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# Are there consistent behavioral differences between sexes and male color morphs in *Pelvicachromis pulcher*?

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## ABSTRACT

Adult sex ratios in the kribensis cichlid (*Pelvicachromis pulcher*) are influenced by environmental conditions during early development. These environmental sex-determining factors may also organize life-long variation in social behavior within each sex. If this is true, then individual differences in behavior may be, at least in part, expressions of the relative strength of sexual differentiation of that individual. As adults, kribensis males take on one of four alternative color morphs. Males of the yellow morph tend toward breeding monogamously and are produced at higher frequency under female-biasing environmental conditions, while males of the red morph tend more towards breeding polygynously and are produced more frequently by male-biasing early environments. Here we test whether these two alternative kribensis male color morphs show consistent behavioral differences as predicted by an underlying behavioral syndrome of relative feminization to masculinization. We compare these males to females in five different behavioral tests: an aggression assay, an open field exploration task, a novel environment emergence task, and three cerebral lateralization tests. We hypothesize that red males will show more exaggerated sex differences across all behaviors. We find that red males are hypermasculinized as predicted with respect to aggressive behavior and activity levels, but not all behaviors follow this pattern. We find no evidence for a common behavioral syndrome underlying personality traits across females, yellow males and red males.

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## 1. Introduction

Males of many species show distinct differences in coloration that also signal alternative life history strategies. Examples include plumage in birds (Lank et al., 1995; Tuttle, 2003), the throat or dewlap color in lizards (Sinervo and Lively, 1996; Moore et al., 1998) and the gill cover color in fish (Martin and Taborsky, 1997). In each of these cases, relatively subtle differences in coloration discriminate between conspecific males that are harem versus monogamous, or territorial versus satellites, or which otherwise differ consistently in their territorial and aggressive behavior. The development and organization of the mechanisms underlying this intrasexual variation are thought to be similar to the development and organization of the mechanisms of intersexual differences (Moore et al., 1998). Males of the lizard *Urosaurus ornatus* have one of many distinct color patterns on their dewlaps; each male's color type is determined around or before the time of hatching

and fixed for life (Hews and Moore, 1995; Hews et al., 1997). Orange–blue males pursue the strategy of being an aggressive territory holder with a harem of several females, while orange males are larger, but less aggressive, non-territorial, and appear to cuckold the harem holders (Moore et al., 1998). Dewlap color does not always show a perfect correspondence to behavior in this species (Zucker and Murray, 1996). A similar polymorphism is seen in the lizard *Uta stansburiana* (Sinervo and Lively, 1996; Zamudio and Sinervo, 2000).

Male ruffs (*Philomachus pugnax*) are either of a lighter or darker plumage variety. There is a strong tendency for dark plumage males to pursue ownership of a lek territory, and light plumage males, an unaggressive satellite strategy (about 15% of males are discordant) (Hogan-Warburg, 1966). The plumage and behavioral polymorphism is genetically determined and fixed for life (Lank et al., 1995; Küpper et al., 2016). A similar behavioral polymorphism, with aggressive and territorial behavioral differences, is seen in white-throated sparrows (*Zonotrichia albicollis*) (Tuttle, 2003).

These discrete color morphs, with their associated suites of behavioral differences, have remained outside the rapidly expanding literature on animal personality. Animal personality is typically

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thought of as consistent individual differences in behavior along continuously varying dimensions of variation within otherwise similar individuals (Briffa and Weiss, 2010; Dingemanse and Dochtermann, 2013). These different dimensions, such as the shy–bold, exploration–avoidance, activity, aggressiveness or sociability dimensions (Réale et al., 2007), may co-vary producing suites of behaviors, grouped into behavioral syndromes (Sih et al., 2004a,b; Dingemanse et al., 2012). The bold–shy dimension is one of the best-studied dimensions of animal personality (Coleman and Wilson, 1998; Toms et al., 2010), while aggressiveness is another dimension that often correlates with it, forming a behavioral syndrome axis from simultaneously more bold and more aggressive individuals to less bold and less aggressive ones (Huntingford, 1976; Bell, 2005; Strandberg et al., 2005; Dingemanse et al., 2007; Norton et al., 2011). In addition to the co-varying behavioral components, physiological or morphological traits may also form part of the syndrome. For example, the pace-of-life syndrome describes an axis of variation in which a range of traits varies from a live-fast-die-young strategy, of fast growth, high metabolism, bold, highly aggressive individuals at one end of the axis to slower growing, more cautious, less aggressive, shy individuals with slower metabolisms at the other end (Réale et al., 2010). Females are expected to benefit more than males from increasing their life span and fecundity, while males are expected to benefit more than females by maximizing their mating opportunities (Bateman, 1948; Trivers, 1972); males are therefore expected to be bolder, more aggressive and grow faster than females. This suggests that we should expect to find a single behavioral syndrome, akin to a pace-of-life syndrome, ranging from faster growing, more aggressive and more bold to slower growing, less bold and less aggressive that should run through both sexes, with males biased to one end and females to the other.

Sex differences in personality, and individual differences within sexes, have been ascribed to variation in the strength of masculinization of the brain (Ryan and Vandenberg, 2002; Hönekopp and Watson, 2011; Auyeung et al., 2013), suggesting a mechanism which produces a single behavioral syndrome along a continuous spectrum of sex. Species in which sex is environmentally determined allow investigation of the relationship of strength of sexual differentiation on adult behavior (Rhen and Crews, 1999; Rhen et al., 2005).

Four alternative male color morphs have been identified in the African kribensis cichlid (*Pelvicachromis pulcher*) based on the color of the male's operculum: yellow, red, blue, and green morphs (Linke and Staack, 1994). The blue and green morphs have been noted in the hobbyist literature (e.g., Linke and Staack, 1994), but remain almost totally unstudied. The two most common morphs, yellow and red, have been said to be fixed for life and genetically based (Heiligenberg, 1965; Martin and Taborsky, 1997). Both the yellow and red morphs can be territorial monogamous breeders, but the red morphs will sometimes breed polygynously. In a semi-naturalistic laboratory environment, about half of red males became harem holders, while the rest formed monogamous pairs, while about a third of yellow males bred monogamously, and the rest were “satellite” males (Martin and Taborsky, 1997). While Martin and Taborsky (1997) compared the behavior of harem-holder, monogamous and satellite males, they did not explicitly compare the behavior of red and yellow males; however, unpublished data by E. Martin cited therein were that red males were more aggressive and active in territorial patrolling.

The proportion of red to yellow males in kribensis is influenced by the same environmental influence, pH, that influences sex during the same critical period, and not merely the result of differential mortality (Rubin, 1985; Reddon and Hurd, 2013). These parallels in effects suggest that the organization and activation of sex dif-

ferences may be mechanistically linked to the organization and activation of male morph differences.

