Masculinizing Effect of Background Color and Cortisol in a Flatfish with Environmental Sex-Determination

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Synopsis Environmental sex-determination (ESD) is the phenomenon by which environmental factors regulate sex-determination, typically occurring during a critical period of early development. Southern flounder (Paralichthys lethostigma) exhibit temperature-dependent sex-determination that appears to be restricted to the presumed XX female genotype with the extremes of temperature, both high and low, skewing sex ratios toward males. In order to evaluate other environmental factors that may influence sex-determination, we investigated the influence of background color and cortisol on sex-determination in southern flounder. Experiments involving three sets of tanks, each painted a different color, were conducted at different temperatures using southern flounder of mixed XX–XY genotype. The studies involved rearing juvenile southern flounder in either black, gray, or blue tanks and sex-determination was assessed by gonadal histology. In both studies, blue tanks showed significant male-biased sex ratios (95 and 75% male) compared with black and gray tanks. The stress corticosteroid cortisol may mediate sex-determining processes associated with environmental variables. Cortisol from the whole body was measured throughout the second experiment and fishes in blue tanks had higher levels of cortisol during the period of sex-determination. These data suggest that background color can be a cue for ESD, with blue acting as a stressor during the period of sex-determination, and ultimately producing male-skewed populations. In a separate study using XX populations of southern flounder, cortisol was applied at 0, 100, or 300 mg/kg of gelatin-coated feed. Fish were fed intermittently prior to, and just through, the period of sex-determination. Levels of gonadal P450 aromatase (cyp19a1) and forkhead transcription factor L2 (FoxL2) messenger RNA (mRNA) were measured by qRT–PCR as markers for differentiation into females. Müllerian-inhibiting substance mRNA was used as a marker of males’ gonadal development. Control fish showed female-biased sex ratios approaching 100%, whereas treatment with 100 mg/kg cortisol produced 28.57% females and treatment with 300 mg/kg cortisol produced only 13.33% females. These results suggest that cortisol is a critical mediator of sex-determination in southern flounder by promoting masculinization. This linkage between the endocrine stress axis and conserved sex-determination pathways may provide a mechanism for adaptive modification of sex ratio in a spatially and temporally variable environment.

Introduction

Gonadal sex is the central component of an organism’s mating strategy, but is not always thought of as an adaptively plastic component of reproduction. Even in the familiar and well-studied mammals and birds, adaptive adjustment of the sex ratio is prevalent. When key aspects of the environment an offspring will inhabit cannot be predicted by cues available to parents, plasticity in sex-determination in those offspring can be selectively advantageous (Charnov and Bull 1977). This appears to be the case for many teleost fishes, particularly in the marine environment where the habitats ultimately occupied by juveniles and adults often are determined after long-range dispersal by a planktonic larval stage. Gonadal development may also be considerably delayed following settlement of larvae into their newly found habitat. If there is variation across
habitats in terms of relative reproductive success expected for males and females developing in those habitats and cues from the physical or social environments predict these differences, then the delay between larval settlement and gonadal differentiation may be viewed as a "window of opportunity" for selectively advantageous sex-determination. These are the general conditions postulated by Charnov and Bull to select for environmental sex-determination (ESD).

Teleost fishes display a wide range of sexual patterns including ESD, genotypic sex-determination, and gradations in between these patterns in response to variation both in the physical and social environments (Ospina-Alvarez and Piferrer 2008). We have been focusing on ESD in the southern flounder (Paralichthys lethostigma), an ecologically and economically important species in the southeastern USA. Like their Japanese congener Paralichthys olivaceus and a number of other flatfish species, southern flounder exhibit temperature-dependent sex-determination (Luckenbach et al. 2009). In Japanese and southern flounder, this is restricted to the presumed XX female genotype with the extremes of temperature favoring development of males (Yamamoto 1999; Luckenbach et al. 2003). For southern flounder, high (28°C) and low (18°C) temperatures produce a preponderance of males, while a mid-range temperature (23°C) favors 1:1 female: male sex ratio (Luckenbach et al. 2003). The precise mechanisms that transduce thermal cues into determination of sex are not known for any species, but accumulating evidence suggests that the endocrine stress-axis, i.e., glucocorticoids, play a critical role in this process (Hattori et al. 2009; Yamaguchi et al. 2010; Fernandino et al. 2012). If so, other environmental factors such as background color that activate the endocrine stress-axis might also affect sex-determination and hence sex ratios in populations.

