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The effect of substrate availability on behavioural and physiological indicators of welfare in the African cichlid (*Oreochromis mossambicus*)

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Abstract

Male African cichlids (*Oreochromis mossambicus*) establish territories on the substrate upon which spawning pits are dug, thus attracting females. The substrate, therefore, plays a very significant role in its lifecycle. The effects of substrate access on behaviour and physiology in captivity were assessed. Mixed-sex, all-male and all-female groups were observed for five days, with and without substrate. Social patterns, behaviour directed towards the substrate, locomotor activity and spatial behaviour were recorded, and haematocrit, plasma cortisol and glucose levels were measured. Substrate inclusion saw a significant increase in behavioural diversity, sexual behaviour of dominant males in mixed groups, pit digging and territoriality whereas a lack of substrate was characterised by increased chafing and inactivity. Vacuum-pit digging was also observed. Frequency of aggression did not differ significantly and female behaviour was not affected by the presence of substrate. For both sexes, no differences in cortisol and glucose levels were found between the two treatments, but haematocrit increased with substrate. The key role played by substrate in territorial males is consistent with the behavioural and physiological data reported. In the absence of substrate, decreased territorial behaviour is contrasted with similar levels of aggression, cortisol and glucose; all of which are suggestive of a stress-related context. Moreover, the exhibition of vacuum activities is a signal that behavioural needs are not being met and may be some form of coping mechanism. These findings, taken in conjunction with the variations in behavioural diversity and inactivity, suggest that the welfare of male cichlids may be adversely affected by the absence of substrate.

Keywords: animal welfare, behavioural needs, cortisol, fish welfare, glucose, substrate

Introduction

The concept of animal welfare remains hugely relevant to all human activities involving live animals, and shapes the manner in which animals are kept and treated in captivity. Housing and husbandry standards as well as legislative requirements have been established in a number of different areas. Although all vertebrates are included, a significant portion of the acquired knowledge is concerned primarily with studies focusing on mammals and birds. However, since fishes constitute a very important resource — not only in aquaculture but also in public aquaria, in research and as pets — fish welfare has emerged as an area of growing interest.

There have been three basic approaches addressing the concept of welfare and its subsequent measurement. One of these is the comparison between natural behaviour and the behaviour expressed under artificial conditions (Duncan & Fraser 1997). Excluding fear responses, it assumes that natural behaviour is positively motivated and promotes biological functioning as it is the best way of portraying the preferences and evolutionary capabilities of a species to adapt to a given environment (Bracke & Hopster 2006).

However, caution must be exercised in interpreting the value of natural behaviour, in terms of the individual's welfare. Certain context-dependent behavioural patterns may disappear in artificial environments without negative consequences for welfare, but the restriction or prevention of those which exclusively derive from an internal motivation (behavioural needs) may impact negatively on welfare (Dawkins 1990). Furthermore, as Barnard and Hurst (1996) have pointed out, some behavioural patterns that may be interpreted at first sight as an indication of poor welfare may be linked inextricably to vital aspects of the species' evolutionary history and should, thus, not be considered to be impairing welfare. A second approach to the concept of welfare relates to bodily function, whereby health indicators (eg mortality, reproductive success) and physiological parameters (eg corticosteroids, blood chemistry) assume great significance as welfare indicators (Broom & Johnson 1993). Although extremely useful in providing information regarding the manner in which the body functions in certain circumstances, this approach should not be used to the detriment of behavioural observations and a detailed evaluation of the animal's

context (Mason & Mendl 1993). For example, glucocorticoids are produced in order to prepare the body to react to a great variety of stressors. However, their generalised use as stress indicators may be limited by a number of factors such as circadian rhythms, age, physiological status, hierarchical position in social groups, etc (Lane 2006). A third approach to the welfare definition and evaluation is the existence of subjective mental experiences, where attention is paid to how the animal perceives the situation it finds itself in (Dawkins 1980). To some authors this encapsulates what welfare is all about (Dawkins 1990; Duncan & Fraser 1997). Unavoidably indirect, the indicators of such mental experiences rely greatly on behaviour. Measurement of preferences and motivation has been the most popular approach in recent years. The exhibition of certain behaviours indicating frustration or conflict have also been used as possible indicators of impaired welfare (Mench & Mason 1997). At present, sophisticated strategies which expand upon the cognitive abilities of animals have been used to address the issue of subjectivity in animal experiences (Mendl & Paul 2004).

In previous decades, the vast majority of studies conducted on fish, relied on health and productivity indicators in aquaculture systems and on studies of stress. Although the existence of subjective experiences in fishes has been a matter of deep controversy (Rose 2002; Sneddon 2003; Sneddon *et al* 2003), there is a considerable body of evidence regarding conscious subjective experiences (Braithwaite & Huntingford 2004; Chandroo *et al* 2004). Fish welfare remains a relatively recent field of research and a number of blindspots in current understanding exist (for a review, see Huntingford *et al* [2006]). Among the areas considered to warrant more attention are a better understanding of fishes' behavioural needs and an improved array of welfare indicators.

In the present study, the African cichlid (*Oreochromis mossambicus*) was used as a model as it is widely used in both aquaculture and research, it is robust, easy-to-keep, easy to breed in captivity and its biology and behaviour are well known (Baerends & Baerends-van Roon 1950; Neil 1966; Fryer & Iles 1972; Trewavas 1983).

In this species, during the breeding season, males aggregate in shallow waters forming arenas. Here, individual territories are defined through the building of spawning pits (nests) on the substrate, to which ripe females will be attracted for spawning (Neil 1966; Nelson 1995). The attainment of specific territory and dominance positions are achieved through aggression and short bouts of combat are reported (Turner 1994; Oliveira & Almada 1996). However, according to Fryer and Iles (1972), agonistic encounters during maintenance of territories have evolved into more threatening displays, which are less deleterious. It is apparent, therefore, that the substrate plays a key role in reproduction and in the regulation of social interaction.

It is generally felt that in captivity the presence of a substrate is also a highly relevant environmental feature in modulating social interaction, as the behavioural repertoire is essentially the same (Baerends & Baerends-van Roon

1950; Pinheiro 1980). A number of authors only report qualitative differences in behaviour. Aggression appears more prevalent because the school spends more time swimming on the dominant animals' nesting areas and individuals remain in permanent contact with each other (Barlow 1974; Munro & Pitcher 1985). The period of time between the onset of courtship and effective spawning may be longer, with males exhibiting a particular sequence of courtship behaviours (tilt-lead-roll) more frequently in captivity than in the wild (Neil 1966).

In some artificial systems, this species, like many other cichlids for which the substrate plays a similar role, is kept in tanks without substrate. The lack of consideration for species-specific needs has adversely affected the welfare of many animals in captivity. It is this concern which has led to the development of environmental enrichment strategies with the primary objective of increasing physical and mental well-being through the promotion of more opportunities for species-typical behaviour (Kreger *et al* 1998).

The aim of the present study, therefore, is to discuss the importance of substrate to the African cichlid through measurement of behavioural and physiological parameters, recorded in relation to substrate availability and, in so doing, contribute to the current paucity of information on this subject.

Materials and methods

Animals and housing

Ninety-two adult African cichlid were used in this study (46 females and 46 males). The experimental fish were part of a stock held at the Instituto de Psicologia Aplicado in Lisbon, Portugal. The stock were maintained in glass aquaria (120 × 40 × 50 cm; length × width × height, 240 l), at a temperature of 26 (± 2)°C and on a light:dark, 12 h:12 h photoperiod. Each tank had a layer of fine gravel substrate, a double-filtering system (sand and external biofilter [Eheim GmbH & Co, KG, Deizisau, Germany]) and a constant airflow into the water. Water quality was analysed weekly for nitrites (0.2–0.5 ppm), ammonia (< 0.5 ppm) (Pallintest kit®, Pallintest Ltd, Tyne & Wear, UK) and pH (6.0–6.2). All individuals were identified by means of a transponder (Trovan Ltd, UK) ID 100; 2.2 × 11.5 mm; length × breadth) and/or a combination of three coloured beads attached to the underneath of the dorsal fin, by a nylon line. Fish were fed daily *ad libitum* with commercial cichlid sticks (ASTRA Aquaria GmbH, Bissendorf, Germany). The experimental conditions in testing aquaria (100 × 40 × 50 cm, 200 l) and isolation aquaria (50 × 25 × 31 cm, 40 l) matched those described for stock conditions.

