AN EXPLORATION OF THE MOLECULAR MECHANISMS OF BEHAVIOR IN *APTERONOTUS LEPTORHYNCHUS*, OR THE BROWN GHOST KNIFEFISH

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Brianna D. Silver

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Molecular Mechanisms of Behavior in *Apteronotus leptorhynchus*, or the Brown Ghost Knifefish

Brianna Silver

Abstract:

Behavioral, molecular, and hormonal mechanisms work together to impact sensory processes, communication, and mate choice. This study investigates the evolution of communication and sexual dimorphism through an analysis of intrasexual variation in behavioral and molecular mechanisms in *Apteronotus leptorhynchus*, or the brown ghost knifefish. Although knifefish behavior is well described, gene expression studies in the brain are fairly novel, and few studies have looked at the correlation between behavior and gene expression in the brain within individuals. The brown ghost knifefish has unique, sexually dimorphic communicative behaviors which involve electric signals and can be systematically quantified by measuring electric organ discharge frequency (EODf) and chirp rates (rapid frequency modulations). In this study, we investigated the hypothesis that intrasexual variation in behavioral and molecular traits will be higher in males than in females due to sexual selection pressures, and that this variation is the result of changes in hormone receptor expression. To this end, we show that behavior is dimorphic in brown ghost knifefish, both in terms of EODf and chirp rates. Males have higher baseline frequencies and chirp rates than females. In addition, there is significantly more intrasexual variation in chirp rates within males. To determine if this sexually dimorphic behavior is due to changes in hormone receptor expression, we looked molecularly at expression of the androgen receptor, and of the estrogen receptors ESR1 and ESR2A. Preliminarily, our data suggest that perhaps receptor gene

expression levels are not dimorphic in the brain, and therefore a different molecular mechanism seems to be driving this dimorphic behavior. Upon further investigation, we found that hormones, and specifically 11-ketotestosterone, seem to be likely candidates. This work supports the hypothesis that behavioral variation is greater in male knifefish, but the presence or absence of molecular dimorphism in the brain specifically could not be confirmed.

Introduction:

This project sought to further characterize the evolution of communication by studying both intersexual and intrasexual variations within weakly electric fish. In doing so, we gained a deeper understanding of the neural underpinnings behind sex differences. Particularly, this study deals with apternotids, a large family of electric fish in South America with neurogenic electric organs.

Sexual selection, dimorphism and evolution

Studying sexual dimorphism and the molecular mechanisms behind it is important because it allows for deeper insight into evolution and sexual selection. Often the driving force behind sexual selection is some kind of dimorphic behavior or trait. This is because dimorphic traits often indicate sexual maturity, and stronger, more evolved mates, thereby creating more viable offspring and furthering the species as a whole.

Many different hypotheses have been put forward in the past as to what may or may not drive sexual selection amongst sexually dimorphic populations. As a result, phylogenetic studies have been conducted in which historical patterns in trait evolution have been investigated. One such study involving the *Physalaemus pustulosus*, or the

Tungara frog, supported the hypothesis that sensory exploitation is the sexual selection mechanism that drives changes within a population (Ryan & Rand 1993). The hypothesis states that sexual selection will always favor the males that evolve signals to match the females' sensory biases. The more extreme the trait, the better, as this demonstrates ability for the male to thrive in spite of the extreme trait's possible practical drawbacks (Zahavi, 1975). This causes males to undergo various evolutionary changes more often than females (Fisher, 1930).

There are numerous examples of this seen both in the past and today in the animal kingdom - for instance in Irish elk. Males of this species grew enormous antlers that were not functional for anything other than attracting a mate. The larger the antlers, the more desirable the mate - however, it became increasingly difficult for the male to survive. In fact, this is hypothesized to be why such an extreme adaptation was seen as desirable – if the male could survive despite having such an impractical trait, they were likely to be a strong, genetically advantageous mate (Fisher, 1930). Irish elk are an example of runaway sexual selection, as this trait evolved quickly and was such a disadvantage the elk are now extinct. Examples of these kinds of rapid male evolutionary changes are seen all over the animal kingdom. Another example is seen in birds of paradise – males of many species have evolved to have flamboyant, unnecessary feathers for no other reason than to attract a mate. These animals are still extant, but it will be interesting to see how they continue to evolve and if their dimorphism continues to become even more extreme.

On the basis of these hypotheses and examples of sexual selection in the past and present, we sought to test the following hypothesis: that males will have higher

intrasexual variation than females in the model organism *Apteronotus leptorhynchus,* or the brown ghost knifefish, because of the frequent and extreme evolutionary changes that have occurred in order to please potential female knifefish mates.