Flounders are benthic predators that rely on camouflage against the bottom for ambushing prey. Considering their cryptic nature, these fishes may be particularly sensitive to background coloration, which has been previously shown to regulate the glucocorticoid stress-axis in fishes (Papoutsoglou et al. 2000; Rotllant et al. 2003; Merighe et al. 2004; Barcellos et al. 2009). We postulate that background color may represent an additional environmental cue that influences sex-determination in fishes, possibly mediated by glucocorticoids. For improving the culture of flounder, it is important to identify those background (tank) colors that might regulate sex and that are often used in commercial operations to maximize the production of faster-growing females. This article examines: (1) the influence of background color both on sex-determination and on whole-body levels of cortisol and (2) the effect of exogenous cortisol on molecular markers of sex-determination during the developmental window when sex in southern flounder is sensitive to temperature.

**Methods and materials**

**Effects of background color on sex-determination and whole-body cortisol**

Animals

In two different years, fish for studies of the effect of background color were spawned at the University of North Carolina at Wilmington, Center for Marine Science (UNCW-CMS) in Wilmington, NC, and then reared at North Carolina State University (NCSU) in Raleigh, NC. Wild-caught southern flounder were maintained at UNCW-CMS and eggs and sperm were collected by strip-spawning from multiple broodstock and pooled for *in vitro* fertilization (Daniels and Watanabe 2002). Fertilized eggs were transported to the NCSU Lake Wheeler laboratory and larvae were reared in a 3000-L recirculating artificial seawater system until 60 days post hatch (dph) (Daniels and Watanabe 2002). Larvae were fed a diet of live feed (rotifers and *Artemia*) and gradually weaned onto a diet of high-protein dry feed (Reed Mariculture, Campbell, CA, USA) that was continued through metamorphosis. All procedures described below were approved by Institutional Animal Care and Use Committees at the University of North Carolina at Wilmington and North Carolina State University.

**Experimental design**

At 60 dph (~15 mm TL), metamorphosed juveniles were transferred into nine, 400-L round fiberglass tanks and stocked at a density of 3900 fish/m². Fish were randomly assigned in triplicate either to gray, black, or blue tanks. Salinity was gradually decreased from 22 ppt to 0.7 ppt. Water-hardness was maintained at 150 mg/l and alkalinity at 200 mg/l. Photoperiod was maintained on a 12L:12D schedule. These environmental parameters are those previously shown to maximize production of females (Luckenbach et al. 2003). For the initial experiment with different colors of tanks, the temperature was maintained at 22.9°C for the entire study to target a 1:1 sex ratio. In the subsequent experiment, animals were stocked at 60 dph (14 mm TL) and temperature was maintained at 18.8°C for the next 50 days (60–110 dph, 14–75 mm TL), then increased to
23.0°C for the remainder of the study. The period during which the fish were exposed to lower initial temperatures coincides with the presumed period when sex-determination occurs [prior to onset of histologically discernible sex-differentiation as defined by Luckenbach et al. (2003) beginning at 75 mm TL]. This should have caused a moderately male-skewed population. Thus, any effects of background color beyond this should provide for a more robust determination of the effects of color on masculinization. Fish were fed a 50% protein diet (1.0–4.0 mm) (Zeigler, Gardners, PA) to satiation four to five times daily. Fish were fed a 50% protein diet (1.0–4.0 mm) (Zeigler, Gardners, PA) to satiation four to five times daily. Fish (n = 30 fish per treatment) were sampled at 0, 34, 65, and 93 days post stocking (dps) with average body sizes of 14, 59, 86, and 104 mm TL, respectively for analysis of whole-body cortisol. For time zero (baseline) whole-body cortisol levels, animals were sampled from stock tanks just prior to transfer to the different background colors on that day. Fish were euthanized by a lethal dose (300 mg/L) of Tricaine (MS-222, Sigma-Aldrich, St. Louis, MO, USA) buffered with sodium bicarbonate, immersed in liquid nitrogen and stored at −80°C for analysis of whole-body cortisol.