Cerebral lateralization has been linked to both aggression and boldness in cichlid fish (Reddon and Hurd, 2008, 2009a). In humans, both aggression and cerebral lateralization have long been thought to be related to prenatal testosterone exposure, or related aspects of sexual differentiation of the brain (Geschwind and Galaburda, 1987; Crespi and Badcock, 2008), suggesting that they should co-vary if they are the products of the same underlying developmental process.

Alternative male reproductive tactics have been linked to relative investment in gonadal tissue (Parker, 1990). Male fish following parasitic reproductive strategies typically have higher gonadosomatic indices than parental morphs (Oliveira, 2006; Taborsky and Neat, 2010). If yellow males are sometimes following a satellite strategy in which they attempt to sneak spawnings, then they may be expected to have proportionately larger gonads. Males following a polygynous mating strategy do not tend to have larger testes than monogamous males (Vahed and Parker, 2012), suggesting that red males' gonads should be unaffected by their tendency to polygynous breeding.

Here we examine growth rate, gonadal investment, aggressiveness, boldness, and cerebral lateralization in yellow and red morph males and compare them to females. We shall test whether the same pattern of differences is seen between females, yellow males and red males across these traits. Our general hypothesis is that when sex differences exist, red males will be hypermasculinized compared to yellow males. This is the expected pattern if there is a single behavioral syndrome which extends through the sexes and male morphs. Alternatively, the male morphs may be products of independent developmental programs that produce totally different personality structures.

## 2. Materials and methods

### 2.1. Test subjects

Subjects were 137 adult kribensis cichlids: 50 females and 87 males descended from pet trade breeding stock, obtained from local suppliers, and housed in a mixed sex stock tank until a week before testing. Fish to be tested were housed in 30-l tanks (50 cm × 27 cm × 30 cm) subdivided by transparent “six-pack” dividers into smaller 16 cm × 12 cm × 27 cm sections each containing one fish. Males and females were alternated within the larger tank such that each fish saw at least one fish of the opposite sex and one of the same sex in the adjacent sections. This will have reduced the size hierarchy and dominance confounds by preventing the physical harassment typical of stock tank housing. Each fish was allowed their own home space by keeping them at a much lower density than typical stock tank housing, and not subjected to the effects of prolonged social isolation that would result from solitary housing. Water temperature was maintained at  $25 \pm 2^\circ\text{C}$  and overhead lighting provided a 12:12 h light:dark cycle for all tanks. Fish were fed once a day, five days a week, with either frozen brine shrimp or dried flake food.

### 2.2. Testing sequence

Each fish was given the same sequence of behavioral tests: first the mirror aggression test, followed by the open field test, then the novel environment emergence test, octagonal mirror lateralization test, circular lateralization test with female stimulus fish, and lastly the circular lateralization test with male stimulus fish. Fish were provided a minimum of a one-week break between tests (ranging up to 17 days), and weighed after each behavior test.

### 2.3. Aggression testing

The aggressiveness assay was conducted using a modified version of Reddon and Hurd's (2008) design. A 30-l tank (50 cm × 27 cm × 30 cm) was divided into two (25 cm × 27 cm) testing halves by an opaque white barrier. Each half contained a piece of PVC piping or a plant for shelter and was filled to a depth of 11 cm. Each end wall had a mirror behind a removable opaque black wall. Trials consisted of allowing a single fish to acclimate in one of the two compartments for 24 hours, followed by the remote raising of the black opaque barrier. The fish were video recorded from the side interacting with their mirror image for an hour. The entire video was scored using JWatcher (Blumstein and Daniel, 2007) for four different aggressive behaviors: biting the mirror, charging the mirror (identified by a quick darting motion towards the mirror), fanning out their dorsal and ventral fins in a lateral display, and beating the mirror with their tails (tail beating). Fish that did not engage in aggressive behavior were winsorized to give a maximum latency time to first aggressive act of 3600 seconds, while counts of zero were recorded for each of the four behaviors.

### 2.4. Open field test

Fish were assayed for activity levels and negative thigmotaxis (preference for staying away from squares next to the tank wall, an index of boldness; Toms et al., 2010) in an open field task following a modified version of Champagne et al.'s (2010) design. The task environment consisted of a 38-l (50 cm × 27 cm × 30 cm) tank filled to a depth of 22 cm with a plastic sheet with grid marks laid out under the tank dividing the tank into 50 equal sized squares (5 cm × 5 cm). Fish were acclimatized in a circular piece of PVC piping (10 cm × 8 cm) covered with a lid in the middle of the tank for 2 min. The PVC pipe and lid were then lifted out remotely, and the fish were video recorded for 5 min. The videos were scored using JWatcher. Fish were scored on the total time spent in each of the middle, edge, and corner squares and the number of squares entered for each of the middle, edge, and corner squares. The proportion of each type of square entered was calculated by dividing the number of squares entered for a given type by the total number of squares entered over the duration of the trial.

### 2.5. Novel environment emergence test

The novel environment emergence task followed a modified version of that used by Brown et al. (2007) and Reddon and Hurd (2009a). The apparatus consisted of a 38-l (50 cm × 27 cm × 30 cm) tank filled to a depth of 11 cm and containing an opaque Plexiglass start box (12 cm × 13 cm × 12 cm) with no ceiling and a door that could be raised remotely. The start box was placed at one end of the tank (about 5 cm out from the end of the tank) with the door facing the empty center of the tank. Subjects were given 2 min to acclimate in the start box before the door was raised to start the test. Latency to emerge was measured by timing how long it took the fish to emerge, past the opercula, from the Plexiglass box shelter into the novel environment (Brown et al., 2007). Fish that had not emerged after 20 min were returned to their home tank for two days before being retested, up to a maximum of ten trials. The data analyzed are only the emergence times of the first 20 min trial winsorized to give a maximum emergence time of 1200 s.

### 2.6. Lateralization tests

The octagonal mirror task used a modified apparatus and procedure of Moscicki et al.'s (2011) design. The apparatus consisted of a large square aquarium (74 cm × 74 cm × 38 cm) containing eight square mirrors (30 cm × 30 cm) arranged in an octagon. A circular

piece of opaque PVC piping, with a diameter of 8 cm, was centered in between the centered internal octagon (41 cm × 41 cm × 20 cm) made of eight opaque Plexiglass rectangles (17 cm × 20 cm) and the external mirror octagon. The apparatus was filled with water to a depth of 11 cm. Trials consisted of a 2 min acclimation period followed by the remote lifting out of the PVC piping start box and a 10 min session photographed from above, every two seconds, using a webcam. Pictures were scored on the number of pictures the fish had a particular eye facing the mirror, the number of pictures where the fish did not move, the number of pictures where the fish could be looking at the mirror with both eyes and the number of pictures where the fish was facing away from the mirror or not visible to the webcam. Lateralization indices were calculated by subtracting the total number of left eye viewings from right eye viewings and divided by the total number of either eye use (i.e., excluding the cases where both eyes could have been used) (per Bisazza et al., 1997; Moscicki et al., 2011). Absolute lateralization indices were calculated by taking the absolute value of the lateralization indices.

