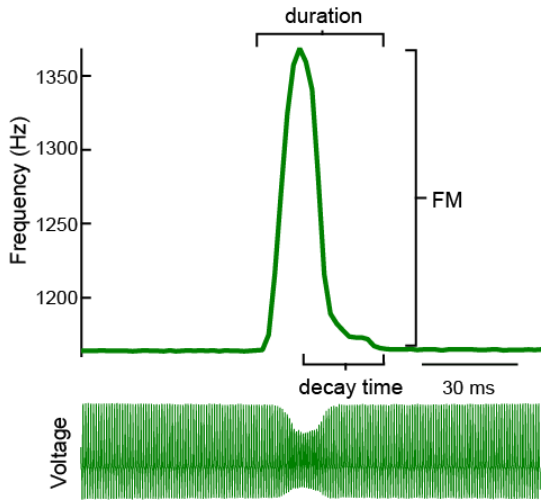


Fig. 1. Single-peaked chirp of an *Adontosterarchus devenanzii* showing chirp parameters used in this study. The EOD trace (bottom) shows the change in head-tail voltage over time. During the chirp, EOD amplitude decreases and EOD frequency rapidly increases (top). Chirp FM is the maximal increase in EOD frequency relative to baseline during the chirp. Chirp duration is the time that elapses between chirp onset and cessation. Chirp decay time is the amount of time that elapses between the peak of the frequency excursion and the end of the chirp. We report relative chirp decay here, which is the ratio of chirp decay time to chirp duration.

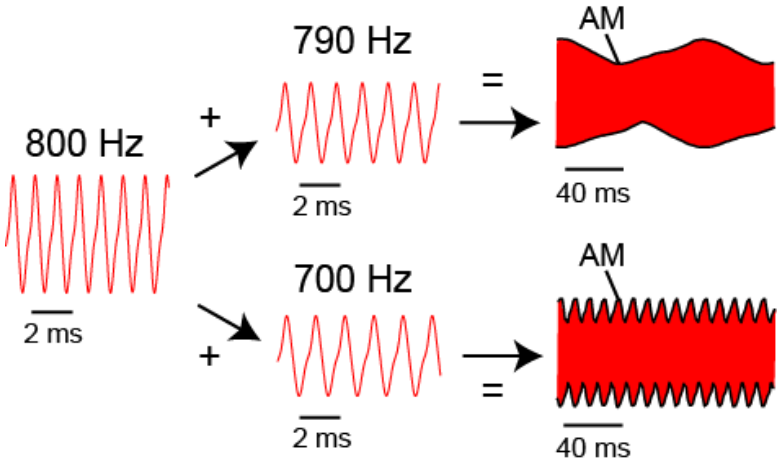


Fig. 2. Interactions of EODs to produce beats. Red traces are the head-tail voltage during the EOD. Beat frequency is determined by the EOD frequencies of the two interacting EODs. Here an 800 Hz EOD was combined with a 790 Hz EOD to create a 10 Hz beat (top). Then the same 800 Hz EOD was combined with a 700 Hz EOD to create a 100 Hz beat (bottom). The AM (amplitude modulation) is indicated with thick black lines on the beats. Note the differing timescales for the EODs and the beats. Also note that neither the EOD waveform nor the beat waveform is precisely sinusoidal.

fish (Scheich, 1977b; Watanabe and Takeda, 1963). The beat frequency is equal to the absolute value of the difference frequency (DF) between the EODs of two interacting fish. The frequency and pattern of the amplitude modulation (AM) created by the interaction of two or more EODs conveys social information that is encoded by amplitude-sensitive electroreceptors (P-units; Nelson et al., 1997; Zakon, 1988). When one fish rapidly increases its EOD frequency during a chirp, the beat frequency increases correspondingly. The regular beat is thus transiently disrupted by a change in the modulation frequency. This transient change in the beat causes the beat's phase to abruptly change. The phase shift is particularly noticeable for chirps lasting less than one beat cycle (Benda et al., 2005; Walz et al., 2013; Walz et al., 2014). Similarly, a decrease in EOD amplitude during a chirp reduces beat contrast. Although EODs and chirping serve different social functions, both signals are produced and detected simultaneously by the same array of electroreceptors, since the chirp is a modulation of the EOD. Chirps can only be detected based on how they disrupt the beat, and thus their perception is likely constrained by the structure of the interacting EODs that produce the beat.

The complicated dynamic created by the co-evolution of EODs and chirps is one potential explanation for the existence of an enormous degree of variation in signal structure between sexes and across different species of weakly electric knifefish (Smith, 2013; Turner et. al, 2007; Zakon & Dunlap, 1999). EODs vary in the broadness of species-typical frequency range and in the shape of the EOD waveform. Some species have EOD waveforms that are nearly sinusoidal; some species have complex, multi-peaked EOD waveforms; and other species have EOD waveforms of intermediate complexity. However, it is currently unclear whether weakly electric fish perceive or

attend to waveform information (Fig. 3; Dunlap & Larkins-Ford, 2003b; Fugère & Krahe, 2010; Kramer & Otto, 1991). Additionally, the relationship between EOD frequency and beat frequency is well understood, but little is known about how EOD waveform affects beat structure (Bullock et al., 1972; Heiligenberg et al., 1978; Scheich, 1977a). In recent years, the ability of the electrosensory system to encode the disruption created by chirps on different beat frequencies has been explored. However, these studies have focused on sinusoidal beats and have not yet considered how chirps interact with the more complex beats that naturally occur when species with complex EOD waveforms interact (Benda et al., 2006; Hupé et al., 2008; Walz et al., 2014). We also do not know how differing EOD waveforms and their interactions with beat frequency affect the conspicuousness of chirps.

Like EOD waveform, species-typical chirp characteristics vary widely. Quantifiable characteristics of chirps include chirp duration, chirp frequency modulation (FM), chirp amplitude modulation (AM), the proportion of time during which the EOD frequency is rising vs. falling during a chirp (chirp relative decay time), and the presence or absence of multi-peaked chirps (Turner et al., 2007). Some species use two or more distinct chirp "types," the best-studied examples of which are the *A. leptorhynchus* and *A. albifrons* "big" and "small" chirps. These chirps are named for their bimodal distributions of chirp FM (Bastian et al., 2001; Dunlap & Larkins-Ford, 2003a; Kolodziejski et al., 2005). Finally, all species studied to date exhibit at least one sexually dimorphic signaling feature in EOD frequency or chirping (reviewed in Smith, 2013). This remarkable degree of naturally occurring variation in the primary communication channel provides a unique opportunity to examine how fitness-enhancing information about sex,

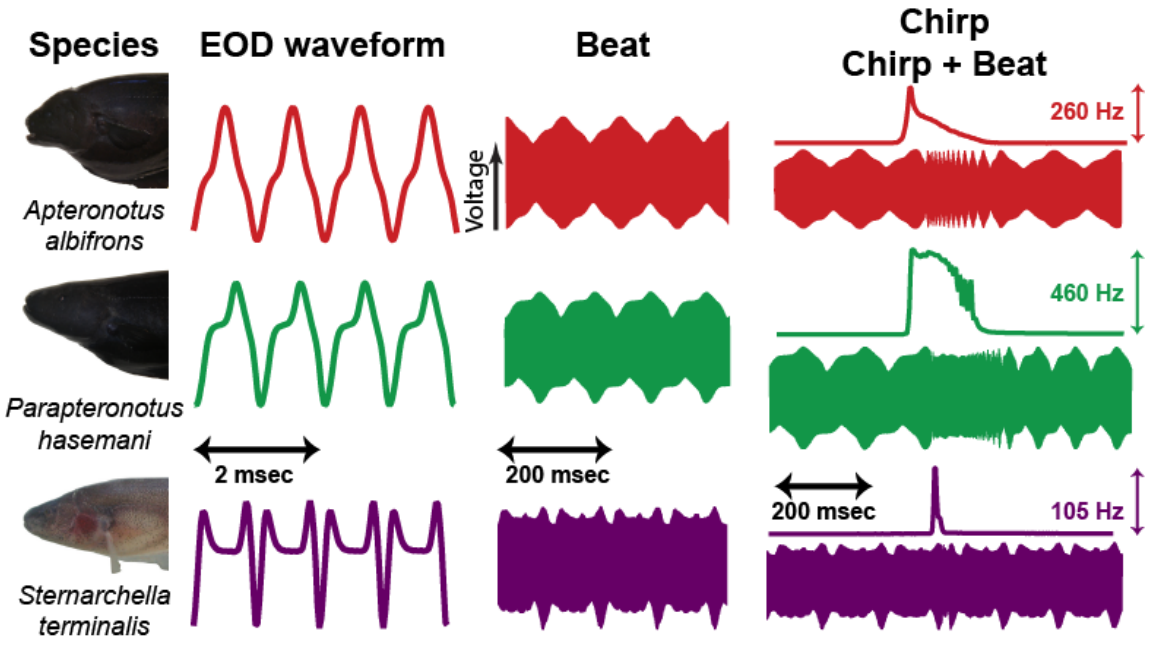


Fig. 3. EOD waveform and chirp structure vary substantially across species. We present a few cycles of three different EODs here to illustrate this variation: a nearly sinusoidal EOD (*A. albifrons*), a moderately complex EOD (*P. hasemani*), and a complex EOD (*S. terminalis*). Each EOD was combined with a temporally stretched copy of itself to generate a 10 Hz beat (middle column). The beat is shown on a longer timescale that encompasses many cycles of the interacting EODs. In the right column, the frequency trace shows the frequency modulation during the chirp (top), which translates into differing patterns of disruptions to the beat (bottom). Note the different frequency scales for each chirp.

species, breeding condition, and motivational state can be extracted from the complex sensory stream created by aggregations of two or more fish. Because chirps are detected as disruptions in the beat created by interacting EODs, changes in EOD properties can necessarily be expected to influence how chirps are detected. Thus, we might expect co-evolution between the properties of EODs and chirps, with chirps maximally conspicuous on conspecific beats in order to enhance the efficacy of this more transient signal. Here we use recordings of many different species of weakly electric fish to simulate social interactions in order to model how parameters of chirps and EODs interact to affect the conspicuousness of these social signals.

2. METHODS

2.1. Animals & Recordings

We analyzed 147 signals from twelve species of South American ghost knifefish that varied across several parameters of EODs and chirping. These species included *Adontosternarchus balaenops*, *Adontosternarchus devenanzii*, *Apteronotus albifrons*, "*Apteronotus" bonapartii*, *Apteronotus leptorhynchus*, *Parapteronotus hasemani*, *Porotergus gimbeli*, *Sternarchella terminalis*, *Sternarchogiton nattereri*, *Sternarchogiton porcinum*, *Sternarchorhynchus roseni*, and *Sternarchorhynchus curvirostris*. EODs and chirps were elicited using playbacks and were characterized in detail in previous studies (Ho et al., 2010; Ho et al., 2013; Kolodziejski et al., 2005; Petzold & Smith, 2015; Turner et al., 2007; Zhou & Smith, 2006). The recordings are available in an online archive of electric fish signal recordings (Electric Fish Signal Archive: http://www.indiana.edu/~efishlab/catalog/). For this study, a chirp (which is a modulation

of the EOD) was included only if it had at least five seconds of unmodulated EOD before and after its occurrence. After a chirp was selected, a stable-frequency unmodulated EOD clip of identical length was taken from the same recording of the same fish to use when simulating social interactions. The sampling rate was 44.1 kHz for each file.

2.2. Extracting the Amplitude Modulation

We used audio editing software (CoolEdit Pro; Syntrillium; Phoenix, AZ, USA) to simulate social interactions by combining recordings of chirps and EODs. The simplest situation was the interaction of two EODs without any chirps. For these simulations, the EOD signal was temporally stretched and resampled to decrease the frequency by 10 Hz or 100 Hz. The amplitude of the unmanipulated EOD was reduced to 30% of its original value, and then added to the stretched EOD. The resulting signals mimic the beat detected by the fish with the lower EOD frequency, since the receiver's own EOD is closer to its electroreceptors (and thus typically higher in amplitude) than the signaler's EOD. For interactions in which we simulated the signaling fish (higher frequency EOD) chirping at the receiver fish (lower frequency EOD), the procedure was exactly the same except that the middle of the unmanipulated EOD signal contained a chirp. Temporally stretching the EOD signal allowed us to precisely standardize the frequency difference between the interacting EODs, and thus produce beats that had specific frequencies. For a subset of EODs, we confirmed that combining EODs with a temporally stretched/compressed copy of themselves produced similar EOD and beat waveforms as combining two unmanipulated EOD signals. We selected two difference frequencies (DF; 10 Hz and 100 Hz) in order to examine how chirp conspicuousness might be affected by the frequency

difference between signaler and receiver across encounters with conspecifics within a species-typical range of EOD frequencies. These two beat frequencies also simulate same-sex (10 Hz beat) and opposite-sex (100 Hz beat) social interactions in the two species from our sample that have sexually dimorphic EOD frequencies (*A. albifrons* and *A. leptorhynchus*; Meyer et al., 1987; Zakon & Dunlap, 1999). The chirp signal was added at four different phases in the unmodulated EOD signal, which resulted in the chirp occurring at four different phases of the beat in the combined signal. For analyses using peak and sum conspicuousness, the conspicuousness results from the four phases were averaged to provide a single conspicuousness value for each chirp. For analyses that looked specifically at variability of conspicuousness across phase, we calculated the standard deviation of the four conspicuousness values for each chirp. We extracted the AM of the combined signals using a two-step method: 1) performing a full-wave rectification in MATLAB (Mathworks; Natick, MA, USA) and 2) using Adobe Audition (Adobe Systems; San Jose, CA, USA) to apply the FFT filter function (low-pass filter cut-off at 400 Hz, Hamming window size 32768). The DC offset was also removed with the Audition software.

2.3. EOD and AM Waveform Comparison

Waveform complexity of the EOD and the AM was quantified by comparing the difference in the power of the second or third harmonic relative to the fundamental frequency (F2-F1 or F3-F1, in dB) in each signal. Each EOD signal was temporally stretched or compressed twice, once to obtain an 800 Hz fundamental frequency and again to obtain an 810 Hz fundamental frequency. These two signals were combined with

a 30% beat contrast, and the AM was extracted as described above by full-wave rectifying, low-pass filtering, and removing the DC offset. Short (~1 second) segments of both the 800 Hz EOD carrier and the AM of the combined EODs were selected in the Adobe Audition software, and a power spectrum was obtained with the frequency analysis tool (Blackman-Harris window size 65536). The power of the fundamental, second, and third harmonics was extracted from the resulting power spectrum, and the relative power of the second and third harmonics was calculated by subtracting the peak power at the fundamental frequency (F1) from the peak power at the second and third harmonic frequencies (F2 and F3). To verify that our calculation of F2-F1 and F3-F1 using the peak power did not introduce artifacts based on the shape of the peaks in the power spectrum, we calculated an average of the highest three power values for each frequency peak on a subset of EODs and AMs (~10%) and used these values to quantify F2-F1 and F3-F1. The values calculated this way were nearly identical to the values calculated using the maximum of each frequency peak. We also verified that the relationship between EOD waveform and AM waveform was robust to differences in beat contrast by examining a different subset of EOD waveforms at 1%, 5%, 10%, and 20% contrast.

2.4. Chirp Conspicuousness

We developed a custom MATLAB script to quantify chirp conspicuousness based on existing methods to compare the similarity of two signals (Gill et al., 2008; Kennedy, 2007; van Rossum, 2001). The algorithm relies on a correlation-like measure to compare the similarity in structure between a section of beat AM with a chirp and a section of beat

AM without a chirp. Specifically, for a portion of chirp signal of length *2l* centered on time *x*, the similarity value *S(x)* between this chirp signal *C(t)* and a beat excerpt *B(t)*, both of which had their mean removed, was:

$$S(x) = \frac{\sum_{t=x-l}^{x+l} W(t)B(t)C(t)}{\frac{1}{2}\sum_{t=x-l}^{x+l} W(t)B(t)B(t) + \frac{1}{2}\sum_{t=x-l}^{x+l} W(t)C(t)C(t)}$$

where W(t) is a Gaussian window with a width of 10, 20 or 40 ms at 10% height.

Note that S(x) has the same numerator as a Pearson correlation coefficient but is normalized by a different denominator. The normalization we use allows for differences in absolute amplitude of a signal to influence S(x); thereby, a decrease in amplitude during a chirp could influence the similarity value. The value of S(x) is critically dependent on the alignment of the two signals. For example, two identical sinusoidal signals compared in antiphase would result in low similarity values. We therefore systematically varied the alignment of the signals to be compared by shifting the beat excerpt by a duration of as much as 1.67 cycles of the regular beat period. For each point *x* in the chirp signal the similarity value $S_{max}(x)$ was taken as the maximum value of *S(x)* across all time shifted comparisons. Our conspicuousness measure is taken as $1\text{-}S_{max}(x)$.

The script generates a conspicuousness curve that depicts conspicuousness of the chirp file across the entire signal. The conspicuousness varies between 0 and 1, with values close to 0 indicating little difference between the beat with and without the chirp, and with values close to 1 indicating substantial chirp conspicuousness (Fig. 4). From these plots, we used two measures of chirp conspicuousness: the peak value of the conspicuousness curve and the sum of all points under the conspicuousness curve from the start of the chirp minus half the window width to the end of the chirp plus half the

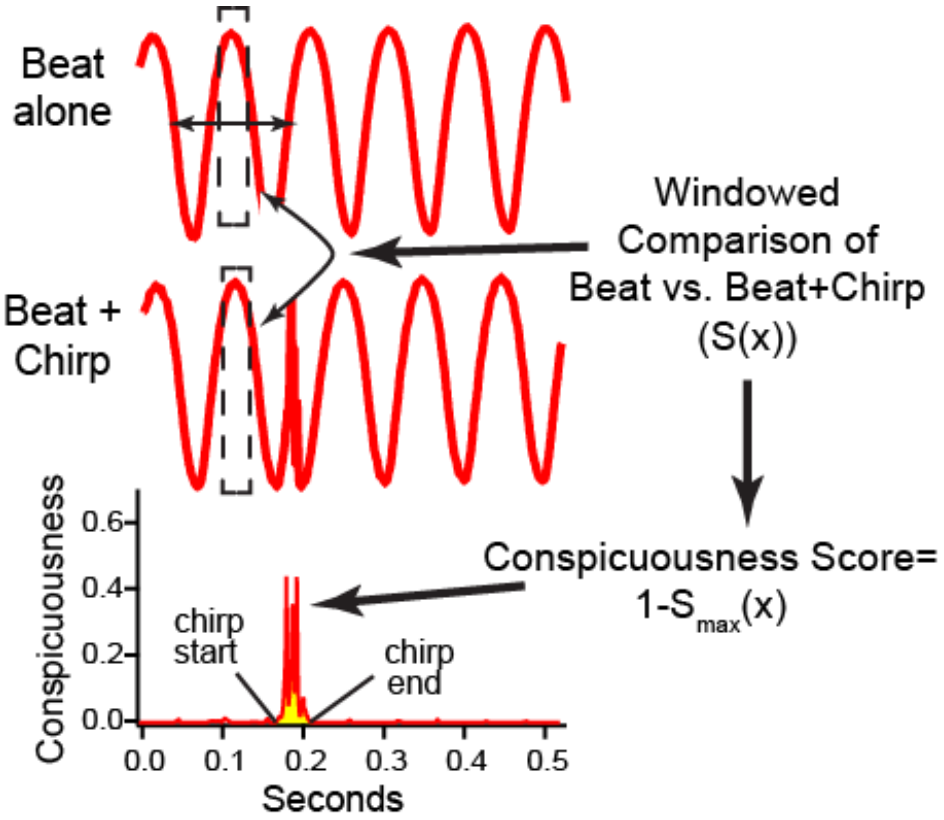


Fig. 4. A simplified schematic of the chirp conspicuousness analysis. The AM (beat) of interacting EODs without a chirp is compared to the AM (beat) of the same EODs interacting with a chirp. The algorithm generates a conspicuousness score that is based on a windowed comparison between the beat alone vs. the beat containing the chirp (S(x), see Methods). The conspicuousness score is plotted across the duration of the signal. We report here peak conspicuousness (the maximal point in this plot) and sum conspicuousness (the area under the curve during the time that the sampling window overlaps the chirp, shaded in yellow here). Note that the red traces shown are the AM, not the original EOD. The specific parameters of the chirp displayed here caused the beat to be shifted in phase after the chirp compared to the unperturbed beat. In other words, the two signals are in phase before the chirp but nearly anti-phase after the chirp. Chirp duration and frequency jointly determine how much phase shift the chirp causes.

window width. The peak value provides information about maximal instantaneous conspicuousness, whereas the sum value is an integrated measure of conspicuousness over the duration of the chirp. These two measures of conspicuousness allow us to make predictions about how fish might detect chirps in natural contexts. We report here the results for the peak and sum values using the 20-ms window size. The peak values for the 10-ms and 40-ms windows are included in the supplementary materials. This range of window sizes was chosen to adequately sample both the beat and the chirp. The windows were long enough to contain a sufficient portion of the low frequency (10 Hz) beat alone to compare it with the beat + chirp and were within a range that would capture details of the disruption in the beat created by the chirps. Furthermore, our choice of window size was influenced by an interest in focusing on the modulation during the chirp, without the beat that precedes or follows the chirp influencing our quantification. Most of the chirps we analyzed had durations in the tens of milliseconds range. Windows that greatly exceeded the duration of the chirps would be dominated by portions of beat rather than the chirp itself. This would be a problem because chirps often cause a phase shift in the beat after the chirp. As a consequence, there is no way to align the beat both before and after the chirp to a segment of beat that does not contain a chirp (see Fig 4). Therefore, for windows much larger than the typical chirp, the conspicuousness value would not reflect how well the chirp modulation stands out against the beat background, but merely how well beat cycles surrounding the chirp can be matched and aligned to the reference beat excerpt.

2.5. Heterospecific Chirp Synthesis

Species-typical chirp parameters and EOD waveform complexity are necessarily confounded by species-specific variation in these signals. That is, each species produces chirps with a specific range of parameters on a species-typical EOD waveform. These characteristics cannot be examined independently by using only naturally occurring signals. To address this problem, we developed a method for synthesizing hybrid chirps that would allow us to decouple EOD and chirp characteristics and independently examine the effects of each component on chirp conspicuousness. Constructing synthetic chirps also allowed us to investigate how chirp structure and EOD waveform interact and to test the hypothesis that chirps would be most conspicuous when they occurred on the background of conspecific EOD beats. The "ChirpSynth" algorithm was implemented in Igor Pro (Wavemetrics Version 4.09; code available on request to GTS (getsmith@indiana.edu)). Briefly, it superimposed the chirp parameters of one species on the EOD waveform of several different species. The resulting hybrid chirps could then be analyzed and compared to the same chirp re-synthesized on its own EOD waveform. The properties of chirps (EOD frequency and amplitude over time during the chirp) were calculated as described previously with autocorrelation window sizes of 3 ms and 67% window overlap (Kolodziejski et al. 2005; Turner et al. 2007). Frequency-vs.-time and amplitude-vs.-time data were then resampled at 44.1 kHz. The frequency vs. time data from the chirp were used to temporally stretch and compress an EOD recording from another fish. The amplitude vs. time data from the chirp were used to scale the EOD recording to impose the amplitude modulation of the chirp on it. The resulting signal has the chirp characteristics of one species and the EOD waveform of the same or another species (Fig. 5).

