

## Sound pressure enhances the hearing sensitivity of *Chaetodon* butterflyfishes on noisy coral reefs

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## ABSTRACT

Butterflyfishes are conspicuous members of coral reefs that communicate with acoustic signals during social interactions with mates and other conspecifics. Members of the genus *Chaetodon* have a laterophysic connection (LC), a unique association of anterior swim bladder horns and the cranial lateral line, but the action of the LC system on auditory sensitivity was previously unexplored. Baseline auditory evoked potential threshold experiments show that *Forcipiger flavissimus* (which lacks swim bladder horns and LC) is sensitive to sound tones from 100 Hz up to 1000 Hz, and that thresholds for three species of *Chaetodon* were 10-15 dB lower with extended hearing ranges up to 1700-2000 Hz. The relatively high thresholds to sound pressure and low pass response near 500 Hz for all four species is consistent with a primary sensitivity to hydrodynamic particle acceleration rather than sound pressure. Deflation of the swim bladder in *Forcipiger* had no measurable effect on auditory sensitivity. In contrast, displacement of gas from the swim bladder horns in *C. multicinctus* and *C. auriga* increased thresholds (decreased sensitivity) by approximately 10 dB with the greatest effect at 600 Hz. The evolution of swim bladder horns associated with the LC system in *Chaetodon* has increased hearing sensitivity through sound pressure transduction in the frequency bands used for social acoustic communication. The close affiliative behaviors that are common in *Chaetodon* and other butterflyfish species facilitate sound perception and acoustic communication at close distances relative to the high background noise levels found in their natural reef environment.

## INTRODUCTION

The perception of communication signals in fishes can involve transduction mechanisms in both the inner ear and lateral line systems. The acoustic field of underwater sound includes a hydrodynamic flow field in close proximity to the source that can accelerate the body of a nearby receiver and directly stimulate the inner ear (Kalmijn, 1988; Braun and Grande, 2008). Within distances of a few body lengths from the source, the local flow field gradient across the skin may also stimulate the mechanosensory lateral line system and provide additional information such as direction of the source (Braun and Coombs, 2010). In addition, sound pressure waves propagate farther distances, penetrate the body of a receiving fish and can induce pulsations of a gas-filled swim bladder, anterior swim bladder horns or bullae if present. These secondary particle motions may also stimulate the inner ear (Schellart and Popper, 1992; Braun and Grande, 2008). Thus, the perception of biologically-relevant sounds by a receiver fish is complex and dependent upon many factors related to the distance and direction of the receiver from the sound producer, the frequency spectrum and intensity of the acoustic field, the configuration of the lateral line system, and the presence of morphological associations between the inner ear and swim bladder.

Several fish groups have independently evolved adaptations that reduce the distance between the gas-filled swim bladder and the inner ear to enhance hearing capabilities. Bilaterally-paired swim bladder horns that approach or contact the otic capsule have evolved independently in several families of acanthopterygian fishes (reviewed by Schellart and Popper, 1992, Braun and Grande, 2008;). Behavior and physiology experiments show a general trend for enhanced sensitivity or extended high frequency range of hearing that is associated with the presence of rostral extensions of the swim bladder among taxa that include holocentrid squirrelfishes (Tavolga and Wodinsky; 1963; Coombs and Popper, 1979), Atlantic cod, *Gadus morhua* (Chapman and Hawkins, 1973), several distant and sister cichlid

genera (Schulz-Mirbach et al., 2012), mormyrids (Yan and Curtsinger, 2000; Fletcher and Crawford, 2001), sciaenid drums and croakers (Ramcharitar et al., 2006; Hodosky et al., 2008), the geriid mojarra (Parmentier et al., 2011b) and others (reviewed in Braun and Grande, 2008). Although this diversity of swim bladder and inner ear associations is widespread among teleost fishes, a detailed assessment of the relationship between swim bladder horn morphology among congener species is lacking especially in relation to the frequency response of the ear and contexts of sounds used in social communication.

Butterflyfishes (family Chaetodontidae) include approximately 130 species in 10 genera that are common inhabitants of coral reefs. The genus *Chaetodon* (~90 species) is distinguished by a laterophysic connection (LC), a unique morphological association between rostral projections of the swim bladder and the lateral line at the posterior margin of the skull (Blum, 1988; Webb, 1998). *Chaetodon* subgenera differ with respect to the dimensions of the horns (length and width), and proximity to the lateral line in the supracleithrum and inner ear. These features are proposed to translate sound-pressure-stimuli into fluid motion in the lateral line canal and particle motion in the inner ear (Webb and Smith, 2000; Webb et al., 2006; Webb et al., 2010), and to favor the efficient perception of auditory information among fish pairs (Tricas et al., 2006). Recent work shows that *Chaetodon*, *Forcipiger*, *Hemitaenichthys* and *Heniochus* produce several forms of pulsed sounds with peak frequencies and bandwidths from <1 Hz to >1000 Hz during social interactions in the field and lab (Tricas et al., 2006; Boyle and Tricas, 2011; Parmentier et al., 2011a; Tricas and Boyle, 2014, *in review JEB*). In *Chaetodon*, this includes very low frequency hydrodynamic stimuli (<1-30 Hz) (Tricas et al., 2006; Hanke et al., 2008; Tricas and Boyle, *in review JEB*) that can likely stimulate the inner ear by whole body accelerations and the mechanosensory lateral line of the receiver fish. Pulsed acoustic sounds from >100-1000 Hz are produced by all genera by a diversity of motor mechanisms (Boyle and Tricas, 2010, 2011; Parmentier et al., 2011; Tricas

and Boyle, *in review JEB*). Of relevance to perception of these acoustic stimuli by *Chaetodon* is the strong pairing of monogamous species that defend territories from conspecific competitors (Reese, 1975; Hourigan, 1989; Tricas, 1989; Roberts and Ormond, 1992). Acoustic stimuli are produced during social interactions and are directed towards both mates and conspecific competitors within distances of a few body lengths. However, the effect of the swim bladder horns on the perception of sounds used in natural behaviors by *Chaetodon* and sister taxa was previously untested.

Butterflyfishes live in a noisy coral reef environment with high levels of background noise from abiotic, vertebrate and invertebrate sources (e. g. Wenz, 1962; Tricas and Boyle, 2014; Lammers et al., 2008) that overlap with the spectrum of their communication sounds (Tricas and Boyle, *in review JEB*). Ambient background noise can have several effects on sound detection and communication in fishes and it is necessary to understand the sound characteristics of their natural habitats to determine their auditory efficiency and behavior (Ladich, 2013, 2014)

In this study we test the hypothesis that the LC system enhances the auditory sensitivity of *Chaetodon*. We compare butterflyfish hearing sensitivity to tone stimuli in three *Chaetodon* species with different LC morphologies to that of *Forcipiger flavissimus* which lacks swim bladder horns and the LC. We use the auditory evoked potential (AEP) technique to test the effects of the rostral horns and swim bladder on hearing sensitivity in the 100–2000 Hz range. We interpret their audiograms with respect to the frequency spectrum of sounds produced during social communication, and the presence of high ambient noise levels recorded in butterflyfish territories on coral reefs. Our results support the hypothesis that the rostral extensions of the *Chaetodon* swim bladder associated with the LC has increased both auditory sensitivity and hearing range that may promote perception of conspecific acoustic

signals in their noisy coral reef environment. We also interpret these finding in relation to the likely co-stimulation of the lateral line.

## RESULTS

### Hearing sensitivity of *Forcipiger* and *Chaetodon*

Auditory thresholds between 100-2000 Hz were obtained for the four study species. Representative AEP waveforms are shown for a 200 Hz stimulus at different intensities for *F. flavissimus* and *C. ornatissimus* (Fig. 1). The AEP waveform is visible above background noise at a stimulus level of 125 dB re:1  $\mu$ Pa for *Forcipiger*, and at a lower intensity of 111 dB re:1  $\mu$ Pa for *C. ornatissimus*. Comparisons of the baseline threshold audiograms show several similarities and differences in the frequency response characteristics among species (Fig. 2A). All *Forcipiger* individuals responded to stimuli from 100-800 Hz, a higher frequency response at 1000 Hz recorded for two individuals (33%), and no responses at higher frequencies. In contrast, all individuals of all three *Chaetodon* species responded to stimuli up to 1000 Hz. An extended frequency response range was apparent for both *C. multicinctus* and *C. auriga* with the maximum recorded frequency response at 1700 Hz for only a few individuals. This frequency response was exceeded by *C. ornatissimus* with hearing thresholds observed for all individuals at 1300 Hz, and a response at 2000 Hz for one individual. Both sound pressure and particle acceleration audiograms (Fig. 2B) further indicate a low pass response (flat sensitivity) to frequencies  $\leq 400$  Hz in *Forcipiger* and below about 500-600 Hz in *Chaetodon*. Audiograms for responses to particle acceleration show a decreasing threshold response with frequency in *Forcipiger* with a minimum threshold of 77 dB re:1  $\mu\text{m s}^{-2}$  at 100 Hz compared to a relatively flat low pass band of 65-75 dB re:1  $\mu\text{m s}^{-2}$  below 500-600 Hz for all *Chaetodon* species. However, the average audiogram threshold curve for *Forcipiger* is 5-15 dB higher than those for *Chaetodon* species in the 200-1000 Hz

stimulus range. A two-way repeated measures ANOVA (GLM) tested for differences in frequency sensitivity and species across the 100-800 Hz band and confirms differences between species and stimulus frequencies with no interaction of factors (Table 1). A post hoc Student-Newman-Keuls test shows that *C. multicinctus* has an overall lower threshold than *Forcipiger* ( $p < 0.05$ ) but the test did not detect sensitivity differences among *Chaetodon* species in this range. The maximum frequency at which auditory thresholds differed between *Forcipiger* and *C. multicinctus* was 600 Hz for both total sound pressure level (25 dB difference = 134.8 vs 109.6 dB re:1  $\mu\text{Pa}$ , respectively) and particle acceleration level (29 dB difference = 93.8 vs 64.9 dB re:1  $\mu\text{m s}^{-2}$ , respectively) (Fig. 2). Because *Forcipiger* is not uniformly sensitive to auditory stimuli  $>800$  Hz, we tested for differences in AEP responses at 1000 and 1300 Hz among the three *Chaetodon* species. Repeated-measures ANOVA (GLM) show that responses differ among frequencies but not among species (Table 1) and was confirmed by the post hoc Student-Newman-Keuls test ( $p < 0.05$ ). While we were able to run the above ANOVA tests on reciprocal transformed normalized data in terms of sound pressure, we were not able to normalize particle acceleration data for these species and frequency comparisons.

