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The ears of butterflyfishes (Chaetodontidae): 'hearing generalists' on noisy coral reefs?

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Analysis of the morphology of all three otolithic organs (sacculus, lagena and utriculus), including macula shape, hair cell morphology, density, orientation pattern, otolith morphology and the spatial relationships of the swimbladder and ear, reveals that butterflyfishes in the genera *Chaetodon* (which has anterior swimbladder horns) and *Forcipiger* (which lacks anterior swimbladder horns) both demonstrate the ear morphology typical of teleosts that lack otophysic connections, fishes that have traditionally been considered to be 'hearing generalists'.

Key words: CT; hair cells; laterophysic connection; otolith; swimbladder.

INTRODUCTION

Coral reefs are characterized by high sound levels originating from both abiotic and biotic sources (Au, 2002). A major source of sounds on reefs are those produced by coral reef fishes (Moulton, 1958; McCauley & Cato, 1998, 2000, 2001, 2002), which communicate acoustically despite high background noise levels. Thus, hearing specializations might be expected among coral reef fishes that communicate acoustically. For instance, members of the holocentrid subfamily Myripristinae (*e.g.* the soldierfishes *Myripristis* spp.) are sound-producing, crepuscular reef fishes (Salmon, 1967; Horch & Salmon, 1973) that possess a well-developed otophysic connection (large, robust anterior swimbladder horns in contact with the thinned wall of the otic capsule) and have enhanced hearing capabilities (lower thresholds and broader frequency range; Coombs & Popper, 1979). Their ears are characterized by very large, dense otoliths (Nelson, 1955; pers. obs.) and highly modified morphology of the sensory macula (hair cell epithelium) in the sacculus, the largest of the three otolithic organs (Popper, 1977). Interestingly, other holocentrids [*e.g.* squirrelfishes, *Sargocentron* Fowler (=*Adioryx*)] lack an otophysic connection (Nelson, 1955), have generalized saccular morphology (Popper, 1977) and unremarkable hearing capabilities (*e.g.* lower sensitivity and narrow range of frequency detection; Coombs

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& Popper, 1979). Thus, it appears that the presence of an otophysic connection is correlated with both enhanced hearing and modified ear morphology. In contrast, the damselfishes (family Pomacentridae) are diurnal, mostly planktivorous or herbivorous, often territorial, and several have been shown to produce sounds in the context of social interactions (Lobel & Mann, 1995; Mann & Lobel, 1995; Lobel & Kerr, 1999; Amorim, 2006; Maruska *et al*., 2007). They have neither an otophysic connection nor modified saccular morphology, and do not demonstrate the enhanced hearing sensitivity or broad frequency responses of the myripristine squirrelfishes, however, which have an otophysic connection (Myrberg & Spires, 1980; Kenyon, 1996; Maruska *et al*., 2007). Thus, it appears that the evolution of otophysic connections with accompanying specialization of the auditory anatomy and physiology (with reference to threshold and frequency) is a strategy that has evolved in only some groups of coral reef fishes that communicate acoustically.

Butterflyfishes (family Chaetodontidae) are prominent members of coral reef fish assemblages worldwide and while their social behaviour has been studied extensively, little was known about their auditory system and their use of acoustic communication until recently. Like damselfishes, they are diurnal, but the majority of butterflyfish species are corallivorous, territorial and monogamous (Roberts & Ormond, 1992). Butterflyfishes in the genus *Chaetodon* L. have a laterophysic connection (LC), a unique association of anterior bilateral swimbladder horns, not with the otic capsules, but with a medial opening in the supracleithral lateral line canals (Webb, 1998; Webb & Smith, 2000; Smith *et al*., 2003; Webb *et al*., 2006), which is thought to enhance sound detection using a novel combination of acoustic inputs to both the lateral line system and inner ear (Webb *et al*., 2006). Recently, sound production has been described in *Chaetodon* and *Forcipiger* Jordan & McGregor (Boyle & Tricas, 2006; Tricas *et al*., 2006), providing a source of biologically significant acoustic stimuli for these fishes.

The correlation between modified ear morphology, the presence of an otophysic connection, and enhanced hearing capabilities among taxa (*e.g.* holocentrids, as well as in sciaenids, clupeoids and otophysan fishes; Schellart & Popper, 1992; Braun & Grande, 2008) suggests that knowledge of the morphology of the ear and swimbladder can be used to predict whether a fish has enhanced auditory capabilities.

Popper (1977) examined the sacculus and lagena of the millet butterflyfish *Chaetodon miliaris* Quoy & Gaimard and reported that it has an unmodified ear similar to that of other percomorph fishes that lack an otophysic connection. Unlike most *Chaetodon* species, which are monogamous, pair-forming corallivores, *C. miliaris* is planktivorous and is characterized by a group spawning strategy (Ralston, 1981). This is likely to influence the ways in which it uses sound. It is now known that *C. miliaris* has an indirect LC like the pebbled butterflyfish, *Chaetodon multicinctus* Garrett, which is defined by an indirect LC with long, wide swimbladder horns (Webb *et al*., 2006). If variation in ear morphology and hearing capabilities exists among *Chaetodon* species with different LC variants (*e.g.* direct *v*. indirect LC, long wide swimbladder horns *v*. long thin horns *v*. short horns, Webb *et al*., 2006), then *C. miliaris* may not be representative of the genus, thus warranting further study of the ear among *Chaetodon* species. Thus, the goal of this study was to describe the ear morphology in three representative species of *Chaetodon* (with long and short swimbladder horns) and in one species of *Forcipiger* (which lacks swimbladder

horns) in order to reveal whether *Chaetodon* species, which are characterized by the presence of a laterophysic connection (LC), are likely to have enhanced auditory capabilities like fishes with otophysic connections.

MATERIALS AND METHODS

Scanning electron microscopy (SEM) and computed tomographic (CT) imaging were used to analyse ear morphology and the spatial relationship of the ear and swimbladder among *Chaetodon* species. Four study species were chosen based on the morphology of their LC (Smith *et al*., 2003; Webb *et al*., 2006): two *Chaetodon* species with long swimbladder horns and either a direct (threadfin butterflyfish *Chaetodon auriga* Forsskål) or indirect (*C. multicinctus*) LC, one *Chaetodon* species with an indirect LC and short horns (ornate butterflyfish *Chaetodon ornatissimus* Cuvier) and one non-*Chaetodon* species (longnose butterflyfish *Forcipiger flavissimus* Jordan & McGregor), which lacks both swimbladder horns and an LC. Fishes were collected in the waters around the island of Oahu, Hawaii (Honolulu, 21° 19′ N; 157° 51′ W) or obtained commercially. All fishes were over anaesthetized in a solution of MS-222 prior to dissection in preparation for SEM analysis. After CT imaging, live fishes were over anaesthetized in a solution of MS-222, injected intraperitoneally and behind the orbit, and then immersion fixed in 10% formalin in sea water to facilitate additional morphological analyses. Histological material from the four study species and CT images of additional species that had been prepared for previous analyses (Webb *et al*., 2006) were also available for study.

