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ORIGINAL ARTICLE

Metabolism, oxidative stress and territorial behaviour in a female colour polymorphic cichlid fish

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Abstract Intrasexual selection on body coloration is thought to play an important role in the evolution of colour polymorphism, but its physiological underpinnings have received limited attention. In the colour polymorphic cichlid *Neochromis omnicaeruleus*, three fully sympatric female colour morphs—a plain morph (P) and two conspicuously coloured blotched morphs, black-and-white blotched (WB) and orange blotched (OB)—differ in agonistic behaviour. We compared routine metabolic rate (when females were housed in social isolation), short-term energetic costs of interacting with a same-colour rival housed in an adjacent transparent chamber and oxidative stress between the three female colour morphs. WB females had a lower routine metabolic rate compared with the other colour morphs. WB females also had a lower active metabolic rate during inter-female interactions than OB females, while OB females used more oxygen per unit aggressive act than the other two colour morphs. However, there were no consistent differences in oxidative stress between the three morphs. Concerted divergence in colour, behaviour and metabolism might contribute to the evolution of these polymorphisms in sympatry.

Keywords Metabolic costs · Oxidative stress · Female-female competition · Sexual selection · Cichlid fish · Lake Victoria

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Introduction

Colour polymorphisms are widespread in several animal taxa such as birds, insects, lizards and fish (Gray and McKinnon 2007; Cole and Endler 2015). In many cases, disruptive, negative frequency-dependent selection is suggested to be involved in the origin and coexistence of colour morphs (Roulin 2004; Sinervo and Calsbeek 2006; Gray and McKinnon 2007; McLean and Stuart-Fox 2014). Disruptive selection is accompanied by poor fitness of intermediate phenotypes while negative frequency-dependent selection favours rare phenotypes. Selection can directly act on colour through social interactions (e.g. mate choice, male-male or female-female conflict), predation risk and thermoregulation (Endler 1980; Majerus 1998; Bittner et al. 2002; Seehausen and Schluter 2004; Grether et al. 2009; Hughes et al. 2013). However, selection can also act on other traits that are correlated with the colour pattern (Brooks 2000; Horth 2003; Ducrest et al. 2008; Gray et al. 2008; McKinnon and Pierotti 2010). For example, sympatric colour phenotypes may differ in agonistic behaviour, immunity and/or mating strategy (McKinnon and Pierotti 2010). An analysis of costs and benefits associated with such traits can help us understand how balancing selection operates to maintain more than one phenotype in wild populations.

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Across species or morphs, variation in colour is often associated with varying levels of social dominance and/or aggressiveness. In a range of taxa, colour phenotypes exhibit inherent differences in aggressiveness (Saino and Scatizzi 1991; Dijkstra et al. 2005; Pryke 2007; Ducrest et al. 2008; Dijkstra and Groothuis 2011). The benefits of elevated aggressiveness are priority of access to mates and resources (West-Eberhard 1979; Berglund et al. 1996; Wong and Candolin 2005), while an important cost is a higher rate of energy expenditure (Briffa and Sneddon 2007). Metabolic rate, the rate at which an animal oxidises substrates to produce energy, is a fundamental measure in the study of behavioural decisions and the evolution of life-history strategies, since the mitochondrial processes that underlie it are thought to impinge on several key physiological processes such as oxidative stress and aging (Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010). Oxidative stress and aging have major effects on a range of important life-history traits, including the expression of secondary sexual characters and the immune system (Monaghan et al. 2009; Garratt and Brooks 2012). Covariance between body coloration and metabolic rate could therefore result in different payoffs between colour morphs, which would be expected to affect the evolution of colour polymorphism. While previous studies have examined variation in metabolic rate across morphs (e.g. Borowsky 1984; Dijkstra et al. 2013), few studies have examined how morph-specific variation in metabolic rate covaries with oxidative stress. While mitochondria are a key source of the reactive oxygen species (ROS) that are a major contributor to oxidative damage to biomolecules such as proteins, lipids and DNA, the connection between mitochondrial activity (and hence metabolic rate) and oxidative stress is not clear, with recent studies reporting positive (Fletcher et al. 2013), negative (Salin et al. 2012) or no (Beamonte-Barrientos and Verhulst 2013) relationship between energy expenditure and levels of oxidative damage, partly due to the variable relationships between mitochondrial oxygen consumption and the production of both ATP and ROS (Salin et al. 2015). It is thus difficult to predict *a priori* whether colour morph differences in behavioural dominance are likely to be paralleled by differences in oxidative stress, and hence potential rates of aging.