Experimental procedures

Animals were grouped into three different sex ratio groups composed of four individuals: all males (MM), all females (FF) and half males/half females (MF). Each replicate included one of each of these three groups, in a total of eight replicates. One MF group was removed from the experiment due to the death of one male during the course of the experiment. Variation in body size within each group was kept as uniform as possible (standard length coefficients of variation:

0.06 [\pm 0.02]% for FF, 0.06 [\pm 0.03]% for females of MF, 0.05 [\pm 0.02]% for males of MF, 0.07 [\pm 0.02]% for MM).

For each replicate, the three described groups were subjected to two conditions in testing aquaria: a) without substrate and b) with a 7 cm layer of sand as a substrate. Prior to experimentation, individuals spent seven days in social isolation, to minimise possible effects of previous social experience and each condition lasted for a period of five days (Oliveira & Almada 1998a). Each replicate took 24 days to be finalised and to protect against any bias, the presentation of the two conditions was balanced among the eight groups (Figure 1).

Behavioural sampling

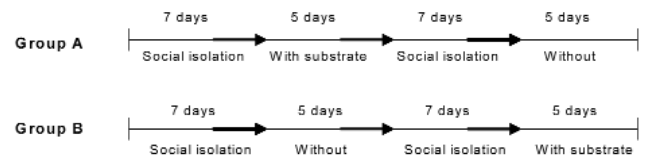
Behavioural sampling was carried out twice daily (1130 and 1530h) during the five days of each condition, in accordance with the following protocol (Martin & Bateson 1993): a) behaviour continuous sampling — a sampling period of 10 min for social interaction and behaviour associated with the substrate; scanning instantaneous sampling — at intervals of 30 s, the position of the four individuals on a grid (with six squares marked on the aquaria glass) was recorded for a total period of 10 min; behaviour continuous sampling — a period of 5 min only for behaviours associated with the substrate; focal continuous sampling — a period of 30 s per individual for locomotor activity (number of times the fish head crossed the lines of the mentioned grid).

Therefore, the daily total sampling effort was 58 min per aquaria. The behaviour patterns identified and recorded in this study have already been used in a number of studies (Oliveira & Almada 1998a) and were originally described by Baerends and Baerends van Roon (1950) and Neil (1966). For analysis, the behavioural patterns were grouped into behavioural categories (Table 1).

Social status

Since territoriality and courtship behaviour are shaped by dominance relationships in this species (Fryer & Iles 1972; Oliveira & Almada 1998a), the determination of social status was judged the best way to identify where possible differences between conditions (with and without substrate) exist. In order to estimate the social status of each individual the method validated by Almada and Oliveira (1997) was followed and adapted to the present data. Using a simulation programme (ACTUS [George F Estabrook, New Hampshire, USA: Estabrook & Estabrook 1989]), the analysis of 4×2 contingency tables (individuals \times number of performed agonistic behaviours/number of received agonistic behaviours) allowed individuals to be classified into three different levels of social status: a) dominants, when the number aggressive acts was larger than that expected by chance ($P < 0.05$); b) subordinates, when the number of received aggressive acts was larger than that expected by chance ($P < 0.05$) and c) intermediates, when the number of performed/received aggressive acts did not differ not significantly different from the simulated one ($P > 0.05$). In order to be able to be used in the statistical analysis, an overall

Figure 1



Schematic representation of the experimental design. Groups were run in pairs (A and B) in order to balance potential order effects of the access to the substrate.

dominance score (OD) was defined being: a) OD = 0, when in both conditions males were never dominant; b) OD = 1, when males were dominant only in one condition and c) OD = 2, when males were dominant in both conditions. According to this, in the all-male groups (MM) there were seven totally dominant males (2), two partially dominant males (1) and 23 males that were never dominant (0). In the mixed groups (MF), there were five totally dominant males (2), two partially dominant males (1) and seven males that were never dominant (0).

Female social status was not considered, as hierarchical relationships seem to be functionless in the context of their natural history. Although there is a reference to a nip-order hierarchy in females of the blue acara cichlid (*Aequidens pulcher*) (Munro & Pitcher 1985), and the occurrence of complex agonistic behaviour (eg displays) among females in aquaria, in nature they tend to spend their time shoaling with the exception of when they depart the schools to visit the males' arenas and incubate.

The aggressive encounters that occurred during this study did not lead to any injuries or fatalities in the males involved. Furthermore, the subordinates' behaviour did not appear to demonstrate any signs of major behavioural restriction (eg movement restriction, excessive submissive postures, feeding inhibition, etc), in which case the procedure for the prospective group would have been discontinued.

Blood sampling and assays of physiological parameters

In order to avoid circadian effects, sampling always took place at the same time (1300–1400h). Only one time point was chosen in order to avoid cumulative anaesthesia and handling stress which would interfere with the results. At the end of each treatment phase (day five), fish were removed individually from the aquarium and lightly anaesthetised (stage two [Ross 2001]) in a solution of MS-222 (tricaine methane sulphonate, Sigma [Sigma-Aldrich Corporate Offices, St Louis, USA]; 200 ppm). Samples of 100–200 μ l of blood were taken from the caudal vein (1 ml syringe; 25G/16 mm needle) and body length (total and standard) and weight were measured. The fish were then placed in aerated water and took between 30 s and one min to recover from the anaesthesia. Induction of anaesthesia and blood sampling took no longer than four min which is

Table 1 Brief description of behavioural patterns and respective categories.

Categories of behaviour [†]	Behavioural pattern [‡]	Description
Asymmetric low-intensity agonistic interactions (ALI)	Frontal display	Facing opponent, fish erects gill covers and branchiostegal membrane, usually with mouth opened.
	Lateral display	In parallel/antiparallel position to opponent, fish fully erects dorsal and anal fins, with spread caudal and pelvic fins, and eventually branchiostegal membrane erected.
	Tail beating	In lateral display and with vigorous body undulations, fish beats tail sideways.
	Approaching	Fish swims towards the opponent to a distance inferior to its body length, eventually in frontal display: it can or cannot touch and/or attempt to bite.
	Biting attempt	Fish approaches opponent with mouth opened and, just before touching its body, swims backwards.
Asymmetric high-intensity agonistic interactions (AHI)	Attacking	Fish swims rapidly and suddenly towards the opponent. It may or may not be followed by touching or biting.
	Chasing	Usually, after approaching or attacking, fish pursues opponent in a very rapid swimming.
	Circling	Fish swims around opponent in circles; it may be followed by more escalated forms of aggression.
	Touching	Fish induces physical contact with opponents' body (head, flanks, fins).
	Biting	Fish approaches or attacks opponent with mouth opened and rams it at any part of the body, but especially the flanks and close to the head.
Symmetrical agonistic interactions (SIM)	Carouseling	In lateral display, opponents circle each other.
	Mouth fighting	Mutual attempt to grip the opponents' jaws; once firmly seized, opponents pull and push with tail beats.
	Pendelling	Two nest holder males, facing each other's heads, rush at each other with dorsal and anal fins closed against the body, suddenly breaking just before contact.
Sexual interactions (SEX)	Tilting	The fish holds its body at an angle of about 30° to the horizontal, with the unpaired fins against the body.
	Leading	While tilting, the male swims in front of the female towards the spawning pit.
	Circling	With the female close to the pit, the male circles around it; when the female joins the male, they circle the pit with the male behind.
	Quivering	The male vibrates the body and presumably ejects sperm.
Behaviours associated to the substrate	Pit digging (DIG)	In a vertical position or at angle of 45°, with mouth opened, male digs a depression on the substrate. Two main movements are included: with head downwards, mouth is pushed against the substrate, filled with particles, which are ejected in the pit periphery and, in deeper pits, fish may swim up the slope pushing the substrate to the periphery.
	Territoriality (TER)	Male hovers near the bottom or remains immobile in touch with the substrate above the spawning pit.
	Inactivity (INA)	Fish remains immobile in touch with the substrate and/or hovering in non-territorial areas.
	Nipping substrate (NIP)	With head downwards, the body at an angle of 45°, and fins half-erected, fish vigorously nips out substrate; sand and particles may be carried some distance before being expelled, while fish explore an apparent wandering route.
	Chafing (CHA)	Fish shoots downwards to the bottom, lays itself on the flank and chafes over the substrate: then it rises and returns to previous position.
	Moving in the bottom (MOV)	Fish swims just above the substrate, very low in the water column.

