AQUATIC SURFACE RESPIRATION, BUOYANCY CONTROL AND THE EVOLUTION OF AIR-BREATHING IN GOBIES (GOBIIDAE: PISCES)

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Summary

The role of a buccal gas bubble, held while performing aquatic surface respiration (ASR; ventilating the gills with surface water during hypoxia), was examined in benthic, intertidal Australian gobies (Favonigobius tamarensis, F. exquisitus, Pseudogobius olorum, Chlamydogobius sp., Mugilogobius paludis, Cryptocentroides cristatus and Arenigobius bifrenatus). Analyses of the forces of lift and weight of the head and body during ASR indicate a hydrostatic role for the bubble. During ASR, lift from the bubble was sufficient to provide neutral or positive buoyancy to the head, anchoring the mouth at the water surface. A buoyancy role was confirmed by experiments demonstrating the ability of some species to alter bubble volume, to compensate either for different body positions or for water densities (salinities). Use of the bubble for aerial respiration by Cryptocentroides, Mugilogobius, Chlamydogobius and Arenigobius was confirmed in hypoxia by the presence of blood-filled capillaries in the buccal subepithelium (mean air–blood barrier less than 30 μm) in areas of the buccal cavity that contacted the bubble. Blood-filled capillaries were rare or absent in normoxia in all species except Mugilogobius. Cutaneous respiration was inferred from the presence of blood-filled capillaries in the dermis and epidermis of emersed portions of the head in Mugilogobius, Chlamydogobius and Arenigobius. The buccal bubble has respiratory and hydrostatic roles and there is support for the hypothesis that ASR and the buoyancy regulation (air-gulping) required to perform it effectively are prerequisite steps in the evolution of air-breathing in these gobies.

Key words: buoyancy, aerial respiration, hypoxia, Gobiidae.

Introduction

Negatively buoyant benthic gobies survive extreme hypoxia by performing aquatic surface respiration (ASR) in shallow water (Gee and Gee, 1991). ASR is a common adaptation for surviving hypoxia (Lewis, 1970; Gee et al. 1978; Kramer, 1983) and involves ventilating the gills with the upper 1–2 mm of surface water, which contains significant amounts of O2 (Burggren, 1982). Gee and Gee (1991) showed that 9 out of 12 species of gobies investigated performed ASR in shallow water (<3 cm) while holding a bubble in their buccal cavity. This bubble was exchanged frequently (<70 s), was present only when the fish was using ASR and was released if the fish was disturbed and temporarily left the water surface. This paper examines the functions of this bubble and assesses the evolutionary implications of bubble-holding during ASR.

Two obvious roles for the buccal bubble are to enhance ASR by further oxygenating the water prior to ventilating the gills (Burggren, 1982) and to provide O2 directly to respiratory tissues in the buccal cavity, as occurs in some gobies (Graham, 1976). In addition, the bubble could play a hydrostatic role by providing lift to the body and positioning the mouth in the surface water, thus facilitating ASR. Buoyancy control during ASR allows fish to optimize their position (benthic species) or swimming mode (midwater species) and to compensate for O2 loss from the swimbladder (which decreases in volume) that occurs during hypoxic stress (Gee, 1986; Chiasson and Gee, 1983; Gee and Ratynski, 1988). Gee and Gee (1991) found that intertidal benthic gobies performed ASR in shallow water in one of the following positions: (1) by beaching the body with the anterior dorsal surface emersed and the mouth just below the water surface, (2) in an arched position with the head tilted up, the snout emersed and the mouth at the water surface, with the body resting on the pelvic, anal and caudal fins, or (3) hanging vertically from the water surface at positive buoyancy or attached to a vertical surface with the fused pelvic fins. Lift from a buccal bubble appeared to be important in the latter two positions, enabling fish to perform ASR effectively by holding the mouth in the surface water.

The objective of our study was to determine whether the buccal bubble has hydrostatic and respiratory functions in benthic gobies. To do this, we describe the amount and distribution of forces of lift and weight on seven species of benthic gobies to make inferences about a hydrostatic role for

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the bubble. Two experiments were completed to confirm a hydrostatic function for the bubble in three species. A histological examination of tissues lining the buccal cavity and of the skin of emersed portions of the head was completed on all species to assess the potential for aerial respiration at these sites.

Materials and methods

Marine gobies were collected from estuaries within the Hawksbury River, NSW, Australia, at either Bobbin Head and Appletree Bay (Cowan Water), or from Careel Bay (Pittwater). Salinities at the point of capture varied from 10 to 33‰. Freshwater Chlamydogobius sp. were progeny of fish taken from Dalhousie Springs (Lake Eyre drainage) and were provided by J. R. Merrick. Although all species were benthic, five possessed a swimbladder (Favonigobius tamarensis, Mugilogobius paludis, Favonigobius exquisitus, Cryptocentroides cristatus and Arenigobius bifrenatus) and two (Pseudogobius olorum and Chlamydogobius sp.) did not. One species, F. tamarensis, did not use a buccal bubble in hypoxia. Gee and Gee (1991) provide details of the locations of the sites of capture and the habitats occupied. In the laboratory, fish were held in aquaria (30–70 l) at a room temperature of 22–23 °C with a natural photoperiod. Initially, salinities were kept to within 5‰ of those at the capture site and later fishes were acclimated to 20‰ (2.5‰ day−1) for at least 5 days prior to testing. Chlamydogobius sp. was held and tested in fresh water. Commercially prepared sea salt (Sera, Heinsberg, FRG) was used with aged tap water to create the appropriate salinity (measured by a hydrostatic method; APHA, 1965). Fish were fed shredded prawns up to 24 h prior to testing.