SCANNING ELECTRON MICROSCOPY

Saccular, lagenar and utricular maculae from adult *C. auriga* (standard length, L_S, 115–153 mm, $n = 3$), *C. multicinctus* (58–76 mm L_S, $n = 4$), *C. ornatissimus* (60–65 mm L_S, $n = 3$) and *F. flavissimus* (98–114 mm L_S, $n = 2$) were prepared for SEM. The head was cut from the body, then a horizontal cut exposed the cranial cavity dorsally and a transverse cut exposed the cranial cavity rostrally. Tissue was then fixed by immersion in cold 2% glutaraldehyde and 4% paraformaldehyde in 0·1 M cacodylate buffer for several days. The brain was removed to expose the inner ears, which were placed into fresh cold fixative for up to several days. Ears from other specimens used for CT analysis, which had been fixed in 10% formalin in sea water previously, were dissected in the same manner. Otolithic sacs were trimmed, pinned into a small silicone-lined dish and dehydrated in an ascending ethanol series. They were then critical point dried with liquid CO_2, mounted on stubs and sputter coated with Au–Pd alloy immediately prior to viewing at 10 kV on a Hitachi (www.hht-eu.com) S-570 scanning electron microscope.

Low power scanning electron micrographs (×150) were used to construct macular montages (Adobe Photoshop; www.adobe.com) in order to determine the size, shape and area of 29 maculae from 12 individuals (one to three maculae per individual). High power micrographs (×3500) were taken at four equally spaced locations along three transects in each macula (see Fig. 1) in order to describe macula shape, pattern of hair cell orientation and hair cell morphology, and to estimate hair cell densities. The number of hair cells in a known area [726 μm^2, the area of a 10 × 12·5 cm (101·6 × 127·0 mm) polaroid photograph] at a magnification of ×3500 was used to calculate hair cell density at each of the 12 macular transect positions in each macula. ANOVA and Bonferroni all pairs comparisons were used to detect differences in hair cell density among positions within transects and among transects within a macula. Level of significance was 0·05 for all statistical tests and results are expressed as mean ± S.D. Macula area (derived from montages) and minimum and maximum hair cell densities (calculated at each of the 12 transect positions and converted to number of hair cells per 100 μm^2) were then used to estimate the size of the total hair cell population in each macula.

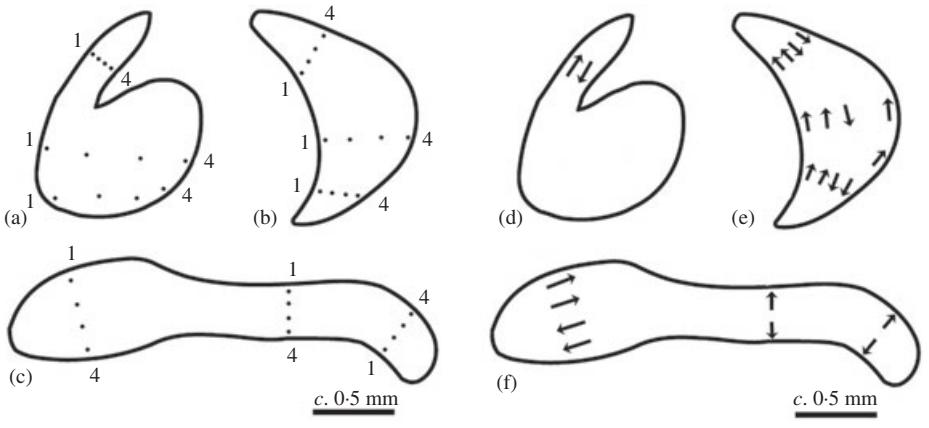

FIG. 1. Shape and hair cell orientation in the (a), (d) utriculus, (b), (e) lagena and (c), (f) sacculus of *Chaetodon* and *Forcipiger*. (a)–(c) Four dots (1–4) in each of the three transects are the locations at which hair cell morphology, density and orientation were assessed. (d)–(f) Hair cell orientations as determined from scanning electron microscope analysis are indicated (⟶).

COMPUTERIZED TOMOGRAPHIC (CT) IMAGING

Between two and four adult individuals of each study species (*C. auriga*, 88–115 mm L_S, $n = 2$; *C. multicinctus*, 58–75 mm L_S, $n = 4$; *C. ornatissimus*, 60 mm L_S, $n = 2$; *F. flavissimus*, 90–114 mm L_S, $n = 2$) were used for two-dimensional CT imaging and three-dimensional reconstruction of the otoliths and swimbladder. In addition, three-dimensional reconstructions of the otoliths and swimbladder of adults of the holocentrid fishes, speckled squirrelfish *Sargocentron punctatissimum* (Cuvier) (90 mm L_S, $n = 1$) and shoulderbar soldierfish *Myripristis kuntee* Valenciennes (125–130 mm L_S, $n = 2$) were examined for comparative purposes. CT scans were carried out on a Siemens Somatom Plus 4 or Siemens Volume Zoom scanner (www.medical.siemens.com) in the Radiology Department of the Massachusetts Eye and Ear Infirmary (MEEI, Boston, MA, U.S.A.) or on a Siemens Volume Zoom scanner at the Woods Hole Oceanographic Institution (WHOI, Woods Hole, MA, U.S.A.). Each fish was positioned on the scanner bed with the left side up in a glass or plastic dish containing an anaesthetic solution of MS-222 in sea water. A topogram (similar to a traditional X-ray image) was obtained to visualize the full extent of the swimbladder in lateral view and then a scan was carried out from the middle of the orbit and through the ear and the entire swimbladder. Fishes were scanned in the rostro-caudal axis using a spiral acquisition protocol at 200 mA/140 kV with a 1·0 collimator width (MEEI) or at 150 mA/120 kV with a 0·5 mm collimator width (WHOI). Data were reformatted at 0·1 mm (WHOI) or 0·5 mm (WHOI and MEEI) to provide full series of transverse slices, which were used to generate series of horizontal and sagittal slices and multiple plane reconstructions with a slice thickness of 0·1 or 0·2 mm.

