

Color change and pigmentation in a color polymorphic cichlid fish

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Abstract In many haplochromine cichlids, body coloration is an important communication cue during social interactions. In some cichlids, individuals can change color, but we have little information about the underlying physiological mechanisms. We examined the regulation of coloration in the color polymorphic cichlid fish *Astatotilapia burtoni* where males are either blue or yellow. Previous studies implicated the melanocortin system, a neuroendocrine center that regulates pigmentation and the stress response, in regulating the color polymorphism in this species. We found that both blue and yellow males express a high density of yellow xanthophores. Dispersal of xanthophore pigments in both yellow and blue morphs occurred within minutes in a dose-dependent manner. Similarly, exogenous α -melanocyte stimulating hormone (α -MSH, a melanocortin hormone) increased yellowness of the body in a dose-independent fashion.

We observed many color changes in males housed in social communities with the proportion of yellow males increasing over the 2-week experimental period. However, color phenotype or color change was not influenced by experimental alteration of the stability of the social hierarchy. The effects of α -MSH suggest that the melanocortin system contributes to the polymorphism in coloration in *A. burtoni* but the role of social interactions and social stress in regulating color remains unclear.

Keywords Sexual selection · Melanocortin receptors · Hormones · Fish · Pigmentation

Introduction

The haplochromine cichlid fish in East Africa is a textbook example of rapid speciation and adaptive radiations (Salzburger 2009; Malinsky et al., 2015; Meier et al., 2017). Cichlids are well-known for their color diversity. While genera can be strongly differentiated in ecology, within genera species can be strikingly similar in ecomorphology but very different in nuptial coloration (Seehausen et al., 2008). The inference is that sexual selection by female mate choice and male–male competition on male color plays a central role in the evolution of haplochromine species richness (Seehausen et al., 2008; Dijkstra & Groothuis, 2011). Competition for mates and

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resources can cause disruptive selection and consequently lead to evolutionary divergence if it coincides with reproductive isolation (Weissing et al., 2011; Tinghitella et al., 2018; Dijkstra & Border, 2018). Male contest competition for access to display sites and mates can have an impact on the evolution of phenotypic and genetic diversity (Seehausen & Schluter, 2004; Grether et al., 2009). For example, in haplochromine cichlids, stronger competition among same-colored individuals than those with different colors can generate negative frequency-dependent and disruptive selection (Seehausen & Schluter, 2004). Male coloration is used as a cue in male contest interactions and in female mate choice decisions (Seehausen & Van Alphen, 1998; Dijkstra et al., 2006; Pauers et al., 2010). The importance of nuptial coloration for communication with rival males and potential mates is illustrated by the fact that haplochromine cichlid males express much brighter nuptial coloration when territorial (Dijkstra et al., 2007).

In many closely related cichlid species or color morphs, males are either yellow or blue, suggesting that yellow-blue color polymorphisms evolved repeatedly in East-African cichlid species flocks (Salzburger, 2009; Maan & Sefc, 2013). Interestingly, in some haplochromine cichlids, males can change color (e.g., from yellow to blue). These species are good candidates to examine the physiological mechanisms underlying this common color dichotomy in haplochromine cichlids. For example, in the Lake Victoria cichlid *Neochromis omnicaruleus* (Seehausen & Bouton), males are either yellow or blue, although most yellow males gradually become blue as they approach sexual maturity or become territorial (Maan et al., 2006). A plastic yellow–blue polymorphism also exists in the cichlid fish *Astatotilapia burtoni* (Günther) from Lake Tanganyika. Like other haplochromine cichlids, this species lives in a lek-like social system in which dominant males defend a spawning territory through acts of aggression and threat displays (Fernald & Maruska, 2012). Subordinate males are non-territorial and show submissive behavior (Fernald & Maruska, 2012; O’Connell & Hofmann, 2012). Dominant males express bright blue or yellow body colors while subordinate males can be pale blue or yellow (Korzan & Fernald, 2007; Korzan et al., 2008, Fig. 1). Color has no detectable heritability in *A. burtoni* (Dijkstra, unpublished data) and the color

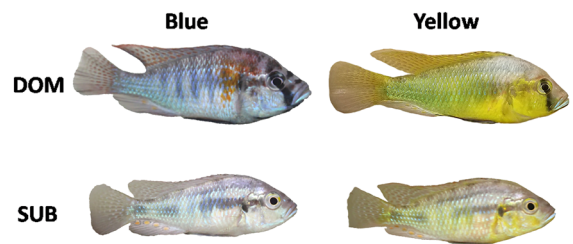


Fig. 1 Typical blue and yellow males. Graphical representation of typical blue and yellow males (*DOM* dominant (top), *SUB* subordinate (bottom))

polymorphism is maintained throughout many generations in many lab populations, although the relative frequency of yellow and blue males can differ dramatically between labs (PDD, personal observation). In *A. burtoni* all males can change colors, a process which typically takes several days in dominant males, but which occurs more rapidly in subordinate males (PDD, personal observation). Color in *A. burtoni* has been suggested to allow males to signal different behavioral strategies in competition for mating territories. For example, yellow males are more likely to become dominant in dyadic contest (Korzan et al., 2008). In territorial males, yellow males tend to initiate more chases towards females and non-territorial males than blue males (Dijkstra et al., 2017; but see Korzan & Fernald, 2007), while blue males tend to perform more border displays with neighboring territorial males (Dijkstra, unpublished data).

It is unknown how social and environmental circumstances influence the expression of yellow or blue coloration. We recently discovered that exogenous α -melanocyte stimulating hormone (α -MSH), an agonist on several melanocortin receptors, can cause a transient increase in yellow coloration in *A. burtoni* by causing pigment dispersion in yellow xanthophores (Dijkstra et al., 2017). The melanocortin system is a neuroendocrine system that regulates body pigmentation and physiological functions including the stress response (Cone, 2006). Given the shared developmental pathway for pigmentation and stress (Ducrest et al., 2008), it is possible that social or environmental stressors can influence the expression of body coloration in *A. burtoni*. Consistent with the idea that stress influences color phenotype is the observation that yellow males have lower cortisol levels than blue males (Korzan et al., 2008; Dijkstra et al., 2017). One important social stressor is losing high dominant status

but we do not know yet how social descent influences color phenotype (Maruska et al., 2013).

In the present study, we examine how α -MSH regulates physiological color change in a dose-dependent manner, compare chromatophore density between morphs, and study how social stress influences the expression of blue versus yellow nuptial coloration in the cichlid fish *A. burtoni*. We test the following specific hypotheses: (1) exogenous α -MSH increases yellowness, and this phenotypic effect of α -MSH on color is mediated by very rapid dispersing effects of α -MSH on the xanthophores in a dose-dependent manner; (2) yellow males have more xanthophores than blue males while blue males have a considerable density of xanthophores; and (3) social stress is linked to color variation and color change.