The impressive species radiations of haplochromine cichlid fish of the Great African Lakes are an important model to understand speciation, adaptive radiation and colour polymorphism (Kocher 2004; Genner and Turner 2005; Seehausen 2006; Fan et al. 2012; Maan and Sefc 2013). Three distinct genetic colour morphs are found in the rock-dwelling Lake Victoria cichlid fish *Neochromis omnicaeruleus* that each also occur in several other species in this radiation: a plain morph (P) and two conspicuously coloured blotched morphs, black-and-white blotched (WB; black blotches on white) and orange blotched (OB; black blotches on orange). In terms of external appearance and ecology, these morphs differ in little other than colour (Seehausen et al. 1999; Magalhaes et al. 2010). P is presumably the ancestral morph, because the distribution of the blotched morphs across Lake Victoria cichlid species is nested within that of the P morph (Seehausen 1996). The WB and the OB morphs are predominantly found in females, presumably because colour morphs in this species have been linked to dominant female-determining genes which are X-linked (Seehausen et al. 1999). Intermediates between these morphs exist but are rare. A long-term series of field data collected between 1991 and 2005 at Makobe Island (western Speke Gulf, Tanzania) suggests all three colour morphs persisted over at least 14 years with fluctuations but with no evidence for directional change (Maan et al. 2008; Magalhaes et al. 2010). Population genetic data and mate choice studies indicate nonrandom gene flow between colour morphs (Seehausen et al. 1999; Magalhaes et al. 2010). Magalhaes et al. (2010) found limited evidence of ecomorphological differentiation between P and blotched morphs. Fish density can be high, and females are often aggressive and territorial when foraging on algae or defending fry (Seehausen et al. 1999; Maan et al. 2008).

In previous studies, we not only showed that females bias their aggression towards rivals of their own colour but that the WB morph socially dominates the other morphs in both dyadic and community settings (Dijkstra et al. 2008, 2009). In theory, the joint action of aggression biases and asymmetric dominance relationships could lead to negative frequency-dependent selection stabilising the colour polymorphism (Seehausen and Schluter 2004; Dijkstra et al. 2010; Lehtonen 2014). More aggressive colour morphs may invade, but their initial dominance advantage may diminish with increasing frequency resulting in more intense within-morph fights. Aggression biases may lead to higher within-morph competition than between-morph competition, conferring a fitness advantage to rare types due to a reduced rate of being attacked (Dijkstra et al. 2010). Given the intense territoriality of *N. omnicaeruleus* females, the differences in dominance between morphs and the commonly found link between dominance and metabolic rate (Burton et al. 2011), we predicted that the colour morphs would also differ in metabolic rate. However, following the finding that in male *Pundamilia* cichlids the more aggressive species in a pair of sibling species had the greater energetic efficiency when fighting (Dijkstra et al. 2013), we also predicted that the morphs might differ in the energetic cost of aggression, and given the possible link between metabolism and oxidative stress, we also predicted that they should differ in levels of oxidative stress.

Here, we compare females of the three colour morphs of *N. omnicaeruleus* in terms of their routine metabolic rate and short-term energetic costs of territorial aggression. Based on the observation that across vertebrates more melanistic individuals have increased resting metabolic rate (Ducrest et al. 2008), we predicted that WB females would have higher

routine metabolic rates than the other colour morphs. We also predicted that WB females would minimise the costs of aggression by using less oxygen for a given level of agonistic activity. Finally, we compared oxidative stress across colour morphs and tested the hypothesis that oxidative stress is linked to metabolic rate and differs among female colour morphs.

Material and Methods

Species

At Makobe Island, about half of the females in the population, but only less than 1.7 % of the males, are blotched (Magalhaes et al. 2010). The three morphs are fully sympatric and ecologically very similar (Seehausen et al. 1999; Maan et al. 2008; Magalhaes et al. 2010), but differ in male and female mating preferences (Seehausen et al. 1999; Pierotti and Seehausen 2007), and mate partially assortatively in the laboratory (Seehausen et al. 1999). Males (but not females) of the WB morph exhibited mating preferences for the WB morph, and males and females of the P morph exhibited strong mating preferences for their own morph (Seehausen et al. 1999; OB morph fish have not been tested in mate choice experiments). Intermediate colour phenotypes occur, suggesting hybridisation between the morphs, but population genetic data suggest nonrandom mating between colour morphs (Magalhaes et al. 2010).

Subjects and housing conditions

We used lab-bred offspring obtained from a stock of wild-caught parental fish collected around Makobe Island, Lake Victoria, Tanzania in 2003 and 2005 (Dijkstra et al. 2009). Fish were reared in sib groups at EAWAG in Kastanienbaum, Switzerland. Mature fish were transferred to the University of Glasgow 7 months prior to the start of the experiments and then housed in individual compartments of aquarium tanks in a recirculating water supply system for at least 1 month before experimentation. To standardise territorial conditions, all females were kept in compartments with at least one other female visible behind a transparent PVC screen. In this housing condition, all females became territorial. Each compartment had a substrate of gravel and contained a PVC tube that the fish used as a hiding place. Note that the respirometry measurements were made in a separate chamber that was not connected to the recirculation of the water supply system (see below). The water temperature in holding aquaria and the respirometry chamber was kept at 28 ±1 °C, and the room in which the fish were housed and where experiments took place was maintained on a 12 light (L)/12 dark (D) cycle. All fish were fed with granular cichlid food (ZM Premium Granular, www.zmsystems.co.uk) once per day. The respirometry measurements took place in the same room where the fish were housed. For each morph, fish were derived from two to three families and we randomly selected between one and four individuals per family. We tested a total of 11 OB females, 9 P females and 14 WB females. Details of the body mass of the test subjects are listed in Table 1.

