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Metabolic divergence between sibling species of cichlids *Pundamilia nyererei* and *Pundamilia pundamilia*

P. D. Dijkstra*†, O. Seehausen‡ and N. B. Metcalfe*

**Institute of Biodiversity, Animal Health and Comparative Medicine, Graham Kerr Building, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, U.K. and ‡Centre of Ecology, Evolution and Biogeochemistry, EAWAG Swiss Federal Institute for Aquatic Sciences, CH-6047, Kastanienbaum, Switzerland*

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This study compared *Pundamilia nyererei* and *Pundamilia pundamilia* males in routine metabolic rate (R_R) and in the metabolic costs males pay during territorial interactions (active metabolic rate, R_A). *Pundamilia nyererei* and *P. pundamilia* males housed in social isolation did not differ in R_R. In contrast to expectation, however, *P. nyererei* males used less oxygen than *P. pundamilia* males, for a given mass and level of agonistic activity. This increased metabolic efficiency may be an adaptation to limit the metabolic cost that *P. nyererei* males pay for their higher rate of aggressiveness compared to *P. pundamilia* males. Thus, the divergence between the species in agonistic behaviour is correlated with metabolic differentiation. Such concerted divergence in physiology and behaviour might be widespread in the dramatically diverse cichlid radiations in East African lakes and may be an important factor in the remarkably rapid speciation of these fishes. The results did not support the hypothesis that higher metabolic rates caused a physiological cost to *P. nyererei* males that would offset their dominance advantage.

Key words: Lake Victoria; male–male competition; speciation.

INTRODUCTION

Sexual selection on male secondary sexual traits can be a powerful force facilitating population differentiation and speciation, and can contribute to maintaining reproductive isolation between sympatric species (Fisher, 1930; Lande, 1981). Identifying the mechanisms that permit the coexistence of several ecologically similar species is a classical challenge in evolutionary biology. Several mechanisms have been proposed that promote coexistence of competing species, such as temporal and spatial niche partitioning and balancing and negative frequency-dependent selection (Schluter, 2000; Vandermeer *et al*., 2002; Sinervo & Calsbeek, 2006).

In several animal taxa, closely related species differ in colouration and little else, often resembling colour variation that distinguishes conspecific colour morphs (Gray & McKinnon, 2007). For colour polymorphic species, it has been suggested that

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†Author to whom correspondence should be addressed at present address: The University of Texas at Austin, Section of Integrative Biology, Institute for Cellular and Molecular Biology, 1 University Station–C0930, Austin, TX 78712, U.S.A. Tel.: +1 512 475 7318; email: pddijkstra@gmail.com

selection on other traits that are correlated with colour pattern may sometimes contribute to the origin and maintenance of colour variation within populations (Brooks, 2000; Horth, 2003). The same may apply to partially hybridizing species that are genetically weakly differentiated and mainly differ in colour pattern. A cost-benefit analysis associated with such traits can help in understanding how balancing selection operates to maintain more than one phenotype in wild populations. Variation in colour is often associated with variation in levels of aggressiveness (Korzan *et al*., 2008; Dijkstra *et al*., 2010) and social dominance (Barlow, 1983; Dijkstra *et al*., 2005; Pryke & Griffith, 2006). The benefits of elevated aggressiveness are priority of access to mates and resources (Wong & Candolin, 2005), while the costs are predominantly physiological, for example, a higher rate of energy expenditure through an increased rate of activity. Metabolic rate (MR), the rate at which an animal oxidizes substrates to produce energy, is an important measure in the study of behavioural decisions and the evolution of life-history strategies, as it directly impinges on several key physiological processes such as oxidative stress and ageing (Monaghan *et al*., 2009; Metcalfe & Alonso-Alvarez, 2010). Correspondingly, in a wide range of taxa closely related species are metabolically differentiated, reflecting (local) adaptation to unique physical features of their habitat or life history, including temperature (Whitehead & Crawford, 2006; Ohlberger *et al*., 2008), ecology and lifestyle (Killen *et al*., 2010), and the trade-off between life span and offspring production (Wiersma *et al*., 2007).

