RESEARCH ARTICLE

Phenotypic differences between the sexes in the sexually plastic mangrove rivulus fish (*Kryptolebias marmoratus*)

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ABSTRACT

To maximize reproductive success, many animal species have evolved functional sex change. Theory predicts that transitions between sexes should occur when the fitness payoff of the current sex is exceeded by the fitness payoff of the opposite sex. We examined phenotypic differences between the sexes in a sex-changing vertebrate, the mangrove rivulus fish (*Kryptolebias marmoratus*), to elucidate potential factors that might drive the 'decision' to switch sex. Rivulus populations consist of self-fertilizing hermaphrodites and males. Hermaphrodites transition into males under certain environmental conditions, affording us the opportunity to generate 40 hermaphrodite–male pairs where, within a pair, individuals possessed identical genotypes despite being different sexes. We quantified steroid hormone levels, behavior (aggression and risk taking), metabolism and morphology (organ masses). We found that hermaphrodites were more aggressive and risk averse, and had higher maximum metabolic rates and larger gonadosomatic indices. Males had higher steroid hormone levels and showed correlations among hormones that hermaphrodites lacked. Males also had greater total mass and somatic body mass and possessed considerable fat stores. Our findings suggest that there are major differences between the sexes in energy allocation, with hermaphrodites exhibiting elevated maximum metabolic rates, and showing evidence of favoring investments in reproductive tissues over somatic growth. Our study serves as the foundation for future research investigating how environmental challenges affect both physiology and reproductive investment and, ultimately, how these changes dictate the transition between sexes.

KEY WORDS: Sex change, Sex differences, Reproduction, Metabolic rate, Behavior, Steroid hormones

INTRODUCTION

A central tenet of life history theory is that reproduction negatively impacts survival (Harshman and Zera, 2007; Roff and Fairbairn, 2007). For example, field-based studies in both brown anole lizards (*Anolis sagrei*) and eastern chipmunks (*Tamias striatus*) have shown that even a single reproductive event can significantly reduce survival (Cox and Calsbeek, 2010; Bergeron et al., 2011). The trade-off between reproduction and survival is mediated, in part, by the costs (e.g. energetic, oxidative stress, behavioral) that

individuals pay to reproduce (Bergeron et al., 2011; Cox et al., 2010; Crossin et al., 2012; Harshman and Zera, 2007). These costs are not equal among individuals and can vary significantly between sexes (Brick and Jakobsson, 2002; Harshman and Zera, 2007; Hayward and Gillooly, 2011; Yong and Grober, 2013). Predators can intercept male courtship signals (Taylor and Ryan, 2013) and competing aggressively to acquire mating opportunities can be energetically costly and carry high risks of injury (Brick and Jakobsson, 2002; Neat et al., 1998). Females accrue costs by supporting energetically expensive ovarian tissues (Cox et al., 2010; Hayward and Gillooly, 2011; Jonsson and Jonsson, 1997; Yong and Grober, 2013). To sustain current reproductive efforts, females may allocate energy away from somatic growth, fat storage or future reproduction (Cox et al., 2010). Maintenance of energetically expensive ovarian tissue often requires elevated metabolic rates, which can subsequently lead to oxidative stress, damaging cellular structures and increasing rates of senescence (Bergeron et al., 2011; Harshman and Zera, 2007).

To maximize reproductive success and reduce the costs of reproduction, a number of animal species have evolved a flexible sexual strategy, functional sex change, which is the morphological, physiological, neural and behavioral transition from one sex to the other (Vega-Frutis et al., 2014). In most cases of sex change, individuals transition once in their lifetime either from male to female (e.g. protandry) or from female to male (e.g. protogyny), but there are exceptions (e.g. 'repetitive sex change': Collin, 2006; Erisman et al., 2013; Munday, 2002; Munday et al., 2006; Rodgers et al., 2007). Several theoretical models have been developed to predict the adaptive significance and evolution of sex change (reviewed in Munday et al., 2006; Erisman et al., 2013; Vega-Frutis et al., 2014). These theoretical models evaluate the costs and benefits underlying an individual's 'decision' to change sex. According to these models, the optimal time for an individual to transition should be when the cost/benefit ratio of the current sex exceeds the cost/benefit ratio of being the opposite sex (Erisman et al., 2013; Munday et al., 2006; Vega-Frutis et al., 2014; Yong and Grober, 2013). To assess the potential costs and benefits of being a particular sex and the timing of the transition between sexes, it is necessary to quantify phenotypic differences between the sexes (Munday et al., 2006). In this study, we sought to quantify phenotypic differences between the sexes in an unusual sex-changing vertebrate system, the mangrove rivulus fish (*Kryptolebias marmoratus* Poey 1880).

The mangrove rivulus is a simultaneous hermaphrodite, possessing both ovarian and testicular tissue in a single ovotestis (Harrington, 1974), and is one of two known self-fertilizing hermaphroditic vertebrates, the other being its sister species *K. hermaphroditus* (Costa et al., 2010; Harrington and Kallman, 1968; Weeks et al., 2006). Mangrove rivulus are androdiecious, with populations consisting only of males and hermaphrodites (Earley et al., 2012; Harrington, 1974; Tatarenkov et al., 2012; Weeks et al.,

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2006). Males are rare in natural populations (<3%), with the exception of some Belize populations in which males reach frequencies above 20% (Turner et al., 2006). Males are categorized either as primary, where embryos develop as males, or secondary, where an adult hermaphrodite transitions to male (Cole and Noakes, 1997; Harrington, 1974). Temperature is one known trigger of primary and secondary male development, although the precise mechanisms remain unknown (Harrington, 1974; Turner et al., 2006). The lifetime fitness costs and benefits of being male or hermaphrodite, or of transitioning between the sexes, have yet to be explicitly examined. Outcrossing can occur between males and hermaphrodites (Lubinski et al., 1995), but never between hermaphrodites (Furness et al., 2015). Outcrossing can be quite prevalent and can drive substantial levels of heterozygosity in some populations (Tatarenkov et al., 2007, 2009), but the most common mode of reproduction for mangrove rivulus is self-fertilization or 'selfing'. Repeated bouts of selfing can result in completely homozygous individuals capable of producing offspring that are genetically identical to the parent and all siblings (Earley et al., 2012; Mackiewicz et al., 2006a,b; Tatarenkov et al., 2012; Avise and Tatarenkov, 2015). Because sex change is environmentally driven and because rivulus produces isogenic lineages via selfing, hermaphrodites and secondary males with identical genotypes can be produced. This allows us to examine sex differences in phenotypic traits while controlling for inter-individual genetic variation (Crews, 1998).

In this study, we examined a suite of physiological (metabolic rates and steroid hormone concentrations), behavioral (aggression and risk taking) and morphological (mass, standard length, gonadosomatic index, fat index and liver index) traits in hermaphroditic and male mangrove rivulus. We hypothesized that there would be significant sex differences in steroid hormone profiles, metabolic rates, behavior and morphology. In a prior study, Minamimoto et al. (2006) determined that hermaphrodites and males differ in their basal steroid hormone concentrations. Males exhibited higher 11-ketotestosterone (KT), a potent fish-specific androgen, and estradiol (E2) concentrations compared with hermaphrodites, while testosterone did not vary between the sexes. In another sex-changing fish, the bluebanded goby (*Lythrypnus dalli*), males and females did not differ in testosterone or cortisol concentrations (Lorenzi et al., 2009). Based on Minamimoto et al.'s (2006) previous findings, we predicted that males would have higher KT and E2 concentrations than hermaphrodites, while testosterone and cortisol would not vary between the sexes. Comparisons of the energetic demands for maintaining ovarian and testicular tissues often reveal ovarian tissue as more costly than testicular tissue (Hayward and Gillooly, 2011; Jonsson and Jonsson, 1997; Yong and Grober, 2013; but see Olsson et al., 1997). In rivulus, the hermaphrodite ovotestis is dominated by ovarian tissue and has small pockets of testicular tissue. The ovotestis is also about twice as large as the male testis. Thus, hermaphrodites have the energetically expensive task of maintaining larger, more complex gonads (both ovarian and testicular tissues) relative to males, which maintain small, less complex gonads (testicular tissue only; Cole and Noakes, 1997; Sakakura et al., 2006). We thus predicted that maintaining both ovarian and testicular tissues should require hermaphrodites to have elevated metabolic rates compared with males. Individuals with higher metabolic rates are hypothesized to be more aggressive and risk prone relative to individuals with lower metabolic rates (pace-of-life syndrome; Biro and Stamps, 2010; Burton et al., 2011; Careau and Garland, 2013). Accordingly, we predicted that if

hermaphrodites exhibit higher metabolic rates than males, they also should be more aggressive and risk prone than males.