### **Action of the swim bladder and horns on hearing sensitivity**

Analysis of horn and swim bladder experiments confirms differences between species and stimulus frequencies. Deflation of the swim bladder in *Forcipiger* had no effect on thresholds across frequencies (Fig. 3A). A two-way ANOVA (frequency and swim bladder as factors, individuals as random subjects) shows no effect on thresholds following deflation of the swim bladder, no change in frequency response, or statistical interactions of these factors (Table 1). Deflation of the swim bladder in *C. ornatissimus*, which has short horns that could not be independently evacuated, appeared to show a 5 dB increase in thresholds at

200 and 400 Hz but we could not test for statistical differences because of low sample size (Fig. 3B). In comparison, displacement of gas from the swim bladder horns and subsequent deflation of the swim bladder increased hearing thresholds in *C. multinctus* and *C. auriga*. Figure 4 shows the AEP responses for an individual *C. multinctus* with a baseline threshold of 107 dB re:1  $\mu$ Pa, and sequential 10 dB increases following gas displacement in the horns and deflation of the swim bladder. Differences in threshold responses at 200, 400 and 600 Hz were found for *C. multinctus* across treatments (Two-way repeated measures ANOVA for subjects with all three treatments at these test frequencies, Table 1, Fig. 3) but there were no differences in thresholds at 100 Hz among treatments (One-way repeated measures ANOVA for subjects with all three treatments at 100 Hz, d.f.=2,2,4;  $F=0.40$ ;  $p=.694$ ). Post hoc Student-Newman-Keuls test shows no differences among horn or swim bladder treatments at 200 Hz, but an increase in threshold from baseline by both treatments (but their thresholds did not differ), and a sequential increase in threshold by swim bladder deflation following displacement of gas from the horns. A similar effect was observed for *C. auriga* at the same test frequencies (Fig. 3D). Deflation of the horns increased thresholds at 100, 200, 400 and 600 Hz but the subsequent deflation of the swim bladder had no additional effect (Two-way ANOVA, Table 1). The reduction in the number of fish that showed responses after manipulation of the horns and swim bladder precluded further statistical tests, but the loss of hearing sensitivity may extend to frequencies >600 Hz as seen in post manipulation audiogram plots for *C. multinctus* (Fig 3C). Comparisons of the net effect of the swim bladder and horns on hearing sensitivity shows these relative effects on *Chaetodon* and *Forcipiger*, and also among *Chaetodon* species (Fig. 5). Taken together, all of the above results indicate that both *Forcipiger* and *Chaetodon* are most sensitive to particle acceleration stimuli. In *Chaetodon* sound pressure actions on the swim bladder and horns enhances



frequency sensitivity from 200-600 Hz and increases the maximum frequency response range to at least 1300 Hz.

### **Ambient noise levels in coral reef territories, sound production and hearing**

Data from the field recordings of ambient noise levels in reef territories of multiband butterflyfish, *C. multinctus*, were compared with data on auditory thresholds and spectral levels of their communication sounds (Tricas and Boyle, *in review JEB*) to provide a first estimate of the potential constraints on hearing perception in their natural setting. The total background noise levels across the full 10 Hz-24 kHz band show variation in intensity of about 10-30 dB among territories and among frequencies that vary with depth (Fig. 6 Background Noise curves). The greatest difference in background noise was observed in territories <6 m deep, with relatively high average noise levels in the 10-100 Hz band compared to territories at deeper sites. Overall, the noise levels are near but below the AEP auditory thresholds to frequency tones. Total noise levels were also below the peak frequency levels reported for the four sounds produced by this species during communication at close distances (Tricas and Boyle, *in review JEB*) as shown by representative sound spectral curve plots in Fig. 6. The tail slap pulse is a very low frequency stimulus associated with accelerations of a local hydrodynamic flow field with a peak frequency of ~10 Hz. The body shake sound is produced infrequently and has a low peak frequency ~29 Hz. The body pulse sound is produced very frequently during social interactions and has an average peak frequency of 137 Hz (median 231 Hz) and bandwidth of 350 Hz that overlaps well with their range of highest frequency sensitivity from 100-600 Hz. A high frequency and broadband click sound is also often produced during the tail slap behavior but has an average peak frequency far above the hearing range of 100-1700 Hz.

The estimated intensity spectrum level ( $SPL_{int}$ ), in which sound intensities are corrected for a 1 Hz band to provide a more realistic comparison of relative intensity across a range of frequencies, confirms that ambient noise levels on the reef substrate (where fish forage in pairs) decrease non-linearly with water depth [inverse polynomial function,  $SPL_{int}=Y_0+(a/\text{depth})$ :  $SPL_{int}=73.36+(22.90/\text{depth})$ ,  $p=0.012$ ] and a similar relationship is found for noise levels measured 1-m above the substrate where fish occasionally swim [ $SPL_{int}=76.10+(16.82/\text{depth})$ ,  $p=0.033$ ] (Fig. 7A). However the parameters for these two curves do not differ (ANOVA:  $Y_0$ , d.f.=1,20,21,  $F=2.10$ ,  $p=0.163$ ;  $a$ , d.f.=1,20,21,  $F=0.374$ ,  $p=0.548$ ) and indicate total sound levels did not differ with depth at the two elevations within a territory. When their respective sound frequency bands are included in the estimation of band noise, the relationship between ambient noise and territory depth differs for the two most common sounds. The noise level within the frequency band of the tail slap sound decreases rapidly with territory depth [ $SPL=(-1.75 \times \text{depth})+127.30$ ,  $p<0.001$ ,  $R=0.68$ ] whereas ambient noise in the body pulse sound band does not change with depth [ $SPL=(-0.083 \times \text{depth})+105.40$ ,  $p=0.782$ ,  $R=0.095$ ] (Fig. 7B). Comparison of these band-specific background noise levels with the 6 dB bandwidth levels for each sound type (from Tricas and Boyle, *in review JEB*) indicates that the signal to noise ratio of the tail slap sound increases with depth of the territory [ $SNR = (1.57 \times \text{depth})+3.70$ ,  $p=0.021$ ,  $R=0.68$ ] but that for the body pulse sound does not change with territory depth [ $SNL=(0.08 \times \text{depth})+10.60$ ,  $p=0.78$ ,  $R=0.10$ ] (Fig. 7C).

## DISCUSSION

This study demonstrates that the auditory sensitivity of *Chaetodon* butterflyfishes is enhanced by the perception of sound pressure and is mediated by the swim bladder and horns associated with the LC. As for most AEP studies we used tone stimuli to determine auditory

thresholds across relevant frequencies. Below we use the audiogram data to interpret their likely sensitivity to the spectra of different sounds that are important for acoustic communication and the effects of ambient noise spectra encountered in their natural coral reef environment. We recognize the limitation that the AEP tone stimulation method shows responses to single brief tones and not more complex sounds used for communication. In addition, the AEP technique can also include direct responses of the lateral line and possibly those mediated by the LC, which we also discuss.