In this study, the metabolic costs in two species of the Lake Victoria haplochromine cichlids of the genus *Pundamilia* were examined. The impressive species radiations of haplochromine species flocks of the Great African Lakes are an important system to understand speciation and adaptive radiation (Kocher, 2004; Genner & Turner, 2005; Seehausen, 2006). Among closely related species, there is large variation in male colour (Seehausen, 2009). Evidence suggests that this variation affects female mate choice. Consequently, sexual selection on colouration by mate choice has been implicated in their colour diversification (Deutsch, 1997; Seehausen *et al*., 1997; Knight & Turner, 2004). Among cichlids, sexual selection is expected to be particularly strong in haplochromines because of exclusively maternal brood care. In addition, male contest competition for access to females can exert selection on male colour indirectly, because social status and territory size are important in mate choice (Maan *et al*., 2004), while colour is also a cue in intrasexual communication (Dijkstra *et al*., 2005; Pauers *et al*., 2008).

The species *Pundamilia pundamilia* (Seehausen *et al*., 1998*a*), *Pundamilia nyererei* Witte-Maas & Witte, 1985 and their hybrids occur at various stages of ecological and genetic differentiation around different islands along a gradient of water clarity in Lake Victoria, East Africa (Seehausen, 2009). Males of *P. nyererei* are crimson dorsally (including the dorsal fin) and yellow on their flanks. Males of *P. pundamilia* are metallic blue on the body and in the dorsal fin. Along the gradient of water clarity the two sibling species go from being one species with phenotypic variation (unimodal, predominantly intermediate between red and blue) to two incipient species, and finally two reproductively isolated sister species, in what has been called a 'speciation transect' (Seehausen *et al*., 1997, 2008). In this study, two frequently hybridizing incipient species that showed the weakest yet still significant biomodality in the distribution of male nuptial colouration and the lowest significant differentiation at neutral loci were used. These characteristics make them the most suitable population to investigate traits under disruptive and divergent selection (Seehausen *et al*., 2008; Magalhaes *et al*., 2009). Only distinctly red or blue males and no intermediate phenotypes were used. Female mating preferences are assortative and largely based on colour (Seehausen & Van Alphen, 1998; Stelkens *et al*., 2008), but this preference for own colour can be weakened or even overridden by variation in territory quality (Dijkstra *et al*., 2008). Males of *P. nyererei* are more aggressive and are more likely to defeat males of *P. pundamilia* in dyadic combats (Dijkstra *et al*., 2005). Across animal species social dominance is associated with elevated fitness (Wong & Candolin, 2005). Despite the dominance advantage of red males red male phenotypes do not appear to displace blue in *Pundamilia*: the geographical distribution of *Pundamilia* species with red male breeding colour is nested within that of species with blue colour (Seehausen & Van Alphen, 1999).

One potential explanation for why red males have not displaced blue males is that their elevated aggressiveness may be associated with physiological costs. These costs may, at least partly, offset the fitness benefit associated with the dominance advantage of red males. Energetic costs are one such example. Territorial males vigorously defend their mating site against competitors and the energetic costs of aggression may influence the relative fitness of red and blue phenotypes.

Here males of *P. nyererei* and *P. pundamilia* were compared in terms of their routine metabolic rate (R_R; *i.e.* when in social isolation) and short-term energetic costs of male–male interactions. By comparing the unit costs of aggression of *P. nyererei* and *P. pundamilia* males, that is, controlling for other factors such as body size and frequency of aggressive acts, the null-hypothesis was tested that *P. nyererei* and *P. pundamilia* males pay the same energetic costs at a given level of aggressiveness. The higher rate of aggressiveness of *P. nyererei* will then result in higher energetic costs, which might help explain the persistence of the blue (*P. pundamilia*) phenotype despite its behaviourally subordinate status.

