AGGRESSIVE PHENOTYPES IN MALAWI CICHLIDS ASSOCIATED WITH V1AR VARIANT

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
B.S. in Biology with the Research Option
in the School of Biological Sciences

Georgia Institute of Technology
December 2016

ACKNOWLEDGEMENTS

I would like to thank Dr. Patrick McGrath and Dr. Todd Streelman for their faith in me as a researcher and the willingness to involve me in their research. I would also like to thank Dr. Nicole Baran, who has personally helped me throughout this endeavor and has taught me a great deal about the scientific process. Lastly, I would like to thank Dr. Eric O'Neill for his interest and assistance in this project and for taking time out of his schedule to assist in solving a particularly difficult problem along the way.

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SUMMARY

The cichlid model provides a great opportunity to explore diversity in behavioral phenotypes. Different groups of Malawi cichlids exhibit distinct patterns of behavior for a variety of scenarios, including aggressive encounters. These cichlids, characterized by the rocky or sandy habitats they occupy, exhibit strong genetic divergence, possessing large numbers of alternatively fixed variants between them. One such variant exists in the gene *avpr1a*, also known as *V1aR*, a major receptor for vasopressin in humans. This gene has been linked to behavioral effects across a variety of animal species, with this specific variant likely to have significant structural implications for the receptor product. Here we investigate the aggressive behaviors of a set of rock and sand hybrid fish for their association with the variant observed in *V1aR*. While specific metrics of aggression showed similar trends in these hybrids compared to those observed in the parental rock and sand species, ultimately these trends were not significant and were inconclusive. However, these results serve as a preliminary investigation of this gene's involvement in cichlid aggressive behavior. In future work, further examination of the locus will be conducted utilizing more precise and powerful methods in order to draw stronger conclusions.

CHAPTER 1

INTRODUCTION

African cichlids stand out as a particularly interesting candidate for many types of research for their remarkable diversification and rapid speciation; the cichlids of Lake Malawi in particular stand out with regard to this explosive diversification, having radiated into 400 different species from a singular ancestor in the past million years (Moran et al, 1994). The sheer number of species allows for incredible numbers of comparisons to be made when examining this adaptive radiation in a variety of fields of research including genomics, morphology, and behavior.

As a behavioral model, Malawi cichlids are well-differentiated into two specific groups based their natural habitat: a rocky or sandy environment (Danley & Kocher, 2001). Malawi cichlid males that live in rocky habitats are fairly territorial, guarding a small, closed-off surrounding that they coax females to mate in all-year round. In stark contrast, sand species do not have an environment conducive to distinct territories; instead, they build bowers, structures made by moving the sand in their environment to create distinct hills or troughs, in order to attract mates during the mating season. These distinctions in mating behavior form the basis for many of the social interactions of cichlid conspecifics, whereby social dominance plays a large role in claiming the best territories and attracting the most mates (Fernald & Hirata, 1977).

As such, a particularly interesting behavioral phenotype to study between the rock and sand groups is their aggressive response to conspecifics. Given their social hierarchies, both groups are evolutionarily inclined to defend the territories and social standings aggressively;

however this competition manifests itself differently between species of rock and sand groups, with the rock group being substantially more aggressive in their response than species in the sand group (Danley, 2011).

As sequencing technology has become more affordable and common, the genomics of these various cichlid groups across the African lakes has become better characterized (Brawand et al, 2010), including specific variants existing between the rock and sand cichlid groups of Lake Malawi. In order to investigate a potential genetic cause for the different aggressive phenotypes between these groups, we examined the set of characterized variants between the two groups for any potential candidates that might have a strong effect. These variants have been alternatively fixed in all the species of their respective groups and therefore are likely to contribute to the difference in phenotypes between these groups. One such candidate that stood out was in *V1aR*, a gene responsible for the production of a receptor for arginine vasopressin (AVP) in fish, a homologue of the mammalian hormone vasopressin. This gene contained a variant with an 11 base pair deletion fixed across sand species in Lake Malawi, which was implicated through domain prediction of the amino acid sequence to completely alter the C-terminal domain of the GPCR protein product via a frameshift (Fig.1). What makes this gene particularly worth investigating is its implication in behavioral phenotypes across a variety of different animal groups, including aggression in small mammals (Gutzler et al, 2010), (Gobrogge et al, 2009), social interactions in songbirds (Baran, Tomaszycki, and Adkins-Regan, 2016), and social ascent in closely related cichlid groups such as *Astatotilapia burtoni* (Huffman et al, 2015).

