PROXIMATE AND ULTIMATE ASPECTS OF ACOUSTIC AND MULTIMODAL COMMUNICATION IN BUTTERFLYFISHES

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ABSTRACT

Communication in social animals is shaped by natural selection on both sender and receiver. Diurnal butterflyfishes use a combination of visual cues like bright color patterns and motor pattern driven displays, acoustic communication, and olfactory cues that may advertise territorial behavior, facilitate recognition of individuals, and provide cues for courtship. This dissertation examines proximate and multimodal communication in several butterflyfishes, with an emphasis on acoustic communication which has recently garnered attention within the Chaetodontidae. Sound production in the genus *Forcipiger* involves a novel mechanism with synchronous contractions of opposing head muscles at the onset of sound emission and rapid cranial rotation that lags behind sound emission. Acoustic signals in *F. flavissimus* provide an accurate indicator of body size, and to a lesser extent cranial rotation velocity and acceleration. The closely related *Hemitaurichthys polylepis* produces rapid pulse trains of similar duration and spectral content to *F. flavissimus*, but with a dramatically different mechanism which involves contractions of hypaxial musculature at the anterior end of the swim bladder that occur with synchronous muscle action potentials. Both *H. polylepis* sonic and hypaxial trunk muscle fibers have triads at the z-line, but sonic fibers have smaller cross-sectional areas, more developed sarcoplasmic reticula, longer sarcomere lengths, and wider t-tubules. Sonic motor neurons are located along a long motor column entirely within the spinal cord and are composed of large and small types. *Forcipiger flavissimus* and *F. longirostris* are site attached and territorial, with *F. flavissimus* engaged in harem polygyny and *F. longirostris* in social monogamy. Both produce similar pulse sounds to conspecifics during territoriality that vary little with respect to communicative context. *Chaetodon multicinctus* can discriminate between mates and non-mate intruders, but
require combined visual and olfactory cues to display a differential agonistic response towards non-mates, which is consistent with the hypothesis that multimodal cues are required for mate recognition and may have important ramifications for the evolution of communication. Results from this dissertation indicate that acoustic communication may be widespread among chaetodontid fishes and involve variable mechanisms convergently similar to distantly related teleosts.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>I. Introduction and Background</td>
<td>1</td>
</tr>
<tr>
<td>II. Sound Production in the Longnose Butterflyfishes (Genus <em>Forcipiger</em>): Relationships with Cranial Kinematics and Muscle Activity</td>
<td>34</td>
</tr>
<tr>
<td>Abstract</td>
<td>34</td>
</tr>
<tr>
<td>Introduction</td>
<td>35</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>38</td>
</tr>
<tr>
<td>Results</td>
<td>45</td>
</tr>
<tr>
<td>Discussion</td>
<td>59</td>
</tr>
<tr>
<td>References</td>
<td>67</td>
</tr>
<tr>
<td>III. Pulse Sound Generation, Anterior Swim Bladder Buckling, and Associated Muscle Activity in the Pyramid Butterflyfish, <em>Hemitaurichthys polylepis</em></td>
<td>71</td>
</tr>
<tr>
<td>Abstract</td>
<td>71</td>
</tr>
<tr>
<td>Introduction</td>
<td>72</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>75</td>
</tr>
<tr>
<td>Results</td>
<td>82</td>
</tr>
<tr>
<td>Discussion</td>
<td>99</td>
</tr>
<tr>
<td>References</td>
<td>109</td>
</tr>
<tr>
<td>IV. Ultrastructure, Innervation, and Distribution of Motor Neurons of Extrinsic Swim Bladder Muscles in the Sound Producing Pyramid Butterflyfish (<em>Hemitaurichthys polylepis</em>)</td>
<td>112</td>
</tr>
<tr>
<td>Abstract</td>
<td>112</td>
</tr>
<tr>
<td>Introduction</td>
<td>113</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>116</td>
</tr>
<tr>
<td>Results</td>
<td>122</td>
</tr>
<tr>
<td>Discussion</td>
<td>133</td>
</tr>
<tr>
<td>References</td>
<td>148</td>
</tr>
<tr>
<td>V. Social System, Territoriality, and Agonistic Sound Production Behavior in the Syntopic Sister Taxa: <em>Forcipiger flavissimus</em> and <em>F. longirostris</em> (Chaetodontidae)</td>
<td>152</td>
</tr>
</tbody>
</table>
Abstract .......................................................................................................................... 152
Introduction ...................................................................................................................... 153
Materials and methods ..................................................................................................... 157
Results ............................................................................................................................... 166
Discussion ......................................................................................................................... 180
References ......................................................................................................................... 190

VI. Multimodal Discrimination Of Mates And Territorial Intruders: Visual and Olfactory Stimuli Influence Territorial Behavior of the Socially Monogamous Pebbled Butterflyfish (Chaetodon multicinctus) ........................................................................................................ 194

Abstract .......................................................................................................................... 194
Introduction ...................................................................................................................... 195
Materials and methods ..................................................................................................... 197
Results ............................................................................................................................... 204
Discussion ......................................................................................................................... 212
References ......................................................................................................................... 219

VII. Conclusions, Significance, and Further Research ....................................................... 223
LIST OF TABLES

I. Introduction and Background

Table 1.1: Sound production mechanisms and behavior in fishes.........................4

II. Sound Production in the Longnose Butterflyfishes (Genus Forcipiger):
    Relationships with Cranial Kinematics and Muscle Activity

Table 2.1: Acoustic properties of *F. flavissimus* and *F. longirostris* sounds.................................................................46

Table 2.2: Kinematics of *F. flavissimus* and *F. longirostris* sound production.................................................................47

Table 2.3: Relationship between *F. flavissimus* cranial kinematics and sounds.................................................................53

Table 2.4: Relationship between *F. flavissimus* body size and sound features.................................................................55

Table 2.5: Relationship between *F. flavissimus* muscle activity, sound features, and cranial elevation........................................57

III. Pulse Sound Generation, Anterior Swim Bladder Buckling, and Associated Muscle Activity in the Pyramid Butterflyfish, *Hemitaurichthys polylepis*

Table 3.1: Acoustic properties of *H. polylepis* sounds.................................84

Table 3.2: *H. polylepis* sonic muscle EMG durations........................................90

Table 3.3: Occurrence of single and multiple *H. polylepis* EMG firing types.................................................................92

Table 3.4: *H. polylepis* sonic and non-sonic muscle EMG onsets relative to sound emission.................................................................95

IV. Ultrastructure, Innervation, and Distribution of Motor Neurons of Extrinsic Swim Bladder Muscles in the Sound Producing Pyramid Butterflyfish (*Hemitaurichthys polylepis*)

Table 4.1: Sonic motor pathways and patterns among teleost fishes.................................140
V. Social System, Territoriality, and Agonistic Sound Production Behavior in the Syntopic Sister Taxa: *Forcipiger flavissimus* and *F. longirostris* (Chaetodontidae)

**Table 5.1:** Social association habits of *Forcipiger longirostris* and *F. flavissimus* ................................................................. 167

**Table 5.2:** Resightings of tagged *Forcipiger flavissimus* ......................... 169

**Table 5.3:** Resightings of tagged *Forcipiger longirostris* .......................... 170

**Table 5.4:** Features of agonistic sounds produced by *F. flavissimus* and *F. longirostris* ............................................................... 174

**Table 5.5:** Discriminant analysis models to discriminate individuals, behavioral context, and species identity in *F. flavissimus* and *F. longirostris* .... 177
LIST OF FIGURES

II. Sound Production in the Longnose Butterflyfishes (Genus Forcipiger): Relationships with Cranial Kinematics and Muscle Activity

**Figure 2.1:** Cleared and stained *Forcipiger flavissimus* and anatomical landmarks for kinematic analysis ........................................ 42

**Figure 2.2:** Acoustic features of typical pulse sounds from *F. flavissimus* and *F. longirostris* recorded in the laboratory .......................... 46

**Figure 2.3:** Kinematic associated with typical pulse sound generation in *F. flavissimus* and *F. longirostris* ........................................ 48

**Figure 2.4:** Kinematic profiles of sound production events from *F. flavissimus* and *F. longirostris* .................................................. 49

**Figure 2.5:** Two representative sound waveforms, electromyograms, and kinematic profiles, sound waveform from *F. flavissimus* sound production events .................................................. 50

**Figure 2.6:** Timing of *Forcipiger flavissimus* muscle activity and cranial elevation relative to sound emission ........................................ 51

**Figure 2.7:** Scatter plots showing the relationship of *Forcipiger flavissimus* acoustic and kinematic features ........................................ 54

**Figure 2.8:** Scatter plots showing the relationship of *Forcipiger flavissimus* acoustic features and body size ........................................ 56

**Figure 2.9:** Scatter plots showing the relationship of *Forcipiger flavissimus* acoustic features and anterior epaxialis activity ........................................ 58

III. Pulse Sound Generation, Anterior Swim Bladder Buckling, and Associated Muscle Activity in the Pyramid Butterflyfish, *Hemitaurichthys polylepis*

**Figure 3.1:** Location of sonic muscle in *Hemitaurichthys polylepis* and EMG recording electrode sites ........................................ 78

**Figure 3.2:** Representative Pyramid Butterflyfish pulse train recorded in the field ........................................ 83
**Figure 3.3:** Acoustic features of individual pulse sounds recorded in the lab. 85

**Figure 3.4:** Relationship between sound production features and body size in Pyramid Butterflyfish. 87

**Figure 3.5:** Kinematic buckling during Pyramid Butterflyfish sound production. 88

**Figure 3.6:** Muscle activity during sound production by Pyramid Butterflyfish. 91

**Figure 3.7:** Relative onset timing of sonic muscles, pulse sound onset, and buckling during Pyramid Butterflyfish sound production. 92

**Figure 3.8:** Pyramid Butterflyfish sound phase relationships between body orientation relative to hydrophone position. 94

**Figure 3.9:** Variability of sound pulse emission rates in Pyramid Butterflyfish. 97

**Figure 3.10:** Variability of sonic muscle firing emission rates in Pyramid Butterflyfish. 98

---

**IV. Ultrastructure, Innervation, and Distribution of Motor Neurons of Extrinsic Swim Bladder Muscles in the Sound Producing Pyramid Butterflyfish (Hemitaurichthys polylepis)**

**Figure 4.1:** Diagram of Pyramid Butterflyfish sonic muscle, innervation, and location of sonic motor neurons. 118

**Figure 4.2:** Cross section of Pyramid Butterflyfish sonic and hypaxial muscle fibers. 124

**Figure 4.3:** TEM cross section of Pyramid Butterflyfish sonic and hypaxial muscle. 125

**Figure 4.4:** Longitudinal TEM micrographs of Pyramid Butterflyfish sonic muscle. 126

**Figure 4.5:** Longitudinal TEM micrographs of Pyramid Butterflyfish white trunk muscle. 127
Figure 4.6: Photomicrographs of Pyramid Butterflyfish spinal cord cross sections with labeled sonic motor neurons……………………….… 129

Figure 4.7: Photomicrographs of Pyramid Butterflyfish spinal cord cross sections with labeled small-type sonic motor neurons………………………………………………………….… 130

Figure 4.8: Histogram of cell area and comparison of cell size between large and small-type Pyramid Butterflyfish sonic motor neuron somata…………………………………….… 132

V. Social System, Territoriality, and Agonistic Sound Production Behavior in the Syntopic Sister Taxa: *Forcipiger flavissimus* and *F. longirostris* (Chaetodontidae)

Figure 5.1: Schematic of setup for *F. flavissimus* and *F. longirostris* field sound production behavior experiments……………….… 160

Figure 5.2: Differential response of resident *F. flavissimus* and *F. longirostris* to caged intruders…………………………………….….… 172

Figure 5.3: Sound waveforms and power spectra of *F. flavissimus* and *F. longirostris* sounds produced in the field……………..… 173

Figure 5.4: Biplots of features used to discriminate individual *F. flavissimus* and *F. longirostris* agonistic sounds…………………………………....…….178

Figure 5.5: Classical MDS biplot of time domain similarity values between *F. flavissimus* and *F. longirostris* sounds……………………………………….179

Figure 5.6: UPGMA dendrogram and classical MDS biplot showing similarity between sound features between individual fish: *F. flavissimus* and *F. longirostris*…………………………………....…….180

VI. Multimodal Discrimination Of Mates And Territorial Intruders: Visual and Olfactory Stimuli Influence Territorial Behavior of the Socially Monogamous Pebbled Butterflyfish (*Chaetodon multicinctus*)

Figure 6.1: Experimental setup of *Chaetodon multicinctus* individual discrimination experiments…………………………………………. 199

Figure 6.2: Proportion of time spent by resident fish to model bottle stimuli in *matched odor experiments*……………………………… 206
Figure 6.3: Proportion of time spent by resident fish to model bottle stimuli in crossed odor first experiments………………………… 207

Figure 6.4: Proportion of time spent by resident fish to model bottle stimuli in intruder scent only experiments……………………….. 209

Figure 6.5: Proportion of time spent by resident fish to model bottle stimuli in mate-odor intruder-visual experiments…………….…. 211

Figure 6.6: Proportion of time spent by resident fish to model bottle stimuli in no olfactory cue experiments……………………….…. 212
CHAPTER I: INTRODUCTION AND BACKGROUND

Proximate and ultimate aspects of animal communication

Communication has important fitness consequences for a variety of animals. True communication involves two participants, sender and receiver, and requires that the signal emitted by the sender alters the probability of a particular response by the receiver to the sender’s benefit (Bradbury and Vehrencamp, 1998). Conspecific communication signals may be used for territorial defense, mate attraction, and recognition of specific individuals, such as kin, competitors, and mates (Alcock, 2001). Thus modes of communication are shaped by selection pressures that drive the coevolution of the signaling individual and the receiver. The efficacy of a communication signal depends on the physical properties of the environment and the sensory abilities of the receiver which may co-evolve and drive the evolution of the signal mechanism (Endler, 1992). Further, natural selection is predicted to act on senders to favor signaling mechanisms that avoid interception by illegitimate receivers (Myrberg, 1981; Brown et al., 2001).

