

REVIEW PAPER

Life in a bubble: the role of the labyrinth organ in determining territory, mating and aggressive behaviours in Anabantoid fishes

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Running headline: *THE LABYRINTH ORGAN AND THE ANABANTOIDS*

The Anabantoids are a group of approximately 137 species of obligatory air-breathing freshwater fishes found in Africa and Southern Asia. All Anabantoids have a pair of suprabranchial chambers that each house an air-breathing organ known as the labyrinth apparatus: a complex bony structure lined with thin, highly vascularised respiratory epithelium. The labyrinth apparatus allows Anabantoids to extract oxygen from air, and is a morpho-physiological innovation that has had a dramatic influence on the behaviour of these fishes. Air-breathing influences a wide range of anabantoid behaviours, including territorial displays, courtship, and breeding and parental care, and also equips these fishes to persist in hypoxic and polluted water. These traits also make Anabantoids successful invaders of novel habitats, a global problem compounded by their popularity in the aquarium trade. By reviewing the functionality and evolution of air breathing in Anabantoids, this study aims to examine the role of the labyrinth apparatus in modulating behaviour within this group. The Anabantoids are a fascinating group and have often been cited as a model organism due to the stereotypical and easily identifiable behaviours that they adopt during social interactions. They also provide a unique opportunity to further our understanding about how fishes adapt their behaviour in response to an extreme environment, whilst limited by their own physiological constraints.

Key words: air-breathing; *Betta splendens*; facultative; gourami; obligate; respiration

Introduction

Air-breathing in fishes

A defining characteristic of the majority of fish species is their reliance upon their gills as the primary organ for gas exchange (Evans *et al.*, 2005). In certain environments, seasonal or
5 continuous aquatic hypoxia has led some species to supplement aquatic oxygen uptake with aerial respiration (Carter, 1931; Carter & Beadle, 1931; Johansen, 1970; Randall et al. 1981; Graham, 1997). To cope with hypoxic environments, evolution has favoured the development of modified breathing structures such as lungs, labyrinth organs, the digestive tract, or the skin (Johansen, 1970; Graham, 1997).

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Air-breathing in fishes first appeared during the late Silurian or early Devonian period, approximately 438-408 million years ago, and has evolved independently in multiple lineages (Barell, 1916; Dutta & Munshi, 1985; Graham, 1997). Today, there are nearly 1,000 species of air breathing fishes, the majority of which are found in bodies of stagnant, tropical
15 fresh water (Graham, 2011). All air-breathing fishes are bimodal breathers, retaining their gills for aquatic gas exchange, and particularly for the excretion of ammonia and carbon dioxide (Johansen, 1968; Randall et al. 1981; Graham, 1997; Randle & Chapman, 2005), while also varying greatly in their dependence on aquatic respiration in normoxia. The greatest degree of diversity among air-breathing fishes is displayed in their different methods
20 of aerial respiration (Johansen, 1970; Herbert & Wells, 2001; Johannsson *et al.*, 2014).

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Air-breathing fishes are on a spectrum, spanning from facultative to obligate air-breathers. Facultative air breathers do not typically breathe air and mostly only do so when dissolved oxygen (DO) is low or when oxygen demands are high (but see McKenzie et al. (2015) for exceptions). In contrast, obligate air breathers use aerial respiration at all times, regardless of
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aquatic conditions or their oxygen demands, due to their reduced gill surface areas. This distinction between facultative and obligate breathers is not always entirely distinct, as many air-breathing fishes can modulate their aerial uptake relative to demand, and aerial uptake varies within individuals (McKenzie *et al.* 2015), between individuals, and between species.

30 For air-breathing fishes, there are stereotyped and distinct reflex behaviours, driven by oxygen-sensitive chemoreceptors, that are associated with the necessity to travel to the surface to gulp air. Trade-offs between the requirements for oxygen and the risk of being predated upon (McKenzie *et al.* 2015), for example, might drive the evolution of unusual behaviours, and create a more complex respiratory situation for such fish species (Shingles *et al.* 2005). As such, air-breathing fish species, like the Anabantoids (Perciformes: Anabantoidei), offer an intriguing model group to understand the evolutionary pressures that have driven these interactions between physiology and behaviour, and how these two key factors are traded off against each other.

40 **The Anabantoids**

The Anabantoids are a group of obligatory air-breathing freshwater fishes found in Africa and Southern Asia that evolved some 60 million years ago (Berra, 2001; Goldstein, 2001; Rüber, 2009). Consisting of roughly 137 species (Fig. 1) across three families (Anabantoidae, Helostomatidae and Osphronemidae), all have a pair of suprabranchial chambers (SBC) that

45 directly extend either side of the 1st epibranchial gill arch into the opercular cavity (Rüber *et al.*, 2004; Kang & Lee, 2010; Huang *et al.*, 2011). Each SBC houses an air-breathing organ (ABO) known as the labyrinth apparatus (Fig. 2): a complex bony structure lined with thin, highly vascularised respiratory epithelium.

Filling most of the SBC, the labyrinth organ's intricate arrangement is due to the many folds
50 that line its surface. This elaborate modification vastly increases the organ's surface area to
enhance oxygen uptake. Various other structural modifications, such as its dorsal location,
ensure that inhaled air can flow into the chamber with relative ease (Shadwick & Lauder,
2006). A 'double-pump' mechanism, along with the short diffusion distance across the
65 capillaries and the large surface area of the labyrinth organ itself, ensure an effective
alternative for acquiring oxygen to aquatic gaseous exchange (Hughes & Singh, 1970).
However, due to its encroachment into the opercular cavity, the labyrinth organ has
fundamentally altered gill size within this group, resulting in relatively smaller sets of gills
compared to non-air-breathing species (Hughes & Singh, 1970; Shadwick & Lauder, 2006).