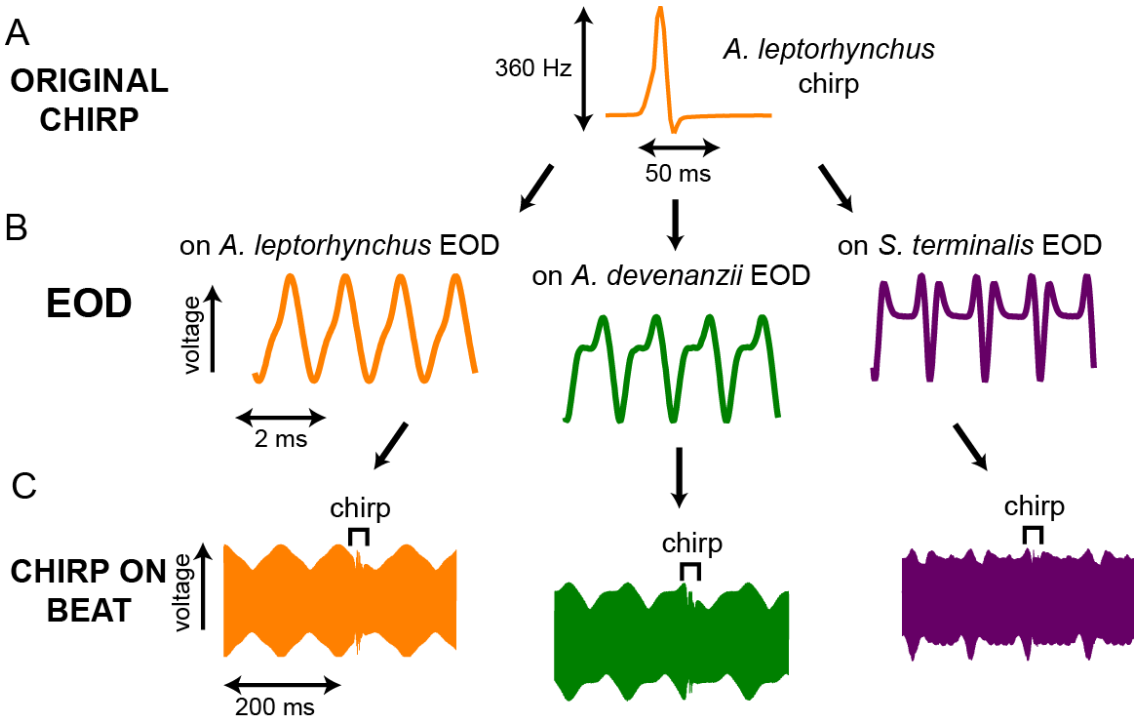


Fig. 5. Synthesis of hybrid chirps. Parameters of A) chirps of one species were used to modulate the frequency and amplitude of B) its own and other species' EODs of varying waveform complexity. C) The hybrid chirp was then combined with an EOD from the waveform donor species to produce 10 Hz beats. The chirp is indicated on each beat with brackets. All EODs and beats are on the same timescales shown for the *A. leptorhynchus* EOD and beat. The chirp shown in this example is less conspicuous on the complex *S. terminalis* beat than on the more sinusoidal beats.

For this analysis, we selected four species that span the range of chirp and EOD waveform diversity: *A. albifrons* and *A. leptorhynchus*, the two most widely studied species, both with two distinct chirp types and relatively sinusoidal EOD waveforms; *A. devenanzii*, a species with a moderately complex waveform and wide variation in chirp duration and which produces both simple chirps and chirps with multiple frequency peaks; and *S. terminalis*, a species with a complex EOD waveform and short, stereotyped chirps (Bastian et al., 2001; Dunlap & Larkins-Ford, 2003a; Kolodziejski et al., 2005; Turner et al., 2007; Zhou & Smith 2006). We used six chirps from *A. devenanzii* and *S. terminalis* and six small chirps and six big chirps from both *A. albifrons* and *A. leptorhynchus*. Each chirp was synthesized on all the EOD waveforms of the other species and also re-synthesized on its own waveform to control for any potential artifacts introduced during the chirp synthesis procedure (Fig. 5). Each synthetic chirp was combined with frequency-shifted EODs from the EOD waveform donor species using 10 Hz and 100 Hz DFs at 30% contrast. Chirps were analyzed for conspicuousness as described above.

2.6. Statistical Methods

Waveform complexity (F2-F1 and F3-F1) of the EOD vs. the AM of interacting EODs was analyzed using a simple linear regression. A six-step forward stepwise regression with an F-to-enter value of 1.00 was used to determine which signal features of the natural chirps and EODs had the largest impact on peak and sum chirp conspicuousness. Chirp FM, chirp duration, chirp relative decay, DF, F2-F1, and F3-F1 were entered into the stepwise regression as predictors. Chirp relative decay describes the

shape of the chirp but is relatively independent of both chirp duration and chirp FM. Chirp AM is tightly correlated with chirp FM both within and across species and thus we did not include it in the analysis to avoid multicollinearity in the regressions (Turner et al., 2007). For all stepwise regression analyses, we transformed chirp FM, chirp duration, chirp relative decay, and peak conspicuousness using a natural log in order to linearize the data. For the synthetic chirps, a three-way repeated measures analysis of variance (ANOVA) was used to examine the effects of chirp species, EOD species, and DF on peak and sum chirp conspicuousness. Fisher's Protected Least Significant Difference tests (PLSDs) were used for post-hoc analyses of significant interaction terms. All statistical analyses were performed with Statistica 7 (StatSoft Inc.; Tulsa, OK).

3. RESULTS

3.1. EOD Waveform and AM Waveform

The distribution of power in the fundamental frequency (F1) and the harmonic frequencies (F2, F3, and F4) of EODs varied substantially across species, as illustrated by the power spectra of *A. albifrons* and *S. terminalis* EODs, which are the two extremes in EOD waveform complexity in our sample (Fig. 6 A&B). Correspondingly, species also varied substantially in the relative power in F1 and higher harmonics of the AM created by the interaction of two conspecific EODs (Fig. 6 C&D). Across all species, we saw a striking relationship between the waveform complexity variables (F2-F1 and F3-F1) for the EOD vs. that of the AM (Fig. 6 E&F). Small differences in waveform complexity for EODs that were of intermediate complexity were linearly transformed into corresponding

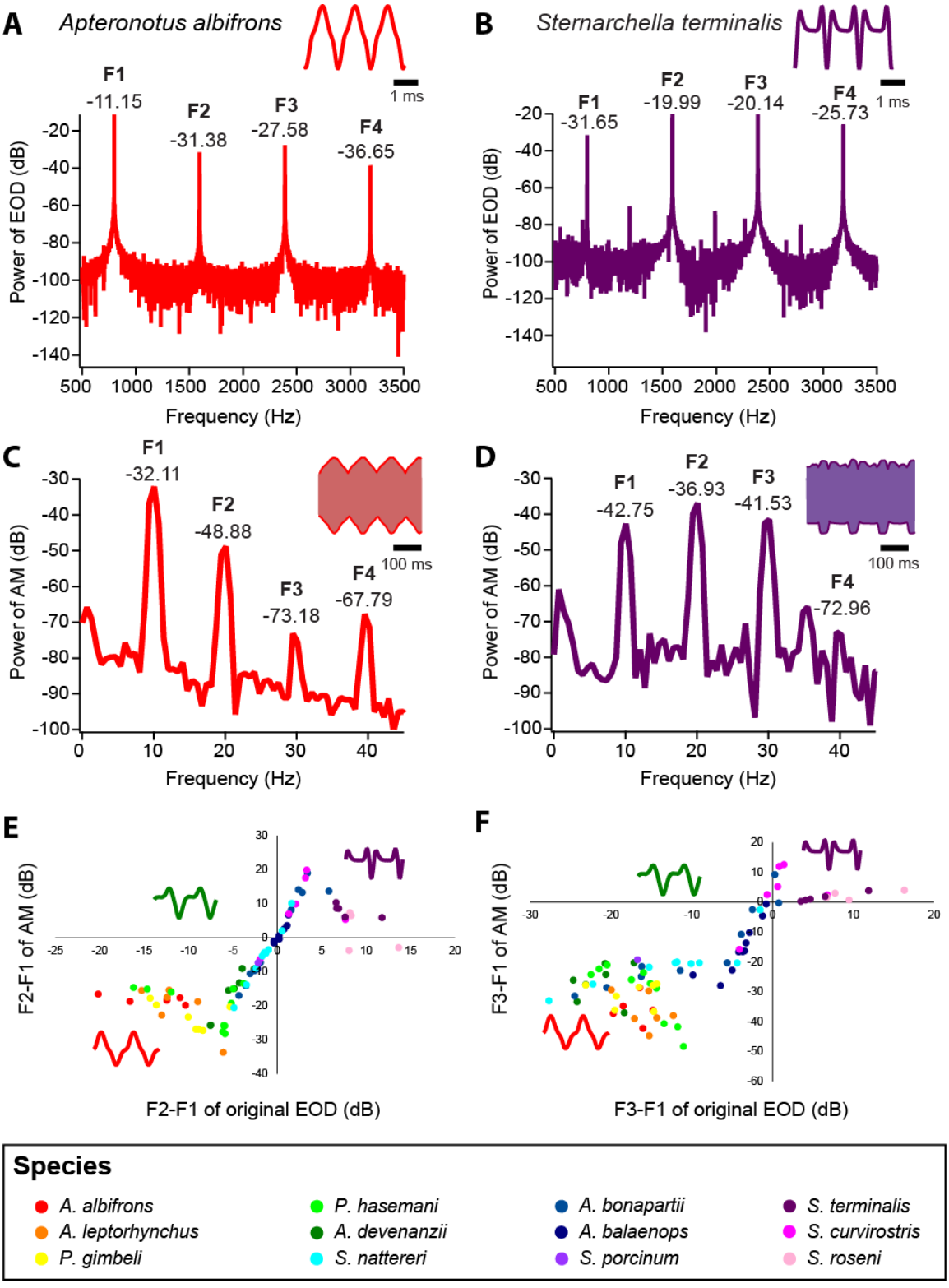

A
Apteronotus albifrons
F1
-11.15
F2
-31.38
F3
-27.58
F4
-36.65
1 ms
Power of EOD (dB)
Frequency (Hz)
B
Sternarchella terminalis
F1
-31.65
F2
-19.99
F3
-20.14
F4
-25.73
1 ms
Power of EOD (dB)
Frequency (Hz)
C
F1
-32.11
F2
-48.88
F3
-73.18
F4
-67.79
100 ms
Power of AM (dB)
Frequency (Hz)
D
F1
-42.75
F2
-36.93
F3
-41.53
F4
-72.96
100 ms
Power of AM (dB)
Frequency (Hz)
E
F2-F1 of AM (dB)
F2-F1 of original EOD (dB)
F
F3-F1 of AM (dB)
F3-F1 of original EOD (dB)
Species
A. albifrons
A. leptorhynchus
P. gimbeli
P. hasemani
A. devenanzii
S. nattereri
A. bonapartii
A. balaenops
S. porcinum
S. terminalis
S. curvirostris
S. roseni

Fig. 6. Comparison of harmonic content in the waveform of the EOD and AM (beat) generated by two interacting EODs 10 Hz apart in frequency. Power spectrum of A) an *A. albifrons* EOD and B) a *S. terminalis* EOD. Both EODs were stretched and re-sampled to 800 Hz. Representative EODs are shown in the upper-right corner of each panel. F1, F2, F3, and F4 show the power spectrum peaks corresponding to the fundamental frequency, second harmonic, third harmonic, and fourth harmonic, respectively. The height of those peaks (in dB) indicates the power in each harmonic. We calculated F2-F1 and F3-F1 to quantify differences in waveform. The same analysis was done for C) the AM of two *A. albifrons* EODs combined at a 10 Hz DF and D) the AM of two *S. terminalis* EODs combined at a 10 Hz DF. The AM is denoted by the top and bottom traces on the combined EODs shown in the upper-right corner of panels C and D. Note the difference in the timescale of the EOD vs. AM traces and in the frequency scale on the X-axis of the power spectra in A vs. C and B vs. D. For *A. albifrons*, both the EOD waveform and the AM generated by the interacting EODs are relatively sinusoidal, and there was much more power in F1 than in F2 or F3. Conversely, for *S. terminalis*, the EODs and the AM are both relatively complex, and there was more power in F2 and F3 than in F1. Note that dB is a logarithmic scale. E) Relationship between F2-F1 in the EOD and F2-F1 of the AM of interacting EODs across individuals from twelve apteronotid species. This analysis arranged each fish according to waveform, with the most sinusoidal EODs on the left and the most complex EODs on the right. F2-F1 of the EOD is strongly correlated with F2-F1 of the AM in species with waveforms of intermediate complexity. Insets show representative waveforms for *A. albifrons* (nearly sinusoidal), *A. devenanzii* (moderately complex) and *S. terminalis* (complex). F) Relationship between F3-F1 in the EOD and F3-F1 of the AM of interacting EODs across the same fish from twelve apteronotid species. A pattern similar to that of F2-F1 emerges for the comparison of F3-F1 of the EOD to F3-F1 of the AM.

differences in the complexity of the beats they created. However, there were both "ceiling" and "floor" effect nonlinearities in the relationship between EOD vs. AM waveform complexity. Thus, small differences in waveform complexity of either relatively sinusoidal EODs or highly complex EODs did not translate into comparable differences in beat complexity. This nonlinearity was robust at other behaviorally relevant beat contrasts ranging from 1% to 30%. Across the entire range of EOD complexity, the linear regression indicates R^2 values of 0.59 for the relationship between EOD waveform F2-F1 vs. AM waveform F2-F1 and 0.68 for the relationship between EOD waveform F3-F1 vs. AM waveform F3-F1. For EODs of intermediate complexity, however, the linear relationship between EOD waveform complexity and AM complexity was much tighter (EOD F2-F1 between -6dB and 4 dB, R^2=0.98; EOD F3-F1 between -7dB and 3 dB, R^2=0.93).

3.2. Conspicuousness of Recorded Chirps

Chirp conspicuousness was influenced by properties of both the chirps themselves and the background EOD beats on which they occurred. Chirp conspicuousness in this context refers to the degree of dissimilarity between two segments of EOD beat, one with a chirp and one without a chirp. Peak conspicuousness measures maximum instantaneous conspicuousness, whereas sum conspicuousness is an integrated measurement of conspicuousness over the duration of the chirp. In order to determine which signal parameters are likely to have the greatest impact on peak chirp conspicuousness across species, we performed a stepwise regression on all recorded chirp samples on both 10 Hz and 100 Hz DFs (beats). Chirp FM, chirp relative decay, and DF (frequency difference

between the EODs) were all significant predictors of peak chirp conspicuousness using the 20-ms window (Table 1). Chirps that had a larger frequency increase were more conspicuous (Fig. 7). Chirp relative decay measures the proportion of time that the frequency was falling during the chirp. High values of chirp relative decay indicate a more abrupt chirp rise and/or a slower return to baseline, and chirps with high values of chirp relative decay were more conspicuous (Fig. 8 A&B). Chirps were also more conspicuous on a slower (10Hz) beat compared to a faster (100Hz) beat (Fig. 9; Walz, 2014). Chirp duration and EOD waveform complexity (F2-F1 and F3-F1) were not significant predictors of peak chirp conspicuousness (Supplementary Figs. 1-3). The effects of EOD and chirp parameters on peak chirp conspicuousness were consistent across analysis window sizes with two exceptions: (1) DF was a significant predictor at the two smaller window sizes but not the larger window size, and (2) the EOD waveform variable F3-F1 was significant at the largest window size (Supplementary Table 1, Supplementary Figs. 1-5).

For the sum conspicuousness measure, chirp FM, chirp duration, chirp relative decay, and the EOD waveform complexity variables all significantly influenced chirp conspicuousness (20-ms window; Table 1). Chirps with greater FM, longer duration, and a prolonged relative decay time were more conspicuous (Fig. 8 C&D, Fig. 10). Chirps on more complex waveforms (higher values of F2-F1 and F3-F1) were less conspicuous relative to chirps on more sinusoidal waveforms (Fig. 11). DF was the only variable that was not a significant predictor of sum chirp conspicuousness.

Table 1. Effects of EOD and Chirp Parameters on Peak and Sum Chirp Conspicuousness (20-ms window, partial correlations)

	Peak (20-ms window)[1]		Sum (20-ms window)[2]	
Signal parameter	Partial correlation	p	Partial correlation	p
Chirp duration	0.06	0.32	**0.60**[3]	**<0.0001**
Chirp FM	**0.72**	**<0.0001**	**0.28**	**<0.0001**
Chirp relative decay	**0.17**	**0.003**	**0.30**	**<0.0001**
DF	**-0.42**	**<0.0001**	-0.01	0.81
F2-F1	-0.002	0.97	**-0.25**	**<0.0001**
F3-F1	0.005	0.94	**-0.22**	**0.0002**

[1] $F(3, 290)=130.4$, $p<0.0001$, R^2 adj=0.57 for the multiple regression model

[2] $F(5, 288)=96.7$, $p<0.0001$, R^2 adj=0.62 for the multiple regression model

[3] Bold values indicate variables included in the respective stepwise regression model.

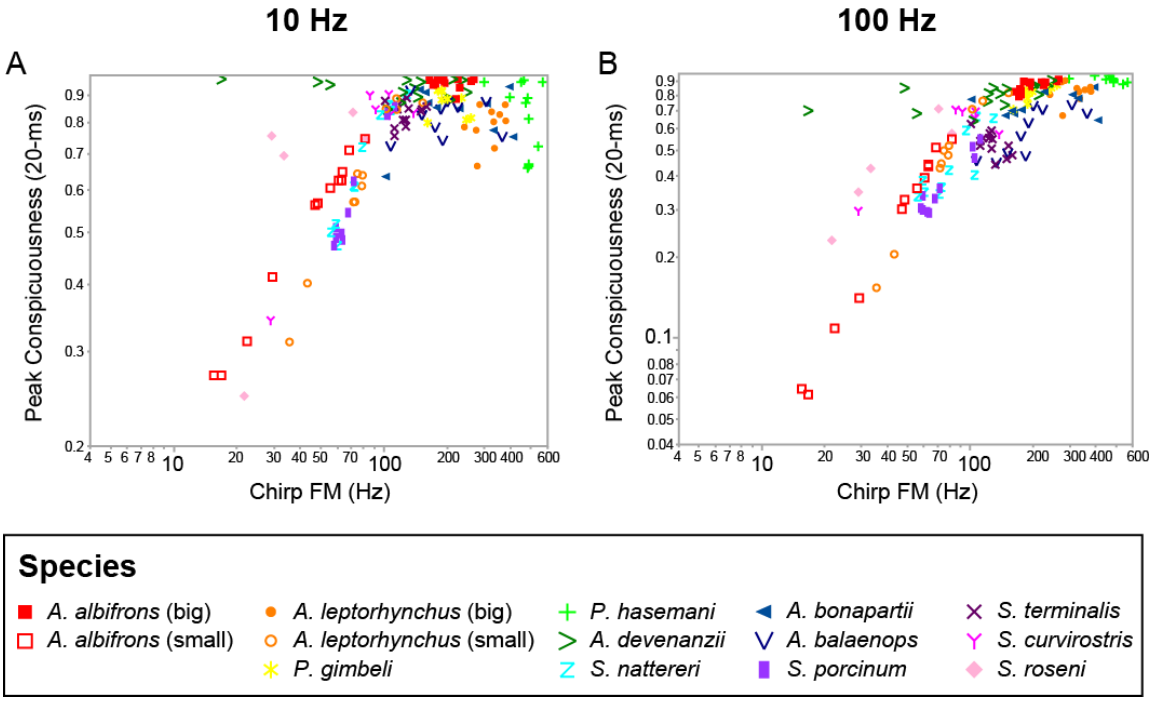


Fig. 7. Contribution of chirp FM to peak conspicuousness of natural chirps on A) a 10 Hz beat and B) a 100 Hz beat. Chirp FM and peak chirp conspicuousness are shown on a log scale. Chirps with large frequency excursions were more conspicuous (partial correlation: 0.72, $p<0.0001$). One notable exception to this pattern was the chirps of *A. devenanzii* (green > symbols), which are highly conspicuousness with low chirp FM values. This may be because these chirps have a complex, multi-peaked structure (Zhou & Smith, 2006).

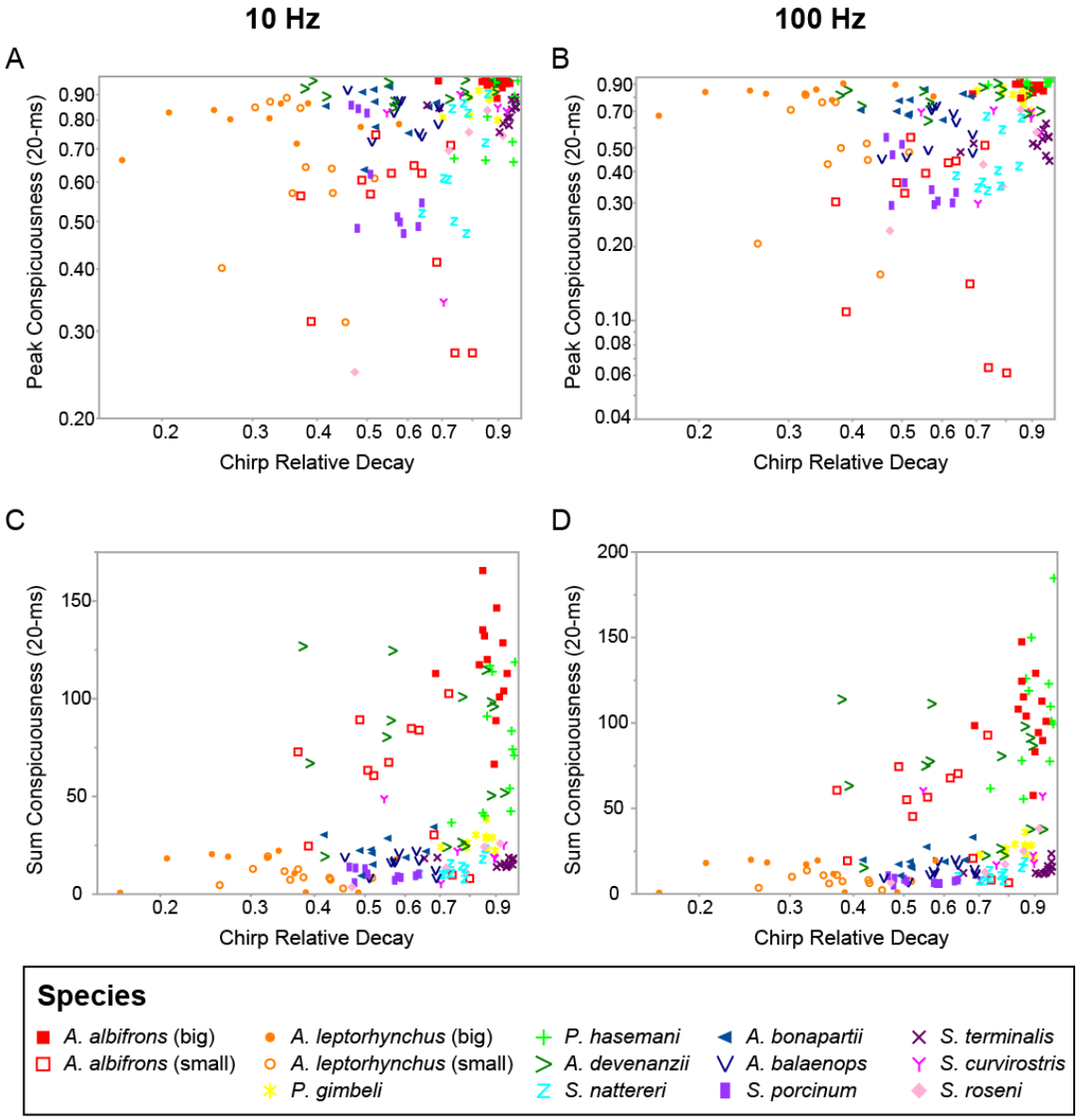


Fig. 8. Contribution of chirp relative decay to peak (A,B) and sum (C,D) conspicuousness of natural chirps on 10 Hz (A,C) and 100 Hz (B,D) beats (20-ms analysis window). Higher values of chirp relative decay (log transformed) were significantly correlated with greater values of peak conspicuousness (partial correlation: 0.17, p=0.003). Additionally, higher values of chirp relative decay were significantly correlated with greater sum chirp conspicuousness (partial correlation: 0.30, p<0.0001). This shows that chirps that rise abruptly and/or return to baseline frequency more slowly are more conspicuous. The outlying clusters in the sum conspicuousness plots (C,D) represent chirps of species with long-duration chirps (*A. albifrons*, *A. devenanzii*, *P. hasemani*) that consequently have larger sum conspicuousness values.

