

# Copy number variation in the melanocortin 4 receptor gene and alternative reproductive tactics the swordtail *Xiphophorus multilineatus*

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**Abstract** Alternative reproductive tactics are powerful examples of how variation in genetics and physiology among individuals can lead to striking diversity in phenotype. In the swordtails (genus *Xiphophorus*), copy number variation (CNV) at the melanocortin 4 receptor (*mc4r*) locus is correlated with male body size, which in turn is correlated with male mating behavior. We measured the relationship between *mc4r* CNV, behavior, and 11-ketotestosterone (11-KT) in *X. multilineatus* to determine whether *mc4r* CNV was associated with other components of male tactics in addition to body size. We confirmed the results of previous studies, showing that

male size increases with *mc4r* CNV and that mating behavior toward females was size-dependent. We also examined agonistic behavior by exposing males to their mirror image and found that male-male displays behavior were size-dependent. Small males were less likely to exhibit an agonistic response, suggesting that alternative reproductive tactics span intrasexual and intersexual contexts. There was no significant association between *mc4r* CNV and behavior or 11-KT hormone titer. *Mc4r* CNV is thus associated with the variation in male body size, but not with other traits independent of body size.

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**Keywords** 11-ketotestosterone · Alternative reproductive tactics · Copy number variation · Melanocortin 4 receptor · P-gene · *Xiphophorus*

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## Introduction

Species with alternative reproductive tactics are classic examples of how several individual traits can be functionally integrated to produce divergent reproductive phenotypes, usually occurring in the male sex. A typical polymorphism consists of a “bourgeois” tactic that attracts and defends females or the resources they require to mate and a “parasitic” tactic that relies on crypsis and sexual coercion to obtain fertilizations (reviewed in Taborsky 1998, 2001; Oliveira et al. 2008a). Tactics often differ in suites of life history, morphological, physiological, and behavioral traits that affect nearly every aspect of their reproductive biology. The mechanisms responsible for such dramatic differences in male phenotype are thought to be mediated by the endocrine

system, which is itself influenced by genetic and environmental factors (reviewed in Emlen 2008; Oliveira et al. 2008b).

In some species, a strong genetic component underlies the production of alternative reproductive tactics (Cade 1981; Shuster and Wade 1991; Lank et al. 1995; Sinervo and Lively 1996; Brockmann and Taborsky 2008). In the trimorphic side blotched lizard *Uta stansburiana*, for example, heritability for male throat color is high ( $h^2 = 0.87$ , Zamudio and Sinervo 2000) and correlated with differences in aggression, mate guarding and sneak mating behavior as well as physiological performance (Sinervo and Lively 1996; Sinervo et al. 2000). Variation in tactics is thought to depend upon the pleiotropic effects of a throat color locus on the endocrine system (Miles et al. 2007), particularly testosterone production, which differs among morphs and alters tactic expression when administered to males in the field (Sinervo et al. 2000). Similarly, breeding experiments have shown territorial, non-territorial satellite, and female mimic (“faeder”) tactics are inherited via a small number of autosomal loci in the shorebird *Philomachus pugnax* (Lank et al. 1995, 2013). A study of a captive population of this species found that territorial males also exhibit higher levels of circulating testosterone than other tactics, but only at times of social instability within the group, such as during lek formation (Morgan 2009).

A recent study of the genetics of sexual maturation in *Xiphophorus* fish (Lampert et al. 2010) provides a new opportunity to understand how genetic variation leads to the integration of traits involved in the reproductive phenotype. *Xiphophorus multilineatus* and *X. nigrensis* are live-bearing poeciliids from the freshwater drainages of northeastern Mexico. As body size increases, males become deeper-bodied, have higher dorsal fins, longer swords (extended rays of the caudal fin) and perform stereotyped courtship displays to entice females to mate. Small males, on the other hand, are slender, have relatively smaller dorsal fins, lack prominent swords, and chase females to force copulation (Zimmerer and Kallman 1989; Ryan et al. 1990; reviewed in Ryan and Rosenthal 2011). Traits important in post-copulatory sexual selection also vary with male reproductive tactic; small male *X. nigrensis* produce ejaculates with a greater proportion of viable sperm and sperm that is longer lived in vitro (Smith and Ryan 2010).