Growout

At 93 dps, fish randomly were culled, counted, and restocked at a lower density of 875 fish/m² to allow for continued optimal growth. Luckenbach et al. (2003) demonstrated that sex-determination is likely to occur prior to 75 mm TL in P. lethostigma and that sex can be determined by microscopic observation of gonads at 120 mm TL. This is consistent with the results of Montalvo et al. (2012) in southern flounder that indicated sex-determination occurs before fish reach 65 mm TL. Therefore, altering stocking densities after the fish exceed 75 mm TL should not affect sex-determination. At 121 dps, 20 fish were removed from each tank (60 per treatment) and tagged by injection with an elastopolymer dye (Northwest Marine Technology, Shaw Island, WA, USA) to identify their original treatment, then transferred to a single black tank (4.3-m diameter) on a recirculating system for the remainder of the experiment. At 163 dps (144 mm TL), fish were euthanized and gonads were removed and preserved in Bouin’s fixative for histological analysis and classified as described by Luckenbach et al. (2003).

Analysis of whole-body cortisol

Analysis of whole-body cortisol was conducted on a subsample of flounder from the second experiment on background color (18.8–23.0°C) at each sampling point during the study (0, 34, 65, and 93 dps). Analysis of whole-body cortisol was adopted from a modified procedure of King and Berlinsky (2006). Heads and guts were removed from frozen juvenile flounder. The bodies were weighed, chopped, and homogenized in a chilled phosphate-buffered solution (PBS) at four times volume to body weight in a benchtop blender (Waring blender base LB10, Waring attachment MC1; Waring Laboratory, Torrington, CT, USA). The homogenate was further diluted 1:1 v:v in PBS and centrifuged at 3000×g for 10 min at 4°C. The supernatant was removed and stored at −80°C until assay. Volumes of 100 μL and 50 μL of supernatant from each sample were incubated with recovery tracer equivalent to 5% of the total counts per minute (CPM), double-extracted with ether and dried under nitrogen gas. The steroid extracts were reconstituted with 300 μL of PBS. One hundred μL of cortisol antibody diluted 1:40,000 (Stock # R4866, University of California at Davis, School of Veterinary Medicine, Clinical Endocrinology Laboratory) and 5000 CPM of 1,2,6,7-3H-cortisol (250 μCi, Amersham Biosciences) were added to each aliquot of 100 μL and 50 μL of reconstituted extract, vortexed, and incubated 2 h at room temperature and then overnight at 4°C. Tubes were placed in an ice-bath and incubated for 15 min with 500 μL of chilled charcoal/dextran solution (1% w/v in 0.1 M PBS; Sigma-Aldrich, St. Louis, MO, USA). Tubes were centrifuged at 3000×g for 15 min at 4°C. The supernatant was poured off into vials containing 4 mL scintillation fluid (BP458-4, Fisher BioReagents, Pittsburgh, PA, USA) with 10% methanol and counted for 5 min in a scintillation counter (Beckmann-Coulter LS6500). Samples were adjusted for recoveries by calculating the value of the extracted recovery tracer for each sample. Sensitivity of the assay was 7.8 pg/tube with the 80% intercept = 15.6 pg/tube, 50% intercept = 93.8 pg/tube, and 20% intercept = 250 pg/tube. The interassay coefficient of variation was 5.6%. Standards ranged from 1.95 to 8000 pg cortisol/tube. Spikes (100 50, and 25 pg/tube) were measured as positive controls with known amounts of cortisol added to stripped, whole-body samples.

Assessment of background color

The CIE (Commission Internationale d’Eclairage; International Commission on Illumination) is the authority on matters related to light and lighting, color, and vision, as well as various aspects of the measurement of color. The CIELAB color space, officially termed the CIE 1976 L* a* b* Color Space,
has three mutually perpendicular opponent-color axes, \(L^*, a^*,\) and \(b^*\) representing lightness, [redness \((+a^*)/greenness \((-a^*)]\) and [yellowness \((+b^*)]/blueness \((-b^*)\)], respectively. The lightness attribute, \(L^*\), varies from zero for perfect black to 100 for perfect white. Black, white, and gray colors are commonly regarded as achromatic colors as they are devoid of hue; their spectral reflectance curves are approximately flat across the visible spectrum, and therefore mainly vary in lightness \((L^*)\). To measure the background color of tanks used in this study, a 552 X-rite portable spectrophotometer (X-rite Inc., Grand Rapids, MI, USA) was employed. A large aperture was used to ensure the surfaces measured were representative of the entire tank. The instrument employed a D65 (1500 lux) daylight simulator lamp, and UV light was excluded during the measurements. Diffuse illumination geometry was used and, due to the glossy nature of the surfaces’ colors, the specular component was excluded to minimize error. Based on the CIELAB color space analysis, the black tank showed an \(L^*\) of 23.55, \(a^*\) of \(-1.02\), and \(b^*\) of \(-3.48\). The gray tank had an \(L^*\) of 43.64, \(a^*\) of 0.00, and \(b^*\) of \(-0.37\). The blue tank had an \(L^*\) of 73.68, \(a^*\) of \(-21.31\), and \(b^*\) of \(-12.71\). The difference between gray and black tanks is mainly due to lightness with the black tanks being darker than the gray ones; however, for the blue tank the difference is due to all three attributes.

