Genomics of Sexual Selection

A Dissertation

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ABSTRACT

Sexual selection is a major driver of rapid phenotypic evolution, often resulting in conspicuous and charismatic traits. How these traits and the preferences for them originate, and how they affect the evolution of an organism's genome, remain largely unknown. This lack of knowledge is problematic because the evolution of complex traits is a vital mechanism that connects seemingly independent topics, such as behavior, genetic variation, reproduction, fitness, competition, and genome evolution allowing them to be incorporated into broader evolutionary theory. As modern methods offer new opportunities, we find ourselves better prepared to dive deep into these facets and provide a more accurate understanding of them. This dissertation aims to understand the role of sexual selection on complex trait and genome evolution. There are three specific areas of study for this dissertation: (1) the evolution of sexually selected traits in the African turquoise killifish (*Nothobranchius furzeri*), (2) the role of male pregnancy in genome and gonad transcriptome evolution, and (3) the role of sex-role-reversal and methodology in shaping our understanding of transcriptome-based analyses. The results from these studies have contributed to our understanding of female preferences in maintaining conspicuous male phenotypes, the role of sexual selection in genome and transcriptome evolution, and the degree to which bioinformatic analyses have greatly influence our understanding of biological processes.

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This research would not have been accomplished without the live animal facility members, who have kept my fish healthy and well fed. This research was also made possible by funding from the National Science Foundation, the University of Idaho, and the Graduate and Professional Student Association at the University of Idaho.

DEDICATIONS

To my family and friends for their love, encouragement, and support; especially to my mother, who has been a valuable underpinning of my success.

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STATEMENT OF CONTRIBUTION

CHAPTER 2: NOTHOBRANCHIUS FURZERI AS AN EMERGING MODEL FOR MATE CHOICE: FEMALE CHOICE REVEALED BY ANIMATIONS

BDJ performed the behavioral tests and part of the behavioral analyses. AF assisted with the behavioral analyses. LRW and BDR created the animations. AGJ and GEC provided intellectual contribution and guidance in experimental design. BDJ wrote this article and included input from all the other authors.

CHAPTER 3: THE EVOLUTION OF THE TESTIS TRANSCRIPTOME IN PREGNANT MALE PIPEFISHES AND SEAHORSES

BDJ and AGJ performed all the genomic analyses included in this project. APA, CMS, ER, and SPF collected the pipefish samples and performed the RNA extractions and sequencing for this project. CHR aided with the alignments used for the molecular evolution analysis. BDJ and AGJ wrote this article and included input from all the other authors.

CHAPTER 4: SENSITIVITY OF TRANSCRIPTOMICS: DIFFERENT POPULATIONS AND METHODOLOGY ALTER CONCLUSIONS IN GULF PIPEFISH (SYNGNATHUS SCOVELLI)

BDJ assembled the transcriptomes and performed all the genomic analyses included in this project. ER collected the pipefish samples and performed the RNA extractions and sequencing for this project. AGJ provided intellectual contribution and guidance in experimental design. BDJ wrote this article and included input from all the other authors.

CHAPTER 1: INTRODUCTION

Reproduction is a fundamental shared feature of life, yet it also entails a remarkably diverse set of modes, selective pressures, and evolutionary outcomes. Selection related to competition for mates or fertilization opportunities, called sexual selection, is known to be a major driver of rapid and complex phenotypic evolution (Andersson 1994). We might have heard the resonant songs of birds on a walk in the park, viewed ostentatiously colored male guppies through the aquarium glass, or seen two male elk bellowing and clashing their antlers together on television. These are all forms of evidence that sexual selection is an intense force in the natural world. In this regard, sexual selection is a key component for understanding how complex traits evolve, are maintained, and shape genome evolution. This mystery is the focus of my research, which aims to understand the role of sexual selection on the evolution of complex traits and genomes. Specifically, my research is broken down into three main study systems: (1) understanding the evolution of sexually selected traits in the African turquoise killifish (*Nothobranchius furzeri*), (2) understanding how the unique mating strategy of male pregnancy in seahorses and pipefish influences the evolution of their gonads, and (3) understanding the degree of sexual dimorphism in pipefish brain transcriptomes.

EVOLUTION OF SEXUALLY SELECTED TRAITS

The African turquoise killifish is sexually dimorphic, and males display bright color patterns that vary across their native range (Cellerino et al. 2015). The force driving the maintenance of these different male color phenotypes was largely unknown. In this chapter, I tested hypotheses regarding female mating preferences for these male traits. This led to the first paper that provided an explanation for the maintenance of these male color traits (Johnson et al. 2020). Male color patterns were retained because females preferred males that displayed certain colors and the strength of this preference was driven by genetic factors. This study was also innovative because it developed and utilized computer animations to investigate preferences. These animations are now available as a public resource, enabling killifish to serve as a model for studying mating preferences.

ROLE OF MALE PREGNANCY IN GENOME EVOLUTION

Fishes from the family Syngnathidae (includes seahorses and pipefish) utilize a novel mating strategy, male pregnancy (Paczolt and Jones 2010). Females transfer eggs to a male's brooding structure during mating, thus eliminating sperm competition between males (Paczolt and Jones 2010). In this chapter, I tested hypotheses on how the evolution of male pregnancy has affected the evolution of genes important to the testis in seahorses and pipefish. This study was noteworthy, as it was the first to address the evolution of genes related to male fertility in a fish with no potential to engage in

sperm competition (Johnson et al. 2022). It also addressed the role of sexual selection in genome-wide patterns of genome evolution.

SEXUAL DIMORPHISM IN PIPEFISH BRAIN TRANSCRIPTOMES

In addition to male pregnancy, pipefish are also relatively unique in that they are sex-role-reversed (Jones et al. 2000; Fritzsche et al. 2021). In this regard, females compete for access to male mates, and sexual selection acts stronger in females (Jones et al. 2000). In species with conventional mating roles, where sexual selection acts stronger on males, there are a surplus of male-biased genes with sex-biased expression (Singh and Kulathinal 2005). Given this observation, we expect that pipefish will display more female-biased genes. I address the degree of sexual dimorphism in pipefish brain transcriptomes within a methodological framework. I assess how well differential expression analyses explain the biological phenomenon of sex-biased expression, and how robust certain transcriptome-based conclusions are given variation in sampling source and analyses used.

Collectively, these studies provide valuable insights into the mechanisms and consequences of sexual selection. They address major questions in the field regarding the degree to which the genome and transcriptome have been influenced by sexual selection. The African turquoise killifish study advances our understanding of the mechanisms behind the maintenance of sexually selected traits, demonstrating that mate choice is a powerful selective force. The study on the evolution of male pregnancy in seahorses and pipefish highlights the importance of sexual selection in driving genome evolution. The study on brain transcriptomes emphasizes the importance of experimental design in investigating and interpreting patterns of sex-biased expression. By investigating diverse aspects of sexual selection, from pre- to post-copulatory mechanisms, these studies contribute to a deeper understanding of how sexual selection shapes the evolution of sexually dimorphic traits, genomes, and gene expression patterns. They offer insights that can be applied across multiple taxa, providing a broader perspective on the role of sexual selection in driving evolutionary change. They also provide a foundation for future sexual selection research using these model systems.

CHAPTER 2: *NOTHOBRANCHIUS FURZERI* AS AN EMERGING MODEL FOR MATE CHOICE: FEMALE CHOICE REVEALED BY ANIMATIONS

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ABSTRACT

The African turquoise killifish (*Nothobranchius furzeri)* is the shortest-lived vertebrate research model. It is also sexually dimorphic, making it suitable for studying sexual selection. We take advantage of a natural tail color polymorphism in males and investigate female responses to computer animations of males that differ in this phenotype. Our findings indicate that GRZ (Gonarezhou) females prefer animated males with traits specific to their strain (a yellow tail with a black band) compared to males exhibiting traits from another strain of the same species (a red tail). When females were simultaneously shown animations of both males, they spent significantly more time on the side of the tank where the yellow-tailed animation was visible, and significantly more time interacting with the yellow-tailed animation. Given these repeatable responses and the availability of genomic resources, *N. furzeri* represents an excellent, untapped model for studying the genetic basis of preferences and reproductive behaviors.

INTRODUCTION

Conspicuous signals used in mate choice have long been recognized to have evolved through the perception of choosers (Darwin 1871). Lesser understood is the evolution of the preferences for the signals themselves and the genetic mechanisms involved. Multiple hypotheses exist, ranging from signals evolving to utilize the pre-existing sensory mechanisms of the choosers, to choosers using a signal as an indicator of overall mate quality (Andersson 1994). While its importance is recognized, studying the evolution of preference is challenging, partially because finding a practical animal model that demonstrates strong mate choice and has functional genomics tools available has been limiting.

The African turquoise killifish, *Nothobranchius furzeri*, has several characteristics that make it an appealing model for the study of sexual selection and mate choice. They were first collected in 1968 from small ephemeral ponds across Zimbabwe and Mozambique and have been maintained in a lab setting as distinctive strains since then (Cellerino et al. 2015). Their native ponds are formed and desiccated by the wet and dry seasons of the region, and as a result, have generated highly structured, inbred populations of *N. furzeri* (Bartáková et al. 2013). These ponds also give insight into this species' uniquely short lifespan, which is one of the shortest of any vertebrate, with certain strains reaching sexual maturity in roughly one month and living only about three months on average (Terzibasi et al. 2008; Kirschner et al. 2011; Kim et al. 2016; Hu and Brunet 2018). Interestingly, male tail color is the primary visual difference between populations, being either red or yellow. The most notable visual difference occurs between the yellow and black banded tails of males originally collected from Gonarezhou National Park in Zimbabwe (GRZ) and the fully red tails of males found throughout Mozambique (MZM) (**Figure 2.1**) (Jubb 1971; Cellerino et al. 2015). Later expeditions into their native range have found ponds where both tail color morphs coexist, and collections from these sites have led to the establishment of other strains (Reichard et al. 2009).

Male tail color in *N. furzeri* has been shown to be strongly influenced by a single Mendelian locus, with yellow being dominant to red (Reichard et al. 2009; Kirschner et al. 2011). That being said, laboratory breeding experiments have shown that crossing individuals from different tail-color strains can yield a yellow-tail morph that develops partial red coloration with advancing age (Valenzano et al. 2009; Kirschner et al. 2011), so other loci are clearly involved in the full determination of tail coloration. In comparison to males, females are smaller and neutrally colored, giving the species conspicuous sexual dimorphism (Cellerino et al. 2015). Mating is preceded by a courtship interaction, where females can decide to approach or avoid a male (Cellerino et al. 2015; Harel et al. 2016). Due to its versatility and practicality in many fields of biology, *N. furzeri* has become an increasingly popular lab model, and considerable effort has been devoted to the

development of numerous tools for genomics and gene-editing studies. For instance, several strains have had their genomes sequenced, tissue-specific transcriptome profiles are available, and efficient CRISPR and transgenic protocols have been developed (Reichwald et al. 2009; Valenzano et al. 2009; Hartmann and Englert 2012; Allard et al. 2013; Petzold et al. 2013; Ng'oma et al. 2014; Harel et al. 2016).

While the turquoise killifish presents a seemingly excellent opportunity to study sexual selection and female mating preferences, it is important that we first determine if females exhibit any mating preferences for male traits. Here, we used live females from the most widely used strain, GRZ, to test their preference for strain-specific male tail color, using visually realistic animated males with color patterns comparable to males from the yellow-tailed GRZ and the red-tailed MZM strains. The use of animated models to assess female mating preferences has been profitable for many teleost species (including *Poecilia latipinna, P. mexicana, Xiphophorus variatus,* and *Gambusia hubbsi*) (Culumber and Rosenthal 2013; Langerhans and Makowicz 2013; Veen et al. 2013; Ingley et al. 2015; Gierszewski et al. 2016, 2018). Such an approach has two tremendous advantages relative to the use of live stimulus males: (1) it gives researchers complete control of male behavior and (2) it allows scientists to alter specific traits of interest quickly and without affecting other aspects of the phenotype. Researchers can also exaggerate trait values outside of their natural distribution to determine the shape of the preference function or to determine if peak preferences lie outside of the natural distribution of the trait. For the present study, the use of animated males allows us to control for all other potential visual or behavioral traits that might differ between the strains, thus isolating the effects of male coloration *per se*.

The objectives of this study are to establish whether female *N. furzeri* respond to animated playbacks of stimulus males and to test for strain-specific preferences based on the caudal fin coloration trait that differs between strains. We hypothesize that females will respond to the animated males, spending more time near males with their strain-specific tail color. Ultimately, we demonstrate that *N. furzeri* has tremendous potential as a model for bridging sexual selection and functional genomics, and that computer animations of stimulus males provide a promising approach to dissect aspects of female preference in this species.

METHODS

HOUSING CONDITIONS

N. furzeri GRZ fish eggs were acquired from the Anne Brunet lab at Stanford University and bred in our lab at the University of Idaho for four generations (under IACUC protocol 2017-53). Eggs

from the fourth generation were hatched, and the fry were reared on a constant supply of freshly hatched *Artemia nauplii.* After four weeks, they were transitioned to Otohime fish diet from Reed Mariculture Inc. and were fed twice a day. After 5 weeks, sexual coloration began to develop in males. Males initially show darker pigmentation across their body before developing yellow tail coloration about a week later. Males that began to develop darker pigmentation were immediately removed from the housing rack, leaving a female-only housing system. Females were housed in pairs in 1.8L tanks on the same housing rack. Each housing rack was on a separate recirculating filtration system. Additionally, females were shielded from viewing any males in the housing facility and all labels or additional placards colored yellow or red were removed from the room. All fish were kept on a 12hr:12hr light:dark cycle.

ANIMATIONS

We used 3DSMax and ZBrush software to create animated 3D digital models of *N. furzeri* (**Video 2.S1 and Video 2.S2**). The models were constructed from still and video reference images of *N. furzeri* from the Jones lab and from additional publications (Cellerino et al. 2015). Fin coloration was adjusted using color sampling from still images of GRZ and MZM male fish (**Figure 2.1**). The colors were non-static, and brightness varied depending on the movement of the fish in relation to a top-down light source. The average color values are reported for the yellow tail (#aba84b) and red tail (#79403f). Animated swim patterns were made to mimic courtship behaviors from previously recorded GRZ males mating with females. The background color was dark (#33404b), and a slow-moving grey fog-like element was superimposed to simulate mild water movement and turbidity, creating a more realistic, non-static environment. We constructed videos that could be played on a seamless loop, in which the position of the digital male was the same at the beginning and end of the video sequence. The video sequence was 18 seconds in duration with a resolution of 1920x1080 at 30.0 fps.

The female's ability to perceive the animation was tested using five females from a cohort separate from those used for the main experiment. Each female was given the option between a screen displaying a yellow-tailed GRZ male animation and a screen displaying the non-static background that did not include a fish. The screen on which either animation was shown was randomized. Video footage of female responses was taken, and the time females spent within 9 cm of either screen was scored.

BEHAVIORAL TEST

Thirty-three females were included in this experiment. Behavioral testing was conducted between 08:00 AM and 11:00 AM within a one-week timeframe. Females were fed in the morning at 07:45 AM and given fifteen minutes rest prior to the start of the experiment. Females were tested one at a time. At the beginning of each trial, the focal female was moved into the behavioral tank and was given 6 minutes alone to acclimate to the tank. The behavioral tank (20 cm W × 50 cm L x 20 cm water depth; **Figure 2.2**) was divided down the middle by a neutral area of 4 cm, between the left and right zones (23 cm each). Videos were displayed on a 17.8 cm LED display at a resolution of 1024 x 600 at 171 ppi. One display was attached to the outside of the acrylic wall adjacent to the left zone, and another display was attached to the outside of the acrylic wall adjacent to the right zone. During acclimation, both screens played the background animation that was used to simulate mild water turbidity (**Video 2.S3**). This animation did not contain any fish. After the acclimation period, one screen was randomly chosen to display the yellow-tailed male animation and the other was assigned the red-tailed male animation. Once the female crossed the neutral zone and viewed both male animations, the recording started and continued for a total of 15 minutes. Females that took longer than 3 minutes to view both males were excluded from the study. During the recording, the researcher stepped out of the room. The behavioral setup was surrounded by acoustic foam panels, to block fish from any unintentional and disruptive sounds or visuals.

Video footage was taken both from a top-down angle and from the side. The screens displaying the animation were not visible from the top-down angle. Side-angle footage was used to verify that both animation videos functioned throughout the behavioral experiment. Behavioral times were taken from the top-down angle footage without visual indications of male placement to prevent any bias in scoring.

STATISTICAL ANALYSIS

Our goal was to determine the amount of time females exhibited two types of behaviors: (1) the time they spent in either side of the tank and (2) the time the females spent closely interacting with the acrylic wall to which the LED screen had been affixed. The first behavior was scored as total time spent on either the left or the right zone of the tank. Between both zones, there was a neutral area of 4 cm (where no screen was present). When the focal female was in the neutral zone, she did not accrue time counting toward the left or right zones. The second behavior was the time the female spent within 5 cm of either the left or right screen, swimming alongside or towards it. Time spent outside of this area was considered neutral; thus, the neutral zone for this behavior included more area

than the first behavior (**Figure 2.2**). In courtship and mating between live *N. furzeri* individuals, receptive females typically follow or swim alongside the male (Cellerino et al., 2015; and personal observation). The inclusion of the second behavior was thus an effort to identify behaviors associated with female mating receptivity.

Scoring of duration for each behavior was done by the first two authors (B.D.J. and A.F.) using BORIS (v7.9.7) (Friard and Gamba 2016). Both scorers were blind to the coloration of each animated male during scoring. Time scores between both authors were then compared using Pearson's correlation coefficient (n=66) for both the time spent on either side of the tank (ρ=0.999, $P < 0.001$) and time spent closely interacting with the screen (ρ=0.993, $P < 0.001$). Inspection of the scatterplots also revealed no major outliers indicative of instances where the two scorers scored the same fish differently. Scores were then averaged for each fish and this value was used for downstream analysis. All data were tested for normality using the Shapiro-Wilks test, and non-parametric tests were used when normality assumptions were not met.

RESULTS

To test the female's ability to perceive the animation, the time females spent between a screen displaying a yellow-tailed GRZ male animation and a screen displaying the non-static background that did not include a fish was scored. On average, females spent dramatically more time interacting with the screen that had a male (average±SEM: 550.4±19.4 seconds; and Shapiro-Wilk test of normality: W=0.83, P=0.139) than with a screen displaying only the non-static background element (38.7±16.6 seconds; W=0.87, P=0.268) (paired t-test: t=14.30, n=5, $P < 0.001$). This result is most likely driven by the increased sensory input provided by the screen displaying a male and was used to confirm that females perceived and reacted to a stimulus on a screen.

For the choice experiment, during the acclimation period, both screens displayed the background video, which did not contain any animated fish. Females typically explored the left and right sides of the behavioral tank, as well as the front and back of the tank, relatively evenly during this period. The screens then displayed animated male fish, randomly chosen to show one of the male morphs. Recording started after the female crossed over the neutral zone once and thus had the opportunity to see both animated males. Females took, on average, 46 seconds to view both males. Females that failed to cross the neutral zone in less than 3 minutes were excluded from the study (2 females). For a typical run of the experiment, females would alternate between following each male closely, exploring the behavioral tank, and watching the males from a distance while hovering.

Females, on average, switched between the left and right zones of the tank 9 times, and closely followed GRZ-phenotype males 19 times and MZM-phenotype males 14 times.

Females (n=33) spent significantly more time on the side of the tank displaying the yellow-tail GRZ colored males, than the red-tailed MZM males (Wilcoxon signed-rank test (paired, two-sided): W=146, n=33, P =0.015; see **Table 2.1** and **Figure 2.3**). Thus, females were seen to associate more with males from their specific GRZ strain. To distinguish behaviors more specific to potential mating receptivity, females were also scored for time spent closely interacting with the screen, either swimming alongside or towards it. Here, females also spent significantly more time interacting with GRZ colored males than their MZM counterparts (Wilcoxon signed-rank test (paired, two-sided): W=124, n=33, P=0.004; see **Table 2.1** and **Figure 2.3**). The two behaviors had a positive correlation coefficient of 0.655, estimated using Spearman's rank correlation (S=16,524, n=66, $P < 0.001$), indicating that females tended to spend more time in the general area of the male with whom they closely interacted.

DISCUSSION

Computer animations have impacted several lines of inquiry in behavioral ecology already, allowing researchers to measure mating preferences, to determine the role of mate choice in speciation, and to gauge male responses to increased sperm competition risk. Here, we assess the preference of GRZ *N. furzeri* females for male tail coloration using animated males with visual traits similar to males of the GRZ strain (a yellow and black banded tail) and the MZM strain (a fully red tail). Females spent significantly more time associating and closely interacting with animated males with yellow tails than with red tails (see **Table 2.1**). During the behavioral trials, females were also seen engaging in other behaviors that were not necessarily associated with mating interests, such as exploration. However, these behaviors tended to occur more often in the vicinity of the male with whom they interacted closely. Important to note is the presence of variation in female preferences, with a handful of females showing preference for red-tail animations. The question of why females from a genetically homogeneous, inbred strain display variation in preference is a potentially interesting topic for future work (Godin and Dugatkin 1995; Wagner 1998). On a related note, it would also be valuable to conduct mate choice trials for MZM females to see whether they prefer color traits from their own strain, or if they prefer the yellow morph as well. For the purposes of our study, we limited ourselves to GRZ females as they are the most popular strain, have more functional genomics tools, and are easier to acquire and maintain in the lab. In addition, we wished to verify that

females respond to computer animations and attend to male coloration before embarking on a large, multi-strain study.

Given the caveat of using animated males, our results are similar to those of other studies in which association times were indicative of mating preference by female fish (Wagner 1998 p. 199; Jeswiet and Godin 2011). However, studies using live male fish could serve to further corroborate these results and potentially shed light on traits not previously known to be involved in mate choice in this species. For future studies, it would be important to validate the responses found here towards computer animated stimuli with live males (Chouinard-Thuly et al. 2017). Use of live fish would also be necessary to study non-visual traits used in mate choice, such as chemical factors or tactile cues, which are known to be important in many species (Schlupp et al. 2010; Thomas 2011) and were not addressed in the present study. However, the use of live males comes with its own set of limitations, such as an inability for the researcher to control for the effects of male behavior on the female and difficulties arising from correlations between the trait of interest and other aspects of the male phenotype. Regardless, our results strongly suggest that female *N. furzeri* respond behaviorally to computer animations of males and exhibit apparent mating preferences based on male appearance, opening the door for more detailed studies of sexual selection in this species.

Our results contribute to a growing effort to use computer animations to delve into the subtleties of fish behavior, using a controlled and manipulatable subject. The studies that have used animated individuals have indicated robust responses from a number of fish species, particularly livebearers and sticklebacks (*Gasterosteus aculeatus)* (Zbinden et al. 2003, 2004; Langerhans and Makowicz 2013; Gierszewski et al. 2016, 2018). Female sailfin mollies (*P. latipinna*), for example, have been confirmed to respond as strongly to an animated male as to a live male or a recording of a live male (Gierszewski et al. 2016). Males were also shown to discriminate between male and female animations, showing an agonistic response toward male animations (Gierszewski et al. 2016).

Sympatric reproductive isolation through female preference has also been explored with the use of animations. For example, the Bahamas mosquitofish (*G. hubbsi*) exhibits within- and among-population geographical variation in body shape (Langerhans and Makowicz 2013). Using animated males to mirror extreme within-population variation in body shape, mate choice trials revealed that females exhibited within-population mating preferences for males with similar body shapes to their own (Langerhans and Makowicz 2013). This mechanism has been hypothesized to contribute to the development of sexual isolation between populations from environments with differing predation pressures (Langerhans and Makowicz 2013). A second example, with a similar experimental approach, comes from the shortfin molly (*P. mexicana*), where females were seen to have stronger

assortative mating preferences based on body shape when populations existed in sympatry rather than allopatry (Greenway et al. 2016). It is hypothesized here that female preference for body shape reinforces reproductive isolation between sympatric populations (Greenway et al. 2016).

Interestingly, animations have also been used to study sperm competition in sticklebacks (*G. aculeatus)*, where males increased their ejaculate size in response to animations of other males who were larger or performing courting behaviors (Zbinden et al. 2003, 2004). Here, males were seen to respond to female-preferred male traits, utilizing this information to alter their response to a potential increase in sperm competition risk (Zbinden et al. 2003, 2004). In general, the use of animated individuals for mate choice trials allows researchers to manipulate visual traits independently of other aspects of the phenotype, including those that might have unknown importance, in a repeatable manner. This flexibility permits rigorous tests of hypotheses that would be virtually impossible to address without computer animations.

One area in which computer animations are certain to contribute as our genomic resources expand is in the study of the genetic basis of mating preferences. As females used in our mate choice trial were never exposed to sexually mature males, their preference for males of their own strain suggests the involvement of a genetic mechanism of preference. Similar results have been seen in other organisms, where it is well known that mating preferences can involve many loci (Majerus et al. 1982; Houde 1994; Godin and Dugatkin 1995; Iyengar et al. 2002; P. Haesler and Seehausen 2005). Even more interesting and less understood, however, are the specific genes that are involved in establishing these preferences, and the gene networks or developmental processes in which they are involved. Finding these specific genes and elucidating their mechanistic roles with genetic manipulations could provide new insights into sexual selection theories that aim to explain how preferences evolve and persist. Given the availability of these genomic tools and resources, an extremely rapid generation time, and the evidence here that females exhibit mating preferences and respond to computer animations of males, the African turquoise killifish is poised to contribute to this important research enterprise.

Figure 2.1

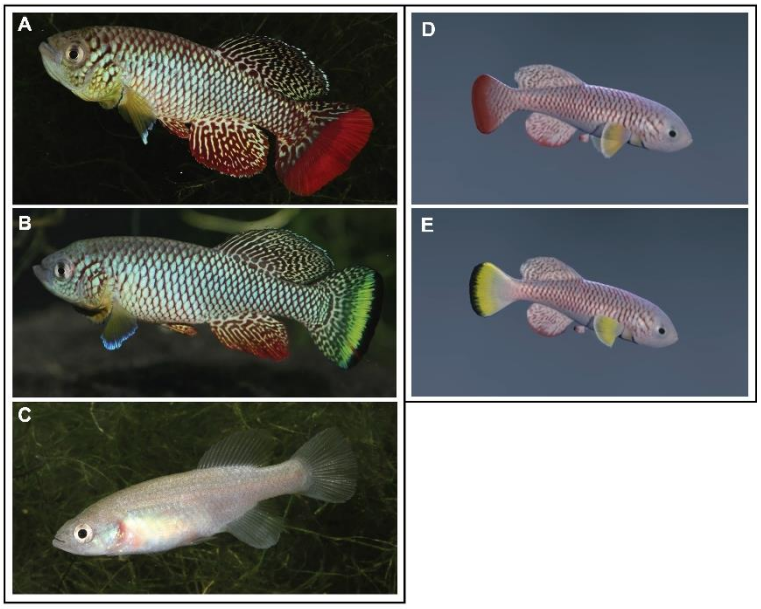

Figure 2.1: *Nothobranchius furzeri* male morphs.