The circular lateralization task, a modified version of the mirror octagon task, comprised a white Plexiglass tank (74 cm × 74 cm × 39 cm) containing three concentric circular rings (diameters 71 cm, 42 cm, and 20 cm) with only the external ring made of opaque Plexiglass, the two internal concentric rings were of clear Plexiglass. An 8 cm removable start box of opaque PVC piping was centered in between the 42 cm clear and 71 cm opaque Plexiglass circular rings. Trials consisted of inserting three stimulus test fish of the same sex into the center ring, allowing the fish to acclimate for 3 min prior to depositing the test fish into the start box and allowing this fish to acclimate for 2 min. Following acclimation, the start box was raised remotely and fish were photographed from above using a webcam every two seconds for 10 min. Each fish was tested in the apparatus twice – once with a male stimulus fish and once with a female stimulus fish, with at least one week between tests. The stimulus fish were each given two days between testing. Individuals were scored on the number of pictures the fish had a particular eye facing the stimulus fish in the center. Lateralization indices and absolute lateralization indices were calculated as in the mirror octagon test.

### 2.7. Postmortem measures

The fish were euthanized after the last behavioral test. Their heads were placed in a 4% paraformaldehyde solution for male color morph identification (Reddon and Hurd, 2013). While male morph may be identified visually in living fish, males may facultatively hide their red or blue coloration, especially when disturbed. We therefore used this postmortem method for definitive classification to make a single, unambiguous classification. Fifteen red males were identified in the sample along with seventy yellow males. An additional two blue morph males were identified in this same manner; we have excluded them from the analyses due to their small number. Gonads were dissected out for confirmation of sex and calculation of gonadosomatic indices (testis mass/whole body mass). All procedures were reviewed and approved by the University of Alberta Animal Care and Use Committee, Project Name "Social Determination of Sex and Social Behavior in a Cichlid Fish," No. AUP00000055, June 1, 2011.

### 2.8. Statistical methods

All statistical analyses were conducted in R (R Core Team, 2015), using the current stable version at the time of analysis (specific version varies with different analyses). Repeated measures ANOVA model comparisons of mass were conducted following the methods of Faraway (2006) calculating the  $\chi^2$  from the difference between the model likelihood estimates. Individual identity was used as a

random factor, and all other variables as fixed effects. When testing for the effect of fish type, time point was a fixed effect in the null model, which was compared to a model which included both time point and the sex/color category. When testing for the effect of time point within each sex/color category, the null model included no fixed effects and was compared to the model including a fixed effect of time point. When testing for the interaction of color by time point, the null model included fixed effects of time point and sex/color category, but no interaction term, the comparison model included the interaction term.

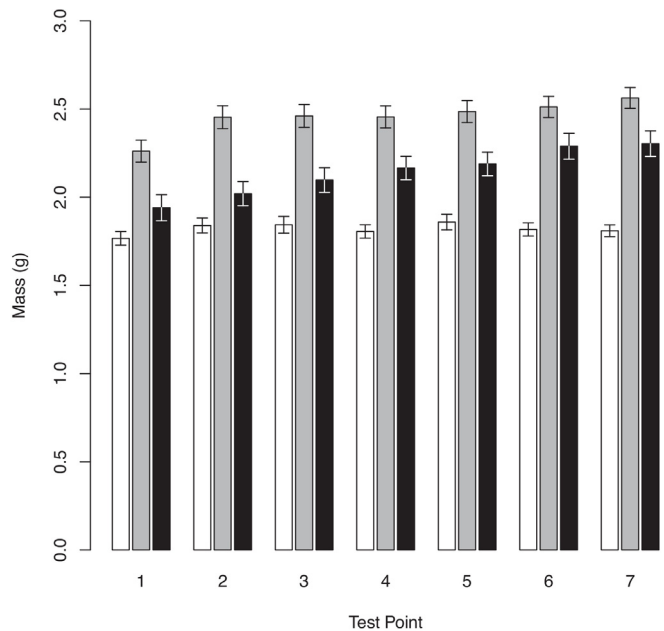
For all behavioral assays, the outcome measures were compared first between females, yellow males, and red males with a one-way ANOVA in the case of single outcome measures (such as emergence time), or MANOVA in the case of a test with multiple outcome measures (as in the aggressive behavior and lateralization measures). If this “top level” analysis of sex/color category differences showed a significant difference, it was followed by analyses of the individual measures (in the case of a “top level” MANOVA) and post-hoc analyses to compare the three groups to each other. Heteroscedasticity robust (Welch–Satterthwaite corrected) methods were used for *t*-tests and one-way ANOVAs (using the default *t*-test() and oneway.test() functions). For post-hoc comparisons of the three sex/color classes we used the pooled variance paired.t.test() function, with the default “holm” adjustment. The outcome variables for the counts for each of the four aggressive acts in the mirror aggression test, the latency to emerge in the novel environment task, the total number of squares entered, and the time spent in non-wall squares in the open field task were all positively skewed and therefore transformed by using square-root of scores before analysis. Data were all plotted using untransformed values to preserve the intuitive sense of the data.

Multicollinearity of dependent variables (when one variable is equal to a combination of other variables) can threaten the assumptions of a MANOVA; moderate correlations between outcome variables are ideal, while high correlations (over  $r = 0.90$ ) pose a threat to validity due to the possibility of collinearity (Tabachnick et al., 2000; Hair et al., 2006). The correlations of the aggressive behavior scores ranged from  $r = 0.33$  to  $r = 0.65$  with a mean of  $r = 0.44$ . The correlations for the dependent measures in the lateralization MANOVA ranged from  $r = 0.13$  to  $r = 0.46$ , with a mean of  $r = 0.28$ .

Behavioral syndromes were investigated through principal component analyses using the princomp() function, from the five behavioral tests (aggressiveness, lateralization, emergence time, activity, and center time) collapsing the number of aggressive acts into a single score by taking the first principal component from the four different aggressive behaviors, and collapsing the three laterality strengths by taking the first principal component from their scores. The sample sizes in our three groups are not big enough to allow for inter-group comparisons of behavioral syndromes using structural equation modelling (Dingemans et al., 2010). Instead, to test for the existence of behavioral syndromes while managing the problem of multiple testing (noted by Dingemans et al., 2010), inter-correlations of these five different behavioral traits were calculated, and the distribution of *p*-values compared to that expected under the null distribution for an omnibus test. The nature of the first and second principal components was then investigated in light of the significance of the behavioral correlations.

### 3. Results

Females, yellow males, and red males showed significant differences in mean body mass over the course of the experiment (repeated measures lmer model comparison of mass as a function of time point and fish identity vs. as function of time point, iden-



**Fig. 1.** Fish mass over the course of the study as a function of sex and male morph. Females (white bars), yellow males (grey bars) and red males (black bars) differed in mean mass ( $p < 0.001$ ). Both yellow and red males gained weight over the course of the study (both  $p < 0.001$ ). Yellow males appeared to gain weight early then slow their rate of growth, while red males seemed to grow more slowly (see text for full statistical treatment).