Comparison of audiograms indicates that *Chaetodon* species are more sensitive than *Forcipiger* to sound stimuli at frequencies between 100-800 Hz, and also have an extended frequency range of hearing. In addition, hearing audiograms for butterflyfishes are similar in several respects to species that are primarily sensitive to particle acceleration rather than sound pressure. Sound pressure thresholds at best frequency were within the low frequency band of 100-600 Hz for all species and ranged from 124 dB re:1  $\mu$ Pa for *Forcipiger* to 110 dB for *C. multicinctus*. These minimum SPL thresholds are similar to those reported for species that lack anterior projections of the swim bladder such as the pumpkinseed sunfish (Wysocki and Ladich, 2003), oyster toadfish (Yan et al., 2000), gobies (Lugli et al., 2003) and larval snappers and jacks (Wright et al., 2010). These SPL thresholds are far greater than those reported by many AEP studies on species with peripheral auditory structures known to enhance sensitivity to sound pressure such as the Weberian apparatus of some cyprinids and catfish (Kenyon et al., 1998; Ladich, 1999; Amoser and Ladich, 2005; Lechner and Laddich, 2008), the otic gasbladder of mormyrids (Yan and Curtsinger, 2000) and the suprabranchial organ of gouramis (Ladich and Yan, 1998), although AEP audiograms are not considered to be directly comparable between studies due to differences in techniques (Ladich and Fay, 2013). Primary sensitivity to particle acceleration in butterflyfish is further indicated by the descending low pass characteristics and relatively flat low pass curves below 500 Hz. The

band of highest frequency sensitivity was from 100-400 Hz for *Forcipiger* and 100-600 Hz for *Chaetodon*. This coincides with the narrower 200-300 Hz band of best sensitivity recorded for larval and juvenile spotfin butterflyfish, *C. ocellatus* (Webb et al., 2012). These bands of best sensitivity are well below the 1-3 kHz range seen in many sound pressure sensitive species with otophysic connections (Ladich and Fay, 2013). The maximum frequency response for some *Forcipiger* individuals extended to 1000 Hz whereas *Chaetodon* species showed sensitivities to 1700 and 2000 Hz. Thus, while all butterflyfishes in this study appear most sensitive to particle acceleration stimuli, *Chaetodon* species showed higher frequency sensitivity than *Forcipiger*.

The differences in response characteristics observed for *Forcipiger* and *Chaetodon* support the hypothesis that the anterior swim bladder horns in *Chaetodon* enhance both the hearing frequency range and thresholds via an added sensitivity to sound pressure stimuli. Deflation of the swim bladder in *Forcipiger* had no effect on auditory thresholds and supports the conclusion for an auditory sensitivity only to particle acceleration. However, displacement of gas from the relatively long swim bladder horns in both *C. multicinctus* and *C. auriga* increased the auditory thresholds between 200-600 Hz and therefore decreased hearing sensitivity to sound pressure in this range (Fig. 4C,D). Scanning electron microscopy and computed tomographic imaging analyses of the maculae, hair cell populations, otoliths and swim bladder of several LC variants reveal that the butterflyfish inner ear has an unremarkable morphology and lacks a physical otophysic connection (Webb et al., 2010). However, the swim bladder horns of both species are long and have equivalent relative lengths when corrected for body size (Woods, 2006). In addition, the swim bladder horns of *C. multicinctus* approach the lagena of the inner ear at close distances (1 mm) in both the transverse and horizontal planes whereas the horns of *C. auriga* approach the lagena at twice that distance (~2 mm) and in only the transverse plane. This closer proximity of the horns to

the inner ear and the presence of a more blunt rostral horn apex found in LC indirect variants indicate a potentially stronger effect of the horns on auditory sensitivity in *C. multincinctus* (and other LC indirect variants) than *C. auriga* (and other LC direct variants) (Woods, 2006). Despite these morphological differences in horn morphology and proximity to the inner ear, our results indicate that the relative effect of the horns on sound pressure sensitivity may be more prominent in *C. auriga*. Displacement of gas from the swim bladder horns in *C. auriga* reduced sensitivity at 200-600 Hz but there was no additional statistical effect on sensitivity after subsequent swim bladder deflation as observed for *C. multincinctus*. Recent work on the hearing abilities of representatives of four cichlid genera with different swim bladder and horn morphologies (Schulz-Mirbach et al., 2012) shows that species with anterior swim bladder horns have improved auditory sensitivities of 20-40 dB (SPLs) between 0.5 and 1 kHz. That work also indicated that swim bladder size was involved in extending the upper frequency range to 3 kHz. Of note in this study is that the highest hearing sensitivity was observed at 2 kHz for *C. ornatissimus*, a species with very short swim bladder horns (LC variant Ind2) but also approach the lagena at a short 1 mm in the transverse plane (Woods, 2006). The physical responses to sound pressure stimuli by these morphological variants of the LC, swim bladder horns, and swim bladder need to be quantified in order to confirm their contribution to butterflyfish hearing.

The relative action of acoustic stimuli on the head and trunk lateral line system was not addressed in this study. Recent work shows that there can be a significant contribution to the AEP from hydrodynamic motions across the lateral line canals especially at lower stimulus frequencies (Higgs and Radford, 2013). Thus, AEP responses at low frequencies should be considered as multimodal rather than only auditory evoked potentials derived from the inner ear. While our AEP responses likely also include a contribution from the lateral line system, the observed changes in threshold following evacuation of gas from the swim

bladder and horns is more likely due primarily to changes in excitation of the inner ear. The LC portion of the lateral line adjacent to the swim bladder horns includes only a few associated neuromasts of the supracleithrum and lateral line canal scales (Webb et al. 2006). Nonetheless, the physical and neurophysiological actions of the LC system on the butterflyfish ear and lateral line need to be clarified as there is likely an integrative role in the detection and processing of information encoded from both systems during butterflyfish acoustic communication.

Recent work shows that our four test species produce several sound types with bandwidths that span four decades from  $< 1$  Hz to  $> 1000$  Hz. The body pulse and other sounds produced by *C. multicinctus* in the field and lab have peak frequency content from 137-466 Hz (Tricas et al., 2006; Tricas and Boyle, 2014; Tricas and Boyle, *in review JEB*) and the pulsed sounds at 346 Hz produced by *C. ornatissimus* (Tricas and Boyle, 2014). Thus sounds produced by both species overlap with their best frequency sensitivities demonstrated in this study. We would expect these to be the most effective sound types to stimulate the swim bladder and LC at close and greater distances from the source. In contrast, the prominent tail slap and other sounds produced during social interactions with conspecifics by *C. multicinctus*, *C. ornatissimus*, *C. auriga* and *Forcipiger* contain hydrodynamic and sound pressure components in the infrasound range of  $<1$ -30 Hz which should not affect horns or swim bladder transduction (Sand and Karlsen 2000). The response of the butterflyfish inner ear to such low frequency linear accelerations needs to be determined to understand the full range of acoustic signals used by these fish in their social interactions. In addition, the discrimination of sound stimuli needs to be determined beyond the small laboratory tank environment so that the particle motion, sound pressure and spatial configuration of communication sounds can be presented to stimulate the ear in more natural ways.

The rostral extensions of the swim bladder may serve other functions in addition to the facilitation of acoustic communication. The connection between the modified swim bladder and ear in the mojarra, *Eucinostomus argenteus*, imparts a high hearing sensitivity but that species apparently does not produce sound for communication (Parmentier et al., 2011). In this case the swim bladder morphology was argued to facilitate body orientation during feeding on small benthic prey and a similar hydrostatic function may occur in coral feeding butterflyfish which must orient with great precision to selectively feed on small coral polyps (Tricas, 1989b). Enhanced hearing sensitivity and frequency range may also function for the detection of predators or prey as was suggested for the highly sensitive otophysans in quiet freshwater habitats (Ladich, 2000). Such potential non-communication functions of the modified associations between the swim bladder, ear and LC need further investigation in relation to the detection of prey, predators, ambient background noise levels and body orientation behaviors.

### **Butterflyfish sound communication on coral reefs**

The best sensitivities for both *Forcipiger* (~125 dB SPL) and *Chaetodon* (~115 dB SPL) are similar to those reported for some other coral reef dwelling damselfish (e.g. Enger and Mann 2005; Maruska et al, 2008) and are higher than that of many non-reef dwelling species (Ladich and Fay, 2013). Although differences in recording techniques preclude direct comparisons, it is possible that the noisy coral reef environment constrains adaptations for higher hearing sensitivity as seen in aquatic environments with less background noise (Ladich 2014). The hearing sensitivity difference between *Forcipiger* and *Chaetodon* is not likely explained by differences in habitat selection because the two species overlap extensively in their distribution over the reef, although this remains to be tested.

The efficiency of acoustic communication by butterflyfish on noisy coral reefs may be facilitated by the selection of optimal acoustic sub-habitats. Ambient sound recordings taken within territories of the coral-feeding multiband butterflyfish, *C. multinctus*, show that total noise levels are higher in shallow areas of the reef (<6 m depth) especially within the lower frequency band of 10-100 Hz (Fig. 6). Such low frequency background noise in shallow reef waters comes from several abiotic sources such as wind, waves, tidal flow and also biological sources (Wenz, 1962; Urick, 1983). This noise spectrum includes the frequency range for the tail slap and body shake sounds which are used during social interactions with mates and other conspecifics (Tricas and Boyle, 2014; *in review JEB*). The intensity of ambient noise near the reef substrate (where butterflyfish pairs swim and feed together on corals) in these shallow territories was approximately 10-15 dB greater than the intensity within territory sites 10-13 m deeper on the reef, and this relationship was maintained when noise levels were adjusted for the frequency band of the tail slap sound (Fig. 7). In comparison, the intensity of ambient noise within the frequency band of the body pulse sound type did not change with territory depth. These differences indicate the possibility for different actions of ambient reef noise levels on the perception of different sound types. Are there fitness advantages related to acoustic communication efficiency (information transfer, useful distance of acoustic communication, etc.) among mates and rivals that establish territories in deeper areas of the reef with lower ambient noise levels? In addition, other studies on ambient reef noise reveal different spectral signatures among reefs and habitats (Simpson et al., 2005; Kennedy et al., 2010; Radford et al., 2014) but have not yet distinguished between the hydrodynamic surge/water turbulence and sound pressure components of sounds that also differ respectively with habitats and depth. Future work should address the acoustic characteristics of reef environments associated with the reef fish distributions and their potential impact on sound perception during acoustic communication.



