ELSEVIER

Physiology & Behavior 89 (2006) 164 – 170

PHYSIOLOGY & BEHAVIOR

Aggressive behaviour and energy metabolism in a cichlid fish, *Oreochromis mossambicus*

Albert F.H. Ros [a,*], Klaus Becker [b], Rui F. Oliveira [a]

[a] *Unidade de Investigação em Eco-Etologia, Instituto Superior de Psicologia Aplicada, Rua Jardim do Tabaco 34, 1149-041 Lisboa, Portugal*
[b] *Department of Aquaculture Systems and Animal Nutrition in the Tropics and Subtropics, University of Hohenheim, Germany*

Received 19 January 2006; received in revised form 4 May 2006; accepted 30 May 2006

Abstract

We have investigated the effect of mirror-elicited agonistic behaviour on oxygen consumption in the Mozambique tilapia, *Oreochromis mossambicus* (Cichlidae). Males exposed to their mirror image showed higher frequencies of both lateral display and tail-beating and escalated aggression more frequently than males exposed to a transparent glass that was used as a control for the presence of a novel object in the tank. This aggressive response was correlated with an increase in oxygen consumption. Overt aggression was highly correlated with display behaviour and with locomotor activity. Bivariate analyses showed high correlation (explaining about 64% of variation) between overt aggression, locomotor activity and metabolic rates. Weakly positive bivariate correlations between displays and metabolic rates turned spurious after partialling out aggression. The data suggest that energetic costs only emerge late during the conflict, when animals escalate their aggressive behaviour.

Keywords: Mirror-elicited aggression; Agonistic display; Metabolic rate; Tilapia; *Oreochromis mossambicus*

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1. Introduction

Competing males use a ritualized sequence of visual, acoustic, and tactile signals that may escalate to overt (physical) aggression during agonistic interactions [1–4]. This sequence has been interpreted as a way to sort out winners and losers at an early stage of the conflict, thereby preventing the escalation of the fights during which both parties incur physical damage ([5–10], but see [11]). In order to prevent cheating at the early stage of conflicts, it is expected that the expressed agonistic signals (e.g. aggression and displays) convey honest information on the relative fighting ability of the opponents. This may occur when their expression has some significant intrinsic cost associated [12,13]. In this paper we investigate the potential energetic costs of such displays.

The metabolic consequences of fighting have been described in detail in *Betta splendens* by Haller [14]. This species directly escalates in staged dyads, and amino acid and glycogen content of the muscles significantly decreases already after 10 min of fighting. Neat et al. [8] showed that fighting *Tilapia zillii* males depleted sugar reserves and accumulated lactate in their muscles. In both these studies, losers were reported to incur higher costs than winners [8,14]. Grantner and Taborsky [15] measured direct oxygen consumption using respirometry in males of another cichlid species, *Neolamprologus pulcher*. In these males, agonistic behaviour increased approximately five times the energy expenditure relative to the basal metabolic rate. These authors used a mirror to elicit aggression in their experiment which has the advantage that during fighting no damage is inflicted on the focal animal and data can be collected on an individual basis [16]. Both studies on cichlids suggest that the high energy expenditure of fighting is restricted to the escalation part of the interaction, which is likely to be a consequence of the increased motor activity associated with the expression of overt aggression [8,15].

The objective of this study was to investigate energetic costs of agonistic behaviour in Mozambique tilapia (*Oreochromis mossambicus*) males. This species was chosen because males show escalated fighting towards their own mirror image [17], and also because individuals of this species readily adapt to staying in the metabolic chambers used in respirometry [18–20]. The experiment was part of a study on the effects of the androgen

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* Corresponding author. Tel.: +351 218811700; fax: +351 218860954.
E-mail address: aros@ispa.pt (A.F.H. Ros).

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doi:10.1016/j.physbeh.2006.05.043

11-ketotestosterone on metabolic rate. Males treated with the androgen had higher basal metabolism [21]. Here we test whether this treatment showed any interaction with the relationship between behaviour and metabolism.