Kallman (1989) suggested that Y-linked allelic variation at the P (“pituitary”) locus underlies variation in

the timing of sexual maturation in males, resulting in differences in both age and size at sexual maturation. Differences in size persist through adulthood as growth is drastically reduced following sexual maturation. Schreibman et al. (1986) showed that the administration of androgens (testosterone or the fish-specific 11-ketotestosterone, 11-KT) during development facilitated the production of pituitary gonadotrophs and initiated sexual maturity in both early and late-maturing male *X. maculatus* genotypes. As with many vertebrates, androgens are critical not only in organizational effects during development but also play a role in activating differences in sexual behavior between and within the sexes (Adkins-Regan 2012). Androgens, however, have not yet been measured in early- and late-maturing males of *Xiphophorus* either during development or in adults.

Recently, Lampert et al. (2010) showed that variation in male size in *X. nigrensis* and *X. multilineatus* is correlated with Y-linked copy number variation (CNV) of the melanocortin 4 receptor (*mc4r*) gene, which encodes a seven transmembrane G protein-coupled receptor that binds  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH). Two allelic classes of *mc4r* were identified based on their amino acid sequences. “A” alleles reside on the X chromosome and are thus found in males and females while “B” alleles are Y-linked and thus not found in females or XX males (Kallman 1984).

*In vitro* experiments by Lampert et al. (2010) showed that the “A” alleles encode a functional protein, while the proteins resulting from the “B” alleles lack a pair of cysteine amino acids, which renders them functionless. Specifically, “B” allele proteins bind the ligand ( $\alpha$ -MSH), but effectively act as a shunt as there is no transduction of signal by the receptor in response to the binding. Lampert et al. (2010) hypothesized that the “B” alleles act as a dominant negative receptor version that probabilistically delays maturation as more copies of the “B” alleles either reduce the formation of functional *mc4r* dimers or sequester more of the ligand needed to result in a receptor response that eventually stimulates the hypothalamic-pituitary-gonadal (HPG) axis and induces maturity.

Here, we ask if *mc4r* CNV influences male mating and agonistic behavior independently from its effects on size. We also examine the relationship between *mc4r* CNV, male behavior, and 11-KT, an androgen known to have important effects on the expression of male behavior in teleost fish (Borg 1994; Oliveira et al 2008b).

## Methods

### Fish collection and maintenance

Male and female *X. multilineatus* were collected at the Río Coy (21° 45' 0" N, 98° 57' 25" W) and Tambaque (21° 41' 6" N, 99° 2' 30" W), Mexico in August 2009. Sexual maturity was confirmed by the presence of a brood spot in females and the development of the intromittent organ in males (Rauchenberger et al. 1990). Fish were transported to the University of Texas at Austin, housed in mixed sex groups on a 14L:10D light cycle, and fed Tetramin© fish flake twice daily and brine shrimp once daily. On weekends fish were fed fish flake and brine shrimp once daily.

### Behavioral trials and hormone collection

#### General procedures

Males ( $n=29$ ) ranged in size from 21.9 to 42.4 mm standard length (SL; tip of snout to the mid-lateral posterior edge of the hypural plate). Male behavior was quantified in two contexts, (1) a mating context with a female, and (2) an agonistic context using a mirror attached to the aquarium to simulate an encounter with another male of similar size. Each male was tested in both contexts in randomized order between 09:00 h and 13:00 h.

Seven days prior to testing, the male was removed from his stock tank and socially isolated in a 21×40×26 cm aquarium equipped with gravel and a sponge filter to provide refuge and filtration. On the day of testing, the male was placed in an 800 ml beaker filled with 100 ml of water for 60 min to collect a pre-trial hormone sample. Steroid hormones diffuse passively from the gills into the water (Scott and Ellis 2007; Scott et al. 2008) and are significantly correlated with plasma levels in the cichlid *Astatotilapia burtoni* ( $r=0.46$ – $0.88$ , Kidd et al. 2010) and the sister species to *X. multilineatus*, *X. nigrensis* ( $r=0.64$ , Ramsey et al. 2011). The hormone collection procedure did not appear to stress the fish as they remained quiescent within the beaker. The male was then placed into the experimental tank and his behavior recorded using a Canon HG10 HD video camera positioned behind a blind (see below).