Effects of cortisol on sex-determination

Animals

For this study, male broodstock with a presumed XX genotype were produced through meiogogenesis (Morgan et al. 2006) and masculinized with high temperature (Luckenbach et al. 2003) at the Lake Wheeler fish laboratory at NCSU. Fish were subsequently grown to maturity and held at UNCW. Sperm from these fish was used for fertilization of eggs stripped from wild-caught females. The resulting all XX progeny were grown to ~40 mm TL (76 dph) at Carolina Flounder, LLC (Wallace, NC, USA) before being transported to the Lake Wheeler laboratory at NCSU.

Although not a focus of this work, it has not previously been clear that southern flounder exhibit a XX/XY system of sex-determination like that in the congeneric Japanese flounder (Yamamoto 1999). We are not able to karyotype our male broodstock, but our results are consistent with those from Japanese flounders. Also, there was a very strong female-biased sex ratio observed in the progeny of meiogynogenetically derived male southern flounder in the absence of cortisol treatment (Fig. 5 in Results). Overall, these observations argue strongly that our meiogynogenetically derived male broodstock have an XX genotype.

Experimental design

Fish (40 mm TL, 76 dph) were transported to the Lake Wheeler laboratory, bulk-weighed, and counted and stocked at a density of 250 fish/m² (125 fish per tank) into recirculating systems, each equipped with four 100-L gray tanks. The four tanks in one system contained groups fed cortisol at 100 and 300 mg/kg feed \((N=2\) tanks per group). Fish stocked in a duplicate system were fed no cortisol and served as controls. Activated charcoal was used to absorb any cortisol liberated into the culture-water. Charcoal was rinsed thoroughly and held in fine-mesh bags (Aquatic Ecosystems). Fish were fed ad libitum four times daily with a commercial feed (Skretting Feeds; New Brunswick, Canada). The recirculating systems were regularly backwashed and replaced with fresh culture-water. Pre-mixed salt (Crystal Sea Marine mix; Baltimore, MD, USA) was added as necessary to maintain similar salinities among systems. Salinity was decreased from 24 ppt to 4 ppt over the duration of the study. Photoperiod was maintained on a 12L:12D schedule. Temperature was maintained at 23.5°C. Fish were sampled at 14, 27, 42, 69, and 78 dph for analysis of gonadal mRNA.

Cortisol feed

Feed was prepared according to the method of King and Berlinsky (2006). Cortisol was dissolved in ethanol and then mixed into unflavored Knox gelatin prepared according to package directions. This mixture was spread evenly over 0.5 kg of feed. Treatment groups included 0, 100, and 300 mg cortisol/kg feed. Fish were fed cortisol-treated feed (and gelatin control feed) once a day every 3 days for 2 weeks up to an average body size of 44 mm TL (0–14 dps), then given a 12-day respite to prevent chronic or pharmacological effects of steroids and potential receptor downregulation. Fish were then fed cortisol again once a day every 3 days for 2 weeks from an average size of 55–76 mm TL (26–42 dps), at which point sex can be determined by molecular markers. All fish were subsequently fed regular feed until termination of the experiment at 78 dph (98 mm TL). For terminal sampling, fish were euthanized by a lethal dose of Tricaine as described above. Gonads were collected from fish at 14, 26, 42, 69 (91 mm TL), and 78 dph and snap-frozen in liquid nitrogen and then stored.
at −80°C until processing. Gonadal tissues from fish from each tank were collected at the termination of the study for assessment of sex-specific mRNA biomarkers and determination of sex ratios.