Measurement of the variables necessary to describe lift and weight and experiments relating to a hydrostatic role for the buccal bubble were completed on fish performing ASR in an aquarium (60 cm × 25 cm × 30 cm) with a sloping bottom (2 cm drop along 7 cm length) that formed a shallow beach at one end. The bottom then dropped 25 cm along 25 cm of its length and the remaining 28 cm of length was at full depth. The shallow beach permitted gobies access to surface water, facilitating ASR.

Lift and weight

Lift and weight were measured on fish while they were using ASR in shallow water in an arched position. Fish (one species at a time; N=8) were placed into the experimental aquarium 18–20 h prior to examination and N2 was bubbled gently through a diffuser stone, reducing O2 levels to less than 1.0 p.p.m. over 6–8 h. One hour prior to examination, sodium sulphite was added (4.5 mg l−1) to reduce O2 content to 0 p.p.m.. Under such conditions, O2 from the atmosphere diffuses into the surface water to a depth of 1–2 mm (Burggren, 1982) and it becomes available for use during ASR. Measurements of buoyancy-related variables commenced after 1 h at 0 p.p.m. dissolved O2.

Mean bubble volume was measured for each fish (±0.001 ml; N=6) by capturing buccal bubbles (using the procedure of Gee and Gee, 1991). Fish were then captured, placed into anaesthetic (0.03 % MS-222; ethyl m-aminothenzoate methane sulphonate) and a number of measurements and calculations were made, but not in the order described below. Total length of fish and length of head (distance from snout to origin of fused pelvic fins) were measured (±0.1 mm). Lift and weight were measured for the total body and then on the head and trunk separately to determine whether lift values of the head and trunk varied independently of each other. The head was separated from the trunk by placing the fish on its dorsal surface, folding the pectoral fins forward, and cutting across the body between the pectoral and pelvic girdles. Measurements of the weight of the head, trunk and total body were made in air and fresh water so that their volume and density could be calculated. The position of the centre of gravity was estimated by trial and error by placing a small pin through the body under the first dorsal fin and allowing the fish, free of all gas inclusions, to rotate freely in water of experimental salinity and temperature. The point at which the fish remained horizontal was used as the position of the centre of gravity. The centre of swimbladder lift was defined as the midpoint along the swimbladder length as determined from X-ray negatives of the intact fish. Distances between these centres and the snout (±0.1 mm) were measured and expressed as a percentage of total length. The position of the buccal bubble was visible through the transparent gular tissues when fish were viewed through the sloping glass bottom of the aquarium. Buoyancy was defined as the proportion of the weight of the fish in water supported by lift from gas inclusions. Buoyancy values were calculated for the body supported by lift from all gas inclusions, for the head supported by lift from the buccal bubble and for the trunk supported by lift from the swimbladder.

Measurements given in 1, 4, 10, 11 and 15 below were made for the body and then for the head and trunk separately to calculate the density of either the body or the head. Measurements of the weight of fish in 2 were made using a submerged pan suspended from a below-the-balance hook. Negatively buoyant fish were placed on the pan; positively buoyant fish were placed under the pan. Care was taken to remove all air bubbles from the buccal and opercular cavities. The following were measured (±1.0 mg): (1) mass of body/head/trunk blotted dry in air; (2) weight of the intact body in water at the experimental salinity and temperature; (3) weight of the body, gas-free, in water at the experimental salinity and temperature; (4) weight of the body/head/trunk, gas-free, in fresh water.

Measurements and calculations relating to forces of lift and weight were made as follows: (5) water density (g ml−1); (6) swimbladder lift (g), 3 − 2; (7) swimbladder volume (ml), 6/5; (8) bubble volume (ml), measured as above; (9) bubble lift (g) = 8 × 5; (10) body/head/trunk volume, gas-free (ml) = 1 − 4; (11) body/head/trunk density (g ml−1) = 1/10; (12)
swimbladder lift per unit trunk volume (g ml\(^{-1}\)) = 6/10; (13) bubble lift per unit head volume (g ml\(^{-1}\)) = 9/10; (14) total lift per unit body volume (g ml\(^{-1}\)) = (7 + 8)/10; (15) body/head/trunk weight in water per unit volume (g ml\(^{-1}\)) = 11 – 5; (16) buoyancy of total body = 14/15; (17) buoyancy of head = 13/15; (18) buoyancy of trunk = 12/15; (19) percentage of head volume emersed = ((13 – 15)/11) × 100. The basis for 6, 7 and 10 is that, in water, air provides a lift of 1 g ml\(^{-1}\).