To minimise observer bias, blinded methods were used when data were recorded and/or analysed. However, this was not possible during recording of behavioural data because the morph phenotype was visible to the observer.

Measurements of metabolic rate

Oxygen consumption rates were determined as the reduction in oxygen concentration over time, using intermittent flow (or open-closed) respirometry (Steffensen et al. 1984; Herskin 1999). The day prior to recording, an individual fish was placed into a 1.68-L respirometry chamber through which flowed a constant current of water, created by 1000 L powerhead pumps (Maxi-jet MJ 1000, www.somhydro.co.uk). The size of the respirometry chamber was 14.5×10.5×11.0 cm (L×W×H). The chamber was submerged in a water bath containing water kept at 100 % oxygen saturation by aerating the water using an air stone. At the same time, a stimulus female fish of the same-colour morph as the focal female was allowed to settle in an identical adjacent chamber in the same water bath but was visually isolated by an opaque screen during the acclimation period and isolation treatment (explained below). The two chambers were 1 cm apart, with their long sides facing each other. Each stimulus female was used no more than once. Focal fish were not fed on the day prior to testing in order to remove any effects related to elevation of metabolic rate that accompanies mechanical and chemical processing of food (Secor 2009). The oxygen concentration in the water exiting from the respirometry chamber was measured using a FIBOX3 Fibre optic oxygen metre (PreSens GmbH, www.presens.de) and associated software Oxyview 5.31 (PreSens GmbH) and LoliResp (LoligoSystems ApS, www.loligosystems.com). Oxygen concentration was measured initially (t_0) when the system was in the open position (i.e. the chamber was receiving a continual pumped supply of aerated water from the water bath). Then the system was closed for ≈15 min so that the water was continually recirculated in a closed loop (volume including pump, chamber and tubes being 1.73 L). During this time, the oxygen concentration in the closed loop was recorded every 5 s, and the behaviour of the fish was filmed on video for later analysis using a Sony Handycam (DCR-SR52). At the end of the period of closure (t_1), the valves were opened, thereby allowing fully aerated water to again be pumped through the chamber. The arrangement of pumps was such that the rate of flow of water through the chamber did not alter noticeably when the system was switched from

Table 1 Rate of movements per hour (for the isolation treatment), attacks per hour and displays per hour (for the social treatment) and body mass (g; for orange blotched (OB), plain (P) and white blotched (WB) female colour morphs of the cichlid *Neochromis omnicaeruleus*)

Parameter	OB	P	WB
Rate of movements	66.56±19.84	85.33±39.77	42.00±20.12
Rate of attack	698.18±107.17	581.81±89.03	569.79±133.82
Rate of display	125.45±19.03	185.09±29.80	129.43±16.07
Body mass	11.61±0.69	10.96±1.16	12.00±0.62
OXY	215.49±14.02	225.12±16.30	209.98±10.92
dROM	10.55±1.80	12.38±1.83	11.84±3.34

Also shown are measures of serum antioxidant defence (OXY; mM of HOCl neutralised) and oxidative damage (concentration of reactive oxygen metabolites (dROM); measured in Carratelli units, see Costantini et al. 2006). All data presented as means±SE

the open to the closed position. At no point did oxygen concentrations drop below 90 % saturation. The flushing time between different measurements was at least 4 min to ensure restoration of 100 % oxygen saturation in the chambers.

Each fish was allowed to settle in the chamber overnight (without food) before measurements began; recordings of oxygen consumption for each fish were then taken in two different situations (both between 0900 and 1000 hours). The first measurement was while the fish was in visual isolation from any other fish (*isolation* treatment). At the start of the isolation treatment, the flow valves were switched to the closed circulation option and the behaviour of the fish was video filmed from the side. After the first measurement, the flow valves were opened to restore 100 % oxygen. Fish were then exposed to the *social* treatment by removing the screen between the focal female and the stimulus female, allowing the two females to see each other and interact while not engaging in physical contact. The sight of another rival fish immediately results in territorial behaviour, but their physical separation prevents hierarchy formation (Dijkstra et al. 2006; Huffman et al. 2012). Females were exposed to females of their own colour because previous studies had shown that females preferentially attack their own colour (Dijkstra et al. 2008), and the aim was to maximise territorial responses in all female colour morphs. Upon removing the screen, the flow valves were closed immediately and the behaviour of the fish was video filmed. On completion of the measurement of oxygen consumption, the focal fish was removed, weighed and returned to its original holding tank. Single oxygen measurements were taken because in a separate study in the cichlid *Pundamilia*, oxygen consumption measurements had very high repeatability (Dijkstra et al. 2013). The isolation treatment always occurred before the social treatment because we were interested in metabolic rate at rest and wanted to avoid taking these measurements when fish are showing respiratory responses to prior territorial defence.

In both the isolation and social treatments, metabolic rate was calculated from the rate of oxygen decline in the closed respirometer over an interval of 5 min, starting 2 min after the loop was closed (to ensure complete mixing of the water). Control trials (ran in empty chambers 10 min before introduction of fish) were performed almost daily on 23 occasions to determine the baseline oxygen consumption rate due to other biological (e.g. bacterial) activity, and this rate was subtracted from the oxygen consumption rate obtained for each fish. Oxygen consumption rate for each fish was corrected using the most recent control trials that took place before the test. The oxygen sensor was calibrated once a week using water from the fully aerated water bath (with no fish present) as the 100 % oxygen standard and a solution of sodium sulphite for the zero oxygen calibration.