60 Aerial respiration in Anabantoids is a multi-faceted process, whereby various instinctive
reflex actions occur nearly simultaneously, and using X-ray films, Peters (1978) was able to
characterise the mechanics of this procedure. Using a double-pump mechanism, ventilation of
the ABO occurs via an alternating action of sucking and pumping of the buccal cavity and
opercular chambers. The double-pump – or biphasic – mechanism involves the expansion and
65 contraction of the opercular chamber, which in turn drives a reversed stream of water up into
the SBC, forcing out any gas still present within. At this point, buccal expansion removes this
gas into the mouth where it can either be exhaled as a bubble if under water, or straight into
the air if at the surface (Graham, 1997). Notably, the chamber is entirely emptied of gas on
exhalation in most instances (Peters, 1978). With biphasic air ventilation, inhalation follows
70 exhalation, and any new air that is superfluous to demands is expelled via the operculae
(Peters, 1978; Graham, 1997). In 1987, using cineradiographic and emg data, Liem (1987)
updated the description of the air-breathing mechanics for most Anabantoids from biphasic
(reversal, expansive), to be described as quadruphasic. This was to include the preparatory

and compressive stages of the breathing process (preparatory, reversal, expansive,
75 compressive). The systemic circulatory system ensures a constant flow of partially
oxygenated blood from the heart, via the anterior gill arches (1st and 2nd arches), to the
labyrinth organ (Fig. 3). Gas exchange occurs while the air bubble is held within the SBC and
partially oxygenated blood flows through it, such that the blood becomes fully oxygenated
before circulating back to the heart (Burggren, 1979).

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Anabantoid fishes are not born with functional labyrinth organs (Mendez-Sanchez &
Burggren, 2014). Air gulping has been observed in the young of various species, though this
behaviour occurs long before the labyrinth apparatus has fully formed (Graham, 1997). The
labyrinth organ grows throughout development, and juvenile fishes remain reliant upon their
85 gills until maturity (Pinter, 1986). Early life experiences, such as access to air, influence the
effectiveness of the labyrinth organ by altering capillary density of the surface epithelium
(Mendez-Sanchez & Burggren, 2014). Individual factors such as fish body size, shape and
skull structure are important in driving labyrinth organ morphology (*i.e.* size, shape and
complexity) yet there is no significant evidence of a pattern in its structure among species
90 (Graham, 1997).

The evolution of the labyrinth organ

The labyrinth organ essentially prevents asphyxiation in fishes that inhabit areas low in DO,
by offering a viable alternative and supplement to aquatic respiration (Graham, 1997). The
95 origins, therefore, of the labyrinth organ tie effectively with the fishes surrounding
environment, with aquatic hypoxia being the most likely candidate in driving its evolution
(Packard, 1974; Blank & Burggren, 2014). Given the array of costs and benefits associated
with ABO evolution, it is difficult to predict the suite of environmental conditions that favour

the evolution of organs such as the labyrinth apparatus. This is further complicated given the
100 evident success of non-air-breathing species in hypoxic habitats, which have forsaken the
development of an ABO altogether despite the low-oxygen conditions. For example, it is
established that many non-air-breathing fishes in Lake Victoria can survive in various
hypoxic areas by developing alternative adaptations such as larger gill surface areas or higher
haemoglobin concentrations (Chapman *et al.*, 2002). Indeed, a persistent theme appears to be
105 that of metabolic efficiency, whereby these species have responded to hypoxia by becoming
more tolerant, rather than developing a new organ altogether.

It is possible to deduce the sequence of events, based on the role of aquatic hypoxia, which
resulted in the development of ABOs like the labyrinth organ (Fig. 2; Blank & Burggren,
110 2014). It is likely that the 1st gill arch of these ancestral variants was located relatively close
to the mouth, and during hypoxic events, fish could use buccal pumping at the water surface
to sustain oxygen uptake (aquatic surface respiration, ASR). ASR would have provided a
selective advantage in the hypoxic environment, and is likely to have been the precursor for
air-breathing (Kramer & Graham, 1976). Given its location, the 1st gill arch was able to
115 extract oxygen from small quantities of air. As provided a selective advantage, the arch
became larger and more vascularised to supplement the increased respiratory demands.
However, the buccal cavity in which the 1st gill arch lies is a relatively small and confined
space that is primarily used for feeding (Fig. 2). As such, a compromise was needed for the
requirements for feeding and ventilation. Thus, with little room to expand the buccal cavity,
120 part of the gill arch extended away from the cavity and developed into its own stand-alone
organ. With time, it became a highly convoluted and vascularised, yet introverted, delicate
mass of bone and capillaries that essentially enhanced the surface area of the organ for

gaseous exchange. This primitive ABO eventually developed into the labyrinth organ that is present today (Randall, 1981; Goldstein, 2001).

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ABOs have provided an opportunity for further diversification as populations of bimodal breathers have been able to thrive in habitats where unimodal gill breathers could not. The lack of competition has facilitated adaptive radiation and consequently allowed them to expand into various ecological niches. In these instances, the ABO has retained the all-
130 important respiratory functions yet there have been dramatic morphological modifications in jaw shape, diet and dentition amongst the various species (Graham, 1997). The labyrinth organ has also permeated into other facets of life, mediating behavioural responses in the Anabantoids. Territorial displays, nest building and parental care are just a few examples of where this group have been influenced by the need to acquire oxygen via aerial gas exchange
135 (Graham, 1997). Some Anabantoid species, like members of the genus *Sandelia*, have even regressed, losing their ABO. Many have retained a complex labyrinth organ to facilitate air-breathing in their typical environmental; others, however, have flourished in more oxygenated habitats and, consequently, the ABO of these members has reverted into a far simpler organ (Helfman *et al.*, 2009).