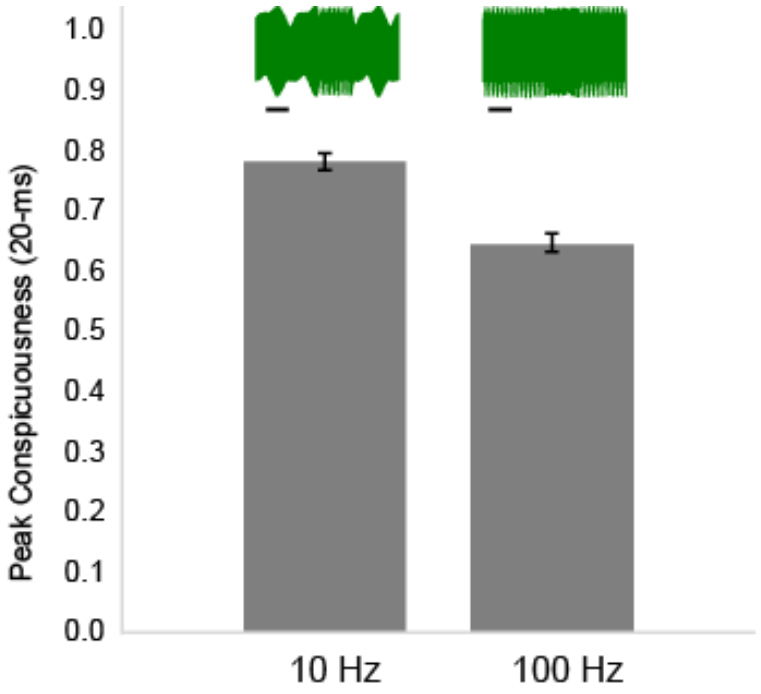


Fig. 9. Effect of DF on peak conspicuousness of 147 natural chirps (20-ms analysis window). Chirps were more conspicuous on a 10 Hz DF than on a 100 Hz DF (partial correlation: -0.42, <0.0001). Insets depict the same *A. devenanzii* chirp on a 10 Hz DF (left) and a 100 Hz DF (right). Scale bar denotes 50 ms. Error bars show one standard error from the mean.

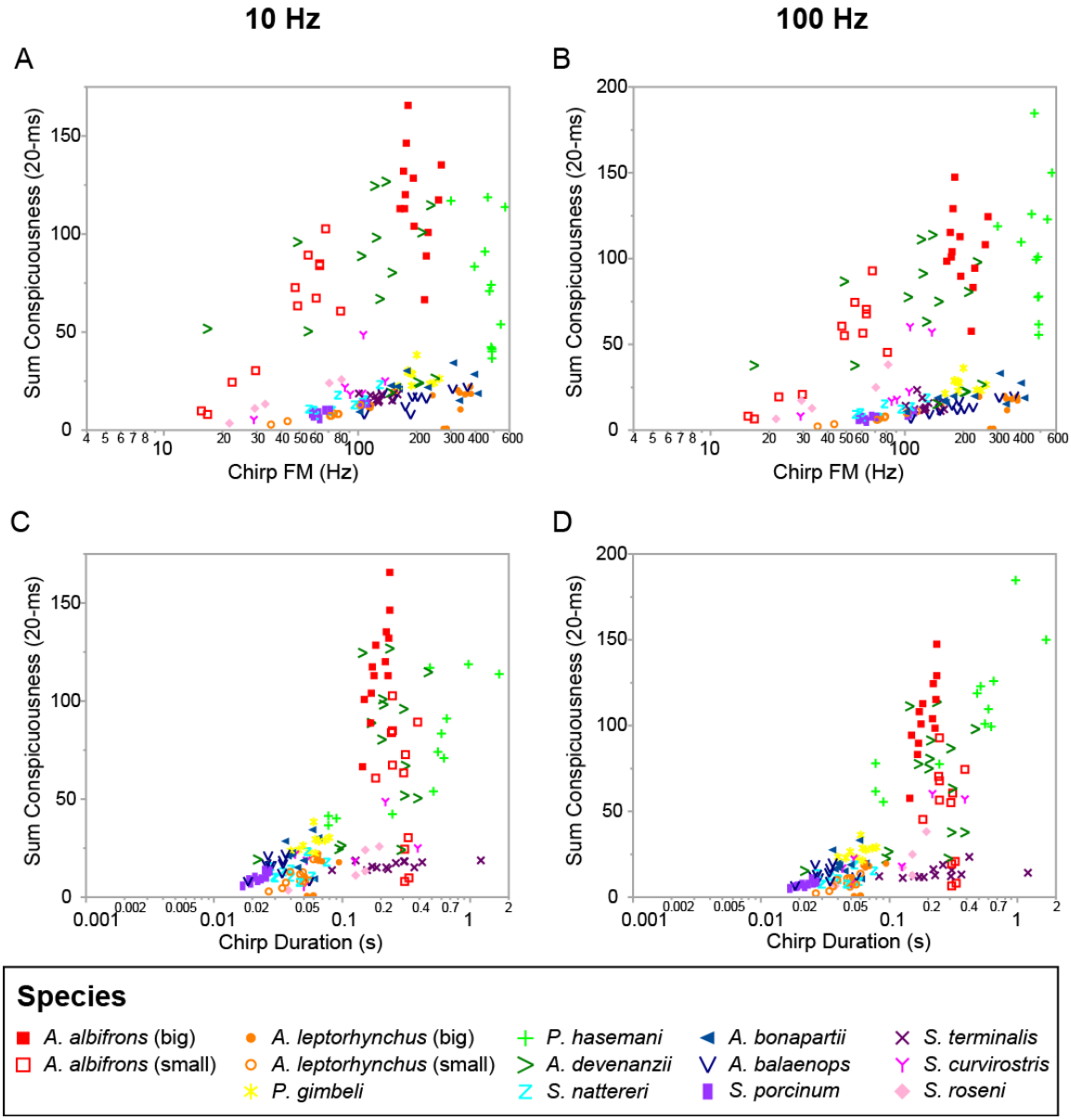


Fig. 10. Contribution of chirp FM (A,B) and chirp duration (C,D) to sum chirp conspicuousness of natural chirps on 10 Hz beats (A,C) and 100 Hz beats (B,D) (20-ms window analysis). Chirp FM (log-transformed) was a significant predictor of sum chirp conspicuousness (partial correlation 0.28, $p<0.0001$). Chirps with greater FM were more conspicuous. Likewise, chirp duration had a strong effect on sum conspicuousness (partial correlation 0.60, $p<0.0001$). Longer duration chirps were more conspicuous.

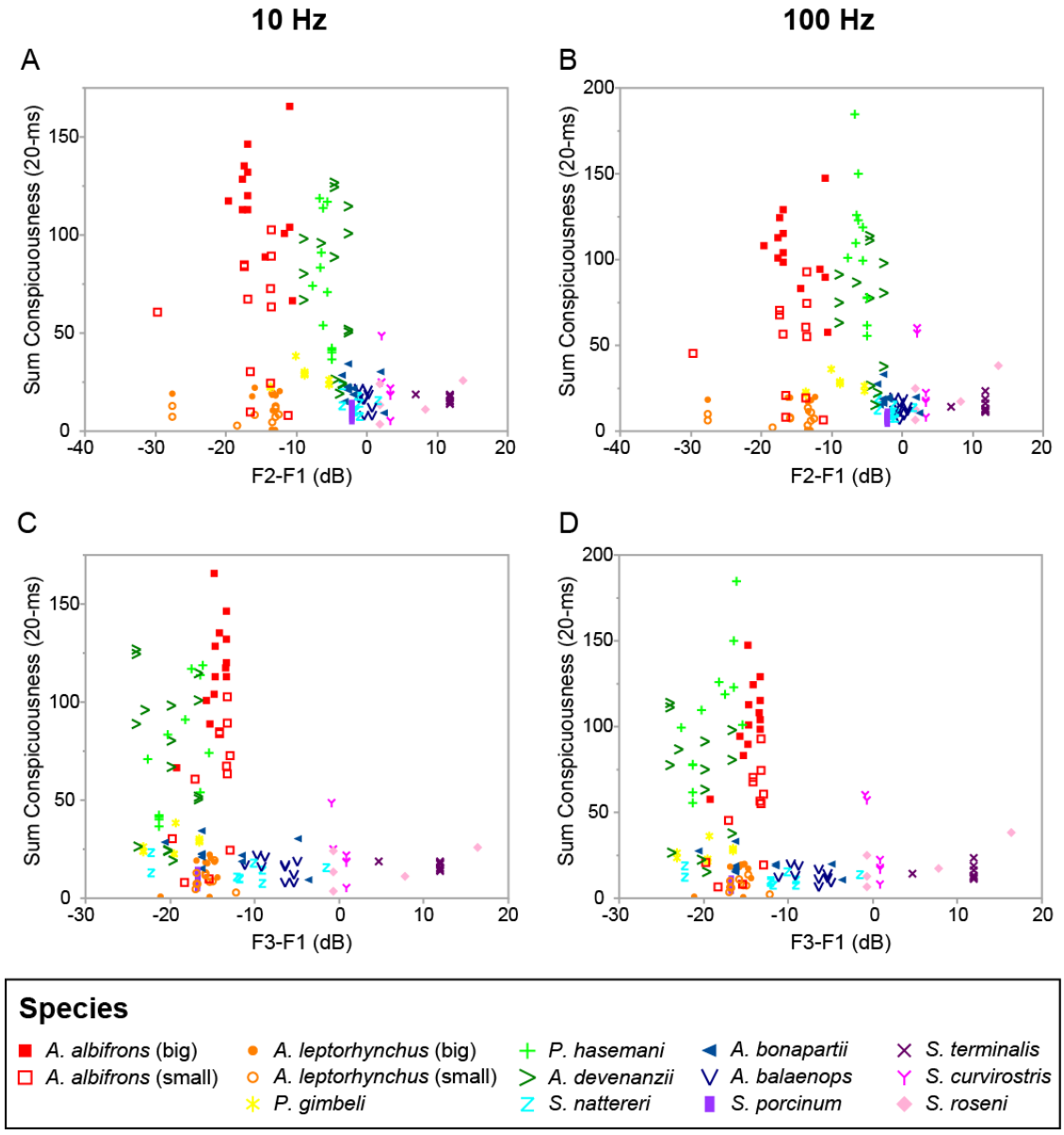


Fig. 11. Contribution of waveform complexity (F2-F1, A,B; F3-F1, C,D) to sum chirp conspicuousness of natural chirps on 10 Hz beats (A,C) and 100 Hz beats (B,D) (20-ms analysis window). Both waveform variables were significant contributors to sum chirp conspicuousness (F2-F1 partial correlation: -0.25, $p<0.0001$; F3-F1 partial correlation: -0.22, $p=0.0002$). Chirps that naturally occur on more sinusoidal waveforms (more negative values of F2-F1) were more conspicuous. Chirps with lower values of F3-F1 were also more conspicuous. These trends appear to be driven largely by the high-frequency, long duration and/or multi-peaked chirps of *P. hasemani* and *A. devenanzii*, which naturally occur on waveforms of intermediate complexity, as well as the big and small chirps of *A. albifrons*.

3.3 Variation in Conspicuousness of Natural Chirps Across Phase

Conspicuousness varies depending on the phase of the beat at which the chirp occurs. We therefore used a separate stepwise regression model to determine which chirp and waveform parameters best predicted variation across phase (calculated as the standard deviation of the peak conspicuousness score). Chirp FM (log-transformed) and DF had the greatest impact on phase-related variation in conspicuousness (Supplementary Table 2). The conspicuousness of chirps with greater FM was more variable across phase. Additionally, chirp conspicuousness across phase was more variable on the 10 Hz DF than on the 100 Hz DF (Supplementary Fig. 6).

3.4. Conspicuousness of Hybrid Synthetic Chirps

The creation of hybrid synthetic chirps allowed us to independently evaluate how chirp conspicuousness is affected by EOD waveform, since this technique enabled us to independently vary EOD waveform while keeping the parameters of a particular chirp constant. Chirp species, EOD species, DF, and all associated interactions significantly affected peak conspicuousness (Table 2). Species-specific chirp structure robustly affected conspicuousness (Fig. 12). Small chirps of both *A. leptorhynchus* and *A. albifrons* were generally less conspicuous than big chirps. Most chirps were less conspicuous on the complex EOD waveform (*S. terminalis*) than on the sinusoidal and intermediate EOD waveforms. The two notable exceptions to this trend were the *A. leptorhyncus* and *A. albifrons* small chirps on the 10 Hz beat, which were more conspicuous on the complex waveform. As with the natural chirps, the hybrid chirps were more conspicuous on a 10 Hz beat than on a 100 Hz beat. Additionally, the decrease in

Table 2. Effects of EOD and Chirp Parameters on Peak Conspicuousness of Hybrid/Synthetic Chirps (20-ms window)

	degrees of freedom	F[a]	p
Chirp Species	5, 30	12.84	<0.0001
DF	1, 30	147.90	<0.0001
DF*Chirp Species	5, 30	14.09	<0.0001
EOD Species	3, 90	25.28	<0.0001
EOD Species*Chirp Species	15, 90	15.27	<0.0001
DF*EOD Species	3, 90	62.68	<0.0001
DF*EOD Species*Chirp Species	15, 90	3.22	0.0003

[a] 3 Factor, Repeated Measures ANOVA

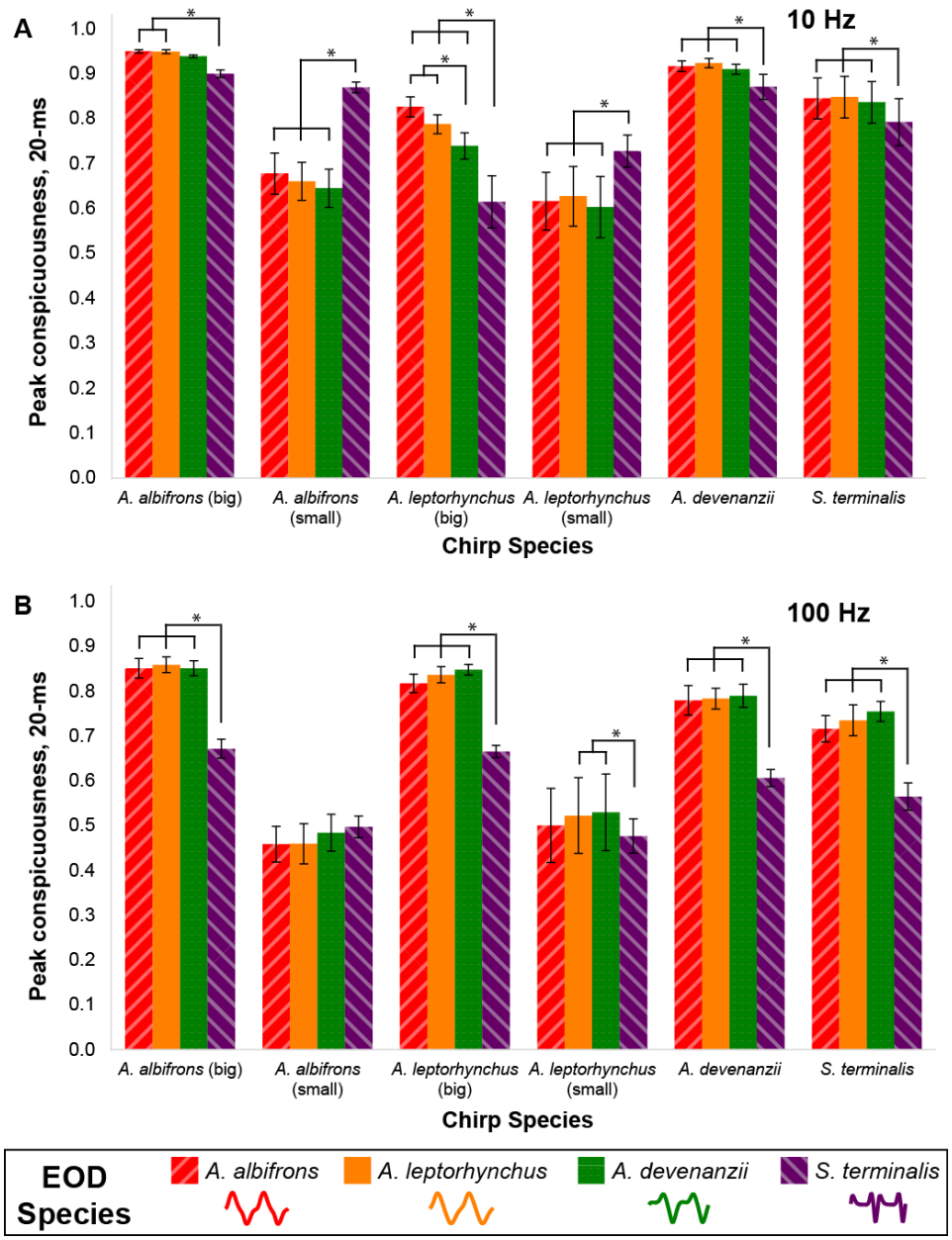


Fig. 12. Peak conspicuousness of hybrid chirps using a 20-ms window. Chirps from four species (including two different types of *A. leptorhynchus* and *A. albifrons* chirps, n=6 from each species/chirp type) were re-synthesized on the waveform of all four species, including the waveform of the species from which the chirp came. The bars within each chirp type are arranged from most sinusoidal (*A. albifrons*) to most complex (*S. terminalis*) EOD waveform. The chirps were combined with an EOD from the waveform donor species to measure peak conspicuousness on a A) 10 Hz beat and B) 100 Hz beat. Asterisks indicate statistically significant differences ($p<0.05$, Fisher PLSD) between the conspicuousness of chirps on different species-specific EOD waveforms. Error bars show one standard error from the mean.

conspicuousness that occurred for most chirps on the complex waveform relative to the sinusoidal or intermediate waveforms was exaggerated on the 100 Hz beat. Finally, we did not see a pattern whereby chirp types were maximally conspicuous on conspecific EODs. In fact, *S. terminalis* chirps had greater mean peak conspicuousness on the three heterospecific EODs than on the conspecific EOD for both DFs, and, at least for the 10 Hz beat, the small chirps of *A. albifrons* and *A. leptorhynchus* were much more conspicuous on the *S. terminalis* EOD waveform. The 10-ms and 40-ms windows showed the same general trends as the 20-ms window (Supplementary Table 1). However, when analyzed with the 10-ms window, the *S. terminalis* chirps were most conspicuous on their own complex EOD at the 10 Hz DF and showed no differences in conspicuousness based on EOD waveform at the 100 Hz DF (Supplementary Fig. 7). In the analysis using a 40-ms window, we saw the same general trends as the 20-ms window, although some of these effects were weakened or missing, likely due to most chirps being near the maximal peak conspicuousness value on most EODs (Supplementary Fig. 8).

Chirp species, EOD species, and DF also significantly influenced sum chirp conspicuousness of the hybrid chirps (20-ms window; Table 3). One of the most striking comparisons for this measure of conspicuousness is the variability in conspicuousness across different chirp species and types (Fig. 13). On both the 10 Hz and 100 Hz beat, *A. albifrons* big and small chirps and *A. devenanzii* chirps were substantially more conspicuous than *A. leptorhynchus* big and small chirps and *S. terminalis* chirps. Interestingly, the *A. albifrons* big and small chirps and *A. devenanzii* chirps were also all more conspicuous on the complex EOD waveforms relative to the sinusoidal or intermediate EOD waveforms. *S. terminalis* chirps also had greater sum conspicuousness

Table 3. Effects of EOD and Chirp Parameters on Sum Conspicuousness of Hybrid/Synthetic Chirps (20-ms window)

3 Factor, Repeated Measures ANOVA Results

	degrees of freedom	F[a]	p
Chirp Species	5, 30	45.63	<0.0001
DF	1, 30	88.79	<0.0001
DF*Chirp Species	5, 30	24.04	<0.0001
EOD Species	3, 90	44.49	<0.0001
EOD Species*Chirp Species	15, 90	10.14	<0.0001
DF*EOD Species	3, 90	37.52	<0.0001
DF*EOD Species*Chirp Species	15, 90	16.28	<0.0001

[a] 3 Factor, Repeated Measures ANOVA

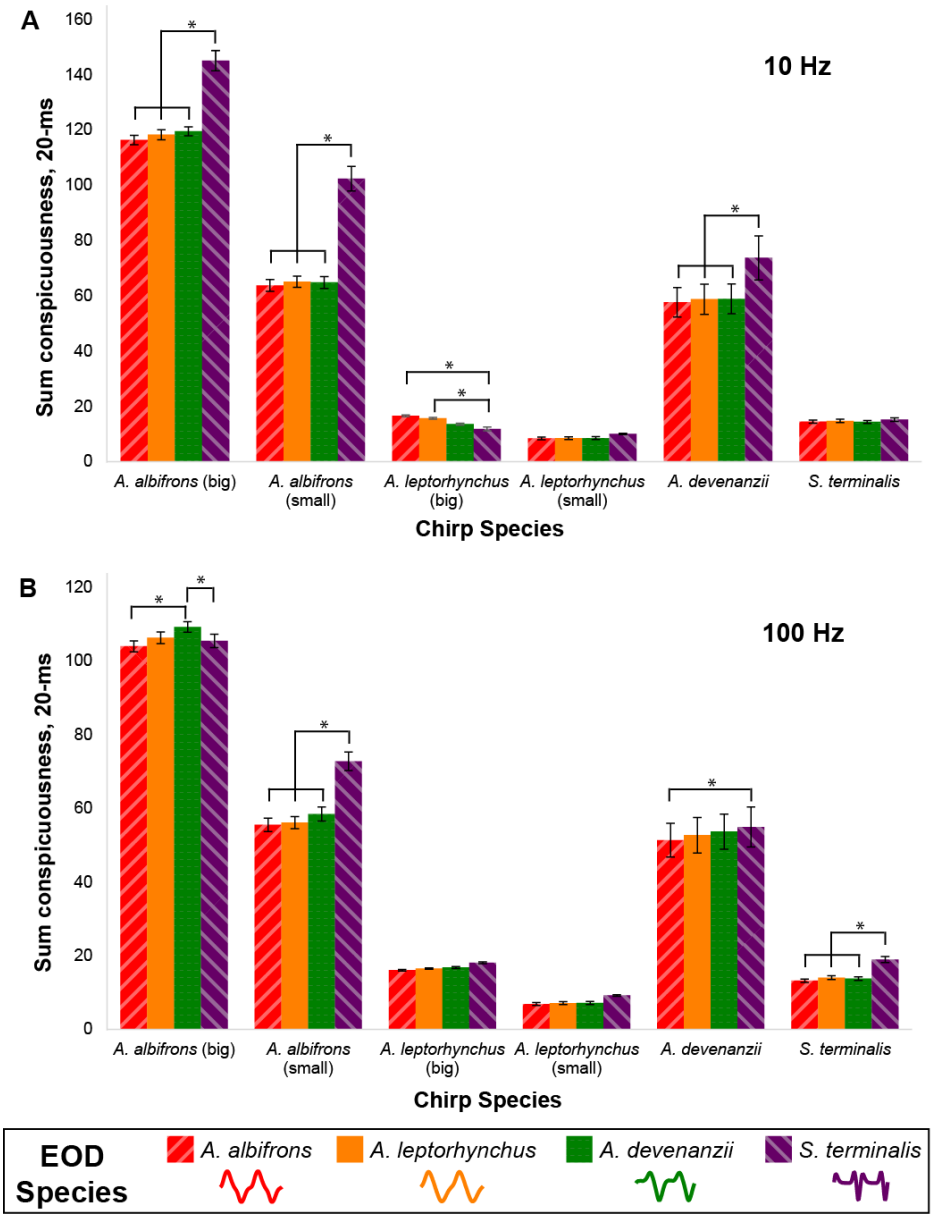


Fig. 13. Sum conspicuousness of hybrid chirps using a 20-ms window. Chirps from four species (including two different types of *A. leptorhynchus* and *A. albifrons* chirps, n=6 from each species/chirp type) were re-synthesized on the waveform of all four species, including the waveform of the species from which the chirp came. The bars within each chirp type are arranged from most sinusoidal (*A. albifrons*) to most complex (*S. terminalis*) EOD waveform. The chirps were combined with an EOD from the waveform donor species to measure sum conspicuousness on a A) 10 Hz beat and B) 100 Hz beat. Asterisks indicate statistically significant differences ($p<0.05$, Fisher PLSD) between the conspicuousness of chirps on different species-specific EOD waveforms. Error bars show one standard error from the mean.

on its own EOD for the 100 Hz DF. Thus, the two measures of conspicuousness (peak and sum) give us different results when examining whether chirps are more or less conspicuous on complex waveforms relative to more sinusoidal waveforms.

