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Parental care and the development of the parent offspring conflict in discus fish (Symphysodon spp.)

Buckley, Jonathan

Frontispiece. A male from a breeding pair of discus fish (*Symphysodon* spp.) providing produced mucus as a source of nutrition to offspring.

Parental care and the development of the parent offspring conflict in discus fish (*Symphysodon* spp.)

by

Jonathan Buckley

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Abstract

Parental care has evolved across the animal kingdom to increase the probability of offspring surviving in an environment fraught with danger. While parental care is common among mammals and birds, it is relatively rare in fish with the vast majority of fish showing no form of parental care at all, whilst those that do, often just provide parental care to developing eggs pre-hatch. The provision of parental care in discus fish (*Symphysodon* spp.) is, therefore, interesting in that parents provide mucus to offspring as a source of nutrition during the first few weeks of care. In mammals this post-birth provision of parental care can lead to the development of the parent offspring conflict. It is, however, possible that this conflict is also present in discus fish. This thesis examines both the interesting parental care strategy of discus fish along with the potential for the parent offspring conflict to develop.

To examine the dynamics of parental care in discus fish, a range of behavioural and mucus composition studies were carried out. The analysis of mucus revealed that similar to mammals, parents provided offspring with an initial high quantity of nutritional and non-nutritional factors including antibodies (IgM), essential ions and hormones. Behavioural studies also revealed that initially parents were highly diligent in providing care to offspring but that after two weeks of care, the behaviour of parents changed making it harder for offspring to obtain mucus. At this point a weaning period was initiated where offspring began spending less time with parents and more time foraging for external food sources. The initiation of this weaning period suggests the presence of the parent offspring conflict and indicates that a point is reached where the energetic demands of offspring are too great and that energy is better invested in to future offspring. Research into the bite size and feeding rate of fry suggest that during the weaning period fry could demand excessive amounts of mucus, which may be energetically unsustainable leading to the observed offspring avoidance behaviour of parents.

As parental care behaviour is known to be intimately associated with mate choice, mate choice behaviour was also assessed in discus fish with the hypothesis that the ability to provide mucus would be selected for by prospective mates. My dietary experiment, which examined the effect of dietary protein on an individual's ability to mate, did not influence mucus quality or mating ability. The mate choice experiment, however, did reveal the importance of hierarchies in discus fish, indicating that dominant individuals were significantly more likely to pair than subordinates. This is similar to that observed in closely related cichlids where the ability to be dominant and protect a territory was indicative of the ability to successfully raise offspring.

In conclusion, the parental care behaviour of discus fish appears to share more similarities with that seen in mammals than that observed in fish. The implications of these findings indicate that parental care in discus fish could be a new model of parent offspring conflict hitherto unseen in fish which could ultimately help our understanding of the evolution of parental care in fish.

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Author's declaration

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Publications:

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We were granted the cover image for this journal volume which featured our image of discus fish surrounded by feeding offspring. This paper was also shortlisted by the editors of The Journal of Experimental Biology for the award of 'most outstanding contribution to the journal'.

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Contribution to public education

A press release for the paper 'Biparental mucus feeding: a unique example of parental care in an Amazonian cichlid' was produced by the Society of Experimental Biology which was then picked up by the BBC. I was then interviewed by a BBC reporter about our findings which was presented in an article on the BBC website under the Earth news section titled 'Tropical fish are mammal-like parents' (November, 2010).

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Signed ..... Date.....

Chapter 1: Parental care and the development of conflict in an Amazonian cichlid: a review

1.1 Abstract

Relative to that observed in most teleost fish, the parental care exhibited by discus fish (*Symphysodon* spp) is unusual in that both parents provide mucus secretions as a form of nutrition to offspring during the first few weeks of post hatch development. The provision of parental mucus results in the rapid development of fry suggesting that this mucus may contain a range of nutritional and non-nutritional factors essential for the fast development of offspring. In this respect this form of parental care behaviour may have more in common with the provision of colostrum and milk observed in mammals than with the parental behaviour normally observed in teleost fish. In mammalian and avian species the post hatch/birth provision of resources can often lead to the development of the parent offspring conflict, an evolutionary conflict stemming from the genetic differences of parents and their offspring, a behaviour that has not yet been described in teleost fish.

This thesis shall examine parental care in discus fish, focusing on both the composition of parental mucus as well as the behaviours of both parents and offspring throughout the period of parental care in order to determine whether conflict can develop over the provision of resources. This review chapter will start by introducing parental care in discus fish before discussing the potential composition of parental mucus. The second part of this review will then focus on the uniqueness of this form of parental care in teleosts, before discussing the potential for conflict to develop between parents and their offspring. Examples from birds and mammals, where parent offspring conflict theory is well established, will then be used to suggest potential avenues of research that could help elucidate the dynamics of parental care in discus fish.

1.2 Introduction

The evolution of parental care across the animal kingdom has produced a myriad of parental care strategies ranging from the simple protection of eggs, as displayed by many reptiles and fish species, to more complex forms of parental care, such as the provision of resources post birth/hatch as seen in many birds and mammals. Parental care has evolved to aid the survival of offspring during a period of critical development and can be defined as any form of behaviour that appears likely to increase the fitness of a parent's offspring. In the broadest sense this definition includes the preparation of nests and burrows, the production of large, heavily yolked eggs and the care of eggs or young inside or outside the parent's body (Clutton-Brock, 1991). The provision of parental care was often thought to be a one way process whereby offspring passively accepted parental care allowing parents to equally distribute resources to all offspring so as to maximise their inclusive fitness. Trivers (1974), however, pointed out that offspring are not always passive and will use a range of physiological and psychological tactics to try and solicit more parental resources than their parents would be willing to provide. This results in the development of the parent offspring conflict, an evolutionary conflict arising from the differences in the optimal fitness of parents and their offspring.

The parent offspring conflict is evident as early as the period of intrauterine development in mammals. Haig et al. (1993) demonstrated the conflict between foetus and parent during gestation and described how foetal genes are selected to draw more resources from the mother than would be optimal for the mother to give. This conflict continues and intensifies throughout lactation, a behaviour which allows the post birth provision of a range of nutritional and non-nutritional factors essential for the growth of offspring (Goldman et al., 1998; Klobasa et al., 1987). As offspring develop there comes a point when it pays for a parent to stop investing in their current offspring so

that resources may be saved for investment in future offspring. The point at which a parent decides to halt parental care is often different to the time at which offspring would be willing stop accepting care, resulting in a weaning period characterized by high levels of parent offspring conflict as demonstrated in gibbons (*Hylobates hoolock*) (DeVore, 1963; Trivers, 1974), rhesus macaques (*Macaca mulatta*) (Hinde and Spencer-Booth, 1971; Trivers, 1974) and langurs (*Presbytis entellus*) (Jay, 1963; Trivers, 1974). Although scope for intrauterine conflict may not be apparent in lecithotrophic animals, there is still scope for conflict in species where parents provide care to young post fertilization (Smith and Fretwell, 1974). Examples of this can be seen in caecilian amphibians (*Boulengerula taitanus*) where the female parent provides a modified skin layer which can be fed upon by young (Kupfer et al., 2006) in a manner analogous to that of lactation in mammals. A similar behaviour is also apparent in several species of Amazonian cichlids within the genus *Symphysodon.* This genus is composed of three very similar species, *Symphysodon discus, Symphysodon aequifasciata* and *Symphysodon tarzoo* which are collectively known as discus fish. In all three of these species, both parents provide offspring with a diet consisting solely of mucosal secretions for the first few weeks of development (Chong et al., 2005). Mucus is secreted across the whole body of each parent allowing free swimming fry to feed off the mucus for up to 30 days post hatch (Hildemann, 1959; Noakes, 1979). The first few weeks of development can arguably be described as the most crucial part of a fish's development and is often a period characterized by high mortality rates due to nutritional requirements not being met (Nislow et al., 2004). The ability of discus fry to survive solely on parental mucus secretions suggests that parental mucus could contain several nutritional and non-nutritional factors similar to those found in mammalian milk during lactation. This form of parental care is rare amongst fish species and it is estimated that only 30 fish species display this form of mucus provisioning (Schutz and

Barlow, 1997). Discus fish may, however, be one of the few species where mucus feeding is an obligate process for the survival of offspring in the wild (Hildemann, 1959; Noakes, 1979; Schutz and Barlow, 1997). The obligate, bi-parental provisioning of mucus in discus fish is fascinating, especially as it has more in common with the parental care exhibited by mammals than it does with the parental care normally exhibited by fish. Research into discus fish parental care, particularly the care associated with parental mucus provisioning, will help gain insight into the dynamics of the conflict associated with bi-parental care as well as help examine the functional properties of parental mucus that enable the survival and development of offspring.

Mucus production is an integral part of the parental care in discus fish. The analysis of the nutritional and non-nutritional factors present in parental mucus will, therefore, be important in determining the role of mucus feeding in relation to the benefits gained by offspring. The first part of the literature review will, therefore, describe both existing information regarding discus fish mucus composition as well as suggesting potential avenues of research that could help quantify and qualify mucus composition. The second part of this review will then concentrate on why bi-parental care is so rare and why conflict can develop. This will then help to pose questions regarding the evolution of care in discus fish and the implications conflict may have for mucus production.

1.3 Nutritional/Non-nutritional composition of mucus

Fish mucus is known to contain many biologically active peptides and proteins which make it an important factor in many common biological functions such as respiration, ionic and osmotic regulation, communication, reproduction, and disease protection (Shephard, 1994). Chong et al. (2005) discovered the existence of a large number of proteins in parental discus fish mucus indicating the ability of parents to provide offspring with an array of nutritional and non-nutritional components. The following

five sections will discuss the potential composition of parental mucus and how this composition may affect the development and growth of discus fish larvae.

1.3.1 Nutritional factors

One of the main components of parental care in mammals involves the maternal provision of a nutritional substance to offspring initially termed colostrum which later on develops into a substance termed milk. The change from colostrum to milk reflects a change in composition which is correlated to the development of young. During early mammalian development colostrum is provided which is rich in carbohydrates, protein and antibodies but low in fat and lactose (Klobasa et al., 1987). High levels of carbohydrate and protein and low levels of fat and lactose reflect the underdeveloped nature of the neonate's digestive system while the provision of antibodies reflects the neonate's lack of immune competency (Goldman et al., 1998; Grosvenor et al., 1993). As offspring develop, milk is then provided which contains lower levels of protein and antibodies which reflect a rise in neonatal immune competency. Milk also contains a higher quantity of fat and lactose reflecting the changing ability of the neonate to digest these substances which are vital for fast growth. The changing composition of maternally-provided care in mammals is linked intimately to the development of offspring and reflects a level of parental care which may also be apparent in discus fish.

One of the first questions posed when assessing parental care in discus fish is the question 'what nutritional value can mucus provide to developing offspring?' The offspring of discus fish display fast growth rates and high survivability due to the parental provision of mucus secretions (Hildemann, 1959; Noakes, 1979). It can, therefore, be hypothesized that mucus secreted by parents contains nutritive factors that allow the rapid growth of larvae. It can also be hypothesized that the provision of parental care is energetically costly to the adults as extended periods of larval care often

negatively affect the subsequent reproductive performances of parents, resulting in decreasing yields of discus fry (Chong et al., 2002a). Discus fish are both a popular and valuable aquarium species farmed relatively intensely around the world. The increasing demand for this aquarium species has led to several studies designed to assess the requirements of offspring so that steps may be taken to rear young without their parents to negate the damaging impact offspring can have on future parental yields.

Research has so far focused on the dietary needs and gut structure of discus larvae so that future larval micro diets may be produced as a replacement for parental mucus. Work concerning the dietary needs and gut structure of discus fish also happens to be a useful starting point for determining the potential nutritional composition of parental mucus. Chong et al. (2002) detailed the formation of the digestive system in *Symphysodon aequifasciata* by chronicling the time at which different proteases and digestive structures become apparent. The digestive system of *S. aequifasciata* larvae is first characterized by the presence of alkaline proteases such as serine proteases, trypsin and chymotrypsin (Chong et al., 2002a). Each enzyme is specific to a different range of amino acids with trypsin hydrolysing peptide bonds to release arginine and lysine and chymotrypsin hydrolysing peptide bonds to release tyrosine, phenylalanine, tryptophan, methionine and leucine. The early digestive system of *S. aequifasciata* larvae is also devoid of an acidic environment and the associated digestive protease pepsin. The absence of both an acidic environment and pepsin, suggests that complex proteins could not be denatured during the early developmental period of larvae (Ronnestad et al., 2003). It may be possible that until an acidic environment develops in the digestive system of larvae, that larvae are reliant on an abundant source of peptides or free amino acids. Amino acids are particularly important for the development of offspring due to their role in anabolism. For example, lysine, one of the amino acids cleaved by trypsin is vital for the production of all protein types and is especially important hormones,

muscle, enzymes and antibody production (Encarnacao et al., 2006; Hevroy et al., 2007). Arginine, another amino acid cleaved by trypsin, also plays an important role in larval development, influencing cell growth, immune function and the release of hormones (Cowey, 1994; Lopezalvarado and Kanazawa, 1994; Mommsen et al., 2001). As well providing the raw material for growth, several authors have highlighted the capacity of amino acids to act as a crucial fuel source during the early life stages of marine teleost fish (Finn et al., 1995; Ronnestad et al., 1993; Ronnestad et al., 2003). Recent work by Chong et al. (2005) demonstrated the presence of the ten essential amino acids in parental discus fish mucus. Out of the ten amino acids found in the mucus, lysine and phenylalanine were present at particularly high levels (Chong et al., 2005). It is, therefore, quite probable that the early needs of developing discus are met by the parental provision of basic peptides and amino acids. To evaluate the potential importance of certain amino acids over others in discus fish, it is possible to look at the whole body amino acid ratio (A/E) of juvenile discus fish as this correlates well with the actual quantitative amino acid requirements of fish. This method is used in diet formulation as the A/E of a fish correlates well with its actual quantitative amino acid requirements (Wilson et al., 1985) and in this instance can be used to gain information about parental mucus composition. The A/E characteristics of juvenile discus fish show high quantitative amounts of the amino acids arginine, leucine, lysine and phenylalanine (Chong et al., 2004). It may, therefore, be expected that parental mucus has a similar composition of amino acids. Although discus offspring may be obligate mucus feeders in the wild, some aquarists and authors have demonstrated the ability to rear discus larvae without parental care. Lim and Wong (1997) managed to rear discus larvae without the need for parental mucus using only the rotifer species *Brachionus calyciflorus.* Ovie and Ovie (2006) found that *B. calyciflorus* had an A/E composition that was high in arginine, leucine, lysine and phenylalanine, a composition, similar to

that of juvenile discus (Ovie and Ovie, 2006). Rotifers are also known to contain pools of free amino acids which can be utilized without energy demanding catabolic processes and so are easy for larvae to access (Lim and Wong, 1997; Ovie and Ovie, 2006; Ronnestad et al., 2003). The success of supplementing parental mucus in an obligate mucus feeder for a live feed rich in amino acids suggests the potential importance of amino acids in parental mucus.

As the digestive system of S. *aequifasciata* offspring develops, there is an eventual progression from an alkaline environment rich in serine proteases to an acidic environment containing several pepsin like proteases. This change occurs 15-20 days after the first day of exogenous feeding and would allow the catabolism of more complex proteins (Chong et al., 2002a). At this point it appears that S. *aequifasciata* offspring are able to utilize more complex nutritional items. Chong et al. (2005) compared parental and non-parental mucus and found distinct differences in composition suggesting that discus fish have an ability to alter the composition of their mucus. It would be interesting to see if the nutritional content of mucus could change to accommodate the development of their offspring's digestive system in a similar fashion to the changing composition of mammalian investment during lactation.

Although the role of amino acids is often thought to be primarily that of protein anabolism and energy allocation, some authors have suggested that they may act as potent attractants or as stimulants of feeding behavior (Carr et al., 1996). Amino acids are known to act as feeding stimulants in several species. Two interesting examples include the herbivorous freshwater *Tilapia zillii* and the marine carnivore *Prionotus carolinus*. The herbivore *T. zillii* feeds predominately on water plants and epiphyton. In this species the amino acids, glutamic acid, aspartic acid, serine and lysine are major stimulants of feeding behavior (Adams et al., 1988; Carr et al., 1996; Johnsen and

Adams, 1986) while the amino acids tryptophan and phenylalanine are the major stimulants of feeding behaviour in the marine carnivore *P. carolinus* (Carr et al., 1996). Both phenylalanine and lysine are abundant in the mucus of parental discus (Chong et al. 2005). It could, therefore, be suggested that both may play a role in the stimulation of feeding in discus offspring which if correct could have important implications regarding the dynamics of the parent offspring conflict, an issue that will be discussed in detail later in the review.

While nutritional factors are important for the development of offspring, a range of non-nutritional factors can also be crucial for the survival of young dependent on parental care. Mammalian offspring in particular are highly dependent on the maternal provision of immunity, a provision which may also be important in discus fish.

1.3.2 Immune factors

The transfer of maternal immunity, like nutrients, is well recognized in mammals, occurring during the intra-uterine period through the placenta and after birth during lactation (Mor and Avtalion, 1990). The active immunity of offspring is only obtained when the lymphoid system is fully mature, a process which does not occur until several weeks after birth leaving the neonate initially susceptible to pathogenic attack (Goldman et al., 1998). The lack of an adaptive immune system in newborn offspring is offset by the maternal provision of defence agents during lactation, a process which involves the horizontal transfer of maternal immunity to offspring via a substance which begins as colostrum and later develops into milk. Colostrum is a specialized mammary gland secretion that contains various components important for immune protection including lymphoid cells, cytokines, growth factors, hormones and immunoglobulins (Adamski and Demmer, 2000; LeJan, 1996). The passive immunity provided by colostrum is vital for the survival of the mammalian neonate; new-born pigs deprived of colostrum for the

first 4 weeks of development show mortality rates close to 100% (Kurse, 1983). The provision of maternally derived immunity is, therefore, crucial in ensuring survival against pathogens in neonate mammals (Bramble et al., 1951; Mor and Avtalion, 1990). The provisioning of passive immunity to offspring occurs until offspring can mount their own adaptive immune response at which point the provision of parental resources changes. This change in composition reflects the changing needs of the neonate and represents a reciprocal relationship where offspring are provided with passive immunity and parents gain a higher inclusive fitness due to increased offspring survival.

It is not just mammalian neonates who are born into an environment fraught with pathogens; fish hatching into an aquatic environment also face similar problems. Like mammals, several species of fish are known to provide some degree of passive immunity to their offspring via egg yolk preserves. The transfer of a maternally-derived passive immunity has been demonstrated in several teleost species where the immunisation of maternal fish led to higher survival rates in offspring (Kawahara et al., 1993; Sin et al., 1994). In the majority of species where maternally-derived immunity has been recorded, the transmission of immunity is vertical and occurs between the mother and her eggs (via the yolk sac). Vaccinated female tilapia (*Oreochromis mossambicus*) demonstrated their capacity to vertically transfer antibodies to their eggs when antibodies raised against bovine serum albumin (BSA) were transferred successfully from female *O. mossambicus* to their offspring (Mor and Avtalion, 1990; Takemura, 1997). BSA antibody activity was initially present in the larvae of vaccinated females before decreasing as the antibody was metabolised by the larvae. It was postulated that immunoglobulin (IgM) is stored in the yolk sac and that the metabolization of maternal IgM is associated with the metabolization of the yolk sac (Takemura, 1993, 1997). Although maternally-derived IgM is quickly metabolised, it is thought that it can still provide a short-term passive immunity for larvae (Takemura,

1997). Studies on the antigenic development of the immune system in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio)* showed that their lymphoid systems are still immature until 3-6 and 3-8 days after hatching and that functional capacity was unlikely (Botham and Manning, 1981; Grace and Manning, 1980). Short term maternally provided immunity could, therefore, serve to provide pathogenic protection during the 3- 8 day period where the immunity of larvae is compromised. This short term immunity provided to offspring is known to occur in several teleost species, many of which only provide parental care to eggs. It is not known whether the vertical provision of maternal immunity occurs in discus fish, however, the extensive provision of parental care exhibited by this species indicates it may be a possibility.

Sin et al. (1994) looked at another possible mode of IgM transfer that may well be applicable to discus fish. Vaccination and challenge trials were carried out in *Oreochromis aureus* to assess the potential for the vertical transmission of maternally derived IgM. While these trials demonstrated that fry from vaccinated parents showed higher survival rates than controls, an increase in survival was also gained when natural mouth-brooding behaviour was allowed. Vaccinated females allowed to mouth brood fry showed higher fry survival rates (95.7% survival) compared to vaccinated females that were prevented from mouth brooding fry (78.4% survival). It has been hypothesized that the increase in survival connected with mouthbrooding behaviour could be due to fry feeding on mucosal secretions within the mouth of female *O. aureus*. This behaviour may be similar to lactation in mammals where maternally provided IgM within colostrum supplies offspring with a passive form of immunity. The mucosal transmission of IgM from parents to offspring may also be occurring in discus fish. Early attempts at breeding discus fish without parental care often resulted in high mortality rates, leading some aquarists to grow discus larvae with antibiotics which apparently aided survival (Shephard, 1994). The absence of parental care may have left

a deficit in the provision of passive immunity gained from mucus feeding. Indeed, several papers have demonstrated the presence of IgM in the cutaneous mucus secretions of several other species of teleost fish (Hou et al., 1999; Shephard, 1994; Zilberg and Klesius, 1997) lending more weight to the possibility of discus fish providing passive immunity to offspring via the vector of mucus.

Proteomic profiling of discus mucus has highlighted the appearance of a c-type lectin, uniquely expressed in parental mucus as opposed to non-breeding parental mucus (Chong et al., 2005). C-type lectins function as the first line of defence in the pre-immune host, where they recognize carbohydrate patterns found on the surface of a large number of pathogenic micro-organisms, including bacteria, viruses, protozoa and fungi (Liu et al., 2007; Nikolakopoulou and Zarkadis, 2006; Russell and Lumsden, 2005; Vasta et al., 1999). Once c-type lectins recognize these pathogenic micro-organisms they can then activate the complement system (Vasta et al., 1999). The composition of discus mucus may also have an effect on the expression of immune related genes. Sanderson and Naik (2000) hypothesised that changes in the environments of vertebrates can alter the expression of immune related genes. The ingestion of parental mucus could be a potent mechanism for altering the environment of cells in most organs, particularly cells within the gastrointestinal tract. Chong et al. (2005) hypothesised that mucus feeding could therefore positively influence the expression of immune related genes in developing fry.

1.3.3 Hormones

The initiation of parental care behaviour in mammals is largely driven by hormones. Prolactin in particular is responsible for a large component of parental care. The release of this hormone initiated by suckling stimulates the parental care behaviour of mammals as it promotes the release of milk to offspring. As well as providing the impetus for

parental care behaviour, hormones can play an important role in the development of offspring. Hormones, like IgM, are another important non-nutritional factor delivered to mammalian offspring during lactation. Hormones and the roles they play in the mammalian mother-infant trophic relationship are well known, with recent research indicating that many of the hormones in milk can survive the environment of the neonate gut. Hormones can then become absorbed into neonatal circulation where they can exert important functions in the developing neonate (Grosvenor et al., 1993).

Similar to that observed in mammals, hormones play a vital part in the neonatal development of fish and are responsible for a wide range of roles and functions (Harris and Bird, 2000; Manzon, 2002). The transfer of hormones in fish, like mammals, is known to occur vertically between the mother and offspring (Ayson and Lam, 1993); in the case of fish this often occurs via the vector of maternally provided egg yolk. Recent research has, however, demonstrated the possibility of hormones being horizontally transferred via the parental provision of mucus in fish. Schutz and Barlow (1997) assessed the composition of mucus in the midas cichlid (*Cichlasoma citrinellum)* (a species that like discus fish provide mucus to offspring during the time of first feeding) and found the presence of three hormones prolactin, growth hormone and thyroid hormone. These hormones are highly multifunctional and play important roles in neonatal development. While research often centres on the vertical transmission of these hormones, the information gained from these studies can be used to infer their roles regarding a possible horizontal transfer via mucus. Thyroid and growth hormones are particularly important in acting synergistically to control the metamorphosis of a variety of species including tadpoles, flatfish, eels and silkworms (Schutz and Barlow, 1997; Takagi et al., 1994). The vertical transmission of thyroid hormone, i.e. passed from mother to offspring as opposed to passed between conspecifics and its subsequent effect on offspring development, has been recorded in several species of fish. Female

rabbitfish, (*Siganus guttatus)* injected with elevated levels of thyroid hormone subsequently transferred the hormone to oocytes which was then present in rabbitfish larvae (Ayson and Lam, 1993). Larvae from females treated with higher levels of thyroid hormone tended to be longer in length and had higher survival rates when compared to the larvae from control fish and those injected with lower quantities of thyroid hormone. Similar results correlating increases in thyroid hormones with desirable properties such as enhanced growth, survival, dry weight and body area have been found in several species of teleost fish including rockfish, (*Sebastes schlegeli*) (Kang and Chang, 2004) striped bass (*Morone saxatilis*) (Brown et al., 1989) and tilapia (*Sarotheroden mossambicus*) (Yamano, 2005). Growth hormone and prolactin are also found in the mucus of the midas cichlid and are known to be pleiotropic, playing a role in both osmoregulation (Björnsson, 1997; McCormick, 1995) and energy metabolism in fish (Leung et al., 1991; Sheridan, 1986). Their roles in osmoregulation have been studied in a number of teleost species and appear to be somewhat antagonistic (Shepherd et al., 1997). Growth hormone increases total levels of gill Na^+, K^+-ATPase activity, consistent with the seawater-adapting role of this hormone in salmonids and cichlids (Borski et al., 1994; McCormick et al., 1995; Shepherd et al., 1997). Conversely, prolactin treatment reduces gill Na^+,K^+-ATPase levels, consistent with its role as a freshwater osmoregulatory hormone (Fujimoto et al., 2006; McCormick et al., 1995; Richman and Zaugg, 1987; Shepherd et al., 1997). Fujimoto et al. (2006) noted that as well as the ability to reduce the rate of water flux in the gills of goldfish, prolactin expanded the mucous cell layers on scales, which was hypothesised as a method to further restrict water inflow by the mucus system.

The close evolutionary relationship and similar life histories shared by the midas cichlid and discus fish, suggest that a similar suite of hormones may also be transferred to discus fish offspring during the first few weeks of mucosal feeding. The post hatch

provision of thyroid and growth hormone to offspring could potentially allow a more rapid rate of metamorphosis and development as seen in rabbitfish, which could presumably enhance the survival of discus offspring by speeding up the time at which offspring spend in a delicate period of development. The parental provision of prolactin could also be particularly important for discus fish offspring due to the ion poor, acidic nature of their natural habitat. Osmoregulation in this environment may be problematic for offspring as their small size results in a considerable surface area for ion loss and water uptake. The provision of prolactin to offspring may aid osmoregulation in this species due to the role of prolactin in preventing ion loss and water uptake (Manzon, 2002). Other than roles in osmoregulation, prolactin has also been demonstrated as playing an important part in the expression of parental behaviour in a range of fish species. The addition of a prolactin inhibitor (bromocriptine) to North American bluegill males (*Leopmis macrochirus*) caused a subsequent reduction in parental care duties such as egg fanning and aggression aimed at predators which ultimately led to a reduction in offspring survival (Kindler et al., 1991). Male and female discus fish, injected with elevated levels of prolactin also demonstrated an increase in egg fanning and mucus cell production (Blum and Fiedler, 1965) demonstrating the importance of prolactin in the initiation of parental care in this species. The up-regulation of prolactin within the skin of parental discus has also been demonstrated suggesting the importance of this hormone for parental care in discus possibly playing a role in ensuring mucus is produced consistently over the period of parental care (Khong et al., 2009).

Parallels can also be drawn between the role of prolactin in mammals and the role of prolactin in discus fish as this hormone appears to increase both the production of milk and mucus respectively (Blum and Fiedler, 1965; Freeman et al., 2000; Khong et al., 2009), both of which act as the sole source of nutrition for mammalian and discus offspring. Since the action of suckling in mammals initiates the release of prolactin,

which in turn provides the impetus for milk production, it would be interesting to see if the same occurred in discus fish i.e. would the bite rate of fry cause an increase in prolactin in adult discus fish, causing a subsequent increase in mucus production.

As well as leading to the production of prolactin, the continual bite rate of discus fry during parental care may also lead to the parental production of cortisol. Personal observations indicate that the biting of fry can eventually lead to signs of epidermal damage on the parents, a process which could initiate a physiological stress response in adults, particularly as fry develop in size and the demand for food increases. Signs of stress such as rapid breathing and visible mechanical damage on the epidermal surface are notable which may be indicative of elevated plasma levels of the stress hormone cortisol. This could well lead to the horizontal transfer of cortisol to offspring as elevated levels of cortisol in the plasma of sea bass (*Dicentrarchus labrax*) (due to stress) were found to correlate to a similar, albeit reduced relative to plasma, increase in mucus cortisol levels (Simontacchi et al., 2008). Prolonged exposure to cortisol is known to have damaging effects in several teleost species and can cause immunosuppression that renders fish vulnerable to pathogens (Campbell et al., 1992; Shankar et al., 2007). The vertical transmission of cortisol from mother to ooyctes has previously been recorded in several teleost species (Campbell et al., 1992; Foo and Lam, 1993). Trout and tilapia offspring suffered reductions in growth and survival due to the vertical transfer of elevated levels of cortisol (Campbell et al., 1992; Foo and Lam, 1993). Research in other species, however, has demonstrated that a reduction in survival was not necessarily correlated with enhanced cortisol levels (Stratholt et al., 1997) and that moderate amounts of cortisol may actually be highly beneficial for juvenile fish. Studies have demonstrated the hypercalcemic role of cortisol in rainbow trout, where cortisol enhanced calcium uptake in cultured gill epithelium (Kelly and Wood, 2008). As described previously, the ion poor nature of the discus natural habitat

would pose problems associated with osmoregulation. The parental provision of cortisol may, therefore, help alleviate these problems due to the role of cortisol in increasing calcium uptake (Bonga and Meis, 1981). When female three-spined sticklebacks (*Gasterosteus aculeatus*) were exposed to the threat of predation, they produced larger eggs with higher levels of cortisol that (post-fertilization) consumed higher levels of oxygen than controls (Giesing et al., 2011). The production of larger eggs has been demonstrated in previous studies to have positive effects on swimming abilities (Ojanguren et al., 1996) and survival (Henrich, 1988) indicating the positive role cortisol may have on offspring development. In addition to the observed changes in physiology, juveniles from mothers exposed to the threat of predation, would shoal closer to their mothers during a mild disturbance. As shoaling behaviour is known to be an effective anti-predatory response in fish (Magurran, 1990) cortisol can be seen to have a positive effect on offspring behaviour (Giesing et al., 2011).

1.3.4 Ions

As alluded to in the previous section, the provision of certain hormones may help increase the survival of discus fish offspring due to their ability to prevent ion loss. Regulating the loss of important ions, such as sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) while limiting water influx are some of the main problems associated with osmoregulation in freshwater teleosts. Freshwater environments are less concentrated than the internal environment of the teleost which can subsequently lead to a loss of ions from the high ion concentration environment (the organism) into a low ion concentration environment (the external media) (Gonzalez et al., 1997; Madara, 1988). The problem of osmoregulation is magnified in freshwater systems such as the Amazon, the natural habitat of discus fish, where the water is particularly ion deficient and acidic. Around 37% of continental South American freshwater drains into the Amazon basin

making it the largest fluvial system in the world (Aride et al., 2007): Water draining through this region passes through dense jungle regions filled with decomposing vegetation which leads to the continuous input of humic and fulvic acids. The input of these acids helps to create the acidic, ion poor water chemistry that characterizes the Amazon River Basin (Gonzalez et al., 1998).

One of the immediate problems of living in an ion poor environment is that ions such as Na^+ and K^+ are simply hard to come by due to their scarcity in the bulk medium (Potts, 1994). The scarcity of ions such as Na^+ and K^+ is exacerbated by the abundance of hydrogen ions (H^+) in the surrounding medium which can compete with Na^+ uptake as the transport of Na^+ into the epithelium is coupled with H^+ extrusion through a Na^+/H^+ antiport (Lin and Randall, 1991; Potts, 1994). Dilute, acidic water can also cause ion loss at paracellular tight junctions effectively causing an even greater efflux of ions (Gonzalez and Dunson, 1989; Gonzalez et al., 1998; McDonald and Rogano, 1986). Paracellular tight junctions depend on the binding of calcium to prevent ion loss. Soft, acidic waters contain low levels of calcium which can ultimately lead to an increase in ion permeability at the paracellular tight junctions. This increase in ion permeability can allow the natural movement of ions from the ion rich, internal environment of the fish to the external, ion deficient environment of the Amazon. The inhibition of ion uptake combined with an increase in ion efflux can be particularly damaging to teleost species. Milligan and Wood (1982) proposed a sequence of events that began with excessive ion loss and led to an increase in arterial blood pressure, a development which would ultimately end in circulatory failure. Larval fish may be even more susceptible to ion loss than adults due to their relatively high surface area. Mucus feeding may, however, help offspring alleviate the problems of obtaining ions. Gradients of ions within the mucus layers of adults can represent a significant barrier against the diffusional efflux of ions (Shephard, 1982, 1994) which if consumed by offspring could help limit the

stress of living in an ion poor environment while potentially imposing a cost to parents; previous experiments have shown that the dietary supplementation of essential ions can help fish adjust to living in an ion poor environment (Smith et al., 1989).

1.4 Wild versus aquarium bred discus

So far the composition of discus mucus has been discussed in relation to the pressures exerted by the Amazonian environment. Discus fish are however a hugely popular ornamental species and have been bred in aquariums for upwards of 50 years. During this period the breeding of discus has been largely driven by novelty with the emphasis being placed firmly on the production of interesting colour and body morphs (Koh et al., 1999a). While selection pressures in the wild may rest on an individual's ability to resist pathogens or avoid predators, the selection pressures imposed by aquarists are based on appearance as opposed to function. Aquarists raise discus fry in sterile conditions, in water distinctly different from the Amazon, with an abundance of well formulated feeds and a distinct lack of predators where mortalities are imposed by the aquarist based on appearance. The potential for this type of selective breeding to cause evolutionary change is not appreciably different from other forms of predator-induced mortality (e.g. Law 1979; Reznick et al. 1990), especially as aquarists impose a strict differential mortality and control on reproduction among different genotypes. One of the consequences of this type of selective breeding, however, is inbreeding.

Inbreeding is essential for obtaining novel colour morphs over a short number of generations but this process ultimately leads to a reduction in genetic heterozygosity, something which can increase the probability of lethally recessive genes being expressed. It can also lead to a reduction in genetic variance as well as inbreeding depression (Falconer, 1981; Hutchings and Fraser, 2008). Studies in rainbow trout (*Oncorhynchus mykiss*) demonstrated that inbreeding led to reductions in growth and

survival while increasing the percentage of fry with developmental abnormalities (Aulstad and Kittelse, 1971; Hutchings and Fraser, 2008). While the appearance of developmental abnormalities is easy to identify, subtle changes in the genotype of aquarium bred discus may be harder to identify.

One of the defining aspects of the discus life cycle is the parental provision of mucus to offspring. In the wild, strong selection pressures select for the ability of individuals to produce mucus that would best serve the survival of their offspring such as the possibly provision of antibodies or ions to combat the harsh environmental conditions. These selection pressures are relaxed in aquarium conditions due to the abundance of formulated food, lack of predation, sterile living conditions and favourable water chemistry. It would, therefore, be feasible for changes to occur in the ability of adults to produce mucus without a noticeable effect on offspring survival. Comparisons between wild and aquarium reared discus would therefore be interesting to elucidate any difference between the two types of discus.

So far, this review has largely proposed the possible benefits gained by the mucosal feeding habits of discus larvae. The stability of the Amazon environment has recently come under question as industry seeks to expand and develop (Herbert, 2005; NewScientist, 2006, 2007), a process which will inevitably affect a large proportion of species within this complex ecosystem. The next section will explore how the mucosal feeding behaviour of discus fish may, as a result of encroaching industry, become disadvantageous.

1.5 Environmental contaminants

One of the many proposed functions of epidermal mucus in fish is that of defence. Defence is provided by mucus, largely due to the ability of mucus to separate the internal and external environment of the fish. This ability is particularly important when

water quality is compromised by contaminants. The presence of contaminants in the natural environment is not uncommon and has led to the hypothesis that epidermal mucus may provide a suitable defence against water contaminants such as metals (Shephard, 1994). One way in which mucus may aid defence is through its ability to bind and precipitate the contaminant (Shephard, 1994). In this respect mucus can be thought of as a barrier which can absorb and prevent metals from entering the internal environment of the fish. The constant production of mucus would allow older, (more highly contaminated), mucus to be eventually sloughed off. Several studies suggest that the rate of mucus production may increase as a response to the presence of heavy metals (Lock and Vanoverbeeke, 1981; Shephard, 1994; Wong et al., 1977) allowing a more rapid removal of metals from the vicinity of the fish.

The recent heavy development of industry, in and around the Amazon basin has been associated with a notable rise in the concentrations of metals such as mercury (Hg) (Uryu et al., 2001), copper (Cu) and cadmium (Cd) (Matsuo et al., 2005). These metals pose considerable problems to fish in most freshwater environments, but can be particularly problematic to fish in the Amazon due to the specific water chemistry of this environment. Acidic freshwater environments are known to increase the toxicity of metals due to the increased solubility of the metal (Shephard, 1994); in an alkaline environment, metals would have to compete with calcium for binding sites on the gills. The Amazon is distinctly lacking in calcium, further increasing the toxicity of pollutants (Playle et al., 1993; Playle et al., 1992). In addition to the problems encountered by adult fish in dealing with contaminants in such acidic ion poor water, the ability of mucus to bind contaminants may well pose problems for discus fish due to the nature of bi-parental care in this species. The accumulation of metals in the mucus of adult discus may well enter the young directly via mucosal feeding. This would have serious

consequences for populations of wild discus fish, especially as industry begins to encroach on the rivers of the Amazon (Matsuo et al., 2005; Uryu et al., 2001).

Cu and Cd are have become increasingly prominent in the Amazon environment with recent rises in Cu associated with effluent disposal around industrialized, heavily populated areas such as Manaus (Matsuo et al., 2005; Sampaio, 2000) while an elevation in Cd has been associated with an increase in petroleum extraction along the Amazon River (Matsuo et al., 2005; Oliveira, 2003). Both metals can be extremely toxic to fish. Copper is known to reduce the influx of sodium (Na^+) (Lauren and McDonald, 1985) while Cd is known to compete with calcium (Ca^{2+}) uptake (Verbost et al., 1989); both of these actions can lead to ion loss and eventual circulatory collapse (Milligan and Wood, 1982). The Amazonian environment may provide a significant defence against Cu due to the large quantity of humic acids and dissolved organic matter (DOM) occurring naturally in the water column. Sufficient quantities of humic acid and DOM can chelate metals such as Cu (Jeong et al., 2007) which effectively reduces the number of free Cu ions in the water column, thereby reducing their potential to inhibit the branchial uptake of Na^+ (Matsuo et al., 2005). Matsuo et al. (2005) examined the effects of Cd and Cu on the Amazonian teleost, tambaqui (*Colossoma macropomum*) and concluded that even the presence of high levels of Cu would do little to affect the uptake of Na^+. The presence of Cd, however, was shown to pose a major threat to tambaqui due to the ability of Cd to severely inhibit Ca^{2+} uptake. The inhibition of Ca^{2+} uptake, if severe enough, could eventually result in hypocalcemia (Verbost et al., 1989), as well as circulatory collapse (Milligan and Wood, 1982). Cd is also known to interfere with several neural Ca^{2+} dependent processes leading to neurotoxicity (Scherer et al., 1997; Sloman et al., 2003) and has also been demonstrated as having adverse effects on species behaviour (Malm, 1998).

Another potential pollutant that could affect Amazonian species is the metal mercury (Hg). Recent gold mining activities over the last fifty years have led to the total environmental emissions of 2000-3000 tonnes of Hg (Fjeld et al., 1998; McKim et al., 1976; Uryu et al., 2001). A large proportion of this mercury has subsequently entered the aquatic environment posing a significant problem for many freshwater species. High levels of mercury are lethal with smaller doses leading to a reduction in sperm viability, egg production and larval survival rate (Naes et al., 1999). Uryu et al. (2001) noted that incidences of Hg poisoning were correlated with both the distance of a species from gold mining sites and the species diet type; omnivores and carnivores were found to be more susceptible to Hg presumably through bioaccumulation. To my knowledge there is no information detailing the position of gold mining operations in relation to known discus fish habitats. The widespread development of gold mining around the Amazon, however, suggests that Hg may well be a potential metal affecting discus fish populations.

The prevalence of petroleum extraction around the Amazon has also led to the increase in ambient levels of polycyclic aromatic hydrocarbons (PAH). PAHs result from the incomplete combustion of carbon containing fuels and are associated with the production of petroleum (Krahn et al., 1984; Varanasi and Gmur, 1981; Varanasi et al., 1981). Aquatic organisms can accumulate PAHs and metabolize them to potentially toxic or carcinogenic products that may be more harmful than the parent structures themselves (Fabacher and Baumann, 1985; Krahn et al., 1984). The carcinogenic nature of PAHs has been reported in several papers where elevated levels of PAH led to an abnormal proliferation of cells (neoplasia, otherwise known as tumours) in the organs of several teleost species (Collier et al., 1986; Johnston and Baumann, 1989; Varanasi et al., 1986). The potential effect of PAHs on discus fish larval physiology is yet to be considered. Different species subjected to the same levels of PAH have shown quite

different responses in relation to the occurrence of tumours. Species that retain more metabolites as adducts and less metabolites in bile exhibit a higher frequency of tumours than species with a low tumour frequency (Johnston and Baumann, 1989). It is likely, therefore, that the ability of discus larvae to retain metabolites as adducts will influence the propensity of this species to develop tumours (Koh et al., 1999a).

Parental care in discus fish has evolved in a largely contaminant free environment to provide offspring with a range of factors essential for growth and development via the provision of mucus. As contaminants associated with industry increase in the Amazonian environment there is potential for parental mucus to act as a sink for contaminants. Mucus feeding in discus could, therefore, reduce the fitness of offspring due to the consumption of mucus containing high concentrations of contaminants. To understand the composition and benefits that a mucus feeding strategy provides to offspring it is also important to understand how this might be affected by the external environment and how some components may be disadvantageous.

1.6 Conflict associated with parental care

Research into the nutritional and non-nutritional components of mucus will help to provide a firm empirical basis for addressing the potential conflicts associated with this interesting form of parental care. Although parental care in discus fish affects both the survival of offspring (Chong et al., 2000; Chong et al., 2005) and the future reproductive ability of parents (Clutton-Brock, 1991; Trivers, 1974), what is not known, is whether there is a degree of conflict associated with the parental provision of mucus. The last part of this review will, therefore, discuss the dynamics of bi-parental care in discus fish with particular focus on the potential conflicts that could arise due to the parental provision of care in this species.

Parental care is a behaviour often characterized by conflict (Dawkins, 1976) and to fully understand the mechanisms of parental care, it is important to consider all aspects of conflict that might arise. Conflict associated with parental care can arise due to:

1) Conflict between parents and offspring.

2) Conflict between offspring

3) Conflict between parents.

1.6.1 Parent –Offspring conflict: offspring solicitation of care

Parental care has evolved because it maximises the selfish genetic interests (inclusive fitness) of the parent (Hamilton, 1964). An organism's success, from the gene's point of view, ultimately depends on leaving behind the maximum number of replicas of its genes present within a population; a concept known as inclusive fitness (Dawkins, 1976; Kolliker and Richner, 2001; Trivers, 1974). In all organisms where parents and offspring are not genetically identical, conflicts of interest will arise over levels of parental investment. Trivers (1974) defined parental investment as anything done by the parent for the offspring that increases the offspring's chance of surviving while decreasing the parent's ability to invest in other offspring. With this definition, mucosal feeding in discus fish can clearly be described as a form of parental investment. As parents only share half of their genes with their offspring, conflict over the amount of investment offered to offspring should ultimately develop. An individual wishing to maximise their inclusive fitness, would want to invest in current offspring up until the point where any further investment would only offer diminishing returns. Any investment past this point would squander energy that would have a greater return if invested into future offspring. Too much parental investment into current offspring will therefore reduce the total number of offspring that an individual could ultimately produce; this equates to a reduction in an individual's inclusive fitness. It is expected

that parents should regulate the amount of care they provide to their current offspring 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. Interestingly in clonal species, this same conflict could not spread due to the genetic interests of the parent and offspring being identical (Clutton-Brock 1991).

On an evolutionary timescale, being attentive to the needs and health of offspring would have evolved due to the mutual benefit of efficient parental investment in relation to the short term needs of offspring. This is the start of an ever-escalating conflict between parents and offspring due to the unequal genetic interests of both parties (Kolliker and Richner, 2001; Trivers, 1974). A gene expressed in offspring that allowed the successful manipulation of parental investment would enhance the inclusive fitness of offspring. Likewise, a gene expressed in parents that allowed parents to efficiently regulate parental investment and resist offspring manipulation would also have similar chances of being transmitted down the germ line (Kolliker et al., 2006). This genetic conflict can be likened to an arms race and is an important factor in determining levels of parent-offspring conflict. The origins of this conflict can be found during the time when communication first occurs between parents and offspring. Parents wishing to efficiently invest care in offspring (and save energy for future offspring) can do so if there is communication between offspring. Maternal provisioning in burrowing bugs (*Sehirus cinctus*) is dependent on the chemical signals produced by offspring (Vergne et al., 2007), where chemicals from poor quality offspring were found to stimulate more maternal care than chemicals from high quality offspring. If the signals produced by the offspring were produced passively, without manipulation, a parent would be able to gauge the exact needs of the offspring. This would allow parents to accurately distribute investment and allow parents to maximise their inclusive fitness. Offspring, however,

are expected to solicit more care than would otherwise be given. Although the parent is much larger than the offspring, offspring are still able to gain more care through manipulation. It is not known whether the chemical signals in the burrower bug are passive (honest) or actively manipulated (dishonest). It is easy to envisage a process whereby healthy offspring produce chemicals which indicate a poor quality status to allow them to gain more parental investment.

Without a form of communication it would be quite easy for a parent to either under or over-provide, both scenarios would reduce the inclusive fitness of the parent. One of the first questions applicable to parental care in discus fish is whether there is communication between parents and offspring. In other species, communication between parents and offspring are exerted through audio (Kilner et al., 1999), visual (Kolliker et al., 2006) and chemical means (DeVore, 1963; Trivers, 1974). It could be possible that the mechanical stimulation of the parental epidermis is itself a mechanism by which offspring communicate need. If parent-offspring communication occurred in discus fish, it would be useful to examine the form and detail of this communication as means of understanding the levels of conflict associated with this relationship.

As mentioned previously there comes a point during the period of parental care when parents should wish to stop investing in current offspring. This heralds the start of the weaning period in mammals and is typically met with conflict as offspring try and solicit investment past the point at which parents would willingly provide. The conflict associated with the weaning period has been observed in a vast array of mammals and can involve offspring carrying out a range of psychological tactics such as crying and feigning injury to encourage more parental care (Balshine-Earn and Earn, 1998). In mammals, parents often ignore offspring during this period and physically prevent young from obtaining milk. It would be interesting to see if there was some sort of

weaning period in discus fish and if so how this would operate. Would it involve a physical behaviour such as aggression or avoidance of offspring, or act through a chemical signal released in the mucus to wean offspring off its content? Understanding the benefits offspring obtain from feeding on parental mucus would also be useful in understanding the potential drivers of conflict in this species. The duration of conflict between parents and offspring can also vary depending on the offspring's potential relatedness to future siblings. When relatedness between broods is low (in a polygamous breeding system), parent-offspring conflict can be expected to be high as offspring have less inclusive fitness to lose by taking away resources from future broods. Likewise, conflict can be expected to be reduced in monogamous breeding systems as different broods will share a greater genetic similarity. The breeding system in discus fish is not fully understood which leaves a lot of questions in regards to the potential dynamics of parent-off spring conflict in this species. There are several aspects of the parent-offspring conflict which are shaped by the type of breeding system utilized. For example the ability of an individual to re-mate can have considerable effects on the levels of parental care offered to offspring (Keenleyside, 1983). Experimental manipulation of the ratio of breeding males to females, also known as the operational sex ratio, in the bi-parental cichlid *Herotilapia multispinosa,* caused significant differences in the amount of care offered to young (Clutton-Brock, 1991; Macnair and Parker, 1979; Parker, 1985). In a population dominated by females, males were much more likely to abandon their mate and offspring for the opportunity to re-mate. When both parents invest and are equally susceptible to solicitation, conflict will generally be higher than under uniparental care (Schradin and Anzenberger, 1999). Several papers and aquarists characterize discus fish as a cichlid displaying monogamy (Magee and Neff, 2006; Neff and Gross, 2001). If this is truly the case in wild discus fish populations, it would negate mate desertion as a factor in parental investment.

Although monogamous behaviour is found in the aquarium environment it would be interesting to see if this behaviour applies equally to the wild scenario.

The perceived genetic relatedness can also be a factor in determining levels of parental investment. Male bluegills (*Lepomis macrochirus*) are the sole providers of parental care but will alter levels of parental investment if paternity is in question (Magee and Neff, 2006; Neff and Gross, 2001). If bluegill males detect paternity lost due to cuckoldry, this signals a reduction in perceived brood quality; males then adaptively lower their levels of parental investment. Conversely, if they detect that their paternity is higher than previously assessed, they adaptively raise their level of parental investment (Mock et al., 2005). This dynamic adjustment during brood rearing is important in species where cuckoldry is prevalent. The proposed monogamous life history of discus would suggest that cuckoldry is absent from the life history of discus and may therefore, not play a role in the dynamic adjustment of parental investment.

An interesting area of future research would be to assess how levels of investment could change in discus fish. In house sparrows (*Passer domestic*) an increase in parental investment would equate to an increase in the provision of food to offspring (Evans, 1996; Legge, 2000). Parental investment in discus fish has so far been classified as the production of mucus, potentially containing several nutritional and non-nutritional factors. Previous research has indicated the changeable composition of mucus in discus fish demonstrating differences between parental and non-parental adults (Chong et al., 2006; Chong et al., 2005) with work in other species demonstrating the significant effect of diet on influencing mucus composition (Saglio and Fauconneau, 1985). Mucus quality and quantity may therefore be factors that could vary between parents. If mucus quality could change to facilitate greater investment, it would be interesting to see

which components of the mucus were elevated i.e. amino acid content, and which components of the mucus were depressed i.e. water content.

1.6.2 Offspring-Offspring conflict

One of the other forms of conflict associated with parental care is the conflict which arises between offspring. Offspring are as equally related to their parents as they are to other siblings provided that the discus mating system is monogamous. Just as conflict is expected between parents and offspring, conflict can also be expected between offspring. Resource based rivalry between offspring is particularly evident in avian species where competition among nestlings can often lead to siblicide (Kacelnik et al., 1995). There is often considerable competition between nestlings for prime positions in the nest; particularly for positions closest to the nest entrance. These positions are often associated with higher intakes of food (Burd et al., 2006). Experimental work with the brood-tending leech (*Helobdella papillornata)* also indicated that there is intra-brood competition for feeding positions. Parental feeding in the leech is facilitated through the female curling up and presenting prey to her offspring. Larger juveniles were attached to the mother in a position which would allow them greater access to food suggesting conflict was occurring between offspring for position (Fraser and Thompson, 1991). Aggressive competition among suckling pigs is also known to occur for access to the prime anterior teats of the mother (Dawkins, 1976). This raises particular questions about the nature of parental provisioning in discus fish. Are there specific areas of either parent which produce greater quality/higher quantities of mucus? If so, will there be higher levels of competition between offspring for access to these sites? Interestingly still, would conflict between offspring cease in clonal species?

So far the conflict discussed has been the conflict associated with related individuals. If conflict occurs between related individuals, it is almost certain that conflict occurs between the two unrelated parents.

1.6.3 Parent-Parent conflict

If there is conflict between individuals who share half of the same genetic material (parent-offspring conflict, offspring-offspring conflict), it can be assumed that there will be conflict between individuals who are completely unrelated (Dawkins, 1976; Schwagmeyer et al., 2002). Bi-parental care represents a compromise between the genetic interests of mothers and fathers (Ezaki, 1988), this is a situation which can lead to conflict and affect the amount of parental investment offered to offspring. Although some form of parental care is not unusual in a freshwater fish, it is, however, unusual for both parents to be involved in care. Out of those fish that show care, only 13 % of externally fertilizing teleost fish display a form of bi-parental care (Gross and Sargent, 1985). Due to the rareness of this form of parental care it might be expected only in circumstances where the benefits of additional care are unusually high or the costs unusually low. The few species that show bi-parental care often carry out extensive parental provisioning of fry. It is, however, in the genetically selfish interests of parents to avoid any form of parental care and in most instances this is possible as one parent may be more than adequate at defending/cleaning/fanning eggs and ensuring a high survival of young (Clutton-Brock, 1991). In some instances though both parents may be required to ensure the survival of young; this can be especially true when parents provide care to mobile fry (Clutton-Brock, 1991; Gross and Sargent, 1985). During the extensive provisioning of care conflict can arise as one parent could gain more from investing less, than if they cooperated. The energy saved in the current brood could then be used for parental investment into future broods. Extreme examples of this parent-

parent conflict, can be seen in species where the desertion of either sex can occur before the care of offspring is over (Balshine-Earn and Earn, 1998; Keenleyside, 1983). Mate desertion is associated with instances where the payoff for desertion is high, such as the ability to breed again (Beissinger, 1987), or when reductions in care have little effect on fitness of the brood (Balshine-Earn and Earn, 1998; Keenleyside, 1983). Although mate desertion in discus fish is unlikely, there may still be subtle forms of conflict. Negotiation of parental investment levels may still be occurring throughout the period of parental care. In most bi-parental species, parents should adjust their levels of care, both to their own and to their partner's condition. Parental duties are switched once from maternal care to paternal care in the mouthbrooding cichlid, *Eretmodus cyanostictus.* The first phase of parental care involves the maternal mouthbrooding of young until a transition phase where the care of young is switched over to the male. Males with a lower condition accepted the care of young at a later date compared to males with a higher condition. The delay of the transition period (as seen with males of a lower condition) subsequently results in a reduced period of male parental investment (McNamara et al., 1999). Discus fish also adopt offspring transfer behaviour, which begs the question of whether negotiation occurs in during the transition of offspring. Offspring, feeding off the secretions of one parent are flicked onto the other parent in one quick movement. Unlike the mouthbrooding cichlid, *E. cyanostictus,* the transition occurs frequently. It could, therefore, be hypothesised that a delay in transition, as seen in *E. cyanostictus*, could be repaid by a delay in transition by the other parent during the next transition. The degree of phenotypic variability within natural discus fish populations could also affect levels of conflict. If there was an asymmetry in condition status between the two parents, there could be elevated conflict over provisioning rates. The higher quality parent could potentially end up investing more energy to compensate for the poorer quality individual. This potential conflict over investment could be

eliminated if individuals of a similar quality paired up. Mate recognition would, therefore, be a highly important behaviour in discus fish. Mate selection has led in most species, to the formation of one 'choosy sex' and one sex trying to compete for mate acquisition (Chapman et al., 1997). Kokko and Johnstone (2002) proposed that in monogamous pairs, where the investments of both sexes are large and not too different from each other, that the conditions for mutual mate choice can then be satisfied. Both parents are involved in the care of offspring and are proposed to carry out the same degree of parental investment. Mutual mate choice may therefore function as a behaviour that could reduce parent-parent conflict. Although the mechanisms of mate choice in discus fish has to the author's knowledge not yet been investigated it is possible that visual signals are important in mate in mate choice decisions.

Intersexual selection, otherwise known as mate choice, is a key component of sexual selection and is responsible for many of the striking secondary sexual traits observed in the animal kingdom (Andersson, 1994). These traits, often taking the form of brightly coloured ornaments have been hypothesised to provide information to prospective mates in regards to either the indirect or direct benefits of mating with them. Indirect mate choice, refers to the selection of a mate to obtain genes for traits related to improved offspring performance (Andersson, 1994). In sticklebacks (*Gasterosteus aculeatus*), indirect mate choice has been observed with females preferentially mating with males containing bright red colouration. The resulting progeny of females that mated with brightly coloured males had a higher resistance to the tapeworm *Schistocephalus solidus* indicating that red colouration in this species may be an honest indicator of genes for parasite resistance (Barber et al., 2000a). While colour can signal the genetic benefits of mating with an individual, colour in other species has also indicated an individual's ability to provide parental care, a direct form of mate choice known as the 'good parent' hypothesis. In female northern cardinals (*Cardinalis*

cardinalis), the provisioning rate of offspring correlates with underwing colour (Linville et al., 1998) as well as the size and darkness of the face mask. In fish, females of the biparental African cichlid (*Pelvicachromis taeniatus*) advertise their readiness to spawn via the extent of their red colouration while fecundity, maternal quality and offspring fitness is indicated by the extent of blue colouration (Baldauf et al., 2011). Although the vast majority of work concerning colour and sexual selection has looked at colour within the visible spectrum (400-800nm), a recent body of work has also demonstrated the importance of wavelengths within the ultraviolet (UV) spectrum. The importance of UV in mate choice trials in a range of avian and fish species including starlings (*Sturnus vulgaris*) (Bennett et al., 1997), zebra finches (T*aeniopygia guttata*) (Hunt et al., 1997), blue tits (*Parus caeruleus*) (Hunt et al., 1999), bluethroats (*Luscinia svecica*) (Johnsen et al., 1998), three spined sticklebacks (*Gasterosteus aculeatus*) (Rick et al., 2006) and guppies (*Poecilia reticulata*) (Kodric-Brown and Johnson, 2002; Smith et al., 2002a) have demonstrated that prospective mates look more attractive when UV wavelengths are present. UV markings have also been demonstrated as providing information about body condition in sticklebacks (Rick et al., 2004) and a wide range of species have been demonstrated to have cones withing the retina that sensitive to UV. Due to the high level of parental investment required by both parents for the survival of offspring, it is possible that visual signals in both the visible and UV spectrum may be important in providing information to prospective mates in regards to the ability to provide care.

1.7 Conclusions

The aim of this study was to evaluate the potential properties of parental mucus and shed light on how this could potentially relate to parental care behaviour in discus fish. It is clear that mucus can be a truly multifunctional substance and could potentially provide many different areas of assistance to offspring. It is also clear that with this bi-

parental provision of care there can be potential for parent-offspring conflict, parent-parent conflict and offspring-offspring conflict. As well as providing useful insights into models of bi-parental care, information gained from this research has applications for both the aquarium trade and the conservation of this species. The functional significance of parental mucus also has several applications for the discus fish farming industry. Discus fish receive considerable attention from the aquarium sector of the aquaculture industry and is one of the most popular and valuable freshwater species being cultured in Malaysia, Thailand and Singapore, often gaining a higher average import price than any other popular farmed species. Quantification and classification of nutritional and non-nutritional substances in mucus will be of significant importance to the discus fish farming industry, particularly in areas of diet formulation and larval husbandry.