MATERIALS AND METHODS

Second generation laboratory-bred offspring were used. The fishes were bred from 41 wild (and presumed outbred) fishes collected in 2003 and 2005 around Kissenda Island, Lake Victoria, Tanzania. Experimental males (61 *P. nyererei* and 60 *P. pundamilia*) were sexually mature and were at least 6 months old. Fishes were reared in sib groups at the Biological Centre in Haren, the Netherlands and EAWAG (Swiss Federal Institute of Aquatic Science and Technology) in Kastanienbaum, Switzerland. Mature fishes were transferred to the University of Glasgow 5 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 standardize territorial condition before males were used in the experiments, all males were kept in compartments with at least one other male visible behind a transparent PVC screen. In this housing condition, all males become territorial. Each compartment had a substratum 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 water bath that was not connected to the recirculation water supply system. The water temperature in holding aquaria and the respirometry chamber was kept at 28° C range ±1° C, and the room in which the fishes were housed and where the experiments took place was maintained on a 12L:12D cycle. All fishes 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 fishes were housed. The body mass (M) of *P. nyererei* males ranged from 6·35 to 23·85 g (mean ± s.e. $= 15{\cdot}73 \pm 1{\cdot}71$ g; $n = 13$) while that of *P. pundamilia* males ranged from 11·71 to 49·9 g (mean ± S.E. = 30·51 ± 2·99 g; $n = 21$). The standard length (L_S) of *P. nyererei* males ranged from 61·0 to 93·0 mm (mean ± S.E. = 79·1 ± 3·0 mm; $n = 13$) while that of *P. pundamilia* males ranged from 71·0 to 113·0 mm (mean ± S.E. = 95·6 ± 3·1 mm; $n = 21$).

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, a fish was placed in 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 cm × 10·5 cm × 11·0 cm (l × w × h). The chamber was submerged in a water bath containing water at 100% oxygen saturation. At the same time, a stimulus fish of the same phenotype as the focal male 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. The two chambers were 1 cm apart, their long sides facing each other. Each stimulus male was used once. Focal fishes were not fed on the day prior to testing in order to remove any effects of specific dynamic action (Secor, 2009) from the measurements of MR. The oxygen concentration of the water leaving the respirometry chamber was measured using a FIBOX3 Fibre optic oxygen meter (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 continuous pumped supply of aerated water from the water bath). Then the system was closed for *c*. 15 min so that the water was continuously 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; www.sony.com). At the end of the period of closure (t_1) the valves were opened, thereby allowing fully aerated water to be pumped again 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 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 taken while the fish was in visual isolation from any other fish (isolation treatment). At the start of the isolation treatment, the flow valves were closed and the behaviour of the fish was video filmed. Most of the fishes were at least occasionally active (*i.e.* spontaneous swimming movements), so their rate of oxygen consumption in the isolation treatment represents routine oxygen consumption rates (R_R, Grantner & Taborsky, 1998). Within 20 min of this isolation measurement, fishes were exposed to the social treatment by removing the screen between the focal male and the stimulus male, allowing the two males to see each other and interact. The sight of another male immediately results in territorial behaviour and an increase in the expression of nuptial colouration and vertical bar blackening in these fishes (Baerends & Baerends-van Roon, 1950). Males were exposed to males of their own colour because previous studies had shown that males of both sibling species preferentially attack their own colour (Dijkstra *et al*., 2010), and the aim was to maximize territorial responses in both sibling species. Upon removing the screen, the flow valves were closed immediately and the behaviour of the fishes 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 consumption measurements were taken in both the isolation and social treatments as a pilot study indicated very high repeatability (two oxygen consumption measurements of the same fish within the same treatment: isolation, $n = 10$, $r^2 = 0·94$; social: $n = 5$, $r^2 = 0·91$: combined, $r^2 = 0·97$).

In both the isolation and social treatments, MR was calculated from the rate of oxygen decline in the closed respirometer over an interval of 8 min, starting 2 min after the loop was closed. Control trials (without fish in the chamber) were performed 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. The oxygen sensor was callibrated 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.

ACTIVITY AND BEHAVIOURAL MEASUREMENTS

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 male passed one of the lines was recorded over a 5 min time interval. In the social treatment, the number of attacks (bites and butts) and display behaviours (frontal and lateral displays) of the focal male towards the stimulus male was quantified over the same time interval (Baerends & Baerends-van Roon, 1950). 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 male. During frontal displays, the focal male extended its dorsal fins, and sometimes pectoral fin and operculum as well, while facing the lateral or frontal side of the stimulus male. During a lateral display, the male extended its dorsal, anal and pelvic fins and positioned itself such that its flank was in front of the head of the stimulus male.