**Table 1**

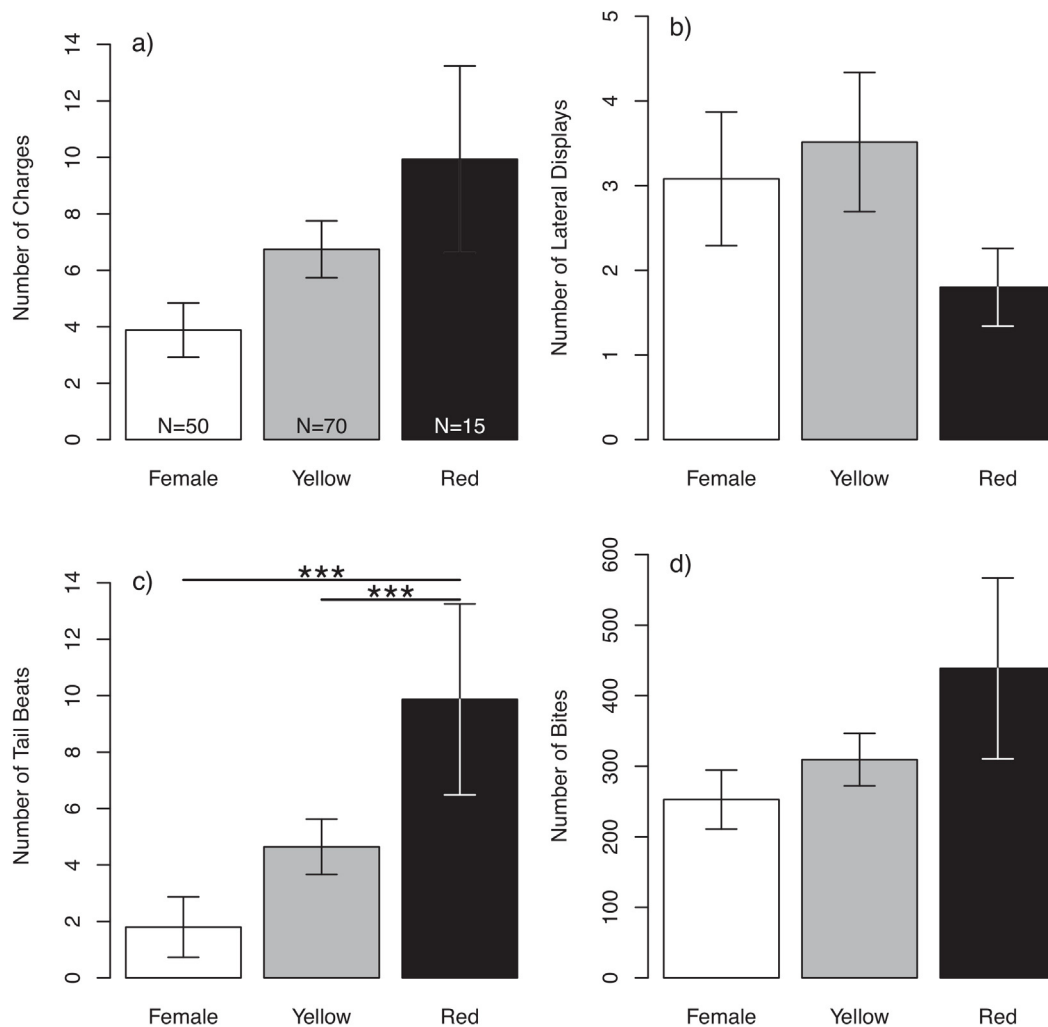
Proportion of females, yellow males and red males which responded aggressively to their mirror image. No significant difference was found ( $\chi^2(2) = 3.20$ ,  $p = 0.20$ ).

	Non-aggressors	Aggressors	% Aggressing
Females	21	29	58%
Yellow males	19	51	73%
Red males	4	11	73%

tity and sex/color category,  $\chi^2(2) = 59.7$ ,  $p < 0.001$ , Fig. 1). Females were the smallest, yellow males were the heaviest, and red males were intermediate in mass. There was no significant effect of time point on female mass ( $\chi^2(1) = 0.93$ ,  $p = 0.34$ ), but yellow males ( $\chi^2(1) = 90.3$ ,  $p < 0.001$ ) and red males ( $\chi^2(1) = 89.3$ ,  $p < 0.001$ ) both showed significant increases in mass over the course of the experiment (tests were model comparisons between models of mass as a function of fish identity vs. identity and time point). There was a significant interaction between testing point and sex/color (repeated measures lmer model comparison of mass as function of sex/color category, time point and individual identity vs. the same model with the additional sex/color by time point interaction term;  $\chi^2(2) = 60.4$ ,  $p < 0.001$ ). Yellow males showed considerable growth early on, between the first and second tests, and then seemed to approach an asymptotic mass. Red males, on the other hand, showed consistent growth throughout, starting with masses that were close to female-typical, and finishing with masses that were much closer to yellow male-typical masses (Fig. 1).

There was no significant difference between females, yellow males and red males in the proportion that showed aggression to their mirror image ( $\chi^2(2) = 3.20$ ,  $p = 0.20$ ; Table 1). A MANOVA revealed significant differences between females, yellow and red males in the number of bites, charges, lateral displays, and tail beats during the mirror presentation test ( $F_{(2,132)} = 2.35$ ,  $p = 0.02$ ; Fig. 2). When analyzed by individual act, there were significant differences between the three classes in the number of charges ( $F_{(2,36.8)} = 3.40$ ,  $p = 0.04$ ) and tail beats ( $F_{(2,35.8)} = 7.39$ ,  $p = 0.002$ ), but not bites ( $F_{(2,37.2)} = 1.43$ ,  $p = 0.25$ ) or lateral displays ( $F_{(2,51.6)} = 0.22$ ,





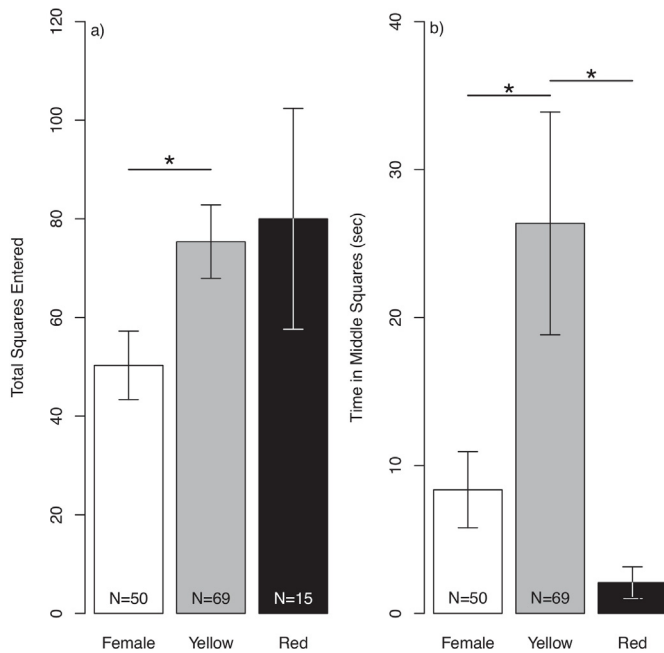
**Fig. 2.** Number of aggressive acts. When the number of aggressive acts showed significant variation between females (white bars), yellow males (grey bars), and red males (black bars), as was the case (a) in the mean number of charges ( $F_{(2,36.8)} = 3.40$ ,  $p = 0.044$ ) and (c) of tail beat displays ( $F_{(2,35.8)} = 7.39$ ,  $p = 0.002$ ), red males were most aggressive and females were least aggressive.