MATERIALS AND METHODS
Animal care and general procedures
All focal individuals were derived from field-caught progenitors determined to be genetically distinct and homozygous at 32 highly polymorphic microsatellite loci (Mackiewicz et al., 2006a,b; Tatarenkov et al., 2012). From hatching, all focal individuals were housed separately in ventilated, 1 l Rubbermaid® containers, filled with 650 ml of 25 ppt salt water (aged tapwater and Instant Ocean® Sea Salt), maintained at 26±1.0°C, and kept under a 12 h light:12 h dark photoperiod (lights on at 07:00 h and off at 19:00 h). Individuals were fed 2 ml live brine shrimp (*Artemia* sp.) nauplii at 17:00 h each day. Forty hermaphrodite–male pairs (40 hermaphrodites and 40 males; N=80) matched for genotype, age (within 1 month of age; 635±25 days, mean±s.e.m.) and generation (number of generations removed from the field, e.g. F2 male paired with F2 hermaphrodite) were generated for this experiment. *Post hoc* analyses showed that the male and hermaphrodite of a pair did not differ significantly in age (paired *t*-test: t_{39}=−0.16, P=0.87). Individuals within a pair were isolated prior to and throughout the experiment, i.e. animals were not physically paired but rather were 'paired' for the purposes of statistical analysis. Because the focus of this study was to examine sex differences in a host of phenotypic traits and to identify potential mechanisms associated with adult sex change, only secondary males (born hermaphrodite but transitioned into male) were used. Hermaphrodites exhibit a mottled grayish color and a pronounced black spot (ocellus) on the dorsal region of their caudal peduncle. Secondary males exhibit mottled orange body coloration, orange fins, a black margin on their caudal and anal fins, and a faded ocellus; primary males exhibit uniform and more brilliant orange coloration with no evidence of having had an ocellus. Males used in this study naturally transitioned from hermaphrodite to male under common garden conditions; transitions were not forced by, for example, changes in temperature. The University of Alabama Institutional Animal Care and Use Committee approved all procedures (IACUC no. 08-309-1, 08-312-01, 11-362-01).

Hormone collection and assay
Steroid hormone collection was conducted using a non-invasive, water-borne hormone collection technique, which has been validated for mangrove rivulus (Earley et al., 2013). This method is preferred over caudal venipuncture and whole-body assays, which would be terminal for species of this size (mass ranges from 0.25 to 1 g). Individuals were transferred to beakers pre-cleaned with ethanol and distilled water and filled with 500 ml of 25 ppt salt water for 1 h, during which time steroid hormones passively diffused across the gills and into the surrounding water (Scott et al., 2008). Collections were run between 09:00 h and 10:00 h to avoid spikes in hormone concentrations due to circadian rhythm, anticipation of feeding, and oviposition (Harrington, 1963). Individuals were returned to their housing containers following hormone collection and remained there until they were acclimated to the behavior-testing arenas (see below).

Water samples were filtered using Whatman filter paper (Grade 1) to remove debris and feces, and then pulled through Waters Sep-Pak C-18 extraction columns (500 mg, 3 cc; primed with 2×2 ml methanol and 2×2 ml distilled water) using a vacuum manifold. Excess salts were removed from the columns with 2×2 ml of distilled water. Columns were stored at −20°C until assays were

performed. Prior to assays, columns were thawed and then excess salts were purged with 2 ml of distilled water. The free fraction of steroid hormones was eluted with 2×2 ml ethyl acetate into 13×100 mm borosilicate vials. Eluted samples were placed in a 37°C water bath and solvent was evaporated with a gentle stream of ultrapure nitrogen gas using an evaporating manifold. Samples were resuspended in 30 µl of 100% ethanol and vortexed for 1 min. Enzyme-immunoassay (EIA) buffer (570 µl; Cayman Chemicals Inc.) was added and the sample was vortexed for 5 min. A serial dilution assay using pooled samples (combination of 45 µl per sample from all resuspended samples) was performed to determine the appropriate dilutions needed to properly assay each hormone. Serial dilutions were performed for each sex separately. For males, E2 and cortisol were assayed at a 1:1 dilution while testosterone and KT were assayed at a 1:4 dilution (100 µl resuspended sample in 300 µl EIA buffer). For hermaphrodites, E2, cortisol and KT were assayed at a 1:1 dilution while testosterone was assayed at a 1:4 dilution (50 µl resuspended sample in 150 µl EIA buffer). Instructions provided by Cayman Chemicals, Inc., were followed strictly. Prepared samples were run on three 96-well EIA plates per hormone with control samples at the beginning and end of each assay plate; controls were the same pooled samples as used for the serial dilution. Intra- and inter-assay coefficients of variation were calculated using the absorbance values of controls on each plate and among plates, respectively. Intra-assay coefficients of variation were (plates 1–3) 0.5%, 0.4% and 1.1% for KT; 2.0%, 5.1% and 1.3%, for testosterone; 2.0%, 0.3% and 1.4% for cortisol; 1.5%, 1.3% and 3.4% for E2. Inter-assay coefficients of variation for E2, cortisol, KT and testosterone were 5.1%, 1.5%, 5.7% and 1.0%, respectively. Parallelism between the standard and serial dilution curves was achieved for all hormones in males (slope comparisons; Zar, 1996, p. 355; E2: $t_9=0.110$, $P=0.92$; cortisol: $t_{11}=0.019$, $P=0.99$; KT: $t_{11}=0.100$, $P=0.92$; testosterone: $t_{11}=0.200$, $P=0.85$) and hermaphrodites (E2: $t_9=0.294$, $P=0.77$; cortisol: $t_{11}=0.252$, $P=0.81$; KT: $t_{11}=1.08$, $P=0.3$; testosterone: $t_{11}=0.052$, $P=0.96$).

Behavioral assays
Individuals were assayed for aggressive and risk-taking (boldness and exploration) behaviors 24 h following hormone collections. Hermaphrodites and males were systematically assigned to experience the aggression or the risk-taking assay first ($N=20$ pairs per sequence); individuals within a hermaphrodite–male pair were assigned the same assay sequence. All behaviors (see below) were quantified from digital recordings using the JWatcher behavioral quantification program (http://www.jwatcher.ucla.edu/). Individuals were returned to their housing containers between behavioral assays.

Aggression was assayed by exposing focal individuals to 3D printed resin rivulus models created using Google Sketchup 8 and printed with Catalyst EX software on a Dimension sst 1200es 3D printer with ABS ivory polymer. Model size was kept constant (2.1 cm standard length) throughout the study and differences in length between focal individuals and the model (0.82±0.39 cm, mean±s.e.m.) were accounted for during statistical analyses. Models were hand painted with acrylic paint to match hermaphroditic rivulus' color patterns (mottled gray). We elected to use the basic color pattern of hermaphrodites for the model as a means of experimental standardization. Whether individual mangrove rivulus respond to opponent color pattern (e.g. determining sex of opponent) and modulate their behavior/physiology accordingly has yet to be elucidated. Models, rather than live opponents, were used to standardize the aggression-eliciting stimulus and because

the animals' response to models predicts actual contest behavior better than other methods (e.g. mirror aggression test; Earley et al., 2000). Focal individuals were acclimated overnight (16 h) in fight tanks. Fight tanks were 31×20×15 cm Plexiglas containers lined on all sides by black corrugated plastic and divided in the center by a removable opaque partition. At 10:00 h the following day, the partition separating the focal individual and the model was removed and the interaction digitally recorded for 30 min. The following behaviors were quantified: (1) latency to resume normal swimming behavior (recover) following disturbance (partition being lifted); (2) latency to approach the model (individual oriented towards and came within one body length of model); (3) latency to threat display (individual orients towards model and flares gill covers); and (4) total number of approaches, threat displays and bites delivered to the model.

Risk taking was assayed using an emergence from shelter/open-field exploration paradigm (e.g. Archard et al., 2012). Individuals were acclimated for 2 h beginning at 10:00 h in an enclosure constructed from opaque black corrugated plastic. The enclosure was a box, measuring 9 cm³, located at the furthest length of the open field, with a removable door facing the open field. An opaque partition prevented the individual from swimming around the enclosure. The open field measured 24 cm long×29 cm wide. The arena was filled with 4 l of 25 ppt salt water. At 12:00 h, the door to the enclosure was removed, which allowed the individual to voluntarily move between the enclosure (i.e. shelter) and an unfamiliar, open-field environment for 30 min. The following behaviors were quantified: (1) latency to first emergence from the shelter; (2) time spent out of the shelter; (3) time spent at the edge of the arena (half of body length within 4 cm of arena wall); (4) time spent in the open-field (half of body length beyond 4 cm of arena wall); (5) number of jolts (rapid change in direction followed by burst swimming); and (6) number of re-entries into the shelter. Overhead lighting conditions created shadows within the arena. The first half of the test arena, closest to the shelter entrance, was shaded while the second half of the arena, furthest from the shelter entrance, was lit. This was an unintended by-product of the arena design but time spent in the lit and shaded areas of the arena was quantified. Usage of shaded areas was considered a risk-averse behavior (shade may conceal individuals from potential dangers) while usage of the lit areas was considered a risk-prone/bold behavior. These categorizations were also consistent with distance from the shelter.