To determine whether variation in activity or movement level (i.e. swimming or inactivity) significantly influenced the measurements of oxygen consumption in the isolation treatment, one short side of the chamber was partitioned into four equal parts, using lines visible on the video recordings (Ros et al. 2004). As a measurement of activity, the number of times the head of the focal female passed one of the lines was recorded over the same 5 min time interval used to calculate the metabolic rate. Similarly, in the social treatment, the number of attacks (bites and butts) and display behaviours (frontal and lateral displays) (Baerends and Baerends-Van Roon 1950) of the focal female towards the stimulus female was quantified over the equivalent 5 min time interval used to calculate the metabolic rate. An attack event was defined as a butt or bite against the wall of the respirometry chamber as the focal fish moved towards the stimulus female. During frontal displays, the focal female extended her dorsal fins, and sometimes pectoral fin and operculum as well, while facing the lateral or frontal side of the stimulus female. During a lateral display, the female extended her dorsal, anal and pelvic fins and positioned herself such that her flank was in front of the head of the stimulus female.

Experimental design for measuring oxidative stress

Seven days after completing the respirometry measurements, females were randomly allocated to one of two conditions: in social isolation, or in a social condition in which females engaged in ongoing territory defence against a rival female (OB: isolation, n=5, social, n=6; P: isolation, n=3, social, n=6; WB: isolation, n=6, social, n=8). We biased our samples to the social treatment where we expected to see the biggest difference. Note that these housing conditions are different from the treatments used in the respirometry setup. In the social condition, females were kept in compartments with at least one other female visible behind a transparent PVC

screen, allowing for ongoing territorial defence throughout the day. This housing condition was exactly the same as the pre-experimentation housing condition prior to placing fish in the respirometer. Females housed in isolation were kept in the same holding aquaria but separated by opaque PVC screens. Females were kept in these arrangements for 6 days to give the fish in the social condition the opportunity to engage in prolonged territorial interactions over several days. Fish were then killed using an overdose of MS-222, the tail was cut off and a blood sample (30–50 μL) was immediately collected from the caudal vein using a heparinised microcapillary that was then transferred to an Eppendorf tube containing a small drop of heparin (<1 μL). The blood sample was then stored on ice and subsequently centrifuged at 13,000 rpm for 10 min. The blood plasma was stored at −80 °C for analysis in the oxidative stress assays. Two samples (both from WB) were unsuitable for analysis (insufficient blood). The balance between oxidants and antioxidants defines the level of oxidative stress of an organism (Finkel and Holbrook 2000). In this study, we did not measure oxidative damage directly, but measured reactive oxygen metabolite (ROM) concentrations in the blood, together with a measurement of overall antioxidant capacity.

Measurement of reactive oxygen metabolites

The serum concentration of ROMs (primarily hydroperoxides, ROOH) was measured by the reactive oxygen metabolite (dROM) test (Diacron, Grosseto, Italy), as in earlier studies of oxidative stress levels in mammals (Brambilla et al. 2002), birds (Costantini et al. 2006, 2013) and fish (Bagni et al. 2007; Dijkstra et al. 2011). For more information, see Costantini et al. (2006). Each serum sample (7.5 μL) was incubated in duplicate for 75 min at 37 °C with 200 μL of 0.01 M acetic acid/sodium acetate buffer, pH 4.8, containing *N*,*N*-diethyl-phenylenediamine as chromogen. Absorbance was read at 490 nm by a Biolinx plate reader. In order to perform the system calibration, 4.5 mM H_2O_2 as reference standard and a reagent blank were used. The intra-assay coefficient of variation (CV) was 7.55 %. To evaluate the inter-assay variation, 12 samples were run once on two different plates and the inter-assay CV was 9.14 %.

Measurement of antioxidant capacity

Serum antioxidant defence was measured in the form of free radical scavenging capacity, quantified as the capability to neutralise the oxidant action of HOCl using the OXY assay (Diacron, Grosseto, Italy). For more information, see Costantini et al. (2006). The serum (2 μL) was diluted 1:100 with distilled water; 5 μL of each diluted serum sample was incubated in duplicate for 10 min at 37 °C with 200 μL of a titrated HOCl solution as oxidant. Then, 5 μL of the same chromogenous solution used for the ROMs determination was added. Absorbance was read at 490 nm as the end-point. Calibration was achieved by using a reference serum able to neutralise 440 μM HOCl. Measurements were expressed as mM of HOCl neutralised. The intra-assay CV was 5.87 %. 15 samples were run once on two different plates and the inter-assay CV was 9.79 %.