Direct observation of a hydrostatic function for buccal bubble

A hydrostatic function for the buccal bubble can be shown if fish adjust bubble volume under conditions that might require different amounts of lift. As some gobies used ASR in both the arched and vertical positions (Gee and Gee, 1991), a hydrostatic function could be demonstrated if fish held a larger bubble when vertical, because it must support not only the weight of the head but also that portion of the trunk that is not supported by the swimbladder. In addition, in species performing ASR while vertical, the bubble volume may vary inversely with the swimbladder volume if a precise total lift (buoyancy) from both gas inclusions is required. Finally, species tolerant of a range of salinities may alter bubble volume inversely with water density (salinity) if maintaining a particular buoyancy is critical.

Bubble volumes in the vertical and arched positions were compared for individual fish in *M. paludis, A. bifrenatus* and *C. cristatus*. The experimental aquarium (described above) was divided across its width. One side contained the shallow beach, allowing surface access in the arched position, while the other was at full depth, allowing surface access only in a vertical position either floating at the water surface or attached to the side. Fish (*N*=2) were exposed to hypoxia (0 p.p.m.; O\(_2\) reduced over 90 min as above) in one portion of the aquarium and then six bubble volumes were measured on each fish. Fish were transferred to the other side (also hypoxic), left for 30 min, and six bubble volumes were again measured. Mean bubble volumes were compared in the vertical and arched positions using the *t*-test for paired observations.

Bubble and swimbladder volumes were compared in *M. paludis* and *C. cristatus* when using ASR in a vertical position to determine whether mean bubble volume (*N*=6) varied inversely with swimbladder volume within individual fish. These data were obtained from fish in the above experiment.

Histological examination

Histological preparations were made of tissues from the buccal cavity and dorsal surface of the head for all seven species to make inferences of their capacity for aerial respiration. Three individuals of each species held in normoxic water (O\(_2\)>8 p.p.m.) were compared with three held for 5.5 h in hypoxic water (dissolved O\(_2\)<0.5 p.p.m.). Fish were killed and placed in Bouin’s fixative for at least 24 h. The heads were then removed, decalcified in a solution of formic acid and formaldehyde, dehydrated in 70 % ethanol, embedded in paraffin, sectioned at 7 μm and stained with Mallory’s solution. Serial cross sections were made through the whole head from the snout to just posterior to the eyes. Microscopic examinations were made of tissues from the surface of the head at the snout and between the eyes and from the roof of the buccal cavity below the eyes, and the occurrence of blood-filled capillaries near the surface was assessed. On one fish from each treatment, the number of blood-filled capillaries was counted over a distance of 500–850 μm to estimate their abundance in the epidermis/dermis and epithelium/subepithelium (connective tissue) at the above sites. To assess further the potential for aerial respiration, measurements were taken of the air–blood barrier, the thickness of the dermis and the epidermis of the skin and of the epithelium and subepithelium of the buccal lining. Measurements also were taken of the blood–water barrier of the secondary lamellae of the gills, for use as a reference. Measurements (using a filar ocular micrometer) were made on one fish from hypoxic water from each species displaying an increase in the number of blood-filled capillaries.

Results

Lift and weight

The centre of swimbladder lift was located just anterior to the centre of gravity in each species and both were located about one-third of the way along the body (Table 1; Fig. 1). The head made up 21–26 % of the body length and 28.3–40.5 % of the body weight. Mean body tissue density varied among species from 1.057 to 1.082 g ml\(^{-1}\) and in each species the density of the head was significantly greater (*P*<0.05; ANOVA) than that of the trunk (Table 1). The centre of bubble lift varied within individual fish from directly below to just behind the eyes (Fig. 1). As water was inspired, the gular region expanded and the anterior portion of the bubble
moved to a position just ventral to the eyes (Fig. 2) and on expiration the bubble moved forward under the eyes. It was contained within the buccal cavity by a buccal valve. The bubble remained anterior to the gill holobranchs at all times.

Buoyancy of gobies varied from negative to near neutral. *Favonigobius tamarensis* (*no buccal bubble*), *P. olorum* and *Chlamydogobius* sp. (*no swimbladder*) were negatively buoyant. However, the remaining species, which possessed both gas inclusions, were at near neutral buoyancy (mean values ranged from 0.92 to 1.06; Table 2). All bubble-gulping species

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### Table 1. Distribution of lift and weight forces and tissue density of gobies studied