Three-dimensional reconstructions of the volume of air in the swimbladder and swimbladder horns were produced with a Shaded Surface Display programme using a range of X-ray attenuation values (1024/−510 Hu, Hounsfield units) appropriate for imaging the air within the swimbladder, the swimbladder horns and some details of the air–tissue boundary. Volume rendering technique was used to visualize the morphology and spatial relationships of the swimbladder, swimbladder horns and otoliths. Density histograms were also obtained from two-dimensional CT slices for saccular, and in some cases, utricular otoliths in six *Chaetodon* species (melon butterflyfish *Chaetodon trifasciatus* Park, spotband butterflyfish *Chaetodon punctatofasciatus* Cuvier, sunburst butterflyfish *Chaetodon kleinii* Bloch, Hawaiian butterflyfish *Chaetodon tinkeri* Schultz, *C. miliaris* and *C. ornatissimus*) and in *F. flavissimus*, as well as *M. kuntee* and *S. punctatissimum* (Family Holocentridae) in order to determine maximum otolith densities.

RESULTS

The ears of three species of *Chaetodon* and of *F. flavissimus* examined are located within paired, well-ossified otic capsules (with an ossified medial wall between them) located at the posterior margin of the skull, ventral to the brain and rostral to the vertebral column. The three semicircular canals and the three otolithic organs (sacculus, lagena and utriculus) were clearly visualized upon dissection. The two maculae neglectae that had been reported in a 17 mm tholichthys of *C. trifasciatus* (Bauchot *et al*., 1989) were not observed in the present study, but deserve further analysis. The cristae of the semicircular canals are visible through the transparent canal walls. Each crista is found in a spherical ampulla, and is composed of a population of densely packed hair cells with very long kinocilia that sit on a raised ridge that runs perpendicular to the axis of the semicircular canal that extends from it.

Histological material and dissections clearly show that the sensory maculae of the right and left lagenae and sacculae are situated on either side of the vertical face of the ossified medial wall between the two otic capsules. In contrast, the utricular maculae sit on the inner surface of spherical sacs that are situated rostral, dorsal and lateral to the sacculae and lagenae, and are more closely associated with the semicircular canals, sit within the neurocranial cavity, and are not enclosed by bone of the otic capsule.

EAR MORPHOLOGY

The morphology of the three otolithic organs (sacculus, lagena and utriculus) and the hair cell orientation patterns in their sensory maculae were found to be similar among the three *Chaetodon* and one *Forcipiger* species, with minor differences. The morphology of the ear reported here is similar to that reported by Popper (1977) for *C. miliaris*, despite the fact that its feeding and reproductive habits are different from that of the majority of *Chaetodon* species.

The sacculus is the largest of the three otolithic organs. Its macula measures *c*. 2–4 mm in length, with an expanded anterior portion and a narrow, elongate posterior portion, which is directed ventrally at its terminus [Figs 1 and 2(a)]. The expanded anterior portion of the macula is *c*. 400–600 μm wide (in its dorso-ventral axis) representing *c*. 30–50% of the total length of the macula (Table I). In *Forcipiger*, the anterior portion of the macula appears to be narrower and longer than it is in *Chaetodon*. In all four species, hair cells are found in four quadrants, each composed of a hair cell population with a different orientation [Fig. 1(b)], the 'standard' hair cell orientation pattern described in the sacculus of other teleosts (Schellart & Popper, 1992). The orientation of hair cells at the edges of the two posterior quadrants follows the contour of the macula as it curves ventrally.

The saccular otolith (sagitta) fills the saccular chamber as it does in *C. miliaris* and other percomorphs, *e.g.* tilapia *Oreochromis* sp., pers. obs., pinecone soldierfish *Myripristis murdjan* (Forsskål), Hawaiian squirrelfish *Sargocentron xantherythrum* (Jordan & Evermann) (=*Adioryx xantherythrus*) (Popper, 1977), and is in the shape of a relatively flat, robust oval. The otolith has a prominent, elongate sulcus of the same size and shape as the sensory macula [Fig. 3(b)] that is filled with a firm gelatinous material. This remained with the otolith upon careful dissection [Fig. 3(d)] and demonstrates staining properties distinct from that of the otolithic membrane as revealed histologically. The surface of the otolith bearing the sulcus is a somewhat