The interval used to estimate oxygen consumption overlapped with the period over which activity was recorded. In approximately half of the instances, however, there was a short delay (maximum of 5 min) between the recording of behaviour and that of oxygen consumption. Although this may have slightly increased variation in the estimations of oxygen consumption per unit activity, this is likely to be minimal as both measurements are highly repeatable at this small timescale (oxygen: correlation between first and second measurement of the same fish in the same housing condition, see above; Dijkstra *et al*., 2006). Moreover, any increased variation will merely make the analyses more conservative.

ANALYSIS

Statistical analyses were implemented in PASW Statistics (version 18; www-01.ibm.com/software/analytics/spss/products/statistics). All variables (except the rate of activity in the isolation treatment and L_S) were $\log_{10}$ transformed to meet assumptions for parametric statistical testing. R_R (*i.e.* routine metabolic rate in isolation) of *P. nyererei* and *P. pundamilia* males were compared while controlling for the rate of movement using an ANOVA. The active metabolic rate (R_A, *i.e.* in the social treatment) was used to compare the energetic costs of the two sibling species while controlling for any differences in agonistic activity. To this end the energetic cost of display and attack behaviour was determined using multiple regression analysis across all fishes (*i.e.* both sibling species combined). The analysis evaluated the extent to which the rate of displaying, the rate of attacking and the male's M explained variation in R_A. Using the regression coefficients for the energetic cost of attacks and displays from this analysis (Table I; fit of the model $r^2 = 0{\cdot}60$), the expected MR for a given fish was calculated, taking into account its M value and the rate at which it performed attacks and displays. The relative metabolic rate (R_D) for each fish was the difference between the expected and actual R_A. Thus fish with higher R_A than expected for their size and rate of agonistic activity had positive values for R_D, while those with respiration rates lower than expected had a negative R_D (Metcalfe *et al*., 1995; McCarthy, 2000). The R_D was compared between *P. nyererei* and *P. pundamilia* in the social treatment using ANOVA.

RESULTS

The oxygen consumption rates relative to M are shown in Fig. 1 for the two different treatments. Not surprisingly, in isolated fish M was a strong positive predictor of R_R (ANOVA, $F_{1,31} = 43{\cdot}74$, $P < 0{\cdot}01$). Males were typically resting at the bottom of the chamber, though they did swim around in the chamber

TABLE I. Regression coefficients values for the effect of aggression (rates of attacks and displays h^{-1}) on oxygen consumption (mg O_2 h^{-1}) in the social treatment of *Pundamilia nyererei* and *Pundamilia pundamilia*. The data are $\log_{10}$ transformed. Also included is the effect of body mass M (g). Analysis based on both species combined ($n = 34$ fish)

Coefficient		P
Intercept	−1·337	<0·01
Rate of attack	0·084	>0·05
Rate of display	0·284	<0·01
M	0·713	<0·001

(Table II; number of movements h^{-1}, mean ± S.E.: 150·4 ± 16·8). Males showed a variety of activity levels ranging from complete inactivity to almost continual swimming movements, and this variation in the rate of movements was a positive predictor of R_R ($F_{1,31} = 5{\cdot}04$, $P < 0{\cdot}05$). Importantly, *P. nyererei* and *P. pundamilia* males did not differ in the rate of movement (Table II, independent *t*-test: $t = 1{\cdot}48$, d.f. = 32, $P > 0{\cdot}05$). In addition, there was no relationship between the rate of movement and M (Pearson, $r = 0{\cdot}096$, d.f. = 34, $P > 0{\cdot}05$). As most males were at least occasionally active, the values for oxygen consumption in this isolation treatment represent R_R, which did not differ significantly between *P. nyererei* and *P. pundamilia* males after correcting for M and level of activity ($F_{1,30} = 0{\cdot}14$, $P > 0{\cdot}05$).