The perception of a sound stimulus in a reef habitat with high background noise would be enhanced by a closer association of two fish (small distance of separation) compared to fish in a habitat with low background noise. Previous studies indicate that signal to noise level ratios are 10-20 dB at threshold and are dependent upon the relative directions of a sound and the ambient noise source (Chapman, 1973; Chapman and Sand, 1974; Hawkins and Sand, 1973; Fay, 1988). The signal to noise levels estimated in the present study were determined from independent measurements of AEP hearing thresholds in the relatively quiet lab environment and compared to empirical field measurements of sound band-specific noise levels (corrected for the frequency range of a specific sound type) in the field. By this method we estimated sound signal to ambient noise level ratios to be 10-20 dB near the source (Fig. 7C). Unfortunately, there is not a rigorous association for all species between hearing thresholds measured by the AEP and behavior assay techniques (Ladich and Fay, 2013) thus the behavioral thresholds to tone stimuli remain unknown, and also to their complex sound waveforms. Behavioral thresholds are needed to determine the maximum distance of separation for the perception of conspecific sounds in the presence of noise in the coral reef environment. The signal jump sound used to attract females to nest sites by male Hawaiian Dascyllus damselfish, *Dascyllus albisella*, was estimated to be detectable by a receiver fish at a distance of 11-12 m from the source where the signal to noise ratio was 5-10 dB (Mann and Lobel, 1997). Unlike damselfishes, butterflyfishes are broadcast spawners that do not form nesting sites. Many *Chaetodon* butterflyfish form monogamous pairs that spend a large portion of their day foraging together separated by only a few body lengths, and are not known to produce acoustic advertisements for mates at great distances. This leads to the conclusion that pairing and other close social behaviors of butterflyfish promote efficient acoustic communication in a noisy reef environment. Future studies on hearing capabilities in the coral reef environment should investigate the reception sounds produced by

conspecifics (rather than tones) to determine the extent that the intense ambient noise that may reduce sound perception. In addition, the potential contribution of sound pressure transduction by the LC to acoustic communication at near and greater distances needs to be assessed to better understand the potential adaptive values for their close affiliative behaviors.

## METHODS

### Study species

Spectral sensitivity was determined for three *Chaetodon* species with different LC morphologies (including long and short rostral swim bladder extensions) that belong to different subgenera (sensu Webb et al., 2006) and *Forcipiger flavissimus*. The threadfin butterflyfish, *C. auriga*, (n=11, SL=116.0±9.7 SD mm) has a direct LC with mucoid connective tissue between the medial opening in the supracleithral lateral line canal, and has long and wide swim bladder horns (LC variant Dir1). The multiband butterflyfish, *C. multicinctus*, (n=9, SL=76.0±6.7 SD mm) has an indirect LC with a layer of epaxial muscle (and no mucoid connective tissue) between the medial opening of the supracleithrum, and long and wide swim bladder horns (LC variant Ind2). The ornate butterflyfish, *C. ornatissimus*, (n=3, SL=89.3±7.3 SD mm) has an indirect LC with short horns that do not closely approach the anterior lateral line or inner ear (LC variant Ind3). The forcepsfish, *Forcipiger flavissimus*, (n=6, SL=106.2±15.6 SD mm) lacks swim bladder horns and a LC (no medial opening in the supracleithrum). Adult fish were acquired from local fish collectors, transported to the lab and acclimated in 40 l tanks with coral/sessile invertebrate food and fresh flow through seawater (25–28 °C) for ≥24 h before experiments were performed.

## Stimulus generation and AEP recordings

The auditory responses of butterflyfishes to sound stimulation were determined using the AEP physiology technique which detects compound neural responses to estimate the hearing sensitivity of fish and used to determine the effect of gas-filled anterior swim bladder and horns on auditory sensitivity (Kenyon et al. 1998; Yan et al., 2000), and we followed recording procedures on reef fish used previously in our lab (Maruska et al., 2007). This non-invasive technique was preferred because frequency thresholds could be rapidly determined and allowed sequential manipulation of the gas-filled swim bladder horns and swim bladder chamber of individual fish.

Experimental fish were immobilized with an injection of pancuronium bromide into the dorsal body musculature, lightly restrained in a mesh harness with a clamp suspended from a PVC frame, positioned slightly below the water surface of a 23 l bucket (29 cm diam x 35 cm high) and ventilated through the mouth with fresh seawater. Stainless steel electrodes (Rochester Electro-Medical, Inc., Tampa, FL, USA; 6–12-k ohm impedance) were sealed on the ends with glue and nail polish so that only ~1-mm of metal was exposed at the tip. The recording electrode tip was inserted 3–5-mm deep into the cranial musculature along the dorsal midline above the brainstem. The reference electrode was inserted into the cranial musculature between the eyes, and a ground wire placed in the water near the fish.

Sound stimuli were generated (and AEPs recorded) with a Cambridge Electronics Design (CED, Cambridge, UK) Micro 1401 controlled by Spike 2 software and a CED 3505 attenuator. Conditioned signals were differentially amplified (UMA 352; Peavey Electronics, Meridian, MS, USA) and sent to an underwater speaker (UW-30; frequency response 100-Hz–10-kHz) placed at the bottom of the test bucket and approximately 28–30 cm below the fish head. A total of eight primary stimulus frequencies from 100–800 Hz were tested for each subject and 1–2 kHz when possible. Acoustic pips at test frequencies of

$\geq 200$ -Hz consisted of 2000 pure tone 20-ms pulses (10-ms plateau with rise and fall times of 5-ms), and at a 100-Hz pulse plateau, rise and fall times of 10-ms. Stimulus artifacts in the AEP recordings were minimized by sequential alternation of the pip phase. Each trial began at a suprathreshold intensity (136–156-dB rms re:1- $\mu$ Pa) and was decreased in 5-dB steps to a sound level below the presumed threshold (90–125-dBrms re:1- $\mu$ Pa). Threshold was determined for each frequency (described below) before moving to the next test frequency. Sound pressure levels produced by the loudspeaker were calibrated with a Brüel and Kjær (Nærum, Denmark) hydrophone (model #8103; sensitivity=211-dB re: 1-V / Pa; frequency response 0.1-Hz–180-kHz) placed in the experimental tank at the position that the fish head normally occupies. We confirmed that SPL was uniform across the position of the fish body. For calibration, pips were presented without phase alternation, and voltage levels of sounds at all frequencies and intensity levels were measured with the hydrophone, Brüel and Kjær Nexus amplifier (Model 2692, sensitivity 10-mV/Pa or 31.6-mV/Pa) and then signal averaged with our Spike 2 routine to determine actual sound pressure levels in dBrms re:1- $\mu$ Pa. We also calibrated the tank for particle acceleration with a EDO Corp. P/N 51815-4, sensitivity 14.1 mV/(m s<sup>2</sup>) accelerometer kindly provided by Joe Sisneros (University of Washington) and Michael Gray (Georgia Tech).

AEP waveforms were differentially amplified (10-000X) and band-pass filtered (1–10-000-Hz) with a differential amplifier (DP-301, Warner Instruments, LLC, Hamden, CT, USA), digitized with on the CED Micro 1401 (described above) running Spike 2 software, and stored on the computer hard drive. Responses to a total of 2000 repetitions were averaged for each sound intensity and test frequency. Power spectra (FFT, 512 or 1024-points) of the averaged waveforms were calculated and examined for peaks at twice the stimulus frequency that results from the opposed orientation of ear hair cells and nonlinearities in the auditory system. Thresholds were determined by both the averaged AEP

trace and power spectrum and were defined as the lowest sound level to show a repeatable AEP trace above background noise, and an FFT peak at twice the stimulus frequency. In cases where the threshold appeared between adjacent 5-dB intensity steps, the threshold level was verified by a repeated test of the averaged AEP.

### **Experimental manipulation of the swim bladder horns and swim bladder**

Each fish was tested for changes in baseline hearing thresholds after manipulation of the swim bladder horns (*C. multicinctus* and *C. auriga*) and/or swim bladder (all four species). After baseline control responses were recorded, gas in the rostral swim bladder horns was displaced (evacuated) by injection of 100  $\mu$ l of Super Poligrip<sup>TM</sup> or Vaseline (petroleum jelly) into the swim bladder horns and hearing thresholds re-tested. The swim bladder was then deflated with a hypodermic needle and syringe and the fish tested one final time. Only pre-manipulation controls and swim bladder deflation thresholds were determined for *C. ornatissimus* which has short inaccessible horns at the rostral end of the swim bladder, and *F. flavissimus* which lacks rostral swim bladder horns. Fish were euthanized after experiments by immersion in MS-222, and fills of the rostral horns and deflation of the swim bladder were confirmed by dissection. We also performed AEP experiments on freshly euthanized specimens to confirm that the AEP responses were abolished and not a recording artifact.