Photos represent MZM (A) and GRZ (B), and a female (C) alongside animated males used in this study (D and E). Photos of *N. furzeri* (A, B, and C) were obtained with permission from (Cellerino et al. 2015).

Figure 2.2

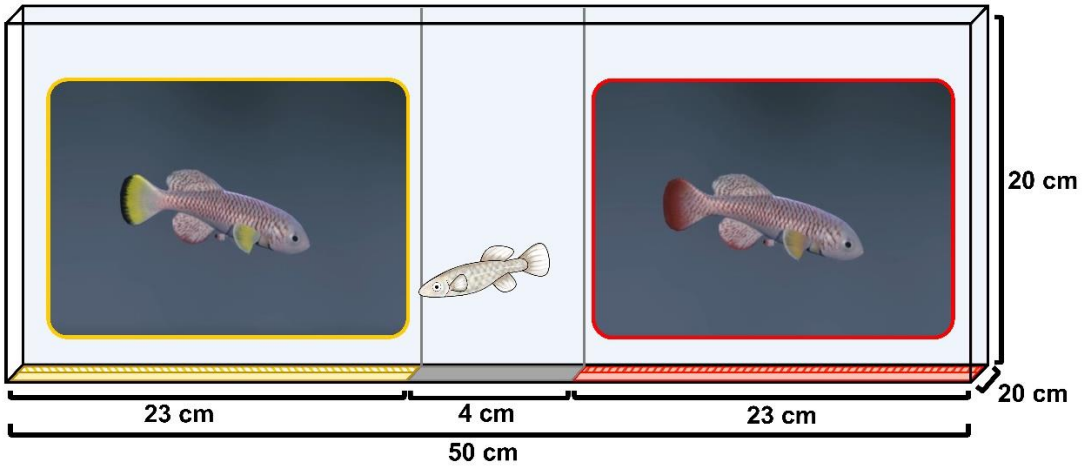

Figure 2.2: Behavioral tank setup.

A behavioral tank setup (20 cm W × 50 cm L x 20 cm water depth) with three zones was used. Male animations were randomly assigned to either the left or right side of the tank. For the scoring of the first behavior, the yellow and red zones (each 20 cm W x 23 cm L) were separated by a neutral area in the middle (20 cm W x 4 cm L). Here, we represent the bottom of each zone with a corresponding color. The scoring of the second behavior only included a subsection of the original yellow and red zones, which was the area 5 cm from the screen (each 5 cm W x 23 cm L). We represent this zone with stripes. The neutral zone for this behavior included the rest of the tank. Recording started once females had entered both the left and right zones, viewing both male options at least once. Time spent in either male animation's zone counted towards that respective male option. Time spent in the neutral zone did not count towards either option.

Figure 2.3

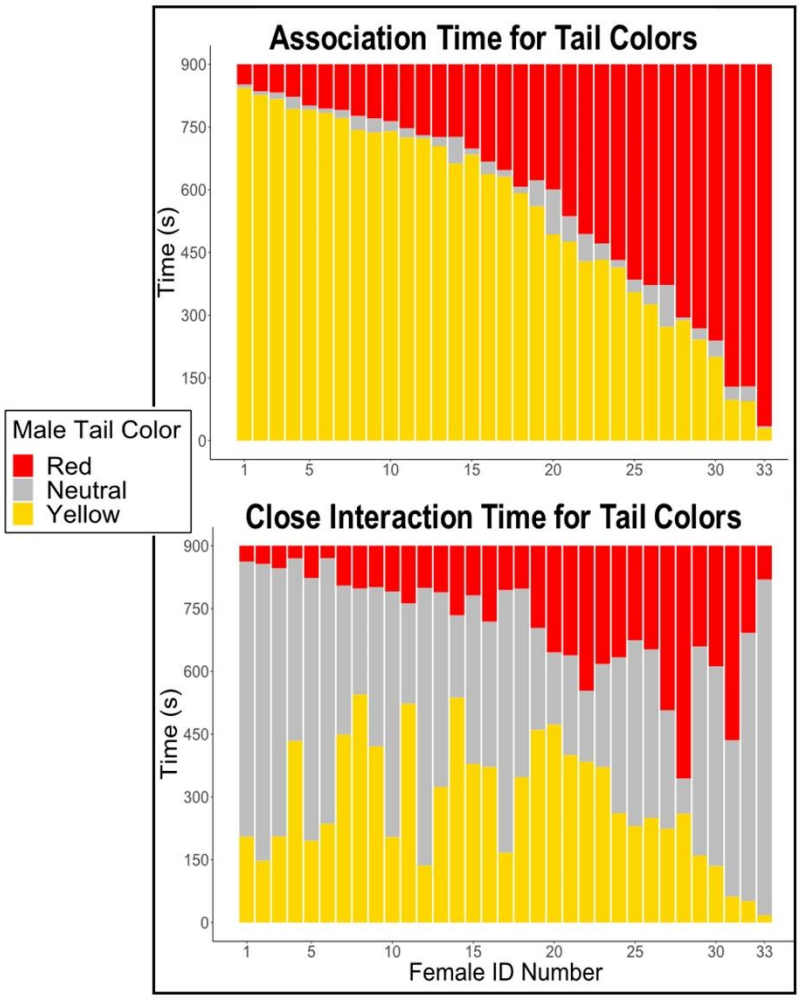

Figure 2.3: Time spend by females in each behavioral test.

The top panel shows the time females spent on the side of the tank with the GRZ yellow-tail male versus MZM red-tail male. The bottom panel shows the time females spent close to or swimming alongside the animated males. The x-axis for both charts displays each individual female tested and the time she spent in each zone. Individual females are shown in the same order in both panels. Time spent in neutral zones is shown in gray.

Table 2.1

	Average Time (± SEM)	Percentage of total time	*P*-value
(Behavior 1): Time female spent on either side of tank			
GRZ-male	543.0 ± 42.2 s	60.3%	0.015
MZM-male	325.0 ± 41.3 s	36.1%	
(Behavior 2): Time female spent closely interacting with screen			
GRZ-male	290.1 ± 25.4 s	32.2%	0.004
MZM-male	182.1 ± 22.2 s	20.2%	

Table 2.1: Behavioral Times

Time reported for two behaviors: (1) the total time females spent on either side of the behavioral tank, and (2) the total time females spent within 5 cm of the screen, swimming alongside or towards it. The GRZ animated males have the yellow and black banded tail color pattern, while the MZM animated males have the red tail color pattern. The values represent the average time (in seconds) GRZ females spend with either male (written with the standard error of the mean), the percentage of total time females spent engaged in either behavior with either male, and the *P*-value determined by a paired and two-sided Wilcoxon signed-rank test (Behavior 1: W=146, *n*=33; and Behavior 2: W=124, *n*=33).

VIDEO 2.S1: *N. furzeri* yellow-tail male digital model.

Video containing the yellow-tail male animation, actively swimming, and displaying, 17.5 MB.

VIDEO 2.S2: *N. furzeri* red-tail male digital model.

Video containing the red-tail male animation, actively swimming, and displaying, 16.9 MB.

VIDEO 2.S3: Fog-like background digital model.

Video containing the fog-like background used in the animations, 7.26 MB.

CHAPTER 3: THE EVOLUTION OF THE TESTIS TRANSCRIPTOME IN PREGNANT MALE PIPEFISHES AND SEAHORSES

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ABSTRACT

In many animals, sperm competition and sexual conflict are thought to drive the rapid evolution of male-specific genes, especially those expressed in the testes. A potential exception occurs in the male pregnant pipefishes, where females transfer eggs to the males, eliminating testes from participating in these processes. Here, we show that testis-related genes differ dramatically in their rates of molecular evolution and expression patterns in pipefishes and seahorses (Syngnathidae) compared to other fish. Genes involved in testis or sperm function within syngnathids experience weaker selection in comparison to their orthologs in spawning and livebearing fishes. An assessment of gene turnover and expression in the testis transcriptome suggests that syngnathids have lost (or significantly reduced expression of) important classes of genes from their testis transcriptomes compared to other fish. Our results indicate that more than 50 million years of male pregnancy have removed syngnathid testes from the molecular arms race that drives the rapid evolution of male reproductive genes in other taxa.

INTRODUCTION

A major goal of evolutionary genomics is to resolve the mechanisms responsible for broad patterns of genome evolution. One well-established observation regarding the evolution of genomes is that male-biased genes, especially those specific to the testis, tend to exhibit a pattern of rapid evolution and stronger signatures of selection compared to other genes in the genome (Meiklejohn et al. 2003; Zhang et al. 2004; Ellegren and Parsch 2007). The typical explanation for this pattern is that rapid evolution is driven either by sperm competition, which occurs when the ejaculates from different males compete with one another to fertilize eggs (Parker 1970, 1984), or sexual conflict, where males are selected to manipulate the reproductive interests of the females in a way that enhances short-term male fitness at the expense of female fitness (Parker 1979, 1984; Yapici et al. 2008). These mechanisms are not mutually exclusive; hence, we may expect sperm competition and sexual conflict to act in tandem in many sexually reproducing organisms.

With respect to sperm competition, we expect sexual selection to target sperm abundance, testis size, sperm characteristics, and seminal fluid proteins involved in male-male competition. In animals where females mate with multiple males per reproductive bout, sperm abundance and testis size are expected to increase as a function of the intensity of sperm competition (Parker 1990; Møller and Briskie 1995; Vahed and Parker 2012). This expectation is so pervasive that testis size has become a metric for the strength of sperm competition in a wide range of taxa, including primates, fish, and birds (Møller 1988; Stockley et al. 1997; Pitcher et al. 2005). Modification to sperm structure or size is also a common occurrence, with hallmark examples coming from *Drosophila*, where sperm can be 10 times the male's body length, as observed in *Drosophila hydei* (Pitnick and Markow 1994; Simmons 2001). Sperm competition risk is also expected to lead to an increase in abundance or recruitment of novel seminal fluid proteins, some of which may have evolved to inactivate sperm from competing males (Zbinden et al. 2003; Ramm et al. 2015; Simmons and Lovegrove 2017; Whittington et al. 2017).

The situation becomes somewhat more complex when we turn our attention to sexual conflict. In this case, males experience selection to manipulate female behavior in a way that enhances male fitness at the expense of the female's fitness (Arnqvist and Rowe 2005). Females, in turn, are selected to resist such male manipulation, potentially leading to an arms race between the reproductive interests of males and females (Arnqvist and Rowe 2005). Compelling examples of sexual conflict come from insects, where males have been shown to produce a range of effects on females, from reducing their mating receptivity, as occurs in katydids (Simmons and Gwynne 1991), to inflicting harm by increasing a female's fecundity beyond her optimal rate, a phenomenon observed

in fruit flies and field crickets (Baumann 1974; Loher 1979; Fowler and Partridge 1989; Arnqvist and Nilsson 2000). These examples, along with many others, have led to an expansion of our understanding of the varied mechanisms that males use to manipulate females, where tactics include mating plugs, seminal receptivity inhibitors, anti-aphrodisiacs, seminal toxins, aggressive sperm, and infertile sperm (Arnqvist and Rowe 2005).

This wide variety and divergence of reproductive phenotypes must ultimately be reflected in changes at the level of the genome. In terms of specific protein-coding genes, both sperm competition and sexual conflict are thought to increase rates of molecular evolution and alter expression patterns as males compete with other males and evolve to overcome female defenses. A large proportion of genes involved in male reproduction appear to evolve rapidly and show signs of positive selection (Gavrilets 2000; Wyckoff et al. 2000; Swanson and Vacquier 2002; Torgerson et al. 2002). Expression patterns can also change in response to increased sperm competition risk, as evidenced by investment plasticity in ejaculate components in the presence of rival males (Fedorka et al. 2011; Simmons and Lovegrove 2017). Moreover, sperm competition and sexual conflict are also expected to lead to a burst of novel genes and gene turnover within the genome, as new genes are recruited to participate in these processes (Zhang et al. 2007; Roberts et al. 2009; Harrison et al. 2015; Whittington et al. 2017).

Although the predictions of sperm competition and sexual conflict theory have been tested extensively in species with polygamous mating systems and strong sexual selection on males, very few studies have addressed these issues in species in which sexual selection and sperm competition are expected to be weak or absent. For example, we might expect species with monogamous mating systems, self-fertilization, parthenogenesis, or sex-role reversal to show different patterns of evolution compared to species with high potential for male-male competition and sexual conflict.

Species with a low potential for sperm competition are expected to evolve several phenotypic traits, such as reduced sperm abundance and testis size, a prediction that has been upheld in monogamously selected experimental populations (Hosken and Ward 2001; Simmons and García-González 2008). Low sperm competition also might relax selection on sperm morphology, potentially resulting in abnormal structure, as observed in the eusocial naked-mole rat (*Heterocephalus glaber*) (van der Horst et al. 2011). Also worthy of mention are the Eurasian bullfinch (*Pyrrhula pyrrhula*), Azores bullfinch (*Pyrrhula murina*), and the Greater Bandicoot rat (*Bandicota indica*), which have unusual sperm shape and low testis weight, leading to the hypotheses that these traits evolved as a consequence of an evolutionary history of monogamy (Durrant et al. 2010; Thitipramote et al. 2011; Lifjeld et al. 2013). The evolution of these testis and sperm-related phenotypes might include a shift

in the rate of molecular evolution in genes typically involved in testis function and the loss or reduced expression of genes with historically male-biased expression. For monogamous systems, testis-related traits (and the underlying genes) might be predicted to experience purifying selection or to be evolving neutrally, because they are either required to maintain a function or would experience relaxed selection due to a lack of sperm competition (Birkhead and Møller 1996; Bauer and Breed 2006; van der Horst and Maree 2014).

In self-fertilizing systems, a significantly smaller transcriptome and the absence of sex-biased genes is also predicted. Such a pattern has been documented in the self-fertilizing nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae* (Thomas et al. 2012), the latter of which was shown to have evolved a smaller genome and experienced the loss of some sexually important genes, such as *male secreted short* genes, which are involved in sperm competition and the production of sperm glycoproteins (Yin et al. 2018). Thus, some of the predictions regarding genome evolution as a consequence of sexual selection and conflict have been supported in at least one system. However, further work is necessary to strengthen our understanding of this phenomenon and to establish how broadly these predictions apply across disparate taxonomic groups and reproductive systems.

Here, we focus on pipefishes and seahorses (family Syngnathidae), in which females transfer eggs to the brood pouches of males and the male carries the developing embryos during an extended pregnancy (Paczolt and Jones 2010). All studies of parentage in pipefishes and seahorses have shown that males are always the genetic fathers of the offspring in their pouch, indicating that sperm competition is absent (Jones and Avise 1997b,a, 2001; Mccoy et al. 2001; Avise et al. 2002). Additional research has revealed the presence of traits typically associated with reduced sperm competition, including low testis weight and low sperm counts (Kvarnemo and Simmons 2004; Piras et al. 2016a). Furthermore, males possess no known mechanism to transfer sperm, seminal fluid, or any other related molecules to the female during mating, removing the testis from a role in sexual conflict. Indeed, the tables are turned, as the female transfers ovarian fluid and eggs to the male during mating (Paczolt and Jones 2010). This highly unusual mating strategy of syngnathids leads to the hypothesis that the male gonad-biased transcriptome will not be a target of selection related to sperm competition or sexual conflict, a pattern that starkly contrasts with most other vertebrates. Syngnathid fishes thus provide a unique opportunity to perform a critical test of the hypothesis that testis-related genes evolve rapidly due to sexual selection and sexual conflict. Here, we address the following two questions: (1) Is the pattern of molecular evolution in genes involved in testis function and sperm competition different in syngnathid fishes compared to fishes with more conventional

reproductive strategies? And (2) how has the suite of genes expressed in the testis been altered by evolution under male pregnancy?

METHODS

MOLECULAR EVOLUTION OF TESTIS GENES

We compared five members of Syngnathidae with two other teleost groups with more common reproductive modes: the livebearing fishes from Poeciliidae and the spawning fishes from Cichlidae. We used assembled genomes for five species of Syngnathidae, including two seahorses (*Hippocampus comes* and *Hippocampus erectus*) that exhibit strict monogamy and three polygamous pipefishes (*Syngnathus scovelli*, *Syngnathus floridae*, and *Syngnathus acus*) (Wilson et al. 2003). The annotated genome for the Gulf pipefish (*S. scovelli*) was obtained through the Cresko Lab website (https://creskolab.uoregon.edu/pipefish/) (Small et al. 2016). We also obtained the assembled *H. erectus* genome reported by Lin et al. (2017) and the assembled (but not annotated) *S. floridae* genome from NCBI (Lin et al. 2017). Annotated genomes were obtained from the NCBI Genome Database for the following species (all accession numbers for this article are listed in **Table 3.S1**): *H. comes*, *S. acus*, and *Pundamilia nyererei* (Brawand et al. 2014); *Maylandia zebra* (Brawand et al. 2014; Conte and Kocher 2015); *Haplochromis burtoni* (also known as *Astatotilapia burtoni*) (Brawand et al. 2014); *Oreochromis niloticus* (Brawand et al. 2014); *Xiphophorus maculatus* (Schartl et al. 2013); and *Poecilia mexicana*, *Poecilia latipinna*, and *Poecilia reticulata* (Künstner et al. 2016).

Genes (n = 24) involved in spermatogenesis, sperm structure, or seminal fluid composition were selected for their expected involvement in sperm competition from previous research, gene ontology predictions, ortholog predictions, and InterPro domain predictions (McGinnis and Madden 2004) (**Data 3.S1**). Orthologs were identified using OrthoDB (v10.1) for all members of Cichlidae and Poeciliidae under study, as well as for *H. comes* (Kriventseva et al. 2019). The ortholog sequence from *H. comes* was then reciprocally blasted (NCBI's TBLASTX and BLASTN) against genome assemblies for *S. scovelli*, *S. floridae*, *S. acus*, and *H. erectus*, which are not represented in the OrthoDB database. The best blast hits, with *E*-values less than 1×10^{-20}, were retained as orthologs for these syngnathid species.

Amino acid sequences were aligned using the package ClustalW (Thompson et al. 1994) implemented in MEGA (version 7.0.26) (Kumar et al. 2016). A phylogenetic tree (**Figure 3.1A**) was reconstructed using the concatenated alignment of all 24 genes using MEGA version 7.0.26 (Kumar et al. 2016). The tree was inferred by using the Maximum Likelihood method based on the JTT

matrix-based model (Jones et al. 1992). Neighbor-Joining and BioNJ algorithms were applied to a matrix of pairwise distances estimated using a JTT model for the heuristic search for the initial tree, and the topology with the best log likelihood value was selected. All positions with less than 95% site coverage were eliminated, leaving a total of 10,321 positions used in the final dataset. The percentage consensus for 1000 bootstrap replicates for a clade is reported, and branch lengths are measured as the number of substitutions per site. The final topology agreed with previous studies (Sanciangco et al. 2016; Hamilton et al. 2017; Rabosky et al. 2018) and the tree was used unrooted, without branch length information, as a reference for downstream molecular evolution analyses. The tree as depicted in **Figure 3.1A** represents the rate of substitutions for the genes of interest between taxa and not a phylogenetic reconstruction based on whole-genome alignments.

The ratio of nonsynonymous to synonymous substitutions (dN/dS or ω) was estimated using models implemented in the codeml program of the PAML package (version 4.9) (Yang 2007). We ran two analyses to test the rate of molecular evolution: a site-model that allows ω to vary over different sites in a gene, and a branch model that allows ω to vary over different branches within our tree. For site-based analyses, we performed each test within each of the following three taxonomic groups: pipefishes and seahorses (*S. scovelli*, *S. floridae*, *S. acus*, *H. comes*, and *H. erectus*), spawning cichlids (*P. nyererei*, *M. zebra*, *H. burtoni*, and *O. niloticus*), and livebearers (*X. maculatus*, *P. mexicana*, *P. latipinna*, and *P. reticulata*). For branch models, genes were aligned across all species (including those within Syngnathidae, Poeciliidae, and Cichlidae). For both site and branch models, we estimated ω and the likelihood ratio test statistic for each possible selection model for the same set of 24 genes involved in male reproduction.

The site models (model = 0) for M0, M1a, M2a, M7, and M8 (NSsites = 0, 1, 2, 7, 8) were run for each gene, within each taxonomic group, and the model of best fit was determined through a likelihood ratio test ($\alpha = 0.05$). We tested each of the three taxonomic groups separately, which provided a model of best fit and an overall ω estimate for each gene for each taxonomic group (72 total analyses). Primarily, we focused on a comparison between three models: a one-ratio model (M0) of purifying selection ($\omega < 1$), a nearly neutral model (M1a) that includes two classes of sites (purifying selection [$\omega < 1$] and neutral [$\omega = 1$]), and a positive selection model (M2a) with three classes of sites (positive selection [$\omega > 1$], purifying selection [$\omega < 1$], and neutral [$\omega = 1$]). Additionally, the beta model with 10 classes of sites ($\omega \leq 1$) (M7) and the beta and ω model with 11 classes of sites (10 with $\omega \leq 1$ and one with $\omega > 1$) (M8) were also run. For genes where M8 was significant, the null hypothesis M8a (NSsites = 8, fix_omega = 1, omega = 1) was also run (Swanson et al. 2003; Wong et al. 2004). Our results concentrate on our M0-M1a-M2a comparisons, because

these provide a more stringent test and are nearly identical to our M0-M7-M8-M8a comparisons (Yang et al. 2000). Results for the likelihood ratio test for all site models, as well as the dN/dS estimates for each gene, can be found in **Data 3.S1**. Additionally, the dN/dS values for all genes were averaged within the three family groups and the variance among genes within each group was calculated.

The branch models (NSsites = 0) for M0 versus M2 (model = 0, 2) were run and the model of best fit was determined through a likelihood ratio test ($\alpha = 0.05$). We tested the Syngnathidae branch for a different value of ω, in comparison to the other species of Cichlidae and Poeciliidae. This approach estimated two separate ω values (one for Syngnathidae and one for all other fish) and a likelihood value, which was compared against a null model (M0) that estimates a uniform ω for all fish. Results for the dN/dS estimates and the likelihood ratio tests for all branch models can be found in **Data 3.S1**.

GENOME-WIDE dN/dS ESTIMATION IN SYNGNATHUS

To compare the rate of molecular evolution of the 24 genes of interest to a genome-wide expectation within the genus *Syngnathus*, we identified single-copy orthologs among four species of pipefish. The genome files for *S. acus*, *S. rostellatus*, and *S. typhle* were obtained from NCBI (**Table 3.S1**), and analyzed along with the *S. scovelli* genome from the Cresko Lab. The genome, annotation, and protein files for *S. acus* were used as a reference species to run MAKER (version 3.01.03) (Cantarel et al. 2008) to annotate the genomes of the other three *Syngnathus* species. *Syngnathus acus* was used as the reference because it has the highest quality annotation of the species listed here.

After genome annotations were obtained for each of our species, OrthoFinder (version 2.5.4) (Emms and Kelly 2019) was used to align proteins and identify common orthologs between species of *Syngnathus*. OrthoFinder produced a list of all single-copy orthologs, which were then aligned at the protein level using MAFFT (version 7.487) (Katoh and Standley 2013). The nucleotide sequences were then retrieved for each protein alignment from the appropriate genomes using PAL2NAL (version 14) (Suyama et al. 2006). These nucleotide alignments were analyzed in codeml (PAML version 4.9) (Yang 2007) using a site model analysis, as described above (MOLECULAR EVOLUTION OF TESTIS GENES in *Methods*). The result was a dN/dS estimate and best fitting model of selection for each gene identified as a single copy ortholog across four members of the genus *Syngnathus*. Alignments with a dN/dS estimate of 999 were checked and removed (eight alignments, leaving a total of $n = 13{,}545$).

GENE TURNOVER IN GULF PIPEFISH TESTES

For this analysis, we addressed the hypothesis that patterns of gene turnover in the testes of syngnathid fishes would show a signature consistent with the loss of sperm competition in pipefishes and seahorses. We used RNA-seq data to identify testis-enriched genes in the Gulf pipefish (*S. scovelli*) and the Japanese pufferfish (*Takifugu rubripes*), which has external spawning. We selected the Japanese pufferfish for this comparison because it likely engages in sperm competition, is a member of the Percomorpha (which also includes the Syngnathidae), has a well-annotated genome, and is one of the few fish with replicated testis and ovary RNA-seq data available (Yamahira 1994). We blasted each set of highly expressed testis genes against the genome of the other species to determine the percentage of ortholog matches for highly expressed, testis-enriched genes. The proportion of matching testis orthologs was then compared to an expected distribution of ortholog matches, which was generated by randomly sampling sets of orthologs from the entire genome (without regard to testis enrichment).

We generated the *S. scovelli* RNA-seq data by using next-generation sequencing on testis tissue samples from seven males and ovary samples from five females. Fish were collected from the Gulf of Mexico, USA (Redfish Bay, TX) in accordance with IACUC approval (2013-0020). Only individuals that showed either a well-developed brood pouch or secondary sexual traits were used to ensure all fish were sexually mature. Fish were euthanized with an overdose of MS-222. RNA was extracted and isolated from both testis and ovary tissues using TRIzol® Reagent (Life Technologies, Carlsbad, CA) (Leung and Dowling 2005). Libraries were prepared using TruSeq mRNA Library Prep Kit version 2 by Michigan State University RTSF Genomics Core and quality was tested using Caliper GX and qPCR methods. All individuals were barcoded and sequenced individually using two lanes of Illumina HiSeq 2500 sequencing, and base calling was done with Illumina Real Time Analysis (RTA) (version 1.17.21.3). The output of RTA was demultiplexed and converted to FastQ with Illumina Bcl2fastq (version 1.8.4) resulting in 150 bp paired end reads used for downstream analyses. Raw paired-end reads from testes and ovaries for the Japanese pufferfish were obtained through the NCBI SRA library (**Table 3.S1**) (Wang et al. 2017; Yan et al. 2018). Trinity (version 2.8.4) (Grabherr et al. 2011) was used for *de novo* assembly of testis-only reads into a separate transcriptome for both *T. rubripes* and *S. scovelli* using default parameters. For this analysis, we used a *de novo* assembled transcriptome instead of a reference genome for two main reasons. The first is using a *de novo* transcriptome ensures that we capture sequences that might not be present in the genome (Grabherr et al. 2011). This can be especially important if genomes have different assembly annotation qualities. The second is that the *S. scovelli* genome was not annotated using testis RNA-

seq data, so although all the genes should be present within the genome, novel transcripts that are important to this specific analysis might not be (Small et al. 2016). Trimmomatic (Bolger et al. 2014) was used to trim reads inside of Trinity, using the settings SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25.

