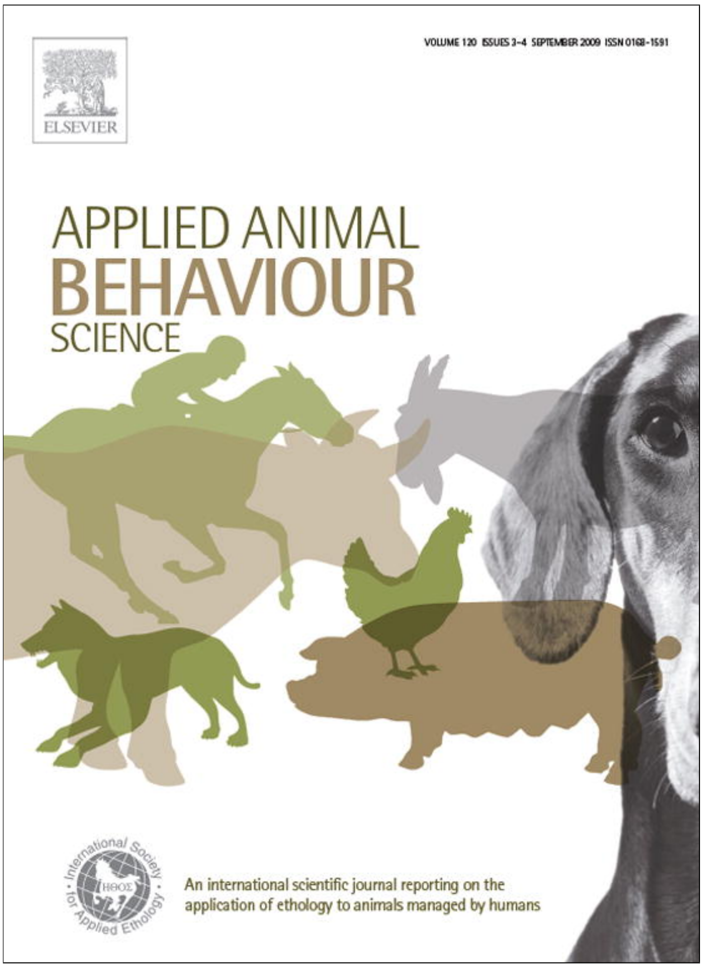

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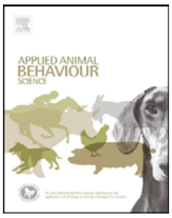

Preference for the presence of substrate in male cichlid fish: Effects of social dominance and context

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ABSTRACT

Many cichlid species dig spawning pits or nests in soft bottoms and exhibit many substrate oriented activities. Despite this fact being of general knowledge, captive cichlids in laboratory and aquaculture set-ups are often kept in the absence of a soft substrate that they can manipulate. This raises a potential welfare issue, depending on how the substrate is valued by cichlid fish. The aim of this study is to assess the importance of substrate for male Mozambique tilapia (*Oreochromis mossambicus*) in social and non-social contexts. Preferences were established as a measure of time spent in two choice compartments, before and after the presence of a female. Locomotory activity, social interactions and substrate-related behaviours were recorded. Results show that dominant males prefer the area with substrate regardless of social context, and that female's presence strengthens this preference. The same preference is not apparent in the subordinate males, except for foraging. To draw conclusions on the importance of substrate to subordinates, preferences should also be assessed in agonistic contexts, during which substrate may serve to displace aggression. These results, together with related previous studies, show that the lack of substrate is particularly deleterious in a reproductive context, and thus it is likely to decrease the welfare state of breeding males of Mozambique tilapia.

1. Introduction

Preference tests started to be developed in the scope of welfare studies in the 70s by Dawkins and others on floor preferences in hens (reviewed by Dawkins, 1980). The aim of preference tests is to assess the choice of an animal faced with a number of available resources. Although these tests have been widely used in fish to investigate aggression, mate choice, parental care, schooling, etc. (e.g. Gonçalves and Oliveira, 2003; Landmann et al., 1999; Schlupp et al., 1999; Schluter et al., 1998; Webster and Hart, 2004), their application to fish welfare is not frequent (Huntingford et al., 2006).