$p = 0.80$ ). Post-hoc comparisons between the classes for each of the significant individual behavior ANOVAs show that red males used more tail beats than yellow males ( $p = 0.0075$ ), or females ( $p = 0.0022$ ) (Fig. 2).

The three groups differed in the time taken to emerge into the novel environment ( $F_{(2,37.9)} = 4.12$ ,  $p = 0.024$ ); post-hoc analyses found no difference between yellow (mean = 559 s, sem = 49 s) and red males (mean = 591 s, sem = 119 s;  $p = 0.80$ ), or red males and females (mean = 800 s, sem = 68 s) but females took longer to emerge than yellow males ( $p < 0.012$  and  $p = 0.22$ , respectively). Half of the 48 females emerged from the start box during the 20 min of this test, while 81% of the yellow males (56 of 69) and 80% of the red males (12 of 15) emerged ( $\chi^2_{(2)} = 13.9$ ,  $p < 0.001$ ).

The three groups differed in the total number of squares entered during the test period ( $F_{(2,36.7)} = 3.38$ ,  $p = 0.045$ ; Fig. 3a); yellow males were significantly more active than females ( $p = 0.041$ ) but not red males ( $p = 0.83$ ), and females did not differ significantly from red males ( $p = 0.35$ ). In addition to these differences in activity levels, there were significant differences in the amount of time each class spent in the middle squares during the test ( $F_{(2,67.7)} = 8.09$ ,  $p < 0.001$ ; Fig. 3b). Post-hoc tests showed that yellow males spent more time away from the walls of the open field task than females ( $p = 0.045$ ) or red males ( $p = 0.045$ ), while red males and females did not differ ( $p = 0.39$ ).

Lateralization was assessed in three different tests, one using the fish's own mirror image as a stimulus, and the other two using three male or three female stimulus fish. When the signed (population level bias) laterality indices for the three different groups were analyzed, for each of these three different stimulus types, none of the nine showed a significant left eye or right eye bias at the  $p = 0.05$  level (Table 2). A MANOVA of group on signed laterality in response to the three stimuli also found no significant effect ( $F_{(2,131)} = 0.48$ ,  $p = 0.82$ ). None of the three groups showed any differences in directional lateralization to any of the stimulus types (Table 3). The lack of directional effects was as anticipated, since it is the strength of lateralization, rather than the direction, that is most often associated with sex and personality traits (Reddon and Hurd, 2008, 2009a, b; Dinsdale et al., 2011). There was a clear and consistent pattern of differences in the absolute strength of lateralization in response to the three stimulus types. All nine of the group-by-stimulus one sample  $t$ -tests were significant with  $p < 0.005$  or less (Table 4), demonstrating individual-level preference for eye use. A MANOVA of sex and color group on strength of this preference in response to the three stimuli showed a significant effect ( $F_{(2,131)} = 3.26$ ,  $p = 0.004$ ; Fig. 4), and all three of the different stimulus sets showed group differences in absolute laterality index (mirror octagon  $F_{(2,74.5)} = 26.9$ ,  $p < 0.0001$ ; Fig. 4a; male stimuli  $F_{(2,57.5)} = 10.7$ ,  $p = 0.0001$ ; Fig. 4b; female stimuli  $F_{(2,76.2)} = 11.6$ ,



**Fig. 3.** Behavior in the open field test. (a) The three groups showed significant differences in total activity (number of squares entered,  $F_{(2,36.7)} = 3.38$ ,  $p = 0.045$ ); females were significantly less active than yellow males. (b) The proportion of time spent in the more exposed center squares differed between the groups ( $F_{(2,67.7)} = 8.09$ ,  $p < 0.001$ ); yellow males spent significantly more time in the center squares.

**Table 2**

Tests for population-level asymmetries in viewing three different stimulus types. There was no evidence for a population-wide side preference to view any of the three stimuli among any of the three types of individuals.

Mirror image			
	<i>t</i>	df	<i>p</i>
Females	-1.0261	49	0.31
Yellow males	-0.051231	69	0.96
Red males	1.5003	14	0.16
Female conspecific			
	<i>t</i>	df	<i>p</i>
Females	0.15875	49	0.87
Yellow males	-1.4529	69	0.15
Red males	-1.5361	13	0.15
Male conspecific			
	<i>t</i>	df	<i>p</i>
Females	-0.82326	49	0.41
Yellow males	-0.29485	69	0.77
Red males	0.934	14	0.37

**Table 3**

Comparison of directional lateralization between sexes and male morphs to different visual stimuli. There were no differences among any of the three types of individuals in their population-wide side preferences to view any of the three stimuli.

Stimulus	Df	<i>F</i>	<i>p</i>
Mirror image	2, 79.9	1.3358	0.27
Male conspecific	2, 60.1	0.8087	0.45
Female conspecific	2, 63.5	0.69182	0.50
Same-sex conspecific	2, 56.8	0.3732	0.69
Opposite-sex conspecific	2, 63.5	0.042834	0.96

$p < 0.0001$ ; Fig. 4c). All three stimulus types showed the same response, with red males showing weaker preferences, and yellow males and females showing comparable, stronger responses.

Gonads were dissected out for confirmation of sex after the end of behavioral testing. Red males were found to have signifi-

**Table 4**

Tests for individual-level deviation from symmetry in preference of eye use on three cerebral lateralization tasks.

