Abstract

Title of Dissertation: Sex and the Evolution of a Double Hermaphrodite

Dissertation directed by: Professor Eric S. Haag, Department of Biology

The *Kryptolebias marmoratus* species complex contains the only known self-fertile hermaphroditic vertebrates. There are three species in this clade and all three live in the mangrove forests across the tropical Americas. All three have individuals with both testis and ovarian tissue in their gonads with two using self-fertility as their main mode of reproduction, and all three have apparent different sex determination and sexual modes. In this dissertation, I explore aspects of sex in these species. *K. marmoratus* is the androdiecious and self-fertile member of the species complex with sequential hermaphroditism. In this species, the control of sex change from hermaphrodite to male is poorly understood. Individuals that were believed to be genetically identical could be raised in the same environment and change sex at drastically different times or not at all. Small fluctuations and variance in the hormonal profiles of individuals was thought to be a potential cause and while androgen dosing can lead to masculinization of both the gonad and the soma, it was not enough to maintain a permanent transition like what is seen in nature. In *K. ocellatus*, the obligate outcrosser of the *K. marmoratus* species complex, it was believed that they were using genetic sex determination to differentiate between males and the females that had hermaphroditic gonads. While we found strong evidence against heteromorphic sex chromosomes, all tests for homomorphic sex chromosomes came back inconclusive due to apparent *K. hermaphroditus* DNA contaminating the dataset. *K. hermaphroditus*, the self-fertile hermaphrodite species with exceptionally rare males, appears to be extending its range further and further south and/or hybridizing with *K. ocellatus* at rates previously underappreciated. The hermaphrodites of the Kryptolebias genus still hold many evolutionary and physiological secrets but can potentially be revolutionary to the understanding of vertebrate sexual development and evolution.

Sex and the Evolution of a Double Hermaphrodite

By

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Chapter 1: Introduction

Kryptolebias marmoratus is an odd fish. First described from Cuban specimens in 1880 (Poey), it was later found across the Caribbean, and has gone through numerous taxonomic re-shufflings (e.g. Lira, Berbel-Filho et al. 2021). *K. marmoratus* started to gain broader attention when it was discovered that its "females" are actually self-fertilite hermaphrodites (Harrington 1961). It and the other species in its species complex are the only known self-fertile vertebrates. In addition, this species is also a sequential hermaphrodite (Harington 1961). As a "double hermaphrodite", *K. marmoratus* is unique among other teleost sequential hermaphrodites. This, as well as other unusual reproductive and physiological adaptations, has led to increasing interest in the scientific community (Orlando, 2012). Here, I first summarize the forms of hermaphroditism in fishes. With this context, I then review the major lines of research on *K. marmoratus,* with special emphasis on its unique reproductive biology, its likely ecological relevance, and its place in the study of sex and hermaphroditism.

1.1 Vertebrate Hermaphroditism

Among all vertebrates, fishes are the only clade with functional hermaphrodites (Smith 1975, Mank and Avise 2006, Mank 2006, Avise 2011). Roughly one percent of teleosts are some kind of hermaphrodite, with the majority of those being sequential (Kuwamura, Sunobe et al. 2020). Simultaneous hermaphrodites also exist but are much rarer (Mank and Avise 2009). Individual fish from generally male-female species can also show mixed sex features, especially in the gonad (Hinck, Blazer et al. 2009). This can be triggered by environmental toxins, but in some cases may reflect a baseline natural intersexuality (Atz 1964, Bahamonde, Munkittrick et al. 2013). These examples of "abnormal hermaphroditism" should be distinguished from what would be a common and reproductively functional feature of the species, and will not be discussed further here (Atz 1964).

1.1.1 Sequential hermaphroditism

There are two forms of sequential hermaphrodites, protogynous and protandrous. Protogynous species start their life as females before transitioning to male, while protandrous species start as male and transition to female. In fish, protogyny is more common, but there are ample examples of both (Avise 2011)(**Table 1.1**). Similarly some species' sex change is reversable, but most are not (Avise 2011)(**Table 1.1**).

Table 1.1: Selected teleost sequential hermaphrodites.

Family	Species (common name)	Sex-change Order	Trigger	Reversible	Citation
Pomacentridae	*Amphiprion clarkii* (Clown Fish)	Male -> Female	Social	Yes	(Miura, Nakamura et al. 2008; Nakamura, Miura et al. 2015)
Pomacanthidae	*Centropyge potteri* (Potter's Angelfish)	Female -> Male	Social	No	(Lutnesky 1994)
Labridae	*Thalassoma bifasciatum* (Blue Headed Wrasse)	Female -> Male	Social	Yes	(Lamm, Liu et al. 2015)
Labridae	*Semicossyphus reticulatus* (Asian seepshead wrasse)	Female -> Male	???	No	(Ochi, Fukui et al. 2017)
Serranidae	*Centropristis striata* (Black sea bass)	Female -> Male	???	???	(Breton, Kenter et al. 2019)
Gobiidae	*Lythrypnus dalli* (Blue Banded Goby)	Female -> Male	Social	Yes	(Pradhan, Solomon-Lane et al. 2015)
Pomacentridae	*Pomacentrus amboiensis* (Ambon Damselfish)	Female -> Male	Social	???	(McCormick 2016)
Latidae	*Lates calcarifer* (Australian Barramundi)	Male -> Female	Size?	No	(Orbán, Shen et al. 2021)
Sparidae	*Acanthopagrus schlegelii* (Blackhead Seabream)	Male -> Female	Age	No	(Wu, Dufour et al. 2021)
Rivulidae	*Kryptolebias marmoratus* (Mangrove Killifish)	Self-Fertile Hermaphrodite -> Male	???	No	(Harington 1961)

The triggers for sex change in sequential hermaphroditic species are generally lumped into two categories: social interaction and size/age. For social species, social hierarchies and interactions with dominant members of a specific sex can dictate the sexes of individuals in their populations (Todd, Liu et al. 2016). One example would be the protogynous species *Thalassoma bifasciatum*, the Blue-headed wrasse, where the loss of a male in the population will trigger the largest female to sex change into a male (Liu, Todd et al. 2017). Another example would be *Amphriprion melanopus*, the cinnamon clownfish.

This species reproduces in monogamous breeding pairs with a larger female, a smaller male and other non-reproductive immature fish as part of the group. Loss of the big female triggers sex change in the male to become the new female, and a new male will mature among the immature fish to become her mate (Roux, Salis et al. 2020).

Age and size are believed to be common triggers due to a reproductive advantage of being a specific size for a specific sex. For example, if males compete vigorously and the larger one always wins, then protogyny should evolve. Conversely, with stable pair bonds, females benefit more from the large size as they can produce more eggs. This overall idea is called the Size Advantage Model, or SAM, and was first proposed by Ghiselin in 1969. A good example of this would be *Acanthopargus schlegeli*, the black porgy, a protandrous marine fish whose size dictates sex in a highly predictable manner (Lee, Du et al. 2001). Mechanisms for social triggers have been linked to stress and cortisol levels in blue-headed wrasse (Casas and Saborido-Rey 2021) however exact pathways for the age and size triggered pathways are less clear with many hormones along the hypothalamic pituitary gonadal and Hypothalamic-pituitary-interrenal axes being proposed (Casas and Saborido-Rey 2021).

While the end points of sex change are generally pure male and female phenotypes, the transition states are less well understood. With regard to the gonad, understanding the process of sexual transformation requires intensive destructive sampling across development, and thus has not been examined for a large number of species. The general pattern of sex change is to degrade initial sex tissues and then develop that of the “terminal sex” (Casas and Saborido-Rey 2021). However, it is often unknown whether spermatogonia in a protogynous species (or oogonia in a protandrous one) arise from dormant yet sexually differentiated germ cell populations, or form from sexually bipotent primordial germ cells that are undifferentiated or reprogrammable (Casas and Saborido-Rey 2021).

Secondary sexual traits such as pigmentation, dimorphic body shapes/ornaments, and behavior generally correlate to gonadal content (Casas and Saborido-Rey 2021). Sex steroid hormones, like other traits, are similarly correlated with phenotype in the expected manner before and after sex change. However, whether they are a cause or consequence of it is not clear in most cases. For example, the protogynous temperate wrasse, *Notolabrus celidotus,* does not initially reduce estradiol levels or aromatase expression

(both generally essential for female differentiation) as it begins to transition (Goikoetxea, Muncaster et al. 2021), whereas there are early decreases in estrogens in the transition of other protogynous species that have been proposed to directly cause transition (Nakamura, Hourigan et al. 1989, Bhandari, Komuro et al. 2003, Kobayashi, Horiguchi et al. 2010). Gene expression during this time has been most extensively studied in the black porgy due to its predictable age-related transition (Lee, Du et al. 2001, Wu, Dufour et al. 2021). However, as RNA sequencing has become more accessible, more species are beginning to be characterized, such as clownfish, the ricefield eel (*Monoperus albus*), orange-spotted grouper (*Epinephelus coioides*), and yellowfin seabream (*Acanthopargus latus*) (Casas, Saborido-Rey et al. 2016, Zhu, Zhang et al. 2021, Fan, Yang et al. 2022, Li, Li et al. 2023).

1.1.2 Simultaneous Hermaphroditism

Less is known about vertebrate simultaneous hermaphrodites than their sequential cousins due to their relative rarity. Simultaneous hermaphroditic species almost always reproduce through reciprocal outcrossing or sperm/egg trading (Avise 2011). An example of this would be *Serranus tortugarum*, the chalk bass. Matting in this species involves rapidly alternating between releasing eggs and sperm with their partner (Hart, Kratter et al. 2016). This use of both sexual roles is not universal. Some species, such as the blue banded goby, will only use one aspect of their hermaphroditic gonads in any given reproductive season (St. Mary 1993).

1.1.3 Kryptolebias marmoratus

K. marmoratus is a unique vertebrate hermaphrodite. Starting its life as a self-fertile simultaneous hermaphrodite before transitioning to be male means this species is neither protogynous nor protandrous (Harrington 1961). Its trigger for sex change is not predictable even for genetically identical fish reared alone in the same environmental conditions (Harrington 1967). *K. marmoratus'* rate of sex change varies in a heritable fashion across its geographic range leading to highly variable sex ratios in different populations (Turner, Fisher et al. 2006). This species' ability to self-fertilize makes its apparent sexual mode seem more similar to that of *Caenorhabditis elegans* than that of any other vertebrate (Harrington 1961). *K. marmoratus*'s combination of self-fertile hermaphroditism and unpredictable sequential hermaphroditism makes it a unique study system that is prime to help the exploration of vertebrate hermaphroditism.

1.2 Ecology & Biogeography

Kryptolebias marmoratus was first described by Poey in 1880 as a member of the Rivulus genus of New World killifish (order Cyprinodontiformes). Kryptolebias species can be found in mangrove forests from southern Florida, the Caribbean, Central America south to southern Brazil (Lin and Dunson 1995, Costa 2016). The exact ranges vary with how one distinguishes *K. marmoratus* from the other taxa that exist within its species complex (discussed below). In their mangrove habitats, they can be found within small ponds, log hollows, and other spaces with small crevices in which to hide, remain moist, and generally avoid the open water column (Harrington and Rivas 1958). Due to their location at the confluence of rainwater runoff and tidal sea water, shallow depth, and abundant decomposing organic litter, mangrove surface water varies greatly in salinity, is relatively low in dissolved oxygen, and high in sulfides (Marchand, Baltzer et al. 2004, Rossi, Tunnah et al. 2019).

Kryptolebias marmoratus has many adaptations that allow it to survive in this brackish and ephemeral aquatic landscape. One feature noted about these fish is their tolerance for a wide variety of salinity levels ranging from sea water to freshwater (Lin and Dunson 1995). The fish fare better in lower salinity environments (McCain, Kopelic et al. 2020) but are able to dynamically adjust their ammonia output and gill morphology to survive in a wide range of salinity and water quality (Grizzle and Thiyagarajah 1987, Rodela and Wright 2006, Rodela and Wright 2006). These adaptations allow these fish to survive outside of water for up to 3 months, depending on humidity (Grizzle and Thiyagarajah 1987, Frick and Wright 2002).

With these adaptations and their self-fertility, it may be that these fish make great colonizers. They can adapt to a wide range of salinity, water quality, and even lack of water entirely, allowing them to live in a wide range of habitats. They also do not need a mate to found a new population. In addition, as previously mentioned, *K. marmoratus* hermaphrodites have the ability to change sex, from self-fertile hermaphrodite into a fertile male (Harrington 1971). This can allow outcrossing after an initial period of effectively clonal growth (Turner, Fisher et al. 2006, Avise 2011) Temperature extremes do limit their range, as mangroves cannot tolerate freezing, and both extremes affect *K. marmoratus* reproductive development in ways that hinder their self-fertility (Turner, Fisher et al. 2006).

1.3 Reproductive physiology and Development

Being self-fertile hermaphrodites, a natural question that was asked was how the physiology of *K. marmoratus* functions with both male and female gametes in production simultaneously. One of the first things noted about *K. marmoratus's* self-fertility was that hermaphrodites fertilized their eggs internally, while males fertilized eggs externally (Harrington 1967, Harrington 1971). It was hypothesized that morphological differences between hermaphroditic and male sperm may underlie this difference (Kweon, Park et al. 1998). Consistent with this hypothesis, co-housed hermaphrodites cannot use their sperm to fertilize other hermaphrodite's eggs (Furness, Tatarenkov et al. 2015). However, whether this is due to behavioral or gametic factors, or both, is unclear.

The gonads of the hermaphrodites in young fish show only signs of female development. Both histologically apparent testes and measurable expression of the male-promoting genes *dmrt1* appear simultaneously at about 25 weeks after hatching (Qu et al. 2020). In the Haag Lab *K. marmoratus* colony, this corresponds with the age at which self-fertile reproduction is initiated, indicating that testis formation occurs only shortly before adulthood. Mature hermaphrodites have both testis and ovary tissue adjacent to each other, with testis generally found alongside the oviduct (Sakakura, Soyano et al. 2006). The balance between testis and ovary can be manipulated via temperature during the larval stage. Low temperatures promote maleness and testicular development and are sufficient to produce individuals that completely skip the hermaphrodite stage and develop directly into males at adulthood (Harrington 1967, Turner, Fisher et al. 2006). Conversely, high temperatures during development suppress self-fertility by inhibiting the development of testis making their gonads more ovarian (Park, Kim et al. 2017). It remains unclear how relevant these laboratory temperature manipulations are for natural sexual development, however.

The factors influencing sequential hermaphroditism in the field are not as well understood. Various populations can range from one to twenty-five percent male in the wild (Cole 1997). Common garden experiments demonstrated that genetic differences between populations influence the variation in sex ratios (Turner, Fisher et al. 2006), but epigenetic factors may also play a role (Ellison, Rodríguez López et al. 2015). While all individuals with the stereotypical bright orange male coloration with black tail bar

(**Figure 1.1**) have complete testis (Soto 1994), what have been described as cryptic males that lack the male patterning yet still have been complete testis have also been described (Marson, Taylor et al. 2019).

Figure 1.1: Hermaphrodite (top) and Male (bottom) *Kryptolebias marmoratus*. These adult specimens are approximately 4 cm in length. Note the prominent ocellus (eye spot) on the dorsal tail region of the hermaphrodite, and the orange pigmentation specific to the mature male. Many males also have a black bar on the ventral margin of the tail fin.