1.8 Project aims and objectives

Discus fish present a unique example of a bi-parental behaviour rarely seen in other species of fish. The aims of this project were to elucidate the dynamics of bi-parental care by studying the relationship between the changing behaviour and physiology of parents and offspring during the breeding period. Specifically the objectives were

- To determine the composition of parental mucus in order to understand the investment by parents and the benefits to offspring.
- To document the behaviour of both parents and offspring to determine whether there is conflict over the provision of care.
- To compare the mucus produced by wild and aquarium bred discus fish with a view to understand the potential effects of selective breeding and the environment on the provision of parental care in this species.
- To see whether fry are adapted to mucus feeding and if so how this achieved.
- To investigate the mechanisms of mate choice and see if mate choice decisions are based on traits that provide information about an individual's ability to provide parental care.

Chapter 2: Biparental mucus feeding in an Amazonian cichlid, a unique example of parent offspring conflict

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2.1 Abstract

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. is unusual among fish species with both parents providing offspring with mucus secretions to feed from after hatch. 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, I investigated the relationship between parents and offspring during a breeding cycle, determining both the mucus composition (total protein, cortisol, immunoglobulin and Na^{+}, K^{+} Cl^{-} and Ca^{2+}) 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 adults took at caring for their young. A peak in mucus antibody provision was seen as offspring reached free-swimming suggesting a role analogous to colostrum provision in mammals. Mucus protein content was lowest during weeks two and three free swimming, and a weaning period, similar to that seen in mammalian parental care occurred when the offspring had been free swimming for around three 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.

2.2 Introduction

The neonatal period is one of the most critical periods of any organism's life due 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, through 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 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 until 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 into 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 which 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, evolved to encourage an extended period of parental care (DeVore, 1963; Mathevon and Charrier, 2004; Trivers, 1972a). It has been proposed that parent-offspring conflict can begin as early as the period of intrauterine development where the foetus interacts with the mother through hormonal communication, signalling the intent of the foetus and 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 (Hildemann, 1959; Noakes, 1979). 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 might be important nutritional factors within parental mucus. Previous studies in *Symphysodon* spp. have 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 (Hatten et al., 2001; Ingram, 1980; Shephard, 1994), where it is 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 auerus* (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. Offspring survival was further increased if parents were allowed to mouth brood their young; it was suggested that the increase in offspring survival could be due to the young feeding on mucus containing antibodies during mouth brooding (Sin et al., 1994). It is, therefore, at least conceivable that IgM is transferred to offspring via parental mucus in discus fish and

that discus 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 also help deliver hormones. In the midas cichlid (*Cichlasoma citrinellum*), the parental mucus which 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 offspring as it could aid ion uptake allowing them to better cope 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). Adult discus fish mucus may, therefore, contain a sufficient quantity of ions which could be obtained by offspring, especially if repeated nipping by young causes the cellular leakage of ions from the epidermis into the mucus, a process which could help offspring obtain essential ions that are otherwise lacking in the environment.

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). Discus fish breeders have long recognized 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, I 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. I also 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.

2.3 Materials and methods

2.3.1 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 (Chens discus, Harrow, London) and transported to the aquarium facilities of the University of Plymouth. Fish were quarantined, wormed (Kusuri discus wormer, Newton Abbot) and then held in groups of 12 in 100 l glass tanks and observed for reproductive behaviours. Fish that formed breeding pairs were separated into their own 100 l glass tanks and allowed to spawn on a plastic breeding cone. All fish were kept in recirculation systems held at constant conditions (temperature (n=3): 29 ± 0.5º C, pH: 7.0 ± 0.5, dissolved oxygen (n=3): 99 ± 0.5%, 12h:12h L:D photoperiod, Ca^{2+} (n=6)21.56 ± 1.26 mg l^{-1}; Na^{+} (n=6) 9.28 ± 0.26 mg l^{-1}; K^{+} (n=6) 1.42 ± 0.02 mg l^{-1}; Cl^{-} (n=6) 15.32 ± 0.76 mg l^{-1}) and fed a beef heart-based, or commercial pellet (Tetra prima granular, Tetra (UK) Southampton) feed once daily to satiation. Hatched young fed solely from their parents' mucus until the final (4^{th}) 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.

2.3.2 Behavioural observations

Behavioural observations began on the first day of free-swimming (FS) and continued daily until the last day of mucus sampling (~35 days post fertilisation). Pairs had between 60 and 120 young in a single brood. 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 one hour 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 parental care behaviours while being observed.

2.3.2.1 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 one hour 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, 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, and also information on the number and duration of each feeding turn.

2.3.2.2 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 calculated. Young that moved out of view during the 30 s were ignored and a new count started.

2.3.3 Mucus physiology methods

2.3.3.1 Mucus sampling

Breeding pairs (n=6) were sampled for mucus at eight time points through a complete breeding cycle as described in figure 1. Each sample corresponded to a distinct stage within the breeding cycle; eggs spawned (E), eggs hatched (H), free swimming fry (FS), free swimming fry + 1 week (W1), free swimming fry + two (W2), + three (W3) and + four weeks (W4). A 'zero' sample was collected at a standardised time point of two

days after the removal of a clutch of eggs. Mucus samples were also obtained from non-breeding fish (NB) which were yet to pair. Mucus samples were obtained using a fragrance and chemical free polyester sponge ('Buff-Puff' facial sponge, 3M Minnesota) cut into 2×2×1 cm sections. To enable later calculation of sample weight, each sponge and a 5 ml syringe were pre-weighed to 0.0001 g. Using a modified shallow net, each fish was individually removed from the tank in such a way that the mucus on one flank was not disturbed by the net or handler. This flank was orientated upwards for 5 s to allow draining of excess water before the sponge was used to swab the mucus from the top half of the fish, based on the methods of Chong et al. (2005) and Schultz and Barlow (1997) to avoid any contamination by excretory products. The sponge containing the mucus sample was then placed within the syringe and reweighed so that mucus sample mass could be attained. Once in the syringe, the plunger was then used to compress the sponge containing sample so that mucus could be squeezed out in to an micro centrifugation tube. After this initial squeeze, 1 ml of distilled water was then added so that a further compression could be used to elute as much mucus as possible. This mucus and water mixture was then vortexed, centrifuged and the supernatant immediately frozen for later physiological analyses. Before physiological assays could be carried out mucus samples were first defrosted on ice, diluted in distilled water and analysed via the following methods.

2.3.3.2 Bradford protein assay

Mucus samples were defrosted on ice, diluted in distilled water and analysed for total protein via the Bradford method (Bradford, 1976).

2.3.3.3 Chloride assay

The determination of chloride via colorimetric assay requires the interaction between sample and a chloride colour reagent (Zall et al., 1956). A chloride colour reagent was produced using a 1:1:13 ratio of mercuric thiocyanate, ferric nitrate and double

disstilled water, respectively. The procedure for the determination of chloride depends on the liberation of the thiocyanate ion from mercuric thiocyanate by the formation of unionized but soluble mercuric chloride. In the presence of ferric ions, the liberated thiocyanate forms a highly coloured ferric thiocyanate proportional to the original chloride concentration. The coloured product can then be read on a spectrophotometer.

2.3.3.4 Ions

The ions, sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) were measured using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). ICP-AES is an emission spectrophotometric technique that utilizes the fact that the excited electrons of a specific element emit energy at a given wavelength. The intensity of this emission is indicative of the concentration of the element within the sample. ICP-AES allows the simultaneous measurement of a wide range of ions, including Na^+, K^+ and Ca^{2+} using a multi element standard. A total sample size of 2 ml is required for ICP-AES measurement. Samples analysed comprised 10 µl of mucus, 100 µl 1 N HNO_3 and 1890 µl of double distilled water.

2.3.3.5 Antibody (IgM) detection and validation.

To detect the presence of IgM within parental mucus a competition Enzyme Linked Immunosorbant Assay (ELISA) was developed and optimised.

Principles of the competition based ELISA

An ELISA is a technique that can be used to detect the presence of either antibodies or antigens. In this instance a competition-based ELISA was used to detect the presence of antibodies (IgM), a technique which essentially competes a known level of IgM against an unknown level of IgM i.e. the sample. The results of two different hypothetical samples are demonstrated in figure 2 to demonstrate the concept of the competition ELISA; one sample containing reduced levels of IgM (steps 1, 2, 3, and 4) and the other demonstrating elevated levels of IgM (steps 1, 5, 6 and 7).

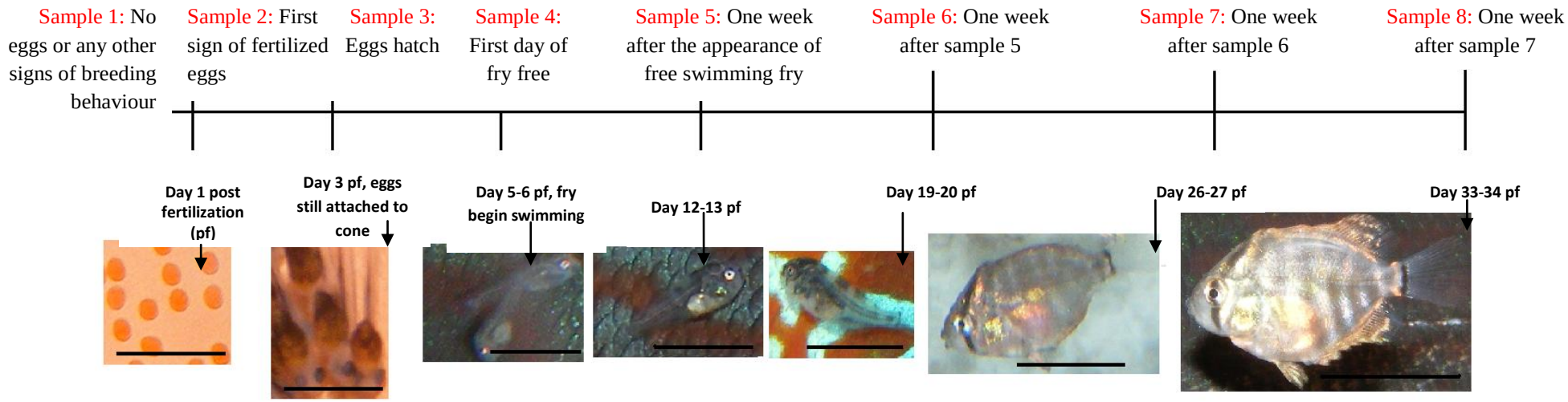


Fig.1.Timeline of discus breeding period, indicating times of mucus sampling and the size of the offspring at the sample point. Scale bar represent 10 mm.

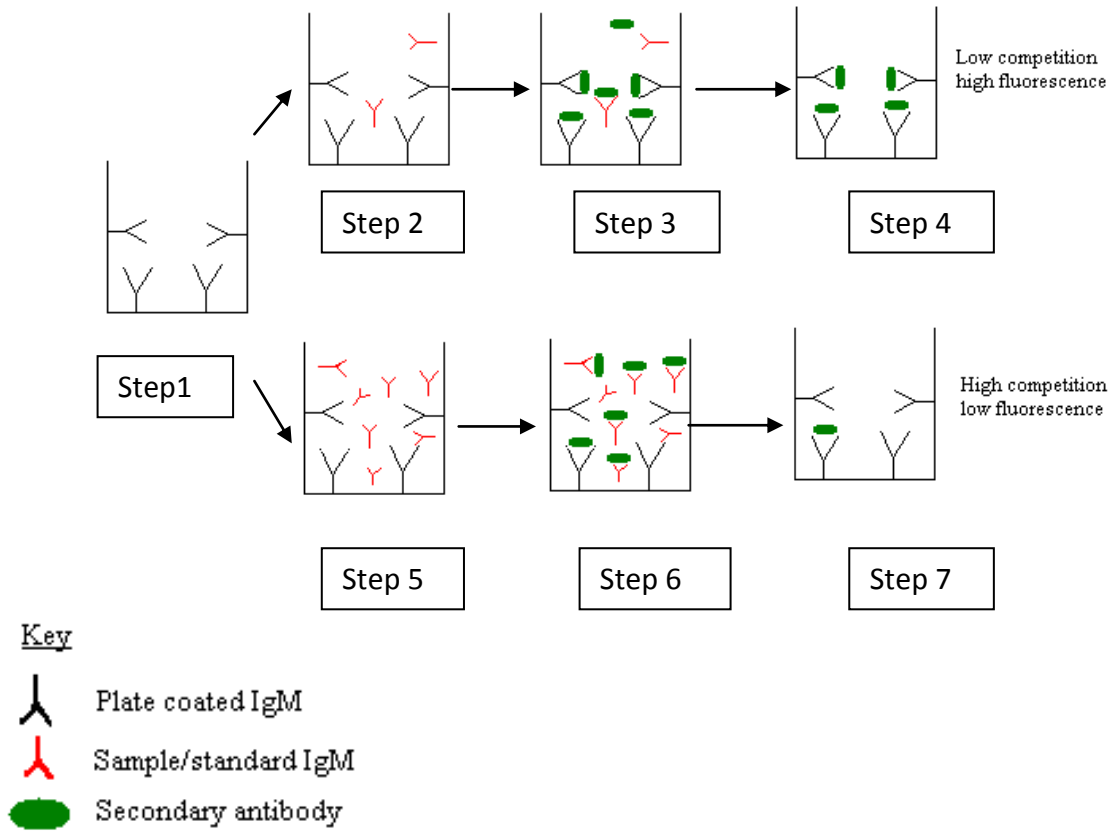


Fig. 2. Diagrammatic representation of a competition ELISA.

Step 1: A known quantity of the IgM of interest is coated to a 96 well plate

Steps 2 and 5: Samples containing an unknown IgM quantity are then added; step 2 depicts a sample with low levels of IgM whereas step 5 depicts a sample with high levels of IgM.

Steps 3 and 6: A known amount of secondary IgM is then added to the plate wells. Secondary IgM binds only to the IgM of interest i.e. the IgM coated to the plate and the IgM present in the sample. IgM coated to the plate competes with the IgM in the sample for the secondary IgM. Step 3 depicts a low level of competition, whereas step 6 depicts a high level of competition.

Steps 4 and 7: After a series of washes, only the plate coated IgM plus attached secondary IgM remain. This can then be reacted with a conjugate, followed by a

substrate to produce fluorescence proportional to the amount of secondary IgM bound to the plate coated IgM. Step 4 depicts a situation where a sample containing low levels of IgM results in low levels of competition. This subsequently leads to a high level of bound secondary IgM which produces a high fluorescent signal. Step 7, however, depicts a situation where a sample containing high levels of IgM, outcompetes the bound IgM. This subsequently results in low levels of secondary IgM being bound to the plate, resulting in a low fluorescent signal.

ELISA development

The development of a competition ELISA first required that a secondary IgM be established with a strong affinity for discus IgM. This was obtained by cross-reacting discus serum (rich in IgM) with a range of IgMs from different species, until one was found which would bind strongly to discus IgM. Discus serum was obtained from a large male, terminally anesthetized using 400 mg l^{-1} of buffered MS222. Approximately 1 ml of blood was then taken from the caudal vasculature, just below the lateral line. The blood, stored in a micro centrifugation tube, was then placed at a slight angle in a fridge and allowed to clot for 12 h. After 12 h the serum was clearly distinguished as the upper, clear coloured supernatant devoid of any clotting factors. Discus serum was then sent to Stirling University on ice and cross-reacted with a number of different IgMs. An Anti-Asian sea bass (AASB) IgM showed the greatest cross-reactivity with discus serum and was, therefore, utilized as the secondary IgM for this ELISA.

Once a suitable secondary IgM had been ascertained, checkerboard assays were then carried out to optimise the quantities of AASB IgM, serum derived plate coated IgM and levels of serum derived IgM which could be used as standards. The competition ELISA methodology used in this study was based on previous work by Magnadottir (1998).

a) ELISA methods

1) 100 µl of purified IgM, diluted in a carbonate based coating buffer was pippetted into the appropriate wells of a 96 well plate and left overnight at 4º C. This known concentration of IgM was coated to the plate so that it could eventually compete with the standard/sample for the secondary antibody. The concentration of plate coated IgM was one of the factors which needed to be predetermined through a series of checkerboard assays.

2) Once the IgM had bound to the 96 well plate, the plate was then washed three times with a low salt wash buffer (LSWB).

3) 250 µl of blocking reagent (5% dried milk Marvel powder made up in PBS) was then pipetted into the appropriate wells and left overnight at 4º C.

4) The plate was then washed a total of three times with LSWB.

5) 100 µl of standard/sample was then added to the appropriate wells of the 96 well plate. Standards and samples were both diluted appropriately in 0.05% PBS-Tween. The concentrations of standards were another one of the factors which required optimisation via a checkerboard assay.

6) 100 µl of secondary IgM (in this instance AASB IgM) was then added to the wells that already contained sample/standard. Once the secondary IgM had been added, competition between the sample/standard and the plate coated IgM for the secondary AASB IgM could then begin. The quantity of secondary AASB IgM needed optimisation; an excess of secondary AASB IgM would cancel out any of the effects of competition as there would be enough secondary AASB IgM to bind to all of the IgM in the sample/standard as well as the plate coated IgM. Too little secondary AASB IgM can also affect the accuracy of the assay.

7) The plate was then incubated for 2 h at 37º C to allow sufficient time for the competition to occur.

8) After incubation the plate was then washed five times with high salt wash buffer (HSWB), with the last wash including incubation at room temperature for 5 min.

9) 100 µl of conjugate diluted 1:400 in 1% bovine serum albumin in LSWB was then added to the appropriate wells and left for 1 h at room temperature.

10) The plate was then washed five times with HSWB; the last wash was incubated for 5 min at room temperature. Washing removes any sample/standard bound to secondary IgM, conjugate complexes, leaving only the plate coated IgM bound to the secondary AASB IgM and conjugate complexes.

11) 100 µl of substrate was then added to the appropriate wells. At this point there was a noticeable colour change from clear to blue as the conjugate, bound to the secondary IgM plate coated IgM complex, reacted with the substrate.

12) 50 µl of stop solution was then added to the appropriate wells, at this point the blue colour turned to yellow and was fixed permanently; the plate was then read via a spectrophotometric plate reader at 450 nm.

In order to optimise the ELISA protocol, a series of checkerboard assays were conducted to fully optimise the concentrations of standards, plate coated IgM, and secondary AASB IgM (Magnadóttir, 1998). Checkerboard assays were carried out before the serum had been purified and quantified so simple dilutions were used to initially optimise the ELISA. All checkerboard assays utilized the same methods as described above differing only in the levels of standard concentration, plate coated IgM and secondary AASB IgM.

b) Checkerboard 1

The first checkerboard ELISA sought to evaluate the interaction between the following range of dilutions. The results of this ELISA can be seen in figure 3.

- Secondary AASB IgM concentrations were 1:12.5, 1:25, and 1:50
- Concentrations of standards were 1:50, 1:100, 1:200 and 1:400
- Plate coated IgM concentrations were 1:50, 1:100 and 1:200

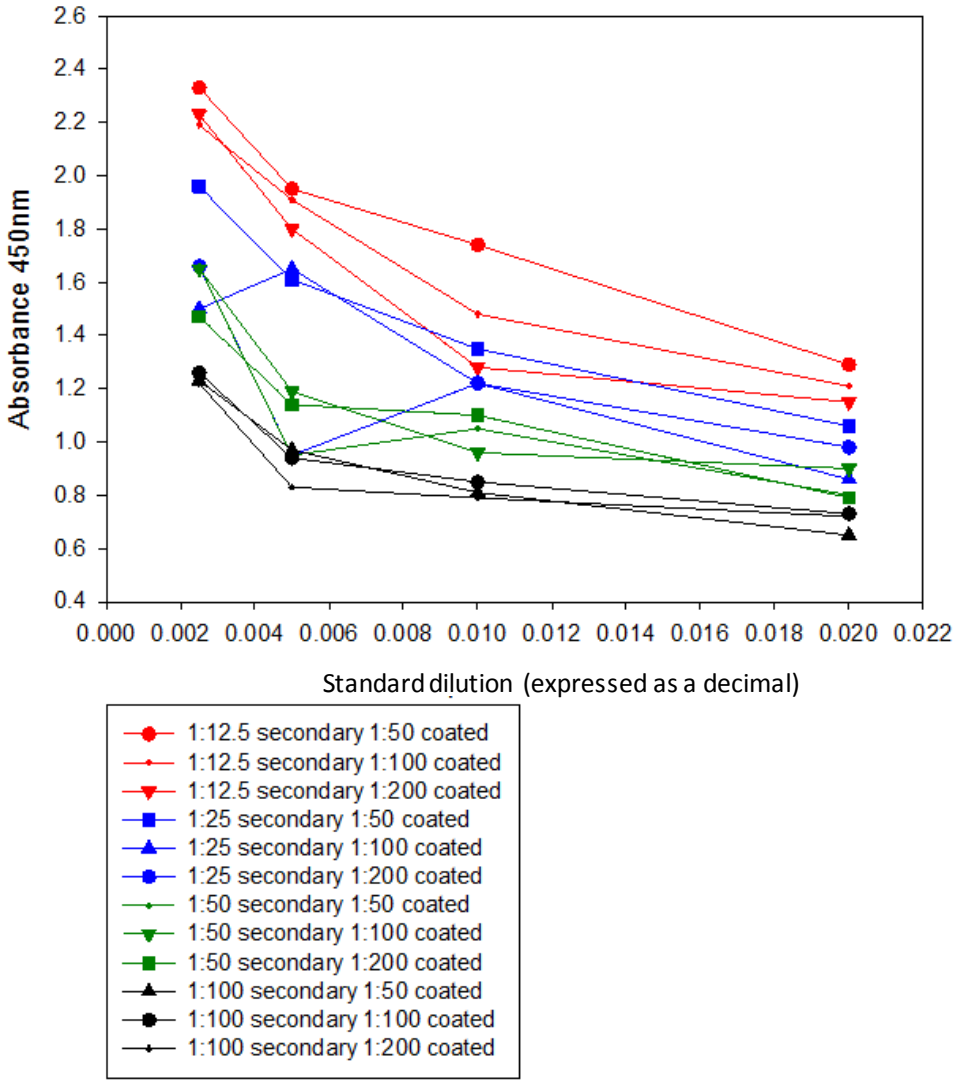


Fig. 3. Absorbance values attained from four different standards (1:50, 1:100, 1:200, 1:400) interacting with four different secondary AASB IgM concentrations (1:12.5, 1:25, 1:50, 1:100) and three different concentrations of plate coated IgM (1:100, 1:200, 1:50).

Secondary AASB IgM: Four different concentrations of secondary AASB IgM were evaluated. The more dilute concentration (1:50) produced a smaller range of absorbance values and was, therefore, deemed inappropriate. Out of all the values 1:12.5 and 1:25 concentrations produced the greatest range of absorbance values. The next ELISA, therefore, utilized two concentrations of secondary AASB IgM at a 1:10 and 1:20 concentration.

Standards: The concentrations used produced a standard curve with a limited range. A larger range could have potentially been achieved if there was a greater level of competition at the top end i.e. a standard of greater concentration than 1:50. The next ELISA utilized concentrations of 1:25, 1:50, 1:100, 1:200, 1:400 as well as a control. The 1:25 concentration was used to produce a lower absorbance value by out-competing the IgM coated to the plate. The use of a control i.e. no competition helped gain a measure of how sensitive the ELISA was to low concentrations of sample IgM.

IgM coated to plate: The IgM used to coat the plate was obtained from discus serum, a reagent which was at a premium. Although the less dilute concentrations of plate IgM (1:50) produced a higher range of absorbance, lower levels of plate coated IgM (1:100 and 1:200) also produced a broad range of absorbance so long as levels of secondary AASB IgM were also concentrated i.e. 1:12.5, 1:25. Since the serum used to coat the plate was at premium, the second checkerboard assay used plate coated IgM concentrations of 1:100, 1:200 and 1:400 as a compromise between absorbance range and serum availability.

c) Checkerboard 2

The second ELISA utilized the following concentrations of reagents based on the results of the 1st checkerboard ELISA.

- Secondary AASB antibody concentrations were 1:10 and 1:20
- Concentrations of standards were 1:25, 1:50, 1:100, 1:200, 1:400 and a PBS based control.
- Plate coated IgM concentrations were 1:100, 1:200 and 1:400

The results from this ELISA can be seen in figure 4.

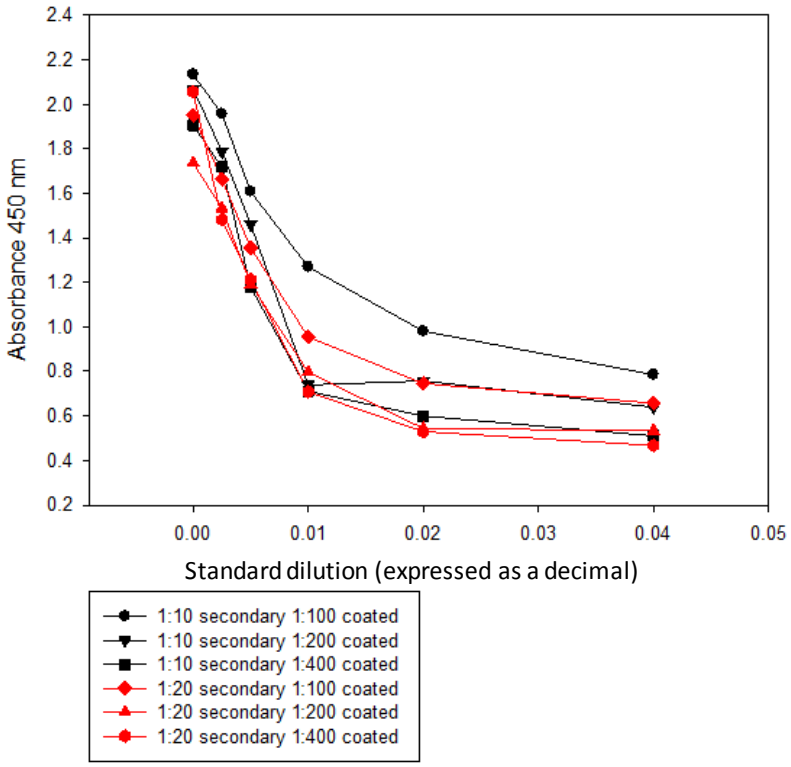


Fig. 4. Absorbance values attained from six different standards (1:25, 1:50, 1:100, 1:200, 1:400 and a control) interacting with two different secondary IgM concentrations (1:10 and 1:20) and three different concentrations of plate coated IgM (1:100, 1:200 and 1:400).

Secondary AASB IgM: Out of the two concentrations, the 1:10 secondary AASB IgM produced the greatest range of absorbance values allowing a greater range of detection. This concentration was utilized for all future ELISAs.

Standards: A much greater range of absorbance values were obtained using the extra 1:25 IgM standard concentration. The inclusion of a control, i.e. no competition, helped elucidate the potential sensitivity of the ELISA. There was a notable difference between the absorbance values of the control (PBS-no antibody) and the 1:400 concentration which indicated that the ELISA should, if needed, be sensitive enough to pick up low levels of IgM within discus mucus.

IgM coated to plate: Plate coated IgM concentration of 1:400 gave one of the greatest ranges in absorbance for both secondary AASB IgM concentrations (1:10 and 1:20). This concentration was then chosen for future assays as it allowed serum to be saved as well helping to attain a broad level of absorbance across the standards.

d) First test with mucus

The next phase of ELISA development involved testing discus mucus samples under the optimal conditions ascertained from the previous two checkerboard ELISAs i.e. a secondary AASB IgM concentration of 1:10 and a plate coated IgM concentration of 1:400.

Mucus samples were taken from a total of five individuals including three males and two females and assayed at a variety of concentrations including undiluted, 1:25, 1:50, 1:100, 1:200 and a 1:400 dilution. All undiluted mucus samples contained recordable levels of IgM that steadily decreased as mucus samples were diluted. The absorbance signals of all undiluted mucus samples were all within the range of the 1:200 and 1:400 serum dilutions that acted as the standards.

The next phase of the ELISA development required quantifying the IgM within the discus serum used in the standards. This would allow the construction of a standard curve of known concentrations of IgM as opposed to dilutions.

e) Obtaining serum

Several adult discus fish were anesthetized with 25 mg ml^{-1} MS222 before 500 µl of blood was taken from the caudal vasculature just below the lateral line. Adults were subsequently revived and suffered no ill effects from the anaesthetic or blood sampling. Blood samples were then allowed to clot overnight in a fridge at 4º C, after 12 h of clotting samples were then centrifuged and the supernatant obtained. The supernatant obtained was serum which contains all proteins not used in blood clotting. Once serum had been obtained the next step involves purifying it to obtain a solution containing just antibodies.

f) Serum Purification

A HiTrap IgM purification column was used to purify serum so that a pure sample of IgM, excluding all other proteins was obtained. Purification involved several steps and required several buffers, specifically:

- Binding buffer: 20 mM sodium phosphate, 0.8 M ammonium sulphate, pH 7.5
- Elution buffer: 20 mM sodium phosphate, pH 7.5.
- Regeneration buffer: 20 mM sodium phosphate, pH 7.5 with 30% isopropanol.

All buffers were passed through a 0.45 µm filter before use in purification.

Before the serum was put through the column a 0.8M concentration of ammonium sulphate was added to the serum to ensure the solubility of serum was high and similar to the binding buffer. Small amounts of ammonium sulphate were stirred into the serum

slowly and continuously. The solution was then passed through a 0.45 µm filter immediately before being purified.

Once the buffers and sample were prepared serum was purified via the following steps:-

1. The HiTrap column was washed with five column volumes (5 ml) of each buffer: binding buffer, elution buffer and regeneration buffer at a flow rate of 1 ml min^{-1} (this flow rate applied to all washes).
2. The column was equilibriated with five column volumes (5 ml) of binding buffer.
3. The sample was applied using a syringe fitted to the luer adapter.
4. The unbound sample was washed out with 15 column volumes (15 ml) of binding buffer or until no material appeared in the effluent.
5. Pure IgM was then eluted with 12 column volumes (12 ml) of elution buffer. Each column volume of elution buffer was collected into an micro centrifuge tube. A Bradford protein assay was carried out on each ml of purified IgM to determine the IgM concentration, since IgM was the only protein left in the purified samples. Only the first three micro centrifuge tube contained useable amounts of IgM and so were combined to produce a stock of purified IgM at a concentration of 478.5µg ml^{-1}.

g) Preparation of standards

Purified serum was diluted in 0.05% PBS-Tween appropriately to produce standards at concentrations of 0, 25, 50 and 100 µg ml^{-1}. An ELISA was then carried out to determine the accuracy of the standard curve with the results plotted in figure 5. The standards produced a linear regression R^2 value of 0.9904 that was deemed reliable for future ELISAs. The next step involved looking into the different ways mucus samples could be processed for use in the ELISA.

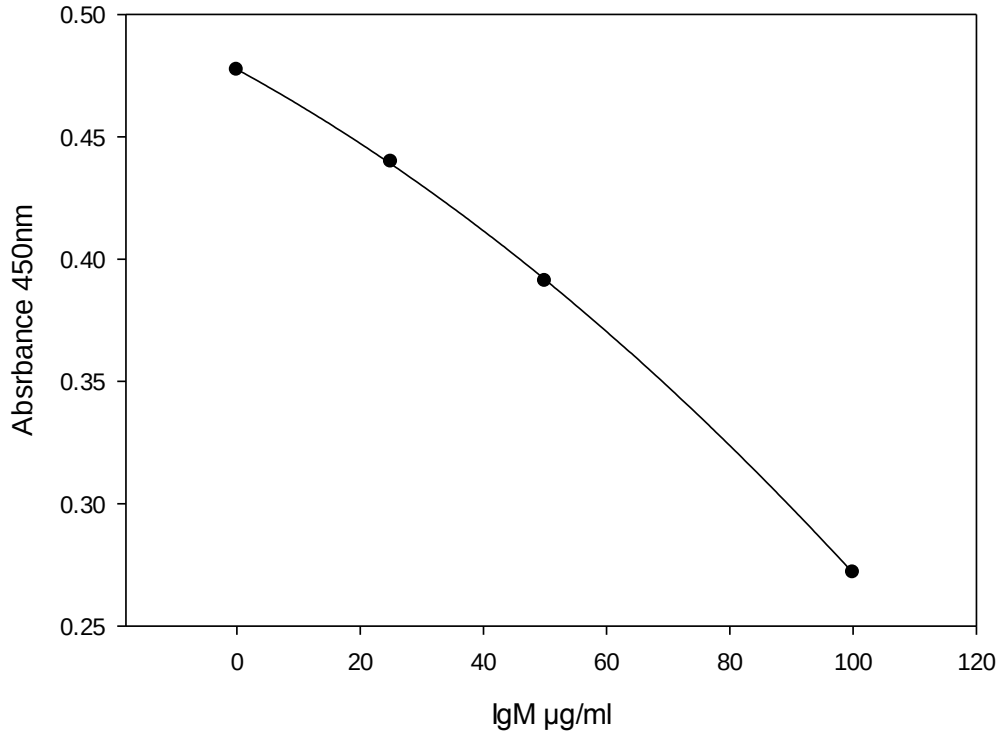


Fig. 5. Standard curve using purified IgM to obtain the absorbance (450 nm) of known IgM concentrations (n=4). R^2 value of 0.9904

h) Sample processing

A key factor that could influence the accuracy of the ELISA involved the processing of parental mucus. The following three factors were, therefore, analysed in regards to their effect on the ELISA.

1. **Mucus elution.** Once mucus was sampled via a sponge it then needed to be eluted into a 1 ml solution. The utilization of PBS and water to elute parental mucus was tested to see if either method affected the results of the ELISA.
2. **Mucus dilution.** To ensure there was sufficient mucus available for other assays it was important to see if it was possible to dilute samples of parental mucus. As well as testing a range of dilutions, the different solvents that mucus could be

diluted in, including PBS-Tween, PBS and distilled water were examined and tested.

3. **Mucus mixing.** Once diluted the sample could either be vortexed or centrifuged. The effect of these two methods was also tested.

Results of eluting mucus in PBS compared to water

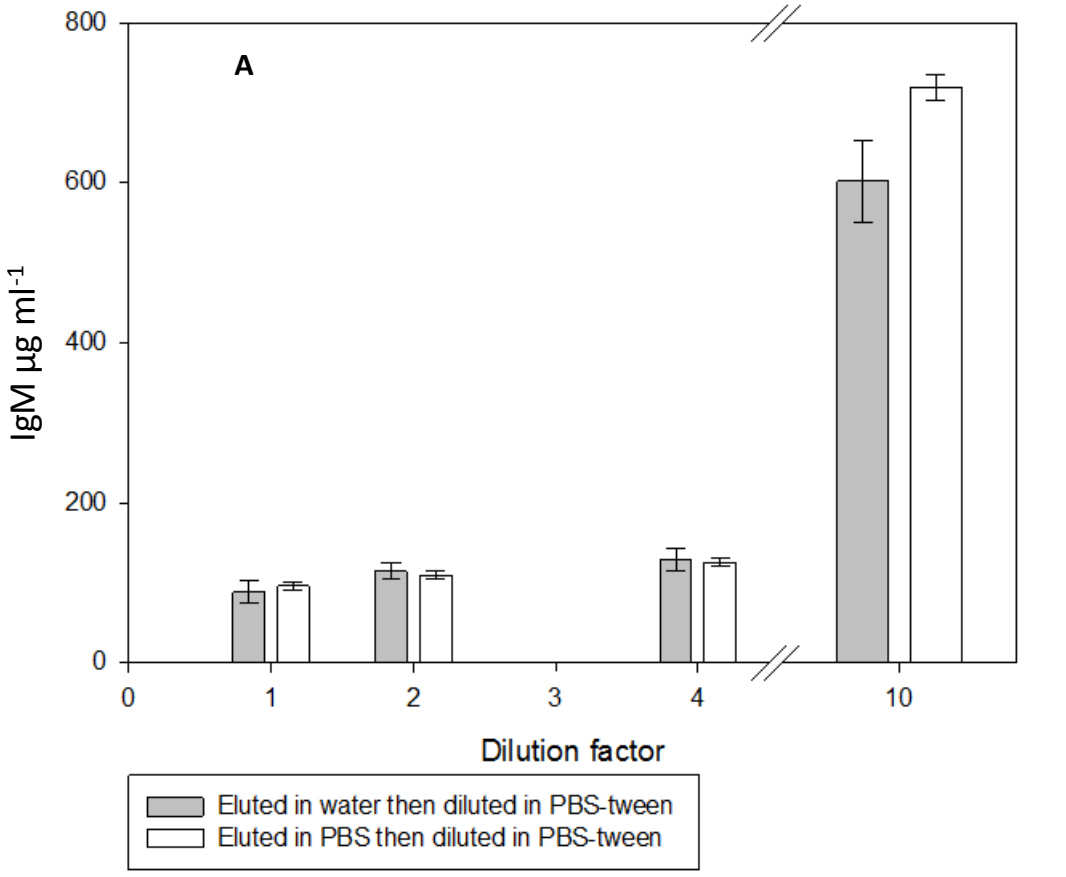
Eluting mucus in water compared to PBS made no discernable difference in IgM concentration (Fig.6 A, B and C). The range of values obtained via mucus eluted with water or PBS were similar. Due to the suite of other assays that would require mucus eluted in water, water was chosen as the solvent to elute mucus in future assays.

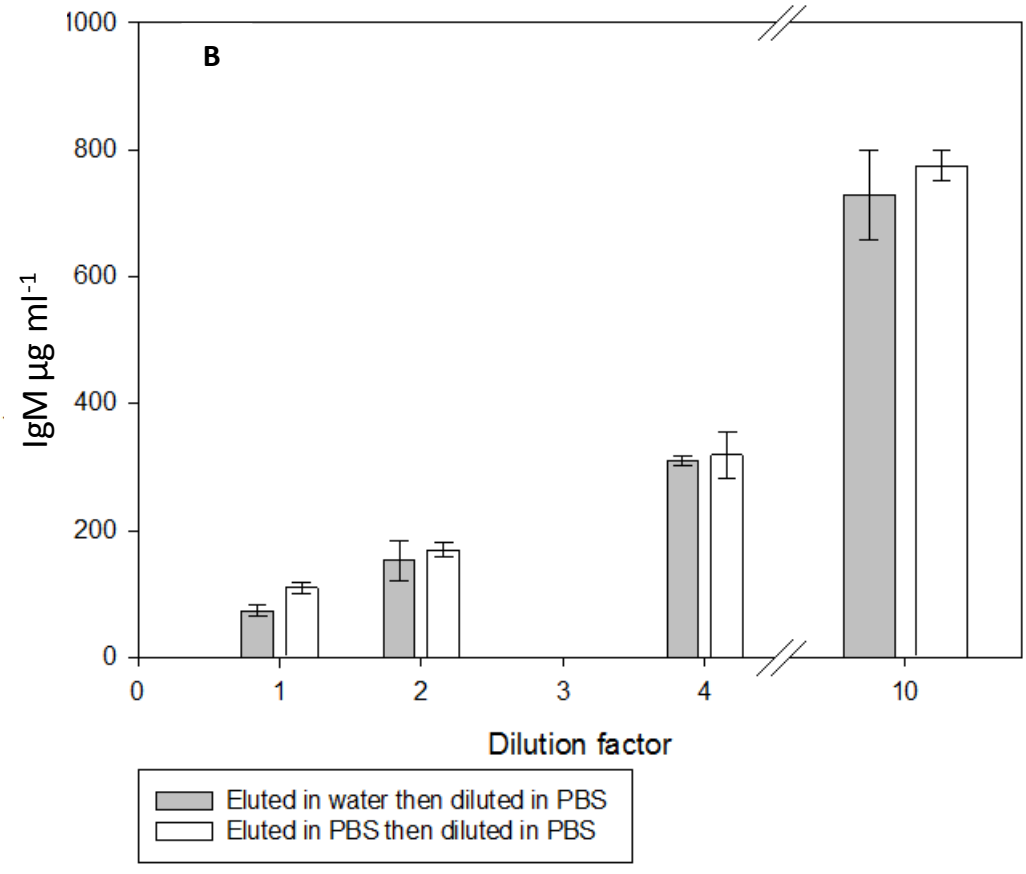
Results of diluting mucus in PBS-Tween compared to PBS and distilled water

Mucus samples diluted in PBS-Tween appeared to produce IgM values, that when back calculated to account for the dilution, were most similar to the 0 dilution IgM values (Fig. 6.A). Since standards are diluted in PBS-Tween it was therefore decided that samples would also be diluted using PBS-Tween. Dilutions of a factor of 10 in all instances produced unrealistic values due to the magnification of error (Fig. 6.A, B and C). Although a dilution factor of 4 with PBS-Tween produced values similar to that expected, a dilution factor of 3 was chosen for future assays to ensure that enough sample was saved for other assays (total protein, ion, cortisol etc) and that the accuracy remained high.

Results of mucus mixing test

The centrifugation of mucus samples produced higher IgM values (102.54 µg ml^{-1} ± 8.73) than the same samples that were vortexed (80.12 µg ml^{-1} ± 8.52). This may be due to the quantity of larger proteins that are removed via centrifugation leaving a more concentrated IgM sample in a similar way that centrifugation removes clotting proteins from blood leaving the IgM rich serum.


A
800
600
400
200
0
IgM µg ml-1
0
1
2
3
4
10
Dilution factor
Eluted in water then diluted in PBS-tween
Eluted in PBS then diluted in PBS-tween


B
1000
800
600
400
200
0
IgM µg ml-1
0
1
2
3
4
10
Dilution factor
Eluted in water then diluted in PBS
Eluted in PBS then diluted in PBS

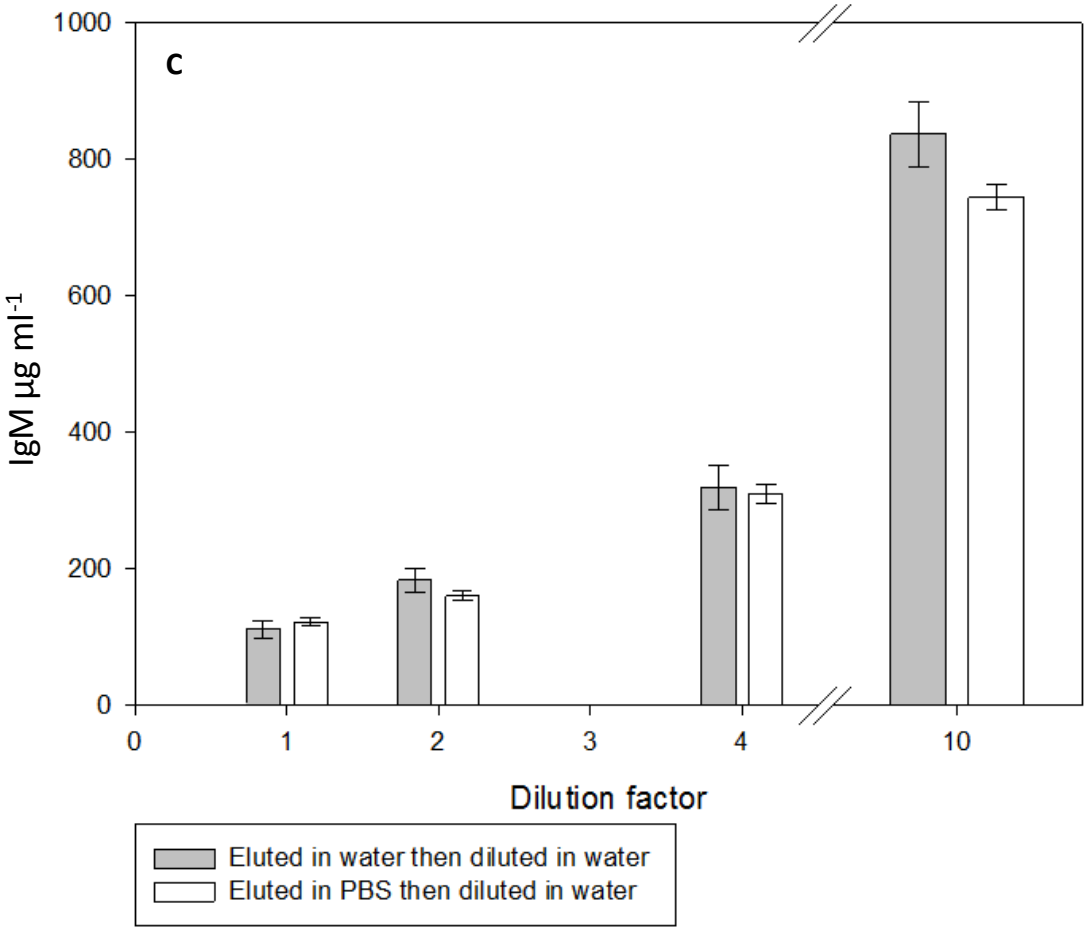


Fig. 6. Mucus samples eluted in either water (n=3) or PBS (n=3) and diluted by a factor of 0, 2, 4 and 10 in either PBS-Tween (A), PBS (B) or water (C). Results are mean ± s.e.m and back calculated to account for their dilution, thus theoretically all IgM µg ml^{-1} should be equal.

Mucus interference

To determine whether there were any factors within discus mucus that may have interfered with the accuracy of this assay, three different samples of mucus from three non-breeding adults were assayed for IgM before and after they were spiked with serum at a known concentration of 80 µg ml^{-1} IgM. Post spike, the three mucus samples were then diluted and measured to check 1:2, 1:4 and 1:10 dilutions for accuracy.

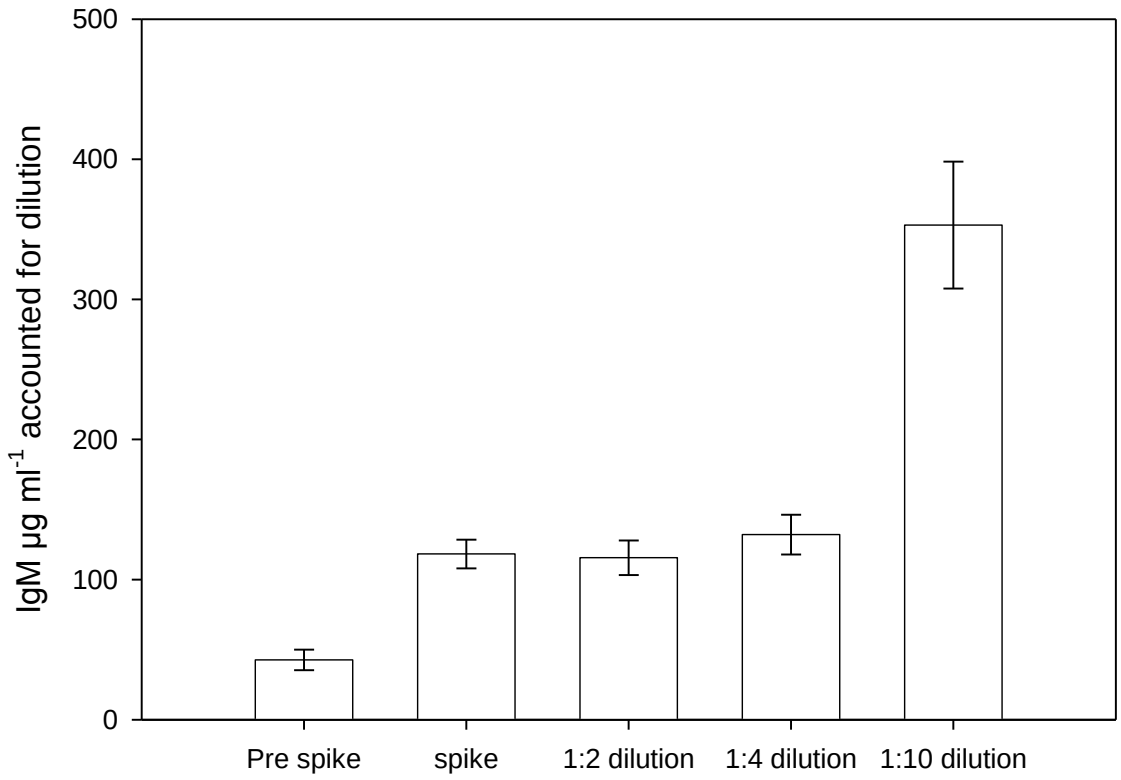


Fig. 7. Mucus samples (n=3) pre and post spike (80 µg/ml IgM) diluted by a factor of 2, 4 and 10. Results are mean ± s.e.m and back calculated to account for their dilution, thus theoretically all IgM µg ml^{-1} should be equal.

An average of 75.54 µg ml^{-1}± 2.31 of IgM of the 80 µg ml^{-1} of IgM was recovered in the spiked mucus (Fig. 7). These results indicate that there are no substances within the mucus that are masking the presence of IgM. The subsequent dilutions also confirmed the previous ELISA by indicating that anything between a 1:2 and 1:4 dilution was adequate for producing a result that when back calculated is congruent with that seen in neat samples. A 1:10 dilution, similar to that observed in the previous ELISA, produced values that were extremely exaggerated.

Once the ELISA had been developed as detailed above, the concentration of IgM within parental mucus was determined.

2.3.3.6 Cortisol

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), of which 250 µl was removed, dried under nitrogen and then re-suspended in phosphate buffered saline containing 0.1% bovine serum albumin (Sigma-Aldrich Co. Ltd, Dorset, U.K.) before analysis.

2.3.4 Statistical Analysis

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

2.3.4.1 Physiology

Physiology data was 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 assessed via a one way ANOVA followed by LSD post hoc analyses. Comparisons between the mucus compositions of breeders at different time points across the breeding period were compared via a repeated measures ANOVA with sex and time as factors. Where significant effects of time were recorded, post-hoc paired *t* tests were used.

2.3.4.2 Behaviour

A repeated measures 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) test was used to assess the differences within each week in terms of how long fry spent associated with each mode of parental care.

2.4 Results

2.4.1 Time on parent

Young spent significantly more time alone (without any parent) in week 4 compared to the first three weeks (RM-ANOVA: $F_{1, 3}$= 4.99, P<0.05, Fig. 8D).Young also spent less time with the female in week four compared to the other three weeks (RM-ANOVA: $F_{1, 3}$= 4.012, P<0.05, Fig. 8B). There were however, no significant differences across the four 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 8A, C). Interestingly, during the first week, young spent more time feeding off the female than the male (One-way-ANOVA: $F_{3, 23}$=4.52, P<0.05).

2.4.2 Change in parental duties

Throughout the period of parental care, parents would regularly change the mode of parental care. In the first two 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 two weeks parents would often swim away from young leaving them on their own; Such behaviours would require the young to actively swim toward parents to feed. The number of changes in the mode of parental care steadily increased after young began feeding in W1, reaching a peak at W3 (Fig. 9) which was significantly different from W1, W2 and W4 (RM-ANOVA: $F_{1, 3}$=5.677, P<0.05).

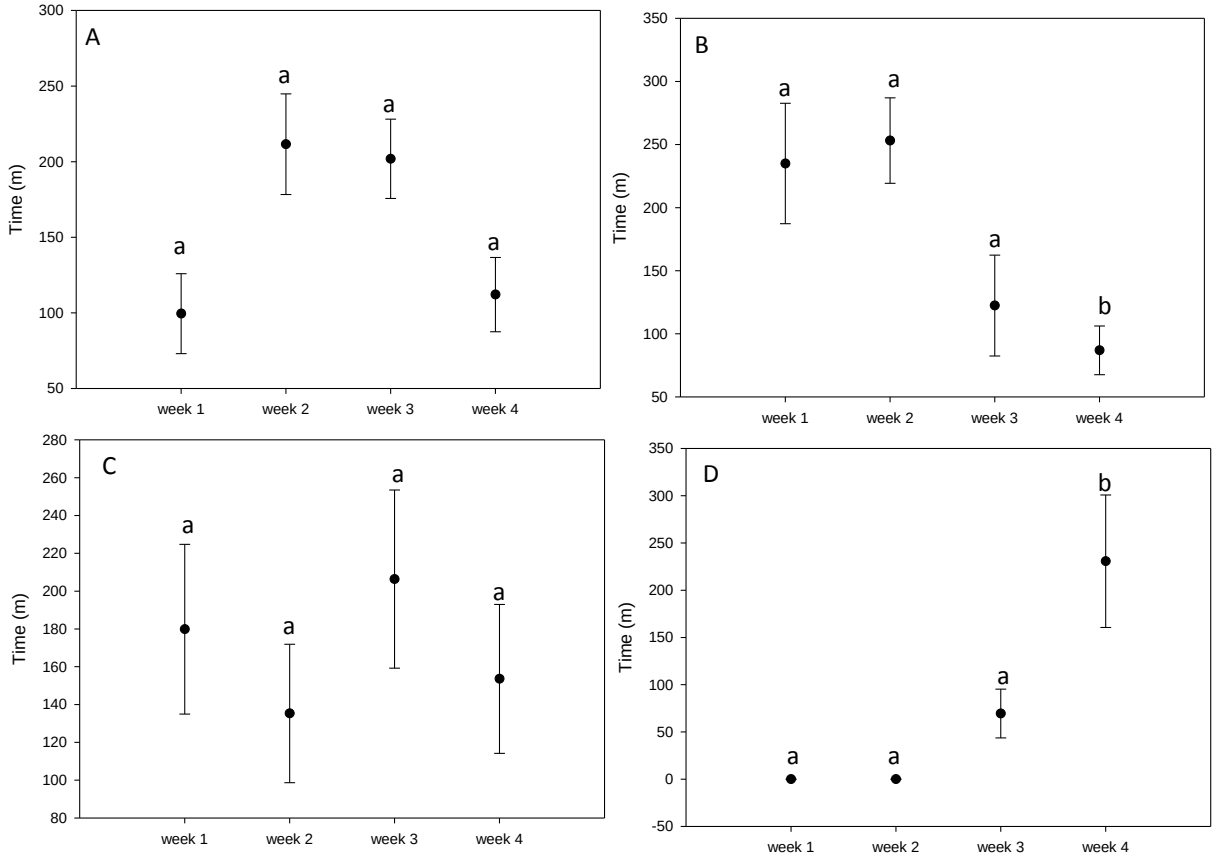


Fig. 8. Time young spent associated with the male parent (A), female parent (B), both parents (C) or neither parent (D). Differences in letters denote a significant difference (paired *t* test; p<0.05; n = 6) where bars that share a letter are not statistically different (mean ± s.e.m. n=6).

2.4.3 Bite Rate

Bite rate significantly increased over time (Repeated Measures (RM)-ANOVA: $F_{1, 20}$=7.933, P<0.05) peaking around day 12-15 before slowly decreasing (Fig. 10). The bite rate of young, however, did not differ significantly (RM-ANOVA: $F_{2, 40}$=0.304, P=1.000) between young feeding off the male or female parent.

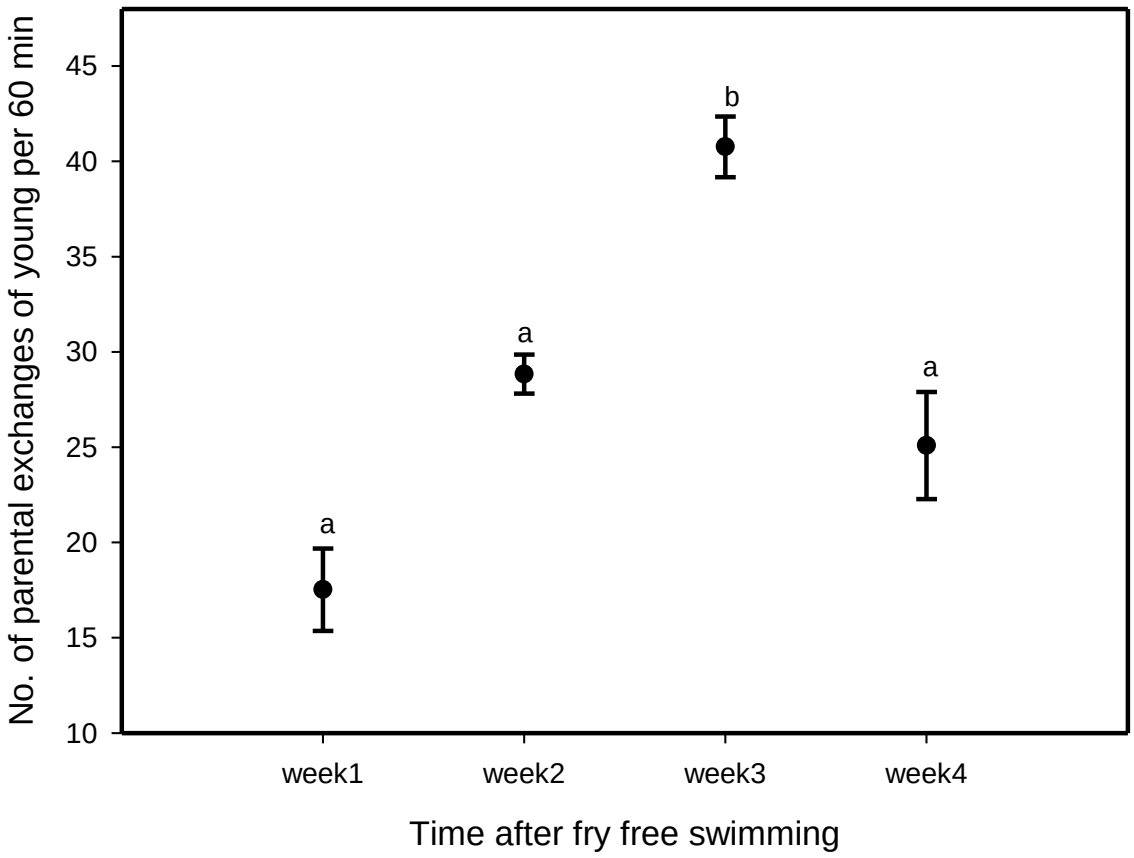


Fig. 9. Total number of incidences within a 60 min observation period where the mode of parental care changed across the four-week breeding period. Differences in letters denote a significant difference (paired *t* test; $p<0.05$) where bars that share a letter are not significantly different (mean ± s.e.m. n=6).

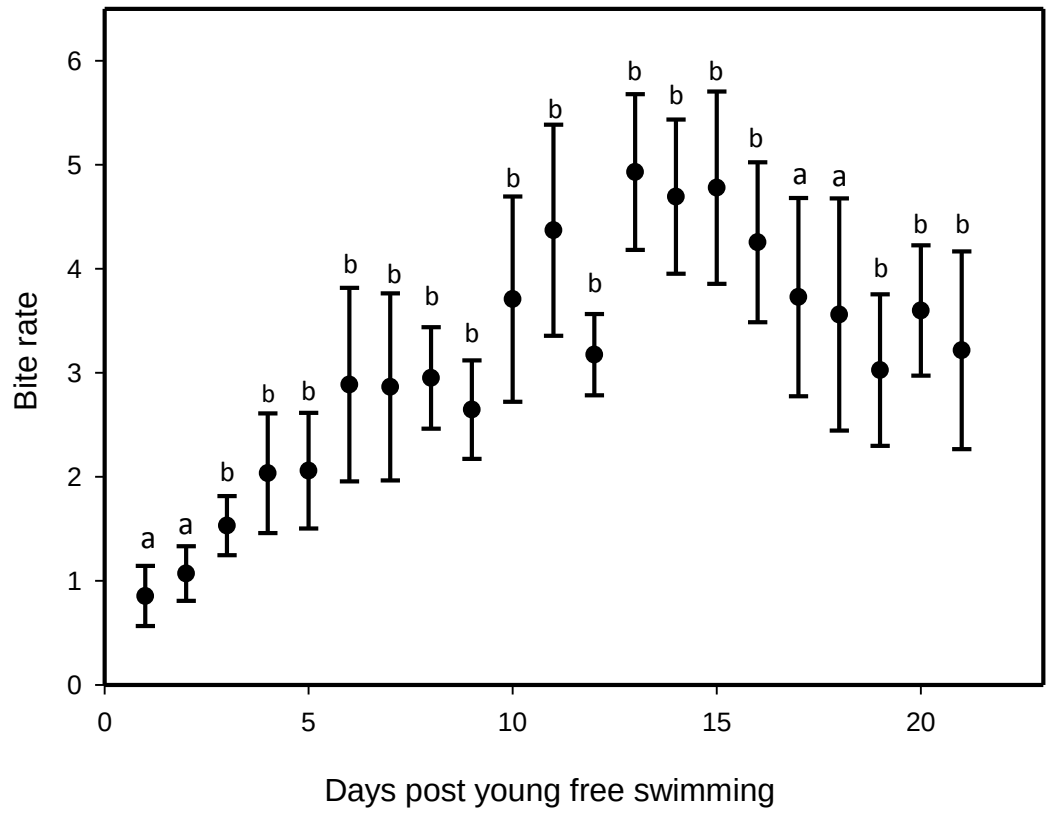


Fig. 10. Bite rate of young per 30 s (mean ± s.e.m. n=10) on both parents over the first three weeks of the breeding period. Differences in letters denote a significant difference between each time point and the bite rate recorded on day 1 (paired *t*-test: p<0.05).