[†] ALI includes all non-reciprocal display patterns of aggression; AHI includes all non-reciprocal overt patterns of aggression; SIM includes all reciprocal patterns of aggression. [‡] Adapted from Baerands and Baerands-van Roon (1950) and Oliveira and Almada (1998a).

the latency for cortisol release into the systemic circulation in response to handling stress (Foo & Lam 1993). The sequence in which fish were removed from the tanks, within each group, did not affect the cortisol levels (two-way-ANOVA, $F_{3,82} = 0.71$, $P > 0.05$).

For each blood sample, two heparinised capillary tubes were used for the haematocrit. These tubes were then centrifuged (3.5 min, 16,000 rpm) and the haematocrit read in accordance with Morgan and Iwama (1997). The remaining blood was centrifuged for 10 min at 3,500 rpm to isolate the plasma, which was then stored at -20°C until assayed for cortisol and glucose.

The free cortisol fraction was extracted from the plasma through addition of diethyl ether as the steroid solvent. The samples were then centrifuged (5 min, 1,000 rpm, 4°C) and frozen (10 min, -80°C) to separate the ether fraction which remained liquid. The steroids were then isolated through evaporation of the ether. This process was repeated twice. Levels of free cortisol fraction were then determined via radioimmunoassay, using the commercial antibody 'Anti-rabbit, Cortisol-3' (ref: 20-CR50, Brand Interchim, Fitzgerald, Montluçon, France) cross-reactivity: cortisol 100%, prednisolone 36%, 11-desoxycortisol 5.7%, corticosterone 3.3%, cortisone $< 0.7\%$. Intra- and inter-assay variability is 6.4 and 4.2%, respectively. Plasma glucose was measured by the enzymatic method with glucose oxidase in accordance with the Randox glucose assay kit protocol (Randox GL 2623, Randox Laboratories, Antrim, UK).

Data analysis

Statistical analysis was conducted in order to assess the effect of substrate on each behavioural and physiological parameter, using multiple repeated measures analyses of variance for males (repeated factor: substrate; independent factors: type of group, overall social status) and two-way repeated measures analysis of variance for females (repeated factor: substrate; independent factor: type of group). When significant differences in the variances between the two conditions were found (Levene's test), data were normalised using the transformations proposed by Zar (1984), namely logarithmic transformation for continuous variables (cortisol), Poisson transformation for frequencies (behavioural patterns) and arcsin transformation for percentages (haematocrit, position in the water column). In the event of data not meeting the parametric assumptions, ANOVAs were still undertaken due to the lack of equivalent non-parametric tests and because the F -statistic is remarkably robust to deviations of normality and heterogeneity of variances (Lindman 1974). Following the ANOVAs, planned comparisons of least squares means were performed between the two conditions (with and without substrate).

An index of behavioural diversity was computed through the adaptation of the ecological Shannon diversity index (Zar 1984; Galhardo *et al* 1996; Wemelsfelder *et al* 2000). Absolute diversity (H) provides a measure of diversity within a given sequence of behaviour. It is represented by the equation:

$$H = -\sum_{i=1}^S P_i \times \ln P_i$$

where, S is the number of behaviours in the sequence and P_i , the proportion of each behaviour. Relative diversity (R) is given by dividing H by H_{\max} (maximum absolute diversity possible within a given condition, ie all possible behaviours each of them occurring in equal proportions). Therefore, relative diversity means the diversity of behaviour in a given environment (H) as a function of the behavioural options available in that environment (H_{\max}) (Wemelsfelder *et al* 2000). To compare the differences between the diversity of behaviour in each of the two conditions, the Wilcoxon matched pairs test was used. Pearson correlations were used to compare social status in both conditions presented (with and without substrate) and also to establish the degree of association between the variation of glucose and cortisol in the tested fish.

A value of $P < 0.05$ was taken for significance in all statistical tests. The statistical package used for analysis was Statistica V.7.1® (StatSoft Inc, Tulsa USA).

Results

Social status

Variation of male social status was independent of the order of substrate presentation (ACTUS: $\chi^2 = 3.36$, $df = 2$, $P > 0.05$). However, a tendency towards a higher social status consistency was noted when substrate was available as the first condition. When an absence of substrate was the first condition tested (half of the groups), 58% of males (13 out of 22) maintained the same social status (four of which were dominant), four gained it (two became dominant) and five lost it (two lost dominance). Social status was correlated between both conditions ($r_s = 0.6$, $n = 22$, $P < 0.01$). However, when presence of a substrate was the first condition tested, more males maintained social status (83%, 20 out of 24) and only four changed it (two gained and two lost). Eight dominant males remained without any change between conditions and, here, social status showed greater correlation between both conditions ($r_s = 0.7$, $n = 24$, $P < 0.001$).

Social behaviour

Having access to substrate did not influence the frequency of agonistic interactions in male groups, with the exception of the intermediate social status ($n = 2$) in the MM group (Figure 2, Table 1). However, an effect was noted on the asymmetric aggression (low and high intensity) in relation to the social status, since these patterns of behaviour were more frequent in the overall dominant males (Figure 2, Table 2). Agonistic behaviour did not change with substrate in females, but it was more expressive in the FF groups than in the MF ones (Figure 3, Table 3).

Regarding sexual behaviour, a clear effect of substrate was noted as was the influence of type of group and social status among males. Frequency of sexual behaviour was higher in the dominant males of MF groups in the presence of substrate (Figure 2, Table 2).

As Figure 2 shows, sexual behaviour was also observed among males in the male groups. In an FF group, *ad libitum*

Table 2 ANOVA repeated measures results for male behavioural and physiological parameters.