<table>
<thead>
<tr>
<th>Species</th>
<th>Centre of gravity</th>
<th>Centre of SB lift</th>
<th>Head as % of body length</th>
<th>Head as % of body weight</th>
<th>Tissue density (g ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. tamarensis</em> (29–47 mm)</td>
<td>34.5±2.4</td>
<td>33.8±1.0</td>
<td>25.9±2.6</td>
<td>40.0±4.7</td>
<td>1.078±0.005</td>
</tr>
<tr>
<td><em>P. olorum</em> (32–39 mm)</td>
<td>34.6±1.2</td>
<td>*</td>
<td>21.0±0.5</td>
<td>28.3±3.6</td>
<td>1.079±0.005</td>
</tr>
<tr>
<td><em>Chlamydogobius</em> sp. (26–32 mm)</td>
<td>33.8±1.6</td>
<td>*</td>
<td>23.4±2.2</td>
<td>40.5±3.1</td>
<td>1.057±0.005</td>
</tr>
<tr>
<td><em>M. paludis</em> (31–49 mm)</td>
<td>35.3±1.4</td>
<td>34.4±1.3</td>
<td>21.8±1.7</td>
<td>36.0±4.7</td>
<td>1.077±0.003</td>
</tr>
<tr>
<td><em>F. exquisitus</em> (36–57 mm)</td>
<td>35.6±1.2</td>
<td>30.0±1.2</td>
<td>24.3±0.7</td>
<td>33.9±1.6</td>
<td>1.082±0.005</td>
</tr>
<tr>
<td><em>C. cristatus</em> (79–115 mm)</td>
<td>34.7±1.0</td>
<td>30.9±0.7</td>
<td>22.3±0.5</td>
<td>37.5±1.3</td>
<td>1.076±0.001</td>
</tr>
<tr>
<td><em>A. bifrenatus</em> (43–79 mm)</td>
<td>34.9±1.6</td>
<td>34.4±0.9</td>
<td>26.0±1.4</td>
<td>36.4±2.7</td>
<td>1.078±0.004</td>
</tr>
</tbody>
</table>

*No swimbladder present.*

Values given are means ± S.D. (*N*=8). Total length is given in parentheses.

Centres of gravity and swimbladder (SB) lift are the distance between the centre and the tip of the snout expressed as a percentage of total length.

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**Fig. 1. Buoyancy values, tissue densities and the distribution of lift and weight in benthic gobies performing aquatic surface respiration (ASR) in the arched position. Ranges in variation of (A) the estimated centre of lift from the buccal bubble, (B) the centre of swimbladder lift and (C) the centre of gravity. The broken line shows the boundary between the head and trunk.**

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*Favonigobius tamarensis* (*no buccal bubble*), *P. olorum* and *Chlamydogobius* sp. (*no swimbladder*) were negatively buoyant. However, the remaining species, which possessed both gas inclusions, were at near neutral buoyancy (mean values ranged from 0.92 to 1.06; Table 2). All bubble-gulping species...
possessed a negatively buoyant trunk, regardless of the presence of a swimbladder (Fig. 1). In the arched position, there was no relationship between bubble volume and swimbladder volume in the four species possessing both. Regression analyses on each of these species showed that the amount of variation in bubble lift due to a regression on swimbladder volume was not significant ($F$-test on $r$) and the slope of the regression line did not deviate from zero ($t$-test on regression coefficient). This indicates that lift derived from the swimbladder and the bubble act independently when the fish is performing ASR in the arched position. The bubble-gulping species *P. olorum* and *Chlamydogobius*, both of which lack a swimbladder, performed ASR (arched position) in a similar manner to those bubble-gulping species with a swimbladder. The non-bubble-gulping species *F. tamarensis* performed ASR when arched but used the beached position most frequently. All of the bubble-gulping species appeared to use bubble lift combined with the flexibility of the body to arch the head upwards, placing the mouth at the water surface. As a result, we considered lift and weight acting on the head to be independent from lift and weight acting on the trunk. The head of all bubble-gulping species was either neutral (*P. olorum*) or positively (remainder) buoyant. The bubble-gulping species had an average of 2.8–7.2% of their head volume above the water surface (Table 2). These data show that during ASR with a buccal bubble present the trunk rests on the substratum while the head is either neutral or positively buoyant and held at the water surface.

**Direct observation of a hydrostatic function for buccal bubble**

Individual *M. paludis*, *C. cristatus* and *A. bifrenatus* averaged a significantly larger bubble volume when vertical than when in the arched position (Table 3). While vertical, the bubble supported not only the weight of the head but also that portion of the trunk not supported by the swimbladder. Bubble lift varied independently of swimbladder lift in *M. paludis* and *C. cristatus* when vertical. In both species, a less than significant portion of the variation in bubble lift was due to a regression on swimbladder lift ($F$-test on $r$) and the slope of the regression line did not deviate significantly from zero. Fish did not vary bubble lift to compensate for variation in swimbladder lift while in the vertical position.

There was no adjustment of bubble volume by *P. olorum* to compensate for variation in water density because bubble lift was similar at all water densities (Table 4). Although body density increased significantly at the highest water density, the buoyancy of the fish increased as a result of the increased water density. In *M. paludis*, bubble lift was significantly lower at 1.016 and 1.032 g ml$^{-1}$ than at 1.0 or 1.047 g ml$^{-1}$ (Table 4) and head density increased significantly with increased water density. This caused the buoyancy of the head to remain similar at 1.0, 1.016, and 1.032 g ml$^{-1}$, indicating a hydrostatic role for the bubble at these densities. At 1.047 g ml$^{-1}$, however, the buoyancy of the head increased significantly because of a significant increase in bubble lift (Table 4).