2.4.4 Mucus IgM concentration

Parental mucus collected at time zero had significantly less IgM (RM-ANOVA: $F_{1,7}$=3.732, P<0.05) than that collected at the time points E, H, FS, W1, W2 and W3 (Fig. 11). 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).

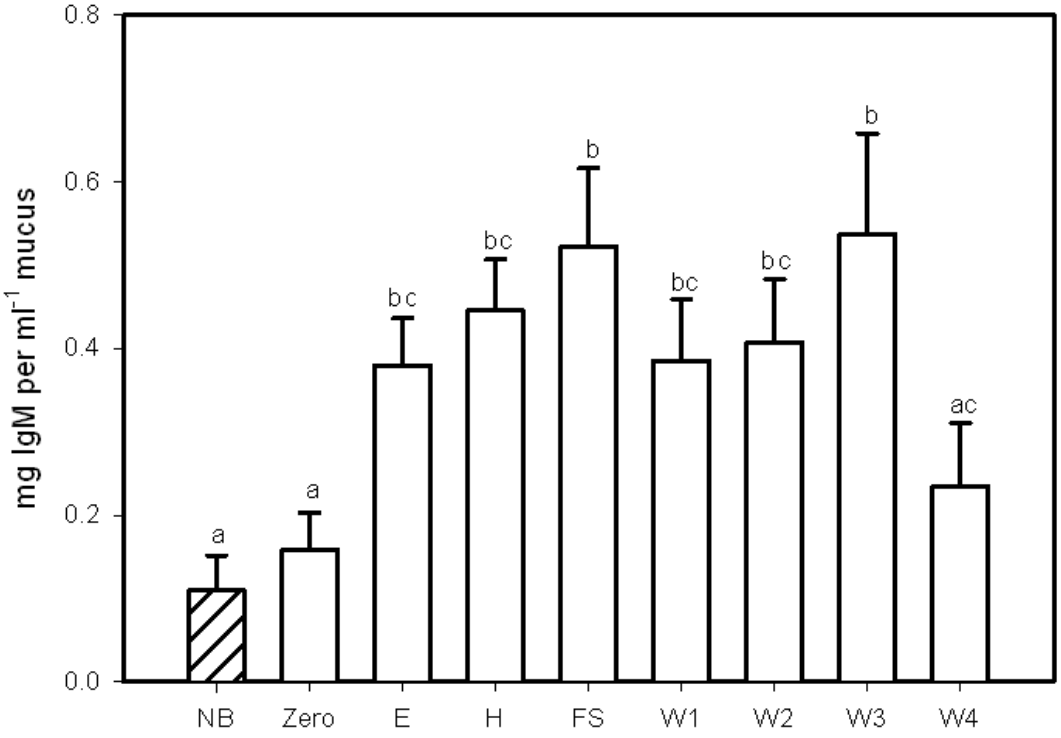


Fig. 11. Total IgM (mean ± s.e.m.) in the mucus of non-breeders (NB) (n=6) and breeding pairs (n=12 including males and females combined) over the breeding cycle. Time points throughout the breeding cycle include a time of no breeding activity (zero) the time eggs were laid (E), the time the eggs hatched (H), the time young became free-swimming (FS), one week (W1), two weeks (W2), three weeks (W3) and four weeks (W4) after young became free-swimming. Differences in letters denote a significant difference (paired *t* test and LSD: p<0.05) where bars that share a letter are not significantly different.

2.4.5 Mucus Total Protein concentration

Parental mucus at W2 and W3 had significantly lower levels of total protein (RM-ANOVA: $F_{1, 7}$=4.006, P<0.05) than mucus taken at the time points E, H and W1 (Fig. 12). The mucus of non-breeders was significantly lower in protein than the parental mucus at the time points E, H, and W1 (one-way-ANOVA: $F_{8, 96}$=2.642, P<0.05). There

was no effect of sex on parental mucus total protein (RM-ANOVA: $F_{1,\ 7}$=0.763, P=0.620).

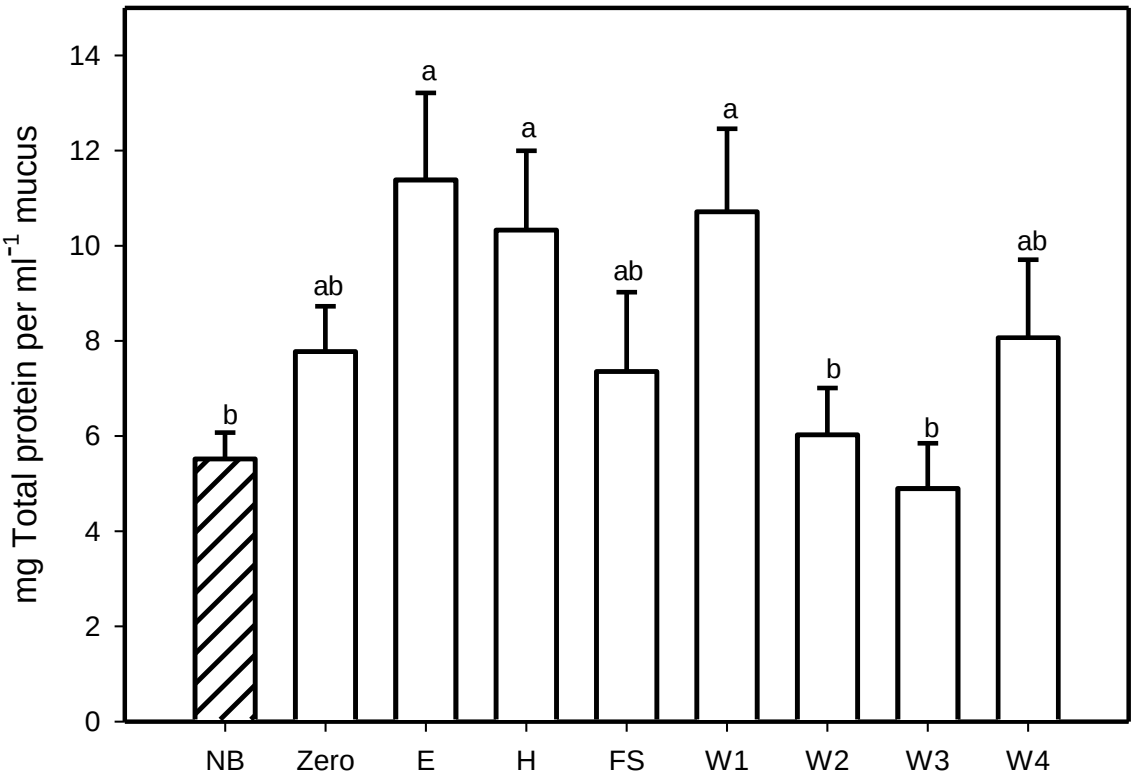


Fig. 12. Total protein (mean ± s.e.m.) in the mucus of non-breeders (n=6) and breeding pairs (n=12) over the breeding cycle. Differences in letters denote a significant difference (paired *t* test and LSD; p<0.05) where bars that share a letter are not significantly different.

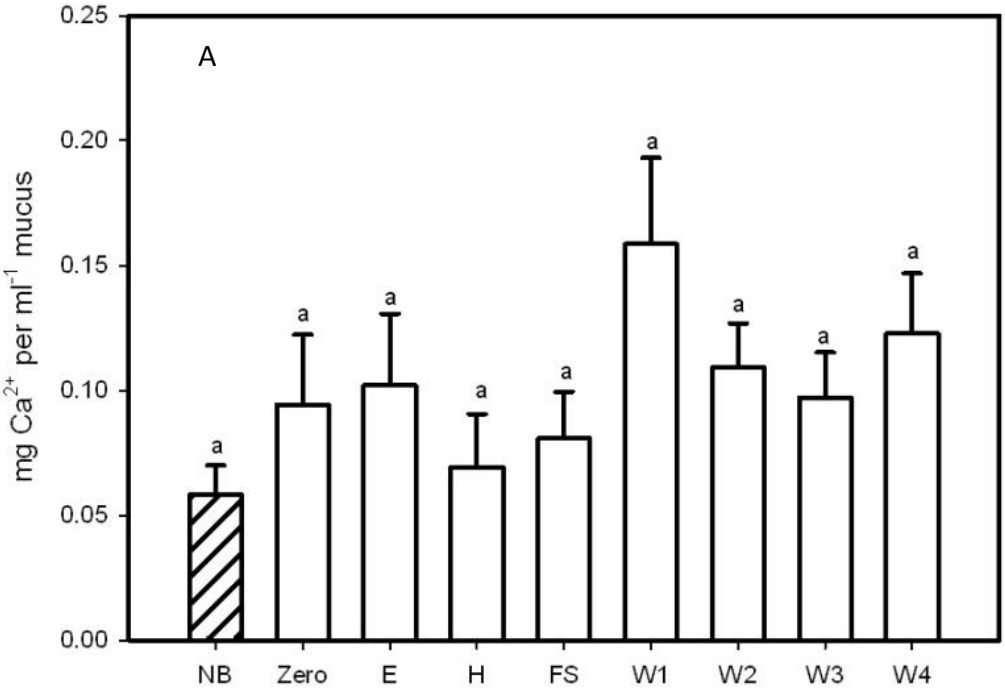
2.4.6 Mucus Ion composition

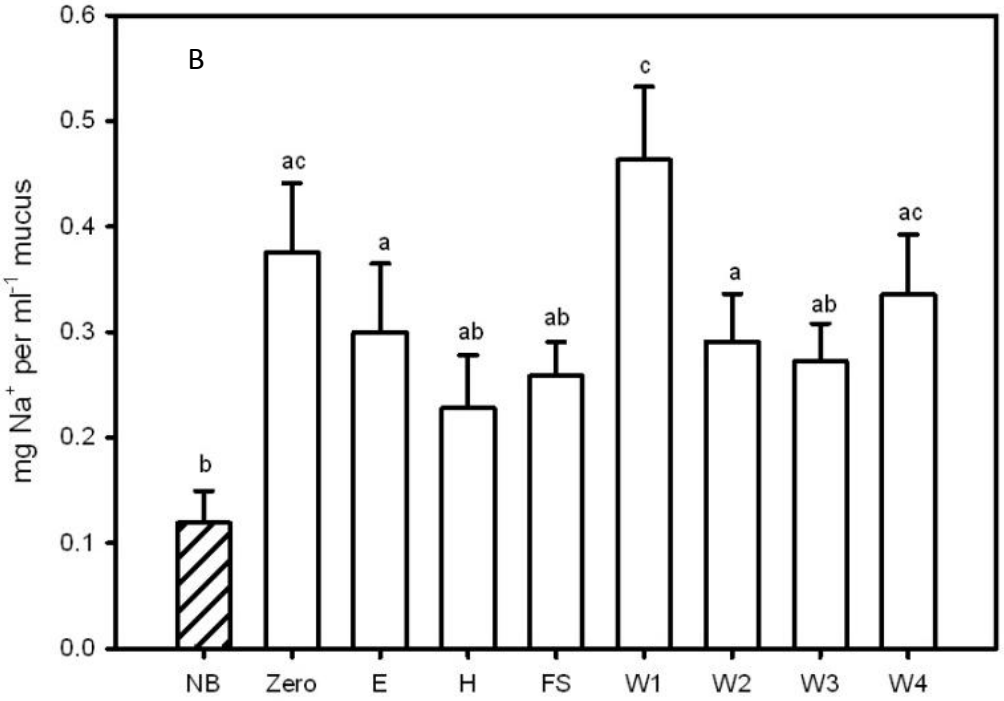
Calcium (Fig. 13A) was the only ion where there were no significant differences between parental mucus taken at different time points (RM ANOVA: $F_{1,\ 7}$=2.333, P=0.139), between breeders and non-breeders (one-way-ANOVA: $F_{8,\ 87}$=1.470, P=0.180). Sodium values (Fig. 13B) during W1 were significantly higher (RM-ANOVA: $F_{1,\ 7}$=3.287, P<0.05) than the time points E, H, FS, W2, W3 and NB. Non-

breeders also had significantly lower levels of Na^+ than zero, E, W1, W2 and W4 (one-way-ANOVA: $F_{8, 97}$=2.956, P<0.05; post hoc LSD). The concentration of K^+ in parental mucus (Fig. 13C) during the zero time point was significantly higher (RM-ANOVA: $F_{1, 7}$=5.274, P<0.001) than 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. Chloride concentrations (Fig. 13D) were significantly higher in parental mucus (RM-ANOVA: $F_{1,7}$=2.666, P<0.05) at the time points zero and FS than during W2 and W3.

2.4.7 Mucus cortisol concentration

Although there were no significant differences in the levels of parental mucus cortisol over time (Fig. 14) (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).


A
mg Ca^{2+} per ml^{-1} mucus
0.25
0.20
0.15
0.10
0.05
0.00
a
a
a
a
a
a
a
a
a
NB
Zero
E
H
FS
W1
W2
W3
W4


B
mg Na^{+} per ml^{-1} mucus
0.6
0.5
0.4
0.3
0.2
0.1
0.0
b
ac
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a
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W1
W2
W3
W4

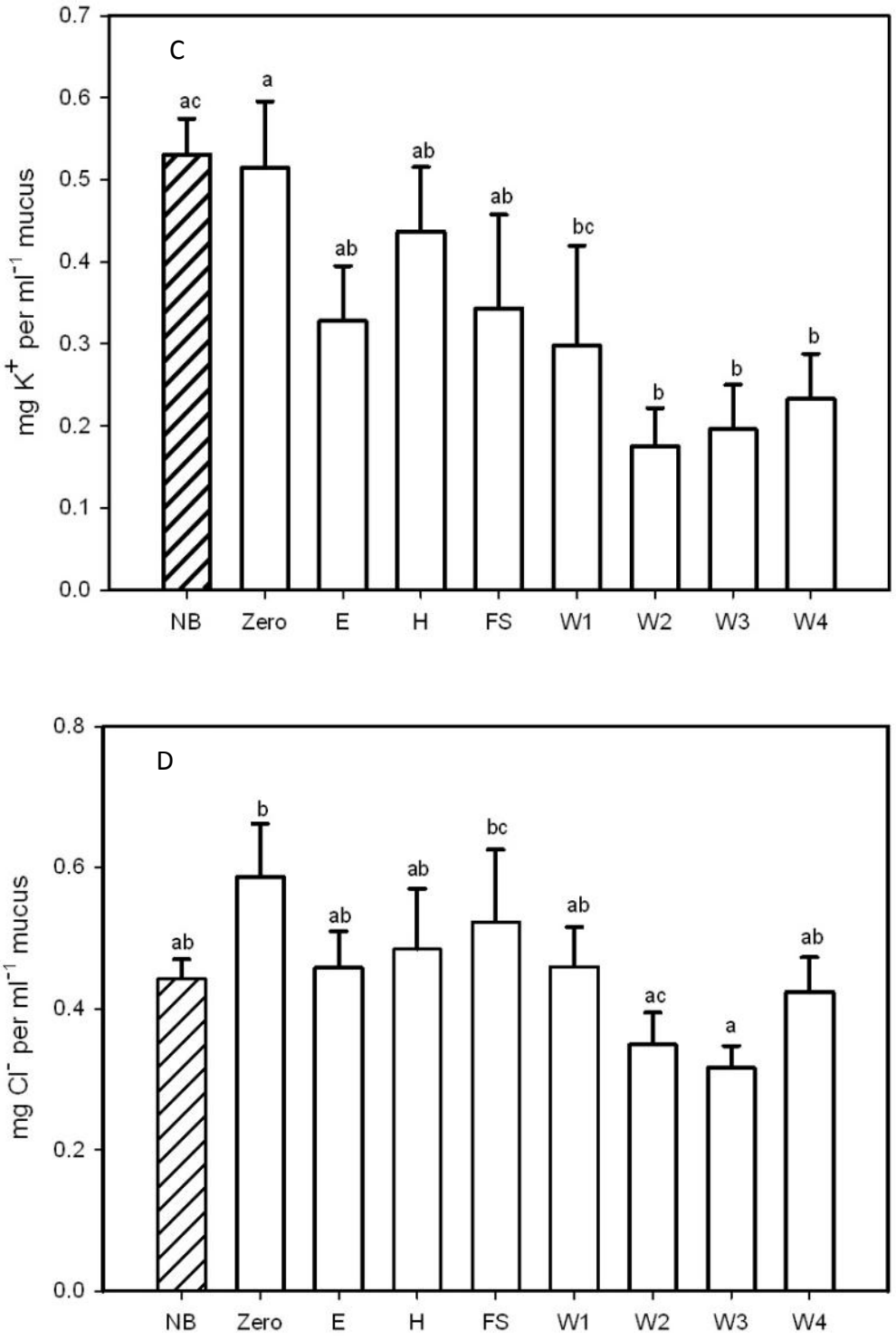


Fig. 13. (A) Calcium (B) sodium (C) potassium and (D) chloride concentrations (mean ± s.e.m.) in the mucus of non-breeders (NB) (n=6) and breeding pairs (n=12) over the breeding cycle. Differences in letters denote a significant difference (paired *t* test and LSD; $p<0.05$) where bars that share a letter are not significantly different.

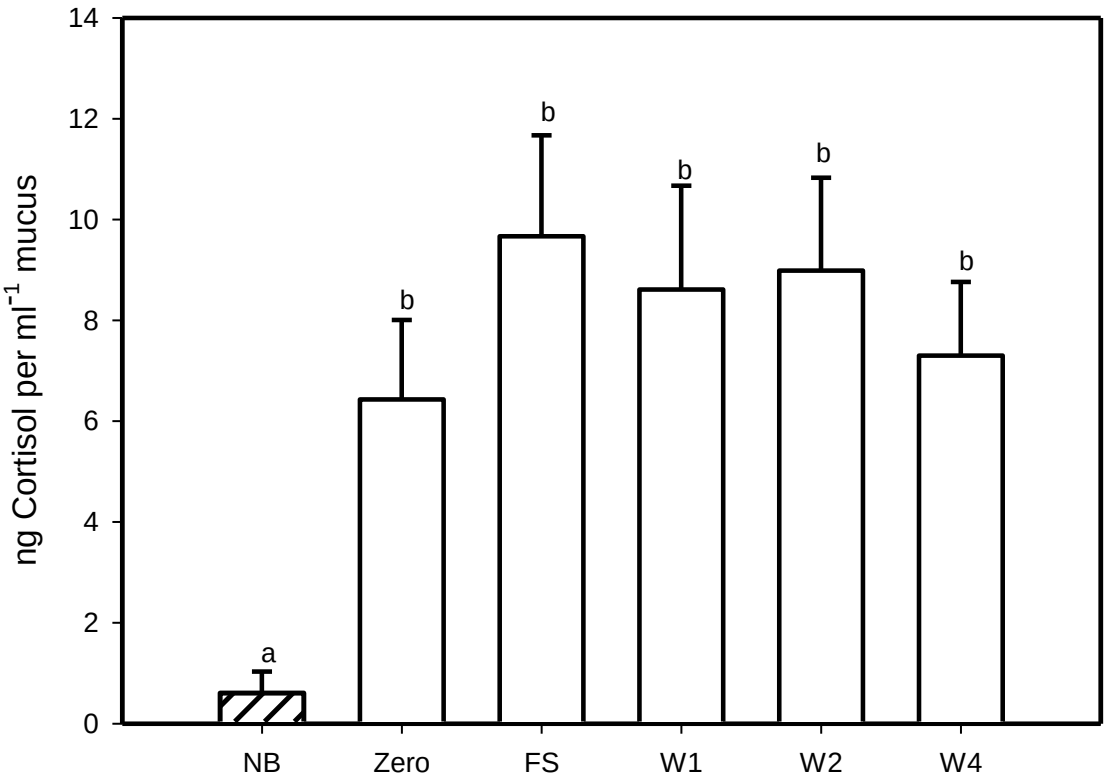


Fig. 14. Cortisol content (mean ± s.e.m.) in the mucus of non-breeders (NB) (n=6) and breeding pairs (n=12) over the breeding cycle. Differences in letters denote a significant difference (post hoc paired *t* tests) where bars that share a letter are not significantly different.

2.5 Discussion

The first two weeks of parental care in discus fish involved both parents spending the vast majority of time associated with their offspring with either one or both parents looking after young simultaneously; young were at no point left alone. During W1, 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 W2 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 two 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 was reflected in the behaviour of young that exhibited a steady increase in bite rate similar to that observed by Chong et al. (2005).

Parental behaviour began to change during W3 with parents opting to leave offspring on their own for short periods of time making it difficult for young to feed on mucus. Week 3 also saw parents frequently changing care duties. The mean duration of each parental care mode in W3 was reduced (30-60 s) compared to that observed during W1 (5-10 min) making it more difficult for young to feed due to the constant movement of both parents. Young were also 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 W3 young began to display foraging behaviours. It remains unclear if the initiation of this change in feeding strategy was a consequence of the observed parental avoidance behaviours, or some 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 independently foraging (Brown, 1984). The bite rate of young also reached a plateau around W2 before declining toward W3 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 (scales in certain areas were raised and scratches appeared to be present), would actively swim away from offspring severely limiting the ability of young to feed. The epidermal damage and stress noted in

adults during W4 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 was introduced in this 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 likely 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 W4 away from their parents.

The change in parental behaviour from that seen in W1 and W2, which involved close attentive contact with young, to the behaviour observed in W3 and W4, 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 increases to the point where parents and offspring are in conflict over the provision of these resources (Trivers, 1974). Parents in other vertebrates with precocial young alter their behaviours to increase the cost of offspring solicitation to thus aid in the development of their offspring's independent foraging (Davies, 1978; Pugesek, 1990; Rehling and Trillmich, 2008; Weary et al., 2008). These observations suggest that this weaning behaviour, although more typically associated with mammals and birds, also occurs in discus fish.

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 breeding fish once eggs were laid, and the increase in mucus IgM levels remained until W4. This suggests the process is endogenously controlled rather than due to IgM leakage following young causing epidermal damage during feeding. The mechanism that reduces parental mucus IgM levels during W4 could be endogenously controlled via a similar suite of hormones 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 four to six 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. (2005) where they identified a C-type lectin in the mucus of breeding discus which is absent in non-breeding individuals. Lectins are responsible for activating the complement system after recognizing pathogenic micro-organisms (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.

Parental mucus during the time points W1, E and H had significantly higher levels of total protein than W2 and W3. The drop in total protein in 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 in 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 non-breeders. The elevation of total protein is probably due to the elevated levels of IgM and 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 Fiedler, 1965), was found to be elevated in the skin of discus parents during the period of parental care (Khong et al., 2009) (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. 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 breeding discus mucus, these fish did have significantly higher levels of cortisol than non-breeders. Cortisol plays a vital role in ionoregulation (McCormick, 2000), which might be an advantage to young developing in an ion-deficient environment.

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 sodium (Na^+), potassium (K^+) calcium (Ca^{2+}) and chloride (Cl^-) for osmoregulation. Fish mucus can help reduce ion loss *via* a gradient of ions within mucus layers (Handy and Maunder, 2009), which represent a significant barrier against their diffusional efflux (Shephard,

1994); it may also provide a possible sink of ions for discus offspring. Out of all the ions assayed Na^+ was the only ion that appeared to be significantly higher in mucus of breeding discus compared to non-breeding fish, possibly suggesting the importance of this ion for developing young. It is likely that is not an accurate representation of the ionic composition of parental mucus from wild discus due to the water chemistry of the aquarium environment. In the Amazon, the water chemistry is highly dilute and acidic, the aquarium environment, however, typically contains a much greater concentration of ions making the parental provision of ions in the aquarium environment potentially less crucial as young can uptake ions across the gills and gut if background levels are appreciably high. Chapter 3 will, therefore, investigate the differences in mucus composition between wild and aquarium reared discus fish.

2.6 Conclusion

Parental care duties in discus fish appear to be shared equally between the male and female, both in regards to 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 then fell to pre-breeding values as young began to feed on other sources. Protein content of the parental mucus was lowest during W2 and W3 mirroring the intensity at which the young fed during this period. A weaning period was observed to occur in W3, which was possibly initiated by a shift in the observed parental behaviour. I 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 in regards to the evolution of this behaviour as well as the sexual selection which precedes this exceptional form of parental care.

Chapter 3: Comparative analysis between the mucus of wild and aquarium bred breeding and non-breeding discus

Data within this chapter has been published in combination with data from chapter 2 in the Journal of Experimental Biology: Buckley, J., Maunder, R. J., Foey, A., Pearce, J., Val, A. L. and Sloman, K. A. (2010). Biparental mucus feeding: a unique example of parental care in an Amazonian cichlid. Journal of Experimental Biology 213, 3787-3795.

3.1 Abstract

The Amazonian cichlid, *Symphysodon* spp. is a hugely popular species within the aquarium trade. This is due in part to both the aesthetic qualities and the unusual form of parental care that this species adopts where fry feed obligatorily from parentally-provided mucus for the first few weeks of development. The interest in this species has led to a huge demand in the sale of aquarium-bred discus fish resulting in the selective breeding of individuals with novel colour patterns. The environmental conditions and selection pressures imposed on aquarium bred fish are markedly different from those experienced by wild discus where pressures related to the ability to survive environmental fluctuations and are more important than appearance. There is, therefore, a possibility that the different pressures imposed on aquarium versus wild discus could manifest themselves in the ability to provide parental care. Here, I investigate the mucus composition (total protein, cortisol, immunoglobulin and Na^+, K^+ and Ca^{2+}) of wild breeders and wild non-breeders so that comparisons with chapter 2 can be made between the ability of wild and aquarium bred discus to provide parental mucus. Elevated levels of Na^+, K^+, Cl^- and total protein were present in the mucus of wild breeders compared to wild non-breeders. Although not significant wild breeders also appeared to have elevated levels of IgM and Ca^{2+} than non-breeders. Interestingly, wild breeders also had significantly greater levels of the ions Na^+, K^+ and Cl^- compared to aquarium-bred breeders potentially due to the difference in water chemistry of their respective environments. There was a trend for wild breeders to have slightly higher levels of IgM possibly due to the selection pressures imposed by the wild environment versus the aquarium environment. Mucus concentrations of the metals copper (Cu), cadmium (Cd) and mercury (Hg) were also assessed, with wild breeders having significantly higher levels of Cu and Hg than non-breeders. The concentrations present,

however, were far below what is known to be hazardous, with the presence of Cu possibly acting as an essential micronutrient.

3.2 Introduction

Discus fish are a freshwater species of cichlid native to the Amazon Basin River system. Like most cichlids, discus show a characteristically high level of parental investment (Clutton-Brock, 1991). This investment involves not only the protection of fry, as is found in a large proportion of cichlids (Barlow, 2002) but also the provision of a nutritionally rich mucosal substance; a substance which is used to sustain discus fry solely for the first few weeks of post hatch development (Chong et al., 2005). This form of parental care, which seems to share several parallels with lactation in mammals (Chapter, 2; Chong et al., 2005), has no doubt added to the allure of this species, making it one of the most valuable species available on the ornamental market (Axelrod et al., 1986; Chong et al., 2000). The world-wide interest in this species has resulted in the formation of commercial discus farms that selectively breed discus for morphological and colour characteristics that appeal to aquarists (Koh et al., 1999a); the resulting colour morphs greatly differ in appearance compared to their wild counterparts. Selective breeding focusing on aesthetic appearance has been carried out in discus farms for several generations which begs the question of whether other characteristics such as the ability to provide parental mucus have also been inadvertently affected. Previous studies focusing on parental care in discus fish have all used farmed varieties as opposed to wild discus (Buckley et al., 2010; Chong et al., 2005); a range of environmental and genetic factors, however, may lead to significant differences between the ability of wild and farmed species to provide parental mucus secretions.

Wild discus fish inhabit the slow moving lakes associated with the Amazon River Basin, an environment characterized by extreme fluctuations in water depth due to

changes in rainfall between the wet and dry season (Crampton, 2008). Changes in water depth from one season to another can be as large as 12 metres, a change which can radically affect the availability of food and shelter, the density of predators and parasites and physicochemical properties of the water such as pH and dissolved oxygen (Crampton, 2008; Junk, 1997). In contrast, the environmental conditions of the aquarium are relatively stable with water chemistry properties, predator/pathogen quantities and diet all being controlled. Research on wild discus have thus far indicated that the diet of adults during the high water period largely consists of a mixture of algal periphyton, fine organic detritus and green plant matter (Crampton, 2008) and that during the low water period the stomachs of discus fish were only partially filled. The diet of wild discus is in stark contrast to the diet of aquarium bred discus, which in my initial study (Chapter 2) were regularly fed a well formulated diet rich in protein, oils, fats and minerals. Previous work in goldfish (*Carassius auratus*) indicated that differences in diet led to distinct changes in the amino acid composition of epidermal mucus (Saglio and Fauconneau, 1985); it could therefore be predicted that differences between the diets of wild and aquarium-reared discus could also result in observable differences in mucus composition. As well as diet, external factors such as water chemistry could alter mucus composition.

The Amazon River Basin collects freshwater from around 37% of continental South America. This water drains through the Amazon passing through dense jungle regions filled with decomposing vegetation leading to the continuous input of humic and fulvic acids. Due to the regional geochemistry of the area, inputs of calcium (Ca^{2+}) are limited resulting in waters that are soft and ion limited (Furch, 1984). The combination of low levels of essential ions such as Ca^{2+} and large inputs of humic and fulvic acids helps to create the acidic, ion poor water chemistry that characterizes the Amazon River Basin

(Gonzalez et al., 1998). Ion concentrations in the Rio Negro, a major tributary of the Amazon River are Na^+= 0.363 ± 0.06, K^+= 0.319 ± 0.02, Ca^{2+} =0.212 ± 0.03, Cl^- =1.65 ± 0.56 (mg l^{-1} ± s.e.m) with pH values reaching 3 or lower (Gonzalez et al., 1997). One of the difficulties of living in an ion poor acidic freshwater environment like the Rio Negro, involves regulating the loss of important ions such as sodium (Na^+), potassium (K^+) and Ca^{2+} while limiting water influx. The difficulties of regulating ion loss in such dilute acidic waters was observed in the angelfish (*Pterophyllum scalare*), a species closely related to discus fish, where the uptake of Na^+ in the dilute waters of the Rio Negro was inhibited by exposure to a pH of 4.0 and completely inhibited at pH 3.5 (Gonzalez and Wilson, 2001). One way in which fish can combat ion loss is to reduce ion efflux through the development of a gradient of ions in epidermal mucus (Handy and Maunder, 2009), a process known to provide a significant barrier against their diffusional efflux (Shephard, 1994). Ions lost by passive efflux to the external environment can also be replaced if they are present in high enough numbers in the environment so that active uptake of ions across the gills can occur: If not present within the environment, they can also be obtained through diet (Smith et al., 1989). In discus, the production of ion gradients within parental mucus may provide a possible sink of ions for offspring to consume, a process which may help offspring alleviate the problems of osmoregulation in an ion poor environment. This idea was investigated in aquarium bred fish in chapter 2, however, a significant increase in breeder mucus ion composition relative to non-breeders was not observed across most of the essential ions (Chapter 2). This may be due to the water chemistry of the University of Plymouth aquarium which is notably different from the Amazon as it lacks the large inputs of humic and fulvic acids typical of the Amazon and contains much higher concentrations of essential ions. The concentration of Ca^{2+} in the water of aquarium discus observed in chapter 2 for example, was much greater (22 mg l^{-1}) than that generally reported in the

Amazon (0.4-7 mg l^{-1}) (Furch, 1984). It is possible that the differences in water chemistry between the wild and aquarium environment could, therefore, lead to pronounced differences in parental mucus ion compositions.

In aquarium bred discus one of the key components of parental mucus appeared to be the provision of IgM to offspring, a provision which could potentially provide offspring with a passive form of immunity for the first few weeks of development (Chapter 2). Several generations of aquarium reared discus have been grown on and bred in aquariums fitted with water filters and UV sterilizers ensuring that the aquarium environment is largely pathogen free. This pathogen free environment combined with the ability to treat most pathogens with a range of over-the-counter drugs suggests that the selection pressures normally exerted by pathogens present in the wild may be reduced in aquarium reared discus fish. While natural environmental pressures could be responsible for marked differences between the mucus of aquarium and wild discus, a series of recent anthropogenic perturbations in the wild could also potentially impact mucus provision in wild discus. Contaminants associated with industries such as gold mining and petroleum extraction are having a noticeable effect on both the communities of flora and fauna as well as the communities of indigenous people associated with the Amazon region (Nascimento et al., 2006; Nevado et al., 2010). In particular the recent heavy development of industry, in and around the Amazon basin has been associated with a notable rise in the concentrations of metals such as mercury (Hg) (Uryu et al., 2001), copper (Cu) and cadmium (Cd) (Matsuo et al., 2005). Although most species are susceptible to the effects of contaminants, discus may be particularly vulnerable due to the unique form of parental mucus provision displayed in this species. One of the many proposed functions of epidermal mucus in fish is that of defence (Shephard, 1994), in particular defence against contaminants. The defence aspect of mucus is provided largely by its ability to separate the internal and external environment of the fish. This is

achieved through the binding and precipitation of pollutants at the mucus layer (Shephard, 1994), an ability which is particularly important when the external environment of the fish is compromised by contaminants. In this respect, mucus can be thought of as a barrier, which can absorb and prevent the pollutant from entering the internal environment of the fish. The presence of contaminants is known to cause an increase in mucus production around the gills and epidermis (Eddy and Fraser, 1982; Lock et al., 1981) which can be sloughed off, minimising the impact of the contaminants on the fish. Mucus also has a property that allows it to bind and sequester metal rich particulates (Smith and Flegal, 1989; Tao et al., 2000) further protecting the fish from coming into contact with the pollutant. The ability of mucus to bind contaminants, however, may well result in the parental care strategy of discus becoming disadvantageous to offspring. If contaminants are present within the environment during the first few weeks of mucosal feeding, it is entirely possible that parental mucus could act as a sink for these pollutants and allow them to be transferred to young. Mucosal feeding in this species, may therefore, lead to fry ingesting high levels of contaminants which could have serious consequences for the populations of wild discus, especially as the Amazonian region becomes ever more industrialized (Matsuo et al., 2005; Uryu et al., 2001).

The aims of this chapter are two-fold:

1) To compare the mucus composition of aquarium reared discus with that of their wild counterparts to determine normal mucus composition parameters of wild and aquarium bred discus fish.
2) To determine the presence and quantity of a selection of metals within the mucus of wild breeders and non-breeders. It was hypothesised that recent anthropogenic perturbations may have resulted in the elevation of metals in the environment which could accumulate in parental mucus.

3.3 Materials and methods

Two research trips to the Amazon River Basin were conducted so that samples of parental mucus could be obtained from wild discus breeding pairs. Once obtained, parental mucus samples were then assayed to elucidate any differences between the parental provisions of wild vs. aquarium bred discus as well as to detect the presence and quantity of metals within wild mucus.

3.3.1 Research trip 1

3.3.1.1 Date of Sampling

Discus begin breeding during the Amazonian dry season, a season characterized by several months of dry weather; water levels associated with the discus habitat during this period drop to just a metre or so in depth. The first research trip was, therefore, planned to coincide with the region's dry season in October/November 2008 to increase the possibility of obtaining mucus samples from discus breeding pairs.

3.3.1.2 Sample sites

The first research trip was planned in conjunction with the Instituto Nacional de Pesquisas da Amazônia (INPA) and focused on sites along the Rio Negro, a river characterized by its highly acidic and dilute properties. Sample sites were located in the slow moving lakes associated with the main body of the Rio Negro. These lakes, in contrast to the main body of the fast flowing Rio Negro, have a very limited flow rate and are filled with a vast array of submerged trees and bushes. Slower rates of flow combined with the vast networks of branches provided by submerged vegetation seem to provide a highly protected habitat for discus. Several fishermen from the local artisanal fishery were hired to help identify the most suitable fishing sites for the expedition. All of the hired fishermen had experience in the catch and export of discus

and possessed a detailed knowledge of the lakes populated by this species. Sampling was split over a period of seven days and involved four different sites.

3.3.1.3 Fishing procedure

Fishing was carried out throughout the night from 19:00 to 05:00. Discus fish were caught by local fishermen using a circular fishing hand net in combination with a high powered flashlight. The flashlight allowed fishermen to see down into the lake (1.4 – 2 m) and assess the presence or absence of discus. The flashlight also induced a hypnotic effect on the fish which allowed them to be caught more easily.

3.3.1.4 Sampling procedure

Once individual discus fish had been caught, a fragrance and chemical free 'buff puff' sponge was used to gently swipe three times across the top half of the fish in order to obtain a sample of mucus. The sponge was then placed in a 5 ml syringe and stored in a water-proof box until sample processing. After the mucus sample had been taken, the individual fish was then stored in a box of river water until the fishermen had finished in that particular area so as to prevent individuals from being re-caught and sampled twice. A total of 6 water samples representative of the areas fished were also collected in a water tight 50 ml plastic container.

3.3.1.5 Sample processing

Mucus samples were processed using the same methods described in chapter 2. After elution, samples were stored at -4^{o} C in a cool box containing a combination of ice and until arrival back at INPA. Upon arrival samples were immediately transferred to a -80^{o} C freezer until they could be analysed at INPA. Throughout the seven day sampling period, only individual discus fish were caught: there were no visible signs of breeding pairs. This was possibly due to the unusually elevated levels of rainfall experienced by

the region during our sampling period. Lake water levels had presumably not dropped low enough to provide the impetus for breeding pairs to form. Over one hundred, individual, non breeding discus fish were caught and sampled during the seven day sampling period. Although breeding pairs could not be obtained, mucus samples from individuals were used to look for the presence of metals and ascertain the pre-breeding composition of wild discus mucus.

3.3.2 Research trip 2

A second research trip was carried out during the month of February when lake water levels had finally dropped to that normally expected for the dry season. Researchers from INPA, trained in collecting mucus samples, travelled back to the discus breeding sites along the Rio Negro to obtain mucus samples from breeding pairs with offspring. A total of four breeding pairs with offspring were sampled, four fry from each pair were also obtained and stored at -80^{o} C until they were shipped to the University of Plymouth.

3.3.3 Mucus Assays

The composition of parental mucus was determined via a range of assays. Mucus assays for total protein and the ions sodium (Na^{+}), potassium (K^{+}), calcium (Ca^{2+}) and chloride (Cl^{-}) were carried out at INPA, and the rest of the assays including IgM, cortisol and the metals copper (Cu), cadmium (Cd) and mercury (Hg) carried out at the University of Plymouth. Samples were, therefore, shipped back to Plymouth for analysis.

3.3.3.1 Total Protein (INPA)

Total protein was measured using the Bradford assay (Bradford, 1976), the same technique as described in chapter 2. Subtle changes to this method, however, had to be

adopted due to differences in lab equipment. The spectrophotometer available at INPA only operated at a small range of wavelengths and could not read samples at the required total protein wavelength of 595 nm. The wavelength 620 nm was used instead. Since the recommended wavelength for reading this assay is 595 nm a comparison between a set of protein standards read at 620 nm and the recommended wavelength of 595 nm was carried out by technicians at Plymouth University. The results from the comparison were sent to the research lab in Manaus, Brazil and indicated that a total protein assay read at 620 nm produced reproducible results up to a concentration of 200 µg g^{-1}. Samples were, therefore, diluted appropriately so that diluted concentrations fell between 0-200 µg g^{-1}.

3.3.3.2 Ions

Mucus ion concentrations, including Na^+, K^+ and Ca^{2+} were measured using flame absorbance spectrometry whereas Cl^- was measured using a colorimetric-based assay. Flame absorbance spectrometry was utilized to measure ion levels instead of the ICP-AES methods utilized in chapter 2. Unlike the ICP-AES, multiple element readings on the same sample could not be carried out using flame absorbance spectroscopy. Three separate assays were, therefore, required for the measurement of the three ions. Atomic absorption spectroscopy works on similar principles to ICP-AES. Fluid samples are injected into a flame where they are then atomized. The atoms then absorb either ultraviolet or visible light (depending on the atom) and make transitions to higher electronic energy levels. The analyte concentration is then determined from the amount of absorption.

3.3.3.2.a Sodium and potassium

A sample volume of 3 ml was required for analysis via the flame absorbance spectrometer. The analysis of Na^+ and K^+ both required that a 40 µl mucus sample was

made up to 3000 µl with double distilled water. All samples were diluted appropriately to read within the standards.

3.3.3.2.b Calcium

Due to the wavelength used to measure Ca^{2+}, there can often be interference due to other ions. To prevent interference, mucus samples were made up to the required volume with 3% of the releasing agent lanthanum chloride ($LaCl_3$) before being read. The analysis of Ca^{2+} required that a mucus sample of 20 µl be made up to 2000 µl with $LaCl_3$. All samples were diluted appropriately (1:3) to read within the standards.

3.3.3.2.c Chloride

Chloride levels were assayed using the methods for the colorimetric assay methods described in chapter 2.

3.3.3.3. IgM

IgM concentrations were assayed using the same methods developed for the ELISA described in chapter 2.

3.3.3.4. Cortisol

Cortisol concentrations were assayed using the same methods described in chapter 2.

3.3.3.5 Metals

Copper (Cu), cadmium (Cd) and mercury (Hg) were measured using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) at the University of Plymouth. A total of 1500 µl was required for ICP-AES measurement which consisted of 20 µl of sample and 1480 µl of double distilled water.

3.3.4 Statistics

IgM and metal data was analysed non-parametrically but everything else met assumptions for parametric analysis.As stated in chapter 2 all data was adjusted per volume of mucus as opposed to mucus total protein. A Kruskall-Wallis test was performed on IgM data followed by a post hoc of six Mann-Whitney U tests used to compare all combinations of IgM data. For all other physiology data a one-way ANOVA followed by an LSD post hoc test was used to compare data from wild breeding/non-breeding discus with aquarium bred breeding/non breeding discus fish. Out of the 90 wild non-breeding discus fish sampled, a total of eight randomly selected mucus samples were used for comparisons between wild breeders (n=8), aquarium-bred breeders (n=8) and a representative sample of aquarium-bred non-breeders (n=8). Mucus composition of wild breeding Brazilian pairs was compared against the mucus values of week three Plymouth aquarium-bred discus; the fork length of young obtained from Brazilian pairs (15 ± 0.8 mm; n=6) was similar to the fork length of Plymouth young during week three of the breeding period (15 ± 0.1 mm; n=6). Copper, cadmium and mercury concentration data concerning wild breeders and wild non breeders was compared via a Mann-Whitney U test.

3.4 Results

3.4.1 Mucus IgM composition

A significant difference was observed between the four different groups (Kruskall-Wallis: $P<0.05$, Fig. 15) with the IgM of aquarium-bred non-breeders having significantly less mucus IgM than aquarium-bred breeders, wild-breeders and wild non-breeders (Mann-Whitney U: $P<0.05$, Fig. 15).

3.4.2 Mucus total protein composition

Wild non-breeders had significantly less total protein within their mucus than wild breeders, aquarium-bred breeders and aquarium-bred non-breeders (one-way-ANOVA: $F_{3, 28}$=4.299, P<0.05, Fig. 16).

3.4.3 Mucus ion composition

Wild breeders had significantly higher mucosal concentrations of Na^+ (One-way-ANOVA: $F_{3, 28}$=3.022, P<0.05, Fig. 17), K^+ (one-way-ANOVA: $F_{3, 28}$=7.364, P<0.05, Fig. 18), and Cl^- (one-way-ANOVA: $F_{3, 28}$=19.013, P<0.0001, Fig. 19), than wild non-breeders, aquarium-bred breeders and aquarium-bred non-breeders. Calcium was the only ion that did not significantly differ between wild and aquarium-bred breeders and non-breeders (One-way-ANOVA: $F_{3, 28}$ = 2.749, P=0.61, Fig 20).

3.4.4 Mucus cortisol composition

Cortisol levels were significantly higher (one-way-ANOVA: $F_{3, 39}$=17.894, P<0.001) within the mucus of aquarium-bred breeders (7.30±1.46) and non-breeders (0.60±0.37) due to cortisol being undetectable in wild bred breeders and non-breeders.

3.4.5 Mucus metal composition

Wild breeders had significantly higher levels of Cu (Mann-Whitney U test: P<0.05, Fig. 21) and Hg (Mann-Whitney U test: P<0.05, Fig. 22) within their mucus than wild non-breeding discus.

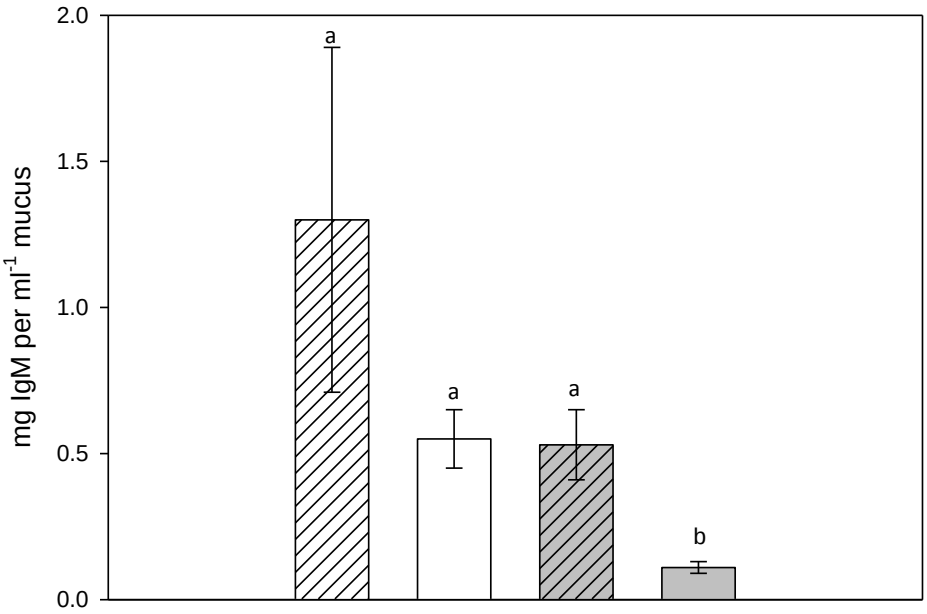


Fig.15. Total IgM (mean ± s.e.m) in the mucus of wild breeders (n=8), wild non-breeders (n=8), aquarium-bred breeders (n=8) and aquarium-bred non-breeders (n=8). Difference in letters denote a significant difference (Mann Whitney U; P<0.05) where bars that share a letter are not significantly different.

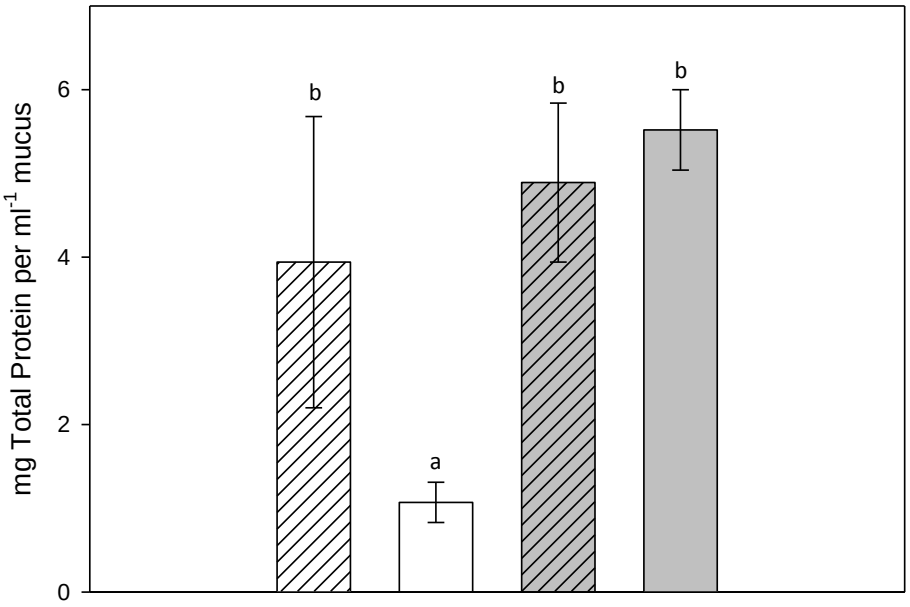


Fig.16. Total protein (mean ± s.e.m) in the mucus of wild breeders (n=8), wild non-breeders (n=8), aquarium-bred breeders (n=8) and aquarium-bred non-breeders (n=8). Difference in letters denote a significant difference (one-way-ANOVA; P<0.05) where bars that share a letter are not significantly different.

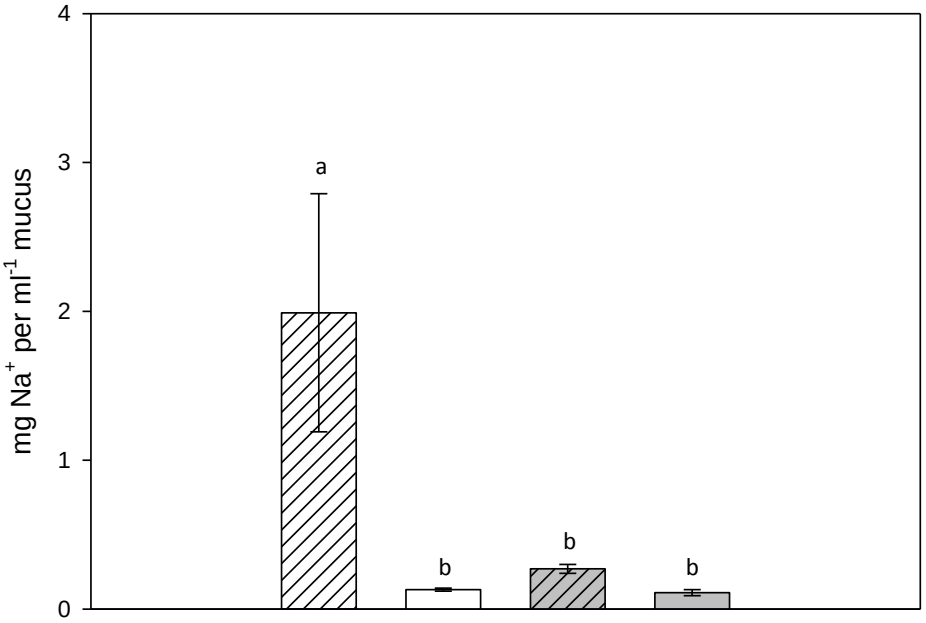


Fig.17. Sodium concentrations (mean ± s.e.m) in the mucus of wild breeders (n=8), wild non-breeders (n=8), aquarium-bred breeders (n=8) and aquarium-bred non-breeders (n=8). Difference in letters denote a significant difference (one-way-ANOVA; P<0.05) where bars that share a letter are not significantly different.

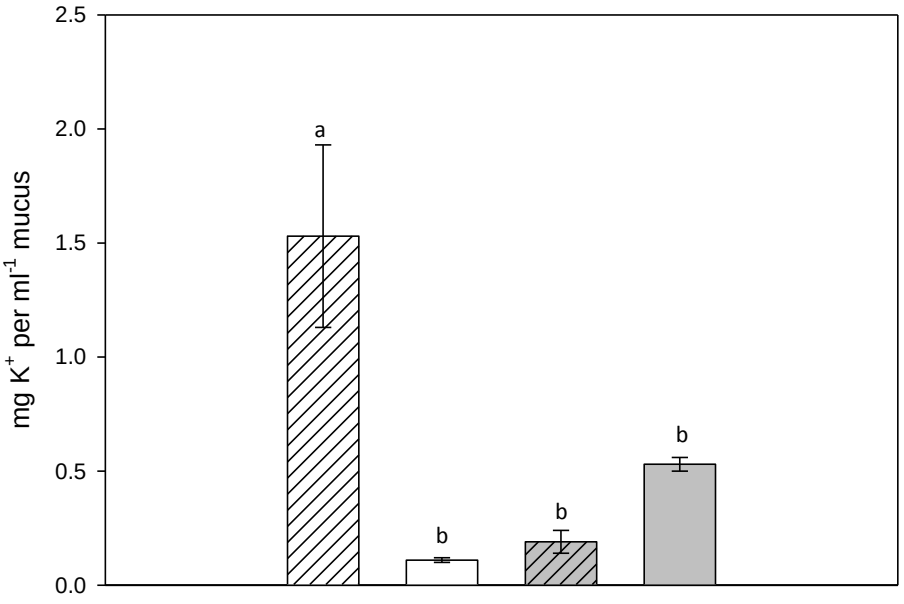


Fig.18. Potassium concentrations (mean ± s.e.m) in the mucus of wild breeders (n=8), wild non-breeders (n=8), aquarium-bred breeders (n=8) and aquarium-bred non-breeders (n=8). Difference in letters denote a significant difference (one-way-ANOVA; P<0.05) where bars that share a letter are not significantly different.

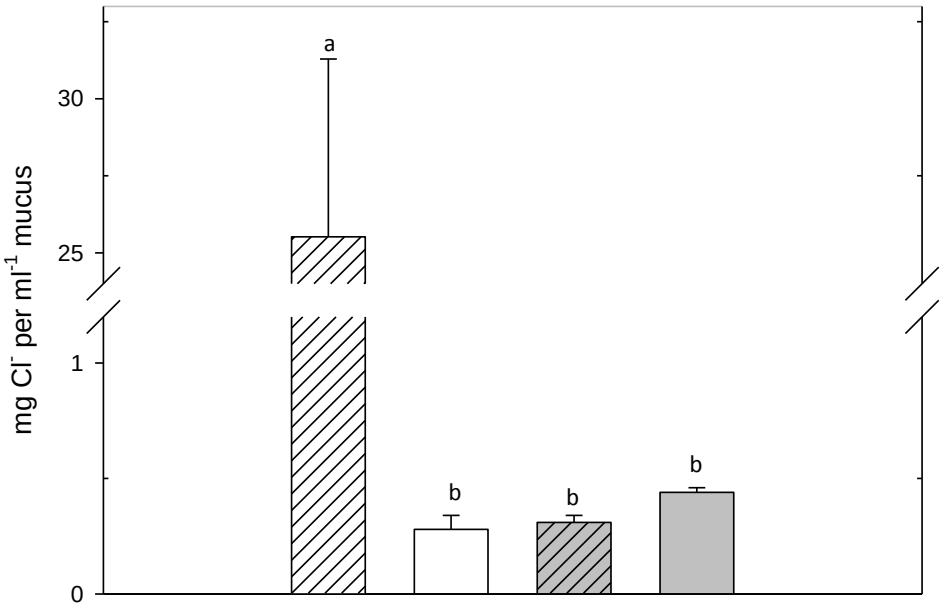


Fig.19. Chloride concentrations (mean ± s.e.m) in the mucus of wild breeders (n=8), wild non-breeders (n=8), aquarium-bred breeders (n=8) and aquarium-bred non-breeders (n=8). Difference in letters denote a significant difference (one-way-ANOVA; P<0.05) where bars that share a letter are not significantly different.

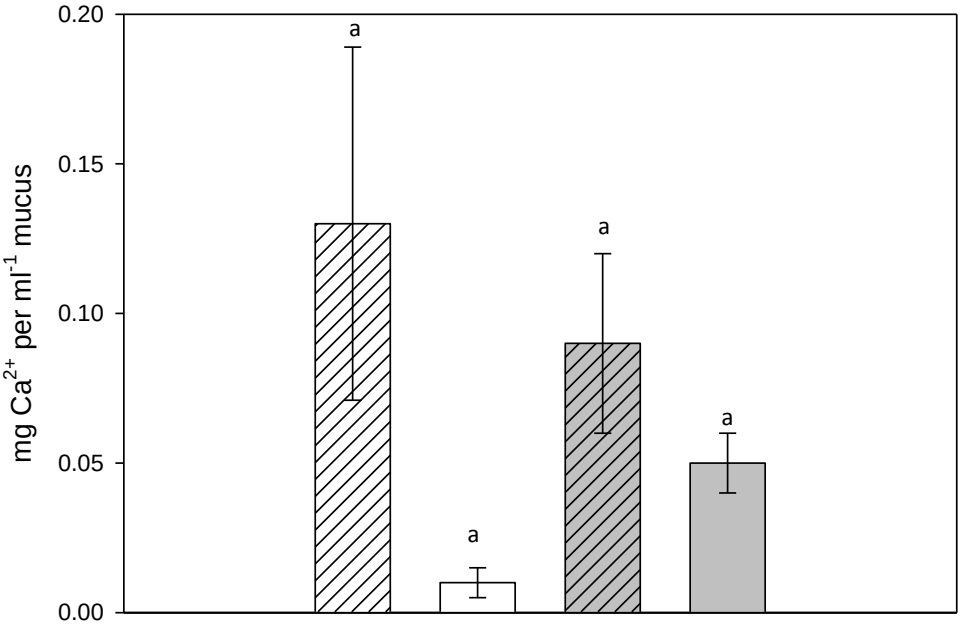


Fig.20. Calcium concentrations (mean ± s.e.m) in the mucus of wild breeders (n=8), wild non-breeders (n=8), aquarium-bred breeders (n=8) and aquarium-bred non-breeders (n=8). Difference in letters denote a significant difference (one-way-ANOVA; P<0.05) where bars that share a letter are not significantly different.

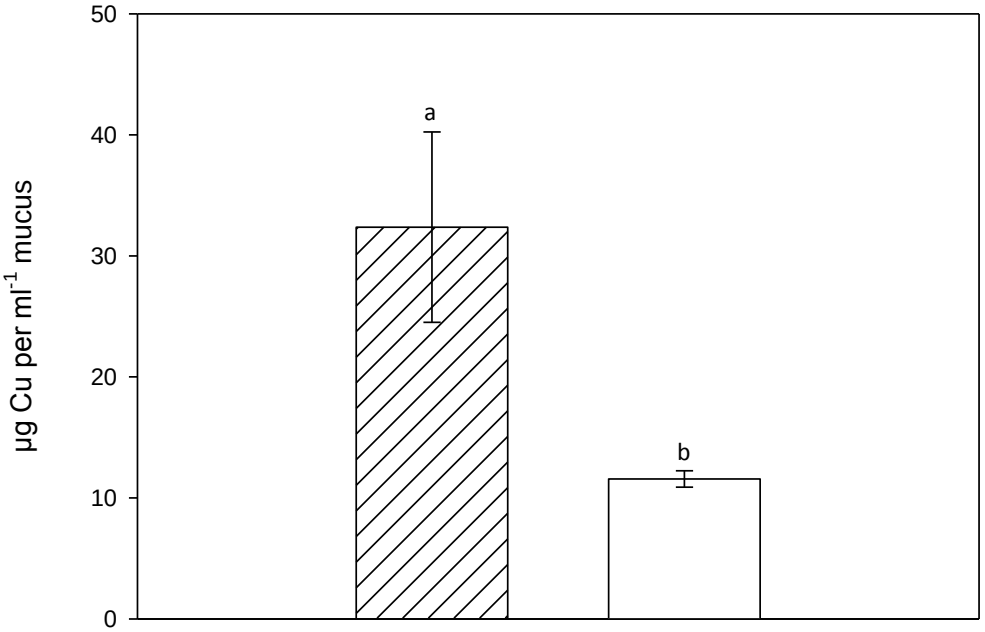


Fig.21. Copper concentrations (mean ± s.e.m) in the mucus of wild breeders (n=8) and wild non-breeders (n=8). Difference in letters denote a significant difference (Mann-Whitney U test; $p<0.05$).