2. Materials and methods

2.1. Animals and housing conditions

Adult *O. mossambicus* males of 2 to 4 years old were kept in mixed sex groups, typically holding two males and three females, in 750–800 l aquaria at the animal housing facilities of the Instituto Superior de Psicologia Aplicada, Lisbon, Portugal [22]. Only males were selected that were reproductively active (see below), and all males had experienced multiple spawnings in the stock aquaria (males may reach sexual maturity at about 9 months of age [23]). Water was continuously aerated and kept at a temperature of 26 °C (±1 °C), and the photoperiod regime was 13 L:11 D.

In half of the animals ($n=14$) the levels of the androgen 11-ketotestosterone (KT) were experimentally elevated by implanting them with a silastic KT implant. The other half ($n=13$) received a silastic implant with castor oil only (for details about surgery see Ros et al. [21]). The levels of circulating KT in both groups were 1.34 ± 1.31 (avg±SD) ng/ml in control males and 2.45 ± 0.83 (avg±SD) ng/ml in KT treated males [21].

At day one of the study, dominant males were identified in stock tanks and caught. These males were individually housed in 12 l aquaria during seven days, visually and chemically isolated from other males. To standardize body condition, during the first six days of isolation they were fed proportionally to their metabolic body mass: 9 g food pellets per $kg^{0.8}$ fish per day (pellets were made at the Department of Aquaculture Systems and Animal Nutrition in the Tropics and Subtropics, University of Hohenheim: 42.0% crude protein, 9.9% crude lipid, 11.3% crude ash, 20.3 kJ gross energy). This was followed by 1 day of fasting to prevent interference of heat increment of feeding on oxygen consumption measurements [24]. Fasting was continued for the three days during which the males were in respirometry.

At experimental day 8, body mass (average 75.8 ± 4.3 g) was measured and they were individually placed in the sealed respirometer chambers that were visually isolated from each other.

At experimental day 11 mirror and control tests were carried out. Two adjacent chambers were sampled for oxygen consumption and fish behaviour was recorded on video for later analyses. Oxygen consumption was sampled once every 3 min per chamber. A reference oxygen value was taken from a chamber without fish before the start of the experiment. To calculate base-line oxygen consumption, two measurements were taken before the start of the mirror or control stimulation. Stimulation started by placement of a mirror or a similar sized glass window on one of the sides of the chambers and ended 36 min later by taking it away. Three hours later the experiment was repeated but the chamber that previously had a mirror treatment now received a glass treatment and vice versa, so that the order of presentation of mirror vs. glass was balanced among all fish. During stimulation, oxygen was measured once every 3 min. Once every 9 min, a reference value was obtained from the chamber without fish. Three consecutive oxygen consumption values were averaged resulting in eight values per individual (four periods of 9 min for mirror and control stimulation). At the end of the experiment (day 12), all males were returned to their original stock tanks.

2.2. Measurements of activity and behaviour

Video recordings were analysed using the software package JWatcher (v. 0.9, Animal Behaviour Laboratory, Macquarie University, Sydney, Australia). The following behaviours were scored: Motor *Activity*: the percentage of the total time in which the animal was not immobile and lying on the bottom of the chamber; Overt *Aggression*: buts and bites which in this set-up were directed towards the side of the chamber where the mirror/glass was placed; *Tail-beating*: a sudden slap of the tail. *Frontal display*: the male is in a swimming position and oriented frontally towards the side of the chamber where the mirror/glass was placed with dorsal fin extended. Observations from video recordings limited observation of extension of the opercula and therefore this aspect of the display was left out of analyses; *Lateral display*: as Frontal display but shown in a lateral orientation in respect to the side of the chamber where the mirror/glass was placed; Other social behaviours such as circling were performed in very low frequency and thus were left out of analyses.