Animal communication is shaped by both proximate and ultimate factors. Proximate causes of biological responses are concerned with relatively immediate reasons for a biological trait or phenomenon, e.g., how a physiological state affects a behavior, while ultimate causes deal with the adaptive and evolutionary history of the trait or phenomenon, e.g. the phylogenetic and adaptive context in which a particular behavior evolved (Mayr, 1961). The proximate causes involved in animal communication can be examined with respect to signaling mechanisms and sensory systems in terms of anatomical descriptions, functional morphology, and physiology of signal transmission.
and reception. Signaling mechanisms and sensory systems utilized in communication may also be examined within an ultimate context, as the anatomical and physiological properties of features involved in communication may be examined within a phylogenetic framework that traces evolutionary changes associated with function. Further, experimental tests on the performance of structures involved in communication can test hypotheses on their adaptive significance. Similarly, the role communication plays in animal’s behavior can also be examined in terms of proximate and ultimate factors. Examinations of proximate effects in communication include the circumstances that elicit signaling from senders and the response of receivers to communication signals. The ultimate aspects of communication involve the adaptive consequences of a particular form of communication for both the sender and receiver and the evolutionary history of the behavioral context and role of the signal.

_Fishes and communication in an aquatic environment_

Fishes largely possess the same suite of senses as other vertebrates. The great diversity of actinopterygian fishes (~26,891 spp.) (Nelson, 2006) and wide range of physical properties exhibited in different habitats occupied by various fish species make them an ideal taxon to examine the sensory ecology of communication. Aquatic environments are able to transmit chemical, acoustic, hydrodynamic, and electric stimuli efficiently. Various aquatic habitats provide different photic environments in accord with depth and turbidity. Ray-finned fishes are equipped with well-developed olfactory and gustatory systems (Hansen and Reutter, 2001); auditory systems that are receptive to particle motion and in some, to sound pressure stimuli (Popper and Fay, 1999); a lateral-
line system that is receptive to velocity and acceleration of local flow fields (Coombs et al., 1992); well developed vision (Myrberg and Fuiman, 2002); and in some taxa, electroreception (Dunlap et al., 1998; Hanika and Kramer, 2000).

**Acoustic communication in fishes**

Acoustic communication by fishes has received less attention from researchers than terrestrial animals, likely because of the added difficulty of study in an aquatic environment (Zelick et al., 1999). Sound production, however, is known from a wide variety of phylogenetically diverse ray-finned fishes (Tavolga, 1971a; Myrberg, 1981; Zelick et al., 1999; Ladich and Fine, 2006; Parmentier and Diogo, 2006; Kasumyan, 2008). Social sound production (sounds hypothesized to have communicative, including distress and warning sounds) is reported from the basal ray-finned sturgeons, and within three major clades of teleosts: the osteoglossomorphs, otomorphs, and euteleostomorphs (Table 1.1). Unlike well-studied soniferous (i.e., sound producing) tetrapod groups, ray-finned fishes do not possess a single homologous organ for sound emission that is common to most members (e.g. anuran larynx, avian syrinx, and mammalian larynx). For this reason, sound production mechanisms are poorly understood among fishes with the exception of a few well-studied groups.

Known sound production mechanisms in fishes can be classified broadly into five mechanistic categories: stridulation, indirect swim bladder mechanisms, extrinsic swim bladder muscle driven mechanisms, intrinsic swim bladder muscle driven mechanisms, and gas expulsion mechanisms (Tavolga, 1971a). Stridulation involves vibration from grinding or friction of hard structures such as teeth, bones, and tendons.
Table 1.1. Sound production mechanisms and behavioral contexts among actinopterygian fishes

<table>
<thead>
<tr>
<th>Classification</th>
<th>Mechanism</th>
<th>Context</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actinopterygii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondrostei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acipenseriformes</td>
<td>Acipenseridae</td>
<td>?</td>
</tr>
<tr>
<td><strong>Neopterygii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Teleostei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoglossomorpha</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoglossiformes</td>
<td>Mormyridae</td>
<td>ExSM&lt;sup&gt;6,17&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Otomorpha</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clupeiformes</td>
<td>Clupeidae</td>
<td>Gas Exp.&lt;sup&gt;96,97&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Clupei</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ostariophysi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Otophysa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cypriniformes</td>
<td>Cobitidae</td>
<td>♀&lt;sup&gt;81&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>♀&lt;sup&gt;39, 41&lt;/sup&gt;</td>
<td>♀courtship&lt;sup&gt;41&lt;/sup&gt;, ♀agonistic&lt;sup&gt;39,41&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Characiphysae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Characiformes</td>
<td>Characidae</td>
<td>GasExp.&lt;sup&gt;65&lt;/sup&gt;, ExSM&lt;sup&gt;21,51,88&lt;/sup&gt;</td>
</tr>
<tr>
<td>Siluriformes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artidae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspredinidae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callichthyidae</td>
<td>pect. strid.&lt;sup&gt;29&lt;/sup&gt;</td>
<td>♀&lt;sup&gt;29&lt;/sup&gt;</td>
</tr>
<tr>
<td>Doradidae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ictaluridae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mochokidae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pimelodidae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Euteleostomorpha</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neoteleostei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eurypterygia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctenosquamata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acanthomorphata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gadaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gadiformes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gadoidei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gadidae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zeacea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zeiformes</td>
<td></td>
<td></td>
</tr>
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<td>distress(^\text{62}), ♀ courtship(^\text{31})</td>
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<td>Chaetodontidae</td>
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<td>terr., agon., distress</td>
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<td>Pomacanthidae</td>
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<td>♂ agonistic &amp; courtship, distress</td>
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<td>Sciaenidae</td>
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<td>Terapontidae</td>
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<td>Diodontidae</td>
<td>pectoral fin drum\textsuperscript{62,84}</td>
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<td>oral jaw teeth strid.\textsuperscript{62}</td>
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\*\*\* indicate large uncertainty of monophyly

**Sonic mechanism abbreviations:**
- ExSM=extrinsic swim bladder muscles
- Gas Exp.=swim bladder gas bubble release
- head stridulation=transduction of supraoccipital with coronet scute
- hyoid jar closure=rapid closure of the oral jaws from an apomorphic ligamentous connection to the interinnis swim bladder muscles
- pectoral girdle vibration=vibration of pectoral girdle by contractions of protractor pectoralis (syn. cephaloclavicularis and cucularis)
- phryn. jaw strid.=stridulation of the pharyngeal jaw tooth plates
- operc. water jet=expulsion of water through the opercular opening
- oral jaws=oral jaw closure mechanism
- oral jaw teeth strid.=stridulation of the oral jaw teeth
- tendon strid.=stridulation of pectoral fin ray tendons against osseous extensions of pectoral fin rays
- trunk muscle=trunk muscle contractions without confirmation of extrinsic swim bladder musculature
- ?=unknown mechanism, pect. strid.=pectoral spine (fused pectoral rays) – pectoral girdle stridulation

**Behavioral context abbreviations:**
- agonistic or agon.=agonistic social sounds
Table 1.1. continued

<table>
<thead>
<tr>
<th>biosonar=echolocation signals</th>
<th>courtship or crtshp.=courtship sounds</th>
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<tbody>
<tr>
<td>distress=distress, startle</td>
<td>feeding=possibly non-social sounds that occur with feeding handling, or warning sounds</td>
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<tr>
<td>nest dfnse=defense sounds from nest-guarding parental care</td>
<td>post. spn.=post spawning sounds made after the spawning event</td>
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<tr>
<td>species recog.=species recognition calls</td>
<td>spwn.=spawning sounds</td>
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<td>terr.=sounds associated with territorial behavior</td>
<td>♂ sounds made predominately by males in this context</td>
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<tr>
<td>‡ sounds made predominantly by females in this context</td>
<td>References:</td>
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Examples include stridulation of the proximal end of the pectoral spine (fused rays rather than true acanthomorph spines) stridulation against a socket of the cleithrum of the pectoral girdle in various catfishes (Gainer, 1967; Ladich and Fine, 1994; Ladich and Bass, 1996; Fine et al., 1997; Kaatz and Lobel, 1999), grinding of the supraoccipital and coronet bone in seahorses (Colson et al., 1998), pharyngeal teeth grinding of haemulid
grunts (Burkenroad, 1930; Moulton, 1958), and plucking of pectoral fin ray tendons by internal, proximal extensions of pectoral fin rays in croaking gourami (Kratochvil, 1978; Ladich and Fine, 1992). Indirect swim bladder mechanisms include various methods of sound generation with anatomical features removed from contact with the swim bladder. Examples include the pectoral fin drumming mechanism over a thin body wall membrane employed by triggerfishes (Moulton, 1958; Salmon et al., 1968) and the hyoid bar oral jaw linkage mechanism recently discovered in anemonefish, in which the jaws snap close as the head is raised (Parmentier et al., 2007). Extrinsic swim bladder muscle driven mechanisms are arguably the most well-known and include sound production systems that involve muscles that originate away from the swim bladder, often from the rostral cranium, pectoral girdle, or both and insert on the swim bladder or in close association to the swim bladder, such as ribs and aponeuroses adjacent to the swim bladder. Contractions, depending on the taxon, likely stretch or compress a portion of the swim bladder and no muscle antagonist is involved. Rather, internal pressure in the swim bladder relaxes the muscle after a contraction. Examples of sonic fishes with extrinsic swim bladder muscles include squirrelfishes and soldierfishes (Winn and Marshall, 1963; Salmon, 1967), several families of catfishes (Tavolga, 1962; Ladich and Fine, 1994; Ladich and Bass, 1998; Ladich, 2001), drums and croakers (Sciaenidae) (Tower, 1908; Ono and Poss, 1982; Hill et al., 1987; Connaughton et al., 2000), and congiopodid pigfish (Packard, 1960). Intrinsic swim bladder muscle driven sound production involves contractions of the swim bladder muscles that originate and insert entirely on the swim bladder. These apomorphic muscles are known from comparatively fewer fish families and are also relaxed by internal swim bladder pressure, rather than antagonistic muscles.
Examples of fishes with intrinsic sonic muscles include toadfishes and midshipmen (Batrachoididae) (Tower, 1908; Greene, 1924), sea robins (Triglidae) (Tower, 1908; Evans, 1973, 1975; Bass, 1985; Bass and Baker, 1991; Connaughton, 2004), Walleye Pollock (Gadidae) (Park et al., 1995; Onuki and Somiya, 2006), John Dory (Zeidae) (Onuki and Somiya, 2004). The homologies of intrinsic and extrinsic muscles in fishes are unknown, but are likely derived from various trunk muscles and likely evolved independently many times (Winterbottom, 1974; Parmentier and Diogo, 2006).

Developmental patterns of swim bladder muscle formation in toadfish have shown that the intrinsic muscles form originally from occipital somites and migrate caudally (Tracy, 1959, 1961). Developmental mycology is lacking, however, from the majority of fish with sonic swim bladder muscles. Gas expulsion sound production mechanisms that arise from forcing gas through the pneumatic duct in species with physostomous swim bladders are reported from eels and catfishes (Dufossé, 1874), and a characid (Nelson, 1964). Recently, gas expulsion sound production was described from an additional posterior pneumatic duct, present in clupeids, that connects the swim bladder with the hind gut (Wahlberg and Westerberg, 2003; Wilson et al., 2004). These sounds have a potential social function, but this hypothesis has not yet been tested. Sculpins (Cottidae) possess a sound production mechanism that does not fit these five broad categories. Cottids, which lack swim bladders as adults, are able to produce sounds with rapid vibration of the pectoral girdle by muscles from behind the head without sound radiation from a swim bladder (Barber and Mowbray, 1956). In summary, a wide array of sound production mechanisms are known from fishes that often directly or indirectly involve the swim bladder (Ladich and Fine, 2006), an organ principally involved in buoyancy.
regulation that has been co-opted repeatedly for use as a sound source. In sound production, swim bladders function as an impedance matching devices that transduces small mechanical movements of the swim bladder to the aquatic medium (Tavolga, 1971a).

Behavioral contexts of sound production

An ultimate factor that governs the evolution of sound production in fishes is the adaptive significance of how acoustic signals function in communication. In order to formulate hypotheses about the function of acoustic signals in communication, detailed observations of natural behavior and experimental manipulations that test for situations that elicit signaling, and playback experiments that test responses of receivers, are needed. Unfortunately, many accounts of sound production among fishes are known only from distress-like calls made towards human observers. Known behavioral contexts of fish sound production include courtship advertisement sounds, prespawning sounds, and spawning sounds (Table 1.1). Some fishes are known to produce strong acoustic signals during courtship that are used for mate attraction in visually-limited environments. Female Plainfin Midshipmen (Batracohididae: Porichthys notatus) display a strong phonotaxis response to the hum sound produced during courtship by males (McKibben and Bass, 1998). Many croakers and drums (Sciaenidae) produce spawning choruses at night before spawning (Guest, 1978; Luczkovich et al., 1999; Ramcharitar et al., 2006; Gannon, 2007; Aalbers, 2008; Parsons et al., 2009). Atlantic Cod and Haddock (Gadidae) also produce nocturnal courtship sounds (Brawn, 1961; Engen and Folstad, 1998; Hawkins and Amorim, 2000; Finstad and Nordeide, 2004; Rowe and Hutchings,
Courtship sounds are used by some highly visual species as part of a multimodal stimulus that occurs with visual communication. Males of many damselfish (Pomacentridae) species are known to produce elaborate courtship sounds that co-occur with visual displays in clear, coral reef environments (Myrberg and Spires, 1972; Mann and Lobel, 1998; Lobel and Kerr, 1999; Maruska et al., 2007; Parmentier et al., 2009). Female Croaking Gourami (Osphronemidae: *Trichopsis vittata*), but not males, produce prespawning courtship sounds (Ladich, 2007). Courtship and spawning sounds may be more widespread among soniferous species that have not been observed and acoustically recorded during reproduction.