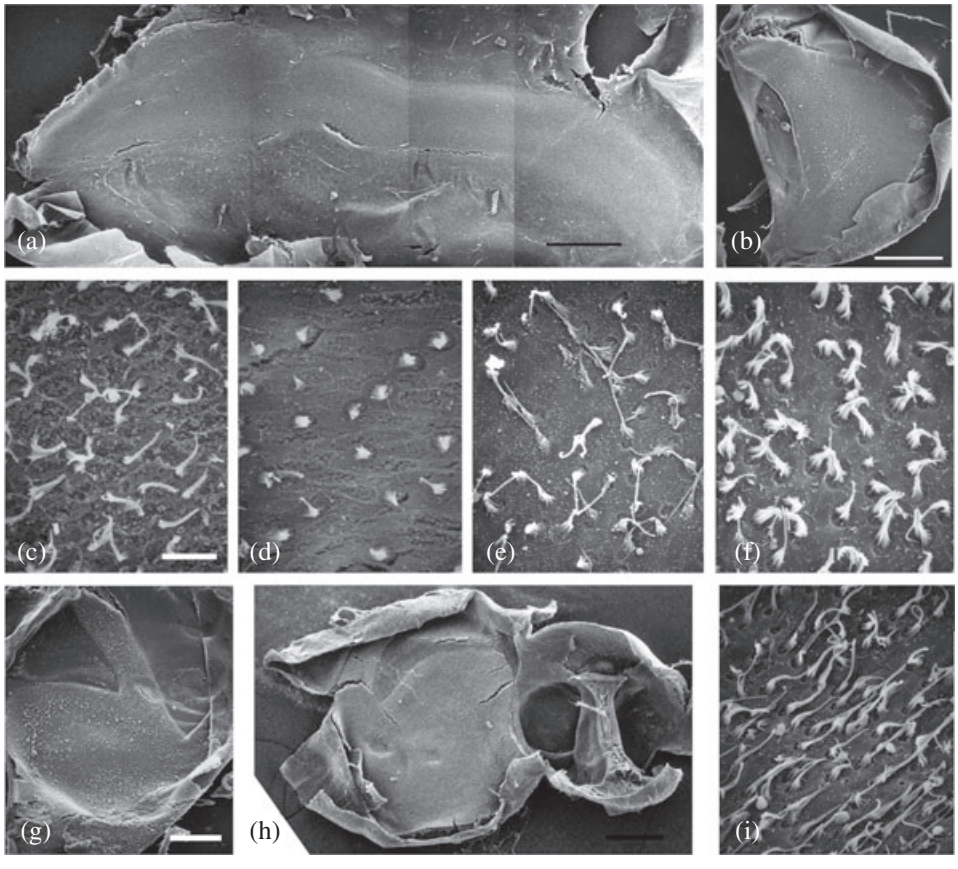

FIG. 2. Morphology of macula and hair cells in the sacculus, lagena and utriculus in *Chaetodon* and *Forcipiger*. (a) Saccular macula in *Chaetodon ornatissimus*, rostral to left. (b) Lagenar macula in *C. ornatissimus*, rostral to left. (c) Hair cells with long kinocilia at periphery of rostral portion of sacculus in *C. ornatissimus*. (d) Hair cells with short kinocilia in central area of rostral portion of sacculus in *C. ornatissimus*; note lower density of hair cells than in (c). (e) Hair cells with long kinocilia at periphery of middle transect of lagena in *C. ornatissimus*. (f) Hair cells with shorter kinocilia in central area of middle transect of lagena in *C. ornatissimus*. There is no statistical difference in hair cell density in (e) and (f). (g) Utricular macula of *F. flavissimus* showing round body and the lacinia that extends caudally and dorsally at a 45° angle from the edge of the body of the utriculus. (h) Utricular macula in *C. multicinctus*, showing lacinia (partially covered) extending from the body of the utriculus at more acute angle than in (g). The utriculus is located immediately adjacent to the opening to the horizontal semicircular canal and its crista which sits on a ridge. (i) Dense hair cells with extremely long kinocilia in the central region of the lacinia in *C. ornatissimus*. Scale bars: (a), (b), (g), (h) = 250 μm; (c)–(f), (i) = 5 μm.

convex. The expanded anterior and curved posterior ends of the elongate sensory macula appear to wrap over the edges of the otolith, with the expanded anterior end of the macula sitting in the dorso-rostral notch at the edge of the otolith. Thus, it does not appear that the saccular macula sits completely in only one plane. The saccular otolith is quite dense with Hu values of 1700–1900 in *Chaetodon* spp. and *Forcipiger* sp. and is thus the densest structure in these fishes. In two species in which the density of both the saccular and utricular otoliths could be determined (*C. ornatissimus* and *C. tinkeri*), the saccular otolith had a maximum density of

TABLE I. Summary data on the size and shape of the sacculus, lagena and utriculus of four butterflyfish species (*Chaetodon auriga*, *Chaetodon multicinctus*, *Chaetodon ornatissimus* and *Forcipiger flavissimus*) derived from scanning electron microscope images of a total of 29 maculae. Lengths and heights are linear measurements (in mm) and do not account for the curvature of the boundaries of the maculae

	Range	Mean	*n*
Sacculus			
Total length (rostro-caudal) (mm)	2·1–3·9	2·7	9
Anterior portion			
Length (rostro-caudal) (mm)	0·9–1·4	1·1	9
Width (dorso-ventral) (mm)	0·4–0·6	0·5	8
Anterior length:anterior width	1·7–2·5	2·2	8
Anterior length:total length	0·3–0·5	0·4	9
Anterior width:posterior width	1·6–2·0	1·7	7
Lagena			
Height (dorso-ventral) (mm)	0·8–1·6	1·0	8
Width (rostro-caudal) (mm)	0·3–0·7	0·5	8
Utriculus			
Body height (dorso-ventral) (mm)	0·5–1·2	0·9	10
Width (rostro-caudal) (mm)	0·6–1·5	1·0	11
Lacinia			
Length (mm)	0·3–1·0	0·6	10
Width (mm)	0·1–0·3	0·2	10

about twice that of the utricular otolith. A similar relationship was found between the much larger and denser saccular and utricular otoliths in *Myripristis* sp. [pers. obs.; see Fig. 4(b)].

The lagena is caudal to the sacculus with which it is contiguous, but a slight constriction between them (the 'sacculo-lagenar foramen'; Jenkins, 1977) demarcates the boundary between them. The lagenar macula sits in the vertical plane and is shaped like a robust crescent moon. It is *c*. 1 mm long (in the rostro-caudal axis) and is approximately two to three times taller (in the dorso-ventral axis) than it is wide [Table I and Figs 1 and 2(b)]. The hair cells in the rostral half of the macula are oriented ventrally, and the hair cells in the caudal half of the macula are oriented dorsally [Fig. 3(b)], but, as in the sacculus, their orientation is modified at the macular periphery with hair cells oriented parallel to its curved edge. A third zone of hair cells was apparent in some specimens at the edge of the macula, but was difficult to characterize.

The lagenar otolith (asteriscus) is similar in size and shape to the lagenar macula and fills the lagenar sac. Its anterior edge closely approaches the posterior edge of the saccular otolith [Fig. 4(b)], thus accounting for the inability to distinguish between them in CT images. In one specimen of *C. auriga,* a firm, transparent, finger-shaped gelatinous structure (approximately the same consistency as the firm material filling the sulcus of the saccular otolith) extended rostrally from the dorsal tip of the lagenar otolith into the saccular sac, running along the lateral surface of the saccular otolith [Fig. 3(c)]. This feature, which is damaged during dissection, is particularly interesting as it may provide a functional linkage between the sacculus and lagena.