Fish welfare has been measured using several physiological and behavioural responses to stress as well as other indicators of organic functioning, such as incidence of diseases, growth and mortality rates (Iwama et al., 1997). However, the interpretation of these approaches in the light of the welfare concept may be very complex, even contradictory. Instead, the preference tests are probably among the most well described approaches to obtain an indirect expression of the animal's mental states (Dawkins, 2004; Duncan, 2006; Mason and Mendl, 1993; Volpato et al., 2007).

Substrate may serve different functional purposes according to the fish species, or even within the same species. Many African cichlids use substrate during the breeding season for nest building (Fryer and Iles, 1972). As some cichlid species have substrate-related feeding habits (Fryer and Iles, 1972), it is also possible that different substrate types have different impacts on foraging. For example, Webster and Hart (2004) show that three-sticklebacks prefer complex over simple substrate for

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foraging purposes. The feeding context also allowed Horstkotte and Plath (2008) to relate different diets with substrate preference in pupfish. Hoglund et al. (2005), in their study of monoamines and avoidance behaviour, have demonstrated the role of substrate in crucian carp as a relevant anti-predator feature of the environment.

In the present study, the relevance of substrate for males of Mozambique tilapia (*Oreochromis mossambicus*) is assessed. This species is an African mouthbrooder cichlid for which substrate is likely to have an important role, at least for territorial males during the breeding season. Males aggregate in leks in shallow waters during this season, where they establish social hierarchies (Neil, 1966; Nelson, 1995). Territorial males are usually bigger and stronger, with larger mouths and therefore more likely to win territorial contests (Oliveira and Almada, 1995). Disputes are mainly related to the acquisition of a territory, a depressions built in the substrate, to which ripped females are attracted for spawning (Fryer and Iles, 1972; Oliveira and Almada, 1996). Mouthbrooding takes place in areas outside the lek (Baerends and Baerends-Van Roon, 1950).

The lack of substrate can potentially disrupt the stability of the social hierarchy and decreases the sexual behaviour of territorial males of Mozambique tilapia (Galhardo et al., 2008). Males try to build nests, even in the absence of substrate and become more inactive when this resource is not available (Galhardo et al., 2008). Therefore, the lack of substrate may affect the welfare of, at least, territorial males during breeding contexts. Yet, these animals are commonly maintained in aquaria without substrate in experimental and aquaculture settings.

The aim of this study is to evaluate the preference for substrate in males of Mozambique tilapia. Due to the mentioned social differences in territories' acquisition, territorial and non-territorial males will be tested. The effect of a female's arrival to the set-up where males were placed alone is also assessed. Our main hypothesis is that territorial males prefer substrate, at least when a breeding context is created.

2. Materials and methods

2.1. Fish

The experimental fish were 29 males (weight: 99 ± 3.1 g) of the species *O. mossambicus*, belonging to a stock held at ISPA. They were maintained in glass aquaria (120 cm × 40 cm × 50 cm, 240 l), at a temperature of 26 ± 2 °C on a 12L:12D photoperiod. All experimental fish were part of eight mixed sex social groups (3–5 males/5–6 females), forming stable social hierarchies. Their social status was identified prior to the experiment. Territorial males adopt a specific nuptial black coloration and a frequent territorial behaviour which includes the nest building and its defence. Each tank had a layer of fine gravel substrate of the same kind as that used in the experimental aquaria. Tanks were supplied with a double filtering system (sand and external biofilter, Eheim) and constant aeration. Water quality was weekly analysed for nitrites (0.2–0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0–6.2). All individuals were marked by means of a combination of three colour beads attached underneath the dorsal fin, by a nylon line. This tagging method was adopted in order to differentiate animals in the stock with minimum manipulation. The experience in this lab is that it does not affect individual or social behaviour, namely the social ranks, and it does not cause local problems. Fish were fed daily *ad libitum* with commercial cichlid sticks (ASTRA).