The hormone collection procedure was then repeated to obtain a post-trial hormone sample. After a 3 day

isolation period the procedure was repeated for the other behavioral context.

#### Mating context

Following the pre-trial hormone collection, the male was placed in a 32×61×40 cm experimental tank with gravel and a female [SL (mm) mean  $\pm$  sd: 32.8 $\pm$ 3.1, range=27.0–39.8] originating from the same population. The test tank was illuminated by a 500W quartz-halogen lamp directed at a Teflon sheet angled above the aquarium to reflect diffuse light into the tank. Fish were allowed to acclimate for 10 min, during which the female remained inside an opaque cylinder to prevent visual and physical access to the male. The cylinder was then lifted and behavior videotaped for 20 min, beginning when the male approached to within 5 cm of the female. The number and duration of male courtship displays, fast chases, and copulation attempts were manually scored from the video using the ethogram in Ryan and Causey (1989). Females were only used once in the experiment.

#### Agonistic context

After the pre-trial hormone sample was taken, the male was returned to his social isolation aquarium for 10 min to acclimate. A mirror was then positioned on the side of the aquarium to simulate an encounter with another male of similar size. Moretz and Morris (2003) found that male *X. multilineatus* respond to their mirror image with behavior similar to that observed in experimental contests with live males, and within-male repeatability of agonistic behavior using this protocol is high ( $r=0.67$ – $0.93$ ). Once the male approached to within 10 cm of his image, his behavior was videotaped for 20 min. The number of bites toward the image and the time spent parallel swimming were manually scored from the video. Bites were conspicuous behaviors where a male approached his image and struck it with his mouth. Parallel swimming is a display behavior where a male glides back and forth parallel to the image, usually with the body held rigid and the dorsal fin raised (Moretz and Morris 2003).

#### Morphometric analysis

Fish were anesthetized with clove oil (1 drop per 100 ml water), fin clipped and photographed with a digital

camera 3 days after the end of the experiment. Standard length was measured from the photos using ImageJ.

### mc4r genotyping

Copy number of *mc4r* alleles was determined as in Lampert et al. (2010). Briefly, DNA was extracted from a fin clip and the number of *mc4r* B-alleles estimated using a denaturing gel gradient (DGGE) approach (DGene system, BioRad, Hercules, CA, USA). Thirty  $\mu$ l of PCR product from individual fish were loaded onto the gel and copy numbers recorded for each individual. We used primers F929 and R5 to determine total *mc4r* copy number, and primers F929 and AR1 to determine the number of A-alleles present in each individual (see Table S2 in Lampert et al. 2010 for details on the primers sequences). Optimal separating conditions were: 6.5 % polyacrylamide solution (37.5:1 ratio acrylamid/bisacrylamid), 30–50 % urea gradient, run temperature 60 °C, runtime 20 h, at 100V.

### Hormone assay

Water-borne hormone samples were processed as in Kidd et al. (2010) except where noted below. Samples were poured through filter paper to remove any debris and stored in polypropylene bottles at  $-20$  °C until extraction. Eleven-KT was extracted by running the samples through Sep-PakPlus C18 columns (55–105  $\mu$ m; Waters Corporation, Milford, MA) fitted to a vacuum manifold and eluted with 4 mL of 100 % EtOH. Samples were then dried under nitrogen gas and stored at  $-20$  °C until the 11-KT enzyme-linked immunosorbent assay (EIA, Cayman Chemicals, Ann Arbor, MI) was performed.

Dried samples were re-suspended in 500  $\mu$ l of EIA buffer [rather than 320  $\mu$ l of assay buffer as in Kidd et al. (2010)] then diluted 1:16 for the assay. All samples and standards were assayed in duplicate then read with a Beckman Coulter DTX 880 Multimode Detector. This procedure gave absorption values within the 20–80 % of the standard curve as recommended by the manufacturer. The average intra-assay coefficient of variation was  $6.5 \pm 4.9$  % and the inter-assay coefficient of variation was 13 %. We validated the 11-KT EIA for *X. multilineatus* by confirming parallelism. Water-borne hormones from five males of different sizes were collected, dried, suspended in 500  $\mu$ l of EIA buffer, pooled and serially diluted. The slope of this line was parallel to the standard curve (comparison of slopes:  $t_8 = -0.01$ ,  $p = 0.99$ ). The

1:16-fold dilution gave the best dynamic range and was chosen for processing all experimental samples.