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Behaviour and the labyrinth organ

Territorial defence

Animals use ritualised threat displays to warn off rivals from a territory, to ensure exclusive access to important resources such as food, shelter or mates. The nature of threat displays
145 observed amongst members of the Anabantoid group during such territorial disputes is ubiquitous – deepening their body colour, erecting their fins, tail-beating and flaring their gill covers (opercula, Fig. 4) – before escalating to chasing and biting the rival (Miller & Miller,

1970; Meliska *et al.*, 1980a; Tooker and Miller, 1980). A key feature of these displays is their intensity, significantly increasing oxygen consumption in order to meet increased metabolic requirements (Castro *et al.*, 2006). The opercular flare, in particular, compounds this condition as it prevents water (an already poor source of DO) from effectively passing over gills and forces an individual into a self-imposed hypoxic state (Abrahams *et al.*, 2005). Due to their costly nature, the opercular flare is only used as an acute response to other males and is swiftly swapped for less costly behaviours, such as fin flaring, against persistent intruders (Forsatkar *et al.*, 2016). As such, the duration of opercular and dorsal fin flaring relates to an individual's condition and can subsequently be used to indicate the winner of an interaction (Simpson, 1968; Evans, 1985; Abrahams *et al.*, 2005). Furthermore, the duration of opercular flaring and tail slashing positively correlates with the frequency of air breathing (Dore *et al.*, 1978; Meliska *et al.*, 1980b), suggesting that these activities increase metabolism or reduce aquatic gas exchange (Alton *et al.*, 2013). Consequently, individuals need to respond to the increased energetic requirements placed on their bodies during these bouts of extreme activity through the rapid acquisition of more oxygen.

This is where the labyrinth organ has been incorporated to meet these demands. During male-male interactions, Anabantoid species use aerial oxygen uptake to supplement their respiratory needs (Fig. 5 (d); Alton *et al.*, 2013), and this is particularly evident during aggressive displays when metabolic rate is increased (Fig. 5 (a)). This is probably as a result of the limited capabilities of their gills *i.e.* their relatively small surface area, and the increase in oxygen requirements as a result of display (Fig. 5; Alton *et al.*, 2013). This is not to say that the labyrinth organ completely fulfils metabolic requirements, and it too has limited surface uptake capabilities (Fig. 5 (c), (e)). To counter this problem, individuals have to take more frequent trips to the surface to take more breaths (Fig. 5 (b)) (Kramer & Graham, 1976;

Graham and Baird, 1982; Chapman and Chapman, 1994; Graham, 1997; Alton *et al.*, 2013).

175 Interestingly, in male Siamese fighting fish *Betta splendens* Regan 1910 the amount of oxygen uptake per breath at the surface does not change significantly when comparing males at rest versus males displaying, suggesting the amount of oxygen uptake per breath is already at a maximal level, and explains why more breaths are required at the surface during display, as opposed to more O₂ per breath (Fig. 5; Alton *et al.*, 2013).

180 Such constraints have resulted in the evolution of what appears to be a ‘gentleman’s agreement’ of synchronous air-breathing between sparring individuals, whereby one male will lead the other to the surface to obtain air (Randle & Chapman, 2005; Chapman & Mckenzie, 2009; Alton *et al.*, 2013). One possible explanation for such behaviour is related to the need to reduce the likelihood of being predated upon from above. The low DO content of
185 the water generally precludes large fishes from being present, and the main threat to most Anabantoids are small piscivorous eating birds, such as members of the Coraciiformes group. Synchronous air-breathing, like shoaling, offers a dilution effect against such predation, reducing the chance for an individual male of being attacked (Kramer & Graham, 1976). However, this behaviour could be construed as unintuitive. A fish that is tiring from intense
190 displaying and needs to replenish oxygen stores from the surface would, in theory, provide an easy target for the other duelling male to attack. However, a failure to successfully win the antagonistic interaction at this point would result in depleted oxygen reserves, and opponent male returning from the surface with an oxygen-laden labyrinth organ. This situation becomes even more intriguing when taking into consideration that O₂ per breath is correlated
195 with body mass (Fig. 6), yet males of different body masses will engage in synchronous breathing.

Ascertaining the fitness benefit of such an unintuitive behaviour is difficult, even more so given that synchronous air-breathing is found throughout non-aggressive species; *Corydora* species are air-breathers that use synchronous air-breathing purely for safety in numbers (Kramer & McClure, 1980). In the case of the Anabantoids, it is possible that the labyrinth organ has facilitated the development of synchronous air-breathing behaviour, as the requirements for surface breathing can relay information about the physiological state of each male. For instance, subordinate males of various bird and fish species adopt appeasement postures to prevent encounters from escalating into harmful events and synchronous air-breathing may be an example of this (Keenleyside, 1979). By following a male to the water surface, a rival is able to defend its territory whilst also reducing the risk of predation and of either coming to physical injury. Individuals who share similar morphologies may also encourage synchronous air-breathing.

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The labyrinth organ facilitates aggressive displays during male-male interactions and also adds another dimension of social interaction through synchronous air-breathing. Social exposure amongst the Anabantoids is tied to the habitat that they reside in, be it isolated in a pond or sharing a body of water with a mass of individuals. Consequently, territorial disputes can be influenced by the degree of prior social stimulation an individual encounters, *i.e.* isolation or combat exposure. Social isolation has been observed to both increase (Davis, 1975; Miley & Burack, 1977; Halperin & Dunham, 1993) and decrease antagonistic tendencies (Clayton & Hinde, 1967; Meliska *et al.*, 1980a) across various Anabantoid species. In one case, social isolation initially decreased the readiness to display in male *B. splendens* but, after priming, males were more aggressive (Halperin *et al.*, 1992). Davis *et al.* (1974) demonstrated that social isolation enhanced male and female paradise fish *Macropodus opercularis* L. 1758 aggression with no effect on the amount of air gulping,

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which in other species has been shown to increase during vigorous displays to supply the requisite oxygen to perform the behaviour. It is possible that metabolic requirements are subject to behavioural modulation, whereby social interactions or predator stimuli override the metabolic response amongst the Anabantoids and other air-breathing species. When isolated, individuals delay surfacing for as long as physiologically possible until the need for air takes priority over risk of predation (Sloman *et al.*, 2009) (Fig. 7). Herbert and Wells (2001) reported that exposure to a model predator resulted in a decrease in air-breathing frequency in the blue gourami *Trichogaster trichopterus* Pallas, 1770, whilst other non-Anabantoid fish species are known to forsake air-breathing until night when the risk of predation is comparatively lower than the day (Grigg, 1965). These examples suggest that there is a trade-off between satisfying metabolic needs and the risk of mortality during states of hypoxia, manifesting as a reduction of air-breathing behaviour (Fig. 7).