Reads from both the testes and the ovaries were then mapped back to the species-specific testes transcriptome using RSEM (version 1.3.1) (Li and Dewey 2011) and the raw contig read counts were recorded. From these datasets, EBSeq (version 3.8) (Leng and Kendziorski 2019) detected differentially expressed contigs between testes and ovaries and generated a posterior probability for each contig. Using a false discovery rate (FDR) of 0.05, only contigs with a posterior probability of differential expression ≥0.95 were kept for analysis. Testis-biased fold changes were calculated per contig as the mean testis expression level divided by the mean ovary expression level. Transcripts that had an expected count of at least 500 reads and a testis-biased fold change of 2 or more were extracted and labeled as highly and differentially expressed. These cutoff values were chosen to provide a substantial sample size while ensuring we chose transcripts that were highly and differentially expressed. A total of 325 transcripts for pufferfish and 831 transcripts for pipefish were identified as highly and differentially expressed in the testis.

The transcripts from each fish were then blasted using NCBI's TBLASTX (McGinnis and Madden 2004) to their own annotated genome file to find a matching annotated gene. The annotated genome for *T. rubripes* was available through the NCBI Genome Database (Aparicio et al. 2002). A match was any sequence that had a blast hit with an *E*-value $\leq 1 \times 10^{-20}$. If a transcript did not have a match in the annotated gene list, the transcript was then blasted against the whole genome. If there was a match in the whole genome file, then the transcript sequence from the *de novo* assembly was used in this analysis. When multiple transcripts matched the same gene, in both the annotated genome and the whole genome file, the match with the smallest *E*-value was used. This was done to prevent potential bias resulting from differences in paralog density or annotation quality.

The corresponding annotated sequences of the highly and differentially expressed transcripts from *T. rubripes* (or the transcript sequences as described above) were then blasted using NCBI's TBLASTX, to the whole genome of *S. scovelli*. An ortholog match was any sequence that had an alignment with an *E*-value $\leq 1 \times 10^{-20}$. The reciprocal search for the *S. scovelli* highly and differentially expressed genes in the whole genome for *T. rubripes* was also performed. This analysis was used to calculate a percentage of orthologs present for testis-biased genes of one species in the other species' genome.

To generate a null expectation of ortholog presence in the reciprocal comparisons between species, we sampled random sets of protein coding genes from the entire genome and performed the same analysis as described above. The number of genes in the set was identical to the number of testis-biased genes in the species of interest. We then used TBLASTX to compare these genes from the species of interest against the whole genome of the other species. The percentage of genes with significant hits was recorded, and we repeated this entire procedure 1000 times to generate a distribution for randomly chosen sets of genes. This distribution represents a null expectation for the proportion of randomly chosen genes that are present in the other species' genome for a sample of genes equal in size to the number of testis-biased genes identified for the focal species. We then compared the proportions for testis-biased genes against this null distribution for each reciprocal comparison between pipefish and pufferfish (**Figure 3.2**).

FUNCTIONAL EVOLUTION OF PIPEFISH TESTES

To compare the testis transcriptome of the Gulf pipefish (*S. scovelli*) to other related species of fish, we first obtained gonadal RNA-seq reads and genome data from six other representatives of the Percomorpha. We also obtained data from zebrafish (which is not in Percomorpha) as an outgroup. Genomes and RNA-seq data were obtained from the NCBI genome and SRA databases for the following species: *T. rubripes*, *Oryzias latipes*, *P. reticulata*, *Paralichthys olivaceus*, *Nothobranchius furzeri*, and *Lates calcarifer* (**Table 3.S1**). We also obtained the zebrafish (*Danio rerio*) genome, with two testis and two ovary datasets (**Table 3.S1**).

We trimmed the testis and ovary RNA-seq reads using Trimmomatic (with settings ILLUMINACLIP:TruSeq3-PE.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:75), and we aligned the reads against assembled genomes using HISAT2 (version 2.1.0) (Kim et al. 2015). Sorted BAM files containing the HISAT2 results were fed into StringTie (version 2.1.3b) (Pertea et al. 2015) to identify and quantify transcripts. Within each species, we ran StringTie separately for each RNA-seq dataset, merged the results into a single merged gff file, and then estimated species-specific abundances against this merged gff transcript file.

For the analysis of the top 1000 testis genes, we ranked all genes identified by StringTie by TPM (transcripts per million), from largest to smallest. For each of the top 1000 genes in terms of TPM, we extracted all transcripts assembled by StringTie. The cutoff value was chosen to provide a substantial sample size while ensuring we chose genes that were most expressed. Each transcript was converted to the protein encoded by its longest open reading frame by using the C++ program fastatoorf. To compare these transcripts against a common reference proteome, we downloaded all

annotated zebrafish proteins from UniProt (Zebrafish-UP000000437_7955) and constructed a local BLAST database. We used BLASTP to compare all retained transcripts against the zebrafish proteome, using an *E*-value cutoff of 1×10^{-20}. For each transcript with at least one hit below the *E*-value cutoff, we retained the zebrafish protein ID of the best hit. If multiple transcripts blasted to the same protein, duplicate entries were removed from the final list. Thus, for each species, we obtained a list of putative zebrafish orthologs that correspond to the top 1000 genes expressed in the testis transcriptome of the focal species. The number of unique genes on the final lists was 824 for *P. olivaceus*, 805 for *T. rubripes*, 746 for *P. reticulata*, 811 for *N. furzeri*, 839 for *O. latipes*, 711 for *L. calcarifer*, 919 for zebrafish, and 805 for Gulf pipefish (mean = 807.5 genes).

For the gene ontology analysis, we used the PANTHER database and associated tools (version 15.0) (Thomas et al. 2003a,b; Mi et al. 2013b). We uploaded testis gene lists from each species under consideration and performed a functional classification based on the zebrafish database. We performed gene ontology analyses for molecular function and biological process by using the curated PANTHER GO-Slim databases. We also performed an analysis using the PANTHER Protein Class database for each species. Results were compiled and compared as percentages of the total hits in each database. For statistical analyses, we calculated expected proportions using the mean across the six non-pipefish Percomorphs. These proportions were used to derive expected values for Gulf pipefish and zebrafish, under the null hypothesis that these species do not differ from Percomorphs with respect to the functional classification of highly expressed testis genes. A χ^2 test was used to test this hypothesis for each of the gene ontology analyses (i.e., biological process, molecular function, and PANTHER protein class). Categories with expected values less than five were lumped for the purposes of this test. In the case of a significant χ^2 test, the number of genes assigned to each category was compared between Percomorphs and either pipefish or zebrafish using a one-sample, two-sided *t*-test, with an FDR correction at 0.05.

For the overrepresentation analysis, we used the PANTHER tools to perform a statistical overrepresentation test. This test was performed using the GO biological process complete dataset implemented in PANTHER. The zebrafish genome was used as the reference gene set, as it is an outgroup relative to all other fish under consideration here. For each species, we uploaded the list of highly expressed testis genes derived from the BLASTP analysis described above. PANTHER returns a fold-enrichment value for each gene ontology category relative to expectations based on the frequency of occurrence of genes in that category in the reference genome. We examined fold-enrichment values for all 25,888 gene ontology categories for all eight species under consideration here. We calculated mean fold-enrichment for each category across the six non-pipefish Percomorphs

and compared pipefish and zebrafish against these values using a simple linear regression. For this analysis, we retained categories that had a mean of at least four genes in Percomorphs (not including pipefish), resulting in a final list of 815 gene ontology categories for comparison with Gulf pipefish and zebrafish. Clusters of genes outside the 95% prediction intervals of the linear regressions were considered to be significant outliers.

MOLECULAR EVOLUTION OF GONAD-BIASED GENES

To connect our preceding analyses together, we investigated the rate of molecular evolution of testis-biased genes from *S. scovelli* and *T. rubripes*, our representative pipefish and spawning fish. The goal was to compare testis-biased genes with ovary-biased genes, and a control set of genes that were highly and evenly expressed between both gonads. As noted above, these two species were the only species within the Percomorpha clade with replicate testis and ovary RNA-seq data.

Genes that were identified by HISAT2 (Kim et al. 2015) and quantified by StringTie (Pertea et al. 2015) (see FUNCTIONAL EVOLUTION OF PIPEFISH TESTES in *Methods*) were used to determine the average TPM value for each gene within testis samples and within ovary samples for *S. scovelli* and *T. rubripes*, separately. To subset genes for the gonad-biased categories, we retained genes with a four or greater fold change (i.e., a $\log_2$ fold change greater than or equal to 2) difference between testis and ovaries. To restrict attention to genes that were highly expressed, we also set an average TPM cutoff. This threshold was a minimum TPM of 100 for *S. scovelli* and 20 for *T. rubripes*. We varied the TPM threshold to keep the sample sizes for the molecular evolution analysis roughly similar across species. In particular, we lowered the TPM threshold for *T. rubripes*, because very few differentially expressed genes met the TPM threshold of 100. To subset genes for the evenly expressed category, we took genes with a $\log_2$ fold change between −0.3 and 0.3. For the evenly expressed genes, the TPM cutoff was a minimum of 50 for *S. scovelli* and a minimum of 20 for *T. rubripes*. Again, we varied the TPM cutoff to control the sample size. Altogether this left a list of highly expressed testis-biased genes, highly expressed ovary-biased genes, and highly and evenly expressed genes across gonads for both *S. scovelli* and *T. rubripes*.

To provide an estimate of molecular evolution for each of these identified genes, the nucleotide sequences from the three lists of genes (highly expressed testis-biased genes, highly expressed ovary-biased genes, and highly and evenly expressed genes in both gonads) as well as their respective ortholog sequences from other species within their genus were aligned. For *Syngnathus*, we used *S. scovelli*, *S. rostellatus*, *S. typhle*, and *S. acus*, following the same pipeline as mentioned above (see GENOME-WIDE dN/dS ESTIMATION IN SYNGNATHUS in *Methods*). For

the *Takifugu* genus, we obtained the genome, annotation, and protein files for *T. rubripes* and *T. flavidus* from NCBI (**Table 3.S1**). Here, single-copy orthologs were identified and aligned using the same pipeline we used for *Syngnathus*. Our ortholog analysis was run within *Syngnathus* and within *Takifugu* to increase the likelihood of finding orthologs between species within each genus.

For the genes of interest that had single-copy orthologs, we used codeml (PAML version 4.9) (Yang 2007) to implement site models as previously mentioned (MOLECULAR EVOLUTION OF TESTIS GENES in *Methods*). The result was a dN/dS estimate for each gene identified as gonad biased or evenly expressed within either *Takifugu* or *Syngnathus*. Genes with a dN/dS estimate higher than 10 were checked and removed if there were no synonymous differences in the alignment (thus inaccurately inflating dN/dS). Ultimately, the following number of genes produced dN/dS estimates within each category: *Syngnathus* testis biased (429), ovary biased (473), and evenly expressed (546); *Takifugu* testis biased (97), ovary biased (151), and evenly expressed (726).

RESULTS

MOLECULAR EVOLUTION OF TESTIS GENES

The phylogeny of the taxa involved in our analysis of molecular evolution is shown in **Figure 3.1A**, and the species-specific distributions of dN/dS values from the sites models are shown in **Figure 3.1B**. These models indicate that the dN/dS of Syngnathidae (0.204 ± 0.029, mean ± SEM) is smaller but not significantly so, compared to those of Poeciliidae (0.269 ± 0.044) and Cichlidae (0.283 ± 0.041) for genes involved in testis-related processes (Mann-Whitney-Wilcoxon test, two-sided, paired, $n = 24$: Syngnathidae vs. Poeciliidae, $P = 0.277$; Syngnathidae vs. Cichlidae, $P = 0.121$; Poeciliidae vs. Cichlidae, $P = 0.473$). We also observe that the Syngnathidae orthologs show a nonsignificant reduction in variance in dN/dS ($\sigma^2 = 0.021$), compared to Poeciliidae ($\sigma^2 = 0.049$; Brown-Forsythe test, $P = 0.242$) and Cichlidae ($\sigma^2 = 0.042$; $P = 0.132$).

To further investigate these patterns, we estimated models of selection for each gene using likelihood ratio tests, as implemented in codeml. For each ortholog in each taxonomic group, we were able to classify its best-fitting evolutionary model as purifying selection, nearly neutral, or positive selection. **Figure 3.1C** shows the percentages of orthologs categorized into each model for Syngnathidae (20.8% purifying selection, 79.2% nearly neutral, and 0% positive selection), Poeciliidae (62.5% purifying selection, 33.3% nearly neutral, and 4.2% positive selection), and Cichlidae (58.3% purifying selection, 29.2% nearly neutral, and 12.5% positive selection). The gene-by-gene results are summarized in **Data 3.S1**. Overall, these results are consistent with the idea that syngnathid fishes have experienced a simultaneous reduction in positive selection and purifying

selection in their testis transcriptomes, showing a pattern of generally weaker selection acting on male reproductive function, compared to other fishes.

With respect to our branch models, we found that 41.7% (n = 10) of the genes showed a different branch rate (significance for M2) for ω between Syngnathidae and the other teleosts (Poeciliidae and Cichlidae; **Data 3.S1**). In eight of these cases, ω was greater for the Syngnathidae branch (with an average ω of 0.218) than the other teleosts branches (average ω of 0.114), and in the other 2 cases ω was greater for the other teleosts (average ω of 0.277) than the Syngnathidae branch (average ω of 0.157). For these 10 genes, the Syngnathidae alignments best fit models of nearly neutral evolution (seven genes) or purifying selection (three genes).

Our genome-wide dN/dS analysis within the genus *Syngnathus* resulted in an average dN/dS value of 0.267 ± 0.002 (mean ± SEM). The percentage of orthologs categorized into each model of selection were as follows: 62.2% purifying selection (M0), 20.2% nearly neutral (M1a), and 17.6% positive selection (M2a). These proportions differ substantially from those observed for the 24 testis- or sperm-associated proteins examined in Syngnathidae (**Figure 3.1C**).

GENE TURNOVER IN GULF PIPEFISH TESTES

For our second question, we examined patterns of gene turnover in syngnathid testes by comparing the presence of orthologs for testis-enriched genes between the Gulf pipefish (*S. scovelli*) and the Japanese pufferfish (*T. rubripes*). Testis-enriched transcripts of the Gulf pipefish were compared against the Japanese pufferfish genome to establish the proportion of pipefish transcripts present in the pufferfish. We also performed the reciprocal comparison, to establish the proportion of pufferfish transcripts present in the pipefish. The proportions of matches for testis transcripts were compared against a null distribution generated by conducting the same procedure a thousand times for randomly chosen sets of genes from each species' genome (regardless of testis enrichment).

Our comparison of testis transcriptomes between the Gulf pipefish and the Japanese pufferfish showed that pipefish testis-enriched genes were significantly more likely to have orthologs in the pufferfish genome compared to subsets of randomly chosen genes (**Figure 3.2**, bottom). In addition, the reverse pattern was seen in the reciprocal comparison, where pufferfish testis-enriched genes were less likely than randomly chosen genes to appear in the pipefish genome (**Figure 3.2**, top). Specifically, 90.6% of pipefish testis-enriched genes were present in the pufferfish genome, whereas a mean of 85.9% of randomly chosen sets of genes had orthologs in pufferfish. However, only 75.4% of pufferfish testis-enriched genes were found in the pipefish genome, despite a mean of 92.9% for randomly chosen sets of pufferfish genes.

The difference in the distributions for reciprocal comparisons for randomly chosen genes likely arises from distinct histories of gene duplication and gene loss or differences in genome quality in terms of assembly and annotation. This would be consistent with findings on the pufferfish lineage (*Tetraodon*), which has been documented to have recently lost genes that arose after the teleost whole-genome duplication event, potentially leading to pufferfish speciation (Taylor et al. 2003; Kai et al. 2011). This historical pattern of genome evolution could contribute in part to the difference in null expectations and underscores the need to interpret the percentage of shared testis orthologs relative to a null distribution.

FUNCTIONAL EVOLUTION OF PIPEFISH TESTES

To examine the evolutionary changes in the testes of Syngnathidae since the origin of male pregnancy, we compared the testis transcriptome of the Gulf pipefish to six other percomorph species, as well as to zebrafish (*D. rerio*), an outgroup species. The results of the GO analysis are shown in **Figures 3.S1–3.S3**. There were slight but nonsignificant differences between the Gulf pipefish and other fish taxa with respect to these GO categories (**Figure 3.S2**). For the protein class analysis, we used the PANTHER Protein Class database (version 15.0). This analysis revealed an increase in expression of genes encoding calcium-binding proteins, membrane traffic proteins, and chaperones for the Gulf pipefish in comparison to the other percomorph species (**Figure 3.3A**). Gulf pipefish also show a dramatically lower number of genes categorized as protein modifying enzymes (**Figure 3.3A**). Notably, the zebrafish transcriptome does not differ significantly from the Percomorphs (excluding pipefish) with respect to any of these categories (**Figure 3.3A**), suggesting that Gulf pipefish testes differ from typical fish testes in terms of the types of proteins encoded by highly expressed genes.

The results of our comparative overrepresentation analysis are shown in **Figure 3.3B,C** The scatterplot indicates a cluster of GO categories that are strongly overrepresented in most Percomorphs (more than threefold enrichment) but are not overrepresented or are missing from Gulf pipefish (**Figure 3.3B**, red arrow). The comparison of zebrafish to Percomorphs contains no such cluster, indicating that the reduction of genes in these GO categories is unique to the Gulf pipefish testis. We examined the functions of these outlying genes (**Figure 3.3B**), as well as the protein-modifying enzymes that were apparently lost from the highly expressed category in the pipefish testis transcriptome (**Figure 3.3A**). Beginning with the protein-modifying enzymes, we compared gene counts for each of the subcategories of protein-modifying enzymes between Gulf pipefish and the other taxa (**Table 3.1**, Panel A). Gulf pipefish show a reduction in genes classified as various types of proteases, nonreceptor serine/threonine protein kinases, and ubiquitin-protein ligases relative to the other species. The overrepresentation analysis reveals a suite of GO categories that have evolved

reduced expression in Gulf pipefish relative to other Percomorphs and zebrafish (**Table 3.1**, Panel B). These GO categories are all related to the assembly and function of the sperm cell's flagellum, or cell division and meiosis.

MOLECULAR EVOLUTION OF GONAD-BIASED GENES

To tie together both our initial questions, we investigated the pattern of molecular evolution on the suite of genes expressed in the testis of pipefish. We calculated dN/dS estimates for genes that were highly expressed and testis biased, ovary biased, or evenly expressed between testis and ovaries for *S. scovelli* and compared these results to those for a percomorph with more conventional reproduction (*T. rubripes*). The following median dN/dS values were obtained for each of these categories: *Syngnathus* testis biased (n = 429, median = 0.218), ovary biased (n = 473, 0.224), and evenly expressed (n = 546, 0.160); *Takifugu* testis biased (n = 97, 0.399), ovary biased (n = 151, 0.253), and evenly expressed (n = 726, 0.224) (**Figure 3.4**; **Table 3.S2**). In *Syngnathus*, the rate of molecular evolution of testis-biased genes did not differ from that of ovary-biased genes (Mann-Whitney-Wilcoxon test, two-sided, unpaired: P = 0.840). This result contrasts with our analysis involving *Takifugu*, where testis-biased genes exhibited a significantly higher dN/dS value compared to ovary-biased genes ($P \leq 0.001$). In fact, the testis-biased genes of *Takifugu* differed significantly from both ovary-biased genes and evenly expressed genes ($P \leq 0.001$), which did not differ significantly from one another (*Takifugu* ovary biased vs. evenly expressed: P = 0.2029).
For *Syngnathus*, however, both testis-biased genes and ovary-biased genes showed significantly elevated dN/dS values compared to evenly expressed genes (*Syngnathus* testis biased vs. evenly expressed, and ovary biased vs. evenly expressed: $P \leq 0.001$).

DISCUSSION

The study of reproductive genes and the proteins they encode has been strongly motivated by arguments related to sperm competition and sexual conflict (Zhang et al. 2007; Harrison et al. 2015; Yin et al. 2018). The underlying notion, which is consistent with much of the published literature, is that male-male competition or an arms race between the sexes drives rapid evolution of the proteins mediating these processes (Simmons 2001; Swanson and Vacquier 2002; Lüpold et al. 2016; Dean et al. 2017; Civetta and Ranz 2019; Liao et al. 2019). However, most of the tests of these ideas have been in species with considerable potential for strong selection on male reproductive function and conflict between the sexes, with a few notable exceptions, which we will discuss further below. Here, we provide a critical test of these predictions by studying molecular evolution and gene turnover in a sexually reproducing, male-pregnant vertebrate species where the testes have no opportunity to play a

role in either sperm competition or sexual conflict. Our results support the interpretation that rapid evolution of male reproductive proteins and rapid gene turnover in the testis is indeed driven by sexual selection and conflict, and that the syngnathid fishes differ from the normal pattern of testis evolution because male pregnancy has diminished the role of the male gonad in sexual selection and sexual conflict.

MOLECULAR EVOLUTION OF TESTIS-RELATED GENES

Our analysis of dN/dS revealed modest values for all species under consideration here. The mean dN/dS values for the 24 testis-associated genes were most similar for cichlids and livebearers, and slightly lower but not significantly so for pipefish. The variance in dN/dS among genes was lower for pipefish than for cichlids and livebearers, but again this result was not statistically significant. None of the genes in this study displayed exceptionally large values of dN/dS, as have been observed in other studies of genes involved in male reproduction. For instance, Wyckoff et al. (2000) studied 18 genes directly involved in male reproduction in primates and found that five of these genes (28%) had dN/dS values >1 (Wyckoff et al. 2000). Other studies of specific gene families involved in male reproduction, such as the *CRISP* (cysteine-rich secretory proteins) genes and *Adam* (a disintegrin and metalloprotease) genes in rodents, also found evidence of positive selection, in some cases with very large dN/dS values (Grayson and Civetta 2013; Vicens and Treviño 2018). However, the study of *CRISP* genes, like ours, found multiple genes with evidence of positive selection despite having dN/dS values <1.

Regardless of the absolute values of dN/dS, the more compelling comparison involves the scrutiny of codeml-estimated models of selection in syngnathids relative to other fishes. Our results for cichlids and poeciliids are remarkably similar. Both groups show a majority of genes (around 60%) with a pattern of purifying selection (i.e., the one-ratio model in codeml was the best fit). About a third of the genes in each group were best fit by a nearly neutral model, and a small number of genes in each group were best described as positively selected (4% and 13% of genes, respectively, in livebearers and cichlids). Thus, in these taxa, codeml classified about two thirds of genes as experiencing some form of selection, either positive or negative. The results for syngnathids were dramatically different. None of these genes were categorized as positively selected in syngnathids and only 20% showed evidence of purifying selection. Rather, the vast majority of genes (79%) best fit a nearly neutral model (i.e., a two-ratio model with a mixture of sites either experiencing purifying selection or evolving according to neutrality). All 24 genes were present in the *de novo* Trinity assembly based on the RNA reads from *S. scovelli* testes (section GENE TURNOVER IN GULF PIPEFISH TESTES), so they are expressed at some level in pipefish testes. We interpret these results

as an indication that selection on testis-expressed genes, be it positive or negative selection, is generally weaker in syngnathid fishes compared to other percomorphs.

The branch models from codeml also were consistent with this interpretation of reduced selection on testis-related genes in syngnathids compared to other taxa. Out of the 24 genes analyzed, 10 showed a significantly different rate of molecular evolution in syngnathid fishes compared to poeciliids and cichlids. For eight of these genes, the syngnathid branch had an elevated dN/dS, consistent with a relaxation of purifying selection in the syngnathid group. The other two genes had reduced dN/dS values in syngnathids, a pattern that could be consistent with several possibilities, such as an increase in purifying selection in syngnathids or a reduction of positive selection on some parts of the proteins in syngnathids. Regardless, many genes in the branch analysis are consistent with the emerging pattern that selection on testis-associated genes is weaker in the syngnathid lineage relative to other percomorphs.

These findings are particularly interesting when compared to the genome-wide molecular evolution estimates, which show that members of Syngnathidae do not have an unusually low baseline of molecular evolution across their genome. For instance, recent studies have examined the strength of selection on genes related to other complex adaptive phenotypes important to the evolutionary success of syngnathids. One example of such a trait involves the independent evolution of spiny body plates, a predator deterrent, across the seahorse phylogeny (Li et al. 2021). A comparison between spiny and nonspiny seahorse lineages revealed 37 genes that were under strong positive selection and had likely roles in teleost skin and scale development (Li et al. 2021). The independent appearance of complex morphological phenotypes such as this, the high diversification rate within the *Hippocampus* clade (Li et al. 2021), and the accelerated rate of nucleotide evolution within *H. comes* in comparison to other teleost genomes (Lin et al. 2016) demonstrate the potential for accelerated evolution of advantageous traits within syngnathids and are in blunt contrast with our findings within the testis-related genes.

GENE TURNOVER IN SYNGNATHID TESTES

We predicted that weaker selection acting upon testes in the pipefish should lead to a loss of orthologs over time (Whittington et al. 2017; Yin et al. 2018). Under this scenario, an ancestral pipefish, shortly after the evolution of male pregnancy, would have found itself in a situation where its testes produced more sperm than necessary to fertilize the eggs in its possession. Its testes also would have been producing unnecessary sperm-related substances that would have aided in sperm competition before the evolution of male pregnancy. Presumably, selection in a pregnant male would

act to reduce the number of sperm and to cease the production of potentially costly reproductive substances that had once been involved in sperm competition but are no longer needed. Consequently, the genes involved in these traits would be subjected to genetic drift or active selection to reduce male energetic expenditures. The result would be a streamlined testis transcriptome that only harbors genes essential to produce just enough sperm to fertilize the eggs received during mating. This scenario agrees well with empirical observations from various syngnathid testes, where phenotypes such as unusually small testis size and low sperm density, along with semicystic spermatogenesis, are predicted to reduce the cost of sperm production (Wilson et al. 2003; Kvarnemo and Simmons 2004; Biagi et al. 2016; Piras et al. 2016b).