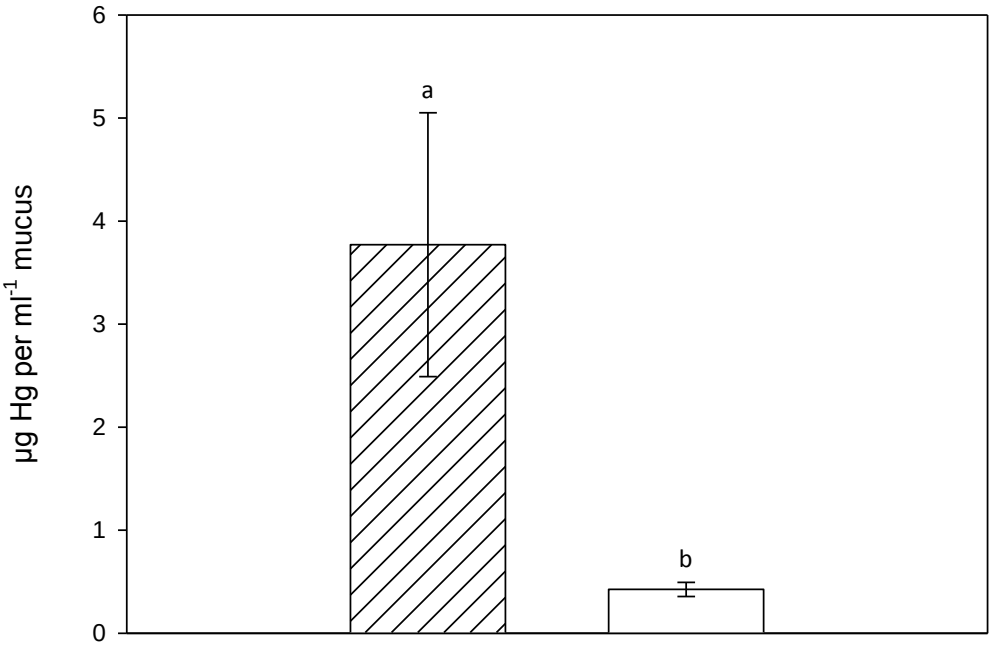


Fig.22. Mercury concentrations (mean ± s.e.m) in the mucus of wild breeders (n=8) and wild non-breeders (n=8). Difference in letters denote a significant difference (Mann-Whitney U test; $p<0.05$).

Table 1. Comparison between the ionic composition of breeding/non-breeding discus mucus and local water chemistry.

		Aquarium			Wild	
	Water (6)	Breeder (8)	non-breeder (8)	Water (6)	breeder (8)	non-breeder (8)
Na^+ (mg/ml^{-1})	9.28 ± 0.26	0.27 ± 0.03	0.11 ± 0.02	3.43 ± 3.43	1.99 ± 1.04	0.13 ± 0.01
K^+ (mg/ml^{-1})	1.42 ± 0.02	0.19 ± 0.05	0.53 ± 0.03	0.46 ± 0.12	1.53 ± 0.49	0.09 ± 0.01
Ca^{2+} (mg/ml^{-1})	21.56 ± 1.26	0.09 ± 0.01	0.05 ± 0.01	0.32 ± 0.06	0.13 ± 0.059	0.01 ± 0.00
Cl^- (mg/ml^{-1})	15.32 ± 0.76	0.31 ± 0.03	0.44 ± 0.02	10.05 ± 4.46	25.52 ± 5.77	0.28 ± 0.06

3.5 Discussion

Differences in mucus total protein were marked, with the mucus of wild non-breeders being significantly lower in protein than that of wild breeders, aquarium-bred breeders and aquarium-bred non-breeders. The lower total protein content of wild non-breeders compared to aquarium bred non-breeders suggests that environmental differences may well play a role in mucus compositions. Crampton et al. (2007) noted that the gut contents of wild discus fish varied considerably depending on the season. Individuals sampled during the high water season exhibited a predominately full stomach while a third of individuals sampled during the low water season had an empty stomach and two thirds having a stomach fullness less than 30%. The variation in gut content analysis suggests an infrequent supply of food which could be responsible for the decrease in mucus total protein as several studies have linked starvation to a reduction in mucus total protein (Heming and Paleczny, 1987; Saglio and Fauconneau, 1985). There is also the possibility that the infrequent availability of food selects for individuals that could limit non-essential mucus production outside of the breeding period leading to the observed decrease in wild non-breeder mucus total protein. The relatively high total protein levels found in aquarium-bred non-breeders is likely due to the constant provision of nutritionally rich formulated food which would remove pressures related to the conservation of resources.

The elevated levels of total protein in wild-breeders compared to wild non-breeders is congruent with what was found in chapter 2 where levels of total protein within the mucus of breeders were significantly higher than in non-breeders. As previously discussed in chapter 2, the difference in total protein levels between breeders and non-breeders is likely due to a large array of protein based substances such as mucins, lectins and to a minor degree some hormones. Schutz and Barlow (1997) assessed the composition of mucus in the midas cichlid (*Cichlasoma citrinellum*) (a species that like discus fish provide mucus to offspring during the time of first feeding) and found the presence of the three hormones prolactin, growth hormone and thyroid hormone (Schutz and Barlow, 1997). These hormones are known to be multifunctional and several studies have demonstrated their ability to aid in the growth and development of young (Brown et al., 1989; Kang and Chang, 2004; Yamano, 2005) as well as playing a role in aiding osmoregulation (Björnsson, 1997; McCormick, 1995) and energy metabolism in fish (Leung et al., 1991; Sheridan, 1986). Due to the relatedness of this species to discus fish it is likely that the protein based prolactin and growth hormone are potentially contributing to the elevated total protein values seen in breeding discus. One hormone that was present in aquarium-bred discus, albeit at low levels, was the steroid hormone cortisol. This hormone, however, was undetectable in wild breeders and non-breeders possibly indicating that rather than playing a role in parental care, cortisol present in aquarium bred discus may actually be an artefact of the aquarium environment or reflect differences in the stress response of wild versus inbred strains of fish.

As well as hormones giving rise to the elevation in wild parental mucus total protein, the parental provision of sugar binding proteins known as lectins could also contribute. A C-type lectin was identified within the mucus of aquarium bred discus fish in a previous study (Chong et al., 2006) and was suggested as playing a role in antimicrobial

defence due to the ability of lectins to bind to the sugar residues of pathogens and activate the complement system. The importance of providing offspring with a source of parentally derived immunity was also highlighted in chapter 2 where high levels of antibodies (IgM) were found within the mucus of aquarium-bred breeders. This study on wild discus further confirmed the presence of high levels of IgM within the mucus of wild breeders and although not significant (possibly due to the low n number) mucus IgM concentration appears to be highest in wild breeders as opposed to non-breeders. Again, although not significant, there was also a hint that wild breeders had higher IgM concentrations than those seen in aquarium-bred breeders. This suggests that parentally provided immunity may be especially important in wild discus fish, possibly due to differences in their respective environments. Unlike a controlled aquarium environment largely free of disease, the Amazon contains a wide spectrum of pathogens that could pose risks to developing young. At the onset of the dry season discus migrate from within the flooded forest to galhadas, areas consisting of submerged trees and branches (Crampton, 2008). Once the water level drops there can be up to a hundred or so discus fish confined to these small areas (Crampton 2008). During this time individuals will pair up and breed but will stay within ‘galhadas’ until the waters have risen during the onset of the wet season. The still moving nature of the water combined with this group living environment could pose several problems for developing young as these conditions are known to increase the transmission of pathogens (Hughes et al., 2002; Poulin, 1999; Trivers, 1985). Young could, therefore, be challenged before their own adaptive immune system has developed leading to a significant drop in survival rates. It, therefore, seems likely that there would be a significant selection pressure for parents to provide offspring with a passive form of immunity during this delicate period, a mechanism that may have been observed in both wild and aquarium discus fish. Interestingly IgM values within the mucus of wild non-breeding discus were also very

similar to that of aquarium-bred breeders with the IgM appearing to contribute to the a large proportion of the total protein. During the sampling of wild non-breeders it was apparent that the vast majority of fish had scars and a high degree of epidermal damage; the presence of high levels of mucosal IgM may, therefore be required to help facilitate the prevention of bacterial colonisation at these sites of damage and be essential for preventing serious infection.

As well as finding evidence that wild discus are potentially negating the threat of pathogens by providing IgM to their offspring, this study also highlights the potential for parents to negate the danger of living in a dilute acidic environment through the mucosal provision of essential ions. The ions Na^+, K^+ and Cl^- were all 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 (Table 1) as the concentration 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}; Table 1) were higher than that 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}; Table 1). The greater 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 a 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 being used for the active uptake of ions (Gatlin et al., 1992).

While the provision of essential ions could help offspring mitigate the ion poor environment the provision of Cu within wild breeder mucus could act as a useful dietary supplement for offspring. Wild breeders had a significant elevation in mucus Cu concentration relative to wild non-breeding discus and while high concentrations can be highly toxic to fish (Handy, 1996) small quantities of Cu can function as an essential micronutrient. The quantity of Cu within wild breeder mucus only reached a concentration of 32.37µg l^{-1} which is considerably lower than the dietary requirement of most teleost fish, a requirement which ranges from 16-730 mg Cu kg^{-1} dw feed depending on the species, feeding regime and life stage (Clearwater et al., 2002). The ingestion of Cu by fry at the concentrations found in this study is, therefore, likely to be beneficial as Cu at low concentrations is important in cellular respiration as well as being a co-factory for over 30 different enzymes (Linder, 1991).

The sequestering of Cu within wild breeder mucus relative to wild non-breeders was also observed with the metal Hg. Hg unlike Cu is not an essential micronutrient in living organisms and can be extremely toxic in fish with low levels adversely affecting reproduction, ultimately leading to death (Eisler, 1987; McKim et al., 1976; Roelke, 1990). Previous work has demonstrated that muscle concentrations of 10-20 µg Hg g^{-1} or more is lethal and that 1-5 µg Hg g^{-1} is sublethal to fish (McKim et al., 1976; Niimi and Kissoon, 1994). Wild breeding discus mucus in this study contained 0.003 µg Hg g^{-1} a thousand fold lower than the sub-lethal dose. Due to the lack of work done on Hg dose rates in Amazonian fry it is difficult to say with certainty what constitutes a safe dose. It is, however, interesting that breeding fish appeared to sequester higher levels of the metals Hg and Cu than non-breeders. While the concentrations of metals within parental mucus were low, the discus fish sampled in this study were taken from sites that were to my knowledge un-contaminated. A recent rise in industry associated with

the Amazon has been associated with a notable rise in the concentrations of metals such as mercury (Hg) (Uryu et al., 2001), copper (Cu) and cadmium (Cd), resulting in certain sites becoming heavily contaminated (Matsuo et al., 2005). If discus were sampled from sites close to areas of contamination it would be interesting to see just how much contaminant could potentially accumulate in mucus as well as looking at the trophic transfer of contaminants from parental mucus through to fry.

3.6 Conclusion

The mucophagus feeding strategy of discus fish, as described in both the current chapter and chapter 2 has most likely evolved over a large period of time to counteract the environmental problems of living in a confined, ion poor, pathogen rich environment. Differences between wild breeders and wild non-breeders were congruent with what was seen in aquarium bred discus (Chapter, 2). The observed differences between wild and aquarium bred discus were most likely a result of a greater range of selection pressures acting on wild fish; selection pressures which include coping with a wide range of pathogens as well as the osmotic challenge of living in an ion poor acidic environment. In this study the concentrations of Cu and Hg within parental mucus were low, with the presence of Cu most likely being beneficial and acting as a dietary supplement in offspring. The ability of parental mucus to possibly sequester metals and other contaminants indicates a potential for trophic transfer of contaminants from parents of offspring highlighting a potential vulnerability in the parental care behaviour. Future work looking at this potential mechanism would, therefore, be desirable.

<u>Further studies</u>

As a result of this work, I was involved in a further study looking at transfer of contaminants to offspring via parental mucus (see Maunder et al., 2011).

Chapter 4: Investigation of novel offspring adaptations to the parental care strategy of the discus fish

Elements of this chapter have been published in Aquatic toxicology: Maunder, R. J., Buckley, J., Val, A. L. and Sloman, K. A. (2011). Accumulation of dietary and aqueous cadmium into the epidermal mucus of the discus fish Symphysodon sp. Aquatic Toxicology 103, 205-212.

4.1 Abstract

While the behaviour of parents is often seen as key in facilitating the provision of parental care, the development of specialised structures in young can often aid parents in their provision of care. In several aquatic species, offspring have evolved a range of transitory structures including specialized dentition used to access parentally-provided food, and cement glands that allow parents to position newly hatched offspring to substrates that are easy to protect. The Amazonian cichlid *Symphysodon* spp. commonly known as the discus fish, is unusual among teleost fish in that parents provide offspring with a substantial amount of care, including both the provision of mucus for the first few weeks of development, as well as protection from predation. Here I investigated the adaptations of discus offspring that could aid parental care behaviour in this species, focusing on dentition and mouth structure of offspring and structure of the cement gland. Similar to that observed in other cichlids, the cement gland in discus fish comprised of six hemispherical volcano protrusions that progressed in a manner previously documented in tilapia and angelfish. Conical unicuspid teeth were present on both the premaxilla and lower jaw of offspring that could enable fry to easily grip and tear off mouthfuls of mucus. The mucus volume consumed by offspring was calculated using the bite rate of offspring in conjunction with mouth size measurements, indicating an initial consumption of 3 µl mucus per day rising to 763 µl mucus consumed per day by the 17th day post fertilization. The benefits of mucus consumption to fry appeared substantial with offspring experiencing an initial specific growth rate of 28.94 (% per day) over the first week of parental care, while gaining an increase in mass of 54.15 mg over the three week period of parental care. Results from this study provide useful information in regards to the development of discus offspring as well as the costs and benefits associated with mucus feeding that underpin the parent offspring conflict in this species.

4.2 Introduction

Parental care is often assumed to be a process actively driven by the behaviour and physiology of the parent while offspring remain passive participants taking in the provisions allocated by parents. Trivers (1974), however, noted that the degree of relatedness between diploid, out-bred parents and offspring is only 0.5 indicating that offspring should be active participants in obtaining parental care. A whole host of experimental studies have since demonstrated that offspring have evolved a range of behavioural and physiological tactics to both co-operate and cause conflict over the act of parental care (Clutton-Brock, 1991). Some of the most well known offspring adaptations to parental care involve the behavioural response of begging as a means to elicit parental care, a behaviour seen in a wide range of mammalian and avian species (Wright and Leonard, 2002). Although behavioural adaptations are often the most prevalent method by which offspring can obtain care, offspring can also have interesting developmental structures that allow parents to better facilitate the provision of care.

In environments characterized by a limited availability of food, or in species where young are altricial and lack the capacity to digest or obtain complex foodstuffs, parental care may evolve to provide offspring with a novel source of nutrition. This provision can require the presence of offspring developmental structures to help consume the parentally provided food as is seen in *Boulengerula taitanus*. Offspring of this oviparous caecilian have specialized dentition, a row of fetal teeth, which they use to peel and consume the outer layer of skin from their mother (Kupfer et al., 2006). These young are altricial and depend entirely on the transformed lipid rich skin produced by their mother. During one week of maternal care, offspring can substantially increase their total length by about 11%, with average individual growth estimated to be about 1 mm a day.

Substantial growth and development is also seen in the embryos of sharks that are either oophagus or adelophophagus. Female oophagus species of shark, such as the porbeagle (*Lamna nasus*), provide embryos with a supply of eggs which act as a source of nutrition for the embryo throughout the period of gestation. In order to consume the eggs, embryos develop temporary fang-like teeth that are used to tear open egg capsules so that the ova within can be consumed (Francis and Stevens, 2000). Similarly in adelphophagus species such as the sand tiger shark (*Carcharias taurus*), embryos also develop fang-like unicuspid teeth which are used to attack and consume fellow siblings until there is just one embryo left in the uterus; fellow siblings in this instance act as a form of nutrition (Lucifora et al., 2002). In both examples, the development of juvenile dentition allows mothers to provide embryos with a novel, albeit high quality food, that will aid development so that, when born, offspring resemble miniature adults better able to cope with a harsh environment.

Parental care in the Amazonian cichlid *Symphysodon* spp, commonly referred to as the discus fish, is also more ellaborate than that observed in most fish as parents protect offspring for several weeks while providing them with a source of nutrition in the form of nutrient rich mucus (Hildemann, 1959; Noakes, 1979). Unlike discus fish, the vast majority of fish receive no parental care (Gross and Sargent, 1985) and have to begin finding their own food, usually of planktonic origin, once they have hatched and their yolk sac has run out. Planktivory in these species is thought to be aided by the presence of a set of first generation jaw teeth used to trap prey. Although the morphology of jaw teeth in adult fish can vary greatly (Barlow, 2002), the morphology of first generation teeth in all bony fish studied so far has been the same in that teeth are unicuspid, conical and present on both the premaxilla and lower jaw (Sire et al., 2002). The first feed of discus fish, however, differs from the planktivorous prey consumed by most fish in that it consists of a viscous mucus layer. This raises the interesting question of whether

newly hatched discus offspring require a specific dentition, to adequately consume parental mucus secretions.

The development of specialized dentition in *B. taitanus*, *C. taurus* and *L. nasus* allows offspring to make large gains in growth through the utilization of novel, parentally provided nutrition. Large gains in growth and development are also noted in discus fish consuming parentally provided mucus. It is not clear, however, how great the gains in growth are and just how much parental mucus is consumed over the period of parental care. Previous research has reported feeding rates of discus offspring (Buckley et al., 2010; Chong et al., 2005) which if coupled with an estimate of bite size and offspring growth rate could be used to discern an estimate of mucus intake over the period of parental care. Previous work also suggested the presence of a weaning period in discus fish, a behaviour associated with the parent offspring conflict which involves parents reducing their investment in young when the energetic costs of parental care begin to outweigh the benefits of an increase in fitness. Understanding both the costs of parental care to adults in terms of the quantity of mucus produced and the benefit to fry in terms of fry specific growth rate (SGR) could allow inferences to be made about the dynamics of the parent offspring conflict in discus fish as well as the importance of mucus feeding as parental care strategy.

As well as providing offspring with a source of nutrition, parental care strategies can include behaviours that allow parents to position offspring in favourable areas away from predators and environmental perturbation. In a range of aquatic species with a larval phase, this parental care behaviour is often aided by the development of transitory structures in larvae termed cement glands. A variety of structurally different cement glands are found in a wide range of teleost, ascidian and anuran species (Britz et al., 2000; Nokhbatolfoghahai and Downie, 2005; Pottin et al., 2010) where they function primarily to attach larvae to a substrate via the secretion of a glue-like substance.

Although the function of the cement gland is similar across species the structure and position of this gland is often quite different and can take the form of a single flat gland (Pottin et al., 2010) a series of individual attachment cells (Britz, 1997; Britz et al., 2000; Britz and Kottelat, 1999; Jones, 1940) or more complex multicellular glands that can consist of several volcano shaped protrusions (Britz, 1997; Britz and Kottelat, 1999; Groppelli et al., 2003; Jones, 1940; Meijide and Guerrero, 2000). This structure is only transitory and after a few days of larval development the structure normally regresses back into the body (Groppelli et al., 2003). In species that provide protection to eggs and larvae, the development of a cement gland in larvae can enable parents to manoeuvre offspring to areas that are more easily protected from predators. In the African pike (*Hepsetus odoe*) parents deposit young within a foam nest. Once hatched embryos suspend themselves below the nest via a cement gland and remain in the vicinity of the spawning site until they have reached a relatively advanced stage of development. This allows parents to efficiently guard the nest and provide protection to their offspring (Merron et al., 1990). Cement glands are particularly common in cichlids and have been detailed previously in a range of substrate brooders (Brinley and Eulberg, 1953; Grier, 1981; Groppelli et al., 2003; Hamlett, 1990; Jones, 1972; Jones, 1937; Kuwamura and Mihigo, 1988; Pottin et al., 2010). In these species, cement glands present in newly hatched larvae are utilized by parents to position larvae into a grouped mass that can be easily protected from predators. Similarly to the aforementioned cichlids, discus larvae are attached to substrates immediately after hatch. Reports from the discus aquarist trade indicate that if disturbed parents will regularly transport and reattach larvae to a vertical substrate in a more secluded area of the aquarium away from the perceived disturbance. The structure used by discus larvae to attach to substrates has so far gone unobserved and, although it is likely to be similar to that seen

in other substrate brooding cichlids, observations are required to detail and track its development.

The aims of this study were twofold: to firstly examine the mouth of fry in order to establish whether specialized dentition was present as well as gaining an estimate of mucus consumption so that combined with larval growth rates the importance of this form of nutrition could be ascertained. Secondly, the structures located around the head that allow larvae to attach to substrates were described, so that comparisons could be made with other substrate brooding cichlids.

4.3 Materials and methods

4.3.1 Experimental fish and husbandry

Brood stock of adult discus fish (*Symphysodon* spp.), originating from a captive bred strain in Malaysia were obtained from a commercial dealer (Will Penman, Plymouth imports, UK) and transported to the aquarium facilities of the University of Plymouth. Fish were quarantined, wormed (Kusuri discus wormer, Newton Abbot) and then held in groups of 12 in 100 l glass tanks and observed for reproductive behaviours. Once mating behaviour was observed and a breeding pair had formed, each pair was removed from the group tank and placed in a separate individual 100 l glass tank complete with a breeding cone. Fish were kept in recirculation systems held at constant conditions (temperature mean ± SE: 29 ± 0.5^{o}C, pH: 7.0 ± 0.5, dissolved oxygen: 99 ± 0.5%, 12h:12h L:D photoperiod, Ca^{2+} 19.03 ± 1.01 mg l^{-1}; Na^{+} 11.32 ± 0.62 mg l^{-1}; K^{+} 2.07 ± 0.04 mg l^{-1}; Cl^{-} 12.35 ± 0.99 mg l^{-1}) and fed a commercial pellet (Tetra prima granular, Tetra (UK) Southampton) feed once daily to satiation. Spawning was initiated via the replacement of two thirds tank water with fresh colder water (26 ± 0.5^{o}C) on a daily basis, a stimulus known in the aquarium trade to induce breeding in Amazonian cichlids. Once eggs had been laid on the breeding cone parents were allowed to care for

their eggs and raise their offspring as normal. The only feed available to fry over the period of parental care involved the mucus produced by both parents. A total of six pairs were used; fry from three pairs were used for histology and scanning electron microscopy and another three pairs were used to ascertain the growth rate of fry.

4.3.2 Scanning Electron Microscopy

The moment of fertilization was taken as 0 h with samples of developing discus fish being taken at the following time points; 3, 4, 5, 6 and 7 days post fertilization (dpf). A total of three fry were taken from one pair at each time point, killed by an overdose of anaesthetic (MS222; 200 mg l^{-1}) and then transferred to chilled fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer with 3.5% sodium chloride) for 48 h prior to further processing. Fry were then dehydrated through a graded ethanol series ranging from 35% through 50%, 70% and 90% to absolute ethanol, prior to desiccation using the critical point drying method (Platt, 1977). Fully desiccated fry were subsequently mounted on a specimen stub using a carbon tab, and coated with ca. 8 nm of gold in an Emitech K 550 sputter coater (working at approximately 5×10^{-6} Torr). The processed specimens were investigated and photographed using a JEOL JSM 5600 scanning electron microscope operated at 15 kV, and a 15 mm working distance.

4.3.3 Determination of mouth size

Approximate mouth height, width and depth (μm) were calculated for fry on 4, 5, 6 and 7 dpf; measurements were not carried out at 3 dpf as there was no recognizable mouth structure present. Height, width and depth measurements of the mouth were taken using lateral and dorsal electron micrographs. To standardise measurements, recognizable set points on the mouth were chosen so that consistent measurements could be made across the 4 days of development. The width of the mouth was calculated as the widest measureable part of the mouth (Fig. 23A). The height of the mouth was measured from

the bottom of the lip on the lower jaw closest to the microscope, through to the top of the lip on the premaxilla via a straight line (Fig. 23B). The depth of the mouth was measured from the far edge of the intersection where the top and bottom lip meet through to the furthest protruding part of the lip (Fig. 23B). Electron micrograph images of the mouth were assessed using Corel Paint Shop Pro (Ottawa, Canada); the zoom function allowed images to be magnified so that a standard 15 cm ruler could be placed against the monitor to record a measurement which could then be converted using the electron micrograph scale bar.

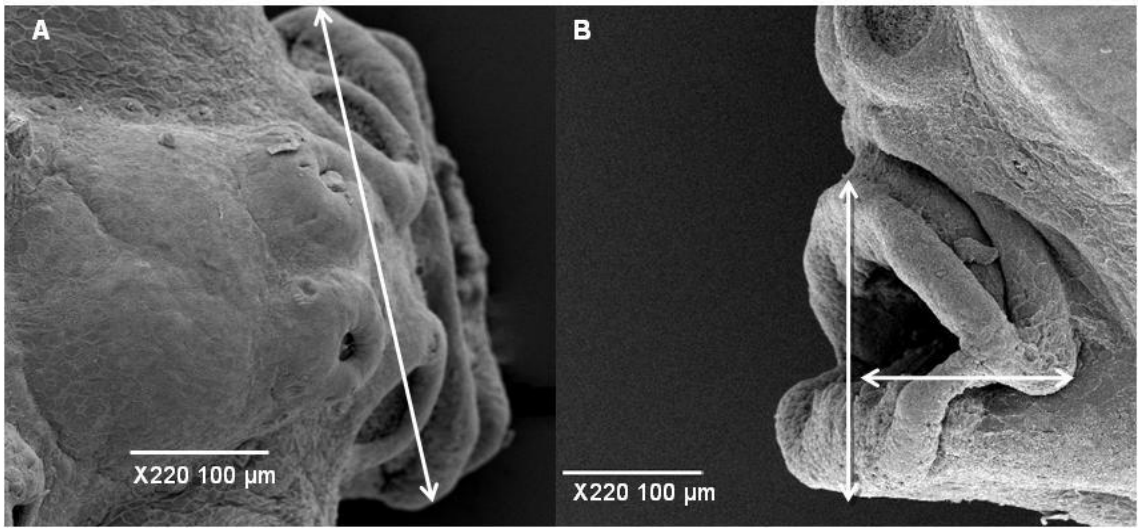


Fig. 23. Dorsal (A) and lateral (B) scanning electron micrographs used to measure the width (A), depth and height (B) of the discus larvae mouth. Arrows depict the lengths measured.

The volume (V, in µl) of a fry's mouth was approximated to a cone with an elliptical (Fig. 24) base and calculated using the formula:

$V = {}^{1}/_{3}\pi hwd$

where h= height, w = width and d = depth.

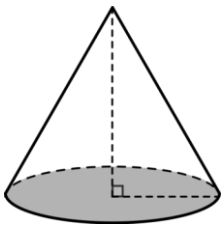


Fig. 24. Diagramic representation of the cone shape that was used to approximate the bite of discus fry.

A total of three fry were measured at each time point to obtain a mean ± SE for the volume of the mouth. Using data available on average length of fry over the 21 day breeding period and our previously published data on average number of bites performed by fry over the breeding period (Figure 3 in Buckley et al. 2010) it was possible to calculate the approximate consumption of mucus by a single fry over 24 h on each day of the breeding period. To take into account the fact that fry do not feed constantly over a 24 h period, and to prevent an overestimation of mucus consumption, values were divided in half. Due to work in chapter 2, where the quantity of protein per ml of mucus was ascertained, it was also possible to back calculate the total quantity of protein potentially consumed by fry over the three week period of parental care.

4.3.4 Specific growth rate of discus fry

A total of three breeding pairs were allowed to raise their young over a period of three weeks. During this time fry were sampled on a weekly basis with the first sample being taken on the day when fry began feeding from their parents. Samples were then taken at weekly intervals until the third week post first feed. A total of 10 fry were gently removed from their parents using a conventional aquarium net, blotted lightly and transferred to a tared 10 ml beaker where they could be accurately weighed via an analytical balance to the nearest 0.01 mg before being gently transferred back to the tank with their parents. The total mass recorded was then divided by the number of fry

in the beaker so that the individual mass (mg) per fry could be ascertained. Behavioural observations were carried out to ensure that the reintegration of fry did not result in a change in parental behaviour. The mass data obtained was then used to generate the specific growth rate of fry over the three weeks of parental care using the equation below as per Fagbenro and Jauncey (1995).

Specific growth rate (SGR) [% growth per day] = 100 x ((In. final mass of fish – In. initial mass of fish)/trial length in days)

4.3.5 Histology

A total of two fry were taken every day from 3 dpf through to 31 dpf and killed via an overdose of anesthetic (MS222; 200 mg l^{-1}) before being transferred to 10 % formal saline fixative and stored for 48 h. Whole specimens were then processed and put through a graded series of solutions beginning with 70% ethanol for 18 h, 90% ethanol for 2 h, IMS for 2 h, absolute alcohol for 2 h, xylene for 2 h and finally hot wax for 3 h. Once samples had been stored in hot wax for 3 h they were embedded in paraffin in a sagittal orientation. As soon as the wax had hardened, sagittal sections were cut at 7 µm with a Leica RM2235 microtome. Once the sections had been cut, they were then mounted onto a slide via a water bath before being stained with haematoxylin and eosin. Once stained, slides were mounted with a coverslip using DPX and left to dry for 24 h. The dentition of discus larvae was observed with a light photomicroscope at x 4, x 20 and x 40 magnification.

4.3.6 Determination of cement gland size and development

The three pairs of structures that comprise the cement gland were each assigned a number (Pair 1, Pair 2 and Pair 3) (Fig. 25), tracked and measured daily over the five day period (3 dpf through to 7 dpf). A total of three fry were used at each time point to obtain a mean ± SE for the height and width of the cement glands that comprise pairs 1,

2 and 3. The two structures that comprise a pair were both measured for their height and width so that a total of two height and width measurements were available for each pair per day. Due to the orientation of the head at 3 dpf it was not possible to calculate the width of cement glands belonging to pair 3. Electron micrograph images of the cement glands were assessed using Corel Paint Shop Pro (Ottawa, Canada) as before. Height and width measurements were standardised by measuring five points that cover the widest and highest parts of the structures (Fig. 26). Dorsal electron micrographs were also used to measure the distance between the two cement glands that comprise a pair (Fig. 27); this measurement was done on each of the three fry, per day, over the 5 day period.

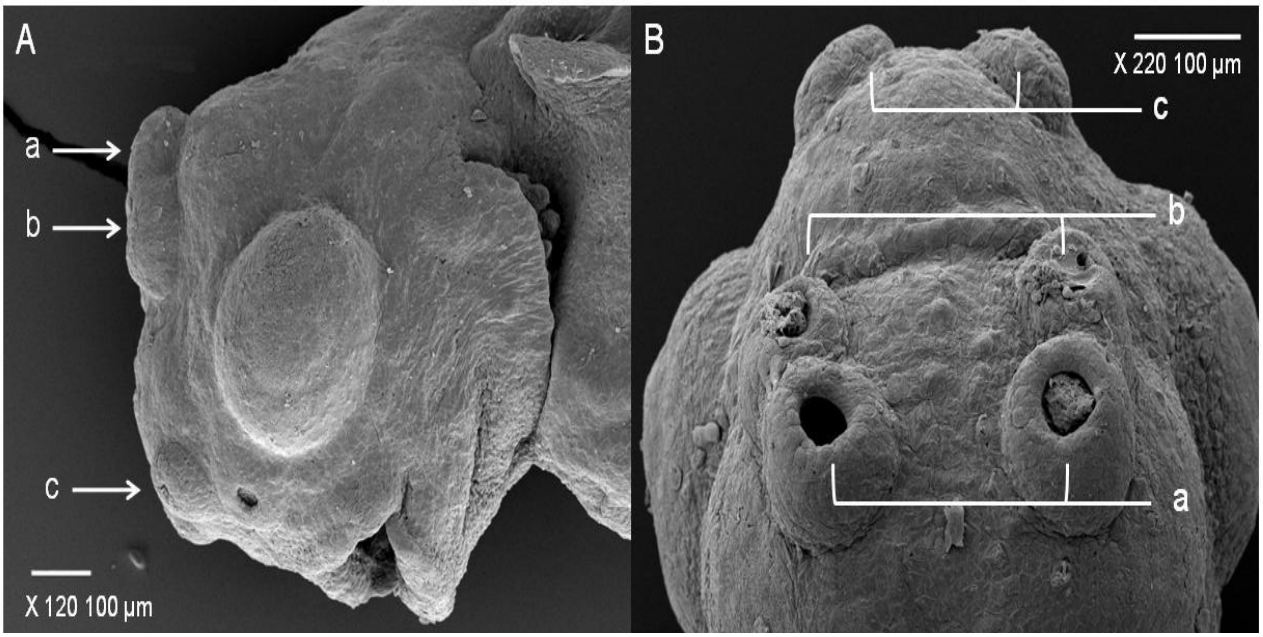


Fig. 25. Lateral (A) and dorsal (B) scanning electron micrographs of day 4 post fertilization *Symphysodon* spp. larvae highlighting the distribution of pair 1 (a), pair 2 (b) and pair 3 (c) of the structures that comprise the cement gland.

4.3.7 Statistical analysis

All data analysed was checked for normality and heterogeneity using a Kolmogorov-Smirnov and Levene's test respectively and conformed to parametric assumptions. A

one-way ANOVA was used to look at differences over time in both the dimensions of the cement gland and the growth of fry. A one way ANOVA was also used to compare the height, width and distance between cement glands, among the three different cement gland pairs on each day. Where significant differences were observed, a Bonferroni post hoc test was used. The level of significance for all tests was taken as $P<0.05$.

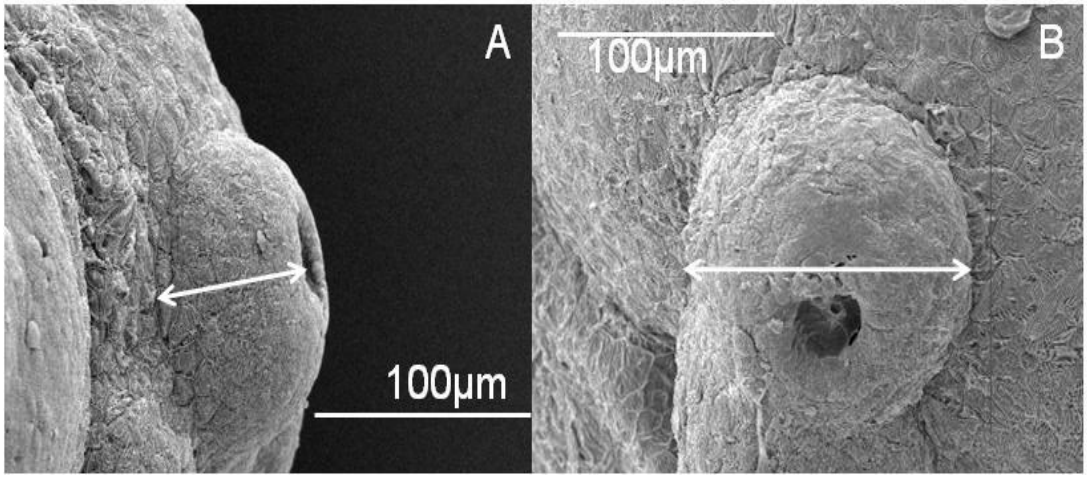


Fig. 26. Scanning electron micrographs used to measure the height (A) and width (B) of the structures that contribute to a functioning cement gland. Arrows depict the lengths measured.

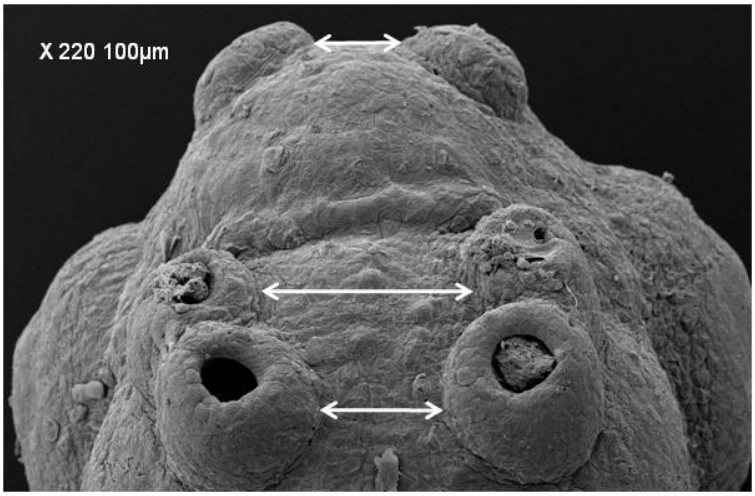


Fig. 27. Dorsal scanning electron micrograph used to measure the distance between the individual cement glands that comprise pair 1, pair 2 and pair 3. Arrows depict the three distances measured.

4.4 Results

4.4.1 Mouth structure

The moment of fertilization was taken as 0 h with the first samples taken at 3 dpf (the time point discus eggs are known to hatch). After hatching, larvae remain attached to a vertical substrate via a structure on their head for a further 3 days; although not free swimming, larvae swing their tails as if swimming. During this period larvae utilize egg yolk stores until the rapid swinging of their tails leads them to break free around 5 or 6 dpf. At this point larvae swim toward their parents where they can begin feeding on mucus. The presence of specialized mucophagus dentition in discus larvae was assessed through a combination of standard histology and SEM analysis. Observations across the initial 5 days (3 to 7 dpf) revealed the opening of the mouth at 4 dpf and the continued development of this structure over the next four days (Fig. 28.A-E). During the first 7 days of development post fertilization there was a distinct lack of specialized dentition but by 8 dpf a number of canine unicuspid teeth had emerged from the epithelium of the premaxilla and lower jaw representing the first generation teeth in discus fish (Fig. 29 A-F). First generation unicuspid teeth were seen throughout the 23 days measured; by 31 dpf the teeth had become less prominent due to the continued development of the lips (Fig. 29 E and F).

4.4.2 Mouth size

Assuming that the inside of the discus larvae mouth is conical, measurements of the height, width and depth revealed a steady increase in mouth volume across the four days (Fig. 30). The bite volume ascertained from 7 dpf larvae was used in conjunction with the bite rate of fry (detailed in chapter 2; Buckley et al. 2010) to determine that the total volume of mucus consumed by 7 dpf fry over the course of 24 h amounted to 3.01 µl. The bite volume was then scaled up proportionally in line with the measured increased

in fork length across a 21 day period. Steady increases in the consumption of parental mucus can be seen throughout the 21 day period with a peak of 763 µl mucus consumed per day seen at day 17 (Fig. 31). Over the 3 week period it would, therefore, be possible for fry to consume a total of 8564 µl of parental mucus. Using protein per ml data from chapter 2 it was then possible to calculate that out of the total 8564 µl of mucus consumed over a three week period, 48.11 mg of that was total protein.

4.4.3 Growth rate of offspring

The growth rate of discus was linear with time (r^2=0.9737; P=0.0133; Fig. 32A) indicating a steady increase in mass over the three week period. The SGR recorded in fry was highest during the first week of mucus feeding reaching a value of 28. Over the next two weeks a drop in SGR was noted with the SGR of weeks 1 and 2 being significantly higher than that observed in week 3 (One-way ANOVA: $F_{1,2}$=32.165, P<0.05, Fig. 32B).

4.4.4 Cement gland

A total of three pairs of hemi-spherical volcano like protrusions resembling cement glands were tracked over the 5 day period. Two pairs of cement glands were situated next to each other and positioned dorsal to the eye (Fig. 33, B1 and 2); these two cement glands, labelled pair 1 and pair 2 were present at 3 dpf. The third pair of cement glands was located anteriorly next to the nasal pit (Fig. 33 B1 and B2) and was also present from 3 dpf. Throughout the 5 days observed, cement glands in pair 1 were significantly wider on days 5 and 6 than on days 3, 4 and 7 (One-way ANOVA: $F_{1,4}$=17.250, P<0.05, Fig. 34A). There were, however, no significant differences in the width of pairs 2 and 3 over time (One-way ANOVA: $F_{1,4}$=2.117, P=0.170, Fig 32. B; $F_{1,4}$=2.394, P=0.167, Fig. 34C).

Variations in cement gland height, however, were much more prominent with all pairs showing a marked change in height over time. Pair 1 cement glands steadily declined in height over time with cement glands at 3 and 4 dpf being significantly higher than at 7 dpf (One-way ANOVA: $F_{1,4}$= 26.452, P<0.05, Fig. 35A). Similarly pair 2 cement glands demonstrated a gradual decreased in size over time with those at 3 and 4 dpf being significantly higher than those at 5, 6 and 7 dpf (One-way ANOVA: $F_{1,4}$= 74.810, P<0.05, Fig. 35B). Unlike the previous two pairs, pair 3 showed a steady increase in cement gland height over time with the cement glands at 3 dpf being significantly smaller than those at 5 and 6 dpf (One-way ANOVA: $F_{1,4}$= 11.069, P<0.05, Fig. 35C). A subsequent drop in height was then noted with cement glands at 7 dpf being smaller than those at 6 dpf (One-way ANOVA: $F_{1,4}$= 11.069, P<0.05, Fig. 35C).

Although the distance between the cement glands that comprise a gland did not differ significantly over time in either pair 1 (One-way ANOVA: $F_{1,4}$= 3.286, P=0.071, Fig. 36A) or pair 2 (One-way ANOVA: $F_{1,4}$= 3.069, P=0.083, Fig. 36B) there was a trend suggesting that the glands grew closer over time. This was especially apparent in pair 3 where the distance between glands at 4 dpf was significantly larger than the distance between glands at days 5, 6 and 7 dpf (One-way ANOVA: $F_{1,4}$= 63.092, P<0.05, Fig. 36C). By 7 dpf the cement gland structures of all pairs had largely regressed back into the head and were no longer as defined or prominent (Fig. 33, E.1 and 2) as they were during the earlier time points (Fig. 33, B1 and B2). Similar to the observations of cement glands in angelfish (Groppelli et al., 2003) it was also apparent that the cement glands in discus larvae were secreting a glue-like substance which was seen emanating from the cement glands at 4 dpf (Fig. 37).

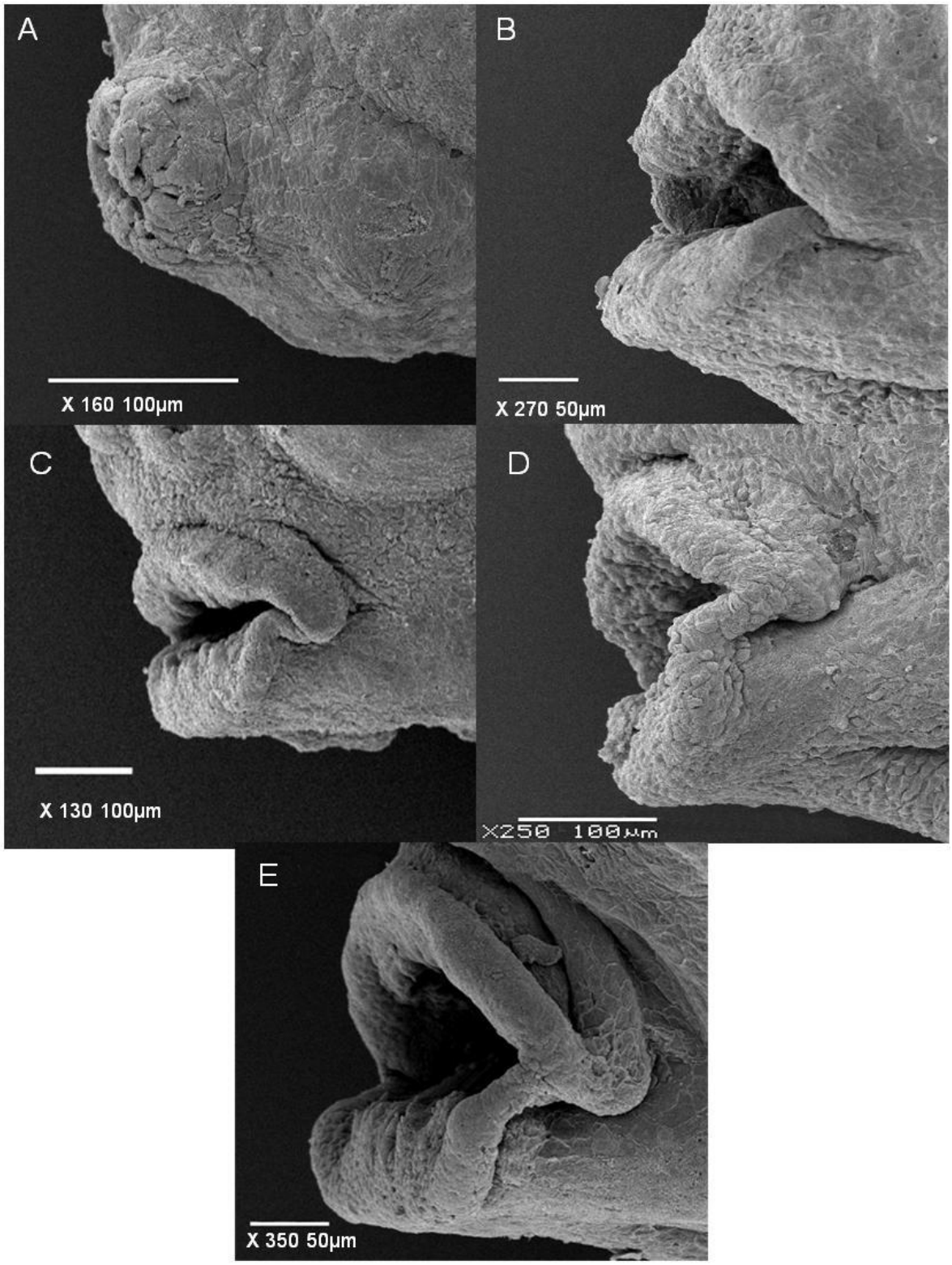


Fig. 28. Scanning electron micrographs showing a lateral view of the developing mouth of *Symphysodon* spp. at the time points 3 (A), 4 (B), 5 (C), 6 (D) and 7 (E) days post fertilization.

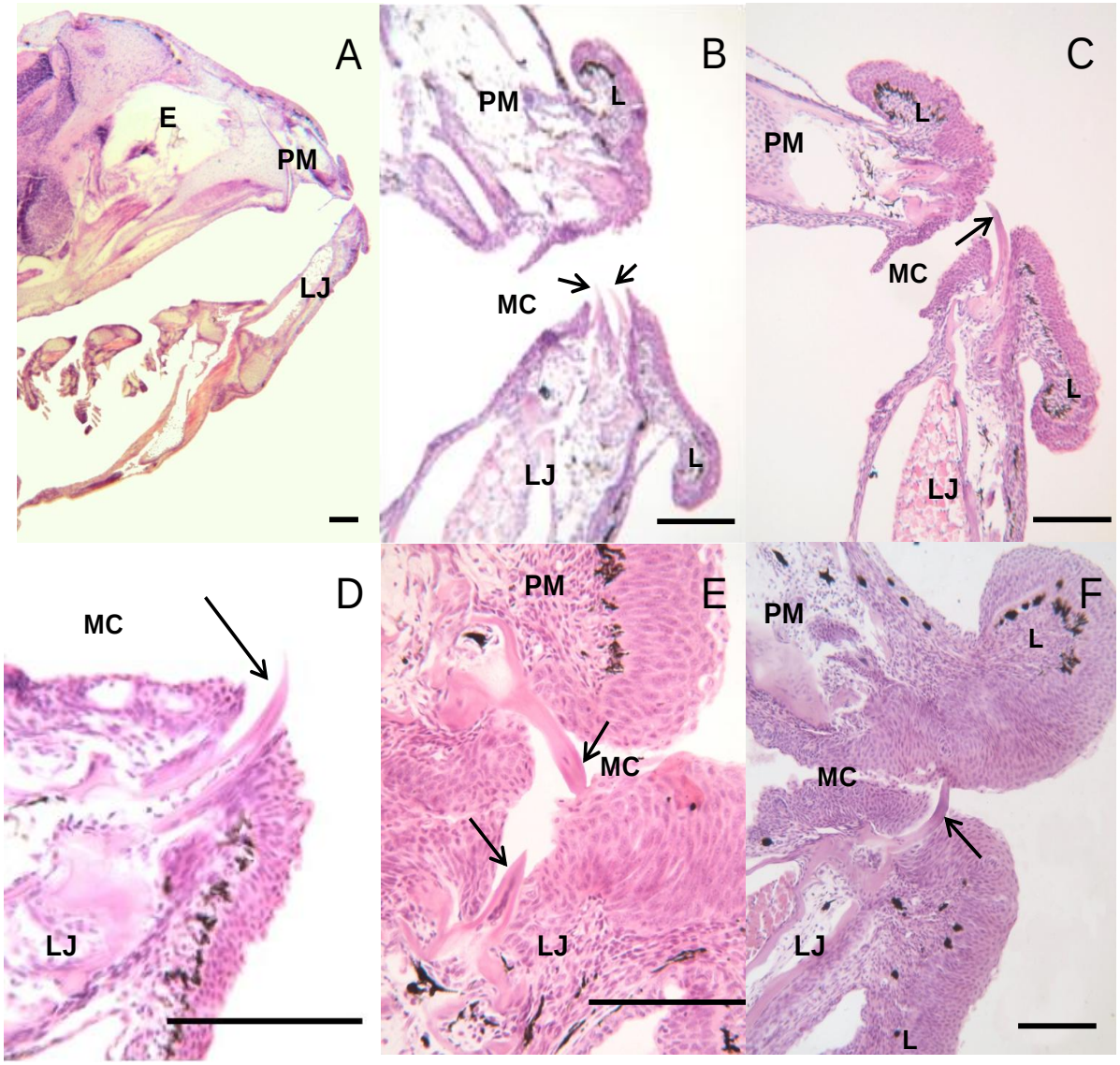


Fig. 29. Sagittal sections of the premaxilla and lower jaw of discus larvae stained with haematoxylin and eosin on days (A) 8, (B) 8, (C) 14 (D) 14, (E) 23, (F) 31 days post fertilization. Scale bar represents 10 µm. Arrows indicate the presence of unicuspid teeth. Abbreviations: E, eye cavity; PM, premaxilla; LJ, lower jaw; L, lips; MC, mouth cavity.

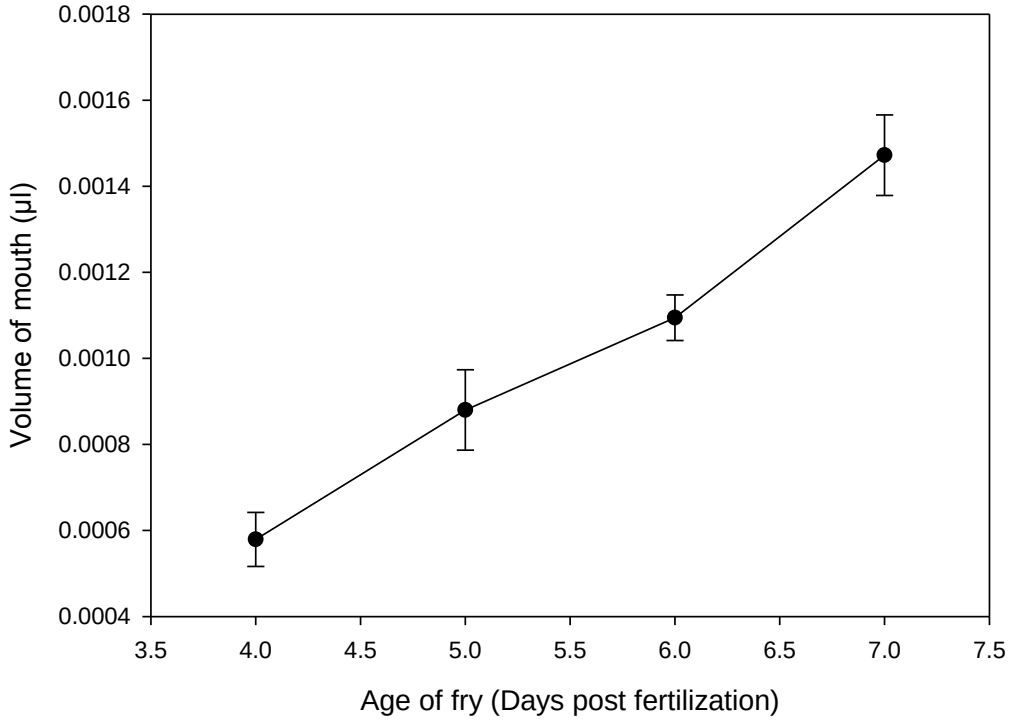


Fig. 30. Increase in the mouth volume (µl) (mean ± s.e.m) of discus larvae (n=3) over a 4 day period from days 4 to 7 post fertilization.

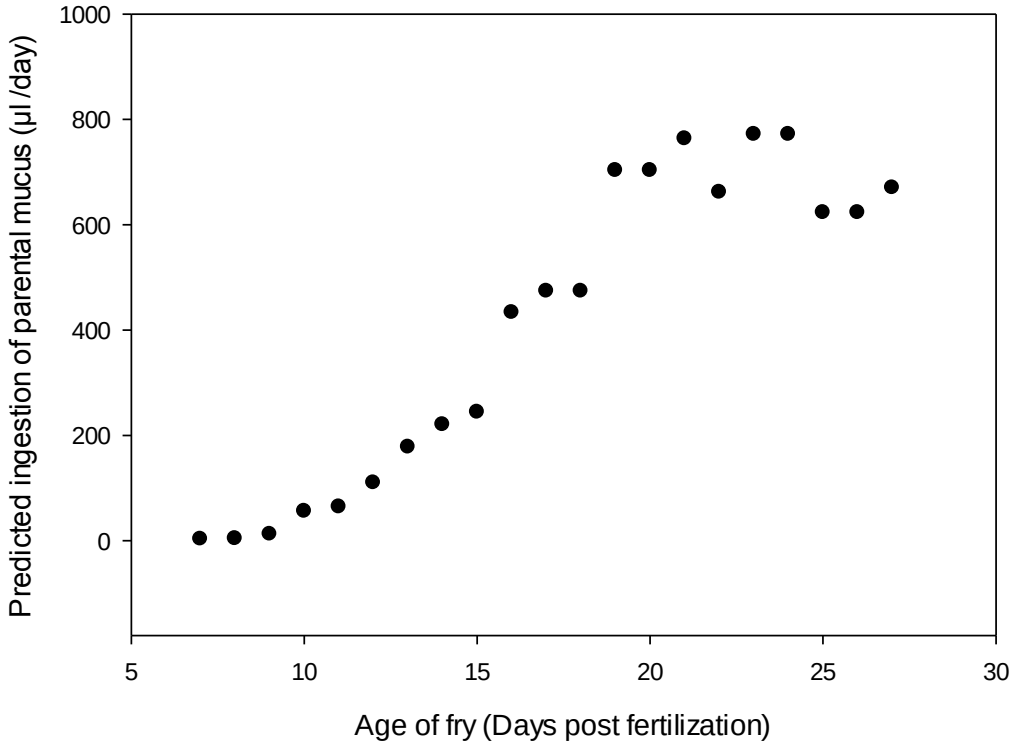


Fig. 31. Predicted daily consumption (µl/day) of parental mucus by *Symphysodon* spp. fry across a 21 day period

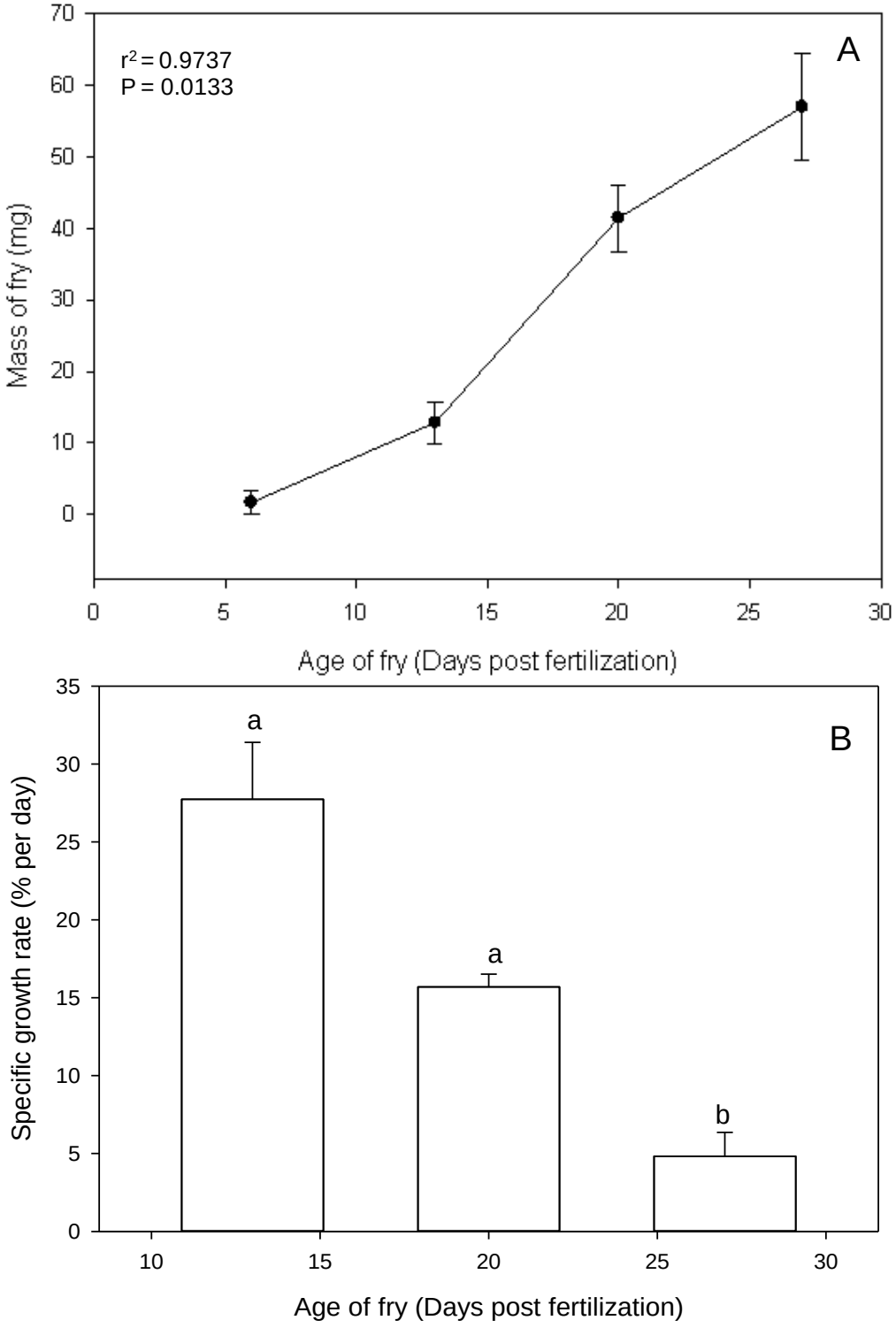
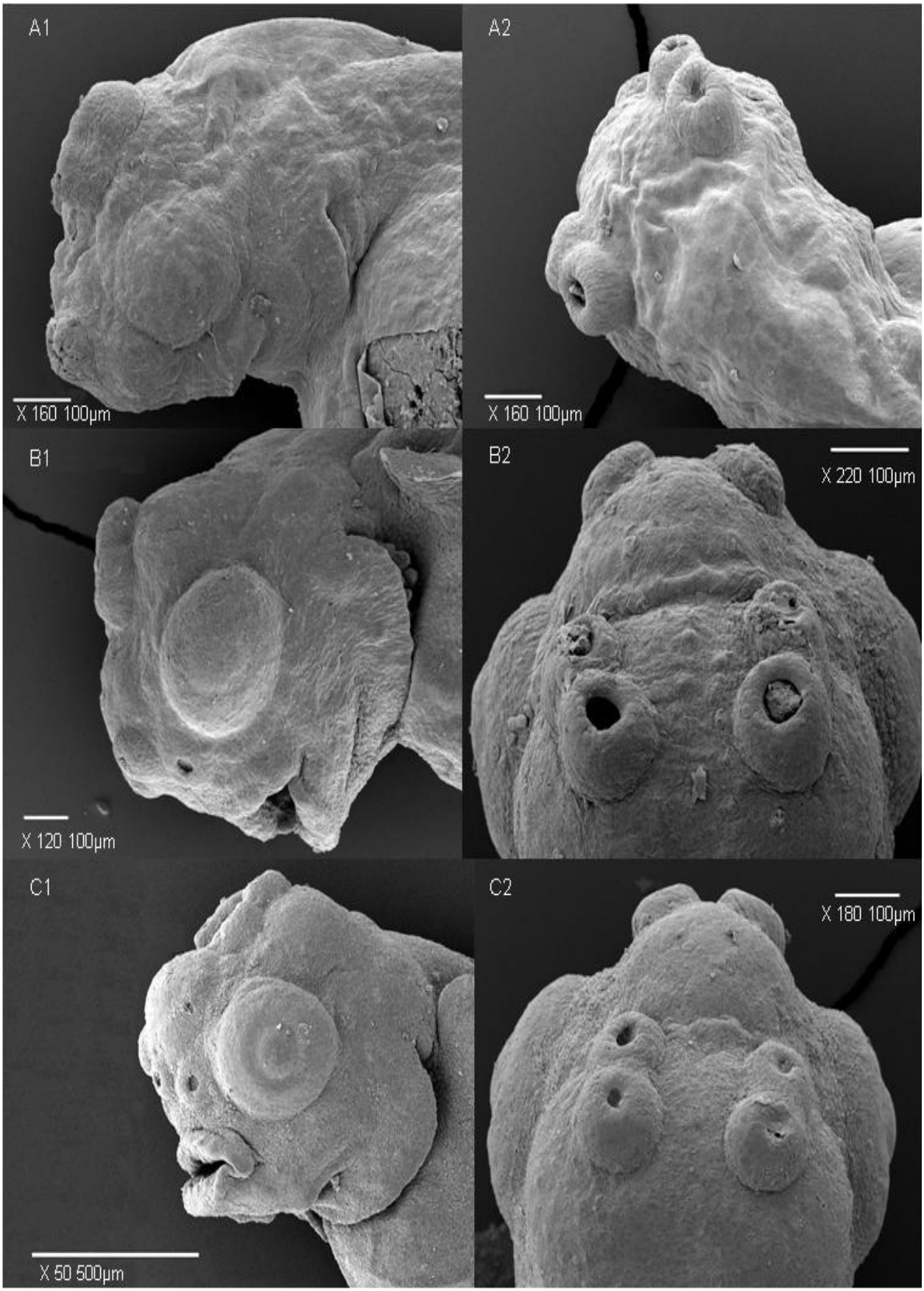


Fig. 32. Increase in mass (mg) (A) (mean ± s.e.m) and change in SGR (B) (mean ± s.e.m) of discus fry (n=30) over a 21 day period covering the period discus fry are known to feed i.e. from 6 dpf to 27 dpf. Difference in letters indicate a significant difference (One-way ANOVA; $p<0.05$) where points that share a letter are not significantly different.