**Isolation of RNA and synthesis of cDNA**

Tissues were homogenized in TRI Reagent® using 6 µL Polycryl carrier (Molecular Research Center, Inc; Cincinnati, OH, USA), according to the manufacturer’s protocol. RNA pellets were resuspended in 20 µL of nuclease-free water. Samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and qualified by 1% gel electrophoresis both prior to and after treatment with DNase. Samples were diluted to 500 ng/µL and treated with DNase (DNA Free, Ambion; Austin, TX, USA). Concentration of samples was adjusted to 250 ng/µL and cDNA was generated using a High Capacity cDNA Reverse Transcriptase kit according to the manufacturer’s directions (Applied Biosystems, Foster City, CA, USA). cDNA was stored at −20°C until further analyses using qRT–PCR.

**Cloning and sequencing of FoxL2 and Müllerian-inhibiting substance**

Partial coding-sequences for southern flounder FoxL2 and Müllerian-inhibiting substance (MIS) were obtained by PCR of genomic DNA derived from pooled ovaries of mature adults using primers designed from Japanese flounder FoxL2 (AB303854) and MIS (AB166791) (Yamaguchi et al. 2010) sequences (Primer Express 3.0 software; Applied Biosystems; Foster City, CA, USA). Following PCR amplification and sequencing for confirmation, deduced nucleotide sequences were translated to amino-acid sequences using the ExPASy Proteomic Server’s translation tool (Gasteiger et al. 2003). ClustalW (v1.83) was employed to align southern flounder FoxL2 and MIS with known sequences from other species (Thompson et al. 2002).

**Quantitative real-time PCR for measurement of mRNA**

Specific primers were designed from southern flounder FoxL2 and MIS sequences using Primer Express 3.0 software (FoxL2 forward primer (FP): GTCCCCGCCAAGTACCT, FoxL2 reverse primer (RP): GGCCCAGGCACTTAG, MIS FP: CTGCCCAGGCCATTGGCA, MIS RP: CAGGACGGCATGGTTGATG). Primers for gonadal aromatase were based on the published sequence for *P. lethostigma* (Luckenbach et al. 2005; P450Arom FP: GAGGCCACAGAGACGGGAA, P450Arom RP: GGGCCCAAACCCAGAC). Primers were verified using a standard curve with pooled cDNA in eight-fold dilutions on a qRT–PCR. Real-time qRT–PCR analysis used Brilliant® III SYBR Green master mix (Stratagene; La Jolla, CA, USA), 1.5 µM forward and reverse primers, and 1 µg cDNA in a total reaction volume of 20 µL. The qRT–PCR cycling parameters were an initial denaturing step at 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Two negative controls, sterile water (“no template”) and no reverse transcriptase (“no amplification”) confirmed lack of contamination by genomic DNA. Only single-gene products were observed from melting curves generated by qRT–PCR. Cycle threshold (Ct) values for experimental samples were transformed using a standard curve of serially diluted cDNA versus Ct values ($R^2=0.93–0.98$) and normalized to reflect the amount of cDNA template per nanogram of total RNA.

**Statistics**

For the study of the effects of background color, sex ratios were analyzed by treatment using a Chi-square goodness-of-fit test using the assumption that normal populations are 50% male regardless of background color (Zar 1996). The treatments were compared with each other using analysis of variance (ANOVA) and then a Student–Newman–Keuls post hoc test. Additionally, cortisol concentrations were analyzed by ANOVA and then a Student–Newman–Keuls post hoc test.

For messenger RNA (mRNA) expression of each gonadal sample from cortisol treatments, females were identified as those having gonads that had a combination of elevated values of aromatase and FoxL2 expression ($\geq 0.35$ ng cDNA/ng total RNA) and reduced MIS expression. Samples showing elevated expression of MIS ($\geq 0.35$ ng cDNA/ng total RNA) and minimal expression of FoxL2 and aromatase were counted as males. Sex was not assigned for gonads that showed low expression ($<0.35$ cDNA/ng RNA) of all three genes. Individuals showing elevation of MIS and either aromatase or FoxL2 were also excluded. Sex ratios were determined separately for each tank, and for each treatment. Chi-square analysis was used to differentiate significant differences among groups. All statistical analyses were performed using SAS 9.1 software (SAS Institute, Cary, NC, USA) and GraphPad Prism (San Diego, CA, USA).
Results

Effect of background color on sex-determination

Based on gonadal histology, in both experiments blue tanks produced significantly higher percentages of males than did either black or gray tanks (Fig. 1). In the first experiment, juvenile flounder were raised at 22.9\degree C and produced 95% males in blue tanks (Chi-square, \(P < 0.0001\)), but ~50% males in the black and gray tanks (Fig. 1A). For the second experiment in which fish were raised initially at 18.8\degree C and then at 23.0\degree C, all three treatments resulted in male-biased sex ratios, but the blue tanks yielded sex ratios (74%, Chi-square, \(P < 0.001\)) that were significantly more biased towards males than was true for the gray and black tanks (Fig. 1B).