Materials and methods

Experimental animals

Astatotilapia burtoni used in this study were descended from a wild-caught stock population. Experimental mixed-sex communities were established (8–10 males and 10–15 females per tank) in 110-l aquaria with five inverted flowerpots as previously described (O'Connell & Hofmann, 2012). All experimental tanks were allowed to settle in for at least 2 weeks. All males were tagged just below the dorsal fin with colored beads attached to a plastic tag (Avery-Dennison, Pasadena, CA). All animal care procedures were approved by Central Michigan University Institutional Animal Care and Use Committee.

Effect of α -MSH on body coloration

This experiment was carried out in mixed-sex communities. We characterized males as dominant (DOM) or subordinate (SUB) as previously described (O'Connell & Hofmann, 2012). Dominant males were defined as guarding a flowerpot and engaging in regular aggressive behaviors with other males (chasing, border displays, and lateral displays) along with chasing and courtship towards females (Fernald, 1977). Subordinate males were characterized as not defending a territory and rarely engaging in aggressive behaviors with other males. We selected DOM males that had been stable in their color and status for at least

2 weeks. Males were treated with saline or three different concentrations of α -MSH using intraperitoneal injection. To quantify yellowness, one researcher (GD) assigned yellow scores using a five-point scale ranging from 0 (no yellow coloration) to 5 (strong bright yellow coloration) (Dijkstra et al., 2017). Yellowness was quantified prior to and 60 min after injection. Solutions of α -MSH (product number M4135, Sigma Chemical Co.) were prepared by dissolving the powder in PBS at a concentration of 0.014225 mg/ml (8.54 μ M) similar to the concentration used in a previous study (Dijkstra et al., 2017). This was then further diluted to 0.0071 mg/ml (4.27 μ M) and 0.00356 mg/ml (2.14 μ M). Global observations were conducted of each community ($N = 10$) to identify color, status, and spatial location of territory (if any) for each male. We treated a total of 10 blue and 13 yellow males with saline and one or several α -MSH treatments. For blue males, we treated 10 with 8.54 μ M α -MSH, 9 with 4.27 μ M α -MSH, 10 with 2.14 μ M α -MSH, and 8 with saline. For yellow males, we treated 13 with 8.54 μ M α -MSH, 12 with 4.27 μ M α -MSH, 13 with 2.14 μ M α -MSH, and 13 with saline. The interval between treatments was at least 1 day and the order of treatment was randomized. One male per tank was treated at any given time. For each test we used the following procedure: before netting the focal male we first removed all flowerpots, then weighed the focal individual. We determined the volume of product to inject (saline or peptide, volume range: 22–49 μ l) based on the individual weight of the focal male and aimed at injecting 25 μ l of solution per 2.85 grams of body weight. Intraperitoneal injections at the base of the anal fins were done using insulin syringes (SoftPack Syringe with Permanently Attached Needle, gauge 28, volume 0.5 ml, needle length 1.3 cm). After injection the focal male was put back in the tank and all flowerpots were returned to their original position. The experimenter (GD) was blind to the treatment given to each focal male. All manipulations and observations took place in the morning and time of testing was held constant within males with respect to the saline and α -MSH treatment.

We used linear mixed models (LMMs) to test the influence of color morph and treatment on the change in yellowness, calculated as the differences between pre-treatment and post-treatment scores (post–pre). Fish identity was included as a random effect. More

details about the statistical analysis are given below in “[Data analysis](#)” section.

Effect of α -MSH on pigment granule dispersal in xanthophores

To examine the cellular effect of α -MSH, we conducted a pharmacological experiment on *A. burtoni* scales. To standardize social status, males were housed individually with visual access to at least one neighboring male (modified from Dijkstra et al., 2011). Males were separated by clear perforated PVC screens and each compartment contained a piece of flowerpot to encourage territorial behavior. Individual scales of yellow and blue males were removed from the lateral side of the body and incubated in 30 μ l K^+ -rich saline for at least 15 min. K^+ -rich saline is a melanosome-aggregating fluid (containing 127.9 mM NaCl, 49.8 mM KCl, 0.64 mM $MgCl_2$, and 1.53 mM $CaCl_2$ in deionized water, modified from Vokey and Burton, 1998). Photographs of chromatophores in individual scales were taken at $20\times$ with a digital camera (Leica DMC2900 Camera) attached to a Leica DM 2000 LED microscope (Leica) using the Leica Application Suite software image acquisition and processing software. Separate scales were bathed in one of three different α -MSH concentration (100, 1000 and 5000 nM) or saline control. After perfusing the scale with the treatment solution, time-lapsed photographs were taken at 20 s intervals for 15 min; the first image was taken 20 s after perfusion.

Images were imported into ImageJ and the xanthophores were selected using the color threshold function in CIE $L^*a^*b^*$ (Commission International de l’Eclairage (CIE)). The CIE $L^*a^*b^*$ color space consists of three parameters: the L^* value (lightness) gives the relative lightness ranging from total black to total white, the a^* value (‘redness’) represents the balance between red and green, and the b^* value (‘yellowness’) represents the balance between yellow and blue. CIE $L^*a^*b^*$ is a standardized, perceptually uniform and device-independent color space and it has been frequently used in fish color quantifications (e.g. Sköld et al., 2008). An added advantage of CIE $L^*a^*b^*$ is that it has a single value (b^*) that constitutes the balance between blue and yellow which facilitated the xanthophore selection in the photographs. We used these threshold values to select the xanthophores in all 45 post-perfusion photographs of the same scale. For

each scale, the percentage xanthophore area in the first image after perfusion was set to zero. The pigment granule dispersion in xanthophores was calculated as the change in percentage xanthophore area relative to the first image. We tested whether the initial rate of pigment dispersion (percentage xanthophore area measured over the first three images, which equals to approximately the first minute post perfusion, during which pigment dispersion increased steadily for both morphs in all α -MSH treatments) was morph and treatment specific using LMMs, with fish identity included as a random effect. We also tested whether maximum α -MSH-induced pigment dispersion differed between color morphs by comparing the percentage xanthophore area after dispersal had ceased (during the final minute, i.e., the final three images) using LMMs. More details about the statistical analysis are given below in “[Data analysis](#)” section.

Xanthophore and melanophore density

To examine the difference between xanthophore and melanophore density in color morphs for *A. burtoni*, we plucked 2–6 scales at the dorsal and ventrolateral (referred to as ventral) region and incubated them in 100 μ l K^+ -rich saline for at least 30 min. We selected males from mixed-sex communities that had been consistent in their color phenotype and status for at least 5 days (> 84% of all males had been consistent in their color phenotype and status for at least 3 weeks). We tested a total of 8 males for each morph and status combination. Photos were taken at $5\times$ using a digital camera (Leica DMC2900 Camera) as described above. The collected images of the scales were fused together into a single image using Photoshop’s merge feature. The number of xanthophores and melanophores were counted from 2 randomly selected dorsal and ventral scales for all males sampled. Density was obtained by dividing the xanthophore or melanophore count by the surface area of the pigmented region of each scale. The experimenter (MT) counting the chromatophores was blind with respect to color morph and status.