The hormonal profile of *K. marmoratus* hermaphrodites is of interest, as both male and female developmental pathways are active simultaneously. Gonadotrophin releasing hormone (GnRH) appears to stay at high expression until sex change to maleness occurs, at which point its presence drops substantially (Rhee, Seo et al. 2008). In addition, when juveniles are dosed with exogenous methyl-testosterone (MT), they skip the hermaphrodite stage and become primary males (Kanamori, Yamamura et al. 2006). The converse, dosing of juveniles with estradiol, is not as effective. While there is a slight apparent disruption in testis development they still become self-fertile and maintain their ability to transition to maleness later in life (Voisin, Kültz et al. 2019). In addition, expression of aromatase (which converts androgens to estrogens and is generally needed for female development) is relatively low in juveniles, but gradually increases throughout adulthood (Lee, Williams et al. 2005).

1.4 Population genetics

Since the discovery of their self-fertility, populations of *K. marmoratus* have been known to be nearly entirely clonal. This was first confirmed with a grafting test by Harrington and Kallman (1968). Tissue from one fish was grafted onto another from the same population and it wasn't rejected, showing that the self-recognition systems used to identify self from non-self could differentiate between members of the same population. This was eventually confirmed using polymorphic DNA microsatellite markers (Mackiewicz,

Tatarenkov et al. 2006), although males allowed for outcrossing in populations that had them (Tatarenkov, Lima et al. 2017). In the near-clonal *K. marmoratus* populations, microsatellite mutation rates are significantly higher than other Cyprinodontiforms (Turner, Davis et al. 1992).

While males do allow gene flow, they only fertilize a small portion of eggs. Lab experiments have shown that pairs in breeding tanks only produce about six percent cross progeny (Mackiewicz, Tatarenkov et al. 2006, Mackiewicz, Tatarenkov et al. 2006). These laboratory rates appear to be similar to crossing rates in the wild (Taylor, Fisher et al. 2001, Mackiewicz, Tatarenkov et al. 2006, Mackiewicz, Tatarenkov et al. 2006). Sex ratios, as previously mentioned, are set by the probability of sex change. The ratios can range from one to twenty-five percent (Cole 1997) and the sex change rates are under at least some genetic control (Grageda, Sakakura et al. 2005).

Karyotyping shows that *K. marmoratus* has 24 chromosome pairs, similar to other *Rivulus* species (Scheel 1972). Recently, genome assemblies have been made to the linkage group level for each chromosome (Kelley, Yee et al. 2016, Rhee, Choi et al. 2017).

1.5 Research and Model usage

Given the near clonal nature of *K. marmoratus*, it has become a well-used study system for many research fields that wish to control for any genetic variation in their studies. The two main fields that have taken advantage of this are behavioral research and toxicology. Studies of aggression and competitive behaviors can attribute individual differences to past experience and not any intrinsic genetic disposition (Earley, Hsu et al. 2000, Earley and Hsu 2008, Hsu, Lee et al. 2008). Other studies have employed *K. marmoratus'* clonality to measure the effects of various toxicological agents, like nonylphenol and ethinyl oestradiol (Tanaka and Grizzle 2002, Lee, Raisuddin et al. 2008, Johnson, Weinersmith et al. 2016). The ability to have a vertebrate species that is immune to inbreeding depression and able to control for genetics, has been a great boon for these fields, and could be exploited in others.

1.6 Taxonomy & Sister species

The taxonomy of the sister species of *K. marmoratus* has a complex history, due to shifts in species and genus names. Originally *K. marmoratus* was described as *Rivulus marmoratus* (Poey 1880), and it had a closely related sister species *Rivulus ocellatus* (Hensel 1868). These two species would only become of

scientific interest after almost another hundred years, when Harrington (1958) discovered self-fertility in this clade. From there, three species were identified and defined in what is known as the *R. marmoratus* species complex, *R. marmoratus, R. ocellatus, and R. caudomarginatus,* with *R. caudomarginatus* being an outgroup to the two sister species (Lin and Dunson 1995). Eventually these and a few other species were shifted into their own genus, Cryptolebias/Kryptolebias (Costa 2004a, Costa 2004b). Subsequently, it was noticed that the taxon that was being called *Kryptolebias caudomarginatus* was actually the same non-selfing species that Hensel had originally described as *R. ocellatus* (Costa 2006, Costa 2011), with the latter species name thus having precedence. After 2011 the obligate outcrosser *K. caudomarginatus* thus took the name *K. ocellatus,* while the self-fertile hermaphrodite species that had been known up to that point as *K. ocellatus* was renamed as *K. hermaphroditus*. From this point onward this review will call the species by their current taxonomic names (**Figure 1.2).**

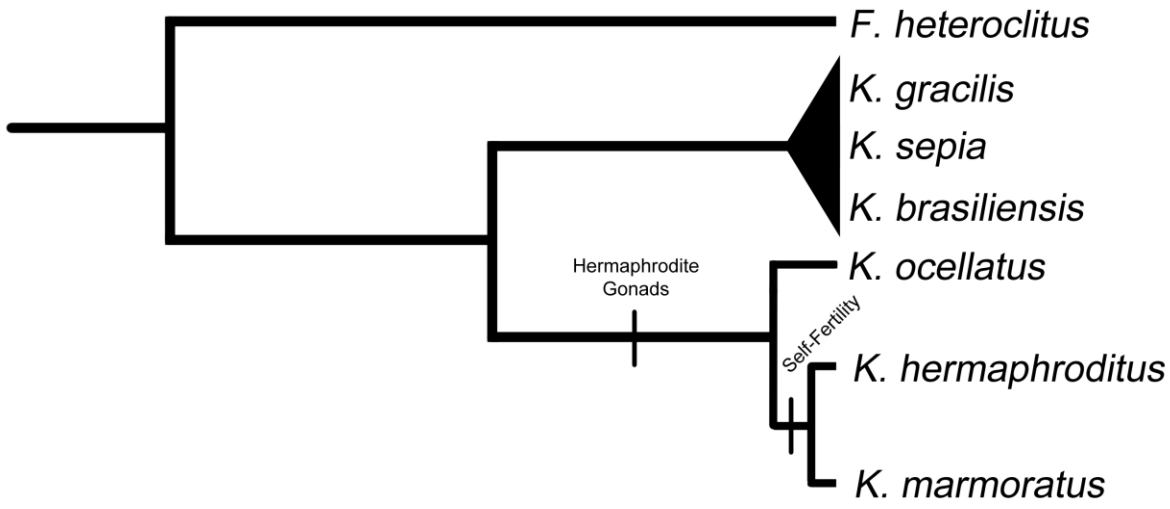

Figure 1.2: Cladogram of *Kryptolebias marmoratus* species complex, non-hermaphroditic Kryptolebias, and the temperate killifish *Fundulus heteroclitus* as an outgroup. Adapted from Costa et al 2017; Vermeulen and Hrbek 2005; and Berbel-Filho, Pacheco et al. 2022. Relationships within non-hermaphroditic *Kryptolebias* have yet to be resolved.

Kryptolebias hermaphroditus is the only other self-fertile hermaphroditic vertebrate species (Tatarenkov, Earley et al. 2012). Similar to *K. marmoratus*, its populations are highly clonal. Males were long thought to not exist in *K. hermaphroditus,* but some were eventually identified in Brazil, the likely source of the

genus' radiation (Berbel-Filho, Espírito-Santo et al. 2016). It is currently unknown whether male *K. hermaphroditus* develop, through sequential hermaphroditism, as in *K. marmoratus,* or as primary males specified by an unknown mechanism during larval life.

While many people identify *K. hermaphroditus* and *K. marmoratus* as separate species, the existence of natural hybrids between them suggests that one could regard them as two lineages of *K. marmoratus* (Costa, Amorim et al. 2017, Tatarenkov, Lima et al. 2017). More recently, researchers have begun to refer to a *K. marmoratus* species complex to acknowledge this ambiguity (Tatarenkov, Lima et al. 2017).

Kryptolebias ocellatus is the outgroup member of the *K. marmoratus* species complex. Unlike the other members of this species complex it is an obligate outcrossing (male-female or gonochoric) species functioning at Hardy-Weinberg equilibrium (Costa 2006). Calling the egg producing members of the species female may be not completely accurate, however. These individuals still have testis tissue within their ovary although that testis apparently isn't able to produce functional sperm (Tatarenkov, Lima et al. 2009)

The members of the species complex have one more feature that connects all of them: they are all interfertile with one another. While mitochondrial sequencing can recover the various species as distinct (Tatarenkov, Lima et al. 2017), they have been found to hybridize in both the lab and in the wild (Tatarenkov, Earley et al. 2021, Berbel-Filho, Pacheco et al. 2022). Hybrid zones exist in areas where their territories overlap as depicted in **Figure 1.3** (Costa, Amorim et al. 2017).

1.6 Conclusion

There are still many unknowns in the *Kryptolebias marmoratus* species complex. How did its self-fertility evolve? How does the simultaneous presence of testis and ovary tissue in one body with a shared circulatory system not antagonize and inhibit one another? How can these three species with drastically different reproductive modes all be interfertile? *Kryptolebias marmoratus* is an odd fish, but its oddities are of great interest for various fields of research and a great boon to many others. In the subsequent

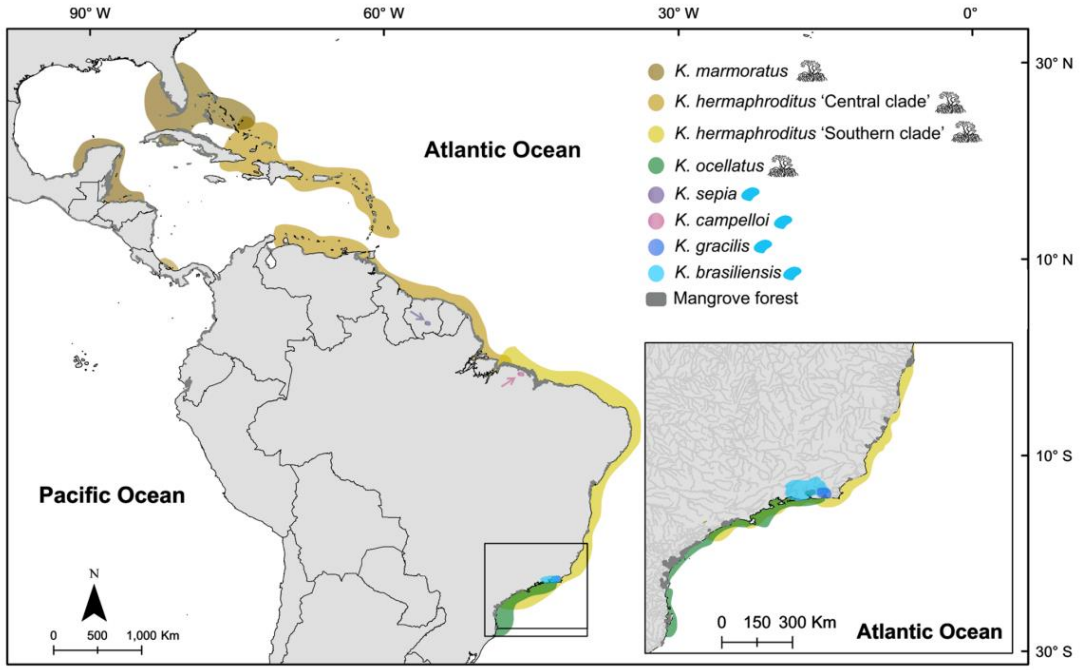

Figure 1.3: Geographic distribution of member of the Kryptolebias genus. Adapted from Costa 2016.

chapters, I explore hormonal aspects of *K. marmoratus* sexual plasticity (Chapter 2) and the genetics of sex determination and hybridization in a Brazilian population of *K. ocellatus* (Chapter 3).

Chapter 2: Hormonal manipulation of sex in adult *K. marmoratus*

2.1 Introduction

Development of different sexes within a species poses a tricky problem. Sexes share the majority or all of their genomes but can have drastically different morphology and behaviors to promote their sexual roles. There are many different ways that species initiate the process of sex-specific development, but some genetic factors that manage it have been conserved (Zarkower and Murphy 2022).

Sexual development in vertebrates is generally guided and reinforced by circulating sexual steroid hormones (Navara 2013). In the teleost fishes, like other vertebrates, sex hormone synthesis runs through the brain/pituitary/gonad axis (**Figure 2.1**). Gonadotropin releasing hormone (GnRH) is produced by the hypothalamus and subsequently binds receptors in the pituitary gland. This triggers the release of luteinizing hormone (LH). LH circulates through the bloodstream to the gonad to trigger the production of

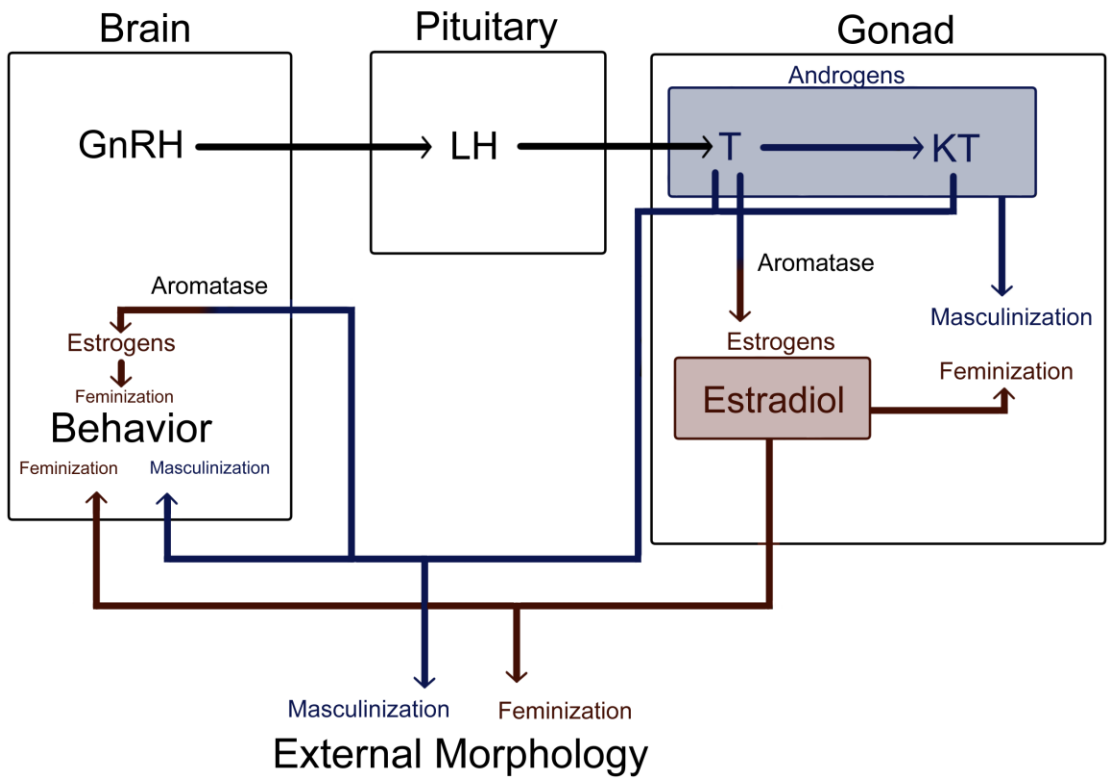

Figure 2.1: Simplified Brain/Pituitary/Gonad axis of vertebrate sex hormones. Modified from Tovo-Neto et al 2018, Baroiller et al 1999, and Liu et al 2017.

sex steroids. The final step in the production of estrogens is the aromatization of androgens by aromatase (a cytochrome P450 enzyme). This can occur in either the gonad or the brain, as aromatase is expressed in both. Not all androgens can be aromatized into estrogens. For example, 11-keto-testosterone (KT) is an un-aromatizable androgen that is produced directly from the aromatizable testosterone. KT has been shown to be a highly active androgen in teleosts apparently binding to one of the two androgen rectepors found in the clade (arβ) (Olsson, Berg et al. 2005, Tokarz, Moller et al. 2015). This pathway is typically described as having alternative male (high androgen) or female (high estrogen) states. However, it is not as simple when both male and female tissue and cell types need to exist within the same body and circulating hormonal environment.