Additional research on this gene in songbirds has demonstrated the importance of critically conserved palmitoylation sites in the cytoplasmic C-terminal domain (Abe et al, 2012). These palmitoylation sites are common post-transcriptional modifications for proteins in the

GPCR family, most commonly observed on cysteine residues in the cytoplasmic tail end of the protein to effect structural changes via additional lipid binding capacity as well trafficking to the cellular membrane (Chini & Parenti, 2009). Such putative sites are also disrupted in the deletion observed here (Fig. 1), strongly suggesting the domain structure is impacted by this genetic variant. Given these features, we sought to characterize a possible connection between the variants of this gene and the different behavioral phenotypes of rock and sand cichlid species.

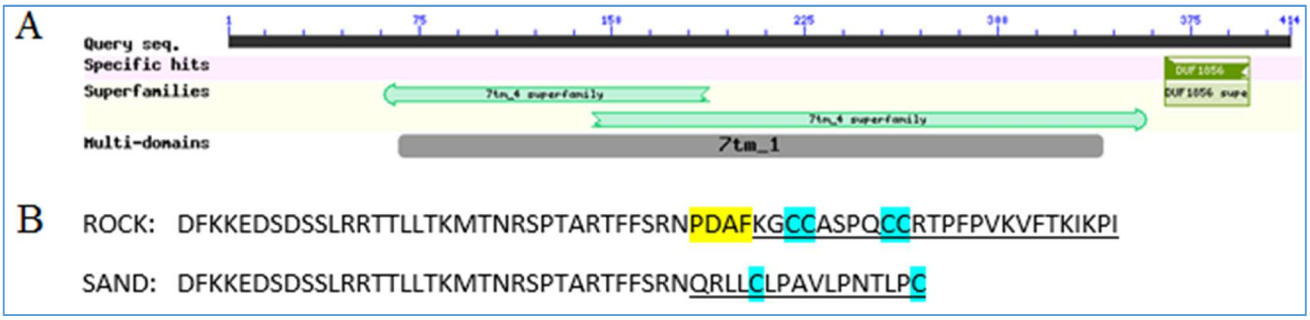

Figure 1. (a) Domain predictions for the *Metriaclima Zebra* (rock) reference sequence of *V1aR*. Variant contains 11 base pair deletion causing frameshift starting at residue 399. (b) Amino acid sequence comparison of predicted cytoplasmic C-terminal region of the two *V1aR* sequences. Highlighted in yellow is the partially and completely deleted residues, followed by the underlined frameshifted region in the sand variant. Cysteine's highlighted in blue represent putative palmitoylation sites, the effect of which has great significance for this domain in GPCRs.

CHAPTER 2

LITERATURE REVIEW

The Relation of Cichlid Aggression and Dominance to Highly Variant Traits in Adaptive Radiation

African cichlid fish are well-characterized for a wide variety of behaviors that are easy to observe and quantify; early literature by Russell Fernald provides such a methodology of recording and reporting the types of behavior observed in specific African cichlids (Fernald, 1977; Fernald & Hirata, 1977), demonstrating a means of investigating the behavioral diversity among the different species as a model for behavioral differentiation. One of the hallmarks and greatest strengths of cichlids as a model for studying behavioral differentiation is their incredibly recent and rapid divergence through adaptive radiation (Kocher, 2004), whereby the gamut of observable traits, from jaw shape, to body and color morphology, to an assortment of behavioral phenotypes are all found dispersed across the phylogenetic tree (York et al, 2015).