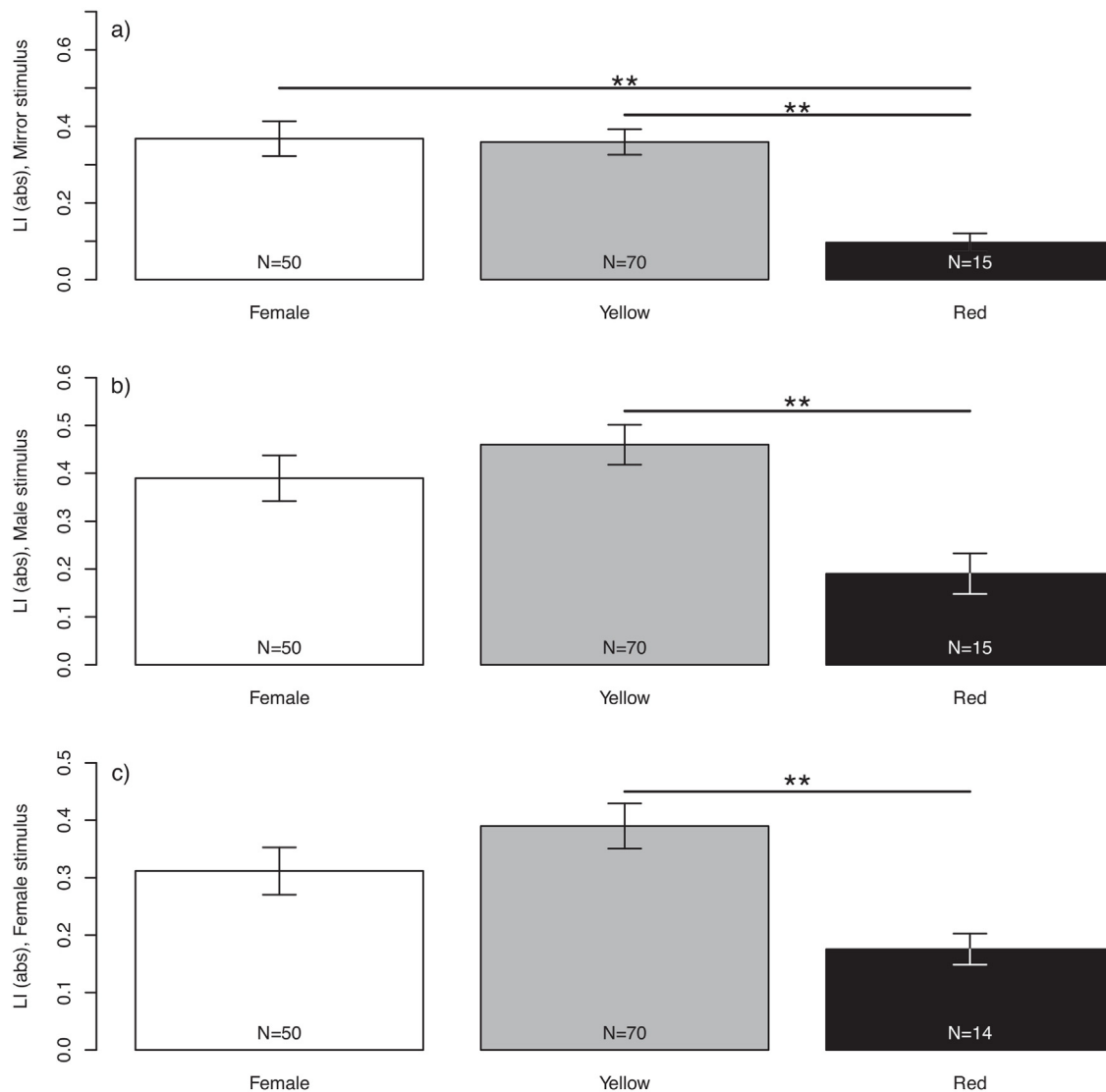
Stimulus	Group	df	<i>t</i>	<i>p</i>
Mirror image	Females	49	8.05	<0.001
Mirror image	Yellow males	69	10.73	<0.001
Mirror image	Red males	14	4.04	<0.001
Male conspecifics	Females	49	8.15	<0.001
Male conspecifics	Yellow males	69	11.03	<0.001
Male conspecifics	Red males	13	4.46	<0.001
Female conspecifics	Females	49	7.58	<0.001
Female conspecifics	Yellow males	69	9.90	<0.001
Female conspecifics	Red males	13	6.59	<0.001

cantly larger GSI than yellow males (Cohen's  $d' = 0.52$ ,  $t_{(35.92)} = 2.13$ ,  $p = 0.04$ ).

The correlations between the five different behavioral dimensions for females, yellow males and red males are shown in Table 5. The  $p$ -values of the thirty correlations (plotted in Fig. 5a) were very strongly positively skewed; the preponderance of small  $p$ -values deviated significantly from the uniform distribution predicted by the null hypothesis (Kolmogorov–Smirnov  $D = 0.44$ ,  $p < 0.0001$ ) demonstrating significant correlations among the behaviors across individuals. If a single behavioral syndrome underlies behavioral variation among all three classes of fish, then we would expect the correlations between any two behaviors to be in the same direction when comparing behavioral correlations in one sex or color morph to another. When comparing females to yellow males, five of the ten correlations are in the same direction in the two types of fish, and five are in the opposite direction. When comparing females to red males, there are four concordant directions of correlation, and six discordant ones. Between yellow and red males there are only three concordant correlations and seven discordant ones. When looking at the strength of these inter-behavior correlations, we see that they are significantly stronger in red males than yellow males or females ( $F_{(2,17.1)} = 4.7$ ,  $p = 0.02$ ; Fig. 5b). The first two principal components were extracted for females, yellow males and red males. These two factors accounted for 31, 33 and 47 percent of the variance in the first factor and 25, 26 and 27 percent of variance in the second factor, respectively, for each of the three types of fish. The loadings of the five behavioral measures onto these two principal components are plotted in Fig. 6. Common to the behavioral syndrome structure of all three types of fish is that PC1 is composed of activity level and thigmotaxis in one direction and emergence time in the other. The relationship of the strength of lateralization to this first factor is consistent, loading along with activity level and thigmotaxis, in the case of females and yellow males, but in the opposite direction in the case of red males. Aggressiveness has little involvement in PC1. The second principal component shows less consistency across the three groups; aggressiveness is the largest contributor in females and red males, but is unimportant in yellow males. PC2 is essentially an activity/thigmotaxis axis in yellow males but an aggressiveness/emergence time axis in red males; in females PC2 is an emergence time/thigmotaxis/aggression axis. There is little evidence of a common structure to behavioral syndromes across the three classes of fish.

#### 4. Discussion

Here we show significant behavioral and morphological differences between two alternative male color morphs in the cichlid *Pelvicachromis pulcher*. Our initial hypothesis was that the two male morphs would show a general pattern in sex differences such that red males would show more exaggerated sex differences than yellow males. This hypothesized pattern of hypermasculinization has weak-to-mixed support. Aggressive behavior and activity levels



**Fig. 4.** Absolute cerebral lateralization. Significant differences were seen in the strength of lateralization when viewing (a) the fish's own mirror images ( $F_{(2,74.5)} = 26.9$ ,  $p < 0.0001$ ), (b) stimulus males ( $F_{(2,57.5)} = 10.7$ ,  $p < 0.0001$ ) and (c) stimulus females ( $F_{(2,76.2)} = 11.6$ ,  $p < 0.0001$ ). In all cases yellow males showed stronger side preferences than red males. In the case of the mirror image stimuli, females also showed stronger preferences than red males.

generally showed the predicted pattern of variation, but the other behaviors did not. There did not appear to be a common behavioral syndrome structure to personality among females, yellow males and red males.