2.2.1. Calorimetric system and oxygen consumption measurements

Energy metabolism was measured using an open flow-through respirometry system [25], designed to record oxygen concentrations sampled from eight different respiration chambers at constant intervals [21]. Water was kept at a constant temperature of 26 °C (±1 °C), filtered over charcoal (Eheim filter, Germany), and oxygenated with an air stone. Each chamber of the respirometer was made from flat, optically clear 12 mm thick acrylic plastic (Perspex) (outside dimensions $154 \times 154 \times 262$ mm). A chamber containing no fish was used as control to correct for possible consumption of oxygen by algae and bacteria present in the water.

Automated continuous flow sampling allowed to measure oxygen consumption of several chambers over a single sensor (CellOx® fitted with stirrer type R2 300 in a through flow cell type D201; WTW GmbH, Germany). The oxygen meter (Oxi 197; WTW GmbH, Germany) was logged to a computer that allowed for automated online acquisition of the data for later analyses.

Metabolic rates were calculated using the method of Niimi [26] which corrects for a time-lag due to washout delays caused by the volume of water in the respiration chamber [25,27]. Oxygen consumption is strongly influenced by the amount of metabolically active tissue in the organism, and therefore scales with body mass with an allometric factor of 0.8 [18,28]. Therefore we report oxygen consumption rates (r) corrected for body mass (M) using this allometric factor ($r \cdot M^{-0.8}$ mmol O_2 h^{-1} $kg^{-0.8}$). After correction no significant correlation of body mass (range 33

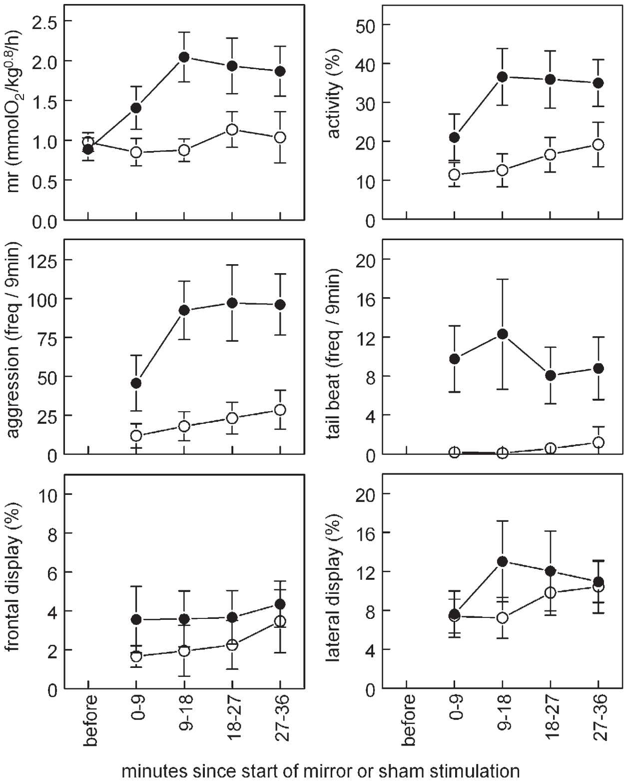

Fig. 1. Time course (average ± SE) of metabolic rate (mr), overt aggression, agonistic behaviour (tail-beating, frontal and lateral display) and motor activity during the mirror or control stimulation experiment. Metabolic rate values have been corrected for differences in metabolic body mass. Lines with filled symbols depict patterns during mirror stimulation; lines with open symbols depict patterns of the control group.

to 149 g) with oxygen consumption rates remained [21] (only subjects with body mass lower than 30 g show a deviation in corrected oxygen consumption rates, Ros and Oliveira unpublished observations).

2.2.2. Statistical treatment

Three subjects were left out of the analyses. In two cases the oxygen consumption measurements were invalid due to blockage of the flow regulators. In one case the wound made during surgery was not closed, which might have interfered with the oxygen and behavioural measurements (the hormonal implant was visible from the outside). Therefore the final sample size was 24 males.