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**Table 2. Lift and weight forces (g ml$^{-1}$) of the body and on the head of gobies performing aquatic surface respiration in an arched position**

<table>
<thead>
<tr>
<th>Species</th>
<th>Body Weight</th>
<th>Body Lift</th>
<th>Body Buoyancy</th>
<th>Head Weight</th>
<th>Head Lift</th>
<th>Head Buoyancy</th>
<th>Percentage of head volume emersed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. tamarensis</em></td>
<td>0.062±0.005</td>
<td>0.043±0.010</td>
<td>0.69</td>
<td>0.074±0.010</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td><em>P. olorum</em></td>
<td>0.063±0.005</td>
<td>0.019±0.004</td>
<td>0.30</td>
<td>0.073±0.009</td>
<td>†</td>
<td>†</td>
<td>0.30</td>
</tr>
<tr>
<td><em>Chlamydogobius</em> sp.*</td>
<td>0.057±0.005</td>
<td>0.036±0.006</td>
<td>0.63</td>
<td>0.063±0.004</td>
<td>0.099±0.017</td>
<td>1.57</td>
<td>4.0</td>
</tr>
<tr>
<td><em>M. paludis</em></td>
<td>0.061±0.005</td>
<td>0.056±0.017</td>
<td>0.92</td>
<td>0.071±0.007</td>
<td>0.114±0.012</td>
<td>1.61</td>
<td>5.7</td>
</tr>
<tr>
<td><em>F. exquisitus</em></td>
<td>0.067±0.006</td>
<td>0.068±0.013</td>
<td>1.01</td>
<td>0.082±0.006</td>
<td>0.144±0.021</td>
<td>1.76</td>
<td>2.8</td>
</tr>
<tr>
<td><em>C. cristatus</em></td>
<td>0.060±0.003</td>
<td>0.058±0.007</td>
<td>0.97</td>
<td>0.080±0.004</td>
<td>0.111±0.017</td>
<td>1.39</td>
<td>2.8</td>
</tr>
<tr>
<td><em>A. bifrenatus</em></td>
<td>0.062±0.004</td>
<td>0.066±0.008</td>
<td>1.06</td>
<td>0.066±0.008</td>
<td>0.144±0.020</td>
<td>2.18</td>
<td>7.2</td>
</tr>
</tbody>
</table>

*No swimbladder; †no bubble-gulping.
Lift acting on the body comes from all gas inclusions, whereas that acting on the head is from the buccal bubble.
Values are means ± S.D. (N=8).
Buoyancy is the proportion of weight supported by lift.
Histological examination

Gobies fell into three distinct groups on the basis of the abundance of blood-filled capillaries at potential respiratory sites (Table 5). In *F. tamarensis*, *F. exquisitus* and *P. olorum*, blood-filled capillaries were either rare or not observed at any site in either normoxic or hypoxic treatments. Striking differences occurred between normoxic and hypoxic treatments in *C. cristatus*, *A. bifrenatus* and *Chlamydogobius* sp., where there was an increase in abundance of blood-filled capillaries in the hypoxic treatment. In *M. paludis*, blood-filled capillaries were abundant in both treatments (Table 5). These four species emersed a portion of the head when performing ASR in hypoxia, but only three showed an abundance of blood-filled capillaries in the emersed areas of the skin (Table 5). In hypoxia, these capillaries extended into the epidermis of *Chlamydogobius* and *M. paludis* (Fig. 3A), while in *A. bifrenatus* they were found only in the dermis. In the hypoxic treatment, capillaries in *M. paludis* formed loops through the epidermis to the skin surface on emersed portions of the head and were more continuous in length than those in normoxia. Blood-filled capillaries were obvious at the boundary between the epithelium and subepithelium in the roof of the buccal cavity beneath the eyes in *C. cristatus*, *M. paludis*, *A. bifrenatus* and *Chlamydogobius* (Fig. 3B). Capillaries were also seen in *A. bifrenatus* in the subepithelium on the sides of the buccal cavity and on the surface of the tongue.

The mean air–blood barrier of gobies with abundant blood-filled capillaries in hypoxia varied among species from 9 to 29 μm in the buccal lining and was less than 25 μm in the skin of the emersed portions of the head for *Chlamydogobius* (between the eyes) and *M. paludis* (snout and between the eyes; Table 6). Although there was a similar abundance of blood-filled capillaries at potential respiratory sites in *M. paludis* in both treatments, the diffusion barrier was much less in hypoxia (Table 6). The water–blood barrier of the gills varied from 2 to 5 μm.

**Discussion**

**Inferences from lift and weight**

These gobies appear to require either a neutrally or a positively buoyant head to perform ASR effectively in an arched position. The head is the most dense portion of the body and, although gobies can rest their negatively buoyant trunk on fused pelvic, anal and caudal fins, about one-third of the body weight lies anterior to the pelvic fins and must be supported by the muscles and skeleton. The anterior portion of the trunk is flexible, allowing the head to be tilted upwards and positioning the mouth in the oxygenated surface water. A considerable amount of lift is required to place the mouth in the surface water since a portion of the head must be emersed because of the prominent snout. The exception was *P. olorum*, which had the greatest flexibility and could tilt its head upwards at such an angle that no part of the head needed to be emersed. Its head was neutrally buoyant. The remaining bubble-gulping species required a positively buoyant head (mean buoyancy between 1.39 and 2.18), emersing 2.8–7.2% of head volume, to place the mouth just below the water surface. This analysis of lift and weight forces provides convincing support for a

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**Table 3. Mean bubble volume in both arched and vertical positions**

<table>
<thead>
<tr>
<th>Species</th>
<th>Number examined</th>
<th>Arched (ml)</th>
<th>Vertical (ml)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. paludis</em></td>
<td>13</td>
<td>0.014</td>
<td>0.020</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>C. cristatus</em></td>
<td>15</td>
<td>0.224</td>
<td>0.336</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>A. bifrenatus</em></td>
<td>5</td>
<td>0.024</td>
<td>0.032</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

P from t-test for paired observations.