Despite the distribution of specific traits not being particularly well-stratified, this distribution is not without some degree of segregation. Many of these traits are found to be assorted phylogenetically in a step-wise fashion, in what could be described as a stages model, whereby traits are segregated in a particular order through the adaptive radiation of cichlid species (Streelman & Danley, 2003). Lastly differentiated in the cichlid phylogeny appears to be courtship behavior, specifically with regard to both how time is most often spent on different activities between cichlid species as well as how frequently they transition into other distinct behavioral events.

Of particular interest is the earlier differentiated trait of habitat in cichlids, which can vary between rock fish (referred to here on as mbuna) and sand fish (Danley & Kocher, 2001), and how it relates to courting behavior. Being the first differentiated trait in cichlid phylogeny, the habitat occupied by species is actually very predictive for many behavioral characteristics such as those in courting. African cichlid fish have a well-documented social hierarchy that consists of dominant and subordinate males that compete over territory (Fernald, 1977). This social hierarchy then plays a large role in behavior, both in the ability to successfully woo a mate as well as success in acts of aggression. The habitat of occupation of a species has a large influence on the nature of these reproductive and aggressive behaviors however. All sand fish species males build bowers as an extended phenotype in order to take part in seasonal leks in attempts to garner the attention of a female (McKaye, 2001). These bowers are integral both in their ability to successfully woo a mate for reproduction as well as in triggering territorial aggression; while dominant males are fiercely protective of their bowers, disruption of these bowers has been shown to decrease the aggressive response (Magalhaes et al. 2013).

This structure-dependent aggression stands in contrast to the non-bower building mbuna species, which acquire and guard territories in a non-temporal manner. In these species, aggression in territorial defense is mediated purely by hierarches of social status, for which size is usually a direct proxy (Oliveira & Almada, 1996). Mbuna are remarkably more aggressive on average compared to sand species due to each male's regular possession of territory as opposed to territory possession dependent on dominance. This is contrasted however by the specificity with which these aggressive acts occur however; Oliveira notes that most aggressive events occur between males of comparable social status in the *Oreochromis mossambicus* mbuna species (Oliveira & Almada, 1996).

The nature of this aggression between those of similar social status appears to be mediated by color recognition in cichlid fish. Just as coloration was one of the highly variant traits between species (Maan & Sefc, 2013) it is also highly variant between the various morphs within a species. Female fish are often drab, while males are usually found with particularly vibrant and unique color patterns usually specific to the species as well as being highly dynamic within them (Deutsch, 1997). Coloration is a trait that males control to a great degree. This variation in color within conspecific males contributes to their sexual selection by females; the more vibrant the color patterns, the better the male's success at reproduction (Jordan et al, 2003). Because of this selective pressure on the basis of color, the distribution of color between males of a population is very indicative of the social dominance hierarchy. Because the most successful fish in bouts of aggression are often the largest, the large fish gains the privilege of expressing the best color patterns for mating. This type of social rank is then immediately evident to other males by the coloration patterns alone (Grosenick et al. 2007). If another male is to have a better chance at courting a female, he would need to adapt more successful colors, but would then likely attract the attention of a male with higher social status who sees his colors as a challenge of dominance.

This male-male aggression on the basis of color presents a window of opportunity into studying the selective pressures for adaptive radiation. The specificity for color appears fairly stringent for both cases of aggressive acts by conspecific males with regard to color similarity as well as in female courting choice (Maan et al., 2004; Pauers et al. 2008). While females may select between males based on color-evident hierarchies, Jordan notes that the female's choice in males doesn't typically span outside of conspecifics (Jordan et al., 2003). With non-dominant males facing pressures to differentiate the expression of color, sympatric speciation could occur quite easily with mutations affecting female preference. Once a female acquires a preference

for a different color pattern, the social pressures on males combined with the opportunity to mate would quickly allow for a speciation on the basis of this color selectivity (Dijkstra et al. 2007).