2.2. Experimental aquaria

The experimental aquarium (100 cm × 40 cm × 50 cm, 200 l) was divided in three distinctive compartments (Fig. 1). The lateral or choice compartments have similar dimensions (40 cm × 39 cm) and the bottom of only one of them was covered with a 7 cm layer of fine gravel. To control for side effects, substrate was placed in each lateral compartment for half of the trials. The central or neutral compartment (20 cm × 19 cm) was the start box, where the male was placed to start the experiment. Behind it, there was a glass box (20 cm × 19 cm × 40 cm) physically isolated from the main tank, where a female is placed. Visual contact with the female compartment was only possible from the lateral compartments, since there was an opaque Plexiglas partition between it and the central compartment.

2.3. Choice test

The choice test was divided in two parts during which six observation periods of 10 min each were conducted. During the first part no female was placed in the female box, with the objective of testing the compartment choice in a non-social context. A male was removed from the stock tank and

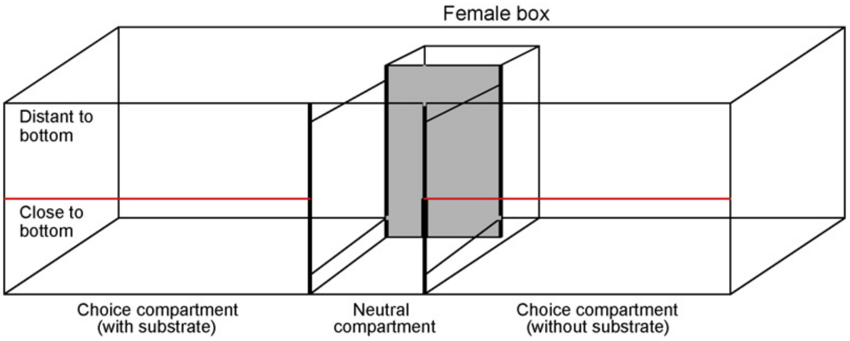

Fig. 1. Representation of the experimental set-up.

placed in the start box, separated from the lateral compartments by two removable transparent Plexiglas partitions. Two minutes later, when it reduced the ventilatory rate, assumed a non-stressed colour pattern (no dark stripes) and started to swim around, the partitions were removed and the male was allowed to swim freely in the experimental set-up. The first observation period was conducted immediately after the start box opening with the objective of following the behaviour during initial acclimation (observation 1). Two behavioural observations without female were respectively conducted 1 h and 2 h later (observations 2 and 3). In the second part of the choice test, a female was placed in the experimental set-up, with the objective of testing the compartment choice in a social/breeding context. A female belonging to the same stock tank (familiar) was placed in the female box, while the male was confined again in the start box. A total number of 29 females were used (one per male). After 2 min, the start box was opened and the behaviour of the male was immediately sampled (observation 4). Two trials on a social context were then conducted 1 h and 2 h after the female has been placed in the set-up (observations 5 and 6).

2.4. Behavioural sampling, exclusion and preference criteria

All observation periods involved a focal and continuous behavioural sampling (Martin and Bateson, 2007). Behaviours sampled are described in Table 1. In each lateral compartment two sub-areas were assigned in relation to their distance to the bottom (close and distant) for constant position sampling (Fig. 1). In this way, four areas were defined: substrate/close, substrate/distant, no substrate/close, no substrate/distant. Time spent in each of these four areas was used as a measure of preference. Latency to enter the first choice compartment was not considered because when the partitions of the start box were lifted, the animals simply entered the compartment to which they were randomly faced to.