4. DISCUSSION

4.1. AM Waveform Contains Information About EOD Waveform

The question of whether EOD waveform – which varies substantially across species – is both discriminable and socially relevant for weakly electric fish has not yet been conclusively established. There is evidence that at least some species can be trained to discriminate between signals based on EOD waveform alone and that untrained fish show a preference for certain EOD waveforms over others (Kramer, 1999; Kramer & Otto, 1988). Additionally, free-swimming *A. leptorhynchus* males chirped more robustly to playbacks of *A. leptorhynchus* EODs compared to sine waves of the same frequency, indicating that waveform may contain social information (Dunlap & Larkins-Ford, 2003b). However, free-swimming *A. leptorhynchus* did not preferentially approach conspecific (quasi-sinusoidal) waveforms relative to heterospecific (complex) waveforms and did not chirp more toward the conspecific waveforms in a chirp chamber (Fugère & Krahe, 2010).

If EOD waveform is indeed socially relevant, there are at least two potential ways in which the information could be used. First, EOD waveform may allow fish to make broad species-level distinctions between conspecific and heterospecific fish. This could be useful in social contexts in which suitable habitat is co-occupied by two or more species of weakly electric fish. In areas with high species richness, EOD frequency

ranges overlap substantially, making EOD frequency alone insufficient for determining the species of a nearby individual (Kramer et al., 1981). Discriminant function analysis suggests that the inclusion of EOD waveform information alongside EOD frequency significantly enhances the power to discriminate among species based on EOD information alone (Turner, 2007). Second, and perhaps additionally, fish could potentially make finer waveform discriminations to get more detailed information about individual characteristics, such as sex or quality, or to make discriminations between species that have very similar EOD waveforms (Kramer & Otto, 1988). Generally, analyses of signal transmission and sensory perception among these animals have focused on the response of P-type tuberous receptors to the AM of the beat (Hopkins, 1976; Hopkins, 1988; Scheich et al., 1973; Walz et al., 2013; but see Stöckl et al., 2014). We demonstrate here that two interacting EODs that differ a lot in waveform should be easily distinguishable based on the AM they produce when they interact. Thus, a species with a very sinusoidal waveform should be able to distinguish a conspecific from a species with a very complex waveform and vice versa. For fish with EODs of intermediate complexity, EOD harmonic content is strongly correlated with AM harmonic content, making the finer extraction of EOD waveform detail from the AM waveform theoretically more feasible. However, because this linear relationship between EOD waveform and AM waveform falls apart for sinusoidal and very complex EODs, species that have EODs at these extremes probably cannot make such fine distinctions of within-species variation in EOD waveform based solely on beat structure.

Nevertheless, if EOD waveform is indeed a biologically relevant signal parameter, there are likely other sources of sensory input (such as information about

phase modulation from T-type electroreceptors) that may allow fish to glean waveform information during an interaction with another fish. Phase modulation, like amplitude modulation, occurs at a frequency that is equal to the DF, and detection of phase modulation is an essential component of the jamming avoidance response (Heiligenberg, 1989; Heiligenberg et al., 1978). The pattern of phase modulation might differ as a function of EOD waveform and could therefore provide information about EOD waveform. Spatial information is also likely to be an important factor in waveform identification. Because the EOD is not spatially uniform, the beat waveform that is perceived by electroreceptors likely varies with where the electroreceptors are located on the fish's body and with the position and orientation of the other fish (Assad et al., 1999). Without additional data, it is difficult to speculate as to whether local variations in waveform would simplify or complicate waveform discrimination. Further behavioral and neurophysiological experiments should explore whether fish are able to discriminate minor and/or major variation in EOD waveform and, if so, examine the neurosensory mechanisms for processing EOD and beat waveforms.

4.2. Chirp Parameters Impact Conspicuousness

Chirp FM was consistently the strongest predictor of peak chirp conspicuousness. Chirp relative decay, chirp duration, and/or DF also influenced chirp conspicuousness, depending on the measure of conspicuousness and the window size used. The two different measures of conspicuousness (peak and sum) were not always in strict agreement and led to different conclusions about the relative conspicuousness of different chirp parameters and chirp types on varying EOD waveforms and DFs. For example, the

sum conspicuousness measure of the hybrid chirps led us to conclude that *A. albifrons* big and small chirps and *A. devenanzii* chirps are substantially more conspicuous than *A. leptorhynchus* big and small chirps and *S. terminalis* chirps, but the peak conspicuousness measure does not support this conclusion. The difference between findings with peak and sum conspicuousness are primarily due to the fact that sum chirp conspicuousness is highly sensitive to chirp duration, and *A. albifrons* and *A. devenanzii* chirps are longer than *A. leptorhynchus* or *S. terminalis* chirps. The relevance of these differences in peak vs. sum estimates of chirp conspicuousness might be resolved by a greater understanding of how the electrosensory system actually encodes "conspicuousness" across the natural range of signal variation. Because peak and sum conspicuousness are two ways of quantifying the same conspicuousness curve, the interpretation of the peak vs. sum measures may tell us something about the relative importance of instantaneous vs. longer-duration deviations in the beat for detecting signals. Similarly, the slight variation we see across our selected window sizes suggests that conspicuousness is likely to vary based on how the electrosensory system samples the amplitude modulation. If chirp-detecting circuits are attuned to disruptions in the beat over short timescales, they would likely perceive chirps in a manner consistent with our shorter windows/peak conspicuousness measure. If, however, chirp-detecting circuits integrate beat structure over longer timescales, they are more likely to perceive chirps in a manner consistent with the longer windows/sum conspicuousness measure.

Chirps often cause a phase shift of the beat after the chirp relative to the phase of the beat before the chirp (see Fig 4 and the corresponding legend). We chose analysis window sizes that were typically on the same order of magnitude as the duration of most

chirps (i.e., 10-40 ms) so that this phase shift did not dominate the conspicuousness value. Our measure of conspicuousness thus focuses on disruption of the beat during the chirp itself, rather than a comparison of beat phase before vs. after the chirp. It is conceivable, however, that this phase shift could serve as a cue to detect the presence of the chirp that would not be well-represented in our conspicuousness measure. If this were so, chirp conspicuousness would be related to chirp properties in a complex way: i.e., small changes in chirp duration or FM would cause very large differences in phase shift/conspicuousness. For example, a small chirp of 14-ms and 60 Hz Gaussian frequency excursion might cause a 180º phase shift, whereas a 17-ms, 90 Hz chirp might cause no phase shift, and increasing chirp size/duration slightly more to 19 ms and 120 Hz chirp would again produce a 180º phase shift. If this phase shift determined how chirps were detected, we might predict that chirp parameters would cluster around those that maximized phase shift and avoid those that produced smaller phase shifts. Such a pattern is not apparent in the chirps of most species, which are largely continuously distributed in FM-duration space (Turner et al. 2007). Behavioral experiments that explicitly test detection of chirps that produce different phase shifts are needed to test whether the disruption of the beat during the chirp or the phase shift of the beat before vs. after the chirp are the critical factors in chirp detection.

In addition to shifting the phase relationship between the beat before vs. after the chirp, chirps themselves can occur at different phases of the beat. We found that chirp conspicuousness varied somewhat depending on beat phase at which the chirps occurred and that variation in chirp conspicuousness across beat phase was influenced by chirp and EOD parameters (Supplementary Fig. 2). This suggests that beat phase, and its

interaction with the structure of chirps and EODs, might influence the detectability of chirps. Findings that chirps are not produced preferentially at particular beat phases and that fish produce similar behavioral responses to chirps occurring at different beat phases suggest that phase might not affect chirp discrimination (Zupanc and Maler 1993; Walz et al. 2013; Aumentado-Amstrong et al, 2015; Metzen et al, 2016). Nevertheless, studies of responses to chirps at different beat phases have typically used quite conspicuous chirp stimuli and measured behavioral preferences rather than explicitly testing whether beat phase affects the sensitivity of the fish to detect the chirps. Behavioral and electrophysiological experiments designed to test chirp detection under more challenging conditions (e.g. subtle chirps on reduced contrast beats and/or in noisy backgrounds) are needed to more fully test whether beat phase influences the ability of fish to detect chirps.

4.3 Hybrid Chirps Reveal the Complex Interplay among EOD Waveform, Chirp Parameters, and DF

Synthesizing hybrid chirps from several species on conspecific and heterospecific EODs allowed us to systematically investigate how signal context influences chirp conspicuousness. Signal context for chirps is dependent on features not just of the chirps themselves but also of the interacting EODs that generate the beat. Our results show that these animals live in complex sensory environments, producing and perceiving signals whose conspicuousness is simultaneously influenced by species-typical chirp parameters, EOD waveform, and beat frequency. We saw species-specific effects of chirp parameters similar to the natural chirp conspicuousness analysis, indicating that species-typical chirps or chirp types that tend toward greater FM and longer duration are more

conspicuous in general and that chirps are usually more conspicuous on a slow beat (10 Hz) than a fast beat (100 Hz). We also saw an interesting effect of EOD waveform in that chirps were often equally conspicuous on the two quasi-sinusoidal EOD waveforms (*A. albifrons* and *A. leptorhynchus*) and the intermediate EOD waveform (*A. devenanzii*) but less conspicuous on the complex EOD waveform (*S. terminalis*). This is the case for all chirp types except *A. albifrons* and *A. leptorhynchus* small chirps, which show the opposite trend. *A. albifrons* and *A. leptorhynchus* small chirps were more conspicuous on the *S. terminalis* EOD waveform on the 10 Hz beat but not the 100 Hz beat. A likely explanation for this exception is based on the fact that the second harmonic of the *S. terminalis* beat has more power than the fundamental frequency, which effectively doubles the DF. Since P-units better encode (i.e., synchronize better to) small chirps as DF increases up to about 50 Hz, small chirps might be more conspicuous on the *S. terminalis* waveform because the distribution of power in the beat waveform makes this effectively more like a 20 Hz DF instead of a 10 Hz DF (Benda et al., 2006; Walz et al., 2014). The pattern of increased conspicuousness of the small chirp on the complex waveform beat disappears in the 100 Hz DF condition because in that case the DF for the *S. terminalis* beat is effectively 200 Hz, and small chirps are not encoded well on very high DFs.

4.4 Co-Adaptation of Chirps and EODs May Be Influenced By Sociality

Regardless of the strong EOD waveform effect, we did not find strong support for a strict co-adaptation of EOD waveform and chirp structure to maximize chirp conspicuousness on conspecific EODs. In other words, chirps were not always more

conspicuous on their own species' EODs. There could be other environmental factors that complicate signal perception and help explain why this is the case. For example, it seems likely that differences in sociality (e.g., degree of territoriality or gregariousness) may play a large role in determining how chirps and EODs are molded by evolution. Our model used two interacting EODs, but many highly social species are routinely found in lively social groups of a dozen or more individuals (Kramer et al., 1981; McNeil et al., 2014). The simultaneous interactions of all these EODs with each other and with the amplitude modulations created by the interactions of these EODs creates a beat structure that is far more complex than that of two interacting EODs. This extraordinarily complex sensory environment likely makes it even more difficult to detect chirps and to discriminate among them. Among territorial species, social interactions are unlikely to occur in the presence of more than one or two other EODs, which would make the extraction of relevant information a less complicated process. Thus, one possibility is that the relationship among chirp parameters, EOD waveform, DF, and conspicuousness is itself influenced by the presence of complex social beats (gregarious species) or more simple beats (territorial species). It is likely, then, that the features of chirps that make them more or less conspicuous are tailored to the unique signal environment (EOD waveform, DF, number of electrically signaling animals nearby) and may differ across physical and social environment. Additionally, the degree of aggression or level of competition for mates within the social structure of a species might optimize sensory processing to emphasize certain chirp types or chirp features. For example, rapid detection may be more important for aggressive chirps used to threaten attack or to appease an attacker, but other features might be useful for attracting or judging the

quality of a mate. One possible way to expand the model of chirp conspicuousness would be to include information about group size, species-typical EOD range, and territoriality in order to model naturally occurring signal contexts. This would allow us to gain more insight into how EODs, chirping, and sociality influence the evolution of electrocommunication across species.

4.5 Chirp Encoding Likely Varies With Species-Typical Chirp Properties

Although our measures of chirp conspicuousness reflect properties of chirps and beats that make the chirps embedded in the beat different from the beat alone (and thus potentially more detectable), the fishes' ability to actually detect chirps may depend both on the signal properties that we analyzed as well as on the environment (e.g. noise) and the sensory systems of receivers. Generally, our evidence suggests that the encoding of chirps by the electrosensory system is likely influenced by the features of the chirps themselves. Recent work in *A. leptorhynchus* shows that the encoding of different chirp types in the electroreceptive periphery is based on a pattern of synchronization (small chirps) or de-synchronization (big chirps) of firing across the population of electroreceptor cells (Benda et al., 2006). Small chirps on slow beats cause P-units to synchronize their firing and big chirps on fast beats cause P-unit firing to desynchronize, relative to the response of P-units to the beat alone. This pattern is thought to enhance the rapid detection of aggressive signals (small chirps on slow beats) while also allowing for the finer discrimination of signal parameters – and thus potentially signaler quality – during courtship signals (big chirps on fast beats; reviewed in Marsat et al., 2012). In the electrosensory lateral line lobe (ELL) of *A. leptorhynchus*, two distinct populations of

cells are responsible for encoding the two chirp types. E-type pyramidal cells encode small chirps on slow beats with a bursting code that is optimized for signal detection but does not allow fine discrimination. Conversely, big chirps are encoded with a graded, heterogeneous code by I-type pyramidal cells that preserve information about fine details of chirps (Marsat et al., 2012). Thus, in the one species in which the electroreceptive encoding of chirps is best studied, there is clear evidence of adaptation of specific sensory mechanisms that link signal structure to how signals are detected and how the information the signals convey is encoded. Importantly, recent evidence suggests that neurons in the midbrain may have invariant responses to chirps at differing beat phases and that phase differences in chirp presentation may not affect behavioral responses (Aumentado-Armstrong, 2015; Metzen et al., 2016). More information about higher-order processing and behavioral responses across species will elucidate whether beat phase in particular is an important factor in the perception of chirps among weakly electric fish.

The current study shows that variation in chirp and EOD parameters (waveform, DF) across species has a strong potential to influence how chirps are embedded in the beat. This raises the question of how and whether electrosensory systems of other apteronotid species might be adapted to species-level variation in chirp structure to optimize chirp detection and encoding of fine differences in chirp structure. Therefore, species with extreme or unusual EODs or chirps may have novel and interesting ways of encoding signals.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation IOS 0950721 (GTS) and IOS 1557935 (GTS, GM), National Institutes of Health T32HD049336 (Common Themes in Reproductive Diversity training grant, JMP), and the Center for the Integrative Study of Animal Behavior at Indiana University. This research was also supported in part by Lilly Endowment, Inc. through its support for the Indiana University Pervasive Technology Institute, and in part by the Indiana METACyt Initiative.

REFERENCES

Assad, C., Rasnow, B., Stoddard, P.K., 1999. Electric organ discharges and electric images during electrolocation. Journal of Experimental Biology 202, 1185-1193.

Aumentado-Armstrong, T., Metzen, M. G., Sproule, M. K. J., Chacron, M. J. 2015. Electrosensory midbrain neurons display feature invariant responses to natural communication stimuli. Plos Computational Biology 11:e1004430.

Bastian, J., Schniederjan, S., Nguyenkim, J., 2001. Arginine vasotocin modulates a sexually dimorphic communication behavior in the weakly electric fish Apteronotus leptorhynchus. Journal of Experimental Biology 204, 1909-1924.

Benda, J., Longtin, A., Maler, L. 2005. Spike-frequency adaptation separates transient communication signals from background oscillations. Journal of Neuroscience 25, 2312-2321.

Benda, J., Longtin, A., Maler, L., 2006. A synchronization-desynchronization code for natural communication signals. Neuron 52, 347–358.

Bullock, T.H., Hamstra, R.H., Scheich, H., 1972. Jamming avoidance response of high-frequency electric fish. 1. General features. Journal of Comparative Physiology 77, 1-22.

Denis, D., Blatrix, R., Fresneau, D., 2006. How an ant manages to display individual and colonial signals by using the same channel. Journal of Chemical Ecology 32, 1647-1661.

Dunlap, K.D., Larkins-Ford, J., 2003a. Diversity in the structure of electrocommunication signals within a genus of electric fish, Apteronotus. Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology 189, 153-161.

Dunlap, K.D., Larkins-Ford, J., 2003b. Production of aggressive electrocommunication signals to progressively realistic social stimuli in male Apteronotus leptorhynchus. Ethology 109, 243-258.

Fortune, E.S., Rose, G.J., Kawasaki, M., 2006. Encoding and processing biologically relevant temporal information in electrosensory systems. Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology 192, 625-635.

Fugère, V., Krahe, R., 2010. Electric signals and species recognition in the wave-type gymnotiform fish Apteronotus leptorhynchus. Journal of Experimental Biology 213, 225-236.

Gill, P., Woolley, S.M.N., Fremouw, T., Theunissen, F.E., 2008. What's that sound? Auditory area CLM encodes stimulus surprise, not intensity or intensity changes. Journal of Neurophysiology 99, 2809-2820.

Hagedorn, M., Heiligenberg, W., 1985. Court and spark - electric signals in the courtship and mating of gymnotid fish. Animal Behaviour 33, 254-265.

Hebets, E.A., Papaj, D.R., 2005. Complex signal function: developing a framework of testable hypotheses. Behavioral Ecology and Sociobiology 57, 197-214.

Heiligenberg, W., 1989. Coding and processing of electrosensory information in gymnotiform fish. Journal of Experimental Biology 146, 255-275.

Heiligenberg, W., Baker, C., Matsubara, J., 1978. Jamming avoidance-response in Eigenmannia revisited - structure of a neuronal democracy. Journal of Comparative Physiology 127, 267-286.

Higham, J.P., Hebets, E.A., 2013. An introduction to multimodal communication. Behavioral Ecology and Sociobiology 67, 1381-1388.

Ho, W.W., Fernandes, C.C., Alves-Gomes, J.A., Smith, G.T., 2010. Sex Differences in the Electrocommunication Signals of the Electric Fish Apteronotus bonapartii. Ethology 116, 1050-1064.

Ho, W.W., Turner, C.R., Formby, K.J., Smith, G.T., 2013. Sex differences in the electrocommunication signals of Sternarchogiton nattereri (Gymnotiformes: Apteronotidae). Journal of Ethology 31, 335-340.

Hopkins, C. D. 1976. Stimulus filtering and electroreception: Tuberous electroreceptors in three species of Gymnotid fish. Journal of Comparative Physiology. 111: 171-207

Hopkins, C.D., 1988. Neuroethology of electric communication. Annual Review of Neuroscience 11, 497-535.

Hupé, G.J., Lewis, J.E., Benda, J., 2008. The effect of difference frequency on electrocommunication: Chirp production and encoding in a species of weakly electric fish, Apteronotus leptorhynchus. Journal of Physiology-Paris 102, 164-172.

Kennedy, H.L., IEEE, 2007. A new statistical measure of signal similarity. Information Decision and Control, 112-117.

Kolodziejski, J.A., Nelson, B.S., Smith, G.T., 2005. Sex and species differences in neuromodulatory input to a premotor nucleus: A comparative study of substance P and communication behavior in weakly electric fish. Journal of Neurobiology 62, 299-315.

Kramer, B., 1999. Waveform discrimination, phase sensitivity and jamming avoidance in a wave-type electric fish. Journal of Experimental Biology 202, 1387-98.

Kramer, B., Kirschbaum, F., Markl, H., 1981. Species specificity of electric organ discharges in a sympatric group of gymnotoid fish from Manaus (Amazonas). Advances in Physiological Sciences 31, 195-217.

Kramer, B., Otto, B., 1988. Female discharges are more electrifying - spontaneous preference in the electric fish, Eigenmannia (Gymnotiformes, Teleostei). Behavioral Ecology and Sociobiology 23, 55-60.

Kramer, B., Otto, B., 1991. Wave-form discrimination in the electric fish Eigenmannia - Sensitivity for the phase differences between spectral components of a stimulus wave. Journal of Experimental Biology 159, 1-22.

Larimer, J.L., Macdonald, J.A., 1968. Sensory feedback from electroreceptors to electromotor pacemaker centers in gymnotids. American Journal of Physiology 214, 1253-1261.

Marsat, G., Longtin, A., Maler, L., 2012. Cellular and circuit properties supporting different sensory coding strategies in electric fish and other systems. Current Opinion in Neurobiology 22, 686–692.

McNeil, E.R., Skinner, M.A., Smith, G.T., 2014. Sociality, reproductive condition, and communication in a weakly electric fish. Integrative and Comparative Biology 54, E316.

Metzen, M. G., Hofmann, V., Chacron, M. J. 2016. Neural correlations enable invariant coding and perception of natural stimuli in weakly electric fish. eLife 2016;5:e12993

Meyer, J.H., Leong, M., Keller, C.H., 1987. Hormone-induced and maturational changes in electric organ discharges and electroreceptor tuning in the weakly electric fish Apteronotus. Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology 160, 385-394.

Moore, D., Liebig, J., 2010. Mixed messages: fertility signaling interferes with nestmate recognition in the monogynous ant Camponotus floridanus. Behavioral Ecology and Sociobiology 64, 1011-1018.

Nelson, M.E., Xu, Z., Payne, J.R., 1997. Characterization and modeling of P-type electrosensory afferent responses to amplitude modulations in a wave-type electric fish. Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology 181, 532-544.

Partan, S.R., Marler, P., 2005. Issues in the classification of multimodal communication signals. American Naturalist 166, 231-245.

Petzold, J.M., Smith, G.T., 2016. Androgens regulate sex differences in signaling but are not associated with male variation in morphology in the weakly electric fish Parapteronotus hasemani. Hormones and Behavior 78, 67-71.

Rose, G.J., 2004. Insights into neural mechanisms and evolution of behaviour from electric fish. Nature Reviews Neuroscience 5, 943-951.

Rowe, C., Guilford, T., 1999. The evolution of multimodal warning displays. Evolutionary Ecology 13, 655-671.

Scheich, H., 1977a. Neural basis of communication in high-frequency electric fish, Eigenmannia-virescens (jamming avoidance-response). 1. Open loop experiments and time domain concept of signal analysis. Journal of Comparative Physiology 113, 181-206.

Scheich, H., 1977b. Neural basis of communication in high-frequency electric fish, Eigenmannia-virescens (jamming avoidance-response). 2. Jammed electroreceptor neurons in lateral line nerve. Journal of Comparative Physiology 113, 207-227.