Statistical analyses

Statistical analyses were implemented in R version 2.14.1 using the plyr and ggplot2 packages. All variables (except the OXY data and rate of movement) were log transformed to meet assumptions for parametric statistical testing. As most females were at least occasionally active, the values for oxygen consumption in the isolation treatment represent *routine metabolic rate* (RMR). RMR of female colour morphs was compared while controlling for the rate of movement and body mass using an analysis of covariance (ANCOVA), with movement (line crosses h^{-1}) and body mass as covariates. The metabolic rate in the social treatment was referred to as *active metabolic rate* (AMR) because in this treatment females showed continual (active) territorial defence. AMR was used to compare the energetic costs of interactions among the three colour morphs while controlling for any differences in agonistic activity. To this end, the energetic costs of display and attack behaviour were determined using multiple regression analysis across all fish (i.e. all colour morphs combined). The analysis evaluated the extent to which the rate of displaying, the rate of attacking and the female's body mass explained variation in AMR. Using the regression coefficients for the energetic cost of attacks and displays from this analysis (Table 2), the expected metabolic rate for a given fish was calculated, taking into account her mass—measured on completion of the measurement of oxygen consumption—and the rate at which she performed attacks and displays during the period of measuring oxygen consumption. A repeated measures ANCOVA was used to compare RMR and AMR in the same fish. The *relative metabolic rate* (rAMR) for each fish was the difference between the expected and actual AMR. Thus, fish with higher AMR than expected for their size and rate of agonistic activity had positive values for rAMR, while those with respiration rates lower than expected had a negative rAMR (Metcalfe et al. 1995; McCarthy 2000; Dijkstra et al. 2013). The rAMR was compared among colour morphs in the social treatment using an ANOVA. Pairwise comparisons were done using Tukey's honestly significant difference (HSD) tests. Measures of oxidative stress (dROM and OXY) were compared among colour morphs while controlling for body mass and rAMR using ANCOVA.

Models were run in a stepwise backward manner, sequentially removing the nonsignificant (interaction) effects using a

Table 2 Regression coefficients for the effect of aggression (rates of attacks and displays h^{-1}) on oxygen consumption (mg $O_2 h^{-1}$) of female *Neochromis omnicaeruleus* cichlids in the social treatment

Parameter	Coefficient	*P* value
Intercept	−1.44373	0.00165
Log(rate of attack)	0.1637	0.00405
Log(rate of display)	0.26883	0.02997
Log(*M*)	0.8657	0.00618

The data are log transformed. Also included is the effect of body mass *M* (g). The fit of the model (r^2) was 0.48. Analysis based on all morphs combined (n=34 fish)

threshold of P=0.1. In all analysis, we verified normality by examining residual plots.

Results

Metabolic rate

In isolated fish, body mass was a strong positive predictor of RMR (ANCOVA, $F_{1, 29}$=6.703, P=0.0015). Females showed a variety of activity levels (rate of movements h^{-1}; mean±SE, 61.4±14.7) ranging from complete inactivity (13 females) to frequent swimming movements. Importantly, females of different colour morphs did not differ in the rate of movement (Table 1, $F_{2, 31}$=0.719, P=0.495). RMR differed significantly between female colour morphs after correcting for body weight and rate of movement (ANCOVA, morph: $F_{2, 29}$= 9.62, P=0.0006; body weight: $F_{1, 29}$=6.703, P=0.015; rate of movement: $F_{1, 29}$=3.901, P=0.058. The residuals of this model are shown in Fig. 1a). This was due to WB females having a lower RMR compared with the other colour morphs (Fig. 1a, Tukey's HSD: P-OB: P=0.82; WB-OB: P=0.002; WB-P: P=0.02).

A repeated measures ANCOVA comparing metabolic rates in the same fish in the isolation vs. social treatments (Fig. 1b) revealed a significantly higher AMR than RMR (effect of treatment: $F_{1, 31}$=122.30, P<0.00001) as well as significant effects of body mass ($F_{1, 30}$=8.76, P=0.006) and colour morph ($F_{2, 30}$=6.76, P=0.0038). Taking data for just the social treatment, female colour morphs differed significantly in AMR (Fig. 1b, ANCOVA, $F_{2, 30}$=3.825, P=0.033), after controlling for the effect of body weight ($F_{1, 30}$=3.448, P= 0.0732. The residuals of this model are shown in Fig. 1b). This was due to WB female colour morphs having a lower AMR than OB females (Fig. 1b, Tukey's HSD: P-OB: P= 0.25; WB-OB: P=0.023; WB-P: P=0.63). Colour morphs did not differ in the rate of aggression (Table 1, ANCOVA, rate of attack, $F_{1, 31}$=1.055, P=0.36; rate of display: $F_{1, 31}$= 1.32, P=0.283).

We compared the rAMR of the three colour morphs in order to compare their energetic costs per unit aggressive act. Relative metabolic rate differed significantly among the colour morphs (Fig. 2, ANOVA, $F_{2, 31}$=5.675, P=0.008), with OB females using more oxygen per unit aggressive act than the other two colour morphs (Fig. 2, Tukey's HSD: P-OB: P=0.028; WB-OB: P=0.012; WB-P: P=0.999).

