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Biparental mucus feeding: a unique example of parental care in an Amazonian cichlid

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SUMMARY

Vertebrates display a wide variety of parental care behaviours, including the guarding of offspring pre and post nutritional independence as well as the direct provision of nutrients during the early development period. The Amazonian cichlid *Symphysodon* spp. (discus fish) is unusual among fish species, in that both parents provide offspring with mucus secretions to feed from after hatching. This extensive provision of care, which can last up to a month, imposes a physiological demand on both parents and gives rise to conflict between the parent and offspring. Here, we investigated the relationship between parents and offspring during a breeding cycle, determining both mucus composition (total protein, cortisol, immunoglobulin, and Na^+, K^+ and Ca^{2+} concentrations) and the behavioural dynamics of the parent–offspring relationship. Over the course of a breeding cycle, a significant increase in offspring bite rate was recorded, with a concomitant increase in the frequency of turns the male and female parent took at caring for their young. A peak in mucus antibody provision was seen as offspring reached the free-swimming stage, suggesting a role analogous to colostrum provision in mammals. Mucus protein content was lowest during the second and third weeks of free swimming, and a weaning period, similar to that seen in mammalian parental care, occurred when the offspring had been free swimming for ~3 weeks. In many ways, the parental behaviour of discus fish is more similar to mammalian and avian parental care than other fish species, and represents an exciting aquatic model for studying the parent–offspring conflict.

Key words: discus fish, cichlid, immunoglobulin, mucus.

INTRODUCTION

The neonatal period is one of the most critical periods of any organism's life, owing to an increased vulnerability to a range of biotic and abiotic factors such as disease, predation and environmental perturbation. To negate this period of heightened vulnerability, many species have evolved parental care strategies to increase survival of offspring (Clutton-Brock, 1991). Parental care strategies occupy a whole spectrum of behaviours from the simple guarding of offspring, as seen in many species of fish, to the parental provisioning of nutrition during the first phases of offspring development, a characteristic of the vast majority of mammalian and avian parental care strategies. In mammals, offspring have access to milk, a substance rich in a range of nutritious and non-nutritious factors that are essential for the survival of the developing neonate (Clutton-Brock, 1991; Klobasa et al., 1987). Colostrum, the initial release of mammalian milk, is high in immunological factors such as cytokines, growth factors, hormones and immunoglobulins (LeJan, 1996), which provide offspring with a passive form of immunity (Goldman et al., 1998). Newborn pigs deprived of colostrum show mortality rates close to 100% (Kurse, 1983), highlighting the importance of this parental provisioning. Milk provided later in development lacks the large quantities of immune factors found in colostrum, as offspring have developed sufficiently by this point to mount their own immune response. The milk is instead rich in fats and lactose to aid offspring growth (Klobasa et al., 1987). The changing composition of maternally provided milk mirrors the changing needs of the neonate in what is a reciprocal relationship between the mother and her offspring. Although mostly detailed in mammals, analogous behaviours are also apparent in other species such as the brooding caecilian amphibian (*Boulengerula taitanus*), where nutrition is provided by the mother *via* a modified layer of maternal skin, which is consumed by her offspring (Kupfer et al., 2006).

The parental provision of nutrients to offspring ultimately leads to the development of the parent–offspring conflict, an evolutionary conflict stemming from the differences in the optimal fitness of parents and their offspring (Trivers, 1974). Parents wishing to maximise their inclusive fitness, should invest in their current offspring, but only up to the point where any further investment would offer diminishing returns. Any parental investment past this point would use energy that would have a greater return if invested in future offspring. It is, therefore, expected that parents should regulate the amount of care they provide to current offspring so as to maximise their own inclusive fitness. Offspring, however, are also concerned with maximising their own inclusive fitness and should seek to solicit more care than a parent is selected to give. It is this period of disagreement that gives rise to parent–offspring conflict, the height of which is often termed the weaning period in many mammals (Clutton-Brock, 1991; Weary et al., 2008). Parent–offspring conflict has been observed in a vast array of mammal and avian species where offspring can be observed carrying out a range of behavioural 'tactics', such as crying and feigning injury, that have evolved to encourage an extended period of parental care (DeVore, 1963; Mathevon and Charrier, 2004; Trivers, 1972). It has been proposed that parent–offspring conflict can begin as early as the period of intrauterine development, where the fetus interacts with the mother through hormonal communication, signalling the intent of the fetus and the response of the mother (Haig, 1993). In

lecithotrophic species, such as most of the bony fish, where there is no intrauterine interaction, parent–offspring conflict can still develop if there is a nutritional dependency of offspring on the parents. The vast majority of bony fish species display no parental care (Gross and Sargent, 1985) and hence there is little scope for the development of parent–offspring conflict. A notable exception to this is the parental care provided by a variety of cichlid species that display behaviours including the post-hatch defence of young; at least 30 species of cichlid are also known to provide mucus for their developing young to feed on (Noakes, 1979; Hildemann, 1959). These nutritional and behavioural allocations maintain parent and offspring contact for several weeks post-hatch and, hence, facilitate the development of parent–offspring conflict.

Mucus feeding confers fast growth rates and high survival to offspring while reducing the ability of parents to invest in future offspring (Chong et al., 2005). Although present in several species of cichlid, it may only be obligate for the survival of offspring in *Symphysodon*, a genus of Amazonian cichlids commonly known as discus fish (Chong et al., 2005). Early attempts by aquarists to raise discus young away from their parents resulted in high mortality rates due to starvation, as young would not feed on live food (Hildemann, 1959; Noakes, 1979). These high mortality rates indicate the importance of parental mucus for the survival of young and suggest that there are important nutritional factors within parental mucus. A previous study of *Symphysodon* spp. has highlighted the presence of a range of amino acids in parental epidermal mucus, indicating the potential for this mucus to act as a source of nutrition for young (Chong et al., 2005). Antibodies such as immunoglobulin M (IgM) have been reported in the mucus of several other species of fish (Ingram, 1980; Hatten et al., 2001; Shephard, 1994), where they are predicted to play a role in the ability of mucus to prevent the colonisation of bacteria, parasites and fungus in adults (Ingram, 1980). Previous work has also hinted at the possibility of post-egg-laying antibody transfer in the tilapia *Oreochromis aureus* (Sin et al., 1994). Challenge trials in this species demonstrated that offspring survival was greatly increased if the mother had been vaccinated prior to egg laying, demonstrating the vertical transmission of antibodies in the egg yolk (Sin et al., 1994). Offspring survival, however, was further increased if the mother was allowed to mouth brood young; although not observed, the increase in offspring survival could be due to young feeding from mucus in the epithelial lining of the mouth, which may potentially act as a source of nutrients and antibodies. It is, therefore, at least conceivable that IgM is transferred to offspring *via* parental mucus in discus fish and that parents provide offspring with a passive form of immunity through the mucosal provision of IgM.