Metabolism assay
Respirometry assays were developed based on protocols detailed in Rosewarne et al. (2016) with modifications. A 0.170 l Bläzka-type swimming respirometer (Model SW10000; Loligo Systems, Tjele, Denmark) was used to measure oxygen consumption rate ($\dot{M}_{O_2}$; mg O_2 kg^{-1} h^{-1}). Water was supplied by submerging the respirometer in an ambient tank (0.9×0.35×0.39 m) filled with 25 ppt salt water. Water temperature was maintained at 26.0±0.1°C using an Artica Titanium 1/15 hp Aquarium Chiller (model no. CD-70391) and a Marineland 50 W submersible heater (model no. CD-71375). An air stone was placed in the ambient tank to maintain oxygen levels at >95% air saturation. Water in the ambient tank was cycled through a separate mechanical filter (Pick-up 2006; Eheim, Deizisau, Germany) and a UV sterilizer (UV-10000; Tetra Pond, Melle, Germany) to maintain water quality and minimize microbial respiration. Oxygen partial pressure in the respirometer was measured using a fiber optic sensor (PreSens, Regensburg, Germany). AutoResp software (Loligo Systems) was used to

control the measurement cycles and calculate $\dot{M}_{O_2}$ based on standard equations (Rosewarne et al., 2016).

All individuals were assayed for maximum metabolic rate (MMR) and a subset (N=18; 9 randomly selected hermaphrodite–male pairs) were assayed for standard metabolic rate (SMR) in addition to MMR. A substantial time requirement for measuring SMR (16 h) and the fact that we had a single respirometer constrained our ability to measure SMR in all focal individuals. Five focal individuals were assayed within a 24 h period. The first four individuals were assayed for MMR only while the final individual of the day was assayed for both MMR and SMR. The day preceding the metabolism assay, all focal individuals were weighed (g) then fasted for 24 h from the end of the behavioral assays to the start of metabolic assays.

An initial background reading without the fish present was taken to account for microbial respiration. Following background reading, the first focal individual, still in its home container, was chased with the blunt end of a fish net handle for 5 min, similar to previous studies (Svendsen et al., 2012, 2014). Immediately afterwards, the focal individual was transferred into the respirometry chamber and the AutoResp software was set to run a single 5 s flush, 120 s wait and 180 s measurement cycle. This cycle captured an individual's peak metabolic rate (i.e. MMR) with a minimum delay from chasing to respirometry. Previous studies have shown that the first measure of metabolic rate after chasing is the highest (Rosewarne et al., 2016) and therefore the best estimate of MMR. Once MMR had been recorded, the focal individual was processed for tissue samples (see 'Tissue processing', below). Three additional focal individuals were then run through the chase and MMR assay protocols. Following the fourth focal individual of the day, a second background reading of microbial respiration was taken and the last individual of the day was submitted to the chase protocol and MMR measurement. However, this individual was not processed immediately following MMR measurement. Instead, it was left in the chamber overnight with the AutoResp software set to continuously run 240 s flush, 120 s wait and 300 s measurement cycles. The focal individual remained in the chamber for 16 h before being removed and processed. To correct for background microbial respiration, a linear regression was used to interpolate the increase in microbial respiration that occurred during the respirometry trials. Using the interpolation, the estimated microbial respiration was subtracted from each measure of metabolic rate (Norin and Malte, 2011). SMR was estimated using metabolic rate data collected over 16 h. Similar to previous studies (Svendsen et al., 2014; Rosewarne et al., 2016), the median of the lowest 10 measurements of metabolic rate with an R^2 value ≥0.92 (calculated by AutoResp software) was used as an estimate of SMR for each individual fish. Aerobic scope (AS), the difference between MMR and SMR (Sandblom et al., 2014), also was calculated for individuals with both MMR and SMR measurements.

Tissue processing

Focal individuals were killed using a lethal dose of sodium bicarbonate-buffered Finquel (tricane methanesulfonate, MS-222). The focal individual was then splayed length-wise and photographed along with a ruler scale. Images were uploaded to ImageJ software (http://rsbweb.nih.gov/ij/) and both standard length (snout to caudal peduncle, to the nearest 0.1 cm) and total length (snout to tip of caudal fin, to the nearest 0.1 cm) were determined. Liver, visceral fat and gonads then were excised from the focal individual and stored in 0.5 ml centrifuge tubes. Liver and visceral

fat tissues were weighed (to nearest 0.001 g) before being flash frozen. Gonads were weighed and then transferred to Bouin's fixative for 30 min before being stored in 70% ethanol.

Statistical analysis

All statistical analyses were run using JMP (v. 7.0.1; SAS Institute Inc., Cary, NC, USA). Sex differences in steroid hormone concentrations, behavior, metabolic rates (SMR, MMR and AS) and organ masses corrected for body size (gonadosomatic index, hepatosomatic index and fat somatic index) were evaluated with ANOVA. When analyzing hormone concentrations, total fish mass (g) was included as a covariate to account for variation in hormone levels due to variation in individual mass; larger individuals may release more hormones because of their greater absolute gill surface areas relative to smaller individuals (Scott et al., 2008). A principal components analysis (PCA) was performed to distill all aggression and risk-taking behaviors to four principal components. Analyses on all behaviors included sequence (e.g. aggression then risk taking) as a main effect to account for order effects. Analyses on aggressive behaviors included size asymmetry as a covariate to account for size differences between the 3D model and the focal individuals. Somatic mass was calculated as total body mass minus gonad mass. To account for variation in organ mass due to variation in total body mass, gonad, liver and fat indices were generated by dividing the absolute mass of these organs by total body mass and multiplying by 100. We performed two separate analyses on fat index: (1) excluding individuals without fat bodies (N=47; 32 males and 15 hermaphrodites) and (2) for all individuals, including those without fat bodies (N=80; 40 males and 40 hermaphrodites). For the second analysis, we utilized a Wilcoxon rank sum test because of the inability to normalize the data. Steroid hormone concentrations (E2, cortisol, KT, testosterone), fat index and MMR were normalized using natural log transformations; all other parameters remained untransformed. Tukey's honest significant difference (HSD) multiple comparison test, which corrects for compounding type I error, was performed on all significant effects within the models. Separate correlation analyses, which included all physiological, morphological and behavioral variables measured for males and hermaphrodites, were performed. Further, a separate Spearman's ρ correlation analysis was performed for fat index when all individuals were included. Sequential Dunn–Sidak adjustments were applied to all P-values to account for compounding type I error that might result from conducting multiple correlations on non-independent data.

RESULTS
Hormones

Water concentrations of some hormones varied significantly between the sexes (Fig. 1). Males had significantly higher E2 ($F_{1,79}$=25.66, P<0.0001) and KT ($F_{1,79}$=31.04, P<0.0001) water concentrations than hermaphrodites (Fig. 1). Water concentrations of cortisol and testosterone did not vary significantly between the sexes (cortisol: $F_{1,79}$=1.70, P=0.20; testosterone: $F_{1,79}$=1.56, P=0.22; Fig. 1).

Behavior

'Explorative' behaviors loaded heavily on principal component 1 (PC1): short latency to emerge from the shelter and extended amounts of time spent outside the shelter, along the edge of the arena and in the lit area (Table 1). 'Low aggressive motivation' behaviors loaded heavily on principal component 2 (PC2): long latencies to recover from disturbance, approach the model and

display towards the model, as well as a low frequency of approaches towards the model (Table 1). 'Aggressive' behaviors loaded heavily on principal component 3 (PC3): short latency to direct threat displays towards the model and higher frequency of approaches, threat displays and bites delivered to the model (Table 1). 'Risk-aversive' behaviors loaded heavily on principal component 4 (PC4): longer times spent in the shaded area and higher frequencies of jolts and shelter re-entries (Table 1). Time spent in the open field, which could be classified as a 'bold/risk-prone' behavior, also loaded heavily on PC4. However, the shaded area encompassed part of the open field and was closest to the shelter; individuals with high PC4 scores spent large portions of their time in the open field directly in front of the shelter, giving them quick access to the shelter. As such, we maintain that PC4 classifies risk-aversive behavior.

There were significant differences between hermaphrodites and males in aggressive and risk-aversive behavior (Table 2). Hermaphrodites were significantly more aggressive towards the model (PC3 scores: males=−0.27±0.08 and hermaphrodites=0.28± 0.20) and more risk averse (PC4 scores: males=−0.26±0.15 and hermaphrodites=0.26±0.15) than males.