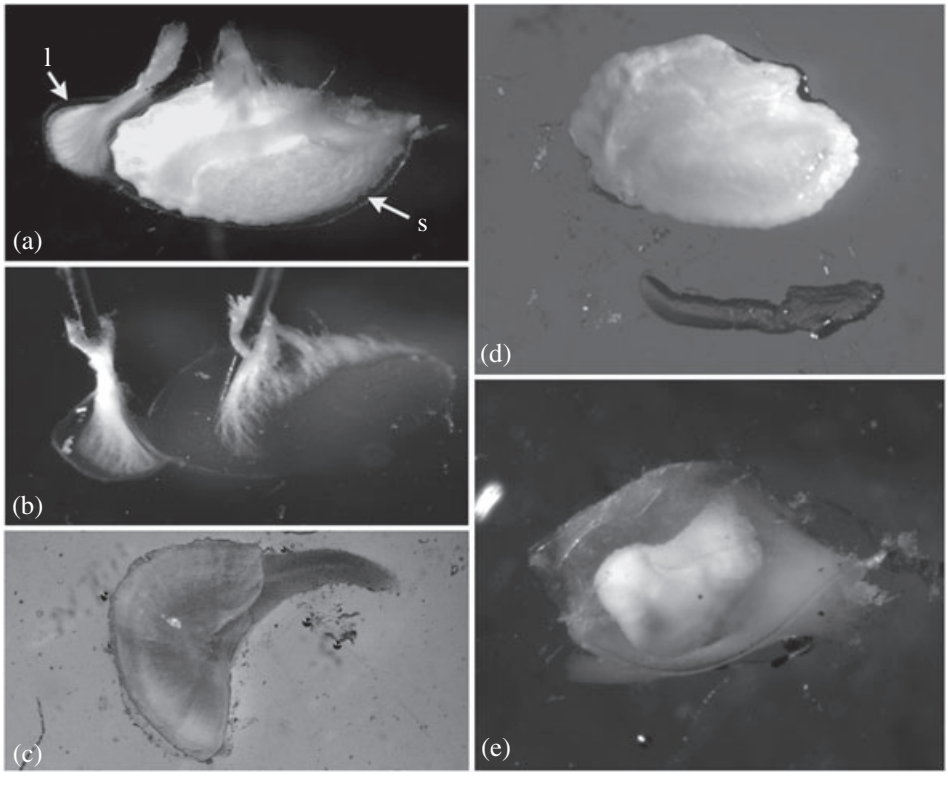

FIG. 3. The sacculus and lagena in *Chaetodon* and *Forcipiger* (a) Medial side of intact sacculus, s, and lagena, l showing the bundle of neurons innervating the two maculae and the white otoliths in *C. multicinctus*. The lagenar otolith is the same shape as its macula. The sulcus of the saccular otolith over which the saccular macula lies is apparent. (b) Same view as (a), but with the saccular and lagenar otoliths removed. (c) Finger-like gelatinous projection extending to the right from rostro-dorsal tip of lagenar otolith in *C. auriga.* (d) A saccular otolith in *Forcipiger* showing the sulcus and the firm gelatinous material removed from the sulcus. (e) Intact utriculus containing its otolith in *C. ornatissimus* (see Table I for dimensions of sensory maculae).

The utricular macula sits on the ventral and medial walls of the curved inner surface of the spherical utricular sac. SEM reveals that the body of the utricular macula is roughly circular, measuring *c*. 1 mm in both rostro-caudal and latero-medial axes [Fig. 2(g), (h)]. An elongate lacinia extends up to 1 mm from its mediodorsal edge and is at least twice as long as it is wide (Table I). In histological section, the utriculus may appear to be divided into two patches of sensory epithelium where the plane of section traverses both the body of the utriculus and the lacinia. The hair cell orientation pattern in the utriculus is complex and was difficult to discern.

The utricular otolith (lapillus) was not visible upon dissection in specimens fixed in formalin in sea water (probably due to its weak buffering capacity), but was well preserved in material fixed in buffered glutaraldehyde–paraformaldehyde. The utricular otolith appeared robust in shape, like a human hand with the fingers curled underneath [Fig. 3(e)]. Upon dissection it was found to have a firm, thick, gelatinous

material wrapped around its edge, which alters its overall shape. The orientation of this otolith with respect to its macula, which sits on the inner surface of the spherical sac, was difficult to discern.

MACULAR SIZE AND HAIR CELL DENSITY

The area of the saccular macula varied considerably among specimens examined (*c*. 0·20–1·27 mm^2; Table II). The area of the lagenar macula was smaller than that of the sacculus and was typically *c*. 0·22–0·35 mm^2, but was *c*. 1·0 mm^2 in the largest specimen examined (*C. auriga*, 153 mm L_S). The area of the utricular macula (0·49 to >1·0 mm^2) is comparable to that of the largest sacculus examined, despite the difference in the shape of the macula.

Estimates of hair cell number were derived from hair cell counts along sampling transects and the macular area was calculated from SEM montages (Table II). Estimates of minimum hair cell numbers were probably influenced by preparation artifact that obscured hair cells, so maximum hair cell number is probably a more accurate reflection of the size of the hair cell population. Maximum hair cell number was *c*. 12 000–65 000 in the sacculus, *c*. 18 000–48 000 in the lagena and *c*. 30 000–87 000 in the utriculus.

Hair cell density varied significantly only among locations in the saccular macula of some individuals of *C. multicinctus* and *C. ornatissimus* (Herman, 2005). When data were pooled from all 12 transect locations in the 29 maculae examined in the four study species, however, hair cell density varied significantly among maculae (ANOVA, d.f. = 2,304, $P < 0{\cdot}001$) and was higher in the lagena (mean = 5·98 ± 1·61 hair cells per 100 μm^2, $n = 8$) and utriculus (mean = 5·49 ± 1·90 hair cells per 100 μm^2, $n = 10$) when compared to that in the sacculus (mean = 3·64 ± 1·30 hair cells per 100 μm^2, $n = 11$; Bonferroni all pairs comparison, $P < 0{\cdot}001$ for both tests). In the sacculus, mean hair cell density varied among the four transect positions (ANOVA, d.f. = 3,118, $P < 0{\cdot}001$). The hair cell densities in the two peripheral positions (1, 4) were not significantly different from one another and the hair cell densities in the two central positions (2, 3) were not significantly different from one another (Bonferroni all pairs comparison, $P > 0{\cdot}05$ for both tests), but hair cell density was significantly higher in peripheral positions 1 and 4 (4·15 ± 1·48 and 4·17 ± 1·44 hair cells per 100 μm^2, respectively) than in central positions 2 and 3 (3·07 ± 0·92 and 3·18 ± 0·89 hair cells per 100 μm^2, respectively; Bonferroni all pairs comparison, $P < 0{\cdot}05$ for all tests). No significant differences were found in hair cell density among the three transects in the sacculus (ANOVA, d.f. = 2,119, $P > 0{\cdot}05$) or in the lagena (ANOVA, d.f. = 2,80, $P > 0{\cdot}05$). In the utriculus, mean hair cell density varied among transects (ANOVA, d.f. = 2,99, $P < 0{\cdot}001$). Apparent differences between hair cell density in the lacinia (6·17 ± 1·96 hair cells per 100 μm^2) and in the two transects in the body of the utriculus (5·08 ± 1·98 and 5·12 ± 1·57 hair cells per 100 μm^2, middle and ventral transects, respectively), however, were not statistically significant (Bonferroni all pairs comparison, $P > 0{\cdot}05$ for all tests). Nevertheless, when the total area of the utriculus is considered (*c*. 1 mm^2), a difference in one hair cell per 100 μm^2 translates into a difference of *c*. 10 000 hair cells in the entire sensory macula, which may indeed be biologically significant.