Data were normalised using the appropriate transformations proposed by Zar [29], logarithmic transformation for continuous variables (oxygen consumption data), Poisson transformation for frequencies (overt aggression and tail-beating), and arcsin transformation for percentages (time per observation period in which animals showed lateral or frontal display, or motor activity). Using these transformations, Kolmogorov–Smirnov tests did not show significant deviation from normality for any of the measurements (all variables $p > 0.10$) taken during mirror stimulation. However, of the measurements during control stimulation, tail-beating and aggression deviated significantly from normality due to a large number of zero values. Due to the lack of accessible equivalent non-parametric methods, we still opted for using parametric ANOVA for testing the effects of mirror stimulation and of the different phases of the contest in these behaviours. For all ANOVAs violations of the sphericity assumption and equality of variances were tested and conservative (Huyn–Feldt) corrections of p-values were used when required.

To test for overall effects of KT treatment and mirror stimulation a multivariate ANOVA was carried out with metabolic rate, the aggressive behaviours (overt aggression, tail-beating, frontal display and lateral display), and motor activity as dependent variables, and with two factors: mirror stimulation (two repeated levels: exposure to mirror and exposure to glass) and period (4 repeated levels: 0–9th min, 9th–18th min, 18th–27th min, 27th–36th min of the test period). KT treatment was entered as independent variable. To further test the significant relationships found in this analysis univariate ANOVAs were calculated.

Pearson correlation coefficient was calculated to describe the possible associations between metabolism and behavioural measurements. As a consequence of our sampling method our database included per individual eight cases including all the different measurement (mirror/glass × four periods). In order to prevent inflation of n-values due to pseudo replication we have used a re-sampling method [30]. A macro was made that used a random generator (Microsoft Excel Rnd function) to select one case per individual. For these 24 selected cases the bivariate correlation coefficients were calculated. This procedure was repeated 500 times and from the resulting coefficients we selected the median and the 25 and 75% quartiles, representing the precision of the estimation of the correlation coefficient in our sample. p-values were calculated based on $n=24$.

All statistics were calculated using the SPSS 13 package (SPSS Inc., Chicago, USA) and Microsoft Office Excel (Microsoft Corporation, USA) and p-values represent two-sided probabilities.

3. Results

3.1. *Effect of KT treatment on energy metabolism and behaviour*

Multivariate analysis showed non-significant for the effect of KT treatment (treatment: Wilks' $\lambda(15,8)=0.705$, $p=0.24$) and all interaction effects with KT treatment (all Wilks' $\lambda(15,8)>0.22$, all $p>0.19$). Further, KT treatment did not have a significant effect on the latency to first overt aggressive behaviour (mean ± SE: KT group = 7.2 ± 2.4 min; control group: 9.1 ± 3.0 min, two sample T-test: $T(22)=0.49$, $p=0.62$), or tail-beating (mean ± SE: KT group = 9.6 ± 3.5 min; control group: 10.1 ± 3.0 min, two sample T-test: $T(22)=0.09$, $p=0.93$). This suggests that the increased KT levels due to KT treatment did not increase the readiness of the focal animals to fight their mirror image in this experiment.

3.2. *Effect of mirror stimulation on energy metabolism and behaviour*

Multivariate analysis showed significant effects for both factors mirror stimulation and period (mirror stimulation: Wilks' $\lambda(1,22)=0.33$, $p<0.001$; period: Wilks' $\lambda(3,20)=0.62$, $p=0.020$), and a trend for an interaction effect between the two (Wilks' $\lambda(3,20)=0.73$, $p=0.088$). We subsequently calculated univariate ANOVAs for testing behavioural and metabolic variables separately. As depicted in Fig. 1, energy metabolism increased two-fold (about five times higher than resting levels in some individuals) from before the experiment to the second 9-min-period ($p<0.001$; Table 1), after which it remained constant. A similar pattern was present for motor activity, overt aggression, tail-beating and frontal display, which all increased during mirror stimulation in comparison to control stimulation (i.e. glass; Fig. 1; all $p<0.05$; Table 1). Lateral display durations were not significantly different between mirror and glass stimulation, but tended to increase during the mirror test, indicating a possible effect of the mirror image (Fig. 1, Table 1).