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Audience effects and sociality

The labyrinth organ, and the requirement for oxygen intake from the surface, has resulted in an intricate series of ritualised displays to communicate information about physiological condition, breeding status and territoriality in most Anabantoid species. Such communications can take place between multiple males, or between males and females. When considering social exposure, it is important to consider that signals are likely to occur within a network and not simply between a single pair comprising a receiver and a signaller (McGregor, 1993; McGregor & Peake, 2000) unlike, for example, the situation which is often studied under artificial circumstances with just two male *B. splendens* present. Signals aimed at one individual can be received by many others within a social network, with a signal travelling past the targeted receiver and interpreted by any other individual present or able to observe the signal or interaction (Zahavi, 1979). Audiences, comprising both males and

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females, can eavesdrop to gain information from an interaction, such as perceived health of an individual, without partaking in the display. This results in no cost or risk to the audience member (McGregor, 1993; Oliveira *et al.*, 1998; Doutrelant & McGregor, 2000; Peake, 2005; Bertucci *et al.*, 2014). The presence of an audience, however, creates higher costs for losers, as the perceived loser from an interaction is more likely to be challenged by the observer. Males, therefore, are expected to alter their behaviour in the presence of an audience (Doutrelant *et al.*, 2001; Matos *et al.*, 2003), and these behavioural alterations in an individual's behaviour in response to being observed by conspecifics are termed audience effects (e.g. Marler *et al.*, 1986).

During territorial displays or bouts of aggression, the costs and benefits of winning or losing vary differently in the Anabantoids, depending on whether the audience is male or female (Matos & McGregor, 2002). When the audience is male, the two duelling males are more aggressive from the start of the conflict, performing more bites and rapidly escalating the conflict to more physical contact as opposed to ritualised displays (Dzieweczynski & Perazio, 2012). A male observer that is watching the interaction adds additional costs to the duelling pair, either through increased energy expenditure as a result of a more intense duel, greater risk of injury, or a carry-over effect as a result of the interaction being observed (Matos & McGregor, 2002). Carry-over effects are primarily dominated by the likelihood of the loser of the interaction being immediately challenged by the audience male (Oliveria *et al.* 1998), while observing females will direct more interest towards the winner (Doutrelant & McGregor, 2000). In the presence of a female audience, males reduce their aggression by decreasing the number of physical attacks, such as biting, and increasing the frequency of visual displays, i.e. tail beating (Doutrelant *et al.*, 2001; Dzieweczynski & Perazio, 2012). Males must make a trade-off between being aggressive enough with the duelling male to win

an interaction, but not scare away a potential female by putting them at risk of harm or being perceived as overtly aggressive (Matos & McGregor, 2002). Moreover, the degree to which an audience alters a male's behaviour depends upon the individual's reproductive state (Dzieweczynski *et al.*, 2005, Dzieweczynski *et al.*, 2006), resources (Dzieweczynski & Walsh, 2010) and familiarity with their opponent and audience (Dzieweczynski & Perazio, 2012; Dzieweczynski *et al.*, 2011; Dzieweczynski *et al.*, 2012; Bertucci *et al.*, 2014).

Audience effects have also been observed in male-female interactions (Dzieweczynski *et al.*, 2009; Dzieweczynski & Walsh, 2010) and female-female interactions (Dzieweczynski *et al.*, 2014a). During courtship with a female, males increase their opercular flaring, fin spreading, and nest monitoring in the presence of an audience male rival. This is thought to be an attempt to outcompete the rival male for the female's attention, while simultaneously reporting their territorial status to the rival male (Dzieweczynski *et al.*, 2009; Dzieweczynski & Walsh, 2010). However, the presence of a rival male can also cause a reduction in courtship behaviours directed at the female, as males chose to behaviour aggressively towards the rival (Bronstein, 1982), rather than display to the female. The differences between these studies may be in part due to individual differences or due to the use of a dummy female in Dzieweczynski *et al.*'s (2009) study, compared to live females by Bronstein (1982) and Dzieweczynski and Walsh (2010). Females also increase their display behaviour in the presence of a male audience in an attempt to advertise their presence to both males (Dzieweczynski & Walsh, 2010).

Encounters between rival males are also known to be affected by prior exposure to a rival (Frey & Miller, 1972; Wallen & Wojciechowski-Metzlar, 1985; Oliveira *et al.*, 1998; Karino & Someya, 2007), facilitating the development of a dominance hierarchy amongst groups.

Prior winners are likely to be more aggressive and win their next interaction (the winner effect), while losers are less aggressive in future conflicts and flee sooner, losing the duel (Dzieweczynski *et al.*, 2012). This reduction in aggression between known individuals is what allows for the formation of dominance hierarchies. Priming (individuals who observed a contest between conspecifics) can also facilitate winning, with primed male *B. splendens* being more aggressive, accompanied by a higher probability of winning, during subsequent encounters than naïve males (individuals with no prior stimulus) (Karino & Someya, 2007). Hollis *et al.* (1995) reported a similar effect amongst male *T. trichopterus*, whereby individuals who had been primed were more likely to defeat their naïve rivals and win later encounters. However, priming does not always have this effect. Halperin *et al.* (1998) found that priming male *B. splendens* caused males to escalate rapidly, but primed individuals lost more interactions than unprimed individuals. Priming was concluded to be a form of cheating and primed individuals became exhausted during interactions and subsequently lost. Moreover, those who lost their first encounter would go on to lose their next encounter, being more passive and more likely to retreat in subsequent encounters, and being under a ‘loser effect’ (Frey & Miller, 1972; Francis, 1983, Hollis *et al.*, 1995). The changes in an individual’s aggressive intensity in future conflicts is likely due to a self-assessment of their own fighting ability (Hsu & Wolf, 2001; Hsu *et al.*, 2009). By winning or losing an individual adjusts how they perceive their fixed fighting ability, regardless of the winner’s prior experience, and subsequently respond differently in future interactions.