Metabolic physiology and morphological traits

MMR was significantly greater in hermaphrodites than in males ($F_{1,79}$=8.01, P=0.0059), but neither SMR ($F_{1,17}$=0.26, P=0.13) nor AS ($F_{1,17}$=0.02, P=0.89) differed between the sexes (Fig. 2). Males were significantly heavier in somatic mass (0.39±0.09 g; $F_{1,79}$=23.22, P<0.0001) and total body mass (0.41±0.09 g; $F_{1,79}$=20.85, P<0.0001), and slightly longer (3.04±0.26 cm; $F_{1,77}$=3.29, P=0.07) than hermaphrodites (0.30±0.07 g, 0.31±

Table 1. Summary of principal component analysis on behavioral variables

Behavior	PC1	PC2	PC3	PC4
Latency to recover	−0.09	**0.80**	0.14	−0.10
Latency to approach	−0.04	**0.91**	−0.05	−0.06
Latency to threat display	−0.08	**0.62**	**−0.52**	−0.06
No. of approaches	0.01	**−0.72**	**0.35**	0.05
No. of threat displays	0.07	−0.08	**0.84**	0.01
No. of bites	0.11	−0.10	**0.88**	0.03
Time to first emergence	**−0.71**	0.17	−0.05	−0.41
Time out of shelter	**0.95**	−0.12	0.10	0.18
Time along edge	**0.93**	0.08	0.02	−0.16
Time in open field	0.18	−0.34	0.15	**0.59**
Time in lit area	**0.92**	−0.10	0.12	−0.12
Time in shaded area	0.32	0.05	−0.05	**0.63**
No. of jolts	−0.28	0.04	0.26	**0.43**
No. of re-entries	−0.16	−0.11	−0.09	**0.76**
Eigenvalue	4.00	2.67	1.69	1.40
% Variation	28.64	19.07	12.08	10.03
Cumulative % variation	28.64	47.71	59.79	69.82

The heaviest loadings on each component are shown in bold. Interpretation of each component is as follows: PC1, explorative; PC2, low aggressive motivation; PC3, aggressive; PC4, risk aversive. % Variation: percentage of variation in the observed behavioral variables explained by each principal component (PC). Cumulative % variation: sum of total percentage of variation in the observed behavioral variables explained by a given PC and its preceding PCs.

0.06 g and 2.93±0.04 cm, respectively). Hermaphrodites had a significantly larger gonadosomatic index (Fig. 3; $F_{1,79}$=21.78, P<0.0001) but a significantly smaller fat index (Fig. 3; $F_{1,46}$=10.31, P=0.002) than males. When all individuals, not just those with
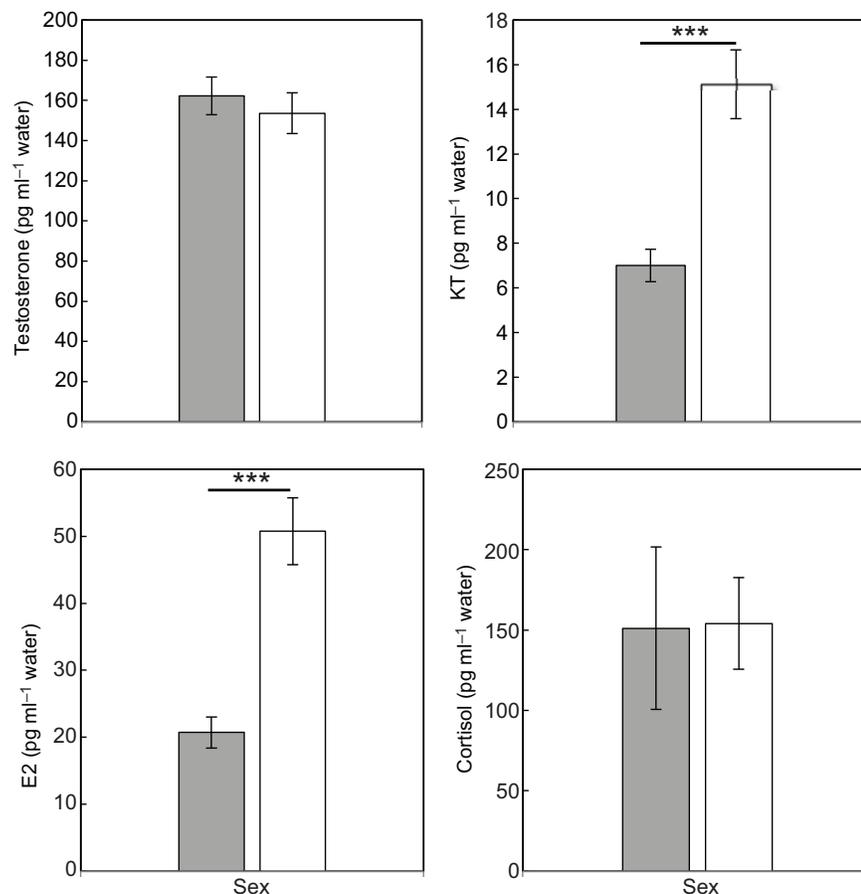

Fig. 1. Comparison of water steroid hormone concentrations between sexes. Mean (±s.e.m.) water steroid hormone concentrations for hermaphrodites (N=40; gray bars) and males (N=40; white bars). E2, estradiol; KT, 11-ketotestosterone. Asterisks indicate a significant difference (***P<0.0001).

Table 2. Analyses of sex differences in behavior

	d.f.	F-value	P-value
Risk prone (PC1)	1,79	1.82	0.15
Sex	1	1.90	0.17
Sequence	1	2.69	0.11
Sex×sequence	1	1.30	0.26
Low aggressive motivation (PC2)	1,79	0.74	0.64
Sex	1	1.74	0.20
Sequence	1	0.01	0.94
Size asymmetry	1	0.89	0.35
Sex×sequence	1	0.99	0.32
Sex×size asymmetry	1	0.47	0.49
Sequence×size asymmetry	1	0.08	0.78
Sex×sequence×size asymmetry	1	0.23	0.63
Aggressive (PC3)	1,79	1.84	0.09
Sex	**1**	**5.90**	**0.02**
Sequence	1	1.34	0.25
Size asymmetry	1	0.76	0.39
Sex×sequence	1	2.61	0.11
Sex×size asymmetry	1	0.10	0.75
Sequence×size asymmetry	1	0.01	0.99
Sex×sequence×size asymmetry	1	0.01	0.99
Risk aversive (PC4)	1,79	2.37	0.07
Sex	**1**	**6.47**	**0.01**
Sequence	1	0.08	0.77
Sex×sequence	1	1.25	0.27

Analyses with significant results ($P<0.05$) are shown in bold. Assay sequence was included in all models to account for potential order effects. Size asymmetry represents a difference in standard length between focal individuals and the 3D model and was only included in models of sex differences in aggressive behavior.

visible fat bodies present, were included, the results were the same; males still had a significantly greater fat index relative to hermaphrodites (Wilcoxon: Z=4.73, $P<0.001$; Fig. S1). A *post hoc*
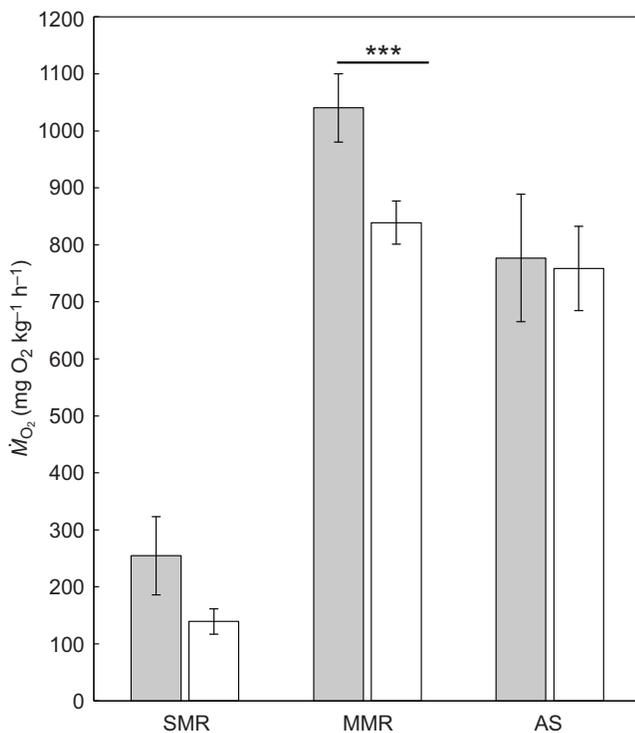
heterogeneity G-test revealed significant sex differences in the frequency of fat bodies within the body cavity (32 males versus 15 hermaphrodites possessed fat stores; G_1=6.22, P=0.013). Hepatosomatic indices did not differ between sexes (Fig. 3; $F_{1,79}$=1.52, P=0.22).