While red and yellow males were equally likely to show aggression when faced with a size-matched apparent rival, it was generally the case that red males used the most aggressive displays, yellow males slightly less and the females least of all. The same pattern held true for general activity levels; females were the least active, yellow males more active, and red males most active. These two results are in agreement with E. Martin's observations that red morph males were more aggressive and patrolled their territories more intensively than yellow morph males (Martin and Taborsky, 1997). Negative thigmotactic behavior in the open field task and cerebral lateralization behaviors did not show the hypothesized pattern of behavioral differences. Yellow males were the most dissimilar, showing more willingness to spend time away from the walls in the thigmotaxis assay than the females and red males, which were most similar to each other. Females and yellow males were similarly strongly lateralized, while red males were

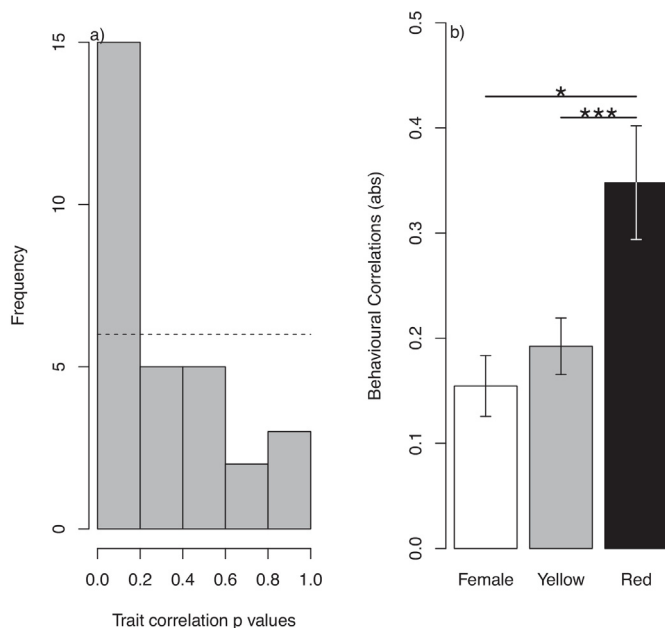
more weakly lateralized. Across the three classes, there was generally the pattern that thigmotaxis/anxiety and lateralization were in agreement, such that stronger lateralization was associated with the bolder behavior of spending more time away from walls, as predicted (Reddon and Hurd, 2009c). The results of the emerge time task were not informative with respect to the hypothesis, as red and yellow males did not differ; both seemed to show floor effects with little opportunity for fish to emerge much faster than they did.

The sex differences found in aggressive behavior were in marked contrast to those seen in a previous study in our laboratory (Reddon and Hurd, 2013) and in some casual observations in the hobbyist literature (Judy, 2005). The present results showed the typical sex difference, in which males are more aggressive than females (Archer, 1988; Andersson, 1994), whereas females were more aggressive in Reddon and Hurd (2013). The assay used here differed from that used by Reddon and Hurd (2013) in that the one hour observation time used here was much longer than the 10 min period of Reddon and Hurd (2013). This difference may account for the discrepancy in effect, if females initially respond with more intensity, but males persist in aggressive behavior over longer peri-



**Table 5**Correlation coefficients between the five behavioral dimensions for females, yellow males and red males. The *p*-values for these correlations are plotted in Fig. 5.

Females (N = 48)				
	Lateralization	Emergence latency	Activity	Negative thigmotaxis
Aggression	0.067	0.219	0.019	0.085
Lateralization		–0.229	0.217	0.189
Emergence latency			–0.275	0.041
Activity				0.204
Yellow males (N = 68)				
	Lateralization	Emergence latency	Activity	Negative thigmotaxis
Aggression	–0.164	0.124	–0.057	–0.163
Lateralization		–0.286	0.282	0.113
Emergence latency			–0.180	–0.315
Activity				–0.239
Red males (N = 14)				
	Lateralization	Emergence latency	Activity	Negative thigmotaxis
Aggression	0.083	–0.276	–0.470	–0.113
Lateralization		0.182	–0.509	–0.503
Emergence latency			–0.376	–0.444
Activity				0.523



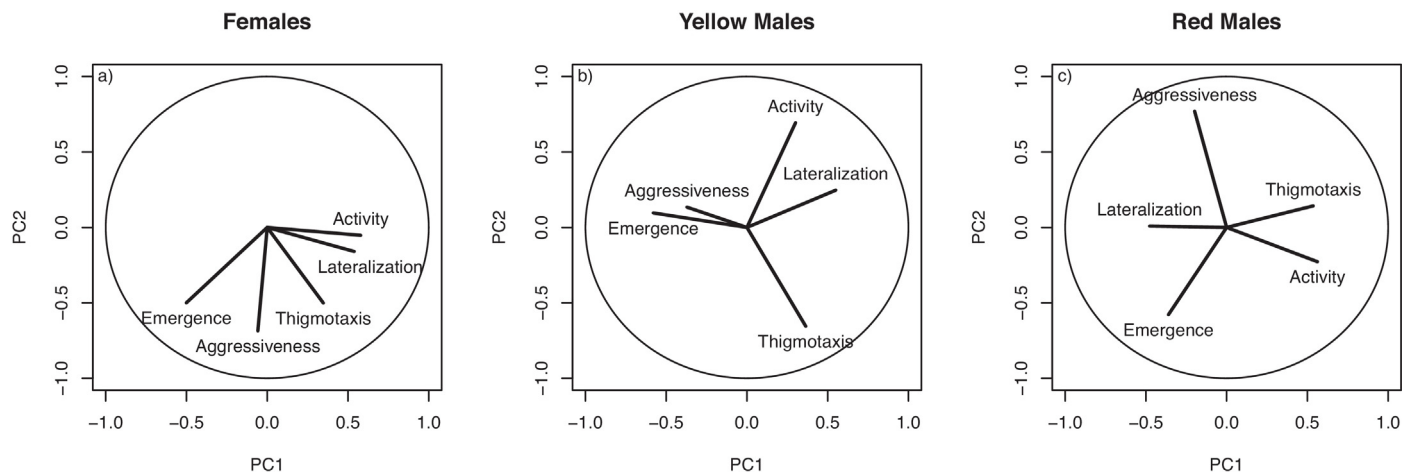
**Fig. 5.** (a) The distribution of *p*-values of the correlations between behavioral dimensions for the three types of fish (see Table 5). The distribution differs significantly from the uniform distribution expected under the null hypothesis given by the dotted line (Kolmogorov–Smirnov  $D = 0.44$ ,  $p < 0.001$ ) indicating the presence of significant covariance between the dimensions. (b) The strengths (absolute values) of the correlations (data from Table 5) differed significantly for the three types of fish ( $F_{(2,17.1)} = 4.7$ ,  $p = 0.02$ ).

ods. Of course, some bias exists in the test; females aggressed against females, yellow males against yellow males, and red males against red males, since they were all responding to their mirror image. One might expect these three classes to convey differing levels of threat or intimidation. That the same proportion of each of these three classes did, and did not, respond aggressively argues that the level of threat was very similar across the three classes. Alternative tests, where individuals to be tested are all presented with the same standardized stimuli, either live fish, or prepared models, would be valuable but far more problematic in reality, as they require both size matching and ensuring that the stimulus animals are equally motivated, or color matching of paint to match

kribensis color perception. In the case of mirror image presentations there is no confounding size effect, nor are there opponents whose faster or slower escalation in behavior influences the behavioral choices of the focal animal.