A1
X 160 100µm
A2
X 160 100µm
B1
X 120 100µm
B2
X 220 100µm
C1
X 50 500µm
C2
X 180 100µm

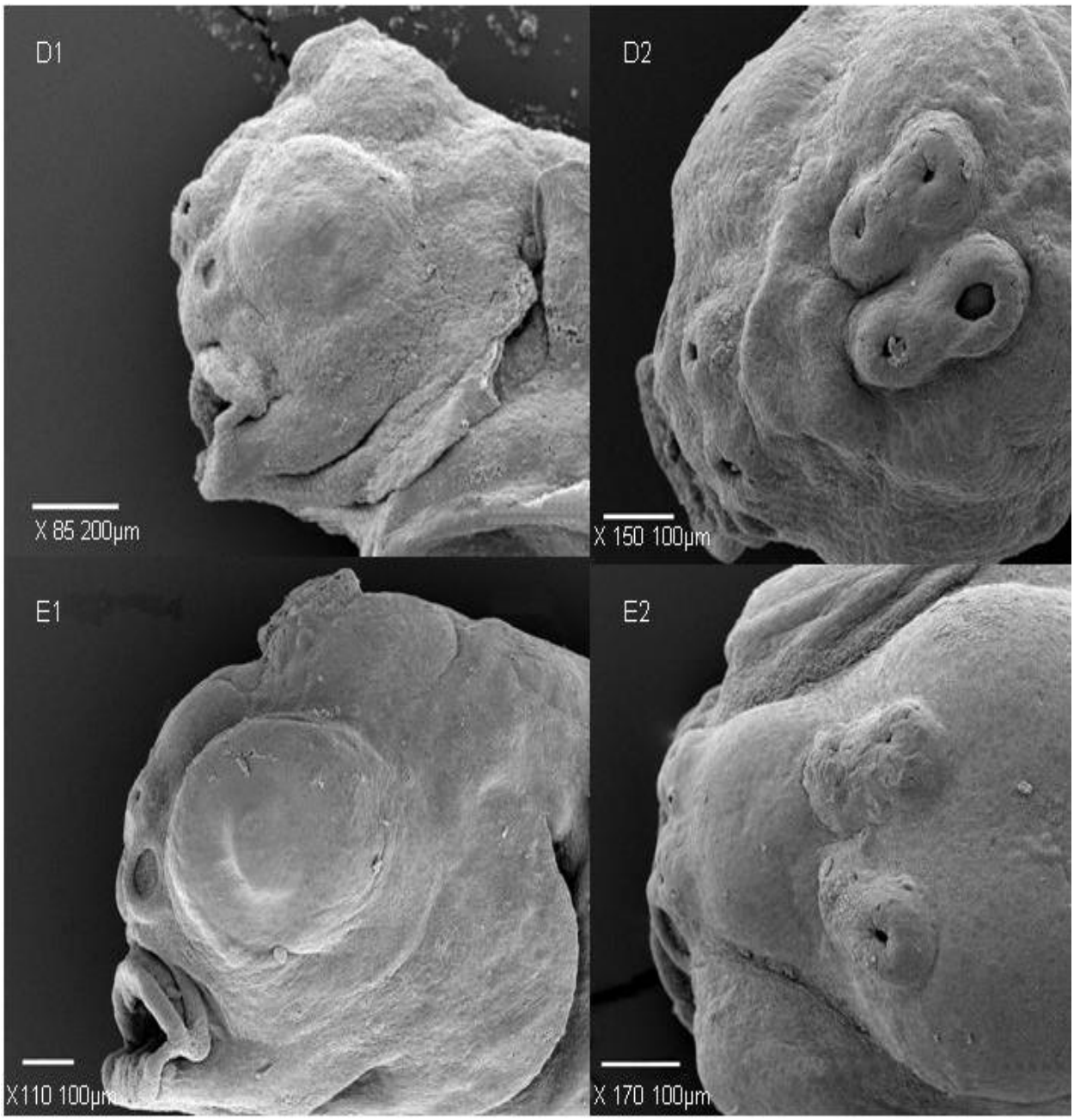


Fig. 33. Lateral (1) and dorsal (2) scanning electron micrographs of *Symphysodon* spp. highlighting the origin and development of three pairs of cement glands on 3 (A1, A2), 4 (B1, B2), 5 (C1, C2), 6 (D1, D2) and 7 dpf (E1, E2).

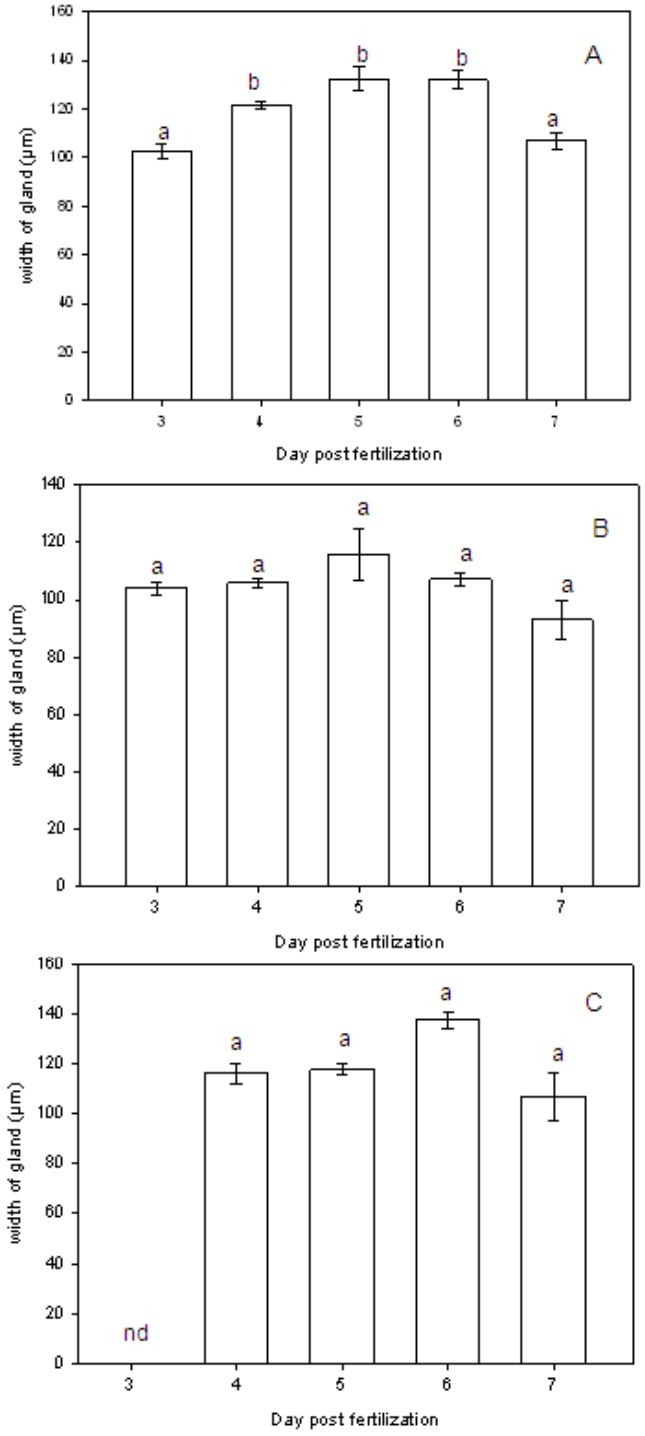


Fig. 34. Width (mean ± s.e.m.) of the paired cement gland structures (n=3) pair 1 (A), pair 2 (B) and pair 3 (C) across the 5 days of development. Differences in letters denote a significant difference (One-way ANOVA; $p<0.05$) where bars that share a letter are not significantly different. Abbreviation: nd, no data available.

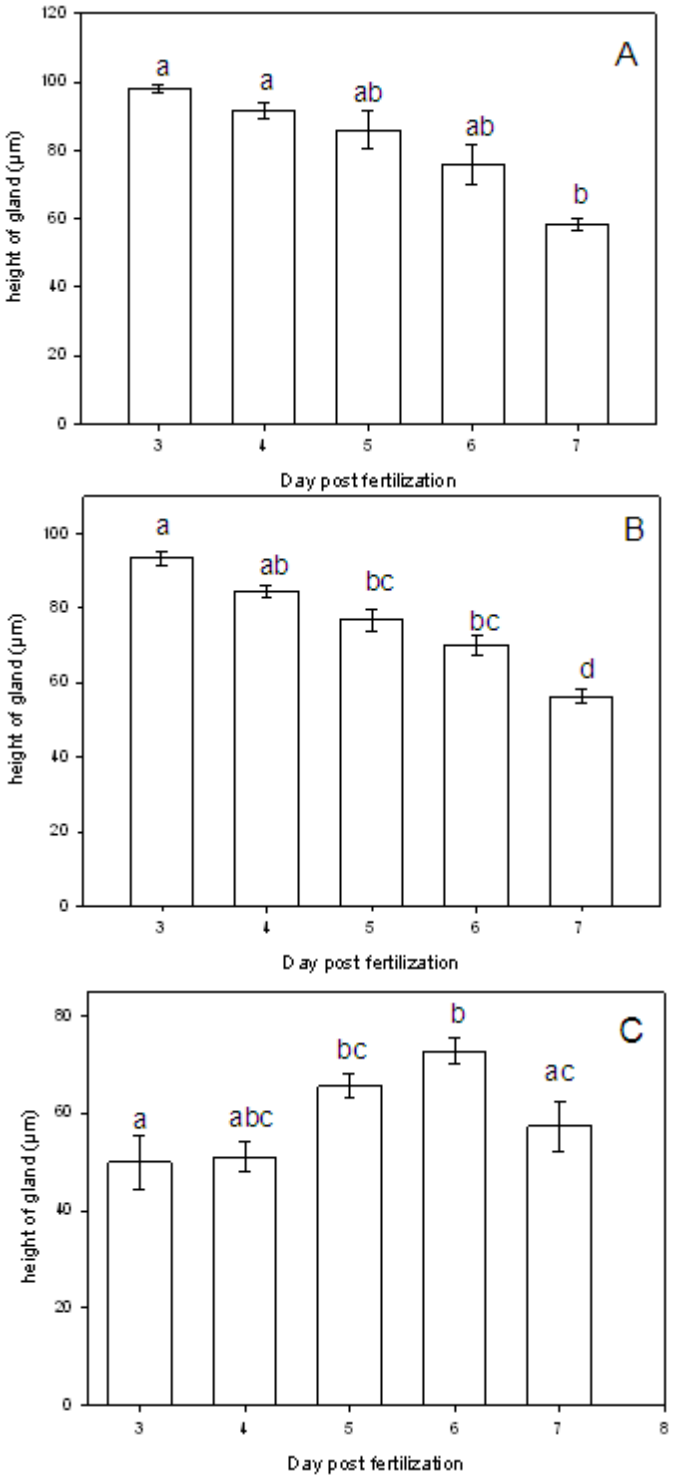


Fig. 35. Height (mean ± s.e.m.) of the paired cement gland structures (n=3) pair 1 (A), pair 2 (B) and pair 3 (C) across the 5 days of development. Differences in letters denote a significant difference (One-way ANOVA; $p<0.05$) where bars that share a letter are not significantly different.

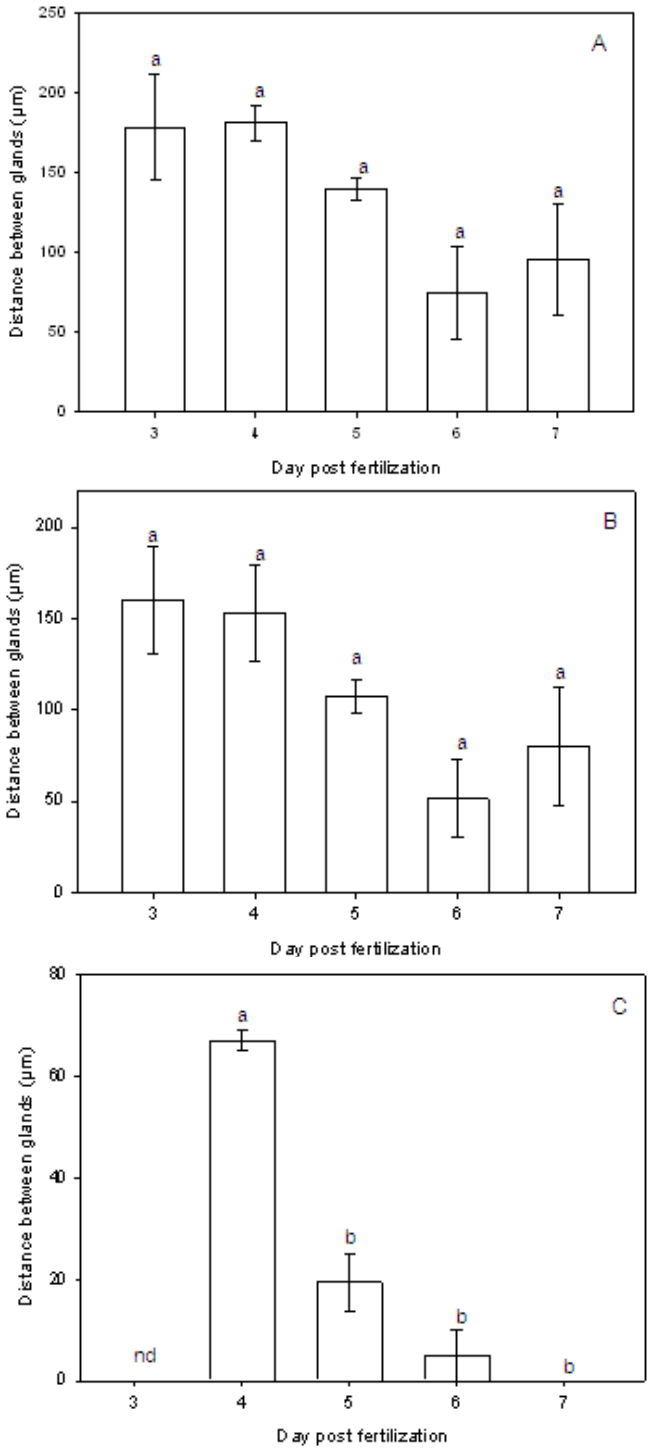


Fig. 36. Distance (mean ± s.e.m.) between the individual cement glands (n=3) that make up pair 1 (A), pair 2 (B) and pair 3 (C) across the 5 days of development. Differences in letters denote a significant difference (One-way ANOVA; $p<0.05$) where bars that share a letter are not significantly different. Abbreviation: nd, no data available.

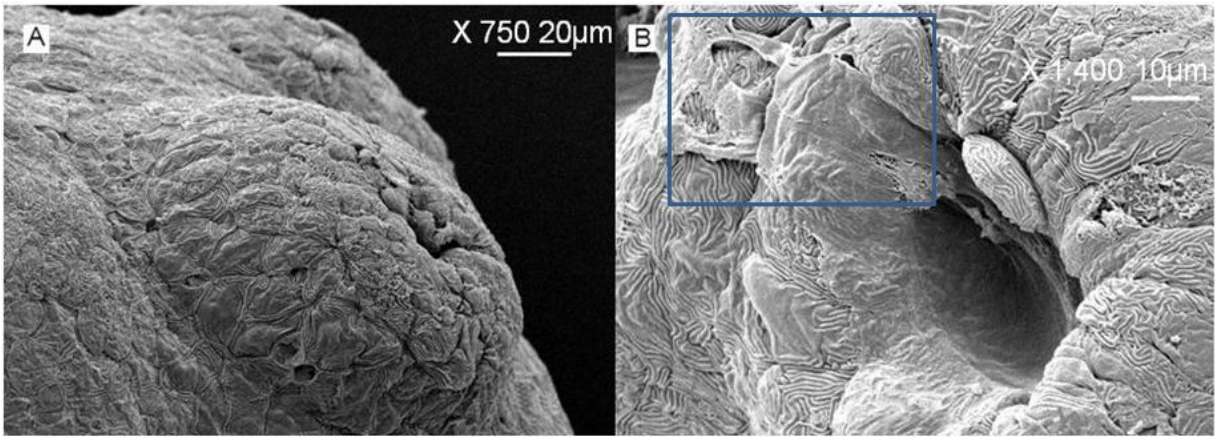


Fig. 37. Electron micrograph of *Symphysodon discus* cement gland at 4 dpf (A). The second image (B) reveals a further magnification of the cement gland highlighting the presence of a secretion around the opening of the structure highlighted via the coloured box.

4.5 Discussion

Teeth of bony fishes are incredibly varied displaying a diverse range of sizes, shapes and numbers (Huysseune and Sire, 1998; Stock, 2001; Trapani et al., 2005). This diversity in bony fish teeth morphology is linked to the wide variety of feeding habits that fish can adopt, a diversity that is exemplified in the cichlids (Barlow, 2002). Tooth morphology in this family can range from widely spaced sharply pointed unicuspids in zooplanktivorous and insectivorous species (e.g. *Cyanoticulapia afra*) to closely packed tricuspids in algal scrapers (e.g. *Labeotropheus fulleborni*) (Streelman et al., 2003). Despite the variety of teeth present in adult fish, the first generation teeth in teleosts have so far always been conical and unicuspid, a morphology thought to aid planktivory, the feeding behaviour by which most fish obtain their first feed. It is, therefore, of particular interest to see whether discus fish had evolved a unique form of first generation dentition to better cope with the consumption of parentally-provided mucus. Similar to that observed in other teleosts, a series of conical, unicuspid teeth were also present in discus fish emerging from the epithelium of the premaxilla and

lower jaw at 8 dpf. This characteristic first generation tooth morphology is even found in species where adults lack oral teeth (Huysseune and Sire, 1998; Kakizawa and Meenakarn, 2003) or when species, such as the cichlids *Metriaclima zebra* and *Labeotropheus fuelleborni* have either bicuspid or tricuspid adult dentition (Streelman et al., 2003) lending weight to the idea that this form of tooth morphology is the norm for all first generation teeth in bony fish (Sire et al., 2002; Streelman et al., 2003). These first-generation teeth were present for the 31 days observed and had not given way to the adult dentition arrangement which involves a reduction in teeth typically consisting of 2-4/1-4 unicuspid slender, apically slightly recurved and pointed cylindrical teeth in the centre of the premaxilla and lower jaw (Kullander, 1986). While a series of rudimentary arranged unicuspid teeth are likely the default for first generation teeth due to their usefulness in trapping planktivorous pray, it is also likely that a mucophagus feeding strategy is aided by the presence of these teeth. Personal observations of mucus feeding behaviour in the aquarium indicate that fry twist their bodies in an exaggerated movement as they remove mouthfuls of mucus from the side of their parents, a behaviour which suggests a level of difficulty to the process. Feeding from a layer of viscous mucus on a relatively flat surface might therefore be aided by a set of teeth which could be used by fry to get some purchase on the mucus before a twisting motion of the body is used to remove a mouthful from the side of the parent. The presence of teeth also explains the high prevalence of epidermal damage seen in parents during the later stages of parental care, damage which may be partly responsible for the initiation of the weaning period in discus fish (Buckley et al., 2010).

The weaning period, a component of the parent offspring conflict, is a behaviour closely associated with mammalian parental care and occurs when parental investment is costly and offspring have developed to a point where any future investment would only offer diminishing returns; at this point parents begin to reduce levels of investment (Weary et

al., 2008). Recent observations suggest the initiation of a weaning period in discus fish during the third week of parental care when the normally attentive behaviour of parents switches to the active avoidance of fry (Buckley et al., 2010). This suggests that at this point a threshold has been reached whereby further parental investment is energetically too costly and no longer offers sufficient benefits to the parent in terms of an increase in fitness. Although part of the energetic cost of parental care in discus fish will undoubtedly come from the mechanical damage caused by fry through the act of mucus feeding, the parental production of nutrient rich mucus could be a significant component of the total energetic cost. An understanding of the cost of parental care in discus fish was attained using a combination of mouth volume and bite rate so that an estimate of the volume of mucus consumed by fry could be gained. Initially the consumption of mucus per fry could potentially amount to around 3 µl per day. While not a high volume, discus can potentially have up to 100 offspring leading to a shoal of fry consuming a maximum of 300 µl over the first day of feeding. As fry develop, so too does their body mass, mouth size and appetite; after a week of growth an individual fry could potentially consume a maximum of 177 µl with a clutch of 100 offspring consuming 17.7 ml of parental mucus a day. At the peak of mucus consumption at 17 dpf individual fry could consume a maximum of 763 µl of mucus with a clutch of 100 potentially consuming a total 76 ml. Even with the burden of mucus production split between both parents, 38 ml of mucus production per parent still appears like a demand that would be hard to meet. Close to the time peak feeding activity is recorded (17 days after first feed) parents are observed actively avoiding the advances of fry showing signs of stress apparent due to a darkening of colour; the energetic cost of feeding offspring at this point may therefore be too great leading to the initiation of the weaning period. It would also be interesting to observe the interaction of offspring during this time as a limited availability of parental mucus could lead to the development of

offspring-offspring conflict. Conflict between offspring when resources are limited is observed across a wide range of species and is particularly prevalent in mammals (Clutton-Brock, 1991). In Galapagos fur seals (*Arctocephalus galapagoensis*) and sea lions (*Zalophus wollebaeki*), mothers wean their single offspring at 2 years. This leads to a situation where up to 23% of all pups are born while an older sibling is still being nursed. During times when food is limited, conflict between siblings over maternal resources can lead to elevated mortality rates in the younger sibling due to either direct aggression or scramble competition with the older sibling (Trillmich and Wolf, 2008). Aggressive competition among suckling pigs is also known to occur for access to the prime anterior teats of the mother (Dawkins, 1976) while the presence of siblicide is especially prominent in birds where nestlings compete for prime positions in the nest as certain positions are associated with a higher intake of food (Burd et al., 2006; Legge, 2000). Throughout a three week period it would potentially be possible for an individual fry to consume a total of 8.5 ml of mucus with 48 mg of that being total protein. While a large proportion of the mucus consumed will be water there will also likely be carbohydrate and fat components such as glucose and triglycerides. Characterizing and quantifying these components in the future would provide further information regarding the evolution of parental mucus provision in discus fish as well as highlight the benefits obtained by fry from consuming a mucus only diet.

Although there is limited information on the energetic requirements of mucus production in fish, one study has looked at the mucus cocoons produced by parrotfish (*Chlorurus sordidus*) (Grutter et al., 2011). The production of mucus cocoons in *C. sordidus* occurs every night and was estimated to require a moderate 2.5% of the daily energy budget of an individual to produce a146 g cocoon (Grutter et al., 2011). While *C. sordidus* produces one thick mucus cocoon per evening, discus fry are observed feeding from parents all day with a potentially high quantity of mucus required to feed

offspring. The energetic cost of mucus production in discus may, therefore, be considerable. As well as understanding the potential costs of parental care, it is also important to understand the benefits gained. Over the total three week period of parental care fry fed solely on parental mucus secretions achieved an average 54.15 mg increase in body mass. The SGR of fry during the first week of mucus feeding was 28.9 per day^{-1} ± 3.7, a figure comparable to the SGRs of other juvenile cichlids such as *Cichlasoma managuense* (34.5 % per day^{-1} ± 12.6) (Gunther and Boza, 1991) and *Cichlasoma doviid* (45.5 % per day^{-1} ± 18.2) (Gunther and Ulloa, 1995). Considering that the above cichlids were fed high quantities of *Artemia* nauplii, the similar SGR achieved by fry feeding on mucus demonstrates the highly nutritive properties of parental mucus as well as the importance of this type of parental care for the survival and development of young when food sources in the wild are low. Future work manipulating the start of the weaning period by altering the costs and benefits of parental care in discus fish could allow an insight into the dynamics of the parent offspring conflict in discus fish validating this species as an interesting new model for addressing the parent offspring conflict in fish.

The high consumption of parental mucus and associated high gains in growth described here also has repercussions for the survival of fry in the wild considering the recent anthropogenic perturbations in the Amazon. A recent surge in illegal gold mining and industry associated with the Amazon has released vast quantities of mercury (Hg) (Uryu et al., 2001), copper (Cu) and cadmium (Cd) (Matsuo et al., 2005) into the vast River systems of the Amazon. Recent work by Maunder et al. (2011; Bound copy of publications) demonstrated that dietary and water borne cadmium could accumulate in the mucus of discus fish at significant concentrations. This has considerable implications for the survival of fry as consumption rates of 0.3 and 11.0 g Cd g^{-1} day^{-1} predicted by Maunder et al. (2011) could have damaging consequences to the survival

of fry, particularly considering that the larval period of fish is one of the most susceptible stages to aquatic contaminants (Brinkman and Hansen, 2007; Lizardo-Daudt and Kennedy, 2008). Due to the importance of discus fish in Amazonian artisanal fisheries it will, therefore, be important for future work to also look at the horizontal transfer of toxicants from parents to offspring as discus fish in particular may be susceptible to pollution events in the Amazon.

The ability of aquatic larvae to attach to substrates during the early phases of development prior to free swimming has been observed across a wide range of species spanning several different classes including the Actinopterygii, amphibians, and ascidians (Gianguzza and Dolcemascolo, 1994; Nokhbatolfoghahai and Downie, 2005). Attachment in these species is facilitated by the development of transitory structures termed cement glands that are composed of two distinct secretory cell types responsible for producing a cement like substance allowing larvae to attach to a substrate (Nokhbatolfoghahai and Downie, 2005; Pottin et al., 2010). The shape, size and distribution of these structures can vary greatly between species but the function always involves the attachment of larvae to a substrate prior to the period of free swimming. While a lot of research has focused on the anuran amphibians and solitary ascidians (Gianguzza and Dolcemascolo, 1994; Nokhbatolfoghahai and Downie, 2005; Pennati et al., 2000), fish from the family Cichlidae (Jones, 1972) have also been found to develop a cement gland with research so far focusing on the cichlids *Pterophyllum scalare* (Groppelli et al., 2003) and *Tilapia mariae* (Pottin et al., 2010). Consistent with that observed in *P. scalare* and *T. mariae*, the cement gland in *Symphysodon* consists of three pairs of volcano hemi-spherical shaped cement glands with two pairs of cement glands being situated next to each other on top of the midbrain positioned dorsal to the eye, with the third pair of cement glands located anteriorally next to the nasal pit on top of the forebrain. All cement gland pairs were present at 3 dpf with each pair showing a

significant change in size and relative position over the course of the 5 days measured. While the variation in cement gland width over time for pairs 1, 2 and 3 was minimal with a decrease in width occurring at 7 dpf, a distinct change in height was noted. The cement glands in pairs 1 and 2 were highest over the initial two days (3 and 4 dpf) before steadily decreasing in size. In contrast to the height change noted in pairs 1 and 2, cement glands in pair 3 were smallest during the first two days before showing a steady increase in size up until 6 dpf after which a drop was subsequently noted at 7 dpf. The change in dimensions of the three pairs is similar to that reported by Gropelli et al. (2003) in that pairs 1 and 2 are largest in the beginning while pair 3 is largest during the later stages of cement gland development. Gropelli et al. (2003) correlated the size of the cement glands with their activity, finding that pairs 1 and 2 were active early on when they were at their largest and that pair 3 was most active toward the later stage of development when it was at its largest. Groppelli et al. (2003) hypothesised that the different rates of cement gland development and activity may be a way to prolong the period larvae can attach to substrates. Similar to that described in angelfish, the initial position of discus larvae after hatch involves the head being bent ventrally (Fig. 33 A1 and B1) with the most active cement gland pairs, 1 and 2, in the most anterior position making them most likely to come in to contact with a vertical substrate. As development proceeds the head begins to straighten resulting in pair 3 becoming the most anterior pair and therefore most likely to come in to contact with a vertical substrate at a point when its activity and size is at its greatest (Fig. 33 C1 and D1). The cement gland apparatus is, however, a transitory structure and by 7 dpf a reduction in both the size and structure of all cement gland pairs had occurred as fry had all become free swimming. Although not significant, there was also a trend suggesting that the cement glands within a pair migrated closer together over time and that by 7 dpf some cement glands were now touching each other while regressing back into the head.

While the function of the cement gland in discus fish clearly lies in its ability to attach fry to a substrate, it is not clear what advantages this structure might provide to larvae in the wild as *in situ* studies on larval behaviour are lacking. The few studies that have attempted to look at wild discus reported that breeding begins as the water levels rise and that eggs are laid on substrates close to the water surface. It is thought that here, eggs benefit from a richer supply of oxygen and can be more easily protected by parents as there is a smaller volume of water available for predators to attack (Crampton, 2008; Lowe-McConnell, 1969). Parental care that involves re-positioning larvae to a more favourable safer position is seen in several species (Peters, 1965; Pottin et al., 2010) and may be particularly important in discus fish. Personal observations in the aquarium indicate that when disturbed parents will often move larvae from the original vertical surface to a more secluded part of the aquarium lending weight to the idea that the cement gland allows parents to reposition and protect offspring. This seems increasingly likely when the range of potential predators in the wild are considered. Densities of predators in the flood plains during the low water season are known to be incredibly high (Crampton, 2008; Goulding, 1980) and although discus begin breeding as the waters rise there may still be a considerably quantity of predators in the near vicinity of the breeding ground. There may also be a threat of larval predation from conspecifics as personal observations in the aquarium environment indicate that if not properly guarded larvae will be consumed by other non-parental discus within the same tank. In the wild discus congregate in large numbers sheltering in coarse woody debris known as ‘galhadas’ during the low water period (Crampton, 2008) and although discus are known to disperse in to the nearby forests as soon as the high water period begins (Crampton, 2008) there may still be a considerable number of conspecifics close to areas where breeding pairs lay eggs.

Recent work across several species has also demonstrated a property of the cement gland beyond basic adhesion. Research in *Xenopus laevis* demonstrated that when activated, pressure sensitive receptors within the cement gland release glutamate to stimulate brainstem GABAergic reticulospinal neurons which in turn inhibit spinal neurons and turn off swimming behaviour (Boothby and Roberts, 1992a; Boothby and Roberts, 1992b). This response is facilitated by the neural connection between the cement gland and the trigeminal ganglion a connection that has been recently demonstrated in several species of fish including in the Mexican cave fish (*Astyanax mexicanus*) and the tilapia (*Tilapia mariae*) (Pottin et al., 2010). Removal of the cement gland in *A. mexicanus* resulted in a 10 fold increase in swimming behaviour revealing an inhibitory function consistent with that seen in *X. laevis*. This evidence suggests that the inhibitory function of the cement gland may be conserved across fish and amphibians (Pottin et al., 2010) which could suggest that this property is also present in discus fish. This behaviour could therefore be important in helping discus larvae conserve energy by reducing movement during periods of attachment while allowing larvae to swim back toward a substrate if unattached.

4.6 Conclusion

Attachment of discus fry to substrates was achieved via a cement gland identical in structure to those described in other closely related cichlids. Although personal observations indicate that parents may use this structure to reposition offspring to favourable areas during disturbances further in situ work would be needed to assess the adaptive properties of this structure. The development of conical unicuspid teeth in discus was similar to that observed in all teleost fry thus far observed. While these teeth may help enable planktivory in most teleosts it is probable that they help facilitate the consumption of parental mucus in discus fish. The consumption of parental mucus resulted in discus fry obtaining high specific growth rates suggesting a high degree of

benefit to fry feeding from parental mucus. The potential for large quantities of mucus to be consumed also indicates the potential for this form of parental care to carry a cost to parents, a cost that may be significant by the third week of care resulting in the initiation of the weaning period. Using information about the costs and benefits of mucus feeding it would interesting for future work to focus on whether the weaning period could be manipulated in discus fish by altering the benefits and costs associated with parental care.

Chapter 5: Does the ultraviolet play an integral role in mate choice in *Symphysodon*?

This work was made possible by the help and guidance of Professor Julian Partridge from the University of Bristol.

5.1 Abstract

The ability of teleosts to perceive parts of the spectrum other than the visible range (400 nm-800 nm) has until recently been largely ignored. Recent evidence, however, has shed light on the prevalence of ultraviolet (UV) perception and in particular its role in mate choice in a number of teleosts. The Amazonian cichlid, *Symphysodon* spp. provides an interesting look at mate choice as this species is highly choosey and yet the clues used to discern mate quality are unknown. As both parents are monomorphic and show no obvious signs to the human eye of quality or gender in the visible spectrum, it is plausible that this information is displayed in the UV spectrum. Here I investigated the ability of adult and juvenile discus fish to perceive UV by analysing retinal histology and lens transmission properties. Retinal histology revealed that adult discus fish did not possess the accessory cones used to detect light within the UV spectrum indicating that UV perception in adult discus fish was unlikely. This was confirmed as T50 optical transmission values of adult lenses were above 400 nm. Interestingly juvenile lenses attained T50 values of 357 nm suggesting that UV perception may be present in juveniles, possibly playing a role in planktivory and location of parents during the period of parental care.

5.2 Introduction

Until recently, only the visual part (400-800 nm) of the spectrum was considered in hypotheses concerning animal vision and intraspecific signalling. The discovery of ultraviolet (UV) perception in a wide range of species such as arthropods (Cronin et al., 1994; Koehler et al., 1987; Li and Lim, 2005), amphibians and reptiles (Loew et al., 1996; Perry and McNaughton, 1991; Sillman et al., 1997), birds (Bennett et al., 1994; Bennett et al., 1997; Bennett et al., 1996; Cuthill et al., 1999; Hunt et al., 1998; Hunt et al., 1999; Hunt et al., 1997; Smith et al., 2002b) mammals (Jacobs, 1992; Jacobs et al., 1991) and more recently fish (Garcia and de Perera, 2002; Jordan et al., 2004b; Kodric-Brown and Johnson, 2002; Losey et al., 1999; Smith et al., 2002a) has led to a recent surge in work concerning the role of UV in intraspecific signalling, especially in the context of sexual selection.

Ultraviolet light refers to a band of electromagnetic radiation composed of short wavelengths between the range 280-400 nm. These wavelengths lie outside the perception range of the majority of animal eyes, including humans. The ability to perceive UV carries a cost, as the absorption of short wavelength electromagnetic radiation can lead to the production of free radicals and active oxygen resulting in DNA damage (Jacobs, 1992; Losey et al., 1999; Sinha and Hader, 2002; Tyrrell and Keyse, 1990). This is a cost which has no doubt limited the distribution of UV perception in the animal kingdom. The majority of eyes in the animal kingdom have, therefore, developed structures to negate the harmful effects of UV consequently a large proportion of the animal kingdom cannot perceive the UV spectrum. Despite the potential negative effects of utilising UV, recent work has shown that this ability is present in certain species where the negative effects are outweighed by the potential benefits gained from the use of UV in sexual selection (Losey et al., 1999).

Mate choice, a key component of sexual selection, occurs when conspecifics communicate either honestly or dishonestly about their quality in order to achieve a mate. Much of the work regarding mate choice has revolved around the communication of quality and sex via clearly visible signals such as elaborate plumage or colour patterns that are visible to the human observer (Bennett et al., 1994), but a key component of mate choice in some species lies within the UV spectrum. Garcia et al. (2002) looked at the effect of UV on female mating preferences in the viviparous amarillo fish (*Giradinicthys multiradiatus*). Female amarillo fish given the option to choose between males seen through a UV+ filter or a UV- filter consistently spent more time near males seen through the UV+ filter. This suggests that UV, in this species, is an important wavelength required for sexual selection. Under the illumination of a strong UV light it was also possible to determine distinct areas on the amarillo fish that reflected heavily in the UV spectrum. Further behavioural work determined that it was indeed these distinct areas reflecting UV that were responsible for the enhanced female preference. Similar mate choice trials have demonstrated the importance of UV in mate choice in a range of avian and fish species including starlings (*Sturnus vulgaris*) (Bennett et al., 1997), zebra finches (T*aeniopygia guttata*) (Hunt et al., 1997), blue tits (*Parus caeruleus*) (Hunt et al., 1999), bluethroats (*Luscinia svecica*) (Johnsen et al., 1998), three spined sticklebacks (*Gasterosteus aculeatus*) (Rick et al., 2006) and guppies (*Poecilia reticulata*) (Kodric-Brown and Johnson, 2002; Smith et al., 2002a). These aforementioned mate choice trials were similar to the work of Garcia et al. (2002) in that individuals (either male or female) were given a choice between conspecifics under conditions where natural levels of UV were either present or absent. Conspecifics were significantly more attractive under natural levels of UV than individuals in UV deprived conditions. Although UV has been demonstrated as an important component of sexual selection in several species of fish, there has, to my knowledge, been only one

study which has correlated UV intensity to mate quality. Rick et al. (2004) demonstrated that the UV-colour of a distinct region of the male three spined stickleback correlated positively with body condition factor. Although this is the only study to directly compare UV-colour against mate quality, the energy requirements of producing UV reflective compounds would most likely be costly enough to represent a viable signal of mate quality.

As well as signalling an aspect of quality, UV has been shown to signal sex in some species. To the human eye, male and female blue tits are monomorphic in appearance. When UV light is present the blue tit becomes sexually dichromatic for multiple regions of plumage which has led to the suggestion that they be called ultraviolet tits (Hunt et al., 1998). Starlings can also be difficult to sex, often appearing monomorphic throughout the non-breeding season and only slightly sexually dichromatic during the breeding season. UV analysis has shown that starlings are indeed sexually dichromatic in the UV spectrum for several regions of their body (Cuthill et al., 1999). Some fish are sexually dichromatic under UV conditions; Cummings et al. (2003) demonstrated that swordtails (*Xiphophorus nigrensis)* display a significant UV sexual dimorphism and that UV is a significant component of their mate choice behaviour.

Despite the potential negative effects of perceiving UV light, many vertebrate species utilize this wavelength for intraspecific signalling. The widespread utilization of UV perception by vertebrate species in sexual selection suggests that there are significant benefits to this behaviour. One proposed benefit involves the idea of a personal communication channel that cannot be accessed by predators. Sexual selection in most species involves one or both of the sexes signalling their potential quality through elaborate colours and ornaments, a behaviour which not only makes them more conspicuous to the desired sex but also to predators (Cummings et al., 2003). It has been suggested that UV may act as a private channel whereby individuals can display

potential quality in a wavelength unseen by potential predators (Banks, 2001). This particular benefit in avian species has been debated heavily due to the potential for avian predators to develop UV perception just as easily, thereby taking away the advantage of using UV. Fish species, however, may well benefit from using UV to avoid appearing conspicuous to predators. UV light is scattered heavily in water and, as such, intraspecific signalling using UV would need to be carried out over short distances which could allow communication but prevent detection by others (Losey et al., 1999). Cummings et al. (2003) demonstrated this in the northern swordtail (*Xiphophorus malinche, X. nigrensis*). UV signalling in this species is almost undetectable to its major predator and its development and importance in courtship displays depend on predator density (Cummings et al., 2003).

If discus fish were to use the UV wavelength for communication, then several factors would have to be present.

1) There would first have to be enough UV present in the environment to be biologically useful.

2) Discus fish would require structures to enable UV reflection for use in communication with other conspecifics.

3) The discus eye would have to possess the ability to perceive UV.

4) There would have to be significant benefits for UV signalling to have evolved to outweigh the negative effects of UV exposure.

The Amazon Basin river system occupies a large part of the South American equatorial region. At the equator, the sun is perpendicular to the surface of the earth for much of the year. This ensures that the region experiences a high degree of UV irradiance so that an ample supply of UV hits the Amazon Basin rivers. Once the UV hits the water it can then potentially be absorbed by organics within the water column. The Amazon Basin

river systems all differ dramatically in their ability to absorb and attenuate UV. Black water rivers such as the Rio Negro are characterized by their dark colour; despite this dark appearance, the low turbidity characteristic of the Rio Negro allows a deep penetration of UV-light (Furch and Otto, 1987; Mounier et al., 1999; Patel-Sorrentino et al., 2004). White water rivers such as the Rio Solimões, however, are characterized by high levels of sediment which may provide a barrier to UV light penetration (but to my knowledge there has been no relevant account of how much UV can penetrate this river system). Other regions, such as the Para State are known for their clear rivers and lakes areas which would most likely provide a deep penetration of UV light. Discus fish, as described in chapter three, are found in the still lakes connected with the main river systems. The slow moving nature of the water in these lakes, as seen on a recent research trip, has the potential to allow sediments to separate out leaving clear water. Even if discus are associated with a river known for high sediment loads i.e. a white water river, there is the potential that the still waters of the discus habitat can help separate out sediment leaving relatively clear water. The discus breeding season occurs during the region's dry season, a period which reduces the discus habitat to less than 1 metre in depth. This reduction in habitat depth would ensure that less UV is scattered or absorbed before being biologically useful to discus. The dry season is also typified by an extended period of calm weather without rainfall which would prevent turbulence and further help the lakes to separate out sediment. Therefore, although to my knowledge there has been no official record of UV levels within the habitat of discus, it is highly likely that there are sufficient levels of UV available for intraspecific signalling to be possible.

If UV signalling were to play a role in discus intraspecific signalling then individuals would require structures to reflect and display UV to conspecifics. Spectral reflectance measurements in several bird and fish species have demonstrated the ability of

iridescent structures to reflect strongly in the UV (Bennett et al., 1997; Rick et al., 2004; Smith et al., 2002b). Both wild and farmed discus possess similar iridescent markings around the face and fins as demonstrated in figure 37. The position of the iridescent markings present in discus is similar to the UV reflective areas found in coral reef fish, located around the head and fin regions (Siebeck and Marshall, 2001); areas which are presented to conspecifics during display behaviours such as courtship.

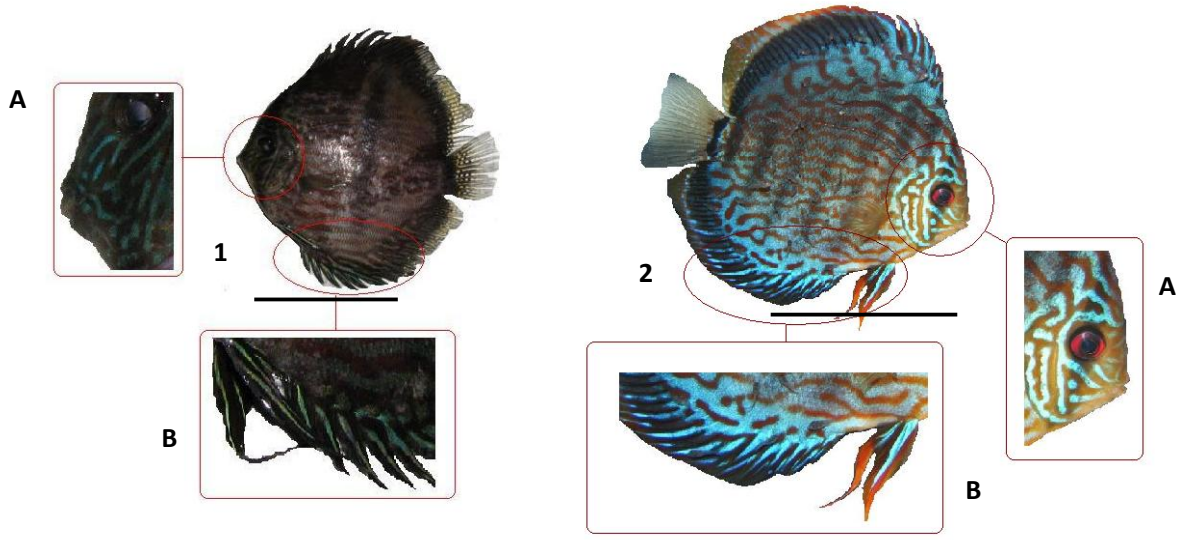


Fig.38 Iridescent structures are present on both wild (1) and aquarium bred discus (2). Structures are located around several areas including the head (A) and pelvic/dorsal fin (B) region. Scale bar represents 10 cm. Pictures taken with a Sony Cybershot DSC-T70.

As well as the ability to display UV signals, discus must also be able to perceive UV. Cone structures within the retina are ultimately responsible for picking up UV wavelengths and relaying them through the optic nerve to the brain, but for the UV wavelengths to hit the cones they must first pass through ocular media such as the lens and cornea. Due to the damaging nature of UV, species that do not utilize UV have developed ocular media which block the transmission of UV wavelengths. The transmission spectra of ocular media such as the lens and cornea can be evaluated for

their T50 values, a value which represents the wavelength at which 50% of the maximum transmission is reached (Losey et al., 1999). Ocular media with T50 values that fall within the UV spectrum can then be assumed to transmit UV wavelengths. If UV can pass through the ocular media it will then hit the retina, the part of the eye responsible for detecting light at different wavelengths which goes towards producing a comprehensive image of the environment. Cone structures embedded in the back of the retina are responsible for the detection of these different wavelengths; if a species can detect UV then it must possess cones specific to wavelengths within the UV spectrum. Carelton et al. (2000) demonstrated the ability of a Lake Malawi cichlid to perceive UV through microspectroscopy. The presence of accessory cones, cones which can be stimulated by wavelengths in the UV spectrum (Miyazaki et al., 2005) is also an indicator of the ability of a fish to perceive UV and can be viewed using standard histological techniques on the retina.

If UV were to play a role in discus intraspecific communication, then there would need to be a significant benefit to this behaviour to outweigh the potential negative effects previously described in this chapter. Several authors have suggested that communication in the UV spectrum could act as a personal communication channel between conspecifics while allowing individuals to remain inconspicuous to predators (Banks, 2001). It could be hypothesized that a similar mechanism may have led to the development of a UV based communication channel in discus and that this may be due to the level of parental care exhibited by the species.

In the majority of vertebrates, particularly mammals and birds, males invest considerably less time in the care of young compared to females; males often display no parental care at all (Clutton-Brock, 1991). Without the energy and time burdens of parental care, males are free to try and maximise their inclusive fitness through extra matings (Dawkins, 1976, 1982). Females on the other hand are left with the time and

energy burdens of parental care which imposes a strict limit on a females' potential inclusive fitness (Trivers, 1974). In this situation a male can potentially mate with tens if not hundreds of females; females, however, are limited in the amount of mating opportunities. Females are, therefore, the limited sex while males are expendable. This expendability allows females to become the choosy sex which in turns leads to the sexual selection of males with desirable traits. This has led to clear sexual dimorphisms in polygamous species such as birds of paradise where choosy females appear drab compared to the brightly ornamented expendable males (Dawkins, 1976). In contrast to polygamous species such as the birds of paradise, discus show no signs of sexual dimorphism and are monomorphic. Unlike polygamous species, parental care in discus requires that both parents aid in the protection, guarding and subsequent feeding of offspring. Both parents thus complete a reproductive cycle together making their inclusive fitness identical. Male and female discus could, therefore, be considered equally choosy and equally valuable with no one sex deemed expendable (Kokko and Johnstone, 2002).

This raises two interesting questions in regards to mate choice in discus. Discus breeders find it difficult to differentiate between male and female discus, and to the human eye it can be difficult to determine the quality of individual discus (with the exception of very unhealthy fish) so how do discus communicate information regarding their gender and quality? Secondly, why is this information not communicated via the optical wavelength? In response to the first question posed, it could be hypothesized that gender and quality are both signalled in the UV as seen in a variety of fish species (Losey et al., 1999). For the second question it could also be hypothesized that communication via UV could allow individuals to appear highly conspicuous to prospective mates (signalling both gender and quality) without compromising an increase in detection by predators. Since both sexes are required for the successful

survival of offspring. It would be hard to imagine an evolutionary stable strategy (ESS) whereby one sex was notably visibly different to the other, thereby making one sex more susceptible to predation. Likewise it would be hard to imagine, in a monogamous system, an ESS where higher quality individuals were visibly more pronounced and thus more susceptible to predation than lower quality individuals. The natural habitat of discus is home to a rich variety of fish species, many of which are piscivorous. The abundance of predators may have resulted in selection pressures for a form of signalling which would be both inconspicuous to predators but highly informative to conspecifics.

The aim of this study was to assess the ability of discus to communicate using the UV spectrum with the hypothesis that their unique form of biparental care, should dictate a form of intraspecific signalling, which would allow the expression of individual sex and quality to conspecifics without gaining the attention of predators. This was addressed through the assessment of the structures and properties of the discus eye. A combination of retinal histology, and the assessment of ocular media transmission properties was used to elucidate the capacity of discus to perceive UV.

5.3 Materials and Methods

5.3.1 Fish and retinal tissue for histology

A non breeding, adult *Symphysodon* spp. fish (140 mm in fork length) was chosen from the stock tank for retinal histology. The fish was placed in a tank covered in a dark cloth so that it could be dark adapted for 4 h before terminal anesthetization (using 400 mg l^{-1} of MS222). Dark adaptation was carried out to help separate the retina from the retinal pigmented epithelium to allow the easier dissection of the retina. Both eyes were then enucleated and placed into a dish of TRIS-buffered saline. A scalpel was used to cut the eyes in half so as to remove the half containing the cornea, lens and fluid vitreous layer leaving the bottom half of the eye cup containing the retina. The retina is roughly 0.5

mm in thickness and can be characterised by its white appearance (Lye et al., 2007). The retina was then slowly removed from the retinal pigmented epithelium using a paintbrush to tease apart the two layers (Lye et al., 2007). Once the retina had been successfully removed it was then topographically divided into several 5 mm sheets using a scalpel and forceps. The sections were then transferred to small tubs filled with Bouin's solution for 24 hours before processing.

5.3.1.1 Tissue processing

Tissue processing involved storing the retinal tissue in a series of solutions beginning with 90% ethanol (1 h), IMS (1 h), Absolute alcohol (2 h), Xylene (1.5 h), Hot wax (1 h).

5.3.1.2 Histological analysis

Once the samples had been stored in hot paraffin wax for 1 h, they were embedded in wax paraffin. Sections were positioned appropriately so that cut sections would produce both tangential and radial sections. As soon as the wax had hardened, tangenital sections were cut at 5 µm. Once the sections had been cut, they were then mounted onto a slide via a water bath before being stained with haematoxylin and eosin. Slides were then mounted with a coverslip using DPX and left to dry for 24 h. Samples were then observed with a light photomicroscope.

5.3.2 Fish and retinal tissue for ocular transmission properties

Two non-breeding sexually mature adults, between 1 and 1.5 years in age and two juvenile discus fish, between 3 and 5 months in age were terminally anesthetized (using 400 mg l^{-1} of MS222) so that the transmission properties of a typical adult and juvenile discus lens could be ascertained. Both eyes were enucleated and the lens dissected away

from the anterior segment of the eye. The lens was then measured using an aluminium insert, designed to fit inside a standard cuvette, in which a 6.0 mm hole (the same diameter as the lens) had been drilled to coincide with the measuring beam of the spectrophotometer, and in which the lens could then be positioned in its normal orientation to the incident light. All lenses were measured in air. A PC UV-VIS scanning spectrophotometer fitted with a Shimadzu ISR-260 integrating sphere assembly to reduce the effects of light scattering by the tissue samples was then used to measure the transmission properties of discus lenses (Hart et al., 1999). A six point rolling average was used to plot transmission data per eye per fish so as to reduce background noise. An average of the four adult eyes and average of four juvenile eyes were used to produce the transmission curves for both adults and juveniles. The standard means of characterising ocular media transmission is to determine the wavelength at which 50% of the maximal transmittance (T50) was reached as this is considered the transmission cut off (Douglas & McGuigan, 1989). This was done using a linear regression similar to the method of Partridge (1989).

5.4 Results

5.4.1 Histology of the retina

The retina of aquarium bred discus had a cone photoreceptor layer with double cones and one type of single cone. From a tangential view of the ventral retina, these cone cells were arranged in a flower-like mosaic pattern, consisting of four double cones (marked red in fig. 39. b) surrounding a central, single cone (marked blue in fig. 39. b). There was no evidence of accessory cones.

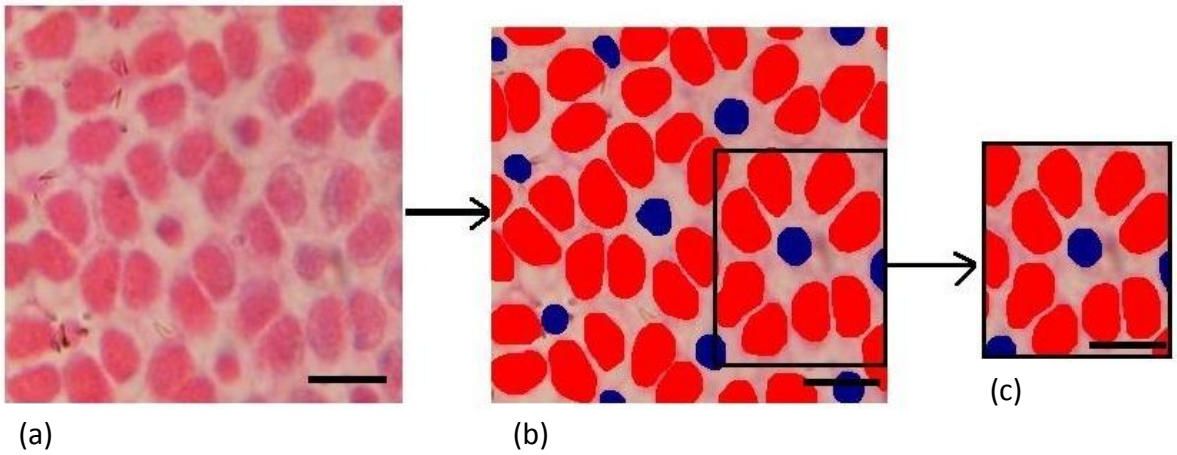


Fig. 39. (a) Tangenital section of the cone photoreceptor layer in the retina of discus. Scale bar = 10 µm. (b) Tracing of section (a), cones marked blue are single cones, and those marked in red are double cones. (c) Typical arrangement of cones, a single cone surrounded by four double cones.

5.4.2 Lens transmission properties

Transmission data from the lenses of adults and juveniles were markedly different. Adult lens transmission profiles produced T50 values of 410 nm (Fig. 40A), 421 nm (Fig. 40B) with an average T50 value of 411 nm (Fig. 40C) indicating an inability to functionally perceive wavelengths within the UV spectrum. The two juveniles tested, however, had T50 values of 356 nm (Fig. 41A) and 357 nm (Fig. 41B) with an average T50 value of 357 nm (Fig. 41C) indicating the potential for UV perception to occur in juveniles.