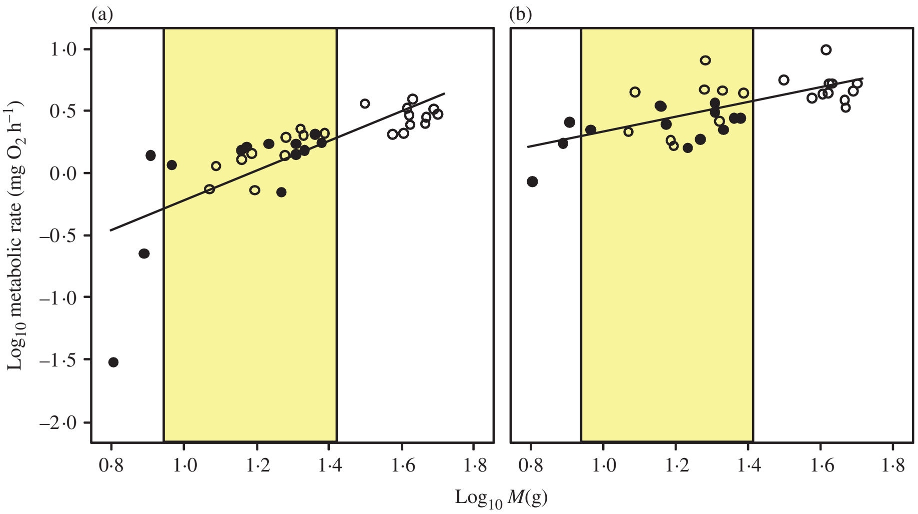

FIG. 1. Oxygen consumption rates in (a) social isolation (routine metabolic rate R_R) and (b) in the social treatment (active metabolic rate R_A) of *Pundamilia pundamilia* (○) and *Pundamilia nyererei* (●) males relative to their body mass (M). MRs were measured as oxygen consumption rates in mg O_2 h^{-1}. The data are $\log_{10}$ transformed. 🟨, the subsample of males that overlap in size (used for comparisons between species). The curves were fitted by (a) $y = 1{\cdot}1679x - 1{\cdot}3695$ ($r^2 = 0{\cdot}57$) and (b) $y = 0{\cdot}5812x - 0{\cdot}2521$ ($r^2 = 0{\cdot}44$).

TABLE II. Number of movements h^{-1}, attacks h^{-1} and displays h^{-1} for *Pundamilia nyererei* and *Pundamilia pundamilia* males. Means ± S.E. shown for both species combined ($n = 34$ fish)

Variable	*P. nyererei*	*P. pundamilia*
Number of movements	119·08 ± 30·64	169·71 ± 18·94
Rate of attack	807·69 ± 129·82	436·57 ± 88·00
Rate of display	323·08 ± 56·36	330·86 ± 43·86

As expected, MR was significantly higher when males were in the social treatment than when in isolation (Fig. 2; repeated measures ANOVA comparing R_R and R_A in the same fish, $F_{1,31} = 38{\cdot}70$, $P < 0{\cdot}01$). M was again a significant predictor of R_A (Fig. 1; $F_{1,31} = 10{\cdot}97$, $P < 0{\cdot}01$), but there was more individual variation about the regression line relating MR to M ($r^2 = 0{\cdot}57$ for isolation treatment *v*. 0·44 for social treatment), due to individual differences in agonistic activity levels when able to see a rival. The number of attacks h^{-1} was mean ± S.E. = 578·5 ± 78·9 (range: 36–1872) and the number of displays h^{-1} was 327·9 ± 34·1 (range: 12–852). The rates of these behaviours are shown in Table I for the two sibling species separately, but not compared statistically for reasons given below.