The influence of color morph, status, and body region on xanthophore and melanophore density was analyzed using LMMs with fish identity used as random effect. More details about the statistical analysis are given below in “[Data analysis](#)” section.

Social stability and color expression

In order to examine how habitat disruption and social stress affects the expression of blue and yellow coloration over time, communities ($n = 15$) consisting of 10 males and 14 females were set up and observed for 2 weeks. Communities had one flowerpot placed in each corner of the tank and one flowerpot placed in the center of the tank. Territorial males typically defend one flowerpot and removing or rearranging the flowerpots can lead to loss of territory (for similar paradigm, see Hofmann et al., 1999). Treatments consisted of stable, unchanging territory arrangements ($n = 7$) and unstable, manipulated tanks ($n = 8$) which experienced a change in flowerpot number and arrangement weekly (Fig. 2). At the time of manipulation (at the end of week 1), flowerpots in unstable communities were reduced from 5 to 3 inducing social descent and social instability. In stable communities, flowerpots were removed and then immediately replaced in the same location to account for handling stress. The experiment was continued for a total of 21 weeks with weekly manipulations of flowerpot arrangement in the unstable communities. However, for the current study we focus on the first flowerpot manipulation to specifically examine the effect of social stress induced by forced social descent. To quantify shifts in color and tenure of male color and

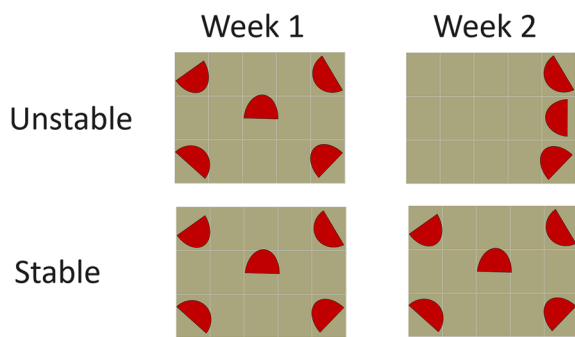


Fig. 2 Timeline for habitat manipulation. Schematic representation of the arrangement of flowerpots from week 1 to week 2 in unstable (top row, $N = 8$) and stable (bottom row, $N = 7$) communities; there were 3 weekly observations of status and color and the flowerpot manipulation took place after the third observation in week 1. Rectangles indicate the aquarium floor (top view) with small circles representing flowerpots used to stimulate territorial defense. Removing the flowerpots from the left side of the aquarium resulted in dominant males from that side of the tank losing territoriality (or they claimed a flowerpot on the other side of the tank)

social status, global observations were taken three times per week with the third observation occurring just prior to manipulation of the flowerpots. During each global observation, we quantified color phenotype as being yellow (all yellow coloration), yellow/blue (mostly yellow with some blue coloration), blue/yellow (mostly blue with some yellow coloration), or blue (all blue coloration). Social rank was assigned during each global observation by characterizing males as dominant (DOM) or subordinate (SUB).

Color phenotypes as well as transitions in color and status over time were analyzed using R v3.4.3 (2017). Only full shifts from yellow to blue were counted as a transition in color. Intermediate shifts in color (shifts between yellow and yellow/blue or between blue and blue/yellow) were not counted as a color transition. The change in proportion of yellow males in each community over time was analyzed using linear mixed models with tank included as a random effect. More details about the statistical analysis are given below in “Data analysis” section.

Data analysis

All analyses were conducted in R v3.4.3 (2017) (R Core Team, 2012). Linear mixed models (LMMs) were implemented using a maximum likelihood protocol (R package lme4), and for all our analyses we selected models that best fit the data using Akaike Information Criterion corrected for small sample sizes (AICc) using the model.sel function (R package MuMIn). Models were fitted containing fixed effects relevant to our hypotheses. The results of all models within 2 AICc of the top model are reported as best-fit models, with P values at a 5% level for significance. We also report 95% confidence intervals of parameter estimates of all fixed effects. In all model summaries, the reference category for fixed effects were determined alphabetically or numerically: blue (vs. yellow, for color morph); DOM (vs. SUB for status), saline control (vs. α -MSH); dorsal (vs. ventral for body region); stable (vs. unstable communities). For all analyses, model residuals were examined to ascertain assumptions of normality and homogeneity of variances were met.

Results

α -MSH increased yellow coloration in a dose-dependent manner in blue males

α -MSH induced increased yellowness in both blue and yellow males (Fig. 3), consistent with previous studies. To test whether exogenous α -MSH had a dose-dependent effect on yellowness, we ran models for yellow and blue males separately. We found one best-fit model for each analysis (Table 1). In yellow males, the increase in yellowness was not dependent on α -MSH concentration. Note that yellow males have a much higher baseline yellow score than blue males, reducing the dynamic range for α -MSH-induced color change. Blue males experienced the greatest increase in yellowness at the 2.14 μ M and 8.54 μ M α -MSH treatments: at the highest α -MSH treatment males became significantly more yellow than the medium α -MSH treatment (LMM, 8.54 μ M vs. 4.27 μ M: 0.54, 95% CI [0.16–0.91], $P = 0.005$), but there was no difference between the highest α -MSH treatment and the lowest α -MSH treatment (LMM, 8.54 μ M vs. 2.14 μ M: 0.14, 95% CI [– 0.23 to 0.51], $P = 0.466$). The lowest α -MSH treatment did not produce a significantly greater increase in yellowness than the medium α -MSH treatment (LMM, 2.14 μ M vs. 4.27 μ M: – 0.40, 95% CI [– 0.81 to 0.01], $P = 0.057$).

α -MSH dispersed the xanthophores within minutes, and to a greater degree in yellow males than in blue males

α -MSH induced rapid pigment dispersion of xanthophores in both yellow and blue males at all concentrations (Fig. 4). Since we were mostly interested in morph-dependent effects on pigment dispersion, we examined the effects of α -MSH treatment for all 3 α -MSH treatments separately. For all 3 α -MSH treatments the final pigment dispersion was higher than the control regardless of morph (LMM, all $P < 0.001$). The initial rate of pigment dispersion was similar at 1000 nM α -MSH for blue and yellow males (Table 2, LMM, effect of morph: 0.10, 95% CI [– 0.14 to 0.34, $P = 0.421$), but was significantly higher in yellow males at 100 nM and 5000 nM α -MSH (Table 2, LMM, dispersion at 100 nM α -MSH: 0.25, 95% CI [0.03–0.47], $P = 0.024$; dispersion at 100 nM α -MSH: 0.36, 95% CI [0.18–0.55], $P < 0.001$). In the final three images, once pigment dispersion had stabilized, all α -MSH concentrations resulted in a higher degree of pigment dispersion in yellow than blue males (Table 3, LMM, all $P < 0.05$). There was no significant difference in final pigment dispersion for either morph based on α -MSH concentration. A visual examination of Fig. 4A suggests that at all three α -MSH concentrations, the maximum pigment dispersion was reached within 7–8 min.