The physiological mechanisms responsible for priming in fishes are still not clear. Although based on hormonal studies on cichlids, such acts stimulate the release of androgens like testosterone that are known to enhance fighting capabilities (Oliveira *et al.*, 2001; Dijkstra *et al.*, 2012). Given the importance of testosterone on other behaviours (*e.g.* parental behaviour;

Kramer, 1971), this raises the question as to the effects of raised androgen levels on the use of the labyrinth organ. Research has yet to focus on this particular field although it is fair to assume that individuals that have recently won an antagonistic interaction will be in a heightened state of aggression and require more frequent trips to the water surface. Mitra and Sapolsky (2012) noted that androgens promote greater predator detection and this would be of great use for air-breathing fishes during states of hyperactivity, ensuring sustained survival compared to docile rivals.

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Courtship display

Courtship displays amongst the Anabantoids are typically dependent on the ability of males to evoke a reproductive state in a female. At the start of the spawning cycle males construct a nest at the centre of their territory (Picciolo, 1964). The presence of the nest can be used by females to identify male territory and stimulates the female to enter, even in the absence of a male (Braddock & Braddock, 1959). On encountering a potential mate, males will chase and vigorously display in an attempt to entice the female towards the nest (Braddock & Braddock, 1959; Bronstein, 1982; Miller & Jearld, 1983). The female is led to the nest by the male who will perform lateral displays to the female and then return to the nest in a zig-zag manner, regularly halting (Miller, 1964; Miller & Jearld, 1983). During courtship displays, males use the same behaviours as in territory defence, however, the frequency and intensity of these behaviours are altered, with males rarely biting (Robertson & Sale, 1975; Simpson, 1968; Forsatker *et al.*, 2016). Both chemical and visual cues are assessed during courtship, with female pheromones eliciting nesting behaviour in male *T. trichopterus* (Cheal & Davis, 1974). Additionally, visual cues are responsible for increased display intensity towards potential mates in males and females (Cheal & Davis, 1974). While courting a female, the male will alternate between displaying and nest building (Bronstein, 1982). Initially, females

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flee and hide, exhibiting submissive vertical bars, but after around 24-hours, females are receptive to the male's displays (Rainwater & Miller, 1968; Bronstein, 1982). However, female *B. splendens* and *Colisa* sp. often will initiate sexual bouts with males (Rainwater & Miller, 1968; Miller & Jearld, 1983). If display movements are successful, the male will circle the female under the nest before eventually mounting the target mid-region, curving the body so that the head and tail touch, as the female turns 180° and lies motionless. From this position, the release and fertilisation of eggs occurs. Known as the Anabantoid embrace, this is a common form of copulation in the group, and typically the male does not surface during this period (Rainwater & Miller, 1968; Bronstein, 1982; Miller & Jearld, 1983; Liengpornpan *et al.*, 2006; Chandran *et al.*, 2013; Biokani *et al.*, 2014). However, if the female flees or is unreceptive to the display, the male increasingly becomes more aggressive towards the female (Rainwater & Miller, 1968).

The intensity of the fin and opercular displays of the males observed throughout these interactions has largely been facilitated by air-breathing at the water surface. Such high-intensity behaviours require more oxygen, and the labyrinth organ allows for rapid acquirement of oxygen in low DO water to ensure individuals are able to *continue* displaying (Alton *et al.*, 2013). This has subsequently had a knock-on effect on the importance of these behaviours as a reliable signal of male health – accurately communicating vitality to potential mates while acting as an indicator of quality to potential rival males (Abrahams *et al.*, 2005). Broadly speaking, healthy individuals are able to sustain an opercular display for a longer amount of time compared to those whose physiological condition has been compromised by disease or poor nutrition. This is because opercular displays are an inherently stressful act, inhibiting aquatic gas exchange by preventing the flow of water across the gills. Individuals of a reduced condition are essentially unable to hold their breath for an extended amount of

time compared to healthy individuals. Therefore, the duration of an opercular spread can represent an honest signal of a male's body condition (Schreck, 1990; Abrahams *et al.*, 2005).

375 The signals that are emitted as a result of aggressive displays, such as duration and intensity of opercular flaring, are not solely for the purpose of the other males, as females eavesdrop on these displays in order to choose a suitable mate (Doutrelant & McGregor, 2000; Desjardins *et al.*, 2012; Bertucci *et al.*, 2014). By observing the intensity of displays between sparring males, females are able to gather important information on the health of potential
380 partners as well as their prospective life success *i.e.* aggressive individuals are better at defending territories and monopolising resources (Dzieweczynski *et al.*, 2014b). This female-orientated male selection highlights the importance of the male opercular display and, in turn, the labyrinth organ, in determining the likely success of a male attracting a suitable mate. Others, however, have questioned the significance of opercular display intensity in mate
385 choice. For example, Dzieweczynski *et al.* (2014) found that female *B. splendens* actually preferred 'lover' males (docile individuals) compared to 'fighter' (aggressive individuals), reasoning that the former are less likely to injure females during mating. Similarly, Kuperberg *et al.* (2009) reported that female *B. splendens* have no preference towards males that exhibit an intense opercular spread. Instead, it was suggested that females are more
390 attracted to other, subtler traits. Tail beating, for example, was the preferred method of display amongst male *B. splendens* in the presence of a female audience (Dzieweczynski & Perazio, 2012). This display may act as an alternative measure of male vitality without the energetic costs associated with opercular flaring. Indeed, males that display at a high intensity have fewer eggs amongst their bubble nests, having invested too much energy in displaying
395 rather than caring for the eggs in their nest (Clotfelter *et al.*, 2006). Nests already containing eggs (Fig. 8) are important for the females of various fish species and it is possible that the

level of male parental care is a more attractive trait (Knapp & Sargent, 1989; Sikkel, 1989; Forsgren *et al.*, 1996). For example, female common gobies *Pomatoschistus microps* Krøyer 1838 prefer males who are more likely to ventilate their young when DO is low (Jones & Reynolds, 1999). Nest fanning is typical amongst males of nest building Anabantoid species (Kuperberg *et al.*, 2009; Huang & Chang, 2011), therefore, the extent to which a male cares for the bubble nest, rather than the intensity of their display, may be a more important criterion when investigating mate-choice.