Six bubble volumes were measured on each fish in both positions.

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**Table 4. Effect of water density (salinity) on mean buoyancy-related variables of Pseudogobius olorum (N=12) and Mugibogobius paludis (N=10)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Water density (g ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td><em>P. olorum</em></td>
<td>(0‰)</td>
</tr>
<tr>
<td>Body density</td>
<td>1.080±0.004*</td>
</tr>
<tr>
<td>Bubble lift</td>
<td>0.018±0.006</td>
</tr>
<tr>
<td>Buoyancy†</td>
<td>0.226±0.080</td>
</tr>
<tr>
<td><em>M. paludis</em></td>
<td>(0‰)</td>
</tr>
<tr>
<td>Head density</td>
<td>1.079±0.009</td>
</tr>
<tr>
<td>Bubble lift</td>
<td>0.141±0.030</td>
</tr>
<tr>
<td>Buoyancy of head</td>
<td>1.799±0.046</td>
</tr>
</tbody>
</table>

Means indicated by an asterisk differ significantly from unmarked means.

†All means differ significantly from each other; P from ANOVA.

Values are mean ± s.d.

Salinity is expressed in parts per thousand.
Buoyancy of gobies in hypoxia

The following results provide additional support. (1) Mean bubble volume varied independently of swimbladder volume (where present) and, for each species, bubble volume was much less variable than swimbladder volume, suggesting a more precise and possibly important hydrostatic function for the bubble than for the swimbladder. (2) The buccal bubble is held only when performing ASR, fish leaving the surface temporarily release their bubble, indicating that its function(s) is related to ASR and is hydrostatic, respiratory or both. (3) Bubble-holding was found only among the negatively buoyant species examined by Gee and Gee (1991) and not among midwater species at near neutral buoyancy during hypoxia.

Table 5. Abundance of blood-filled capillaries in the epidermis and dermis of emersed portions of the head (snout and between the eyes) and the epithelium and subepithelium of the roof of the buccal cavity

<table>
<thead>
<tr>
<th>Species</th>
<th>Buccal bubble present</th>
<th>Head emersed in ASR</th>
<th>Snout Normoxia</th>
<th>Hypoxia</th>
<th>Between eyes Normoxia</th>
<th>Hypoxia</th>
<th>Buccal cavity Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. tamarensis</td>
<td>No</td>
<td>No</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. exquisitus</td>
<td>Yes</td>
<td>Yes</td>
<td>0</td>
<td>0 *</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P. olorum</td>
<td>Yes</td>
<td>No</td>
<td>0</td>
<td>0 *</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. cristatus</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>A. bifrenatus</td>
<td>Yes</td>
<td>Yes</td>
<td>0</td>
<td>***</td>
<td>0</td>
<td>***</td>
<td>0</td>
<td>***</td>
</tr>
<tr>
<td>Chlamydogobius sp.</td>
<td>Yes</td>
<td>Yes</td>
<td>0</td>
<td>*</td>
<td>0</td>
<td>**</td>
<td>0</td>
<td>***</td>
</tr>
<tr>
<td>M. paludis</td>
<td>Yes</td>
<td>Yes</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

Tissues were not examined on the snout of C. cristatus.

ASR, aquatic surface respiration.

Capillaries per 100 \( \mu m \): 0, none; *, rare (<0.35); **, present (0.35–0.70); ***, abundant (>0.70).

Table 6. Air–blood diffusion barrier of skin on emersed portions of the head and of the lining of the buccal cavity, thickness of the skin and buccal lining, and diffusion barrier of gills in four bubble-gulping species

<table>
<thead>
<tr>
<th>Location</th>
<th>C. cristatus</th>
<th>A. bifrenatus</th>
<th>Chlamydogobius sp.</th>
<th>M. paludis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snout Barrier</td>
<td>75 (61–86)</td>
<td>61 (46–81)</td>
<td>26 (6–108)</td>
<td>102 (33–125)</td>
</tr>
<tr>
<td>Epidermis</td>
<td>64</td>
<td>70</td>
<td>90</td>
<td>107</td>
</tr>
<tr>
<td>Dermis</td>
<td>17</td>
<td>11</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Between eyes</td>
<td>48 (41–59)</td>
<td>60 (40–89)</td>
<td>24 (9–54)</td>
<td>19 (0–31)</td>
</tr>
<tr>
<td>Epidermis</td>
<td>36</td>
<td>54</td>
<td>44</td>
<td>103</td>
</tr>
<tr>
<td>Dermis</td>
<td>14</td>
<td>25</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>Buccal cavity</td>
<td>19 (10–41)</td>
<td>29 (15–50)</td>
<td>20 (11–25)</td>
<td>9 (8–15)</td>
</tr>
<tr>
<td>Epithelium</td>
<td>11</td>
<td>28</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Subepithelium</td>
<td>14</td>
<td>14</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Gills Barrier</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Measurements (in \( \mu m \)) are from one fish held in hypoxia, except for M. paludis where values from one individual from both hypoxia and normoxia are compared.