Further opportunities to investigate the relationship between aggression and other traits in cichlid adaptive radiation might exist in the comparison of specific behaviors between different species. A model for speciation frequency could be developed on the basis of male-male aggression; closely related species that show higher levels of aggression between conspecifics of similar color would be predicted to have had more recent and more frequent speciation events in the phylogenetic tree. Such levels of aggression could be measured via quantifying events in aggression in something like a mirror stimulus, which has been shown to evoke aggression by the presentation of a similarly-colored male (Ruzzante, 1992). Likewise, less aggressive species or species with less specificity in their aggressive behaviors would be predicted to face less selective pressures to speciate on the basis of color differentiation, and could also be observed in the phylogenetic tree. This comparison could be made across the cichlid population distribution for trait segregation in the stages model, such as between species of similar color and body morphology as well as between mbuna and sand fish populations, in order to determine how robust the model is between significantly similar and different populations as well as to determine the nature of genetic segregation for levels of aggression alongside other well-characterized phenotypic traits.

CHAPTER 3

METHODS AND MATERIALS

Subjects:

This study examines African cichlid fish from Lake Malawi that are differentiated specifically by their preferred habitat in the wild, a rocky and well-structured substrate versus that of a sandy lake bottom. Prior behavioral data is utilized, consisting of 2 or more replicates for 4 different rock species: *Cynotilapia afra cobue* (Cobue), *Metriaclima Zebra* (MZ), *Labeotropheus fuelleborni* (LF), and *Petrotilapia chitimba thick bar* (Petro), and 3 different sand species: *Copadichromis virginalis* (CV), *Mchenga conophoros* (MC), and *Tramitichromis intermedius* (TI). These different fish have also been bred in lab to generate specimens of F1, F2, and F3 hybrid lineages. For this specific study, 20 different F2 and F3 hybrid male specimens were examined via a behavioral assay to measure aggression, each with an initially unknown genotype for the gene of interest, *V1aR*. Additionally, 2 parent fish specimens (Petro and MC) as well as one F1 hybrid are utilized as genotyping controls for verification of the genotype post-assay.

Behavioral Assay:

Data collected for analysis were acquired from mirror assays, a method of stimulating an aggressive response from a cichlid fish in response to their own reflection (Ruzzante, 1992). Each fish was given 6 days after being transferred to the experimental tanks to habituate and

naturally become dominant in their environment. These experimental tanks had room for two separate trials and as such were divided in half to prevent interactions between fish in separate trials. Each fish had an environment consisting mostly of sandy substrate but with the addition of a sole flowerpot to act as a potential fixation point for territorial rock-species males to claim as territory (Fig. 2). Each trial would then consist of 15 minutes of recording responses to a mirror placed directly adjacent to the flower pot in the tank.

Figure 2. Image of mirror assay conditions. Each tank was segmented into two halves, with one subject male placed in both sides of the partition. A broken flowerpot placed adjacent to the partition was used in each tank as a point of fixation for fish to develop territoriality for. The rest of the tank consisted only of a thin sand substrate, an airstone, and for partitions on the right side of the tank, a stand pipe in the corner. Fish were allowed to acclimate to this environment for 6 days prior the mirror stimulus being introduced.

Video Coding Parameters:

The videos resulting from the mirror assays were coded with an ethogram used in prior mirror assays conducted for parent rock and sand species. In ascertaining the aggressive response in the mirror test, 4 specific behaviors were coded for in each video. This included a proximity to the mirror stimulus, which was defined as the specimen being within 2 inches (or approximately the length of the flower pot next to the mirror) of the mirror, time spent within the flower pot (as a metric of non-confrontation with the stimulus despite being within proximity), instances of frontal attacks on the mirror (consisting of singular events defined by the pressing of the jaw on the mirror stimulus followed by a closing of the jaw before a second instance could be recorded), and lastly lateral displays (a form of displaying in which a fish presents its colors to would-be mates or males challenging their dominance by fanning their fins outward while positioned laterally within their view). This coding was conducted on Noldus Observer software whereafter the data was exported for statistical analysis in R for comparisons being made between experimental groups for the different genotypes determined, as well as to the original parent data.