Apteronotus leptorhynchus - the brown ghost knifefish

A. leptorhynchus is endemic to South America, approximately 15 – 20 cm in length, and is weakly electric. It produces an electric organ discharge frequency (EODf) that it uses for communication and echolocation. This helps them to navigate their natural murky water habitats, such as the Amazon River. EODf is caused exclusively by firing of the pacemaker nucleus region of the brain, and the rate at which the pacemaker nucleus fires is directly related to a fish's EODf. Different knifefish species have different ranges of EODf, with the brown ghost knifefish having a range roughly of ~650 – 950 hertz (Figure 1).

Another behavior of knifefish is what is often referred to as "chirping," or rapid modulations in EODf. Chirps are often observed between communicating knifefish, and each particular knifefish species has a different kind of "chirp signature.".

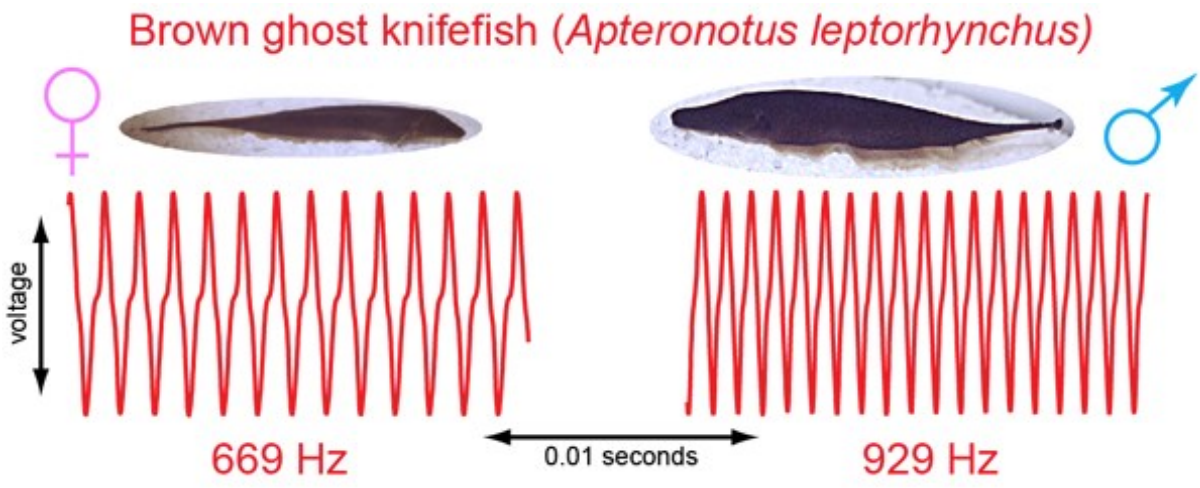

Figure 1. A female and male brown ghost knifefish, and an example of their respective EODf traces (red). Image credit: G. Troy Smith.

In the brown ghost knifefish, EODf and chirping have been shown to be sexually dimorphic; it is known that males have a higher EODf than females, and that males often chirp more frequently (Kolodziejski et al., 2005; Dunlap et al., 1998). It seems that the more dimorphic the traits, or the higher the male's EODf and chirp rate, the more sexually mature the animal is, and the higher the levels of the fish hormone 11-ketotestosterone (11-KT) (Dunlap et al., 1998). However, little research has been done to systematically quantify the EODf and chirp rate variation within sexes. It has already been shown that in *Apteronotus albifrons*, different subpopulations show different levels of dimorphism in EODf (Ho et al., 2013). Therefore, further investigation of intrasexual differences may lend insight into how subpopulations develop, and eventually speciate. In this study, we not only looked at brown ghost knifefish behavior, but also at possible mechanisms causing this dimorphic behavior.

Through a molecular lens

Where there is behavioral dimorphism, there must also be a molecular dimorphism. To look at possible causes of brown ghost knifefish dimorphic behaviors, we primarily investigated gene expression levels of androgen and estrogen receptors. We reasoned that as hormones are known to be key regulators in behavior in many other species (Dunlap et al., 1998), investigating the levels of those receptors involved in hormonal mechanisms may shed light on possible mechanisms driving these behavioral differences. In addition, we looked at differences in hormonal levels, since it is possible that while receptor levels are not dimorphic, the levels of the hormones themselves may be. If these hormones are sexually dimorphic in expression, they may be driving differences in the pacemaker. It is also possible that both receptor expression

and hormone expression are dimorphic, or neither, in which case a different molecular mechanism may be driving this behavior.