Phenotypic correlations

Correlation analyses revealed significant, positive correlations among steroid hormone concentrations. KT correlated positively with testosterone (r_{80}=0.31, $P<0.0001$) and E2 (r_{80}=0.62, $P<0.0001$). The positive correlation between testosterone and E2 (r_{80}=0.42, P=0.0005) was rendered non-significant by Dunn–Sidak corrections (Table S1). ANCOVA with sex as a main effect, hormone concentration as a covariate, and their interactions, was performed to determine sex differences in the relationships among hormones. This revealed significant sex×hormone interactions (Fig. 4; testosterone versus E2: $F_{1,79}$=6.45, P=0.013; testosterone versus KT: $F_{1,79}$=9.82, P=0.003; KT versus E2: $F_{1,79}$=7.50, P=0.008). Only males exhibited significant, positive correlations between the steroids. There was a significant, negative correlation between somatic mass and gonad index (r_{80}=−0.44, $P<0.001$). An ANCOVA with sex as a main effect and somatic mass as a covariate revealed a significant effect of the sex×somatic mass interaction on gonad index ($F_{1,79}$=13.25, $P<0.001$). Only hermaphrodites exhibited a significant, negative correlation between somatic mass and gonad index (Tables S2 and S3). MMR correlated negatively with somatic mass (r_{80}=−0.56, P=0.0001), mass (r_{80}=−0.56, P=0.0002) and standard length (r_{80}=−0.53, P=0.0001; Table S1). ANCOVA did not reveal significant sex×somatic mass ($F_{1,79}$=3.18, P=0.08), sex×total mass ($F_{1,79}$=3.63, P=0.06), or sex×standard length ($F_{1,79}$=1.39, P=0.24) interactions. These correlations were likely an artifact of correcting MMR for individual size differences. Liver and fat indices were positively correlated (r_{80}=0.57, P=0.0001; Tables S1 and S2). ANCOVA revealed a significant
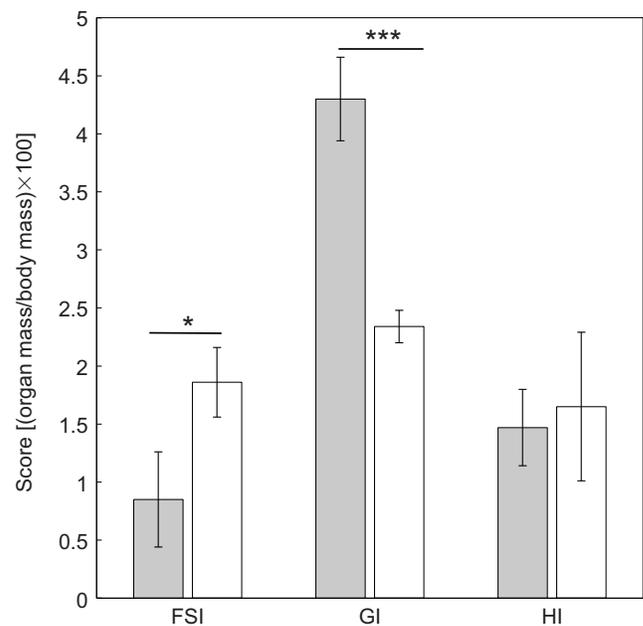

Fig. 2. Comparison of metabolic rates ($\dot{M}_{O_2}$) between sexes. Mean (±s.e.m.) standard metabolic rate (SMR), maximum metabolic rate (MMR) and aerobic scope (AS) for hermaphrodites (N=40; gray bars) and males (N=40; white bars). Asterisks indicate a significant difference (***$P<0.0001$).

Fig. 3. Comparison of somatic indices between sexes. Mean (±s.e.m.) organ index score for hermaphrodites (N=40; gray bars) and males (N=40; white bars). FSI, fat somatic index; GI, gonadosomatic index; HI, hepatosomatic index. Asterisks indicate a significant difference (*$P<0.05$, ***$P<0.0001$).

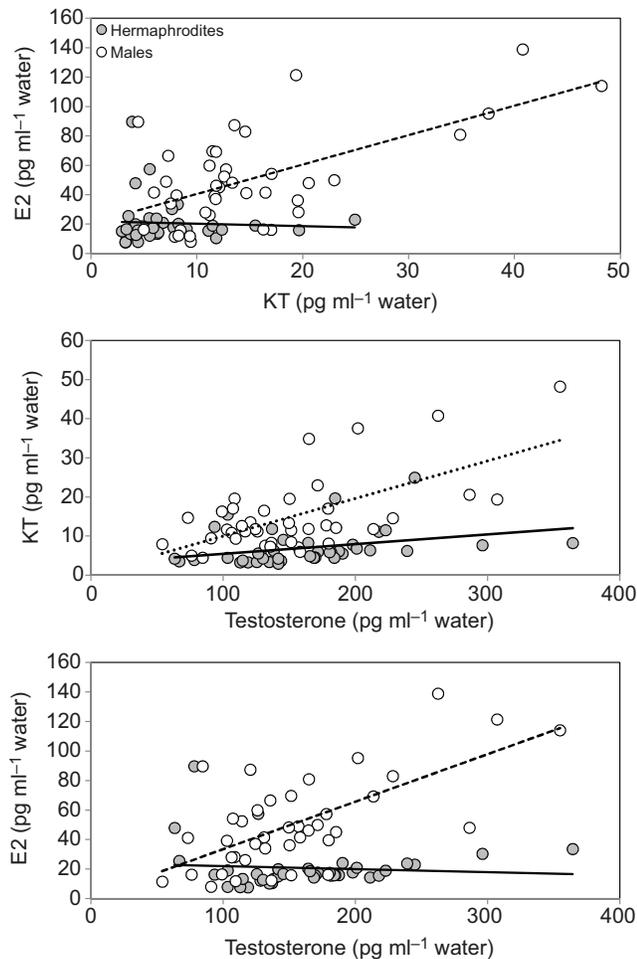**Fig. 4. Correlations of water steroid hormone concentrations between sexes.** Water steroid hormone concentrations for hermaphrodites (*N*=40; gray circles and solid lines) and males (*N*=40; white circles and dashed lines).

sex×fat index ($F_{1,47}$=6.29, *P*=0.02) interaction effect on liver index; males were the only sex to exhibit increased liver indices with increasing fat indices (Tables S1, S3, S4). Further, total mass, somatic mass and standard length correlated positively in both males and hermaphrodites (all *P*<0.0001; Tables S1–S4). All other correlations between metabolic rates, morphological traits and behaviors were non-significant (all *P*>0.05; Tables S1–S4), and none of these traits correlated significantly with hormones for hermaphrodites or males.

DISCUSSION

Sex change is theorized to have evolved as a strategy for coping with sex-specific costs of reproduction (Collin, 2006; Erisman et al., 2013; Munday, 2002; Munday et al., 2006; Rodgers et al., 2007; Vega-Frutis et al., 2014). Selection should favor transitioning between sexes when fitness payoffs of the current sex are outweighed by the fitness payoffs of the opposite sex (Collin, 2006; Erisman et al., 2013; Munday, 2002; Munday et al., 2006; Rodgers et al., 2007; Vega-Frutis et al., 2014). The power of the rivulus system in investigations of sex change is that this fish is a simultaneous hermaphrodite with the ability to self-fertilize (Earley et al., 2012; Mackiewicz et al., 2006a,b; Tatarenkov et al., 2012). This rare reproductive feature grants researchers the opportunity to generate isogenic lineages and investigate sex differences in

phenotypic traits while controlling for inter-individual genetic variation (Crews, 1998).

We used individuals that differed only in their sex (male or hermaphrodite); animals were matched for age, generations reared in the laboratory, handling and, most importantly, genotype. We found that hermaphrodites were significantly more aggressive and more risk averse, had higher maximum metabolic rates and had higher gonadosomatic indices relative to males. In contrast, males had higher levels of both E2 and KT, were heavier (total mass and somatic mass) and longer, and had greater visceral fat stores compared with hermaphrodites. Further, hormones were significantly positively correlated in males but not hermaphrodites. Our findings support our hypothesis that there would be significant differences in fitness-related traits between the sexes. Utilizing this information, we have generated a number of testable predictions regarding when and why sex change in the mangrove rivulus system should be favored by selection. Testing these predictions will require rigorously controlled manipulative studies, which we hope will emerge as a result of our findings.