Variables	G [†]	OD [‡]	S [†]	G × OD [‡]	S × G [†]	S × OD [‡]	S × G × OD [‡]
ALI	0.40, ns	46.45, <i>P</i> < 0.001	0.37, ns	1.60, ns	3.39, ns	1.83, ns	0.92, ns
AHI	0.59, ns	80.89, <i>P</i> < 0.001	2.28, ns	0.54, ns	0.76, ns	0.61, ns	1.91, ns
SIM	0.30, ns	0.77, ns	0.01, ns	0.05, ns	1.83, ns	0.05, ns	0.56, ns
SEX	13.24, <i>P</i> < 0.001	27.87, <i>P</i> < 0.001	9.33, <i>P</i> < 0.01	5.16, <i>P</i> < 0.05	8.44, <i>P</i> < 0.01	6.53, <i>P</i> < 0.01	7.08, <i>P</i> < 0.01
DIG	0.20, ns	16.94, <i>P</i> < 0.001	71.99, <i>P</i> < 0.001	1.39, ns	0.11, ns	22.70, <i>P</i> < 0.001	7.14, <i>P</i> < 0.01
TER	4.56, <i>P</i> < 0.05	21.69, <i>P</i> < 0.001	32.39, <i>P</i> < 0.001	0.58, ns	5.89, <i>P</i> < 0.05	5.87, <i>P</i> < 0.01	1.68, ns
CHA	2.53, ns	5.22, <i>P</i> < 0.05	9.11, <i>P</i> < 0.01	0.84, ns	0.95, ns	1.69, ns	0.21, ns
INA	2.00, ns	0.88, ns	7.34, <i>P</i> < 0.01	0.01, ns	1.90, ns	1.51, ns	0.15, ns
COR	0.85, ns	0.71, ns	0.01, ns	0.07, ns	0.03, ns	0.12, ns	0.16, ns
GLU	0.00, ns	0.97, ns	0.67, ns	0.19, ns	0.17, ns	0.05, ns	0.63, ns
HMT	0.00, ns	0.27, ns	27.82, <i>P</i> < 0.001	0.04, ns	0.04, ns	0.06, ns	0.07, ns

S: substrate (with, without); G: group (MM, MF); OD: overall dominance (0, 1, 2); ALI: asymmetric low-intensity aggression; AHI: asymmetric high-intensity aggression; SIM: symmetrical aggression; SEX: sexual interactions; DIG: pit digging; TER: territoriality; CHA: chafing; INA: inactivity; COR: cortisol; GLU: glucose; HMT: haematocrit. [†] $F_{1,40}$; [‡] $F_{2,40}$.

Table 3 Two-way ANOVA repeated measures results for female behavioural and physiological parameters.

Variables	G [†]	S [†]	S × G [†]
ALI	13.24, <i>P</i> < 0.001	0.08, ns	3.25, ns
AHI	4.80, <i>P</i> < 0.05	0.17, ns	1.73, ns
CHA	1.61, ns	6.30, <i>P</i> < 0.05	0.59, ns
INA	1.09, ns	7.54, <i>P</i> < 0.01	3.92, ns
COR	0.97, ns	3.53, ns	1.76, ns
GLU	0.00, ns	1.13, ns	0.03, ns
HMT	0.02, ns	56.04, <i>P</i> < 0.001	0.36, ns

[†] $F_{1,44}$.

observations showed two females building a nest, courting each other and spawning and, one of them, incubating unfertilised eggs which were eaten later. In mixed groups, no intra-sex courtship was observed.

Behaviours associated with the substrate

Pit-digging behaviour was more frequent in males with elevated social status (dominants once or twice in the two conditions), with access to the substrate. This behaviour was performed equally in MM and MF groups. Territorial behaviour varied in a similar manner, apart from the fact that a more marked difference was seen in the MF groups (Figure 4, Table 2). Nesting and territorial behaviour were also observed in the absence of substrate. For this reason, the frequency of territorial behaviour without substrate by the dominant males of MM groups did not differ significantly from the 'with substrate' condition. Both patterns of

behaviour were conducted by five dominant males (total number of dominant males = 12) and their frequency is also represented in Figure 4.

For both sexes, chafing was significantly higher in the absence of substrate. Among males, this difference was particularly relevant in the dominants of MM groups (Figure 4, Table 2). Females exhibited more chafing in the FF groups (Table 3).

An increased frequency in patterns of inactivity was observed in the absence of substrate, in both sexes of mixed groups (males: Figure 4, Table 2; females: Figure 3, Table 3).

No relevant substrate-dependent differences were found in locomotory patterns in either males or females.

Behavioural diversity

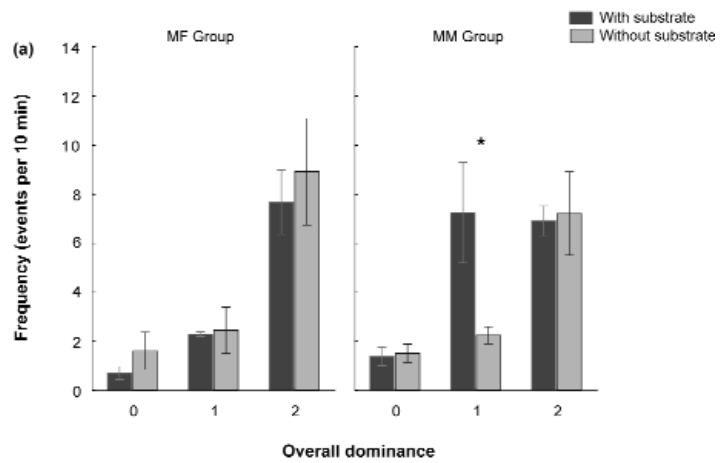
Substrate availability appears to increase behavioural diversity in males, but this difference is significant only in the MM groups (mixed groups: Wilcoxon matched pairs test, $Z = 1.66$, $P = 0.09$; MM groups, $Z = 2.25$, $P = 0.02$) (Figure 5). Among the females, substrate does not appear to influence behavioural diversity but in the FF group there is a tendency for greater diversity without substrate (Wilcoxon matched pairs test, FF: $Z = 1.78$, $P = 0.08$) (Figure 5).

Cortisol and glucose levels and haematocrit

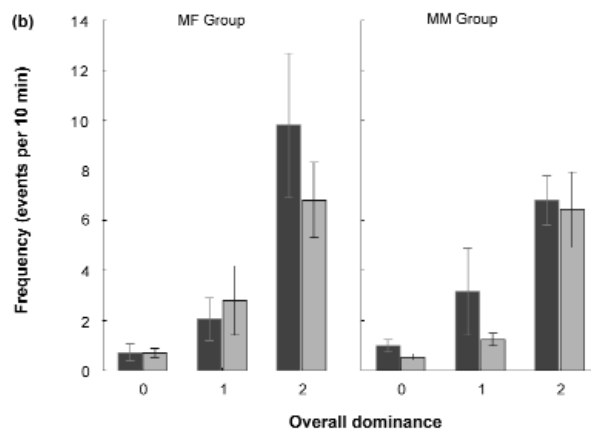
Access to substrate did not influence cortisol or glucose levels of either males or females (Figure 6, Tables 2 and 3). However, the latter had a tendency to show higher cortisol levels in MF groups that had substrate (Figure 6). Cortisol and glucose levels were correlated with each other in males ($r = 0.23$, $n = 88$, $P < 0.05$) but not in females ($r = 0.14$, $n = 82$, $P > 0.05$). The haematocrit values were higher with substrate for both males and females.

Figure 2

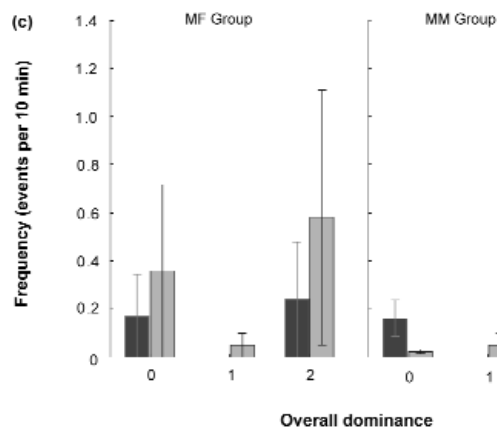
Mean (\pm SE) male social behaviour for (a) Asymmetrical low-intensity agonistic interactions. * $P < 0.05$. Overall dominance, 0: never dominant; 1: dominant in one condition; 2: always dominant.



Mean (\pm SE) male social behaviour for (b) Asymmetrical high-intensity agonistic interactions.



Mean (\pm SE) male social behaviour for (c) Symmetrical agonistic interactions.



Mean (\pm SE) male social behaviour for (d) Sexual behaviour. *** $P < 0.001$.