Hypothesis

In this project, we ultimately sought to discover whether male or female brown ghost knifefish contain more EODf and chirp rate variation, and whether this is correlated at the molecular level to the expression of androgen and estrogen receptors. We hypothesized that males are more likely to express higher levels of variation (i) behaviorally in their EODf and chirp rates and (ii) molecularly in the gene expression levels of their androgen and estrogen receptors as well as (iii) hormonally in 11-ketotestosterone (11-KT) and testosterone (T) levels. This is because we believe the males are undergoing more changes to try to meet the preferences of the choosier female fish. This is in line with the sensory exploitation hypothesis set forth by Ryan & Rand (1993). Ultimately, we can use these fish to test the validity of this hypothesis, and the variation or lack thereof that we observe intrasexually can guide us in understanding past evolutionary events as well as predicting future ones.

Methods:

Behavioral Assays

The behavior of ten male brown ghost knifefish and ten female brown ghost knifefish was analyzed using chirp recordings. For the weeks prior to testing, fish were put in holding tanks with lowered conductivity to simulate the rainy season, which triggers reproductive maturity and signals the breeding season. Conductivity was lowered by increasing water flow rates into the tanks. For testing, fish were placed in dark temperature-controlled tanks held at about 26 degrees Celsius attached to an

amplifier that amplified electric signals and transferred them into sound, which was then recorded onto the computer. Movement was limited by placing fish in mesh hammocks. Fish were then allowed to acclimate to the tank for about 45 minutes to one hour. They were then played the following playback stimuli frequencies in randomized order and their responses recorded: -150 Hz, -20 Hz, -5 hz, +20 Hz, and +150 Hz, relative to the fish's own baseline EODf. This was to simulate a fish of the same sex and close to their size (-5 hz), one of a fish of the same sex but a different size (+/- 20 hz), and one of a fish of the opposite sex (+/- 150 hz). After a four minute long baseline recording in which the fish was not presented a stimulus, each experimental trial was a four minute recording that included one minute of pre-stimulus (no stimulus) two minutes of playback stimulus (stimulus on) and one minute of post-stimulus (stimulus off). Each trial was separated by ten minutes and the order in which the stimuli were presented was randomized. Procedure adapted from Ho et al. (2013).

Gene Expression Assays

Due to technical difficulties, the results of the original 10 males and 10 females used above did not produce enough data to determine if the molecular data and behavioral data correlated. Instead, pilot data from Adam R. Smith using 5 males and 5 females was used as reference. These fish were weighed, sacrificed, and their brains and gonads removed. The pacemaker nucleus and gonads were analyzed. The samples were stored in RNAlater and then the Agilent Absolutely RNA spin protocol was used to extract RNA. RNA was then reverse transcribed into cDNA and real time qPCR was performed, followed by a primer/probe qPCR assay (IDT). The results of the real time PCR was analyzed for threshold points and compared to the expression of two

housekeeper genes: those for beta-actin and the 18s rRNA subunit. Statistical analysis for significance was determined using ANOVA and the Brown-Forsythe test.

Hormonal Assays

Blood samples were collected from the original 10 males and 10 females right after they were sacrificed. These samples were used in testosterone assays. In addition, the blood from the Adam R. Smith sample fish was also assayed for 11-KT. The blood was centrifuged for five minutes in order to separate out the blood plasma. This plasma was extracted and stored at -20 degrees Celsius until testing. The actual assays were enzyme immunoassays performed following the manufacturer's protocols (Cayman Chemical, Ann Arbor, MI). Procedure adapted from Ho et al. (2013).

Results and Discussion:

Behavior was found to be dimorphic, with males chirping significantly more than females