In contrast to expectation, the R_D in the social treatment was significantly higher for *P. pundamilia* males than for *P. nyererei* males (*P. pundamilia* males: mean 0·037 ± 0·031; *P. nyererei* males: mean −0·058 ± 0·031; independent t-test: $t = -2{\cdot}04$, d.f = 32, $P < 0{\cdot}05$). This suggests that, while their non-aggressive routine metabolic costs are similar, *P. pundamilia* males use more oxygen for a given level of aggressive behaviour than do *P. nyererei* males. Variation in agonistic activity, however, makes comparison of the metabolic costs of the sibling species difficult, especially as (1) oxygen consumption per unit of behavioural act depends on the type of behaviour, (2) larger males tended to employ more display behaviour relative to attack behaviour than smaller males (ANOVA, effect of M: $F_{1,31} = 7{\cdot}21$, $P < 0{\cdot}05$) and (3) the mean M of *P. nyererei* sampled was smaller than that of *P. pundamilia* ($t = 3{\cdot}70$, d.f. = 32, $P < 0{\cdot}01$). The analyses were repeated using the subsample of males that were comparable in size in order to check that the apparent difference in energetic costs of aggression of the two sibling species was not due to size differences (Fig. 1; *P. nyererei* males: range from 9·22 to 23·85 g, mean ± S.E. =18·24 ± 1·24 g, $n = 10$; *P. pundamilia* males: range from 11·71 to 24·30 g, mean ± S.E. = 17·34 ± 0·91 g, $n = 10$). In this subsample, the two sibling species did not differ in M value (independent t-test: $t = 0{\cdot}37$, d.f. = 18, $P > 0{\cdot}05$), in the rate of attack ($t = 1{\cdot}20$, d.f. = 18, $P > 0{\cdot}05$), the rate of display ($t = -0{\cdot}32$, d.f. = 18, $P > 0{\cdot}05$) or the proportion that display relative to total agonistic behaviour ($t = 1{\cdot}59$, d.f. = 18, $P > 0{\cdot}05$). As with the analysis of the full dataset, *P. nyererei* and *P. pundamilia* males in this subsample did not differ in R_R ($F_{1,17} = 0{\cdot}01$, $P > 0{\cdot}05$) after controlling for M (effect of M: $F_{1,17} = 8{\cdot}22$, $P < 0{\cdot}05$; effect of rate of movement: $F_{1,16} = 0{\cdot}34$, $P > 0{\cdot}05$). Moreover, the R_D in this size-matched subsample was significantly higher for *P. pundamilia* males than for *P. nyererei* males (*P. pundamilia* males: mean ± S.E. = 0·092 ± 0·049;

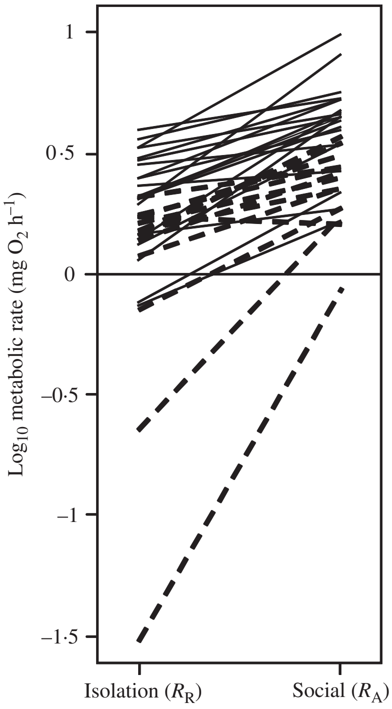

FIG. 2. Oxygen consumption rates in social isolation (routine metabolic rate R_R) and in the social treatment (active metabolic rate R_A). MRs were measured as oxygen consumption rates in mg O$_2$ h^{-1}. The data were log$_{10}$ transformed. Plotted are individual values for *Pundamilia pundamilia* (——) and *Pundamilia nyererei* (- - - -) males.

P. nyererei males: mean ± S.E. $= -0{\cdot}047 \pm 0{\cdot}024$; independent *t*-test: $t = -2{\cdot}53$, d.f. $= 18$, $P < 0{\cdot}05$), again showing that *P. pundamilia* males use more oxygen for a given level of aggressive behaviour than do *P. nyererei* males of the same size.

DISCUSSION

In this study, R_R (when males were housed in isolation) and short-term energetic costs of intermale interaction between males of a sympatric sibling species pair of cichlids (partially hybridizing *P. nyererei* and *P. pundamilia*) were compared. It was hypothesized that *P. nyererei* males would pay an energetic cost for being more aggressive, and that this might constitute a trade-off with their documented social dominance advantage over *P. pundamilia* males.

Pundamilia nyererei and *P. pundamilia* males housed in social isolation did not differ in R_R corrected for M and level of activity. This is in contrast to several studies reporting that MRs may vary between populations or groups of fishes that differ in aggression or colour. For instance, Borowsky (1984) found that tailspot melanophore morphs of the platyfish *Xiphophorus variatus* (Meek, 1904) had 25% lower routine metabolism than unpatterned males; Meyer *et al.* (2006), however, showed that melanin-pattern morphs did not differ in MR. Lahti *et al*. (2002) compared standard MR (comparable to R_R in this study) in allopatric populations of brown trout *Salmo trutta* L. 1758 that vary in aggressiveness and found a positive correlation between aggressiveness and standard MR that was consistent with the relationship at the individual level in other salmonids (Metcalfe *et al*., 1995; Cutts *et al*., 1998; Yamamoto *et al*., 1998).

