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†This study is dedicated to the memory of Stephanie A. Flores, who died in a tragic accident only two weeks before her graduation from UT Austin.

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The melanocortin system regulates body pigmentation and social behaviour in a colour polymorphic cichlid fish†

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The melanocortin system is a neuroendocrine system that regulates a range of physiological and behavioural processes. We examined the extent to which the melanocortin system simultaneously regulates colour and behaviour in the cichlid fish *Astatotilapia burtoni*. We found that yellow males are more aggressive than blue males, in line with previous studies. We then found that exogenous α-melanocyte-stimulating hormone (α-MSH) increases yellowness of the body and dispersal of xanthophore pigments in both morphs. However, α-MSH had a morph-specific effect on aggression, with only blue males showing an increase in the rate of aggression. Exogenous agouti signalling peptide (ASIP), a melanocortin antagonist, did not affect coloration but reduced the rate of aggression in both colour morphs. Blue males had higher cortisol levels than yellow males. Neural gene expression of melanocortin receptors (*mcr*) and ligands was not differentially regulated between colour morphs. In the skin, however, *mc1r* and pro-opiomelanocortin (*pomc*) *β* were upregulated in blue males, while *asip 1* was upregulated in yellow males. The effects of α-MSH on behaviour and body coloration, combined with morph-specific regulation of the stress response and the melanocortin system, suggest that the melanocortin system contributes to the polymorphism in behaviour and coloration in *A. burtoni*.

1. Introduction

Aggressive competition between males can be a major diversification force [1–5]. When a male secondary sexual trait, such as body coloration (pigmentation), is used as a cue in these interactions, competition to gain access to females or resources can have an impact on the evolution of phenotypic and genetic diversity [2,3]. Stronger competition among same-coloured individuals than those with different colours can generate negative frequency-dependent selection [2]. In many animal clades, discrete colour variation within species is strongly correlated with variable aggression, whereby one morph is intrinsically more aggressive than the other [3,5]. Such asymmetries in agonistic behaviour between morphs or species influences the extent to which competing phenotypes can coexist, change the shape of frequency-dependent selection, and may influence agonistic character displacement [2,3,5]. However, the physiological mechanisms underlying trait covariance are often poorly understood.

It is widely recognized that hormones are critically involved in the adaptation and evolution of complex traits such as body pigmentation and social behaviour [6,7]. Endocrine systems link seemingly unrelated traits, and changes in hormone levels can affect multiple traits [8,9]. While correlated changes in suites of traits

may be adaptive (e.g. [4,10,11]), hormonal pleiotropy may also constrain evolutionary change when selection on a given trait has the potential to alter other characters [6,7].

The melanocortin system is a neuroendocrine system that links body pigmentation to a suite of behavioural and physiological functions across vertebrates [12]. The peptide hormone pro-opiomelanocortin (POMC) serves as a precursor for several (neuro)peptides including α- melanocyte-stimulating hormone (α-MSH), adreno-corticotrophin (ACTH) and β-endorphin [9,12,13]. The action of melanocortins is mediated by activation of a family of melanocortin receptor subtypes (MC1R through to MC5R [12]; in fishes: [14]). The *pomc* gene is expressed in the pituitary (which releases melanocortin peptides into the blood stream), the brain and peripheral tissues, including the skin. α-MSH stimulates the synthesis of black eumelanin in skin melanocytes by activating the MC1R. In teleost fishes, aggregation and dispersion of pigments in chromatophores can cause rapid changes in body colour and is under hormonal and neuronal regulation [15]. Pigment dispersal increases body pigmentation while pigment aggregation results in less body pigmentation [15]. The neuropeptide α-MSH is known to have strong dispersing effects in most fish chromatophores [15,16].

Aside from the action of α-MSH in the skin, it also binds to other MCRs including those expressed in the brain, where it regulates social behaviour, appetite and stress physiology [9,12]. α-MSH acts as a neurotransmitter in the brain where it can modulate behaviour mainly via central MC3R and MC4R in a variety of ways (e.g. by modulating the dopaminergic reward system [17]). Agouti signalling peptide (ASIP) and Agouti-related peptide (AgRP) have diverse functional roles in feeding, pigmentation and background adaptation mechanisms [9]. ASIP is an inverse agonist of the MC1R and the MC4R receptors. During development it helps to establish the dorsal–ventral pigmentation in mammals and fishes (ASIP1 in fishes) [18]. In teleosts, ASIP has been shown to antagonize the melanophore dispersing effect of α-MSH *in vitro* [18]. In numerous polymorphic vertebrate species it has been found that darker individuals are more aggressive and less sensitive to stress than their paler counterparts, consistent with the pleiotropic regulation of body pigmentation and other traits by the melanocortin system (Ducrest *et al.* [9]).