EODf is Sexually Dimorphic

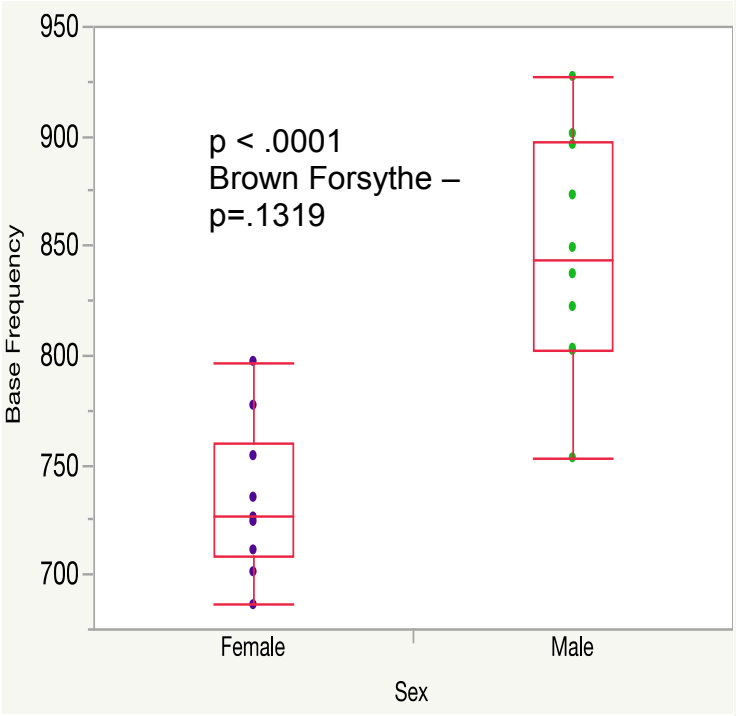

Figure 2. EODf (in Hertz) is confirmed to be sexually dimorphic, with males having higher EODf and as well as more intrasexual variation. Statistical tests: ANOVA dimorphism and Brown – Forsythe for intrasexual variation.

Chirp Number is Sexually Dimorphic

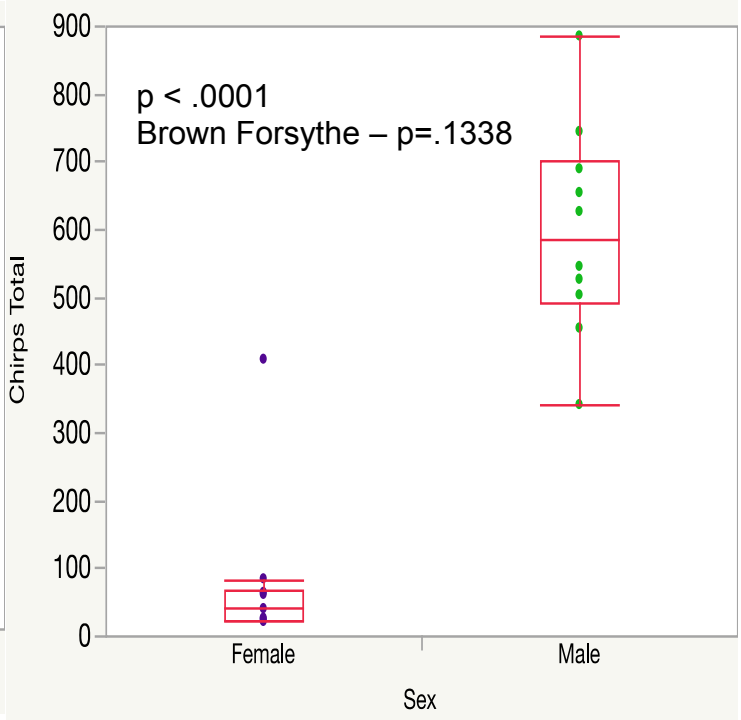

Figure 3. Males are found to chirp substantially more than females when prompted with various other EODfs as stimuli, and also show more intrasexual variation. Statistical tests: ANOVA dimorphism and Brown – Forsythe for intrasexual variation.

In order to investigate behavioral levels of dimorphism and intrasexual variation, we recorded EODf and chirp number for each of the ten individuals in a separate tank known as a chirp chamber. Once the fish had become acclimated to its new tank, we first took a raw recording of the fish in its natural state, thereby getting its individual EODf. EODf was found to be sexually dimorphic (Figure 2). Our behavioral data confirmed that males have significantly higher EODfs than females. Males also showed more intrasexual variation, although this difference was not statistically significant (Figure 2).

Next we stimulated the fish with different frequencies to mimic the presence of other fish in the tank and recorded their response by counting the number of chirps, or rapid frequency modulations. Males were found to chirp significantly more than females (Figure 3). Once again, males showed higher levels of intrasexual variation, but not to a statistically significant degree. With this data, our hypothesis that behavior is dimorphic has been confirmed, however, our hypothesis that males would have higher levels of intrasexual variation is not.

Why do males have higher EODfs? This could simply be so that fish can identify others of the opposite sex. In addition, it is hypothesized that the higher the male fish's EODf, the more sexually mature the male is. However, in comparing base frequency to gonadosomatic index (mass of gonads/mass of fish), we did not find a significant positive correlation ($p = .5576$).