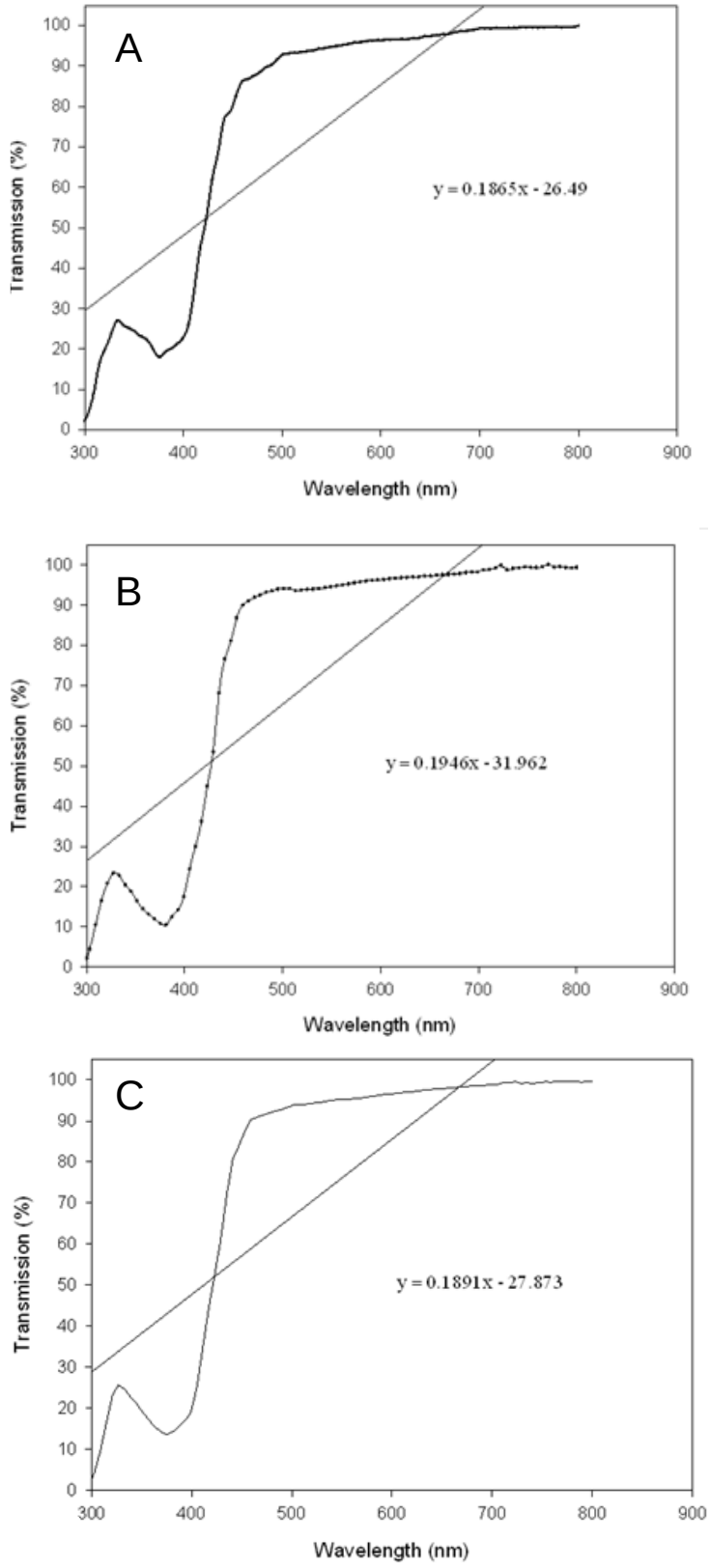


Fig. 40. Transmission profiles with attached linear regression of two lenses from two separate adults (A-B). The average transmission profile was then obtained from these two adults (C).

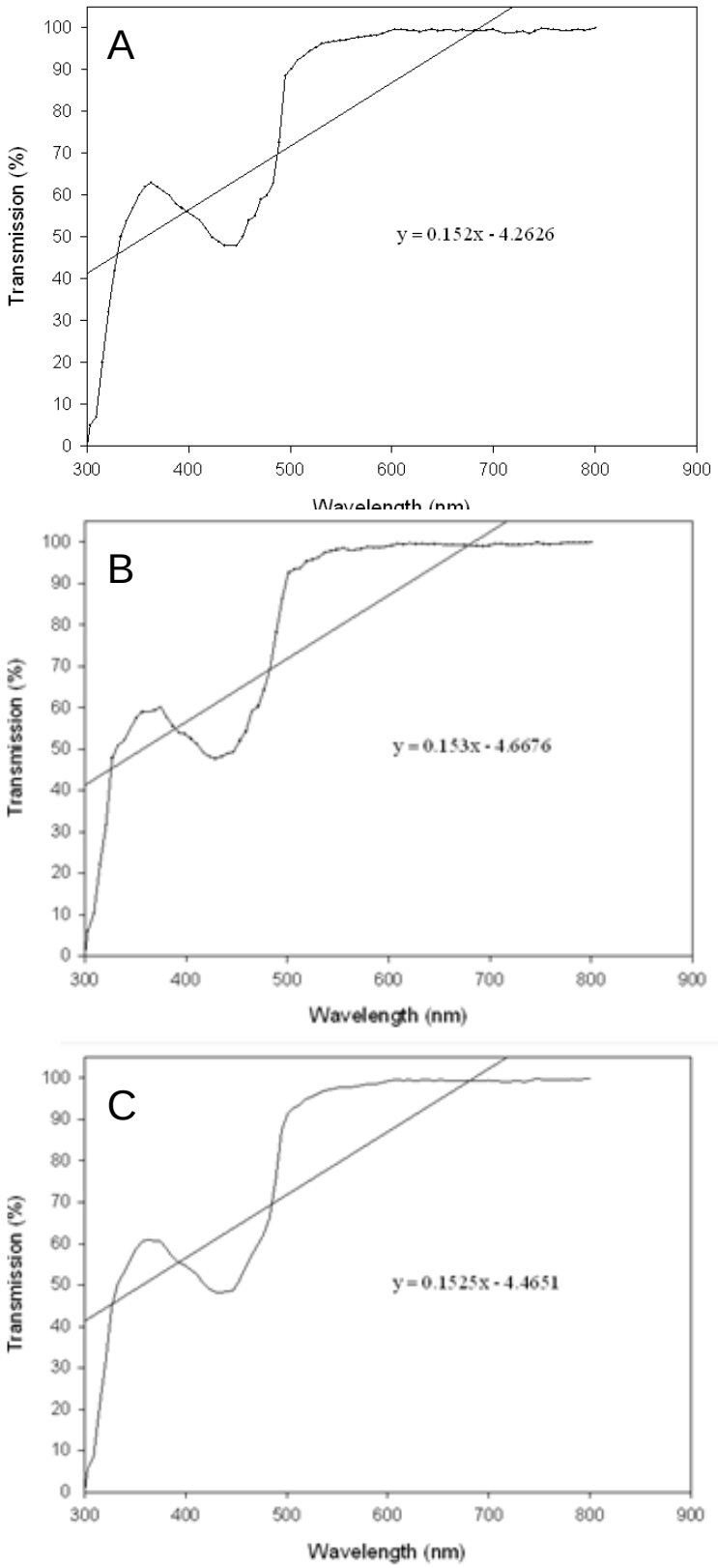


Fig. 41. Transmission profiles with attached linear regression of two lenses from two different juveniles (A-B). The average transmission profile was then obtained from these two juveniles (C).

5.5 Discussion

Mate selection, an integral part of the life history of sexually reproducing organisms, has evolved in many species to incorporate a wide variety of visual, audio, behavioural and olfactory cues. The quality to which these cues are displayed and transmitted to conspecifics plays an important part in determining an individual's potential inclusive fitness, it is, therefore, important to consider as many of these cues as possibly when assessing sexual selection. The visual aspect, in particular the visible spectrum (400-800 nm), is one of the most researched cues when assessing sexual selection (Bennett et al., 1994), the UV spectrum (280-400 nm), however, is often under considered. So that a comprehensive picture of sexual selection in discus fish could be ascertained the ability to perceive UV was first researched.

Retinal histology revealed that the retina of adult discus lacked accessory cones structures that have previously been associated with UV vision in other teleosts (Miyazaki et al., 2005). The lens transmission spectra data from adult discus fish also revealed an average T50 value of 411 nm which indicate that most UV light (280-399 nm) is absorbed by the lens and, therefore, not incorporated into the range of perception of adult discus. Since previous work has determined that the T50 is the cut off at which a wavelength can be functionally useful (Douglas & McGuigan, 1989) it is likely that adult discus fish can not perceive UV. Future work on sexual selection concerning visual cues can, therefore, concentrate on the visible part of the spectrum (400- 800 nm) in this species. Although lens transmission spectra data of adults revealed a lack of UV perception, lens transmission data of 3-5 month old juveniles revealed a T50 value of 357 nm suggesting that UV may be a component of perception in juvenile discus fish. Although further work such as microspectroscopy and behavioural experiments would be needed to confirm that juveniles can utilize the UV spectrum it seems probable considering that UV perception in juvenile fish is thought to be relatively common

(Browman et al., 1993; Jordan et al., 2004b). The presence of UV perception in juvenile discus but absence in adults is not uncommon in fish and is seen in a range of species including rainbow trout (*Oncorhynchus mykiss*) and African cichlids (Carleton et al., 2000; Flamarique, 2001). Several authors have proposed that the UV perception can contribute to the detection of prey during visually guided foraging behaviour (Jordan et al., 2004b) perhaps through contrast enhancement between an ultraviolet-absorbing target (e.g. a zooplankter) and a background rich in ultraviolet veiling illumination (e.g. the upper layers of non-dystrophic water bodies) (Bowmaker and Kunz, 1987; Loew et al., 1993). Once offspring are weaned off parental mucus in the wild it is likely that zooplankton would be the most abundant food source available to offspring: UV perception could, therefore, be highly adaptive in aiding planktivory.

Although it appears that adults can not perceive UV, wild and aquarium bred discus do possess iridescent purple/blue markings as depicted in figure 37. These markings are highly reflective in the UV spectrum and are present in other species that utilize UV such as starlings (Bennett et al., 1997; Rick et al., 2004; Smith et al., 2002a). These markings are generally on the dorsal fin, face and anal fin of adults and although they do not appear to provide information during sexual selection they may aid first feeding fry. During the period of parental care the central disc of both parents darkens considerably, the iridescent markings on the edge of the dorsal and anal fins and face are, however, still prominent. It would, therefore, be interesting, if studying first feeding, to see if these iridescent markings present on adults had any function in first feeding such as directing fry to their parent's side to feed in a manner similar to that seen in flowers, where UV markings are used to help insects navigate to pollen (Thompson et al., 1972).

Understanding the visual system of a species is crucial when examining colouration whether it is in relation to sexual selection, crypsis, warning coloration or mimicry etc. The ability of other animals to perceive wavelengths outside of the visible spectrum has often been ignored in experiments concerning colouration resulting in studies that fail to account for what is actually being perceived by the species in question. This study demonstrated that the visual world of adult discus fish lacks a UV component allowing future work concerning sexual selection to focus on colouration within the visible spectrum.

5.6 Conclusion

Parental care in discus fish involves both parents providing mucus secretions that are obligate for the survival of fry. The large amount of parental investment from both parents dictates that mate choice should be important for both males as well as females. Currently little is known about the mechanisms of mate choice in discus fish, however, future work concerning the role of visual clues in mate choice can focus on signals within the visible spectrum (400-800 nm). The potential for juveniles to perceive UV raises some interesting questions in relation to the benefits juveniles gain from utilizing the potentially harmful UV wavelengths. One interesting function may relate to the iridescent structures present on adults; UV perception in juveniles may allow young to accurately locate the flank of parents to begin mucus feeding. Future work could help elucidate some unique adaptations regarding parental care and UV perception in young.

Acknowledgements

I would like to thank Dr Julian Partridge from the University of Bristol for help and guidance with the analysis of discus lens ocular transmission properties.

Chapter 6: The effect of diet on mate choice in *Symphysodon* spp.

6.1 Abstract

In species where parental care is important for the survival of offspring, the selection of a mate will often act on traits that predict the ability of a prospective mate to provide parental care. In discus fish (*Symphysodon* spp.) both parents provide offspring with extensive levels of parental care, offering both protection and the provision of parentally provided mucus that acts as nutrition for offspring during the first few weeks of development. Due to the importance of parental mucus for the survival of offspring it could be hypothesised that the ability of an individual to produce good-quality mucus could be selected for during mate choice in discus fish. The aims of this study were firstly to investigate the effect of diet on the ability of individuals to produce parental mucus and secondly to evaluate whether mucus quality was a trait selected for during mate choice. After a two month dietary trial where individual young adults were fed either a 50% or 20% protein diet, there were significant differences in growth but not in mucus quality between the two groups. The subsequent mate choice experiment which introduced individuals from both dietary treatments to the same breeding tank, saw individuals pair up independent of diet, but assortatively based on the SGR obtained during the initial dietary period. The difference in SGR obtained during the initial dietary period appears to be linked to the formation of social hierarchies, with dominant fish able to monopolise food and obtain a higher SGR. Comparisons between the colour and physiological characteristics of those fish that paired with those that remained unpaired revealed differences normally observed between dominant and subordinate fish suggesting that a large component of mate choice in discus fish appears to be related to the social status of an individual. This mate choice behaviour is similar to that observed in closely related cichlids and may be selected for in discus fish due to the ability of a dominant individual to better protect offspring from both predators and other discus.

6.2 Introduction

Intersexual selection, otherwise known as mate choice, is a key component of sexual selection and is responsible for many of the striking secondary sexual traits observed in the animal kingdom (Andersson, 1994). These traits, often taking the form of ornaments or behaviours, have been hypothesised to provide information to prospective mates with regards to either the indirect or direct benefits of mating with them. Indirect mate choice refers to the selection of a mate to obtain genes for traits related to improved offspring performance (Andersson, 1994) while direct mate choice refers to the selection of a mate based on traits that relate to their ability to increase the fitness of the choosy sex. Examples of indirect mate choice include the behaviour exhibited by both the chinook salmon (*Oncorhynchus tshawytsch*) and the pink salmon (*O. gorbuscha*) where females delay spawning in the presence of small males to allow time for larger males to displace them (Berejikian et al., 2000; Blanchfield and Ridgway, 1999). The subsequent offspring of females mated to large males grow much faster (Beacham and Murray, 1988) a trait thought to be highly adaptive, as faster growing offspring could potentially better avoid predation (Parker, 1971). Since males do not provide parental care in this species it is thought that female choice is driven by the indirect benefits of mating with a large male and that large males equate to good genes (Berejikian et al., 2000). Colour is also used to signal genetic quality to prospective mates; Barber et al. (2000a) demonstrated that females of the three spined stickleback (*Gasterosteus aculeatus*) preferentially mated with highly coloured males and that the resulting progeny had a higher resistance to the tapeworm *Schistocephalus solidus*. Red colouration may, therefore, be an honest indicator of genes for parasite resistance in this species (Barber et al., 2000a). While several studies have found evidence for indirect mate choice (Møller and Alatalo, 1999; Reynolds and Gross, 1992) the theory has proved controversial with some research questioning the theory's explanatory power and

instead citing direct mate choice as having the greater impact on sexual selection in wild populations, especially in species with high levels of parental care (Arnqvist and Kirkpatrick, 2005; Hadfield et al., 2006; Kirkpatrick and Barton, 1997; Kirkpatrick and Ryan, 1991).

The benefits associated with direct mate choice refer to the increase in fitness the choosy sex can obtain through direct material advantages such as increases in survival and fecundity (Hadfield et al., 2006). In species where parental care is important for the survival of offspring, a potentially important form of direct mate choice involves the selection of a mate based on their ability to provide parental care: This form of selection is known as the 'good parent' hypothesis (Hoelzer, 1989; Kokko et al., 2003). A wide range of traits including size, courtship behaviour and colour have been linked to the ability of an individual to provide parental care. In several species, females choose large males as the size of males is positively correlated with the ability to provide parental care (Downhower and Brown, 1980; Keenleyside et al., 1985; Noonan, 1983; Rogers and Barlow, 1991). Courting behaviour has also been linked to parental care ability in a number of fish species. Females of the fifteen spined stickleback (*Spinachia spinachia*) preferred males that had a higher body shake frequency during courtship, a behaviour that was linked to the ability of a male to fan eggs at a more frequent rate, in shorter bouts, resulting in a higher proportion of eggs hatching (Östlund and Ahnesjö, 1998). Similar results were observed in the damselfish *Stegastes partitus* where males with higher courtship rates were preferred by females as they carried out better parental care and had a higher hatching success (Knapp and Kovach, 1991). The colour of specific regions of individuals may also indicate parental care ability. In female northern cardinals (*Cardinalis cardinalis*), the provisioning rate of offspring correlates with underwing colour (Linville et al., 1998) as well as the size and darkness of the face

mask (Jawor et al., 2004). Male plumage colour positively correlates with nestling provisioning in both the house finch (*Carpodacus mexicans*) (Hill, 1991, 2002) and the cattle egret (*Bulbulcus ibis*) (Krebs et al., 2004). In fish, females of the biparental African cichlid (*Pelvicachromis taeniatus*) advertise their readiness to spawn via the extent of their red colouration while fecundity, maternal quality and offspring fitness is indicated by the extent of blue colouration (Baldauf et al., 2011). Therefore, in species where parental care is important for the survival of offspring, it appears that direct mate choice, based on qualities that predict parental care ability, is particularly important during sexual selection.

The discus fish, *Symphysodon* spp. is one of the most popular ornamental species within the aquarium trade (Chong et al., 2002b) with commercial demand existing for both the wild type as well as the vast array of selectively bred colour morphs available from discus farms (Koh et al., 1999b). While a large part of the appeal of discus fish undoubtedly comes from its impressive aesthetic appearance, enthusiasts are also drawn to this species because of its fascinating parental care behaviour (Hildemann, 1959). Parental care in this species involves both parents providing mucus containing antibodies (Buckley et al., 2010), lectins (Chong et al., 2005), amino acids (Chong et al., 2005), hormones (Buckley et al., 2010; Khong et al., 2009) and ions (Buckley et al., 2010) to offspring over the first few weeks of development. While the relative importance of these components to the development of offspring is unknown, it is likely that they play an important role in helping offspring negate environmental perturbations and pathogens, much in the same way that maternal milk aids the mammalian neonate develop. Similarly to the provision of milk in mammals (Kurse, 1983), mucus feeding is obligate for the survival of offspring and results in fry attaining high specific growth rates (SGR) (Chapter 4). The costs of delivering parental care in discus fish, like in

mammals, is likely to be high as extended periods of parental care have been observed to reduce the ability of parents to invest in future offspring (Chong et al., 2005) as well as result in the development of the parent offspring conflict (Buckley et al., 2010). The selection of an appropriate mate to share the burden of parental care would, therefore, appear to be particularly important in discus fish.

In most species the unequal provision of care results in the development of intense competition for mates in the less caring sex allowing the other limiting sex to be more choosy (Trivers, 1972b). In discus fish, the biparental, obligate nature of care predicts that mate choice in this species will be mutual (Kokko and Johnstone, 2002). Reported operational sex ratios of wild discus conform to a 1:1 ratio (Crampton, 2008; Rossoni et al., 2010), indicative of a species with mutual mate choice and high levels of parental care (Kokko and Johnstone, 2002). If the 'good parent' model of mate choice is present in this species then both parents could be expected to select a mate based on their ability to provide parental care. One of the key components of providing care in discus fish relates to the production of parental mucus by both parents. The transmission of information relating to an individual's ability to produce mucus may, therefore, be a useful component of discus sexual selection.

Fluctuations in mucus total protein during the parental care period indicate that mucus composition can be altered. A previous study manipulating the dietary protein levels in diets for discus fish, demonstrated that inadequate protein (350 g kg^{-1}) reduced the growth and condition of individuals (Chong et al., 2000). It is feasible that dietary manipulations in discus adults could also affect the quality of mucus they produce; indeed in goldfish (*Carassius auratus auratus*), mucus composition reflects the diet of the individual (Saglio and Fauconneau, 1985). Comparisons between wild discus mucus

and aquarium-bred mucus revealed differences in mucus total protein values (Buckley et al., 2010) a result probably related to differences in diet. If potential parental mucus quality is important for sexual selection it may be linked to an easily observed trait. The pairing behaviour of discus fish involves both the male and female displaying their fins, a behaviour that appears to help individuals display their colour which could be an important way of transmitting information in this species. Observations of wild discus indicated that differences in the health of individuals were visually apparent with those infected with the parasite *Braga cichlae* appearing a lot duller and darker in appearance (Crampton, 2008). While colour appears to transmit information relating to parasite burden in discus fish, it may also be important in transmitting information about an individual's condition and therefore ability to facilitate parental care as is seen in other species of fish (Baldauf et al., 2011). While colour in the UV spectrum (300-400 nm) has been demonstrated as playing a pivotal role in signalling mate choice information in some fish species (Banks, 2001; Garcia and de Perera, 2002; Kodric-Brown and Johnson, 2002; Losey et al., 1999), work in chapter 5 indicated that adult discus fish cannot perceive this wavelength. Thus, colour within the visible spectrum (400-800 nm) may be important for choice in discus fish, and is known to be extremely important in providing information to prospective mates in related species (Barlow, 1983; Elmer et al., 2009).

The first aim of this study, was to assess the impact of diet on the ability of discus fish to produce parental mucus with the hypothesis that discus fish fed a diet lacking in protein would have a reduction in body condition and a decreased ability to provide parental mucus highlighted *via* a reduced mucus total protein concentration. The second aim was to evaluate the mate choice decisions of discus fish with the hypothesis that when two dietary groups are introduced to the same tank and allowed to pair,

individuals of a similar quality will select each other and that the ability to provide parental care will be reflected in external traits such as size and colour characteristics.

6.3 Materials and Methods

6.3.1 Experimental fish and husbandry

A total of 30 juvenile discus fish (*Symphysodon* spp.) of the same age and colour morph 'red turq' were obtained from a commercial dealer in Malaysia and transported to the aquarium facilities of the University of Plymouth. Fish were quarantined, wormed (Kusuri discus wormer, Newton Abbot) and then held in three groups of 10 for 2 weeks until it was clear that there was no disease or parasites imported with the fish; during this period fish were fed frozen bloodworm while they acclimated to their new environment. After the acclimation period, fish were graded by size and gender so that two groups of fish consisting of six males and six females, of equal size, could be moved to two separate 100 L tanks. Both tanks were positioned adjacent to each other and connected to the same recirculation system so that water quality parameters would be equal for each group (temperature mean ± SE: 29 ± 0.5^{o}C; pH: 7.0 ± 0.5; dissolved oxygen: 99 ± 0.5%; 12 h:12 h L:D photoperiod; Ca^{2+}: 24.60 ± 0.98 mg l^{-1}; Na^{+}: 7.69 ± 0.44 mg l^{-1}; K^{+}: 2.01 ± 0.01 mg l^{-1}; Cl^{-}: 18.37 ± 0.51 mg l^{-1}). Tanks were left bare with no items that could be used to generate a territory to prevent the early initiation of breeding behaviour. Fish were anesthetised in 25 mg l^{-1} MS222, weighed (g), their fork length measured (cm) and photographed (see below). One 100 L tank was then assigned to a 20% and the other 50% diet treatment (see below) and fed at 5% body mass per day. Water quality measurements showed no changes over the experimental period.

After 2 months fed on their respective diets, fish were sampled for mucus and then anaesthetised, weighed, measured (fork length) and photographed again. After fish had recovered from the anaesthetic, individuals from both dietary treatments were placed

into the same 500 L tank containing a breeding cone and observed daily for the formation of breeding pairs; during this time all fish were fed the same 50% protein diet. Discus fish in the wild are very social animals and live in large groups and so a large breeding group was necessary for the formation of pairs. Additionally, holding groups of adult breeding discus in less than 10 for long periods previously resulted in the deterioration of health as aggressive interactions were more intense with smaller tank numbers. Due to the high cost of discus fish (£100 per breeding adult), it was, therefore, not feasible to control for tank effects in the present study. However, while the sensitivity of the large group behavioural experiment used here may be reduced by practical limitations, the advantages of using natural group densities to observe mate choice behaviour in discus compared to the traditional pair-wise mate choice experiments used for other fish species far outweighs the potential loss in statistical sensitivity.

The formation of a breeding pair was identified by observation of courtship behaviours. These typically involved the male and female swimming towards each other at an angle of 45-60º with all fins extended as far as possible (Fig. 42). As the male and female pass each other the trajectory changes to a downward angle. This behaviour is repeated several times and is often accompanied by aggressive behaviour aimed at other tank mates in order to secure a breeding territory, which in this case was the area surrounding the breeding cone. Once a pair had formed it would inhibit the formation of other pairs within the communal tank. As a result of this, pairs were removed from the tank and sampled once they had formed so that the next pair could form; any individuals left, even if they had not paired, were sampled after two months. Sampling of pairs or unpaired individuals required them to be removed from the tank, anesthetised and mass, fork length and a photograph again recorded. After the photograph was taken, individuals were then terminally anesthetised (400 mg L^{-1} MS222). A heparinised

syringe was used to extract 40 µl of blood by caudal venipuncture. A further sample was used to fill a micro centrifuge tube and centrifuged at 10,000 rpm for 5 min so that the proportion of packed red blood cells could be calculated to determine the hematocrit value. A further 20 µl of blood was transferred to a 10 ml centrifuge tube and filled with 5 ml of Drabkins' solution for the measurement of haemoglobin by the Drabkins' method (Drabkin and Austin, 1935). Fish were then dissected and liver and spleen mass recorded. Over a period of 2 months, fish were removed as they formed breeding pairs with any non-pairing fish sampled at the end of the 2 month period.

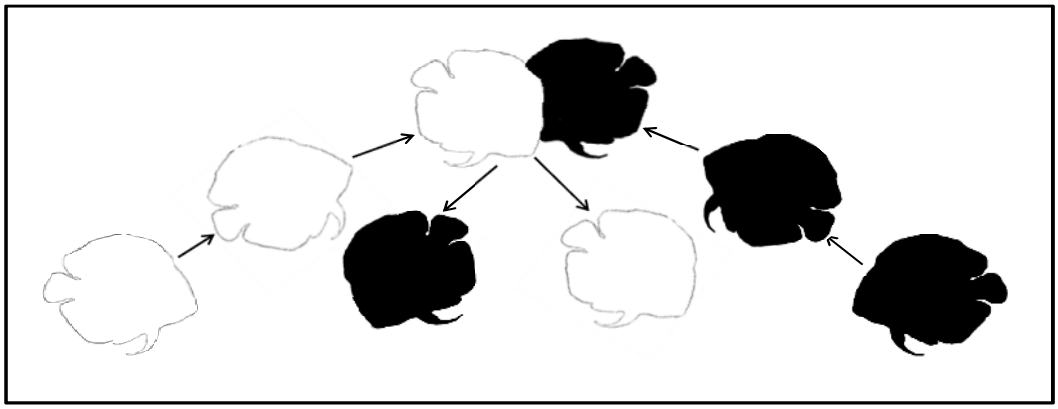


Fig. 42. Courtship behaviour in discus fish. This behaviour involved the female (illustrated in white) and male (illustrated in black) carrying out a controlled swimming pattern indicated by the arrows in the figure.

Diet production and analysis methods

6.3.2 Diet Production

Two diets were used in the present study. The optimal dietary protein requirement of discus fish is 50% protein (Chong et al., 2000). One diet had an optimum 50% protein content while the other had a sub-optimum 20% protein content. A total of 5 kg of each diet was produced using Feedsoft © (Feedsoft Corporation, USA) linear least cost formulation software to formulate the two diets based on either 50 % or 20 % protein. The ingredients used to formulate each diet are described in Table 2. Both diets were cold pressed without steam in a PTM 6 (Plymouth Tropical Marines, Plymouth, Devon,

UK). All the ingredients were ground and sieved to less than 1 mm^2 before being thoroughly mixed. The oil and water fractions were added and the diet extruded through the smallest die (2-4 mm). The resultant pellets were dried in a temperature controlled cabinet at 45º C until total moisture <10%. All diets were then stored at 4º C until use.

Table 2. Quantity and composition of ingredients used to formulate 3 Kg of 50% and 20% protein diets.

Ingredient	50% diet (g)	20% diet (g)
LT94	2083.33	833.33
Corn starch	770.66	1915.66
Fish oil	71	176
PNP vitamin mix	60	60
Carboxymethyl cellulose	15	15

6.3.3 Proximate analysis

A sub-sample of each diet was analysed to confirm that the composition of each diet was as intended, sub-samples were analysed for protein, lipid, ash and moisture content (Table 3).

Table 3. Percentage of protein, lipid, ash and moisture within the 50% and 20% protein diets.

	% Protein	% Lipid	% Ash	% Moisture
Optimal diet	51.68	39.65	7.44	1.23
Sub-optimal diet	22.43	45.16	27.18	5.23

Physiological methods

6.3.4 Growth

Fish were deprived of feed for 24 h then removed from their tank via a shallow bottomed net, blotted dry and transferred to a tared container of system water so that their mass could be determined to the nearest 0.001 g. After weighing, fish were also placed on a flat measuring board so that their fork length could be determined to the nearest 1 mm.

The mass of fish at the different time points was then utilized to calculate the specific growth rate (SGR) as per Fagbenro and Jauncey (1995) via the following equation.

Specific growth rate (SGR) [% per day] = 100 x ((In. final mass of fish – In. initial mass of fish)/trial length in days)

Where In. is the natural log.

6.3.5 Hepatosomatic and relative spleen index

At the end of the experiment, terminally anaesthetised fish had their liver and spleen removed and transferred to a tared slide so that their mass could be determined to the nearest 0.001 g. Liver and spleen masses were then used to calculate the hepatosomatic index (HSI), as well as the relative spleen index (RSI) using the following equations.

Hepatosomatic index (HSI) = Liver mass/body mass x 100.

Relative spleen index (RSI) = Spleen mass/body mass x 100

6.3.6 Total Protein Bradford assay

Mucus samples were taken from all fish after the two month dietary trial using the same methods described in chapter 2. Mucus samples were defrosted on ice, diluted in distilled water and analysed for total protein via the Bradford method (Bradford, 1976).

6.3.7 Photography and colour analysis

The photography protocol was based on a recent study by Stevens et al. (2007) which highlighted the problems with using digital photography to study animal colouration. The following parameters were controlled and standardised:

Preparation of fish: To reduce stress, fish were photographed in a standard (3 cm) depth of water in a 300 ml glass container. This also meant that individuals were photographed in the same medium that other conspecifics would view them. Individual fish were anaesthetised with 25 mg L^{-1} MS222 prior to photography and placed on their right hand side which alleviated the need to tag or mark individuals for identification as their distinctive patterns could instead be used to identify individuals.

Lighting: To ensure standardised light conditions prevailed throughout the experimental period, digital photographs were taken inside a room with no natural light. This ensured that both the quantity and type of light could be controlled. Light was standardised using a lighting rig with two Nikon 60 W incandescent bulbs fixed in position to give an equal level of lighting and eliminate the need for a flash. Camera position in relation to the fish was also standardised using an attachment to the lighting rig which ensured the camera was 50 cm away from the fish. The fixed position of the camera also produced a crisp, non-blurry image.

Camera type: A Nikon d70 single-lens reflex (SLR) camera fitted with nikon 28-105 mm AF-D was used in the present study to ensure that the camera performed no 'automatic' adjustments of the image that might bias the results (Stevens et al., 2007).

Camera settings: The following camera settings were all specifically adjusted to allow standardisation.

- File type: All images were saved as RAW files as this file type contains all the original image information, unlike JPEGs which compress images, losing and skewing the image data.
- Aperture: An aperture value of F8 was chosen to allow as much light as possible to reach the image sensor.
- ISO: An ISO value of 200 was used. This setting ensured the image sensor of the camera was less sensitive to light providing a much sharper and detailed image. The reduction in image sensor sensitivity was counteracted by the subject being adequately lit as described above.
- White balance: A white balance specific to the incandescent bulbs used to light the fish was selected.
- Focus: All images were focused manually by the operator.

6.3.7.1 Camera calibration

Even with the adjustment and standardization of the above settings, camera calibration was still required to produce images that reflect real world colours rather than those produced and manipulated by the algorithms of the camera's CCD (charge coupled device; the sensor used to produce the camera's image). In order to produce images that truly represent colours present in the real world, the output of the red (R), green (G) and blue (B) wavelengths must be linearly related to light intensity.

While digital SLR cameras are approximately linear for each of the three wavelengths, steps were taken to linearize the camera used in this experiment. Linearization was achieved *via* the use of a Munsell x-rite colour standard (Fig. 43) that was photographed under the same conditions as the subjects at the beginning of each sample day. The resulting RAW file of the colour standard was then opened in Adobe Photoshop CS5 where the intensity of the R, G and B wavelengths for the white, midpoint grey and

black swabs on the colour standard could be checked using the colour picker tool. The colour picker was set to record the RGB values of a 50 x 50 pixel area to obtain an average for that area. With true linearity, the intensity values for each of the three colour standards should read 244 for the white standard, 122 for the midpoint grey and 50 for the black. The values obtained in this study were slightly skewed with some of the wavelengths being notably decreased/increased at different reflection levels suggesting that the camera was not truly linear.

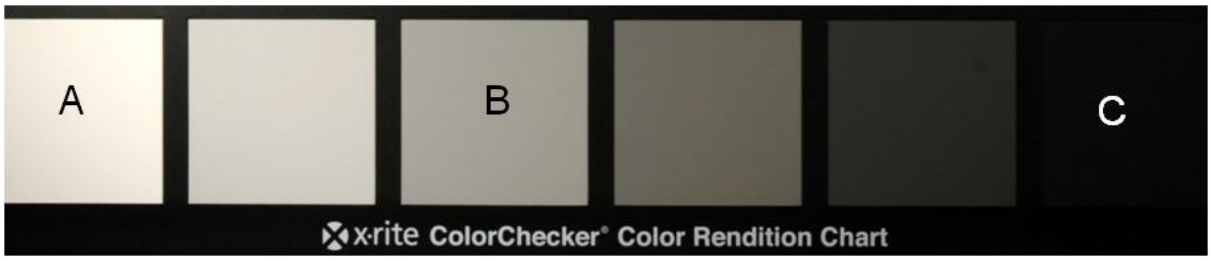


Fig. 43. Munsell X-rite colour checker standard used for calibration. Panel A, the white standard: R, B and G=244; panel B, the midpoint standard: R, B and G=122; panel C, the black standard: R, B and G = 50.

Calibration of the image was conducted in Photoshop where the colour picker tool was used to select and highlight the white (Fig. 43 A), midpoint grey (Fig. 43 B) and black (Fig.43 C) standards so that any changes in the RGB values of these standards could be viewed instantly. The exposure, colour balance and curves of the image were then adjusted until the RGB intensity values of the image matched those of the colour standard. These settings were then saved as a calibration pre-set which was then applied to all images taken on that day. The process of calibration was conducted at the start of every sample date to ensure that variations in light or camera bias would not affect comparisons between sample days.

6.3.7.2 Image analysis

Images were analysed in Photoshop once the calibration preset of that day had been applied to the image. Four areas of the discus fish were chosen for analysis including the operculum region (Fig. 44A), dorsal fin region (Fig. 44B), anal fin region (Fig. 44C) and central disc region (Fig. 44D).

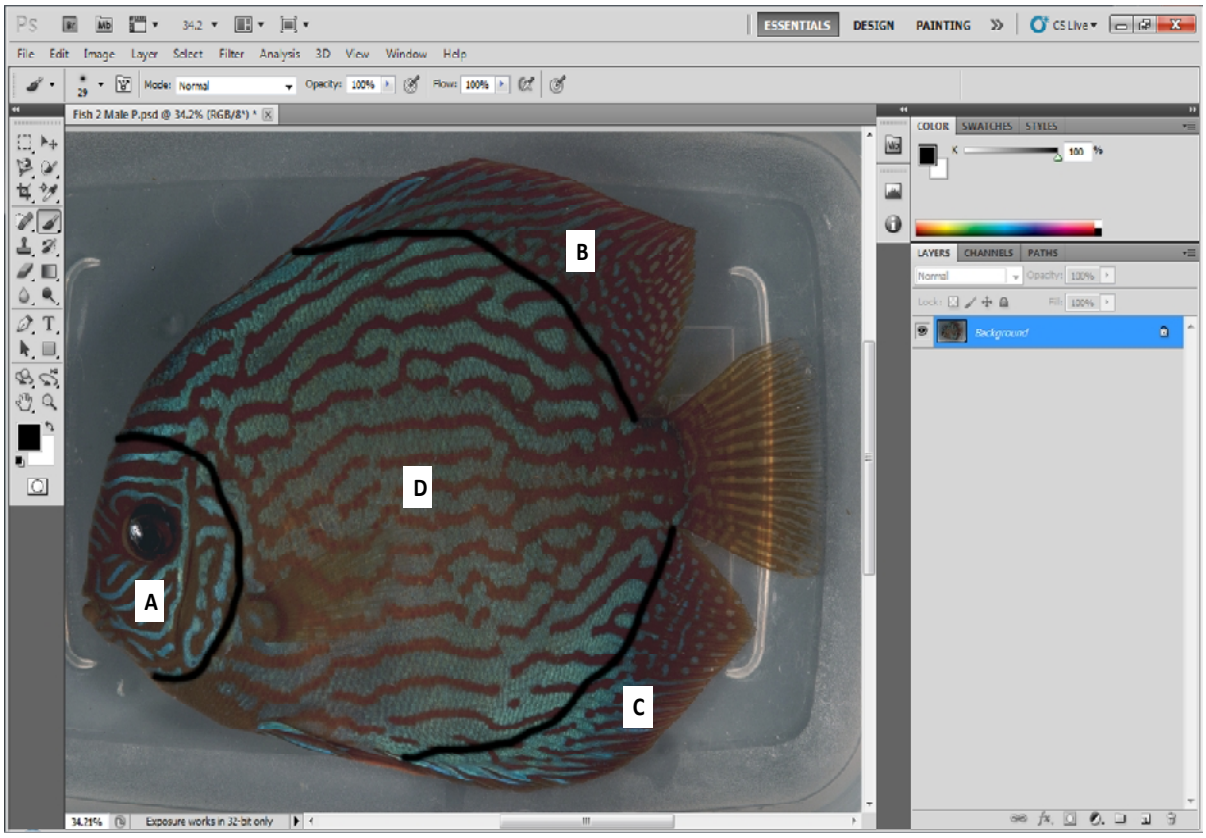


Fig. 44. The areas measured on discus fish. The black lines drawn on using the Photoshop paint tool demarcate the operculum region (A), dorsal fin region (B), anal fin region (C) and the central body region (D) that were analysed for their RGB values using Photoshop.

The predominant colours of ‘red turq’ discus fish are red and blue. Measurements were therefore taken to determine both the quantity of red and blue in specific regions of each fish as well as the brightness of these colours. The colour checker tool was set to

measure the RGB component of a 5 x 5 pixel area as marked out in figure 45. This ensured that for each measurement a 25 pixel RGB average was obtained.

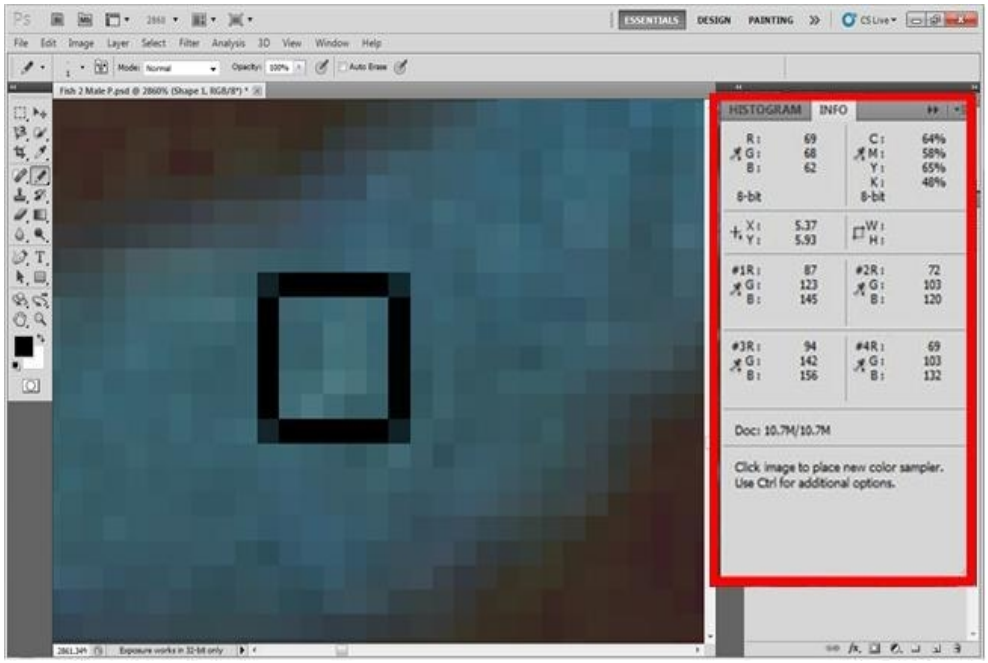
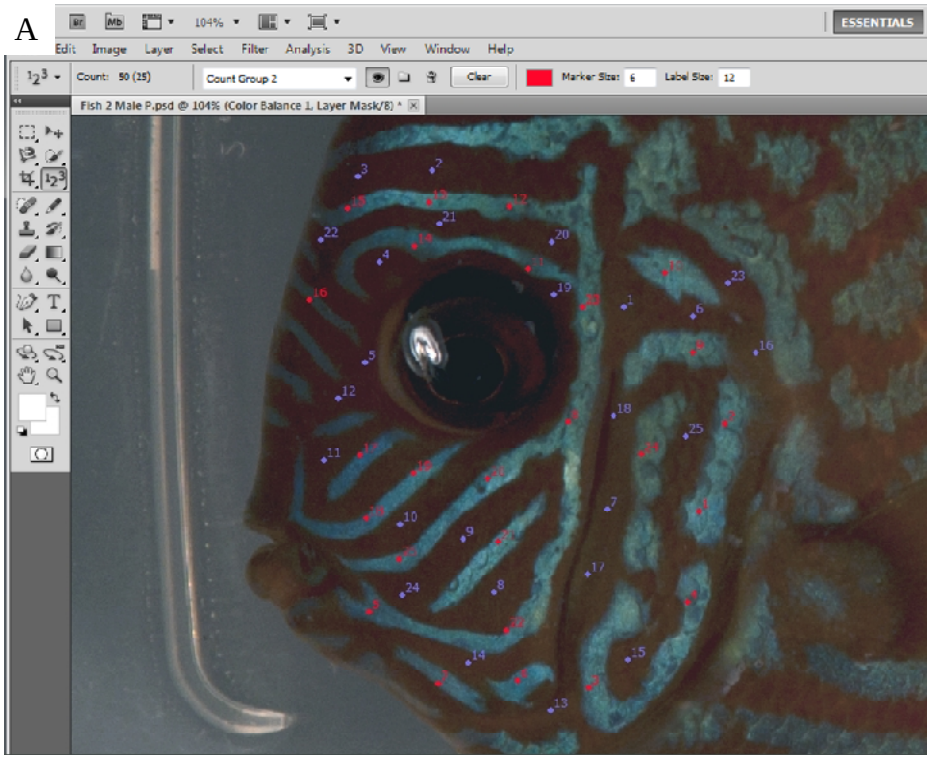


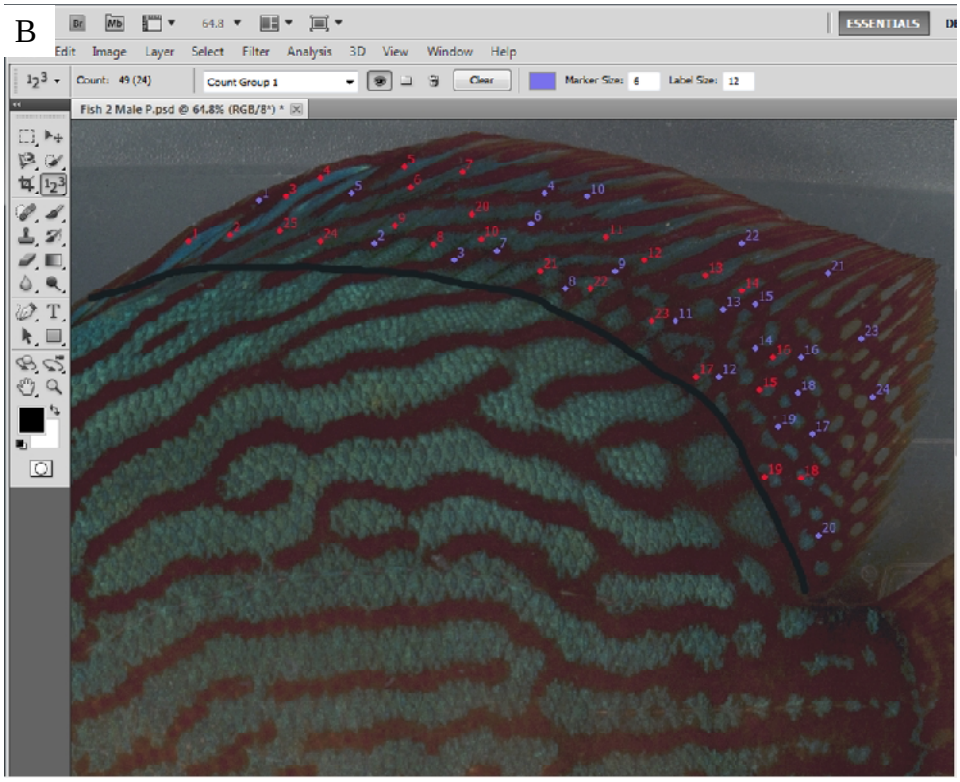
Fig. 45. Close up image of a 5 x 5 pixel area of blue colouration on the operculum marked out by the black box. Each colour measurement taken would consist of the average RGB values of a 25 pixel area. The RGB values for each area selected is displayed in the control box highlighted in red.

A total of 25 measurements were taken for both red and blue markings in the operculum region (Fig. 46A), dorsal fin region (Fig. 46B), anal fin region (Fig. 46C) and central body region (Fig. 46D). As demonstrated in figure 45 the sample points were spread haphazardly across each region to obtain a reliable average of RGB values for that particular region. Although the sample points were not taken in a truly random fashion, potentially introducing variation into which pixels were chosen, the large number of pixels per area (625 pixels in total) buffered these small variations.

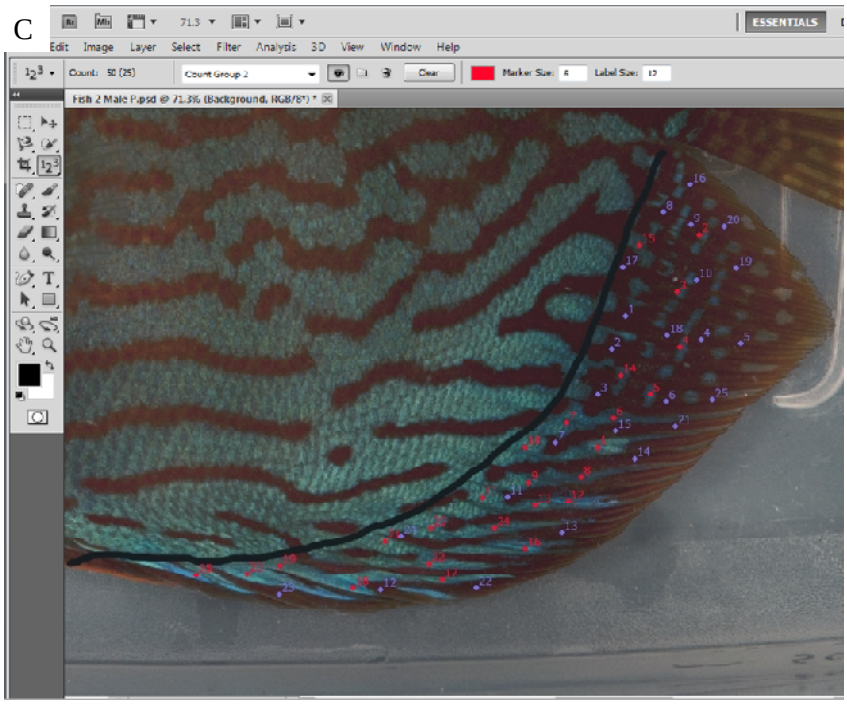
A


ESSENTIALS
Edit Image Layer Select Filter Analysis 3D View Window Help
Count: 50 (25)
Count Group 2
Clear
Marker Size: 6
Label Size: 12
Fish 2 Male P.psd @ 104% (Color Balance 1, Layer Mask/8) *

B


ESSENTIALS
Edit Image Layer Select Filter Analysis 3D View Window Help
Count: 49 (24)
Count Group 1
Clear
Marker Size: 6
Label Size: 12
Fish 2 Male P.psd @ 64.8% (RGB/8*) *

C



D

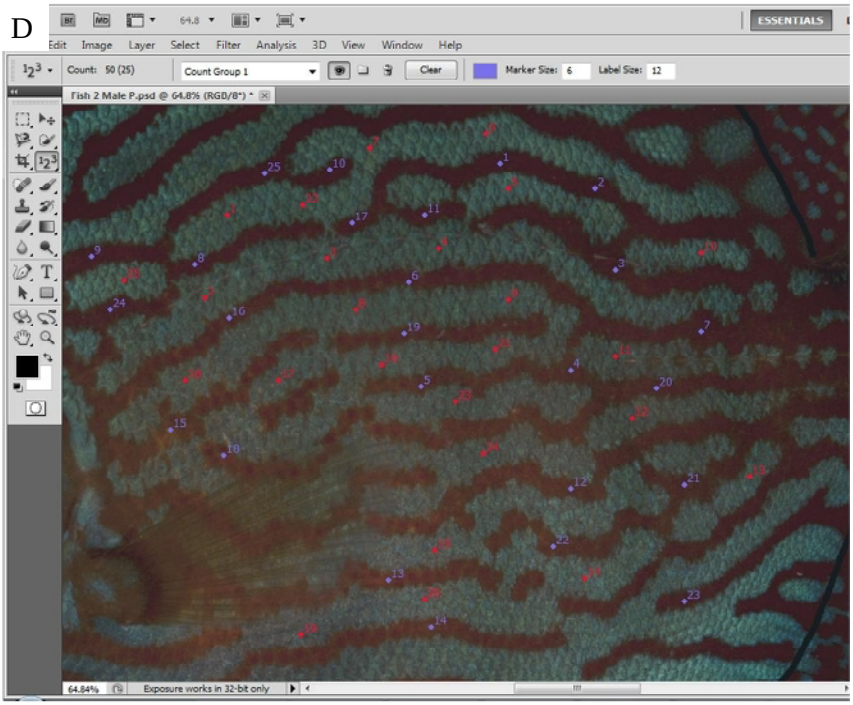


Fig. 46. Regions utilized for colour analysis including the operculum region (A), dorsal fin region (B), anal fin region (C) and central body region (D). In each region there are two sets of 25 marked points representing the 25 colour sample sites for the red markings and 25 colour sample sites for the blue markings.

Once RGB values were obtained for a particular region it was then possible to determine the red index or 'redness' of an area as well as a blue index or 'blueness' of an area *via* the following equations.

Red index = R/(R+B+G)

Blue index = B/(R+B+G)

The blue and red index values operate on a scale whereby a blue index value of 1.0 would indicate that the colour of that area was pure blue and lacked any red or green components whereas a value of 0 would indicate a colour lacking the blue component. The brightness of both the red and blue areas was also calculated by obtaining the greyscale value *via* the following equation.

Greyscale value = R+G+B/3

The greyscale operates on a scale from 0 (black) to 255 (bright white). By taking an average of the R, G and B values from a region it is possible to calculate how light or dark that region is. Two colours may have the same blue index but could have a different greyscale value indicating that one of the blues was brighter than the other (Table 4).

Table 4. Comparison of two blue patches of the same blue index but different greyscale values

	Light blue	Dark blue
R	100	39
G	149	64
B	247	101
Greyscale	165.33	68
Index	0.49	0.49

6.3.8 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. The statistics package SPSS (IBM®SPSS v19.0; Armonk, New York) was used for all analysis.

6.3.8.a Effect of diet

Comparisons between the two dietary groups over the two month dietary trial were obtained using a repeated measures ANOVA (RM-ANOVA) with time (pre and post-trial) and diet type (50% vs 20%) as the two factors. This compared values from the start of the experiment with those at the end of the dietary trial, before fish were placed together and allowed to pair. This statistical test was applied to the mass and colour characteristics data. Comparisons between SGRs and total mucus protein of the two dietary groups were carried out using a one-way ANOVA.

6.3.8.b Mating result

A 2x2 contingency table was used to determine whether individuals fed the same diet would preferentially mate to address the hypothesis that individuals of a similar quality would pair. To determine whether colour or physiological parameters (growth, hematocrit, haemoglobin, HSI, RSI) influenced whether a fish successfully paired, a two-way ANOVA with diet (50% vs 20%) and mating result (paired *versus* non-paired fish) as factors was utilized. Due to the sequential removal of pairs, final samples were taken over a range of different times. To examine whether differences present at the end of the dietary trial, before the fish were placed in the breeding tank, may have influenced which fish paired, the SGR taken at the end of the dietary trial of fish that paired was compared with the SGR of fish that did not pair via a one-way ANOVA. To

examine whether the individuals that paired carried out assortative mating based on the SGR attained after the dietary trial, a Pearson product-moment correlation was carried out.

6.4 Results

For convenience, results are split into two sections, the first dealing with the effects of diet on the physical and colour characteristics of discus fish and the second section looking at whether individuals of similar quality were more likely to pair.

6.4.1. Effect of diet

6.4.1.1 Physical characteristics

A significant interaction between time and diet on mass was observed (RM-ANOVA, $F_{1,20}$=7.714, P<0.05). A post-hoc analysis revealed that mass did not differ between dietary groups at the start of the trial (one-way ANOVA, $F_{1, 21}$=4.443, P<0.05) but that fish fed the 50% diet had a significantly higher mass by 7.22 g at the end of the 2 months (one-way ANOVA, $F_{1, 21}$=0.190, P=0.668). This was confirmed by the higher SGR present in the 50% protein dietary group (one-way-ANOVA, $F_{1, 21}$=8.319, P<0.05; Fig. 47). Mucus total protein quantities, however, did not differ significantly between the two dietary groups at the end of the two month dietary trial (one-way-ANOVA, $F_{1, 21}$=0.138, P=0.714; Fig. 48).

6.4.1.2 Colour characteristics

Comparisons between the colour characteristics of discus fish after the 2 month dietary trial revealed very few significant differences among many of the regions measured (Table 5). There were, however, significant differences in some of the blue index and blue/red greyscale values of the operculum, dorsal fin and anal fin regions which are below investigated further.

Table 5. Effect of diet, time and diet × time on discus colour characteristics.

	Diet		Time		Diet × Time	
	$F_{1, 20}$	P	$F_{1, 20}$	P	$F_{1, 20}$	P
Operculum blue index	0.672	0.422	26.610	**<0.001**	17.046	**<0.001**
Operculum blue greyscale	0.006	0.940	8.899	**<0.05**	0.005	0.945
Operculum red index	0.257	0.618	2.469	0.132	3.656	0.070
Operculum red greyscale	0.956	0.340	2.538	0.127	0.086	0.772
Dorsal fin blue index	0.399	0.535	7.181	**<0.05**	5.770	**<0.05**
Dorsal fin blue greyscale	0.037	0.850	0.340	0.857	0.206	0.655
Dorsal fin red index	0.103	0.923	1.343	0.260	0.357	0.518
Dorsal fin red greyscale	0.558	0.464	0.450	0.833	0.013	0.909
Anal fin blue index	0.074	0.789	3.350	0.569	1.458	0.241
Anal fin blue greyscale	0.739	0.400	5.312	**<0.05**	0.141	0.712
Anal fin red index	0.660	0.426	0.784	0.386	4.083	0.007
Anal fin red greyscale	0.894	0.356	8.115	**<0.05**	0.325	0.575
Body blue index	0.117	0.736	2.982	0.100	0.980	0.757
Body blue greyscale	2.114	0.162	0.026	0.874	1.479	0.238
Body red index	0.017	0.897	0.094	0.762	0.017	0.897
Body red greyscale	0.112	0.741	1.479	0.238	0.192	0.349

Operculum region

A significant interaction between time and diet on operculum blue index was observed (RM-ANOVA, $F_{1, 20}$ 17.046, P<0.001; Fig. 49A). Post-hoc analysis revealed that there was no significant difference between diets at the start of the dietary trial (one-way-ANOVA, $F_{1, 20}$=1.138, P=0.299; Table 5) but that after the 2 month dietary trial fish fed the 50% protein diet had a higher operculum blue index than fish fed the 20% diet (one-way-ANOVA, $F_{1, 20}$=5.401, P<0.05; Fig. 49A). While there was no interaction between time and diet (RM-ANOVA, $F_{1, 20}$=0.005, P=0.945; Table 5) and no effect of diet (RM-ANOVA, $F_{1, 20}$=0.006, P=0.940; Table 5) on operculum blue greyscale values. The effect of time was significant (RM-ANOVA, $F_{1, 20}$=8.899, P<0.05; Fig. 49B) with operculum blue greyscale values being significantly higher in fish from both diets at the end of the 2 month dietary trial.

Dorsal fin region

There was a significant interaction between time and diet on dorsal fin blue index values (RM-ANOVA, $F_{1, 20}$=5.777, P<0.05; Table 5). Post-hoc analysis revealed that while there was a significant rise over time in the blue index value of fish fed the 50% protein diet (paired t-test, t(10)=-3.460, P<0.05) the same significant increase was not apparent in fish fed the 20% protein diet (paired t-test, t(10)=-0.203, P=0.843). Despite a greater increase over time in the dorsal fin blue index of fish fed the 50% diet, comparisons between both dietary groups at the start (one-way-ANOVA, $F_{1, 20}$=0.616, P=0.442; Fig. 50) and at the end of the 2 month dietary (one-way-ANOVA, $F_{1, 20}$=2.350, P=0.141; Fig. 50) were not significant.

Anal fin region

There was no interaction between time and diet on anal fin blue greyscale values (RM-ANOVA, $F_{1, 20}$ 0.141, P=0.712; Table 5) or anal fin red greyscale values (RM-ANOVA, $F_{1, 20}$=0.325, P=0.575; Table 5). Diet, also had no effect on anal fin blue (RM-ANOVA $F_{1, 20}$=0.739, P=0.400; Table 5) or anal fin red greyscale values (RM-ANOVA, $F_{1, 20}$=0.894, P=0.356; Table 5). There was, however, a significant effect of time on both anal fin blue and red greyscale values. Anal fin blue greyscale values were significantly higher in fish from both diets at the end of the 2 month dietary trial (RM-ANOVA, $F_{1, 20}$=5.312, P<0.05; Fig. 51A) while anal fin red greyscale values were significantly lower in fish from both diets at the end of the 2 month dietary trial (RM-ANOVA, $F_{1, 20}$=8.115, P<0.05; Fig. 51B).

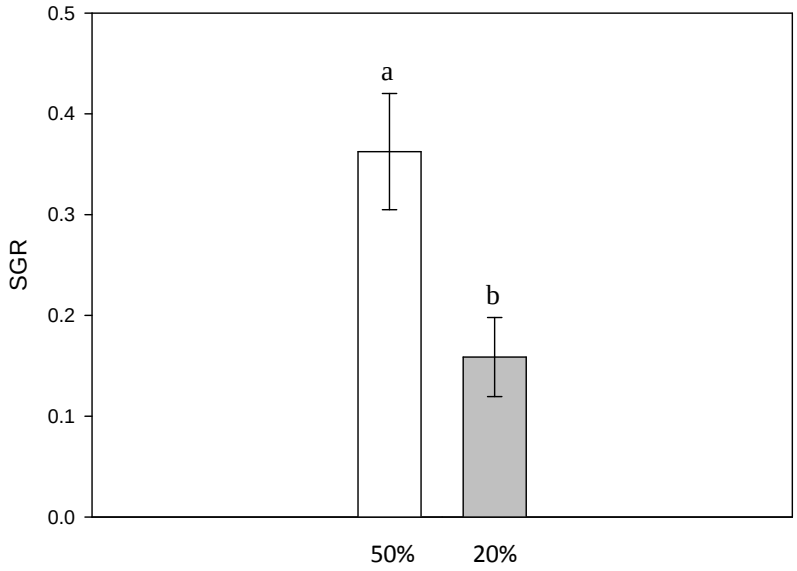


Fig. 47. Comparison between the SGR of fish fed either a 50% (N=11) or 20% (N=11) protein diet. Different letters denote a significant difference (one-way-ANOVA, P<0.05); bars that share a letter are not significantly different. Data are means + s.e.m.