We chose to test this prediction by comparing the genes upregulated in the Gulf pipefish testis to those upregulated in the testis of the pufferfish (*T. rubripes*). We found that 90.6% of the testis-upregulated syngnathid genes were present in the pufferfish genome. This percentage is higher than the expectation based on randomly chosen genes (**Figure 3.2**, bottom). Our interpretation of this result is that the syngnathids have retained genes that are essential for testis function, because syngnathid males, like males of other fish, must still produce sperm to reproduce. These genes, as they are essential for reproduction, have also been retained by pufferfish, resulting in nearly all testis-enriched genes in syngnathids having orthologs in the pufferfish genome.

In contrast, the reciprocal comparison, which involved looking for testis-upregulated pufferfish genes in the pipefish genome, resulted in a substantially smaller proportion of hits (75.4%). This value is much less than the proportion of hits observed for randomly chosen genes (**Figure 3.2**, top). This result indicates that many testis-upregulated genes in pufferfish either have sequences that are not similar enough to be identified by our BLAST-based comparison or are missing altogether in pipefish. This pattern can be interpreted in several different ways, all of which are consistent with a reduction of selection acting on the testes of syngnathids. Syngnathids may have simply lost some genes that are involved in sperm competition. Alternatively, stronger selection acting upon the testes of the pufferfish could have led to an increase in recruitment of novel genes (Harrison et al. 2015; Whittington et al. 2017; Schmitz et al. 2020). Another possibility is that rapid evolution of testis-expressed pufferfish genes, driven in part by sperm competition or sexual conflict, could have resulted in so much sequence evolution that the ortholog was no longer identifiable in our cross-species comparison. All these interpretations are consistent with a relaxation of selection on genes associated with syngnathid testes, much like the results of our molecular evolution investigation.

FUNCTIONAL GENOMICS OF PIPEFISH TESTES

Our analyses of gene ontology and overrepresentation revealed intriguing changes during the evolution of the syngnathid testis. The most noteworthy change detected by our gene ontology analysis was a loss of protein-modifying enzymes, cysteine and serine proteases, and metalloproteases, in the highly expressed testis genes of the Gulf pipefish relative to other percomorphs and our zebrafish outgroup. Interestingly, these types of proteases are heavily involved in testis development, spermatogenesis, sperm capacitation, and sperm-egg binding (Gurupriya and Roy 2017). Cysteine proteases are involved in sperm capacitation (Lee et al. 2018) and serine proteases are a major component of the acrosome (Klemm et al. 1991). Metalloproteases have been shown to be involved in mouse testis development (Guyot et al. 2003) and are associated with sperm quality and ejaculate volume in dogs and humans (Shimokawa et al. 2002; Tentes et al. 2007). Within syngnathids, a zinc-dependent metalloprotease named *patristacin* has been found to be highly expressed in the brood pouch of pregnant males, suggesting a novel role for metalloproteases in male pregnancy (Harlin-Cognato et al. 2006; Whittington et al. 2015; Small et al. 2016; Lin et al. 2017). The *patristacin* gene family has further expanded through duplication within syngnathids, with specific *patristacin* genes showing distinct regulation patterns within the brood pouch depending on the stage of pregnancy (Lin et al. 2016, 2017; Small et al. 2016). In this regard, metalloproteases have an integral role to brood pouch function, and might have a less pivotal role in the function of the testis.

Our gene ontology analysis also showed a reduced number of nonreceptor serine/threonine protein kinases in pipefish testes compared to those of other fishes. This category of genes has been shown to play a role in sperm capacitation (O'Flaherty et al. 2004), and specific kinases, such as protein kinase A (PKA), can be found in the acrosomal cap and sperm flagellum in mammals (Pariset and Weinman 1994; Visconti et al. 1997). PKA activity is also involved in a series of events that leads to tyrosine phosphorylation, which is necessary for other processes within a mature spermatozoon (O'Flaherty et al. 2004; Signorelli et al. 2012; Gangwar and Atreja 2015).

The final category of gene with substantially reduced representation in pipefish testes relative to other fish testes is ubiquitin-protein ligases. These enzymes attach ubiquitin molecules to target proteins and mark them for degradation by the proteasome, and male mice knockouts of specific ubiquitin-protein ligases have nonmature spermatozoa and reduced fertility (Rodriguez and Stewart 2007). This ubiquitin-proteasome system has also been implicated in capacitation, sperm-zona pellucida penetration, elimination of defective sperm, and destruction of sperm mitochondria (Amaral et al. 2014; Zigo et al. 2019). Overall, our gene ontology analysis indicates that pipefish testes have

experienced a reduction in expression of genes involved in the development and function of spermatozoa and possibly development of testes.

The overrepresentation analysis suggests a similar evolutionary pattern and is best interpreted in light of the comparison of pipefish with other percomorphs. For each individual species, the analysis indicates gene ontology classes that are represented at a higher rate in the transcriptome compared to the expectation based on the frequency of the gene ontology class in the whole genome (in this case the zebrafish genome). The scatterplot of overrepresented gene ontology categories in pipefish versus other percomorphs allowed us to identify a cluster of gene categories that were overrepresented in percomorphs but not in pipefish. By also comparing results for zebrafish, where we saw no such cluster, we were able to establish that this lack of overrepresentation in pipefish was restricted to the syngnathid lineage. The gene ontology categories that were overrepresented in the testis transcriptomes of percomorphs but not in the testes of pipefish are nearly all involved in two main functions: meiosis and the assembly of the sperm flagellum (**Table 3.1**, Panel B). The gene ontology categories associated with microtubules could be involved in either meiosis or the sperm flagellum, as microtubules are structural components of flagella and are also involved in movement of the chromosomes during cell division (Simerly et al. 1995). Extracellular transport is not obviously involved in either meiosis or the flagellum, but this is a higher level category that contains multiple cilium-related lower level categories, suggesting that this result may also be due to a lack of genes in the pipefish dataset associated with the flagellum.

Overall, the gene ontology and overrepresentation analyses paint a clear picture of aspects of the pipefish testis transcriptome that differentiate it from the transcriptomes of other percomorphs. Pipefish express fewer genes involved in processes related to sperm function, such as capacitation and sperm-egg interactions. In addition, pipefish testes show a pattern of reduced expression of genes related to meiosis and to the function of the sperm flagellum. It is important to note that our analysis targeted the top 1000 expressed genes in the testis of each species under consideration, so a lack of a gene in our analysis indicates that it is no longer expressed at a high enough level to be in the top 1000 testis-expressed genes, not necessarily that it is absent from the genome. Thus, our interpretation is that the pipefish testes have evolved to produce such a low number of sperm that many of the genes related to sperm development and function are now either absent or still necessary, but expressed at such low levels that they are no longer a major component of the pipefish testis transcriptome (such as those responsible for the formation of the flagellum), a pattern that differs markedly from the majority of other percomorphs. This interpretation is supported by empirical findings that show most syngnathids have sperm with a cylindrically shaped head and a long flagellum, properties that

correlate with internal fertilization (Watanabe et al. 2000; Van Look et al. 2007; Piras et al. 2016b). These adaptive traits likely point toward sperm moving to unite with the eggs through the viscous ovarian fluids the female has deposited into the male (Watanabe et al. 2000; Van Look et al. 2007; Piras et al. 2016b). However, various syngnathids have been documented with both extremely low density of sperm and low testis weight (Watanabe et al. 2000; Kvarnemo and Simmons 2004; Van Look et al. 2007; Dzyuba et al. 2008).

GENOME-WIDE MOLECULAR EVOLUTION OF GONAD-BIASED GENES

To tie together our initial questions and analyses, we investigated the pattern of molecular evolution in the suite of genes expressed in the testis of *Syngnathus* and compared these results to those for genes that were ovary biased and evenly expressed between both gonads. We repeated this comparison using *Takifugu* to allow a comparison between pipefish and a taxonomic group with a more conventional reproductive mode. The dN/dS values in *Syngnathus* testis-biased genes did not differ significantly from dN/dS values for ovary-biased genes, and both were elevated in comparison to values for genes evenly expressed across gonads. This pattern differed substantially from that observed in *Takifugu*, where testis-biased genes differed significantly from both ovary-biased and evenly expressed genes. This result further supports the idea that testis-biased pufferfish genes have a faster rate of molecular evolution than pipefish in part due to sperm competition or sexual conflict. Syngnathid fish remarkably differ from the normal pattern of testis evolution, where testis-biased genes show a slightly elevated rate of molecular evolution most likely due to their tissue-specific nature, but not because testis are under stronger selection than ovaries (Duret and Mouchiroud 2000).

IMPLICATIONS AND CONCLUSION

Our study is noteworthy because it is the first to address the evolution of genes related to male fertility and the testis transcriptome in sex-role-reversed syngnathid fishes, which, as noted above, have no potential to engage in sperm competition. We find a pattern consistent with gene loss, a relaxation of selection, and a reduction in expression of genes involved in the key functions of the testis, such as sperm production, sperm capacitation, and acrosome assembly. At face value, this result affirms the general pattern in the literature that sexual selection and sexual conflict are driving forces behind the rapid evolution of male-biased genes in most taxa.

Syngnathid fishes, although unusual in having male pregnancy, are not the only species in which we might expect a reduction in the efficacy of selection on male reproductive function. Not much work has been done in this arena, but the few studies available have contributed to a better understanding of how deviations from a typical gonochoristic system with high potential for sexual

selection on males can affect the evolution of gonads and male-biased genes at the level of the genome. For instance, Pauletto et al. (2018) studied the molecular evolution of reproductive genes in a sequentially hermaphroditic fish, the gilthead sea bream (*Sparus aurata*), which first matures as male and then transitions to female (Pauletto et al. 2018). They found that all sex-biased genes showed an elevated rate of dN/dS relative to unbiased genes and that female-biased genes were evolving more rapidly than male-biased genes, a reversal of the pattern observed in most other sexually reproducing species. The authors attribute this pattern to a change in the nature of selection on female function as a result of hermaphroditism. Another example of a non-gonochoristic system contributing to our understanding of genome evolution comes from *Caenorhabditis* nematodes. Several studies have compared genome evolution between selfing and outcrossing species of *Caenorhabditis*. These studies show that selfing species tend to lose genes over time, particularly those that were historically strongly sex biased (Thomas et al. 2012; Yin et al. 2018). Much like the present study, these studies also suggest that sexual selection and conflict are extremely important mechanisms of genome evolution in a wide array of taxa, while also highlighting that much work remains to be done and that an effort should be made to address these issues in taxa with diverse modes of reproduction.

As genetic information becomes more accessible, more comparative genomics and transcriptomics studies like these might help shape our predictions and illuminate specific gene families that might expand or contract in the face of different selection intensities. Clades that have both sexual and asexual lineages that have recently branched might prove particularly useful, such as *Poecilia* that includes the Amazon molly (*P. formosa*) that reproduces by gynogenesis (Schartl et al. 1995) or the several species of parthenogenic lizards where multiple asexual branches can be compared (Cole 1975; Murphy et al. 2000). In addition, sex-role-reversed taxa, such as other syngnathid species and sex-role-reversed birds, still have much to offer to this research enterprise (Fritzsche et al. 2021). By drawing on a wide variety of taxa and studying them with the plethora of modern research tools now available, future work should be able to definitively resolve the impacts of sexual selection and sexual conflict on genome evolution.

Figure 3.1

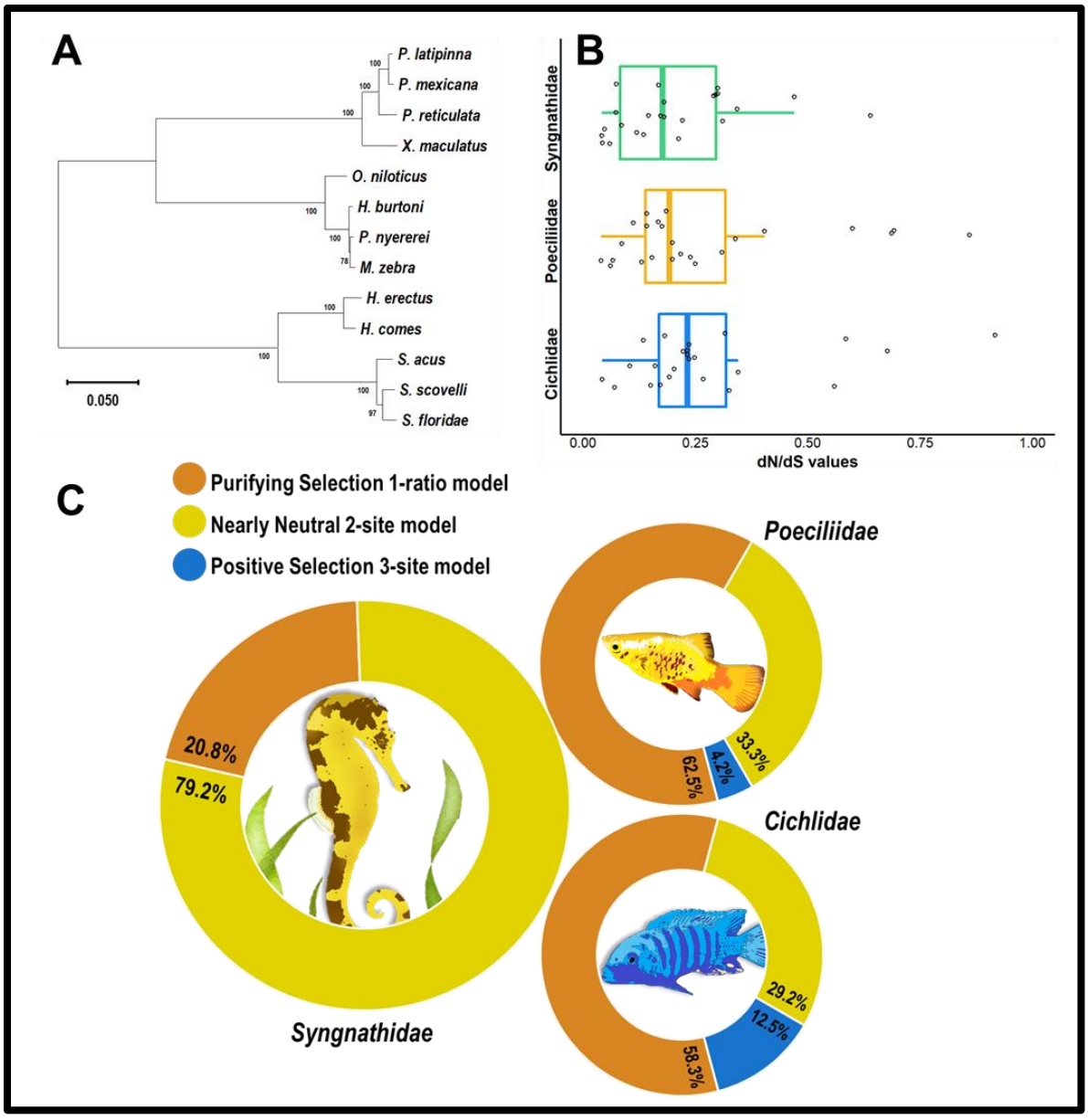
A
P. latipinna
P. mexicana
P. reticulata
X. maculatus
O. niloticus
H. burtoni
P. nyererei
M. zebra
H. erectus
H. comes
S. acus
S. scovelli
S. floridae
0.050
B
Syngnathidae
Poeciliidae
Cichlidae
0.00
0.25
0.50
0.75
1.00
dN/dS values
C
Purifying Selection 1-ratio model
Nearly Neutral 2-site model
Positive Selection 3-site model
20.8%
79.2%
Syngnathidae
Poeciliidae
62.5%
4.2%
33.3%
Cichlidae
58.3%
12.5%
29.2%

Figure 3.1: Rates of molecular evolution among Syngnathidae, Poeciliidae, and Cichlidae.

(a) A maximum likelihood phylogeny for species included in our analysis of molecular evolution, with bootstrap support indicated for each node. Branch lengths are proportional to the number of substitutions per site for the set of 24 genes with an expected involvement in sperm competition or sexual conflict. For these genes, we also show for each taxon studied (b) the median dN/dS values (box plots indicate interquartile range) and (c) the percentage of genes (same set of 24 genes for each taxonomic group) that fit each model of selection as inferred by codeml. The three models are as follows: a one-ratio model (M0) of purifying selection ($\omega < 1$), a nearly neutral model (M1a) that includes two classes of sites (purifying selection [$\omega < 1$] and neutral [$\omega = 1$]), and a positive selection model (M2a) with three classes of sites (positive selection [$\omega > 1$], purifying selection [$\omega < 1$], and neutral [$\omega = 1$]).

Figure 3.2

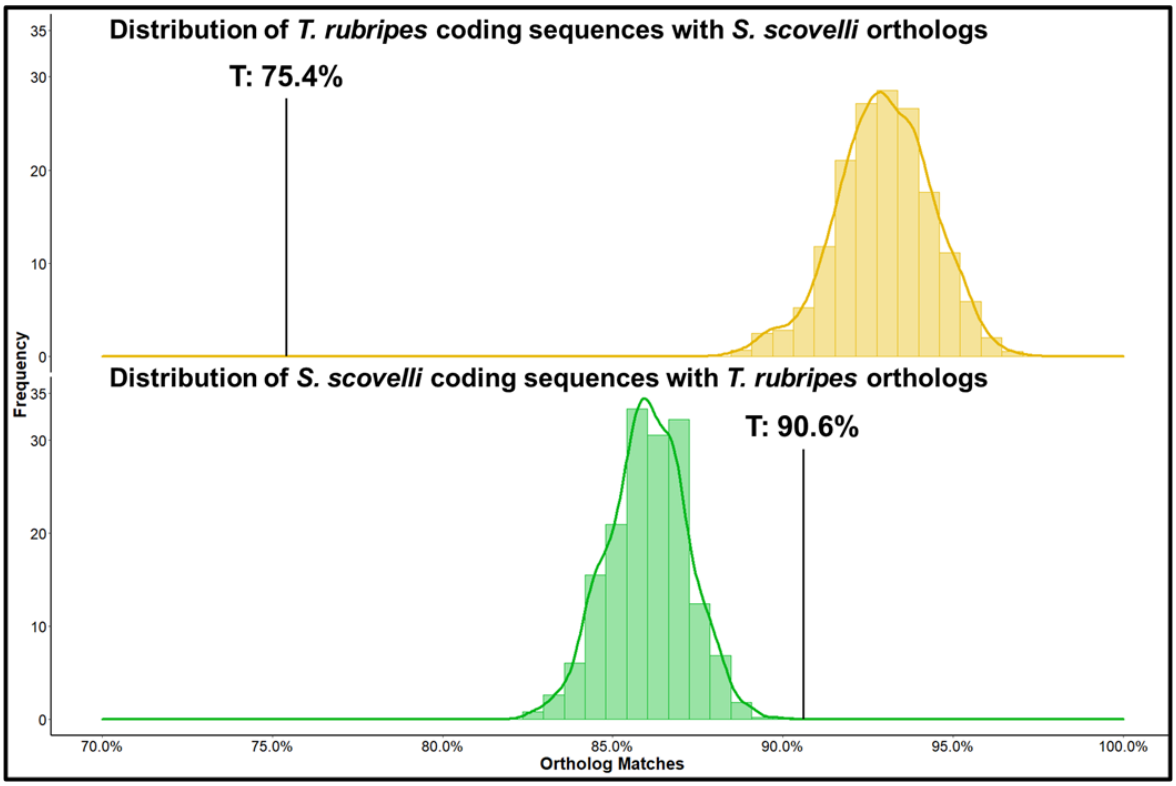

Figure 3.2: Gene turnover between *Syngnathus scovelli* and *Takifugu rubripes* testis transcriptomes.

Distributions represent the expected number of sequences with orthologs in the counter-species by randomly sampling 1000 times across all coding sequences, given a sample size equal to the number of testis-biased (T) genes in the focal species. Black bars represent the percentage of highly expressed testis-biased genes with orthologs in the counter-species. Top: Pufferfish testis-biased genes are less likely than expected to be present in the pipefish genome. Bottom: Pipefish testis-biased genes are more likely than randomly chosen genes to be present in the pufferfish genome.

Figure 3.3

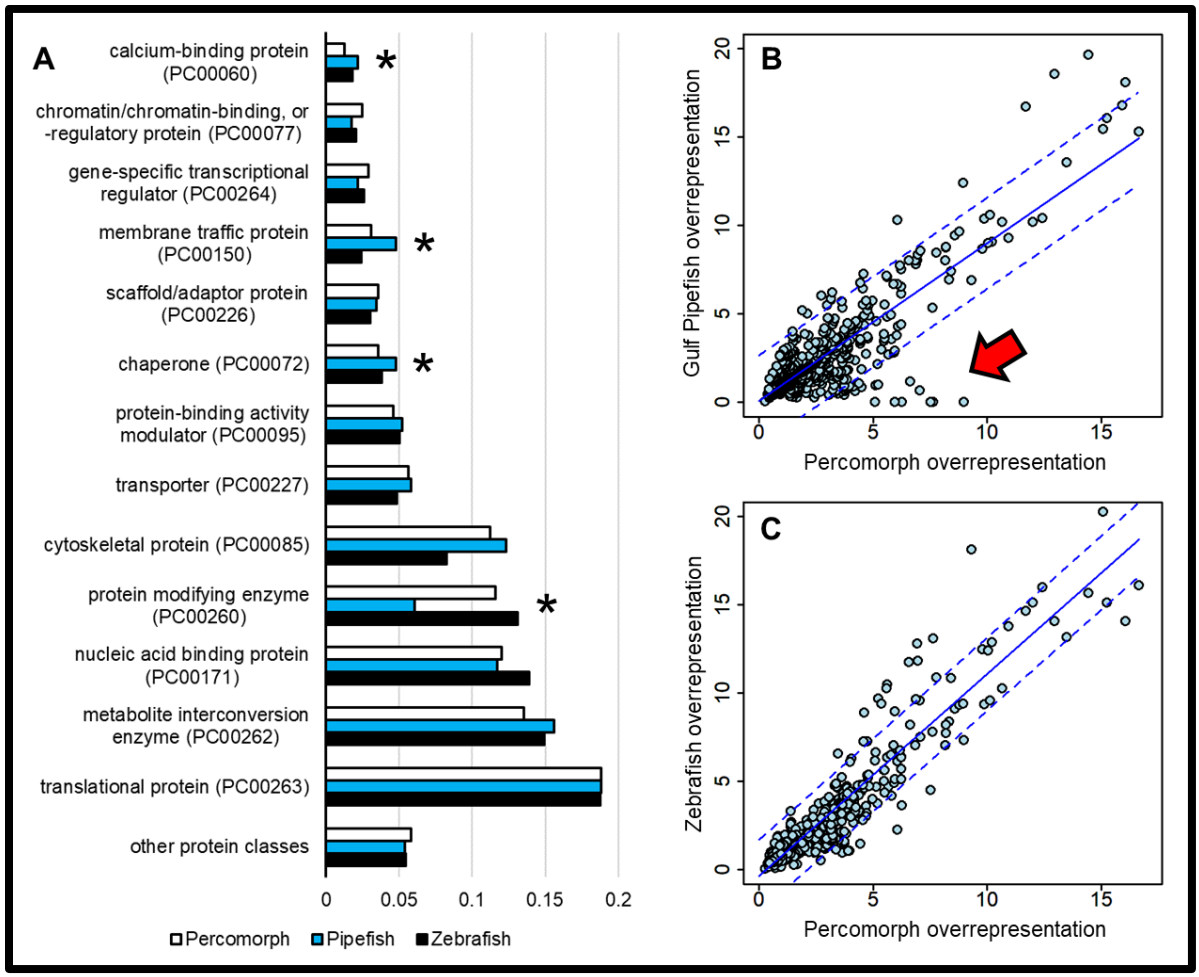

Figure 3.3: Functional evolution of pipefish testes.

(a) Gene ontology (GO) analysis using the PANTHER protein-class database on highly expressed Gulf pipefish testis genes. Pipefish differ significantly from other Percomorphs ($P = 0.019$), whereas zebrafish do not differ from Percomorphs ($P = 0.628$). Asterisks indicate tests that are significant at an FDR of 0.05 (one-sample, two-sided *t*-test). (b) PANTHER overrepresentation analysis comparing pipefish against mean Percomorph values for GO categories (with a mean ≥4 genes in Percomorphs). (c) PANTHER overrepresentation analysis for zebrafish compared to Percomorphs. In panels (B) and (C), lines indicate linear regression (solid) and 95% prediction intervals (dashed). A noticeable cluster of GO categories is below the 95% prediction interval in pipefish (red arrow, **Table 3.1**).

Figure 3.4

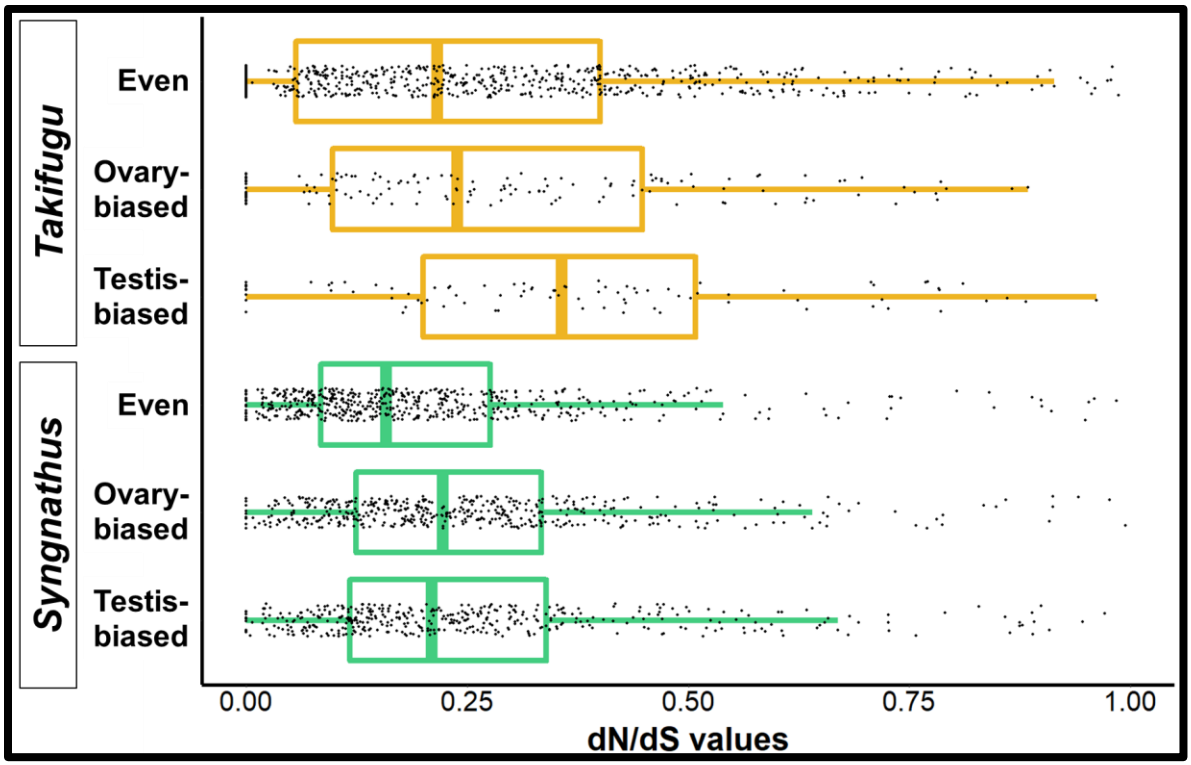

Figure 3.4: Molecular evolution of gonad-biased genes in *Syngnathus* and *Takifugu*.