Estimates of average hearing thresholds and standard error were calculated for each species from data on individual fish. Normal hearing sensitivity was determined by pooling thresholds from individuals not used for manipulation experiments and also baseline control data for fish in which the swim bladder or horns were subsequently manipulated. Repeated measures general linear models (rmGLM) were used in order to test for species-level differences in threshold at each frequency. Because multiple frequencies were tested for each individual, thresholds at each frequency are not statistically independent. Thus we used

rmGLM in which individual fish were used as a random subject factor, frequency and species were used as fixed factors, and threshold as the response: individual fish were nested within species, and species and frequency were crossed. Because the hearing range varied among species, two separate tests were also conducted. One rmGLM tested for differences in thresholds among all species and at frequencies between 100 and 800 Hz. A second test examined thresholds among all three *Chaetodon* species (but not *F. flavissimus*), between 1000 and 1300 Hz. Experiments that tested the effect of the swim bladder and horns on hearing thresholds were also analyzed with rmGLM separately for each species in which individual fish was a random subject factor, frequency and experimental phase (pre-manipulation, horn injection [where applicable], swim bladder deflation) were fixed factors, and threshold was a response. In these experiments individual fish are crossed with treatment and frequency, and the factors treatment and frequency are crossed. Post hoc pairwise multiple comparisons were performed with the Student-Newman-Keuls method. All statistical analyses were performed using SigmaPlot (v11.0 and 12.5).

### **Reef noise and communication sound band estimates**

In order to determine the variation in ambient noise levels encountered by butterflyfishes in their normal coral reef habitats, we first recorded background sound within 11 *C. multinctus* territories using scuba at Puako Reef, Hawai'i. Prior to recordings, focal butterflyfish pairs and territory boundaries were determined from direct observed over a 4-6 week period. A stereo portable digital audio tape recorder (Sony DAT PCM-M1) in a custom housing (Sexton Corporation) with two ATI MIN 96 hydrophones was placed within an open area near a central location of each territory. A constant gain was used for recordings and compared to a calibrated tone of known voltage amplitude. One hydrophone was placed  $\leq 10$  cm above the substrate and the second hydrophone suspended directly 1 m above in the water

column to provide simultaneous recordings at two depths. Depth of fish territories (at the location of the substrate hydrophone) ranged from 2.4 to 12.8 m with various mixtures of dead coral, rock and live coral substrates. Recordings of approximately 5-min duration were made on 24 August 2004 between 1455 and 1615 h. After deployment of the recording apparatus, scuba divers left the area to eliminate bubble noise in the recordings. Background sound recordings were downloaded to a computer and reviewed on Cool Edit Pro software. Approximately one minute of continuous recording was identified for analysis that represented the frequency spectrum and intensity for each territory locations, except for one territory, for which only 16 seconds of recording could be analyzed because of anomalous hydrophone interference. Noise estimates were calculated from a 16,384 point FFT using the ‘scan’ button in Cool Edit Pro. Estimates of spectrum level (dB re: 1  $\mu\text{Pa}^2/\text{Hz}$ ), which provides a standardized estimate of frequency-specific noise, were obtained by subtracting 4.67 dB (10 log BW) to adjust for the 2.93 Hz bandwidth of the FFT. Estimates of octave band sound pressure levels (center frequencies 15.6, 31.3, 62.5, 125, etc.) for each territory were obtained by taking the average among linear values for the FFT bins that correspond to the upper ( $f_u$ ) and lower ( $f_l$ ) cutoff frequency of the octave band. The averaged value was then converted to a dB scale and the amplitude was adjusted based on the bandwidth of the octave band and original FFT bins (2.9 Hz) in order to determine SPL dB re 1  $\mu\text{Pa}$  for each octave band:  $10\log(f_u - f_l) - 10\log(2.9)$ . In addition, we calculated the background noise of frequency bands that correspond to the majority of spectral energy of the two most common sounds of *C. multicinctus*: the tail slap and body pulse (Tricas and Boyle, *in review JEB*). The average minimum and maximum frequencies within 6 dB of peak of sounds from recorded in a laboratory study (Tricas and Boyle, *in review JEB*) were used to represent the bandwidth of the tail slap and body pulse, 2-18 Hz and 21-414 Hz, respectively. For each territory at the substrate and 1m above, estimates of noise levels obtained for these two bands

were determined from the average linear noise values from noise measurements described above for the corresponding frequencies. The average was then converted to a dB value and adjusted by adding  $[10\log(\text{sound bandwidth}) - 10\log(2.9)]$ . We then estimated the total pressure level across the respective bandwidth for each sound type. Signal-to-noise ratio (SNR dB) for each of these two sound types at each territory and recording location (substrate and 1m) were estimated by taking the average sound pressure level recorded in the laboratory (Tricas and Boyle, *in review JEB*) for the tail slap (131 dB) and body pulse (116 dB) and subtracting the corresponding estimated sound type band noise level.

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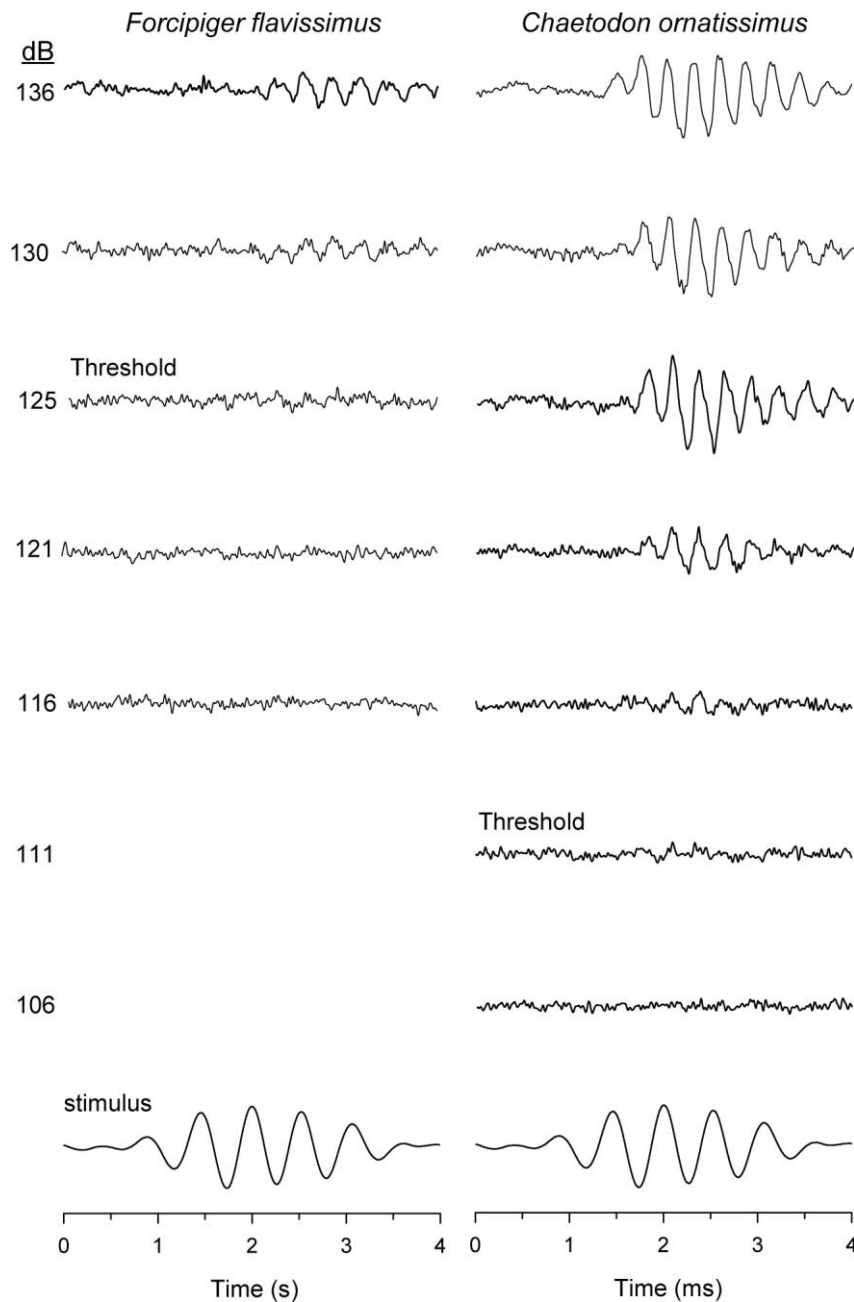
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Table 1. Statistical comparisons of audiograms by two-way ANOVA with repeated measures (general linear model). MS = Mean square. See text for additional details.