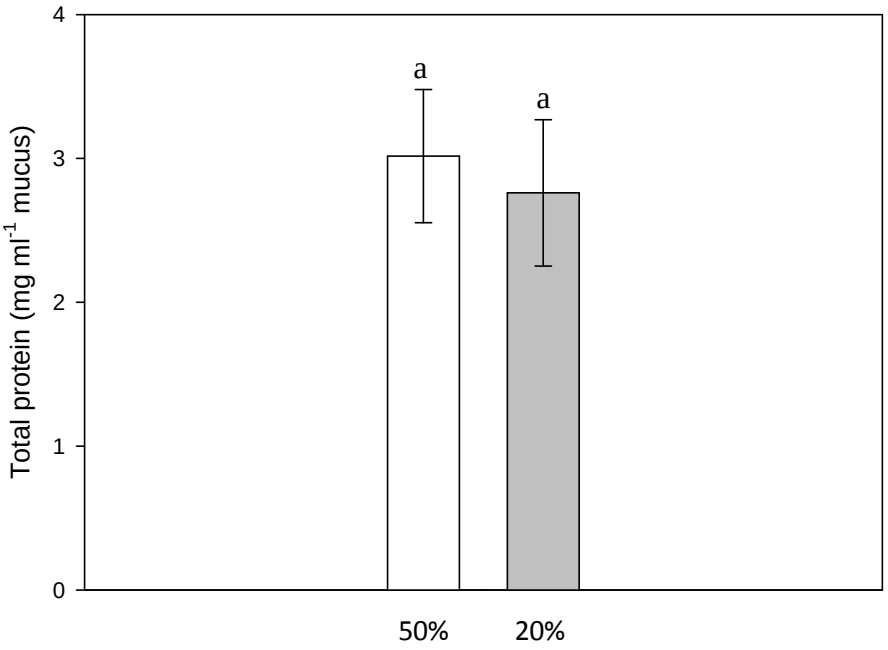


Fig. 48. Comparison between the mucus total protein of fish fed either a 50% (N=11) or 20% (N=11) protein diet. Different letters denote a significant difference (one-way-ANOVA, P>0.05); bars that share a letter are not significantly different. Data are means + s.e.m.

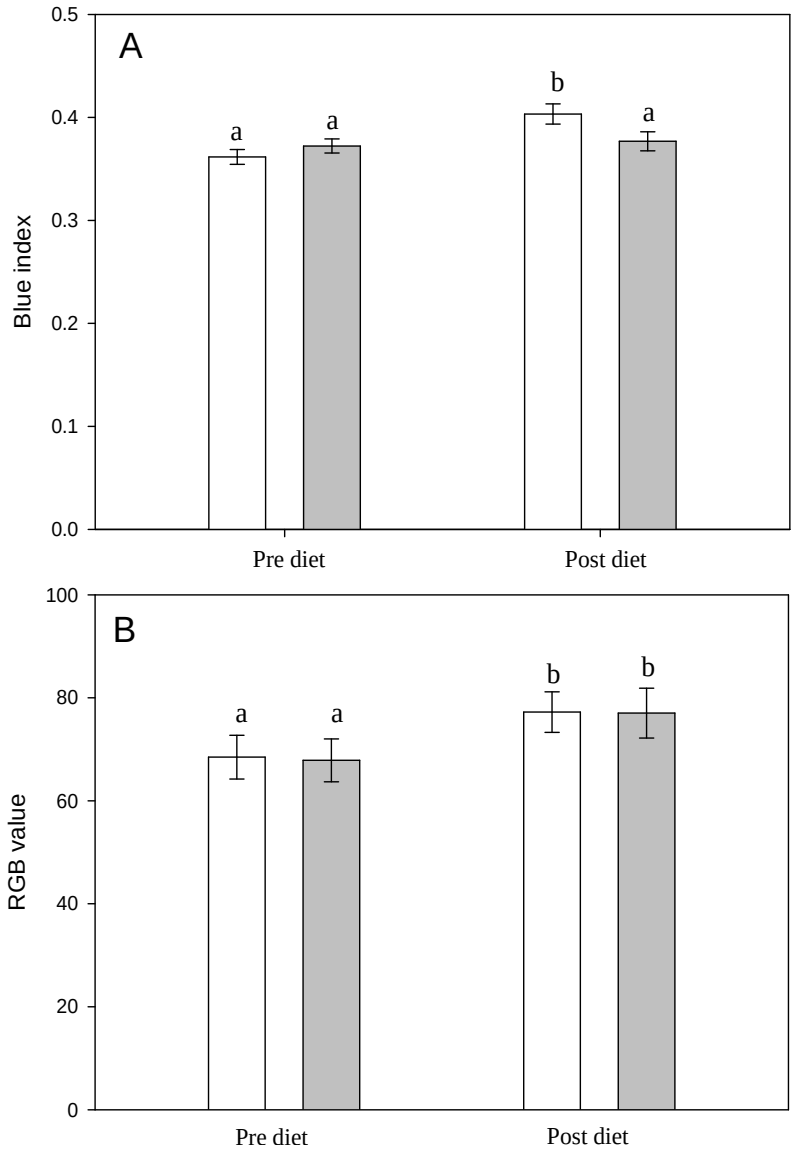


Fig. 49. Operculum colour characteristics of fish fed either a 50 % □ (N=11) or 20 % ■ (N=11) protein diet over a two month period. Colour characteristics: blue index (A) and blue greyscale (B). Different letters denote a significant difference (RM-ANOVA, P>0.05); Data are means + s.e.m. Pre-diet is the start of the experiment and post diet is after 2 months of feeding on either 50 or 20% protein, before being placed into the breeding tank.

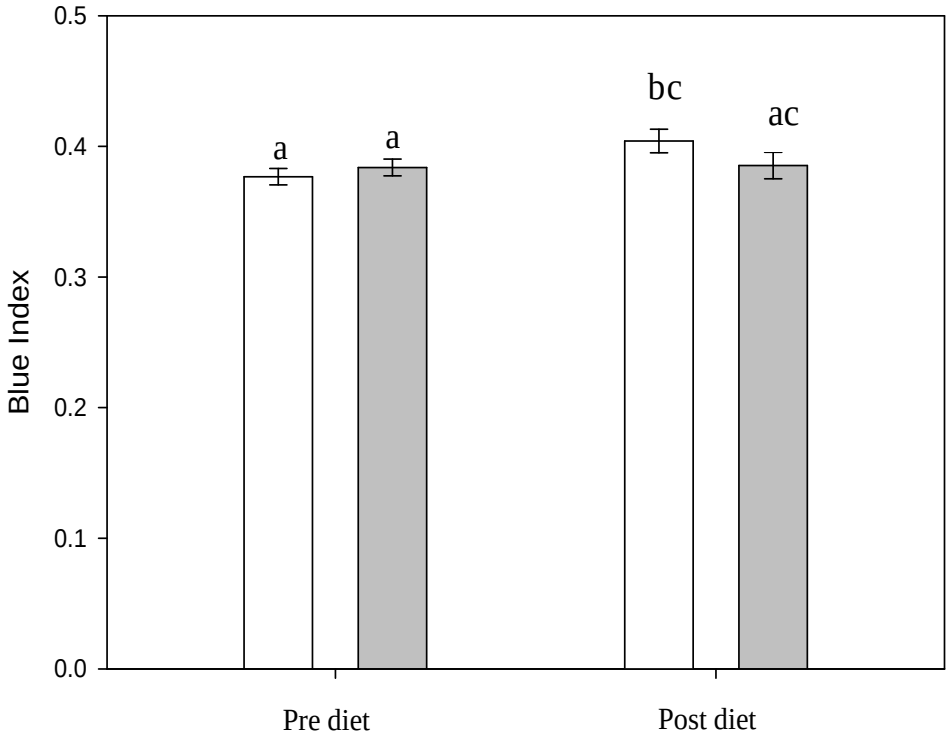


Fig. 50. Dorsal fin blue index colour characteristics of fish fed either a 50% □ (N=11) or 20%■ (N=11) protein diet over a two month period. Different letters denote a significant difference (RM-ANOVA, P>0.05); Data are means + s.e.m. Pre-diet is the start of the experiment and post diet is after 2 months of feeding on either 50 or 20% protein, before being placed into the breeding tank.

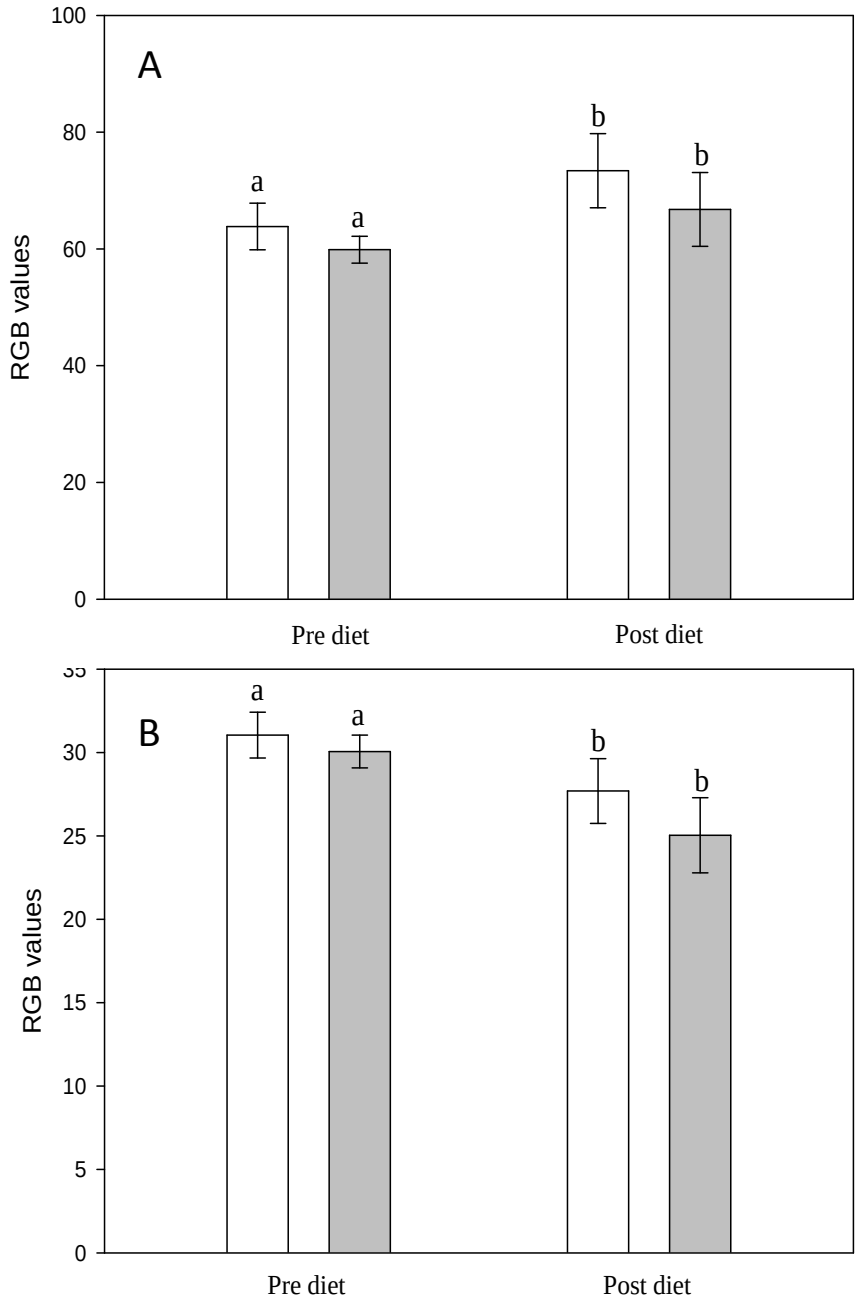


Fig. 51. Anal fin colour characteristics of fish fed either a 50% □ (N=11) or 20% ■ (N=11) protein diet over a two month period. Colour characteristics: anal blue greyscale (A) and anal red greyscale (B). Different letters denote a significant difference (RM-ANOVA, P>0.05); Data are means + s.e.m. Pre-diet is the start of the experiment and post diet is after 2 months of feeding on either 50 or 20% protein, before being placed into the breeding tank.

6.4.2 Mate choice results

6.4.2.1 Physiological characteristics

The diet on which individuals were fed for 2 months pre-pairing, and the resulting changes in physiology, did not predict mate choice (Fishers exact 2x2 contingency table, P=0.472). However, overall, fish that successfully paired within the 2 month mate choice period had a higher mass (two-way-ANOVA, $F_{1, 21}$=14.060, P<0.05; Fig. 52) and hepatosomatic index (two-way-ANOVA, $F_{1, 21}$=19.459, P<0.05; Fig. 53) than those fish that did not pair. After 2 months in the mate choice tank when all fish were being fed on the 50% protein diet, there were no longer any differences in mass (two-way-ANOVA, $F_{1, 21}$=1.241, P=0.280) or hepatosomatic index (two-way-ANOVA, $F_{1, 21}$=1.719, P=0.206) between dietary treatments at time of sampling. Fish that paired also had a lower relative spleen mass (two-way-ANOVA, $F_{1, 21}$=49.798, P<0.001; Fig. 54) and hematocrit (two-way-ANOVA, $F_{1, 21}$=4.511, P<0.05; Fig.55) compared with unpaired fish. While diet did not have a significant effect on haematocrit values following 2 months in the breeding tank (two-way-ANOVA, $F_{1, 21}$=1.836, P=0.192), it did have a significant effect on relative spleen values (two-way-ANOVA, $F_{1, 21}$=9.365, P<0.05) with a significant interaction between diet type and mating result (Two-way-ANOVA, $F_{1, 21}$=10.647, P<0.05). Mating result (two-way-ANOVA, $F_{1, 21}$=1.650, P=0.215; Fig. 56) and diet (two-way-ANOVA, $F_{1, 21}$=13.992, P=0. 692) had no significant effects on haemoglobin levels. Although diet did not predict which fish would pair, the resulting SGR of fish at the end of the 2 month dietary trial (i.e. before being placed in the breeding tank) was significantly higher in those fish that eventually formed breeding pairs relative to those that failed to pair (one-way-ANOVA, $F_{1, 21}$=4.643, P=0.044). Positive assortative mating based on the SGR of individuals after the two month dietary trial was also observed across the seven pairs, with individuals that attained a higher

SGR pairing first and with successive pairs having similar but lower SGRs (Pearson product-moment correlation, r=0.813, P=0.026).

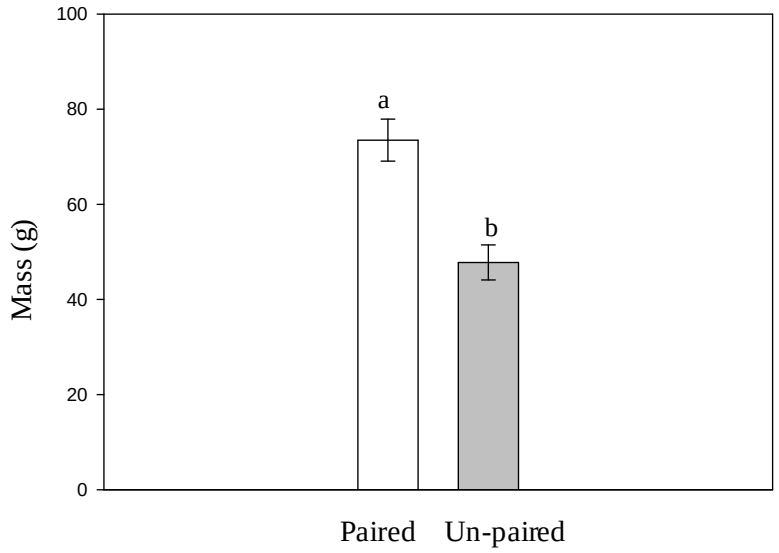


Fig. 52. Body mass of paired (N=14) *vs* un-paired (N=8) fish. Different letters denote a significant difference (two-way ANOVA, P>0.05); Data are means + s.e.m.

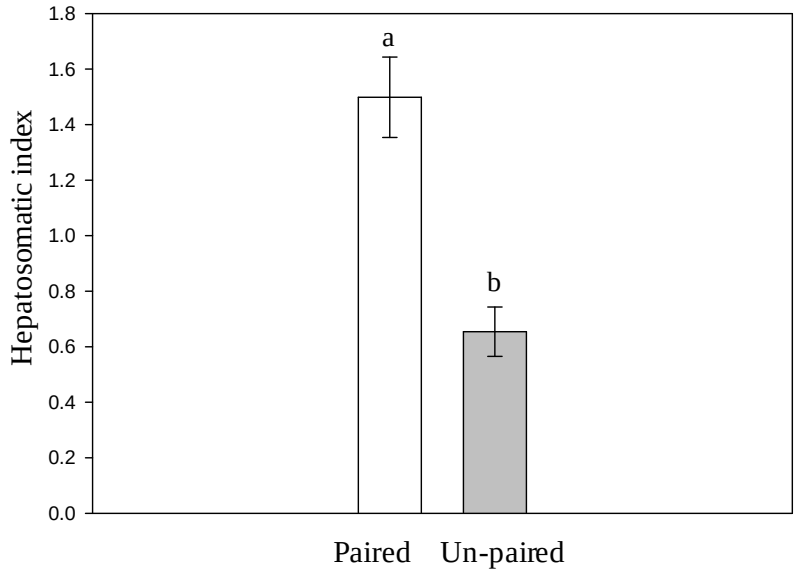


Fig. 53. Hepatosomatic index of paired (N=14) *vs* un-paired (N=8) fish. Different letters denote a significant difference (two-way ANOVA, P>0.05); Data are means + s.e.m.

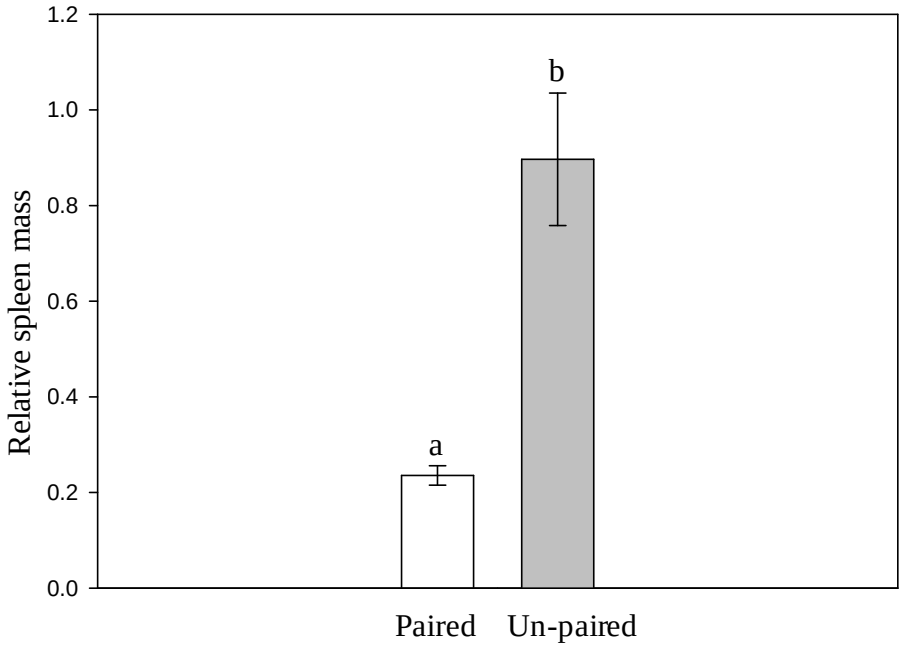


Fig. 54. Relative spleen mass of paired (N=14) *vs* un-paired (N=8) fish. Different letters denote a significant difference (two-way ANOVA, P>0.05); Data are means + s.e.m.

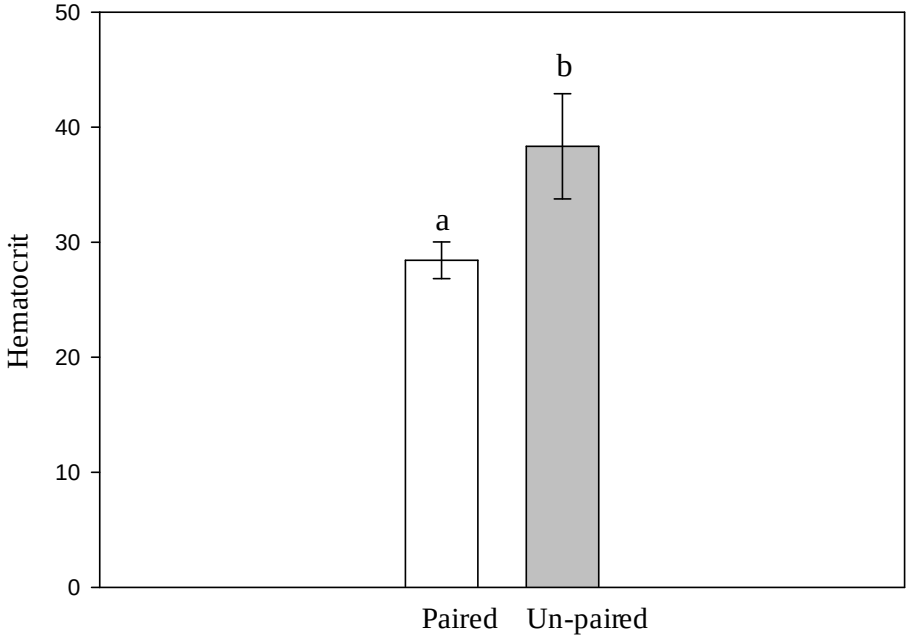


Fig. 55. Hematocrit of paired (N=14) *vs* un-paired (N=8) fish. Different letters denote a significant difference (two-way ANOVA, P>0.05); Data are means + s.e.m.

6.4.2.2 Colour characteristics

Operculum colour characteristics

A significantly higher operculum blue index (two-way-ANOVA, $F_{1,\ 21}$=20.321, P<0.001; Fig. 56A) and operculum blue greyscale value (two-way-ANOVA, $F_{1,\ 21}$=18.490, P<0.001; Fig. 56B) was observed in paired fish relative to non-paired fish. The effect of diet on operculum blue index values (two-way-ANOVA, $F_{1,\ 21}$=0.361, P=0. 555) and operculum blue greyscale values (two-way-ANOVA, $F_{1,\ 21}$=0.856, P=0. 367) was not significant by the end of the time in the breeding tank. No significant difference in red index values (two-way-ANOVA, $F_{1,\ 21}$=0.036, P=0.851) or red greyscale values (two-way-ANOVA, $F_{1,\ 21}$=2.299, P=0.147) was found between paired and non-paired fish. After 2 months in the breeding tank, diet had no effect on operculum red index values (two-way-ANOVA, $F_{1,\ 21}$=0.250, P=0. 623) or operculum red greyscale values (two-way-ANOVA, $F_{1,\ 21}$=1.201, P=0.288).

Dorsal fin colour characteristics

Mating result (two-way-ANOVA, $F_{1,\ 21}$=4.246, P=0.054) and diet (two-way-ANOVA, $F_{1,\ 21}$=0.001, P=0.974) had no effect on dorsal blue index values. While diet did not affect dorsal blue greyscale values (two-way-ANOVA, $F_{1,\ 21}$=0.106, P=0.748), paired fish had significantly higher dorsal blue greyscale values relative to non-paired fish (two-way-ANOVA, $F_{1,\ 21}$=9.299, P<0.05; Fig. 57). There were no significant effects of mating result on red index values (two-way-ANOVA, $F_{1,\ 21}$=0.381, P=0.545) or red greyscale values (two-way-ANOVA, $F_{1,\ 21}$=1.223, P=0.283). Diet had no effect on red index values (two-way-ANOVA, $F_{1,\ 21}$=0.319, P=0.579) or red greyscale values (two-way-ANOVA, $F_{1,\ 21}$=1.223, P=0.283).

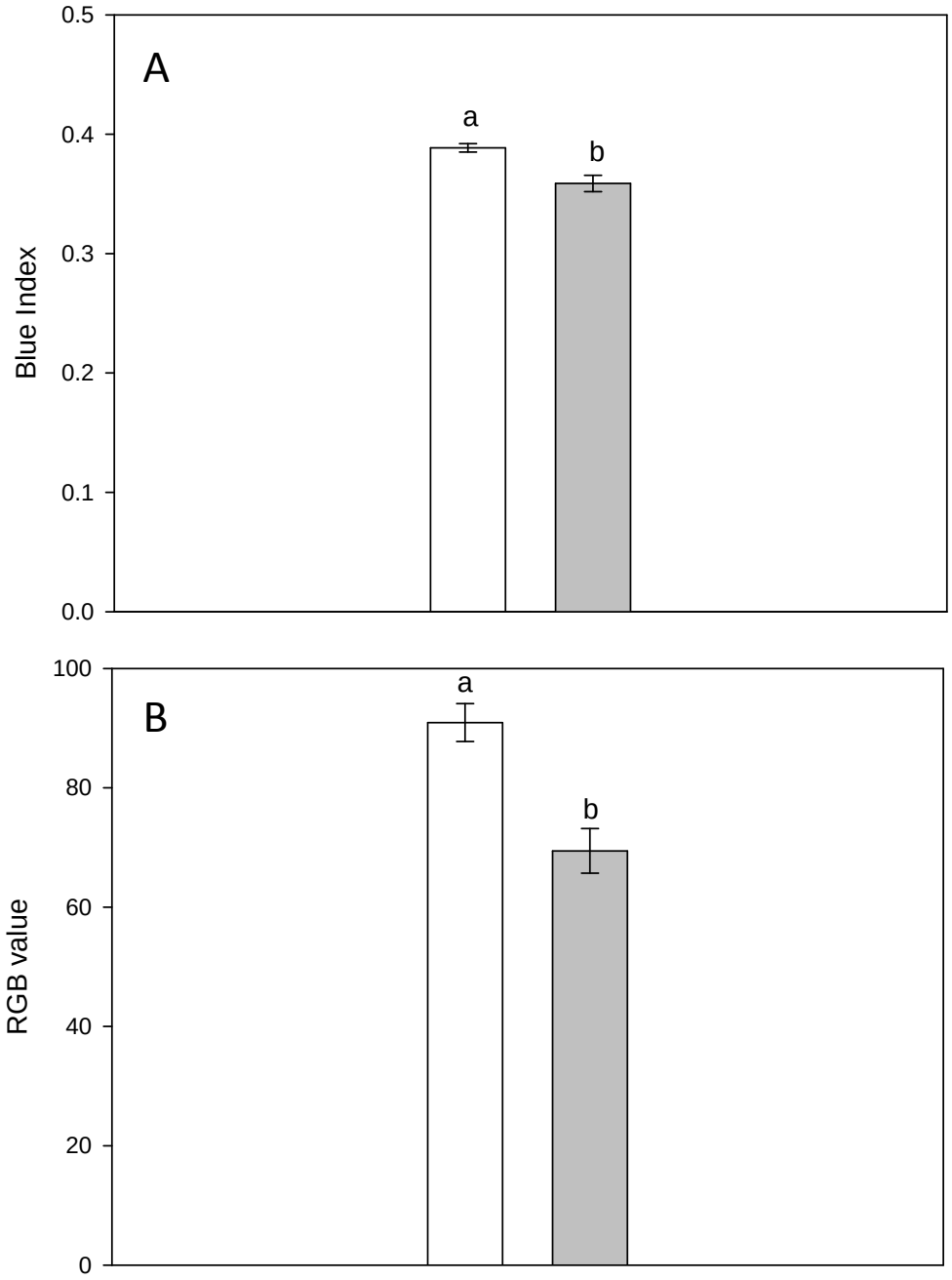


Fig. 56. Operculum colour characteristics of paired ☐ (N=14) *vs* un-paired fish ■ (N=8). Operculum blue index (A), operculum blue greyscale (B). Different letters denote a significant difference (two-way-ANOVA, P>0.05); Data are means + s.e.m.

Anal fin colour characteristics

Mating result (two-way-ANOVA, $F_{1, 21}$=2.203, P=0.155) and diet (two-way-ANOVA, $F_{1, 21}$=0.516, P=0.482) did not have significant effects on anal blue index values. While diet did not affect anal blue greyscale values (two-way-ANOVA, $F_{1, 21}$=2.286, P=0.148) paired fish had significantly higher anal blue greyscale values relative to non-paired fish (two-way-ANOVA, $F_{1, 21}$=17.106, P<0.05; Fig. 58). There were no significant effects of mating result on red index values (two-way-ANOVA, $F_{1, 21}$=0.23, P=0.880) or on red greyscale values (two-way-ANOVA, $F_{1, 21}$=4.135, P=0.057). Similarly, diet had no significant effect on red index values (two-way-ANOVA, $F_{1, 21}$=0.100, P=0.755) or on red greyscale values (two-way-ANOVA, $F_{1, 21}$=0.210, P=0.652).

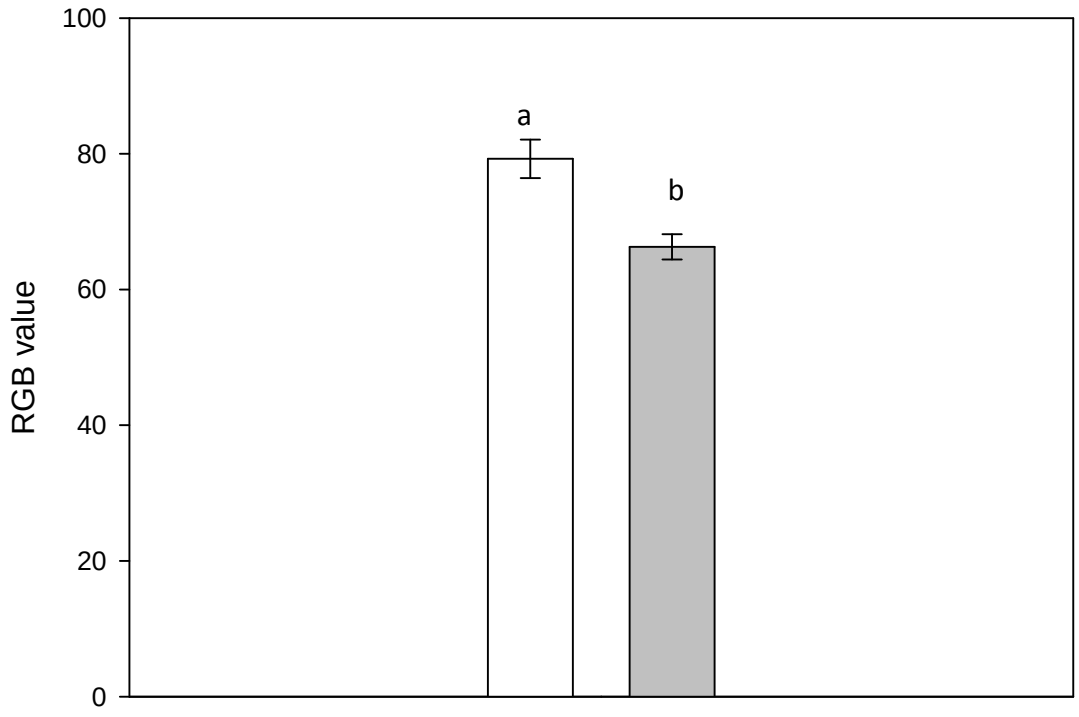


Fig. 57. Dorsal blue greyscale characteristics of paired ☐ (N=14) *vs* un-paired fish ■ (N=8). Different letters denote a significant difference (two-way-ANOVA, P>0.05); Data are means + s.e.m.

Body colour characteristics

Similar to that observed in other regions, paired fish had a significantly higher body blue (two-way-ANOVA, $F_{1, 21}$=26.177, P<0.001; Fig. 59A) and body red (two-way-ANOVA, $F_{1, 21}$=28.323, P<0.001; Fig. 59B) greyscale value than non-paired fish while diet had no effect on either body blue (two-way-ANOVA, $F_{1, 21}$=1.443, P=0.245) or body red greyscale values (two-way-ANOVA, $F_{1, 21}$=0.977, P=0.359). Body blue index (two-way-ANOVA, $F_{1, 21}$=3.677, P=0.071) and body red index (two-way-ANOVA, $F_{1, 21}$=3.671, P=0.071) were unaffected by mating results. Similarly, diet had no effect on body blue index values (two-way-ANOVA, $F_{1, 21}$=2.390, P=0.140) or body red index values (two-way-ANOVA, $F_{1, 21}$=1.795, P=0.197).

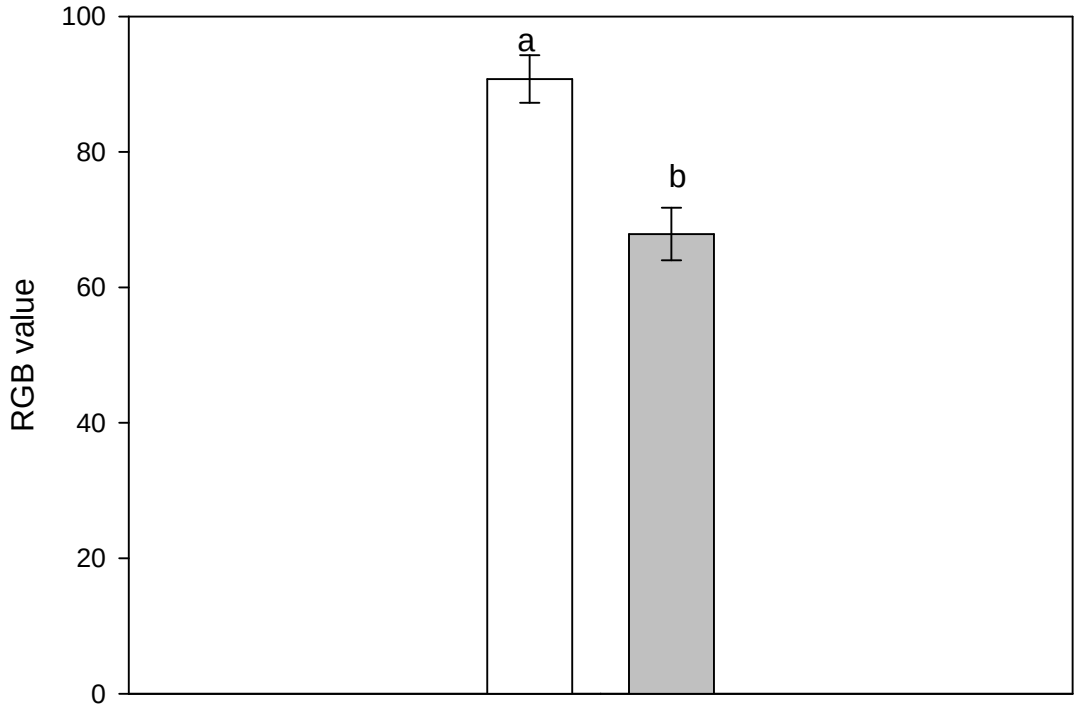


Fig. 58. Anal fin blue greyscale characteristics of paired ☐ (N=14) *vs* un-paired ■ fish (N=8). Different letters denote a significant difference (two-way-ANOVA, P>0.05); Data are means + s.e.m.

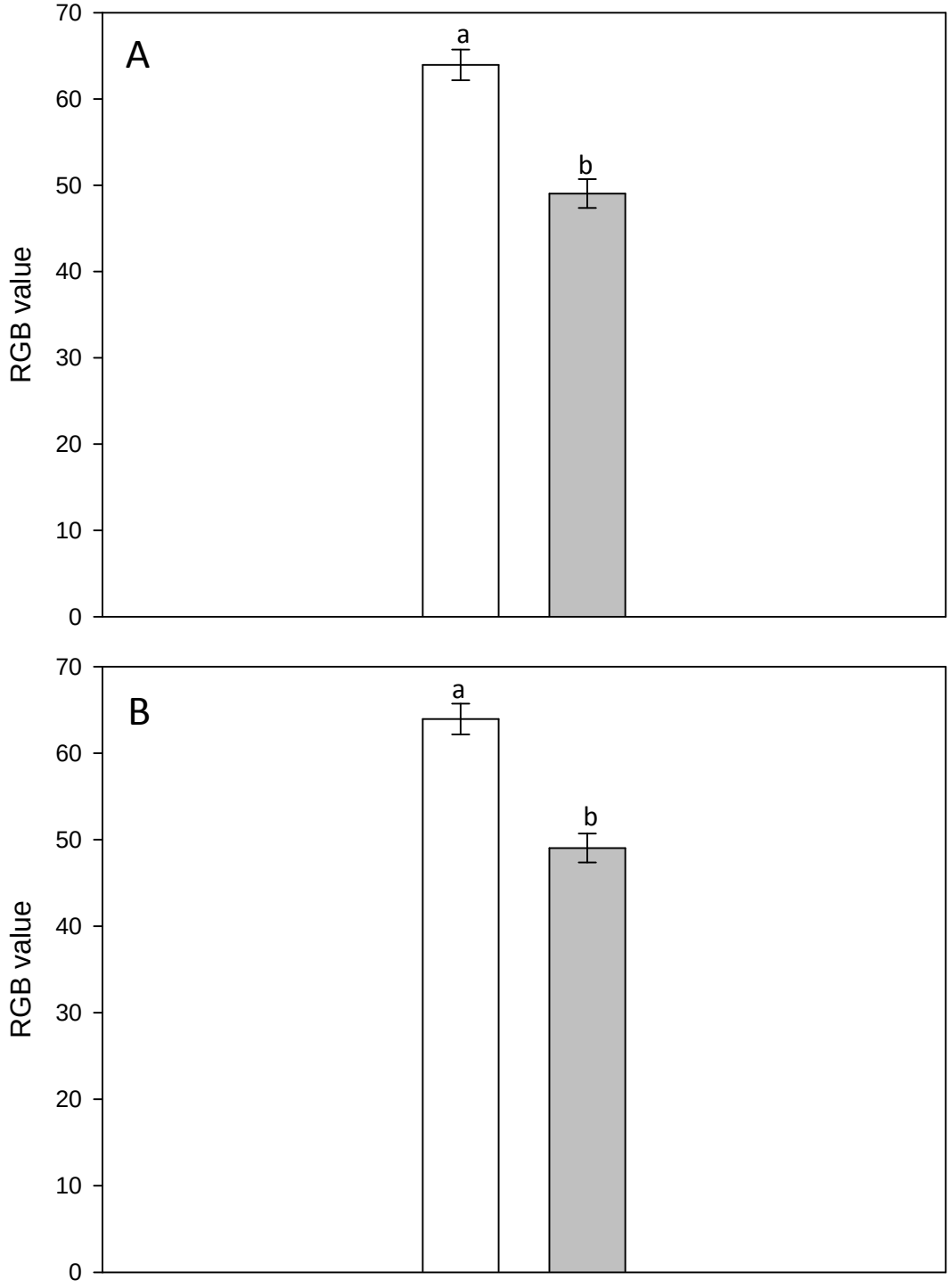


Fig. 59. Body colour characteristics of paired ☐ (N=14) *vs* un-paired fish ■ (N=8). Colour characteristics: body blue greyscale (A) and body red greyscale (B). Different letters denote a significant difference (two-way-ANOVA, P>0.05); Data are means + s.e.m.

6.5 Discussion

6.5.1 Effect of diet

Similar to that observed by Chong et al. (2000) discus fish fed a diet consisting of 50% protein for two months were in better condition and had significantly higher fork lengths, mass and SGRs than those fed the 20% diet. Despite marked differences between the growth characteristics of the two dietary groups, mucus total protein concentrations did not differ significantly. This was surprising as diet was hypothesised as one of the key factors that resulted in the observed difference in mucus total protein between wild non-breeders and aquarium-bred non-breeders (Buckley et al., 2010). Comparisons between the non-breeders in this present study and those of previous studies indicate that discus fed the commercially developed Tetra Prima diet in chapter 2 had higher mucus total protein values (5.52 mg ml^{-1} ± 0.48) than the average found in this present study (2.89 mg ml^{-1} ± 0.34) which in turn was higher than the mucus total protein values observed in wild discus (1.07 ± 0.24 mg ml^{-1}). The lower mucus total protein values of wild discus is most likely a reflection of both the infrequent food supply associated with the Amazon dry season and the nutritional content of their diet, which consists of algal periphyton, fine organic detritus and green plant matter (Crampton, 2008). The average mucus total protein value obtained in this study was over 2 mg ml^{-1} lower than that observed in aquarium-bred non-breeding discus fed the specially formulated tetra prima diet (Chapter 2, 5.52 mg ml^{-1} ± 0.48; Buckley et al., 2010). While the protein content of the Tetra Prima diet (47.5%) was similar to the 50% protein diet used in this study, the type of protein and processing technique used in each diet was different which can lead to differences in protein digestibility (Cheng and Hardy, 2003; Gomes et al., 1995). These differences in protein digestibility may have led to the differences in mucus total protein observed between this chapter and that recorded in chapter 2. While diet is likely one of the main influences affecting mucus

total protein concentration (Saglio and Fauconneau 1985), other factors such as environment and population genetics cannot be ruled out when explaining the differences in mucus protein between different laboratory studies and in wild populations.

Colour in fish is generally determined by either the deposition of pigments such as carotenoids (Barber et al., 2000b) or though the structural arrangement of iridophores (Denton and Nicol, 1966; Doucet and Meadows, 2009; Hawkes, 1974; Losey et al., 1999). Carotenoids are largely responsible for the red colouration of fish. They cannot be synthesized *de novo* (Chatzifotis et al., 2005) and must instead be obtained through the diet. Although the dietary consumption of carotenoids is one of the key mechanisms for enhancing red colouration (Kodric-Brown, 1989), studies have found that the condition of fish (Baube, 1997; Candolin, 1999; Candolin, 2000) and the production of stress hormones can greatly influence red colouration (Loiseau et al., 2008; Mougeot et al., 2010), with many studies discovering rather unintuitively that food deprived fish often have brighter red ornaments (Candolin, 1999; Candolin, 2000). Despite the reduced growth characteristics of discus fed the 20% protein diet red colouration did not differ between the two dietary groups, a result that is perhaps not surprising due to the absence of carotenoids in either diet. While diet had little effect on red colouration, small but significant differences were observed in the blue colouration located on the operculum and dorsal fin. The blue regions located on the operculum, dorsal fin and anal fin in this particular colour morph of discus have an iridescent property suggesting that the blue colour is either part or wholly structural in nature. While structural colour is not necessarily reliant on diet like carotenoid-based colours, some studies have indicated that condition may have an impact on this colouration in both birds (Keyser and Hill, 1999; McGraw et al., 2002) and fish (Cogliati et al., 2010). While the body and anal fin region did not differ, the operculum and dorsal fin showed an elevated blue

index value in fish fed the 50% diet possibly suggesting a particular link between the blue colouration of these regions and condition.

6.5.2 Mate choice

After the two month dietary trial, both treatment groups were introduced to the same aquarium tank complete with a breeding territory and allowed to pair up naturally. Over the next two month period a total of seven pairs formed breeding pairs leaving eight individuals as non-breeders. Diet did not appear to influence mate choice decisions as only one pair consisted of individuals from the same diet, the rest featured males and females from the two different dietary groups. Although diet had very little impact on mate choice decisions, there was a significant difference in both the physiological and colour characteristics of those fish that eventually paired compared with those that did not.

Fish that paired had a higher SGR at the end of the initial two month dietary trial compared to those fish that would ultimately fail to pair. This suggests that during the initial dietary trial there were individuals within both dietary treatments that were better able to monopolise food, a characteristic behaviour of dominant individuals. Although efforts were made to reduce the formation of hierarchies during the first two month dietary trial, personal observations suggest that fish had adopted dominant and subordinate roles within each dietary tank prior to the mate choice experiment. The formation of social hierarchies within each dietary group could have then had a subsequent bearing on the mate choice experiment as prior dominance is known to increase the likelihood of future dominance (Beacham and Newman, 1987; Beaugrand and Zayan, 1985; Rhodes and Quinn, 1998). The development of dominant individuals within each dietary treatment may, therefore, have masked the effect of diet as dominant fish within the 20% protein diet may have monopolised the food source and consumed larger quantities of the diet negating the impact of the lower protein percentage. The

development of subordinates within the 50% dietary tank may have also resulted in these fish having a lowered probability of competing against dominants from the 20% protein tank due to prior experience effects, even though they may have been in better condition and thus better able to facilitate parental care. Supporting the assumption that mate choice decisions were based on social status, physiological characteristics of paired vs non-paired fish at the end of the mate choice trial were consistent with the observed differences between dominant and subordinate fish (Filby et al., 2010; Sloman and Armstrong, 2002).

At the end of the mate choice trial, paired fish were significantly larger than non-paired fish suggesting that these individuals were better able to win fights and control food sources; similar differences in size have previously been noted between dominant and submissives in aquarium studies (Allee et al., 1948; Filby et al., 2010; Sloman et al., 2000a) where dominant fish can both monopolise food sources as well as supress the feeding rates of subordinate fish (Abbott and Dill, 1989; Griffiths and Armstrong, 2002; Sloman and Armstrong, 2002). The HSI of paired fish at the end of the experiment was also significantly higher than non-paired fish indicating greater energy stores in the liver (Lambert and Dutil, 1997). Relative to dominant fish, lower HSI values are also observed in subordinates in other fish species (Guderley and Couture, 2005; Sloman et al., 2000b, 2001b) where the reduction in relative liver size is thought to be indicative of the need for enhanced mobilization of energy stores. In subordinates this may occur due to the catabolic actions of cortisol (Filby et al., 2010; Lambert and Dutil, 1997): a stress hormone released during the agonistic interactions typically received by subordinates (Sloman et al., 2001a). As a result of stress from aggressive interactions with dominant fish, subordinate fish may also have higher haematocrit and haemoglobin values due to splenic contraction (Ferraz and Gomes, 2009; Gallaugher and Farrell, 1998); a process

where the spleen releases stored erythrocytes to allow an increase in oxygen for burst swimming used during periods of aggression (Gallaugher and Farrell, 1998; Primmett et al., 1986). Un-paired fish in the present study had significantly higher haematocrit values and there was a trend towards higher haemoglobin values in non-paired fish. The relative spleen size of un-paired fish was also significantly higher than that of paired fish. While previous research indicates that spleen size decreases in subordinate individuals (Peters et al., 1980) an enlarged spleen is indicative of stress related to parasite infection in fish (Arnott et al., 2000; Barber et al., 2000b; Huang et al., 1999). While no parasites were observed, an enlarged spleen may be an early indicator of increased susceptibility to parasites in subordinates.

Differences in colouration were also observed between paired and non-paired fish. The blue colouration around the operculum, dorsal fin, anal fin and body in paired fish was significantly brighter than in non-paired fish and there was also a trend toward a brighter red colouration in paired fish. It is difficult to link the colour of these regions to the ability of an individual to provide parental care or to diet, but the darkening of colour in non-paired fish may be linked to their subordinate role. Darkening in subordinates has been observed in several species of fish (Beeching, 1995; Höglund et al., 2000; O'Connor et al., 1999) and is thought to be mediated by the production of the pigment melanin, produced during periods of stress by the melanocyte stimulating hormone (Höglund et al., 2000). The darkening of colour in non-paired fish may, therefore, signal their subordinate social status, thereby minimizing potential future conflicts (O'Connor et al., 1999).

The importance of social status for prospective mate choice decisions in discus fish, indicated by the successful pairing of fish with dominant characteristics, was also

highlighted by the presence of an observed positive assortative mating strategy based on the SGR of individuals obtained during the initial two month dietary trial. The first few individuals that formed pairs consisted of males and females with similarly high SGRs with all successive pairs containing males and females with similar albeit decreasing SGRs. If the SGR of fish is indicative of an individual's ability to dominate a territory and monopolise food it could, therefore, be seen as a proxy for dominance. This would then suggest that dominance is an integral part of mate choice decisions in discus fish. In the angelfish (*Pterophyllum scalare*) (Cacho et al., 2006) and the midas cichlid (*Cichlasoma citrinellum*) (Rogers and Barlow, 1991) females prefer larger, aggressive, territorial and experienced males that when mated with tend to an increase in egg survival (Cacho et al., 2006; Rogers and Barlow, 1991) suggesting that in these species dominance is a trait selected for due to the benefits it confers to offspring survival.

In the wild, there is the potential for extensive social communication between conspecific discus fish in breeding aggregations that could allow the formation of social hierarchies. These hierarchies could then allow individuals to assortatively pair up based on characteristics linked to dominance. Observations in the aquarium indicate that unpaired discus will predate the eggs of a breeding pair with studies in the wild indicating that predators are extremely abundant during the time of year discus begin to breed (Crampton, 2008; Goulding, 1980); the ability to be dominant and control a territory may, therefore, significantly aid offspring survival as observed in the closely related angelfish and midas cichlid.

6.6 Conclusion

Mucus total protein values were unaffected by diet in this study suggesting that when diet digestibility and formulation is controlled for differences in dietary protein are not reflected in mucus composition. Although mucus total protein values were not affected by diet in this study, comparisons between the values obtained in this study, and those

obtained in previous studies where diets were notably different, suggesting that factors other than protein content can have marked effects on mucus total protein values. Further dietary manipulation trials focusing more on digestibility and nutritional content may, however, be needed to confirm this. While the two month initial dietary trial resulted in those fish fed the 50% protein diet having a higher SGR than those fed the 20% protein diet, this did not influence the mate choice decisions of discus fish with fish from both groups pairing together. Instead mate choice appeared to be influenced by the social position that individuals had attained during the two month dietary trial. Fish that paired attained a significantly higher SGR than unpaired fish during the initial two month dietary trial suggesting the ability of paired fish to monopolise food sources, a behaviour consistent with that observed in dominant individuals. Further analysis of paired individuals indicated that individuals assortatively paired relative to the SGR attained during the initial dietary trial. At the end of the mate choice trial, differences between the physiological and colour characteristics of paired fish vs unpaired fish were also pronounced and consistent with the differences observed between dominant and subordinate individuals suggesting that, similar to that observed in closely related cichlids, social status was important in mate choice decisions. While benefits of mating with a dominant individual in discus fish are unknown, it is likely that aggressive and territorial behaviours could help with the protection of offspring in an environment characterised by high levels of predation and competition.

Chapter 7: Thesis discussion

7.1 Parental care and the development of the parent offspring conflict

Numerous parental care strategies have evolved across the animal kingdom to maximise the genetic interests of the parent and ensure the survival of progeny (Clutton-Brock, 1991). The provision of parental care during this critical period of development can help provide offspring with benefits such as protection from predators (Dominey, 1981; Forslund, 1993), the provision of an optimum external environment (e.g. heat or oxygen) (Maruyama et al., 2008; Nowak et al., 2000) and the provision of a source of nutrition (Jarvis, 1981; Klobasa et al., 1987). Parental care behaviour is often most notably associated with mammals and birds where behaviours such as the provision of milk in mammals or crop milk in birds is easily observable. It is in these species that perhaps the vast majority of work concerning parental care has been carried out, work which ultimately led to Robert Trivers formulating the concept of the parent offspring conflict (Trivers, 1974). While the provision of parental care was initially thought to be a one-way process whereby offspring passively accepted parental care allowing parents to equally distribute resources to all offspring; Trivers (1974) proposed that offspring should be in conflict with parents as to the level of care that is offered. Inspired by the earlier work of Hamilton (1964) where the concept of inclusive fitness was first proposed, Trivers pointed out that offspring are not always passive and will use a range of physiological and psychological tactics to try and solicit more parental resources than their parents would be willing to provide. The basis for this assumption is derived from the fact that the degree of relatedness between parents and their offspring is only 0.5 between outbred, diploid animals and that parents and offspring should ‘disagree’ about the level of investment. Any parent wishing to maximise their inclusive fitness, would want to invest in current offspring up until the point where any further investment would only offer diminishing returns. Any investment past this point would squander energy that would have a greater return if invested into future offspring. Too much

parental investment into current offspring will therefore reduce the total number of offspring that an individual could ultimately produce; equating to a reduction in an individual's inclusive fitness. It is, therefore, expected that parents should regulate the amount of care they provide to their current offspring so as to maximise their own inclusive fitness. Implicit in Trivers's theory, however, is the suggestion that where offspring are able to modify their parents' behaviour, they may reduce the parent's fitness by shifting the level of investment away from their parent's optimum toward their own.

Examples of conflict are especially prominent in primate research; in chimpanzees (*Pan troglodytes*) mother-infant conflicts occur during feeding, grooming, travelling, evening nesting, suckling, and mating (Clark, 1977; Goodall, 1968; Horvat and Kraemer, 1982; Maestripieri, 2002). This conflict starts when a mother begins to reject the advances of her offspring, at which point the infant will often display many elements of depression and during the final month of suckling, regress to infantile behaviours such as whimpering and tantrums (Maestripieri, 2002). In pigs, sows initially spend all their time associated with their piglets allowing them to suckle at regular nursing bouts. As time progresses the sow initiates less nursing bouts and will often try and spend more time away from the piglets in order to wean them off maternally provided milk (Drake et al., 2007; Jensen and Rece´n, 1989). In many farms sows do not have the space to move away from their piglets which results in the piglets initiating nursing bouts via udder massage. To combat this, the sow will often avoid udder massage by increasing the time spent lying down teats obscured (Drake et al., 2007; Weary et al., 2008). This conflict over resources has also been observed during the period of intrauterine development in humans (Haig, 1993). During this period the placenta secretes allocrine hormones that decrease the sensitivity of the mother to insulin thus making a larger supply of sugar available to the fetus. The mother responds by increasing the level of

insulin in her bloodstream, which is in turn counteracted by the placenta that produces insulin degrading enzymes (Haig, 1993). In mammals the pre-birth conflict can occur due to the intimate connection of the fetus with the mother; in lecithotrophic species such as fish this connection is absent.

In most fish, the initial embryo-mother contact is absent, negating the development of this early form of conflict. While parental care is present in fish, it often involves behaviours such as the cleaning, fanning or protection of eggs from predators, a behaviour that often ends before offspring hatch and become free swimming (Clutton-Brock, 1991; Gross, 2005). In these species where parental care ends before offspring hatch, the parent can allot the amount of care they want to give to offspring, without offspring being able to interact and cause conflict over this decision (Carlisle, 1982; Sargent and Gross, 1993). In around 30 species of cichlid, however, parental care continues past the point where eggs hatch and parents provide both protection and a form of nutrition to offspring for the first few weeks of development (Schutz and Barlow, 1997). While this behaviour occurs to varying degrees across these 30 species, it is most prominent in the Amazonian cichlid *Symphysodon* spp. where parental care is obligate for the survival of fry (Chong et al., 2005). Both the male and female of this species, commonly referred to as discus fish, produce a nutritious form of mucus that is secreted over the surface of the body that fry feed off for the first few weeks of development (Hildemann, 1959; Noakes, 1979). Like that observed in mammals, my research has highlighted how the behaviours associated with the provision of parental care in discus fish can also lead to the development of conflict between parents and their offspring (Buckley et al., 2010).

To further understand the dynamics of parent offspring conflict in discus fish it would be interesting to try and determine the costs and benefits of care to both adults and fry to try and affect the point at which weaning is initiated. The manipulation of diets for example could be used to produce breeding adults raised on either an optimum or sub optimum diet. If those raised on the suboptimum diet are in a poorer nutritional condition, then due a perceived reduction in available energy for mucus production, suboptimum parents could wean offspring early. If the benefits of feeding on mucus are manipulated i.e. an excess of live food provided thereby reducing the benefit of mucus feeding, then offspring may wean themselves early as food is abundant. Previous work by Chong et al. (2005) indicated that this may be the case as the addition of *Artemia* did reduce the bite rate of offspring. Future work designed to investigate the way in which the timing of the weaning period can be manipulated will not only help provide details about the mechanics of conflict but also validate the formation of the parent offspring conflict in discus fish.

As well as understanding the dynamics of conflict between parents and offspring it would be interesting to see if conflict between offspring could also develop (Clutton-Brock, 1991). Offspring are as equally related to their other siblings as they are to their parents and similar to the development of conflict between parents and offspring, conflict can also develop between siblings. The consumption of mucus is such that by the 2^{nd} to 3^{rd} week, offspring demand may exceed parental production. A shortage in available mucus may then lead to the kind of aggression normally observed in mammals. In pigs for example, suckling pigs aggressively compete for access to the prime anterior teats of their mother (Dawkins, 1976) and in birds conflict between offspring can also lead to siblicide (Kacelnik et al., 1995). As well as conflict over access to mucus, there could also be conflict over the area mucus is obtained from. Like the prime anterior teats of sows there may be prime positions on adult discus that either

secrete mucus at a higher rate and so provide a richer access to mucus or allow a greater deal of protection from predators during feeding. A more in depth behavioural analysis of offspring may allow a greater insight in to the potential development of offspring-offspring conflict.

7.2 Composition of parental mucus

While the immediate benefits of mucus feeding relate to a substantial increase in offspring growth rate, analysis of parental mucus revealed a suite of components that could provide benefits to discus fry above that of just growth (Buckley et al., 2010). Although it was only possible to look at the behaviour of aquarium bred discus, it was possible to measure and compare the mucus of aquarium reared discus with that of their wild counterparts (Chapter 3). While wild discus have to face the selection pressures associated with survival in the wild environment, aquarium bred discus have an entirely different set of pressures due to aquarists imposing differential survival based on traits related to appearance. Comparing the mucus physiology of wild vs aquarium bred discus consequently allowed an insight into the different pressures associated with the wild and aquarium environment.

One component present in both the mucus of wild and aquarium-bred discus was the antibody IgM. Concentrations of IgM in aquarium discus became elevated as soon as eggs are laid and continued to stay elevated until the 4^{th} week of parental care where a drop in concentration was then noted. In mammals, the neonate experiences a time during early development where their adaptive immune system is not yet mature and where pathogens could potentially become problematic (Goldman et al., 1998). Mammals negate this potentially immune compromised period through the constant provision of a maternal based immunity during lactation (Adamski and Demmer, 2000; Klobasa et al., 1987; Kurse, 1983; LeJan, 1996). The elevation of mucosal IgM during

the initial period of parental care suggests that like the provision of antibodies in mammalian milk, the provision of IgM within parental mucus may provide offspring with a passive form of immunity. It would be interesting to see if the development of an adaptive immunity in discus offspring occurs during week 3 when parents begin to wean offspring off parental care as this would resemble the close knit relationship observed in mammals. Tracking the development of the adaptive immune system in fry could be achieved through *in situ* hybridisation where the expression of genes related to VDJ recombination (Variable, Diverse and Joining gene segments) could help distinguish when the adaptive immune system of offspring becomes active (Corripio-Miyar et al., 2007; Huttenhuis et al., 2005; Willet et al., 1997). In particular, I identified the RAG-1 gene as being crucial for the generation of mature B and T lymphocytes (Lam et al., 2004; Willet et al., 1997), a process essential for the initiation of the adaptive immune system. Unfortunately after exploratory work where I was able to use genetic probes designed for zebrafish (the genetic similarity between species was such that zebrafish probes were useable) to carry out *in situ* hybridisation, I could not obtain enough discus breeding pairs to carry out a study looking at the ontogeny of the immune system. This was frustrating as I had also developed a methodology that would have also allowed me to measure the IgM present within fry so that I could investigate whether parentally provided IgM within the mucus was being utilized by fry during a period where their adaptive immune system was not yet functioning.