TABLE II. Hair cell densities, macular areas and estimated numbers of hair cells (HC) in the sacculus, lagena and utriculus of *Chaetodon* and *Forcipiger* species. Minimum and maximum hair cell densities are calculated from hair cell counts by scanning electron microscope (SEM) at all 12 transect positions in each macula. Macular areas are derived from digital measurements of SEM montages of entire maculae. Estimated minimum and maximum number of hair cells in a macula is the product of the macular area and minimum and maximum hair cell density, respectively

		Sacculus			Lagena			Utriculus		
Species	L_S (mm)	HC density (number per $10^3\mu m^2$)	Area (10^3 μm^2)	Estimated number of HC	HC density (number per 10^3 μm^2)	Area (10^3 μm^2)	Estimated number of HC	HC density (number per 10^3 μm^2)	Area (10^3 μm^2)	Estimated number of HC
Chaetodon auriga	115	11–60	1069	11 760–64 140				26–51	1090	28 340–55 590
	152	14–50	1271	17 790–63 550						
	153	22–43	1123	24 710–48 290	22–48	996	21 910–47 810			
Chaetodon multicinctus	58				40–87	248	9920–21 580			
	65	22–54	627	13 790–33 860	45–63	312	14 140–19 660	34–73	739	25 130–53 950
	75	23–56	958	22 030–53 650	43–77	236	10 150–18 170			
	76							39–81	679	26 480–56 000
Chaetodon ornatissimus	60	32–74	539	17 250–39 890	48–85	336	16 130–28 560	47–94	815	38 310–76 610
	62				45–85	258	11 610–21 930			
	65				55–88	223	12 270–19 620	43–121	717	30 830–86 760
Forcipiger flavissimus	103	23–54	977	22 470–52 760	51–81	347	17 100–28 110	47–85	621	29 190–52 790
	114	10–43	790	7900–33 970				28–62	485	13 580–30 070

MORPHOLOGY OF CILIARY BUNDLES OF THE HAIR CELLS

Variation in the morphology of the ciliary bundles of the hair cells was noted in SEMs of all three sensory maculae in the four study species, but preparation artifact only allowed a qualitative analysis of these data. The hair cells at the periphery of the saccular macula (transect positions 1 and 4) [see Fig. 1(a)] have a kinocilium that appears to be much longer than the stereocilia [Fig. 2(c)]. In the centre of the macula (transect positions 2 and 3), the hair cells have kinocilia that are short and appear to be not much taller than the stereocilia [Fig. 2(d)]. A distinct transition between the peripheral and central hair cell populations can be seen around the entire macula. The hair cells in peripheral positions in the lagena resemble those in the sacculus, but appear to have fewer stereocilia [Fig. 2(e)], while in the centre of the lagena, the kinocilia are shorter, but appear to have more stereocilia [Fig. 2(f)]. In the body of the utriculus, hair cells at the periphery have long kinocilia, while those in the centre have kinocilia that appear to be just a bit longer than the longest stereocilia. The hair cells in the lacinia have very long kinocilia [Fig. 2(i)], which appear to be two or more times longer than their longest stereocilia, and are thus most similar to the hair cells in the cristae of the semicircular canals.

SPATIAL RELATIONSHIPS OF THE SWIMBLADDER AND EAR

Imaging using CT revealed a swimbladder diameter of 7–14 mm and a swimbladder volume of 500–2000 mm^3 among the specimens of the four study species. Horn length appeared to vary among *Chaetodon* species. *Chaetodon auriga* (direct LC, long horns) had a mean horn length of 5·5 mm and *C. multicinctus* (indirect LC, long horns) had a mean length of 3·5 mm; the larger size of the *C. auriga* individuals examined contributed to this disparity. The horns in *C. ornatissimus* (indirect LC, short horns), not surprisingly, had a mean length of only 1·5 mm. The anterior horns of the swimbladder (mean volume of *c*. 5–20 mm^3) contribute only a fraction of the total volume of air in the swimbladder.

Three-dimensional reconstruction of the swimbladder and otoliths (Fig. 4) illustrates the relationships of the swimbladder horns to the ears. When distances were corrected for L_S, the minimum distance from the swimbladder or horns to the inner ear appeared to be smaller for *Chaetodon* (in the transverse plane) than for *Forcipiger* (in the horizontal and sagittal planes; C. F. Woods, unpubl. thesis). The saccular otoliths of *Chaetodon* are not much larger than those of *Forcipiger*, especially when the large saccular otoliths in *Myripristis* (which has a well-developed otophysic connection; Nelson, 1955) are compared to the small saccular otoliths of *Sargocentron*, which lacks an otophysic connection. Three-dimensional reconstructions in frontal view clearly show that the simple anterior swimbladder horns in *Chaetodon* spp. [Fig. 4(d), (e)] sit dorsal and lateral to the otoliths, unlike the swimbladder horns in *Myripristis* that wrap around and come into contact with the otic capsule [Fig. 4(a), (b)], which characterize the otophysic connection in this genus.

DISCUSSION

The morphology of the three otolithic organs (sacculus, lagena and utriculus) and the hair cell orientation patterns in their sensory maculae were found to be similar