Hermaphroditism is the condition where single individuals exhibit female and male traits over the course of their life. Sequential hermaphrodites like the clown fish, *Amphiprion*, start their life as a specific sex, and after some cue will transition to the other (Iwata, Nagai et al. 2008). The cue can be social, environmental, or physiological (Avise 2011). No matter how the transition is triggered, the sexual determination pathways in the gonads are triggered to replace the production of one gamete type with the other.

Simultaneous hermaphroditism is when a single individual can produce both sperm and eggs within the same time period. This can manifest as the organism having ovary and testis functions separated in time and/or space, like in *Caenorhabditis elegans,* or having a gonad that contains both ovarian and testis tissue (L'Hernault 2009). Simultaneous hermaphroditism requires a balance or separation between the male and female developmental pathways (Avise 2011). While simultaneous hermaphroditism can be found in many organisms, the rarest version is self-fertile hermaphroditism. Self-fertile hermaphrodites can fertilize their eggs with their own sperm. Within plants this is relatively common with about 62-84% of temperate and 35-70% of tropical hermaphrodites regularly self (Jarne and Charlesworth 1993). In animals it is rarer, with marine invertebrates having the highest rate of hermaphroditism. Even in these, however, only about 38% show any signs of selfing (Jarne and Charlesworth 1993). Nevertheless, organisms such as *Arabidopsis thaliana* and *Caenorhabditis elegans* are common laboratory systems that were chosen over strictly outcrossing relatives in part because they are self-fertile. This makes

genetic studies easier, since a founding heterozygote can produce homozygous offspring, and they are naturally resistant to inbreeding depression (Tang, Toomajian et al. 2007, Thomas, Woodruff et al. 2012).

Kryptolebias marmoratus, the mangrove killifish, is found from the north coast of Brazil northward through Central America and the Caribbean, and as far North as the southern tip of Florida (Harrington 1961). This species is notable as it is both a self-fertile simultaneous hermaphrodite and a sequential hermaphrodite (Harrington 1961, Harrington 1971, Avise and Tatarenkov 2015). The hermaphrodite is drab in color, similar to females of related species. Its gonads are structured primarily as ovary, but with small pockets of testis tissue along the oviduct (Harrington 1961, Kanamori, Yamamura et al. 2006). While this testis enables hermaphrodites to internally fertilize their own eggs, it appears that hermaphrodites are unable to outcross with other hermaphrodites (Hee, Eun et al. 1998, Furness, Tatarenkov et al. 2015).

In most strains of *K. marmoratus,* a minority of adult hermaphrodites change sex into males. After sexual transition to males, their gonads are entirely testis, their pigmentation and behavior become similar to that of males of gonochoric relatives, and they gain the ability to externally fertilize the rare unfertilized eggs laid by the hermaphrodites.

Given that hormones circulate body-wide, how do *K. marmoratus* hermaphrodites manage to maintain stable, functional ovary and testis tissues simultaneously within the same body? How do they manage to break this balance to transition fully to males? From a research perspective, can masculinizing hormone perturbations initiate sex change in *K. marmoratus* when it would not otherwise occur? Prior research suggests they might. In gonochoristic or species of fish that have separate and distinct sexes, such as the Nile tilapia or the zebrafish, adult females can be irreversibly transformed into males by application of the aromatase inhibitor fadrozole (Takatsu, Miyaoku et al. 2013, Sun, Jiang et al. 2014). Similarly, adult females of the honeycomb grouper, a protogynous sequential hermaphrodite, can be pushed into premature maleness by application of 11-keto testosterone, or KT (Bhandari, Alam et al. 2006). Further, the synthetic androgen 17-alpha-methyl testosterone (MT) permanently masculinizes juvenile *K. marmoratus*, so that they skip the hermaphrodite to form functional males at sexual maturity (Kanamori, Yamamura et al. 2006).

The above precedents suggest two alternative hypotheses for how *K. marmoratus* control sex change, and how they will respond to masculinizing agents. First, hermaphrodites may harbor substantial between-individual variation in testis content. Since the somatic gonad is a major source of sex steroids (Tokarz, Möller et al. 2015), hermaphrodites bearing a greater initial testis endowment could experience a positive androgen feedback that leads to irreversible commitment to male fates. I term this the "androgen snowball" hypothesis. Alternatively, because they maintain mosaic male and female gonads for long periods, *K. marmoratus* hermaphrodites may be unusually resistant to ectopic androgen treatments or have other methods to recapture excess androgens and prevent their masculinizing effects. In this case, we expect either no effect or a reversible effect from these treatments. Like most teleost, *K. marmoratus* has two androgen receptors, ARα and ARβ, allowing the possibility for divergence in expression patterns and/or subfunctionalization (Lynch and Force 2000) in responses to androgens (**Figure 2.2**). I will show that while, androgens do lead to presentational and gonadal masculinization they are not sufficient to lead to full stable sex change without reversion. While the androgen snowball hypothesis is not fully supported there must be other factors necessary for complete sex change.

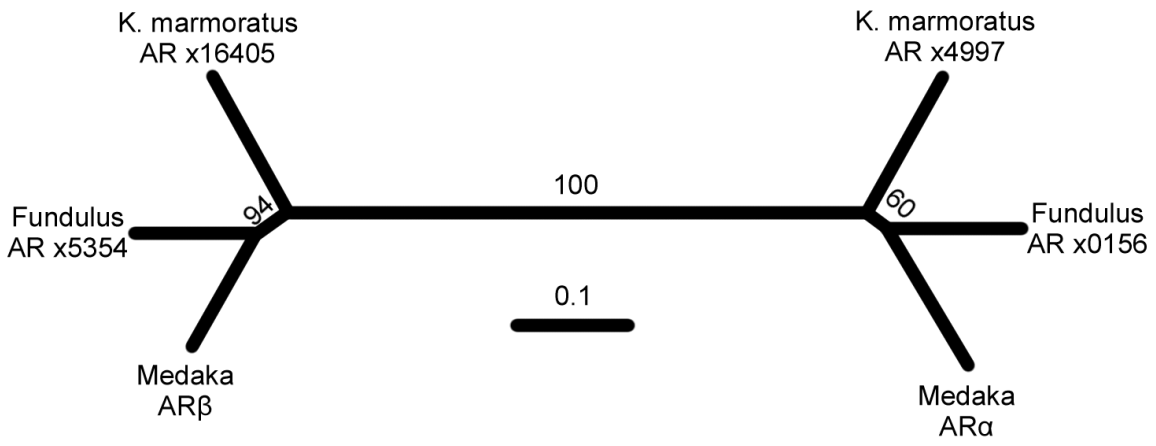

Figure 2.2: Androgen Receptors of select Teleosts. Neighbor-joining tree of full-length predicted androgen receptor (AR) proteins from *K. marmoratus, Fundulus heteroclitus, and Oryzias Latipes* genomes. (Ogino, Katoh et al. 2009, Whitehead 2009, Kelley, Yee et al. 2016). AR-α orthologs form a clade at the right, while AR-β orthologs are at left. Numbers of form "xN" are the final digits of the Ensembl gene prediction from which the protein is derived. Numbers at nodes indicate bootstrap support.

2.2 Methods

2.2.1 Fish care

K. marmoratus were raised individually in 750 ml of brackish water in 1 L food-grade polypropylene tanks (Rubbermaid). Water was prepared with distilled water supplemented with Instant Ocean Sea Salt at 12.5ppt. Light cycles were set at twelve hours on twelve hours off with water temperatures maintained between 26.5-28.5ºC. Fish were fed 6 days out of the week with *Artemia* brine shrimp nauplii and monitored daily for health and signs of external morphological changes. All husbandry procedures and animal protocols were approved by the UMD IACUC.

2.2.2 Hormone manipulations

Hermaphroditic adults were judged by presence of posterior ocellus and ongoing oviposition, lack of male-characteristic orange coloration and ventral melanin tail bars, and recent history of oviposition. Those that were of 2 years of age or older were selected, and their tanks were moved to a common shelf, isolated from the rest of the colony (though in the same room). After a complete water change, the water in their tanks was dosed with either MT to bring final tank concentrations to 100 ng/ml or 50 ng/ml, KT to 50 ng/ml, or the aromatase inhibitor fadrozole to 5 ng/ml. Hormone stocks were dissolved in DMSO at 40ng/ul and stored frozen (-20° C), and then diluted in culture water just prior to addition to tanks. Because the rate of hormone inactivation in the water was unknown and complete changes of water can be stressful, we implemented a partial replacement scheme: Every 10 days, half the tank's water was removed and replaced with fresh aquarium water and re-dosed to bring the concentration of the hormones back to their starting concentrations assuming all active hormones were metabolized or otherwise broken down. If instead hormone persisted with no loss, the day 10 change would elevate the hormone concentration to 1.5 times the target dose, and the second (day 20) to 1.75 times the target. The likely dose is between these two extremes, but it was not measured.

A total of forty-nine fish were used for this experiment. Thirty-nine fish in total were treated as above with the initial setup and partial exchanges on days 10 and 20. Twelve were treated with 100ng/ml of MT, nine were given the other three treatments each, and ten were used as controls. On day 30 the fish were removed completely from the hormone-treated water and returned to fresh aquarium water. Some fish

were euthanized while others were left to be observed for up to 60 days post-treatment. Untreated control hermaphrodites were chosen using the same criteria, and experienced water changes and colony position identical to hormone-treated fish.

2.2.3 Hormone immunoassays

The hormonal assays procedure is based on previous non-invasive hormonal assay methods (Scott, Hirschenhauser et al. 2008, Scarsella, Duque et al. 2016, Houslay, Earley et al. 2019, Houslay, Earley et al. 2022). Fish treated with fadrozole were placed in 500 ml of clean water for 30 minutes immediately following the end of their 30-day exposure. After thirty minutes the fish were returned to their prior containers, now without the fadrozole, and the water they were incubating in was filtered using Whatman grade 1 filter paper to remove large particulate and then adjusted to pH 3 using formic acid.

One Waters C18 500mg solid phase extraction column was primed for each fish by flushing it with two washes of 2 ml HPLC grade methanol followed by two washes of 2 ml of ultrapure water. The hormones were then extracted from the water samples using a vacuum pump and passing through the column. The columns were then purged with a 2ml wash of ultrapure water to remove extra salt. The hormone was then eluted with two washes of 2 ml HPLC-grade ethyl acetate. The elution solvent was evaporated at 37C with a gentle stream of nitrogen gas passing over the samples though an evaporation manifold. The hormones were then resuspended using the proprietary buffers provided in the Cayman Chemical ELISA kits for testosterone (#582701), KT (#582751), and estradiol (#501890) following the protocol provided in said kits. Hormone levels were measured in a BioTek Epoch plate reader via a regression compared to standards.

2.2.4 Histology

Fish were euthanized with 0.2% of bicarbonate-buffered tricaine (MS222) in culture water, followed by decapitation. The visceral mass of the fish including gonad and intestines were immediately fixed using Bouin's fixative for 24hrs, followed by a 24hr bath of 70% ethanol to remove excess fixative. The tissue was then exposed to increasing concentrations of ethanol up to 100% and then cleared with Histoclear II (*Electron Microcopy Sciences)*. The tissue was embedded in paraffin, sectioned at 8 µm thick parallel to the dorsal-ventral body axis, and bound to positively charged slides (Fisher Permafrost Plus) using a slide

warmer. Most slides were stained either using the hematoxylin and eosin protocol detailed in *Animal Tissue Techniques 4th Edition*, or the toluidine blue protocol from the same source (Humason 1979).

2.2.5 Image Analysis

Stained sections were photographed under 3.15x magnification using a Zeiss AxioCam digital camera and Zen Blue microscope imaging software. If the gonad was larger than the view field, then multiple images were composited together using the Zen Blue live panorama function to get a complete image of the tissue. Total cross-sectional area of the section as well as area of testis, oocytes, oviduct, and amorphous/discolored tissue were measured using ImageJ (Schneider, Rasband et al. 2012). Testis was determined by the presence of highly nucleated tissues with little cytoplasm along the edge of the oviduct as is characteristic of testis in the species. Oocytes were larger cells as well as cells with large nuclei. The oviduct was the open tube that ran laterally down the ovotestis. Amorphous and discolored tissue were tissues that were pigmented through melano-macrophage or showed signs of degradation and lack of cellular structures.

The functional area of a gonad section was defined as the total cross-sectional area minus the area taken up by the oviduct lumen using imagej lasso tool (Schneider, Rasband et al. 2012). Testis was characterized by a brighter blue color with many densely packed nuclei. Ovary was measured as the area of oocytes that are darker blue to purple and circular. The smaller oocytes are smooth and can have a clear nucleus while the larger ones have more web like interiors. Discolored tissue was characterized as tissue with melanin like pigmentation. A section's proportions of testis, ovary, and amorphous/discolored tissue were calculated by dividing their areas by the functional area of the gonad. To allow direct comparisons between fish and groups of fish, we normalized the cross-sectional areas for each tissue type for differences in body size. The normalization factor was found by squaring the ratio of the total body length of a focal fish to the average length of all *K. marmoratus* individuals used in this study. This factor was then applied to the total areas of each tissue type for each fish. All treatment groups were compared by each trait individually using a Kruskal-Wallis rank sum test. This was followed by a Dunn's post hoc test with p-values adjusted using the Benjamini-Hochberg method with a false discovery rate of 0.05 (Benjamini and Hochberg 1995).