Using testis and ovary RNA-seq data from *S. scovelli* and *T. rubripes*, we identified genes that were highly and evenly expressed across gonads (even), and genes that were highly expressed in one gonad (ovary-biased and testis-biased). The dN/dS value was estimated using alignments from the genomes of species within *Syngnathus* (*S. scovelli*, *S. acus*, *S. rostellatus*, *S. typhle*) and *Takifugu* (*T. rubripes* and *T. flavidus*). The box plots report the median dN/dS value and the interquartile range for genes within each of the six groups.

Figure 3.S1

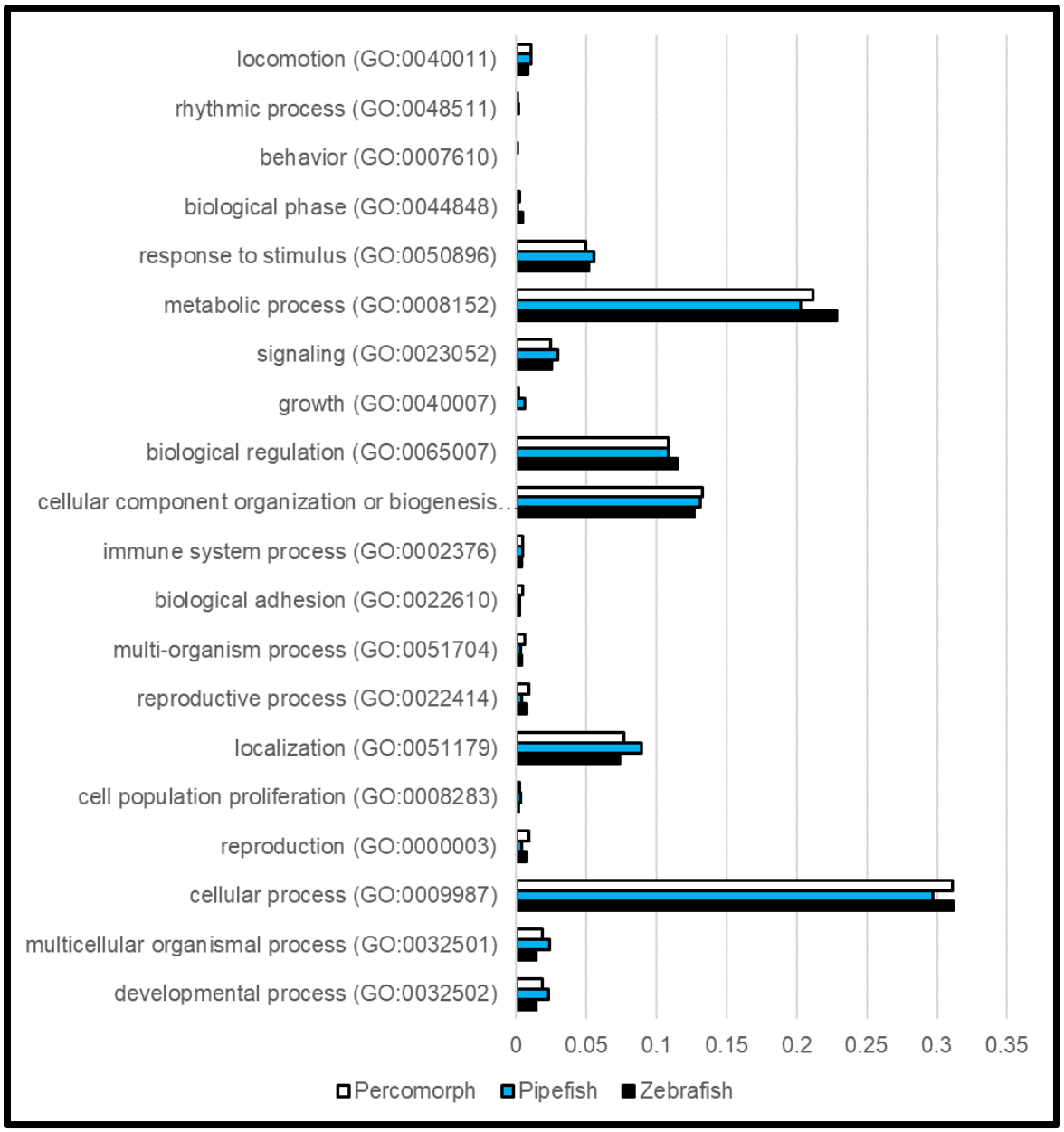

Figure 3.S1: Gene ontology analysis for biological process of highly expressed testis genes in percomorphs (excluding pipefish), Gulf pipefish, and zebrafish.

In terms of biological process, our gene ontology analysis detected no significant differences between percomorphs, Gulf pipefish, and zebrafish for the highly expressed testis genes examined here.

Figure 3.S2

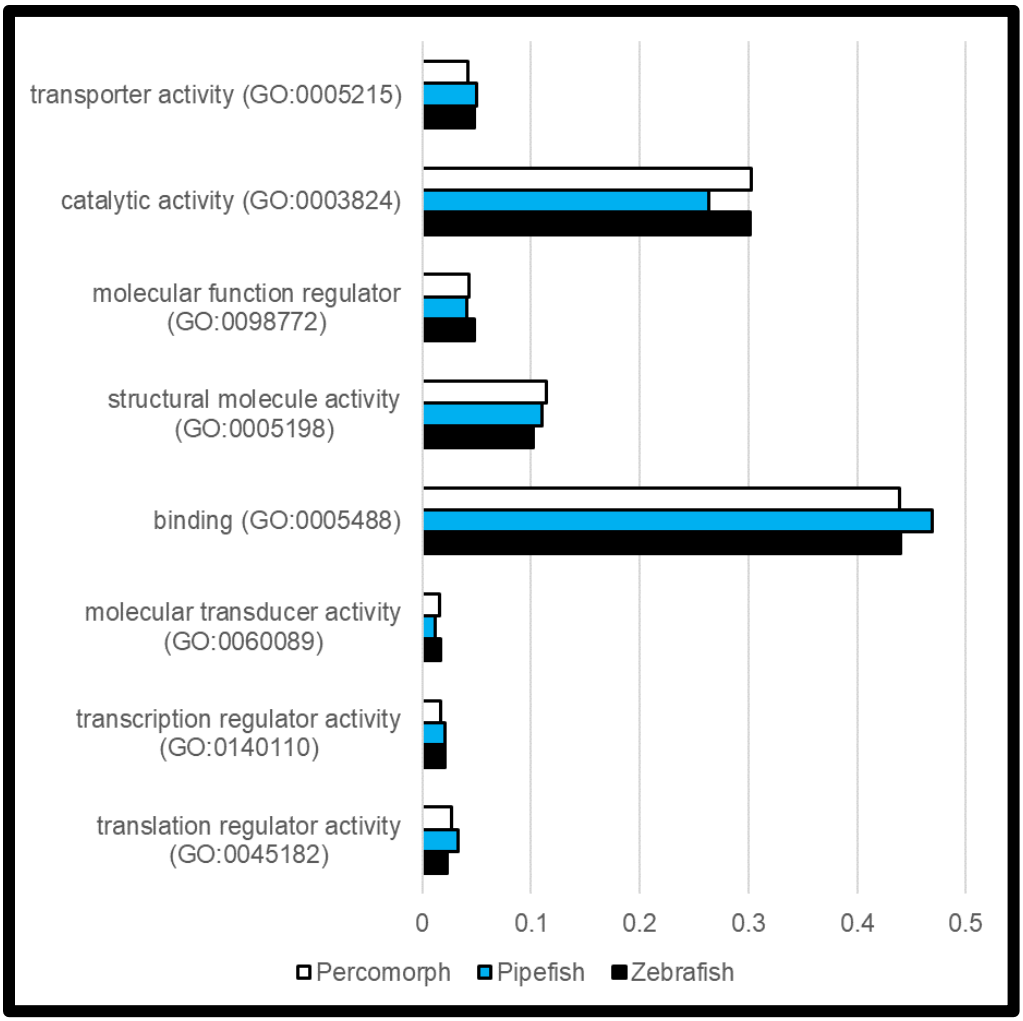

Figure 3.S2: Gene ontology categories for molecular function for highly expressed testis genes in percomorphs, Gulf pipefish, and zebrafish.

We saw no major differences among fish taxa with respect to these gene ontology categories, except for a small but non-significant increase in Gulf pipefish of genes categorized as binding and a non-significant decrease in genes categorized as catalytic activity.

Figure 3.S3

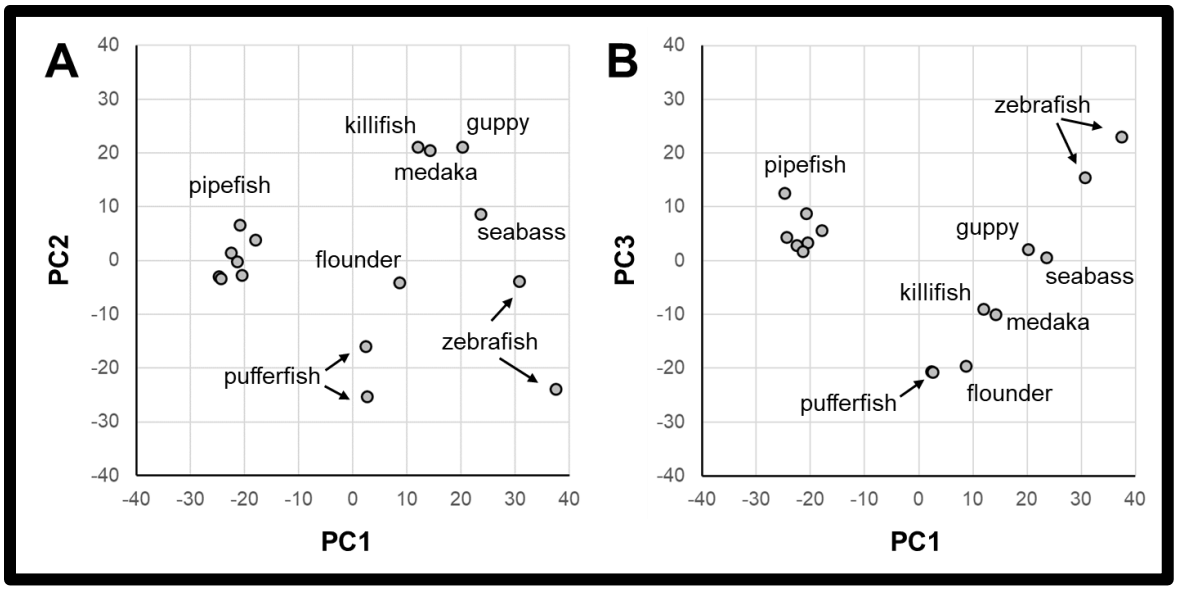

Figure 3.S3: Gene expression across all testis transcripts.

PCA of the testis transcriptomes from Gulf pipefish, zebrafish, and six percomorph species. Gulf pipefish are represented by seven biological replicates, five of which were pregnant males and two of which were non-pregnant males. Zebrafish and pufferfish each had two biological replicates, and all other species (flounder, guppy, killifish, medaka, and seabass) have a single biological replicate. All transcripts identified by StringTie were blasted against a custom OrthoDB database containing zebrafish orthogroups at the level of vertebrates. Raw count values were summed across transcript for each orthogroup in each biological replicate, before being normalized by the total number of counts per sample. Only orthogroups with non-zero values for all species were retained (N=1,674), and a principal components analysis was performed by using prcomp() in R. The left panel (A) shows a plot of PC1 versus PC2, and the right panel (B) shows a plot of PC1 versus PC3. These three principal components explain approximately 67% of the variance. The first principal component clearly separates pipefish from the other percomophs and zebrafish. The non-pregnant pipefish are contained within the pregnant pipefish group, so this analysis does not clearly separate males based on pregnancy status.

Table 3.1

A. PANTHER Protein Class	**Gulf Pipefish Gene Count**	**Mean Percomorph Gene Count**	**Zebrafish Gene Count**
Cysteine Protease (PC00081)	3	7.3	7
Metalloprotease (PC00153)	2	4.5	9
Non-Receptor Serine/Threonine Protein Kinase (PC00167)	3	10.5	10
Protease (PC00190)	9	4.7	3
Protein Phosphatase (PC00195)	2	1.8	0
Serine Protease (PC00203)	2	6.5	16
Ubiquitin-Protein Ligase (PC00234)	7	15.5	20
Protein Modifying Enzyme (PC00260)	0	0.3	0
B. Gene Ontology Biological Process	**Gulf Pipefish Enrichment**	**Mean Percomorph Enrichment**	**Zebrafish Enrichment**
axonemal dynein complex assembly (GO:0070286)	0	7.64	13.08
cilium or flagellum-dependent cell motility (GO:0001539)	0	8.98	7.35
cilium-dependent cell motility (GO:0060285)	0	8.98	7.35
microtubule-based protein transport (GO:0099118)	0	6.27	3.63
protein transport along microtubule (GO:0098840)	0	6.27	3.63
intraciliary transport (GO:0042073)	0	7.52	4.51
chromosome organization involved in meiotic cell cycle (GO:0070192)	0	5.09	6.16
homologous chromosome segregation (GO:0045143)	0	5.97	8.96
axoneme assembly (GO:0035082)	0.68	7.04	9.59
transport along microtubule (GO:0010970)	0.42	3.75	1.48
meiotic cell cycle process (GO:1903046)	0.41	3.08	3.92

Table 3.1 cont'd

meiotic nuclear division (GO:0140013)	0.48	3.47	4.62
mitotic sister chromatid segregation (GO:0000070)	0.64	4.23	2.82
meiosis I (GO:0007127)	0.6	3.63	5.22
meiosis I cell cycle process (GO:0061982)	0.55	3.32	4.77
microtubule-based movement (GO:0007018)	0.65	3.79	3.27
microtubule bundle formation (GO:0001578)	1.17	6.63	8.19
cilium movement (GO:0003341)	0.95	5.12	6.63
nuclear division (GO:0000280)	0.7	3.74	3.88
mitotic spindle organization (GO:0007052)	0.85	4.49	4.45
epithelial cilium movement involved in extracellular fluid movement (GO:0003351)	1	5.22	9.68
extracellular transport (GO:0006858)	1	5.22	9.68
meiotic chromosome segregation (GO:0045132)	0.67	3.41	4.69

Table 3.1: Underrepresented PANTHER protein classes and GO biological process categories in Gulf pipefish testes compared to other fishes.

(A) Gulf pipefish testes have significantly fewer protein-modifying enzymes (broken-down by PANTHER protein class) than those of other fishes. (B) GO categories, with fold-enrichment values, that were significantly low outliers for Gulf pipefish testes in the PANTHER overrepresentation analysis (**Figure 3.3B**, arrow). Shown are categories with a mean of at least threefold overrepresentation in Percomorphs, and at least fivefold more overrepresentation in Percomorph transcriptomes, on average, relative to the pipefish transcriptome.

Table 3.S1

Species	Accession Number from NCBI
Genome accession numbers	
Syngnathus floridae	GCA_010014945.1
Hippocampus comes	GCA_001891065.1
Syngnathus acus	GCA_901709675.2
Pundamilia nyererei	GCA_000239375.1
Maylandia zebra	GCA_000238955.5
Haplochromis burtoni (*Astatotilapia burtoni*)	GCA_000239415.1
Oreochromis niloticus	GCA_000188235.2
Xiphophorus maculatus	GCA_000241075.1
Poecilia mexicana	GCA_001443325.1
Poecilia latipinna	GCA_001443285.1
Poecilia reticulata	GCA_000633615.2
Takifugu rubripes	GCA_901000725.2
Oryzias latipes	GCA_002234675.1
Paralichthys olivaceus	GCA_001970005.2
Nothobranchius furzeri	GCA_001465895.2
Lates calcarifer	GCA_001640805.1
Danio rerio	GCA_000002035.4
Takifugu flavidus	GCA_003711565.2
Syngnathus rostellatus	GCA_901007895.1
Syngnathus typhle	GCA_901007915.1
SRA library accession numbers	
Syngnathus scovelli	BioProject containing all testis and ovary samples from this project: PRJNA850415
Takifugu rubripes	testis: SRR5059294, SRR5816364, SRR5816366; ovary: SRR5059347, SRR5816365, SRR5816367
Oryzias latipes	testis: SRR1524281; ovary: SRR1524280
Poecilia reticulata	testis: SRR1140963; ovary: SRR1137868
Paralichthys olivaceus	testis: SRR3525051; ovary: SRR3509719
Nothobranchius furzeri	testis: ERR879039; ovary: ERR879038
Lates calcarifer	testis: SRR1791598; ovary: SRR1791597
Danio rerio	testis: SRR8286602, SRR8286603 ovary: SRR8286604, SRR8286604

Table 3.S1: Table of accession numbers used in this study.

This table includes all the NCBI accession numbers (genome and SRA RNA-seq libraries) for the species used in this study.

Table 3.S2

For *Syngnathus*:	**Testis-up**	**Even Expressed**	**Ovary-up**
Total # of genes	429	546	473
Average dN/dS	0.2935	0.2193	0.2730
Median dN/dS	0.2178	0.1602	0.2237
Positive Selection (3-site model)	M2a: 81 = 18.9%	M2a: 85 = 15.6%	M2a: 80 = 16.9%
Neutral (2-site model)	M1a: 99 = 23.1%	M1a: 91 = 16.7%	M1a: 100 = 21.1%
Purifying Selection (1-ratio model)	M0: 249= 58.0%	M0: 370 = 67.7%	M0: 293 = 61.9%
For *Takifugu:*	**Testis-up**	**Even Expressed**	**Ovary-up**
Total # of genes	97	726	151
Average dN/dS	0.5424	0.3183	0.3603
Median dN/dS	0.3988	0.2237	0.2531
Positive Selection (3-site model)	M2a: 28 = 28.9%	M2a: 195 =26.9%	M2a: 32 = 21.2%
Neutral (2-site model)	M1a: 1 = 1.0%	M1a: 2= 0.3%	M1a: 3 = 2.0%
Purifying Selection (1-ratio model)	M0: 68 = 70.1%	M0: 529= 72.9%	M0: 116 =76.8%

Table 3.S2: Summary of dN/dS and model of selection results of gonad-biased genes between the *Syngnathus* and *Takifugu* genera.

This table includes the molecular evolution results for genes that were identified as being highly expressed and biased to one of the gonads, or highly and evenly expressed between both gonads. The dN/dS value and best fitting model of selection were estimated using alignments from the genomes of species within *Syngnathus* (*S. scovelli, S. acus, S. rostellatus, S. typhle*) and *Takifugu* (*T. rubripes* and *T. flavidus*). We focus on the M0-M1a-M2a comparisons because these provide a more stringent test (Yang et al., 2000).

DATA 3.S1: Molecular evolution values and gene ontology

Molecular evolution estimates for each gene of interest, including dN/dS values and model of selection likelihood ratio values. Also includes GO function for each gene.

DATA 3.S2: Gene ontology for differential expressed pregnancy genes

Gene list for corresponding upregulated transcripts in pregnant vs non-pregnant pipefish with PANTHER classifications.

CHAPTER 4: SENSITIVITY OF TRANSCRIPTOMICS: DIFFERENT POPULATIONS AND METHODOLOGY ALTER CONCLUSIONS IN GULF PIPEFISH (*SYNGNATHUS SCOVELLI*)

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ABSTRACT

Transcriptome analysis has become a central tool in the study of functional genomics and evolutionary mechanisms. However, variation in the samples collected and the analyses used can greatly influence results, potentially compromising insights into the phenomenon under study. Here, we evaluate differences in the brain transcriptome between female and male Gulf pipefish (*Syngnathus scovelli*) (Redfish Bay population: $N = 15$, and Port Lavaca population: $N = 7$). We perform comparisons between results from entire pipelines for brain transcriptome assembly, quantification, and analysis. We also offer a unique biological comparison between two wild populations. Our results demonstrate important shortcomings with current experimental approaches. We found high variation within our results that was driven by both technical differences between pipelines and biological differences between pipefish population samples. For our analysis of highly expressed genes, we found that the methods used influenced the accuracy of the genes identified and that genes within the same pipeline were more similar (average ± SEM: 0.81 ± 0.07) than any other comparison. For our differential expression analysis, we found that both population and methodology influenced the quantity and consistency of genes identified. In the context of these results, we offer suggestions to current experimental design that may increase the robustness of transcriptome-based conclusions. In particular, the use of a reference-guided assembly and increased sampling were found to improve resistance to noise or error.

INTRODUCTION

The characterization of transcriptomes by RNA-sequencing is an increasingly exciting and invaluable tool, as it provides a key complement to other genomic techniques by allowing researchers to examine the full catalog of genes expressed in any tissue of interest. Gene expression studies can provide us with an extremely detailed summary of numerous biological processes and phenomena, such as regulation (Park et al. 2006), epigenetics (Foret et al. 2009), gene networks (Ko et al. 2006), cell differentiation (Tadjali et al. 2002; Raouf et al. 2008), pathogen resistance (Barakat et al. 2012; Matić et al. 2016), life cycles (Arbeitman et al. 2002; Whittington et al. 2015), aging (Davie et al. 2018; Hu et al. 2020), population-level variation (Ng'oma et al. 2014), and evolutionary comparisons between species (Johnson et al. 2022), to list a few. Moreover, they give us an insight into the specific genes that correlate with processes of interest and allow us to develop testable hypotheses.

The most popular method for assessing transcriptomes, RNA-seq, has the advantage of versatility and ease of use, because transcripts can be assembled and quantified without a reference genome (*de novo*) or aligned and mapped to a reference genome (guided assembly). Both strategies balance their value with unique drawbacks. For *de novo* assemblies, an expression profile can be created without investing in a sequenced genome and it can also potentially detect novel transcripts, but requires substantial computational power, higher sequencing depth, and is sensitive to sequencing and assembly bias or errors (Trapnell et al. 2010; Martin and Wang 2011). Assembling with a reference genome can be useful when there are minor gaps in transcript sequences, contamination, sequencing artifacts, or when genes of low expression are of interest, but requires the availability of a high-quality genome (Denoeud et al. 2008; Martin and Wang 2011; Kim et al. 2015, 2019). Furthermore, both methods can be used on the same RNA-seq dataset in an effort to harvest the benefits offered by both assembly techniques (Wang and Gribskov 2017; Johnson et al. 2022). Once a transcriptome is assembled and its transcripts quantified, several downstream analyses can be used to provide an informative summary of the data. One common line of inquiry is a differential expression analysis, which compares the expression profiles of distinct experimental groups. Popular comparisons occur between the sexes, different treatment groups, and within-organism tissue types (Giacomello et al. 2017).

Multiple programs and pipelines have been developed to help quantify and compare the large datasets produced by RNA-seq approaches. Depending on the type of dataset, pipelines are normally customized by the individual researcher to produce the best result (Hölzer and Marz 2019). This customization can also create disadvantages, such as reduced opportunities for comparisons among datasets from multiple sources. Additionally, within each pipeline, programs can be tailored to run a

different suite of parameters, normalization algorithms, and filtering criteria, all of which can be difficult to replicate even when using the same set of programs. Inevitably, presenting an accurate or precise representation of the transcriptome for a sample can be impeded by several innate program biases or user errors that compound to skew the final result (Rapaport et al. 2013; Seyednasrollah et al. 2015; Wang and Gribskov 2017; Hölzer and Marz 2019).

In addition to these issues introduced from the analyses, and perhaps lesser understood, is the repeatability of results from an organismal perspective. It is generally expected that samples collected from similar conditions should have comparable expression profiles, and that species- or tissue-specific profiles should emerge, especially when the number of samples increases (Rapaport et al. 2013; Seyednasrollah et al. 2015; Giacomello et al. 2017). However, in practice there can be substantial variation among the transcriptomes of distinct samples, indicating some form of overlooked disparities between individuals (Crawford and Oleksiak 2007; Alvarez et al. 2015). This variability can make it difficult to categorize parts of an expression profile as a species-specific expectation, individual variation, transient variation, or noise. These differences can make downstream comparisons difficult to interpret or apply to a broader concept. Disconcertingly, a starting transcriptome dataset can provide different ending results depending on the samples collected and analyses used, affecting our understanding of the larger biological phenomenon we initially wished to study.

For this study, we compared the brains of female and male Gulf pipefish (*Syngnathus scovelli*) between two populations using RNA-seq data. One population was collected from Redfish Bay, Texas (TX), USA for the purposes of this comparison study. The other population was collected from Port Lavaca, Texas, USA from a previously published study by Beal et al. (2018). We assembled and quantified transcripts for each population both with and without a reference genome and conducted differential expression analyses between females and males for each assembly pipeline. We aimed to determine (1) the consistency of results between different pipelines, and (2) how comparable two populations of the same species are. We hypothesized that differing methods would reduce consistency between results, but that expression profiles within a sex would be more similar than between the sexes. To conduct these analyses, we selected a few of the most popular programs, which resulted in twelve analysis pipelines. Our intention was to provide a case study to improve our understanding of how well differential expression analyses explain a biological phenomenon and how robust certain transcriptome-based conclusions can be to variability in sample source and analysis method.