It is interesting to contemplate why it may be that males chirp more. Is it a sign of aggression? Or is it simply a form of communication and mate calling? Perhaps it could be both, and it still isn't known precisely why knifefish chirp. Although for analysis

purposes we pooled together the number of chirps recorded from all of the different stimuli, it was observed that fish chirped the most when played the stimuli that was closest to their own frequencies. This would perhaps lend itself to the theory that chirps are a sign of aggression and a signal to fish at similar frequencies that they should back off, as similar frequency fish are likely of similar size and possibly even dominance levels. At this point, this is mainly speculation however, and further investigation will be needed to understand the purpose of this sexually dimorphic behavior.

Molecular receptor expression was not found to be dimorphic in either the pacemaker or the gonads

First we looked at receptor gene expression in the pacemaker relative to the expression of housekeeper genes beta-actin and the 18s rRNA. The levels of receptor gene expression in the pacemaker did not correlate with EODf in either males or females (Figure 4).

Furthermore, we found that androgen receptor expression in the gonads did not correlate with EODf in either males or females (data not shown). However, estrogen receptor 1 expression correlated with EODf in females (Figure 5). The higher the estrogen receptor 1 expression, the lower the female's EODf. It perhaps could be the case that as the female becomes more sexually mature, she expresses higher levels of estrogen receptor 1 in the gonads and this in turn causes a lowering of her EODf, making it more sexually dimorphic. Receptor expression effect on chirps could not be quantified because full behavioral tests were not run on these samples.

EODf vs. Androgen and Estrogen Receptor 1 Expression in the Pacemaker

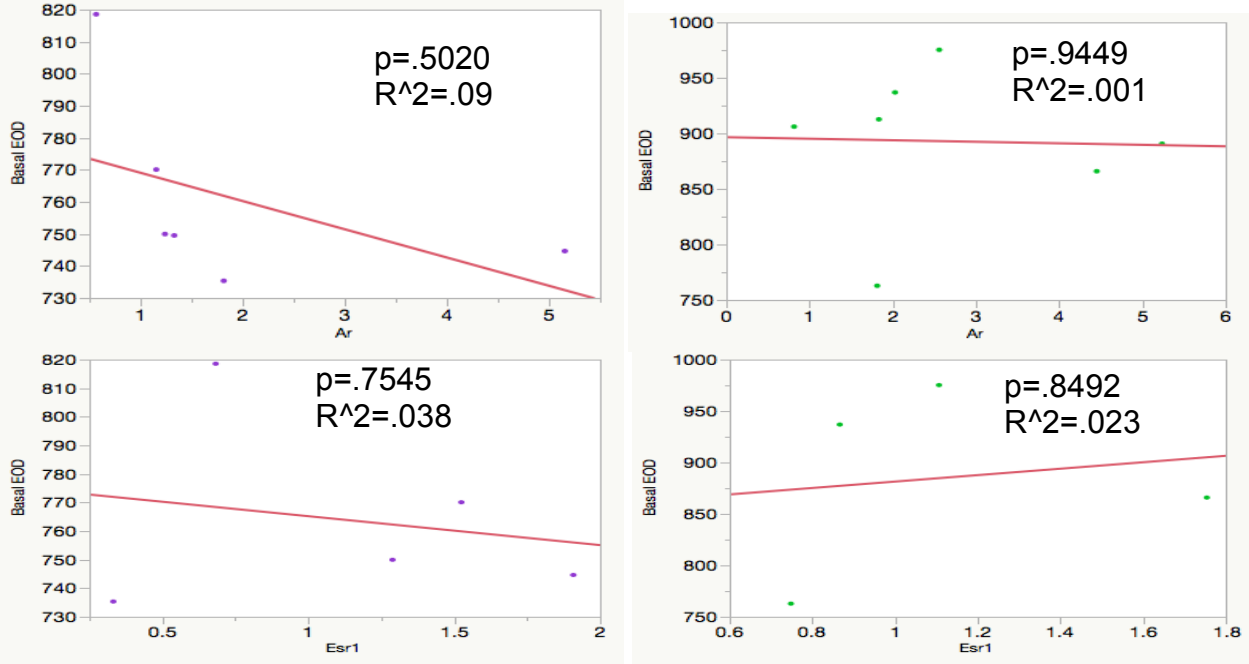

Figure 4. Androgen and Estrogen Receptor 1 expression in the pacemaker does not correlate with EODf. Top left – female androgen receptor expression vs. EODf. Top right – male androgen rector expression vs. EODf. Bottom left – female estrogen receptor 1 expression vs. EODf. Bottom right – male estrogen receptor 1 expression vs. EODf. Estrogen receptor 2A expression levels in the pacemaker were negligible. Statistical tests: ANOVA.