Analysis of whole-body cortisol

At 34 dps, fish in the blue tanks (18.1 ± 1.69 ng/g) had significantly higher levels of whole-body cortisol than the baseline levels (13.36 ± 0.59 ng/g) taken before stocking (\(P < 0.001\), Fig. 2). Cortisol levels for fish in the gray and black tanks did not differ from the initial baseline level. Cortisol levels at subsequent sampling points (65 and 93 dps) were not significantly different among fish from tanks of different colors or compared to the baseline level, or to each other.

Cloning of FoxL2 and MIS

The partially cloned cDNA coding sequences for FoxL2 (575 bp) and for MIS (238 bp) were translated and assessed. The deduced partial FoxL2 sequence (190 amino acids) of southern flounder displayed high amino acid identity (identical amino acids) with \(P.\) olivaceus (100%) and was strongly conserved generally [\(Xenopus\) laevis (87.43%), \(Gallus\) gallus (84.29%) followed by \(Rattus\) norvegicus (78.53%), and \(Homo\) sapiens (78.01%)]. The translated southern flounder MIS sequence of 90 amino acids (~15% of the full sequence) showed high amino-acid identity (98.7%) with the sequence of Japanese flounder.

Effect of cortisol on sex-determination

The gonadal mRNA expression of MIS, FoxL2, and aromatase was highest in flounder of 75 mm TL through 120 mm TL. In a comparative analysis of the expression levels of these three genes, individuals that show the lowest levels of MIS also have elevated FoxL2 and aromatase mRNA in their gonads. Conversely, other individuals with elevated gonadal MIS mRNA values have the lowest level of FoxL2 and aromatase mRNA (Fig. 3 A–C). Fish with low MIS and high FoxL2 and aromatase mRNA were deemed females and individuals with high MIS and low FoxL2 and aromatase expression levels were considered males (Fig. 4). The mean percentage of females produced from replicate groups provided the control diet (no added cortisol) was 91% (note that both parents were XX for these fish). In contrast, cortisol caused a significant dose-dependent masculinization of treated groups (Chi-square, \(P < 0.0001\)). The group with a low treatment of cortisol (100 mg cortisol/kg feed) had a sex ratio of 28.57% females: 71.43% males, while the high-cortisol group (300 mg cortisol/kg feed) had a ratio of only 13.33% females: 86.67% males (Fig. 5).

Discussion

In this study, we showed that blue background color has a significant masculinizing effect on southern flounder populations and that this effect is correlated with an increase in endogenous cortisol. When early
rearing takes place at temperatures of 23°C, a population of these normal southern flounder offspring (not produced from gynogenetically derived XX fathers) should develop as 50% males and 50% females, whereas early rearing at 18°C should produce sex ratios biased toward males (Luckenbach et al. 2003). In our initial experiment on background color and at 23°C, both gray and black tanks produced sex ratios of ~50:50, whereas blue tanks produced strongly male-biased sex ratios (Fig. 1A). In the subsequent experiment on background color, fish were raised at an average temperature of 18.8°C for the first 50 days and thereafter raised at 23.0°C during the time when gonads are already differentiating. As predicted, all three background colors yielded a proportion of males that was different from 50%. Interestingly, the blue tanks yielded sex ratios that were significantly more male-biased (74%) than did the gray or black tanks (Fig. 1B). These two experiments with different cohorts of offspring provide strong evidence that background color influences sex-determination in southern flounder. It is not clear what aspect of color, overall lightness \( L^* \), or perhaps the color itself \( a^*, b^* \), is responsible for this effect.

The masculinizing effect seen in blue tanks was correlated with elevations in cortisol levels. The effects of background color on cortisol levels have been observed in a variety of fish species (Papoutsoglou et al. 2000; Rotllant et al. 2003; Merighe et al. 2004; Barcellos et al. 2009). Cortisol levels were elevated relative to baseline values \( (0 \text{ dps}) \) in southern flounder from blue tanks at 34 dps, but not at subsequent sampling periods, while no such increase was observed for gray or black tanks (Fig. 2). Importantly, these fish were an average size of 59 mm TL at 34 dps, which is just before the 65 mm size when cortisol levels begin to increase.