### Statistical analysis

Statistical analyses were conducted in R v2.12.1 (R Core Development Team 2011). Shapiro-Wilk tests for normality and Levene's test for heterogeneity of variances were used to verify the assumptions of the statistical tests. When data could not be transformed to meet these assumptions, non-parametric methods were used.

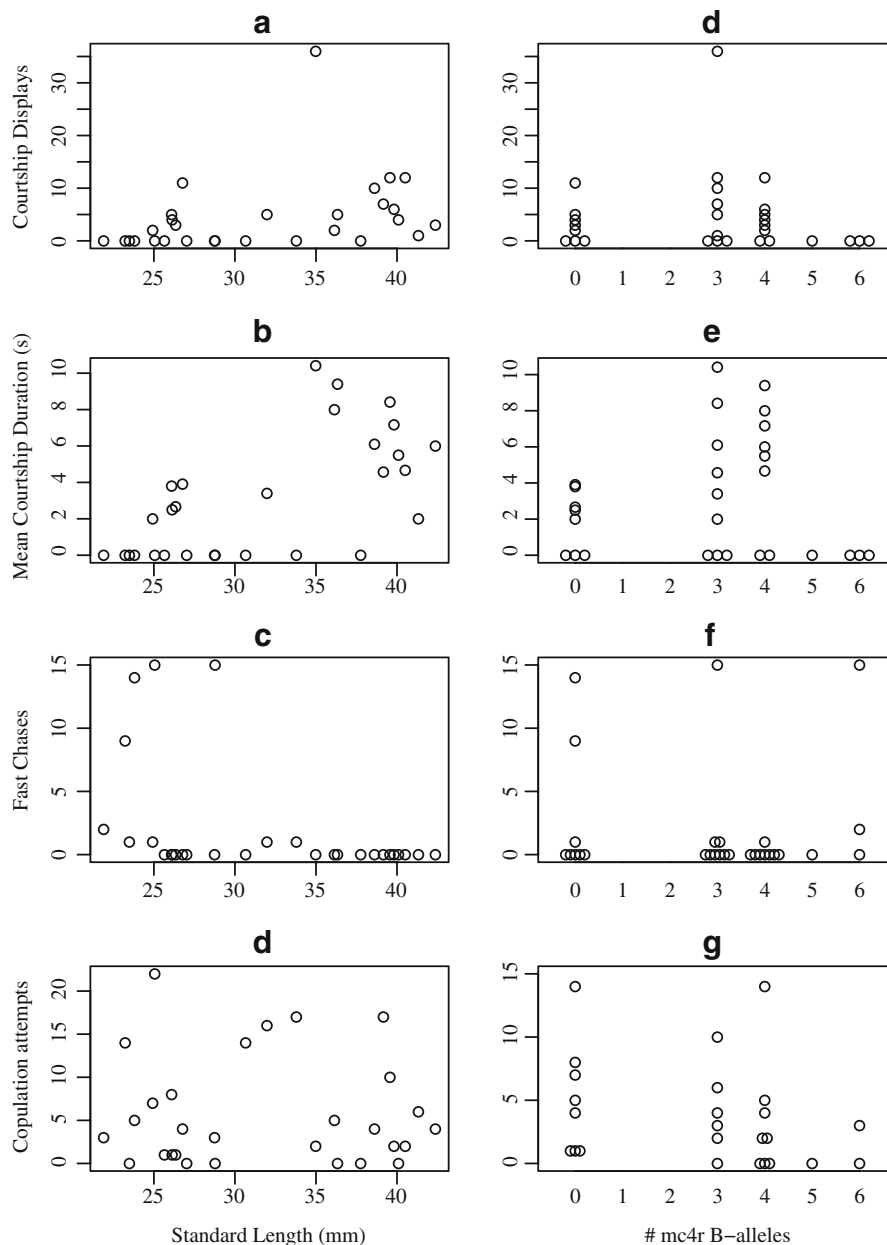
Spearman rank or Pearson partial correlations were used to examine the relationships between male size, behavior, and *mc4r* copy number. Fish sourced from both populations were pooled for analysis as there were no significant differences in behavior (all  $p > 0.05$ ), standard length ( $F_{1,27} = 0.82$ ,  $p = 0.37$ ), *mc4r* copy number ( $F_{1,27} = 0.00$ ,  $p = 0.98$ ), or 11-KT concentration ( $F_{1,27} = 0.19$ ,  $p = 0.66$ ). Eleven-KT concentrations in pre-trial and post-trial water samples did not differ significantly in either behavioral context (see Results), thus the average 11-KT values were used as an estimate of the amount of circulating 11-KT during the trial. The repeatability of 11-KT concentration was calculated using the variance components of a mixed model with male ID as a random effect and no fixed factors (Nakagawa and Schielzeth 2010).