It is well established that maintaining ovarian tissue is energetically expensive whereas maintaining testicular tissue is relatively inexpensive (Hayward and Gillooly, 2011; Jonsson and Jonsson, 1997; Yong and Grober, 2013; but see Olsson et al., 1997). Maintaining ovarian tissue has negative impacts on individual survival, growth, immunocompetence and future reproductive efforts (Bergeron et al., 2011; Cox and Calsbeek, 2010; Cox et al., 2010). Hermaphroditic rivulus must maintain both ovarian and testicular tissues while males only need to maintain testicular tissue (Cole and Noakes, 1997; Sakakura et al., 2006). Our findings suggest that higher maximum metabolic rates in hermaphrodites might support energetically expensive activities (e.g. aggression) while maintaining both types of gonadal tissue. Further, hermaphrodites appear to be allocating more energy towards gonad maintenance and function and less towards somatic growth – a classic life history trade-off (e.g. Arendt and Reznick, 2005; Cox et al., 2010) – as evidenced by their smaller sizes and the significant negative correlation between gonad size and somatic mass that characterized hermaphrodites but not males. Males are liberated from the costs of maintaining energetically expensive ovarian tissue, exhibit lower maximum metabolic rates, and allocate more resources towards growth and energy storage in the form of visceral fat reserves and larger liver mass. We suspect that the cost of maintaining ovarian tissue is a key factor underlying the transition between hermaphrodite and secondary male. We predict that when the benefit of possessing ovarian tissue is reduced by energetic demand (resource limitation, competition) or when age/senescence increases the costs of maintaining ovarian tissue (Kirkwood and Austad, 2000; Finch and Holmes, 2010), transitioning into male should be favored.

It has been hypothesized that individuals with high metabolic rates should have to forage more frequently, which would be supported by more risk-prone behavior, and/or fight more vigorously for resources relative to individuals with lower metabolic rates (pace-of-life syndrome; Biro and Stamps, 2010; Burton et al., 2011; Careau and Garland, 2013). Our results provide partial support for this hypothesis. Hermaphrodites had higher MMR and were more aggressive but also more risk averse than males. The observed behavioral differences between the sexes may be related to differences in both energetic demands and local abundance. In mangrove rivulus populations, males are significantly less abundant than hermaphrodites, comprising less than 25% of the population and typically a far lower percentage

(1–2%; Turner et al., 2006). Further, hermaphrodites oviposit almost daily throughout the year rather than during a single season, which should promote mate-seeking sessions in males (Harrington, 1963). Ample mating opportunities thus exist for males, provided that hermaphrodites sometimes forego selfing and opt to outcross with males, which is supported by population-level heterozygosity data (Mackiewicz et al., 2006a,b; Tatarenkov et al., 2012). Ample mating opportunities should reduce the fitness benefits for males to engage in costly aggression (Biro and Stamps, 2010; Burton et al., 2011; Careau and Garland, 2013), a strategy that should be most beneficial when resources are scarce (Arnott and Elwood, 2008; Dubois and Giraldeau, 2005). However, selection should favor males that search for mating opportunities, which would be facilitated by more risk-prone behaviors and greater propensities to leave the safety of a shelter. Given the local abundance of hermaphrodites and their ability to self-fertilize, intense competition is likely to occur over limited access to shelter and food but not mates; competition among hermaphrodites over mates has yet to be documented. Having to maintain energetically expensive gonadal tissues should further increase the value of energy-rich resources (i.e. food) for hermaphrodites. Taken together, these factors should favor heightened aggressiveness in hermaphrodites (Biro and Stamps, 2010; Burton et al., 2011; Careau and Garland, 2013). Although hermaphrodites need to navigate their environment to forage, they do not need to leave the safety of a shelter to actively seek a mate. As such, being more risk averse (i.e. remaining close to a shelter) should be favored by selection because it may reduce the likelihood of encountering predators and other environmental risks.

An individual's social environment can significantly influence the probability of transitioning between sexes in many species (reviewed in Munday et al., 2006; Vega-Frutis et al., 2014; Rodgers et al., 2007; Lorenzi et al., 2009). In protogynous systems, removal of the male from his harem induces a sex change in the dominant female (Lorenzi et al., 2009). In a repetitive sex changer, the bluebanded goby, individuals change sex based on their social status, which is determined during the formation of male-dominated social hierarchies (Rodgers et al., 2007). For mangrove rivulus, it is possible that the level of competition (low to high) might mediate a hermaphrodite's 'decision' to change sex. Based on our findings, we would predict that when local abundance of hermaphrodites and, ultimately, competition for resources exceeds a threshold level it would be favorable for a hermaphrodite to transition into a male. Mangrove rivulus could be considered a quasi-protogynous system with hermaphrodite to male transition. Interestingly, in most cases of protogynous sex change it is the socially dominant female that transitions (Lorenzi et al., 2009; Rodgers et al., 2007; Vega-Frutis et al., 2014). Here, we are predicting that the weaker/subordinate individuals – those who cannot compete for resources needed to maintain ovarian tissue – would be more likely to transition to male.

A result that we found perplexing was that MMR but not SMR or AS differed significantly between the sexes. We predicted that all measures of metabolic rate (SMR, MMR and AS) would vary significantly between the sexes because hermaphrodites face the energetically costly task of maintaining both ovarian and testicular tissues. There was a non-significant trend for hermaphrodites to have higher SMR than males, which provides qualitative support for our prediction. It is possible that with a larger sample size, this trend would have achieved significance. A *post hoc* power analysis indicated a strong effect size (Cohen's D=0.755) for sex differences in SMR but low power ($1-\beta$=0.325); a sample size of 38 per sex would provide adequate power ($1-\beta$=0.9) to achieve statistical significance. Alternatively, recent work in shorthorn sculpins

(*Myoxocephalus scorpius*) found that SMR, but not MMR, fluctuated significantly with changing environmental conditions, and that AS fluctuated as a result of environmentally induced changes in SMR (Sandblom et al., 2014). It is possible that in mangrove rivulus, SMR and ultimately AS are highly flexible in both hermaphrodites and males. This flexibility may allow both sexes to cope with changing environmental conditions (Sandblom et al., 2014) and might mask variation in SMR and AS between sexes. Sex differences in MMR could represent variation in how the sexes manage metabolic demands (e.g. maintenance of reproductive tissues) under challenging conditions, and could be explained at the proximate level by sex differences in stress responsiveness. The stress response often results in a dramatic change in metabolic activity, which allows the animal to manage situations that perturb homeostasis (Dlugosz et al., 2012; Martins et al., 2011; Øverli et al., 2005). Although we measured baseline cortisol levels, and found no correlation between baseline cortisol and metabolic rates (i.e. MMR, SMR or AS), we did not measure stress-induced (i.e. forced swimming) changes in cortisol. Sex-specific variation in the acute stress response could manifest as sex differences in MMR, which might indicate that selection has favored different physiological strategies for stress coping in hermaphrodites and males that might be linked to the costs of maintaining reproductive tissues.

Elevated levels of androgens in males relative to females (or hermaphrodites) have been documented in numerous sex-changing teleost fishes (reviewed in Godwin, 2010), although there are some exceptions (Lorenzi et al., 2008). Androgens are an essential mediator of the development and activation of male-specific behavioral expression (Apfelbeck et al., 2011; Arnold and Breedlove, 1985; Burmeister and Wilczynski, 2000; Godwin, 2010; Wingfield, 2005). It was, however, peculiar that males had elevated levels of estradiol compared with hermaphrodites, which is inconsistent with findings in most other sex-changing teleosts (Godwin, 2010). There has been a surge in research investigating the role of estrogens in the development, maintenance and functionality of testicular tissue (reviewed in Carreau and Hess, 2010; Hess, 2003; Hess and Carnes, 2004). Leydig cells within the testes are responsible for producing androgens but also have the ability to produce estrogens. The cytochrome P450 enzyme (aromatase), which is responsible for the conversion of testosterone into estrogens, has been repeatedly discovered in testicular tissue (Hess, 2003; Hess and Carnes, 2004). Furthermore, estrogen receptors (ERα, ERβ) are expressed in a number of male-specific tissues including the testes and epididymal epithelium, which indicates that male-specific tissues are responsive to estrogens (Carreau and Hess, 2010). Loss of estrogen production/reception within the testes can significantly impede spermatogenesis, causing malformation of sperm and decreased fertility (Hess, 2003; Hess and Carnes, 2004). Male mangrove rivulus possess large, fully developed gonads composed completely of testicular tissue while hermaphrodites possess only a small fraction of the testicular tissue that males possess (Cole and Noakes, 1997; Sakakura et al., 2006). We find it reasonable to hypothesize that estrogens in male mangrove rivulus are partially responsible for the functionality of the male reproductive tract. Further, steroid hormones are key regulators of the sex-change process (reviewed in Godwin, 2010). For example, elevation of KT or inhibition of E2 production can induce protogynous sex change (female to male) in blackeyed gobies (*Coryphopterus nicholsii*; Gist et al., 2007). Based on our findings, we predict that increases in E2 and KT may mediate the sex change from hermaphrodite to secondary male in mangrove rivulus. It is also likely that sex differences in E2 are age dependent.

Our study and that of Minamimoto et al. (2006) found that males had higher E2 levels than hermaphrodites when the animals were, on average, greater than 635 days old. Johnson et al. (2015), however, showed that hermaphrodites have higher E2 than males when aged 330–550 days.