METHODS

PIPEFISH COLLECTION AND RNA SEQUENCING

Gulf pipefish (*Syngnathus scovelli*) were collected from coastal seagrass beds in Redfish Bay, Texas on May 22 and June 5, 2013. Fish were brought into the lab and paired for mating. Pregnant males were sacrificed at day six of their pregnancy, when eyespots on the offspring first develop. This method was done to control for the effects of the distinct stages of pregnancy and to collect data on males that were mid-way through their pregnancy. Sexually mature adults (five females, five non-pregnant males, five pregnant males) were euthanized during midday hours with an overdose of tricaine methanesulfonate (MS222) in accordance with IACUC approval (2011-51). We included both pregnant and non-pregnant males in our sampling efforts because to determine the innate differences between female and male brain expression, male expressional differences should not be confounded with their pregnancy status. Brain tissue was dissected while coated in RNAlater (Ambion) and immediately transferred to a -80 freezer for storage. RNA was extracted and isolated using TRIzol® Reagent (Life Technologies, Carlsbad, CA) (Leung and Dowling 2005). Libraries were prepared by Michigan State University RTSF Genomics Core using TruSeq mRNA Library Prep Kit v2 and quality was evaluated using Caliper GX and qPCR methods. All individuals were barcoded and sequenced individually using two lanes of Illumina HiSeq 2500 sequencing, and base calling was done using Illumina Real Time Analysis (RTA) (v1.17.21.3). The output of RTA was demultiplexed and converted to FastQ with Illumina bcl2fastq (v1.8.4) resulting in 100 bp paired-end reads. The average number of raw reads per sample was 11,948,040.

Comparison brain RNA-seq data was gathered from the NCBI Sequence Read Archive (SRA) BioProject ID number PRJNA389920, which included samples of *S. scovelli* (three females and four pregnant males) collected from Port Lavaca Bay, Texas in June 2015 (Beal et al. 2018). Libraries from this dataset were prepared by the University of Texas Southwestern center using Illumina TruSeq RNA library kits. Sequencing was done on an Illumina HiSeq 2000 system resulting in 100 bp paired-end reads (Beal et al. 2018). The average number of raw reads per sample was 22,929,985.

READ TRIMMING

All reads were trimmed using Trimmomatic (v0.36) before assembly (Bolger et al. 2014). Trimming included removal of Illumina-specific adapters, and removal of bases from the start and end of the read that were below a threshold for quality (LEADING: 3, TRAILING: 3). Sliding window trimming was also used to moderate read quality (SLIDINGWINDOW: 4:15), and short

reads were also dropped from the analysis. Two thresholds were specified for the minimum length of a read. The first approach replicated methods from Beal et al. (2018), where reads shorter than 30 nucleotides were removed (MINLEN:30). For the second method, reads shorter than 75 nucleotides (MINLEN:75) were trimmed, thus removing a larger portion of short reads. Incorporating shorter read length does not incur any benefits when short reads occur multiple times within a longer target (Bolger et al. 2014). Short reads can also create ambiguity or be misassembled especially when they cover repetitive regions (Cahais et al. 2012). Samples from both populations (Redfish Bay, TX and Port Lavaca, TX) were trimmed using each of the methods, resulting in four separate trimmed read datasets (**Figure 4.1**).

TRANSCRIPTOME ASSEMBLY

For this study, we selected one representative assembler for each of two methods: *de novo* assembly (i.e., assembling without a reference genome), and genome-guided assembly. For our *de novo* assembler we selected Trinity (v.2.8.5) (Grabherr et al. 2011; Haas et al. 2013). Trinity has been repeatedly recognized as a leading *de novo* assembler with highly accurate unigene production and fairly accurate assembly of highly expressed transcripts (Honaas et al. 2016; Hölzer and Marz 2019). For our genome-guided assembly, we used HISAT2 (v2.1.0) (Kim et al. 2015, 2019) a popular tool that balances alignment rate completeness with shorter runtimes (Musich et al. 2021). In addition to our two assemblers, three overall methods of transcriptome assembly were independently used: (1) *de novo* assembly with Trinity using only a subset of samples, (2) *de novo* assembly with Trinity with the inclusion of all samples, and (3) genome-guided assembly with HISAT2 using the available reference genome for the Gulf pipefish (Small et al. 2016). For each of the three methods, only samples within the same population were assembled into a set of transcripts, resulting in six separate reference transcriptomes **Figure 4.1**.

For the first method of *de novo* assembly, we used one male and one female sample, each having the highest number of quality trimmed paired-reads for their sex (as specified in (Beal et al. 2018)). Specifically, we used samples SSF2 (female) and SSP13 (male) from the Redfish Bay population. We also used SRR5783122 (female) and SRR5783120 (male) from the Port Lavaca population (as specified in (Beal et al. 2018)). The reads used for this assembly were trimmed using the parameter MINLEN:30 (as previously described). This procedure was done to best replicate the methodology used from the previous study (Beal et al. 2018).

The second method of *de novo* assembly with Trinity included all the samples collected within each population (but each population was assembled separately). This method was also used as

Trinity recommends the use of all samples to generate a single reference assembly (Grabherr et al. 2011; Haas et al. 2013). Using all samples ensures a more complete assembly and will include any transcripts that are expressed in only a subset of samples (Grabherr et al. 2011; Haas et al. 2013). The reads used for this assembly were trimmed using the parameter MINLEN:75. In both cases, Trinity was run using default parameters for paired-end reads.

The third method used HISAT2 for mapping and aligning reads to the reference Gulf pipefish genome (obtained from: https://creskolab.uoregon.edu/pipefish/ (Small et al. 2016)), and all samples from within each population were used (each population was mapped separately). The reads used for the HISAT2 assembly were trimmed using the parameter MINLEN:75. The alignment rate of reads to the reference assembly is reported in **Table 4.S1.**

QUANTIFICATION OF READS

Two methods of read quantification were used: (1) RSEM (v1.3.1) and (2) StringTie (v2.1.3b). RSEM was used to quantify reads against the *de novo* assembled transcriptomes (Haas et al. 2013). Transcript references for RSEM were prepared using the *de novo* assembly, and gene expression levels for paired-end reads were estimated using default parameters. A data matrix containing the expected counts of each transcript for each sample was generated for each *de novo* assembled transcriptome.

StringTie was used to quantify reads against the transcriptome assembled with HISAT2 using the reference Gulf pipefish genome (Pertea et al. 2016). The reference annotation file for the *S. scovelli* genome was not used. The relatively poor annotation quality of the *S. scovelli* genome could potentially introduce bias during the assembly and quantification of transcripts. StringTie was run separately for each sample within a population and then merged into a single gff file. Transcript abundances were estimated using the merged transcript gff file. Together, both methods resulted in transcript quantification against each of the six separate reference transcriptomes (**Figure 4.1**).

ANALYSIS OF HIGHLY EXPRESSED GENES

For this analysis, we identified and compared genes (or unique transcripts for *de novo* assemblies) with the highest expression value in each pipeline, for each sex. For each of the six main transcript quantification pipelines (**Figure 4.1**), we separately calculated the average transcripts per million (TPM) value for each gene within samples of the same sex. This resulted in separate average TPM values for males and females for each of the six transcriptomes.

To understand the expressional variation present between samples, we conducted a principal component analysis for each of the six main transcript quantification pipelines (**Figure 4.S1**). Average TPM values, as described previously, were filtered, and genes with an average TPM ≥ 25 within a sex and pipeline were retained. These genes were log transformed (log (TPM +1)), and the function prcomp() from the R package stats (v3.6.2) was used to perform a principle component analysis.

We then investigated the top twenty genes with the highest average TPM value for each of the twelve lists. These top genes were isolated, and their corresponding nucleotide sequence was extracted using the C++ program FastaRecordExtractor2. For genes assembled against the genome using HISAT2 and quantified with StringTie, multiple transcript sequences can be represented by a single gene. In these cases, we only used the longest transcript sequence. Each transcript was converted to the protein sequence encoded by its longest open reading frame with the C++ program FastaToORF. The protein sequences from *S. scovelli* were BLASTed (BLASTP) against the NCBI non-redundant coding sequences database. The best resulting BLAST hit with an E-value less than $1x10^{-20}$ was retained (**Data 4.S1**).

All matching protein IDs from NCBI were used to build a database, and the occurrence of each protein ID was recorded as present or absent for each of the of the twelve lists. A pairwise Cramér's V was used to measure association between the twelve groups, using the R package scorecardModelUtils (v1.0). The resulting Cramér's V values were then plotted as an association matrix (**Figure 4.2**).

DIFFERENTIAL EXPRESSION ANALYSIS BETWEEN THE SEXES

For this step, we selected two programs based on their different statistical algorithms for evaluating differential expression, popularity, and ease of use (Soneson and Delorenzi 2013). The first, edgeR (v3.38.1), normalizes using its TMM method, fits expression data to a negative binomial model, and uses a variant of the exact test to determine differential expression (Robinson et al. 2010). The second, EBSeq (v1.36.0), uses median normalization, also uses a negative binomial model to fit expression data, but employs an empirical Bayesian approach for differential expression analysis (Leng and Kendziorski 2019). Additionally, three methods were used for differential expression analysis: (1) edgeR using TPM values, (2) edgeR using expected counts, and (3) EBSeq using expected counts (**Figure 4.1**). The differential expression techniques were used for each of the six separate quantified transcriptomes, resulting in twelve examinations of the original data (**Figure 4.1 and 4.3**).

The first method was an effort to best replicate the methodology used from Beal et al. (2018). After quantification, transcripts with a TPM value of less than one in at least three of the samples were removed. The TPM values of the remaining transcripts were used for differential analysis within edgeR. During the analysis, transcripts were filtered again by CPM value and transcripts with a CPM value of less than one in at least three of the samples were removed. This additional filtration step did not result in the removal of additional transcripts, due to the previous filtration step; however, it was maintained as part of the pipeline for consistency.

The second method used the expected count of the transcripts for differential analysis with edgeR. Expected counts were used instead of the TPM values, as edgeR does not recommend that predicted transcript abundances be used instead of actual read counts (Robinson et al. 2010). The data in this method were also pre-filtered, and transcripts with an expected count of 0 in at least one female sample and one male sample were removed. This method removes transcripts that might have an overall low or variable expression for both females and males. Filtering reads using a normalized count that accounts for differences in library sizes (such as CPM), is typically preferred (Robinson et al. 2010); however, this step would be prudent in cases where there is a non-zero threshold. Differential expression was determined using the built-in exact test followed by a Benjamini-Hochberg false discovery with an alpha value of 0.05.

For our third method, we used EBSeq, and this method also required the expected count for each transcript. To obtain library size factor, we used the median normalization approach, and ran the analysis with five iterations. The number of iterations was determined to be sufficient after checking hyper-parameter estimate convergence (Leng and Kendziorski 2019). This resulted in the calculation of posterior probabilities of differential expression (PPDE). Transcripts were considered differentially expressed if they met the alpha value cutoff of 0.05 (or a PPDE≥0.95).

IDENTIFYING THE RELATIONSHIP OF DIFFERENTIALLY EXPRESSED GENES

For this analysis, we compared the genes identified as differentially expressed between female and male samples, across each pipeline (24 total gene groups). For each of the twelve major differential expression pipelines (**Figure 4.1**), we subset the differentially expressed genes based on which sex had the higher expression and referred to them as female-biased or male-biased. This method left 24 lists of differentially expressed genes (**Figure 4.3**). For these transcripts, we extracted their corresponding nucleotide sequence using FastaRecordExtractor2, and converted them to their protein sequence as previously described, using FastaToORF.

The protein sequences were BLASTed (BLASTP) against the *Syngnathus acus* proteome (GCF_901709675.1), with an E-value cutoff of $\leq 1x10^{-20}$ (**Data 4.S2**). *S. acus* was used as our *Syngnathus* reference because it had a higher quality annotation than that of *S. scovelli* and both species are closely related (Wilson and Orr 2011). This assigned each transcript from the six transcriptomes a common reference protein ID. All matching protein IDs from *S. acus* were used in a pairwise Cramér's V to measure associations between all possible gene groups within each one of the 24 sex-biased categories. The resulting Cramér's V values were then plotted as an association matrix (**Figure 4.4**).

Additionally, matching *S. acus* protein IDs appearing in at least three or more pipelines with the same sex bias were compiled into a list (**Data 4.S3**). These differentially expressed genes were considered more robust and included 84 female-biased and 73 male-biased proteins.

SIMILARITY OF FUNCTIONAL PROTEIN CLASSES

To further examine the identified differentially expressed genes in each pipeline, we also investigated their corresponding protein classes. To identify the protein class for each differentially expressed gene, we obtained and used the zebrafish (*Danio rerio*) proteome from UniProt (Zebrafish-UP000000437_7955) and constructed a local BLAST database. We used BLASTP to search for all differentially expressed genes within the zebrafish proteome, using the best matching hit with an E-value cutoff of $\leq 1x10^{-20}$. Duplicate matches within a differentially expressed group (24 gene groups) were removed.

For this gene ontology analysis, we used the PANTHER database (v17.0) and its associated tools (Thomas et al. 2003a,b; Mi et al. 2013b). The PANTHER database uses the annotation from 143 genomes, classifying proteins based on evolutionary relationship, multiple sequence alignments, and statistical models (Mi et al. 2013b,a). This method can provide a robust and accurate approach to understanding protein function in a wide range of organisms (Thomas et al. 2003a). We uploaded the zebrafish protein IDs that corresponded to each set of the differentially expressed genes and performed a functional classification analysis. Here, zebrafish was used as the reference gene set, as it is the closest related organism with protein classification data available within the database. For the functional classification analysis, zebrafish protein IDs are matched with their PANTHER protein class, which provides information on the function of the protein. The extent of similarity between the protein classes was measured by counting the occurrence of a protein class within each of the 24 differentially expressed groups. Protein classes that were identified in at least half of all female-biased, or all male-biased, differentially expressed groups were reported (**Table 4.2**).

DIFFERENTIAL EXPRESSION ANALYSIS BETWEEN MALES

We conducted a differential expression analysis between pregnant and non-pregnant males within the Redfish Bay population of *S. scovelli*. For this analysis, we focused on transcripts that were identified using a genome-guided assembly (HISAT2) and quantified using StringTie. We used the differential expression analysis program edgeR (**Table 4.S2**) and EBSeq (**Table 4.S3**). Both programs were run as previously mentioned for the genome-assisted pipelines. The differentially expressed transcripts were then searched for (BLASTP) in the *S. acus* genome. Protein matches that had an E-value of less than $1x10^{-20}$ were retained as orthologs.

RESULTS

NUMBER OF UNIQUE TRANSCRIPTS IN ASSEMBLIES

Six separate transcriptomes were assembled using two populations and three methods: (1) *de novo* assembly using a subset of samples, (2) *de novo* assembly with the inclusion of all samples, and (3) genome-guided assembly with the inclusion of all samples. The number of unique transcripts identified for each of the transcriptomes greatly varied (**Table 4.1**). Transcriptomes assembled *de novo* had a greater inflation of unique transcripts than assemblies based on a reference genome. There were also greater numbers of unique transcripts for transcriptomes assembled using the Redfish Bay population samples. The transcriptomes that contained the most realistic estimates, based on the number of protein coding genes in the Gulf pipefish genome (Small et al. 2016), were those assembled using the reference genome.

ANALYSIS OF HIGHLY EXPRESSED TRANSCRIPTS

Twenty genes with the highest average TPM value per sex for each of the six transcriptomes were BLASTed (BLASTP) against the NCBI non-redundant coding sequences database. One comparison, containing the highest expressed genes from female Port Lavaca samples assembled using Trinity *de novo* (with the inclusion of all samples) did not have any significant NCBI matches. For this reason, we did not include it in the analyses for this section. The association between the resulting genes for each pipeline was measured using a pairwise Cramér's V. The resulting values can range from 0 to 1, where 0 indicates no association between the genes for each pipeline and 1 indicates complete association (**Figure 4.2**).

We found the average association value for comparisons between populations (**Figure 4.2**, inside gray box) (average ± SEM: 0.15 ± 0.02) to be less than the average association value within either population (**Figure 4.2**, outside the gray box) (Redfish Bay: 0.27 ± 0.09, and Port Lavaca Bay:

0.17 ± 0.09). The most similar groups were female and male genes within the same pipeline (0.81 ± 0.07). Given these results, genes identified as highly expressed are more likely to be influenced by the methodology used to assemble and quantify the transcriptome. While the average association values within both populations was higher than between populations, this result was mainly driven by the high association value within the same pipeline. This outcome suggests that the genes with the highest expression in a transcriptome might not accurately reflect the true biological state of the tissue.

Additionally, the top twenty expressed genes identified by any of the *de novo* methods were less likely to have a BLAST match (39.4% of genes had a significant BLAST hit) in the NCBI database than genes identified by from genome-guided assemblies (81.3% of genes had a significant BLAST hit) (**Figure 4.2**). Significant matches from *de novo* transcriptomes were also more likely to be from a non-syngnathid source, suggesting the inclusion of contamination or misalignment, than matches from transcriptomes assembled with a reference (**Data 4.S1**). While this difference was substantial, all pipelines contained matches to bacterial sources, indicating some degree of susceptibility to contamination regardless of assembly method.

SEX-BIASED EXPRESSION

Twelve pipelines were used to produce separate lists of differentially expressed genes, with three main methods for calculating differential expression: (1) edgeR using TPM values, (2) edgeR using expected counts, and (3) EBSeq using expected counts. The number of differentially expressed genes identified greatly varied between pipelines (**Figure 4.3**). Noticeably more differentially expressed genes were identified in pipelines where EBSeq was implemented, regardless of the methods used for transcriptome assembly and quantification. EBSeq detected more differentially expressed genes than edgeR, with an average increase of 1,167%. Additionally, the Port Lavaca population was characterized by more differentially expressed gene detections for each pipeline comparison, with an average increase of 594%, despite having fewer unique transcripts identified during assembly and quantification.

When comparing the number of sex-biased differentially expressed genes for females and males, no consistent signal was found. Pipelines using the Port Lavaca population produced more male-biased (four pipelines) than female-biased differentially expressed genes (two pipelines), whereas the pipelines were equally split for the Redfish Bay (three pipelines produced more female-enriched genes and three produced more male-enriched genes). It is worth noting that the use of only pregnant males in the Port Lavaca samples might have created a strong signal for pregnancy-related

genes, if such genes normally have an expression comparable between females and non-pregnant males. Additionally, edgeR pipelines identified more genes under female-biased expression whereas EBSeq pipelines produced more male-biased genes.

CONSISTENCY OF DIFFERENTIAL EXPRESSION BETWEEN PIPELINES AND POPULATIONS

Differentially expressed genes identified as female- or male-biased were compared against a common proteome (*S. acus*), and the association between each pipeline was measured using a pairwise Cramér's V, as previously mentioned (**Figure 4.4**). For both female- and male-biased genes, we found the average association value for comparisons between populations (inside gray box) (average ± SEM: 0.05 ± 0.01) to be less than the average association value within either population (outside the gray box) (Redfish Bay: 0.10 ± 0.02, and Port Lavaca Bay: 0.10 ± 0.02). Use of different methodology when comparing within a population yielded more similar results than when comparing between two populations; however, nearly all comparisons had low association values.

We also compared the corresponding protein IDs from *S. acus.* There were several differentially expressed genes that were repeatedly identified in at least three pipelines for the same sex bias, and this included 84 female-biased and 73 male-biased protein names. There were no differentially expressed genes that were repeatedly identified in all pipelines for the same sex bias. Interestingly, five differentially expressed genes were identified in both the female-biased and male-biased categories (**Data 4.S3**). These five differentially expressed genes were identified from the two *de novo* assemblies of the Port Lavaca population and were found as both female-biased and male-biased in the same pipeline. Upon further inspection of each of the five genes, a differential expression signal was driven by the presence of two different transcripts (as identified by Trinity) of the same gene that are preferentially expressed by the different sexes. No such result was found in the Redfish Bay population. Additionally, like the highly expressed gene results, differentially expressed genes identified using *de novo* methods were less likely to have a match to a *S. acus* protein (66.3% of genes had a significant BLAST hit) than genes identified using genome-guided assemblies (81.5% of genes had a significant BLAST hit) (**Data 4.S3**).

SIMILARITY OF FUNCTIONAL PROTEIN CLASSES

Differentially expressed genes were also matched with a corresponding protein class from within the PANTHER gene ontology database. Protein classes that were identified in at least half of all differentially expressed groups from a single sex bias (≥ six pipelines) are reported in **Table 4.2**. Five functional classes were identified for both female and male-biased DEGs: scaffold/adapter

protein, ubiquitin-protein ligase, DNA-binding transcription factor, non-receptor serine/threonine protein kinase, and transporter. There were 16 unique functional classes only identified in female differentially expressed genes, and seven unique functional classes only identified in male differentially expressed genes.

MALE PREGNANCY-BIASED EXPRESSION

For our differential expression analysis between pregnant and non-pregnant males from the Redfish Bay population, we limited ourselves to the genome-guided transcriptome assemblies, as these were seen to be more robust and accurate. We used both edgeR and EBSeq to identify significantly differentially expressed genes. EdgeR identified ten transcripts biased for non-pregnant males and 11 transcripts biased for pregnant males (**Table 4.S2**). EBSeq identified a total of 66 transcripts biased for non-pregnant males and 63 transcripts biased for pregnant males (**Table 4.S3**). We then identify the *S. acus* protein orthologs for our differentially expressed genes. Protein matches are presented with their protein name, NCBI reference number, and the edgeR calculated log fold change (logFC) or the EBSeq calculated posterior fold change (PostFC) difference between males.

Six transcripts were identified as differentially expressed from both edgeR and EBSeq analyses. In non-pregnant males, prophet of pit-1 (PROP) paired-like homeobox 1, cartilage intermediate layer protein 1, a putative gonadotropin-releasing hormone II receptor, mucolipin-3 isoform X1, and aromatase isoform X1 were upregulated. In pregnant males, only histidine N-acetyltransferase was consistently identified as upregulated.

DISCUSSION

The use of transcriptomic analyses has become an increasingly popular option and has yielded important results that influence our understanding of biological mechanisms and processes. However, these innovative techniques can also produce convoluted or misleading conclusions depending on the experimental design approach. In this paper, we compare the results of entire pipelines for brain transcriptome assembly, quantification, and analysis. We also offer a unique biological comparison between two populations of the Gulf pipefish (*S. scovelli*) to determine how transcriptome results compare across two wild populations of the same species. Here we discuss our findings with suggestions on how to improve current experimental design and increase the robustness of transcriptome-based conclusions.

INFLATION AND NOISE IN DE NOVO TRANSCRIPTOMES

The number of unique transcripts identified for each transcriptome depended on (1) alignment against a reference genome and (2) the number of samples included (**Table 4.1**). The transcriptomes that contained the most realistic estimates of gene number, based on the number of protein coding genes in the Gulf pipefish genome, were those assembled using the reference genome. Reference-based assemblies typically offer a more conservative but precise approach to assembly than *de novo*, which relies on finding overlapping regions between reads for assembly. This constraint is a known limitation of *de novo* assembly, as transcript variation in the absence of a reference can be nearly impossible to reconstruct into a single accurate transcript (Grabherr et al. 2011; Haas et al. 2013). Trinity typically provides accurate estimations of transcript isoforms; however, variation as a result of sequencing errors, gene duplication, heterozygosity, or widespread repetitive sequences can be nearly impossible to disentangle while using short read data (Grabherr et al. 2011; Haas et al. 2013; Honaas et al. 2016). These sources of ambiguity can inflate the number of unique transcripts, as observed here, where *de novo* assemblies produced far more unique transcripts than there are genes in the Gulf pipefish genome (Honaas et al. 2016).

In our analysis of highly expressed genes, we found that *de novo* assemblies were also more likely to produce highly expressed transcripts from sources of contamination or misalignment. Genome-guided assemblies identified highly expressed transcripts that were more likely to align back to a syngnathid-specific gene. In this regard, the weaknesses of a *de novo* assembly can have a significant impact on the interpretation of results, such as identification of highly expressed genes or the reliability of discovering novel transcripts.

The number of unique transcripts also differed between populations, and there was a greater number of unique transcripts identified in the Redfish Bay population. The average sequence depth differed between populations (Redfish Bay: 11,948,040 and Port Lavaca: 22,929,985). Increased sequencing depth can improve statistical analysis, and this benefit is usually maximized at around 20 million reads (Wang et al. 2011). Given this information, we would expect to see a larger number of unique transcripts identified for the Port Lavaca population. As this was not the case, the difference in sample numbers is likely the contributing factor to the imbalance of unique transcripts identified. The Redfish Bay population had more samples (15 samples) than the Port Lavaca population (seven samples) and was more likely to include less frequently expressed transcripts and transcripts only expressed by non-pregnant males. It is also likely that by increasing the number of samples the variation of reads likewise increased, potentially compounding assembly errors.

The inconsistencies presented here are some the reasons why others have encouraged the combined use of more than one assembly method, especially with the inclusion of a reference-guided assembly (Silva et al. 2013; Lischer and Shimizu 2017; Wang and Gribskov 2017). In cases where a genome is not easily available, there are still strategies that can improve the proportion of accurate transcript predictions for both guided and *de novo*, assemblies. The use of paired-end reads or long-read data can aid in finding overlapping regions and resolving ambiguities, which would be especially important for *de novo* assemblies (Grabherr et al. 2011). Once sequenced, filtering by length and coverage can also increase accuracy (Cahais et al. 2012).

COMPARISON OF EXPRESSION PROFILES BETWEEN POPULATIONS

Transcriptomics has the potential to identify crucial differences between populations of the same species. The most common population-level comparison studies investigate phenotypic plasticity in adaptation between two populations of the same species exposed to different environmental factors (Xu et al. 2016; Herrmann et al. 2018; Wang et al. 2020). Understanding the variation in expression among populations also contributes to our understanding of heritable variation, speciation, and evolution (Whitehead and Crawford 2006; Whitehead et al. 2010; Müller et al. 2011; Wang et al. 2020). When phenotypic variation through modulation in gene expression is evident, comparisons between two experimental groups can yield dramatic differences. Lesser understood is the similarity of transcriptomes between samples or members of the same species where phenotypic variation is expected to be low. The two populations selected for this study are only about 128km apart by way of ocean and likely experience similar average environmental conditions for most of their lives. The populations were sampled two years apart during a similar summer timeframe.