Confidence intervals for repeatability were determined using a parametric bootstrap (Nakagawa and Schielzeth 2010). Inflation of type I error due to multiple statistical tests was controlled by correcting  $p$ -values by the false discovery rate (FDR; Benjamini and Hochberg 1995). All statistical tests were two-tailed.

## Results

### Male size and behavior

As previously documented by Zimmerman and Kallman (1989), the number and duration of courtship bouts significantly increased with male size while the number of fast chases declined with male size (Fig. 1, Table 1). The number of copulation attempts was not associated with male size but varied widely among males (mean  $\pm$  s.d.:  $7.83 \pm 8.85$ , range = 0–37; Fig. 2, Table 1). Two males (27.0 mm SL and 37.8 mm SL) did not perform any sexual behavior.



**Fig. 1** Male mating behavior. Relationships with male standard length (**a, b, c, d**) are depicted on the *left* and relationships with *mc4r* B-allele copy number (**d, e, f, g**) are on the *right*

Agonistic display behavior was rare among the smallest males and then increased, resulting in a positive relationship between time spent parallel swimming and male size (Fig. 2, Table 1). Six of the 29 males never interacted with their mirror image and two males interacted with their image for less than 46 s, spending the 20-min trial swimming back and forth across the

tank. All of these males were small (range: 21.9–26.8 mm SL, Fig. 2).

To examine whether the probability of parallel swimming (or not) varied with male size, we fit a binomial generalized linear model and found smaller males were less likely to parallel swim, while *mc4r* CNV had no effect (standard length,  $\beta \pm SE = 1.6 \pm 0.8$ ,  $X^2 = 20.92$ ,

**Table 1** Partial Spearman rank-correlations between male size, behavior, *mc4r* B-allele copy number and 11-ketotestosterone

	Courtship bouts	Courtship duration	Copulation attempts	Fast chases	Bites	Parallel Swimming	Controlled variable
Standard length (SL)	<b>0.63</b>	<b>0.66</b>	-0.15	<b>-0.62</b>	<i>0.45</i>	<b>0.59</b>	<i>mc4r</i>
<i>mc4r</i> copy number	-0.43	-0.27	-0.20	0.21	-0.11	-0.06	SL
Average 11-KT	-0.45	0.27	-0.07	-0.23	0.29	-0.32	SL
Change in 11-KT	0.02	0.05	-0.29	0.37	0.18	-0.03	None

The last column refers to the variable controlled in the statistical analysis. Correlations in bold were significant ( $p < 0.05$ ) after false discovery rate correction, while correlations in italics were no longer significant after false discovery rate correction