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Parental care

Parental care is dominant throughout the Anabantoids, occurring in 16 out of 19 genera (Rüber *et al.*, 2006). The forms it can take are also remarkably diverse, ranging from substrate spawners (*Sandelia*; Cambray, 2004), to plant (*Osphronemus*; Rüber *et al.*, 2006) and bubble nesters (*Pseudosphromenus*; Chandran *et al.*, 2013). Mouth-brooders are also common (*Betta*, Cole *et al.*, 1999), whilst there are some species that display no parental care altogether and simply free spawn (*Ctenopoma*, Cole *et al.*, 1999). Parental investment amongst Anabantoid species is less varied, however, and is largely orientated towards the males with relatively few examples of female and biparental care (Rüber *et al.*, 2006; Zworykin, 2012).

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Stagnant freshwater is home to most, if not all, bubble nesting fish species (Hostache & Mol, 1998; Rüber *et al.*, 2004). Theoretically, the stress of aquatic hypoxia in a static and confined environment favours the development of enriching bubble nests that facilitate successful embryonic development, and allow sufficient oxygen availability. Conversely, species that have adapted to fast-flowing streams generally prefer mouth-brooding as bubble nests are harder to create in strong water currents (Oppenheimer, 1970). The relationship between

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parental form and environment in reality, however, is not always so clear. Rüber *et al.* (2004) found many species of mouth-brooding and bubble nesting Anabantoid species in the same body of water.

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Based on the assumption that free spawning is the plesiomorphic parental condition of the Anabantoids (Rüber *et al.*, 2006), it is likely that bubble nesting evolved first from this state and other parental forms (like mouth-brooding) subsequently followed. The labyrinth organ is not only fundamental to the development of bubble nesting, but is also a central component of later parental care iterations. The organ accommodated the shift towards an investing form of parental care that enhanced offspring survivorship, which allowed further extensions of parental care that reflected particular environmental niches – be it increased surface predator presence or water currents. It is possible that mouth-brooding initially started as a result of extended nest disturbances from predators, whereby eggs were retained for longer during oral transport to ensure protection (Rüber *et al.*, 2004).

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Bubble nest building by male fishes is one of the most commonly associated brood-guarding methods amongst Anabantoids (Bailey & Burgess, 1999), and the development of this behaviour is coupled with the presence of the labyrinth organ. During the reproductive period, species like male *B. splendens* will gulp air from the surface and mix it with mucous found in the buccal cavity, exhaling mucous laden bubbles amongst surface vegetation to create a floating bubble nest (Fig. 8) (Kang & Lee, 2010). The male fish will mate with a female beneath the nest, placing eggs amongst it or allowing them to free float upwards once copulation is complete. The male will nurture and care for the fertilised eggs and newly hatched larvae throughout this period until they leave (Bronstein, 1982). The bubble nest provides additional benefits to the embryos such as added protection from bacterial infection,

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and supplying oxygen and nutrients (Jaroensutasinee & Jaroensutasinee, 2001; Kang & Lee, 2010). Even after hatching, bubble nests serve a purpose. Larvae use wart-like appendages on their heads to attach themselves to the bubbles within the nest to provide added protection
450 from predators (Britz & Cambray, 2001).

Auditory sensitivity and communication

Fishes have evolved a diverse selection of mechanisms for acoustic communication, allowing individuals to emit and hear sounds throughout various contexts *i.e.* during social interactions
455 or when under attack (Kasumyan, 2009). Species are classed as either hearing ‘generalists’ or ‘specialists’ – the latter having enhanced auditory sensitivity and broader frequency ranges relative to the former (Yan, 1998). Largely found in freshwater, hearing specialists owe their enhanced hearing capabilities to specialised structures, which acoustically couple air-filled cavities to the inner ear (Ladich, 2000).

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Some Anabantoid fishes are capable of producing stridulating sounds during social interactions, grinding their pharyngeal teeth to get the desired effects (*e.g.* Bischof, 1996; Ladich & Yan, 1998). Members of the genus *Trichopsis*, however, have developed a novel method of sound production through rapid pectoral fin beating that is regularly used during
465 agonistic interactions (Daugherty & Marshall, 1976; Ladich & Yan, 1998). Indeed, the unique structure of the SBC found within *Trichopsis* and other Anabantoid species greatly enhances hearing abilities and facilitates the development of such behaviours (Yan, 1998). The SBCs ability to retain air, lateral location to the inner ears and thin membranous layers ensure that it is highly sensitive to changes in sound pressure, which can then be transmitted
470 directly to the ear (Ladich & Yan, 1998).

Several Anabantoid species are hearing specialists, producing broadband sounds with high-pitched frequencies (0.8-2.5 kHz) and being sensitive to high-frequency sounds (0.1-5 kHz). Anabantoids exhibit distinct interspecific differences in hearing limits (Ladich & Yan, 1998).
475 This is probably due to differences in the size of the SBC rather than to structural differences of the inner ear (*e.g.* number of hair cells). Indeed, smaller species belonging to *Macropodus* and *Trichopsis* can only take small air bubbles into their pharyngeal cavity, which provide higher resonant frequencies and so greater hearing sensitivity than species that retain larger bubbles (Ladich & Popper, 2001).