Comparisons between wild and aquarium bred fish were also interesting in that although not significant (possibly due to the low n number I managed to obtain) wild discus appeared to potentially have a greater concentration of mucosal IgM (Chapter 3). This suggests that there are maybe more stringent selection pressures in the wild related to surviving pathogens than there are in the sterile environment characterised by most aquariums. As well as differences related to the presence of pathogens, wild discus also

pair and breed in vastly different social situations which itself could lead to an increased presence and transmission of pathogens. There are typically two seasons in the Amazon, the dry season and the wet season (Crampton, 2008; Furch, 1984; Junk, 1997). During the dry season discus seek refuge and shelter from predators in the woody confines of natural structures called 'galhadas' located in the still lakes associated with the Amazon River (Crampton, 2008). During the dry season large congregations of discus can seek shelter within a galhada where individuals will often form breeding pairs. It is during this period that group living combined with still moving water could lead to an increase in the transmission of pathogens (Hughes et al., 2002; Poulin, 1999; Trivers, 1985). Since breeding occurs toward the end of the dry season it is possible that there are risks of offspring being challenged before their own adaptive immune system has developed leading to a significant drop in survival rates. It, therefore, seems likely that there would be a significant selection pressure for parents to provide offspring with a passive form of immunity during this delicate period, a mechanism that may have been observed in both wild and aquarium discus fish.

7.3 Offspring adaptations to parental care

While the behaviour and mucus composition of discus fish have evolved to provide offspring with high levels of care, developmental structures present in offspring seemingly aid the ability of parents to provide care. One of the interesting behaviours I observed during the initial period of parental care involved the interaction between parents and their newly hatched offspring. During the initial 5-6 days of development, fry stay attached to the vertical substrate they were initially laid on via their cement gland. During this time parents will clean away dead eggs, provide aeration, protection and if during this time a threat is perceived, parents will collect fry within their mouths and diligently move and reattach them to a more secluded part of the aquarium. It is not known, however, how parents use this ability *in situ* and how regularly parents move fry

and whether the movement of fry is always due to perceived threats of predation or whether environmental changes due to fluctuations in water height can also cause this.

Another interesting developmental structure in fry is the presence of conical unicuspid teeth that were present around the 8th day post fertilization. These unicuspid teeth have been found on all teleost fry so far investigated (Sire et al., 2002; Streelman et al., 2003) where their role has been predicted to aid planktivory. In discus fish, these unicuspid teeth may function to aid in the removal of bitefuls of mucus from the sides of their parents. Observations of mucus feeding indicate that fry will twist and writhe during their bite motion indicating some difficulty to the process of feeding. The presence of fang like teeth may, therefore, be vital in obtaining enough purchase on mucus so that it can be removed. This raises the fascinating question of whether the evolution of mucus feeding would have been possible without fry already having developed teeth. If not, the presence of these unicuspid teeth in the ancestors of discus fish may have allowed the development and evolution of this interesting and novel form of parental care.

The ability of fry to perceive UV wavelengths may also aid parental care in discus fish. During the analysis of the optical transmission properties of discus lenses, it was discovered that while an adult lens would block UV wavelengths, a juvenile lens would allow this wavelength to pass through, highlighting the possibility of UV perception in juvenile discus fish. The ability to perceive UV light has been demonstrated in a wide range of juvenile fish and has been demonstrated as aiding planktivory as UV perception is great at enhancing contrast (Jordan et al., 2004a), a skill needed to pick out plankton from the water column: In discus fish it may also be used to help navigate fry to their parent's side. In wild fish, bands of iridescent blue were present on the dorsal and anal fins of adults; it would be interesting to see if this iridescent blue (a colour that reflects highly in the UV (Prum and Torres, 2003)) is used as some kind of visual clue akin to a fluorescent sign highlighting a target to help fry locate their parent and feeding

area during the first few days of free swimming. Manipulation of lighting conditions so that parent seeking behaviour could be analysed under UV present and UV absent conditions would help to explore this idea.

7.4 Mate choice

The evolution of this fascinating form of parental care in discus fish also poses some intriguing questions in regards to mate choice in this species as a lot of reproductive behaviours are associated intimately with parental care behaviours (Andersson, 1994; Clutton-Brock, 1991). In discus fish, parents invest a large amount of energy and care into offspring which suggests that potential parents should be picky about whom they mate with. Mating with a lower quality individual could mean they would have to expend a higher proportion of energy in young than if they mated with an individual of equal or greater quality. In species where the provision of parental care is essential for the survival of offspring, mate choice becomes particularly important (Andersson, 1994). One strategy used to predict the quality of a potential mate involves assessing traits that predict their ability to provide parental care, a behaviour known as the 'good parent' hypothesis (Hoelzer, 1989; Kokko et al., 2003). This hypothesis was tested in discus fish through the use of a dietary trial designed to create two groups of discus varying in their ability to produce mucus with the hypothesis that individuals better able to produce high quality mucus will preferentially pair. The results of this trial indicated that the predominate factor for whether a fish paired successfully or not was due to whether or not their social position was one of a dominant or a submissive.

While social position was responsible for the observed mating strategy in discus fish in my experiment, the experiment was limited due to both the expense of fish (limiting replicas) and the space requirements needed for this species. It would be interesting to interrogate mate choice more in this species as other cues may be responsible for mate

choice as well. Olfaction in particular has been demonstrated as having a considerable effect on mate choice decisions in other species, as fish, like mammals, utilize smell as an indicator of genes related to immunity (Landry et al., 2001; McConnell et al., 1998; Milinski, 2003). Fish have been observed to pair with an individual whose genes on the MHC complex compliment theirs in a way that will provide their resulting progeny with an effective immune system (Milinski, 2003). Considering the group living conditions of discus fish and the vast array of pathogens present in their environment, it could be hypothesized that olfaction related to the MHC complex may, therefore, be present in discus fish. While work in chapter 6 demonstrated that the dominant fish were selected for during mate choice and that these fish were much brighter than subordinate fish, it would be useful for further work to interrogate the use of colour in discus mate choice, to see if it there are subtleties in colouration linked to other traits related to aspects of health and nutrition.

One of the interesting aspects of mate choice in discus fish involved pair bonding behaviour. This involved a ritualised swimming behaviour as described in chapter 6, followed by aggression directed at conspecifics. Ritualised swimming behaviour would occur frequently after pairing and especially before the laying of eggs where it seemed to serve the purpose of strengthening the bond between parents. In mammals pair bonding behaviour and parent offspring bonding behaviour is mediated by a range of hormones including prolactin and oxytocin (Bales, 2005) both of which could serve similar roles in discus fish. Links between the presence of the hormone prolactin and parental care behaviour have already been observed in discus fish with previous work demonstrating the up-regulation of the hormone prolactin in the epidermis of parental discus fish (Khong et al., 2009) and an earlier study demonstrating that upon injection, discus fish would begin the production of mucus (Blum and Fiedler, 1965). It is interesting that the presence of prolactin induces both the production of milk in

mammals and the production of mucus in discus fish and that the roles of milk and mucus appear to be analogous. As well as inducing the production of milk/mucus, this hormone is also known to induce parental care behaviour in both mammals (Bales, 2005) and fish (Kindler et al., 1991). This raises the question of whether other hormones related to parental care in mammals have similar functions in discus fish. Along with prolactin, the hormone oxytocin is crucial for the initiation of pair bonding behaviour both between mates and between parents and offspring (Bales, 2005; Bales and Carter, 2002; De˛biec, 2007; Williams et al., 1994). While oxytocin is not present in discus fish, its homologue isotocin is, and has been implied in the modulation of sex typical vocalizations during mate choice in the midshipman fish (*Porichthys notatus*). It could be hypothesised that the importance of both parents for the successful rearing of offspring is such that a mechanism whereby both parents felt urged to stay together and cooperate would be present. In mammals this bonding behaviour is mediated by hormones such as oxytocin, in discus fish it could potentially be mediated by its homologue isotocin. Future work focusing on this hormone and how it changes during mate choice and throughout the period of parental care could help offer an interesting insight into the endocrinology of discus fish and highlight potential similarities between the bonding behaviour of mammals and that of discus fish.

7.5 Conclusion

While discus fish are a complicated and difficult species to work with, the rewards are great as their complex behaviours related to the provision of parental care provide a new model for the analysis of the parent offspring conflict. Although my research has demonstrated the presence of a conflict, hitherto unseen in fish, there is still much work to be done to identify the mechanisms of the parent offspring conflict with dietary and environmental manipulations required to fully gauge the extent of conflict in this species.

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

Jonathan Buckley[1,*], Richard J. Maunder[1], Andrew Foey[1], Janet Pearce[1], Adalberto L. Val[2] and Katherine A. Sloman[1,3]

[1]School of Marine Science and Engineering, University of Plymouth, Plymouth PL4 8AA, UK and [2]Department of Ecology, Laboratory of Ecophysiology and Molecular Evolution, INPA, Manaus 69060-001, Brazil and [3]School of Science, University of the West of Scotland, Paisley PA1 2BE, UK

*Author for correspondence (jonathan.buckley@plymouth.ac.uk)

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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

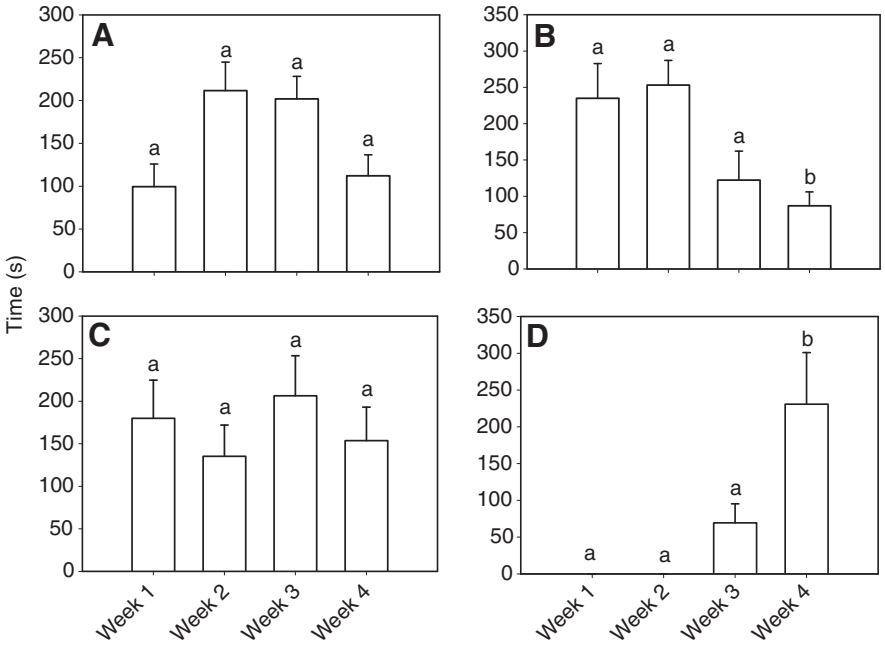

Fig. 1. Time discus fish young spent associated with the (A) male parent, (B) female parent, (C) both parents or (D) neither parent. Different letters denote a significant difference (paired *t*-test, P<0.05, N=6); bars that share a letter are not statistically different. Data are means + s.e.m.

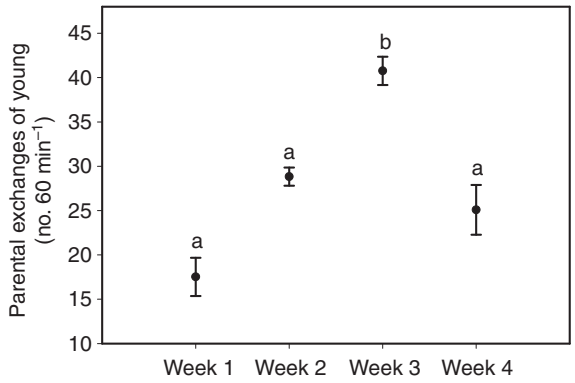

Fig. 2. Total number of incidences within a 60 min observation period where the mode of parental care in discus fish changed across the 4-week breeding period. Different letters denote a significant difference (paired *t*-test, $P<0.05$, $N=6$); bars that share a letter are not significantly different.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

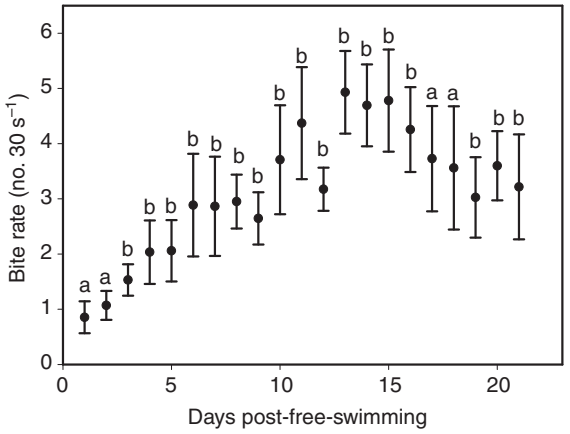

Fig. 3. Bite rate of discus fish young per 30 s on both parents over the first 3 weeks of the breeding period. Different letters denote a significant difference between each time point and the bite rate recorded on day 1 (paired *t*-test, $P<0.05$, $N=12$). Data are means ± s.e.m.

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).

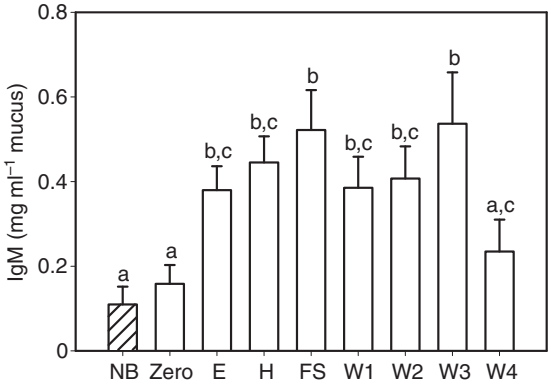

Fig. 4. Total IgM in the mucus of discus fish non-breeders (NB; $N=8$) and breeding pairs ($N=12$, males and females combined) over the breeding cycle. Time points throughout the breeding cycle include: no breeding activity (zero), eggs spawned (E), eggs hatched (H), free-swimming (FS) young, and free-swimming 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).

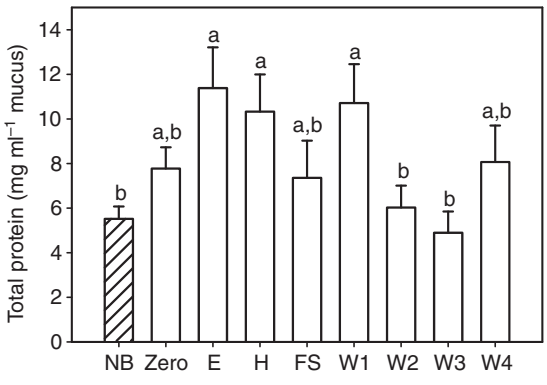

Fig. 5. Total protein in the mucus of discus fish non-breeders (NB; N=8) and breeding pairs (N=12) over the breeding cycle. Different letters denote a significant difference (paired *t*-test and LSD *post hoc*, P<0.05); bars that share a letter are not significantly different. See Fig. 4 for breeding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

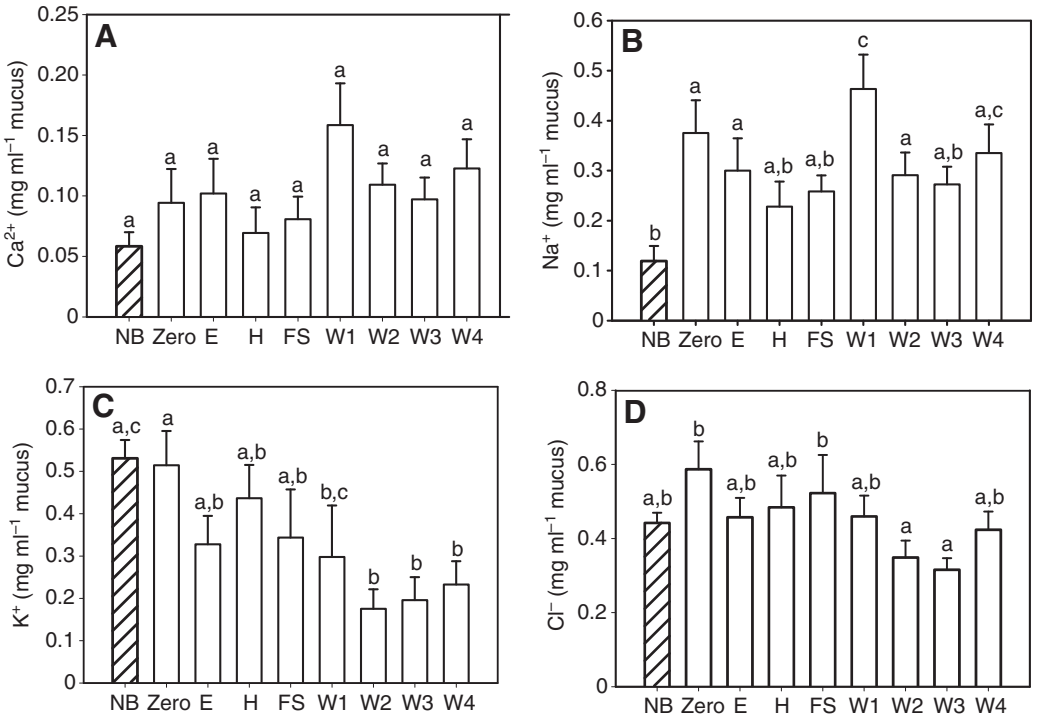

Fig. 6. (A) Calcium, (B) sodium, (C) potassium and (D) chloride concentrations in the mucus of discus fish non-breeders (NB; *N*=8) and breeding pairs (*N*=12) over the breeding cycle. Different letters denote a significant difference (paired *t*-test and LSD *post hoc*, $P<0.05$); bars that share a 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

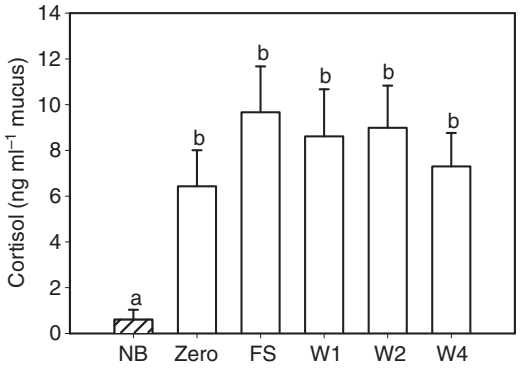

Fig. 7. Cortisol content in the mucus of discus fish non-breeders (NB; *N*=8) and breeding pairs (*N*=12) over the breeding cycle. Different letters denote a significant difference (paired *t*-tests and LSD *post hoc*, $P<0.05$); bars that share a letter are not significantly different. See Fig. 4 for 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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Accumulation of dietary and aqueous cadmium into the epidermal mucus of the discus fish *Symphysodon* sp.

Richard J. Maunder[a,*], Jonathan Buckley[a], Adalberto L. Val[b], Katherine A. Sloman[c]

[a] School of Marine Science and Engineering, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK
[b] Department of Ecology, Laboratory of Ecophysiology and Molecular Evolution, INPA, Manaus, Brazil
[c] School of Science, University of the West of Scotland, Paisley, PA1 2BE, Scotland, UK

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ABSTRACT

The discus fish *Symphysodon* sp. is an Amazonian cichlid with a unusual form of parental care where fry obligately feed from parental mucus for the first few weeks of life. Here, we investigated the possible impact of environmental cadmium on this species, particularly with respect to mucus contamination. We exposed groups of fish to cadmium either through their food (400 mg kg^{-1}) or through the water (3 $\mu g\,l^{-1}$) for 4 weeks, and measured tissue concentrations and ATPase activities at weekly intervals. Cadmium significantly accumulated in all tissues (except for muscle) after 7 days, and tissue concentrations increased until the end of the experiment. Significant alterations in ATPase activities of intestine and kidney were observed at day 7 and 14, but no alterations in gill ATPase activities occurred. The epidermal mucus showed a high accumulation of cadmium from both exposures, but particularly from the diet, indicating that dietary cadmium can be transferred from gut to mucus. Combining this data with approximations of fry bite volumes and bite frequencies, we constructed daily estimates of the cadmium that could potentially be consumed by newly hatched fry feeding on this mucus. These calculations suggest that feeding fry might consume up to 11 $\mu g\,g^{-1}\,day^{-1}$, and hence indicate that this species' dependency on parental mucus feeding of fry could make them particularly susceptible to cadmium contamination of their native habitat.

1. Introduction

The risk posed by the severe toxicity of cadmium (Cd) to wildlife (Burger, 2008) and the fact that the majority of this trace metal's current biogeochemical cycling is driven by anthropogenic activities (Nriagu and Pacyna, 1988; Morel and Malcolm, 2005), has led to it being considered a priority pollutant for environmental research (Campbell, 2006). Exposure of fish to cadmium elicits a wide range of toxicological effects, particularly in early life stages (Wren et al., 1995). A key mechanism of freshwater and dietary Cd toxicity is thought to occur through its high affinity for branchial and intestinal calcium (Ca^{2+}) binding sites (Verbost et al., 1989; Schoenmakers et al., 1992). Impact on Ca^{2+} homeostasis through Cd competition at such sites disrupts both active Ca^{2+} absorption and Na^+/K^+-ATPase facilitated Na^+/Ca^{2+} exchange (Schoenmakers et al., 1992; Flik et al., 1994). Subsequent reductions in absorbed Ca^{2+} can be severe and Ca^{2+} may be mobilised from other sites to replace the lost influx at gills and intestine. Hypocalcaemia-induced death, abnormal development, and bone deformities have all been associated with Cd-induced disruption to Ca^{2+} homeostasis (Witeska et al., 1995; Miliou et al., 1998; Williams and Holdway, 2000). Cadmium exposures can also lead to a range of more subtle effects on ionoregulation, osmoregulation, haematology, reproduction and behaviour (Scott et al., 2003; Sloman et al., 2003; Chowdhury et al., 2004; Szczerbik et al., 2006; Remyla et al., 2008).

The Amazon basin is an ecosystem that supports the richest species diversity of fish on the planet (Val and Almeida-Val, 1995; Henderson and Robertson, 1999), and Cd contamination has been proposed as a key environmental issue in some areas of this region (Matsuo et al., 2005; Matsuo and Val, 2007). Many of the acute and chronic Cd toxicity effect concentrations (e.g. 96-h LC_{50} for rainbow trout parr of 1 $\mu g\,l^{-1}$ (Chapman, 1978)) are exceeded by the aqueous Cd concentrations recorded in the region, up to 10 $\mu g\,l^{-1}$ (Oliveira, 2003) in industrial areas and concentrations of up to 2.3 $\mu g\,l^{-1}$ in areas of high flow (Konhauser et al., 1994). There are also reports of very high Cd sediment concentrations in some sites (up to 2 g kg^{-1}; Nascimento et al., 2006) which may provide scope for transfer of this Cd into surface waters. The general characteristics of Amazonian water chemistry might also serve to exacerbate

* Corresponding author. Current address: Brixham Environmental Laboratory, Astrazeneca, Freshwater Quarry, Brixham, Devon, TQ5 8BA, UK.
E-mail addresses: richard.maunder@astrazeneca.com (R.J. Maunder), jonathan.buckley@plymouth.ac.uk (J. Buckley), dalval@inpa.gov.br (A.L. Val), katherine.sloman@uws.ac.uk (K.A. Sloman).

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the severity of a Cd exposure relative to other environments; the low Ca^{2+} concentrations in the extremely soft waters that predominate (Furch, 1984) further increase Cd competitive binding to Ca^{2+} sites at the gill (Playle et al., 1993; Meinelt et al., 2001). Similarly, the low pH (e.g. pH < 4.5 in areas of the Rio Negro (Seyler and Boaventura, 2003) may increase Cd solubility and mobility, hence maintaining relatively higher dissolved Cd concentrations (Nelson and Campbell, 1991; Scheuhammer, 1991). In contrast, the biological availability and toxicity of Cd to fish in this environment might be mitigated by the relatively high concentrations of humic substances (Petersen et al., 1986) found particularly in the Rio Negro, although lower levels of humic substances are found in other areas of the Amazon basin. High levels of aqueous cadmium can lead to contamination of sediments and uptake into benthic invertebrates and algae (Wren and Stephenson, 1991) resulting in Cd as a dietary as well as an aqueous challenge to Amazonian fish. Despite these concerns, and the huge biological wealth represented within the Amazonian basin, few studies have investigated the impact of Cd on Amazonian fish.

One species that may be particularly susceptible to Cd contamination is the discus fish *Symphysodon* spp., a cichlid that is hugely popular within the region's thriving aquarium trade (Bayley and Petrere, 1989; Andrews, 1990). Its interest in terms of response to toxicants stems from an unusual reproductive strategy involving an obligate stage of parental mucus feeding undertaken by the fry (Hildemann, 1959; Buckley et al., 2010). During the first few weeks of life, fry feed from mucus secreted by both parents all over their bodies. Accumulation of Cd and other metals in the mucus of aqueous exposed fish is well reported (Shephard, 1994) and exposure to metals in the water increases mucus production in skin and gills (Lock and Vanoverbeeke, 1981; Eddy and Fraser, 1982) through an increased density and size of mucus producing goblet cells (Shephard, 1994; Wu et al., 2007). Mucus binds metal-rich particulates, and there are proposed processes of chemical sequestration of specific metals which might influence the bioavailability of the bound metals (Smith and Flegal, 1989; Tao et al., 2000). These processes result in a significant and rapid accumulation (<hours) of metal in mucus (McKone et al., 1971; Handy and Eddy, 1989). Such accumulations may result in the fish being exposed to a higher concentration of toxicant than would occur through the ambient water alone and might influence uptake kinetics, as has been shown for intestinal mucus (Glover and Hogstrand, 2002). The increase in mucus production also, however, facilitates an excretory process through the sloughing away of the metal contaminated mucus, a process which appears to be aided by behavioural alterations such as rapid start–stop swimming and 'coughing' (Skidmore, 1970; Shephard, 1994). A similar excretory process has been proposed for orally ingested metals or those experimentally injected into the body cavity (Varanasi and Markey, 1978; Handy, 1992) but the internal pathways from gut to epidermal mucus are unclear. Therefore, it is possible that *Symphysodon* spp. concentrate both aqueous and dietary borne metal contaminants into epidermal mucus. For a species where epidermal mucus provides a lifeline for offspring survival, this could have serious implications for population fitness.

The aim of the current study was to document the accumulation of aqueous and dietary Cd into the major compartments of *Symphysodon* individuals and to assess the potential impact for offspring feeding from mucus. To do this it was necessary to understand how metals are bioaccumulated in the mucus. In addition, to understand some of the physiological consequences of Cd exposure, ATPase activities of the gill, intestine and kidney were measured. As many previous studies have shown that Cd interferes with Ca^{2+} ATPase activity, and we have previously demonstrated that *Symphysodon* provide their offspring with sodium ions in epidermal mucus rather than calcium ions (Buckley et al., 2010), we focused on the effects of cadmium on Na^+K^+ ATPase and associated Mg^{2+}-dependent ATPase activity.

2. Materials and methods

2.1. Experimental animals

Symphysodon sp. ($n = 96$ fish; total length = 88 ± 0.5 mm, mass = 15.6 ± 0.3 g (mean ± SEM)) were sourced from an in house stock reared at the University of Plymouth. Fish were held in a recirculation system at 29 ± 1 °C on a 12:12 h light:dark photoperiod, with measured water chemistry of Ca^{2+}: 21.6 ± 1.3; Na^+: 9.3 ± 0.3; K^+: 1.4 ± 0.1; Cl^-: 15.3 ± 0.7 (mg l^{-1}; mean ± SEM) and fed *ad libitum* until required for an experiment. Two weeks prior to the start of each experiment, four groups of 12 fish were individually weighed (to 0.01 g) and moved to one of four glass 100 l exposure aquaria where they were fed a commercial feed (Tetra Discus bits, Tetra UK, Hampshire, UK) twice a day at a ration of 2% body mass day^{-1}. For both dietary and aqueous exposures, two tanks were used as controls and two tanks as experimental treatments.

2.2. Dietary exposure

Groups of 12 fish were fed either a control, or Cd-contaminated diet. The diets were formulated by spray coating Tetra Discus bits with a gelatine only (control diet) or gelatine and $Cd(NO_3)_2{\cdot}4H_2O$ mixture at a nominal Cd concentration of 400 mg kg^{-1} (Cd diet) following similar methods to those of Shaw and Handy (2006). Actual concentrations were analysed by inductively coupled plasma emission spectrophotometry (ICP-AES; Varian Liberty 200). Experimental fish were fed twice a day at a ration of 2% body mass day^{-1}. Three fish from each of the four tanks were terminally sampled (see below) at time = 0, and then again at each of weeks 1, 2 and 4 during the exposure ($n = 6$ per treatment at each time point).

2.3. Aqueous exposure

During the aqueous exposure, all fish were fed the control diet at the same ration as in the dietary exposure but exposed to either control or Cd-contaminated water. This was achieved by making concentrated stocks (blank for control, 0.823 g of $Cd(NO_3)_2{\cdot}4H_2O$ for Cd in 100 ml of Millipore DH_2O) which were then diluted 50 times with tank water before dosing at 0.05 ml l^{-1} to achieve the nominal concentrations of 3 μg Cd l^{-1}. Based on the measured water chemistry parameters, it was likely that the Cd was present mainly as the free Cd^{2+} ion. Fish were sampled throughout the exposure as for the dietary exposure.

In both dietary and aqueous exposures, tanks were cleaned twice a day by siphoning waste 1 h after the feeding periods, which formed part of a 50% water exchange designed to maintain high water quality in the semi-static exposures. The replenished water was then dosed with the control or Cd stock to maintain the exposure concentrations within those tanks. Water samples were taken from both experiments to confirm actual concentrations. A total of 24 water samples were taken from the middle of each tank by pipette before and after the cleaning/water exchange. The samples were made up to a final volume of 10 ml in a 15 ml tube that contained 100 μl of HNO_3 (Fisher Scientific, Trace Metal Grade) and stored at room temperature before analysis.

2.4. Tissue sampling and processing

The sampling protocol was consistent for both dietary and aqueous experiments. Fish were not fed for 48 h prior to sampling

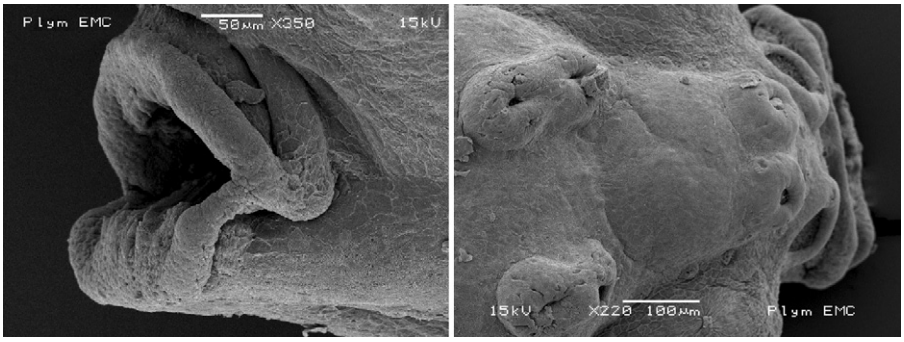

Fig. 1. SEM images showing (A) a lateral view and (B) a dorsal view of the mouth of a *Symphysodon* sp. fry that had just begun free swimming (age approximately 72 h post-hatch).

to minimise food content in the intestine. Fish were individually caught in a net and processed in turn. Initially, a mucus sample was taken by swabbing each flank with a polyurethane sponge. The sponge was placed into the barrel of a pre-weighed 1 ml syringe, weighed to 0.0001 g to determine the mass of the mucus sampled and then eluted into a 10 ml tube containing 50 μl HNO_3 (Fisher Scientific, Trace Metal Grade) by three consecutive 1 ml washes of Millipore DH_2O. Each wash was forced out of the sponge by the syringe plunger before the next was added; 3 ml removed all traces of protein from similar sponges in a trial run. The mass of the mucus sampled was converted to a volume (i.e. 1 g = 1 ml) and concentrations of Cd later expressed as $\mu g\,ml^{-1}$ protein. Previous studies on *Symphysodon* sp. have demonstrated that mucus protein concentration varies, particularly during the breeding season (Buckley et al., 2010), and so expression of mucus contents per ml is more reliable. Each mucus, water and HNO_3 sample was thoroughly mixed and stored at 4 °C until required for analysis. The fish was then killed by an overdose of anaesthetic (MS222; 200 mg l^{-1}), blotted dry and weighed (to 0.01 g) and measured (total length, to 0.1 cm) before blood samples were taken from the caudal vasculature using heparinised syringes. The blood was immediately centrifuged at ~5000 × *g* for 3 min and plasma transferred to a pre-weighed 1.5 ml tube. The samples were made up to a total volume of 1 ml with Millipore DH_2O, mixed thoroughly and stored at 4 °C until analysis. Two gill filament samples (outer two filaments from both sides), a section of liver, the whole kidney (split into two samples), and two mid-sections of intestine (which were clear of food/faeces) and a muscle sample from the shoulder were then dissected and each individually weighed to 0.0001 g in pre-weighed 1.5 ml tubes. The sampled tissue sections were the same for each fish. The left hand side gill filaments, one of the intestine samples and half of the kidney were then immediately snap frozen in liquid nitrogen and stored at −80 °C until analysis of ATPase activities (see below). The remaining samples were digested in 1 ml of 1 N HNO_3 (which for all was > 5 times the volume of the tissue) at 50 °C for 48 h. All acidified mucus, plasma, tissue and water preparations were briefly centrifuged and the supernatant analysed for Cd content by ICP-mass spectrophotometery (Thermo Scientific X Series 2).

2.5. Calculation of theoretical cadmium consumed by fry

Once we had determined accumulation of cadmium in the mucus of parents exposed to waterborne and dietary cadmium, we could calculate the theoretical amount of mucus and, therefore, cadmium that an individual fry would consume during the time that it was feeding from its parents. To do this, firstly, three discus fry were sampled from the clutch of a pair of breeding discus fish held at the University of Plymouth. The fry aged approximately 3 days post hatch were caught in a net just as they began to free swim and killed by an overdose of anaesthetic (MS222; 200 mg l^{-1}). The whole bodies were prepared for examination by SEM following previously published methods (Lovell et al., 2005) and photographed using a JEOL JSM 5600 scanning electron microscope operated at 15 kV, and a 15 mm working distance (Fig. 1).

Approximate mouth length, width and depth were then calculated based on these images and the volume (*V*, in μl) of a fry's mouth was approximated to a cone with an elliptical base, calculated using the formula:

$V = 1/3\pi lwh$ where l = length, w = width and h = height. Using data available on average length and mass of fry over the 21 day breeding period and our previously published data on average number of bites performed by fry over the breeding period (Fig. 3 in Buckley et al., 2010) we were able to calculate the approximate consumption of cadmium by a single fry on each day of the breeding period according to the following equation:

$$\mathrm{Cd} = \frac{Vbc}{m}$$

where V = volume of mouth (μl), b = number of bites of mucus taken by fry per day, c = estimated cadmium concentration in parental mucus ($\mu g\,\mu l^{-1}$) and m = mass of the fry (g). m was obtained from previously recorded mean masses of fry of age 0, 7, 14 and 21 days post free swimming, and extrapolated masses for the days between these points.

2.6. Na^+/K^+ and Mg^{2+}-dependent ATPase activities

High affinity Na^+/K^+ and Mg^{2+}-dependent ATPase activities were measured using a method adapted from the protocol of McCormick (1993). The assay uses an enzyme reaction coupled to the hydrolysis of ATP in which the oxidation of reduced NADH is directly recorded. Values were recorded as enzymatic units of ATPase per mg protein (specific activity) and presented as activity compared to control tissues. Protein was measured using the Bradford method (Bradford, 1976). Samples of gill, kidney and intestine were removed from −80 °C storage and defrosted on ice. Samples were homogenised in an ice cold disrupting buffer (150 mM sucrose, 10 mM EDTA, 50 mM HEPES, 2.41 mM sodium deoxycholate, pH 7.3) at 1:10 wet sample mass to buffer volume using a pre-cleaned and ice cold Kontes pellet pestle homogeniser for 30 s or until tissue was fully homogenised. Homogenates were centrifuged (4 °C, 5000 × *g*) for 10 min and the supernatant analysed for Na^+/K^+ and Mg^{2+}-dependent ATPase activity as described by Zaugg (1982). The supernatant (10 μl) and 50 μl of a salt solution (189 mM NaCl, 42 mM KCl, 10.5 mM $MgCl_2$, were added to four wells of a 96 well microplate. Two wells then received a reaction buffer containing ouabain and two received the ouabain-free reaction buffer (2.8 mM PEP, 3.5 mM ATP sodium salt, 0.4 mM NADH 50 mM HEPES, 4 units ml^{-1} LDH, 5 units ml^{-1} PK (±0.5 M ouabain) pH 7.3). The rate of NADH oxidation was measured every 9 s for 10 min at 340 nm at

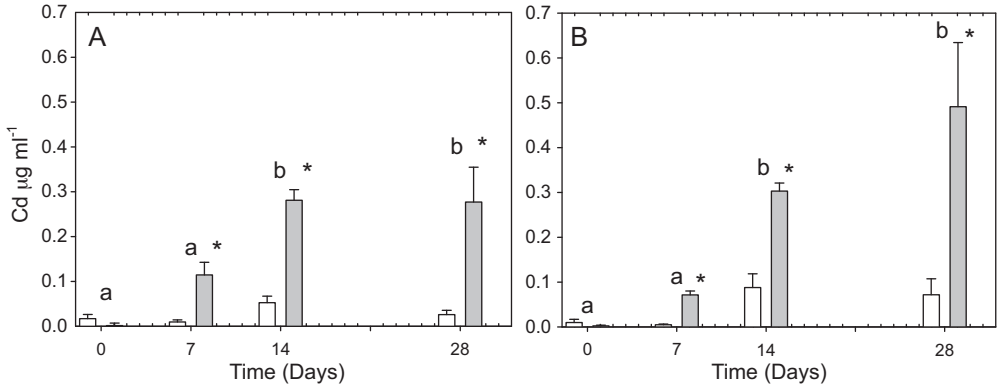

Fig. 2. Plasma (A) and mucus (B) Cd concentrations (mean ± SEM, $n = 6$; µg Cd ml^{-1}) from fish fed a control (□) or Cd enriched diet (■). Asterisk (*) indicates significant differences to the control within each time point, whereas different letters (a and b) indicate significant differences in Cd concentrations between time points (ANOVA (with Post hoc), $p < 0.05$).

25 °C, and the linear section of the oxidation versus time reaction was selected for calculation of enzyme activities. The difference in NADH oxidation between the ouabain and ouabain free media was considered the Na^+/K^+ dependent ATPase activity while the Mg^{2+}-dependent ATPase activity was represented by the ouabain insensitive fraction.

2.7. Statistical analysis

No significant differences were observed between the tank replicates for each treatment so the data were combined. Comparisons of tissue accumulation and enzymatic activities between the control and Cd exposed groups through time were conducted by a two way ANOVA followed by Post hoc Tukey test. Water concentrations from control and exposed tanks from both experiments were compared by Student's *t*-tests. SPSS Statistics 17 was used throughout and the limit of significance was set as $p < 0.05$.

3. Results

3.1. Cd exposure concentrations

Background concentrations of Cd in uncontaminated food were $1.14 \pm 0.10\ \mu g\ g^{-1}$, $n = 20$ (mean ± SEM), while the Cd concentration of the Cd enriched diet was $386.5 \pm 7.4\ \mu g\ g^{-1}$, $n = 20$. On average an individual fish consumed 0.3 g of food per day and, therefore, received an approximate dose of 120 µg of Cd per day, with the exception of the two days before sampling. In both experiments, there was no significant difference in the aqueous Cd concentration through time (week groups; $p > 0.05$) so the mean values for the entire exposure are reported. In the dietary exposure, the mean aqueous Cd concentrations ($1.08 \pm 0.15\ \mu g\ l^{-1}$ ($n = 24$)) were significantly elevated above those in the control diet tanks ($0.06 \pm 0.02\ \mu g\ l^{-1}$, ($n = 24$); $p < 0.01$), indicating that there was some leaching of Cd from the food or fish into the water. However, these concentrations were significantly ($p < 0.05$) below those which occurred in the aqueous exposure ($2.78 \pm 0.28\ \mu g\ l^{-1}$ ($n = 24$)). Cd concentrations in aqueous control tanks were $0.01 \pm 0.00\ \mu g\ l^{-1}$ ($n = 24$).

3.2. Plasma and mucus Cd accumulation

Plasma and mucus Cd concentrations are presented in Figs. 2 and 3. Fish from both experiments showed plasma and mucus Cd concentrations that were significantly greater ($p < 0.05$) than control levels throughout the exposures. A general trend of increasing Cd concentration through time was observed in plasma and mucus of both experiments, indeed the mucus Cd concentrations through time closely matched those found in the plasma (Figs. 2 and 3). In the dietary exposure (Fig. 2), the plasma and mucus concentrations were approximately one order of magnitude higher than those in the aqueous exposure (Fig. 3), despite the higher aqueous Cd concentration occurring in the aqueous exposure.

3.3. Tissue Cd accumulation

No mortalities occurred in either of the experiments and there were no significant differences in the tissue Cd concentrations of the control fish compared to those at the beginning (time zero) of the experiments ($p > 0.05$). The muscle concentrations showed no accumulation above control levels (data not shown). In the other tissues, as in the plasma and mucus, a general trend of increasing tissue Cd accumulation through time was observed in both the dietary and aqueous exposures, with the highest concentrations being attained in the final (28 day) sample (Table 1). The exceptions to this were dietary exposed liver and gill, which showed a reduction in tissue concentration between 14 and 28 days. The highest tissue concentration at day 28 in the dietary exposure was observed in the intestine ($25.9\ \mu g\ g^{-1}$) some 250-fold increase above control levels. The tissue concentrations in the dietary exposure at this time point were ranked as intestine > kidney > liver > gill > mucus > plasma > muscle (Table 1 and Fig. 2). The highest tissue concentration at day 28 of the aqueous exposure occurred in the kidney ($2.7\ \mu g\ g^{-1}$). This is a lower Cd tissue concentration than was observed in the dietary experiment, and this trend was reflected in all of the other tissues (16 – 1.5-fold lower) except for the gill which showed comparable levels (Table 1). The Cd tissue concentrations at day 28 in the aqueous experiment were ranked as kidney > liver > intestine > gill > mucus > plasma > muscle (Table 1 and Fig. 3).

3.4. Estimation of discus fry Cd ingestion

A value of $0.5\ \mu g\ ml^{-1}$ was used as an estimate of the concentration of cadmium present in parental mucus. This was the mean value of cadmium recorded in the mucus following the 28-day dietary exposure (Fig. 2B). Theoretical consumption of cadmium by fry as they feed from parental mucus is shown in Fig. 4 with an estimated mean over the 21 days of $6.1\ \mu g\ Cd\ g^{-1}\ day^{-1}$.

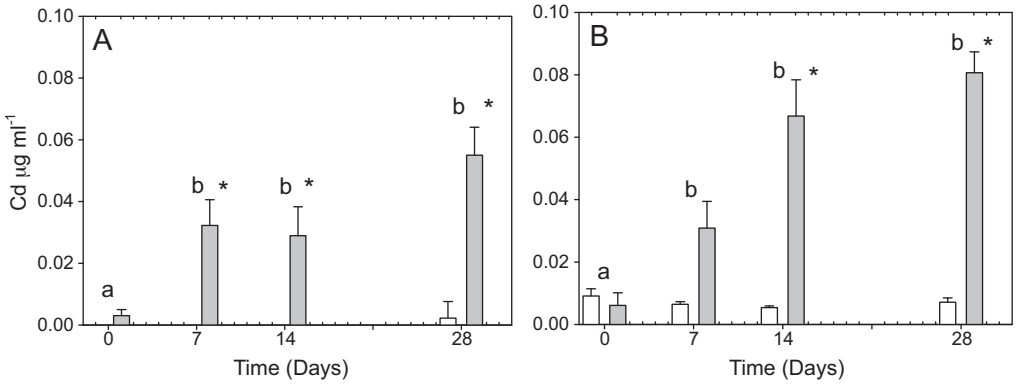

Fig. 3. Plasma (A) and mucus (B) Cd concentrations (mean ± SEM, $n = 6$; µg Cd ml^{-1}) from control (□) and Cd exposed (■) fish following an aqueous Cd exposure. Asterisk (*) indicates significant differences to the control within each time point, whereas different letters (a and b) indicate significant differences in Cd concentrations between time points (ANOVA (with Post hoc), $p < 0.05$). Note Y axis scale compared to Fig. 2.

Table 1
Tissue Cd concentrations (mean ± SEM, $n = 6$) expressed as µg Cd g^{-1} wet tissue following an aqueous or dietary Cd exposure.

Exposure	Time point	Treatment	Tissue			
			Liver	Gill	Kidney	Intestine
Aqueous	Time 0	Control	0.05 ± 0.01	0.01 ± 0.00	0.21 ± 0.02	0.09 ± 0.01
		Exposed	0.04 ± 0.01	0.02 ± 0.00	0.13 ± 0.01	0.09 ± 0.01
	7 days	Control	0.07 ± 0.02	0.02 ± 0.00	0.20 ± 0.04	0.08 ± 0.01
		Exposed	0.64 ± 0.12*	0.95 ± 0.11*,a	1.07 ± 0.16*,a	1.03 ± 0.14*,a
	14 days	Control	0.07 ± 0.02	0.02 ± 0.00	0.26 ± 0.03	0.01 ± 0.02
		Exposed	1.10 ± 0.12*,a	1.03 ± 0.09*,a	1.23 ± 0.15*,a	0.86 ± 0.16*,a
	28 days	Control	0.08 ± 0.01	0.01 ± 0.00	0.25 ± 0.03	0.10 ± 0.01
		Exposed	2.06 ± 0.58*,a	1.52 ± 0.19*,a	2.70 ± 0.47*,a	1.57 ± 0.39*,a
Dietary	Time 0	Control	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.05 ± 0.01
		Exposed	0.04 ± 0.01	0.01 ± 0.00	0.04 ± 0.01	0.03 ± 0.01
	7 days	Control	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.01
		Exposed	0.91 ± 0.11*	1.36 ± 0.40*,a	0.51 ± 0.06*	6.63 ± 1.40*,a
	14 days	Control	0.34 ± 0.12	0.03 ± 0.01	0.16 ± 0.04	0.14 ± 0.04
		Exposed	4.47 ± 0.39*,a	2.19 ± 0.33*,a	6.68 ± 0.54*,a	17.90 ± 6.56*,a
	28 days	Control	0.31 ± 0.15	0.03 ± 0.01	0.18 ± 0.05	0.14 ± 0.04
		Exposed	3.20 ± 0.47*,a	0.83 ± 0.30*,a	9.55 ± 2.97*,a	25.91 ± 9.31*,a

* Significant difference compared to the control within each time point.
[a] Indicates significant differences in Cd concentrations compared to the zero time point (ANOVA, $p < 0.05$).

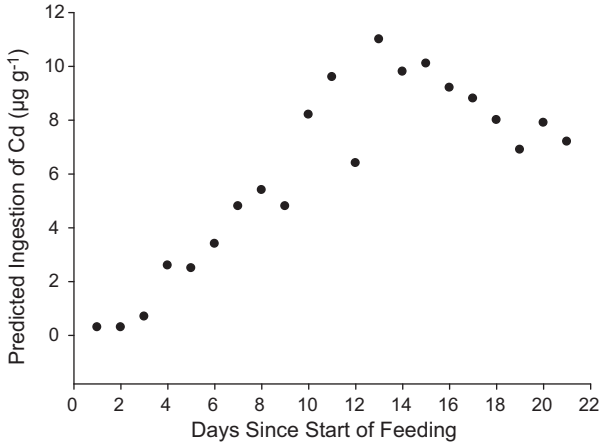

Fig. 4. Predicted daily Cd consumption (µg g^{-1}) by *Symphysodon* sp. fry feeding on contaminated (0.5 µg Cd ml^{-1}) mucus for 21 days.

3.5. Na^+/K^+ and Mg^{2+}-dependent ATPase activities

In the aqueous exposure, Na^+/K^+ ATPase activities were variable between individuals, but significant ($p < 0.05$) inhibitory effects of approximately 50% occurred in the intestine on day 7 and 14 of the exposure. The same time points saw activations of Mg^{2+}-dependent ATPase of 65 and 89% respectively (Table 2). In the dietary exposure, the Na^+/K^+-ATPase activity in the kidney of exposed fish was inhibited by 60 and 70% at days 7 and 14 respectively.

4. Discussion

High accumulation of Cd in mucus occurred in both the aqueous and dietary exposures. Mucus concentrations observed in the dietary exposure were ten-fold higher than those in the aqueous exposure, and while there was a measureable aqueous concentration of Cd in this dietary exposure, the levels were significantly below those in the aqueous exposure. Therefore, the higher mucosal Cd concentrations observed in the dietary exposure indicate that the dietary sourced Cd was transported from the gut into the epidermal mucus of this species. The plasma concentrations in the dietary exposure were similarly high, so gut to blood transfer and subsequent transport of Cd to mucus seems likely. This transport could involve pathways known to facilitate the storage and mobilisation of Ca^{2+} in the skin and scales of teleosts (Takagi et al., 1989) to replace inhibited Ca^{2+} uptake (e.g. Berntssen et al., 2003). Once in the skin, incorporation into goblet cells may occur with consequent transfer to mucus. However, the mechanisms involved in this transport are unknown. The binding at these various stages of transport, or the direct transfer of Cd from water into mucus might be altered by the intestinal Cd exposure itself. Altered uptake kinetics of metals at one site following exposure at another has been well

Table 2
Mean concentrations of high affinity Na^+/K^+ and Mg^{2+} dependent ATPase activities (% enzymatic units mg^{-1} protein compared with control; $n = 6$) in the gill, intestine and kidney of discus fish undertaking an aqueous or dietary exposure to Cd. The control activity was set at 100% for each tissue and time point, and the exposed values are expressed in relation to the control.

	Sample			
	Zero	7 days	14 days	28 days
Aqueous				
Gill				
Na^+/K^+-ATPase	109.7 ± 15.0	118.3 ± 36.2	139.3 ± 35.6	134.8 ± 20.0
Mg^{2+}-ATPase	109.1 ± 8.2	120.2 ± 11.0	141.8 ± 9.5	94.2 ± 12.0
Intestine				
Na^+/K^+-ATPase	96.9 ± 22.7	47.5 ± 11.1*	55.3 ± 14.0*	57.6 ± 24.8
Mg^{2+}-ATPase	140.2 ± 15.4	164.5 ± 20.2*	188.8 ± 20.4*	111.5 ± 16.1
Kidney				
Na^+/K^+-ATPase	70.1 ± 39.2	61.4 ± 27.6	57.5 ± 21.4	53.7 ± 26.4
Mg^{2+}-ATPase	92.1 ± 27.4	91.1 ± 9.0	135.6 ± 14.0	108.9 ± 21.2
Dietary				
Gill				
Na^+/K^+-ATPase	119.2 ± 35.4	138.2 ± 33.3	79.7 ± 3.9	116.5 ± 75.2
Mg^{2+}-ATPase	74.5 ± 8.1	86.2 ± 15.0	78.5 ± 4.5	114.8 ± 11.7
Intestine				
Na^+/K^+-ATPase	41.9 ± 18.0	128.3 ± 30.4	98.6 ± 63.3	33.5 ± 30.5
Mg^{2+}-ATPase	111.2 ± 15.4	74.3 ± 20.2	79.2 ± 20.4	112.8 ± 16.1
Kidney				
Na^+/K^+-ATPase	85.8 ± 17.6	37.1 ± 5.4*	26.3 ± 5.9*	84.2 ± 32.2
Mg^{2+}-ATPase	103.1 ± 19.4	101.1 ± 21.9	131 ± 32.5	134.2 ± 7.0

The mean ($n = 6$) specific activities of Na^+/K^+-ATPase in control tissues over both experiments at individual timepoints ranged from 60 to 208 (gill), 10 to 192 (intestine) and 26 to 174 (kidney) percent of the overall mean activity for those tissues. The mean ($n = 6$) specific activities of Mg^{2+}-ATPase in control tissues over both experiments at individual timepoints ranged from 77 to 121 (gill), 31 to 186 (intestine) and 39 to 168 (kidney) percent of the overall mean activity for those tissues.

* Significant ($p < 0.05$) differences between the exposed and control group at that time point. There were no significant differences over time within a tissue.

reported (Niyogi and Wood, 2003) and it is possible that the intestinal exposure might have caused an enhanced mucosal uptake in the dietary exposed fish.

Reports of dietary Cd excretion through the skin or mucus in other wildlife are relatively common. e.g. the mucus of land snails (Notten et al., 2006), through the shedding of skin in snakes (Jones and Holladay, 2006) and in the shed feathers of birds (Movalli, 2000). Similar examples exist in fish; coho salmon injected with Cd (Varanasi and Markey, 1978) and rainbow trout fed Cd (Handy, 1992) were found to have measurable levels of Cd in epidermal mucus. However, these examples did not establish mucus uptake rates from aqueous-only experiments, and hence cannot completely rule out mucus accumulation from an unintended water-borne exposure.

The accumulation of Cd into other tissues was relatively high when compared to available data from other species. In the dietary exposure, the organ Cd concentrations attained were much higher than those reported in Cd exposed rainbow trout (Handy, 1993; Szebedinszky et al., 2001) fed much higher Cd contaminated diets (1.5–10 g kg^{-1}) for similar durations. The organ concentrations were more comparable, but slightly lower than another Amazonian teleost, the tambaqui, *Colossoma macropomum* fed up to 400 mg kg^{-1} Cd for up to 45 days (Matsuo and Val, 2007). The dietary accumulation of Cd in the various tissues showed an interesting decrease in the liver and gill concentrations at the final timepoint, the same time at which the kidney and intestinal Cd concentrations increased. This could be interpreted as evidence of a change in the internal handling of Cd. It has been previously shown, for example, that metallothionein can act as a detoxification mechanism (Roesijadi and Robinson, 1994), where previously stored Cd is mobilised for excretion. In the aqueous exposure, the Cd accumulation in the liver, intestine and kidney were comparable to those reported in rainbow trout (McGeer et al., 2000a). We have previously shown that the natural environment of these fish (Rio Negro, Amazonia) has lower ion concentrations that those recorded in our laboratory's experimental water (Buckley et al., 2010). These lower ion concentrations in the wild (e.g. mean laboratory Ca^{2+} 21.56 mg l^{-1} versus *Symphysodon* natural habitat Ca^{2+} 0.33 mg l^{-1}) and associated reduced buffering capacity could result in wild fish showing an increased accumulation from those observed in the aqueous exposure of this study.

The Cd concentration of the mucus of the parents has potential implications for feeding fry. While Cd is known to penetrate the chorion of developing embryos in other species (Witeska et al., 1995), this structure is thought to offer some protection against the toxic effects of aqueous Cd (Williams and Holdway, 2000). It is the early lifestage fry that are often considered to be the most sensitive stage to aqueous exposure (e.g. Brinkman and Hansen, 2007; Lizardo-Daudt and Kennedy, 2008) and in *Symphysodon* it is this stage that begins feeding from the parental mucus. There is very little information available on the toxicity of dietary borne metals to early life stage fry, perhaps due to the difficulty in conducting such experiments, which makes comparisons to other studies in determining potential toxicity of dietary Cd to early life stage fry difficult. Using the level of cadmium we found in parental mucus during cadmium exposure, we were able to estimate the dose that feeding fry would receive. Based on the level of 0.5 μg Cd ml^{-1} found within the dietary study, we estimated that a fry would consume between 0.3 and 11.0 μg Cd g^{-1} day^{-1} with a maximum intake of cadmium occurring around day 13 post hatch. This could be a significant dose of dietary cadmium when compared with studies in adult and juvenile teleost dietary trials, especially when we consider that fry at first feeding are likely to be considerably more susceptible to toxicants. Our waterborne exposure produced a 10-fold lower accumulation in parental mucus and so we would expect a concomitant 10-fold decrease in fry exposure. During our adult cadmium exposure, we used a dietary load of 400 mg kg^{-1} fed at 2% body weight per day. This ration equates to 8 μg Cd g^{-1} day^{-1}, and hence is comparable to the dose that we estimate will be subsequently passed on to fry. We therefore suggest that it seems likely that exposure to Cd at this critical stage will cause detrimental effects, as are reported from aqueous exposures on early life stages (Jezierska et al., 2009). The dietary dose received by discus larvae might put them at a higher risk than those of other species

developing in a contaminated area, as they may be exposed both through the ambient water concentration and also through a concentrated dose in the diet.

The exposure of the intestinal tract and gills to Cd can impact ATPase activity in the exposed tissues. Effects are likely to be different between species and organs (Benson et al., 1988), and the results of aqueous Cd on gill ATPase activities from other studies are varied. No alteration of branchial Na^+/K^+-ATPase activity occurred in a similar ($3\,\mu g\,l^{-1}$) exposure in rainbow trout (McGeer et al., 2000b), or an acute exposure of tilapia (Garcia-Santos et al., 2006). Inhibition of branchial Na^+/K^+-ATPase but activation of Mg^{2+}-dependent ATPase was observed in carp *Cyprinus carpio* exposed to a very high aqueous concentration ($1.6\,mg\,l^{-1}$; de la Torre et al., 2000), and Lionetto et al. (2000) observed that Na^+/K^+-ATPase activity in homogenates of eel *Anguilla anguilla* gill and intestine were inhibited *in vitro* by Cd, but that Mg^{2+}-dependent ATPase activities were unaffected. These varying results make it difficult to draw general conclusions, but for discus fish in this study, the observed impacts on ATPase activities in the intestine and kidney would have implications on the ability to actively transport ions at these sites and hence might reduce uptake of ions (Matsuo et al., 2005) and impact osmoregulation (McCarty and Houston, 1976). In particular, the specialised transfer of Na^+ and K^+ ions into the mucus of this species when providing food for their fry (Buckley et al., 2010) might make them particularly susceptible to alterations in ATPase activities. Changes in the provision of these ions to the fry, either through the inability to uptake from the environment, or impaired ability to transport them between tissues might lead to ion-deficiency problems for the feeding fry. Potential effects on adults and fry would presumably be compounded by the ion poor waters of their native habitats.

In conclusion, discus parents accumulate cadmium in their mucus through both waterborne and dietary exposure, the latter suggesting a route for gut to mucus transfer. In addition to causing physiological alterations in adults, such as impacts on ATPase activities, we estimate that first feeding fry would consume a potentially physiologically significant amount of cadmium through mucus feeding. It is clear that there is a lack of knowledge of dietary toxicity in early life stages of teleost fish in general which is of particular interest in this species. Future studies will consider the accumulation of parentally borne toxicants in *Symphysodon* fry.

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