Genotyping:

Each specimen tested by mirror assay was fin-clipped to acquire a DNA sample in order to ascertain the genotype for the gene of interest. This DNA was purified using a Qiagen DNeasy Blood and Tissue Kit and then amplified via polymerase chain reaction for genotyping. Although the alternate allele in sand species for *V1aR* was a deletion, initial attempts via PCR to resolve the genotype via fragment size differences were complicated by the deletion's position at the end of the gene and primer design difficulties. Ultimately, the genotype was resolved with

Sanger sequencing by utilizing a second primer set (Forward: GTTGCGAGGTTCACAGAAGC, Reverse: TCAGGTCAAAGCAAAAGGTTCG) designed for a larger product size.

CHAPTER 4

RESULTS

Of the twenty hybrid cichlids tested, only seven were genotyped to be homozygous for either allele at the *V1aR* locus. In addition to this limited number, three of the twenty cichlids had to be excluded from the dataset for a complete lack of any activity that could be coded by the consistent guidelines followed for behavior characterized, including one of these seven homozygous at the *V1aR* locus. This left only three homozygous data points for both of the *V1aR* alleles from which to draw conclusions.

Previous mirror assays were conducted on the parental species of these hybrids, including ten rock assays and eight sand assays. These data points are included in Figure 3 for the sake of comparison; however, only the data of the three hybrid allelic groups are tested for significance. All comparisons were tested for significance with a Kruskal-Wallis H Test; since all 4 failed to achieve significance within a p-value of 0.05, no post hoc test was needed.

Mann-Whitney U tests of the pre-existing rock and sand parent data found significant differences between lateral display times (Fig. 3A, $p = 0.009$) and latency by the specimens to attack the stimulus (not shown, $p = 0.03$). Additionally, a non-significant trend in the difference between total frontal attacks on the stimulus for the parents (Fig. 3C) was anticipated to reach significance based both on prior research[4] and non-experimental observation. Strong outliers, however, proved to an impediment to confirming this hypothesis with significance. Following these results, four specific comparisons were made with new hybrid data made available from

behavioral coding. Although more comparisons could have been made with the data, these most strongly followed-up on the important results from the parental data.

The most significant of these results, the lateral display disparity, was still observed as a non-significant trend for comparisons of specimens homozygous with the rock and sand alleles (Fig. 3A). In contrast, however, the frontal attack trend was not observed with any confidence compared to the parent comparisons (Fig. 3C). One expectation in data analysis that individual specimen frontal attack counts and lateral display times might reveal some correlation was also examined; it was anticipated through initial observation of the hybrid data that different individual behavioral tendencies might co-occur in the hybrids, such as that individuals with higher proclivity for lateral display time exhibited lower counts of frontal attacks and vice versa. While this was still observed for the parental data set and included large intermediates for the hybrids as a whole, the *V1aR* homozygotes demonstrated no such significant correlation (Fig. 3B). The proximity to stimulus metric was included at the end, despite its extreme lack of significance in the parental set, for being a largely necessary component of data analysis as a prerequisite for both frontal attack and lateral display behavior. The possibility existed that this metric might demonstrate a difference to be observed in the hybrid dataset not observed in the parents; however, no such determination could be made for this (Fig. 3D) or other less important metrics.