As well as possibly being a vector for IgM transfer, parental mucus could help deliver hormones. In the midas cichlid *Cichlasoma citrinellum*, the parental mucus that it provides for its offspring to feed upon contains several hormones, including growth hormone, thyroid hormone and prolactin (Schutz and Barlow, 1997). These hormones have a wide variety of roles and are especially important in developmental processes (Schutz and Barlow, 1997; Takagi et al., 1994). The close evolutionary relationship of the midas cichlid and discus fish suggests that these hormones are likely to be present in discus fish parental mucus. Other hormones may also be present; cortisol (Simontacchi et al., 2008) and the androgen 11-ketotestosterone (Schultz et al., 2005) have both been found in the epidermal mucus of fish at levels that correlate with plasma concentrations.

Feeding behaviour of offspring results in epidermal damage which could initiate a stress response in parents; cortisol may be transferred to offspring *via* parental mucus. Cortisol, although typically known as a stress hormone, also aids ion uptake in several species of teleost (McCormick, 2000). The parental provision of cortisol could be advantageous to discus young in coping with the osmoregulatory challenges presented by their natural ion-poor Amazon environment. Additionally, parental mucus may act as a direct source of ions. Freshwater fish replace ions lost by passive efflux to the external environment through the active uptake of ions across the gills or through the diet (Smith et al., 1989). Experimental diets rich in ions help satisfy the osmoregulatory requirements of fish kept in freshwater, allowing energy normally used in osmoregulation to be used for growth (Gatlin et al., 1992). Mucus layers in freshwater teleosts help to reduce ion loss across the surfaces of fish (Shephard, 1994), as gradients of ions within mucus represent significant barriers against the diffusional efflux of ions (Shephard, 1994). Mucus of adult discus fish may, therefore, contain a sufficient quantity of ions to allow feeding offspring to obtain ions typically absent in their natural environment, especially if repeated nipping of young causes cellular leakage of ions from the epidermis into the mucus.

Unlike in mammals, where nutritional demands are met solely by the mother, in discus fish both parents are responsible for providing mucosal secretions (Chong et al., 2005; Hildemann, 1959). Parental care duties are shared between parents, but how this affects the dynamics of parent–offspring conflict in discus fish is unknown. There may be a peak in conflict between parents and offspring, as in mammals, before parental care is slowly relinquished as offspring develop (Clutton-Brock, 1991). Breeders of discus fish have long recognised that parents that provide mucus for offspring for longer than a week will have a reduced number of subsequent broods (Chong et al., 2005). This suggests a substantial cost attached to parental care in this species and that there is scope for the development of parent–offspring conflict.

Mucus feeding in discus fish represents an unusual parental care strategy in fish, with many similarities to other vertebrate forms of care. The aim of the present study was to investigate the dynamics of the parent-offspring interaction in discus fish. Firstly, we analysed the composition of parental mucus over the typical period of parental care to understand its physiological value to offspring with the hypothesis that it contained essential nutritional and non-nutritional factors. We also compared the mucus composition of laboratory and wild Amazonian discus fish to determine whether inbreeding for the aquarium trade alters mucus composition. Finally, we observed the behaviour of parents and offspring throughout the 4-week period that young fed from their parents, herein referred to as the breeding period, to test the hypothesis that discus fish represent an example of parent–offspring conflict in fish and to see whether interactions between parents and offspring change during the course of the breeding period.

MATERIALS AND METHODS

Experimental fish and husbandry

A brood stock of adult discus fish *Symphysodon* spp., originating from a captive bred strain in Malaysia, were obtained from a commercial dealer and transported to the aquarium facilities of the University of Plymouth. Fish were quarantined, wormed (Discus Wormer; Kusuri, Newton Abbot, UK) and then held in groups of 12 in 100-litre glass tanks and observed for reproductive behaviours. Fish that formed breeding pairs were separated into their own 100-litre glass tanks and allowed to spawn on a plastic breeding cone. All fish were kept in recirculation systems held at constant conditions (temperature 29±0.5°C, pH 7.0±0.5, dissolved oxygen 99±0.5%, 12 h:12 h light:dark

photoperiod, Ca^{2+} 21.56±1.26 mg l^{-1}, Na^{+} 9.28±0.26 mg l^{-1}, K^{+} 1.42±0.02 mg l^{-1}, Cl^{-} 15.32±0.76 mg l^{-1}) and fed a beef-heart-based or commercial pellet (Tetra prima granular; Tetra, Southampton, UK) feed once daily to satiation. Hatched young fed solely from their parents' mucus until the final (fourth) week of parental care when their diet was supplemented with newly hatched *Artemia* nauplii. All procedures in this study were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Behavioural observations

Behavioural observations began on the first day of free-swimming and continued daily until the last day of mucus sampling (~35 days post-fertilisation). Two behavioural parameters were measured consecutively each day, including the distribution of young on the parents and the bite rate of young. Both behavioural measurements were recorded by eye at least 1 h after the parents were fed to avoid any bias introduced by parental movements during feeding. Blinds to prevent the fish from noticing the observer were not necessary, as preliminary studies showed that discus carry out natural parental care behaviours while being observed.

Distribution of parental care

In this case, parental care was defined solely as the parents allowing young to feed from their epidermal mucus. For a period of 1 h, young were observed as a whole group and their feeding habits were recorded. The observed feeding habits fell into one of four clear states: young feeding solely from the male, young feeding solely from the female, young feeding from both parents and young not feeding from either parent. These were recorded as 'male', 'female', 'both' and 'none', respectively. These observations produced data detailing the total time each parent spent feeding young, as well as information on the number and duration of each feeding turn.