Oxidative stress

We tested whether morph, body mass or rAMR had an effect on oxidative damage (dROM) or antioxidant defences (OXY) (Table 3). Housing condition (i.e. isolation vs. social treatment) in the period leading up to sampling had no effect on any of the physiological measures (P>0.1). We found a marginally nonsignificant interaction between morph, body mass and rAMR explaining variation in dROM (Table 3). OXY was negatively related to body mass but unrelated to morph or relative active metabolic rate (Table 3).

Discussion

In the present study, we compared three sympatrically occurring female colour morphs of the same species of cichlid fish for their routine metabolic rate when fish were housed in isolation, active metabolic rate during inter-female interactions and levels of oxidative stress. We hypothesised that, given the known behavioural dominance asymmetries (Dijkstra et al. 2009), the three female colour morphs would differ in those physiological parameters.

WB females, which are socially dominant over P and OB females in dyadic combats and group settings (Dijkstra et al. 2009), had lower RMR and AMR than the OB females after controlling for body mass and the level of activity. In addition, WB females had a lower RMR than P females. These findings are in contrast to our prediction and the widely reported positive association between resting metabolic rate and social dominance. For example, in the freshwater prawn *Macrobrachium rosenbergii* the outcome of aggressive interactions could be predicted from the resting metabolic rate before hierarchy formation (Brown et al. 2003). Lahti et al. (2002) compared standard metabolic rate in allopatric populations of trout that varied in aggressiveness and found a positive correlation between aggressiveness and standard metabolic rate consistent with the relationship previously found in related species at the individual level (Metcalfe et al. 1995; Cutts et al. 1998; Yamamoto et al. 1998). The WB morph is more melanized than OB and P, and its lower metabolic rate is in contrast to the prediction that across vertebrates more melanistic individuals have increased resting metabolic rate (Ducrest et al. 2008). However, this prediction was based on a study on great tits (*Parus major*) and pied flycatchers