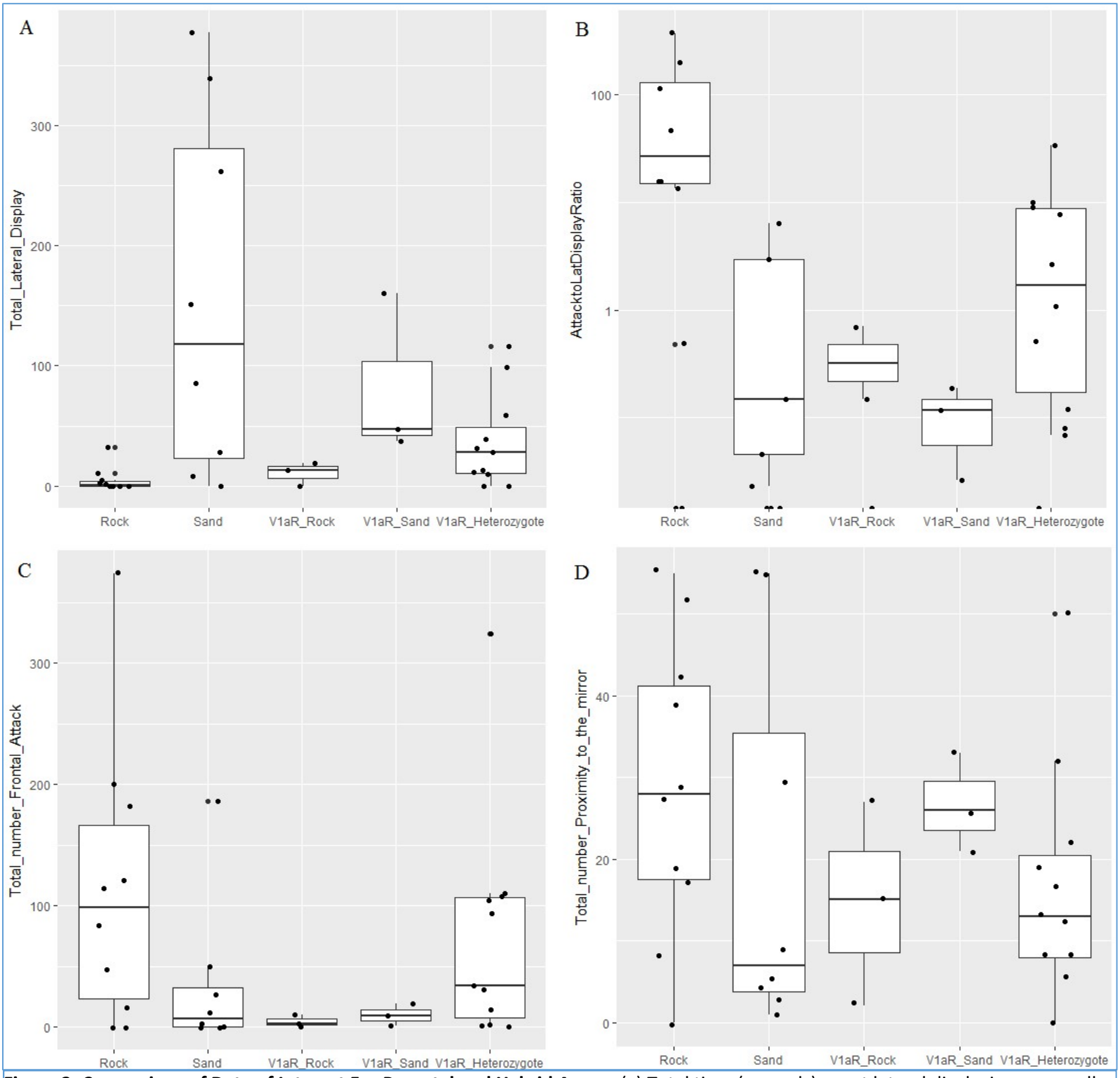

Figure 3. Comparison of Data of Interest For Parental and Hybrid Assays (a) Total time (seconds) spent lateral displaying across all assays, hybrid p-value = 0.1391. (b) Ratio of individual assay count of frontal attacks versus total seconds of lateral displaying, hybrid p-value = 0.2907. (c) Total frontal attack count across all assays, hybrid p-value = 0.1787. (d) Total time (seconds) of proximity to the mirror stimulus across all assays, hybrid p-value = 0.261.

CHAPTER 5

DISCUSSION

We anticipated that our data would demonstrate that the differences in aggressive behavior between the parental rock and sand cichlid species would be partially explained by the genotype of the *V1aR* allele in our tested hybrid specimens. However, despite providing some interesting data, these tests were inconclusive and did not support our hypothesis. The trends observed suggest that some metrics of aggressive behavior, especially lateral display time, could be impacted by the *V1aR* loci, but our means of testing this in this experiment are insufficient to make this assessement with high confidence. Furthermore, other observed differences in behavior between the rock and sand cichlids were absent in their entirity from any association with the *V1aR* alleles in tested hybrids.