2.3 Results

2.3.1 Gonad anatomy of untreated fish

Understanding the variation present within untreated control hermaphrodites is necessary to be able to analyze the effects of the various hormones and hormone modifiers. All untreated hermaphrodites that were examined had both ovarian and testis tissue, while maintaining the stereotypical brown hermaphrodite coloring (**Figure 2.3A**). However, sites of testis tissue were not bilaterally symmetrical in their anterior/posterior position (**Figure 2.3B-C**). In addition, there was no clear trend of testis or ovary

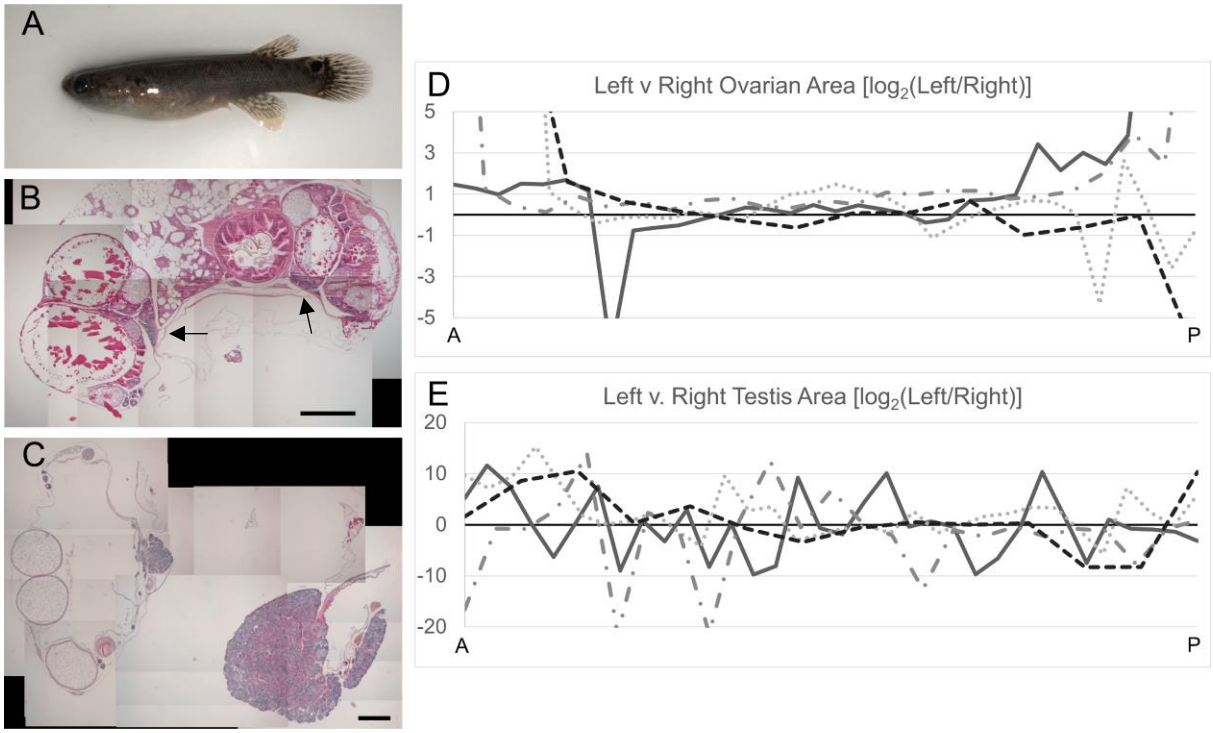

Figure 2.3: Variation in sex allocation in the *K. marmoratus* hermaphrodite gonad. A. External view of adult hermaphrodite (~4 cm long), representative of those used for both gonad comparisons and hormone treatments. B & C: Sections of gonads from separate hermaphrodites, showing the extreme range of testis content. Scale bar = 1mm. B: Largely ovarian section, with small testis pockets indicated with arrowheads. C. A mostly testicular section, with one gonad (right) completely testis, and the other (at left) with mature oocytes (o) and only a small area of testis (arrowhead). D&E: The $\log_2$-transformed fraction of ovarian (D) or testis (E) tissue in the left and right gonads of five separate individuals. Each line represents a separate individual. Anterior of the fish is on the left while posterior is on the right.

localization from anterior to posterior between various hermaphrodites (**Figure 2.3C-D**). Untreated post-transition fish (i.e., secondary males) with bright orange coloration were also examined. All that were

examined had complete testis, with clearly distinguishable spermatocytes and spermatogonia (**Figure 2.4B**).

2.3.2 Impacts of MT on hermaphrodites

Externally, all 21 MT-treated fish from both the 50ng/ml and 100ng/ml groups acquired an orange tint to their coloration as well as a black bar on their tail fin that is characteristic of males (**Figure 2.4**). The tail

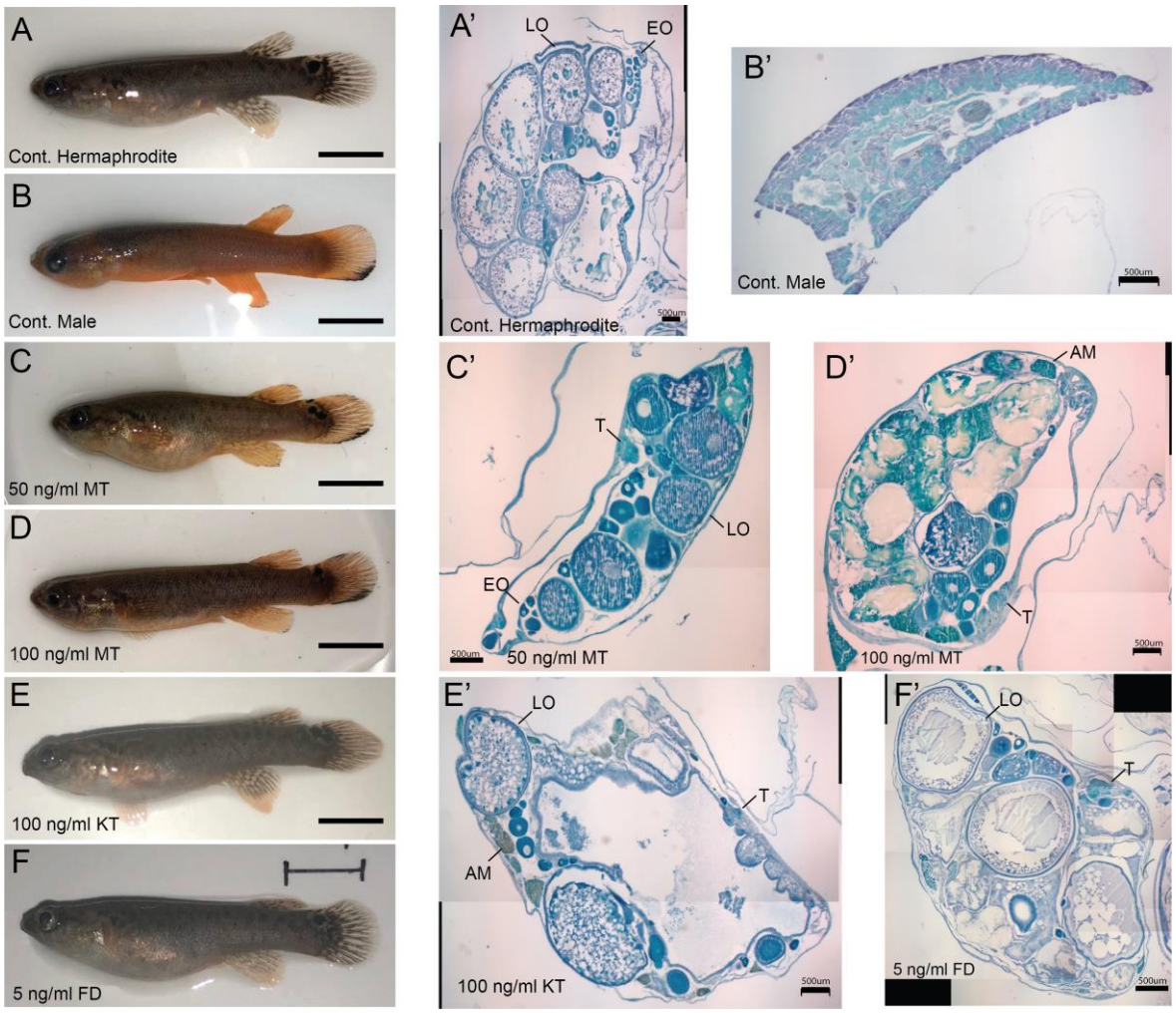

Figure 2.4: External and internal impacts of exogenous androgen administration. A-F: External coloration of fish after 30 days of the indicated treatments. Scale bar = 1 cm. A'-F': Histological sections of gonads of fish from each treatment, all stained with toludine blue. Various gonad elements are labeled. T=Testis; EO=Early Oocytes; LO=Late Oocytes; AM=Amorphous/Melanized tissue. Scale bar = 500um

bar appeared between the sixth and tenth day of exposure to MT, while the orange tint would only start appearing by the fifteenth to twentieth day. Fish treated with 50 ng/ml MT lost their coloration and

resumed laying viable self-fertile offspring within thirty days after removal of the hormone. Those treated with 100 ng/ml MT maintained their orange coloration for over 60 days after removal from the hormone, but it faded by day 120, and most began to lay eggs again (**Figure 2.5**). Their tail bars persisted for up to 6 months post treatment.

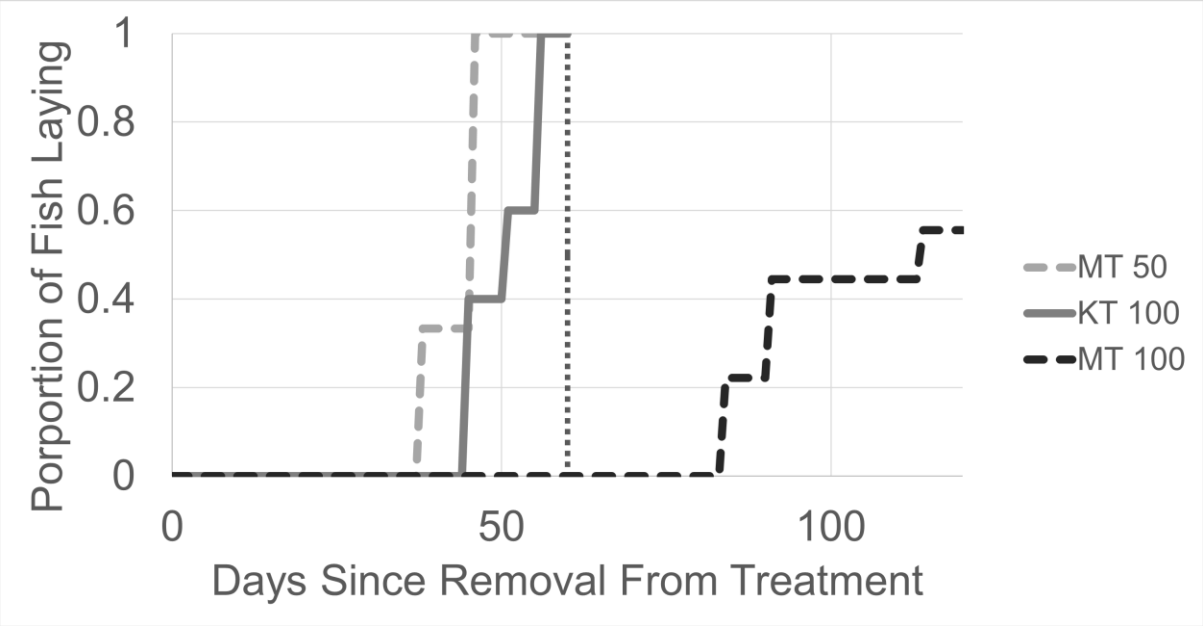

Figure 2.5. Recovery of self-fertility in post-MT hermaphrodites. Vertical dotted line represents day 60 when the majority of individuals were euthanized. MT 50 (N=9); MT100 (N=9); KT100 (N=5).

Internally, the gonads of fish treated with 100 ng/ml of MT showed a range of effects. There was a significant decrease in normalized cross-sectional area, ovary percentage, and oocyte number, while both testis and amorphous/melanized tissue took up a significantly higher proportion of the gonadal volume compared to controls (**Figure 2.6**). Based on studies of other teleost fish, the pigmented tissue is likely an indicator of oocyte atresia (Blazer 2002) and the presence of melano-macrophages (Kumar and Joy 2015). Despite its ability to induce external pigment changes, 50ng/ml of MT did not significantly change any gonad trait other than oocyte count versus the controls.

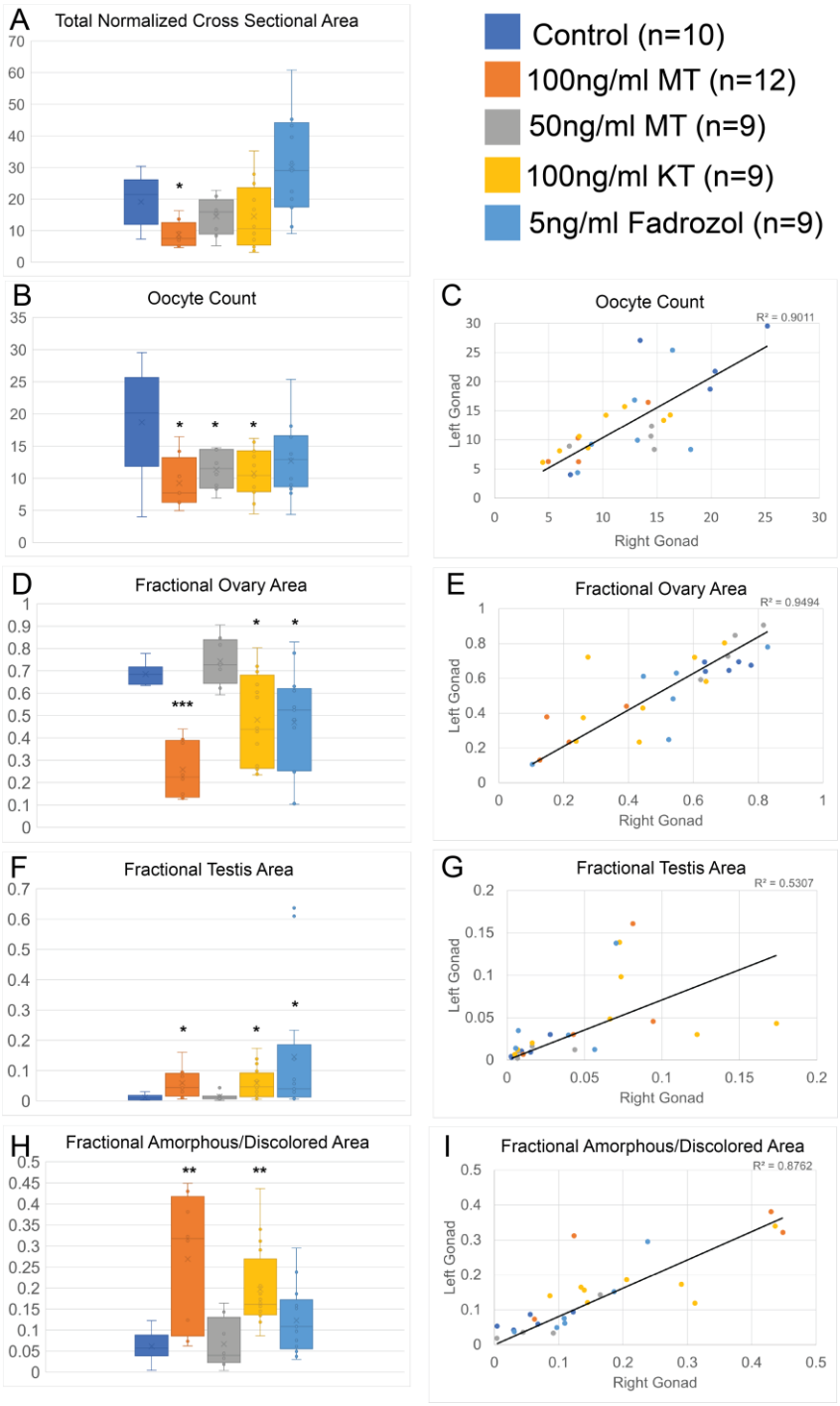

Figure 2.6: Impacts of hormone treatments on gonad histology. A: Total gonad cross-sectional area, normalized for total fish size. B, D, F, & H: Average area per section of gonad arm for the category given. C, E, G, & I: Scatter plot of the relationship between the left and right gonad arms within the same individual. Line is a linear regression of all treatments with R^2 value listed in top corner. B&C: average oocyte count of every stage between early and late. D&E: Percent of gonad volume that is taken up by oocytes. F&G: Percent of gonad volume that is testis. H&I: percentage of gonad that is taken up by amorphous/discolored tissue. P-values compared to control *<0.05; **<0.005; ***<0.0005.