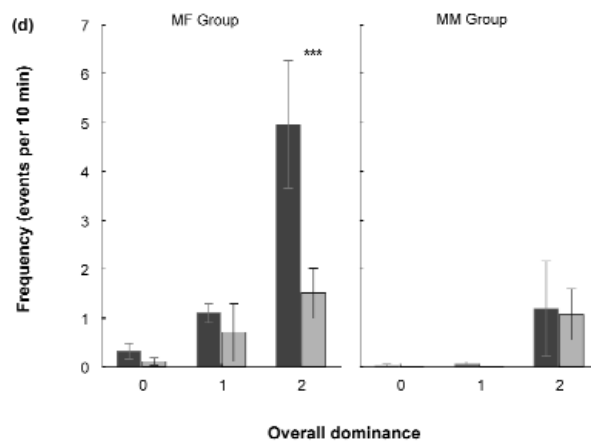
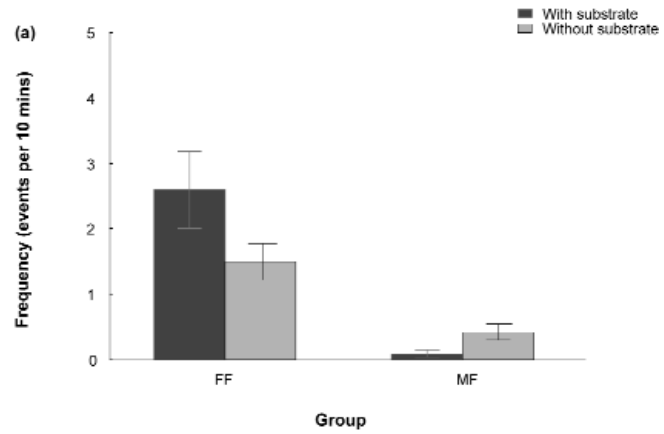
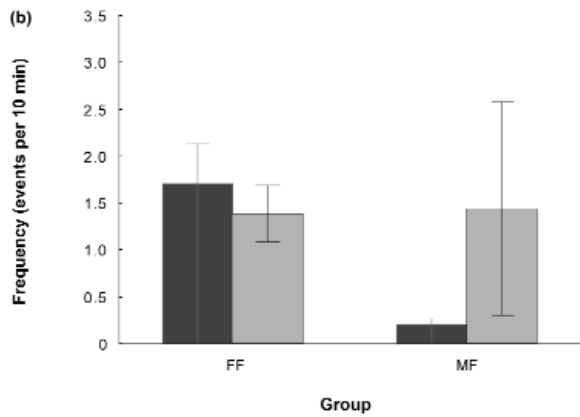


Figure 3

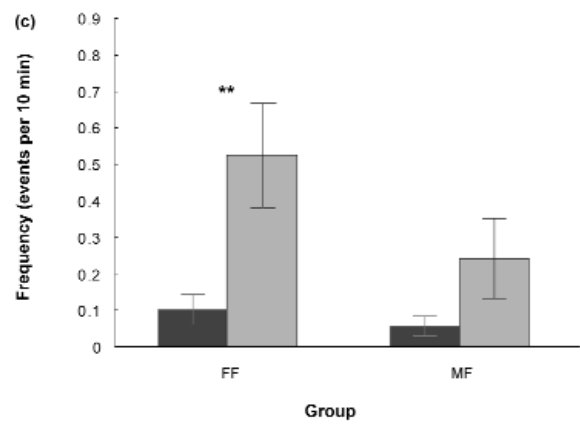
Mean (\pm SE) female behaviour for (a) Asymmetrical low-intensity agonistic interactions.



Mean (\pm SE) female behaviour for (b) Asymmetrical high-intensity agonistic interactions.



Mean (\pm SE) female behaviour for (c) Chafing. ** $P < 0.01$.



Mean (\pm SE) female behaviour for (d) Inactivity. * $P < 0.05$.

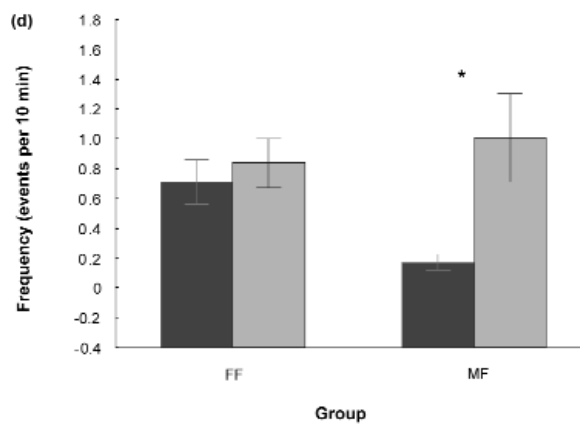
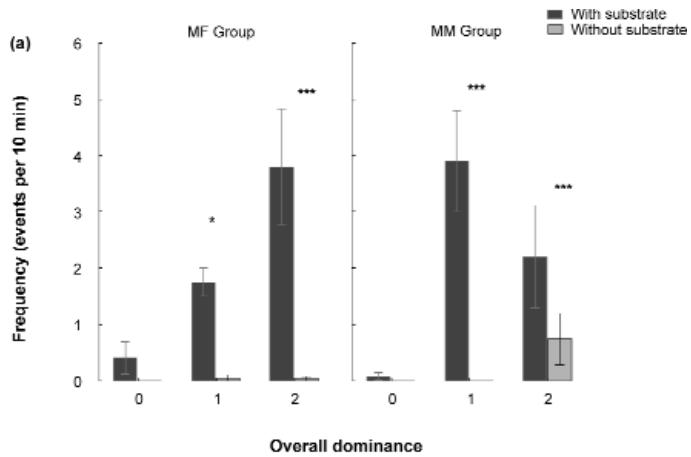
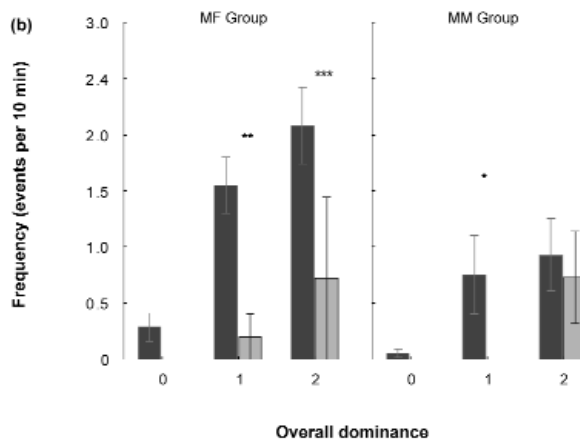


Figure 4

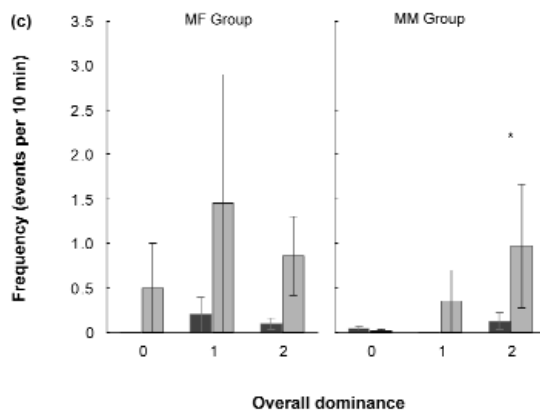
Mean (\pm SE) male behaviour associated with the substrate for (a) Pit digging. * $P < 0.05$; *** $P < 0.001$.



Mean (\pm SE) male behaviour associated with the substrate for (b) Territorial behaviour. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



Mean (\pm SE) male behaviour associated with the substrate for (c) Chafing. * $P < 0.05$.