EODf vs. Estrogen Receptor 1 Expression in Gonads

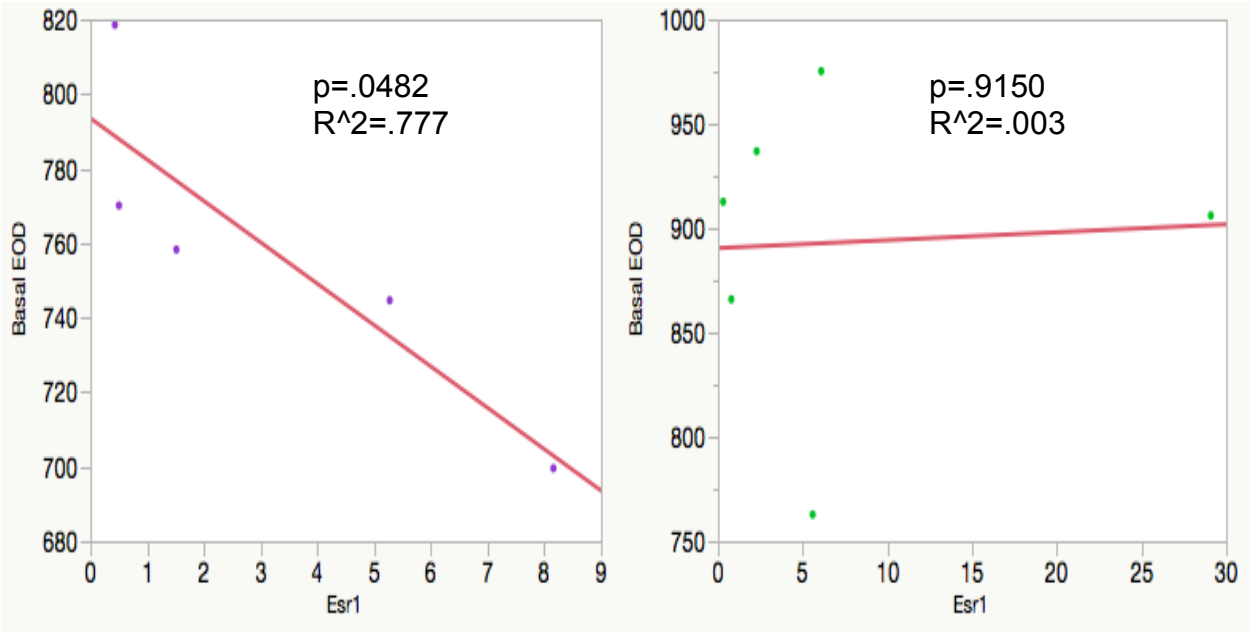

Figure 5. Estrogen receptor 1 expression in the gonads correlates with EODf in female. As seen in the left panel, as estrogen receptor 1 expression goes up, EODf goes down. Male EODf, as seen in the right panel is not affected. Statistical tests: ANOVA.

Estrogen receptor 2A expression was limited to the gonads of both sexes, and not observed in the pacemaker or even the whole brain (data not shown). These results taken together suggest that perhaps receptor expression is not dimorphic in the brain and therefore not likely to be the cause of the dimorphic behavior.

Neither androgen nor estrogen receptor expression was found to be dimorphic in the pacemaker or the gonads. Gene expression of androgen, estrogen 1 and estrogen 2A receptors was not found to be significantly different between the sexes, and showed similar levels of intrasexual variation (data not shown). This seems to suggest that it is not the receptor gene expression alone causing the observed dimorphic behavior, and so further possible molecular causes were investigated.

11-KT concentration was found to be dimorphic and correlate with EODf in males

11-KT (11-ketotestosterone) concentration was found to be hugely dimorphic, with males having significantly higher amounts. In addition, intrasexual variation was extremely dimorphic, with males having much more variation than females (Figure 6). 11-KT significantly correlates with EODf in males, but not