2.3.3 Impacts of KT and fadrozole on hermaphrodites

As MT treatments failed to trigger irreversible sex change, we conducted follow-up studies with the natural androgen, KT, and the aromatase inhibitor, fadrozole. Fadrozole is not a hormone but its ability to competitively bind to and inhibit aromatase production of estrogens means its introduction should promote androgen production while simultaneously inhibiting estrogen production. Neither exogenous KT nor fadrozole triggered any noticeable external coloration change in the fish during or after exposure (**Figure 2.4**). Nevertheless, fish treated with exogenous KT had significantly less ovary content and more testis and amorphous/discolored tissue content in their gonad than the control while the normalized gonad size wasn't affected (**Figure 2.6**). 5ng/ml of fadrozole increased fraction of testis tissue, but caused no changes in relative gonad size, ovarian or amorphous tissue content (**Figure 2.6**).

Because it was not clear how the sex steroid profile of a simultaneous hermaphrodite would react to aromatase inhibitor, we sought to measure it directly using hormone immunoassays. 5ng/ml of fadrozole led to a decrease in both endogenous testosterone and estradiol in the fish, while KT was not changed (**Figure 2.7**).

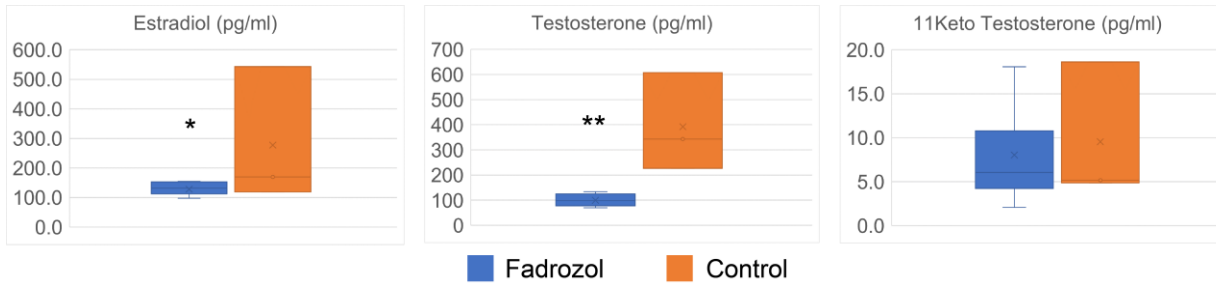

Figure 2.7 Impact of aromatase inhibition on sex steroids. Terminal hermaphrodites treated with 5ng/ml of fadrozole (N=7) or untreated control individuals (N=3) were compared. Estradiol, Testosterone, and 11Keto-Testosterone concentrations were derived from ELISA tests of water in which live, post-treatment fish were incubated for 30 min. P-values *<0.05; **<0.005.

2.4 Discussion

2.4.1 Inadequacy of the "androgen snowball" hypothesis

We hypothesized that the amount of testis, and the androgen levels this presumably dictates, varies between individuals. If testis development is sensitive to androgen levels, as indicated by previous studies (Kanamori, Yamamura et al. 2006), this would cause positive feedback between androgen levels

and masculinization of the gonad to drive sex change (**Figure 2.1**). The initial observation of heterogeneity of testis content across individuals (**Figure 2.3**) is consistent with that. The variation of gonadal content seen in *K. marmoratus* hermaphrodites mirrors the similarly variable timing of sex change in even genetically identical fish reared in a common environment (Davis, Taylor et al. 1990). This can be extended to within-individual heterogeneity between the left and right gonad, indicating there is a stochastic component to both the initial formation and subsequent elaboration of testis. However, in subsequent experiments a simple positive feedback model does not seem to be supported.

The treatment with MT was inspired by the finding of Kanamori et al. (2006) that MT was capable of completely masculinizing juveniles. The highest dosage of MT given in my experiments (100 ng/ml) was at least four times the levels sufficient for juvenile masculinization, and the duration of exposure was three times as long. While external pigmentation did become male-like (**Figure 2.4**), and there were signs of masculinization in the gonad (**Figure 2.6**), this high dose was unable to produce a full male. Furthermore, such treated males lost male pigmentation and transitioned back to self-fertility (**Figure 2.5**). Lowering that dose to 50ng/ml, still twice the level sufficient for juvenile masculinization, induced almost no signs of gonad masculinization, with a drop in oocyte count being the only significant change (**Figure 2.6**). However, this lower dose of MT was capable of triggering male coloration externally. The ability of exogenous MT to alter pigmentation without similar masculinization of the gonad may reflect direct action of hormone on androgen receptors in skin pigment cells that is independent of the endogenous brain-gonad axis. However, it is possible that our histological assays simply fail to capture gonad changes that are required for pigment masculinization.

The incomplete and reversible MT-triggered masculinization led us to look at other androgens and hormone modifiers. KT was an obvious next choice, as it is unable to be aromatized into an estrogen and it has high activity in teleost fish (Borg 1994). Disrupting the activity of aromatase with fadrozole was also used, as it was expected to both increase androgen levels (by blocking their conversion) and to lower estrogen levels (**Figure 2.1**). This could give a more natural sex change if lowering levels of estrogen were more impactful than increasing androgen levels. Both of these approaches have been able to masculinize other teleosts, (Bhandari, Higa et al. 2004, Kroon, Munday et al. 2005). However, neither KT

nor fadrozole triggered full sex change in my experiments. Both treatments showed some facets of masculinization in the gonad but failed to fully masculinize any individual (**Figure 2.5, 2.6**).

Teleosts are known to have 2 androgen receptors, ARα and ARβ (Ogino, Katoh et al. 2009; Figure 2.2). They have divergent responses to androgens (Ogino, Tohyama et al. 2018). As expected from their expression in the gonad of Japanes eels (Todo, Ikeuchi et al. 1999), and from mouse knockouts (Yeh, Tsai et al. 2002, Chang, Chen et al. 2004, De Gendt, Swinnen et al. 2004, Holdcraft and Braun 2004), zebrafish androgen receptors are necessary for normal testis development and spermatogenesis(Tang, Chen et al. 2018). Surprisingly, however, this requirement is not true of all teleosts. In medaka (*Oryzias latipes*), XY fish with single and double androgen receptor knockouts have normal male gonads and sperm. While these receptors seem to have lost their role in testis development and spermatogenesis, they each have important, and different, roles in masculine behaviors and morphology (Ogino, Ansai et al. 2023). In *K. marmoratus,* a similar subfunctionalization may explain the differences in coloration with KT reacting strongly with one receptor and not the other.

None of the above results are fully consistent with the apparent roles of androgen receptor in *K. marmoratus,* given MT is both sufficient to masculinize juveniles (Kanamori, Yamamura et al. 2006), yet insufficient in adult hermaphrodites. While *K. marmoratus* androgen receptors have a role in early testis specification and spermatogenesis that is more similar to mammals and reptiles, the androgens may be functioning on the gonad indirectly, and may change their levels with age. This suggests the difference in effects of KT and MT may be due to different responses of the two androgen receptors, with MT acting directly on the skin pigmentation and the gonad, while KT only binds to receptors in the gonad.

Fadrozole treatment reduced secreted estradiol levels, as expected, but also led to a simultaneous drop in secreted testosterone. This unexpected finding may be due to a negative feedback loop that prevents the over expression of testosterone when estrogen production is interrupted. The fact that 11Keto-Testosterone can be synthesized directly from testosterone may also be a factor since KT levels were not affected even as testosterone had significantly dropped. Overall, fadrozole exposure did not shift secreted sex steroids in a consistently masculinizing direction. A limitation of this result, however, is that our hormone assay is indirect, and may not precisely reflect serum steroids.

All of this together is inconsistent with the androgen snowball hypothesis being the sole sex change mechanism. Hormonal imbalance may still be able to fully masculinize with an even higher dose or longer exposure time. However, given that substantial shifts toward testis fail to initiate full sex change, it appears something else must happen in conjunction with testis growth and hormonal changes to sustain the masculinization process. There may be a missing unknown signal that regulates a pathway upstream of gonad sex that is needed in addition to hormone shifts to allow complete masculinization. This potential trigger for a change could help explain the variation of sex change timing within *K. marmoratus* strains (Sakakura and Noakes 2000, Mackiewicz, Tatarenkov et al. 2006, Costa 2016).

When monitoring testis development after hormone-mediated masculinization, there appears to be a point where either the left or right gonad develops testis at a rapid rate, while the other maintains a primarily ovarian character (**Figure 2.6G**). The percent of variance in the testis content of one gonad that is explained by the other (R^2 value) is dramatically lower than that of other metrics, and especially for the fish with elevated testis content. This indicates each gonad behaves somewhat independently as masculinization progresses. Consistent with this, one overtly hermaphroditic control fish examined had a complete testis on one side, and an ovo-testis on the other (**Figure 2.3C**). If hormone-mediated sex change is in some way mirroring natural sex change, then this may suggest that it is testis that is leading and causing the asymmetry initially.

2.4.2 Gonad and External Phenotype Discordance

The de-synchronization between gonad sex allocation and external coloration as seen in the fadrozole and KT treated individuals helps clarify some apparent contradictions about how *K. marmoratus* males develop. We observed that all unmanipulated individuals with male coloration have complete bilateral testes (Figure 1.3B; Costa 2016). However, MT-treated fish had external masculine coloration even with the 50 ng/ml dosage, which barely had any gonadal masculinization. Further, both KT and fadrozole treated fish were partially masculinized in their gonads but had no external coloration change (**Figure 2.3**). This suggests that during natural sex change, there may be a required level of circulating androgens for coloration change that is only reached with complete gonad masculinization. This would line up with the discovery of cryptic male *K marmoratus* where individuals have full testis but lack the stereotypical

male coloration (Marson, Taylor et al. 2019). These individuals might be secondary males whose gonads have completed transition but who's hormonal composition has not fully transitioned to that of a male. Thus, male coloration could potentially be described as an androgen mediated secondary effect of gonad physiology.

2.4.3 Tentative model of K. marmoratus sex change

Based on my results and those of others, I propose a tentative model for the order of events in natural transition of *K. marmoratus* hermaphrodites into males:

Step I: Transition commitment signal. An unknown factor commits a fish to begin the transition process. As sibling fish from inbred lines reared in isolation still vary, this signal is unlikely to be genetic or social. A modest elevation of testis content within the gonad is not sufficient to induce it. Levels of cortisol or of other hormones in the brain may change with age, and if so this would provide clues as to the nature of this currently unknown signal. Another protogynous fish, blue-headed wrasse, triggers sex change though changes in stress and cortisol levels (Perry and Grober 2003). Removal of the dominant male reduces cortisol in larger females, in which transition is de-repressed. *K. marmoratus* may work similarly, and measuring cortisol levels throughout their life might be able to predict sex change. The source of stress may include environmental factors, such as light levels or diet. However, all fish were kept in a 12-12 light-dark cycle and received equal feeding, yet we still see variability in sex change among individuals from highly inbred lineages.

Step II: Oocyte atresia. Both doses of MT induced significant reductions in oocyte number and ovarian area, and significant increases in amorphous and/or melanized tissue. Melanized ovarian tissue during oocyte atresia (resorption) has been described in other fish, and ascribed to melanomacrophage infiltration (Kumar and Joy 2015). This likely explains the significant drop in total gonad area in the high MT dose. My data suggest that an early impact of MT is loss of oocytes via a similar mechanism.

Step III: Gonad masculinization. In response to androgens, the gonads gradually develop testis. As shown in **Figures 2.3C** and **2.6G**, this can occur on rather independent timetables in each gonad of an individual, and to a great extent in the absence of external pigmentation changes.

Step IV: Secondary Secondary Sex Characteristics. After gonad remodeling is complete, endogenous androgen levels rise to levels sufficient to trigger formation of the ventral caudal fin tail bar, fading of the caudal ocellus, and concentration of carotenoids in the posterior skin. My treatments were sufficient to mimic this independent of endogenous gonadal steroids but faded when exogenous modulators were withdrawn, and gonadal masculinization ceased.

2.5 Conclusion

Gonad transition in *Kryptolebias marmoratus* can be partially controlled through hormone modification, but hormone exposure alone is insufficient to allow the complete transformation from hermaphrodite to male. This indicates the Androgen Snowball Hypothesis is inadequate, and that other factors must be at play for sex change to occur. The unpredictability in their sequential hermaphroditic transition between genetically identical individuals is also mirrored in the unpredictability in their gonadal content between individuals, and even between the left and right gonad arms of the same individual. All of this makes *Kryptolebias marmoratus* in a way “consistently inconsistent” in its sexual characteristics. Further exploration should focus on upstream (i.e. brain and pituitary) regulators of gonad development and the hormonal signaling pathways. One possible approach would be to compare GnRH and LH levels between juveniles of typical strains that form males rarely and slowly with those that form males early and often, as in the Twin Cays, Belize (Mackiewicz, Tatarenkov et al. 2006, Turner, Fisher et al. 2006). Longer-term androgen treatments may also be informative.

Chapter 3: Search for K. ocellatus Sex Chromosomes and Species Complex Hybridization

3.1 Introduction

3.1.1 Introduction

In sexually reproducing animals, individual organisms may either specialize in sperm production (males), in egg production (females), or produce both gamete types (hermaphrodites). With two or more sexual forms encoded by the same genome, there needs to be a way to reliably determine which sex an individual will be within the species. This sex determination can come in various forms. One version is Genetic Sex Determination (GSD), which is one of the better understood methods of sex determination. In GSD the presence, absence, or copy number of specific genes will determine if an individual develops as a male, female, or hermaphrodite (Beatty 1970). The specific genes can vary from lineage to lineage but the core function of determining sex is shared between all (Nagahama, Chakraborty et al. 2021). Other organisms employ Environmental Sex Determination (ESD), where there are no genetic differences between the various sexes. Sex is instead determined by external factors such as temperature during incubation, social status, age, or some other factor outside of genetic sequences (Bull 1983).

GSD and ESD are not mutually exclusive, as species can have genotypes that will influence the sex of an individual, but still leave them susceptible to external factors that may overwrite that genotype's influence (Stöck, Horn et al. 2011, Holleley, O'Meally et al. 2015). There could even be multiple genotypes that all have some influence on the sex of an individual (Gammerdinger and Kocher 2018). No matter the system that is used, in large populations an equal sex ratio will be generally favored by selection (Fisher 1958, Hamilton 1967).