The adaptive radiations of haplochromine cichlid fishes in the East African Great Lakes provide textbook examples of rapid diversification through natural and sexual selection [19]. Several species display covariance in body coloration and territorial aggression [20]. The cichlid fish *Astatotilapia burtoni* from Lake Tanganyika lives in a lek-like social system in which dominant males defend a spawning territory. Subordinate males are non-territorial and lack bright coloration [21,22]. Dominant males express bright blue or yellow body colours while subordinate males can be pale blue or yellow [23,24]. The yellow-blue colour polymorphism has evolved repeatedly in East African cichlid species flocks [25,26]. In *A. burtoni*, colour is correlated with agonistic behaviour and hormone profile, but males can switch between colours [23], making it a unique system to study the hormonal regulation of the polymorphism in coloration and behaviour.

In this study, we examine how the melanocortin system simultaneously regulates colour, behaviour, hormones and gene expression in the cichlid fish *A. burtoni*. We will test the following specific hypotheses: (i) exogenous α-MSH increases yellowness of the body and increases aggression, while ASIP has the reverse effect; (ii) the phenotypic effects of α-MSH on colour are mediated by the dispersing effects of α-MSH on the xanthophores; and (iii) at the level of gene expression, the melanocortin system is more activated in yellow males than in blue males in both the brain and the skin.

2. Material and methods

(a) Experimental animals

Astatotilapia burtoni used in this study were descended from a wild-caught stock population and were group housed (eight males and 12 females per tank) in 110 l aquaria as previously described [22]. All males were tagged just below the dorsal fin with coloured beads attached to a plastic tag (Avery-Dennison, Pasadena, CA). All procedures comply with current laws in the USA. For all experiments, the experimenters were blind to the treatment.

(b) Quantitative behavioural analysis

Quantitative behavioural measurements consisted of frequency of display of various behaviours described elsewhere [27] during a five observation period: *aggression* was based on the sum of: chase and bites to males and females; border threats (usually towards other dominant males), lateral display towards male and females; *courtship* was based on the sum of courtships (lead swims and quivers). For all experiments, we calculated the frequency of behaviour per 1 min interval.

To test for behavioural differences between yellow and blue morphs, we examined the rate of aggression and the rate of courtship of dominant males in a meta-analysis of eight experiments carried out by different members of the Hofmann laboratory (for more details, see the electronic supplementary material, Method S1).

To analyse the rate of aggression and the rate of courtship, we performed a linear mixed model (LMM) analysis with 'tank' and 'experiment' as random effects and 'morph' as fixed effect. More details on the statistical analysis are given below in §2f.

(c) Pharmacology: effect of α-melanocyte-stimulating harmone and agouti signalling peptide on behaviour and body coloration

A detailed protocol can be found in the electronic supplementary material, Method S2. In brief, we treated dominant and subordinate males with saline, α-MSH or ASIP intraperitoneal injection. Quantitative behavioural measurements were recorded (as described in §2b) 30 min (α-MSH) or 70 min (ASIP) after injection. To quantify yellowness, we used a five-point scale ranging from 0 (no yellow coloration) to 5 (strong bright yellow coloration). Yellowness was quantified before and 70 min after the injection. The intervals between hormone injection and behavioural or colour measurements were chosen based on when we expected the strongest effect (for more details see the electronic supplementary material, Method S2).

We tested whether α-MSH and ASIP affected behaviour and body coloration using the differences between pre-treatment and post-treatment scores (post-pre). We used LMMs to test the influence of colour morph and treatment on aggression (natural log transformation [ln $(X + 1)$]) or yellowness. More details about the statistical analysis is given below in §2f.

(d) Pharmacology: effect of α-melonocyte-stimulating harmone on pigment dispersal

To examine the cellular effect of α-MSH, we conducted a pharmacological experiment on *A. burtoni* scales. Individual scales of yellow and blue males were removed from dorsal and ventrolateral