Scheich, H., Bullock, T. H., Hamstra, R. H. Jr. 1973. Coding properties of two classes of afferent nerve fibers: High frequency electroreceptors in the electric fish, Eigenmannia. Journal of Neurophysiology. 36: 39-60

Smith, A.A., Millar, J.G., Hanks, L.M., Suarez, A.V., 2013. A conserved fertility signal despite population variation in the cuticular chemical profile of the trap-jaw ant Odontomachus brunneus. Journal of Experimental Biology 216, 3917-3924.

Smith, G.T., 2013. Evolution and hormonal regulation of sex differences in the electrocommunication behavior of ghost knifefishes (Apteronotidae). Journal of Experimental Biology 216, 2421-2433.

Stöckl, A., Sinz, F., Benda, J., Grewe, J., 2014. Encoding of social signals in all three electrosensory pathways of Eigenmannia virescens. Journal of Neurophysiology 112, 2076-2091.

Turner, C.R., Derylo, M., de Santana, C.D., Alves-Gomes, J.A., Smith, G.T., 2007. Phylogenetic comparative analysis of electric communication signals in ghost knifefishes (Gymnotiformes : Apteronotidae). Journal of Experimental Biology 210, 4104-4122.

van Rossum, M.C.W., 2001. A novel spike distance. Neural Computation 13, 751-763.

Walz, H., Grewe, J., Benda, J., 2014. Static frequency tuning accounts for changes in neural synchrony evoked by transient communication signals. Journal of Neurophysiology 112, 752-765.

Walz, H., Hupé, G.J., Benda, J., Lewis, J.E., 2013. The neuroethology of electrocommunication: How signal background influences sensory encoding and behaviour in Apteronotus leptorhynchus. Journal of Physiology-Paris 107, 13-25.

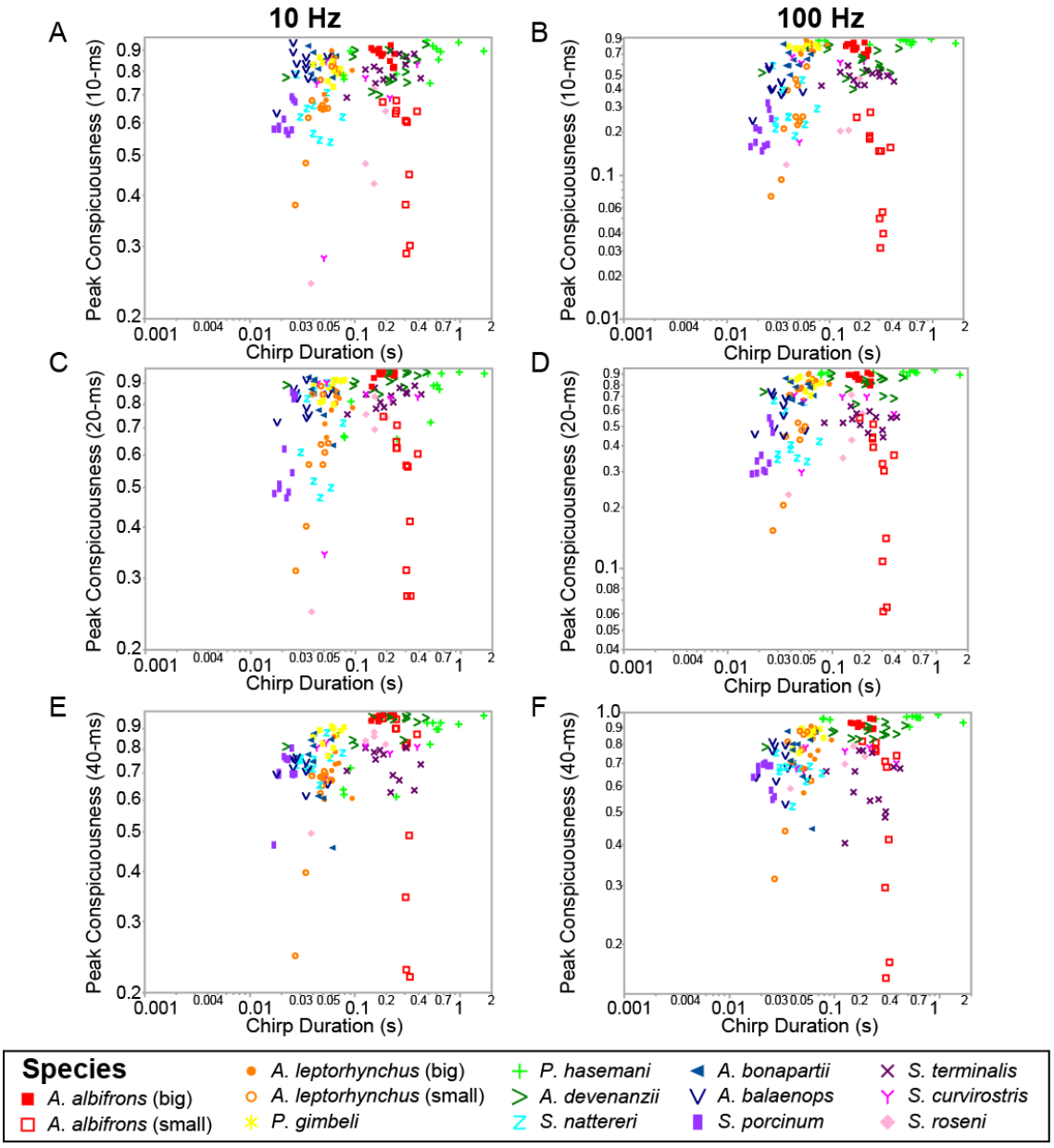
Watanabe, A., Takeda, K., 1963. The change of discharge frequency by A.C. stimulus in a weak electric fish. Journal of Experimental Biology 40, 57-66.

Zakon, H.H., 1988. The electroreceptors: Diversity in structure and function, in: J. Atema, Fay, R.R., Popper, A.N., & Tavolga, W.N. (Ed.), Sensory Biology of Aquatic Animals, 1st ed. Springer-Verlag, New York, pp. 813-850.

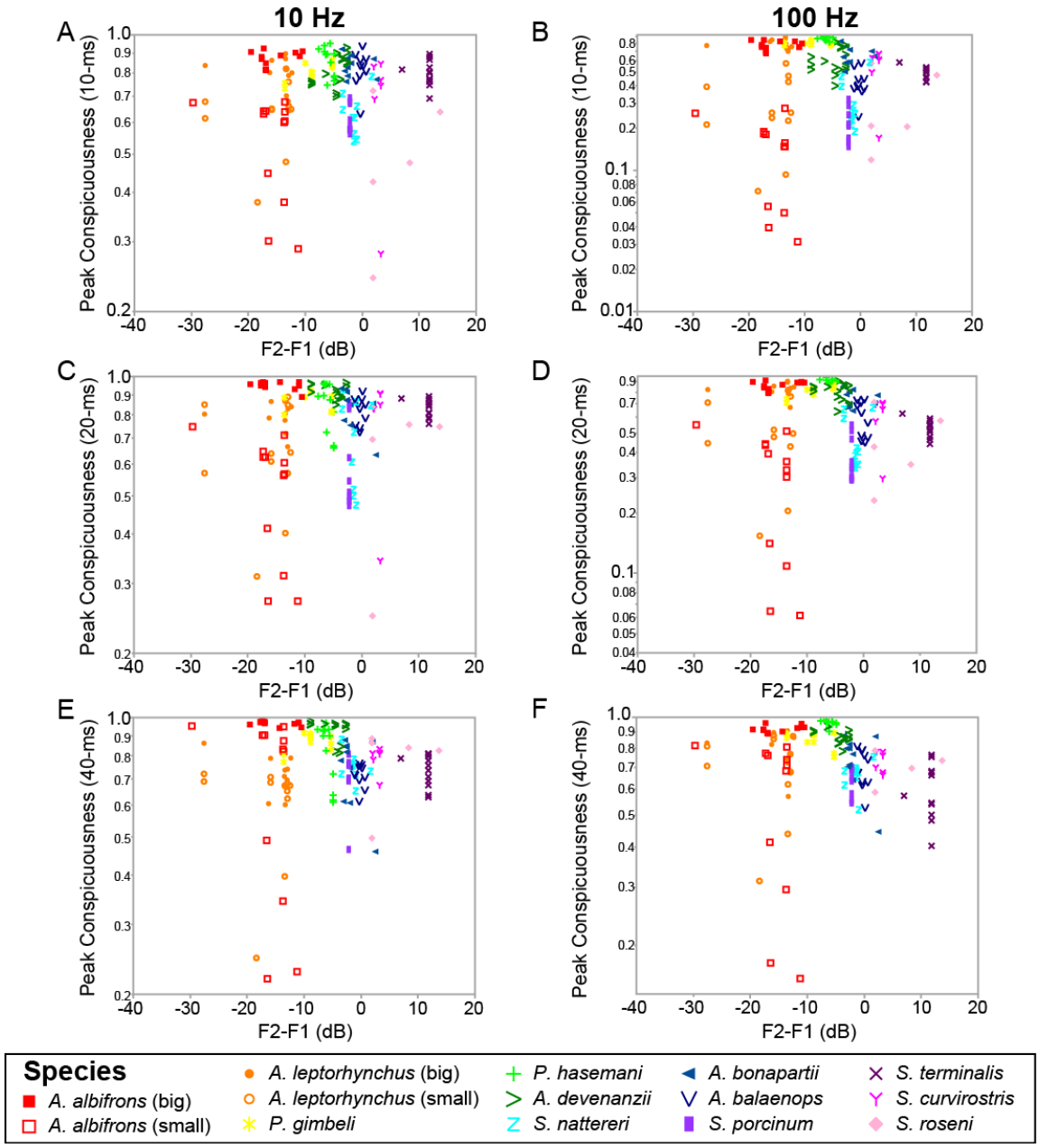
Zakon, H.H., Dunlap, K.D., 1999. Sex steroids and communication signals in electric fish: A tale of two species. Brain Behavior and Evolution 54, 61-69.

Zhou, M., Smith, G.T., 2006. Structure and sexual dimorphism of the electrocommunication signals of the weakly electric fish, Adontosternarchus devenanzii. Journal of Experimental Biology 209, 4809-4818.

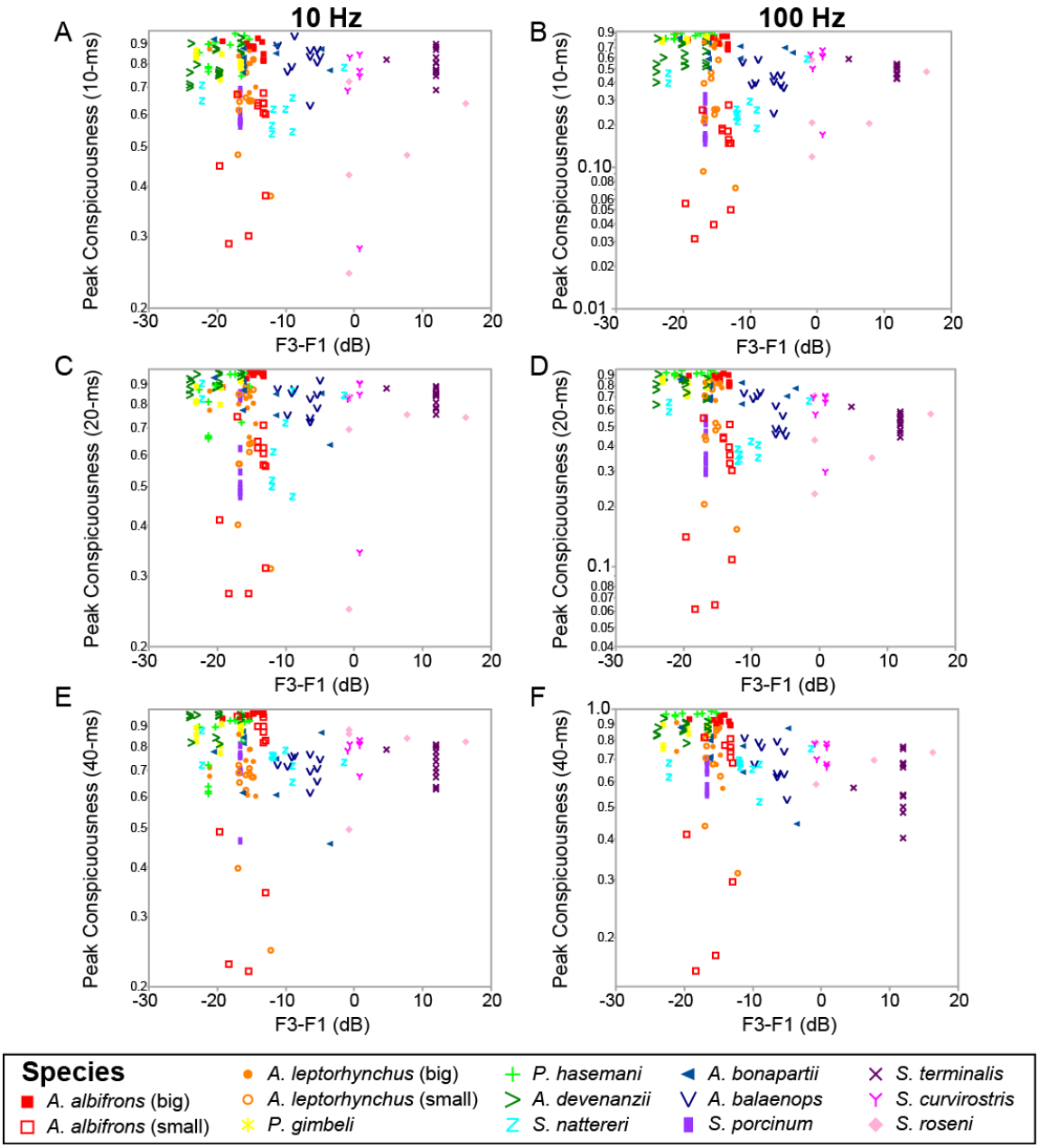
Zupanc G.K.H., & Maler L. 1993. Evoked chirping in the weakly electric fish Apteronotus leptorhynchus—a quantitative biophysical analysis. Canadian Journal of Zoology 71, 2301–2310.



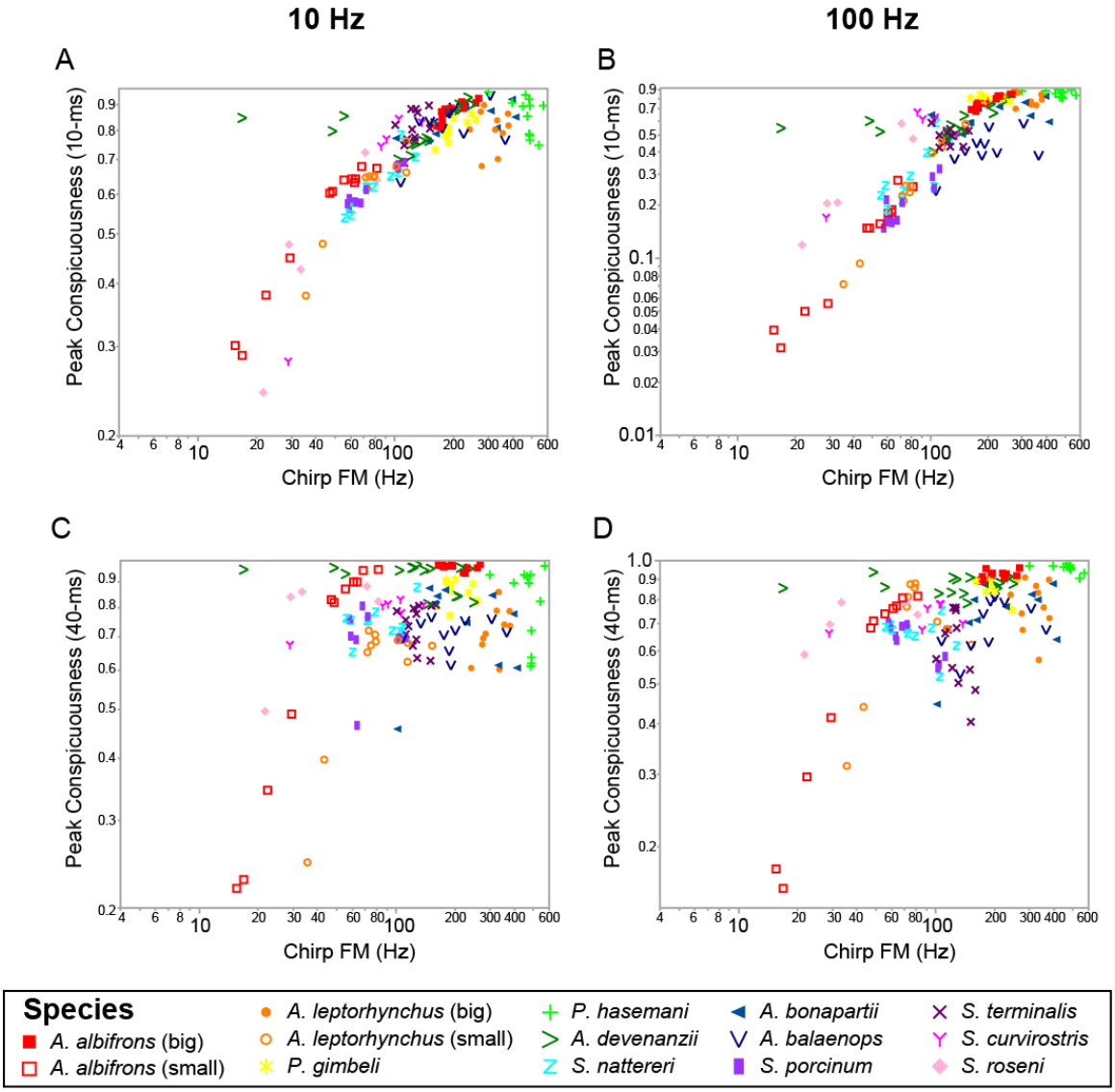
Supplementary Fig 1. Contribution of chirp duration to peak conspicuousness of natural chirps analyzed using a 10-ms window (A,B), a 20-ms window (C,D), and a 40-ms window (E,F) on 10 Hz beats (A,C,E) and 100 Hz beats (B,D,F). Chirp duration (log-transformed) was not strongly associated with peak conspicuousness when analyzed using either a 10-ms or 20-ms windows (partial correlations<0.1, p>0.30). Chirp duration was included in the stepwise regression model of peak conspicuousness using the 40-ms window, but the association was not statistically significant (partial correlation: 0.09, p=0.11). In general, longer duration chirps tended to be more conspicuous with the 40-ms window. The notable exception was the *A. albifrons* small chirps, which have moderately long durations but are relatively inconspicuous, perhaps because they have little FM.



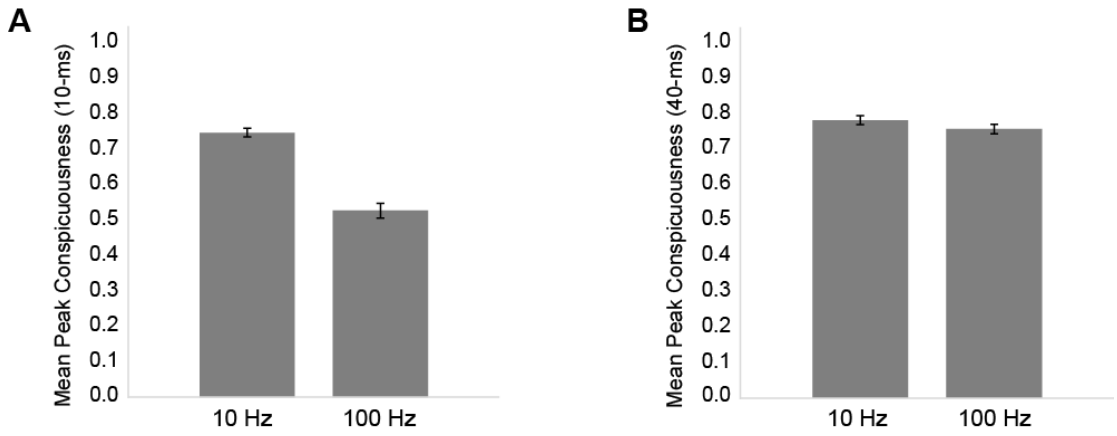
Supplementary Fig 2. Contribution of the waveform complexity variable F2-F1to peak chirp conspicuousness of natural chirps on 10 Hz beats (A,C,E) and 100 Hz beats (B,D,F) analyzed with the 10-ms (A,B), 20-ms (C,D), and 40-ms (E,F) windows. Unlike for sum chirp conspicuousness (Fig. 10), F2-F1 was not correlated with peak conspicuousness at any window size (10-ms partial correlation: 0.006, p=0.92; 20-ms partial correlation: -0.002, p=0.97; 40-ms partial correlation:-0.04, p=0.54).



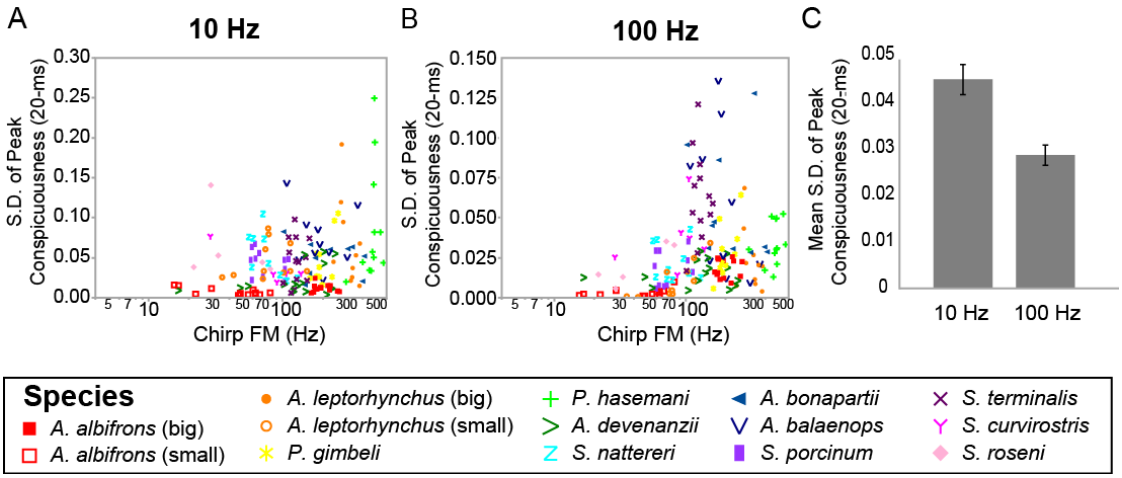
Supplementary Fig 3. Contribution of the waveform complexity variable F3-F1to peak chirp conspicuousness of natural chirps on 10 Hz beats (A,C,E) and 100 Hz beats (B,D,F) analyzed with the 10-ms (A,B), 20-ms (C,D), and 40-ms (E,F) windows. The waveform variable F3-F1 was not correlated with peak conspicuousness for the 10-ms and 20-ms window sizes (10-ms partial correlation: 0.08, p=0.17; 20-ms partial correlation: 0.005, p=0.94). However, F3-F1 was negatively correlated with peak conspicuousness at the largest window size (40-ms partial correlation: -0.16, p=0.006).