Six males (4 territorial and 2 non-territorial) were excluded from trials since they have shown high indicators of stress, namely a characteristic colour pattern (body and eye dark stripes), high ventilatory rates and very high immobility. The excluded fish spent more than 85% of the time in the start box or in the first compartment to which they moved, and remained immobile during the first two sampling periods. The remaining sample was constituted by 23 males, of which 11 were dominants and 12 subordinates. All animals visited both compartments during the acclimation period and thus their choices were fully informed. Preference was defined as a fish spending more than 50% of time in one of the choice compartments, whether close or distant to the bottom (Schluter et al., 1998). These criteria were not applied to observations 1 and 4, which were both periods of acclimation to a new event.

2.5. Data analysis

For analysis, observations 2 and 3 and observations 5 and 6 were pooled together since preliminary analysis have shown no differences between them (Wilcoxon Matched Pairs Tests were run for observations 2/3 and 5/6 for behavioural patterns and use of area, $P > 0.05$). Discrimination of sub-areas (close or distant to the bottom) was analysed using the Wilcoxon Matched Pairs Test. Analysis of the frequencies of pit digging among the different observation periods was carried out with Friedman ANOVA. Time spent in the different areas of the experimental set-up as well as time spent performing specific behaviours in the two social contexts were analysed using a repeated measures analyses of variance (two repeated factors: social context, choice areas; categorical factor: social status). When there were significant differences in the variances between the two conditions (Levene's test), data were normalised using the transformations proposed by Zar (1984), namely Poisson transformation for frequencies (total number of behavioural patterns) and *arcsin* transformation for percentages (duration of behavioural patterns). Even, if the data did not meet the parametric assumptions, ANOVAs were still

Table 1
Brief description of the behaviour patterns and respective categories.

Categories of behaviour	Behavioural pattern[a]	Description
General activity	Swimming	Fish progresses through the water with body undulation and fins movements.
	Hovering	Fish remains motionless over the substrate or the bottom of the tank.
	Inactive	Fish remains motionless in touch with the substrate or the bottom of the tank.
Social interactions	Non-specific interactions	Fish touches or swims very close to the female box. While very close, it can or not synchronise the behaviour with the female.
	Courting	Includes tilting, where the fish holds body at an angle of about 30° with the horizontal, with the unpaired fins against the body, and leading where it swims in front of the female towards the spawning pit.
	Aggression	Fish performs agonistic displays, bites or tail strokes the female box walls.
Substrate-related behaviours	Nipping	With head downwards, body at an angle of 45°, and fins half-erected, fish thoroughly nips out substrate; sand and particles may be carried to some distance before being expelled, while fish describes an apparent wandering route.
	Pit digging	In vertical position or at an angle of 45°, with mouth opened male digs a depression on the substrate. With head downwards, mouth is pushed against substrate, filled with particles, which are ejected in the pit periphery.
	Dragging	Fish moves with the body at a slight angle and the inferior jaw in permanent touch with the bottom.

[a] Adapted from Baerends and Baerends-Van Roon (1950); Oliveira and Almada (1998a).

undertaken due to the lack of equivalent non-parametric tests and also because the *F*-statistic has been shown to be remarkably robust to deviations of normality and heterogeneity of variances (Lindman, 1974). Following the ANOVAs, planned comparisons of least squares means were performed where relevant.

A value of $P < 0.05$ was taken for significance in all statistical tests. All analysis was performed using the statistical package Statistica V.8.0® (StatSoft Inc, USA, 2205).

3. Results

3.1. General behaviour and preferences

Before the female's placement in the experimental set-up, males spent the majority of the time engaged in locomotory activities. This pattern changes with the female's arrival, due to the increase of social interactions and behaviours directed to the substrate (mainly pit digging). In the last observation period, locomotory and substrate-related activities increase, while levels of interactions tend to decrease.