In this study, we sought to identify sex differences in a number of phenotypic traits in the sex-changing mangrove rivulus. Our results indicate that hermaphrodites invest energy predominantly towards reproduction as evidenced by their larger gonadal tissue and higher maximum metabolic rate, smaller size (total mass, somatic mass and length) and smaller fat stores. Males appear to invest in a different way, favoring energy storage and growth, as evidenced by their lower metabolic rates, larger size and presence of abundant fat stores. We suspect that sex differences in behavior result from the interaction between sex-specific energetic demands and a population structure that exposes hermaphrodites and males to very different pressures related to competition, feeding and mating (pace-of-life; Biro and Stamps, 2010; Burton et al., 2011; Careau and Garland, 2013). Lastly, we have identified significant, sex-specific endocrine differences that may be responsible for mediating phenotypic expression (e.g. metabolic rate, behavior, gonad maintenance). Prior work in the mangrove rivulus system has demonstrated that temperature plays a significant role in the transition of hermaphrodites into males (Harrington, 1974; Turner et al., 2006). Currently, this is the only factor identified as a putative mechanism of sex change in mangrove rivulus. Our findings provide an excellent opportunity to explore why exposure to high temperatures during adulthood mediates the transition between sexes. Metabolic rate significantly correlates with temperature in both ectotherms and endotherms (Clarke and Pörtner, 2010; da Silva et al., 2013). For mangrove rivulus, elevated ambient temperatures likely increase metabolic rate; if an individual cannot maintain an elevated metabolic rate, physiologically or behaviorally, and support ovarian function, then transitioning from hermaphrodite to male should be favored. Future work that investigates temperature-induced changes in metabolic rate and its link to sex change may elucidate the proximate mechanism(s) underlying sex change in mangrove rivulus.

References
Apfelbeck, B., Stegherr, J. and Goymann, W. (2011). Simulating winning in the wild—The behavioral and hormonal response of black redstarts to single and repeated territorial challenges of high and low intensity. *Horm. Behav.* **60**, 565-571.

Archard, G. A., Earley, R. L., Hanninen, A. F. and Braithwaite, V. A. (2012). Correlated behaviour and stress physiology in fish exposed to different levels of predation pressure. *Funct. Ecol.* **26**, 637-645.

Arendt, J. D. and Reznick, D. N. (2005). Evolution of juvenile growth rates in female guppies (*Poecilia reticulata*): predator regime or resource level? *Proc. R. Soc. B Biol. Sci.* **272**, 333-337.

Arnold, A. P. and Breedlove, S. M. (1985). Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. *Horm. Behav.* **19**, 469-498.

Arnott, G. and Elwood, R. W. (2008). Information gathering and decision making about resource value in animal contests. *Anim. Behav.* **76**, 529-542.

Avise, J. C. and Tatarenkov, A. (2015). Populations genetics and evolution of the mangrove rivulus *Kryptolebias marmoratus*, the world's only self-fertilizing hermaphroditic vertebrate. *J. Fish Biol.* **87**, 519-538.

Bergeron, P., Careau, V., Humphries, M. M., Réale, D., Speakman, J. R. and Garant, D. (2011). The energetic and oxidative costs of reproduction in a free-ranging rodent. *Funct. Ecol.* **25**, 1063-1071.

Biro, P. A. and Stamps, J. A. (2010). Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? *Trends Ecol. Evol.* **25**, 653-659.

Brick, O. and Jakobsson, S. (2002). Individual variation in risk taking: the effect of a predatory threat on fighting behavior in *Nannacara anomala*. *Behav. Ecol.* **13**, 439-442.

Burmeister, S. and Wilczynski, W. (2000). Social signals influence hormones independently of calling behavior in the treefrog (*Hyla cinerea*). *Horm. Behav.* **38**, 201-209.

Burton, T., Killen, S. S., Armstrong, J. D. and Metcalfe, N. B. (2011). What causes intraspecific variation in resting metabolic rate and what are its ecological consequences? *Proc. R. Soc. B Biol. Sci.* **278**, 3465-3473.

Capossela, K. M., Brill, R. W., Fabrizio, M. C. and Bushnell, P. G. (2012). Metabolic and cardiorespiratory responses of summer flounder *Paralichthys dentatus* to hypoxia at two temperatures. *J. Fish Biol.* **81**, 1043-1058.

Careau, V. and Garland, T., Jr. (2013). Performance, personality, and energetics: correlation, causation, and mechanism. *Physiol. Biochem. Zool.* **85**, 543-571.

Carreau, S. and Hess, R. A. (2010). Oestrogens and spermatogenesis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **365**, 1517-1535.

Clarke, A. and Pörtner, H.-O. (2010). Temperature, metabolic power and the evolution of endothermy. *Biol. Rev. Camb. Philos. Soc.* **85**, 703-727.

Cole, K. S. and Noakes, D. L. G. (1997). Gonadal development and sexual allocation in mangrove killifish, *Rivulus marmoratus* (Pisces: Atherinomorpha). *Copeia* **1997**, 596-600.

Collin, R. (2006). Sex ratio, life-history invariants, and patterns of sex change in a family of protandrous gastropods. *Evolution* **60**, 735-745.

Costa, W. J. E. M., Lima, S. M. Q. and Bartolette, R. (2010). Androdioecy in Kryptolebias killifish and the evolution of self-fertilizing hermaphroditism. *Biol. J. Linn. Soc.* **99**, 344-349.

Cox, R. M. and Calsbeek, R. (2010). Severe costs of reproduction persist in Anolis lizards despite the evolution of a single-egg clutch. *Evolution* **64**, 1321-1330.

Cox, R. M., Parker, E. U., Cheney, D. M., Liebl, A. L., Martin, L. B. and Calsbeek, R. (2010). Experimental evidence for physiological costs underlying the trade-off between reproduction and survival. *Funct. Ecol.* **24**, 1262-1269.

Crews, D. (1998). On the organization of individual differences in sexual behavior. *Am. Zool.* **38**, 118-132.

Crossin, G. T., Phillips, R. A., Trathan, P. N., Fox, D. S., Dawson, A., Wynne-Edwards, K. E. and Williams, T. D. (2012). Migratory carryover effects and endocrinological correlates of reproductive decisions and reproductive success in female albatrosses. *Gen. Comp. Endocrinol.* **176**, 151-157.

da Silva, G. S. F., Glass, M. L. and Branco, L. G. S. (2013). Temperature and respiratory function in ectothermic vertebrates. *J. Therm. Biol.* **38**, 55-63.

Dlugosz, E. M., Harris, B. N., Saltzman, W. and Chappell, M. A. (2012). Glucocorticoids, aerobic physiology, and locomotor behavior in California mice*. *Physiol. Biochem. Zool.* **85**, 671-683.

Dubois, F. and Giraldeau, L.-A. (2005). Fighting for resources: the economics of defense and appropriation. *Ecology* **86**, 3-11.

Earley, R. L., Hsu, Y. and Wolf, L. L. (2000). The use of standard aggression testing methods to predict combat behaviour and contest outcome in *Rivulus marmoratus* dyads (Teleostei: Cyprinodontidae). *Ethology* **106**, 743-761.

Earley, R. L., Hanninen, A. F., Fuller, A., Garcia, M. J. and Lee, E. A. (2012). Phenotypic plasticity and integration in the mangrove rivulus (*Kryptolebias marmoratus*): a prospectus. *Integr. Comp. Biol.* **52**, 814-827.

Earley, R. L., Lu, C.-K., Lee, I.-H., Wong, S. C. and Hsu, Y. (2013). Winner and loser effects are modulated by hormonal states. *Front. Zool.* **10**, 6-19.

Erisman, B. E., Petersen, C. W., Hastings, P. A. and Warner, R. R. (2013). Phylogenetic perspectives on the evolution of functional hermaphroditism in teleost fishes. *Integr. Comp. Biol.* **53**, 736-754.

Finch, C. E. and Holmes, D. J. (2010). Ovarian aging in developmental and evolutionary contexts. *Ann. N. Y. Acad. Sci.* **1204**, 82-94.

Furness, A. I., Tatarenkov, A. and Avise, J. C. (2015). A genetic test for whether pairs of hermaphrodites can cross-fertilize in a selfing killifish. *J. Heredity* **106**, 749-752.

Gist, D. H., Bradshaw, S., Morrow, C. M. K., Congdon, J. D. and Hess, R. A. (2007). Estrogen response system in the reproductive tract of the male turtle: an immunocytochemical study. *Gen. Comp. Endocrinol.* **151**, 27-33.

Godwin, J. (2010). Neuroendocrinology of sexual plasticity in teleost fishes. *Front. Neuroendocrinol.* **31**, 203-216.

Harrington, R. W., Jr. (1963). Twenty-four-hour rhythms of internal self-fertilization and of oviposition by hermaphrodites of *Rivulus marmoratus*. *Phys. Zool.* **36**, 325-341.