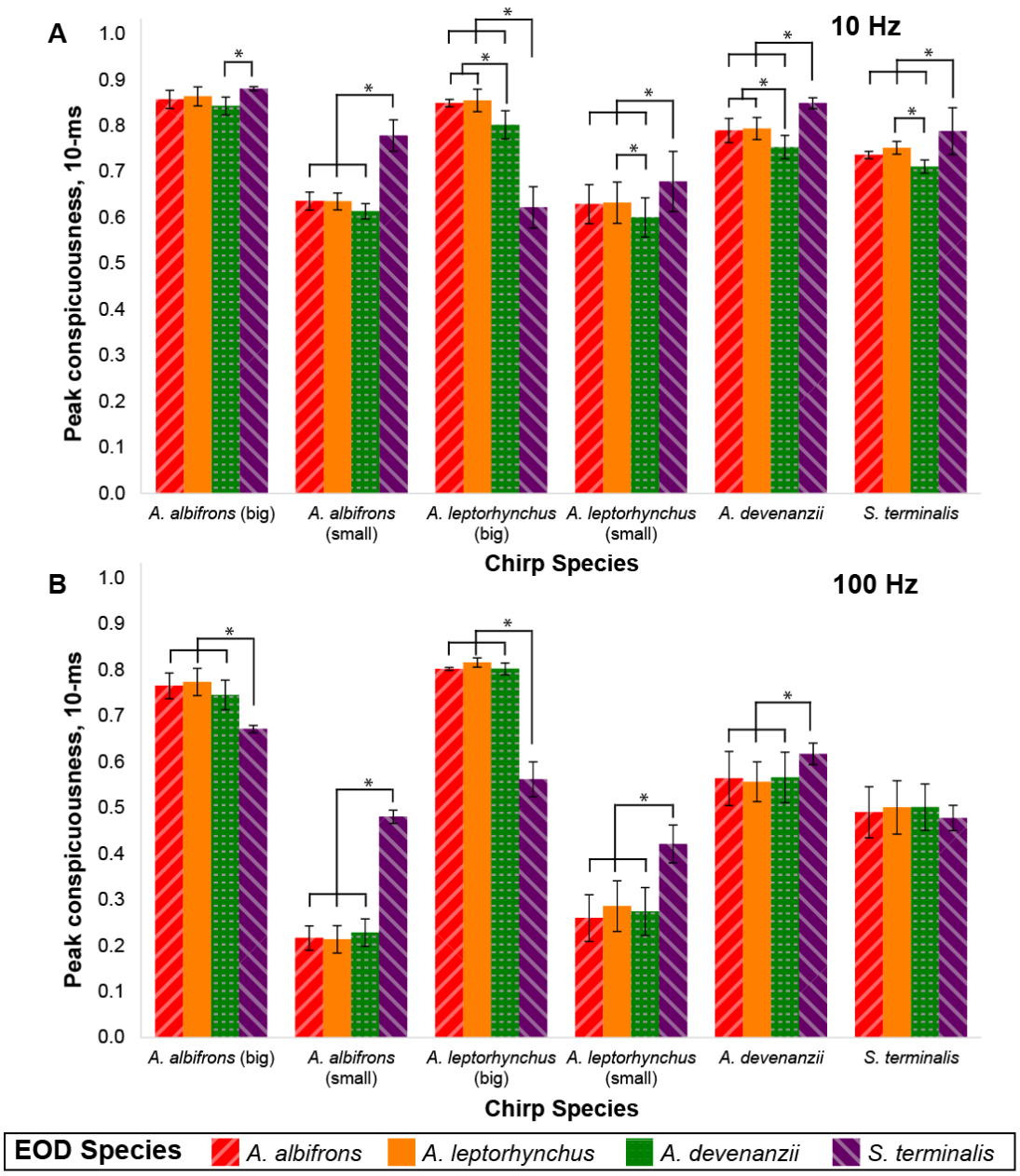
Supplementary Fig 4. Contribution of chirp FM to peak conspicuousness of natural chirps analyzed with a 10-ms window (A,B) and a 40-ms window (C,D) on 10 Hz beats (A,C) and 100 Hz beats (B,D). Chirp FM (log-transformed) was strongly associated with peak conspicuousness when using a 10-ms analysis window. Chirps with large frequency excursions were more conspicuous (partial correlation: 0.74, $p<0.0001$). A similar but somewhat less robust pattern emerges for the relationship between chirp FM and peak conspicuousness when using the 40-ms analysis window (partial correlation: 0.44, p $p<0.0001$).



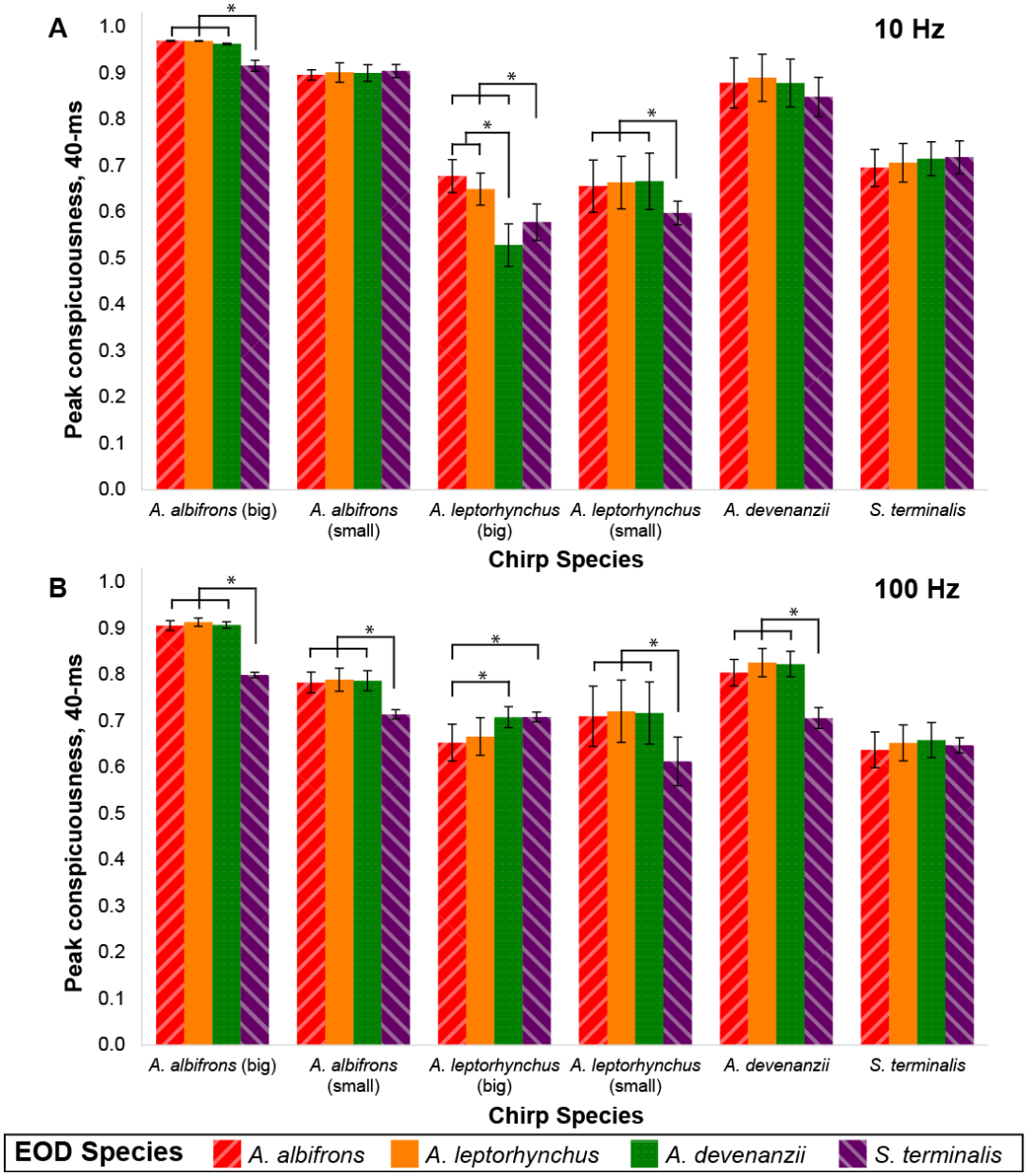
Supplementary Fig 5. Effect of DF on peak conspicuousness analyzed using a 10-ms window (A) or a 40-ms window (B). DF significantly affected peak conspicuousness when using the 10-ms window (partial correlation: -0.60, p<0. 001; 147 chirps). DF was included as a predictor in the 40-ms analysis but was not significant (partial correlation: -0.08, p=0.15; 147 chirps). Chirps tended to be more conspicuous on 10 Hz beats compared to 100 Hz beats. Error bars show one standard error from the mean.



Supplementary Fig 6. Contribution of chirp FM (A,B) to variation across phase. We calculated the standard deviation (S.D.) of the peak value (20-ms window) across four different phases of the beat in order to determine which chirp or EOD parameters have the greatest impact on variation in conspicuousness. Chirp FM (log-transformed) was the strongest predictor of peak conspicuousness S.D. (partial correlation: 0.39, p<0.001). Higher chirp FM led to more variation in chirp conspicuousness across phase. C) DF also influenced variability in chirp conspicuousness (partial correlation: -0.43, p<0.001; 147 chirps). Chirp conspicuousness varied more across phase on 10 Hz beats compared to 100 Hz beats. Error bars show one standard error from the mean.



Supplementary Fig 7. Peak conspicuousness of hybrid chirps on a A) 10 Hz beat and B) 100 Hz beat (10-ms window). Chirps from four species (including two different types of *A. leptorhynchus* and *A. albifrons* chirps, n=6 from each species/chirp type) were re-synthesized on the waveform of those four species, including the waveform of the species from which the chirp came. The bars are arranged from most sinusoidal (*A. albifrons*) to most complex (*S. terminalis*) EOD waveform. The chirps were combined with an EOD from the waveform donor species to measure peak conspicuousness. Asterisks indicate statistically significant differences ($p<0.05$, Fisher PLSD) between the conspicuousness of chirps on different species-specific EOD waveforms. Error bars show one standard error from the mean.



Supplementary Fig 8. Peak conspicuousness of hybrid chirps on a A) 10 Hz beat and B) 100 Hz beat (40-ms window). Chirps from four species (including two different types of *A. leptorhynchus* and *A. albifrons* chirps, n=6 from each species/chirp type) were re-synthesized on the waveform of four species, including the waveform of the species from which the chirp came. The bars are arranged from most sinusoidal (*A. albifrons*) to most complex (*S. terminalis*) EOD waveform. The chirps were combined with an EOD from the waveform donor species to measure peak conspicuousness. Asterisks indicate statistically significant differences ($p<0.05$, Fisher PLSD) between the conspicuousness of chirps on different species-specific EOD waveforms. Error bars show one standard error from the mean.

Chapter 5.

Conclusion

Encoding of EODs

The EOD is the simplest signal produced by electric fish and is the foundation upon which socially induced modulations occur. EOD frequency is a socially relevant signal parameter that continually conveys certain types of information (such as sex or dominance status) depending on species (reviewed in Smith, 2013). Species-typical EOD frequency ranges overlap substantially among species living in sympatry, which means that EOD frequency does not provide sufficient information for species recognition (Kramer et al., 1981). However, species recognition based on EOD alone may become more feasible when fish combine two or more properties of the EOD, such as frequency and waveform (Turner et al., 2007). Since all wave-type electric fish continuously produce an EOD, the EODs of other fish in the environment cannot be detected directly but instead must be encoded in the AM of interacting EODs. Fish encode the EOD frequency of others by comparing the timing of the zero-crossings of the combined EODs with the timing of the zero-crossings of the fish's own EOD across the beat's predictable changes in amplitude (Heiligenberg et al., 1978). My analysis shows that information about EOD waveform is also encoded in the shape of the beat but that the relationship between EOD waveform and AM waveform is not linear across the entire natural range of EOD waveforms. Thus, species may vary in their ability to perceive and use waveform information in intraspecific and interspecific interactions.

In Chapter 3, I showed that the EODs of *Distocyclus conirostris* span a relatively wide range of low frequencies. This frequency range (89-199 Hz) potentially presents a problem for explaining how these fish are able to detect the EOD frequency of conspecifics. EOD frequency determination has been elucidated using electric fish with higher EOD frequencies, most notably *Eigenmannia* and *Apteronotus leptorhynchus* (Heiligenberg et al., 1978; Scheich, 1977). Electrophysiological studies have shown that P-type tuberous receptors distributed across the fish's body encode the amplitude modulation (AM) created by the interaction of two or more fish (Bastian, 1986; Zakon, 1986). P-type electroreceptors fire probabilistically in response increasing signal amplitude. T-type electroreceptors, which are the other type of electroreceptors unique to weakly electric fish, encode the timing of zero-crossings as the voltage of electrical signals changes from negative to positive. To determine whether a social partner has an EOD higher or lower in frequency relative to their own, fish combine information from both P-type and T-type electroreceptors. If the zero-crossings of the AM lag behind the zero-crossings of the fish's EOD when the amplitude of the AM is rising, then the social partner has a higher EOD frequency. If the zero-crossings of the AM are advanced relative to the zero-crossings of the fish's EOD when the amplitude of the AM is rising, then the social partner has a lower EOD frequency (reviewed in Rose, 2004).

In species with high EOD frequencies such as the Apteronotidae, the P-type electroreceptors essentially function as low-pass filters by removing the high-frequency carrier (EOD) from the signal they encode. This is the same technique I used in Chapter 4 to extract the AM from the combined signals of two fish in a simulated social interaction. However, to avoid problems with aliasing, the cut-off frequency of the low-pass filter

must be no greater than approximately half the frequency of the carrier. This presents a potential problem for the ability of *D. conirostris* to encode the beat because the EOD (carrier) frequency for species with low frequency EODs is of the same order of magnitude as the difference in frequency between two EODs, and the AM is thus aliased on the low-frequency carrier. For example, even with the small sample size of *D. conirostris* presented here, the frequency difference between the fish with the lowest EOD frequency (89 Hz) and the fish with the highest EOD frequency (199 Hz) is 110 Hz. Thus, it would be impossible for the low-frequency fish (89 Hz) to determine the EOD frequency of the high-frequency fish using the P-type electroreceptor as a low-pass filter on the AM. For fish with high-frequency EODs such as *Apteronotus leptorhynchus*, issues caused by aliasing may occur much less frequently, perhaps only during interactions between a high-frequency male (1000 Hz) and a low-frequency female (600 Hz; Zakon & Dunlap, 1999). In this case, the difference frequency would be 400 Hz, which is encroaching upon the frequency range of the female's carrier signal but may still be sufficiently different to determine EOD frequency using the currently described neural mechanisms. Given that EOD frequency is sexually dimorphic in *Sternopygus*, another genus of electric fish with low-frequency EODs, we can assume that EOD frequency is probably still socially relevant for species with low EOD frequencies and thus must somehow be extracted by the nervous system (Hopkins, 1974). It appears likely, then, that *D. conirostris*, *Sternopygus*, and other species with low-frequency EODs use some alternative method of extracting EOD frequency information, perhaps by somehow directly encoding the EOD of a social partner. This potential evolution of alternative neural mechanisms for signal processing may not be too surprising when we consider the

diversity of neural mechanisms that have evolved to produce the signals themselves, including the different techniques for producing a jamming avoidance response that I discussed in Chapter 3. Similarly, the comparison of harmonic content in EODs and AMs across species that I presented in Chapter 4 shows that EOD waveform is not linearly encoded in the beat for all species. This suggests that different species may vary in how they signal and interpret the information content of the EOD. Thus, studying waveform perception in the nervous system and via behavior may prove fruitful in understanding how the EOD functions as a social signal.

Sexual Dimorphism in Chirping

All species of weakly electric knifefish studied to date show at least one sexually dimorphic signal feature. For example, in several species (most notably *A. leptorhynchus*, *A. albifrons*, and *Eigenmannia*), the sex of the signaler is communicated through hormonally mediated, sexually dimorphic EOD frequencies (Dunlap et al., 1998; Dunlap & Zakon, 1998; Meyer et al., 1987; Zakon & Dunlap, 1999). At least one species of *Eigenmannia* may also produce sexually dimorphic EOD waveforms (Kramer, 1985; Kramer, 1999). Although the majority of weakly electric knifefish species do not have sharply delineated sex differences in EOD parameters, across species there are numerous chirp parameters that differ between the sexes. These parameters include chirp duration, degree of chirp frequency modulation, rate of chirp production, and the presence or absence of multiple chirp frequency peaks (Ho et al., 2010; Ho et al., 2013; Kolodziejski et al., 2007; Petzold & Smith, 2016; Zhou & Smith, 2006).

The remote and challenging habits in which electric fish live has made characterizing their natural repertoire of behaviors exceedingly difficult. Across animal taxa, males generally incur greater costs in signaling because they face greater demands in acquiring mates and defending resources from same-sex conspecifics (reviewed in Magnhagen 1991; Zuk & Kolluru 1998). This is likely true for electric fish as well. Hagedorn & Heiligenberg (1985) used aquarium observations to describe a male chirping regime that appeared to help coordinate spawning in *Apteronotus leptorhychus*. Similarly, *Eigenmannia virescens* appeared to aggregate in dominance hierarchies in which one male dominated other males and gained exclusive mating opportunities in part through the production of courtship and aggressive signals (Hagedorn & Heiligenberg, 1985). Thus, we might expect that intra- and inter-sexual selection would boost the conspicuousness of male chirps relative to female chirps, leading to the sexual dimorphism we observe in many species (Smith, 2013). To test this hypothesis, I re-analyzed the chirp conspicuousness of four of the sexually dimorphic species described in Chapter 4 including *Apteronotus albifrons* (big and small chirps), *Apteronotus leptorhynchus* (big and small chirps), *Adontosternarchus devenanzii*, and *Parapteronotus hasemani*. The new analysis examined the effect of sex and difference frequency (DF; 10 Hz or 100 Hz) on conspicuousness using a two-way analysis of variance (ANOVA) for each species. For all species, I supplemented the dataset with additional female chirps to achieve a sample size of at least six chirps per sex for each species. The only exception was female *A. leptorhynchus* big chirps, which are extremely rare and thus could not be included due to the insufficient sample size.

In almost all of the species I examined, chirps produced by males were more conspicuous than chirps produced by females. There was no statistically significant interaction term between sex and DF for any species using either the peak or sum conspicuousness measures, which indicates that differences in conspicuousness between the sexes were similar on both the 10 Hz and 100 Hz beats. Only *A. leptorhynchus* chirps, which are extremely short, showed a sex difference using the peak measure, which is perhaps not surprisingly given the finding in Chapter 4 that greater chirp duration increases sum (but not peak) conspicuousness (Fig. 1; Table 1; Table 2). Although female *A. leptorhynchus* do occasionally produce big chirps, I was only able to include male *A. leptorhynchus* big chirps in my sample. Since primarily males produce big chirps and big chirps are conspicuous than small chirps (Chap. 4), it is likely that males have been selected to produce more conspicuous chirps. Additionally, male *A. leptorhynchus* small chirps were also more conspicuous than female *A. leptorhynchus* small chirps. Male *A. leptorhynchus* produce small chirps during aggressive interactions with same-sex conspecifics. They also produce a mix of big and small chirps during courtship. This could suggest that the evolution of more conspicuous male chirps among *A. leptorhynchus* may have been driven by both intra- and/or inter-sexual selection (Hagedorn & Heiligenberg, 1985; Bastian et al., 2001).

A. albifrons and *A. devenanzii* produce longer duration chirps than *A. leptorhynchus,* and these species exhibited significant sex differences in conspicuousness using the sum measure (Fig. 2). Male *A. devenanzii* produce complex chirps with multiple peaks in frequency modulation whereas female *A. devenanzii* chirps have single frequency peaks (Zhou & Smith, 2006). This is one interpretation for why male *A.*

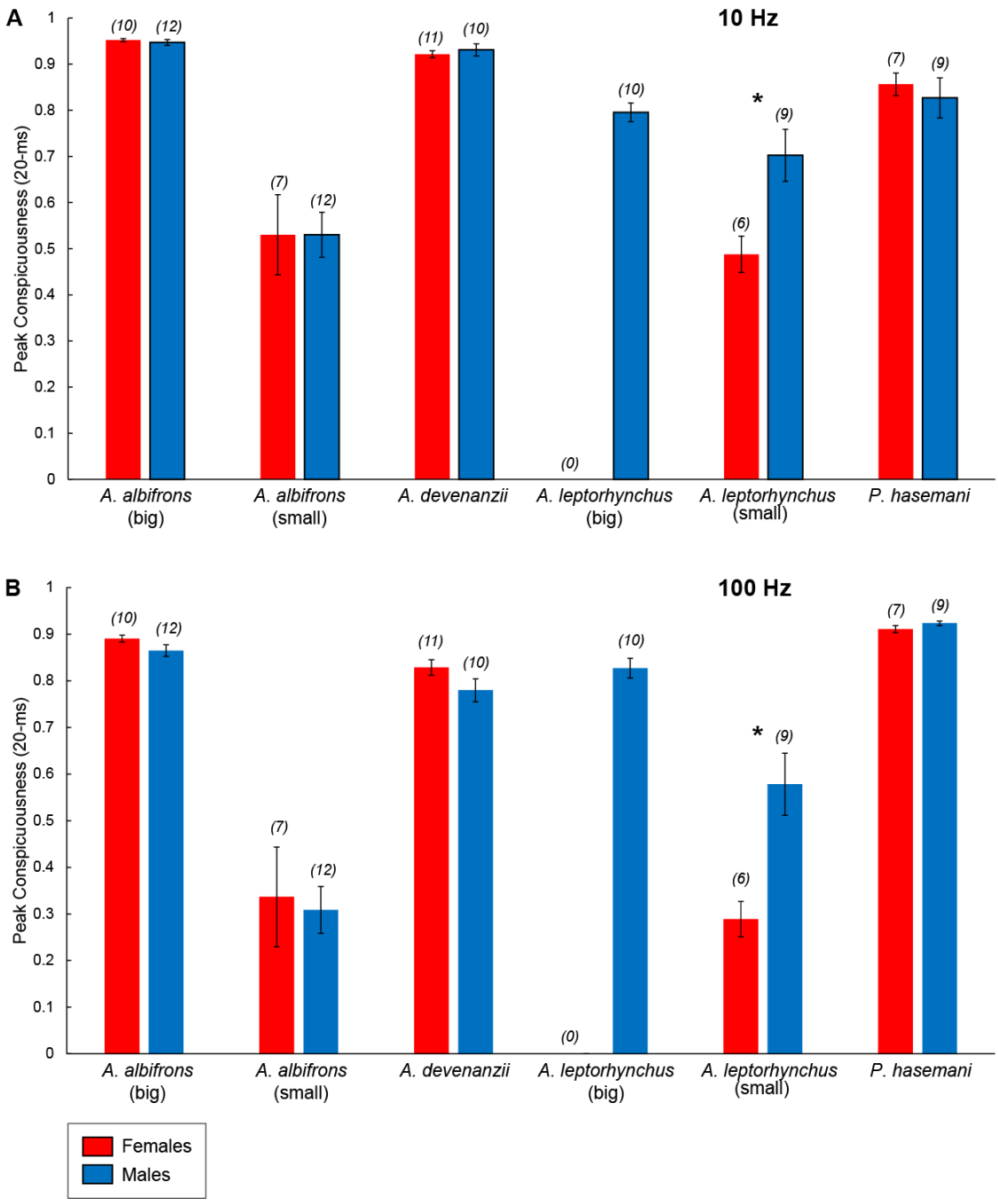


Fig. 1. Peak chirp conspicuousness of male and female chirps on A) 10 Hz and B) 100 Hz difference frequencies. Although all these species have some aspect of the chirping that is sexually dimorphic, only *A. leptorhynchus* showed a sex difference. Small chirps produced by males are more conspicuous than small chirps produced by females. Sample size is indicated above each bar.