The use of space seems to be different in the two choice compartments in terms of being close or distant to the bottom (Fig. 2). Without substrate, the males do not seem to prefer being close or distant to the bottom both without (Wilcoxon Matched Pairs: $Z = 1.07$, P = ns) and with female (Wilcoxon Matched Pairs: $Z = 1.03$, P = ns). On the contrary, in the substrate compartment, males spend more time close to the substrate in both social contexts (Wilcoxon Matched Pairs, no female: $Z = 3.50$, $P < 0.001$; female: $Z = 4.08$, $P < 0.001$).

When analysing the effect of social status on the use of the four areas, there is a clear preference of the territorial males for being in the compartment with substrate, and close to it, regardless of the social context (Fig. 2; Table 2). This difference is accentuated after the female's arrival. The non-territorial males do not show this preference in any of the social contexts under analysis (Fig. 2; Table 2).

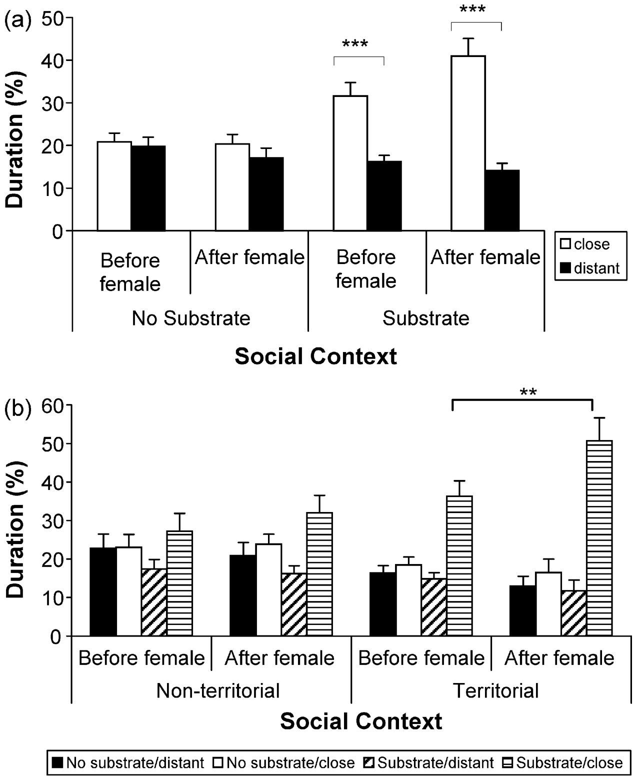

Fig. 2. Total time spent (a) close and distant to the bottom in both choice compartments 1 h before and after the female's arrival (b) in different areas by territorial and non-territorial males in the two social contexts. $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2
ANOVA repeated measures results for time spent in specific behaviours. SS—social status (territorial/non-territorial); SC—social context (courtship: female arrival/after female; interactions: before female/female arrival/after female; other variables: before/after female); A—area (with substrate, close/distant; without substrate, close/distant; nipping and dragging: with, without substrate).

	SS F1,21	SC F1,21	A F3,63	SSxSC F1,21	AxSS F3,63	AxSC F3,63	SSxAxSC F3,63
Total time	4.54, $P < 0.05$	0.18, ns	15.44, $P < 0.001$	2.73, ns	3.77, $P < 0.05$	3.88, $P < 0.05$	1.01, ns
Swimming	0.12, ns	15.30, $P < 0.001$	9.94, $P < 0.001$	4.41, $P < 0.05$	3.44, $P < 0.05$	1.29, ns	0.66, ns
Hovering	2.27, ns	0.92, ns	7.32, $P < 0.001$	0.07, ns	0.58, ns	0.95, ns	0.12, ns
Courtship	6.81, $P < 0.05$	19.78, $P < 0.001$	16.92, $P < 0.001$	0.00, ns	4.63, $P < 0.01$	7.97, $P < 0.001$	0.20, ns
	SS F1,21	SC F2,42	A F3,63	SSxSC F2,42	AxSS F3,63	AxSC F6,126	SSxAxSC F6,126
Interactions	8.54, $P < 0.01$	27.56, $P < 0.001$	2.74, $P = 0.05$	3.61, $P < 0.05$	2.10, ns	4.63, $P < 0.001$	2.52, $P < 0.05$
	SS F1,21	SC F1,21	A F1,21	SSxSC F1,21	AxSS F1,21	AxSC F1,21	SSxAxSC F1,21
Nipping	0.08, ns	0.04, ns	23.56, $P < 0.001$	0.04, ns	0.43, ns	1.85, ns	2.53, ns
Dragging	0.54, ns	6.32, $P < 0.05$	6.32, $P < 0.02$	1.00, ns	0.09, ns	12.28, $P < 0.01$	1.60, ns