Fig. 3 Injection of α -MSH causes increased yellowness in male *Astatotilapia burtoni*. Yellowness defined on a scale from 0 (completely blue) to 5 (completely yellow). Increase in yellowness due to α -MSH in blue (left) and yellow (right) males for each α -MSH concentration. Shown are the mean + SE of the differences between pre-treatment color and post-treatment color (post-pre). Sample sizes: blue, $n = 10$, yellow, $n = 13$

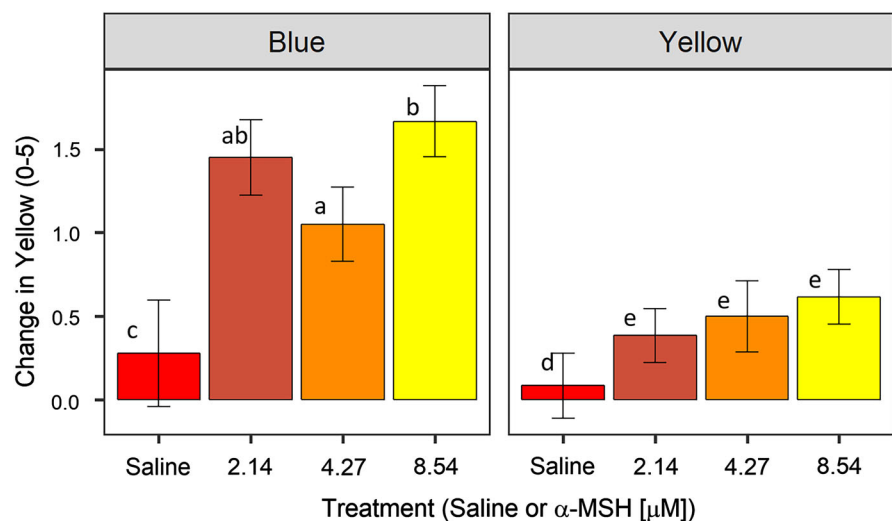


Table 1 The effect of exogenous α -MSH on yellow body coloration

	Yellowness increase in yellow males			Yellowness increase in blue males		
	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value
Fixed parts						
(Intercept)	0.08	0.13–0.30	0.464	0.20	– 0.26 to 0.66	0.414
2.14 μ M α -MSH	0.30	0.01–0.59	0.047	1.25	0.83 to 1.68	< 0.001
4.27 μ M α -MSH	0.42	0.11–0.72	0.010	0.85	0.43 to 1.28	< 0.001
8.54 μ M α -MSH	0.53	0.25–0.82	< 0.001	1.39	0.96 to 1.82	< 0.001
Random parts						
σ^2	0.136			0.217		
$\tau_{00,id}$	0.009			0.303		
N_{id}	13			10		
ICC _{id}	0.060			0.582		
Observations	49			38		
R^2/Ω_0^2	0.305/0.298			0.786/0.780		
AIC	55.954			80.104		

Bold indicates significant correlations ($p < 0.05$)

The best-fit linear models explaining increase in yellowness for blue and yellow males separately. The effect of each α -MSH concentration is indicated relative to the saline control. Shown are the within-group variance (σ^2), the between-group variance (τ_{00}), the sample size of the random effects (experiment and tank), and the intraclass coefficient correlation (ICC). The sample size (observations) as well as the R^2 and Ω_0^2 values of the model are also indicated

Chromatophore density varied by color morph, status and body region

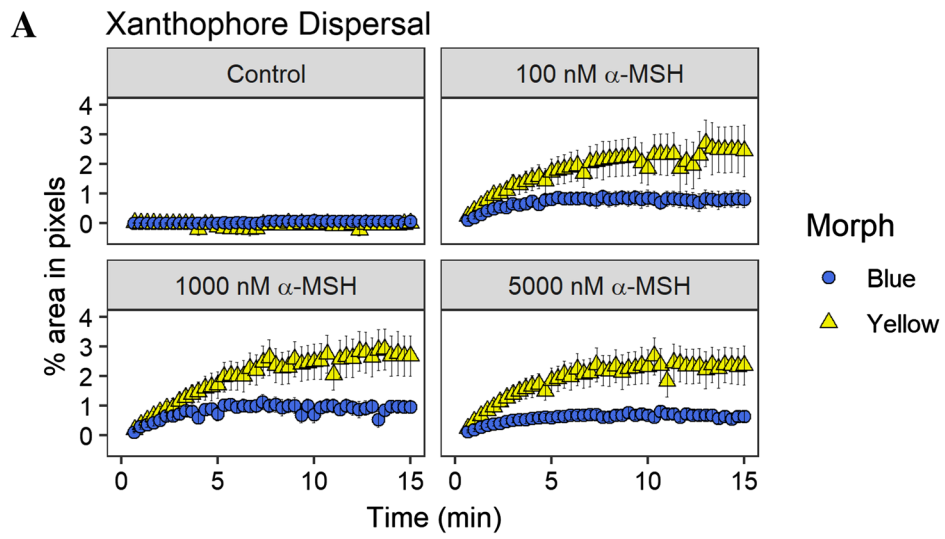
For both xanthophore and melanophore density, the best-fit model included a significant three-way interaction effect for status, morph, and side (Fig. 5, LMM, Xanthophore: -26.62 , 95% CI [-38.55 to 14.69], $P < 0.001$; Melanophore: -16.65 , 95% CI [-29.34 to 0.395], $P = 0.011$). To test the effect of color on chromatophore density, we analyzed the data separately for DOMs and SUBs.

When comparing models to explain variation in xanthophore density in DOMs, the best-fit model included a significant interaction effect of side and morph explaining variation in xanthophores (Table 4). Xanthophore density was higher in yellow males than in blue males, and this difference was greater on the ventral side. In SUBs one of the best-fit models included significant effects of side and morph, while the other best-fit model contained a non-significant interaction effect of side and morph (Table 5). As in DOMs, the xanthophore density was higher in yellow SUBs compared to blue SUBs, while overall the xanthophore density in SUBs was higher in the dorsal side compared to the ventral side.