Bite rate

An individual offspring was selected at random and observed for 30 s. The number of bites to the parents' epidermal mucus during this period was counted *via* operator observation. Individual bites were obvious; the offspring would turn towards the parent, bite at the mucus and twist or shake their body to aid removal. The count was repeated for 10 young feeding from each parent and a mean bite rate was calculated. Young that moved out of view during the 30 s were ignored and a new count was started.

Mucus sampling

In laboratory studies, the same breeding pairs (N=6) were sampled for mucus at eight time points over a complete breeding cycle. Each sample corresponded to a distinct stage within the breeding cycle; eggs spawned (E), eggs hatched (H), free-swimming young (FS), and free-swimming young + 1 week (W1), + 2 weeks (W2), + 3 weeks (W3) and + 4 weeks (W4). A 'zero' sample was collected from the breeding parents during another spawning cycle at a standardised time point of 2 days after the removal of a clutch of eggs. The zero samples reflected a period of time when parents were known to be sexually mature but were not currently engaged in breeding activity. Mucus samples were also obtained from non-breeding (NB) fish that were yet to pair. Mucus samples were obtained using a method similar to that of Schultz et al. (Schultz et al., 2007), whereby mucus was collected onto a pre-weighed polyester sponge (Buff-Puff facial sponge; 3M, St Paul, MN, USA) cut into 2×2×1 cm sections. Fish were removed from the tank using a shallow net, and their upward flank – which was undisturbed by the catching process – was orientated upwards for 5 s to drain before the fish was swabbed with the sponge, removing approximately 30% of the mucus from one side of the parent. The pre-weighed sponge containing mucus was returned to a pre-weighed syringe and weighed to 0.0001 g so that mucus sample mass and, therefore, volume could be ascertained. The syringe was then used to push as much of the sampled mucus out of the sponge and into a 1.5 ml Eppendorf tube; 1 ml of distilled water was then added to the syringe and forced through the sponge to elute any remaining mucus. This mucus and water mixture was then vortexed and centrifuged (13,000 ***g*** for 5 min), and the supernatant was immediately frozen (–80°C) for later physiological analyses. The effect of mucus sampling was clearly visible on parents, as the epidermis appeared lighter in areas where mucus had been removed. Normal colour, however, had returned after 1 h, suggesting that the mucus had been replaced. The quick regeneration of mucus coupled with the decision to only sample mucus once a week suggests that sampling had a minimal impact on the parental mucus available for offspring.

Wild fish

A total of 90 non-breeding wild adult fish were sampled from the Rio Negro, upstream from Barcelos (00°42′02″S, 062°54′27″W). Fish were caught individually by local fisherman using flashlights and hand nets during seven nights of fishing between 29 October and 5 November 2007. Mucus was sampled as described above; however, in the field, eluted mucus samples were stored on ice until arrival back at the lab where they could then be stored at –20°C. Fish were measured using a 30 cm ruler and returned to the water. Water samples were also taken at six representative sites for ion analysis (Ca^{2+} 0.32±0.06 mg l^{-1}, Na^{+} 3.43±1.02 mg l^{-1}, K^{+} 0.46±0.12 mg l^{-1}, Cl^{-} 10.05±4.46 mg l^{-1}). Mucus samples were taken from breeding discus as described above between 11 and 21 February 2008. A total of four breeding pairs with offspring were sampled. Ten young from each pair were also obtained and stored at –20°C until they were shipped to Plymouth.

Measurement of young

The fork length of the young from a non-experimental pair at the University of Plymouth was recorded every 3 days for the same period as experimental fish (from free-swimming young to 4-week-old juveniles). Six young of unknown age were also measured at one time point from a wild breeding pair.

Physiological analyses

IgM

Levels of specific antibodies in the mucus of brood fish were measured using a competition ELISA as described by Magnadottir (Magnadottir, 1998) for measuring total IgM in fish. Blood samples were taken from brood fish *via* the caudal vasculature and left at 4°C overnight to clot; the serum samples were then collected and stored at –20°C. Serum was purified using a HiTrap IgM purification column (GE Healthcare, Amersham, UK); the resulting IgM fractions were combined and read using a Bradford protein assay to assess IgM concentration. Purified IgM was diluted 1:400 in a carbonate-bicarbonate buffer and 100 µl was added per well to coat a 96-well immunoplate (Nunc MaxiSorp, Rochester, NY, USA). After 18 h at 4°C, non-fixed IgM was removed by washing the plate three times with a low salt wash buffer (LSWB), pH 7.3, containing 5% Tween-20. Uncoated sites were blocked overnight at 4°C with 5% milk powder diluted in phostphate buffered saline (PBS) before being washed three times with LSWB. Mucus samples were diluted 1:3 in PBS containing 0.05% Tween-20; 100 µl of the sample was then added to the plate and competed against 100 µl of cross-reacting anti-Asian

sea bass monoclonal IgM diluted 1:10 in a 1% bovine serum albumin (BSA) solution at 37°C for 2 h. Any unbound mucus IgM anti-fish complexes were removed with five plate washings of high salt wash buffer (HSWB), pH 7.7, containing 10% Tween-20. Subsequently, the plate was incubated for 1 h at room temperature with 100 µl anti-mouse IgG peroxidise conjugate diluted 1:400 in 1% BSA in LSWB. Non-reactive conjugate antibodies were removed with five rinses of HSWB. Tetramethyl benzidine (TMB) peroxidise substrate was then added at a volume of 100 µl per well and the reaction was stopped with 50 µl of stop solution (1.8 mol^{-1} H_2SO_4). The absorbance was then read at 450 nm on an Optimax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Trout mucus was used as a negative control.

Protein, ions and cortisol

Mucus samples were defrosted on ice, diluted in distilled water and analysed using previously reported methods. Total protein concentration was measured using the Bradford method (Bradford, 1976). The concentrations of Na^+, K^+ and Ca^{2+} were measured by inductively coupled plasma atomic emission spectroscopy (Varian 725-ES ICP optical emission spectroscopy; Varian, Santa Clara, CA, USA). Chloride concentrations were measured by a colorimetric assay as described by Zall et al. (Zall et al., 1956). Mucus cortisol concentrations were analysed by a commercial ELISA (DRG Diagnostics, Marburg, Germany). Cortisol from standards and samples was extracted by vortex mixing with ethyl acetate (300 µl:300 µl of sample:ethyl acetate; Fisher Scientific, Pittsburgh, PA, USA), of which 250 µl was removed, dried under nitrogen and resuspended in PBS containing 0.1% BSA (Sigma-Aldrich, Dorset, UK) before analysis.