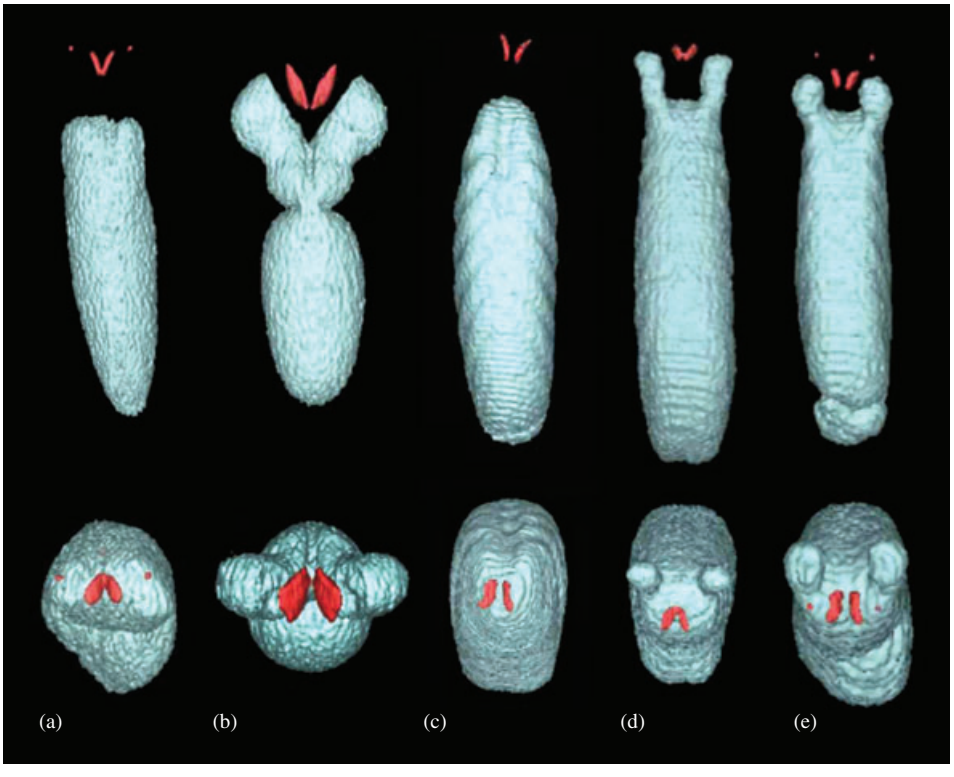

FIG. 4. Three-dimensional reconstruction of computed tomographic imaging slices demonstrating the relationship of otoliths (red) to the volume of air within the swimbladder (white, diameter *c*. 1 cm) in dorsal (top) and frontal (bottom) views of (a), (b) two species of holocentrids and (c)–(e) three species of chaetodontids: (a) *Sargocentron* sp. (family Holocentridae, subfamily Holocentrinae), (b) *Myripristis* sp. (family Holocentridae, subfamily Myripristinae) (note that the swimbladder horns extend rostrally and wrap around the otic capsules containing the very large saccular neuromasts), (c) *Forcipiger flavissimus* (family Chaetodontidae), (d) *Chaetodon auriga* (family Chaetodontidae, subgenus *Rabdophorus*) and (e) *Chaetodon multicinctus* (family Chaetodontidae, subgenus *Exornator*). In (d), (e) *Chaetodon*, the cylindrical horns extend rostrally, but are situated dorsal and lateral to the otic capsules. Large otoliths are the sagittae (saccular otoliths), smaller otoliths [visible only in (a) and (e)] are the lapillae (utricular otoliths).

among the three *Chaetodon* and one *Forcipiger* species examined. These aspects of ear morphology are similar to those reported by Popper (1977) for *C. miliaris*, despite the fact that its feeding and reproductive habits are different from that of the majority of *Chaetodon* species. Thus, it is expected that ear morphology is indeed the same among all members of the genus and perhaps among all members of the Chaetodontidae.

Dissection and SEM data revealed that the saccular, lagenar and utricular otoliths all have dense gelatinous structures associated with them. A clear, dense gelatinous material that is in contact with, but is distinct from, the otolithic membrane (in which the ciliary bundles of the hair cells are embedded) fills the sulcus of the saccular otolith. A relatively thick gelatinous material (similar to a feature reported in a bluefin tuna *Thunnus thynnus* (L.); Song *et al*., 2006) partially covers the irregularly shaped

utricular otolith. A long finger-like structure extends from the dorsal tip of the lagenar otolith along the lateral surface of the saccular otolith, and may provide an unappreciated functional linkage between the lagena and sacculus. Such gelatinous structures, which are easily lost during fixation and dissection, will need to be considered in future considerations of structure–function relationships in the ears of fishes.

The ears of fishes may receive acoustic stimuli either directly, *via* whole body acceleration or indirectly *via* the swimbladder (if one is present), and are thus considered to be sensitive to both the particle displacement and sound pressure components of an acoustic stimulus, respectively. The relative contributions of particle displacement and pressure, however, are not well understood and it is still widely debated whether it is the mere presence of a swimbladder or the presence of modifications of the swimbladder (*e.g.* otophysic connection and anterior swimbladder horns) that is necessary for the ear to be pressure sensitive (Popper & Fay, 2010). Nevertheless, it is generally accepted that the presence of an otophysic connection (linking the swimbladder and inner ear) allows the generation of a secondary particle displacement field by the swimbladder in response to an incoming sound pressure wave.

Otophysic connections take a number of forms among taxa (Schellart & Popper, 1992; Braun & Grande, 2008) and their presence is correlated with both modifications in ear morphology (*e.g.* shape and hair cell orientation pattern in the sacculus) and enhanced hearing capabilities (defined by lower thresholds and broader frequency response, and the reception of sound pressure *via* the swimbladder). As a result, fishes with otophysic connections have been considered to be 'hearing specialists'. In contrast, fishes that lack an otophysic connection (and those that lack a swimbladder entirely) tend to have unremarkable auditory capabilities and have been considered to be 'hearing generalists' (Schellart & Popper, 1992; Popper *et al.*, 2003; Popper & Hastings, 2009). This dichotomy is probably a good first approximation of the functional attributes of the auditory system of fishes. Popper & Fay (2010), however, have recently proposed that this dichotomy be abandoned and suggest that variation in the auditory capabilities of fishes lies along a continuum defined by the relative contributions of particle displacement (*via* whole body acceleration), and pressure sensitivity (*via* the generation of a secondary particle displacement field by the swimbladder, which oscillates in response to an incoming sound pressure wave). The detection of particle displacement by the otolithic organs of the inner ear is the only mechanism for hearing in fishes lacking swimbladders. Pressure sensitivity is provided by the swimbladder (in bony fishes only), especially when it is brought into the close vicinity of, or is mechanically linked to, the inner ear (*e.g.* swimbladder horns and otophysic connections, respectively). Popper & Fay (2010) review those fish taxa for which these data are available but indicate that species can be placed along this continuum only with the results of the appropriate experiments designed to reveal the relative contributions of particle displacement and sound pressure stimuli.

How can the morphological data presented here be used to construct testable hypotheses concerning the auditory capabilities of butterflyfishes? The swimbladder horns of *Chaetodon* spp. (which define the laterophysic connection) do not have an intimate association with the otic capsule as in fishes with an otophysic connection. Rather, the swimbladder horns only approach the ears (within *c.* 1–2 mm), but in doing so, may enhance pressure sensitivity. With reference to ear morphology, the shape of the elongate saccular macula and the 'standard' hair cell orientation pattern

in both *Chaetodon* (regardless of the type of laterophysic connection and length of the swimbladder horns) and *Forcipiger* are similar to that reported for teleosts that lack otophysic connections, *e.g.* oscar *Astronotus ocellatus* (Agassiz) (Popper & Hoxter, 1984), *Oreochromis* sp., Atlantic salmon *Salmo salar* L., *S. xantherythrum*, yellow perch, *Perca flavescens* (Mitchill), bluegill *Lepomis macrochirus* Rafinesque and Hawaiian dascyllus *Dascyllus albisella* Gill (Popper, 1977), and is distinct from those species that have otophysic connections, *e.g.* a mormyrid (Fritzsch, 2000), clupeiforms, goldfish *Carassius auratus* (L.) (Lanford *et al.,* 2000), a catfish (Fritzsch, 2000) and *M. murdjan* (Popper, 1977). Furthermore, the relative height of the rostral portion of the saccular macula in *Chaetodon* spp. and *Forcipiger* sp. revealed in this study (height:length ratio; Table I) is similar to that in two species in the family Sciaenidae [southern kingcroaker *Menticirrhus americanus* (L.) and spot *Leiostomus xanthurus* Lacepède] with unremarkable hearing capabilities (defined by threshold and frequency), but is not as exaggerated as that of the expanded rostral portion of the sacculus in two other sciaenid species with enhanced auditory capabilities [Atlantic croaker *Micropogonias undulatus* (L.) and spotted weakfish *Cynoscion nebulosus* (Cuvier); Ramcharitar *et al*., 2001]. Thus, the morphological data presented here suggest that *Chaetodon* and *Forcipiger*, and probably the remainder of the chaetodontids, have hearing capabilities similar to that of species that lack otophysic connections.