Males experienced an increase in MR in the social situation (R_A) compared to when they were isolated (R_R). The magnitude of this increase (averaging 101%) was similar to other socially induced responses in fishes (Cummings & Gelineau-Kattner, 2009), but was not as high as that seen with other metabolic responses of cichlids to an agonistic challenge (300%; Grantner & Taborsky, 1998). In contrast to this study Grantner & Taborsky (1998) compared socially induced responses to standard MR (taken at night) and used a mirror test which typically leads to severe escalation of the fight (Oliveira *et al*., 2005).

In the social treatment, *P. nyererei* and *P. pundamilia* males did not differ in their rate of aggressiveness. This is in contrast to previous research in which *P. nyererei* males were found to be more aggressive, both when housed individually and when housed in communities (Dijkstra *et al*., 2006, 2011). It could be related to unique features of this study, such as pre-challenge housing conditions (this study: social isolation; Dijkstra *et al*., 2006, 2011: social). The increase in MR in the social situation is most likely due to the energetic requirements of territorial defence (Grantner & Taborsky, 1998; Ros *et al*., 2006), which involves various body movements including bursts of swimming, biting and extending the fins and operculum. Additionally, when males are exposed to a rival they intensify their colouration, flushing dark vertical bars on the flanks and face (Baerends & Baerends-van Roon, 1950). Although the energetic costs of colour intensification are not known, contraction and expansion of melanophores are energetically costly (Rozdzial & Haimo, 1986).

Considering the large number of studies of resting and R_R, it is surprising how little is known about how aggressiveness can affect energy metabolism (Speakman & Selman, 2003; Careau *et al*., 2008). Although some attempts to study this relationship have been made in fishes (Grantner & Taborsky, 1998; Castro *et al*., 2006; Ros *et al*., 2006), this study is the first to compare MRs, in a territorial context, between two sibling species that differ in male breeding colour but share the same mating strategies. In contrast to expectation, for a given mass and level of agonistic activity, *P. pundamilia* males used more oxygen than *P. nyererei* males, or in other words, *P. nyererei* males used oxygen more efficiently when being territorial. This increased metabolic efficiency should mitigate the metabolic cost of the higher rate of aggressiveness seen in *P. nyererei* males compared to *P. pundamilia* males (Dijkstra *et al*., 2010). Caution is required with interpreting the finding that *P. nyererei* and *P. pundamilia* males differ in metabolic efficiency due to problems with metabolic scaling (Killen *et al*., 2010). The results, however, remained the same when focusing only on the size-matched subsample.

Metabolic differentiation between closely related species has been described in other fish species, usually reflecting local adaptation to clinal variation in temperature (Whitehead & Crawford, 2006; Ohlberger *et al*., 2008) or conditions of hypoxia (Melnychuk & Chapman, 2002). Differentiation in MR has also been linked to variation in physical activity. For example, Ohlberger *et al*. (2008) found that two coregonid species differ in temperature-related active metabolism during swimming. Thus, it appears that divergent selection can influence the physiology-based competitive strengths of fish populations, contributing to population divergence and possibly even speciation.

Metabolic efficiency, a fundamental and potentially heritable component of fitness, may be favoured by selection (Watson & Lighton, 1994), especially when the time required for a particular activity is high, as is the case for territorial defence in cichlids (Maan *et al*., 2004). The animals were bred in a common laboratory environment, so the metabolic differences in this study are likely heritable and imply genetic differences between *P. nyererei* and *P. pundamilia* males at the incipient species status. The effects of plastic physiological responses, however, cannot be ruled out (Dufty *et al*., 2002; Westerterp, 2003; Høydal *et al*., 2007).