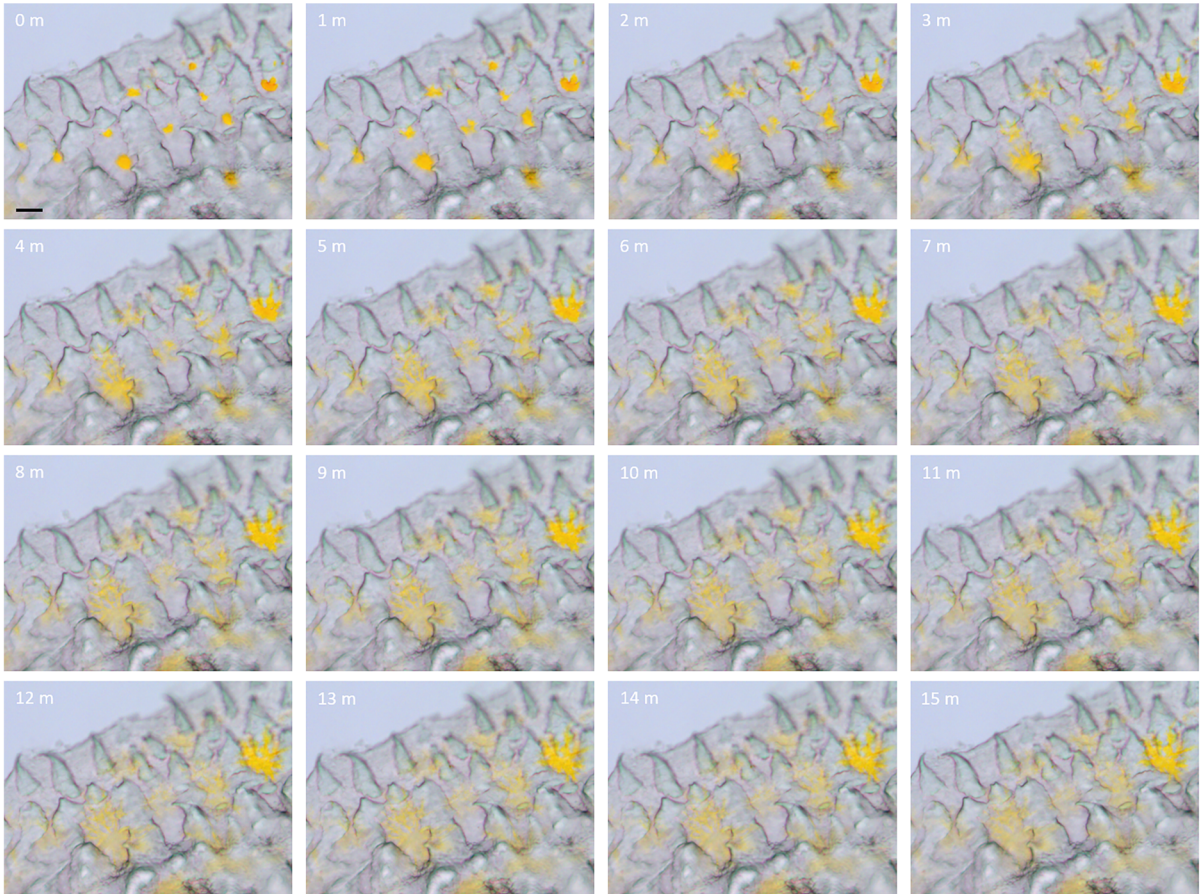
When comparing models to explain variation in melanophore density in DOMs, we found a single best-fit model with a significant side and morph interaction effect (Table 4). A visual examination of the plots shows that blue DOMs have a higher density of melanophores than yellow DOMs, and that melanophore density is higher in the dorsal side. Although it was not one of the best-fit models, a LMM without the interaction effect resulted in significant effects for side and morph (LMM, effect of morph: -12.98 , 95% CI [-23.06 to 2.91], $P = 0.015$; effect of body position: -57.38 , 95% CI [-63.09 to 51.67], $P < 0.001$). In SUBs we found two best-fit models with one producing significant effects of side and morph, while the other best-fit model retained a non-significant interaction effect of side and color (Table 6). As in DOMs, melanophore density was higher in blue SUBs than in yellow SUBs, while overall melanophore density in SUBs was higher in the dorsal side compared to the ventral side.

Social instability and color phenotype

We examined color and status changes from the third global observation just prior to the flowerpot



B



◀ **Fig. 4** Xanthophore pigment dispersion by α -MSH **A** Xanthophore pigment dispersion by α -MSH is dose- and morph-dependent. Pigment granule dispersion in xanthophores was measured as percentage scale area that was yellow based on images that were taken every 20 s for 15 min post-treatment. Pigment dispersion at all α -MSH concentrations was higher than controls. Shown are the mean + SE. **B** Time-lapse composite image of a scale from yellow male perfused with 5000 nM α -MSH over 15 min. Image of scale 20 s after perfusion used as zero point for standardization purposes

manipulation (referred to as week 1) to the final observation at the end of week 2 (referred to as week 2). In the unstable communities, we observed more dominant males transitioning to subordinate status (12 males in unstable communities; 5 males in stable communities; Table 7). In the unstable tanks, all occupied flowerpots that were removed led to the previous flowerpot owner descending in status ($n = 8$) or descending and ascending again by claiming one of

Table 2 Initial xanthophore pigment dispersion rate with varying concentrations of α -MSH

	100 nM			1000 nM			5000 nM		
	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value
Fixed parts									
(Intercept)	0.02	− 0.16 to 0.19	0.850	− 0.02	− 0.18 to 0.15	0.854	0.02	− 0.13 to 0.17	0.790
Morph	0.03	− 0.22 to 0.28	0.817	0.04	− 0.21 to 0.28	0.766	0.02	− 0.20 to 0.24	0.872
Time	0.28	0.13 to 0.44	< 0.001	0.38	0.22 to 0.55	< 0.001	0.25	0.12 to 0.38	< 0.001
Morph × time	0.25	0.03 to 0.47	0.029	0.10	− 0.14 to 0.34	0.425	0.36	0.18 to 0.55	< 0.001
Random parts									
N_{Fish}	23			23			23		
Observations	67			68			67		
R^2/Ω_0^2	0.890/0.887			0.836/0.829			0.914/0.912		
AIC	− 20.022			− 15.853			− 39.206		

Bold indicates significant correlations ($p < 0.05$)

Shown are the within-group variance (σ^2), the between-group variance (τ_{00}), the sample size of the random effects (experiment and tank), and the intraclass coefficient correlation (ICC). The sample size (observations) as well as the R^2 and Ω_0^2 values of the model are also indicated

Table 3 Final xanthophore pigment dispersion at varying concentrations of α -MSH

	100 nM			1000 nM			5000 nM		
	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value
Fixed parts									
(Intercept)	0.80	− 0.24 to 1.83	0.144	0.96	0.06–1.85	0.047	0.64	− 0.15 to 1.42	0.124
Morph	1.69	0.20 to 3.18	0.037	1.72	0.43–3.02	0.016	1.69	0.56 to 2.82	0.007
Random parts									
N_{Fish}	23			23			23		
Observations	66			68			67		
R^2/Ω_0^2	1.000/1.000			1.000/1.000			0.999/0.999		
AIC	− 65.112			− 50.330			− 29.714		

Bold indicates significant correlations ($p < 0.05$)

Shown are the within-group variance (σ^2), the between-group variance (τ_{00}), the sample size of the random effects (experiment and tank), and the intraclass coefficient correlation (ICC). The sample size (observations) as well as the R^2 and Ω_0^2 values of the model are also indicated