Sound production is associated with agonistic behavior in a variety of fishes (Table 1.1). Agonistic behaviors are behaviors on the fight or flight continuum (Scott and Fredericson, 1951), such as fighting, fleeing, and threats. Fish sound production is known from a variety of agonistic behaviors (Ladich and Myrberg, 2006) that include include food competition, nest defense, territoriality, and distress. The highly soniferous family Holocentridae (squirrelfishes and soldierfishes) produce sounds during territorial behavior and when confronted by a potential predator (Winn et al., 1964; Salmon, 1967). Several species of triggerfishes (Balistidae) produce sounds during aggressive intraspecific interactions (Salmon et al., 1968). In addition to courtship sounds, many damselfishes produce sounds associated with territory and nest defense (Myrberg, 1981, 1997; Santiago and Castro, 1997; Parmentier et al., 2006a; Maruska et al., 2007). Thus, adaptive functions of acoustic signaling, in many cases, are not limited to only one context. Fish that have evolved the morphology and neural circuitry necessary for sound emission may produce sounds in different behavioral contexts with potentially different
communicative functions. In these circumstances, the sounds may be similar, different as a result of slight differences in neural motor patterning associated with a particular behavioral context, or different because of slight differences in the sound production morphology (e.g. from seasonality of sonic muscle condition). For example, fish species such as weakfish (Sciaenidae), produce spawning choruses (Luczkovich et al., 1999; Perkins, 2001), but also produce distress sounds when held (Connaughton et al., 2000). This species experiences hypertrophic changes in the sonic muscle mass during the spawning season (Connaughton and Taylor, 1994; Connaughton et al., 1997; Connaughton et al., 2002), and thus changes to the muscles involved in distress sounds as well. Sound production in some fish taxa is known mainly from unusual anatomical features and distress sounds that are produced during handling stress. For example, the sea robins (Triglidae) have long been appreciated for their apomorph intrinsic swim bladder muscles involved in sound production for distress (Dufossé, 1874; Tower, 1908; Connaughton, 2004). Other behavioral contexts of agonistic sound production have been shown recently for captive sea robins during competition for food (Amorim and Hawkins, 2000; Amorim et al., 2004) and it is possible that sound emission is involved in other important communicative functions for fishes in this family which has not been studied extensively in the field.

In summary, acoustic communication in fishes is known from a variety of contexts. Most examples observed are courtship and spawning related sounds, territorial sounds, intraspecific aggression sounds, nest defense sounds, and distress sounds in the presence of a natural predator or when handled by humans (Table 1.1). Behavioral observations of many species are limited and often have the caveats associated with
observation of behavior in an artificial setting of a laboratory, so observations of sound emission in a particular context does not preclude the possibility that sounds are used to convey other types of information. Sound production in many species may be used in multiple contexts as evidenced by the fact that many species that produce courtship sounds also produce agonistic and distress sounds. As sound production in fishes is widespread and likely evolved multiple times (Ladich and Fine, 2006), the original communicative function for which acoustic signaling evolved may vary among taxa. The evolution of sound production musculo-skeletal morphology and neural circuitry within a taxon, further may have provided the opportunity for more behavioral functions of acoustic signals for communication under natural selection. Thus, additional contexts of acoustic signaling may be exaptations, which are selectively advantageous features that originally evolved for a different role (Gould and Vrba, 1982). For example, sound production that originally evolved for predator deterrence may be co-opted for an additional role in courtship signaling.

*Butterflyfishes*

Butterflyfishes (Chaetodontidae) are an appropriate taxon for the study of social communication. As conspicuous members of coral reefs and other nearshore environments in warm temperate and tropical seas (Allen et al., 1998), they are amenable to study in the field, have a diverse range of feeding ecologies (Bouchon-Navaro, 1986; Tricas, 1989a; Pratchett, 2005; Bellwood et al., 2009), and display diverse musculo-skeletal morphologies associated with their feeding ecology (Motta, 1982, 1985, 1989; Ferry-Graham et al., 2001a; Ferry-Graham et al., 2001b). Further, species from this
family occur in a wide range of social associations that range from socially monogamous and often territorial pairs to gregarious shoals (Reese, 1975; Fricke, 1986; Tricas, 1989b; Roberts and Ormond, 1992; Kosaki, 1999; Strang, 2005). The prevalence of these diurnally active fishes in clear waters on coral reefs, along with the bright and conspicuous color patterns of many species, has promoted research on communication that involves visual modalities (Ehrlich, 1977; Hailman, 1981; Meadows, 1993). Recent attention to the morphology of the anterior swim bladder morphology and lateral line among subgenera within the speciose genus *Chaetodon* indicates that acoustic and hydrodynamic stimuli for communication may be common within the family. Members of the genus possess anterior swim bladder bullae that project towards the otic region of the neurocranium and may transducer acoustic pressure to the ears (Webb et al., 2006), though recent evidence on the ultrastructure of chaetodontid otolithic endorgans; the saccule, lagena, and utricle of the inner ear; does not show evidence of apomorphic maculae often seen in otophysic fishes (species with connections from the swim bladder to the inner ear) with enhanced auditory abilities (Webb et al., 2010). Further, *Chaetodon* possesses a connection between the lateral-line and swim bladder (laterophysic connection) that occurs at the lateral bulla surface and medial supracleithrum lateral-line canal and varies among species with regard to swim bladder proximity and type of tissue (muscle, mucoid connective tissue and kidney) between the bulla and canal neuromast (Webb, 1998; Webb and Smith, 2000; Webb et al., 2006). The laterophysic connection potentially may transduce acoustic pressure stimuli to the trunk lateral line. Social sound production behavior was described recently for a socially monogamous and territorial butterflyfish, *Chaetodon multicinctus* (Tricas et al., 2006). Thus, acoustic
communication may be a prominent feature of social communication within the Chaetodontidae that has been unexplored. The complex behavioral ecology of butterflyfish in which many species are territorial and maintain long-term pair bonds with mates, and must recognize conspecifics including mates and nearby territorial holders, make them an ideal group to examine aspects of multimodal communication.

_Butterflyfish evolution and biogeography_  

The butterflyfish family Chaetodontidae comprises approximately 122 species that are found world-wide in tropical and warm temperate seas, with greatest representation in the Indo-West Pacific (Nelson, 2006). Butterflyfishes are hypothesized to have evolved from non-coral reef, deeper dwelling (50-200 m) ancestors and based on phylogenetic evidence of the distributions of extant taxa, may have colonized coral reef habitats at least two times in their evolutionary history (Bellwood and Wainwright, 2002). Fossil chaetodontid fish are known from the Oligocene and Miocene (Carnevale, 2006; Micklich et al., 2009), and thus were present within the Tethys ocean. Two robust molecular phylogenetic hypotheses support a family origin in the early Eocene from an area now corresponding to the western Pacific, and multiple subsequent invasions into the Atlantic (Fessler and Westneat, 2007; Bellwood et al., 2009). The Chaetodontidae contains two monophyletic clades that differ somewhat in morphology and feeding ecology: the butterflyfish clade, which contains the genus _Prognathodes_ and the speciose genus _Chaetodon_; and the long-snouted bannerfish clade (Fessler and Westneat, 2007; Bellwood et al., 2009), which contains the genera _Amphichaetodon, Chelmon, Chelmonops, Coradion, Forcipiger, Hemitaurichthys, Heniochus_, and _Johnrandallia_. 
Social sound production was described first from a member of the genus *Chaetodon* (Tricas et al., 2006). The sister taxon to butterflyfishes comprises the angelfishes (Pomacanthidae) (Bellwood et al., 2009), for which two species are also reported to produce sounds during agonistic social interactions (Moulton, 1958). Chaetodontids are part of a broader, unresolved group of percomorph-like fishes (Percomorphacea incertae sedis) based on the taxonomy proposed by Wiley and Johnson (2010). Other taxa often included as outgroup taxa for Chaetodontidae in phylogenetic analyses include Drepanidae, Scatophagidae, Kyphosidae, Microcanthidae, and Ephippidae (Blum, 1988; Fessler and Westneat, 2007; Bellwood et al., 2009). Of these potential outgroups to chaetodontids and pomacanthids, an ephippid, *Chaetodipterus faber*, is reported to produce distress sounds with intrinsic swim bladder musculature (Burkenroad, 1931).

In summary, butterflyfishes represent a diverse radiation of fishes likely of west Pacific origin and sister to the pomacanthids. The higher level relationships of these two percomorph taxa are still unresolved. Sound production behavior is known in detail from one species, *Chaetodon multicinctus*. Accounts of sound production also exist for pomacanthids and an ephippid. Detailed accounts of sound production from teleosts related to chaetodontids, however, are largely lacking.

*Butterflyfish social behavior*

Butterflyfishes have garnered attention from researchers interested in their widespread pairing behavior. Social monogamy is displayed by many species within the family (Hourigan, 1989; Roberts and Ormond, 1992; Whiteman and Côté, 2004) and is unusual among teleost fishes, especially given that butterflyfishes do not have parental
care. Monogamous fishes without parental care are often strongly site attached and involved in joint territorial defense (Barlow, 1984; Whiteman and Côté, 2004), both characteristics that typify many butterflyfish species that have been studied (Hourigan, 1989; Tricas, 1989b; Roberts and Ormond, 1992; Righton et al., 1998). Social monogamy is known mostly for members of the genus *Chaetodon*, for which many species are corallivorous (Reese, 1975; Hourigan et al., 1988; Bellwood et al., 2009). Fewer detailed accounts of monogamy are known from the long-snouted bannerfish clade, with the exception of long-term pair fidelity in a bannerfish, *Heniochus intermedius* (Fricke, 1986). In addition to social monogamy, harem polygyny is known for some species of butterflyfish (Hourigan, 1987; Yabuta and Kawashima, 1997) and several species of pomacanthid angelfishes (Hourigan et al., 1989; Sakai and Kohda, 1997). The harem mating system in butterflyfishes and angelfishes, like that of monogamous species, involves territorial defense. Harem systems reported for two butterflyfish species, however, involve feeding territories defended only from conspecifics of the same sex (Hourigan, 1987; Yabuta and Kawashima, 1997). In these harems, males occupy larger territories that overlap several female territories. Lastly, some species of butterflyfishes are affiliative and can occur in moderately dense shoals and schools (Allen et al., 1998). Schooling often occurs in planktivorous species that consume non-defendable resources (Hourigan, 1989). Like corallivory, which is widespread and likely evolved independently several times (Bellwood et al., 2009), pairing behavior and schooling likely evolved repeatedly within the family.

Multimodal communication is likely to be important for maintenance of social cohesiveness in butterflyfishes. Butterflyfishes are largely diurnal, with the exception of
*Chaetodon lunula* and *C. quadrimaculatus*, which sometimes forage at night (Hourigan, 1987; Allen et al., 1998). In addition, their laterally compressed bodies and conspicuous coloration (Ehrlich, 1977; Kelley and Hourigan, 1983; Meadows, 1993) indicate the likely importance of visual communication. Further evidence comes from behavioral observations that show strong agonistic responses that begin with directed swimming from a large distance (Reese, 1975; Tricas, 1985). Territorial butterflyfish are known to produce complex communication displays and greeting rituals that have a strong visual component (Reese, 1975; Yabuta, 1999, 2000; Yabuta, 2002). Recently, social sound production was documented in a territorial monogamous butterflyfish during agonistic behaviors associated with territorial defense and between pair members when fish were in distress (Tricas et al., 2006). The behaviors associated with these sounds may have strong acoustic (sound pressure and particle motion likely to stimulate the inner ears) and hydrodynamic (i.e., lateral line) components (Tricas et al., 2006). The laterally compressed bodies of fishes in this family may be especially well suited to produce strong hydrodynamic components at close range. Further, the close interactions of social butterflyfishes during pairing and agonistic territorial behavior may involve chemical communication that employs olfaction. Observations of butterflyfish spawning (Lobel, 1978, 1989; Yabuta, 1997) and during courtship-like behavior (Londraville, 1990) indicate that male fish approach the urogenital region at very close proximity that could be important for the transmission of olfactory cues.

The nature of territoriality and butterflyfish mating systems is consistent with the hypothesis that multimodal communication is critical for the maintenance of these social behaviors. For long-term monogamous or harem associations to persist, fish must be
able to discriminate conspecific mates and non-mates. Otherwise, social bonds would weaken access to spawning partners. Further, monogamous and harem butterflyfishes tend to be territorial species that defend access to food resources. The ability to discriminate between conspecific neighbors (i.e., dear-enemies, familiar neighbors with established territory boundaries), non-familiar conspecifics that pose a competitive threat if granted access to defendable resources, and mates, is ultimately important for the reproductive fitness of butterflyfish. Communication between conspecifics is likely to facilitate recognition of mates and non-mates. Visual signals evidenced by conspicuous color patterns, body shape, and behavioral responses from fish that occur at large distances (>5m) when in apparent visual contact, are clearly important for communication of territorial behavior and pairing with mates. At shorter distances; acoustic, hydrodynamic, and chemical stimuli may provide additional cues for receiver fish to assess the motivation of another fish. These cues may have been under heavy selection pressure to evolve into signaling mechanisms for communication.