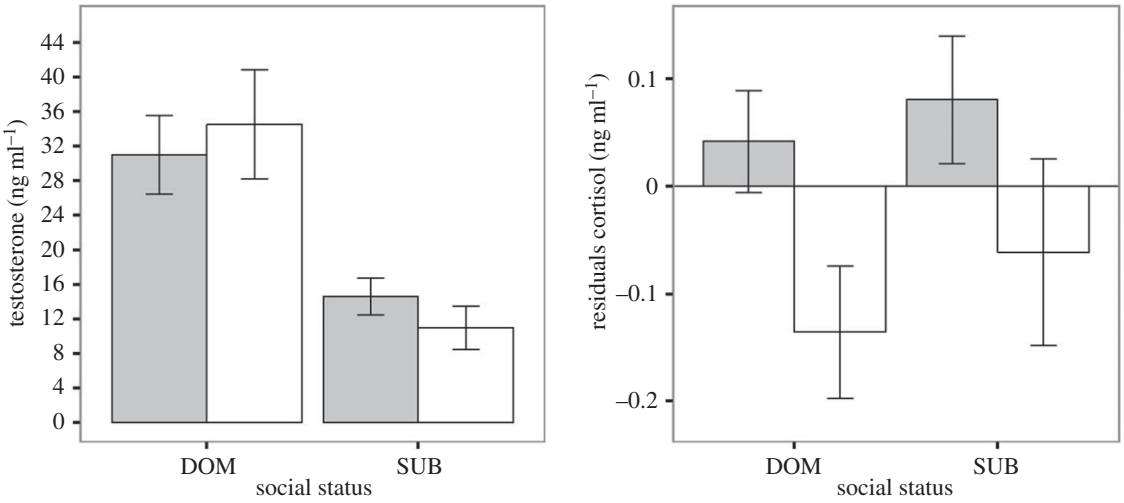

Figure 1. Hormone levels are influenced by colour and status. Circulating testosterone levels (nanogram per millilitre) were significantly higher in dominant (DOM) males compared with subordinate (SUB) males, but there was no difference between yellow (open) and blue morphs (grey). Yellow males had significantly lower circulating cortisol levels (nanogram per millilitre) than blue males. Residuals of circulating cortisol (nanogram per millilitre) were calculated from a linear regression of the log-transformed cortisol (nanogram per millilitre) values over the duration of restraint stress. Shown are the mean ± s.e. Sample sizes, dominant males: yellow, $n = 16$, blue, $n = 15$; Subordinate males: yellow, $n = 9$, blue, $n = 26$.

side (referred to as 'ventral' for brevity) and incubated at different concentrations of α-MSH concentration (0.1, 10 and 1000 nM) or saline control. Photographs were taken before and after 30 min of treatment. The pigment-dispersing activity was calculated for 10 randomly selected chromatophores as the proportion increase in chromatophore area after 30 min incubation relative to baseline area. The influence of colour morph, social status and body region on pigment-dispersing activity of α-MSH was analysed using LMMs. For detailed protocol, see the electronic supplementary material, Method S3.

(e) Tissue collection

Dominant and subordinate males were observed in several communities as described above. The behaviour of each male was quantified for 5 min on the day of tissue collection. All focal fish were removed from their tanks and transferred to the working station in a 1 l bucket (for the restraint stress procedure described below). Blood was drawn and brain dissected and processed as described elsewhere [22]. Skin tissue comprising an area of 0.5 × 0.5 cm was dissected from the dorsal and the ventral side of the body, transfered into RNAlater at 4°C overnight, and then stored at −20°C until RNA extraction.

We measured circulating cortisol and testosterone levels and examined gene expression patterns in the preoptic area (POA) and the dorsal and ventral skin. Details on hormone assays, RNA isolation from the skin, RNA isolation from the POA, reverse transcription and quantitative real-time PCR (qRT-PCR) can be found in the electronic supplementary material, Method S4.

Before the blood draw, all focal fish were subjected to a restraint stress procedure in a 1 l bucket (ranging from 1 to 7.5 min, from the time between initial disturbance in the home tank to the time of the blood draw). To explain variation in CORT (log-transformed) or testosterone (square-root transformed), we performed an LMM analysis. Candidate models included 'tank' as random effect, and restraint stress duration, colour morph, body weight and status as fixed effects. We used linear models to test the influence of colour morph and social status (for skin data only) on gene expression. To examine for morph-dependent covariance patterns, we calculated Pearson's correlation coefficients between gene expression (*pomc* β and *crf* only), behaviour, and hormones, and plotted this as heatmaps (see the electronic supplementary material, Method S5 for more details).

(f) Data analysis

Details of our statistical analyses can be found in the electronic supplementary material, Method S6. In brief, all analyses were conducted in R v. 3.2.1 (R Core Team, 2012). We used linear models or LMMs for analyses where we needed to include a random grouping effect (e.g. fish identity). We selected LMMs, implemented using a maximum-likelihood protocol, that best-fit the data using Akaike information criterion corrected for small sample sizes (AICc). The results of all models within two AICc of the top model are reported because these are considered candidate best-fit models. We also report 95% confidence intervals (CIs) of parameter estimates of all fixed effects.

3. Results

(a) Yellow dominant males are more aggressive than blue dominant males

Yellow males were significantly more aggressive than their blue counterparts (LMM, effect of morph: 0.93, 95% CI[0.31–1.55], $p = 0.003$; see figure and model summary in the electronic supplementary material, Results S1) but morphs did not differ in the rate of courtship behaviour.

(b) Cortisol but not testosterone is differentially regulated between colour morphs

The selection procedure for analysing variation in testosterone levels resulted in two best-fit models to be considered. In both best-fit models, status had a significant effect with dominant males displaying higher testosterone levels than subordinate males (figure 1 and for model summaries see the electronic supplementary material, Results S2). The interaction between status and restraint stress duration was not significant (table 1).