Table 1. Peak conspicuousness ANOVA results

Species	degrees of freedom	F	p
A. devenanzii			
Sex	1	1.46	0.23
Difference Frequency	1	55.78	<0.0001*
Sex * Difference Frequency	1	3.18	0.08
A. albifrons (big)			
Sex	1	3.31	0.08
Difference Frequency	1	73.44	<0.0001*
Sex * Difference Frequency	1	1.51	0.23
A. albifrons (small)			
Sex	1	0.04	0.84
Difference Frequency	1	8.99	0.005*
Sex * Difference Frequency	1	0.04	0.84
A. leptorhynchus (small)			
Sex	1	18.59	0.0002*
Difference Frequency	1	7.65	0.01*
Sex * Difference Frequency	1	0.41	0.53
P. hasemani			
Sex	1	0.09	0.76
Difference Frequency	1	7.57	0.01*
Sex * Difference Frequency	1	0.59	0.45

* indicates a statistically significant difference, $p < 0.05$

Table 2. Sum conspicuousness ANOVA results

Species	degrees of freedom	F	p
A. devenanzii			
Sex	1	36.22	<0.0001*
Difference Frequency	1	0.80	0.38
Sex * Difference Frequency	1	0.26	0.61
A. albifrons (big)			
Sex	1	13.63	0.0007*
Difference Frequency	1	2.98	0.09
Sex * Difference Frequency	1	0.03	0.86
A. albifrons (small)			
Sex	1	4.91	0.03*
Difference Frequency	1	0.62	0.44
Sex * Difference Frequency	1	0.05	0.83
A. leptorhynchus (small)			
Sex	1	13.62	0.001*
Difference Frequency	1	1.53	0.23
Sex * Difference Frequency	1	0.04	0.83
P. hasemani			
Sex	1	1.93	0.18
Difference Frequency	1	8.71	0.006*
Sex * Difference Frequency	1	0.02	0.89

* indicates a statistically significant difference, $p < 0.05$

devenanzii chirps were more conspicuous than female chirps. Similarly, both big and small chirps produced by male *A. albifrons* had greater sum conspicuousness values than those produced by their female counterparts. However, male *P. hasemani* chirps were not more conspicuous than female *P. hasemani* chirps using either measure. This is particularly surprising given that male *P. hasemani* chirps last longer than female *P. hasemani* chirps (Chap. 2; Petzold & Smith, 2016) and duration is a significant predictor of sum conspicuousness (Chap. 4). A larger sample size of *P. hasemani* chirps could potentially help clarify whether there is indeed a difference in conspicuousness between the sexes.

Effect of Context on Chirp Encoding

As I have shown previously, chirp conspicuousness and the efficacy of signal transmission via chirping are affected by EOD context. Up until now, the effect of chirps on beat structure has only been studied in *A. leptorhynchus*, which produce somewhat unusual short duration chirps (reviewed in Walz et al., 2013). *A. leptorhynchus* is particularly interesting in the context of chirp/beat interactions because *A. leptorhynchus* EOD frequencies are sexually dimorphic. Male *A. leptorhynchus* have higher EOD frequencies (800-1000 Hz) than female *A. leptorhynchus* (600-750 Hz; Kirschbaum 1983; Meyer et al., 1987). This means that same-sex interactions generally occur on relatively slow beats, but opposite-sex interactions generally occur on relatively fast beats. The sensory system of *A. leptorhynchus* appears to be adapted to encode chirps in the appropriate context. At the level of the periphery, single-unit recordings show that electroreceptors (P-units) encode small chirps on slow beats by firing in synchrony

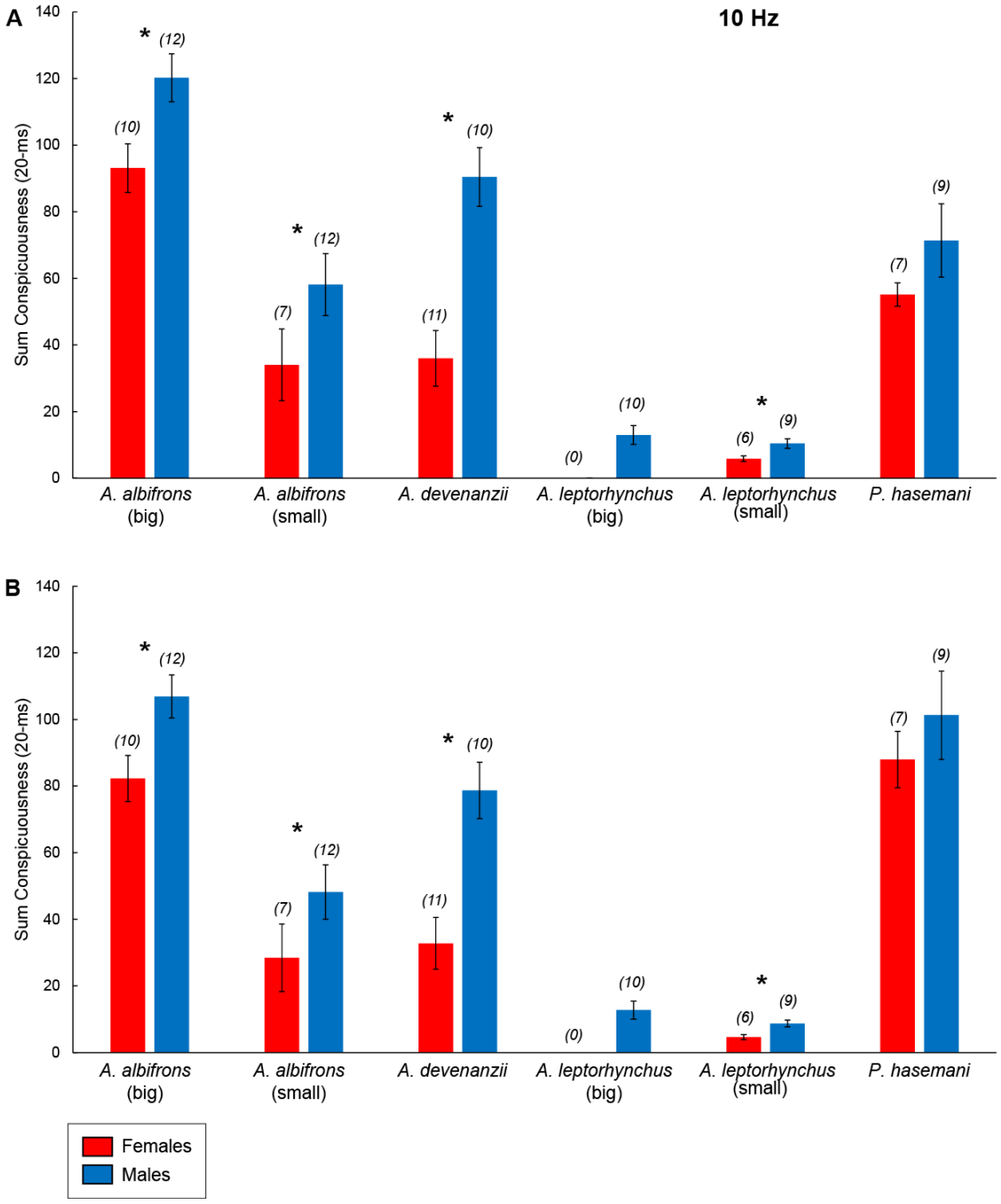


Fig. 2. Sum chirp conspicuousness of male and female chirps on A) 10 Hz and B) 100 Hz difference frequencies. When using the sum conspicuousness measure, nearly all species showed a sex difference. Without exception, the direction of the sex difference showed that chirps produced by males are more conspicuous than the chirps produced by females. Both male and female *P. hasemani* chirps were very conspicuous, but they did not differ significantly from each other. Sample size is indicated above each bar.

(Benda et al., 2006). This beat/chirp combination is the most likely context for intra-sexual aggression and probably enhances the fish's ability to quickly detect small chirps. P-units encode large chirps on fast beats by desynchronizing and again, given the sexually dimorphic EOD frequency of *A. leptorhynchus*, larger chirps typically occur on fast beats. Given that *A. albifrons* also produces sexually dimorphic EOD frequencies and both big and small chirps, we might expect a similar pattern of electroreceptor firing. However, both *A. albifrons* small and big chirps are much longer in duration than *A. leptorhynchus* small and big chirps, which changes how many cycles of the beat are disrupted by each chirp type. Disrupting multiple beat cycles would make both *A. albifrons* chirp types stand out more dramatically on both opposite-sex and same-sex beats (Walz et al., 2014). Additionally, male and female *A. albifrons* both produce big chirps more frequently to DFs that signify same-sex interactions than on DFs that signify opposite-sex interactions, which suggests that big chirps are not courtship signals as they are in *A. leptorhynchus* (Kolodziejski et al., 2007). Such nuance in chirp production and electroreception will likely require expanding our repertoire of electrophysiology subjects in order to understand species-level diversity in signaling.

The complexity of an animal's social and physical environment creates selective pressures for and against certain categories of signals. The specific characteristics of each environment affect the shape of the signals and, over time, differing selective pressures can lead to and/or reinforce speciation events (Grether et al., 2009; Gröning and Hochkirch, 2008). Ryan (1998) reviews potential mechanisms by which receiver biases might evolve. One possibility is that mate choice preferences for traits that indicate health or quality might transfer into preferences for related aspects of the phenotype.

Additionally, sexually selected complex signals in particular may evolve to reduce habituation to an otherwise repetitive signal. This seems particularly relevant for electrocommunication since all weakly electric knifefish continually produce an EOD. Modulations of the EOD (such as chirps) are the only way to disrupt the monotony of the signal and attract the attention of the receiver. Of course, communication signals are not the only source of input to an animal's sensory system, which means that the demands of sensing one type of information (finding prey items or detecting predators, for example) can alter how readily an animal perceives communication signals with a particular set of properties. The confluence of all these factors have shaped the communication signals across species and between sexes, which has led to the immense diversity that exists today. By studying the properties of signals and the social contexts in which animals produce them, we can generate predictions about perception, behavior, and sensory systems. This multi-pronged approach to understanding the complex nature of animal communication from all angles will enhance our understanding of how information is transmitted between and among organisms and how this information is used to enhance the fitness of the animals involved.

Social Complexity and Signal Transmission

One final consideration in the analysis of communication signals is the extent to which sociality affects signal transmission and detection. In my analysis of waveform information content and chirp conspicuousness (Chap. 4), I examined only interactions between two individuals. This was a necessary simplification to validate the effectiveness of the approach and because it allowed me to make equivalent comparisons across

species. However, weakly electric fish show wide variation in the degree to which they affiliate with conspecifics, including some species that are territorial and typically found alone and other species that preferentially clump into large social groups even when they could potentially occupy a larger space (Hopkins, 1974; McNeil, 2014; Stamper et al., 2010). We know from other animal communication systems that the number of signaling conspecifics can shift signal properties and the timing of signaling, which can alter receiver behaviors. For example, female grey treefrogs (*Hyla chrysoscelis*) respond more readily to conspecific mate-attraction signals when the signals are produced during brief reductions in chorus-like noise, but this response is apparently constrained by neural signal detection mechanisms that rely on temporal properties of the call (Vélez & Bee, 2011). Isolating and attending to social stimuli while filtering out irrelevant noise (including noise created by other social signals) is a substantial challenge for the receiver's sensory system. This challenge has been studied in many species including humans and has been referred to as the "cocktail party effect" (Aubin & Jouventin, 1998; Bee, 2008; Bee & Micheyl, 2008). Electric fish likely present an extreme case since all animals in the environment are continuously producing EODs. This constant barrage of signals would likely exert a strong selective pressure on the design of short-duration social signals (e.g., chirps) and on the electrosensory system's ability to filter context-relevant chirps from background EODs. Given the wide range of social contexts and group sizes in which these communication systems have evolved, it is likely that there are interesting differences across species in signal and sensory processes that will become clear only when territorial/affiliative behavior and group size can be taken into account.

References

Aubin, T., & Jouventin, P. (1998). Cocktail–party effect in king penguin colonies. *Proceedings of the Royal Society of London B: Biological Sciences, 265*(1406), 1665-1673.

Bastian, J. (1986). Electrolocation. In: *Electroreception,* edited by T. H. Bullock and W. Heiligenberg. New York: Wiley, 577-611.

Bastian, J., Schniederjan, S., & Nguyenkim, J. (2001). Arginine vasotocin modulates a sexually dimorphic communication behavior in the weakly electric fish Apteronotus leptorhynchus. *Journal of Experimental Biology, 204*(11), 1909-1924.

Bee, M. A. (2008). Finding a mate at a cocktail party: spatial release from masking improves acoustic mate recognition in grey treefrogs. *Animal Behaviour, 75*(5), 1781-1791.

Bee, M. A., & Micheyl, C. (2008). The cocktail party problem: what is it? How can it be solved? And why should animal behaviorists study it? *Journal of comparative psychology, 122*(3), 235.

Benda, J., Longtin, A., & Maler, L. (2006). A synchronization-desynchronization code for natural communication signals. *Neuron, 52*(2), 347–358. doi:10.1016/j.neuron.2006.08.008

Dunlap, K. D., Thomas, P., & Zakon, H. H. (1998). Diversity of sexual dimorphism in electrocommunication signals and its androgen regulation in a genus of electric fish, Apteronotus. *Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology, 183*(1), 77-86. doi:10.1007/s003590050236

Dunlap, K. D., & Zakon, H. H. (1998). Behavioral actions of androgens and androgen receptor expression in the electrocommunication system of an electric fish, Eigenmannia virescens. *Hormones and Behavior, 34*(1), 30-38. doi:10.1006/hbeh.1998.1460

Grether, G. F., Losin, N., Anderson, C. N., & Okamoto, K. (2009). The role of interspecific interference competition in character displacement and the evolution of competitor recognition. *Biological Reviews, 84*(4), 617-635. doi:10.1111/j.1469-185X.2009.00089.x

Gröning , J., & Hochkirch, A. (2008). Reproductive interference between animal species. *Quarterly Review of Biology, 83*(3), 257-282. doi:10.1086/590510

Hagedorn, M., & Heiligenberg, W. (1985). Court and spark - electric signals in the courtship and mating of gymnotid fish. *Animal Behaviour, 33*, 254-265.

doi:10.1016/s0003-3472(85)80139-1

Heiligenberg, W., Baker, C., & Matsubara, J. (1978). Jamming avoidance-response in Eigenmania revisited - structure of a neuronal democracy. *Journal of Comparative Physiology, 127*(3), 267-286.

Ho, W. W., Fernandes, C. C., Alves-Gomes, J. A., & Smith, G. T. (2010). Sex Differences in the Electrocommunication Signals of the Electric Fish Apteronotus bonapartii. *Ethology, 116*(11), 1050-1064. doi:10.1111/j.1439-0310.2010.01823.x

Ho, W. W., Turner, C. R., Formby, K. J., & Smith, G. T. (2013). Sex differences in the electrocommunication signals of Sternarchogiton nattereri (Gymnotiformes: Apteronotidae). *Journal of Ethology, 31*(3), 335-340. doi:10.1007/s10164-013-0382-0

Hopkins, C.D. (1974a). E*lectric communication in the reproductive behavior of Sternopygus macrurus* (Gymnotoidei). *Zeitschrift fur Tierpsychologie*, 35, 518-535.

Kirschbaum, F. (1983). Myogenic electric organ precedes the neurogenic organ in apteronotid fish. *Naturwissenschaften*, 70, 205-207.

Kolodziejski, J. A., Sanford, S. E., & Smith, G. T. (2007). Stimulus frequency differentially affects chirping in two species of weakly electric fish: implications for the evolution of signal structure and function. *Journal of Experimental Biology, 210*(14), 2501-2509. doi:10.1242/jeb.005272

Kramer, B. (1985). Jamming avoidance in the electric fish Eigenmannia - harmonic-analysis of sexually dimorphic waves. *Journal of Experimental Biology, 119*, 41-69.

Kramer, B. (1999). Waveform discrimination, phase sensitivity and jamming avoidance in a wave-type electric fish. *Journal of Experimental Biology, 202*(10).

Kramer, B., Kirschbaum, F., & Markl, H. (1981). Species specificity of electric organ discharges in a sympatric group of gymnotoid fish from Manaus (Amazonas). *Advances in Physiological Sciences, 31*, 195-217.

Magnhagen, C. (1991). Predation risk as a cost of reproduction. *Trends in Ecology & Evolution, 6*(6), 183-185. doi:10.1016/0169-5347(91)90210-o

McNeil, E.R., Skinner, M.A., Smith, G.T., 2014. Sociality, reproductive condition, and communication in a weakly electric fish. *Integrative and Comparative Biology 54*, E316.

Meyer, J. H., Leong, M., & Keller, C. H. (1987). Hormone-induced and maturational

changes in electric organ discharges and electroreceptor tuning in the weakly electric fish Apteronotus. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology, 160*(3), 385-394. doi:10.1007/bf00613028

Petzold, J. M., & Smith, G. T. (2016). Androgens regulate sex differences in signaling but are not associated with male variation in morphology in the weakly electric fish Parapteronotus hasemani. *Hormones and Behavior, 78*, 67-71. doi:10.1016/j.yhbeh.2015.10.014

Rose, G. J. (2004). Insights into neural mechanisms and evolution of behaviour from electric fish. *Nature Reviews Neuroscience, 5*(12). doi:10.1038/nrn1558

Ryan, M. J. (1998). Sexual selection, receiver biases, and the evolution of sex differences. *Science, 281*(5385), 1999-2003. doi:10.1126/science.281.5385.1999

Scheich, H. (1977). Neural basis of communication in high-frequency electric fish, Eigenmannia-virescens (jamming avoidance-response) .1. Open loop experiments and time domain concept of signal analysis. *Journal of Comparative Physiology, 113*(2), 181-206.

Smith, G. T. (2013). Evolution and hormonal regulation of sex differences in the electrocommunication behavior of ghost knifefishes (Apteronotidae). *Journal of Experimental Biology, 216*(13), 2421-2433. doi:10.1242/jeb.082933

Stamper, S. A., Carrera, E., Tan, E. W., Fugere, V., Krahe, R. and Fortune, E. S. (2010). Species differences in group size and electrosensory interference in weakly electric fishes: Implications for electrosensory processing. *Behavioural Brain Research* 207, 368-376.

Turner, C. R., Derylo, M., de Santana, C. D., Alves-Gomes, J. A., & Smith, G. T. (2007). Phylogenetic comparative analysis of electric communication signals in ghost knifefishes (Gymnotiformes : Apteronotidae). *Journal of Experimental Biology, 210*(23), 4104-4122. doi:10.1242/jeb.007930

Vélez, A., & Bee, M. A. (2011). Dip listening and the cocktail party problem in grey treefrogs: signal recognition in temporally fluctuating noise. *Animal Behaviour, 82*(6), 1319-1327.

Walz, H., Grewe, J., & Benda, J. (2014). Static frequency tuning accounts for changes in neural synchrony evoked by transient communication signals. *Journal of Neurophysiology, 112*(4), 752-765. doi:10.1152/jn.00576.2013

Walz, H., Hupe, G. J., Benda, J., & Lewis, J. E. (2013). The neuroethology of electrocommunication: How signal background influences sensory encoding and behaviour in Apteronotus leptorhynchus. *Journal of Physiology-Paris, 107*(1-2), 13-25. doi:10.1016/j.jphysparis.2012.07.001

Zakon, H.H. (1986). The electroreceptive periphery. In: *Electroreception*, edited by T. H. Bullock and W. Heiligenberg. New York: Wiley, 103-156.

Zakon, H. H., & Dunlap, K. D. (1999). Sex steroids and communication signals in electric fish: A tale of two species. *Brain Behavior and Evolution, 54*(1), 61-69. doi:10.1159/000006612

Zhou, M., & Smith, G. T. (2006). Structure and sexual dimorphism of the electrocommunication signals of the weakly electric fish, Adontosternarchus devenanzii. *Journal of Experimental Biology, 209*(23), 4809-4818. doi:10.1242/jeb.02579

Zuk, M., & Kolluru, G. R. (1998). Exploitation of sexual signals by predators and parasitoids. *Quarterly Review of Biology*, *73*(4), 415-438. doi:10.1086/420412

Chapter 6.

Teaching Authentic Scientific Communication Skills in an Undergraduate Laboratory Course

Jacquelyn M. Petzold[1] and Katherine Dowell Kearns[2]

Affiliations:
[1]Department of Biology, Indiana University
[2]Center for Innovative Teaching & Learning, Indiana University

"If we teach only the findings and products of science – no matter how useful and even inspiring they may be – without communicating its critical method, how can the average person possibly distinguish science from pseudoscience?"

Carl Sagan, *The Demon-Haunted World: Science As a Candle in the Dark*

Abstract

Laboratory courses are an integral part of undergraduate science education because they provide students with the ability to actively engage with scientific concepts. However, recently lab courses have been criticized for lacking scientific authenticity and thereby missing an opportunity to build students' critical thinking skills. We describe here a reimagining of an existing undergraduate biology lab to emphasize scientific experimentation and argumentation without substantially altering the closed-ended laboratory exercises themselves. Our approach is scalable and can be adapted to be taught alongside conventional sections of a lab course. We incorporated a flipped classroom approach into a weekly discussion session in order to engage students in a writing-workshop-style classroom environment. Students used scaffolded assignments and deliberate practice to learn about scientific communication and experimental design. We provide here example assignments and thoughts about how to adapt our approach to other contexts.

Introduction

Undergraduate students – even those with science-related majors – struggle to understand how scientific arguments are structured and how scientific arguments can be deconstructed and critiqued (Johnson & Pigliucci, 2004; Rowe et al., 2015; Walker et al., 2002). Recent analyses of biology education at the postsecondary level highlight the critical importance of teaching scientific inquiry in order to give students the skills to understand the world and to make informed decisions about their place within it (AAAS, 2011). These skills are crucial for science majors and non-majors alike since all citizens must make decisions about food, health, energy, and the environment. Given the rapid and accelerating pace at which scientific data is produced, it is becoming increasingly important to balance teaching scientific content matter with teaching scientific inquiry skills. Obviously, these two aims are not mutually exclusive, since scientific inquiry is guided by scientific subject matter and enhances students' ability to assimilate and synthesize new information. By learning how to access primary science sources and to evaluate the evidence support scientific claims, students are better-equipped to gain meaningful, deep subject knowledge about the biological topics that are interesting or relevant to them and to see learning about science as a life-long, self-initiated pursuit.

Laboratory classes are a staple of undergraduate science education because they are an ideal venue for exposing students to science as the process by which principles about the world are elucidated. Implicit in the rationale behind the development of laboratory courses is the assumption that exposure to the tools and techniques of science in a laboratory setting will necessarily translate into a deeper understanding of the scientific method. Yet this may not always be the case (Alberts, 2005; Handelsman et al.,

2004; Kuhn, 1993). Undergraduate laboratory courses in particular have been increasingly criticized for relying on closed-ended "cookbook" exercises that do not allow students to generate and analyze real data. Even the most elegantly designed and eloquently explained closed-ended laboratory exercises take away the opportunity for students to grapple with experimental design in a way that parallels real-life scientific research. The prescriptive steps in closed-ended labs can often be followed with little regard for the underlying meaning or scientific principles, which reduces the potential impact of labs as a means by which lecture concepts are reinforced. Additionally, closed-ended experiments are designed to produce data that gives students a pre-determined "correct" answer, and students who understand the theoretical subject matter generally know what data they are "supposed" to produce. Although this practice may streamline data analysis and interpretation, such laboratory exercises deprive students of the chance to mentally wrangle with the maddening ambiguity of real experimental data and to formulate conclusions based solely on their interpretation of those data.

Many instructors in the sciences have successfully responded to these criticisms by developing novel, engaging, open-ended laboratory exercises that are driven by student-developed hypotheses and experimental designs (Brownell et al., 2012; Goldey et al., 2012; Sundberg et al., 2005). These types of large-scale revisions to the laboratory notebook may not always be feasible for a variety of practical, financial, or institutional reasons, but smaller-scale interventions can have meaningful changes in students' attitudes and skills and can potentially motivate larger-scale course revisions. Many of the most successful non-overhaul approaches involve engaging students with the scientific literature. For example, Van Lacum and colleagues (2014) describe a teaching

strategy that highlights the common features of the scientific literature as a genre. This approach helped students decode the structure of scientific arguments by deconstructing the "rhetorical moves" made by the authors of primary articles. Such focused analyses of otherwise opaque and jargon-filled scientific writing helped students concentrate on commonalities of scientific argumentation, including anticipating and defusing potential critiques and rebuttals. The emphasis on structure demonstrated that scientific writing has a defined purpose, a predictable format, and a specific set of rhetorical techniques that can be applied in the students' own writing (Deiner et al., 2012).