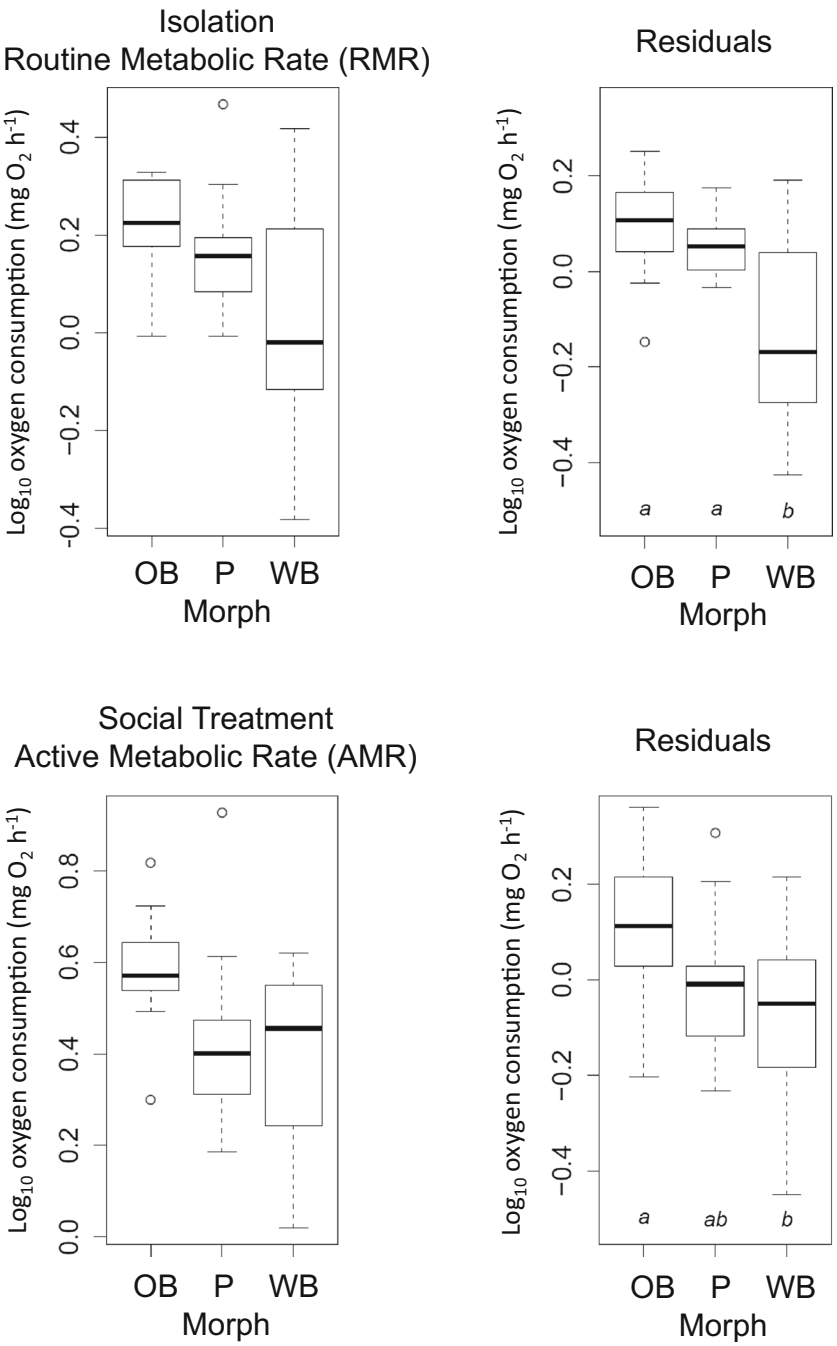

Fig. 1 Boxplots of oxygen consumption rates (mg $O_2 h^{-1}$) for OB, P and WB females in **a** isolation (routine metabolic rate (*RMR*)) and **b** social treatment (active metabolic rate (*AMR*)). Shown are the medium, upper and lower quartile, minimum and maximum values and outliers (more than 3/2 times of the upper quartile, or less than 3/2 times of the lower quartile). Shown are the log-transformed values (*left*) and the residuals (*right*) of these values after controlling for the effect of body mass and (in (**a**)) rate of movement. Pairwise comparisons between colour morphs (using Tukey's HSD tests) are indicated at the *bottom in letter(s)*; morphs that do not share a letter are significantly different. *OB* orange blotched, *P* plain, *WB* black-and-white blotched

(*Ficedula hypoleuca*) (Røskaft et al. 1986) where the degree of melanism was not only related to resting metabolic rate but also social dominance. The lower metabolic rate of WB females is consistent with the results of Borowsky (1984), who found that tailspot melanophore morphs of the platyfish *Xiphophorus variatus* had 25 % lower routine metabolism than unpatterned males (but see Meyer et al. 2006).

Aggressiveness can affect energy metabolism in a range of different animal species (Grantner and Taborsky 1998; Speakman and Selman 2003; Castro et al. 2006; Briffa and Sneddon 2007; Careau et al. 2008; Dijkstra et al. 2013; Seebacher et al. 2013). In contrast to our expectation, for a given body size and level of agonistic activity, OB females used more oxygen than the other female colour morphs or, in other words, OB females were less metabolically efficient when performing territorial behaviours. It is unlikely these metabolic differences are driven by variation in stimulus fish behaviour because morphs did not differ

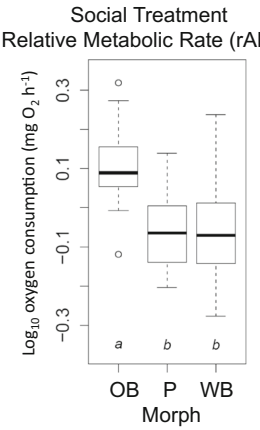

Fig. 2 Boxplots of the relative active metabolic rate (*rAMR*) expressed in oxygen consumption rates (mg $O_2 h^{-1}$) for OB, P and WB females in the social treatment. The rAMR for each fish was the difference between the expected metabolic rate and actual AMR. The expected metabolic rate was calculated using regression coefficients explaining variation in metabolism based on the rate of displaying, the rate of attacking and the female's body mass (see Table 2). rAMR was significantly higher for OB females than for P and WB females, indicating that OB females use more oxygen when performing the same rate of aggressive acts than females of the other colour morphs of similar size. Shown are the medium, upper and lower quartile, minimum and maximum values and outliers (more than 3/2 times of the upper quartile or less than 3/2 times of the lower quartile). Pairwise comparisons between colour morphs (using Tukey's HSD tests) are indicated at the *bottom in letter(s)*; morphs that do not share a letter are significantly different. *OB* orange blotched, *P* plain, *WB* black-and-white blotched

Table 3 Results of ANCOVAs examining the effects of body mass (*M*) and relative active metabolic rates (rAMR) on reactive oxygen metabolites (dROM) and antioxidant capacity (OXY)

Factor	*df*	*F* value	*P* value
dROM			
Morph	2, 20	0.450	0.64
rAMR	1, 20	0.184	0.67
M	1, 20	0.226	0.64
Morph×rAMR	2, 20	2.281	0.13
Morph×*M*	2, 20	1.191	0.32
rAMR×*M*	1, 20	0.931	0.35
Morph×*M*×rAMR	2, 20	3.226	**0.06**
OXY			
Morph×*M*×rAMR	2, 20	0.198	0.82
Morph×rAMR	2, 22	0.078	0.93
M×rAMR	1, 24	2.780	0.11
rAMR	1, 25	0.085	0.77
Morph×*M*	2, 26	2.405	0.11
Morph	2, 28	0.363	0.70
M	1, 30	7.784	**0.0091**

Significance levels are reported for nonsignificant terms before they were removed from the model during the backward elimination process, and for significant terms in the final model (terms shown in bold; retained if $P<0.1$)

in agonistic behaviour (Table 1). Metabolic efficiency, a fundamental and potentially heritable component of fitness, may be favoured by selection (Watson and Lighton 1994), especially when much time is required for a particular activity, as is the case for territorial defence in algae scraping cichlid species (Maan et al. 2008). It should be noted that, while agonistic behaviours are driven by rapid body movements which are largely powered by anaerobic metabolism in white muscle (Marras et al. 2010), the oxygen debt must subsequently be paid off. Although aggression in *N. omnicaeruleus* clearly involves an increase in oxygen consumption, as indicated by the positive relationship between activity and oxygen consumption in the regression analysis, the period over which the oxygen debt arising from agonistic behaviours is paid off may not perfectly match the time span of our respirometry measurements. This may account for the imperfect correlation between aggression and oxygen consumption.