The assumption for designing an expression-based analysis is that within-experimental-group variation is less than between-experimental-group variation. In this regard, we assume selective pressures experienced by one sex should be relatively similar across populations that experience similar environmental conditions. As a result, our expectation was that brain expression profiles within a sex should be more similar than between the sexes. To evaluate this idea, we looked at both the consistency of genes with the highest expression, and those that were differentially expressed. In our comparison of the brain expression profiles between female and male Gulf pipefish, we find little consistency in both comparisons.

For our analysis of highly expressed genes **(Figure 4.2)**, we found that the methodology used dramatically influenced the accuracy of the genes identified. Top genes identified by *de novo*

transcriptomes included a high percentage of genes that did not match any pipefish or seahorse gene sequences on NCBI, indicating a high susceptibility for noise or contamination. The top genes identified for both sexes within a pipeline were also more similar than those identified for the same sex across pipelines. It would be reasonable to assume that females and males share highly expressed genes, like those that maintain basic cellular functions, such as in the case of housekeeping genes. In fact, identifying genes with uniform expression across sexes for a specific tissue would be extremely useful for comparison studies interested in molecular evolution and selection. However, the expectation would then be the presence of similarities between all groups, regardless of methodology used, which was not the case here. That being said, a handful of universally regarded housekeeping genes were identified within our dataset, such as actin, glyceraldehyde-3-phosphate dehydrogenase, and tubulins (Eisenberg and Levanon 2013). Other reoccurring genes with likely housekeeping functions were: 60s ribosomal protein, elongation factor 1-alpha, fructose-bisphosphate aldolase C-B, heat shock proteins, hemoglobin subunits, cytochrome c oxidase subunit 2, NADH-ubiquinone oxidoreductase chain 5-like, myelin basic protein, polyubiquitin, protein phosphatase 1 regulatory subunit, and ubiquitin carboxyl-terminal hydrolase 37-like **(Data 4.S1)**. These reoccurring genes might be useful for identifying taxon-specific housekeeping genes, which is currently lacking (Faheem and Khaliq 2019).

For our differential expression analysis **(Figure 4.3)**, we found two main methodology-based effects. The first was that there were more differentially expressed genes detected in the Port Lavaca population despite the identification of fewer unique transcripts. Furthermore, the Port Lavaca population had the widest range of differentially expressed gene counts across pipelines. As previously mentioned, the lower sample size in the Port Lavaca population is a likely driver of this variation. This idea would be supported by other technical studies which have pointed out the relationship between sample number and precision, calling for the use of caution in interpreting results from low sample numbers (Baccarella et al. 2018).

The second observed bias was that EBSeq always detected more differentially expressed genes than edgeR for each pipeline. Preceding reports have demonstrated that the optimal statistical power for EBSeq skews towards fewer samples with higher sequencing depth than edgeR (Ching et al. 2014). In this regard, EBSeq should more accurately detect a higher percentage of differentially expressed genes in the Port Lavaca population. However, EBSeq provided the most dramatic swing of differentially expressed gene count estimates between pipelines.

BIOLOGICAL CONCLUSIONS FROM DIFFERENTIAL EXPRESSION ANALYSES

Our differential expression analysis yielded distinct lists of differentially expressed genes depending on the pipeline and population. These results contrasted with a previous finding, where the number of shared differentially expressed genes between different assembly methods was higher (about 70%) (Wang and Gribskov 2017). The association value between all variables was low, indicating little intercorrelation and high variation between pipelines. Pragmatically, differentially expressed genes present in one pipeline could not be used to identify differentially expressed genes in another pipeline. In total, 84 female-biased and 73 male-biased genes were identified in at least three pipelines with the same sex bias. This number dropped to 15 female- and eight male-biased genes present in at least four pipelines, three female- and two male-biased genes present in at least five pipelines, and 0 of the same biased genes for either sex present in at least six, or half, of the pipelines.

For the differentially expressed genes identified in at least three pipelines, five of the 84 female-biased and 73 male-biased genes were the same (**Data 4.S3**). Meaning, the same five genes were identified as being both female- and male-biased. As previously mentioned, these five differentially expressed genes were identified from *de novo* assemblies of the Port Lavaca population. This signal was driven by the presence of two different transcripts (as identified by Trinity) of the same gene that are preferentially expressed by different sexes. Given that these transcripts were only identified in the Port Lavaca population, it is possible that the male-biased transcript is specifically biased for pregnant males. These five genes were retbindin (Genc et al. 2020), protein SSUH2 homolog (Xiong et al. 2017), beta-arrestin-1 isoform X3 (Fan et al. 2003), mu-type opioid receptor (Martini et al. 1989), and histone-lysine N-methyltransferase EZH2 isoform X1 (Zhang et al. 2015), which all have different functions in the central nervous system. One of these genes, mu-type opioid receptor, shows expression differences between the sexes during several stages of brain development (Martini et al. 1989) and sex-specific functional differences in rodents (Mague et al. 2009). There is also prior research documenting sex-specific use of alternative transcripts, which might be similar to what was observed here (Scali et al. 2005; McIntyre et al. 2006). This information is difficult to assess in relation to our study, as functional differences of certain genes may be taxon- or species-specific and this signal was seen in only one of the studied populations.

Differentially expressed genes were also matched with a corresponding protein class from within the PANTHER gene ontology database. For our analysis, we recorded PANTHER protein classes that were identified in at least half of all female-biased or male-biased pipelines (**Table 4.2**). As mentioned in the results, five functional classes were identified for both female- and male-biased differentially expressed genes. The relationship between these protein classes and sex-specific brain

function is unclear but select ubiquitin ligases (Kawabe and Stegmüller 2021) and non-receptor serine/threonine protein kinase (Zhang et al. 2002) have implications in healthy brain function and development.

For the unique PANTHER functional classes identified for both sexes (**Table 4.2**), there was precedent for the overexpression of certain protein functional classes that were identified in this study. Specific dehydrogenases have sexually dimorphic expression in the brains of catfish (*Heteropneustes fossilis*), where both sexes had elevated levels depending on the brain region and the different stages of spawning (Mishra and Chaube 2017). In males, ribosomal proteins have been shown to have biased expression and hypermethylation in the brains of male zebrafish (*Danio rerio*), which was hypothesized to be related to male-specific sexual and aggressive behaviors (Santos et al. 2008; Chatterjee et al. 2016). Ribosomal proteins are also found to be associated with male-specific brain development and sexual behaviors in zebra finches (Ping Tang and Wade 2006) and mice (Smagin et al. 2018). In contrast, Gulf pipefish are sex-role reversed, where females compete for access to males (Berglund and Rosenqvist 2003). In this regard, the upregulation of ribosomal proteins in male pipefish might entail a different male-specific behavior and would be difficult to interpret.

Functional genomics research has been mostly limited to select candidate genes in highly popular model organisms such as mice or zebrafish. This inadequacy makes extrapolating any functional information from specific genes nearly impossible when working with non-model organisms or genes that are not highly conserved. In realistic scenarios where one pipeline is used for differential expression, identification of sex-biased differentially expressed genes can lead to overreaching conclusions that establish biological significance for why a gene is biased towards a particular sex. This is concerning, given how methodology or transcriptional noise can influence the consistency and outcome of results.

It is also worth noting the lack of similarity between the differentially expressed genes identified in all pipelines for this analysis and those identified in the previous paper (from which we obtained the Port Lavaca RNA-seq reads) that also studied sex-biased expression in Gulf pipefish brains, even when similar analysis methods were used (Beal et al. 2018). In the present analysis, we did not find consistent support for the idea that there is a higher proportion of male-biased genes. In systems where key behaviors have a significant role in the mating success of only one sex and decrease fitness of the other sex, there is an expectation that sexual conflict could lead to sex-biased expression (Ellegren and Parsch 2007). For example, the ability to make and hold a nesting territory is essential for the reproductive success of territorial black-faced blenny (*Tripterygion delaisi*) males.

Males must attract a female and provide sole parental care to the eggs within his nest (Schunter et al. 2014). When comparing brain expression profiles between territorial males and females, territorial males had more upregulated differentially expressed genes (Schunter et al. 2014). In the context of these high-reward behaviors, sexual selection can favor more sex-biased genes that benefit the sex under stronger selection (Ellegren and Parsch 2007).

Tissues under intense sexual selection and sexual conflict, such as those involved in reproduction or secondary sexual signaling have repeatedly demonstrated sex-biased expression and rapid evolution (Ellegren and Parsch 2007). Given this information, we would expect to see more female-biased genes in Gulf pipefish as sexual selection is stronger for females (as also discussed in Beal et al. (2018)) (Connallon and Knowles 2005). There are several potential explanations for why the number of identified sex-biased genes were not found to be consistent for either sex. Previous studies have documented that both courtship time and ornamental banding influence female mating success (Partridge et al. 2013; Flanagan et al. 2014). It is unknown whether the strength of selection is greater on one trait or the other, and it is possible that females experience more intense selection on non-behavioral traits such as banding. In this case, it would be possible that the behavior of courtship display and mate choice are both under equal selection in both females and males (Kirkpatrick and Ryan 1991). This scenario would lead to sex-biased genes for both sexes, with neither sex having an overwhelming majority. Another explanation is that sex-biased expression during embryo development results in structural differences before sexual maturation (Gegenhuber and Tollkuhn 2020). Under this scenario, similar expression of a gene between the sexes can be associated with different behavioral responses, such as in túngara frogs (*Physalaemus pustulosus*), where females and males both expressed egr-1 in different mating behavior contexts (Hoke et al. 2008). Other factors such as differential RNA processing, mRNA longevity, selective inhibition or activation of mRNA translation, or microRNA regulation of translation, might affect sexes differently and would not be easily detected by the RNA-seq methods used here (Gilbert 2010; Drewe et al. 2013). Lastly, it might also be likely that within Gulf pipefish there is larger than expected expressional variation from heterogeneity in samples. In this case, increasing sample size, only sampling individuals with known mating success, and a single-cell RNA-seq approach to control for proportion of different cell types might help reduce variation (Price et al. 2022).

BETTER PRACTICES

While there are preemptive hurdles to incorporating any bioinformatics component, such as cost and expertise, the largest concerns with transcriptome analyses stem from problematic experimental designs. Generally, (1) noisy or inconsistent results or (2) the lack of a hypothesis-

driven analyses (that has no *a priori* predictions) are common and prominent weaknesses. There are a handful of strategies that can significantly increase the return on investment in the RNA-seq approach.

Consistency in results can be maximized by (1) decreasing heterogeneity in samples (Seyednasrollah et al. 2015; Price et al. 2022), (2) increasing sample size (Rapaport et al. 2013; Soneson and Delorenzi 2013; Seyednasrollah et al. 2015), and (3) using a reference genome or long-read RNA-seq data (Grabherr et al. 2011). Decreasing heterogeneity in samples can include many different approaches from collecting samples in consistent environmental conditions and standardizing the dissection protocol, to sampling a single cell type across samples in a population with low genetic variation (Soneson and Delorenzi 2013; Seyednasrollah et al. 2015; Price et al. 2022). This consideration should be taken when designing an experiment, and researchers should consider factors most important and reasonable for their taxa. An experimental design that (1) isolates a single cell type or controls for the proportion of cell types, (2) uses a population with low genetic variation, (3) can control environmental settings, (4) has experimental groups with noticeable phenotypic differences, (5) can obtain five or more samples per group, and (6) has a high-quality genome reference, will likely have results that are more consistent. However, an experimental design that incorporates all these elements is usually unrealistic and can severely limit the variety of taxa that can be studied. It would reduce the ability to study non-model systems, which are usually the most relevant organisms in the fields of ecology and evolution. Instead, experimental designs should focus on addressing factors that improve the number of samples and the consistency between them, while balancing practical considerations.

Transcriptomics shows us the versatility of the genome and its ability to produce complex systems within the framework of a single genomic story. However, it can also lead us to a distorted understanding of important biological mechanisms based on superficial or noisy evidence. Genomics and transcriptomics are best used within a hypothesis-driven analysis, to understand an established phenomenon. Their use as a basic exploratory tool without strong *a priori* predictions can have extremely diminishing returns. Moreover, the intrinsic variation present within transcriptome data should be considered when formulating realistic hypotheses and interpreting results. In our analysis, we found many of our results varied depending on the pipeline used. Each pipeline, when interpreted by itself at face value, gave an imprecise (or even incorrect) understanding of the biological differences between females and males. However, we also found several consistencies between pipelines that we believe were robust when specific techniques were used. Specifically, we found more consistent and credible results from reference-based transcriptomes and when sample size

increased. To benefit from *de novo* methods, the use of pre-filtering criteria should be heavily considered, while also aiming to decrease sample heterogeneity and increase sample size. As a growing and evolving field, the availability of useful resources coupled with the reduced cost of sequencing, should foster the growth of productive analyses for multiple fields.

Figure 4.1

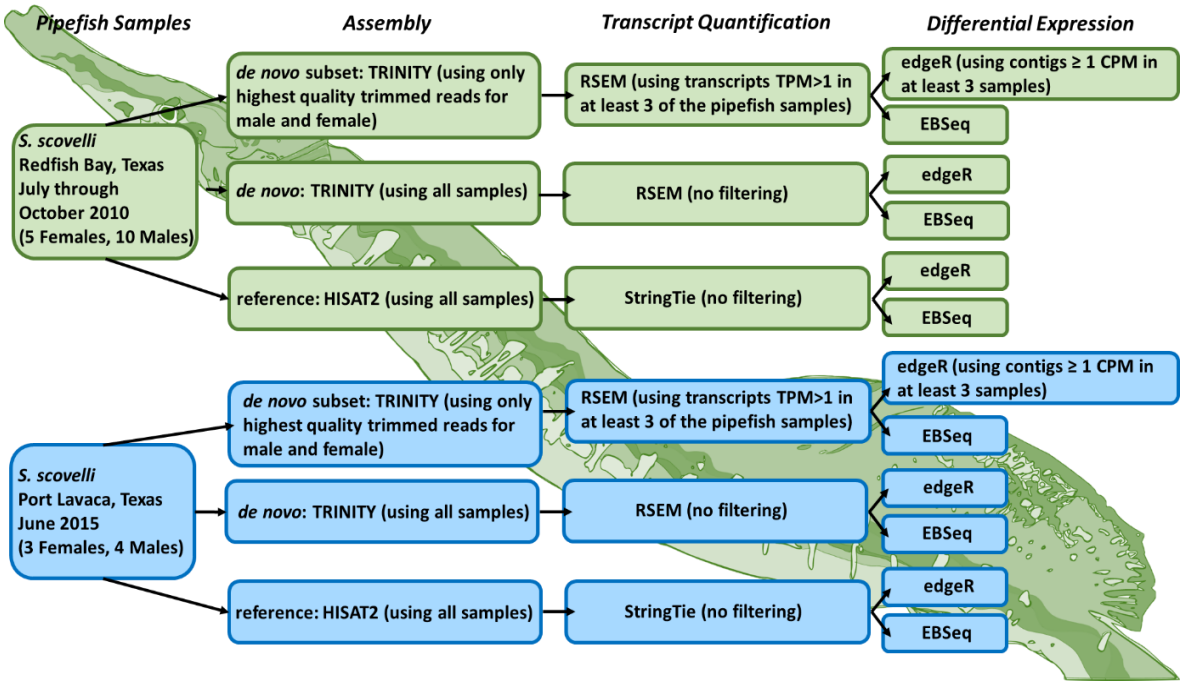

Figure 4.1: Analysis pipeline.

For this study, we compared two pipefish population datasets, and between multiple analysis pipelines. Pipefish were collected from two different location sites: Redfish Bay, TX and Port Lavaca, TX (Beal et al. 2018). Transcriptomes were assembled either *de novo* using TRINITY or against a reference genome using HISAT2. Transcriptomes assembled using a *de novo* subset, were adapted from (Beal et al. 2018), and the pipeline was used to recreate and compare results from this original study. Transcripts were then quantified using RSEM for *de novo* transcriptomes or StringTie for reference-guided assemblies. Both EBSeq and edgeR were used for all assembly pipelines, resulting in 12 final lists of sex-biased genes.

Figure 4.2

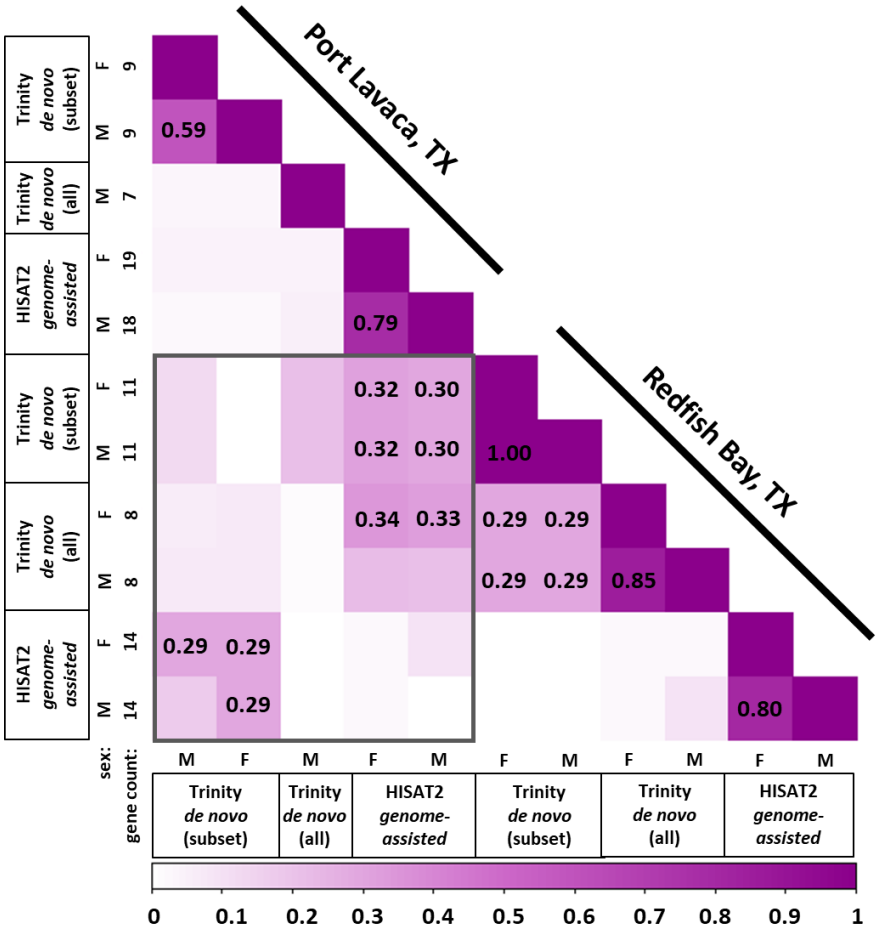

Figure 4.2: Association of populations and methods based on top twenty genes with the highest expression.

For each method, twenty genes with the highest expression (measured as TPM) were searched for against the NCBI non-redundant protein database. Those genes that significantly matched a protein (E-value$\leq 1\times10^{-20}$) were retained (the number labelled as "gene count"), and the similarity and relationship of genes within each pipeline were measured by performing a pairwise Cramér's V tests between each method. The Cramér's V value ranges from 0 to 1, where 0 corresponds to no association between variables and 1 corresponds to complete association between variables. Here, only values ≥ 0.25 have been labelled. The portion of the plot within the outlined region corresponds to comparisons made between populations, the portion outside of this region corresponds to comparisons made between methods testing a single population.

Figure 4.3

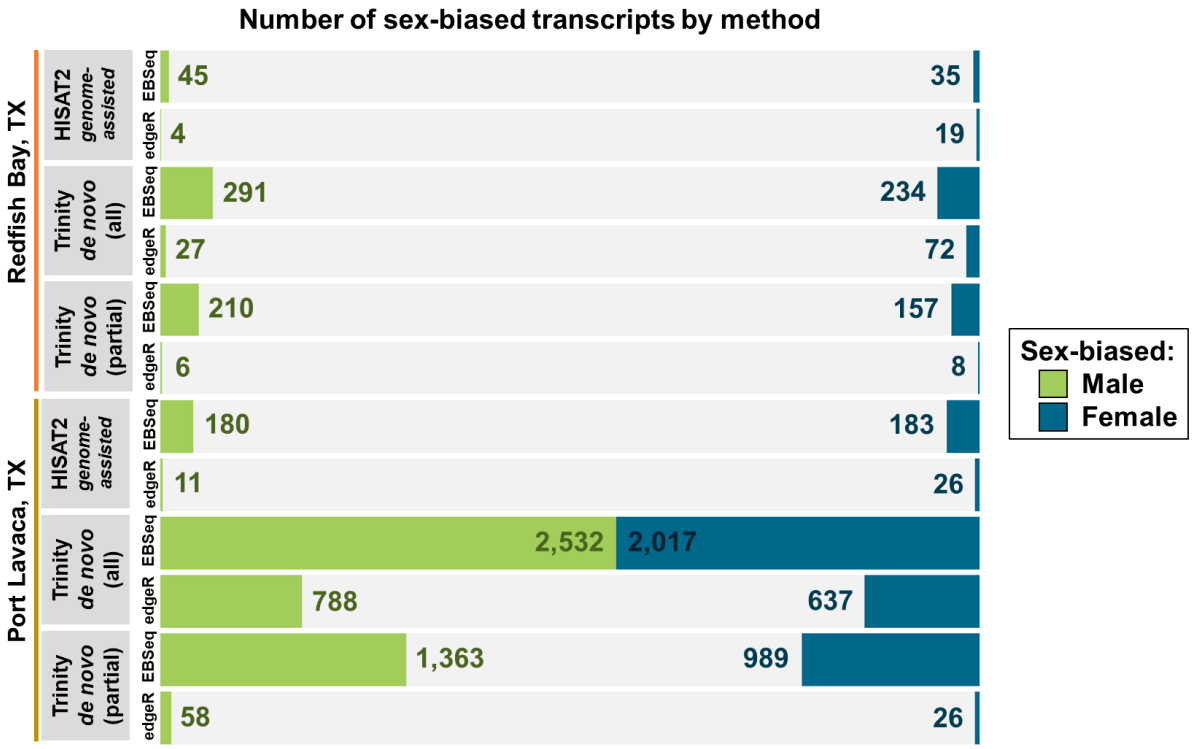

Figure 4.3: The number of transcripts that are significantly differentially expressed, based on each population and analysis pipeline used.

Transcripts are also divided into male-biased or female-biased depending on which sex they had an overall greater expression in. Gulf pipefish were collected from two different location sites: Redfish Bay, TX and Port Lavaca, TX (Beal et al. 2018). Transcriptomes were assembled either *de novo* or against a reference genome. Additionally, either all samples were included and used for the assembly (all) or one male and one female samples with the highest number of quality trimmed reads (partial). The differential expression analysis was conducted using either edgeR or EBSeq. Significantly differentially expressed is defined as having a False Discovery Rate (FDR) ≤ 0.05 (or a Posterior Probability of Differential Expression (PPDE) ≥ 0.95).

Figure 4.4

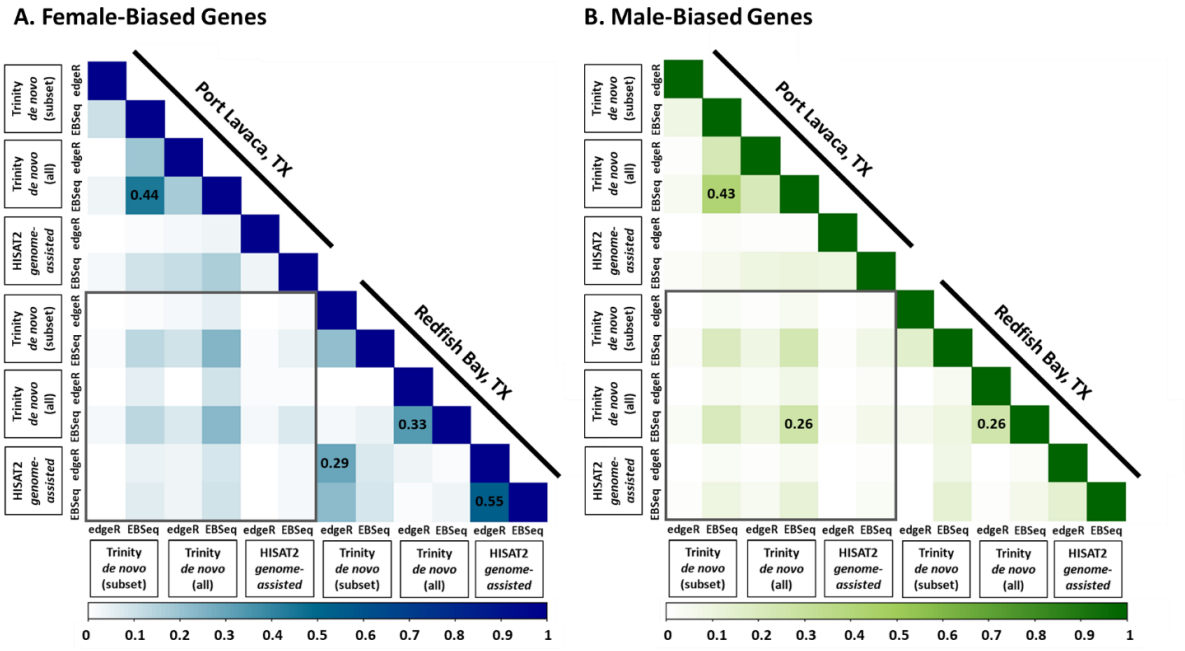

Figure 4.4: Association of populations and methods based on differentially expressed genes.

For each method, a list of differentially expressed genes was produced that was either female or male biased. The similarity and relationship of differentially expressed genes within each pipeline were measured by performing a pairwise Cramér's V tests between each method. The Cramér's V value ranges from 0 to 1, where 0 corresponds to no association between variables and 1 corresponds to complete association between variables. Here, only values ≥ 0.25 have been labelled. The portion of the plot within the outlined region corresponds to comparisons made between populations, the portion outside of this region corresponds to comparisons made between methods testing a single population.