Values are means (N=10) and ranges are shown for air–blood or water–blood barriers.

There was no detectable variation at the gills.

Tissues at the snout of C. cristatus were not examined.

hydrostatic role for the buccal bubble. The following results provide additional support. (1) Mean bubble volume varied independently of swimbladder volume (where present) and, for each species, bubble volume was much less variable than swimbladder volume, suggesting a more precise and possibly important hydrostatic function for the bubble than for the swimbladder. (2) The buccal bubble is held only when performing ASR, fish leaving the surface temporarily release their bubble, indicating that its function(s) is related to ASR and is hydrostatic, respiratory or both. (3) Bubble-holding was found only among the negatively buoyant species examined by Gee and Gee (1991) and not among midwater species at near neutral buoyancy during hypoxia.

Direct confirmation of a hydrostatic role for the buccal bubble

Direct evidence of a buoyancy role for the buccal bubble was indicated when C. cristatus, M. paludis and A. bifrenatus increased their bubble volume significantly in the vertical position when compared with the arched position. Gee and Gee (1991) found that species using a vertical position frequented tunnels of other species and crevices in intertidal areas of estuaries. They concluded that the ability to use a vertical position, either floating at positive buoyancy or attached to a vertical surface by the fused pelvic fins at near neutral buoyancy, was adaptive to performing ASR in such a potentially hypoxic microhabitat.
Swimbladder volume varied considerably within species. In the vertical position, an inverse relationship between swimbladder and bubble lift was expected but was not found. The lack of such a relationship may be the result of experimental conditions. Fish were held in an open aquarium and individuals were sampled repeatedly, possibly interfering with the accuracy of bubble volume regulation. These fishes are easily disturbed when performing ASR and in nature they live in a variety of cover (Gee and Gee, 1991) and are secretive in habit.

Bubble lift in *P. olorum* did not vary significantly between water densities of 1.0 and 1.047 g ml$^{-1}$. As a result, buoyancy increased with water density, although this was partially offset by an increase in tissue density at 1.047 g ml$^{-1}$. In this species, there is no direct evidence supporting a hydrostatic role for the bubble. This does not exclude such a role because it indicates only that this species does not adjust bubble lift to compensate for differences in water density. A hydrostatic role for the bubble was confirmed in *M. paludis*, which reduced bubble lift significantly as water density increased from 1.0‰ to 1.032 g ml$^{-1}$, creating a similar buoyancy at these densities. At 1.047 g ml$^{-1}$, however, bubble lift increased and was similar to that at 1.0 g ml$^{-1}$. This distinct reversal of the trend strongly suggests a dual role for the buccal bubble. At the lower water densities, the hydrostatic and respiratory roles are compatible with the bubble providing lift to the head and oxygen to both buccal water and respiratory tissues in the roof of the mouth. At the highest water density (salinity), O$_2$ demand could be greatly increased as a result of the increased costs of osmoregulation (Rao, 1968). Under such conditions, the

Fig. 3. Location of capillaries in *Mugil gobius paludis* from hypoxic water. (A) Skin from emersed portions of the head between the eyes from two fish and (B) from the lining from roof of the buccal cavity. *ep*, epidermis; *d*, dermis; *e*, epithelium; *s*, subepithelium; *m*, mucus cell; *c*, capillary. Scale bar, 20 μm.
respiratory role may take precedence and bubble volume is enlarged.

Evidence for air-breathing

In *C. cristatus*, *A. bifrenatus*, *Chlamydogobius* sp. and *M. paludis* held in hypoxia there was a greatly increased abundance of blood-filled capillaries in the lining of the roof of the buccal cavity and in the skin of emersed portions of the head. These were on average less than 30 μm from the surface of tissues contacting air and frequently less than 15 μm. Blood-filled capillaries were rare or absent in these areas in fish from normoxia, except for *M. paludis*. Diffusion barriers for respiratory organs such as gills or lungs are typically 0.1–10 μm (Feder and Burggren, 1985). Diffusion barriers in amphibians, which rely on cutaneous respiration for a significant proportion of O₂ uptake, are typically 10–50 μm (Czopek, 1965; Burggren and Mwalukoma, 1983). We infer from this that these four species have some capacity for aerial respiration.