**Dissertation aims and scope**

The purpose of this dissertation research is to examine the proximate and ultimate factors of acoustic and multimodal communication within representative butterflyfishes. Acoustic communication in fishes is widespread, production of social sounds has evolved repeatedly within actinopterygians, and sound production mechanisms of many taxa are completely unknown or poorly understood. Sound production behavior was described only recently in butterflyfish, but is likely widespread. This family provides an opportunity to examine mechanisms of sound production in order to determine if
morphological features similar to other soniferous teleosts are used for sound generation. This research also examines whether butterflyfishes have evolved novel features and kinematic patterns for the generation of sounds. The relationship between functional performance and signal prediction is tested and examined with respect to predictions of signal honesty. Predictions of the function of acoustic signals are tested in the field with respect to the social behavior of two butterflyfish species, which previously were not well-studied. Lastly, this research explores the role of additional sensory pathways in social communication in butterflyfishes.

Specific aims:

1) Determine the kinematics, muscle activity patterns, and functional performance of sound production in two closely related species of the long-snout bannerfish clade (Chapter II)

This research examines agonistic sound production in two species of related butterflyfishes in the long-snout bannerfish clade: *Forcipiger flavissimus* and *F. longirostris*. The functional morphology of acoustic signals in one butterflyfish, the forcepsfish *Forcipiger flavissimus*, are examined with a novel approach similar to methods employed by researchers that have examined mechanics of aquatic feeding in order to test for a relationship between sound production biomechanics and acoustic signal features. Hydrophone recordings of agonistic sounds were made in synchrony with high-speed video to examine kinematics associated with sound emission of the head and body. Electromyography was employed to examine the activity of muscles associated with cranial motion that occurs when both of these species produce sounds.
This chapter describes a novel mechanism of sound production in teleost fishes and examines the relationship between kinematics, body size, and acoustic features.

2) Test of extrinsic sonic muscle activity during pulse train sound production in Pyramid Butterflyfish (Chapter III)

This chapter examines sound production behavior and motor activity in the Pyramid Butterflyfish, *Hemitaurichthys polylepis*. The butterflyfish genus *Hemitaurichthys* is a member of a clade that includes bannerfishes (*Heniochus*) and Barberfish (*Johnrandallia nigrirostris*). This clade is sister to *Forcipiger* and is estimated to have diverged 9.9-19.4 ma (Bellwood et al., 2009). Sound production in *H. polylepis* is dramatically different than the cranial kinematic sound production behavior examined in Chapter II. During sound production in *H. polylepis*, extrinsic sonic muscles at the anterior swim bladder contract and occur with strong, non-burst like electromyograms (EMGs) that are indicative of synchronous contractions. During sound emission, the anterior swim bladder buckles inward with the production of each pulse. These extrinsic swim bladder muscles allow Pyramid Butterflyfish to produce rapid pulse trains, unlike the cranial elevation pulses produced in the genus *Forcipiger*, in which head-bob like motions cannot be repeated in fast succession. The sonic motor pattern of *H. polylepis*, thus bears more similarity with teleosts that produce pulse train sounds.

3) Pyramid Butterflyfish sound production morphology, muscle ultrastructure, and motor innervation (Chapter IV)

This chapter examines the morphology of Pyramid Butterflyfish, (*Hemitaurichthys polylepis*) sound production muscles, skeletal morphology, muscle
ultrastructure, muscle innervation, and motor neurons in order to identify unique patterns and potential adaptations for sound production in this taxon. Sonic production muscles in fishes require rapid synchronous contractions in order to produce sounds. Transmission electron microscopy (TEM) was used to examine the ultrastructure of pyramid butterflyfish sound production muscles to determine if similar or novel modifications of the sonic muscles are present in these fishes. Pathway tracing of sonic motor neurons also was conducted in order to determine the location of motor neurons involved in sound production and to relate results to the patterns known from other sonic fishes.

4) Social behavior and sound production in Forcepsfish and Longnose Butterflyfish (Chapter V)

This chapter examines the mating systems of the Forcepsfish (*Forcipiger flavissimus*) and Longnose Butterflyfish (*F. longirostris*) on reefs of west Hawaii Island. Predictions of the hypothesis that sound production is used in territorial behavior between social groups were tested. Sounds between these related, sympatric species were also compared to test the prediction that sounds from similar congeneres would differ in a consistent manner in order to facilitate behavioral isolation of individuals from each species. This study used a combination of detailed field observations by divers on scuba, tagging fishes and resurveys, and field experiments in which conspecific fish were introduced into the home range areas of other fishes in order to test for agonistic behavior consistent with territoriality. Sounds were recorded during agonistic intruder experiments and analyzed for differences between context (sex of intruder fish, sounds produced to intruders, sounds produced to mates) and between species.
5) Behavioral discrimination of mates and territorial intruders: effects of multimodal stimuli (Chapter VI)

Monogamous and harem butterflyfishes must discriminate routinely between mates and non-mates. Many studies have been conducted on behavioral recognition and discrimination of individuals using teleosts in the laboratory with species that may not need to routinely distinguish individual conspecifics. Butterflyfishes are a taxon for which individual discrimination is likely to be important for most individuals of social species living on coral reefs. These experiments test the agonistic behavioral response of fish in the field when presented simultaneously with their mates and with a non-familiar conspecific of similar size within their feeding territories. Predictions of the hypothesis that multimodal sensory modalities facilitate recognition of mates are tested by experiments that limit or alter available cues and signals. These experiments examine the contribution of visual and chemical cues available for discrimination of individuals.

Organization of dissertation

Chapters II-VI are each written as separate manuscripts for publication. Thus some repetition of introductory, discussion, and referenced material was unavoidable.

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CHAPTER II:
SOUND PRODUCTION IN THE LONGNOSE BUTTERFLYFISHES (GENUS
*FORCIPIGER*): RELATIONSHIPS WITH CRANIAL KINEMATICS AND
MUSCLE ACTIVITY

ABSTRACT
A variety of teleost fishes have evolved sound production mechanisms that do not require
intrinsic or extrinsic swim bladder musculature. We examined the kinematics of the
cranium, median fins, and caudal peduncle in both species of the genus *Forcipiger:*
Forcepsfish *F. flavissimus* and Longnose Butterflyfish *F. longirostris*. We found that
during agonistic behavior towards conspecifics, both species produce single pulses
associated with rapid cranial rotation that follows the onset of sound emission. Cranial
elevation velocities and accelerations exceed those reported in rapid prey strikes of many
ram and suction feeding fishes. Electromyography (EMG) indicated activity of anterior
epaxialis, sternohyoideus, and A1 and A2 adductor mandibulae in close association with
the onset of sound emission, preceding visible cranial elevation. The observed kinematic
and muscle activity pattern is consistent with sound production in which epaxial activity
begins to rotate the head dorsally while a linkage between the pectoral girdle is
maintained via simultaneous activity by the adductor mandibulae and sternohyoideus
musculature in order to pull the girdle and ribs anteriorly, which are in close association
with the swim bladder. We tested predictions of the hypothesis that acoustic signals are
indicators of kinematic performance and body size. In Forcepsfish, variation in sound
duration and sound pressure level (SPL) is explained in part by cranial elevation velocity
and epaxial EMG duration. Body size, however, explains most variation in duration and
SPL. These observed associations indicate that Forcepsfish sounds may be accurate
indicators of size and condition and perhaps reflect resource holding potential.
INTRODUCTION

Teleost fishes independently have evolved a variety of mechanisms for social sound production that include use of musculo-skeletal elements beyond the more familiar extrinsic and intrinsic swim bladder mechanisms of many soniferous fishes (Ladich, 2001; Ladich and Bass, 2003). Examples include pectoral fin tendon plucking of the Croaking Gouramy *Trichopsis vittata* (Kratochvil, 1978), pectoral girdle vibration of sculpins (Cottidae), (Barber and Mowbray, 1956; Bass and Baker, 1990), drumming of the pectoral fins against the body in triggerfishes (Balistidae) (Moulton, 1958; Salmon et al., 1968), and the stridulation of pharyngeal tooth plates in grunts (Haemulidae) (Burkenroad, 1930; Moulton, 1958). Recently, a functional morphological study has documented the production of pulse sounds in an anemonefish (*Amphiprion clarkii*) that involves elevation and coincident lowering of the hyoid bar, and a unique ligament that closes the jaw following high amplitude cranial elevation (Parmentier et al., 2007). The anemonefish sound production mechanism may involve exaptations of kinematics and morphological features that evolved for feeding (Parmentier et al., 2007). In taxa with sound production mechanisms co-opted from structures typically involved in other activities such as feeding, the interaction between anatomical features and the properties of the emitted sound are often unknown.

Numerous studies have examined the functional morphology and evolutionary trends involved in cranial and oral jaw kinematics of prey capture by teleost fishes (Ferry-Graham and Lauder, 2001). Rapid cranial elevation is associated with ram and suction feeding of many species (Bergert and Wainwright, 1997; Ferry-Graham et al., 2001a; Svanbäck et al., 2002; Gibb and Ferry-Graham, 2005; Flammang et al., 2009).
High-speed video can test for kinematic associations between timing of movement of predator and prey (Ferry-Graham et al., 2001a; Wainwright et al., 2001; Konow and Bellwood, 2005; Flammang et al., 2009) and of kinematics and suction pressure generated within the buccal cavity (Svanbäck et al., 2002). Additionally, muscle function during a feeding strike has been examined with electromyography (EMG) to determine timing of activity (Grubrich and Wainwright, 1997; Konow et al., 2008; Van Wassenbergh et al., 2008) and with sonomicrometry to examine muscle strain (Carroll and Wainwright, 2006). Similar approaches can be used to determine the kinematic and muscle activity relationships in fishes that utilize cranial elements in sound emission.

Social sound production recently was described for two species of the coral reef dwelling butterflyfish family (Chaetodontidae). *Chaetodon multicinctus*, a socially monogamous species, produces several classes of sounds during agonistic interactions with conspecifics in defense of coral feeding territories and towards mates in a putative distress call context (Tricas et al., 2006). The direct mechanism for sound emission in *C. multicinctus*, however, is unknown. *Hemitaurichthys polylepis* is a non-territorial butterflyfish that produces sounds during courtship that involve inward buckling over the anterior swim bladder and activity of hypaxial musculature in the same region (Boyle and Tricas 2010). Sounds from *H. polylepis* are of comparable spectral content to *C. multicinctus*, but are known from different social contexts than *C. multicinctus* and occur in rapid trains in a manner similar to soldierfish (Holocentridae) sounds. These recent findings indicate that diversity in sound production mechanisms and behavioral contexts likely exist within the family Chaetodontidae.
The chaetodontid genus *Forcipiger* comprises two Indo-Pacific species: the Forcepsfish *F. flavissimus* and Longnose Butterflyfish *F. longirostris* (Allen et al., 1998). On the Kona coast of Hawaii, the Forcepsfish is socially haremic and the Longnose Butterflyfish is socially monogamous. Both species defend territories against conspecifics (KSB unpublished). In aquaria, agonistic sounds are emitted readily towards conspecifics. Sound emission accompanies a rapid, stereotyped motion that involves cranial elevation. The relationship between the kinematics involved in sound production is unknown and may have important consequences for signal costs and honesty (Fitch and Hauser, 2002; Mitchell et al., 2008). Honest signals reflect properties of the signaler (e.g., body size or health) that may be of importance to receivers (e.g., mates or territorial rivals). Variation in the ability to generate movements required for signal production, i.e., kinematic performance, may be reflected in features of the sound.

In this study, we describe the cranial, body, and median fin kinematics involved in sound emission for the genus *Forcipiger*. Additionally, we test predictions of the hypothesis that acoustic signals are indicators of 1) kinematic performance, 2) body size, and 3) muscle activity. We test for relationships between sound features (frequency and two correlates of sound loudness, duration and sound pressure level) and cranial elevation kinematics, body size, and activity of several muscles associated with movement of the head, oral jaws, and hyoid of teleost fishes via EMG. Results from this study support a relationship between acoustic features and body size and cranial elevation velocity in this sound production mechanism which is previously unknown among teleost fishes.
MATERIALS AND METHODS

Sound production kinematic experiments

Experiments were conducted in the laboratory on 14 Forcepsfish *Forcipiger flavissimus* Jordan & McGregor and four *F. longirostris* (Broussonet), two species with broad Indo-Pacific distributions (Randall, 2007), obtained from Hawaiian waters by commercial suppliers. Experiments were conducted in a flow through 110 L aquarium (76 cm wide, 30 cm deep, 46 cm high). During experiments, water was shut off to minimize background noise, and water level was lowered to about 20 cm (43% of aquarium capacity). In order to elicit sound production, a single fish was placed within the aquarium first and a second fish was introduced some time later (typically about 30 minutes) and fish were allowed to maintain visual contact (separated by a thin piece of acrylic) or were allowed to interact when not too aggressive. All experiments took place at a water temperature between 25 and 28°C.