While the modified morphology of the saccular macula appears to be predictive of enhanced hearing capabilities, other aspects of ear morphology neither support nor refute this notion. The robust crescent moon shape of the lagena in *Chaetodon* and *Forcipiger* is similar to that found in fishes that lack otophysic connections (Popper, 1977). It should be noted, however, that lagenar morphology is modified in species with (*C. auratus*, Popper, 1983; a catfish, Fritzsch, 2000) and without [several myctophids, Popper, 1977; European hake *Merluccius merluccius* (L.), Lombarte & Popper, 1994] otophysic connections. The round shape of the utriculus and the presence of an elongate lacinia are also typical of most other teleosts, regardless of the presence or absence of an otophysic connection *e.g.* zebrafish, *Danio rerio* (Hamilton) (Bang *et al*., 2001), *C. auratus* (Platt, 1977), *M. merluccius* (Lombarte & Popper, 1994), several anabantoids (Ladich & Popper, 2001) and sleeper goby *Dormitator latifrons* (Richardson) (Lu & Popper, 1998). A small or indistinct lacinia has only been reported in a few species *e.g.* a freshwater gadiform, the burbot *Lota vulgaris* (L.) (Flock, 1964) and several deep sea elopomorphs (Buran *et al*., 2005), but this may be an indication of particular auditory modifications associated with invasion of habitats that are atypical for the larger taxa to which these species belong (Gadiformes and Elopomorpha, respectively).

Patterns of hair cell density and morphology of the apical ciliary bundles of the sensory hair cells in both *Chaetodon* and *Forcipiger* reveal heterogeneity such that hair cells at the periphery of the sacculus tend to be denser with longer kinocilia than hair cells in the central region of the macula. This accounts for the large range of estimated hair cell numbers shown in Table II. Similar patterns have been reported in *C. auratus* (Saidel *et al*., 1995), *Perca* (Enger, 1976), *A. ocellatus* (Popper & Hoxter, 1984; Chang *et al*., 1992; Popper *et al*., 1993), *Oreochromis* sp. (pers. obs.) and other teleosts (Popper, 1977). The functional significance of this is not well understood (Saunders & Dear, 1983; Platt & Popper, 1984; Chang *et al*., 1992; Presson *et al*., 1992). Interestingly, several anabantoids, which have air bubbles located close

to their ears (considered to be an otophysic connection, Schellart & Popper, 1992), demonstrate regional heterogeneity in both hair cell morphology and density (Ladich & Popper, 2001), but among four species of sciaenids, only the two species with unremarkable ear and swimbladder morphology (and no swimbladder horns) demonstrate such heterogeneity (*M. americanus* and *L. xanthurus*), while the two species with modified ear and swimbladder morphology (*M. undulatus* and *C. nebulosus*) do not (Ramcharitar *et al*., 2001). Clearly, the significance of hair cell heterogeneity to auditory capabilities warrants further study.

The anatomical data presented here, which suggest that *Chaetodon* and *Forcipiger* have unremarkable hearing capabilities (with regard to threshold and frequency range), are supported by preliminary physiological studies on the hearing capabilities of chaetodontids that use auditory brainstem response or auditory evoked potentials to construct audiograms. Juvenile spotfin butterflyfish *Chaetodon ocellatus* Block (a Caribbean species with the same swimbladder horn and LC morphology as *C. miliaris* and *C. multicinctus*; Smith *et al*., 2003) have a relatively narrow audiogram with best frequency at 100–200 Hz (J. F. Webb, N. Kelly, N. Cicchino, R. M. Walsh, B. Casper & D. A. Mann, unpubl. data.), which is not unusual for teleosts lacking an otophysic connection. Boyle & Tricas (2006) showed that the best sensitivity for adult *C. multicinctus*, *C. auriga* and *F. flavissimus* is 200–600 Hz. This is a bit higher than that reported for *C. ocellatus* juveniles, but may be attributable to some combination of methodological differences, species differences and ontogenetic effects. The data presented here demonstrate no notable differences in ear or otolith morphology among *Chaetodon* species with long (*C. multicinctus* and *C. auriga*) and short (*C. ornatissimus*) swimbladder horns. Boyle & Tricas (2006), however, have shown that the two species with long swimbladder horns had auditory thresholds ranging from 104 to 117 dB while *Forcipiger* (which lacks swimbladder horns) was less sensitive (by 11–16 dB) at these frequencies, suggesting that the long swimbladder horns and their close proximity to the ears, as shown in the current study, contribute to some enhancement of pressure sensitivity in *Chaetodon*. The experiments that would reveal the relative contributions of particle displacement and pressure sensitivity, which would allow the placement of butterflyfishes on Popper & Fay's (2010) continuum have yet to be conducted.

It is concluded that the origin and diversification of the laterophysic connection in *Chaetodon* (a synapomorphy of the genus; Smith *et al*., 2003) occurred in the absence of modifications in ear morphology and evidence for the enhancements of auditory capabilities like those in species with otophysic connections. Nevertheless, the fact that these fishes produce sound demonstrates that acoustic communication is important. The dependence on auditory communication (coupled with visual communication) probably favoured the evolution of pairing behaviour, a strategy that would increase the efficiency of communication in noisy coral reef environments (Boyle & Tricas, 2006). Such behaviour is likely to exploit sensitivity to both particle displacement (with input to both the ear and lateral line system, given the short distances between animals) and pressure reception (*via* the swimbladder horns that generate a secondary particle displacement field in the vicinity of the ears). Whatever the mechanism or mechanisms of acoustic detection in these fishes, this raises the question of how butterflyfishes respond to increasing levels of anthropogenic sound on naturally noisy coral reefs, which are likely to affect the social and reproductive behaviours of these important coral reef fishes.

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