Table 1
Mirror stimulation statistical analyses results

Variables	ANOVA repeated factors		
	MIS	Period	MIS × period
Metabolic rate	$F1,23=32.0$, $p<0.001$	$F4,92=5.66$, $p<0.001$	$F4,92=5.86$, $p<0.001$
Aggression	$F1,23=31.6$, $p<0.001$	$F3,69=12.2$, $p<0.001$	$F3,69=1.75$, $p=0.18$
Tail-beat	$F1,23=35.6$, $p<0.001$	$F3,69=0.44$, $p=0.73$	$F3,69=0.60$, $p=0.59$
Frontal display	$F1,23=5.35$, $p=0.030$	$F3,69=3.99$, $p=0.019$	$F3,69=0.36$, $p=0.74$
Lateral display	$F1,23=1.25$, $p=0.28$	$F3,69=3.72$, $p=0.016$	$F3,69=1.52$, $p=0.22$
Activity	$F1,23=18.7$, $p=0.001$	$F3,69=7.62$, $p<0.001$	$F3,69=1.93$, $p=0.14$

For four variables the sphericity assumption did not hold. Therefore p-values were corrected using Huyn–Feldt epsilon.

Table 2
Pearson correlation coefficients ($N=24$) based on randomized sampling (500 iterations) of the database (eight cases per individual: four during mirror stimulations and four during control stimulation)

	Activity	Aggression	Tail-beat	Frontal display	Lateral display
Activity					
Aggression	$r=0.90$ (0.88–0.92)				
Tail-beat	$r=0.54$ (0.46–0.61)	$r=0.59$ (0.51–0.66)			
Frontal display	$r=0.79$ (0.72–0.84)	$r=0.62$ (0.55–0.69)	$r=0.43$ (0.33–0.54)		
Lateral display	$r=0.86$ (0.82–0.89)	$r=0.60$ (0.51–0.67)	$r=0.32$ (0.24–0.41)	$r=0.66$ (0.57–0.73)	
Metabolic rate	$r=0.76$ (0.70–0.81)	$r=0.80$ (0.75–0.84)	$r=0.56$ (0.49–0.62)	$r=0.62$ (0.52–0.69)	$r=0.55$ (0.45–0.62)

Shown are median with between brackets the values corresponding to the 75% percentiles.

Within the mirror treatment metabolic rate, motor activity, overt aggression and lateral display, increased significantly from the first to the second 9-min-period (paired T-test, $T(23)>2.77$, $p<0.011$), but such an increase was not seen in tail-beating and frontal display (Fig. 1, paired T-test, $T(23)<1.02$, $p>0.32$).

To test whether behavioural activity is costly correlation coefficients between each behaviour and metabolic rate were computed. Tail-beating was neither significantly correlated with frontal nor with lateral display (Table 2, lower quartile: $p>0.11$). All other aggressive behaviours were highly correlated amongst each other (Table 2, the lowest quartile of r reached a p-value below 0.05). There were significant positive correlations between both motor activity and metabolic rates and the different aggressive behaviours (Fig. 2, Table 2). Overt aggression showed the highest correlation: 64% (calculated from the correlation coefficient) of the variation in metabolic rates can be explained by variation in aggression. After partialling out aggression, no significant correlation was left between the residual metabolic rates and other display behaviours (all $r<0.18$, $p>0.40$).