The aquarium was illuminated with four 500W quartz halogen lights and an acrylic sheet with a 1cm grid was placed behind the fish. Sound production events were pre-trigger recorded at 600 frames per s, 432X192 pixels with a Casio Ex-F1 Exilim camera. All image sequences used in analyses had a resolution of at least 7.1 pixels per cm and 75% of all sequences had a resolution $\geq$ 10 pixels per cm. Fish were represented in all image sequences at a minimal resolution of 75 pixels per body length (standard length, SL) with 75% of all observations $\geq$ 120 pixels per SL. This resolution allowed for cranial elevation rotation estimates (see Results) at a resolution of at least 1.7 degrees per pixel, with 75% of all observations with resolutions of 0.71 degrees per pixel or greater. A flasher circuit with LEDs was recorded visually by the camera while square
pulses were digitized and recorded simultaneously on the hydrophone channel in Spike 2 in order to synchronize video data with sound and electromyography (EMG) data (see below).

A calibrated Brüel and Kjaer 8103 hydrophone (-211 dB re: 1V/µPa connected to a Nexus conditioning amplifier with 60 dB gain) positioned approximately 3 cm from the aquarium end was used to detect fish sounds. A CED micro 1401 data acquisition system (Cambridge Electronic Design) and Spike 2 software was used to digitally record sounds on a computer (initial rate sampled initially at 40 kHz). Cool Edit Pro 2.0 software was used to low pass filter and downsample files to 4 kHz with the ‘high quality’ setting. The resulting bandwidth (0-2kHz) was well below the minimum resonance frequency of 4574 Hz estimated for the 20 cm water depth in the aquarium (Akamatsu et al., 2002).

**EMG experiments**

EMGs were recorded from several candidate muscles of *F. flavissimus* in order to determine associations with sound production. Two to four candidate muscles were tested within free-swimming subjects. Candidate muscles were chosen based on preliminary analysis of high-speed video during sound emission that revealed a rapid cranial elevation component (see Results) and based on observations of a related chaetodontid, *Hemitaurichthys polylepis* (Boyle & Tricas, 2010). EMG recording electrodes (Figure 1) were placed in the anterior epaxial musculature (EP) in seven individuals, approximately 0.5 cm posterior to the supraoccipital bone and at a dorso-ventral level approximately 50% of the dorso-ventral axis of the supraoccipital. EMG electrodes were placed in the sternohyoideus (SH) near the caudal portion of the urohyal,
approximately 1-2 mm off the midline, in five individuals. EMG electrodes (two) were placed in the A1 (AM1) and A2 (AM2) subdivision of the adductor mandibulae in two individuals. In one individual, an EMG electrode was inserted into tail epaxial musculature midway between the caudal end of the body cavity and caudal peduncle (TEP), approximately one cm above the midline. Based on observations of muscle activity during sound production in *H. polylepis* (Boyle & Tricas, 2010) an electrode was placed in anterior hypaxial (HP) musculature caudal to the pectoral girdle and rib of the 4th vertebra, at a level approximately 25% along the ventral-dorsal axis of the supracleithrum.

Bipolar recording electrodes that consisted of pairs of 0.05 mm insulated tungsten wire (California Fine Wire) inserted into a 28 gauge hypodermic needle with tips (1mm) exposed and bent back to form opposing hooks. Prior to electrode implantation, fish were anesthetized in 100 mg/L of tricaine methanesulfonate (MS-222, Argent Labs) and ventilated with seawater and anesthetic solution while the electrodes were implanted and the hypodermic needle tips were removed. A loop of surgical suture thread was placed in dorsal trunk musculature, tied around and glued to the electrodes with cyanoacrylate in order to provide strain relief and prevent dislodgement of the electrodes. Fish were revived by ventilation with seawater and placed back in the aquarium after a recovery of approximately 30 minutes.

**Muscle function experiments**

Several manipulative experiments were conducted to demonstrate a direct role of muscle activity to sound emission. Experiments were conducted after recording several sounds
from the same individual fish prior to manipulation. In order to examine the role of the sternohyoideus firing in muscles to pulse sound emission during headbob sounds (see Results), fish were anesthetized with MS-222 (as above) and the sternohyoideus was inactivated with an injection of 30 μL of 2.0% Lidocaine in the muscle. An initial injection of 30 μL 0.2% lidocaine (Crawford and Huang, 1999) did not appear to cause any inactivation to the sternohyoideus. To test efficacy, 30 μL of 0.2% lidocaine was injected into the abductor superficialis of the pectoral fin. No reduction in pectoral fin activity was observed, so a 30 μL injection of 2.0% lidocaine was tested which did result in a temporary (~20 min.) cessation of activity. A 2.0% 30 μL injection was then administered to both right and left sternohyoideus muscles in the same individual. The experiment was repeated with the same volume and full lidocaine dosage in a second individual fish. After apparent recovery (100 minutes later), a 30 μL injection was administered in the right and left anterior epaxialis musculature in the same fish.

Additionally, sound production was recorded from three individuals prior to and after sternohyoideus transection. Fish were deeply anesthetized with MS-222 and a cut was made across sternohyoideus fibers, perpendicular to and deep to the lateral edge of the caudal portion of the urohyal, below the opercle. Fibers of the sternohyoideus insert on the broad, lateral face of the urohyal and thus the perpendicular cut was expected to weaken the linkage between the pectoral girdle and hyoid bar, but not sever all fibers of the muscle.

Kinematic analyses

Quicktime movie files were converted to tiff image stacks and analyzed with Image J
software. X-Y position coordinates were calculated for six skeletal features based on external landmarks (Figure 2.1): 1) the tip of the premaxilla, 2) the dorsal portion of the neurocranium (estimated from external morphology), 3) dorsal margin of the pectoral fin base (reference point), 4) origin of the anal fin, 5) distal tip of anal fin spine II, and 6) dorsal margin of the caudal peduncle. An initial starting reference position 10 frames (0.0167 s) before visible onset of the sound waveform was used to estimate change in cranial elevation angle, change in angle between landmarks 1-2-3 from the initial starting reference, premaxillary protrusion estimated as a change in distance from landmark 1-2 from the initial starting reference, anal fin erection angle was estimated as the change in angle 3-4-5 from the starting reference, and caudal peduncle elevation angle was

Figure 2.1  Cleared and stained *Forcipiger flavissimus* specimen showing the skeletal elements, location of swim bladder (outlined by dotted line), points digitized for kinematic analyses (circles), and EMG recording electrode locations (squares).
estimated as the change in angle 2-3-6. Digitization noise from kinematic data was reduced with a 4th order Butterworth zero phase-shift low-pass filter (e.g. Van Wassenbergh et al., 2007) of 100 Hz and velocity and acceleration were calculated.

Several variables were calculated for cranial elevation, premaxillary protrusion, anal fin erection, and caudal peduncle elevation kinematic data: 1) the maximum cranial elevation angle, maximum premaxillary protrusion, minimum anal fin erection angle, and minimum caudal peduncle elevation angle; 2) the time of position at extreme relative to sound onset; 3) the greatest linear velocity for premaxillary protrusion and greatest angular velocity for all other features; and 4) the greatest linear acceleration for premaxillary protrusion and greatest angular velocity for all other features. Values for 3 and 4 were calculated for the negative phase of anal fin erection and caudal peduncle elevation.

**Sound and EMG analyses**

Sound waveforms were examined aurally and visually with Cool Edit Pro 2.0 software. Sound duration was determined by visual inspection of sounds relative to background noise. Custom Matlab 7.0 programs were used to measure peak-to-peak sound pressure level (SPL dB re: 1μPa) and to estimate power spectra with a zero-padded 1024 point fast Fourier transforms (FFT) with a Hanning window. The following spectral features were determined from 512 frequency values and relative amplitudes values obtained from FFTs:

1) peak frequency, the frequency value with greatest relative amplitude
2) proportion of bandwidth within 10 dB of peak (proportion of BW)

\[
\text{proportion of BW} = \frac{\text{no. of frequencies} \geq (0.316 \times \text{maximum relative amplitude})}{512}
\]

3) minimum frequency 10 dB from peak (10 dB min)

\[
10 \text{ dB min} = \text{lowest frequency} \geq (0.316 \times \text{maximum relative amplitude})
\]

4) maximum frequency 10 dB from peak (10 dB max)

\[
10 \text{ dB max} = \text{greatest frequency} \geq (0.316 \times \text{maximum relative amplitude})
\]

5) median frequency within 10 dB of peak (median frequency)

\[
\text{median frequency} = \text{median of frequencies with amplitudes} \geq (0.316 \times \text{maximum relative amplitude})
\]

EMG oscillograms were rectified with Spike 2.0 software. Muscle activity was estimated by calculating time periods for which the rectified waveform was three times the average background noise level.

**Statistical analyses**

Means and standard error were determined from averages of each individual for sound and EMG features. Multiple regression models were used to assess correlations between acoustic features (dependent variables) and cranial kinematic variables (independent variables). Separate tests were conducted for each acoustic feature (median frequency, sound duration, SPL) and cranial kinematic variables (maximum angle, time of maximum angle, maximum angular velocity, maximum angular acceleration). Additional regressions between acoustic features and body size (SL) and between acoustic features and muscle activity also were conducted. All regressions models included an individual
fish subject factor, and were first tested with an interaction term that was removed from the model when $P>0.05$. When data failed to meet assumptions for normality and homogeneity of variance, they were $\log_{10}$ or rank-transformed. Multiple comparisons from the separate regression models were corrected with a sequential Bonferroni procedure (Rice, 1989). Sternohyoideus muscle function experiments were analyzed with a general linear model (GLM) with a random subject factor and a two sample t-test was used to test the effect of lidocaine on anterior epaxial musculature in one individual. All statistical tests were conducted with Minitab v. 13.31 software.

**RESULTS**

A total of 218 Forcepsfish (*F. flavissimus*) and 14 Longnose butterflyfish (*F. longirostris*) pulse sounds were obtained during agonistic interactions in which conspecifics were in close proximity. Both species produced similar sounds. However, *F. longirostris* individuals were less aggressive in the aquarium and less prone to produce sounds. Sounds from both species were of short duration and of low frequency, with most energy below 750 Hz (Figure 2.2, Table 2.1).

High speed video revealed a stereotypical kinematic pattern that occurred for both species and was closely associated with sound emission onset. During sound emission, both species were found to elevate the cranium rapidly (Table 2.2, Figure 2.3). Cranial elevation from these ‘headbob’ sounds was characterized by moderate cranial elevation angles relative to the pectoral girdle and body, and high angular velocities and accelerations (Table 2.2) and these elevations occurred for all sounds recorded on video for both species. In all observations, cranial elevation occurred after the initial onset of
Figure 2.2. Acoustic features of typical pulse sounds from *Forcipiger flavissimus* (A, C, D) and *F. longirostris* (B, D, E). Oscillogram (A, B), spectrogram (C, D), and power spectrum (E, F). Note the similarity between sounds from both species and the concentration of acoustic intensity from 100-500 Hz. Spectrogram settings: 1024 point FFT, 2.5 % window length, 95% window overlap.

Table 2.1. Acoustic properties of sounds.

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<th>proportion of BW</th>
<th>peak frequency (Hz)</th>
<th>10 dB min (Hz)</th>
<th>10 dB max (Hz)</th>
<th>Median Frequency (Hz)</th>
<th>SPL (dB)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Forcipiger flavissimus</em></td>
<td>14</td>
<td>218</td>
<td>3-29</td>
<td>0.098±0.010</td>
<td>0.06±0.01</td>
<td>100±21</td>
<td>16±6</td>
<td>333±42</td>
<td>145±20</td>
<td>132±2</td>
</tr>
<tr>
<td><em>Forcipiger longirostris</em></td>
<td>4</td>
<td>14</td>
<td>2-7</td>
<td>0.074±0.007</td>
<td>0.03±0.01</td>
<td>62±33</td>
<td>3±2</td>
<td>156±62</td>
<td>74±32</td>
<td>127±2</td>
</tr>
</tbody>
</table>