Mean (\pm SE) male behaviour associated with the substrate for (d) Inactivity. ** $P < 0.01$; *** $P < 0.001$.

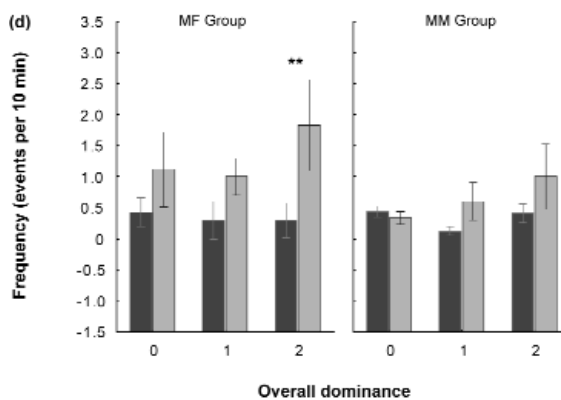
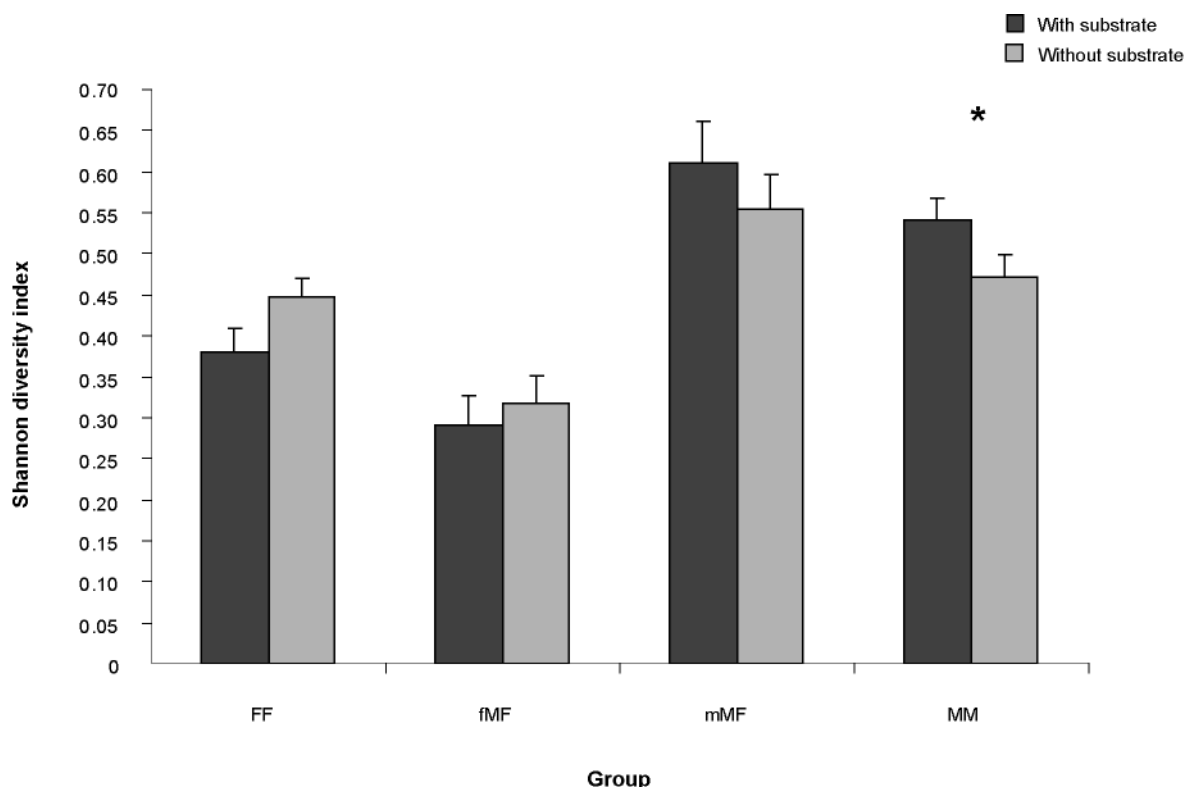


Figure 5



Mean (\pm SE) behavioural diversity. * $P < 0.05$, Wilcoxon matched pairs test.

Discussion

Male social status was correlated in both conditions, especially when presence of substrate was the first condition presented. Substrate availability did not influence the agonistic interactions of both sexes but increased sexual behaviour of dominant males in MF groups. Similarly, pit digging and territorial behaviour were more frequent with substrate, although these also occurred without this resource. Chafing and inactivity were more frequent in the absence of substrate in both sexes. Behavioural diversity decreased in males, but not in females. Physiological data show no differences in cortisol and glucose plasma concentration between the two conditions but haematocrit clearly increased with substrate.

Behavioural parameters

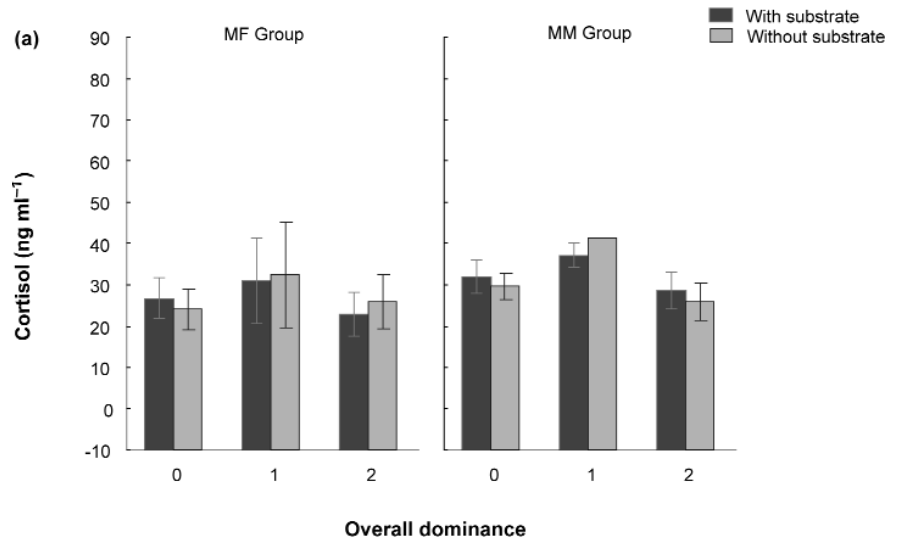
The majority of the males assumed the same social status regardless of substrate availability. However, this relationship was clearer in the groups where substrate was presented first. In these instances, 83% of males retained their previous social status, and all dominants kept their status, even after a period of seven days of social isolation. Since defining social status is important in the African cichlid in order to gain access to and defend territories in the substrate (Fryer & Iles 1972), this result suggests this resource has the potential to strengthen dominance-subordinate relationships.

Sexual behaviour increased in dominant males of MF groups in the presence of a substrate which suggests that the substrate may be an environmental facilitator for the exhibition of such behaviour. Dominant males in MM groups also exhibited sexual behaviour but this was not dependent on substrate. Oliveira and Almada (1998c) suggested that courtship among males may be associated with a low partner selectiveness in the early stages of the courtship process and that, for the courted males, this may reduce aggression.