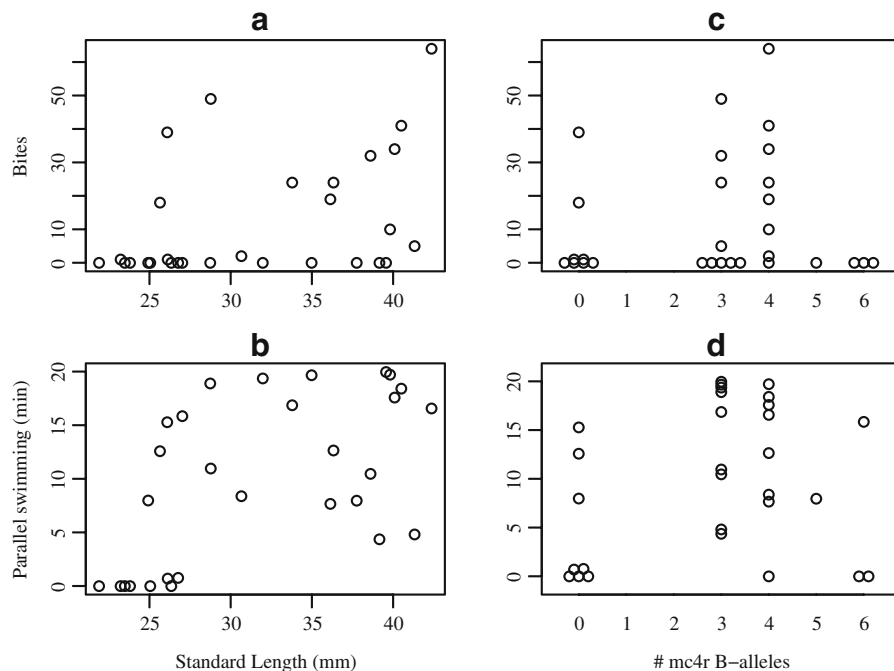
$p < 0.001$ ; # B alleles,  $\beta \pm SE = -0.30 \pm 0.36$ ,  $X^2 = 0.73$ ,  $p = 0.39$ ). The probability of parallel swimming increased steeply at a threshold male size, with the 50 % predicted probability of exhibiting the behavior occurring at 25.2 mm SL (Supplementary Fig. 1). Scoring the two males who displayed for less than 46 s as “not parallel swimming” increased this estimate to 26.1 mm SL.

The number of bites toward the mirror image also increased with male size in a similar fashion; however, the correlation was not statistically significant after controlling for multiple comparisons (Fig. 2, Table 1). The probability of biting increased with male size

(standard length,  $\beta \pm SE = 0.16 \pm 0.08$ ,  $X^2 = 5.33$ ,  $p = 0.05$ ; # B alleles,  $\beta \pm SE = -0.34 \pm 0.27$ ,  $X^2 = 1.94$ ,  $p = 0.16$ ), however the likelihood of escalating the agonistic interaction to biting rose much more gradually with male size than that observed for parallel swimming (Supplementary Fig. 1).

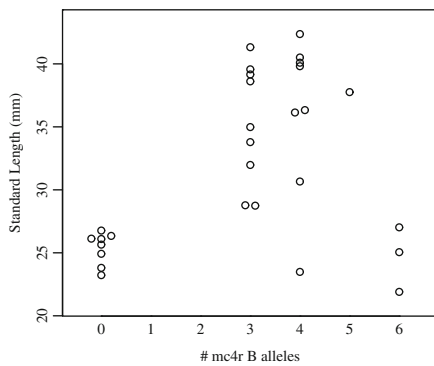
*mc4r* copy number and male size

There were 0 to 6 copies of the *mc4r* B-alleles in our sample of 29 males. Copy number increased significantly with male size ( $r = 0.37$ ,  $p = 0.05$ , Fig. 3). One



**Fig. 2** Male agonistic behavior. Relationships with male standard length (a, b) are depicted on the left and relationships with *mc4r* B-allele copy number (c, d) are on the right





**Fig. 3** Relationship between standard length and *mc4r* B-allele copy number. Open circles are from Lampert et al. (2010) and closed circles are from this study

anomaly was the presence of three small males with six B alleles. Two of these individuals were from the Tambaque population and one individual was from the Río Coy population. The correlation with these males excluded from the analysis ( $r=0.74, p<0.001$ ) was in closer agreement to that found in Lampert et al. 2010 ( $r=0.79$ ). These three males were retained in all analyses.

#### mc4r copy number and behavior

We next asked whether *mc4r* copy number could explain any variation in male behavior after statistically controlling for the relationship between behavior and male size. We found *mc4r* CNV did not correlate with mating behavior or agonistic behavior independent of male size (Table 1).

#### Androgen levels, male size, and mc4r copy number

Eleven-KT concentration increased with male body size, as would be expected if larger fish exude a greater volume of hormone through their gills (linear mixed model: standard length,  $t=3.08, p=0.003$ ). As a consequence, we used partial Spearman rank correlations to examine the relationship between 11-KT concentration and male behavior, controlling for male size. None of the behaviors were correlated with circulating 11-KT concentration except the number of courtship displays (Table 1), but this size-corrected correlation was not significant after controlling for multiple comparisons.