Statistical analysis

All data analysed were checked for normality and heterogeneity using a Kolmogorov–Smirnov and Levene's test, respectively, and conformed to parametric assumptions.

Physiology

Physiology data were adjusted per volume of mucus as opposed to mucus total protein content. Total protein varies considerably as part of the breeding process (Chong et al., 2005), and so was not seen as an accurate and consistent way of adjusting physiological values. Two types of comparisons were carried out on physiological data. Comparisons between the mucus of non-breeders and breeders (with all time points combined) were obtained using a one-way ANOVA followed by least significant difference (LSD) *post hoc* analyses. Comparisons between mucus composition in breeders at different time points across the breeding period were carried out using a repeated measures ANOVA (RM-ANOVA) with sex and time as factors. Where significant effects of time were recorded, *post hoc* paired *t*-tests were used. Each physiological parameter measured was compared between breeding and non-breeding fish from Brazil and Plymouth within a one-way ANOVA. Of the 90 wild non-breeding discus fish sampled, a total of 12 representative mucus samples were used for comparisons between wild breeders (N=8), aquarium-bred breeders (N=8) and aquarium-bred non-breeders (N=12). Mucus composition of wild-breeding Brazilian pairs was compared against that of week 3 Plymouth aquarium-bred discus; the fork length of young obtained from Brazilian pairs (15±0.8 mm; N=6) was similar to that of Plymouth young during week 3 of the breeding period (15±0.1 mm; N=6).

Behaviour

An RM-ANOVA was used to assess the effect of time across the breeding period on bite rate, number of parental care changes and the time offspring spent associated with each mode of parental care. Where significant effects of time were apparent, *post hoc* paired *t*-tests were used. A one-way ANOVA (LSD *post hoc*) was used to assess the differences within each week in terms of how long young spent associated with each mode of parental care.

RESULTS

Time on parent

Young spent significantly more time alone (without any parent) in week 4 compared with the first 3 weeks (RM-ANOVA, $F_{1,3}$=4.99, P<0.05, N=6; Fig. 1D). Young also spent less time with the female in week 4 compared with the other 3 weeks (RM-ANOVA, $F_{1,3}$=4.012, P<0.05, N=6; Fig. 1B). There were however, no

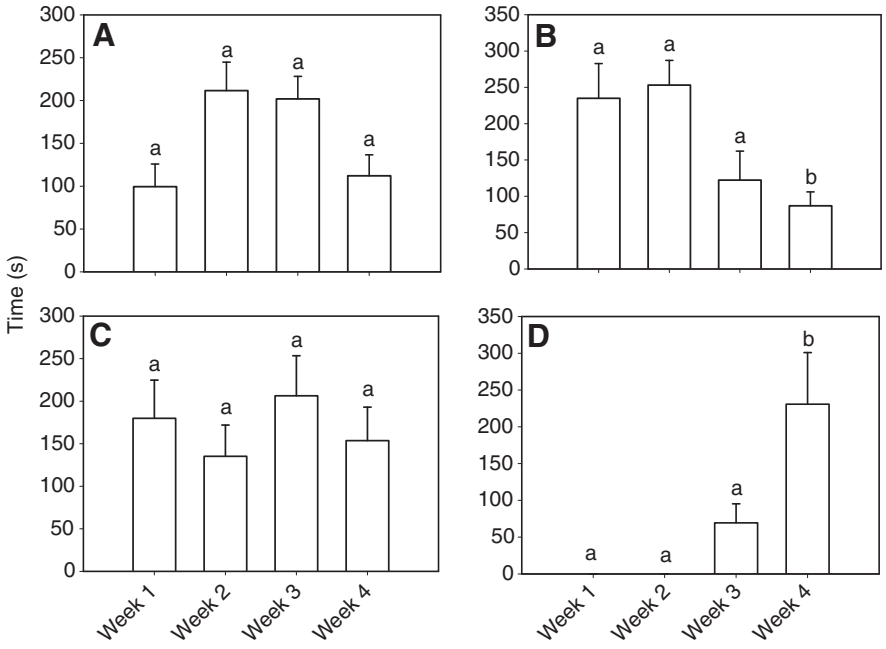

Fig. 1. Time discus fish young spent associated with the (A) male parent, (B) female parent, (C) both parents or (D) neither parent. Different letters denote a significant difference (paired *t*-test, P<0.05, N=6); bars that share a letter are not statistically different. Data are means + s.e.m.

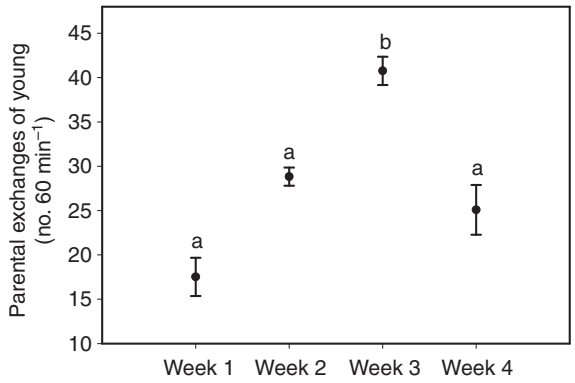

Fig. 2. Total number of incidences within a 60 min observation period where the mode of parental care in discus fish changed across the 4-week breeding period. Different letters denote a significant difference (paired *t*-test, $P<0.05$, $N=6$); bars that share a letter are not significantly different. Data are means ± s.e.m.

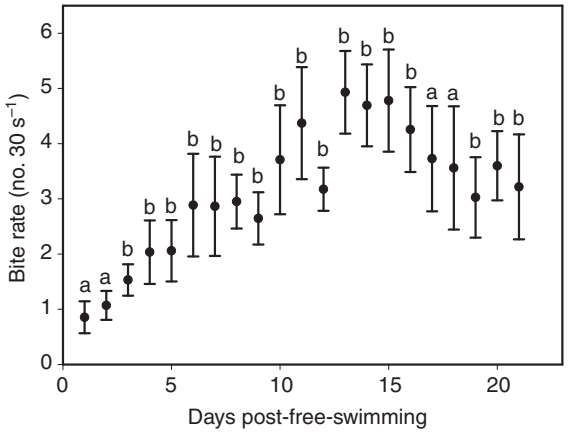

Fig. 3. Bite rate of discus fish young per 30 s on both parents over the first 3 weeks of the breeding period. Different letters denote a significant difference between each time point and the bite rate recorded on day 1 (paired *t*-test, $P<0.05$, $N=12$). Data are means ± s.e.m.

significant differences across the 4 weeks in terms of how long young were associated with males or with both parents (RM-ANOVA, male, $F_{1,3}=1.54$, $P=0.25$; both, $F_{1,3}=0.28$, $P=0.84$; Fig. 1A,C). Interestingly, during the first week, young spent more time feeding off the female than off the male (one-way-ANOVA, $F_{3,23}=4.52$, $P<0.05$, $N=6$).