N = number of individual fish observed, n = number of sounds, Range = range of n observed per individual fish.
Data are presented as means of individual fish averages ± s.e.m.
Table 2.2. Summary of kinematic features of *Forcipiger flavissimus* and *F. longirostris* sound production.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>n</th>
<th>n range</th>
<th>Position at extreme</th>
<th>Time of position at extreme</th>
<th>Velocity</th>
<th>Acceleration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial elevation max</td>
<td></td>
<td></td>
<td></td>
<td>degrees</td>
<td>s</td>
<td>degrees s(^{-1})</td>
<td>degrees s(^{-2})</td>
</tr>
<tr>
<td><em>F. flavissimus</em></td>
<td>14</td>
<td>146</td>
<td>3-29</td>
<td>6.3 ± 0.3</td>
<td>0.057 ± 0.003</td>
<td>932 ± 52</td>
<td>191521 ± 12269</td>
</tr>
<tr>
<td><em>F. longirostris</em></td>
<td>4</td>
<td>14</td>
<td>2-7</td>
<td>6.2 ± 1.1</td>
<td>0.070 ± 0.006</td>
<td>588 ± 108</td>
<td>116894 ± 22141</td>
</tr>
<tr>
<td>Premaxillary protrusion max</td>
<td></td>
<td></td>
<td></td>
<td>cm</td>
<td>s</td>
<td>Cm s(^{-1})</td>
<td>Cm s(^{-2})</td>
</tr>
<tr>
<td><em>F. flavissimus</em></td>
<td>14</td>
<td>146</td>
<td>3-29</td>
<td>0.11 ± 0.01</td>
<td>0.056 ± 0.004</td>
<td>24 ± 1</td>
<td>7809 ± 332</td>
</tr>
<tr>
<td><em>F. longirostris</em></td>
<td>4</td>
<td>14</td>
<td>2-7</td>
<td>0.76 ± 0.13</td>
<td>0.075 ± 0.010</td>
<td>79 ± 10</td>
<td>13973 ± 1818</td>
</tr>
<tr>
<td>Anal fin elevation max</td>
<td></td>
<td></td>
<td></td>
<td>degrees</td>
<td>s</td>
<td>degrees s(^{-1})</td>
<td>degrees s(^{-2})</td>
</tr>
<tr>
<td><em>F. flavissimus</em></td>
<td>14</td>
<td>129</td>
<td>3-27</td>
<td>-8.3 ± 0.8</td>
<td>0.059 ± 0.003</td>
<td>-1609 ± 340</td>
<td>-390294 ± 83113</td>
</tr>
<tr>
<td><em>F. longirostris</em></td>
<td>4</td>
<td>13</td>
<td>1-7</td>
<td>-6.0 ± 1.8</td>
<td>0.100 ± 0.018</td>
<td>-497 ± 44</td>
<td>-147562 ± 5565</td>
</tr>
<tr>
<td>Caudal Peduncle elevation max</td>
<td></td>
<td></td>
<td></td>
<td>degrees</td>
<td>s</td>
<td>degrees s(^{-1})</td>
<td>degrees s(^{-2})</td>
</tr>
<tr>
<td><em>F. flavissimus</em></td>
<td>14</td>
<td>130</td>
<td>2-27</td>
<td>-3.3 ± 0.3</td>
<td>0.062 ± 0.002</td>
<td>-396 ± 33</td>
<td>-90653 ± 6508</td>
</tr>
<tr>
<td><em>F. longirostris</em></td>
<td>4</td>
<td>14</td>
<td>2-7</td>
<td>-1.4 ± 0.7</td>
<td>0.058 ± 0.017</td>
<td>-194 ± 35</td>
<td>-53033 ± 5742</td>
</tr>
</tbody>
</table>

N = number of individual fish observed, n = number of sounds, n Range = range of n observed per individual fish
‘Position at extreme’ refers to the maximum extension during cranial elevation, premaxillary protrusion, anal fin elevation, or caudal peduncle elevation. See Methods for details.
Data are presented as means of individual fish averages ± s.e.m.

Sound emission and the latency to maximum elevation relative to sound onset was highly variable (Figure 2.4). Sound emission kinematics of *F. flavissimus* tended to also co-occur with erection of the anal fin spines and rays and elevation of the caudal peduncle (Table 2.2, Figure 2.3 and 2.4). *Forcipiger longirostris* individuals, however, did not erect the anal fin during sound emission and had moderate and inconsistent elevation of the caudal peduncle (Table 2.2, Figure 2.3 and 2.4). Additionally, *F. longirostris*, typically protruded the upper jaw (premaxilla) and dentary (observed but not quantified here) during headbob sounds in contrast to *F. flavissimus*, which tended to move the jaws very little during cranial elevation (Table 2.2, Figure 2.3 and 2.4).

EMG recordings revealed burst activity of several muscles with close association to sound emission and before visible cranial elevation (Figure 2.5). Sound emission was
Figure 2.3. Kinematics associated with typical pulse sound generation in *Forcipiger flavissimus* and *F. longirostris*. Superimposed images from two separate frames from video (600 fps) recorded during sound emission with arrows to indicate main direction of motion from *F. flavissimus* (A) and *F. longirostris* (B). Motion profiles (see Methods for details) of *F. flavissimus* (C) and *F. longirostris* cranial elevation (D), *F. flavissimus* (E) and *F. longirostris* anal fin erection (F), *F. flavissimus* (G) and *F. longirostris* premaxillary protrusion (H), and *F. flavissimus* (I) and *F. longirostris* caudal peduncle elevation (J). Dotted lines indicate time periods of the two superimposed images. Note the rapid cranial elevation that occurs for both species, the anal fin erection and caudal peduncle elevation that occurred typically only for *F. flavissimus*, and premaxillary protrusion that occurred typically only for *F. longirostris*. 
Figure 2.4. Kinematic profiles from sound production events: cranial elevation in *F. flavissimus* (A) and *F. longirostris* (B), anal fin elevation in *F. flavissimus* (C) and *F. longirostris* (D), caudal peduncle elevation in *F. flavissimus* (E) and *F. longirostris* (F), and premaxilla protrusion in *F. flavissimus* (G) and *F. longirostris* (H). Each color is from the loudest sound recorded on video from different individual fish. Events are aligned by the start of sound emission which occurs 0.0167 seconds on the time axis. Note that both species produce a strong cranial elevation component in which the time of maximum elevation occurs well after the onset of sound production and that the latency until maximum elevation is highly variable in relation to the onset of sound emission.
Figure 2.5. Kinematic profiles, sound waveforms, and rectified EMGs from *Forcipiger flavissimus* that show typical timing of muscle activity, sound emission, and cranial elevation. Example from an individual in which EMG recording electrodes were placed in the anterior epaxialis (epaxial), sternohyoideus, A1 subdivision of the adductor mandibulae (AM1), and A2 subdivision of the adductor mandibulae (AM2) (A). Example from a different individual in which the epaxial and sternohyoideus EMG electrodes were implanted (B). Note the onset of activity from these muscles near the occurrence of sound emission and the variable delay until cranial elevation.
characterized by brief activity of EP, SH, AM1, and AM2 and was closely associated with the initial onset of sound emission before cranial elevation (Figure 2.6). Additional EMGs in one individual showed activity towards the tail by TEP for some, but not all (12 of 18) sound events. Timing of TEP, like EP, was very close to the onset of sound emission, but trended towards lagging behind the EP (EP mean start relative to sound onset -0.00008 ± s.d. 0.00368 s, TEP -0.00071 ± 0.00322, paired t-test, n=12, T=2.01,
In the same individual, HP musculature, a region shown to be active during sound production in *H. polylepis* (Boyle & Tricas, 2010), was found to fire for only 6 out of 18 events, and to lag behind the onset of EP (mean EP onset \(-0.00133 \pm 0.00151\) s vs. HP mean \(0.00632 \pm 0.00517\) s, paired t-test, \(n=6, T = -4.61\) \(P=0.006\)).

Several kinematic features of cranial elevation were associated with frequency, duration, and intensity features of sound emission in *F. flavissimus* (Table 2.3). Maximum cranial elevation acceleration was correlated positively with median frequency (Table 2.3, Figure 2.7). For all of these relationships, individual differences contributed significant variation to the model and several significant interaction terms indicate differences between the kinematic relationships among individuals (Table 2.3). No significant relationships were found for *F. longirostris*, however, the power of analysis was likely low for this species because of the low sample size of individuals and replicates.

A substantial amount of the variation in acoustic signals of *F. flavissimus* was explained by body size differences (Table 2.4). There was a positive trend between median frequency and body size, and a significant interaction term that indicates a different relationship effect among individuals (Table 2.4, Figure 2.8). Sound duration and intensity were both positively correlated with body size (Table 2.4, Figure 2.8). The SPL vs. SL regression model contained a significant interaction term that indicates a difference in the intensity-body size relationship among individuals.

Activity of the anterior epaxial musculature was correlated with features of pulse sounds and with cranial elevation (Table 2.5). EP activity duration was correlated
Table 2.3. Relationships between individual cranial elevation kinematic features and sound features.

<table>
<thead>
<tr>
<th></th>
<th>Forcipiger flavissimus</th>
<th></th>
<th>Forcipiger longirostris</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>F</td>
<td>Total Test P-value</td>
<td>Individual P-value</td>
</tr>
<tr>
<td><strong>Median f</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Angle</td>
<td>2,143</td>
<td>2.95</td>
<td>0.055</td>
<td></td>
</tr>
<tr>
<td>Max Angle Time</td>
<td>2,143</td>
<td>1.02</td>
<td>0.365</td>
<td></td>
</tr>
<tr>
<td>Max Velocity</td>
<td>2,143</td>
<td>5.45</td>
<td>0.005</td>
<td>0.820</td>
</tr>
<tr>
<td>Max Acceleration</td>
<td>2,143</td>
<td>5.97</td>
<td><strong>0.003</strong></td>
<td>0.622</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Angle</td>
<td>3,142</td>
<td>8.45</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Max Angle Time</td>
<td>2,143</td>
<td>4.62</td>
<td>0.011</td>
<td>0.003</td>
</tr>
<tr>
<td>Max Velocity</td>
<td>3,142</td>
<td>11.03</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Max Acceleration</td>
<td>3,142</td>
<td>9.47</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td><strong>SPL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Angle</td>
<td>3,142</td>
<td>10.37</td>
<td><strong>&lt;0.001</strong></td>
<td>0.014</td>
</tr>
<tr>
<td>Max Angle Time</td>
<td>2,143</td>
<td>1.46</td>
<td>0.236</td>
<td></td>
</tr>
<tr>
<td>Max Velocity</td>
<td>3,142</td>
<td>15.35</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Max Acceleration</td>
<td>3,142</td>
<td>11.48</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
</tr>
</tbody>
</table>

Rows show results from individual multiple regression models to test the relationship between features of cranial elevation kinematics and frequency (median frequency), duration, and intensity (sound pressure level) of the sound.

Individual fish were kept in all models and individual x variable interactions were kept in the model when interaction term P<0.05. Bolded P values represent statistical relationship after sequential Bonferroni correction.
Figure 2.7. Scatter plots of individual *Forcipiger flavissimus* acoustic and kinematic features. Sound duration and SPL vs. maximum cranial elevation angle (A). Sound duration and SPL vs. maximum cranial elevation velocity (B). Median frequency, sound duration, and SPL vs. maximum cranial acceleration (C). Sounds recorded from different individuals are represented by different symbols. Note the positive association between these kinematic and acoustic features.
positively with sound duration and positively and most strongly with sound intensity (Table 2.5, Figure 2.9). Individual differences contributed significant variation to both models, however, no significant interaction terms were present. In addition, there was a non-significant trend towards a positive relationship between median frequency and EP activity (Table 2.5). EP activity was positively correlated with maximum cranial elevation velocity (Table 2.5, Figure 2.9). No other relationships were found between activity of other muscles tested by EMG and sound features or cranial elevation.

Evidence of a ventral cranial-pectoral girdle linkage for pulse sound emission was provided from lidocaine and muscle transection experiments. Application of 2% lidocaine to left and right sternohyoideus musculature in two individuals reduced the sound duration (GLM on sound duration ranks with random subjects factor: lidocaine effect \( F_{1, 20} = 361.00, P=0.033 \), individual \( F_{1, 20} = 11000, P=0.006 \), individual-lidocaine interaction \( F_{1, 20} = 0.00 P=0.950 \), back-transformed mean rank pre-lidocaine 0.119 s,
Figure 2.8. Scatter plots of *F. flavissimus* acoustic features and body size. Median frequency vs. standard length (SL) (A), sound duration vs. SL (B), and sound pressure level (SPL) vs. SL (C). Sounds recorded from different individuals are represented by different symbols. The relationship between median frequency and SL is influenced strongly by an interaction between body size and individuals. Note the strong positive relationship between SPL and SL.
Table 2.5. Relationships between muscle activity and 1) sound features and 2) cranial elevation.

<table>
<thead>
<tr>
<th></th>
<th>d.f.</th>
<th>F</th>
<th>Total Test P value</th>
<th>Individual P value</th>
<th>Variable P value</th>
<th>Relationship</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median f</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP</td>
<td>2, 73</td>
<td>7.21</td>
<td><strong>0.001</strong></td>
<td>0.928</td>
<td>0.004</td>
<td>+</td>
<td>16.5</td>
</tr>
<tr>
<td>SH</td>
<td>2, 50</td>
<td>1.68</td>
<td>0.197</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM1</td>
<td>2, 19</td>
<td>9.37</td>
<td><strong>0.002</strong></td>
<td><strong>0.001</strong></td>
<td>0.181</td>
<td>51.0</td>
<td></td>
</tr>
<tr>
<td>AM2</td>
<td>2, 18</td>
<td>7.76</td>
<td>0.004</td>
<td>0.005</td>
<td>0.673</td>
<td>46.3</td>
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</tr>
<tr>
<td><strong>Sound duration</strong></td>
<td></td>
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</tr>
<tr>
<td>EP</td>
<td>2, 73</td>
<td>6.92</td>
<td><strong>0.002</strong></td>
<td><strong>0.001</strong></td>
<td><strong>0.002</strong></td>
<td>+</td>
<td>15.9</td>
</tr>
<tr>
<td>SH</td>
<td>2, 50</td>
<td>1.32</td>
<td>0.275</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM1</td>
<td>2, 18</td>
<td>1.38</td>
<td>0.277</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM2</td>
<td>2, 18</td>
<td>1.62</td>
<td>0.225</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP</td>
<td>2, 73</td>
<td>27.48</td>
<td>&lt;<strong>0.001</strong></td>
<td>&lt;<strong>0.002</strong></td>
<td>&lt;<strong>0.001</strong></td>
<td>+</td>
<td>43.0</td>
</tr>
<tr>
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<td>2, 50</td>
<td>0.16</td>
<td>0.850</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM1</td>
<td>2, 18</td>
<td>1.38</td>
<td>0.276</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM2</td>
<td>2, 18</td>
<td>1.28</td>
<td>0.302</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Max. Cranial Velocity</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP</td>
<td>2, 71</td>
<td>19.08</td>
<td>&lt;<strong>0.001</strong></td>
<td>0.521</td>
<td>&lt;<strong>0.001</strong></td>
<td>+</td>
<td>35.0</td>
</tr>
<tr>
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<td>2, 48</td>
<td>0.53</td>
<td>0.595</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM1</td>
<td>2, 16</td>
<td>3.63</td>
<td>0.050</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM2</td>
<td>2, 16</td>
<td>2.49</td>
<td>0.115</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rows show results from individual multiple regression models to test the relationship between muscle activity from EMG and 1) sound features: frequency (median f), duration, and intensity (sound pressure level) and 2) cranial elevation (maximum cranial elevation velocity).