In addition to examining the average level of circulating 11-KT, we also examined the change in 11-KT concentration over the course of the trial. There was no

significant change in 11-KT concentration (linear mixed model: pre-trial versus post-trial 11-KT concentration,  $t=-0.08, p=0.93$ ; mating versus agonistic context,  $t=0.63, p=0.53$ ;  $t=0.03$ , trial x context interaction,  $t=0.03, p=0.97$ ). Repeatability of 11-KT concentration across all trials was moderate ( $r=0.56, 95\%, CI=0.35, 0.70$ ). The change in 11-KT concentration during the trial was also unrelated to male behavior (Table 1).

Finally, 11-KT concentration was not significantly correlated with *mc4r* CNV, and the relationship between 11-KT and *mc4r* CNV did not differ between the mating and agonistic context (linear mixed model: *mc4r* copy number,  $t=-0.21, p=0.83$ , context,  $t=-1.12, p=0.26$ , *mc4r* copy number x context interaction,  $t=1.30, p=0.20$ , standard length covariate,  $t=3.59, p<0.001$ ).

#### Discussion

We found that *mc4r* copy number was positively correlated with male body size in *X. multilineatus*, but not with behavior when the relationship between *mc4r* and with body size was statistically controlled. *Mc4r* CNV and behavior were also not associated with waterborne levels of 11-KT. Reproductive behavior, however, was correlated with body size as previously described by Zimmerer and Kallman (1989). Larger males exhibited longer and more courtship behavior, while the fast-chase tactic was restricted to the smallest males. A new finding from our study was that agonistic behavior was less likely to be exhibited below a threshold size, suggesting that alternative reproductive behavior in *X. multilineatus* span both intersexual and intrasexual contexts.

Small male *X. multilineatus* might have little to gain by engaging in agonistic behavior because of the importance of size in determining the outcome of male-male contests (Morris et al. 1995a). Most small males did not interact with their mirror image despite being presented with an opponent of the same size, which generates the most aggression in this species (Morris et al. 1995a). Other traits important in agonistic interactions are also lacking in small males, such as melanic bars along the body flank (Zimmerer and Kallman 1988), that are intensified during male-male contests and preferred by females (Morris et al. 1995b). The suppression of both morphological and behavioral traits used in agonistic encounters thus appear to be integrated components of the reproductive tactic. Intrasexual contests can entail

significant energetic and opportunity costs (Briffa and Sneddon 2007; Knell 2009), and by avoiding such interactions, small males might allocate time and energy toward other tasks.

The coordinated expression of alternative male phenotypes is thought to be achieved by pleiotropic effects of endocrine system (reviewed in Oliveira et al. 2008b). In *Xiphophorus*, *mc4r* is thought to initiate sexual maturation via its interaction with the HPG axis (Lampert et al. 2010). In addition to its functions in energy homeostasis (Gantz and Fong 2003), *mc4r* has also been shown to affect sexual behavior in rat (Van der Ploeg et al. 2002), however the extent to which this is true in other taxa has not been investigated.

In our study, *mc4r* CNV was not associated with reproductive or agonistic behavior when its relationship with body size was statistically controlled. The lack of an effect of *mc4r* on behavior independent of size has at least two explanations. First, *mc4r* might not interact at all with the neural networks that govern these behaviors as has been suggested in mammals, where *mc4r* knock-outs took longer to initiate sexual contact and did not ejaculate (Van der Ploeg et al. 2002). Alternatively, pleiotropy might make the downstream effects of *mc4r* on body size and behavior difficult to separate without a pharmacological manipulation. More detailed information on the pathways linking *mc4r* CNV, body size, and behavior, and an experimental manipulation of those pathways, is required to further explore this possibility.

The effects of *mc4r* CNV are likely mediated through androgens, which have large physiological effects on both expression of alternative reproductive tactics and sexual maturation in teleosts (reviewed in Borg 1994; Oliveira et al. 2008b). We examined 11-KT specifically because unlike testosterone, bourgeois males have higher circulating levels of 11-KT in almost all (13/16) of the species examined (Oliveira et al. 2008b). In *X. maculatus*, administration of 11-KT and testosterone to juveniles initiates sexual maturation, including changes in the number and reactivity of pituitary gonadotrophs and luteinizing hormone-releasing hormone (LHRH)-expressing cells in the brain (Schreibman et al. 1986). *Mc4r* CNV presumably influences the timing and/or quantity of 11-KT production, which could influence behavior via organizational effects during development or activational effects in adulthood.