![Fig. 2 Effect of gray, black, and blue background colors on concentrations of whole-body cortisol in southern flounder raised at 19°C until 50 dps and then at 23°C thereafter \( (n = 30 \text{ per tank color per time point}) \). Significant differences between treatments are indicated by different letters; \( p < 0.001 \).](https://academic.oup.com/icb/article-abstract/53/4/755/636781)

![Fig. 3 Gonadal mRNA expression levels (cDNA/total ng RNA) as a function of total body length (in millimeters) from exogenous treatment with cortisol. Females are indicated by circles \( (\text{white} = 0 \text{ mg/kg, red} = 100 \text{ mg/kg, green} = 300 \text{ mg/kg cortisol}) \) and males are indicated by squares \( (\text{black} = 0 \text{ mg/kg, blue} = 100 \text{ mg/kg, yellow} = 300 \text{ mg/kg cortisol}) \). (A) Female expression of aromatase ranged from 0.3267 to 183.5 cDNA/total ng RNA \( (\text{mean} \pm \text{SEM} = 22.17 \pm 4.242) \). Male expression of aromatase ranged from 0.01 to 12.87 cDNA/total ng RNA \( (\text{mean} \pm \text{SEM} = 0.6151 \pm 0.2625) \). (B) Female expression of FoxL2 ranged from 0.01 to 5084 cDNA/total ng RNA \( (\text{mean} \pm \text{SEM} = 288.1 \pm 122.0) \). Male expression of FoxL2 ranged from 0.01 to 2.857 cDNA/total ng RNA \( (\text{mean} \pm \text{SEM} = 0.2119 \pm 0.0474) \). (C) Male expression of MIS ranged from 0.3506 to 62.5 cDNA/total ng RNA \( (\text{mean} \pm \text{SEM} = 15.44 \pm 2.637) \). Female expression of MIS ranged from 0.01 to 2.57 cDNA/total ng RNA \( (\text{mean} \pm \text{SEM} = 0.1770 \pm 0.0378) \). As shown above, males showed considerably higher MIS mRNA values than did females, and cortisol-treated males showed significantly higher levels than did non-treated individuals.]
some juveniles (presumptive females) begin to produce elevated levels of P450arom mRNA both in the laboratory and in field populations (Luckenbach et al. 2005). It is hypothesized that cortisol can inhibit conversion of testosterone into estrogens (and therefore inhibit normal differentiation into females), possibly by suppression of P450arom (Kitano et al. 2000; Perry and Grober 2003; Yamaguchi et al. 2010). It is also known that treatment with estrogen can rescue the masculinizing effect of cortisol in medaka (Kitano et al. 2012). These observations from other species and our finding of elevated cortisol in blue tanks suggest that this steroid may be interfering in the biosynthesis of estrogens and in female sex-determination in southern flounder as well. Consistent with this hypothesis, we also show a masculinizing effect of exogenous cortisol treatment on sex-determination in southern flounder based on expression of conserved molecular markers of sex-determination.

Previous work shows that FoxL2 promotes transcription of aromatase and is expressed primarily in the gonads of females and not in males during sexual differentiation (Pannetier et al. 2006; Alam et al. 2008; Yamaguchi et al. 2007, 2010). Likewise, aromatase is causally linked to female sex-differentiation in Japanese flounder (Kitano et al. 1999, 2000) and its ovarian expression rises and remains elevated during sexual differentiation of southern flounder females, but not that of males (Luckenbach et al. 2005). MIS shows a pattern of expression opposite that of aromatase (Fernandino et al. 2008). Japanese flounder, black porgy (Acanthopagrus schlegeli), and pejerrey (Odontesthes bonariensis) show a sexually dimorphic pattern in MIS expression, increasing during testicular development and declining with ovarian differentiation (Yoshinaga et al. 2004; Wu et al. 2010; Yamaguchi et al. 2010). We obtained partial sequences of FoxL2 and MIS in southern flounder that show high amino acid identity with other vertebrate sequences and nearly 100% identity with Japanese flounder. We evaluated the differential patterns of expression of these genes and of aromatase (Luckenbach et al. 2005) to determine the sex of individuals exposed to cortisol. In most gonads that were evaluated, there was a clear dimorphism in expression of FoxL2 and aromatase relative to MIS mRNA levels, but some individuals had low levels of expression for all three genes, suggesting an
undifferentiated state. Based on these results and on patterns in other species discussed above, we classified individuals with elevated gonadal FoxL2 and aromatase mRNA and low expression of MIS as females and those exhibiting the opposite pattern as males (Fig. 4). Additionally, our previous work on southern flounder shows that expression of gonadal aromatase mRNA is a good predictor of gonadal phenotype. Determinations of sex ratios using this marker produce results identical to those obtained by gonadal histology (Lunkenbach et al. 2005). Interestingly, we found that the difference in magnitude of FoxL2 expression between the sexes is 28 times greater than that for aromatase, suggesting it to be an even more robust biomarker for predicting sex.