EP = anterior epaxialis, SH = sternohyoideus, AM1 = A1 subdivision of the adductor mandibulae, AM2 = A2 subdivision of the adductor mandibulae.

Individual fish were kept in all models. No individual x variable interactions were kept in the model because all interaction terms had P>0.05.

Bolded P values represent statistical relationship after sequential Bonferroni correction.

Post-lidocaine 0.108 s). Sound intensity, however, was not affected by lidocaine administration (GLM on sound intensity, lidocaine effect F₁, 20 = 0.11, P=0.748).

Similarly, transection of the right and left sternohyoideus in three individuals reduced sound duration (GLM on sound duration ranks with random subjects factor: muscle transection effect F₁, 70 = 349.70, P=0.022, individual F₁, 70 = 436.54, P=0.002,
Figure 2.9. Scatter plots of *Forcipiger flavissimus* acoustic and kinematic features vs. anterior epaxialis muscle activity. Sound duration vs. anterior epaxialis EMG duration (epaxial duration) (A), sound pressure level (SPL) vs. epaxial duration (B), and maximum cranial elevation velocity vs. epaxial duration (C). Sounds recorded from different individuals are represented by different symbols. Note the positive relationship between sound duration and epaxial duration, SPL and epaxial duration, and maximum cranial elevation velocity and epaxial duration.
individual-transection interaction $F_{1, 70} = 0.10 \ P=0.905$, back-transformed mean rank pre-transection 0.075 s, post-lidocaine 0.066 s), but an overall drop in intensity was not observed with GLM on sound intensity, muscle transection effect $F_{1, 70} = 1.32, P=0.345$).

Some direct evidence of epaxial musculature contribution to sound emission was found after application of 2% lidocaine to right and left epaxial musculature of one individual, after recovery from a previous sternohyoideus injection. After application of lidocaine to the anterior epaxialis, sound duration was reduced (two-sample t-test, $T=3.18$, d.f.=13, $P=0.007$, pre-epaxial injection mean 0.189 ± s.d. 0.051 s, postinjection 0.115 ± 0.040 s). Intensity also was reduced following injection (two sample t-test, $T=2.27$, d.f.=13, $P=0.041$, pre-epaxial injection mean 135.7 ± s.d. 3.72 dB, postinjection 130 ± 5.27 dB).

There was evidence for recovery over time after epaxial injection, as cranial elevation velocity was strongly and positively associated with time past injection (simple linear regression, $F_{7} = 15.75 \ P=0.007$, $R^2 = 72.4\%$) and no sounds were emitted until 30 minutes after injection. The range of cranial elevation was low during the first 48 minutes following injection (max 3.3º, mean 1.9º).

**DISCUSSION**

This study demonstrated a relationship between Forcepsfish sound features of frequency, duration, and sound pressure level with cranial rotation kinematics. Stronger correlations were found between sound features and body size. Sound production in the Forcepsfish involves a rapid elevation of the head for which the sound frequency, duration, and SPL are related to aspects of maximum angular extension, velocity and acceleration. Visible rotation of the head occurs after sound emission thus maximal extension typically results
well after sound onset (57 ms), but is highly variable and indicative of a potential
decoupling between sound generation and a mechanism responsible for releasing
potential energy transferred to the cranium. Both Forcepsfish *F. flavissimus* and
Longnose Butterflyfish *F. longirostris* produced sounds with a similar, delayed rapid
cranial rotation, although no relationships between signal features and kinematics or body
size were observed for *F. longisrostris*. We suspect, however, that low power and sample
size for *F. longirostris* (in part from reduced motivation to produce sounds in aquaria)
may obscure a similar relationship of body size and kinematics as observed in *F.
flavissimus*. Several apparent differences exist between the two species during sound
emission events, as Forcepsfish sound production typically included anal fin erection and
caudal elevation, while Longnose Butterflyfish typically protruded the oral jaws during
cranial rotation. Results from this study indicate that agonistic sounds from Forcepsfish
may convey information about the signaling animal such as body size and condition.

In this study, body size was correlated positively with duration and SPL, while a
positive trend was found between body size and median frequency, in which strong
individual and body size-individual interaction terms were present. Several studies have
found correlations between body size of fishes and sound features. The positive trend
between frequency and body size found in this study, however, is unusual and runs
counter to results reported from other teleost fishes with a variety of sonic mechanisms.
Negative correlations between frequency content and body size have been reported for a
mormyrid (*Pollimyrus adspersus*) with an extrinsic swim bladder muscle (Crawford et
al., 1997), an armored catfish (*Corydoras palateus*) with a pectoral fin stridulation
mechanism (Pruzsinszky and Ladich, 1998), , the osphronemid Croaking Gourami
(Trichopsis vittata) (Ladich, 1998) which has a pectoral fin strumming mechanism, and the sciaenid Weakfish (Cynoscion regalis) (Connaughton et al., 2000) and Atlantic Croaker (Micropogonias undulatus) (Gannon, 2007), which have extrinsic swim bladder muscles. The pomacentrid Bicolor Damselfish (Stegastes partitus) (Myrberg et al., 1993) and Hawaiian Dascyllus (Dascyllus albisella) (Lobel and Mann, 1995), and the blenniid (Parablennius parvicornis) (De Jong et al., 2004), species with sonic mechanisms unknown at this time, also have negative correlations between sound frequency and body size. Frequency content in well-studied toadfishes (Batrachoididae) is correlated with muscle contraction rate and is independent of body size (Demske et al., 1973; Fine et al., 2001). Negative correlation between body size and frequency has led to hypotheses of swim bladder resonance contributions to sound spectral content (Myrberg et al., 1993). An alternative explanation, based on the observation of swim bladder behavior as highly damped, is that larger fish with larger swim bladder muscles take a longer time to complete a contraction, which results in a lower frequency sound (Connaughton et al., 2002). The basis for the positive correlation between body size and frequency observed in this study is not known, but perhaps it is related to differences in the cranial kinematic abilities, or size-related allometric increases in muscle mass, as cranial elevation acceleration has a weak positive relationship with frequency content.

The correlations between body size and SPL and between body size and sound duration in this study is consistent with the results from other studies of unrelated fishes with different sonic mechanisms. Positive body size relationships have been found for Oyster Toadfish (Opsanus tau) SPL (Fine et al., 2001), Weakfish pulse duration and SPL (Connaughton et al., 2000), Croaking Gourami SPL (Ladich, 1998), and Sand Gobies
(Pomatoschistus minutus) (Lindström and Lugli, 2000). A hypothesis that explains this consistent relationship is that the swim bladder is a sound radiator, which increases in surface area with body size, and if swim bladder oscillation velocities remain constant with body size, volume velocity and sound pressure level would increase from the surface area increase (Fine et al., 2001).

Beyond body size, cranial rotation velocity and acceleration explained the variation seen in Forcepsfish sound duration and amplitude. This occurrence of rapid cranial elevation immediately following sound emission is previously unknown among teleost sound production and involves motion similar to teleosts that use rapid movements for prey capture during feeding. Cranial elevation is a major component of stereotyped prey capture in teleost fish feeding, occurs in taxa that span the ram-suction feeding continuum, and involves a series of movement of cranial elements in order to lower the hyoid and expand the buccal cavity (Gibb and Ferry-Graham, 2005). The rapid cranial elevation velocities and accelerations seen during sound production rival and exceed (>2X) those reported from feeding studies of voracious predators like largemouth bass (Svanbäck et al., 2002). Based on maximum cranial elevation displacement and time to maximum cranial elevation data from a cyprinid, poeciliid, osphronemid, two chaetodontids, four labrids, a centrarchid, a paralichthyid, a pleuronectid, and a syngnathid (Gibb and Ferry-Graham, 2005), Forcepsfish sound production cranial elevation is faster than typical feeding strikes from all but Danio rerio and Syngnathus leptorhynchus. Cranial rotation during Forcepsfish and Longnose Butterflyfish sound production measured from this study also involved faster cranial elevation than has been shown for the same species during feeding (Ferry-Graham et al., 2001a; Ferry-Graham et
al., 2001b). The seahorse and pipefish family Syngnathidae, however, has been shown to produce cranial elevations during feeding that far exceed those measured during sound production in *Forcipiger* (Van Wassenbergh et al., 2008; Flammang et al., 2009; Roos et al., 2009). The relationship between suction feeding performance and cranial kinematics examined in largemouth bass (*Micropterus salmoides*) has revealed strong correlations between buccal suction forces and weaker correlations with the distance prey are moved towards predator via suction (Svanbäck et al., 2002). Muscle activity patterns may help explain the kinematic patterns observed during sound production as they have for teleost feeding.

EMG data measured during this study demonstrated a link between epaxial activity and sound duration, SPL, and to a lesser extent, sound frequency. Epaxial activity was also associated with an increase in cranial elevation velocity. Studies on muscle activity during teleost feeding have demonstrated a pattern that often involves simultaneous activity of the anterior epaxial musculature and the sternohyoideus, which serve respectively to raise the neurocranium and lower the hyoid in order to initiate opening of the oral jaws and expand the buckle cavity, followed by subdivisions of the adductor mandibulae in order to close the jaws (Lauder et al., 1986; Westneat and Wainwright, 1989; Grubrich and Wainwright, 1997; Alfaro and Westneat, 1999). Activity of anterior epaxial muscle from this study typically occurred for 17 ms, comparable to durations seen in feeding studies from other fishes in which this musculature is active as the head is elevated, such as centrarchids (Lauder et al., 1986; Grubrich, 2000), parrotfishes (Alfaro and Westneat, 1999), and Sling-jaw Wrasse (Westneat and Wainwright, 1989). Activity from the sternohyoideus and both
subdivisions of the adductor mandibulae, however, differed from the pattern seen in these fish feeding studies in that activity tended to occur closer to the onset of epaxial activity and tended to be of shorter duration. This observation is consistent with the hypothesis that epaxial driven elevation of the head results in an antero-dorsal motion of the pectoral girdle, along with postcleithral bones and ribs, which lie in close association with the swim bladder as tension is maintained between the hyoid and cleithrum via sternohyoideus activity, and between the hyoid and neurocranium via activity of the adductor mandibulae. Activity of the adductor mandibulae near the onset of sound emission and epaxial activity is predicted by this hypothesis and would be expected to differ from the later onset and longer duration pattern associated with closing the jaws at the end of a feeding strike. Manipulation of a cleared and stained specimen shows that elevation of the head produces forward movement of the pectoral girdle and anterior pleural ribs. Further support for this hypothesis occurred from experiments when the sternohyoideus was inactivated with lidocaine or transected and sound durations were reduced. These experiments did not mute sounds, but bone and connective tissue linkages remained and the muscle transection procedure may have allowed some fiber attachments to remain at the extreme caudal end of the urohyal. In the extreme case of rapid cranial rotation seen in pipefish, the onset of epaxial activity has been shown to occur far earlier (from 300 ms to nearly 0.5 s) than the beginning of cranial rotation, and thus provided strong evidence for a power-amplification system (Van Wassenbergh et al., 2008) similar to that seen in other animals with ballistic-like movements, such as plethodontid salamanders (Deban et al., 2007). Cranial elevation during Forcepsfish sound production typically occurs after epaxial activity, but likely results from much less
power amplification because epaxial activity duration in this study was correlated with cranial elevation velocity unlike the independent relationship found in pipefish. Further, epaxial activity loaded for much shorter periods in this study, with likely far less potential energy stored in connective tissue elements. Epaxial activity in this study consistently occurred immediately before sound emission, yet cranial elevation occurred with variable latency relative to sound onset, epaxial onset, and epaxial onset. This observation is consistent with the hypothesis that cranial elevation occurs as a by-product of sound production and after antagonistic activity of the sternohyoideus and adductor mandibulae ceases which releases the head.

The cranial elevation pattern of sound production observed in this study is unusual, both within the Chaetodontidae and among teleosts more broadly. Social sound production was documented only recently in butterflyfishes. Territorial sounds of unknown mechanistic origin are known for Chaetodon multicinctus (Tricas et al., 2006), and the pyramid butterflyfish Hemitaurichthys polylepis, a member of a closely related genus to Forcipiger, produces sounds via a hypaxial musculature buckling mechanism over the anterior swim bladder (Boyle and Tricas, 2010). Recent phylogenetic hypotheses place the genus Forcipiger as basal within a clade that includes Hemitaurichthys, Heniochus, and Johnrandallia and is estimated to have separated between 9.9 and 24.2 mya (Fessler and Westneat, 2007; Bellwood et al., 2009). The outgroup to this clade includes four genera (Fessler and Westneat, 2007; Bellwood et al., 2009), thus more are analyses of sound production functional morphology within these additional taxa are needed to determine whether the cranial elevation sound production behavior is a derived condition within Forcipiger.
In anemonefish, *Amphiprion clarkii* (Pomacentridae), cranial elevation is also a major component of sound production. However, sound emission occurs near peak cranial elevation, rotation of the neurocranium occurs over a longer period, sound emission results from closing of the oral jaws, and sounds consist of pulse trains (Parmentier et al., 2007). Sound emission also occurs during the rapid cranial elevation of seahorse feeding (Bergert and Wainwright, 1997; Colson et al., 1998). It is not known, however, if sounds have an agonistic or courtship social function and the mechanism of seahorses involves stridulation of the supraoccipital and coronet (Colson et al., 1998). Sound production in *Forcipiger* does not likely involve stridulation, as sounds tend to be of lower frequency and more similar to swim bladder driven sounds.