The selection procedure for analysing variation in cortisol levels resulted in two best-fit models to be considered. Both models retained restraint stress duration and colour morph as significant effect (LMM, effect of restraint stress duration: 0.17, 95% CI[0.13–0.20]; for figure and model summary, see the electronic supplementary material, Results S3), suggesting that blue males have higher cortisol levels than yellow males

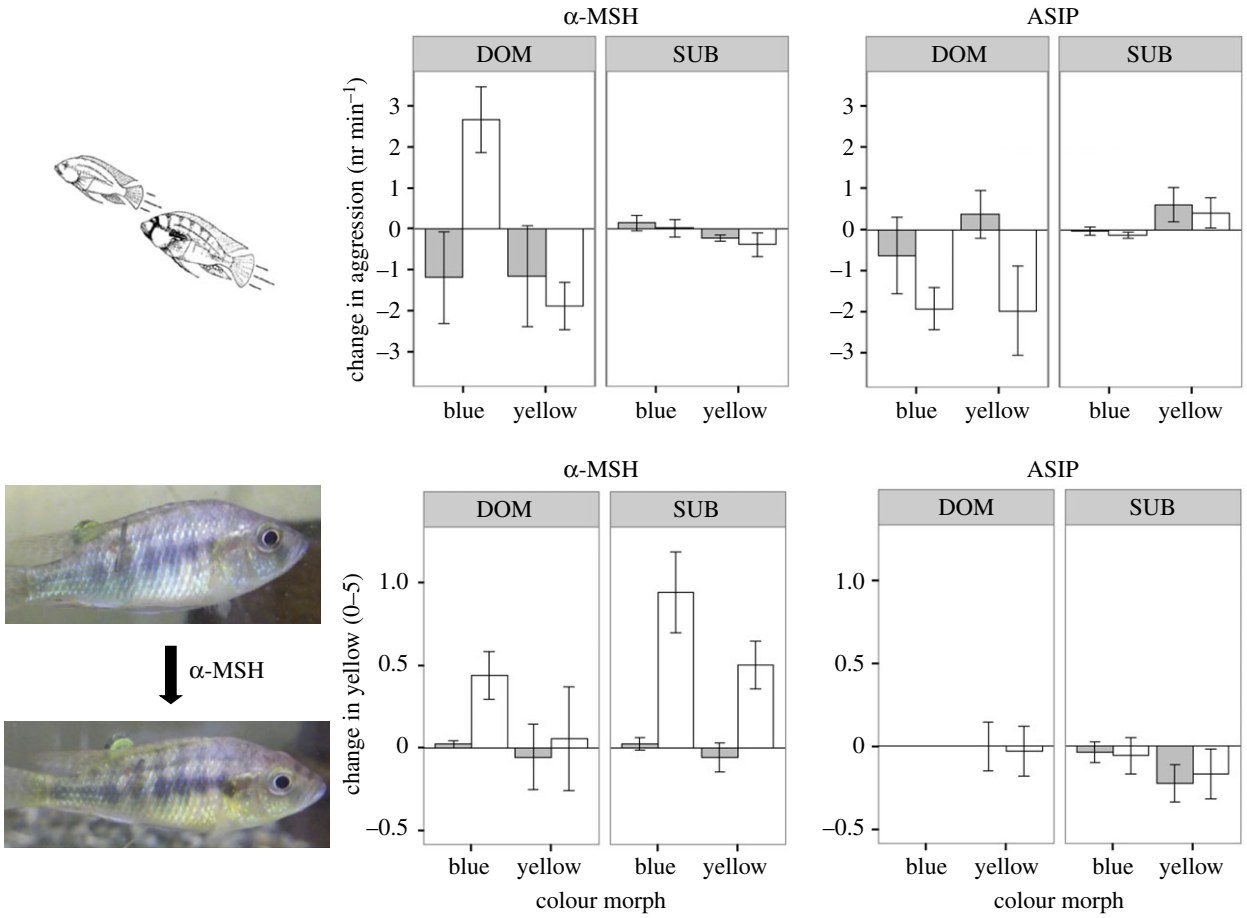

Figure 2. (Top) α-MSH but not ASIP has morph-specific effects on aggression. α-MSH increased the rate of aggression in blue males but not in yellow males. The change in behaviour in response to α-MSH (open) and saline control (grey) was calculated as the differences between pre-treatment (=baseline) and post-treatment scores (post-pre). ASIP reduced the rate of aggression in both colour morphs. The cartoon on the left shows a territorial male chasing a female (Maruska & Fernald). Sample sizes for α-MSH experiment, dominant males: yellow, $n = 9$ (saline), $n = 9$ (α-MSH), blue, $n = 11$ (saline), $n = 11$ (α-MSH); subordinate males: yellow, $n = 10$ (saline), $n = 10$ (α-MSH), blue, $n = 9$ (saline), $n = 12$ (α-MSH). Samples sizes for ASIP experiment, dominant males: yellow, $n = 7$ (saline), $n = 7$ (ASIP), blue, $n = 9$ (saline), $n = 9$ (ASIP); subordinate males: yellow, $n = 3$ (saline), $n = 5$ (ASIP), blue, $n = 5$ (saline), $n = 3$ (ASIP). (Bottom) α-MSH but not ASIP increases yellowness. The change in yellow score (post-pre) in response to α-MSH or ASIP (open) and saline control (grey). The degree of yellowness was determined using a five-point scale: 0 (no yellow coloration) to 5 (strong bright yellow coloration). ASIP did not influence yellow coloration. To the left, a representative photo of a blue SUB before (top) and 1 h after (bottom) α-MSH injection is shown. Sample sizes for α-MSH experiment, dominant males: yellow, $n = 6$ (saline), $n = 6$ (α-MSH), blue, $n = 8$ (saline), $n = 8$ (α-MSH); subordinate males: yellow, $n = 9$ (saline), $n = 9$ (α-MSH), blue, $n = 8$ (saline), $n = 11$ (α-MSH). Samples sizes for ASIP experiment, Dominant males: yellow, $n = 6$ (saline), $n = 6$ (ASIP), blue, $n = 8$ (saline), $n = 8$ (ASIP); subordinate males: yellow, $n = 3$ (saline), $n = 5$ (ASIP), blue, $n = 5$ (saline), $n = 3$ (ASIP).