3.2. Locomotor activity

Territorial males prefer to swim in the substrate compartment, close to the gravel, regardless of social context (Table 2). In these males, the female arrival tends to decrease the time spent swimming in areas distant to the bottom. Non-territorial males do not express any preference.

Time spent hovering is not influenced by the female and there are no marked differences between social status. However, in general hovering tended to occur in the substrate compartment (Table 2).

Inactivity was a rare behavioural pattern in the males that were not excluded from the trials.

3.3. Social interactions

Courtship was intense immediately after the female's placement in the experimental set-up. Therefore, the males' behaviour was analysed immediately at female's arrival and 1 h after it.

On female's arrival, males of both social status exhibited the highest levels of courtship behaviour (Fig. 3). In this observation period, territorial males used both compartments, because they swam constantly from one to the other, while subordinates exhibited more courtship in the non-substrate compartment. In the subsequent observation, subordinates no longer courted the females, while the dominants' courtship became mainly performed in the substrate compartment (Fig. 3; Table 2).

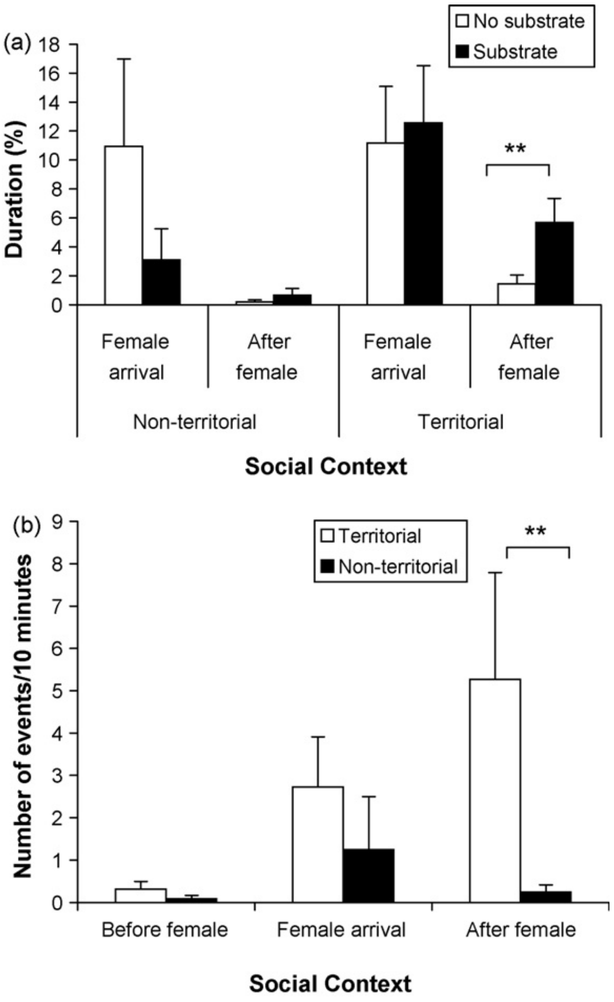

Fig. 3. Activities of territorial and non-territorial males (a) time spent courting in the two compartments immediately after female arrival (female arrival) and 1 h later with the female in the set-up (after female). (b) Pattern of pit digging in the different social contexts. $^{**}P < 0.01$.