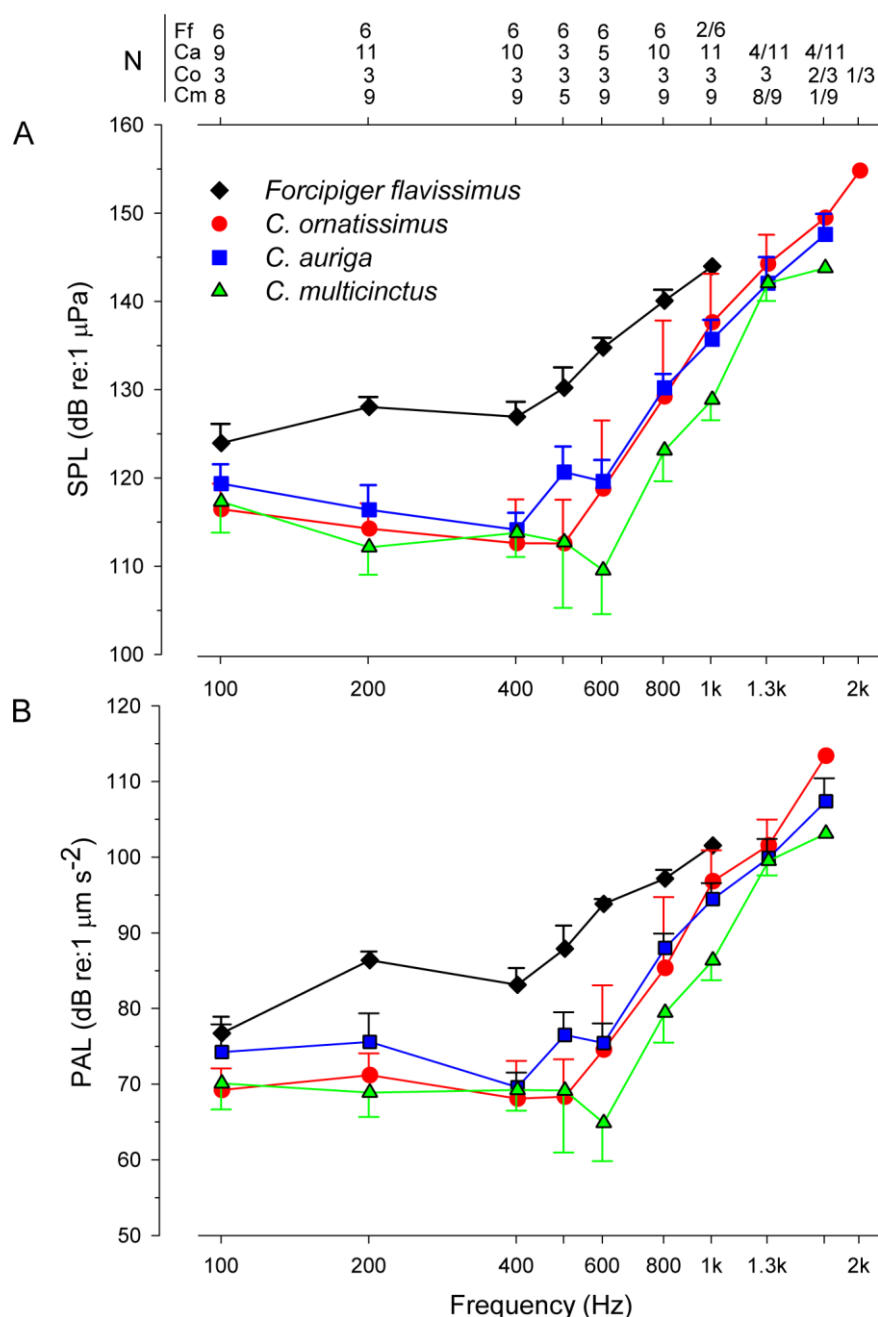
		Sound Pressure Level			
		df	MS	F	P
<b>All species 100-800 Hz</b>					
	Species	3	0.0000068	4.86	<b>0.009</b>
	Individual	23	0.0000015		
	Frequency	5	0.0000022	12.94	<b>&lt;0.001</b>
	Frequency x species	15	0.00000028	1.64	0.077
	Error	97	0.00000017		
<b>All <i>Chaetodon</i> 1 and 1.3k Hz</b>					
	Species	2	99.39	2.57	0.111
	Individual	14	40.01		
	Frequency	1	299.85	19.86	<b>0.001</b>
	Frequency x species	2	47.58	3.15	0.087
	Error	10	151.00	15.10	
<b><i>Forcipiger flavissimus</i> Swim Bladder Deflation</b>					
	Individual	5	775.30		
	Treatment	1	32.33	2.10	0.207
	Treatment x individual	5	15.40		
	Frequency	3	92.50	4.65	<b>0.017</b>
	Frequency x individual	15	19.90		
	Treatment x frequency	3	4.03	0.62	0.616
	Error	15	6.55		
<b><i>C. multinctus</i> Horn Fill and Swim Bladder Deflation</b>					
	Individual	3	188.06		
	Treatment	2	454.24	31.39	<b>&lt;0.001</b>
	Treatment x individual	6	14.47		
	Frequency	2	5.07	0.22	0.808
	Frequency x individual	6	22.94		
	Treatment x frequency	4	81.41	4.77	<b>0.015</b>
	Error	12	17.05		
<b><i>C. auriga</i> Horn Fill and Swim Bladder Deflation</b>					
	Individual	3	0.00000061		
	Treatment	2	0.0000018	22.92	<b>0.002</b>
	Treatment x individual	6	7.9x10 <sup>-8</sup>		
	Frequency	3	0.00000013	0.67	0.59
	Frequency x individual	9	0.0000002		
	Treatment x frequency	6	0.00000013	2.75	<b>0.045</b>
	Error	18	4.7 x10 <sup>-8</sup>		

Probabilities of a type 1 error < 0.05 are indicated in bold.

## Figures



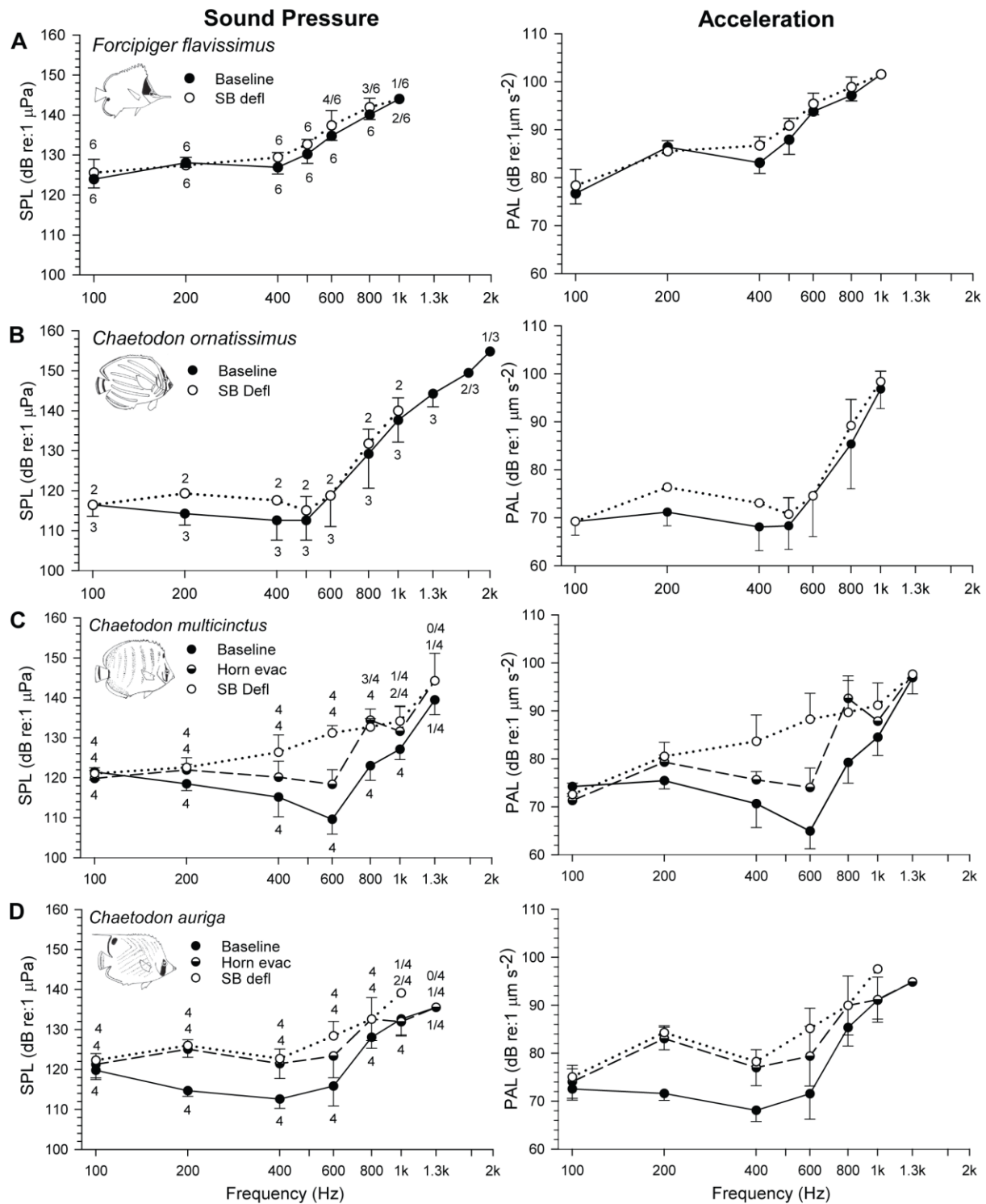
**Figure 1.** Representative auditory evoked potential (AEP) waveforms used to determine intensity thresholds for butterflyfish species at 200 Hz stimulation. A) AEP waveforms for the forcepsfish, *Forcipiger flavissimus*, show a stimulus threshold at 125 dB re:1  $\mu$ Pa (left, 3<sup>rd</sup> trace from top). The ornate butterflyfish, *Chaetodon ornatissimus*, shows a lower threshold intensity at 111 dB re:1  $\mu$ Pa (right, 6<sup>th</sup> trace from top). Bottom traces show the stimulus waveform recorded by the hydrophone at the position of the fish head.