4. Discussion

In this study, overt aggression, characterized by fierce motor activity followed by nibbling of the wall of the aquarium close to the mirror, showed a positive correlation with energy expenditure, explaining about 64% of its variation. Tail-beating and lateral displays were the most abundant agonistic displays during mirror-elicited aggression trials. However, and despite the correlation between these displays and overt aggression was

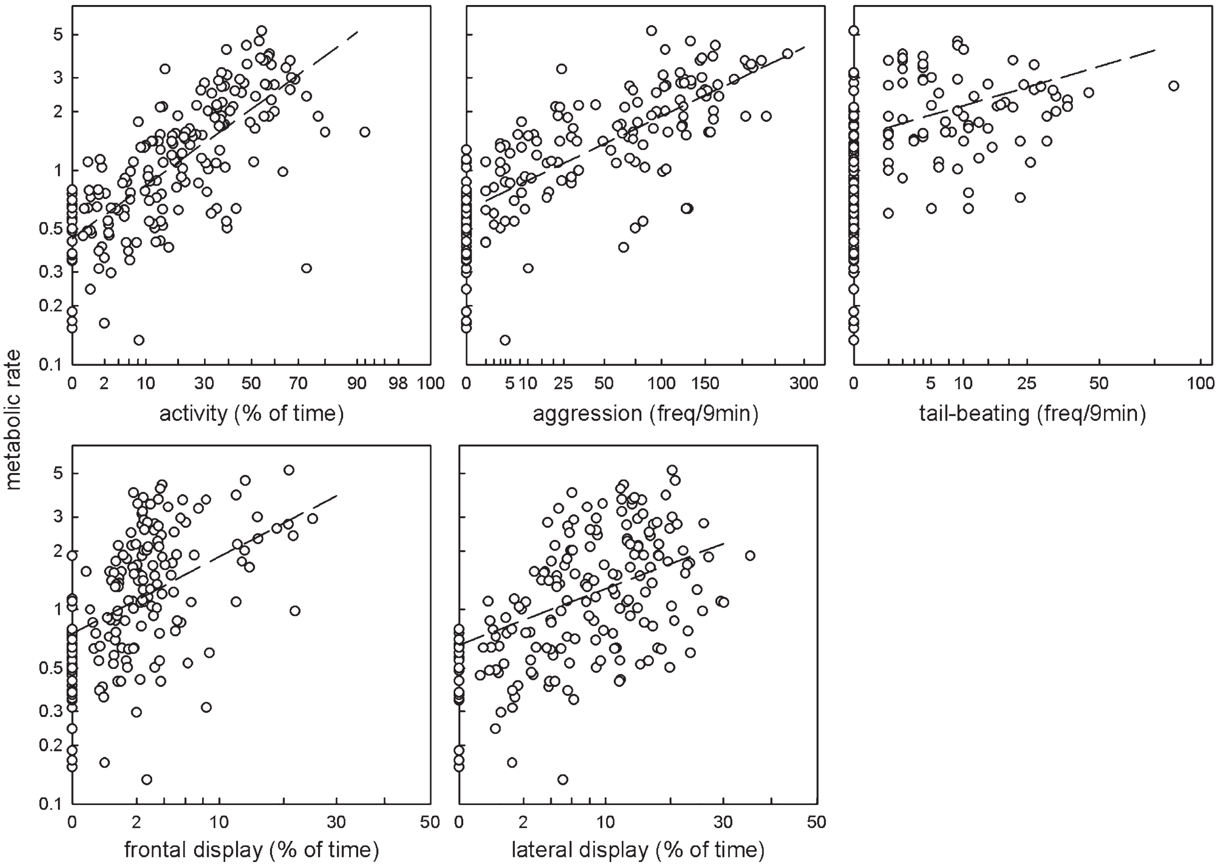

Fig. 2. Correlations between individual scores of metabolic rate (in mmol O_2 h^{-1} $kg^{-0.8}$), activity, aggression and tail-beating. In this graph eight values per individual ($n = 24$) are depicted. In order to prevent inflation of n-values by pseudo replication a re-sampling procedure has been carried out to calculate regression coefficients (see Materials and methods section).

highly significant, no significant correlations were found between these displays and energy metabolism. The weakly positive correlation between lateral display and metabolic rates lost significance after partialling out overt aggression. This suggests that these displays have relatively low energetic costs in comparison to the normal variation in metabolic rates. Therefore, the energetic costs of fighting behaviour seemed only to start playing a role during escalation, and thus relatively late in the fight.