'Non-specific' social interactions were also intense immediately after the female's arrival. Therefore, the males' behaviour was analysed in three social contexts: before the female's placement (for a baseline comparison), on its arrival and some time after it. Non-territorial males spent the highest proportion of time in 'non-specific' interactions immediately after the female's arrival. These interactions occurred especially in both sub-areas of the non-substrate compartment (Table 2). Territorial males did not tend to engage in this kind of interactions and when doing so, they did not choose a specific area or compartment (Table 2).

3.4. Behaviours related to the substrate

Pit digging occurred exclusively on the substrate compartment and mainly by the territorial males after the female's arrival (Friedman ANOVA, $\chi^2 = 7.29$, $df = 2$, $P < 0.05$; Mann–Whitney, female: $Z = 3.00$, $P < 0.01$) (Fig. 3).

Nipping on the bottom was particularly carried out in the substrate compartment by males of both social status and irrespective of social context (Table 2). On the other hand, dragging on the bottom was almost exclusively exhibited in the non-substrate compartment by all males when the female is not present (Table 2).

4. Discussion

4.1. General preferences

There was a clear preference for substrate by territorial males regardless of the social context created by the presence of a female in the set-up. Substrate preference by territorial males was identified when analysing a number of behaviours, namely: patterns of activity (swimming and hovering), social interactions (courtship) and substrate-directed behaviours (pit digging, nipping). This preference was even more expressive when they engage in reproductive behaviours (courtship and pit digging). Non-territorial males did not express a substrate preference, except for nipping on the substrate.

The locomotory activity of territorial males occurred especially over the substrate independently of the social context, while courtship and pit digging were behaviours incremented by the female's arrival. This fact is in line with the natural behaviour of Mozambique tilapia where, prior to courtship, a male is tuned to find and defend a territory, establishing a social hierarchy with the remaining males in the lek (Fryer and Iles, 1972; Oliveira and Almada, 1998a). In this case, as the male had already acquired the dominant status in the stock, it is likely that it was highly motivated for territorial and sexual behaviours, which are both substrate-related, even before the female's arrival. Males spent most time courting the female immediately after it had been placed in the aquarium. In this period, no substrate preference was shown. However, in subsequent observations, dominants maintained their motivation for courtship, together with an increased time digging the spawning pit. Then, their preference for substrate while courting became clear and more meaningful in the context of this species reproductive behaviour. Courtship has a number of distinctive behavioural patterns, an important part of which are related to the spawning site, such as leading the female to the nest or circling it (Oliveira and Almada, 1998b). Furthermore, males were seen to intersperse courtship with dig pitting.

Despite the fact that in natural conditions, subordinates may also establish territories, though in periphery of the lek and less successfully (Fryer and Iles, 1972), the constraints created by artificial conditions, may totally inhibit territorial behaviours. In this study, subordinates did not show a preference for substrate neither through their general locomotor activity nor during social interactions. Despite these males have been tested without dominant males, their reproductive subordinate status brought from the stock may have decreased the reproductive motivation. In fact, while territorial males remained motivated to court the female for hours, non-territorial males have shown a completely different pattern of social interactions. Immediately after the female's arrival, they have attempted to court and exhibited the highest level of 'non-specific' social interactions. However, their motivation to interact soon decreased substantially, as judged by the low levels of interactions and no courtship in subsequent observations. It is interesting to note that during this period subordinates clearly spent their time interacting in the non-substrate compartment instead of sharing the time between compartments (as was the case for dominants). This fact suggests that subordinates may have a perception (simply conditioned or more cognitively elaborated) that substrate is a resource that "belongs" to territorial males, in this way actively avoiding it when interacting with females.