**Figure 2.** Hearing threshold audiograms for *Forcipiger flavissimus* and three *Chaetodon* species as measured by the auditory evoked potential technique. A) Audiograms measured as total sound pressure level (SPL) are similar among *Chaetodon* species with lower thresholds and an extended response range to 1700 Hz for all species and to 2000 Hz for *C. ornatissimus*. B) Audiograms measured as particle acceleration level (PAL) show similar relationship between *Chaetodon* and *Forcipiger* across most tested frequencies. We did not

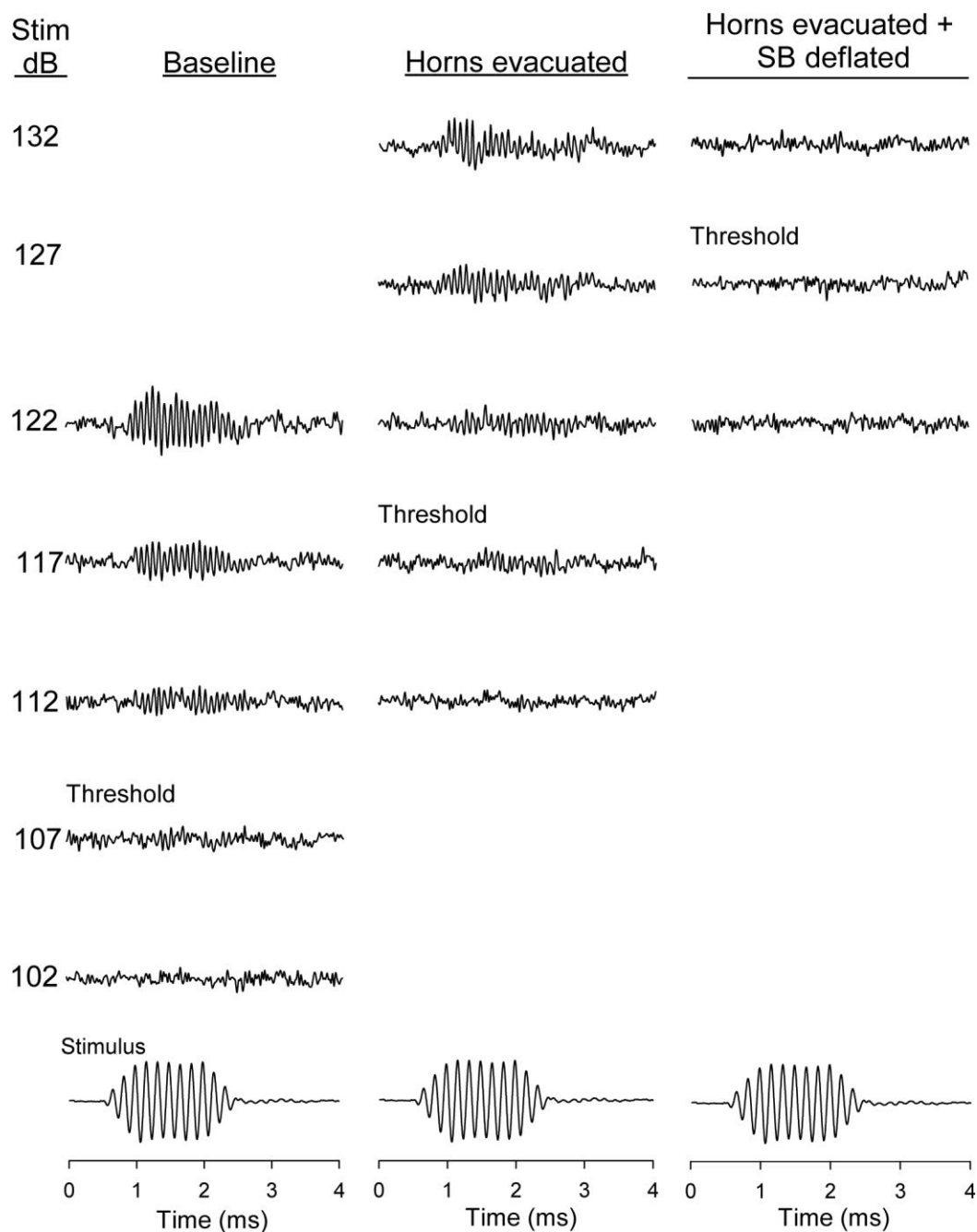


calibrate the accelerometer at 2000 Hz, thus that data point is lacking for *C. ornatissimus*. N = sample size of fish tested at each frequency. Fractions indicate the proportion of tested fish that showed a response. Data are means and SE.

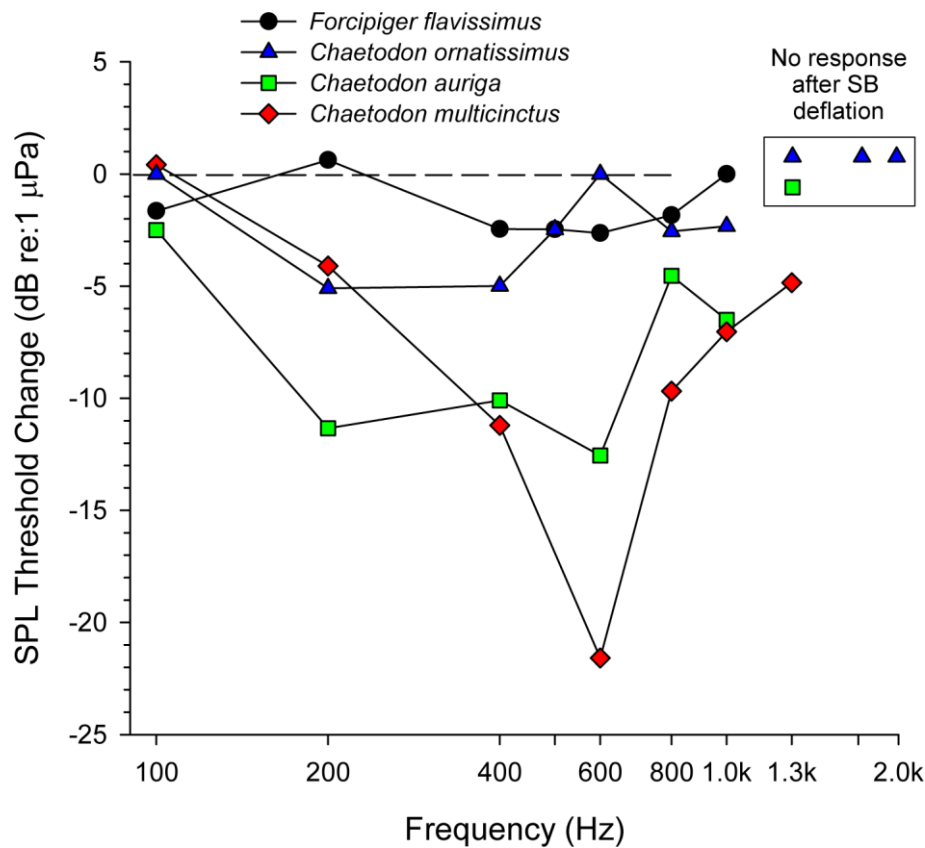


**Figure 3.** The effect of the gas filled swim bladder horns and chamber on hearing sensitivity in *Chaetodon* butterflyfish with different laterophysic morphologies as determined by auditory evoked potential technique. A) The forcepsfish, *Forcipiger flavissimus*, which lacks an LC and swim bladder horns shows no change in normal AEP threshold (solid circles)

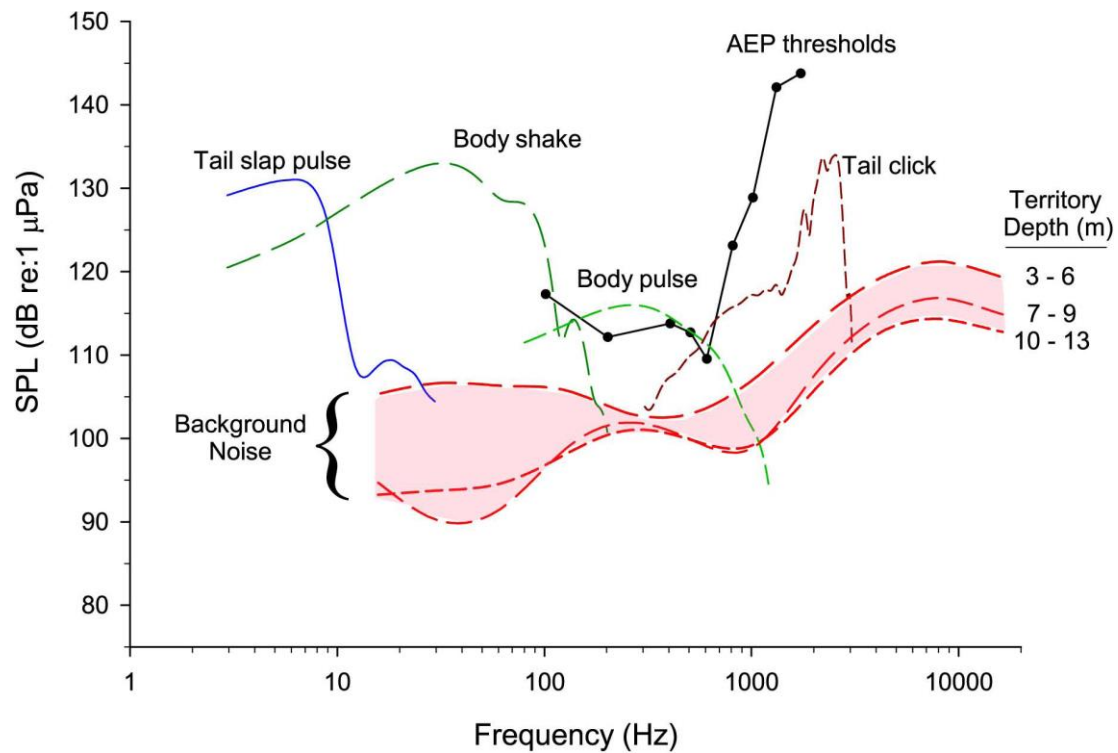
following deflation of the swim bladder (open circles). B) The ornate butterflyfish, *C. ornatissimus*, has short swim bladder horns with an indirect connection to the LC that we were not able to manipulate. The baseline thresholds extended to 2000 Hz and appear to increase by approximately 5 dB in the 200-400 Hz band following deflation of the swim bladder. C) The multiband butterflyfish, *C. multicinctus*, has long swim bladder horns with an indirect connection to the LC. Baseline thresholds increased in the 200-600 Hz band after gas was evacuated from the swim bladder horns (half-filled circles) with a maximum increase of 10 dB at 600 Hz. Subsequent deflation of the swim bladder demonstrated further threshold increases most notable at 600 Hz. D) The threadfin butterflyfish, *C. auriga*, has long swim bladder horns with a direct connection to the LC. Baseline thresholds increased by about 10 dB at 200-600 Hz after gas was evacuated from the swim bladder horns and swim bladder. AEP threshold data are provided in relation to sound pressure level (left column) and particle acceleration level (right column). Data are means and SE among individuals. Numbers at dots indicate sample size at each test frequency, or fraction of test subjects for which an AEP was recorded.