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Species such as *B. splendens* and *M. opercularis* primarily rely on visual cues to communicate and only incidentally through sound. Despite this, these species share similar hearing sensitivities and inner ear morphology to species that rely primarily on vocal communication. This suggests that enhanced hearing abilities in the Anabantoids evolved
485 prior to, or independently of, the evolution of the sound-producing mechanism observed in *Trichopsis*. Based on this assumption, it is then fair to say that the SBC is primarily an adaptation to oxygen-depleted water and the improvement of hearing appears to be, at most, a by-product of this process rather than an additional means of communication (Ladich & Popper, 2001). Enhanced auditory detection for fishes, especially in murky water, is
490 advantageous. Shallow water has a high attenuation rate for low frequencies effectively meaning individuals can only hear if the source is very close (Rogers & Cox, 1988). Whilst quiet environments such as ponds or lakes provide a great opportunity to listen out for your surroundings, at least when compared to noisy and turbulent areas found on coasts or reefs. The connection between inner ear and air-filled cavities such as the SBC favours this, and
495 allows fish to detect sudden pressure waves emanating from either approaching predators or

changes in water currents that otherwise would be undetected due to background noise (Amoser & Ladich, 2005).

Species which have evolved to extend their auditory sensitivity thus increase their chances of survival through greater predator and prey detection (Ladich, 2000). Although established as an ABO, the labyrinth apparatus has provided an opportunity for Anabantoid species to take advantage of the sedentary habitats that they are typically found in and develop an additional predator detection measure and alternative means of communication.

505 **Current trends and outlook**

Domestication

B. splendens offer perhaps the most well-known example of domestication in the Anabantoids. Selectively bred for hundreds of years, domesticated *B. splendens* strains bear little resemblance to their wild relatives, with huge variations in fin size, structure and body colour (Tlustý, 2002; Sriwattanakulchai *et al.*, 2010). Strains bred for sport fighting, in particular, are known for their heightened levels of aggression (Verbeek *et al.*, 2008). However, it is not yet clear what the effect of such behaviour is on air-breathing behaviour amongst these fishes.

515 Verbeek *et al.* (2008) reported that fighter strains have a proactive coping style towards stress and when faced with an inescapable stimuli such as receding water, immobilise themselves almost immediately whereas other strains continue to struggle. Immobilisation is an adaptive response linked to an individual's metabolic rate *i.e.* by barely moving in a hypoxic environment, individuals increase their likelihood of survival. Successful fighter fish are reared in isolated tanks in order to stimulate abnormal levels of aggression and this raises an

important question as to the effects of chronic isolation on fishes – is there a behavioural adaption (*e.g.* change in air-breathing frequency), or is there a physiological adaption (*e.g.* change in labyrinth organ morphology) to reflect their preference for aggression? Verbeek *et al.* (2008) noted the importance of hormones such as serotonin on the proactive approach to stress, although this was in relation to non-social stimuli rather than during male-male interactions. Thus, future research needs to highlight the effects of domestication during male-male interactions through the consideration of air-breathing behaviour, an adaptation known to facilitate energetically demanding behaviours.

530 **Invasive species**

The release of invasive species can have a profound impact on the ecology of communities. Novel traits enable such species to outcompete native rivals over already limited resources. This effect is particularly common amongst aquatic ecosystems, where newly introduced exotic fish species significantly alter native fish populations (Sanches *et al.*, 2012). For instance, the giant gourami *Osphronemus goramy* Lacépède 1801 is well known for its high fishery and aquaculture value and has been introduced throughout South East Asia with great success (Morioka *et al.*, 2013). However, the rapid growth rate of the species together with its voracious feeding habits and air-breathing capabilities, have provided a competitive advantage over native species (Knight, 2010). This effect has been accentuated further by *O. goramy*'s ability to carry pathogens, which native species are highly susceptible to (Whittington & Chong, 2007).

Species such as *B. splendens* and *M. opercularis* are particularly popular in the international aquarium trade. This has led to the issue of aquarium dumping into local bodies of freshwater in non-native countries that has also impacted native species (Magalhaes & Jacobi, 2013). In

particular, there have been reports across Australian media outlets where *B. splendens* have been found in large numbers in freshwater, specifically in Fogg Dam and the Adelaide River Floodplain. *B. splendens* ability to outcompete and predate on other native species, as well as possibly introduce disease, has raised great concerns amongst the local human population (Bray, 2011). Hybridisation is an additional issue associated with invasive species, whereby species mate with their wild equivalents diluting the wild genetic stock and possibly resulting in genetic extinction of such species (Pimentel, 2011).

Anabantoid fish are air-breathers and this trait, along with parental behaviour and territorial aggression, can provide a competitive edge over native species. They are able to ensure the survival of their young through keen parental care (*e.g.* bubble nesting or mouth-brooding) and, with the labyrinth organ, are better able to survive in stagnant or even polluted bodies of freshwater, where other species would otherwise struggle (Rahim *et al.*, 2013; Knight & Balasubramanian, 2015).

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Pollution

South Asia and Africa have witnessed a rapid human population increase and subsequent urbanisation in recent years. Excessive use of pesticides, industrial chemicals and fertiliser has followed suite, polluting large sections of freshwater through agricultural or industrial run-off and significantly impacting the species that inhabit these regions (Natarajan, 1981; Dutta & Munshi, 1985; Afsar *et al.*, 2012). Anthropogenic noise compounds this further by evoking stress, masking auditory senses and damaging auditory organs in species that depend heavily on alternative means of communication. Commercial and small-scale fisheries accentuate this effect by exclusively targeting Anabantoid species (Morioka *et al.*, 2010).

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Such factors might suggest that the Anabantoids are in a particularly precarious position. However, ecological surveys indicate otherwise: these aspects have had only a minor effect on population sizes and that their labyrinth organ actually allows them to survive and even thrive under these conditions (Liengpornpan *et al.*, 2007). For instance, Welcomme and
575 Vidthayanon (2003) found that *T. trichopterus* was found throughout polluted regions of the Mekong River. Whilst Ling *et al.* (2004) reported that the fish composition of Bakong River and its tributaries were dominated by the Anabantoids, seemingly able to flourish within the systems stagnant, organically polluted waters. In addition to these findings, there are many more reports of various Anabantoid species flourishing in river systems rife with effluence
580 throughout South East Asia (*e.g.* Kowasupat *et al.*, 2012a, 2012b; Rahim *et al.*, 2013).