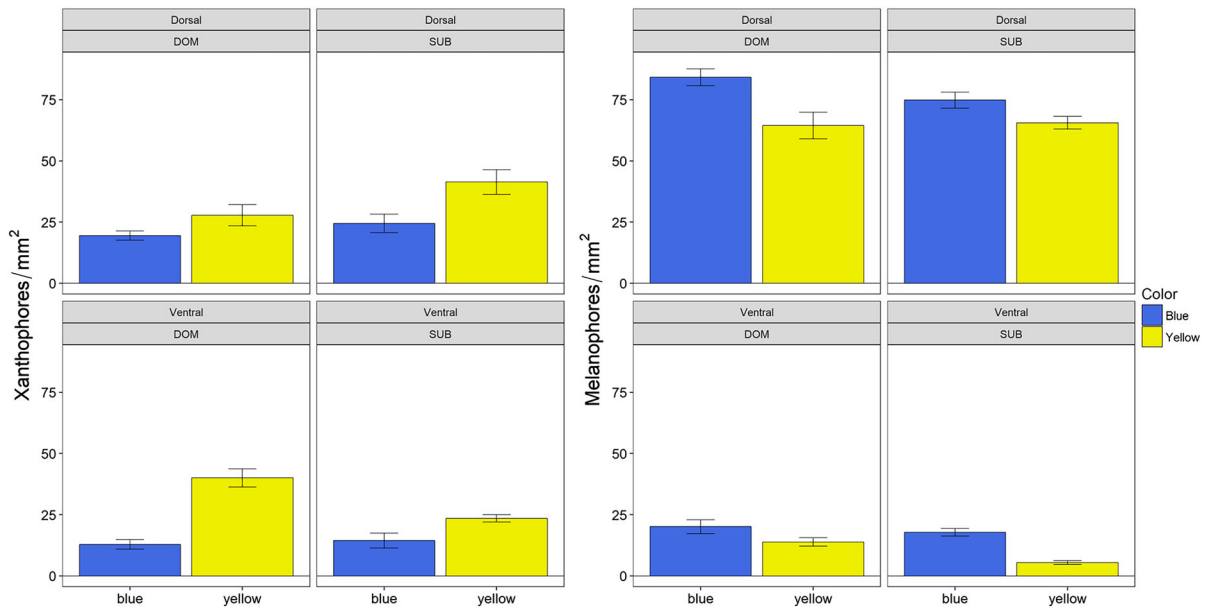


Fig. 5 Melanophore and xanthophore density. Melanophore and xanthophore density of scales selected from the dorsal and ventral side of blue and yellow males in dominant (DOM) and

subordinate males (SUB). The data is based on the average chromatophore density of two scales selected from each side of experimental fish. Shown are the mean + SE

Table 4 Xanthophore and melanophore density in dominant males

	Xanthophore density			Melanophore density		
	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value
Fixed parts						
(Intercept)	19.46	11.93 to 27.00	< 0.001	84.21	76.13 to 92.28	< 0.001
Side	− 6.61	− 12.23 to − 0.99	0.024	− 64.11	− 71.72 to − 56.50	< 0.001
Color	8.35	− 2.31 to 19.00	0.129	− 19.72	− 31.14 to − 8.30	0.001
Side × color	18.82	10.87 to 26.77	< 0.001	13.47	2.70 to 24.23	0.017
Random parts						
σ^2	65.793			120.631		
$\tau_{00, Fish}$	85.343			75.486		
N_{Fish}	16			16		
ICC_{Fish}	0.565			0.385		
Observations	64			64		
R^2/Ω_0^2	0.800/0.795			0.908/0.908		
AIC	490.724			520.417		

Bold indicates significant correlations ($p < 0.05$)

Indicated are the effect of morph (blue vs. yellow) and body region (side: dorsal vs. ventral). Shown are the within-group variance (σ^2), the between-group variance (τ_{00}), the sample size of the random effects (experiment and tank), and the intraclass coefficient correlation (ICC). The sample size (observations) as well as the R^2 and Ω_0^2 values of the model are also indicated

the flowerpots ($n = 5$) on the right side of the tank (Table 7). In four cases, dominant males in remaining flowerpots in unstable tanks were evicted and forced to

descend by one of the newly displaced dominants (Table 7). Overall, 65% of dominant males in unstable tanks experienced at least one status change,

Table 5 Xanthophore density in subordinate males

	Xanthophore density			Xanthophore density		
	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value
Fixed parts						
(Intercept)	24.51	16.02 to 32.99	< 0.001	26.46	18.25 to 34.66	< 0.001
Side	– 10.08	– 16.36 to – 3.79	0.003	– 13.98	– 18.56 to – 9.40	< 0.001
Color	16.87	4.88 to 28.87	0.009	12.97	1.83 to 24.12	0.027
Side × color	– 7.80	– 16.69 to 1.09	0.093			
Random parts						
σ^2	82.314			87.388		
$\tau_{00, \text{Fish}}$	108.682			107.414		
N_{Fish}	16			16		
ICC_{Fish}	0.569			0.551		
Observations	64			64		
R^2/Ω_0^2	0.778/0.772			0.764/0.758		
AIC	505.300			506.171		

Bold indicates significant correlations ($p < 0.05$)

Indicated are the effect of morph (blue vs. yellow) and body region (side: dorsal vs. ventral). Shown are the within-group variance (σ^2), the between-group variance (τ_{00}), the sample size of the random effects (experiment and tank), and the intraclass coefficient correlation (ICC). The sample size (observations) as well as the R^2 and Ω_0^2 values of the model are also indicated

Table 6 Melanophore density in subordinate males

	Melanophore density			Melanophore density		
	Estimate	Conf. int.	<i>P</i> value	Estimate	Conf. int.	<i>P</i> value
Fixed parts						
(Intercept)	74.83	69.86 to 79.81	< 0.001	75.63	70.94 to 80.31	< 0.001
Side	– 57.05	– 61.81 to – 52.29	< 0.001	– 58.64	– 62.03 to – 55.25	< 0.001
Color	– 9.23	– 16.27 to – 2.20	0.012	– 10.83	– 17.00 to – 4.65	0.001
Side × color	– 3.18	– 9.91 to 3.54	0.357			
Random parts						
σ^2	47.106			47.950		
$\tau_{00, \text{Fish}}$	27.928			27.717		
N_{Fish}	16			16		
ICC_{Fish}	0.372			0.366		
Observations	64			64		
R^2/Ω_0^2	0.960/0.960			0.959/0.959		
AIC	459.624			458.476		

Bold indicates significant correlations ($p < 0.05$)

Indicated are the effect of morph (blue vs. yellow) and body region (side: dorsal vs. ventral). Shown are the within-group variance (σ^2), the between-group variance (τ_{00}), the sample size of the random effects (experiment and tank), and the intraclass coefficient correlation (ICC). The sample size (observations) as well as the R^2 and Ω_0^2 values of the model are also indicated

compared to 34% in stable tanks. These data suggest that the flowerpot manipulation triggered social rank changes and increased social instability.