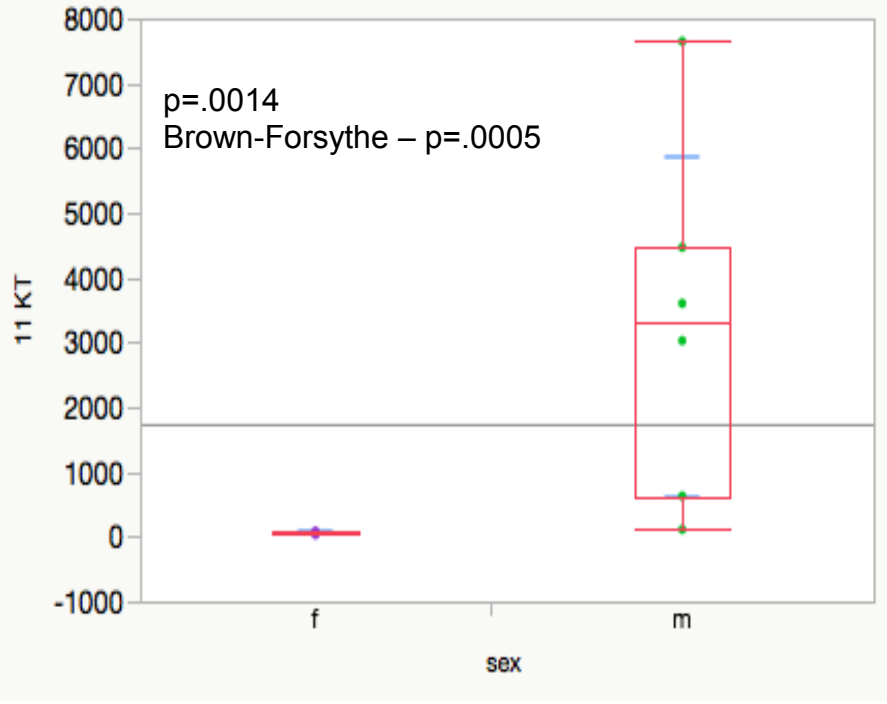

Figure 6. Males have significantly more 11-KT than females. Males also show higher levels of intrasexual variation. Statistical tests: ANOVA and Brown-Forsythe.

females. The higher the 11-KT concentration, the higher the EODf in males (Figure 7). Once again, chirps could not be analyzed for correlations to 11-KT because these fish were not fully tested behaviorally. Finally, 11-KT concentration appears to have no correlation with receptor gene expression levels in any apparent way (data not shown). Testosterone concentration was not found to be sexually dimorphic (data not shown).

11-KT Concentration vs. EODf in males and females

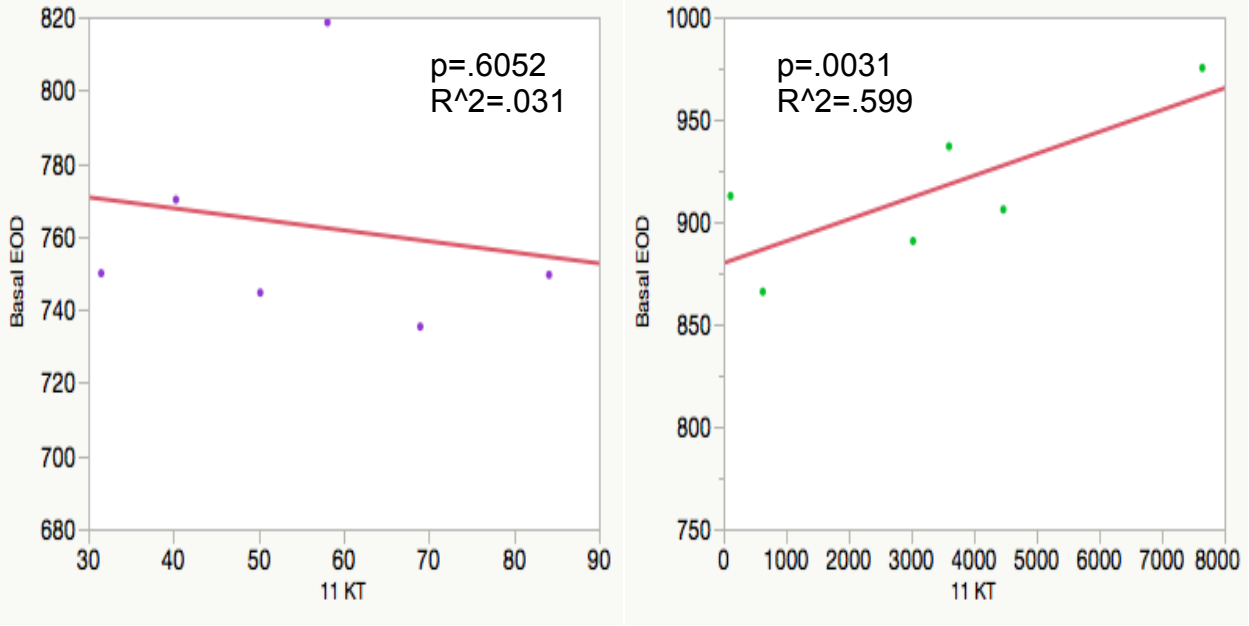

Figure 7. 11-KT concentration correlates with EODf in males (right panel) but not in females (left panel). The higher the levels of 11-KT in males, the higher the EODf. Statistical tests: ANOVA.