In a previous paper we described that a treatment with the androgen 11-ketotestosterone increased basal routine metabolism, leading to an increased metabolism especially during the night period [21]. Here we show that slightly increased levels of this androgen, which is the active metabolite of testosterone in fishes, did not result in increased agonistic activity and metabolism in a mirror test. This result is relevant in questioning whether increased basal metabolic rates are necessary to anticipate fighting, a consequence thereof, or unrelated to aggressiveness [31,32].

Although slightly masked by the high frequency of aggression typical for mirror tests in this species [17], it can be conjectured from the results that agonistic displays in comparison with overt aggression are relatively more abundant at the earlier stages than at the end of the fight. This is in agreement with a classical study on display behaviour in *O. mossambicus* [1] and with studies on several other species of fish that show that lateral display and tail-beating are followed by higher intensity displays like frontal display, mouth-fighting display and finally overt aggression [3,4,33]. The sequential structure of fighting does suggest that displays such as lateral display and tail-beating are used as signals in the primary assessment of fighting abilities, based on which opponents might settle the conflict without proceeding to escalated fighting. Lower energetic costs during this primary assessment in comparison with escalated fighting are consistent with sequential assessment theory [34]. Interestingly, in some animals metabolic rates show a five-fold increase during escalation above the initial levels [15]. Such an increase in metabolic rates is close to the maximum level of sustainable energy metabolism in vertebrates [35,36] and thus escalation might be viewed as an endurance test [11].

Zahavi [37], proposed an alternative mechanism to energetic costs for the displays to function as honest signals that states that displays help opponents to assess relative differences in body size. Asymmetries in body size are expected to play an important role in male–male competition because larger males have repeatedly been shown to be more successful in establishing and defending high quality territories or spawning areas [38,39], and experimentally staged dyads between male cichlids show that relatively small differences in body size predict fighting outcome [3,40–42]. The absence of obvious asymmetries might explain why males so readily escalate aggressively towards their mirror image, as occurred here [31]. Testing such a signalling function of displays could be achieved in video-playbacks by presenting different sizes of the same displaying opponent and recording the latencies of the vocal males to attack the video image [43]. Other alternative hypotheses are that displays, due to differences in internal states (e.g. reproductive state, prior fighting experience or social status), signal the readiness and/or ability of the individuals to fight [4,44,45].

In summary our results do not reveal energetic costs associated with the performance of visual displays shown in initial phases of fights. This result suggests that the efficiency of energy production, such as metabolic scope, does not play a role during initial assessment. The results are consistent with sequential assessment with increasing energetic costs in the course of a conflict due to an increase in energetically costly overt aggressive behaviour. Differences in endurance to perform highly costly aggressive behaviour may play a decisive role in final assessment during escalated fighting.

Acknowledgements

RFO kindly provided the facilities for a sabbatical leave of KB at ISPA (Lisbon). The research and the sabbatical leave of KB were funded by a VW-Foundation grant, which also provided an assistant fellowship for AFHR to work within the scope of this grant. This study is part of a research grant funded to RFO by the Portuguese Foundation for Science and Technology — FCT (POCTI/BSE/38484/01). RFO's research is also supported by the Plurianual Program of FCT (R&D Unit 331/94). Two anonymous referees of P&B have given valuable comments in order to improve the manuscript. Procedures involving animals are in compliance with the "Principles of animal care", publication No. 86-23, revised 1985 of the National Institute of Health, USA, and with the current laws of Portugal.

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