We found that early and periodic application of cortisol dramatically masculinized fish in a dose-dependent fashion. Consistent with this hypothesis, we also show a masculinizing effect of exogenous cortisol on sex-determination in southern flounder, based on expression of conserved molecular markers of sex-determination. Indeed, the proportion of females declined to 29 and 13% in those fish receiving 100 and 300 mg/kg cortisol, respectively, while the control cohort was 91% female (Fig. 5). This effect on sex-determination does not appear to be due to strongly negative effects on health as treatment with cortisol did not influence somatic or skeletal growth (data not shown) or significantly affect mortality (79–98% survivorship in cortisol treatments relative to 93% for controls). Thus, it would appear that the doses and temporal manner in which cortisol was applied likely yielded concentrations of hormone reflecting responses associated with a brief period of moderate stress, rather than one representing a chronic stress, or one associated with application of pharmacological steroids that suppress appetite and growth in fish (Barton and Iwama 1991; Bonga 1997; Lawrence 2007). Our results are in agreement with those found for Japanese flounder, in which cortisol was shown to masculinize fish during continuous treatment with steroids over a 70-day period (Yamaguchi et al. 2010). This group also established that levels of whole-body cortisol increase in fish exposed to masculinizing temperatures, consistent with the hormone-mediating, temperature-sensitive effects on sex-determination.

The data from this study and the emerging view from other studies of ESD in fishes suggest that cortisol may be the physiological mediator not only of the sex-determining effects of temperature, but also of background color and perhaps other, as yet undefined, environmental variables. The mechanisms whereby cortisol might masculinize fish during the window of sex-differentiation are uncertain, although evidence suggests the hormone could inhibit aromatase expression and instead activate pathways to development of males (Gardner et al. 2004). In Japanese flounder, a FoxL2 site, and response elements for estrogen receptor and cAMP lie upstream of the aromatase promoter (Yamaguchi et al. 2007) and cortisol has been shown to directly interfere with cAMP-dependent activation of aromatase transcription (Yamaguchi et al. 2010). Alternatively, as suggested by Fernandino et al. (2012), cortisol instead may directly affect masculinization of the gonad, with suppression of aromatase expression being a consequence, rather than a cause, of this masculinization. These authors found that cortisol dose-dependently stimulated 11-KT production and increased expression of hsd11b2 in pejerrey testes in vitro (Fernandino et al. 2012). The stimulation of 11-KT production could be suppressed by treatment with RU486, an antagonist of the glucocorticoid receptor. This suggests an alternate hypothesis: that cortisol may be masculinizing the gonad, with decreases in aromatase expression being a consequence, rather than cause, of differentiation of males (Fernandino et al. 2012). Studies addressing the effects of cortisol both on FoxL2 expression and on MIS expression could help address this possibility of direct masculinization.

In summary, this study shows that intermittent application of cortisol results in a significant male bias without affecting growth, suggesting that even short-term stress during the critical period of sex-determination can cause masculinization of genetic females in this species. Additionally, we show that blue background color, as well as increased cortisol during the window of sex determination, skews sex ratios toward males. Background color, in addition to temperature, has now been shown to significantly affect ESD in southern flounder and our findings suggest cortisol mediates these environmental effects. There is a range of other environmental factors, yet to be examined, that potentially could impact sex-determination. If cortisol is a key mediator of ESD, other environmental variables that represent stressors with impacts on glucocorticoid signaling during sex-determination could impact population sex ratios and should be investigated.

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