It is possible that the metabolic differentiation between *P. nyererei* and *P. pundamilia* males is a pleiotropic effect of one or several neuroendocrine pathways that have diverged between the two sibling species. For example, skin pigmentation has been suggested to be closely linked to energy homeostasis through melanocortin pathways, possibly resulting in metabolic differentiation between colour phenotypes (Ducrest *et al*., 2008). Second, hormones, in particular steroid hormones that influence aggressiveness may have pleiotropic effects on anabolic processes (Tsai & Sapolsky, 1996). Consistent with this idea is the finding that experimental elevation of the teleost-specific androgen 11-ketotestosterone increased metabolism in another African cichlid *Oreochromis mossambicus* (Peters 1852) (Ros *et al*., 2004). *Pundamilia nyererei* and *P. pundamilia*, however, do not differ in their hormone profiles of 11-ketotestosterone, testosterone and cortisol (Dijkstra *et al*., 2011, 2012), so it seems unlikely that these hormones mediate the sibling species differences in metabolism.

Agonistic behaviours are driven by rapid body movements which are largely powered by anaerobic white muscles (Marras *et al*., 2010). Although aggression clearly involves an increase in oxygen consumption, as indicated by the positive relationship between activity and oxygen consumption, this effect is likely to extend beyond the time span of the measurements as the oxygen debt is being re-paid. This may account for the imperfect correlation between aggression and oxygen consumption.

In the experiments, *P. pundamilia* males were on average larger than *P. nyererei* males. While in some populations *P. pundamilia* males indeed attain a larger body size than *P. nyererei* males (Seehausen *et al*., 1998*b*), data from the laboratory suggests that *P. nyererei* and *P. pundamilia* males do not differ in growth rate (van der Sluijs *et al*., 2008; P. D. Dijkstra, unpubl. data). The size disparity between *P. pundamilia* and *P. nyererei* therefore reflects the availability of fishes at the time of the experiment, and the key results remain the same when focusing on the overlapping size range, ruling out size as a cause of the difference in metabolism of the two sibling species.

Previous studies showed that *P. nyererei* males experience a social dominance advantage in dyadic combat with size-matched *P. pundamilia* males (Dijkstra *et al*., 2005). It was hypothesized that *P. nyererei* and *P. pundamilia* males would pay the same energetic costs at a given level of aggressiveness. The higher rate of aggressiveness of *P. nyererei* males (Dijkstra *et al*., 2010) would then result in higher energetic costs, which might offset their advantage in aggressive interactions. In this study, however, *P. nyererei* males reduced the energetic cost of their intrinsic higher rates of aggressiveness by being metabolically more efficient than *P. pundamilia* males. It is difficult to evaluate whether this results in *P. nyererei* males paying a lower total energetic cost of social interactions than *P. pundamilia* males, as this will depend on their relative rates of aggression in the natural environment. Other factors are probably necessary to stabilize coexistence of the two colour types. For example, it is possible that there is a physiological cost to producing or displaying the red colouration (Clotfelter *et al*., 2007), and it has recently been shown that *P. nyererei* and *P. pundamilia* males differ in immune function and oxidative stress (Dijkstra *et al*., 2011). Finally, *P. nyererei* males might be more easily detected by visual predators. While this remains untested for *Pundamilia*, Maan *et al*. (2008) found that red and orange morphs of the related species *Neochromis omnicaeruleus* (Seehausen *et al*., 1998*a*) suffer higher mortality than other morphs under avian predation. Finally, *P. nyererei* and *P. pundamilia* males at Kissenda Island show subtle differentiation in vertical distribution and ecomorphology (Magalhaes *et al*., 2009; Seehausen, 2009). Thus, energetic demands, metabolism, immune function, sexual selection, differential predation risk and resource partitioning may all interact to stabilize coexistence of the two *Pundamilia* species.

Sexual selection, including male–male competition, has been implicated in the dramatic diversification of East African cichlids, but its physiological underpinnings have received limited attention (Kocher, 2004; Seehausen & Schluter, 2004). Here, sibling species divergence in agonistic behaviour was linked to metabolic differentiation in a pair of fully sympatric sibling species of a haplochromine. Asymmetric aggression between conspecific colour morphs and sister species appears to be common in East African cichlids (Genner *et al*., 1999; Dijkstra *et al*., 2010). Correlated (perhaps pleiotropic) divergence in behaviour and physiology might be widespread in the dramatically diverse cichlid radiations in East African lakes and may be a relevant factor in the remarkably rapid speciation of these fishes.

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