Eleven-KT, however, was not correlated with *mc4r* CNV, suggesting that if the quantity of 11-KT is influenced by *mc4r* CNV it is likely restricted to the juvenile period.

Surprisingly, 11-KT levels were also uncorrelated with reproductive and agonistic behavior, in contrast to other studies that used similar methodology to measure hormone concentration (Gabor and Grober 2010; Dijkstra et al. 2011; Sessa et al 2013).

At least four non-mutually exclusive explanations may account for this observation. First, other endocrine factors besides 11-KT, such as neuropeptides or glucocorticoids (reviewed in Knapp 2003; Bass and Forlano 2008) might play a role in the expression of alternative reproductive tactics in *X. multilineatus*. The number and size of the gonadotropin releasing hormone (GnRH)-containing neurons in the brain, for example, differ between male tactics in some teleosts (Hofmann 2006), including between small and large male *X. maculatus* (Halpern-Sebold et al. 1986). Arginine vasotocin is another neuropeptide that influences tactic differences in behavior and morphology in teleosts (Bass and Grober 2001) as well as in other taxa (reviewed in Knapp 2003; Bass and Forlano 2008). Whether arginine vasotocin also influences tactic expression in *X. multilineatus* warrants further investigation.

Second, 11-KT might have organizational effects on the brain but have a less pronounced role in adults. In species like *X. multilineatus* where sex determination is genetic and tactics are fixed, organizational effects have been proposed to be more important than in species where males rapidly switch between tactics (Moore 1991; Moore et al. 1998). Surprisingly few studies have examined the organizational effects of androgens in species with alternative reproductive tactics. An exception is in the tree lizard *Urosaurus ornatus*, where progesterone and testosterone increase the proportion of the aggressive orange-blue dewlap male morph, but only when administered during a critical period in the juvenile stage (Hews et al. 1994; Hews and Moore 1996). It is not known, however, whether the organizational effects of androgens also affect adult behavior in addition to dewlap morphology. Administration of 11-KT and testosterone to juvenile males that differ in *mc4r* copy number and assessing their behavior as adults would provide insight on whether such organizational effects on behavior are evident in *X. multilineatus*.

Third, it is possible that differences in local environment, such as 11-KT receptor density or reactivity in the brain, rather circulating levels of 11-KT *per se*, might lead to differences in behavior. In juvenile *X. maculatus*, for example, the reactivity of LHRH-expressing neurons



is greater in early compared to late maturing P-genotypes following testosterone (but not 11-KT) administration (Schreibman et al. 1986). Variation in receptor type or distribution thus might influence the expression of alternative reproductive tactics in *X. multilineatus*.

Finally, it could be that we did not detect an effect of circulating 11-KT on behavior because waterborne hormone levels do not correlate with serum levels in *X. multilineatus*. While correlations between waterborne and circulating hormone concentrations are robust in the cichlid *A. burtoni* ( $r=0.88$  for 11-KT, Kidd et al. 2010) and female *X. nigrensis* ( $r=0.80$  for estrogen, Ramsey et al. 2011), we were unable to measure serum levels of 11-KT in male *X. multilineatus* because extracting a sufficient quantity of plasma would require more animals than were available, particularly in the smaller size classes. Nevertheless, future experiments will need to establish this relationship.

In summary, our results are consistent with a role of *mc4r* CNV in determining male size at sexual maturity (Lampert et al. 2010), but its influence on the expression of alternative reproductive behavior warrants further investigation. While much is known about the interaction between the HPG axis and the initiation of sexual maturation, the extent to which sexual maturation and behavior result from shared or independent physiological processes is a pressing question in evolution. Furthermore, behavioral traits in *Xiphophorus* and other species with “fixed” tactics do exhibit a degree of phenotypic plasticity (Zimmerer and Kallman 1989; Sinervo et al. 2000; Mills et al. 2008), which needs to be integrated into our understanding of the underlying physiology and genetics. The coordinated expression of behavior, physiology, and morphology that result in alternative reproductive tactics undoubtedly arises from a constellation of genes and interacting physiological processes. Future work should identify the precise role of *mc4r* in the integration of these individual traits into a complex phenotype.

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experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

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