and that the rate of cortisol secretion in response to an acute stressor is the same for yellow and blue males. Status did not have a significant effect on cortisol levels (for model summaries see the electronic supplementary material, Results S2). We ran a linear regression of log-transformed cortisol levels against restraint stress duration and examined the residuals to further explore differences in cortisol levels between morphs. In dominant males, residual cortisol levels were significantly higher in blue males than in yellow males. This was not the case in subordinate males (figure 1 and for model summaries see the electronic supplementary material, Results S2).

(c) Melanocortin receptor pharmacology modulates behaviour and colour

(i) α-melanocyte-stimulating hormone had a morph-specific effect on aggressive behaviour

In subordinate males, we did not detect an effect of α-MSH on behaviour (figure 2 and for model summaries see the electronic supplementary material, Results S4). However, dominant males treated with α-MSH exhibited significant changes in aggression compared with saline-treated males in a colour morph-dependent fashion (figure 2 and for model summaries see the electronic supplementary material, Results S4); blue males treated with α-MSH exhibited a significant increase in aggression compared with saline controls whereas α-MSH did not affect aggression in yellow males (LMM, effect of treatment in blue males on pre-post: 3.85, 95% CI[1.31–6.4], $p = 0.003$; in yellow males: −0.72, 95% CI[−2.47–1.04] $p = 0.445$). α-MSH treatment did not affect courtship behaviour in dominant males in a colour morph-dependent fashion (electronic supplementary material, Results S5). In dominant males ASIP treatment significantly reduced aggression (for model summaries see the electronic supplementary material, Results S4), and this effect was not influenced by colour morph (colour morph × treatment: −1.067, 95% CI[−3.53–1.39], $p = 0.408$). ASIP did not affect courtship behaviour (electronic supplementary material, Results S5), indicating that its action is fairly specific.