In this article, we (a biology graduate teaching assistant and an instructional consultant) describe a course revision that fosters intellectual engagement in a laboratory course without substantially modifying the existing laboratory notebook. We show how scaffolding writing and experimental design exercises gives students a deeper understanding of how scientific arguments are structured and how scientific evidence is used to support claims.

Aims

We focused on three main goals for the semester:

1. Formulating and deconstructing scientific arguments
2. Designing logical experiments, with an emphasis on hypotheses and controls
3. Analyzing and presenting data using basic statistical and graphing skills

Course Context

This study took place during fall semester of 2015 and spring semester of 2016 at a large research university in the Midwestern United States. All procedures were approved by the Indiana University Institutional Review Board. The course we targeted was BIOL-L113, which is an introductory biology laboratory course with no required co-requisite lecture component. Students are required to either have completed or be in enrolled in one of the two introductory biology courses for majors, although there is minimal coordination among the topics in the lecture course and the laboratory. The laboratory course is coordinated by two experienced biology instructors who are unaffiliated with the lecture course. These biology instructors wrote the laboratory curriculum and mentor approximately twenty-five teaching assistants (TAs) who are responsible for the day-to-day laboratory teaching duties. Each section of the laboratory is run by two TAs: a head TA who supervises the three-hour laboratory period and teaches the weekly fifty-minute discussion section, and an assisting TA who also helps monitor the three-hour laboratory period. The laboratory is a survey of classic biology techniques (pipetting, chromatography, spectrophotometry, etc.) and concepts (photosynthesis, genetics/mutation, animal behavior, etc.). TAs are traditionally encouraged to use the fifty-minute weekly discussion section to provide a mini-lecture or refresher tutorial on the week's lab topic. Although the course is intended as an introduction to laboratory skills in the life sciences, the enrollment typically includes a broad swath of undergraduate students at all levels, based on factors such as the competitiveness of securing a spot in the course and personal preference or procrastination in registering for it.

Independent Project

The culminating experience of the L113 laboratory is the Independent Project, which was originally conceptualized as a student-designed modification of a previous lab exercise but has since broadened to include experiments on any biological topic, provided that the course budget and departmental resources can supply the necessary materials and equipment. The Independent Project is a unique opportunity for undergraduate students to engage deeply with a scientific question that intrigues them. It also serves as a vehicle to learn about scientific research, from generating hypotheses to communicating results. However, previous experience with the course suggested to us that students in the class often did not understand how to develop and test a scientific question. We decided to focus on using the scientific literature to help students draw connections between the writing they were doing about the laboratory exercises ("lab reports") and the writing that is central to how scientists communicate with each other. Through this, we hoped to increase students' argumentation skills while also enhancing their ability to find and assimilate new scientific knowledge.

Teaching Authentic Scientific Communication

We sought to improve students' scientific reasoning and writing skills in a mostly closed-ended laboratory course without changing the laboratory exercises themselves. To do this, we shifted the emphasis of class time during the weekly fifty-minute discussion period from lecturing about biology content to a more collaborative workshop format. The "flipped" approach to instruction has proven useful in a variety of educational contexts because it allows students to practice new skills in class with the support of their

instructor and peers. More passive forms of information delivery are then shifted to time outside of class (reviewed in Rotellar & Cain, 2016 and Betihavas et al., 2016). Before the beginning of the semester, we curated a collection of resources on all the major topics for the lab, with a special emphasis on interesting videos and interactive online activities. We posted these resources to the course's learning management system, organized chronologically by lab manual exercise.

Students entering the class likely had certain expectations about how laboratory courses "ought" to be taught based on previous experience. Thus, we felt it was critical to be transparent about the changes we were making and how our expectations would change their day-to-day experience in the class. On the first day of the discussion section, the students were told about the flipped format during a larger discussion about course goals that emphasized the central theme surrounding practicing scientific communication and learning about science as a process. Because so much of what we were teaching in the course would emphasize the importance of using reliable evidence to support decision-making, we briefly shared with students some of the research that supports innovative teaching and learning strategies in the development of scientific expertise (e.g., Armstrong et al., 2008; Ericcson et al., 1993; Knight & Wood, 2005; Rowe et al., 2015). Students were encouraged to regularly read the explanatory material in the lab manual, to use the online resources, and to ask questions whenever they needed more information. We hoped to enhance students' meta-cognitive abilities (i.e., monitoring their own knowledge base) in order to make them less reliant on passive instructor-driven information transfer.

Starting with the first week, each fifty-minute discussion period was divided into three sections: a five- to ten-minute introduction in which the instructor explained a new concept, gave brief instructions, and/or reminded students about upcoming deadlines, a thirty-five or forty-minute "workshop" time in which students worked in pairs or groups on a structured activity, and a five- to ten-minute wrap-up in which students discussed the work they had just completed, asked questions, or received follow-up information. The goal of the workshop time was for students to develop or practice a skill with the expectation that the activity could be completed during class time and would not become homework. Scaffolding the in-class activities allowed students to attempt to master the same skill or task repeatedly, with increasingly more difficult material and decreasing levels of guidance and direction.

Examples of In-Class Activities

Below we provide some representative examples of the types of assignments students received in class during the discussion sessions:

Basic structure of a primary article

Read an assigned short (one-page) primary article that does not have subheadings (e.g., Greene & Gordon, 2003). Assign each paragraph to the section in which it would appear in a lab report or a longer-format primary article (Abstract, Introduction, Methods, Results, and Discussion). Identify the controls (explicitly stated in the article) and attempt to put into words the purpose of each control (i.e., what comparisons the controls allow the researchers to make). Write a concise abstract for the article.

Experimental design using everyday ideas

Decide with your group how you would empirically test a superstition or "old wives tale" from a list provided in class. Consider which variable(s) to manipulate, how many test subjects/groups you would need, and whether each experiment you are proposing is ethical. This assignment is adapted from Hoefnagels (2003) and is particularly effective if students attempt to design their experiment before the instructor offers any instruction or pointers about experimental design.

Deconstructing a scientific paper

Examine the rationale behind two or more experiments presented in a longer primary article (e.g., Berdoy et al., 2002). Interpret graphs and speculate as to why the authors presented the data the way they did. Before reading the Discussion section, put the provided ideas in an order that you believe starts from the most specific and moves to the most general interpretation of the data. This assignment is a single pdf that contains the primary article interspersed with the relevant questions for each section. Some of the figure captions were deleted so that students would have the chance to practice interpreting the graphs.

Issues in data visualization

During this class, the entire class was presented with a sequence of puzzling or poorly designed graphs (abundantly available on the internet). Student were given time to look at the graphs and to jot down some criticisms before discussing each graph as a class. Some

students were quick to point out that certain graphs weren't necessarily "wrong," which led to an enlightening conversation about how the choices made by people when creating data visualizations can cause readers to draw particular (and sometimes erroneous) conclusions. This showed that sometimes there are multiple graph formats that can work for a particular dataset but that some choices might be better or more informative than others. In this class, students also read an article with tips for interpreting scientific claims (Sutherland et al., 2013) and answered a few questions to explain their thoughts on evaluating evidence.

Independent project planning

One major goal of building students' skills and confidence with scientific communication, experimental design, and data analysis across the semester was to better position them to take advantage of the Independent Project. By having a more comprehensive understanding of how science is conceptualized and communicated, we hoped that students would expand upon a personal interest in biology or to answer a question they found intriguing, rather than choose an experiment more or less at random. To facilitate this, we built in several assignments to lead up to the formal Independent Project proposal, most notably a brainstorming document to use while perusing the scientific literature in class and a final assignment right before beginning the Independent Project that ensured students could explicitly state their hypothesis and the variables they intended to manipulate and measure. For undergraduate students, one potential roadblock to designing inventive experiments is the fear that the experiment will "fail" in some way and thus have a detrimental impact on their grades. We attempted to ameliorate this in

two ways: 1) by explicitly and repeatedly explaining that the experimental design of the independent project would be graded on whether it was a logical test of the hypothesis, not whether it produced the expected results, and 2) by incorporating a "creativity" item into the grading rubric to reward students for taking the time to develop an interesting experiment. This seemed to alleviate some of the students' anxieties about whether their experiment would work the way they anticipated. Describing real-life "failures" in scientific research also helped allay some of these concerns. Student completed their Independent Projects over a two-week period in the laboratory classroom, with the guidance and supervision of the two TAs. Once students collected their data, they wrote a formal lab report (due approximately two weeks later) and gave a 10-minute presentation on their results during the last week of class.

Assessment of Student Learning

We recruited three experienced graduate-level writing center tutors to serve as raters on the Independent Project lab reports that were written by the students during both semesters that we taught the modified course. Before scoring, the three raters attended an hour-long norming session in which they used sample lab reports not included in the study to learn how to consistently apply the rubric. The scoring rubric can be found in Appendix A. Raters were given approximately two weeks to read and score the lab reports. Each lab report was read by all three raters. The raters assigned a numerical value for each rubric category to the lab reports, where 1= "Not Great," 2= "Okay," and 3= "Great." For each student, the three scores from the three raters were averaged to give one score in each rubric category. These scores are presented in Table 1.

Rubric Scoring Reflection

Across both semesters, the rubric scores were fairly high in each category. There are no glaring deficits in performance in any of the areas we measured. Scores were similar across semesters, which could suggest that the two populations of students had similar experiences with the flipped class format. The rubric scores for hypothesis formulation were particularly high. This was likely because students were prescribed a specific formula for hypothesis-writing, which was to state two (or more) clearly defined variables and describe the predicted relationship between/among them. Dirrigl & Noe (2014) note that giving students these types of fill-in-the-blank template statements is one scaffolding method that shows students how to use language to form arguments that will be transferrable to other future scientific writing tasks. Additionally, stating a hypothesis for an experiment, either one described in the literature or one designed by the student, was an activity that students engaged in repeatedly across the semester.

Table 1. Mean rubric scores across both semesters (1= "Not Great," 2= "Good," 3= "Great).

Rubric Item	Fall 2015 Scores	Spring 2016 Scores
Abstract	2.1	2.0
Introduction	2.2	2.3
Discussion	2.0	2.1
Hypothesis	2.5	2.4
Authentic Use of Scientific Literature	2.0	2.1

Instructor Reflections

Perhaps the largest hurdle in considering the shift from an instructor-focused, lecture-driven classroom to a student-focused, activity-driven classroom is the loss both of strict instructor control and of day-to-day predictability in the classroom. Yet in our experience, this spontaneity also became one of the greatest rewards. By giving students time to think about their projects and explore the scientific literature during the discussion section, students were much more likely to spontaneously ask questions or pitch ideas to each other and to the instructor. These lively dialogues would never have happened if each student planned their Independent Project by themselves outside of class. This more conversational approach allowed the instructor to express enthusiasm or offer suggestions in a personalized way that would not otherwise be appropriate for a lecture setting.

Although we did not explicitly measure students' biology content knowledge in any of the areas covered by the closed-ended activities in the lab manual, our sense of the general quality of the non-Independent Project lab reports suggests that students did not suffer noticeable deficits in understanding the basic principles that would have otherwise been covered in the discussion through lectures. Additionally, by charging each students with the responsibility of monitoring their own understanding of the scientific content areas, they were empowered to ask each other and the instructor about the concepts they did not understand, which often led to more dynamic and individually tailored informal instruction experiences before/after class or during the periodic lulls in the lab procedures. Presumably some students also spent more time reading the introductory

material for each activity in the lab manual because they did not have the expectation that the information would be fed to them in a lecture format. Future iterations of this instructional approach could measure outside preparation more explicitly.

One of the more frustrating responses from this experience was that some students still expressed dissatisfaction with doing "busy work" during the discussion sections. Even though we tried to be transparent from the first day of class about why we thought it was beneficial for students to practice designing experiments and writing about science in class, we perhaps could have found other ways to make this unifying theme more explicit. Regardless, it is expected that some students would resist a change in the status quo, and teaching to student preferences does not always translate into greater learning gains or richer educational experiences.

Conclusions

Students can learn higher-order scientific inquiry skills with minimally invasive changes to a standard undergraduate introductory laboratory course. Shifting the emphasis from scientific principles to scientific philosophy allows students to expand their perspective on what constitutes a scientific "fact" and how such "facts" are discovered, refined, and sometimes supplanted by future investigations. We believe this shift could be helpful for other laboratory courses, particularly those that do not have strong topical ties to a specific lecture course. However, we suggest that all students can benefit from more authentic, scaffolded assignments that allow them to see the connections and rationale that are abundantly apparent to experts. Students who engage with laboratory courses that guide them through the process of designing experiments,

interpreting data, and writing about their results will be better positioned to be critical and savvy consumers of scientific information through graduation and beyond.

Acknowledgments

Thanks to Aviva Hakanoglu, Colleen Pawlicki, and Nancy Smith for scoring student lab reports. Thanks also to Leslie Drane, John Paul Kanwit, Keely Cassidy Stueve, and Jo Ann Vogt for administrative support.

References

Alberts, B. (2005). A wakeup call for science faculty. *Cell, 123*(5), 739-741.

American Association for the Advancement of Science (AAAS). 2011. Vision and Change in Undergraduate Biology Education: A Call to Action. Washington, DC. http://visionandchange.org/finalreport

Armstrong, N. A., Wallace, C. S., & Chang, S.-M. (2008). Learning from writing in college biology. *Research in Science Education, 38*(4), 483-499.

Berdoy, M., Webster, J. P., & Macdonald, D. W. (2000). Fatal attraction in rats infected with Toxoplasma gondii. *Proceedings of the Royal Society of London B: Biological Sciences, 267*(1452), 1591-1594.

Betihavas, V., Bridgman, H., Kornhaber, R., & Cross, M. (2016). The evidence for 'flipping out': A systematic review of the flipped classroom in nursing education. *Nurse Education Today, 38,* 15-21. doi:10.1016/j.nedt.2015.12.010

Brownell, S. E., Kloser, M. J., Fukami, T., & Shavelson, R. (2012). Undergraduate biology lab courses: comparing the impact of traditionally based "cookbook" and authentic research-based courses on student lab experiences. *Journal of College Science Teaching, 41*(4), 36-45.

Deiner, L. J., Newsome, D., & Samaroo, D. (2012). Directed self-inquiry: A scaffold for teaching laboratory report writing. *Journal of Chemical Education, 89*(12), 1511-1514.

Dirrigl Jr, F. J., & Noe, M. (2014). The student writing toolkit: enhancing undergraduate teaching of scientific writing in the biological sciences. *Journal of Biological Education, 48*(3), 163-171.

Ericsson, K. A., Krampe, R. T., & Tesch-Römer, C. (1993). The role of deliberate practice in the acquisition of expert performance. *Psychological review, 100*(3), 363.

Goldey, E. S., Abercrombie, C. L., Ivy, T. M., Kusher, D. I., Moeller, J. F., Rayner, D. A., . . . Spivey, N. W. (2012). Biological inquiry: a new course and assessment plan in response to the call to transform undergraduate biology. *CBE-Life Sciences Education, 11*(4), 353-363.

Greene, M. J., & Gordon, D. M. (2003). Social insects: cuticular hydrocarbons inform task decisions. *Nature, 423*(6935), 32-32.

Handelsman, J., Ebert-May, D., Beichner, R., Bruns, P., Chang, A., DeHaan, R., . . . Tilghman, S. M. (2004). Scientific teaching. *Science, 304*(5670), 521-522.

Hoefnagels, M. 2003. Using superstitions and sayings to teach experimental design. Pages 325-327, in Tested studies for laboratory teaching, Volume 24 (M. A. O'Donnell, Editor). Proceedings of the 24th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 334 pages.

Johnson, M., & Pigliucci, M. (2004). Is knowledge of science associated with higher skepticism of pseudoscientific claims? *The American Biology Teacher, 66*(8), 536-548.

Knight, J. K., & Wood, W. B. (2005). Teaching more by lecturing less. *Cell Biology Education, 4*(4), 298-310.

Kuhn, D. (1993). Science as argument: Implications for teaching and learning scientific thinking. *Science Education, 77*(3), 319-337.

Rotellar, C., & Cain, J. (2016). Research, Perspectives, and Recommendations on Implementing the Flipped Classroom. *American Journal of Pharmaceutical Education, 80*(2), 9.

Rowe, M. P., Gillespie, B. M., Harris, K. R., Koether, S. D., Shannon, L.-J. Y., & Rose, L. A. (2015). Redesigning a general education science course to promote critical thinking. *CBE-Life Sciences Education, 14*(3), ar30.

Sundberg, M. D., Armstrong, J. E., & Wischusen, E. W. (2005). A reappraisal of the status of introductory biology laboratory education in US colleges & universities. *The American Biology Teacher, 67*(9), 525-529.

Sutherland, W. J., Spiegelhalter, D., & Burgman, M. A. (2013). Twenty tips for interpreting scientific claims. *Nature, 503*(7476), 335-337.

Van Lacum, E. B., Ossevoort, M. A., & Goedhart, M. J. (2014). A teaching strategy with a focus on argumentation to improve undergraduate students' ability to read research articles. *CBE-Life Sciences Education, 13*(2), 253-264.

Walker, W. R., Hoekstra, S. J., & Vogl, R. J. (2002). Science education is no guarantee of skepticism. *Skeptic (Altadena, CA), 9*(3), 24-29.

Appendix A. Rubric used to score student Independent Project lab reports

		Great (3)	Okay (2)	Not Great (1)
	Abstract	Abstract encapsulates the ***key*** ideas for each section (Intro, Methods, Results, Discussion)	Abstract summarizes the main ideas for each section (Intro, Methods, Results, Discussion) but also includes some extraneous detail	Abstract does not clearly summarize the main ideas for all four sections (Intro, Methods, Results, Discussion)
Hourglass Shape (Structure of Scientific Argument)	**Introduction**	Introduction structure is (in order): (1) broad main scientific concept (2) a narrower scientific idea (3) hypothesis (or other specific indication of what lab report is about)	Introduction has some narrative structure but does not adhere to the formula described in "Great"	Introduction is not organized as a logical argument or is too short to see a flow of ideas
	Discussion	Discussion structure is (in order); (1) interpretation (meaning) of results (2) comparison of results with primary literature (3) broader conclusion about a scientific phenomenon, in light of results	Discussion has some narrative structure but does not adhere to the formula described in "Great"	Discussion is not organized as a logical argument or is too short to see a flow of ideas
	Hypothesis	Hypothesis is formulated as: (1) logical predicted relationship between/among (2) clearly defined, measurable variables.	Does not meet 1 of 2 criteria: (1) direction of relationship not clear (e.g. "changes" or "affects"), **or** (2) variables are poorly defined (i.e., it is not clear what will be measured)	Does not meet either criteria
	Authentic Use of Scientific Literature	Uses the primary literature to motivate (Introduction) and explain (Discussion) the current experiment.	Competently summarizes primary source(s), but in a way that does not explicitly motivate (Introduction) and/or explain (Discussion) the current experiment	References to the primary literature are tangential/unrelated or insufficient

Curriculum Vitae

Jacquelyn M. Petzold

jpetzold@indiana.edu

EDUCATION

2010-2016 Doctor of Philosophy
Evolution, Ecology, & Behavior Program
Indiana University, Bloomington, IN

2007-2008 Master of Library Science
School of Library & Information Science
Indiana University, Bloomington, IN

2003-2007 Bachelor of Science, Biopsychology & Chemistry
Grand Valley State University, Allendale, MI

RESEARCH EXPERIENCE

2010-2016 Graduate Student, Indiana University
Supervised by Dr. G. Troy Smith
Dissertation: "Diversity in the structure of signals produced by South American weakly electric knifefish"

2006 Summer Undergraduate Research Fellow
Supervised by Dr. Kenneth McCarson
Project: "Effects of estrogen on acute pain sensitivity in rats"

TEACHING EXPERIENCE

2010-2016 Associate Instructor, Indiana University
Spring 2013 & 2016, Fall 2015: Introductory Biology Laboratory
Fall 2014: Biology of the Senses
Fall 2013: Research & Professional Ethics
Fall 2010, 2011, & 2012: Evolution and Diversity
Spring 2011: Vertebrate Zoology

2009-2010 Science Librarian, Assistant Professor
University of Nebraska, Lincoln, NE

RESEARCH PUBLICATIONS

Petzold, J.M., Marsat, G., Smith, G.T. (in press). Co-adaptation of electric organ discharges and chirps in South American ghost knifefishes (Apteronotidae). Journal of Physiology – Paris, http://dx.doi.org/10.1016/j.jphysparis.2016.10.005

Petzold, J.M. & Smith, G.T. (2016). Androgens regulate sex differences in signaling but are not associated with male variation in morphology in the weakly electric fish *Parapteronotus hasemani*. *Hormones & Behavior, 78*: 67-71

RESEARCH POSTER PRESENTATIONS

Petzold, J.M. & Smith, G.T. (2014). Encoding of species-specific waveform of electric fish signals in the amplitude envelopes created by social interactions. Poster presented at the Society for Integrative and Comparative Biology annual meeting, Austin, TX.

Petzold, J.M. & Smith, G.T. (2012). Androgens regulate differences in chirp duration in the weakly electric fish *Parapteronotus hasemani*. Poster presented at the Tenth International Congress of Neuroethology biennial meeting, College Park, MD.

Petzold, J.M. & Smith, G.T. (2012). Chirp parameters signal sex but not male quality in the weakly electric fish *Parapteronotus hasemani*. Poster presented at the Society for Integrative and Comparative Biology annual meeting, Charleston, SC.

STEM EDUCATION PUBLICATIONS

Petzold, J., Winterman, B., & Montooth, K. (2010). Science seeker: A new model for teaching information literacy to entry-level biology undergraduates. *Issues in Science and Technology Librarianship*. Online at: http://www.istl.org/10-fall/refereed2.html

STEM EDUCATION POSTERS & PRESENTATIONS

Wiltbank, L., Keesom, S., **Petzold J.**, Kearns, K., & Winterman, B. (2014). Developing information literacy skills and assessing program goals across the biology curriculum. Group presentation at The Edward C. Moore Symposium on Excellence in Teaching, Indianapolis, IN

Petzold, J., Wiltbank, L., Keesom, S., Kearns, K., & Winterman, B. (2013). Enhancing student learning and assessing program goals in the undergraduate biology curriculum. Poster presented at the Center for Innovative Teaching & Learning Fall Celebration, Bloomington, IN

Keesom, S., **Petzold, J.**, Wiltbank, L., Kearns, K., & Winterman, B. (2013). A transferable model for information literacy integration and assessment in large undergraduate programs. Group presentation at the Indiana University Information Literacy Colloquium, New Albany, IN

FELLOWSHIPS, SCHOLARSHIPS, AND AWARDS

- Seminar in Ecology & Environmental Biology, voted "Best Seminar," Fall 2015
- Center for the Integrative Study of Animal Behavior Fellowship Award, 2015
- Common Themes in Reproductive Diversity Pre-Doctoral Fellowship, 2013-2014
- Indiana University Provost's Office Women in Science Travel Award, 2013
- Indiana University Biology Department Travel Award, 2012
- Center for the Integrative Study of Animal Behavior Student Travel Award, 2012
- Special Libraries Association Biomedical and Life Sciences Student Travel Award, 2008
- Indiana University School of Library & Information Science Merit Scholarship, 2007-2008
- Charles A. and Charles H. Davis Fellowship in Scientific Information, 2007-2008
- Grand Valley State University Presidential Scholarship, 2003-2007

SERVICE AND OUTREACH

2011-present	WonderLab Volunteer
2011-2015	Member of the promotions committee for the Indiana University Animal Behavior Conference, including two years as chair
2015	Co-organizer of the Communication Counts symposium at the Indiana University Animal Behavior Conference
2011-2015	Host for the Indiana University Department of Biology's graduate recruitment weekend
Summer 2014	Co-organizer of the biology module for Girls Inc. Summer Science Camp
Summer 2013	Co-organizer of the Foundations in Science & Mathematics summer enrichment program for local middle and high school students