In the buccal cavity, capillaries were found only in the subepithelium and were similar to those described by Schöttle (1931) for *Gobius joco*, *G. auratus*, *G. carinus*, also aquatic bubble-gulping species. However, she found the amphibious gobies *Boleophthalmus boddarti*, *B. viridis* and *Periophthalmus schlosseri* to be more specialised, possessing more numerous capillaries that penetrated into the epithelium. In the latter species, ridges were present on the roof of the buccal cavity, increasing its area. The bubble-gulping goby *Gillichthys mirabilis* also possesses such a specialised capillary bed in the buccal roof (Todd and Ebeling, 1966). Air-breathing appears to have evolved relatively recently in the species studied here because the buccal lining does not show the same degree of specialisation described above. Blood-filled capillaries were found in the dermis of emersed portions of the head in *A. bifrenatus* and in the epidermis of *M. paludis* and *Chlamydogobius*. Aerial cutaneous respiration was suggested for gobies by Schöttle (1931) and by Al-Kadhomiy and Hughes (1988), who described dermal capillaries in the snout (50 μm from the surface) and in the inner lining opercula (9 μm from the surface). Cutaneous respiration is common among marine air-breathing fishes and contributes to a significant proportion of O₂ uptake (Graham, 1976), but the occurrence of capillaries in the epidermis is rare (Feder and Burggren, 1985). In addition to the gobies described by Schöttle (1931), such capillaries have been described in *Rivulus ocellatus marmoratus*, where they occur as close as 1 μm to the surface (Grizzle and Thiagararajah, 1987).

Evolutionary implications of ASR and buoyancy control

Has the change of buoyancy brought about by bubble-gulping during ASR contributed to the evolution of air-breathing? We believe so and propose the sequence of events shown in Fig. 4. Initially, gobies used ASR in shallow water in a beached position (Fig. 4A). In such a fixed location, fish are more vulnerable to predation and the breathing movements of the fish in a fixed location disrupt the thin oxygenated surface layer of water, dispersing the dissolved O₂. As a result, fish increase the frequency and amplitude of opercular pumping to ventilate their gills with more water. Fish gradually evolve through steps 1–5, and the final result is the ability to breath air in a variety of microhabitats (Fig. 4B). Evidence supporting this explanation is found in the considerable variation seen among gobies in their responses to hypoxia. The different species examined here illustrate the steps involved in the above sequence. The behaviour of *F. tamaresensis* (non-bubble-gulper), which utilised both beached and arched positions (Gee and Gee, 1991), illustrates the starting point (Fig. 4A) and steps 1–2 (Fig. 4). *Favonigobius exquisitus* along with *P. olorum* represent steps 3-4 (Fig. 4), as these species use a buccal bubble but show no development of capillaries for respiration. The more highly specialised *C. cristatus*, *A. bifrenatus*, *Chlamydogobius* sp. and *M. paludis* illustrate step 5 and the endpoint (Fig. 4B).

Buoyancy change during ASR as a prerequisite step in the evolution of air-breathing in other teleost fishes

ASR is a very common response in temperate and tropical fishes (Gee et al., 1978; Kramer and McClure, 1982), while air-breathing has evolved independently in numerous teleosts from many orders of freshwater and marine fishes. We believe that ASR and the accompanying buoyancy changes could have contributed to the evolution of aerial respiration in some midwater air-breathing physostomes (e.g. Osteoglossiformes, Kramer et al., 1978; Ghosh et al., 1986; Characiformes, Graham et al., 1977; Kramer et al., 1978; Siluriformes, Browman and Kramer, 1985; Gymnotiformes, Liem et al., 1984; Salmoniformes, Gee, 1981) and physoclist (e.g. Perciformes, Hughes and Singh, 1970; Samy and Reddy, 1978; Schuster, 1989).

Some fish in each of the above groups frequently experience hypoxia and, in performing ASR, require buoyancy correction because (1) ASR frequently requires a greater buoyancy than that required in normoxia (Gee, 1986; Chiasson and Gee, 1983; Gee and Ratynski, 1988) and (2) O₂ diffuses from the swimbladder during hypoxic stress, reducing swimbladder volume (Gee, 1986; Gee and Ratynski, 1988). Secretion of swimbladder gases to increase buoyancy under these conditions is slow, but gulping gas would be more rapid and respiratory tissues could potentially develop in those areas where gas could be most conveniently sequestered. In physoclist, this would be in the buccal or branchial cavities, while in physostomes with a functional pneumatic duct it would be in the pharynx/swimbladder. In physoclist, air is probably gulped accidentally into the buccal and branchial chambers during ASR, whereas it results in hydrostatic and respiratory (oxygenating buccal water) advantages. Carter (1931) and Hora (1935) recognised the close link between ASR and the development of respiratory tissues in these areas. However, midwater physostomes probably gulped air into the swimbladder during hypoxia in response to a decreased swimbladder volume, caused by diffusion of O₂ and a need for an increased buoyancy to perform ASR. This would be
followed by selection for an increased capacity for uptake of swimbladder $O_2$ and more frequent air-gulping, which would fulfil both a hydrostatic and a respiratory role for the swimbladder. This explanation was suggested in part by Carter (1931) and Carter and Beadle (1931) and was used by Graham et al. (1978) to explain the evolution of air-breathing in characins. Other hypotheses, however, are required to explain the evolution of air-breathing in teleost fish more completely, particularly for those benthic air-breathing species with subterminal mouths (Gee, 1976). These ideas support the hypothesis that, during ASR, accidental air-gulping by midwater physoclists and buoyancy control in midwater
physostomes enhances ASR and has led to the evolution of aerial respiration in several groups of fishes.

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