The majority of the males retained their original color phenotype (Table 8, Figs. 6, 7). We observed two males that were yellow dominant in week 1 who

Table 7 Post-manipulation strategies in stable and unstable communities

Stable tanks					Unstable tanks					
Tank	DOM =	SUB ↑	DOM +	DOM -	Tank	DOM ↓	DOM =	SUB ↑	DOM +	DOM -
B1	3	0	0	0	C1	2	2	0	0	0
E1	1	0	1	1	D1	1	1	0	0	0
L1	3	2	0	1	J1	0	0	1	2	1
N1	4	0	0	0	K1	2	2	0	0	0
O1	0	2	0	2	Q1	2	2	0	0	0
P1	3	0	1	1	W1	0	0	0	1	2
V1	2	1	0	0	X1	0	0	0	1	0
Total	16	5	2	5	Y1	1	1	0	1	1
					Total	8	9	1	5	4

DOM ↓	DOM =	SUB ↑	DOM +	DOM -
DOM lost territory due to flowerpot being removed (left side of tank)	DOM maintained same flowerpot after manipulation (right side of tank)	SUB rose by taking empty flowerpot or taking flowerpot from DOM	DOM took flowerpot from another DOM (after losing flowerpot in unstable tank)	DOM loses flowerpot to another DOM or SUB

Table 8 Rank and color morph transitions in stable and unstable communities

Stable					Unstable				
	DOM Blue	DOM Yellow	SUB Blue	SUB Yellow		DOM Blue	DOM Yellow	SUB Blue	SUB Yellow
DOM Blue	8	0	1	0	DOM Blue	6	0	8	0
DOM Yellow	0	10	1	3	DOM Yellow	0	7	1	3
SUB Blue	0	1	19	6	SUB Blue	0	0	25	10
SUB Yellow	0	3	3	15	SUB Yellow	0	2	7	11

The left columns show the status and color just prior to the flowerpot manipulation (week 1), the top rows shows the status and color on the final day of observation at the end of week 2 (this is 1 week post manipulation)

became blue subordinate in week 2, one in each treatment. There was one subordinate blue male in week 1 that became yellow dominant in week 2 in a stable community. We found that the proportion of yellow and blue males was not significantly influenced by treatment (LMM, time × treatment effect: -0.02 , 95% CI $[-0.09$ to $0.05]$, $P = 0.583$). We repeated this analysis using all global observations during the

2-week experimental period. There was a general trend of an increased proportion of yellow males over this 2-week period (LMM, effect of time: 0.03 , 95% CI $[0.01$ – $0.06]$, $P = 0.012$). However, this effect was not influenced by treatment (LMM, time × treatment effect: -0.02 , 95% CI $[-0.06$ to $0.01]$, $P = 0.213$).

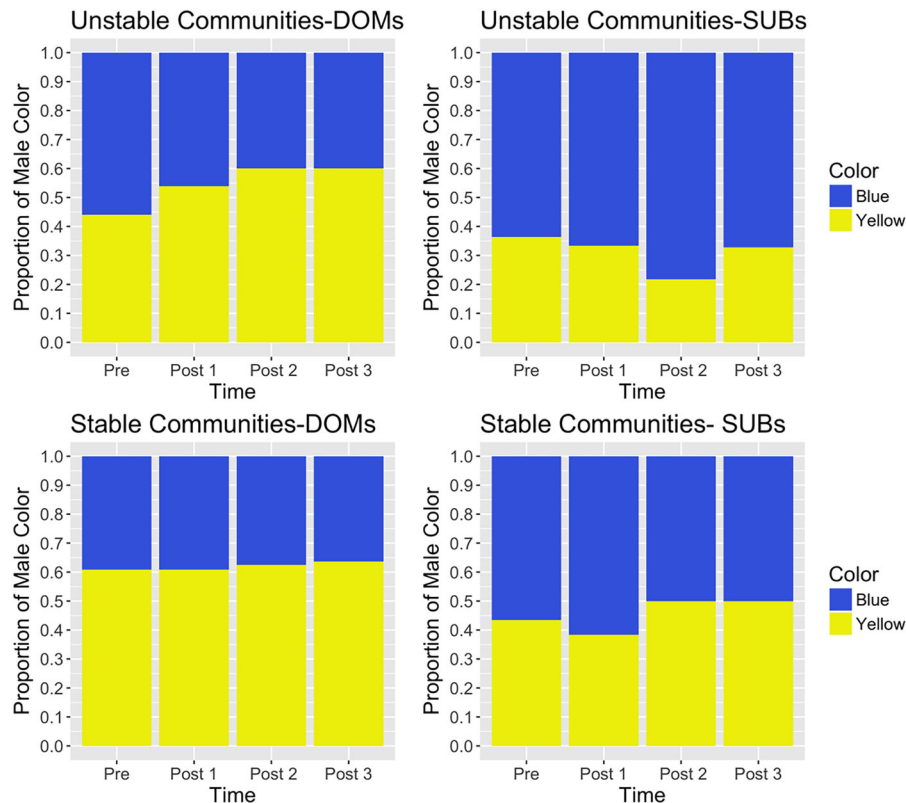


Fig. 6 Proportion of male color after descent. Proportion of yellow and blue males was observed before (pre) and up to 1 week after (post 3) flowerpot manipulation. DOM males (left)

and SUB males (right) were compared in stable (bottom) and unstable (top) communities. No significant differences in color proportion were found following manipulation

Discussion

Natural and sexual selection on male coloration in haplochromine cichlids has received a lot of attention in the literature (Salzburger, 2009; Meier et al., 2017). Coloration is an important target in mate choice and male–male interactions (Dijkstra & Groothuis, 2011; Ding et al., 2014; Selz et al., 2014). The yellow–blue color dichotomy is common in haplochromine cichlids, but we have limited understanding of the physiological basis of these color differences. In the current study, we characterized color variation in *A. burtoni* where males are either yellow or blue.

We first confirmed that the melanocortin hormone α -MSH can influence the expression of yellow coloration. Our in vivo pharmacology data show that α -MSH increased yellow coloration in both yellow and blue males, consistent with a previous study (Dijkstra et al., 2017). In contrast to expectation, there was no dose-dependent effect of exogenous α -MSH on

the degree of the color change for yellow males. However, in blue males, we observed a more pronounced increase in yellow coloration at both the low and high concentration relative to the medium concentration of exogenous α -MSH. While non-linear effects are not uncommon in endocrine systems, it is difficult to interpret these dose-dependent effects of exogenous α -MSH on coloration. The color change triggered by exogenous α -MSH was transient and males displayed their original coloration by the end of the day (personal observation, GD). This rapid and transient effect of α -MSH is consistent with the fact that this hormone acts via increased levels of the second messenger cAMP.