Rapid change from one sex determination system to another creates a problem. Sexually reproducing species must reliably produce both sperm and eggs while the transition between one sex determination system to another is effected. Going between GSD and ESD or an XY and ZW system requires complex epistatic relationships between various genes and environmental triggers that still result in competent sperm and egg production at any stage during the transition (van Doorn 2014).

3.1.2 Reproductive variation in the Kryptolebias marmoratus species complex

In the new world killifish family Rivulidae, there is a clade that had a rapid change in sexual systems. This is the *Kryptolebias marmoratus* species complex (Costa 2004a, Costa 2004b, Tatarenkov, Lima et al. 2009). While the genus *Kryptolebias* houses many species that follow a standard male/female outcrossing sexual mode, the three species in this group that live in mangrove forests have undergone drastic changes in sexual mode: *K. marmoratus, K. hermaphroditus*, and *K. ocellatus* (Costa, Amorim et al. 2017). Found in southern Florida, the Caribbean, and the Atlantic coast of Central and South America, they are all less than 5 million years diverged from one another and have been reported to be able to interbreed (Berbel-Filho, Tatarenkov et al. 2021, Tatarenkov, Earley et al. 2021).

K. marmoratus is a self-fertile hermaphrodite, and together with *K. hermaphroditus* comprise the only such vertebrates known. While males are optional for reproduction, they remain relatively common in *K. marmoratus* populations (Harrington 1961). However, these males are not genetically determined, but instead develop as a product of sequential hermaphroditism (see Chapter 1), in which they arise from older hermaphrodites (Harrington 1971). Male *K.* marmoratus are identifiable by their more orange coloring and a black bar on the ventral sided of their tail fin. These males have been shown to be able to produce viable offspring with *K. hermaphroditus* hermaphrodites (Mackiewicz, Tatarenkov et al. 2006, Tatarenkov, Earley et al. 2021). No territory is shared between *K. marmoratus* and *K. ocellatus* so no natural interspecies breeding has been reported between those species (Berbel-Filho, Tatarenkov et al. 2021).

K. hermaphroditus is another self-fertile hermaphrodite, and the closest relative of *K. marmoratus* (Turner, Fisher et al. 2006, Tatarenkov, Lima et al. 2009). Besides being largely allopatric, another important difference is that there are few or no males in wild *K. hermaphroditus* populations (Berbel-Filho, Espírito-Santo et al. 2016). Their reproduction is thus similar to that of *K. marmoratus*, but with even less opportunity for outcrossing. In areas where they co-exist with *K. ocellatus*, hybridization between the two species has been shown (Berbel-Filho, Pacheco et al. 2022). Via mitochondrial genotyping, it has been hypothesized that this mostly occurs with the rare male *K. hermaphroditus* fertilizing *K. ocellatus* eggs (Berbel-Filho, Tatarenkov et al. 2021).

Unlike its self-fertile relatives, *K. ocellatus* is an obligately outcrossing species. Populations are made up of females and males (Tatarenkov, Lima et al. 2009). Though *K. ocellatus* sex determination system has not been previously characterized, it maintains a 1:1 sex ratio, with sex set at maturity and not changing. It is thus potentially retains a XY-type GSD system similar to those reported in more distantly related Cyprinodontiformes (Murphy and Collier 1996, Arezo, Papa et al. 2014, Myosho, Takehana et al. 2015, Mansoori, Ebrahimi et al. 2017, Franchini, Jones et al. 2018). *K. ocellatus* males are visually different from their self-fertile sister species due to their increased size and females produce eggs that can only be fertilized by males, as would be expected from any other obligate outcrosser. However, histological analysis of female gonads has shown testis tissue within them (Tatarenkov, Lima et al. 2009). They do not seem to use this testis or sperm for anything and are thus functionally females (Costa 2006).

Evolving from a male/female obligate outcrosser to a sequential and simultaneous hermaphrodite like *K. marmoratus* is a complex change, one that presumably had intermediate forms. One simple model would start with homogametic (XX) females developing testes and evolving the ability to self-fertilize as hermaphrodites. While outcrossing would still be possible, populations founded by single hermaphrodites would lack the Y of heterogametic males, and thus lack males entirely. Over time the Y could be lost from most or all of the species, leaving only XX hermaphrodites whose X chromosomes act as autosomes. Prolonged, exclusive selfing creates effectively clonal populations (Felsenstein 1974) that are slow to adapt (Morran, Parmenter et al. 2009, Becks and Agrawal 2012) and subject to the gradual accumulation of deleterious mutations known as Muller's Ratchet (Muller 1932, Muller 1964). In such Y-free populations, males may have later re-evolved by sequential hermaphroditism—the sex change of older hermaphrodites. Perhaps initially rare, sex change became a feature of all populations in *K. marmoratus.* Collectively, the *Kryptolebias marmoratus* species complex appears to include taxa at three different stages of the hypothesized evolutionary scheme.

If the above model is correct, we expect that outcrossing relatives of the self-fertile *Kryptolebias* still use an XY genetic sex determination system. Here I report my examination of genomic variation in a Brazilian population of *Kryptolebias ocellatus*, the obligate outcrosser that serves as a near outgroup to the sex-

changing *K. marmoratus*. Though I set out to find the ancestral sex chromosomes in the species, the study necessarily became an exploration of interspecies hybridization.

3.2 Methods

3.2.1 K. ocellatus collection

Wild caught *K. ocellatus* individuals were collected just south-west of Santos, Brazil (-24.4, -47.0) in collaboration with Dr. Cláudio de Oliveira (Instituto de Biociências/UNESP, Botucatu), and his lab. 52 of these individuals were sexed via dissection, of which 25 were male and 27 were female. Collections were done with appropriate local permitting. In addition, one adult male and one adult female lab-reared specimens were kindly provided by Dr. Ryan Earley (Univ. of Alabama), and sexed via dissection as well.

3.2.2 DNA Isolation and sequencing

Tail tissue of one male and one female *K. ocellatus* preserved in ethanol (the gift of Dr. Ryan Earley) was collected, and DNA was purified from tissues using proteinase K digestion and phenol chloroform extraction (Yin, Schwarz et al. 2018). DNA was used to create separate libraries using NEBNext Ultra II DNA Library Prep Kit for Illumina (#E7645) The libraries were then sequenced using an Illumina NextSeq 1000 at a read depth of about 50x coverage for each.

A separate set of sex-specific libraries was made with DNA from a pool of 25 male and a pool of 25 female individuals from the Brazilian collection. The individuals from the prior preps were not included in the pooled libraries. The pool libraries were then sequenced using an Illumina NextSeq 1000 at 30x coverage for each pool. All reads were trimmed using Trimmomatic version 0.36 before being used for any other analytical method (Bolger, Lohse et al. 2014).

3.2.3 K. ocellatus short-read assembly

Assemblies were made from the single male and single female Illumina datasets using soapDenovo2 with a max read length of 200, average insert size of 200, minimum aligned length of 32, and a read length cutoff of 100 (Luo, Liu et al. 2012).

3.2.4 Mapping to reference genome

The reads from the individual male and female *K. ocellatus* were aligned to the linkage group-level *K. marmoratus* genome assembly (ASM164957v2) using BWA version 0.7.17 with default parameters (Li and Durbin 2009). Read depth was then measured along the genome using SAMtools version 1.14 *depth* on the resulting SAM files (Li, Handsaker et al. 2009). For clarity the read depth along the genome was averaged over the entire genome in 10kb non-overlapping windows. The average depth across the genome was used to normalize the read depths for the male and female datasets.

3.2.5 Sex-linked polymorphisms

The reads from the pooled 25 males and 25 females were aligned to the *K. marmoratus* genome (assembly ASM164957v2) using BWA version 0.7.17 (Li and Durbin 2009). The resulting SAM files were then fed into popoolation2 version 1201 to measure allele frequencies across the genome. They sync file output was used with SexSNPfinder (Gammerdinger, Conte et al. 2019) to locate various SNPs that were fixed in one sex but heterozygous in the other. Multiple settings were used for SexSNPfinder in regard to what the program considered sequencing errors and what was defined as fixed. Error culling was used from 5 or less allele count to no error culling. Fixed allele frequencies were set between 0.9 and 1.

For sex-specific K-mer analysis, *Jellyfish* version 2.3.0 *count* (Marçais and Kingsford 2011) was used to count the number of 22mers within the reads from the pool of 25 males and the pool of 25 hermaphrodites. Jellyfish dump was then used output the 22-mers and their counts. The male and female 22-mers were compared using a custom python script to report any 22-mer that was unique to males or females. All 22-mers that only showed up once were removed from the analysis. Given the 3x more male specific 22-mers and the distribution of their copy number histogram, we took 22-mers that appeared between 10 and 30 times across the pool. These 22-mers of interested were mapped to the de novo *K. ocellatus* assembly using BLASTN to find the scaffolds they were from (Altschul, Gish et al. 1990). The best hit scaffold was then blasted against the *K. marmoratus* genome to map the 22-mers to a linkage group and to get their approximate location relative to the other analysis methods used.

3.2.6 Mitochondrial and nuclear genotyping of individuals

Primers COI-F and COI-R (Tatarenkov, Lima et al. 2017) were used to amplify and sequence the Cox1 sequence of the fifty individuals used for the pooled sequencing in addition to two additional females. The male and female from the initial individual assemblies were used as presumed pure *K. ocellatus* controls. Nuclear microsatellite genotyping was done using Primers R3, R23, R37, and R90 (Mackiewicz, Tatarenkov et al. 2006) to amplify sites of interest within the nuclear genome. R23, R37, and R90 have been shown to target microsatellite loci with different copy numbers between *K. ocellatus* and *K. hermaphroditus,* and were used to differentiate sequences from the two species (Berbel-Filho, Tatarenkov et al. 2021). The allele counts received from these three loci were used in conjunction with the homo/heterozygosity in a chi-squared test against what would be predicted under Hardy-Weinberg equilibrium. Using this method, any potential hybrids can be identified if they have both *K. ocellatus* and *K. hermaphroditus* alleles present from any of the measured markers.

3.3 Results

3.3.2 Tests for heteromorphic sex chromosomes

Normalized male and female depths averaged about 50x (**Figure 3.1**). If a heteromorphic sex chromosome existed, we expect an over-representation of sites with about half (25x) coverage in one sex. However, no such secondary mode at 25x was observed. Across the entire genome, male and female read depths track each other tightly, with no discrete domain of any linkage group depth dropping to read coverage half of the average in one sex, as would be expected for a heteromorphic sex chromosome (**Figure 3.2**)

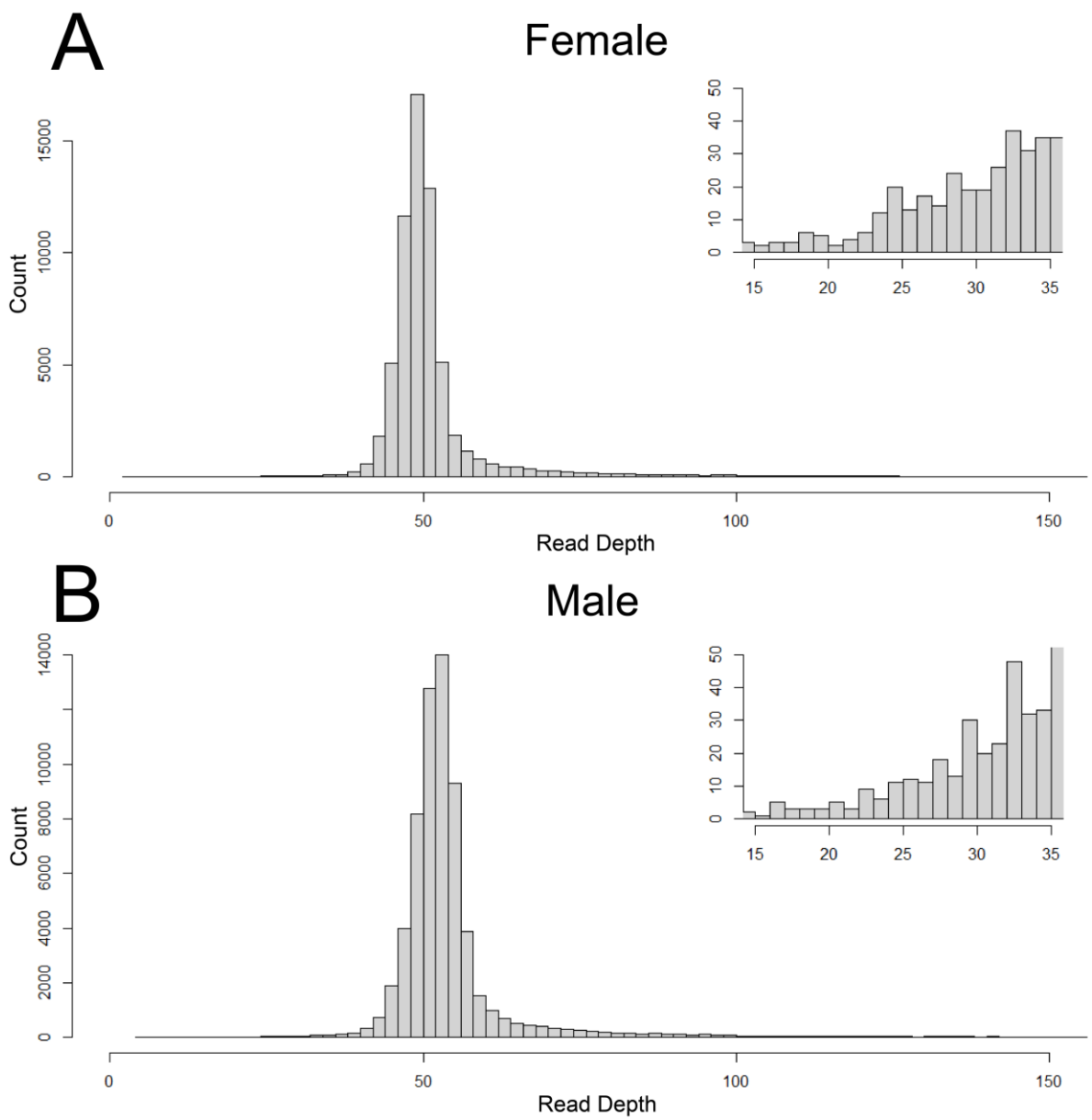

Figure 3.1. Distribution of mapped read depths of 10-kb non-overlapping windows across whole genome for a female (A) and male (B) *K. ocellatus* individuals mapped onto the *K. marmoratus* reference. Bin size = 2. Inset bin size = 1.

3.3.3 Tests for homomorphic sex chromosomes

There were 116,393,219 male-specific and 35,501,791 female-specific 22-mers that appeared at least twice. Analysis of one million random 22-mers from those pools show that a majority of them only appear twice, rather than the expected average of 15X for a sex-specific variant. The major difference between the 22-mer distributions for the two sexes is the length of their tails, with males having more higher copy 22-mers (**Figure 3.3A & 3.3B**). When localizing male-specific 22-mers that appeared between 10 and 25 times, as would be expected of Y reads when the whole genome was sequenced at 30x coverage, no linkage group was vastly overrepresented when controlled for length of linkage group (**Figure 3.3C**).