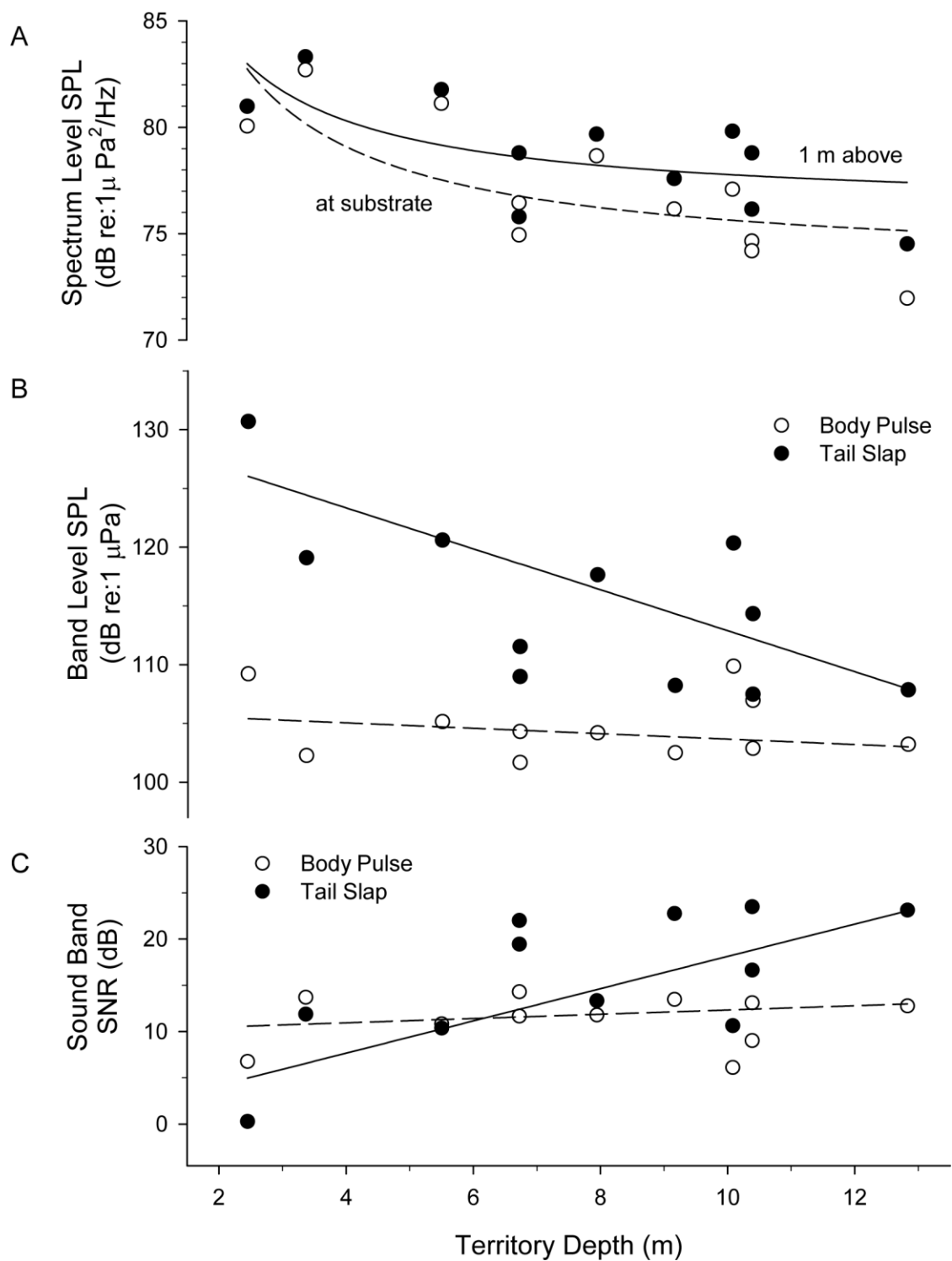
**Figure 4.** Relative change in AEP thresholds after sequential displacement of gas from the swim bladder horns and then evacuation of gas from the swim bladder in an individual *Chaetodon multicinctus* at 600 Hz stimulation. Baseline threshold is at 107 dB re:1  $\mu$ Pa. Displacement of gas from the swim bladder horn by a small injection of gel increased the threshold to 117 dB re:1  $\mu$ Pa. Subsequent evacuation of gas from the swim bladder increased the threshold to 127 dB re:1  $\mu$ Pa. Bottom traces show the stimulus waveform recorded by the hydrophone at the position of the fish head.



**Figure 5.** Summary of relative changes in the average sensitivity thresholds to sound pressure stimuli following deflation of the swim bladder in four butterflyfish species. Averaged response changes for individual fish across stimulus frequencies showed no statistical differences following swim bladder deflation in *Forcipiger flavissimus* (black circles) which lacks a laterophysic connection (LC) and the associated swim bladder horns. Different magnitudes of decreased thresholds were observed for *Chaetodon ornatissimus* (blue triangles) which has short swim bladder horns, and *C. auriga* (green squares) and *C. multicinctus* (red diamonds) both of which have long and wide swim bladder horns of the LC. Note that the peak effects on swim bladder deflation (including evacuation of the swim bladder horns) for all *Chaetodon* species ranged from 200-600 Hz with smaller effects at higher frequencies. Responses were lost for all test individuals in some species at higher frequencies following swim bladder deflation (box). Reference at 0 dB (dashed line) indicates relative baseline threshold level for each test subject.



**Figure 6.** Hearing thresholds and spectra of vocalizations used during social communication by the multiband butterflyfish, *Chaetodon multicinctus*, and the spectra of ambient noise in fish territories at Puako Reef, Hawai'i. Fish AEP thresholds determined in the lab were tested from 100-2000 Hz and show a low pass sensitivity below 600 Hz (black dots). Spectra of four representative sound types are shown. Best hearing sensitivity as determined by the AEP technique is nearest to the band of the body pulse sound used commonly in social interactions (light green medium dash curve). The infrasound tail slap (solid blue line curve) and body shake (dark green long dash curve) pulse are produced during vigorous agonistic interactions but sensitivity to these low frequency particle acceleration stimuli remain to be determined. The broadband high frequency tail click sound (dark red short dash curve) is likely beyond their hearing capabilities. The average ambient background octave noise bands are shown for 11 territories recorded at sites 2.5 – 12.8 meters deep (red dashed lines at bottom, range = shaded area) and illustrate higher noise levels in the fish hearing range in territories in shallow habitats  $\leq 6$  m deep. Data for representative sound intensities are taken from Tricas and Boyle (*in review JEB*).



**Figure 7.** The potential action of sound-band-specific ambient noise on the transmission of communication sounds in coral reef territories of the multiband butterflyfish, *Chaetodon multicinctus*, at Puako Reef, Hawai'i. A) Total ambient noise (in the 10 Hz - 24k Hz band)

measured within territories where fish pairs forage over the reef surface (open dots) are similar to levels measured 1-m up into the water column where fish also swim less frequently (solid dots). B) Background noise band pressure within the predominate frequency range (top 6 dB of the power spectrum) of the body pulse (21 - 414 Hz) and tail slap sounds (2-18 Hz) indicate that the background noise levels change with territory depth for the tail slap but not for the body pulse sound. C) Estimated ratios of signal to noise (SNR) of sound amplitudes near the source to ambient noise levels for the frequency bands of the body pulse and tail slap sounds at different territory depths. Perception of the tail slap but not the body pulse sound should improve at deeper territory locations.