Change in parental duties

Throughout the period of parental care, parents would regularly change the mode of parental care. In the first 2 weeks this was done *via* the exchange of young by a well-orchestrated body flick, transferring young from one parent to another. However, during the last 2 weeks, parents would often swim away from young, leaving them on their own; such behaviours would require the young to actively swim towards their parents to feed. The number of changes in the mode of parental care steadily increased after young began feeding in week 1, reaching a peak at week 3 (Fig. 2), which was significantly different from weeks 1, 2 and 4 (RM-ANOVA, $F_{1,3}=5.677$, $P<0.05$, $N=6$).

Bite rate

Bite rate significantly increased over time (RM-ANOVA, $F_{1,20}=7.933$, $P<0.05$, $N=12$) peaking around day 12 to day 15 before slowly decreasing (Fig. 3). The bite rate of young, however, did not differ significantly (RM-ANOVA, $F_{2,40}=0.304$, $P=1.00$) between young feeding off the male or female parent.

IgM

Parental mucus collected at time zero had significantly less IgM (RM-ANOVA, $F_{1,7}=3.732$, $P<0.05$, $N=12$) than that collected at the time points E, H, FS, W1, W2 and W3 (Fig. 4). The elevation in parental mucus IgM over the breeding period was maintained until W4, when a drop was noted. IgM at W4, however, did not differ significantly from the zero time point. There was no effect of sex on parental mucus IgM (RM-ANOVA, $F_{1,7}=0.518$, $P=0.817$). Levels of IgM within the mucus of non-breeding fish were significantly lower than in breeding fish at all points in the breeding cycle, with the exception of time points zero and W4 (one-way ANOVA, $F_{8,96}=3.397$, $P<0.05$, $N=12$ or 8; Fig. 4). Wild-breeding fish from Brazil demonstrated significantly higher levels of IgM than wild non-breeders, aquarium-bred breeders and aquarium-bred non-breeders (one-way ANOVA, $F_{3,39}=3.219$, $P<0.05$, $N=12$ or 8; Table 1).

Total protein

Parental mucus at W2 and W3 had significantly lower levels of total protein (RM-ANOVA, $F_{1,7}=4.006$, $P<0.05$, $N=12$) than mucus taken at the time points E, H and W1 (Fig. 5). The mucus of non-breeders was significantly lower than the parental mucus at the time points E, H and W1 (one-way ANOVA, $F_{8,96}=2.642$, $P<0.05$, $N=12$ or 8; Fig. 5). There was no effect of sex on parental mucus total protein (RM-ANOVA, $F_{1,7}=0.763$, $P=0.620$). Comparisons between wild and aquarium-bred fish highlighted significantly lower levels of total protein within the mucus of non-breeding wild fish as opposed to wild-breeding fish, aquarium-bred breeding fish and aquarium-bred non-breeding fish (one-way ANOVA, $F_{3,39}=5.077$, $P<0.05$, $N=12$ or 8; Table 1).

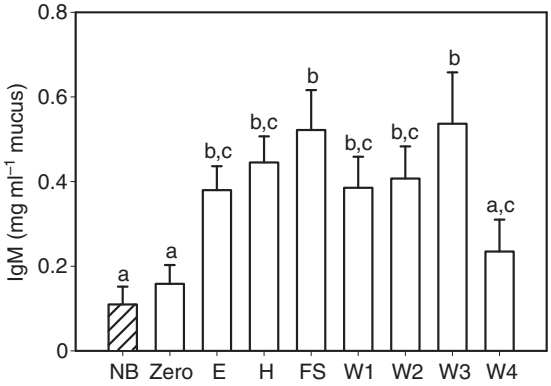

Fig. 4. Total IgM in the mucus of discus fish non-breeders (NB; $N=8$) and breeding pairs ($N=12$, males and females combined) over the breeding cycle. Time points throughout the breeding cycle include: no breeding activity (zero), eggs spawned (E), eggs hatched (H), free-swimming (FS) young, and free-swimming young + 1 week (W1), 2 weeks (W2), 3 weeks (W3) and 4 weeks (W4). Different letters denote a significant difference (paired *t*-test and LSD *post hoc*, $P<0.05$); bars that share a letter are not significantly different. Data are means + s.e.m.

Table 1. Comparison of parental mucus from wild Amazonian and aquarium-bred discus breeders and non-breeders at 3 weeks post free-swimming

	Brazil		Aquarium-bred	
	Non-breeders (*N*=12)	Breeders (*N*=8)	Non-breeders (*N*=12)	Breeders (*N*=8)
Total protein (mg ml^{-1})	1.01±0.20^{a}	3.93±1.73^{b}	5.51±0.48^{b}	4.89±0.95^{b}
IgM (mg ml^{-1})	0.57±0.074^{a}	1.29±0.58^{a}	0.10±0.03^{a}	0.53±0.12^{a}
Na^{+} (mg ml^{-1})	0.13±0.01^{a}	1.99±1.04^{a}	0.11±0.02^{a}	0.27±0.03^{a}
K^{+} (mg ml^{-1})	0.09±0.01^{a}	1.53±0.49^{a}	0.53±0.03^{a}	0.19±0.05^{a}
Ca^{2+} (mg ml^{-1})	0.01±0.00^{a}	0.13±0.059^{b}	0.05±0.01^{a}	0.09±0.01^{b}
Cl^{-} (mg ml^{-1})	0.28±0.06^{a}	25.52±5.77^{b}	0.44±0.02^{b}	0.31±0.03^{a}
Cortisol (ng ml^{-1})	n.d.	n.d.	0.60±0.37^{b}	7.30±1.46^{a}

Data are presented as means ± s.e.m. Letters denote significant differences (one-way ANOVA and LSD *post hoc*; P<0.05). n.d., not determined.