We found that rAMR predicted variation in dROM in a complex morph-and body weight-specific manner, but this effect was retained in the final model as a nonsignificant term. We therefore did not find strong support for morph-specific levels of oxidative stress. This is surprising given the morph differences in metabolism and pigmentation, both of which have been linked to variation in oxidative stress. For example, in the booted eagle (*Hieraaetus pennatus*) plasma total antioxidant capacity was higher in darker females than in lighter females (Galván et al. 2010). However, the connection between mitochondrial activity (and hence metabolic rate) and oxidative stress is not clear (Salin et al. 2012; Beamonte-Barrientos and Verhulst 2013; Fletcher et al. 2013; Salin et al. 2015) and future studies are needed to shed more light on the relationship between oxidative stress and metabolic rate in *N. omnicaeruleus* colour morphs. In a previous study in the sibling species *Pundamilia nyererei* and *Pundamilia pundamilia*, also of Lake Victoria, we found that the more aggressive species, *P.* nyererei, experienced more oxidative stress than its less aggressive counterpart, but only when territorial (Dijkstra et al. 2011).

Our results show that several measures of metabolic rate are different among colour morphs. Although metabolic rate can be influenced by diet and environmental conditions (Chapman et al. 2002; Figueiredo-Silva et al. 2013), the metabolic differences in this study are likely heritable and imply genetic differences between the three female colour morphs since the animals were bred and raised in a common laboratory environment. Mate choice appears to reduce the frequency of hybridisation between the colour morphs of *N. omnicaeruleus* (Seehausen et al. 1999; Magalhaes et al. 2010), perhaps

permitting the maintenance of heritable adaptive divergence in metabolic traits.

In addition to direct genetic differences underlying variability in metabolic rate between colour morphs, there might be a physiological mechanism pleiotropically linking pigmentation to various physiological functions, including metabolism through shared physiological pathways in vertebrates (Ducrest et al. 2008). The OB and WB morphs have more conspicuous melanin patterns than the P morph (WB in the near surface, OB in deeper skin layers, Seehausen et al. 1999). Although the molecular nature of the genes underlying the colour polymorphisms in *N. omnicaeruleus* is not yet known (Roberts et al. 2009), perhaps these genes and their downstream targets influence both metabolism and pigmentation through shared pathways in the melanocortin system (Ducrest et al. 2008; Emaresi et al. 2013; Li et al. 2014). More specifically, melanocortins and their endogenous inverse agonist and antagonists (agouti signalling peptide and agouti-related peptide) have major developmental and physiological effects on pigmentation (Zhang et al. 2010; Manceau et al. 2011), but they also have an important role in the control of energy homeostasis, thyroid hormone production and lipid and glucose metabolism/homeostasis (Ducrest et al. 2008).

The polymorphism in *N. omnicaeruleus* system has inspired models of sympatric speciation by sexual selection and sex ratio selection (Lande et al. 2001; Kocher 2004, see also (Seehausen et al. 1999; Pierotti and Seehausen 2007; Maan et al. 2008; Pierotti et al. 2008). Many factors contribute to the evolution and maintenance of the colour polymorphism in *N. omnicaeruleus*. First, behavioural studies show that there is individual and morph-specific variation in the strength of female and male mate preferences (Seehausen et al. 1999; Pierotti et al. 2009); this is supported by genetic data indicating nonrandom mating between colour morphs (Magalhaes et al. 2010). Second, colour is an important cue in aggressive interactions, and it has been suggested that female-female competition may generate negative frequency-dependent sexual selection, supporting the syntopic coexistence of colour morphs (Dijkstra et al. 2008, 2009). Third, colour morphs experience differential predation risk, with OB being more conspicuous to visually guided predators (Maan et al. 2008; but see Roberts et al. 2009). Fourth, both blotched colour phenotypes are linked to dominant female determining genes, resulting in blotched females producing broods with female-biased sex ratios and possibly unfit YY individuals (Seehausen et al. 1999; Roberts et al. 2009). Finally, eco-morphological differentiation suggests that subtle resource partitioning may contribute to the maintenance of the polymorphism. WB females had a significantly wider snout than OB and P females, while OB females had a significantly smaller pre-orbital depth than P (Magalhaes et al. 2010). Maan et al. (2008) found that the proportion of blotched females increased with increasing water depth hinting at subtle spatial segregation. It is also interesting to note that predation risk is known to influence metabolic rate. For example, the Trinidadian guppy (*Poecilia reticulata*) shows both evolutionary and plastic metabolic responses to variation in predation risk. High predation risk populations have evolved lower resting metabolic rates compared with populations from a low-predation environment (Handelsman et al. 2013). Although speculative, it is possible that the observed patterns in metabolism in *N. omnicaeruleus* are related to morph-specific responses to predation risk.

In this study, we showed that female colour morphs differ in metabolic rate and efficiency. How these metabolic differences are linked to fitness and interact with other factors affecting the evolution of the female colour polymorphism is a promising avenue for future research.

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