In teleost fish, aggregation and dispersion of pigments in chromatophores can cause rapid changes in body color and is under hormonal and neuronal regulation (Leclercq et al., 2010; Sköld et al., 2013). Pigment dispersal increases body pigmentation while pigment aggregation decreases body pigmentation

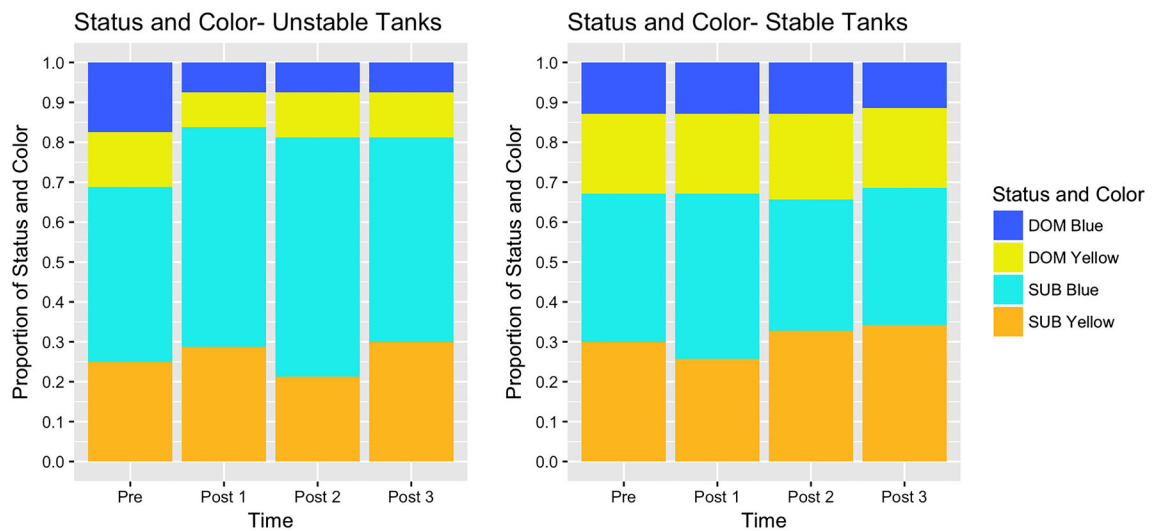


Fig. 7 Proportion of male status and color after descent. Proportion of male status and color is displayed before (pre) and up to a week after flowerpot manipulation (post 3). Status and color for all males (150 total) were observed for 2 weeks in unstable (left) and stable (right) communities. Males were

categorized at each observation as either DOM blue (blue), DOM yellow (yellow), SUB blue (cyan), or SUB yellow (orange). No significant differences in status and color proportion were found following manipulation

(Sköld et al., 2013). We confirmed that the α -MSH-induced increase in yellowness is due to pigment dispersion of the xanthophores. In our in situ xanthophore dispersion scale experiment, we found that exogenous α -MSH caused very rapid dispersion in the xanthophores. Maximum dispersion was achieved within ~ 7 – 8 min, but there appeared to be a dose-dependent effect on dispersion rate such that at the lowest and highest concentrations of α -MSH the xanthophores of yellow males dispersed significantly faster than those of blue males (although there was no difference between treatments on final dispersal). We found that compared to blue males, yellow males were able to disperse their xanthophores over a larger percent of the available area, most likely due to the fact that they have larger xanthophores compared to blue males (Dijkstra et al., 2017). Our results are consistent with the effects of α -MSH on pigment dispersion in other fish (Kobayashi et al., 2012; Sköld et al., 2013). Previous studies quantified the degree of chromatophore dispersion using an index, (e.g., 1–5, Hogben & Slome, 1931), often using a single pre and post hormone manipulation scoring of dispersion index. We used a digital color selection method that not only quantitatively measured the degree of dispersion but also provided insight into the temporal dynamics of α -MSH-induced pigment dispersion in

xanthophores. Finally, it is important to note that color change is mediated by many chromatophore types, including light-reflecting iridophores which are known to show aggregation in response to α -MSH (Ligon & McCartney, 2016). Understanding how α -MSH influences different chromatophore types and their interaction is an exciting avenue for future studies.

We showed that yellow and blue color morphs in *A. burtoni* differ in chromatophore density. Overall, blue males tended to have more melanophores than yellow males, while yellow males, not surprisingly, had a higher density of yellow xanthophores compared to blue males. However, blue males expressed a considerable amount of xanthophores ranging (depending on status and body region) from approximately a third to almost two-thirds the xanthophore density found in yellow males. The presence of xanthophores explains why blue males are capable of expressing yellow coloration in response to hormones that trigger pigment dispersion in the xanthophores. It also means that blue males keep these xanthophores in an aggregated state under normal circumstances. We recently found that melanocortin signaling is stronger in the skin of blue males than in yellow males, with higher expression levels of POMC [which encodes the peptide hormone pro-opiomelanocortin (POMC), the

precursor of melanocortin (Cone, 2006; Ducrest et al., 2008; Harris et al., 2014)], melanocortin receptor 1, and lower expression level of agouti signaling peptide, and antagonist on several melanocortin receptors, including *mcr1* (Cerdá-Reverter et al., 2011). One reason yellow males are yellow is that they keep the xanthophores in a dispersed state. This is surprising given the lower level of melanocortin signaling in yellow males, which is typically linked to pigment aggregation (Kobayashi et al., 2012; Sköld et al., 2013). It is, however, possible that yellow males have higher levels of circulating α -MSH although a preliminary analysis suggests this is not the case (Dijkstra, unpublished data). In addition, other endocrine factors are also important in this process (Leclercq et al., 2010), and more detailed studies are needed to understand the physiological and developmental basis of yellow and blue coloration in *A. burtoni*.

Color change has been extensively studied in poikilotherms in the context of background color adaptation, mimicry, and ontogenetic color shifts (Cortesi et al., 2016; Henning et al., 2013; Kang et al., 2016). In *A. burtoni* the expression of yellow and blue nuptial coloration is not linked to background color or sexual maturation. While many studies have focused on how changes in social status alters hormone profiles (Maruska & Fernald, 2010) and reproduction (Fernald & Maruska, 2012), there has been little attention given to how status changes may alter coloration. We hypothesized that social stress caused by social descent alters color phenotype. However, although many males changed color over the course of the experiment, with the proportion of yellow males generally increasing over time, this effect was not related to social stability treatment. All descending males ($n = 23$) retained their original dominant coloration after transitioning to subordinate status after the manipulation. These findings are consistent with a previous study (Korzán et al., 2008) that found simultaneous transitions in status and color are rare. We also hypothesized that increased short term competition and social instability brought on by rapid loss of available territories would lead to a change in the relative number of yellow and blue males. However, we found that community coloration did not significantly change after the habitat disruption, suggesting that increased competition or social instability does not influence the expression of color

phenotype. It is possible that repeated social stress over longer periods of time would have an effect on the expression of color phenotype. Future experiments should focus on these potential long-term effects on color phenotype.

In conclusion, we found evidence that the melanocortin system regulates physiological color change in *A. burtoni*. We characterized chromatophore density and found morph-specific patterns in melanophore and xanthophore density, with a surprisingly high xanthophore density in blue males. Social stress resulting from social instability did not influence the expression of color phenotype. Future studies should explore how long-term social stress and changes in melanocortin signaling influence the expression of color phenotype in *A. burtoni*.

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