Figure 4.S1

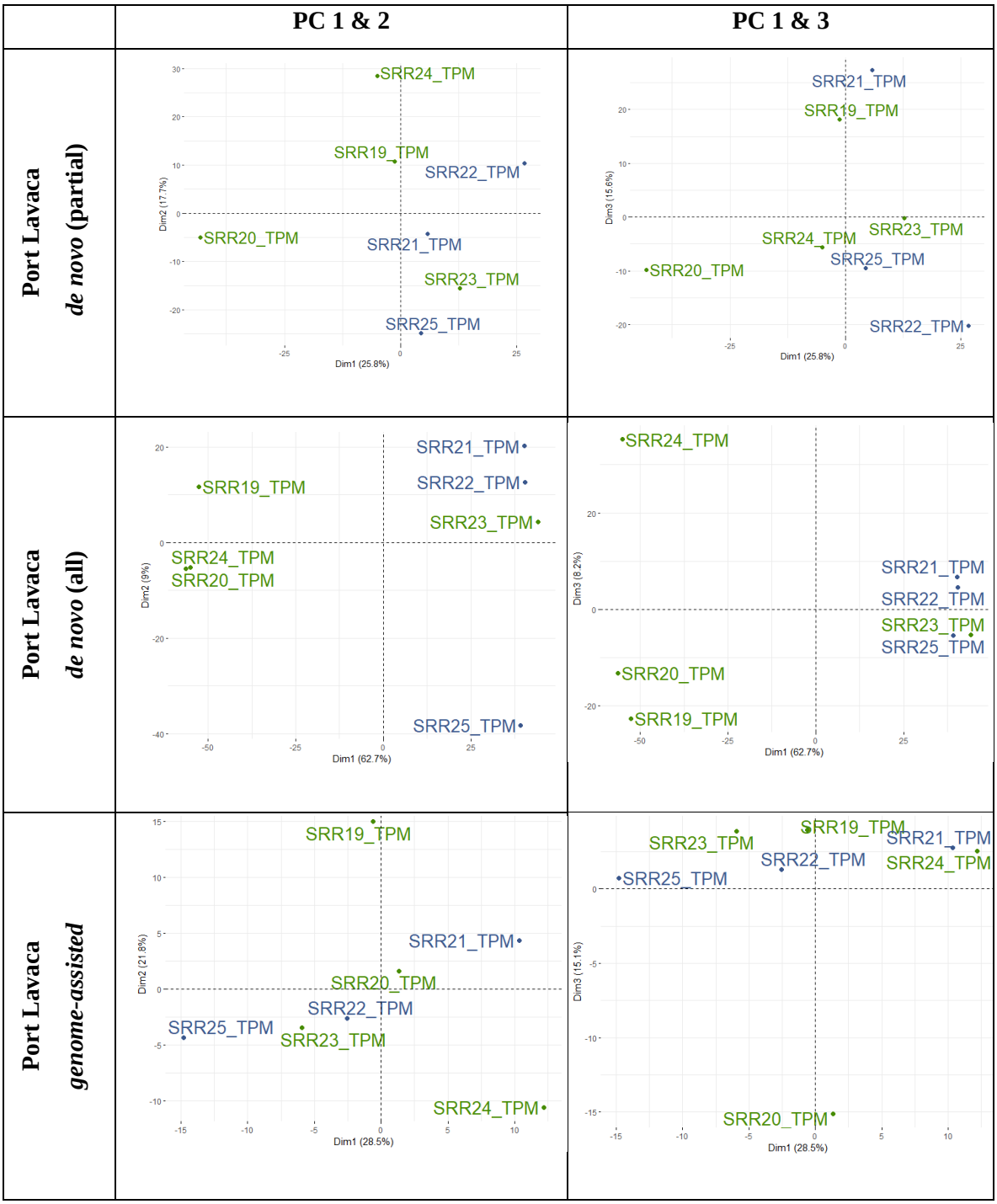
PC 1 & 2
PC 1 & 3
Port Lavaca de novo (partial)
Port Lavaca de novo (all)
Port Lavaca genome-assisted
SRR24_TPM
SRR19_TPM
SRR22_TPM
SRR20_TPM
SRR21_TPM
SRR23_TPM
SRR25_TPM
Dim1 (25.8%)
Dim2 (17.7%)
Dim3 (15.6%)
Dim1 (62.7%)
Dim2 (9%)
Dim3 (8.2%)
Dim1 (28.5%)
Dim2 (21.8%)
Dim3 (15.1%)

Figure 4.S1 cont'd

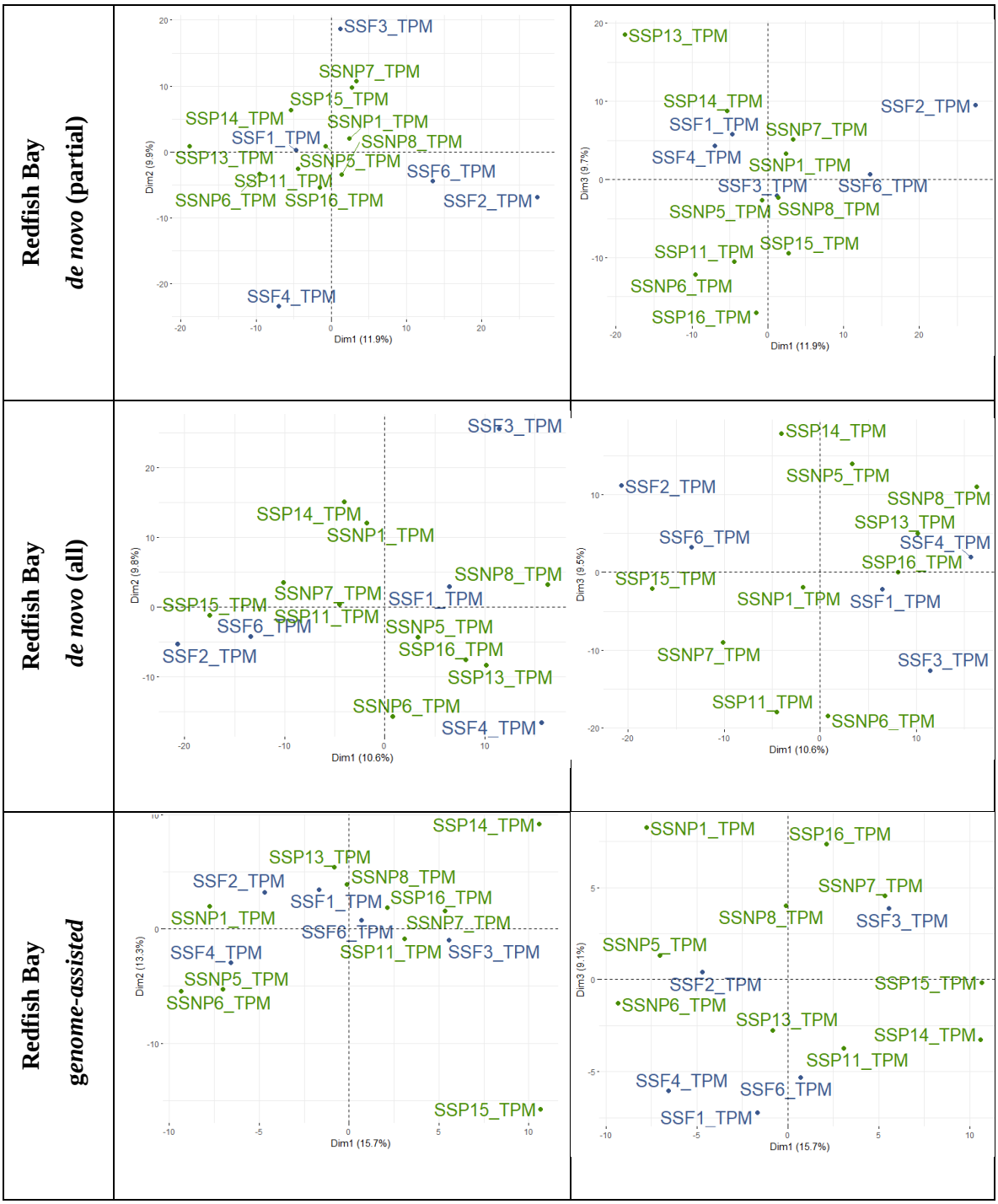

Figure 4.S1: Principal component analysis between samples.

Principal component analysis between samples. To generate points, TPM values for each transcript were initially filtered by an average TPM ≥ 25 within a sex and population and then log transformed (log (TPM +1)). Female samples are labelled as dark blue and male samples are labelled as light green. The plots on the left-hand column represent the first two principal components. The plots on the right-hand column represent the first and third principal component.

Table 4.1

Sample Population	Method	Number of genes identified	Number of unique transcripts identified
Redfish Bay	*de novo* subset	-	160,100
	de novo	-	318,830
	against reference	24,348	52,056
Port Lavaca	*de novo* subset	-	117,137
	de novo	-	227,764
	against reference	22,157	41,949

Table 4.1: The number of genes and unique transcripts identified for each transcriptome.

This is compared to the predicted number of protein coding genes in the reference Gulf pipefish genome (20,841) (Small et al. 2013).

Table 4.2

A. Female-biased	B. Male-biased
primary active transporter (PC00068)	scaffold/adaptor protein (PC00226) †
transmembrane signal receptor (PC00197)	DNA-binding transcription factor (PC00218) †
dehydrogenase (PC00092)	non-receptor serine/threonine protein kinase (PC00167) †
scaffold/adaptor protein (PC00226) †	**ribosomal protein (PC00202)**
cysteine protease (PC00081)	transporter (PC00227) †
membrane traffic protein (PC00150)	**C2H2 zinc finger transcription factor (PC00248)**
microtubule or microtubule-binding cytoskeletal protein (PC00157)	**DNA metabolism protein (PC00009)**
oxygenase (PC00177)	**guanyl-nucleotide exchange factor (PC00113)**
ubiquitin-protein ligase (PC00234) †	**membrane trafficking regulatory protein (PC00151)**
actin or actin-binding cytoskeletal protein (PC00041)	**RNA metabolism protein (PC00031)**
DNA-binding transcription factor (PC00218) †	**small GTPase (PC00208)**
glycosyltransferase (PC00111)	ubiquitin-protein ligase (PC00234) †
ligand-gated ion channel (PC00141)	
metalloprotease (PC00153)	
non-motor actin binding protein (PC00165)	
non-receptor serine/threonine protein kinase (PC00167) †	
protease inhibitor (PC00191)	
protein phosphatase (PC00195)	
transporter (PC00227) †	
viral or transposable element protein (PC00237)	
voltage-gated ion channel (PC00241)	

Table 4.2: The PANTHER protein classes that are significantly and differentially expressed in at least half of all pipelines (≥6 pipelines).

Differentially expressed genes are further separated based on which sex they were biased towards, **(A)** female-biased or **(B)** male-biased. (†) Indicate the same PANTHER protein class appearing in both female-biased and male-biased lists, while bold lettering indicates a unique PANTHER protein class.

Table 4.S1

Sample ID	Sex	Pregnancy status	Read alignment rate to reference assembly (%)
A. Redfish Bay			
SSF1	**F**	**-**	78.93%
SSF2	**F**	**-**	79.58%
SSF3	**F**	**-**	80.15%
SSF4	**F**	**-**	76.92%
SSF6	**F**	**-**	80.44%
SSNP1	**M**	**No**	78.26%
SSNP5	**M**	**No**	81.56%
SSNP6	**M**	**No**	81.30%
SSNP7	**M**	**No**	78.67%
SSNP8	**M**	**No**	78.07%
SSP11	**M**	**Yes**	79.22%
SSP13	**M**	**Yes**	80.41%
SSP14	**M**	**Yes**	78.88%
SSP15	**M**	**Yes**	80.19%
SSP16	**M**	**Yes**	81.25%
B. Port Lavaca			
SRR5783121	**F**	**-**	75.06%
SRR5783122	**F**	**-**	74.19%
SRR5783125	**F**	**-**	73.52%
SRR5783119	**M**	**Yes**	72.86%
SRR5783120	**M**	**Yes**	74.19%
SRR5783123	**M**	**Yes**	73.29%
SRR5783124	**M**	**Yes**	73.59%

Table 4.S1: Description of samples used and the alignment rate of reads for each of the three assemblies.

This table includes data for the population sampled in (A) Redfish Bay, TX and (B) Port Lavaca, TX.

Table 4.S2

	Protein Name	**NCBI Reference**	**logFC**
	Database: *Syngnathus acus*		
Pregnant	glycylpeptide N-tetradecanoyltransferase 1-like	XP_037134325.1	7
	arachidonate 12-lipoxygenase 12R-type-like	XP_037112719.1	5.97
	protein phosphatase 1 regulatory subunit 3E	XP_037103286.1	2.25
	cytochrome P450 1A1	XP_037103748.1	1.41
	heat shock protein HSP 90-alpha	XP_037100027.1	0.53
	histidine N-acetyltransferase	XP_037118646.1	0.43
Non-Pregnant	beta-2-glycoprotein 1-like isoform X2	XP_037112838.1	6.15
	PROP paired-like homeobox 1	XP_037127012.1	3.79
	cartilage intermediate layer protein 1	XP_037104489.1	3.21
	LOW QUALITY PROTEIN: putative gonadotropin-releasing hormone II receptor	XP_037103067.1	3.14
	mucolipin-3 isoform X1	XP_037131270.1	2.94
	aromatase isoform X1	XP_037109509.1	1.28
	FH1/FH2 domain-containing protein 1 isoform X4	XP_037103567.1	0.8

Table 4.S2: Differential expression analysis (using edgeR) between pregnant and non-pregnant males within the Redfish Bay population of *S. scovelli*.

For this analysis, we focused on transcripts that were identified using a genome-guided assembly (HISAT2) and quantified using StringTie. We used the differential expression analysis program edgeR. The differentially expressed transcripts were then searched for in the *S. acus* genome (using BLASTP), whose genome currently offers the best annotation quality for the genus. Protein matches that had an E-value of less than 1x10-20 are presented here with their protein name, NCBI reference number, and the edgeR calculated log fold change (logFC) difference between males.

Table 4.S3

	Protein Name Database: *Syngnathus acus*	NCBI Reference	PostFC
Pregnant	uncharacterized protein C1orf112 homolog	XP_037104626.1	3.45
	transcription factor AP-2-epsilon isoform X2	XP_037120415.1	1.74
	vasoactive intestinal polypeptide receptor-like isoform X1	XP_037131080.1	1.47
	iroquois-class homeodomain protein IRX-1a	XP_037129928.1	1.47
	BCL6A transcription repressor a isoform X2	XP_037106518.1	1.40
	homeobox protein engrailed-2a	XP_037135413.1	1.38
	retinoic acid receptor alpha-A isoform X1	XP_037134354.1	1.35
	lymphoid enhancer-binding factor 1 isoform X3	XP_037107410.1	1.35
	histidine N-acetyltransferase	XP_037118646.1	1.34
	double C2-like domains delta isoform X1	XP_037113444.1	1.33
	single-stranded DNA-binding protein 3-like isoform X2	XP_037097230.1	1.33
	lectin mannose-binding 2-like a isoform X2	XP_037121295.1	1.32
	transcription factor COE3 isoform X4	XP_037128593.1	1.30
	DNA repair protein complementing XP-A cells isoform X2	XP_037107072.1	1.30
	transmembrane protein 178B	XP_037099685.1	1.30
	5-hydroxytryptamine receptor 3A-like isoform X2	XP_037113140.1	1.30
	AP-4 complex subunit sigma-1	XP_037098334.1	1.29
	tRNA selenocysteine 1-associated protein 1-like isoform X2	XP_037131785.1	1.29
	zinc finger MYND domain-containing protein 11 isoform X2	XP_037131107.1	1.28
	DNA-binding protein SATB1 isoform X3	XP_037129364.1	1.28
	uncharacterized protein LOC119129304 isoform X2	XP_037118360.1	1.28
	neuregulin 2b isoform X3	XP_037116929.1	1.27
	rho-associated protein kinase 1 isoform X1	XP_037132779.1	1.27
	rab effector MyRIP isoform X1	XP_037135765.1	1.26

Table 4.S3 cont'd

Pregnant	interleukin enhancer-binding factor 3 homolog isoform X1	XP_037112279.1	1.25
	choline-phosphate cytidylyltransferase B isoform X2	XP_037135274.1	1.25
	ubiquitin-associated and SH3 domain-containing protein B-like isoform X2	XP_037125685.1	1.25
	stathmin-like	XP_037120476.1	1.24
	NACHT and WD repeat domain-containing protein 2 isoform X1	XP_037116041.1	1.24
	proline-rich protein 12 isoform X1	XP_037111647.1	1.24
	putative monooxygenase p33MONOX	XP_037115989.1	1.23
	LOW QUALITY PROTEIN: lysine-specific demethylase 5C	XP_037097569.1	1.22
	protein FAM117B	XP_037121000.1	1.22
	U2 snRNP-associated SURP motif-containing protein	XP_037126261.1	1.22
	histone deacetylase complex subunit SAP130a isoform X2	XP_037105785.1	1.22
	collagen type I alpha 1a isoform X1	XP_037111387.1	1.21
	sine oculis-binding protein homolog A isoform X2	XP_037096664.1	1.21
	signal-induced proliferation-associated 1-like protein 2	XP_037125164.1	1.20
	poly(U)-binding-splicing factor PUF60-like	XP_037131393.1	1.20
	TOX high mobility group box family member 2 isoform X1	XP_037107167.1	1.20
	WD repeat and FYVE domain-containing protein 2	XP_037095806.1	1.18
	cAMP-dependent protein kinase catalytic subunit alpha	XP_037105655.1	1.17
	heterogeneous nuclear ribonucleoprotein A1-like	XP_037129907.1	1.17
	activity-dependent neuroprotector homeobox b	XP_037121969.1	1.17
	cyclin-I isoform X1	XP_037122325.1	1.17
	vang-like protein 1	XP_037096442.1	1.17
	neuroplastin b isoform X1	XP_037104059.1	1.17
	reticulon-4 receptor isoform X1	XP_037113877.1	1.17

Table 4.S3 cont'd

Pregnant	LIM domain-binding protein 2a isoform X1	XP_037117589.1	1.17
	netrin receptor UNC5D-like isoform X3	XP_037121510.1	1.16
	E3 ubiquitin-protein ligase UHRF2-like	XP_037121909.1	1.15
	succinate-semialdehyde dehydrogenase mitochondrial	XP_037130857.1	1.14
	FK506-binding protein 1-like	XP_037131962.1	1.13
	kin of IRRE-like protein 3 isoform X3	XP_037124222.1	1.13
	zinc finger X-chromosomal protein isoform X1	XP_037126268.1	1.13
	apoptotic chromatin condensation inducer in the nucleus	XP_037131466.1	1.07
	lissencephaly-1 homolog A	XP_037123721.1	1.07
Non-Pregnant	LOW QUALITY PROTEIN: putative gonadotropin-releasing hormone II receptor	XP_037103067.1	8.06
	PROP paired-like homeobox 1	XP_037127012.1	7.94
	cartilage intermediate layer protein 1	XP_037104489.1	7.05
	mucolipin-3 isoform X1	XP_037131270.1	6.56
	LOW QUALITY PROTEIN: stereocilin-like	XP_037103360.1	5.11
	prostaglandin F2-alpha receptor	XP_037105928.1	3.10
	oligodendrocyte-myelin glycoprotein-like	XP_037126147.1	2.98
	docking protein 4 isoform X2	XP_037102583.1	2.93
	plasmalemma vesicle associated protein b isoform X1	XP_037104543.1	2.64
	aromatase isoform X1	XP_037109509.1	2.43
	armadillo repeat-containing protein 2 isoform X4	XP_037101150.1	2.37
	extracellular matrix protein 2 isoform X2	XP_037107754.1	2.26
	kunitz-type protease inhibitor 1a	XP_037097490.1	2.15
	nucleobindin-2-like	XP_037109191.1	2.15
	pro-MCH	XP_037099401.1	2.09
	solute carrier family 43 member 2b isoform X1	XP_037124998.1	1.92
	plakophilin-3a isoform X1	XP_037109443.1	1.88
	LOW QUALITY PROTEIN: protein diaphanous homolog 1-like	XP_037116993.1	1.77
	harmonin isoform X2	XP_037110158.1	1.65
	macrophage mannose receptor 1	XP_037136072.1	1.64

Table 4.S3 cont'd

Non-Pregnant	transmembrane protease serine 3 isoform X1	XP_037136572.1	1.63
	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2 isoform X2	XP_037101210.1	1.59
	protein Wnt-7a	XP_037127798.1	1.53
	coiled-coil domain-containing protein 149-A isoform X3	XP_037118281.1	1.53
	CD81 antigen-like	XP_037109645.1	1.53
	6-phosphofructo-2-kinase/fructose-2 6-bisphosphatase 2-like isoform X1	XP_037120628.1	1.53
	von Willebrand factor A domain-containing protein 1	XP_037136014.1	1.50
	sine oculis-binding protein homolog A-like isoform X2	XP_037101394.1	1.45
	DNA replication factor Cdt1	XP_037126751.1	1.44
	peptidyl-prolyl cis-trans isomerase FKBP10-like	XP_037134129.1	1.43
	splicing factor 3B subunit 4	XP_037119471.1	1.41
	uncharacterized protein si:ch211-1a19.3	XP_037105460.1	1.38
	potassium voltage-gated channel subfamily C member 4	XP_037099522.1	1.38
	tyrosine-protein kinase receptor Tie-1 isoform X1	XP_037105847.1	1.38
	parvin alpha a	XP_037109918.1	1.37
	minor histocompatibility antigen H13 isoform X1	XP_037101816.1	1.35
	phospholipid transfer protein	XP_037108448.1	1.34
	PR domain zinc finger protein 12-like	XP_037113897.1	1.32
	catechol O-methyltransferase B	XP_037107663.1	1.32
	cytosolic 5'-nucleotidase 3 isoform X1	XP_037129678.1	1.31
	reticulocalbin-1	XP_037103898.1	1.31
	aldehyde dehydrogenase mitochondrial-like	XP_037129721.1	1.31
	matrix metalloproteinase-14b isoform X2	XP_037131914.1	1.27
	proteasome subunit beta type-3	XP_037115400.1	1.23
	drebrin-like protein A	XP_037115891.1	1.23
	high mobility group protein B1a isoform X1	XP_037124800.1	1.21
	40S ribosomal protein S2	XP_037112553.1	1.20

Table 4.S3 cont'd

Non-Pregnant	peroxisomal biogenesis factor 19	XP_037132183.1	1.16
	sterol regulatory element-binding protein 1	XP_037111488.1	1.16
	transcription factor Sox-19a-like	XP_037118293.1	1.15
	serine/threonine-protein phosphatase 6 catalytic subunit	XP_037115440.1	1.13

Table 4.S3: Differential expression analysis (using EBSeq) between pregnant and non-pregnant males within the Redfish Bay population of *S. scovelli*.

For this analysis, we focused on transcripts that were identified using a genome-guided assembly (HISAT2) and quantified using StringTie. We used the differential expression analysis program EBSeq. The differentially expressed transcripts were then searched for in the S. acus genome (using BLASTP), whose genome currently offers the best annotation quality for the genus. Protein matches that had an E-value of less than 1x10-20 are presented here with their protein name, NCBI reference number, and the log real fold change (log_realFC) difference between males.

DATA 4.S1: Top 20 BLAST hit table

BLAST results from the top twenty expressed genes from each pipeline.

DATA 4.S2: Orthologs of differentially expressed genes in *Syngnathus acus*

NCBI gene ID matches from *S. acus* correspond to each of the differentially expressed genes, from each pipeline. This file is organized by population, pipeline, and sex-biased expression.

DATA 4.S3: Frequently occurring differentially expressed genes

List of differentially expressed genes that appear in three of more pipelines as female-biased or male-biased.

CHAPTER 5: CONCLUSIONS

Sexual selection is a significant mechanism driving complex trait evolution and genome evolution. This dissertation focuses on addressing this overarching theme, utilizing fishes that have evolved unique mating systems and complex traits. The role of sexual selection in selecting for and maintaining these traits and the overall impact on the genome has been the focus of this dissertation's three main chapters.

The main contribution of the first study is that it largely supports the notion that there is a genetic component for mating preferences. In *Nothobranchius furzeri*, males display a complex color pattern on their tail (Cellerino et al. 2015). This trait is sexually dimorphic and not present in females. There is also natural variation within this trait as male *N. furzeri* can have a predominantly yellow or red tail, depending on their population of origin (Cellerino et al. 2015). Females intrinsic to populations with yellow-tail males were tested for their mating preference. These females had no prior exposure to living males, or the colors yellow or red, and overwhelmingly preferred yellow-tailed males (Johnson et al. 2020). This finding supports similar results (Majerus et al. 1982; Houde 1994; Godin and Dugatkin 1995; Iyengar et al. 2002; P. Haesler and Seehausen 2005), but also illuminates, in part, the degree of contribution to which these genetic mechanisms shape mating preferences. In addition, this study resulted in the creation of a tool that enables anyone to freely produce and modify a lifelike animated killifish model. This tool has created opportunities for the use of killifish as a model system for studying mating preference (Johnson and Jones 2023). Researchers can target specific traits of interest without affecting other aspects of phenotype and evaluate exaggerated phenotypes that lie outside natural trait variation (Johnson and Jones 2023).

For the second study, the primary contribution was to highlight the importance of sexual selection in driving genome-wide patterns of evolution. This study strongly supports the idea that sexual selection and sexual conflict play a convincing role in elevating rates of molecular evolution in male-biased genes in species with conventional sex-roles (Mank 2017). In pipefish and seahorses, males have guaranteed paternity (Jones and Avise 1997b,a, 2001; Mccoy et al. 2001; Avise et al. 2002), and do not engage in sperm competition. This mating systems offers a unique opportunity to test predictions of sexual selection and sexual conflict theory in a system unconfounded by conventional sex roles (Jones et al. 2000; Fritzsche et al. 2021). If sexual selection and sexual conflict drive rapid rates of evolution, as seen in male-biased genes from species with conventional sex-roles, then male pipefish should not show the same pattern of transcriptional and molecular evolution. Indeed, we find that male pipefish exhibit relaxed selection, gene loss, and reduced expression of genes involved in key functions of the testis (Johnson et al. 2022).

For the third study, the main contributions were to establish that there is limited evidence for sexual dimorphism in pipefish brain expression profiles, and that variation in methodology greatly impacts our understanding of biological phenomena. When comparing the results of a differential expression analysis between distinct populations and bioinformatic analysis pipelines, *de novo* assembled transcriptomes inflated the number of unique transcripts beyond a reasonable amount. These *de novo* assemblies also contained proportionally higher numbers of transcripts originating from noise, contamination, or sequencing errors. Additionally, populations where fewer individuals were samples had the highest variation in results. In this chapter, several aspects of common transcriptomic experimental designs are discussed and practices that provide more robust results are recommended.

In summary, this doctoral research has focused on the connections between reproductive behaviors, sexual selection, evolution, and genomics. It has provided novel insights into how sexual selection can greatly influence evolution at the level of the genome and transcriptome.

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