Harrington, R. J. (1975). Sex determination and differentiation among uniparental homozygotes of the hermaphroditic fish *Rivulus marmoratus* (Cyprinodontidae: Atheriniformes). In *Intersexuality in the Animal Kingdom* (ed. R. Reinboth), pp. 249-262. Berlin: Springer-Verlag.

Harrington, R. W., Jr and Kallman, K. D. (1968). The homozygosity of clones of the self-fertilizing hermaphroditic fish *Rivulus marmoratus* Poey (Cyprinodontidae, Atheriniformes). *Am. Nat.* **102**, 337-343.

Harshman, L. G. and Zera, A. J. (2007). The cost of reproduction: the devil in the details. *TREE* **22**, 80-86.

Hayward, A. and Gillooly, J. F. (2011). The cost of sex: quantifying energetic investment in gamete production by males and females. *PLoS ONE* **6**, e16557.

Hess, R. A. (2003). Estrogen in the adult male reproductive tract: a review. *Reprod. Biol. Endocrinol.* **1**, 52.

Hess, R. and Carnes, K. (2004). The role of estrogen in testis and the male reproductive tract: a review and species comparison. *Anim. Reprod.* **1**, 5-30.

Johnson, E. L., Weinersmith, K. L. and Earley, R. L. (2015). Changes in reproductive physiology of mangrove rivulus *Kryptolebias marmoratus* following exposure to environmentally relevant doses of ethinyl oestradiol. *J. Fish Biol.* doi:10.1111/jfb.12814.

Jonsson, N. and Jonsson, B. (1997). Energy allocation in polymorphic brown trout. *Funct. Ecol.* **11**, 310-317.

Kirkwood, T. B. L. and Austad, S. N. (2000). Why do we age? *Nature* **408**, 233-238.

Lorenzi, V., Earley, R. L., Rodgers, E. W., Pepper, D. R. and Grober, M. S. (2008). Diurnal patterns and sex differences in cortisol, 11-ketotestosterone, testosterone, and 17β-estradiol in the bluebanded goby (*Lythrypnus dalli*). *Endocrinology* **155**, 438-446.

Lorenzi, V., Carpenter, R. E., Summers, C. H., Earley, R. L. and Grober, M. S. (2009). Serotonin, social status and sex change in the bluebanded goby Lythrypnus dalli. *Physiol. Behav.* **97**, 476-483.

Lubinski, B., Davis, W., Taylor, D. and Turner, B. (1995). Outcrossing in a natural population of a self-fertilizing hermaphroditic fish. *J. Hered.* **86**, 469-473.

Mackiewicz, M., Tatarenkov, A., Taylor, D. S., Turner, B. J. and Avise, J. C. (2006a). Extensive outcrossing and androdioecy in a vertebrate species that otherwise reproduces as a self-fertilizing hermaphrodite. *Proc. Nat. Acad. Sci. USA* **103**, 9924-9928.

Mackiewicz, M., Tatarenkov, A., Turner, B. J. and Avise, J. C. (2006b). A mixed-mating strategy in a hermaphroditic vertebrate. *Proc. R. Soc. B Biol. Sci.* **273**, 2449-2452.

Martins, C. I. M., Castanheira, M. F., Engrola, S., Costas, B. and Conceição, L. E. C. (2011). Individual differences in metabolism predict coping styles in fish. *Appl. Anim. Behav. Sci.* **130**, 135-143.

Minamimoto, M., Sakakura, Y., Soyano, K., Akaba, Y. and Hagiwara, A. (2006). Plasma sex steroid levels and steroidogenesis in the gonad of the self-fertilizing fish Rivulus marmoratus. *Environ. Biol. Fishes* **75**, 159-166.

Munday, P. (2002). Bi-directional sex change: testing the growth-rate advantage model. *Behav. Ecol. Sociobiol.* **52**, 247-254.

Munday, P. L., Buston, P. M. and Warner, R. R. (2006). Diversity and flexibility of sex-change strategies in animals. *Trends Ecol. Evol.* **21**, 89-95.

Neat, F. C., Taylor, A. C. and Huntingford, F. A. (1998). Proximate costs of fighting in male cichlid fish: the role of injuries and energy metabolism. *Anim. Behav.* **55**, 875-882.

Norin, T. and Malte, H. (2011). Repeatability of standard metabolic rate, active metabolic rate and aerobic scope in young brown trout during a period of moderate food availability. *J. Exp. Biol.* **214**, 1668-1675.

Olsson, M., Madsen, T. and Shine, R. (1997). Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proc. R. Soc. B Biol. Sci.* **264**, 455-459.

Øverli, Ø., Winberg, S. and Pottinger, T. G. (2005). Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout-a review. *Integr. Comp. Biol.* **45**, 463-474.

Rodgers, E. W., Earley, R. L. and Grober, M. S. (2007). Social status determines sexual phenotype in the bi-directional sex changing bluebanded goby *Lythrypnus dalli*. *J. Fish Biol.* **70**, 1660-1668.

Roff, D. A. and Fairbairn, D. J. (2007). The evolution of trade-offs: where are we? *J. Evol. Biol.* **20**, 433-447.

Rosewarne, P. J., Wilson, J. M. and Svendsen, J. C. (2016). Measuring maximum and standard metabolic rates using intermittent-flow respirometry: a student laboratory investigation of aerobic metabolic scope and environmental hypoxia in aquatic breathers. *J. Fish Biol.* **88**, 265-283.

Sakakura, Y., Soyano, K., Noakes, D. L. G. and Hagiwara, A. (2006). Gonadal morphology in the self-fertilizing mangrove killifish, *Kryptolebias marmoratus*. *Ichthyol. Res.* **53**, 427-430.

Sandblom, E., Grans, A., Axelsson, M. and Seth, H. (2014). Temperature acclimation rate of aerobic scope and feeding metabolism in fishes: implications in a thermally extreme future. *Proc. R. Soc. B Biol. Sci.* **281**, 20141490.

Scott, A. P., Hirschenhauser, K., Bender, N., Oliveira, R., Earley, R. L., Sebire, M., Ellis, T., Pavlidis, M., Hubbard, P. C., Huertas, M. et al. (2008). Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. *Behaviour* **145**, 1307-1328.

Svendsen, J. C., Steffensen, J. F., Aarestrup, K., Frisk, M., Etzerodt, A. and Jyde, M. (2012). Excess posthypoxic oxygen consumption in rainbow trout (*Oncorhynchus mykiss*): recovery in normoxia and hypoxia. *Can. J. Zool.* **90**, 1-11.

Svendsen, J. C., Genz, J., Anderson, W. G., Stol, J. A., Watkinson, D. A. and Enders, E. C. (2014). Evidence of circadian rhythm, oxygen regulation capacity, metabolic repeatability and positive correlations between forced and spontaneous maximal metabolic rates in lake sturgeon *Acipenser fulvescens*. *PLoS ONE* **9**, e94693.

Tatarenkov, A., Gao, H., Mackiewicz, M., Taylor, D. S., Turner, B. J. and Avise, J. C. (2007). Strong population structure despite evidence of recent migration in a selfing hermaphroditic vertebrate, the mangrove killifish (*Kryptolebias marmoratus*). *Mol Ecol.* **16**, 2701-2711.

Tatarenkov, A., Lima, S. M. Q., Taylor, D. S. and Avise, J. C. (2009). Long-term retention of self-fertilization in a fish clade. *Proc. Natl. Acad. Sci. USA* **106**, 14456-14459.

Tatarenkov, A., Earley, R. L., Taylor, D. S. and Avise, J. C. (2012). Microevolutionary distribution of isogenicity in a self-fertilizing fish (*Kryptolebias marmoratus*) in the Florida Keys. *Integr. Comp. Biol.* **52**, 743-752.

Taylor, R. C. and Ryan, M. J. (2013). Interactions of multisensory components perceptually rescue túngara frog mating signals. *Science* **341**, 273-274.

Turner, B., Fisher, M. and Taylor, D. (2006). Evolution of 'maleness' and outcrossing in a population of the self-fertilizing killifish, *Kryptolebias marmoratus*. *Evol. Ecol. Res.* **8**, 1475-1486.

Vega-Frutis, R., Macías-Ordóñez, R., Guevara, R. and Fromhage, L. (2014). Sex change in plants and animals: a unified perspective. *J. Evol. Biol.* **27**, 667-675.

Weeks, S. C., Benvenuto, C. and Reed, S. K. (2006). When males and hermaphrodites coexist: a review of androdioecy in animals. *Integr. Comp. Biol.* **46**, 449-464.

Wingfield, J. C. (2005). A continuing saga: the role of testosterone in aggression. *Horm. Behav.* **48**, 253-255.

Yong, L. and Grober, M. (2013). Sex differences in the energetic content of reproductive tissues in the Blackeye Goby, *Rhinogobiops nicholsii*. *Enviro. Biol. Fish.* **3**, 321-328.

Zar, J. H. (1996). *Biostatistical Analysis*. 3rd edn. London: Prentice-Hall.