Ions

Calcium (Fig. 6A) was the only ion where there were no significant differences between parental mucus taken at different time points (RM ANOVA, $F_{1,7}$=2.333, P=0.139), between breeders and non-breeders (one-way ANOVA, $F_{8,87}$=1.470, P=0.180) or between wild and aquarium-bred breeders and non-breeders (one-way ANOVA, $F_{3,35}$=2.731 P=0.60; Table 1). Sodium values (Fig. 6B) during W1 were significantly higher (RM-ANOVA, $F_{1,7}$=3.287, P<0.05, N=12) than at the time points E, H, FS, W2, W3 and for NB. Non-breeders also had significantly lower levels of Na^{+} than breeders at the time points zero, E, W1, W2 and W4 (one-way ANOVA and *post hoc* LSD, $F_{8,97}$=2.956, P<0.05, N=12 or 8; Fig. 6). Comparisons between wild and aquarium-bred fish also demonstrated significantly higher levels of Na^{+} within the mucus of wild-breeding fish (one-way ANOVA, $F_{3,39}$=4.128, P<0.05, N=12 or 8; Table 1). The concentration of K^{+} in parental mucus (Fig. 6C) during the zero time point was significantly higher (RM-ANOVA, $F_{1,7}$=5.274, P<0.001, N=12) than at the time points W1, W2, W3 and W4. Non-breeders also had significantly higher levels of K^{+} than breeders at time points W2, W3 and W4 (one-way ANOVA, $F_{8,97}$=2.485, P<0.05, N=12 or 8; Fig. 6). Comparisons between wild and aquarium-bred fish demonstrated significantly higher levels of K^{+} in wild parental mucus compared with that of wild non-breeders and aquarium-bred breeders and non-breeders (one-way ANOVA, $F_{3,39}$=9.830, P=<0.001, N=12 or 8; Table 1). Chloride concentrations (Fig. 6D) were significantly higher in parental mucus (RM-ANOVA, $F_{1,7}$=2.666, P<0.05, N=12) at the time points zero and FS than at W2 and W3. Wild breeders had significantly greater levels of Cl^{-} in their mucus than aquarium-bred discus (one-way ANOVA, $F_{3,39}$=26.070, P<0.001, N=12 or 8; Table 1).

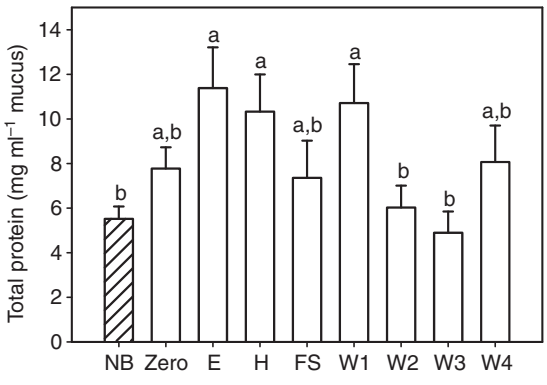

Fig. 5. Total protein in the mucus of discus fish non-breeders (NB; N=8) and breeding pairs (N=12) over the breeding cycle. Different letters denote a significant difference (paired *t*-test and LSD *post hoc*, P<0.05); bars that share a letter are not significantly different. See Fig. 4 for breeding stage definitions. Data are means + s.e.m.

Cortisol

Although there were no significant differences in the levels of parental mucus cortisol over time (Fig. 7) (RM-ANOVA, $F_{4,1}$=0.446, P=0.775), cortisol in the mucus of breeders was significantly higher than in non-breeders (one-way ANOVA, $F_{5,64}$=2.686, P<0.05, N=12 or 8; Fig. 7). Aquarium-bred breeders also had significantly higher levels of cortisol than wild breeders and non-breeders (one-way ANOVA, $F_{3,39}$=17.894, P<0.001, N=12 or 8; Table 1).

DISCUSSION

Parental care behaviour

The first 2 weeks of parental care in discus fish involved both parents spending the vast majority of time associated with their offspring with either one of the parents looking after young or both parents looking after young simultaneously; young were at no point left alone. During week 1, offspring spent significantly more time on females than males, although this was influenced by one female in particular, who, during the first week of care, aggressively prevented the male from looking after offspring. This female did, however, relent in her defence of offspring during week 2, when the male was allowed to take part in parental care duties. In these first two weeks, the frequency at which parents would swap duties – i.e. between the modes of male only care, female only care, both parents caring or neither parent caring – was relatively low, with parents often looking after young for 5–10 min at a time, allowing young a reliable area to feed from. When switching from one mode of care to another during the first 2 weeks, parents would execute a well-orchestrated flick, transferring young from one parent to another. These high levels of parental care behaviour observed in adults were reflected in the behaviour of young, which exhibited a steady increase in bite rate, similar to that observed by Chong et al. (Chong et al., 2005).

Parental behaviour began to change during week 3, with parents opting to leave offspring on their own for short periods of time, thus making it difficult for young to feed on mucus. Week 3 also saw parents frequently changing the mode of parental care. The mean duration of each parental care mode in week 3 was reduced (30–60 s) compared with that observed during week 1 (5–10 min), making it more difficult for young to feed owing to the constant movement of both parents. Young were no longer exchanged by a well-orchestrated flick; instead, parents would actively swim away from young, leaving them on their own. This resulted in young actively seeking their parents as well as the observation that, during week 3, young began to display foraging behaviours. It remains unclear whether the initiation of this change in feeding strategy was a consequence of the observed parental avoidance behaviours or some