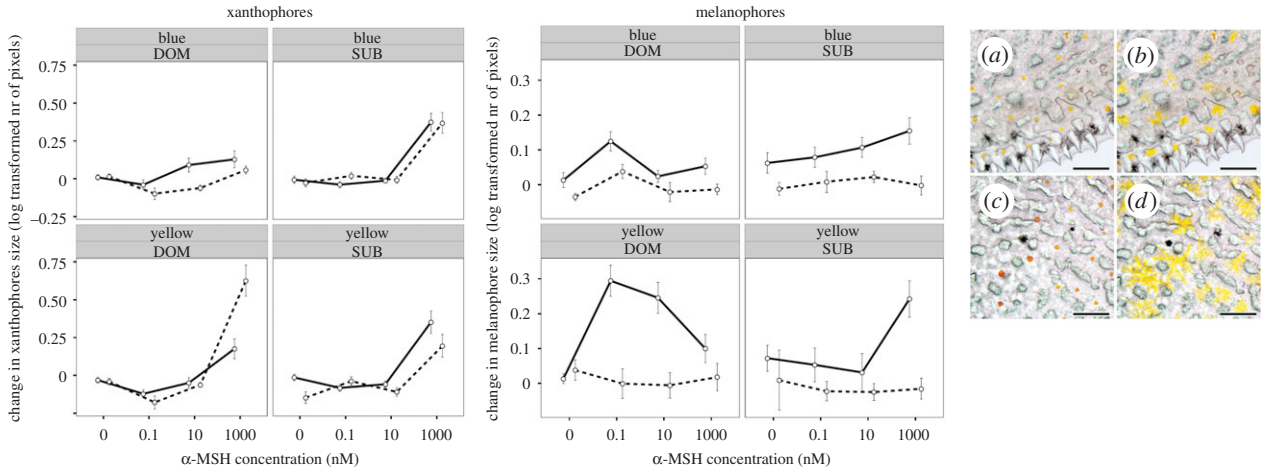

Figure 3. α-MSH disperses pigments in the xanthophores. Pigment-dispersing activity of α-MSH on xanthophores (left) and melanophores (middle) in yellow and blue males for each α-MSH concentration, body region (ventral: dashed line; dorsal: solid line), and status (dominant (DOM) males and subordinate (SUB) males). Shown are the mean ± s.e. of the differences between pre-treatment chromatophore size (=baseline) and post-treatment size (post-pre). (Right) Microphotographs showing rapid pigment-dispersing activity in xanthophores in a scale from the dorsal skin of dominant *A. burtoni* male (*a*,*b*: blue; *c*,*d*: yellow) before (*a*,*c*) and after (*b*,*d*) exposure to 1000 nM α-MSH. Magnification: X20. Scale bar, 0.1 mm. Sample sizes, dominant males: yellow, $n = 5$, blue, $n = 10$; subordinate males: yellow, $n = 5$, blue, $n = 10$.

(ii) α-melanocyte-stimulating hormone increased yellow body coloration

α-MSH increased the degree of yellowness (figure 2). In all four best-fit models the effect of treatment on colour change was significant suggesting that α-MSH increased the degree of yellowness compared with saline controls (for model summaries see the electronic supplementary material, Results S6). Importantly, this effect on coloration did not depend on colour morph (for model summaries see the electronic supplementary material, Results S6). In one of the best-fit models, α-MSH increased yellow coloration more in subordinates than in dominants (for model summaries see the electronic supplementary material, Results S6). By contrast, exogenous ASIP did not increase the degree of yellowness (figure 2, LMM with treatment as fixed effect, −0.02, 95% CI[−0.15–0.12], $p = 0.825$). The model selection procedure resulted in two models with limited explanatory power (both were within less than two ΔAICs of the null model without fixed effects), and, not surprisingly, neither morph nor status were retained as significant fixed effects (effects of morph or status Ps > 0.12). Using a quantitative digital analysis of colour photographs of test subjects, we confirmed the effect of α-MSH on yellowness (see the electronic supplementary material, Results S7).

(iii) α-melanocyte-stimulating hormone dispersed chromatophores in skin scales

α-MSH had a strong dispersing effect on the xanthophore pigments at its highest concentration (figure 3, for complete model summaries, see the electronic supplementary material, Results S8). As can be seen in the figure, the dose-dependent dispersion of α-MSH was dependent on status, morph and body region. In subordinate males, α-MSH had a highly significant pigment-dispersing effect on the xanthophores (LMM, effect of highest α-MSH concentration: 0.38, 95% CI[0.32–0.43], $p < 0.001$). In dominant males, the strongest pigment-dispersing effect of α-MSH was seen in the ventral side of yellow dominant males (LMM, morph × treatment × body region: 0.52, 95% CI[0.29–0.75], $p < 0.001$). α-MSH had variable effects on pigmentary dispersion of the melanophores across groups with pigment-dispersing effects in the dorsal side of dominant males at intermediate α-MSH concentration and higher concentrations in subordinate males (figure 3; for complete model summaries, see the electronic supplementary material, Results S8).

(d) Melanocortin system expression is differentially regulated between colour morphs in the skin but not the preoptic area

In the POA, none of the candidate genes were differentially expressed between yellow and blue males (linear model, Ps > 0.2, for figure and model summaries, see the electronic supplementary material, Results S9). Given the known interaction between *pomc β*, *crf*, cortisol and behaviour, we tested whether these variables covaried in a morph-specific manner using linear models. We detected no evidence for morph-specific correlations (Ps > 0.5; for heatmaps see the electronic supplementary material, Results S10). When combining yellow and blue males, plasma cortisol was a significant positive predictor of both *pomc β* transcript levels (linear model, $F_{1,10} = 7.867$, $p = 0.019$) and *crf* transcript levels ($F_{1,11} = 8.112$, $p = 0.019$).