Perhaps such studies are overlooking the subtle consequences of aquatic pollution. Indeed, research typically concentrates on linking the effects of toxicants to an organism's physiology, specifically as to whether it induces mortality. However, exposure to minor
585 concentrations of contaminants may not overtly harm an organism and may only slightly alter how it reacts in its surroundings, thus influencing their behaviour. Given that concentrations of pollutants are rarely high enough to induce mortality and that behaviour serves as an important link to physiological processes, investigating an organism's behaviour may be ideal for studying the subtle effects of environmental contamination (Scott & Sloman, 2004).

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The use of pharmaceutical products such as contraceptives, antidepressants and antibiotics is on the increase. These medicinal compounds are capable of entering aquatic systems through waste water treatment plants, remaining biochemically active and resulting in noticeable effects on the morphology, physiology and behaviour of organisms residing within
595 (Dzieweczynski & Hebert, 2012). Behaviours that are reliant upon hormones such as

courtship displays and aggression are especially susceptible to these substances (Zala & Penn, 2004). Thus, exploring behavioural responses appear ideal for assessing the effects of aquatic contaminants on fish populations. For instance, Clotfelter and Rodriguez (2006) investigated the effects of exposure to environmentally relevant concentrations of phytoestrogens on male *B. splendens* and found that it suppressed aggressive behaviour. Whilst exposure to endocrine disrupting chemicals (EDCs) such as 17 α -ethinylestradiol (EE2, commonly used in contraceptives) can reduce behavioural consistency amongst *B. splendens* (Hebert *et al.*, 2014) and their ability to communicate with multiple individuals simultaneously (Dzieweczynski & Buckman, 2013).

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Several studies have also indicated the negative effect of Selective Serotonin Reuptake Inhibitors (SSRI) such as fluoxetine, an active ingredient in Prozac, on fish behaviour. For instance, Lynn *et al.* (2007) found that fluoxetine reduced display intensity amongst male *B. splendens*, whilst others have reported its impact on movement and levels of aggression between conflicting males (Dzieweczynski & Hebert, 2012; Bogdan *et al.* 2012; Kohlert *et al.*, 2012). Forsatkar *et al.* (2014) found similar impairments amongst female *B. splendens* following exposure to fluoxetine, significantly reducing overall reproductive performance. Of particular interest, Greaney *et al.* (2015) found that fluoxetine exposure altered male *B. splendens* ability to communicate with multiple individuals simultaneously, being unable to alter the duration of an opercular display regardless of the audience type. Moreover, exposed males reduced opercular displays duration when faced with a rival, essentially reducing aggression amongst these individuals. Conversely, the number of tail beats produced by a male, a more docile behaviour associated with courtship displays, was unaffected by fluoxetine exposure. These findings suggest that fluoxetine exposure has a context dependent impact on fish behaviour, and more importantly, suggests broader implications beyond that of

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the contaminated individual, extending across the network and possibly interfering with the fitness of the species. By decreasing aggression through fluoxetine exposure, inaccurate information on an individual's quality and fighting ability will be conveyed to a female. Female *B. splendens* show preference towards less aggressive males (Dzieweczynski *et al.*, 625 2014b), and this could enhance the reproductive success of affected males who would otherwise be overlooked (Greaney *et al.*, 2015).

Given their resistance to wastewater treatment processes, pharmaceutical compounds are now ubiquitous across aquatic ecosystems. As such, there are wide implications to an organism's 630 physiology *e.g.* reduced immune function (Ardia & Clotfelter, 2006) and disrupted reproductive development (Hano *et al.*, 2011). However, it is the subtle effects on behaviour that is appears to be most detrimental in at least *B. splendens*. Inadvertent pharmaceutical pollution is affecting mating success by interfering with the reproductive system – males are not as aggressive or are effectively communicating with rivals and target females. 635 Consequently, females are not reaching a reproductive state nor is territory defence being maintained, which may lead to the collapse of established reproductive hierarchies. Future research needs to concentrate further on the impact of such compounds on fish species on various levels of exposure. Organisms in the wild may experience fluoxetine and other SSRIs at an acute or chronic level, and by investigating this, a true understanding of the impacts of 640 SSRI exposure can be gained (Greaney *et al.*, 2015).

Conclusion

Anabantoid fishes have bimodal respiration, with aquatic respiration via the gills supplemented by aerial respiration at the water surface, using the labyrinth organ. The evolution of the labyrinth organ was probably driven by the hypoxic conditions in which

these species live. Since its formation, the labyrinth organ has provided the means for the
645 Anabantoids to diversify and thrive where unimodal water breathing fishes could not.
However, the implications of the presence of the labyrinth organ are significantly more far-
reaching, impacting the morphology, behaviour and physiology of the Anabantoids.

Intense defence displays significantly increase the oxygen requirement of displaying males,
650 which is met by increased synchronous air-breathing trips. This subsequently impacts male
mating success as females can observe displays between rivals, using them as an indicator of
male quality. Additionally, air-breathing has influenced the evolution of a diverse range of
parental care behaviours, most notably bubble nesting, and as a by-product of its evolution,
air-breathing has enhanced the hearing abilities of the Anabantoid species to provide an
655 alternative form of communication and additional methods of predator detection.

The advantages provided by the labyrinth organ, however, have been shown to have a
detrimental effect where Anabantoid species are invasive. The characteristics that make these
species able to inhabit hypoxic conditions can also provide them with a competitive
660 advantage over native species and seemingly a resistance to the effects of pollution. Although
studies indicate Anabantoids are found in polluted areas, the effect of this pollution is little
explored, beyond mortality as an endpoint. Indeed, research into pharmaceutical pollutants,
such as antidepressants, reveals a substantial impact upon aggression and communication
within *B. splendens*. Reduced aggression and an inability to communicate to multiple
665 individuals has wider consequences extending across the social network and potentially
interfering with the fitness of the species.

We are grateful to D. Jones for granting permission for using the photographs in Figure 3. A. King provided
useful discussions.

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