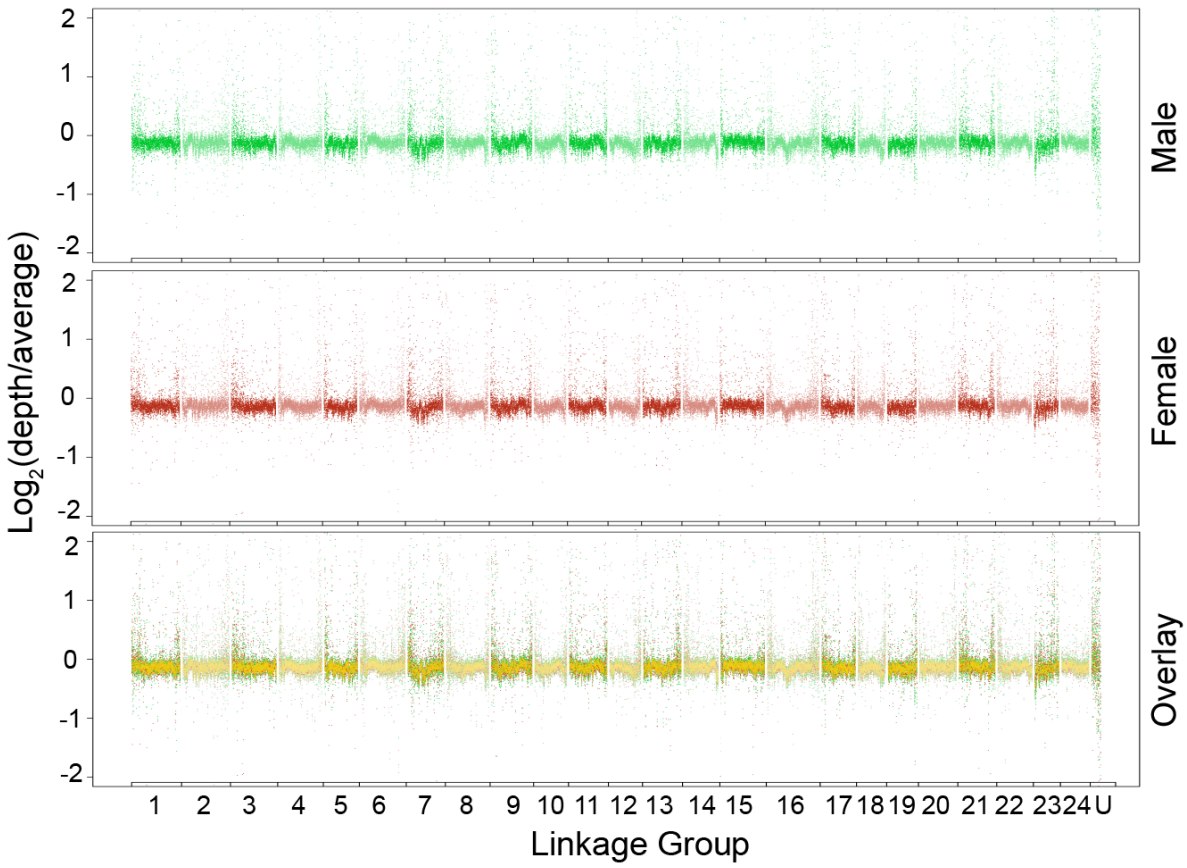

Figure 3.2. Read coverage of male vs. female K. ocellatus using the K. marmoratus reference genome. No difference in read depth seen on any linkage group. 10-kb non-overlapping window depth across the genome.

I used the program SexSNPFinder (Gammerdinger and Kocher 2018) to identify polymorphisms whose allele frequencies differed between male and female pools in a way that would be consistent with sex linkage. For example, candidate X-linked sites would be fixed for one allele in females, but at roughly

50% in males. No matter the stringency used, there were 30-50x more XY-suggestive SNPs than ZW-suggestive SNPs (**Table 3.1**). However, these SNPs mapped evenly to all linkage groups, no matter the stringency (**Figure 3.4**). In addition, when the localization was compared to the genome annotation, the less stringent SNPs fell everywhere across every linkage group, including within exons of many protein-coding genes (**Figure 3.5**). Thus, there is indeed a difference in heterozygosity between the male and female pools, but it is not localized to any one linkage group.

Table 3.1. Sex-SNP counts

	F >0.9 Min <= 5	F> 0.95 Min <= 5	F >0.95 Min <=2	F=1 Min<0
XY SNPs	5,322,070	1,598,331	163,081	32,111
ZW SNPs	136,912	27,573	3,340	1,079

3.3.4 Evidence of interspecies hybridization in both sexes

The high levels of male hetrozygosity observed throughout the genome suggested several possibilities, including sex-specific ploidy differences, hybridization between distinct lineages or species, or species contamination of the sampled population. When aligning the pooled reads to the mitocondrial gene COX1, two primary haplotypes appeared at about a 50/50 ratio in both sexes, one corresponding to *K. ocellatus* and the other to *K. hermaphroditus* (**Figure 3.6A**). When 5 individuals of each sex were individually sequenced, the ratio of hermaphroditus and ocellatus haplotypes were maintained (**Figure 3.6B**). When pooled data were mapped to the contig containing the nuclear gene *ALDH*, a similar patterning of haplotypes was seen **(Figure 3.6C**).

To get a more complete picture of the sampled population, I determined the individual genotypes of all 50 sequenced individuals for three nuclear microsatellites that have been shown to differ between *K. hermaphroditus* and *ocellatus* but not within species (Mackiewicz, Tatarenkov et al. 2006) (**Table 3.2**).

The apparent mixture of *K. hermaphroditus* and *K. ocellatus* nuclear markers is consistent with the pooled sequencing results (**Figure 3.6A & 3.6B**). In conjunction with the COX1 sequences (**Figure 3.6B**), sixteen of the fifty-two individuals that were collected were identified as hybrids. Of the remaining thirty-nine, twelve showed only *K. ocellatus* alleles at both the nuclear and mitochondrial loci, and twenty-seven had only *K. hermaphroditus* alleles. None of the hybrids were heterozygous for markers from both species at every locus, revealing that none of them were F1s. Multiple *K. ocellatus* alleles were seen in two of the three loci, suggesting that there was either back crossing or multiple hybridization events that had occurred in the population.

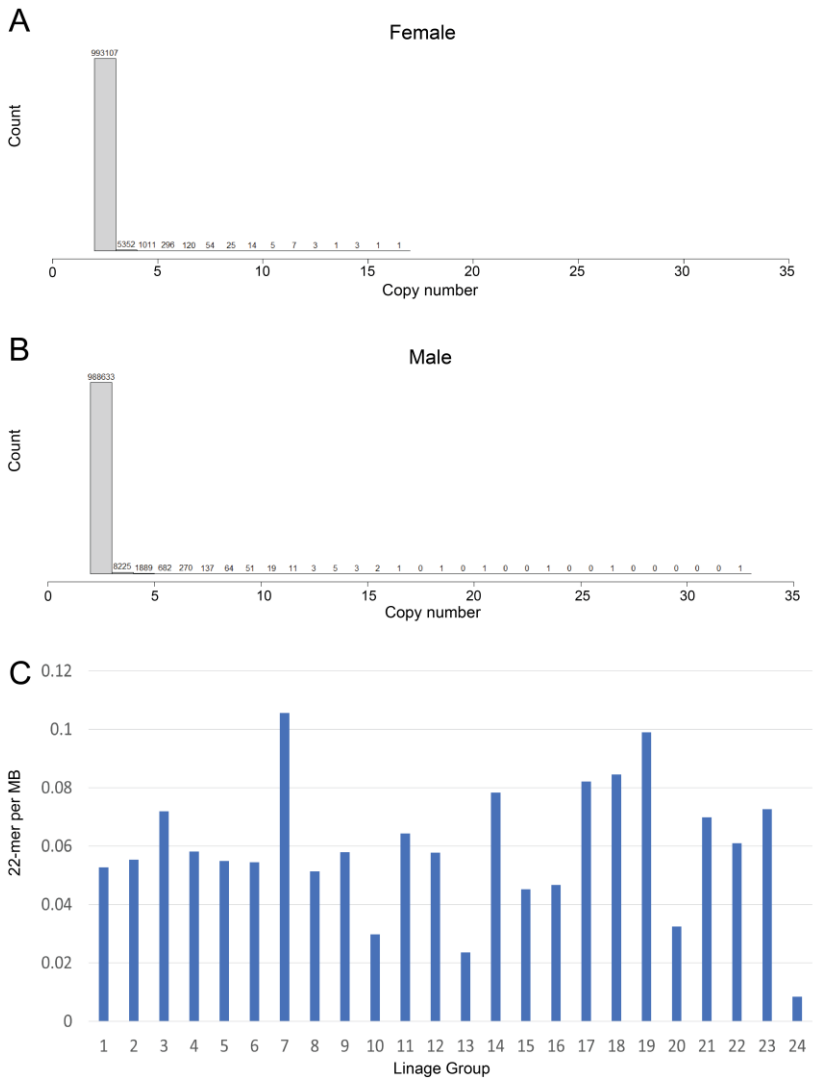

Figure 3.3: Lack of single localization of sex specific 22-mers. A & B: Histogram of one million random female (A) and male (B) specific 22-mers. C: localized linkage group for male specific 22-mers that had copy numbers between ten and twenty-five.

In addition, there was an overabundance of homozygosity at the loci examined in both the confirmed hybrids as well as the other fish in the pool. The number of hybrids was significantly lower than what you would expect from a panmictic population functioning at Hardy-Weinberg equilibrium (**Table 3.3**). This was true for both females and males.

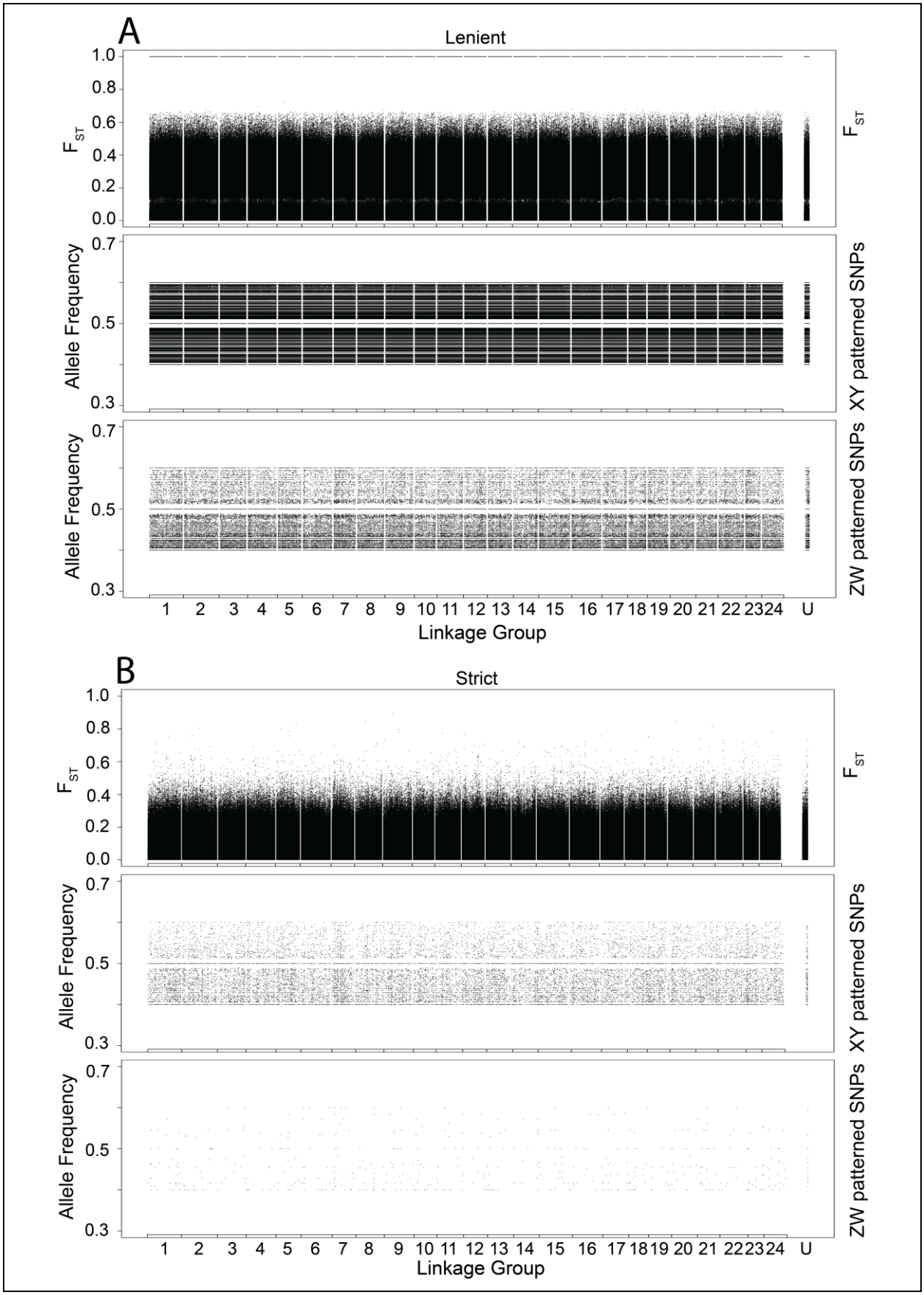
A
Lenient
F_{ST}
Allele Frequency
XY patterned SNPs
ZW patterned SNPs
1.0
0.8
0.6
0.4
0.2
0.0
0.7
0.5
0.3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 U
Linkage Group
B
Strict
F_{ST}
Allele Frequency
XY patterned SNPs
ZW patterned SNPs
1.0
0.8
0.6
0.4
0.2
0.0
0.7
0.5
0.3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 U
Linkage Group

Figure 3.4. Fst & Sex SNP localization across the genome. A: Localization of SNPs and Fst when the Fixed allele ratio > 0.9 and culling alleles that appeared five times or less. B: Localization of SNPs and Fst when the Fixed allele ratio = 1 and no allele count culling.

Figure 3.5. Annotation view of localization of XY suggestive SNPs. Two sites on linkage group 2 (A) and 12 (B) showing the location of XY SNPs identified with a Fixed allele ratio = 1 and no error culling on top in light blue, XY SNPs identified with a Fixed allele ratio > 0.9 and culling alleles that appeared five times or less in dark blue, and Male specific 22-mers.