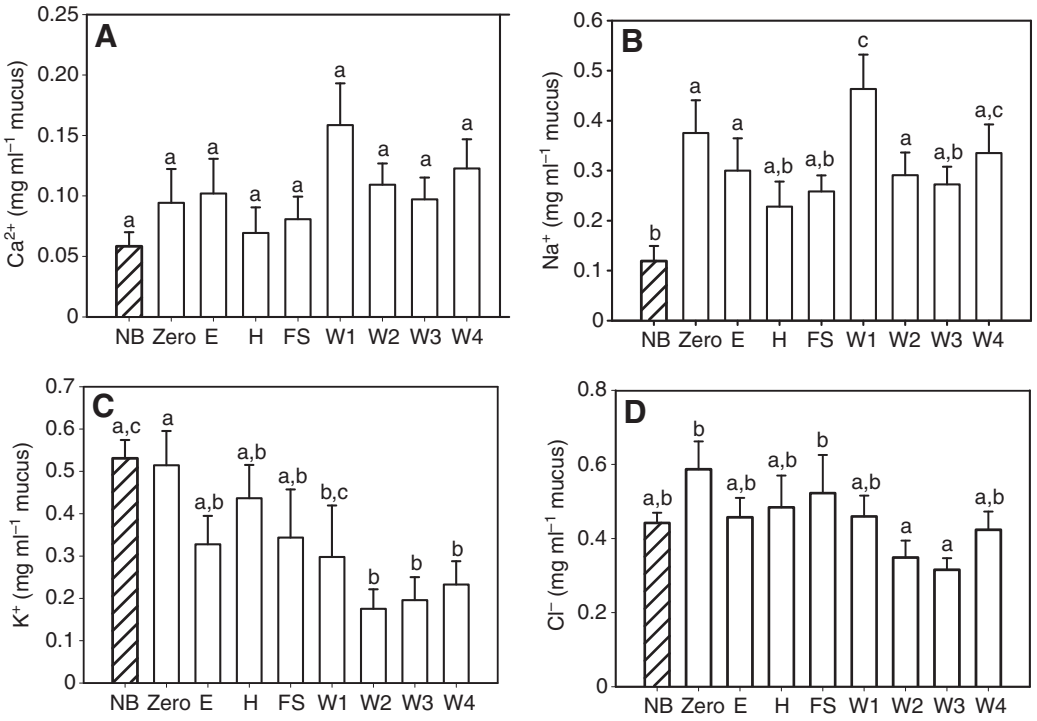

Fig. 6. (A) Calcium, (B) sodium, (C) potassium and (D) chloride concentrations in the mucus of discus fish non-breeders (NB; *N*=8) and breeding pairs (*N*=12) over the breeding cycle. Different letters denote a significant difference (paired *t*-test and LSD *post hoc*, $P<0.05$); bars that share a letter are not significantly different. See Fig. 4 for breeding stage definitions. Data are means + s.e.m.

other underlying developmental change during this period. It is likely that the young were also developing anti-predator behaviours, allowing them to spend more time foraging independently (Brown, 1984). The bite rate of young also reached a plateau around week 2 before declining towards week 3, suggesting that the change in parental behaviour was affecting the ability of young to feed.

Week 4 showed a further increase in the amount of time young spent alone, as parents – now displaying obvious signs of epidermal damage – would actively swim away from offspring, severely limiting the ability of young to feed. The epidermal damage and stress noted in adults during week 4 combined with the lack of feeding opportunities for young raised welfare concerns, which resulted in the addition of *Artemia*. The presence of *Artemia* is known to reduce the bite rate of young (Chong et al., 2005), but they were introduced in the present study at a time when parents had already begun to avoid the feeding advances of young. The addition of *Artemia* provided young with a planktivorous food source, as would occur in their natural environment. Although young could still attempt to feed from parental mucus, constant parental movement during this period appeared to ensure that foraging on *Artemia* was energetically more efficient. This behaviour resulted in a decrease in the number of times parents changed the mode of parental care, as young spent the vast majority of week 4 away from their parents.

The change in parental behaviour from that seen in weeks 1 and 2, which involved close attentive contact with young, to the behaviour observed in weeks 3 and 4, which involved parents gradually impeding the feeding of young, suggests a period of conflict and the presence of a weaning period similar to that observed in many birds and mammals (Weary et al., 2008). As offspring grow and develop, requiring a greater amount of resources, the cost to parents of providing these resources increases to the point where parents and offspring are in conflict over the provision of these resources (Trivers, 1974). Parents in other vertebrates alter their behaviours to increase the cost of offspring solicitation, to aid in the development of independent foraging in their offspring (Davies, 1978; Pugesek, 1990; Rehling and Trillmich, 2008; Weary et al., 2008). Our observations suggest that this weaning behaviour, although more typically associated with mammals and birds, also occurs in discus fish.

Mucus composition

In addition to the behavioural changes observed during the period of parental care, alterations in mucus composition occurred. IgM, a component of the vertebrate adaptive immune system, has been previously found in the mucus of a range of fish species (Ingram, 1980; Hatten et al., 2001; Shephard, 1994). This study demonstrated its presence within the mucus of both breeding and non-breeding discus fish. Interestingly, IgM levels were elevated in the mucus of

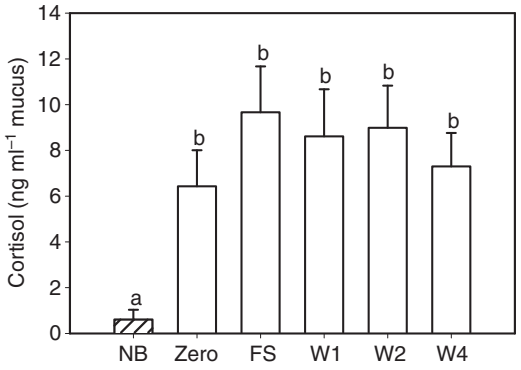

Fig. 7. Cortisol content in the mucus of discus fish non-breeders (NB; *N*=8) and breeding pairs (*N*=12) over the breeding cycle. Different letters denote a significant difference (paired *t*-tests and LSD *post hoc*, $P<0.05$); bars that share a letter are not significantly different. See Fig. 4 for breeding stage definitions. Data are means + s.e.m.

breeding fish once eggs were laid, and the increase in mucus IgM levels remained until W4. This suggests that the process is endogenously controlled rather than being due to IgM leakage following epidermal damage caused by young during feeding. The mechanism that reduces parental mucus IgM levels during W4 could be endogenously controlled *via* a similar suite of hormones to those that initiate the change in parental behaviour observed in W3, although it could also be initiated *via* a reduction in offspring bite rate. The parental production of IgM within mucus appears to be cyclical, similar to the passive provision of immunity seen in mammals during lactation, when antibodies are provided to offspring until they are able to develop their own adaptive response (Adamski and Demmer, 2000; Klobasa et al., 1987). Although it is not yet known how long it takes for the development of a functional adaptive immune system in young discus fish, the drop in parental mucus IgM by W4 may indicate that this is a period when the young can begin to produce their own adaptive immune response. This is in agreement with studies of other fish, for example in zebrafish *Danio rerio*, where it takes 4 to 6 weeks for the adaptive immune system to become functional (Lam et al., 2004). The composition of parental mucus has also been reported by Chong et al., who identified a C-type lectin in the mucus of breeding discus that is absent in non-breeding individuals (Chong et al., 2005). Lectins are responsible for activating the complement system after recognising pathogenic microorganisms (Russell and Lumsden, 2005). Although their functional properties within parental mucus are yet to be elucidated, they may well be transferred to offspring, offering another form of pathogenic protection.