In the skin of both subordinate and dominant males the expression of *asip 1*, *mc1r*, *mc5r* and *pomc β* varied according to colour morph and body region (for figures and LMM results see the electronic supplementary material, Results S11). To specifically test for the effect of morph on gene expression level, we built models containing only morph and dorsal–ventral as fixed factors because with the exception of *mc5r* the expression of all genes differed dramatically between the dorsal and ventral side. The expression of *mc1r* was significantly higher in blue males than in yellow males (LMM, effect of morph: −0.23, 95% CI[−0.31 to −0.14], $p < 0.001$). The expression of *mc5r* did not significantly differ between colour morphs (effect of morph: −0.06, 95% CI[−0.26–0.14],

$p = 0.55$). The expression of *asip 1* was significantly higher in yellow males than in blue males (effect of morph: −0.18, 95% CI[0.02–0.35], $p = 0.035$). *Pomc β* expression was significantly higher in blue males than in yellow males (effect of morph: −0.21, 95% CI[−0.32–−0.10], $p = 0.001$).

4. Discussion

Correlated traits are central to understanding the evolution and maintenance of individual variation [28] and discrete polymorphisms [29]. Moreover, divergence in correlated traits may play a crucial role in population differentiation and speciation [10,30]. However, the molecular and physiological mechanisms underlying these correlated traits are often poorly understood. Endocrine systems are known to underlie seemingly unrelated traits [6,31] and one particularly compelling system that underlies body pigmentation and a range of physiological and behavioural functions is the melanocortin system [9,12].

In this study, we examined how the melanocortin system simultaneously regulates colour and behaviour in the cichlid fish *A. burtoni*. We first confirmed that yellow males are more aggressive than blue males, and that blue males have higher cortisol levels than yellow males. We then found that exogenous α-MSH increases yellow body coloration by dispersing the pigment organelles in xanthophores. To our surprise, exogenous α-MSH had a morph-specific effect on aggression, with only blue males showing an increase in the rate of aggression in response to exogenous α-MSH. Exogenous α-MSH increased yellowness within minutes after administration (P. D. Dijkstra 2012, personal observation), consistent with the rapid, largely non-genomic effect of this peptide hormone [14]. Collectively, these findings suggest that the melanocortin system is differentially activated in blue and yellow males. In contrast with expectation, we found no morph differences in gene expression patterns in the POA. In the skin, however, *mc1r*, *pomc β* and *asip 1* expression levels were all morph-specific, suggesting that the melanocortin system is indeed differentially activated in blue and yellow males.

(a) Covariance in behaviour, hormones and body coloration

We performed a meta-analysis of eight studies which showed that in *A. burtoni* dominant yellow males are more aggressive than dominant blue males. This finding is consistent with the higher social dominance of yellow males previously described in a dyadic setting [23]. We also found that blue males exhibit higher levels of the stress hormone cortisol than yellow males during a restraint stress procedure, suggesting that blue males have higher cortisol levels and that these higher cortisol levels are maintained during an acute stressor. These findings are consistent with the observation that in *A. burtoni* subordinate blue males have higher cortisol levels when housed in a compartment with a dominant male [23]. Our findings add to a growing body of literature showing that phenotypic divergence is often associated with differential regulation of hormone synthesis (e.g. [32–34].

We found evidence that the melanocortin system influences both agonistic behaviour and body coloration. Exogenous α-MSH, a broad melanocortin agonist, had a morph-specific effect on behaviour with only blue dominant males showing an increase in the rate of agonistic behaviour after α-MSH injection. In addition, we found that exogenous ASIP, a melanocortin antagonist on the MC1R and the central MC4R reduced aggression in both colour morphs. α-MSH and ASIP did not affect courtship behaviour, indicating that its action on aggression is specific. The rapid effects of ASIP and α-MSH on behaviour were specific and are probably mediated by melanocortin receptors in the central nervous system. Aggression is a complex trait that is modulated by several interrelated neuroendocrine pathways [35,36]. Future studies should shed more light on central melanocortin signalling and modulation of aggression.

Exogenous α-MSH increased yellow body coloration with this effect being stronger in blue males. This means that blue males have a sufficient density of xanthophores to express yellow coloration but that they maintain the xanthophores in an aggregated state under normal circumstances. Using analysis of pigmentation responses in scales taken from a defined location on the fish body, we found that exogenous α-MSH disperses the pigments of xanthophores suggesting that the phenotypic (*in vivo*) effects of α-MSH are mediated by its direct pigment-dispersing action. Our results are consistent with the effects of α-MSH on pigmentation in other vertebrates, including the barfin flounder *Verasper moseri* [37] and the two-spotted goby *Gobiusculus flavescens*, [38] (see also [15,39]). Pigmentary responses of α-MSH in the melanophores were more complex; we observed pigment dispersion at intermediate α-MSH concentrations in dominant males and at higher concentrations in subordinate males, but these effects were only evident in the dorsal skin. We failed to detect an effect of exogenous ASIP on body coloration. In the barfin flounder, xanthophores exclusively express the MC5R (but see an example with exclusive expression of MC1R in goldfish, [40]). If this is the case in *A. burtoni*, ASIP is unlikely to have an effect because it has very weak binding to this MCR subtype. Future experiments should test whether ASIP can inhibit the pigmentary responses of α-MSH in *A. burtoni* scales.