Many complications in both the experimental design process and the inconsistency in cichlid performance in our assay are potential factors for the lack of conclusivity provided by this data. This investigation was conducted as a probing follow-up to the previous parental dataset, and many of the limitations were the result of a lack of preparatory period for generating stronger hybrid candidates. Foremost, a dearth in sample size regarding specimens homozygous for the gene of interest precludes the possibility of observing with any significant difference in attributable to the allele, barring the highest of effect sizes. Unfortunately due to the logistics of this experiment, this limitation was somewhat unavoidable in the model, given the amount of time required to breed and raise new cichlid hybrids in comparison to simpler animal models. Given the segregation of alleles after mating, twenty potential hybrid specimens results in only a handful of homozygous hybrids; three or four of each homozygote is simply insufficient for observations of data as variant and quantitative as aggression behavior.

This is further complicated by the lack of segregation at the locus for the specific hybrids available. A different gene of interest with a sizeable deletion variant in sand cichlids, *Irx1b*, was investigated in parallel to *V1aR* as another potential candidate for its implication in brain development. Much to our surprise, our genotyping revealed strong linkage between the homozygotes of both genes, with most of the homozygotes for a rock or sand allele sharing the same variant on the other gene. This lack of segregation limits our ability to glean any specific contribution by either gene over the other, and in this way, the implication of the *V1aR* allele in the behavior we investigated could be as much a result of some contribution by the locus at large as it is the specific protein sequence change in our gene of interest. Given that our specimens for these assays were mostly F2 and F3 hybrids, stronger segregation could be achieved with successive generations being bred, but this method runs into the same pitfall as before with regards to the time investment.

Regarding the assay itself, it should be reiterated that three of these twenty fish had to be excluded from the dataset for a complete lack of all codable activity. It was in our judgment that these fish were anomolies, as such inactivity had also been observed in the parental set as well, and was not representative of what could be deemed “normal” assay behavior. These abnormalities included specimens spending the full duration of the assay fixated on inappropriate stimuli, such as the stand pipe in the tank or the opposite side wall of the tank which, when clear, presented in a slight reflection that these fish alternatively acted upon. Any specimen that exhibited the minimum codable response in the assay was kept in the dataset, but the fact that these distractions kept some fish from exhibiting any response to the assay at all suggests that a stronger assay that reins in these deviations is needed, otherwise an even larger sample size will need to be substituted as compensation.

Lastly, there exists the possibility that this variant actually has no effect in our aggression assay despite our observations. Even with the random assortment of genes in our hybrid set, three data points in two of the hybrid groups means that strong noise alone could have provided us with our dataset for any of the observed comparisons. Such a possibility affects our ability to draw strong conclusions even from unsupportive data. It is therefore imperative that future experimentation include stronger genetic controls against such noise in order to provide the conclusions provided by the data.

One such follow-up to better elucidate the effects of this gene might include forgoing the time constraints of breeding a better set of hybrids in favor of CRISPR gene editing. With the increasing ubiquity of CRISPR Cas9 as a means of modifying genomes in vivo, successful inculcation of the deletion variant in a rock background eliminates the potential for noise from other variants existing the genome, including non-random associations that occur by linkage. With the right guide sequence and injection method, sufficiently high numbers of modified spawn could be produced that could be assayed as soon as they reach maturity.

With such a option available, we have the opportunity to use an alternative assay as a means of comparison of aggressive behavior as opposed to the mirror test. We have previously run dominance assays that consist of two conspecifics interacting after simultaneous intrusion by means of removing a partition separating them. Although this assay provides a much more diverse set of interactions to observe, these could not be replicated for hybrids due to a lack of symmetry between the specimens. With gene-edited fish, we can utilize conspecifics that have both been modified to maintain this symmetry in order to observe the difference in responses between modified and unmodified fish. Although this assay tends to be less predictable in terms of maintaining consistent responses to the stimulus over a long period of time, the likelihood of some form of interaction is far greater compared to anomolous mirror assays. This

type of assay also better replicates real conditions in the wild for aggressive encounters, strengthening any potential claims that arise from this data about their consequences in nature. It is our hope that with a stronger experimental follow-up to this investigative probe we might better determine the consequences of this variant and potentially many others in a behavioral context.

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