Despite the results related to non-territorial males, it is inappropriate to conclude that substrate is not relevant to them. In the present study, the social environment was created by a female, thus promoting reproductive behaviour in the motivated males. In fact, in this context, substrate was not particularly relevant to subordinates. But it is well known how preferences may change with different contexts, physiological states, life cycle stages, etc. as reviewed by Bateson (2004). Therefore, the importance of substrate for subordinates may well change in a different social context. For example, in the actual presence of dominant males, it is possible that substrate would become a target for displaced aggression for subordinates, which in that case might be regarded as an important resource to decrease social stress in the aquarium (Galhardo et al., 2008). The use of substrate, in the form of digging or foraging, or as a moderator of aggression and of levels of social stress has been suggested by some authors for different cichlid species (Barlow, 1974; Heiligenberg, 1965; Munro and Pitcher, 1985; Oliveira and Almada, 1998a).

In a non-social context, all males have shown similar patterns of nipping and dragging. Nipping occurred mainly in the substrate compartment and regardless of the female's presence, which shows that gravel may offer an additional opportunity for foraging and exploration in any context. Dragging occurred mainly when the female was not yet in the aquarium and almost exclusively in the non-substrate compartment. This behavioural pattern, and its context of occurrence, is suggestive of some type of inspecting-like behaviour. If this is the case, it denotes that a bottom without substrate is somewhat an unfamiliar environment deserving inspection.

This study has two methodological aspects that deserve to be addressed. The first is related to the non-balanced order of female's presentation to the focal male. In fact, the aim of the present study was to detect changes in the substrate's use in a fish before and after a female's arrival. To balance the presentation order would not be adequate because the duration of the male's internal effects induced by the female's presence would not be controllable. In any case, the different results between territorial and non-territorial animals in this study show that there were no biases created by order effects. It would be interesting to undertake a complementary study where males would be separately tested for substrate's preference in isolation and in a social context, to confirm the preference for substrate of territorial males in a more permanent social context. A second methodological aspect is related to the use of familiar-looking gravel as the substrate option in this study. The choice for familiar environments is one of the identified problems in interpreting animals' preferences (Fraser and Matthews, 1997). For the animals under study, the 'familiar-looking' floor likely includes a number of different properties: the existence of removable particles, the texture, the colour, reflexes or shadows created by the particles. To simply change the size of the particles would not necessarily eliminate the 'familiar-looking' aspect and, furthermore, this lab's experience is that different gravel sizes, as long as they are still removable, have no impact on fish behaviour towards the substrate. Therefore, an attempt to remove the familiarity components of the substrate from the experimental design, would risk the adoption of a solution with no biological meaning for these animals. In any case, in this particular study, 'familiarity' did not become a confounding factor as the results show that only the territorial males had a clear preference for gravel.

4.2. Conclusion and implications for welfare

Substrate is a multiple-function resource for Mozambique tilapia. It is important for territoriality and reproduction, and it seems to provide additional opportunities for foraging and exploration. As discussed, territoriality and reproductive behaviour are natural behavioural features of this species and fundamental for their ecological success. Recently vacuum nest building has been observed in this species in the absence of substrate (Galhardo et al., 2008), suggesting that nest building should be viewed as a behavioural need in cichlid fish (Dawkins, 1988, 1990; Jensen and Toates, 1993). These aspects, together with the expressed preference for substrate by territorial males, allow the conclusion that substrate is a relevant resource for these males because it is the most appropriate environmental outlet for pit digging behaviour. Therefore, its lack of availability is likely to decrease the welfare of dominant males. In the reproductive context, substrate does not seem to be a valuable resource for subordinates, but this fact should be further investigated in agonistic contexts where substrate may be important in the aggression displacement as previously discussed. In non-social contexts, substrate increases environmental complexity, offering new opportunities for foraging and exploration. It seems to promote behavioural diversity and reduce inactivity, which are both signs of increased welfare (Galhardo et al., 2008).

In conclusion, substrate seems to be important for the welfare of *O. mossambicus* males, based on previous knowledge of cichlids natural behaviour (e.g. Fryer and Iles, 1972) and comparative laboratory studies with and without this resource (Galhardo et al., 2008). The present preference study clearly confirmed the substrate relevance for territorial males.

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