The lateralization tests also made use of mirror image stimuli, i.e., of the same type as the focal animal, for one test, and all animals were shown live stimulus animals in the same order. That the result for all three types of stimuli showed the same pattern of results argues strongly that the tests were not sensitive to these details. While each of the behavior tests was separated by at least a week, we cannot say with certainty that behavior in one test did not influence the next, biasing later tests. Randomizing the order of testing would have spread any order effects out equally over all the tests. Only a more complicated design, requiring a larger sample size, could have measured and accounted for any order effects of one test on each subsequent one.

The difference between yellow and red males in their gonadosomatic indices (GSI) is more difficult to place on a scale of relative masculinization. The difference seen, with red males having relatively larger testes than yellow males, is in the opposite direction than one might naively expect. In the classic sneaks-vs-guarders sperm competition game, one male role guards their female mate, while the other attempts to sneak copulations, and greater investment in sperm production is expected in sneaker males than guards (Parker, 1990). Parasitic male morph fish tend to be small, and have a high GSI, compared to the “bourgeois” territorial parental morphs (Taborsky and Neat, 2010; Vahed and Parker, 2012). Since it is yellow males who tend to the sneaker and satellite alternative reproductive strategies (Martin and Taborsky, 1997), this would suggest that we should expect yellow males to have higher GSI (Oliveira, 2006). However, kribensis males have more than two strategies, and yellow males do not fit the general pattern of having the small body size of a specialized sneaker morph. If it is the case for kribensis, as it is for the *Uta stansburiana* lizards, that the satellite males cuckold polygynous males rather than monogamous males (Sinervo and Zamudio, 2001), then monogamous males should have the least need for investment in sperm competition, and the lowest GSI. In the socially breeding cichlid *Neolamprologus pulcher*, subordinate helper males also have lower GSI than territory-owning breeder males (Fitzpatrick et al., 2006). In the present case, yellow kribensis males in the laboratory all seem readily capable of monogamous breeding, whereas the subordinate helper male *N.*



**Fig. 6.** Plots of the five different behavioral scales' contribution to the first two principal components (PC) of behavioral variation for (a) females, (b) yellow males and (c) red males. PC1 accounted for 31, 33 and 47% of variation in females, yellow males and red males, respectively, while PC2 accounted for 25, 26 and 27%, respectively.

*pulcher* have typically undergone reproductive suppression as part of a reversible temporary strategy.

Our data show a slower rate of growth, in terms of body mass, for the red males than the yellow ones. The latter seem to reach a size plateau sooner. The length of time the weight data spanned does differ between fish, because the boldness task took differing times to complete. This potential bias should have the effect of increasing the apparent rate of growth of females compared to males (since females required more days to complete the emergence task) which is the opposite of the actual effect seen. Size differences in our fish notwithstanding, informal observations in the laboratory suggest that red males eventually grow to reach larger sizes than yellow males (pers. obs.). It is expected that faster-growing animals should be more prone to dangerous behavior, and be more aggressive (Stamps, 2007). We see the opposite relationship between the yellow and red males that one would expect; the faster-growing yellow males are not more aggressive. We do see the expected relationship between growth rate and negative thigmotaxis; the faster-growing yellow males spent more time in the dangerous squares away from the walls.

Since male reproductive strategy does not coincide perfectly with color morph in *kribensis* (Martin and Taborsky, 1997) – just as it does not in *U. ornatus* (Zucker and Murray, 1996; Hogan-Warburg, 1966) – we might expect GSI and behavior differences to be most clear when males are classed by reproductive strategy, rather than color morph. It seems hard to imagine, however, that the life history “decision” to attempt to breed monogamously or polygynously will not depend greatly on the specific environment, physical in terms of potential nest site number and location, and social in terms of the specific females available to be courted and the number and qualities of male competitors. The behavioral option for a red male to breed monogamously or polygynously, or for a yellow male to breed monogamously or pursue a satellite strategy is likely far more plastic than the option of becoming red or yellow to begin with. If this is the case, then these behavioral and morphological differences between male color morphs should be expected to reflect the different life history strategies most common to their morphs. The existence of different male morphs means that there must be different patterns of costs and benefits for the alternative life history strategies across some other variable (Taborsky and Brockmann, 2010). In the case of *kribensis*, we suggest that the two strategies are likely skewed across ages, with larger, older red males achieving greater success to offset poorer reproductive success when young. That early exposure to low pH biases the sex ratio towards males suggests that low pH exposure when young results in fish that are

expected to be more competitive as adults (Trivers and Willard, 1973). That the ratio of red to yellow males also increases with early exposure to low pH suggests that red males will be even more competitive than yellow males. We find clear evidence of behavioral syndromes in the fact that the strengths of correlations between different behaviors (seen in Table 5) are significantly higher than expected otherwise (Fig. 5a). On the other hand we see no evidence of a common behavioral syndrome shared across the three types of fish. There are as many, or more, discordant directions of correlation between behaviors across the three types of fish as there are concordant correlations. A clear difference emerges between the three types in the strength of the intercorrelations of behavior; red males show significantly stronger correlations. This suggests that red males are selected for a more tightly organized behavioral syndrome, with sharper costs for deviating from the more delineated dimension of variation (Dingemans et al., 2007; Stamps, 2007).

Overall, the results do not support an across-the-board hyper-masculinization of behavior in red male *kribensis* cichlids. While aggressiveness and activity level does follow this general pattern, there appear to be other dimensions of variation, including that of cerebral lateralization and the boldness-related behaviors of thigmotaxis and latency to emerge into the novel environment behaviors. Rather like previous studies that found behavioral syndrome differences between sexes (e.g. Han et al., 2015; Debecker et al., 2016), we found no evidence of a common behavioral syndrome structure to yellow and red males and females. Experimental induction of behavioral variation by manipulation of environmental sex-determining factors, as in Reddon and Hurd (2013), should allow tests to determine which, if any, of these behavioral dimensions or behavior syndromes are linked to sex determination, or independent of it, and whether syndrome differences between morphs are mediated by sex determination. Neuroendocrinological variables remain unexplored in this system. That orange males in the *U. stanisburiana* system have circulating androgen levels about 50% above yellow or blue males (Sinervo et al., 2000) suggests that gross circulating steroid hormone levels may be important in this system, too. Future work will also need to examine variation in nonapeptide signaling (Oldfield et al., 2013; O'Connor et al., 2016) with respect to individual differences in behavior and male morph.

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