Taken together, the simultaneous effects of α-MSH on behaviour and body pigmentation are consistent with a pleiotropic mechanism in the melanocortin system contributing to the correlation between colour and behaviour in *A. burtoni*.

(b) Morph-specific patterns of gene expression

The morph-specific effect of α-MSH suggests that yellow males have a higher melanocortin tone than blue males assuming that the relationship between aggression and the strength of central melanocortin signalling is curvilinear. Specifically, we hypothesized that yellow males express higher levels of *pomc* or *mcr* paralogues in the brain than blue males, and that the dynamic range for central melanocortin receptor transmission (and downstream effects on aggression) by α-MSH is higher in blue males than in yellow males. Given the pigment-dispersion and body coloration effects of α-MSH, we also hypothesize that yellow males display a higher melanocortin activity in the skin than blue males. In order to test these hypotheses, we measured mRNA levels in the POA, a major neuroendocrine centre, of genes related to the melanocortin system. We found no differences in gene expression levels between morphs. Yellow and blue males may differ at the level of posttranscriptional regulation of mRNA products and/or peptide modifications [41]. For example, Hu & co-workers [42] found that all three *pomc* paralogues were expressed in the brain and pituitary of *A. burtoni* but only

the peptide products of *pomc α1* were found in the pituitary. In the skin, the melanocortin system was more activated in blue males than in yellow males, in contrast with expectation: *mc1r* and *pomc β* expression levels were higher in blue males than yellow males, while *asip 1* expression levels were higher in yellow males than blue males. This finding is in contrast with the polymorphic Midas cichlids (*Amphilophus* sp.) where the gold morph, which has more xanthophores, upregulated the *mc1r* in comparison to the normal (dark) morph [43].

We are faced with a paradox here: blue males show a higher melanocortin tone than yellow males in the skin in terms of mRNA levels of *mc1r*, *pomc β* and *asip 1*. However, experimentally activating the melanocortin system using exogenous α-MSH led to an increase in yellow body coloration. It is important to note that this colour effect was a rapid physiological effect that disappeared after several hours as the hormone was degraded (P. D. Dijkstra 2012, unpublished data). It is well known that the melanocortin system does not only influence physiological colour changes driven by pigment granule migration, but also morphological colour changes that involve chromatophore proliferation, survival and differentiation [44]. Future studies should investigate how α-MSH influences these morphological colour changes in yellow and blue males.

Trait covariance can come about via a variety of mechanisms involving the melanocortin system. In the tawny owl (*Strix aluco*), two prohormone convertase genes whose products cleave POMC were differentially regulated in the feathers of colour morphs that differ in the degree of melanism [45]. Provided that such morph-specific expression patterns exist in other tissues, this could cause pigmentation to be linked to other traits such as the stress response. Another possible mechanism involves regulation via melanocortin receptors. There are many classic examples of mutations in the *mc1r* gene associated with variable coat colour, possibly owing to the fact that *mc1r* variability is free of pleiotropic effects because of its restricted expression in the skin ([46], but see [47]). It is important to note that we have found no evidence linking the polymorphism in *A. burtoni* with genotypic variants of melanocortin receptors.

5. Conclusion

Sexual selection, including male-male competition, has been implicated in the dramatic colour diversification of East African cichlid fishes [2], but the physiological underpinnings of this process has received limited attention. In this study, we have shown that the melanocortin system regulates both body coloration and behaviour in *A. burtoni*, and that yellow and blue males differ in plasma cortisol levels and, at the transcript level, in activity of the melanocortin system in the skin. Asymmetric aggression between conspecific colour morphs has been documented in several East African cichlid fish species, and the yellow-blue colour dichotomy is very common in a range of cichlid clades [26]. A better understanding of how the melanocortin system influences body coloration and other key life-history traits would be instrumental to advancing our understanding of the processes that drive the remarkably rapid speciation of these fishes.

Ethics. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

Data accessibility. Data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.702mg [48].

Authors' contributions. P.D.D. and H.A.H. conceived and designed the study; P.D.D. and S.A.F collected the behavioural and colour phenotyping data; S.M.M., R.M.H. and P.D.D. collected the molecular data; A.A.R., R.S.D. and P.D.D. collected the chromatophore data; P.D.D. and R.M.H. carried out the statistical analysis; P.D.D. drafted the manuscript with input from H.A.H. All co-authors contributed to the final version of the manuscript and gave approval for its publication with the exception of S.A.F.

Competing interests. The authors declare no competing interests.

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