Conclusions:

Based on this preliminary data, it would appear as though hormones are regulating the dimorphic behaviors of brown ghost knifefish, while receptor expression levels in the pacemaker nucleus may not be. This data indicates that it is the hormone levels that are variable, and not the amount of gene expression of various receptors. It would seem as though while a baseline level of receptor expression is needed to propagate the signal, the limiting factor in this behavioral pathway is the level of

hormone itself. This suggests that the large degree of intrasexual variation of 11-KT levels contributes to evolution of male knifefish behavior to please the sensory systems of female knifefish. It will be interesting to see in future studies whether or not 11-KT levels correlate with chirp rates. In a study done by Ho et al. (2013) in the black ghost knifefish (*Apteronotus albifrons*) it was found that 11-KT administered over a period of two weeks significantly masculinized the EODf of the fish. The extent to which the 11-KT affected the EODf however varied greatly between the two populations treated (those from the Amazon and those from the Orinoco). The treatments were not found to alter chirp rates in either population. In the future, it could be interesting to conduct a similar study with the brown ghost knifefish. This would allow us to observe the effects of 11-KT in different populations and build off of the data we currently have to further our understanding of sexual dimorphism in knifefish.

It would also be useful to repeat the experiments outlined here once again. Ultimately, we had to work with two separate data sets in order to have enough information to make cohesive conclusions. As it was my first time carrying out many of the experiments, they did not always go smoothly. Therefore, the data presented here comes from two different sets of fish and experiments - molecular data was pilot data collected by Adam R. Smith, as well as the 11-KT data, while the behavioral and testosterone assay data was collected by myself. This means we could not correlate the molecular data to the complete behavioral data, which is a crucial missing piece to this puzzle. In rerunning the experiment with a consistent data set, it would be possible to then see how different levels of receptor expression or 11-KT do or do not affect chirp

rates. In addition, the smaller sample size of the pilot molecular data decreases the statistical value of those results.

In the future, there are certain mistakes I have made that I would know to look out for and certain ways in which this experiment could be improved when repeated. For example, it was my first time carrying out much of the molecular work. Reverse transcribing mRNA to cDNA is a sensitive process, and having only practiced one time before collecting data, it seems likely I lost samples at this step. In addition, the first attempt yielded cDNA that did not have a high enough concentration to be picked up by the qPCR, or at least not when prepared by someone with moderate and not expert pipette skills, such as myself. Even after correcting for concentration, many of the samples showed up as having null concentrations or reached significant levels at very late cycles. This meant that most of the molecular data I collected gave null or inconsistent results, even amongst the control genes. qPCR is also a time sensitive process, and one at which I did not get quick enough at until a few tries. As time went on, I began to get more accurate and consistent readings. However, I was working with a very small sample size of some very small fish. These small samples meant very little room for error, and my first time through the process I made a few errors that I couldn't really afford. In addition, in some of the smaller fish, and particularly in the smaller females, the pacemaker nucleus of the brain did not yield enough RNA for any sort of meaningful qPCR results, even in the hands of more experienced scientists. This means it might be necessary in the future to make ultra-low input libraries of individual pacemaker nucleus expression across a variety of fish, though this process may require a lot of funding.

It also must be taken into account that despite consistently lowered tank conductivity, some of the animals may have been at different levels of sexual maturity compared to others, as possibly indicated by the varied gonadosomatic index of our samples. This could affect a myriad of things – including our behavioral results and hormonal results, and in particular the levels of instrasexual variation observed. While we aimed to use fish that were sexually mature, many were no doubt small even for brown ghost knifefish and had questionable EODfs (EODf is widely regarded as a way of identifying male versus female knifefish, with more dimorphic EODfs indicating more sexually mature individuals. It is however not an absolutely perfect technique). In addition, testosterone levels of my sample set were quite low and nearly negligible, which also may indicate a lack of sexual maturity. It also isn't clear if across the data set the fish tested were all of the same populations. While I had a goal sample size of 10 males and 10 females, that many animals were only tested successfully for behavior – the molecular data set presented here has 5 different males and 5 different females. So, in addition to a lack of overall cohesiveness of data, the sample size was fairly small, making it hard to draw solid conclusions and identify all of the possible trends and correlations. In the future, if carried out correctly and with a larger sample size, then the experiment would lend itself to more cohesive results and deepen our nascent understanding about the interconnectivity of gene expression, hormone levels, and behavior in the brown ghost knifefish.

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