IgM concentrations in the mucus of wild-breeding discus fish were greater than those found in aquarium-bred breeders. This suggests that parentally provided immunity may be especially important in wild discus fish, possibly owing to differences in their respective environments. Unlike a controlled aquarium environment, the Amazon contains a wide spectrum of pathogens that could pose risks to developing young. Group living, as occurs in wild discus (Crampton, 2008), might increase the probability of newly hatched offspring coming into contact with pathogens (Hughes et al., 2002; Poulin, 1999). Interestingly, IgM concentrations within the mucus of wild non-breeding discus were very similar to those of aquarium-bred breeders. During the sampling of wild non-breeders, it was observed that the vast majority of fish had scars and some epidermal damage; the presence of high levels of mucosal IgM may help facilitate the prevention of bacterial colonisation at sites of epidermal damage.

Parental mucus at the time points W1, E and H had significantly higher levels of total protein than at W2 and W3. The drop in total protein at W2 and W3 may be due to the increased feeding rate of offspring. By this point, young were considerably larger and had much higher bite rates than young at W1. If the elevated feeding rates were greater than the rate of parental mucus production, this would result in a drop in total protein. Parental mucus generally had higher concentrations of total protein than the mucus of non-breeders. The elevation of total protein is probably due to the elevated levels of IgM and, possibly, other factors, such as hormones similar to those found in the mucus of the midas cichlid (Schutz and Barlow, 1997). The mechanism behind the elevation of total protein during the egg stage, in preparation for offspring feeding, is likely to be similar to the mechanism behind IgM elevation involving some kind of hormonal regulation. Prolactin, a hormone known to increase mucus production and initiate parental care behaviour in discus fish (Blum and Fielder, 1965), was found to be elevated in the skin of discus parents during the period of parental care (Khong et al., 2009). This may be one of several hormones involved in the initiation of both the behavioural and physiological response to parental care observed after eggs are laid. Of all the fish sampled, total protein was lowest in wild non-breeders. These lower levels of protein could be due to the differences in selection pressures between the aquarium and wild environment. An irregular food supply (a property of most wild environments) and the need to conserve energy could favour energetically efficient individuals in terms of their mucus production. In an aquarium environment, where fish are generally fed to satiation, non-breeding fish may afford higher mucus protein concentrations than their wild counterparts.

Cortisol was present within the mucus of aquarium-bred discus, albeit at low levels. Although there was no effect of time on the quantity of cortisol within the mucus of breeding discus, the mucus of these fish did contain significantly higher levels of cortisol than that of non-breeders. Cortisol plays a vital role in ionoregulation (McCormick, 2000), which might be an advantage to young developing in an ion-deficient environment. However, in contrast to aquarium-bred fish, cortisol concentrations in the mucus of wild breeders and non-breeders were undetectable. Consequently, rather than the cortisol detected in aquarium breeders playing a role in parental care, the presence of cortisol may be an artefact of the aquarium environment or reflect differences in the stress response of wild *versus* inbred strains of fish.

As well as providing a source of immunity, nutrition and, potentially, hormones, parental mucus may help offspring cope with the demands of the acidic, ion-poor environment of the Amazon. One of the major problems associated with fish living in ion-deficient environments is the need to regulate the uptake and loss of ions such as Na^+, K^+ Ca^{2+} and Cl^- for osmoregulation. Fish mucus can help reduce ion loss *via* a gradient of ions within mucus layers (Handy and Maunder, 2009), which represents a significant barrier against their diffusional efflux (Shephard, 1994); it may also provide a possible sink of ions for discus offspring. Na^+, K^+ and Cl^- were significantly higher in the mucus of wild breeders as opposed to wild non-breeding fish, aquarium-bred breeders and aquarium-bred non-breeders. The difference in the ionic composition of parental mucus between wild breeders and aquarium-bred breeders may be due to the water chemistry of their respective environments. The concentrations of ions within the aquarium environment (Ca^{2+} 21.56±1.26 mg l^{-1}, Na^+ 9.28±0.26 mg l^{-1}, K^+ 1.42±0.02 mg l^{-1}, Cl^- 15.32±0.76 mg l^{-1}) were higher than those recorded in the wild (Ca^{2+} 0.325±0.06 mg l^{-1}, Na^+ 3.43±1.02 mg l^{-1}, K^+ 0.46±0.12 mg l^{-1}, Cl^- 10.05±4.46 mg l^{-1}). The concentration of ions within the aquarium environment may be such that offspring can uptake ions *via* their gills and, subsequently, do not require a parental mucus donation of ions. Conversely, the water chemistry of the natural environment may exhibit an extreme lack of ions to the point where parents have to provide young with a dietary source of ions *via* parental mucus. Such provision of ions to young may allow energy to be diverted to growth, as opposed to the active uptake of ions.

CONCLUSIONS

Parental care duties in discus fish appear to be shared equally between the male and female, with regard to both the parental behaviour directed toward offspring and the provisioning of IgM, total protein, ions and cortisol within parental mucus. The dynamics of parental behaviour and mucus physiology throughout the breeding period share several similarities with that seen in mammalian parental care. Cyclical provision of IgM within parental mucus peaked as young reached the free-swimming stage and then fell to pre-breeding values

as young began to feed on other sources. Protein content of the parental mucus was lowest at W2 and W3, mirroring the intensity at which the young fed during this period. A weaning period was observed to occur at W3, which was possibly initiated by a shift in the observed parental behaviour. We conclude that the reproductive strategy of discus fish has more similarities with that of mammals and birds than other fish species. This poses interesting questions with regard to the evolution of this behaviour as well as the sexual selection that precedes this exceptional form of parental care.

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