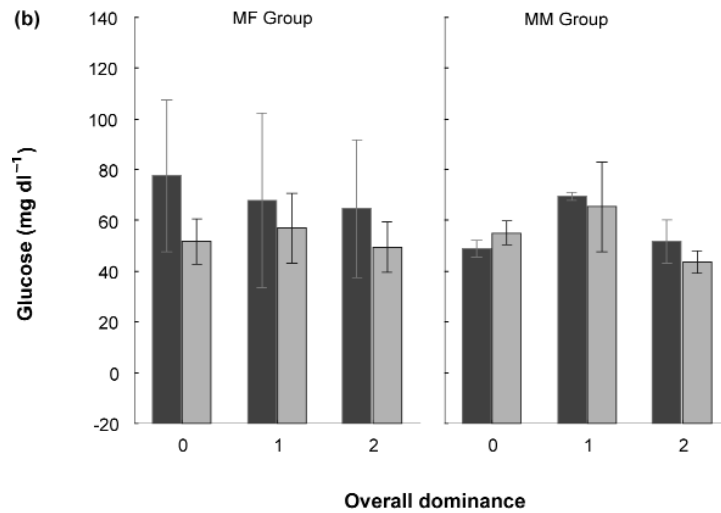
Symmetric aggressive interactions (fights) have been associated with initial group formation and the establishment of social status (Oliveira & Almada 1998a) and substrate availability did not affect their frequency. Asymmetric aggression of low intensity (displays) was higher in the intermediate social status of males in the MM groups. This could suggest that, for individuals such as these, tank minus substrate would be more beneficial. However, as the attainment of a social position is very important for reproductive success, aggressive encounters (especially displays, like these) are a part of their full species-specific repertoire and have evolutionary value. In captive conditions, however, care must be taken to avoid escalated fights from which the subordinates may struggle to escape. Among dominant males, asymmetric aggression was invariant with and without substrate. Overt

Figure 6

Mean (\pm SE) physiological parameters for
(a) cortisol levels in males.



Mean (\pm SE) physiological parameters for
(b) cortisol levels in females.



Mean (\pm SE) physiological parameters for
(c) glucose levels in males. * $P < 0.05$;
** $P < 0.01$; *** $P < 0.001$.

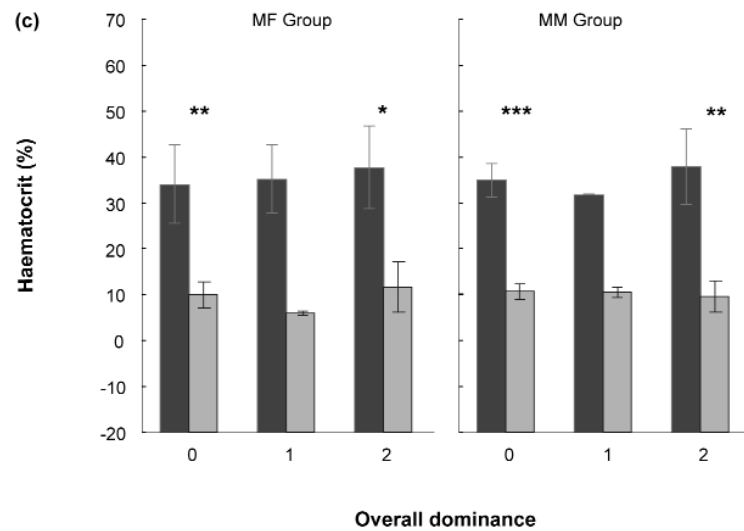
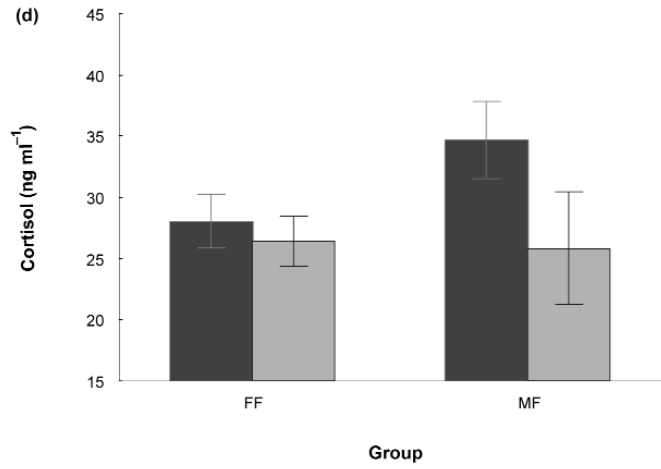
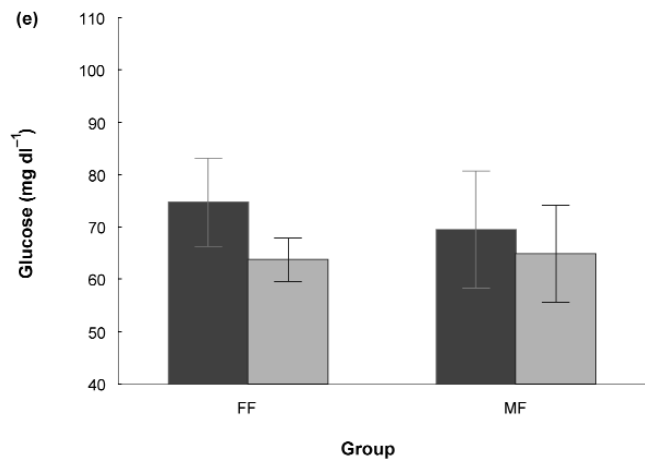


Figure 5

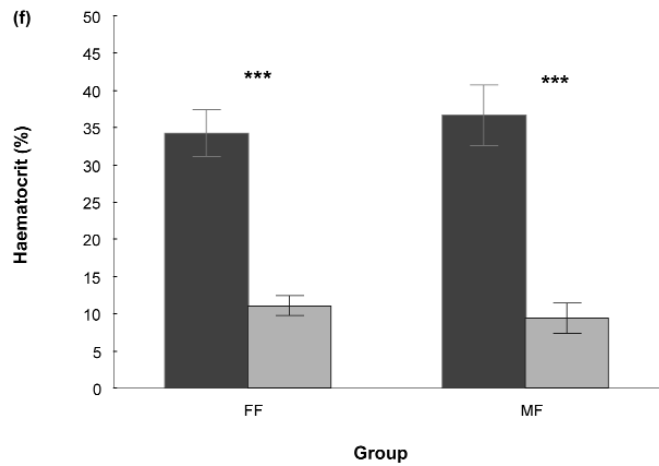
Mean (\pm SE) physiological parameters for (d) glucose levels in females.



Mean (\pm SE) physiological parameters for (e) haematocrit in males.



Mean (\pm SE) physiological parameters for (f) haematocrit in females. *** $P < 0.001$.



aggression appears to have a function in establishing and defending the nest site (Oliveira & Almada 1998a) but no variation occurred with substrate. This suggests it may have been stimulated by factors other than the competition for the nest/territory. In the African cichlid, agonistic and sexual behaviour are part of the same behavioural axis with the androgen 11-ketotestosterone being implied in both behaviours (Borges *et al* 1998). The high frequency of overt aggression without substrate may be related to this hormonal association and to a high motivation to breed, in an environment where the lack of substrate does not facilitate it. Additionally, some authors (Heiligenberg 1965; Barlow 1974; Oliveira & Almada 1998a; Mendonça 2006) have suggested that substrate may have a regulatory role on aggression and that digging behaviour could play a role as a re-directed pattern of aggression. Displaced aggression, towards tank mates (Clement *et al* 2005) and in the form of foraging (Munro & Pitcher 1985) have also been reported for other cichlid species.

Among females, also, no differences in aggression were observed with the availability of substrate, but increased aggression was noted among the FF groups. *Ad libitum* observations of FF groups also showed occasional nest building, courtship and incubation of unfertilised eggs by ovulated females. It is possible that aggression is the result of a high breeding motivation that does not find the appropriate social context in which to be expressed. Aggression among females has been reported exclusively in the breeding context, particularly during pre-spawning or brooding (Neil 1966; Oliveira & Almada 1998b); also corresponding to two peaks of testosterone during the females reproductive cycle (Smith & Haley 1988).