3.4 Discussion

3.4.1 Lack of heteromorphic sex chromosomes

With regard to sex chromosomes in *K. ocellatus,* the most definitive finding from this study is the lack of any evidence for heteromorphic sex chromosomes. All linkage groups in both male and female individuals maintained a similar average read depth and there is also no sign of divergence in read depth when comparing males and females (**Figure 3.2**). These results cannot exclude the possibility of a novel sex-linked sequence in *K. ocellatus* that is not present in the *K. marmoratus* reference assembly. Such sequences have been reported in other teleost fish, either as sex-specific attached sequence or as a supernumerary sex-

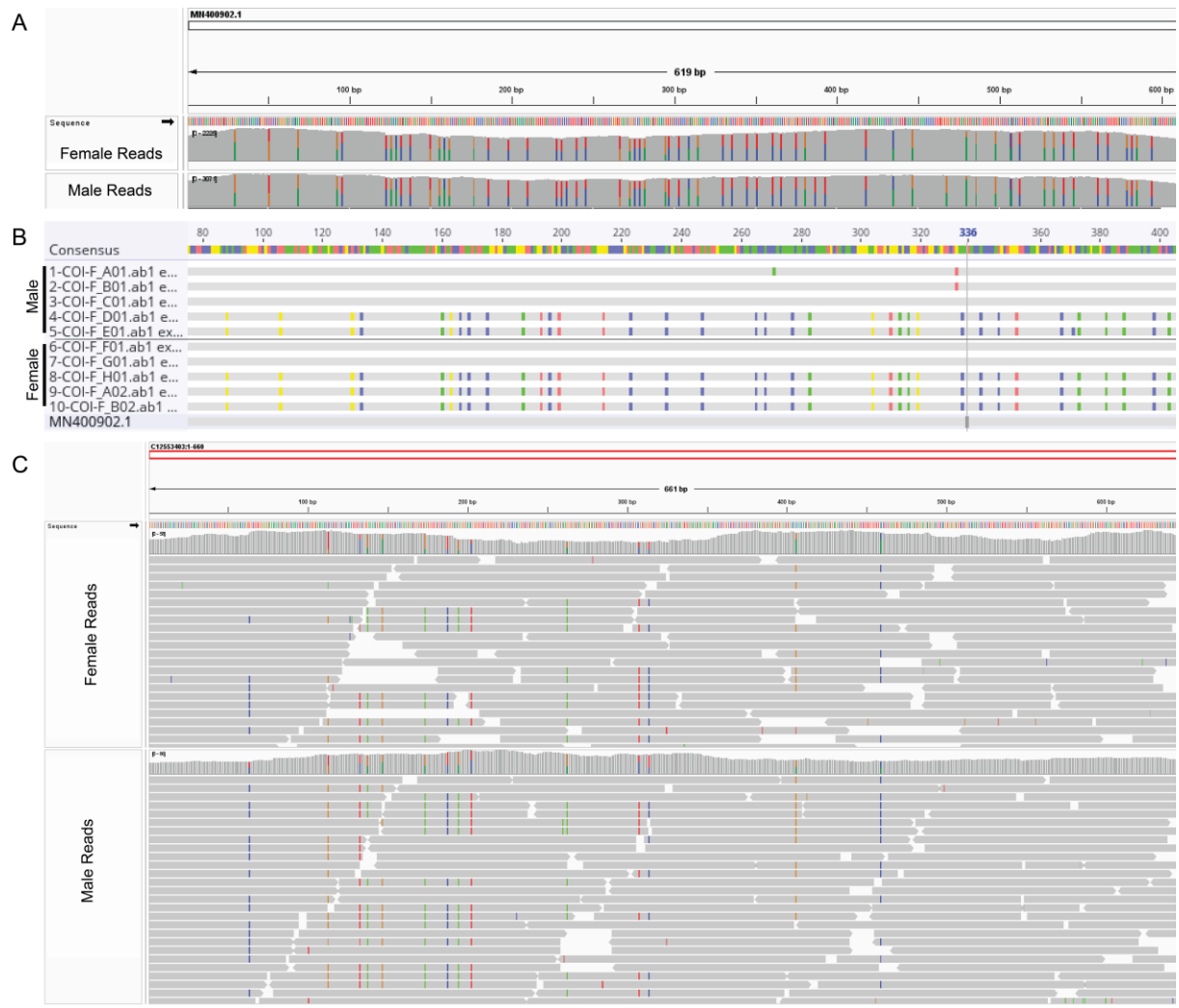

Figure 3.6: **Hybrid/mixed haplogroups in the pooled reads**. A: Alignment of male and female pools to the *K ocellatus* COX1 sequence. B: individual sequences for 5 males and 5 females. C: Alignment of male and female pooled reads to a *K ocellatus* contig that is believed to contain a copy of ALDH.

specific B-chromosome (Clark, Conte et al. 2017, Conte, Clark et al. 2021). However, for my analysis to completely miss such an element in male *K. ocellatus,* it would need to be devoid of any sequences that are alignable with the euchromatic *K. marmoratus* assembly.

With a lack of apparent heteromorphic sex chromosomes, I turned my attention to signatures of homomorphic sex chromosomes, which are common in teleosts (Myosho, Takehana et al. 2015). Superficially consistent with an XY system, there are over twice as many male-specific 22-mers and 30

Table 3.2: Individual genotyping results for three nuclear microsatellite markers (Mackiewicz, Tatarenkov et al. 2006) and mitochondrial COX1 genotype.

	R23		R37		R90		Mitotype	Hybrid?
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2		
Male 1	350	350	170	170	190	190	*K. ocellatus*	
Male 2	290	350	170	170	190	190	*K. ocellatus*	*Confirmed*
Male 3	350	350	170	170	190	190	K. ocellatus	
Male 4	350	350	170	170	190	190	K. ocellatus	
Male 5	350	350	170	170	190	190	K. ocellatus	
Male 6	290	290	400	400	250	250	K. *hermaphroditus*	
Male 7	370	370	170	170	190	190	*K. ocellatus*	
Male 8	290	290	400	400	250	250	K. *hermaphroditus*	Confirmed
Male 9	400	350	170	170	190	190	K. *ocellatus*	
Male 10	350	350	170	170	190	190	K. *ocellatus*	
Male 11	290	290	400	400	250	250	K. *hermaphroditus*	
Male 12	290	290	400	400	250	250	*K. hermaphroditus*	
Male 13	350	350	400	400	failed	failed	K. *ocellatus*	*Confirmed*
Male 14	290	290	170	170	190	190	K. *hermaphroditus*	*Confirmed*
Male 15	290	290	400	400	250	250	*K. hermaphroditus*	
Male 16	290	290	400	400	250	250	K. *hermaphroditus*	
Male 17	290	290	100	100	250	250	K. *hermaphroditus*	*Confirmed*
Male 18	350	350	170	170	190	190	K. *hermaphroditus*	Confirmed
Male 19	290	290	400	400	250	250	K. *ocellatus*	*Confirmed*
Male 20	290	290	400	400	250	250	K. *hermaphroditus*	
Male 21	350	350	170	170	190	190	K. *ocellatus*	
Male 22	290	290	400	400	250	250	*K. hermaphroditus*	
Male 23	290	290	400	400	250	250	K. *hermaphroditus*	
Male 24	290	290	400	400	250	250	K. *hermaphroditus*	
Male 25	290	290	400	400	250	250	*K. hermaphroditus*	
Male 26	290	290	400	400	250	250	K. *hermaphroditus*	
Male 27	290	290	400	170	250	250	K. *hermaphroditus*	*Confirmed*
Female 1	290	290	400	400	250	250	K. *hermaphroditus*	
Female 2	290	290	400	400	250	250	K. *hermaphroditus*	
Female 3	290	390	170	170	250	190	*K. ocellatus*	*Confirmed*
Female 4	350	350	400	170	190	190	K. *hermaphroditus*	*Confirmed*
Female 5	290	290	400	400	250	250	K. *hermaphroditus*	
Female 6	290	290	400	400	250	250	K. *hermaphroditus*	
Female 7	290	290	400	400	250	250	K. *hermaphroditus*	
Female 8	290	290	400	400	250	250	K. *hermaphroditus*	
Female 9	290	290	400	400	250	250	*K. ocellatus*	*Confirmed*
Female 10	290	290	400	400	250	250	*K. hermaphroditus*	
Female 11	290	290	400	400	250	250	K. *hermaphroditus*	
Female 12	290	290	400	400	250	250	K. *hermaphroditus*	
Female 13	290	290	400	400	250	250	K. *hermaphroditus*	
Female 14	290	290	400	400	250	250	K. *hermaphroditus*	
Female 15	350	350	170	170	190	190	*K. hermaphroditus*	*Confirmed*
Female 16	290	290	400	170	250	250	K. *hermaphroditus*	*Confirmed*
Female 17	350	350	170	170	190	190	K. *ocellatus*	
Female 18	290	290	400	170	250	250	*K. hermaphroditus*	*Confirmed*
Female 19	290	350	170	170	190	250	K. *ocellatus*	*Confirmed*
Female 20	290	290	400	400	250	250	K. *hermaphroditus*	
Female 21	290	290	400	170	250	250	*K. hermaphroditus*	*Confirmed*
Female 22	290	290	400	400	250	250	*K. hermaphroditus*	
Female 23	350	400	170	170	190	190	K. *ocellatus*	
Female 24	350	350	170	170	190	190	K. *ocellatus*	
Female 25	290	290	400	170	250	250	K. *hermaphroditus*	*Confirmed*

to 50 times more XY-suggestive SNPs than ZW-suggestive SNPs (**Table 3.1**). These numbers by

themselves would suggest a Y chromosome somewhere in the genome. However, these SNPs and 22-mers are distributed rather uniformly across the entire genome (**Figure 3.3C & 3.4**). In addition, these sex-biased markers fall within exons of various non-sex related genes on every linkage group (**Figure 3.5**). This suggests there is a difference in genome-wide heterozygosity between the sexes, yet it is not due to sex determination.

Table 3.3: X^2 results against expected values under Hardy-Weinberg equilibrium

	R23 X^2 Value (DF)	R23 p-value	R37 X^2 Value (DF)	R37 p-value	R90 X^2 Value (DF)	R90 p-value
Males	49.81267 (9)	1.17×10^{-07}	49.95421 (5)	1.42×10^{-09}	25.02818 (2)	3.67×10^{-06}
Females	19.88504 (9)	0.018635	7.683075 (2)	0.021461	13.87561 (2)	9.70×10^{-4}
Whole population	95.00517 (14)	4.27×10^{-14}	81.50215 (5)	4.07×10^{-16}	41.2948 (2)	1.08×10^{-09}

3.4.2 Evidence of recent hybridization with K. hermaphroditus

Cytochrome oxidase I (COX1) is a mitochondrial gene that can be used as a “barcode” to distinguish *K. ocellatus* and *K. hermaphroditus* (Tatarenkov, Lima et al. 2017). The pooled sequences aligned to COX1 showed that the nominally *K. ocellatus* pools were in fact substantially contaminated with *K. hermaphroditus* DNA (**Figure 3.6A**). Both sexes harbored *K. hermaphroditus* alleles at roughly the same rate. When COX1 from individual fish was sequenced, two out of the five males and three of the five females had *K. hermaphroditus* COX1 haplotypes (**Figure 3.6B**). A representative nuclear gene, aldehyde dehydrogenase (ALDH), also showed a similar mix when aligning the pools, with two specific haplotypes (**Figure 3.6C**). In addition, when individually sequenced, 13 out of the 52 individuals showed both *K. ocellatus* and *K. hermaphrodtitus* genetic markers and significantly higher rates of homozygosity.

With this much integration of *K. hermaphroditus* DNA and the apparent shift away from Hardy-Weinberg, it will be impossible to identify any sex chromosomes that *K. ocellatus* may have using our current dataset. However, a wild population this hybridized allows us to examine the speciation and sexual mode evolution of the *K. marmoratus* species complex from a novel angle.

3.4.3 Potential cryptic selfing in hybrid population

I observed a similar extent of interspecies hybridization in both the female and male pools (**Figure 3.6**). While a surprising observation, it does not explain increase in male-specific k-mers or heterozygosity I observed. One possibility is that "female" hybrids may have some ability to self-fertilize. *K. ocellatus* females from nominally non-hybridized populations are already known to have testis tissue within their gonads (Tatarenkov, Lima et al. 2009). Perhaps the introduction of DNA from the self-fertile *K. hermaphroditus* could allow XX hybrids to both mate and to self to some extent, while fish retaining a dominant male-determining Y chromosome (from *K. ocellatus*) would always be cross-progeny that develop as males. Such limited selfing could be rare enough to not substantially skew the sex ratio, yet still lower the heterozygosity of the females relative to males.

The nuclear markers seem to potentially contradict the above scenario. Both males and females show significant deviation from Hardy-Weinberg equilibrium due to an excess of homozygotes. Again, this may be the product of inbreeding, and potentially of selfing (**Table 3.3**). More nuclear markers would need to be examined to confirm this, as the ones used here were chosen specifically due to the existence of species-specific alleles. If both males and females in this population are the product of selfing, it may suggest that hybrid males may be using environmental sex determination, or sequential hermaphroditism similar to the third sister species in their clade, *K. marmoratus*, and thus not have any genetic or genomic differences between them and females of the same species.

3.4.4 Hybrid crosses and subsequent generations

Being a hybrid population, there is a question of when did this hybridization event occurred and how many times it has happened. When a hybrid cross happens, all heteromorphic sites that vary between the two different parent species would necessarily be heterozygous. No individual observed matches that description (**Table 3.2**). Therefore, all hybrid individuals examined must be of F2 or further generations. Unfortunately, due to the existence of selfing and lack of F1s, its impossible to determine the sex of the parental species in the original crosses and potential back crosses. In addition, there are no reported data on how linked the three sites examined are, confounding our ability to measure generation count further. If we assume that the hybrids are in fact selfing and the sites measured are unlinked, 87.5% of all

heteromorphic sites should be homozygous by the time you reach the F4 generation yet about 92% of the sites I measured were homozygous suggesting that these hybrids may be F4 or higher. Also, the concordant nature of the majority of the nuclear sites being from one species or the other suggests that the hybrids may be also backcrossing to the parent species they more closely resemble genetically. This species concordance would more simply suggest that the sites are linked and thus have a higher probability to travel together. It would still suggest that the hybridization event is older than F4 but the probability of hybridization being more recent would go up.

Previous studies have hypothesized that hybridization between *K. ocellatus* and *K. hermaphroditus* occur exclusively from *hermaphroditus* males fertilizing *ocellatus* eggs (Berbel-Filho, Tatarenkov et al. 2021). While I cannot reject this, I have found nine hybrid individuals with *K. hermaphroditus* mitotypes (**Figure 3.6B, Table 3.2**). The few scenarios where this could occur include if there was back crossing with a male hybrid to a hermaphrodite *hermaphroditus*, or the original hybridization event involved a hermaphrodite *hermaphroditus* and the subsequent mitotype was preserved through selfing or backcrossing. Either way, it suggests that hermaphrodite *hermaphroditus* individuals may be more involved in hybridization than previously reported.

3.5 Conclusion

My data allow me to reject the possibility of heterormorphic sex chromosomes in *Kryptolebias ocellatus*. With regard to homomorphic sex chromosomes, however, hybridization with *K. hermaphroditus* in my sample population has largely drowned out any sex chromosome signals that may exist, making any definitive claims impossible. I note, however, that linkage group seven has the highest density of both male-specific 22-mers and XY sex-patterned SNPs, and thus is the best candidate. Finally, the pattern of nuclear genotypes suggest that the interspecies hybridization has led to back crossing to both species and may have led to self-fertile hermaphroditism in both. Along with other recent studies (Berbel-Filho, Tatarenkov et al. 2021, Tatarenkov, Earley et al. 2021, Berbel-Filho, Pacheco et al. 2022), this work highlights the fluid nature of “species” in *Kryptolebias,* and suggests that the evolution of one sexual mode into another may be reversible through hybridization in this clade.

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