As expected, male pit digging and territorial behaviour followed a similar pattern and were generally more frequent with substrate. As mentioned above, establishment and maintenance of territories plays a very important role in the sexual and agonistic behaviour of this species. It is probably due to this that certain dominant males performed these behaviours even in the absence of substrate. Vacuum activities, such as these, have been used as possible indicators of behavioural needs, ie behaviours that are internally motivated and that can cause frustration and disturbance when prevented (Manning & Dawkins 1992). For this reason, they have also been interpreted in the context of animal welfare, despite lingering doubts surrounding them and the link with the occurrence of actual suffering (Dawkins 1990).

Chafing increased significantly without substrate, mainly among males of higher social status but also among females of FF groups (where more aggression was also noted). Chafing may be performed to release parasites or particles from the body surface and it may be associated with fighting animals which leave clouds of particles in suspension (Pinheiro 1980). However, the possibility that this behaviour is undertaken as a displacement activity in response to an adverse context (eg a lack of substrate in which to dig the nest in the context of male social interactions) should also be

considered and evaluated in the scope of specific work. The above conclusion was reached in light of the administration of two thyroid hormones, thyrotropin-releasing hormone and 3-Me-His2-TRH, in the jewel cichlid (*Hemichromis bimaculatus*) (Christ 1984). Behavioural patterns exhibited in displaced contexts may serve as coping mechanisms, revealing conflict, frustration or a disturbance of some description (Mench & Mason 1997). When prolonged or intense, observation of such patterns may be an indication of poor welfare (Dawkins 1980).

Inactivity in non-territorial areas without substrate was greater for both sexes, especially in MF groups. It is possible that some of the behaviours originally directed towards the substrate were replaced by an increased immobility in its absence. Fish can rest when they are satiated or there are no predators in the surrounding area and sleep has been reported for the African cichlid (Shapiro & Hepburn 1976). Activity and inactivity patterns can be very plastic in fishes, even at the individual level, and are modulated by a number of factors such as light intensity, temperature, shoal size, predation risk or intraspecific competition (Reebs 2002). For this reason, resting behaviours in fish, as in other animals, are not necessarily indicators of poor welfare. However, when prolonged or performed in unusual contexts, they can be a sign of barren environments (Broom & Johnson 1993). In this study, as no other condition changed but substrate availability, it is suggested that this increased immobility may be a response to a poorer environment.

Males showed a tendency for greater behavioural diversity with the presence of substrate. The fact that behavioural diversity did not reach levels of significance in MF groups is probably attributable to the smaller sample number which served to amplify the strong differences in behavioural diversity between dominants and subordinates, diluting the final result. It would appear that the presence of substrate stimulates a number of behaviours that, by definition, disappear from the non-substrate environment. This would ultimately lead to a reduction of the motivational drive to perform substrate-linked behaviours. However, the exhibition of vacuum activities seems to suggest that this motivation is still present, even without substrate. Behavioural diversity has been used often in the assessment of welfare, on the basis that the greater the diversity of behaviour, the greater the likelihood animals have of making use of their species-specific repertoire (Wemelsfelder *et al* 2000). However, caution must be exercised regarding its interpretation because certain functional behaviours (eg courtship, pit digging and other territorial patterns) may be replaced by non-related behaviours (eg hovering and immobility in non-territorial areas) or even by possible coping mechanisms (eg vacuum digging and territorial behaviours, chafing), despite demonstrating behavioural diversity. Therefore, it is the actual nature of the behaviours in question, and not merely their general diversity that is crucial in interpreting possible indicators of welfare.

Physiological parameters

Cortisol was not found to vary between both conditions. Specific variations of cortisol related to group composition or social status were also not found. Glucocorticoids, such as cortisol, have been used as primary indicators of stress and are affected by a multitude of factors (Lane 2006). Cortisol may be elevated during mating as a result of aggression, conflict and other endocrine changes that occur in the sexual context. Social status may also have an effect on both dominants (Correa *et al* 2003) and subordinates (Gilmour *et al* 2005). However, Earley *et al* (2006) point out that this relationship is not always clear since cortisol may vary with a number of subtle characteristics related to social dynamics or housing system. Furthermore, individual characteristics may lead to different behavioural and physiological coping strategies within the same social status, (Clement *et al* 2005). In light of this, it is probably the case that the social dynamics intrinsic to each group may have a combined effect on the lack of a cortisol pattern found in this study; the presence or absence of substrate being an indirect factor affecting the social interactions. Despite the lack of significant differences, there was a tendency towards increased cortisol in females of MF groups with substrate which may be the result of increased sexual context in these aquaria.

Elevated glucose is a secondary response to stress and has been used in a variety of fish species as an indicator of stress (Cnaani *et al* 2004). In this study, levels of glucose were equivalent to those expressed in stress-induced studies (air exposure: 45 [\pm 13.2] mg dl⁻¹); maintenance in high densities: 30–69 mg dl⁻¹ [Silveira-Coffigny *et al* 2004]). Glucose and cortisol levels were correlated in males but not in females. They did not vary significantly between both substrate conditions. The actual relationship between cortisol and glucose is still not fully understood in fish (Mommsen *et al* 1999). It is generally accepted that cortisol stimulates the release of glucose into the bloodstream, which may explain the correlation found in this study for these two physiological variables in males. This relationship, though, is not clear as plasma glucose concentration does not always increase with higher cortisol levels and can also vary with a number of factors, including physiological state, external disturbances to the animal or even the nature of the stressors involved (Mommsen *et al* 1999; Barreto & Volpato 2006). These aspects or the simple fact that males and females perceive the same stimuli (eg substrate) differently may be linked to the lack of a correlation in the females.

Haematocrit was clearly higher in fish with access to substrate compared to those without. It is apparent that haematocrit levels are often elevated during short-term stress as a way of increasing oxygen supply in response to a higher metabolic demand (Cnaani *et al* 2004), but it still remains unclear whether it can be used as a welfare indicator on a longer term basis (Broom & Johnson 1993). In this instance, the observed increase may be linked to reduced inactivity in both sexes and an increase in energy demand associated with nest digging in males.

Animal welfare implications

Aggression related to territoriality plays a crucial role in the survival and reproductive success of male African cichlid (Fryer & Iles 1972). Therefore, any assessment of welfare needs to take this into account; analysing aggressive behaviour in its appropriate functional and evolutionary context (Barnard & Hurst 1996). A naturalistic approach to welfare allows us to suggest, perhaps, that for males the lack of substrate does impact on their natural behaviour. This study allowed identification of a number of behavioural changes that occurred in the African cichlid when in the presence or absence of substrate which can be summarised as follows: natural territorial behaviour, possible coping behaviours, immobility and behavioural diversity. For males lacking substrate, territorial behaviour and subsequent breeding were affected; decreased sexual behaviour, pit digging and hovering over the nest (territoriality). The nature of aggression also changed as it was not based on competition for territories. This change may have negative implications for welfare as the aggression lacks its natural outcome (territory acquisition and defence) and subordinates may find it difficult to avoid agonistic encounters due to the nature of aquaria. Without substrate, some behavioural patterns were exhibited with a putative coping function. These included vacuum pit digging and territoriality and, eventually, chafing in the absence of substrate. The relevance of pit digging as a behavioural need should be further assessed through the development of preference and motivational studies. Immobility also increased as did behavioural diversity in males, suggesting a change in the usual behavioural time budget due probably to an impoverished environment. The effect of substrate availability is not apparent for females but a more specific study should be carried out in ovulating females for which the substrate could be more significant.

When physiological indicators are analysed together with behaviour in the presence of substrate, they seem to be consistent with the natural function of sexual and territorial behaviour. However, without substrate, this relationship is less clear for cortisol and glucose.

In conclusion, a lack of substrate affects males' behaviour, reducing certain patterns that are crucial to their fitness in the natural environment, and increasing others that may be linked to a high motivation to perform substrate-dependent behaviours. These facts suggest a negative effect on welfare and a requirement for further investigation.

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