The relationships observed between fish size and to a lesser extent, cranial kinematics, of Forcepsfish sound production may have important considerations for signal honesty during acoustic communication. Honest signals provide some degree of accurate information about the signaler itself (Fitch and Hauser, 2002). Results from this study demonstrate that SPL, sound duration, and to a lesser extent, frequency are reliable indicators of body size. Additionally, these sound features are correlated directly with cranial elevation velocity and acceleration. Body size effects on sound intensity, which are likely linked with swim bladder size, are not likely subject to deceptive signaling strategies, as swim bladder size is constrained by its function as a buoyancy organ. Our observations on cranial rotation kinematics, however, lead us to hypothesize that fish may be able to vary SPL, duration, and frequency in part with increased cranial elevation performance. Experiments in oyster toadfish (*Opsanus tau*) indicate that the apomorphic mate calling sound produced by intrinsic swim bladder muscles incurs little overall
energetic costs (Amorim et al., 2002), but are fatigue limited as a result of local glycogen depletion (Mitchell et al., 2008). The metabolic costs associated with the much shorter duration cranial rotation sounds produced by Forcepsfish are unknown, but are expected to be far lower than those of toadfish. It may, however, be possible that cranial kinematic performance is indicative of fish condition. Future experiments that evaluate the behavior of receivers in the presence of different acoustic signals should be conducted to test predictions of the ultimate function of sound production and signal honesty in *Forcipiger*.

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CHAPTER III:
PULSE SOUND GENERATION, ANTERIOR SWIM BLADDER BUCKLING, AND ASSOCIATED MUSCLE ACTIVITY IN THE PYRAMID BUTTERFLYFISH, HEMITAUROICHTHYS POLYLEPIS

ABSTRACT
Acoustic behaviors are widespread among diverse fish taxa but mechanisms of sound production are known from relatively few species, vary widely and convergent mechanisms are poorly known. We examined the sound production mechanism in Pyramid Butterflyfish, Hemitaurichthys polylepis, a member of the socially and ecologically diverse reef fish family Chaetodontidae. This fish produces pulse trains at dusk during social interactions likely related to mate attraction and courtship. In laboratory experiments sound production was synchronized to high-speed video to determine body movement associated with sound generation. In addition, electromyography (EMG) recordings tested activity of six candidate muscles. Fish produced individual pulses with mean peak frequency of 97 Hz in rapid succession. EMG experiments show that anterior hypaxial muscles contract at high bilaterally synchronous rates (up to 120 Hz) in near perfect association with rapid inward buckling visible outside the body over the anterior swim bladder. Muscle activity often showed EMG doublets that occurred within the time of a single sound pulse but was not sustained. Buckling and sound pulse rates correlated strongly ($R^2 \approx 100\%$) and sound pulse rate measured over two successive pulses (maximum of 38 pulses s$^{-1}$) was lower than muscle firing rate. These results show extrinsic swim bladder muscles of $H. polylepis$ involve single contractions that produce pulses in a manner similar to distantly related teleosts, but involve a novel doublet motor neuron firing pattern. Thus the Pyramid Butterflyfish sound production mechanism is likely convergent with several percomorph taxa and divergent from other sound producing butterflyfishes (genus Forcipiger).

INTRODUCTION

Teleost fishes have evolved a wide-array of mechanisms for the production of sound in acoustic communication. It is likely that these diverse mechanisms evolved independently several times (Ladich, 2001; Ladich and Bass, 2003, 2005). Some evidence exists for a conserved developmental pattern of vocal musculature and innervation among ray-finned fishes and tetrapods (Bass et al., 2008), however, data on developmental morphology of actinopterygian fishes are limited to few taxa. While sound production is not described for most fishes, acoustic communication is wide-spread and occurs among phylogenetically diverse lineages (Ladich, 2001). Despite the independent origins of fish sound production structures, many species utilize muscle driven contractions of the compressible, gas filled swim bladder as a sound source (Zelick et al., 1999).

Swim bladder muscles for sound production are classified as either intrinsic or extrinsic based on their association with the swim bladder (Tavolga, 1971). Intrinsic swim bladder muscles insert entirely on the swim bladder. However, extrinsic swim bladder muscles originate elsewhere on the body such as the occipital region of the neurocranium, on trunk musculature and associated bones, or both and insert on or are positioned adjacent to the swim bladder. The antagonistic mechanism for these sonic muscles is the swim bladder tunic (Demski et al., 1973). Sound production muscles in teleost fishes have evolved independently and their homologies with generalized teleost musculature are not known entirely, however, in many cases they appear to be derived from hypaxial or epaxial trunk musculature (Winterbottom, 1974).

Fishes with intrinsic sonic muscles, such as the batrachoidid Oyster Toadfish *Opsanus tau* (Skoglund, 1961; Fine et al., 2001) and Plainfin Midshipman *Porichthys*
notatus\) (Cohen and Winn, 1967; Bass and Baker, 1991), often are characterized by high, synchronous, contraction rates that correspond to the fundamental sound frequency. In sea robins (Triglidae), however, antiphasic bilateral firing produces sounds with fundamental frequencies at twice the contraction rate (Bass and Baker, 1991; Connaughton, 2004). In the few species examined with extrinsic swim bladder muscles, such as Southern Pigfish \textit{Congiopodus leucopaecilus} (Packard, 1960), squirrelfishes \textit{Holocentrus rufus} and \textit{H. adscensionis} (Winn and Marshall, 1963; Gainer et al. 1965), Bigscale Soldierfish \textit{Myripristis berndti} (Salmon, 1967), and Weakfish \textit{Cynoscion regalis} (Connaughton et al., 2000) pulse emission rates correspond to the firing frequency of the muscles. The fundamental frequency of these single muscle twitch type sounds are hypothesized to be related more to the duration of muscle contraction, rather than the resonance of the swim bladder which is highly damped by surrounding fish tissues (Connaughton et al., 2000) and can be modeled as an impedance matching device between the sonic musculature and surrounding water medium (Sprague, 2000). Thus, the firing rate of Weakfish muscles sustained over a fish call is much lower (20 Hz) than those of toadfish (200 Hz) (Connaughton et al., 2000).

The butterflyfish family (Chaetodontidae) includes 11 genera and approximately 122 species that occur on reefs in tropical and temperate seas (Nelson, 2006). Several members of this family were shown recently to produce social sounds (Tricas et al., 2006; Boyle and Tricas, 2009). In \textit{Chaetodon multicinctus}, agonistic sound production includes hydrodynamic stimuli produced in part from strong caudal flexion, pulsatile sounds similar to sounds that involve the swim bladder, and broadband click like signals that are consistent with a stridulatory mechanism (Tricas et al., 2006), but the proximate
mechanisms remain unclear. Within the butterflyfish bannerfish clade (sensu Fessler and Westneat, 2007), members of the genus *Forcipiger* produce short, pulsatile sounds that are associated with rapid dorsal elevation of the head, anterodorsal motion of the ventral pectoral girdle and dorsal elevation of the caudal skeleton that elongate the body cavity and likely stimulate sound emission from the swim bladder (Boyle & Tricas 2009, unpublished data).

The presence of extrinsic or intrinsic swim bladder musculature is not yet reported for any butterflyfishes. Several morphological studies exist on the swim bladder and associated musculoskeletal morphology of the chaetodontid genera (*Chelmon, Forcipiger, Hemitaurichthys, and Johnrandallia*), in the context of putative lateral line and auditory function of the laterophysic connection that is unique to *Chaetodon* (Webb, 1998; Webb and Smith, 2000; Smith et al., 2003; Webb et al., 2006). Despite the morphological diversity in features of potential importance for sound reception, descriptions of structures around the swim bladder associated with sound production are unknown.

In this study, we describe and compare the gross anatomy, muscle activity and sound production of the extrinsic swim bladder sonic mechanism for the Pyramid Butterflyfish, *Hemitaurichthys polylepis*. We examine the spectral and temporal patterns of sound emission in recordings taken in the field on Hawaiian coral reefs and the laboratory. In the laboratory, we recorded sounds synchronized with high-speed video to determine the pattern of movement of the body and underlying swim bladder. Electromyography was conducted on free-swimming fish to identify 1) the muscle activity associated with sound emission, 2) bilateral synchrony of activity, and 3) the
relationship between muscle firing, sound emission, and body movement. These experiments provide evidence for an extrinsic swim bladder sonic mechanism that is divergent from the related genus *Forcipiger* but similar to mechanisms reported in distantly related teleost fishes.

**MATERIALS AND METHODS**

**Field observations of sound production behavior**

Field and laboratory experiments were conducted on Pyramid Butterflyfish, *Hemitaurichthys polylepis* (Bleeker), a zooplanktivorous species with an Indo-Pacific distribution (Randall, 2007). Fish were observed during summer months (June-August) 2008 and 2009 along a reef drop-off at approximately 20m depth on the west coast of Hawaii island (Puako 19°58'6"N 155°51'11"W). Fish were observed during periods of intense courtship activity from 15:00 to 19:00 hours by divers using closed circuit rebreathers to mitigate bubble exhaust noise associated with scuba. Field temperatures ranged between 24-28°C. Fish behavior and sounds were recorded on digital video tape with either a Sony TRV-950 camera (manual audio gain control) in an Amphibico housing connected to an external hydrophone (HTI min96, High-Tech Inc.) extended from the camera on a 1m pvc tube or with a Canon Optura camera (automatic audio gain control) in an Amphibico housing and hydrophone. Video and audio recordings were imported on to a pc computer and audio recordings extracted with Cool Edit Pro 2.0. Tonal camera hum noise from the Sony TRV 950 was digitally filtered with a notch filter at 149.8 Hz, 100 dB attenuation, with the super narrow notch width setting in Cool Edit
Pro 2.0. Sound files were analyzed in the same manner as laboratory sound data (see below).

**Laboratory sound production experiments**

Fish for laboratory experiments collected from the main Hawaiian Islands by commercial suppliers. Experiments were conducted in a 110 L aquarium (76 cm wide, 30 cm deep, 46 cm high) with flow-through seawater (which was turned off during experiments) at a temperature of 28°C. Water level was kept low (about 20 cm deep, 43% of aquarium capacity). Fish sounds were elicited from solitary fish in the aquarium when either an observer approached the aquarium in a well-lit room or by introducing a conspecific into the aquarium. These sounds were pooled in the analysis because no differences were observed between their acoustic features (one individual produced multiple sounds in both behavioral contexts, Mann-Whitney tests for differences in duration, peak frequency, median frequency, sound pressure level $P > 0.05$). Sounds from fish in the aquarium were detected with a calibrated Brüel and Kjaer 8103 hydrophone (-211 dB re: 1V/µPa connected to a Nexus conditioning amplifier with gain set to 31.6 mV/Pa) positioned approximately 3 cm from the aquarium end wall. Sounds were recorded digitally with a CED Micro 1401 (Cambridge Electronic Design) and Spike 2 software sampled initially at 40 k samples s$^{-1}$. Sound files were then low pass filtered in Cool Edit Pro 2.0 and downsampled at 4 kHz using the high quality setting, This spectrum is well below the minimum resonance frequency of 4574 Hz calculated for the aquarium at the 20 cm water depth (Akamatsu et al., 2002).
Body kinematics associated with sound production were recorded on high speed video. Subjects were illuminated in the aquarium with four 500 W quartz halogen lights. During experiments, sound production events were pre-trigger recorded at 300, 600, and 1200 frames per second, 512X384, 432X192, and 336X96 pixels, respectively, using a Casio Ex-F1 Exilim camera. Sound and electromyography (EMG) data (see below) were synchronized to video with a flasher circuit in which LEDs were recorded visually by the camera and while square pulses were digitized and recorded simultaneously on the hydrophone channel in Spike 2.

EMG recording experiments

Contraction activity was determined for candidate sonic muscles by EMG recordings in free swimming subjects. Bipolar recording electrodes were made from pairs of 0.05 mm insulated tungsten wire (California Fine Wire) in which the insulation at the tip (1mm) of each wire was removed, inserted into a 28 gauge hypodermic needle and exposed tips bent back into hooks. Fish were anesthetized with 100 mg/L of tricaine methanesulfonate (MS-222, Argent Labs) and ventilated with seawater and anesthetic solution while the electrodes were implanted and the hypodermic needle tips were removed. A loop was inserted in the dorsal trunk musculature with surgical silk suture thread, tied, and glued with cyanoacrylate around both electrodes for strain relief in order to prevent dislodgement of the electrodes. EMGs were amplified in a four channel differential amplifier (AM systems) with 10,000X gain, band-pass filtered between 100 and 5000 Hz, with a 60 Hz notch filter. Up to four concurrent EMGs were digitized with the CED Micro1401 with Spike 2 at 10 kHz.
Based on observations of a related butterflyfish *Forcipiger flavissimus* (Boyle & Tricas unpublished), EMG electrodes were placed (Figure 3.1) in the anterior epaxial musculature (fish 1 and 2) approximately 0.5 cm caudal to the supraoccipital bone, in the sternohyoideus (fish 1 and 2) at the level of the caudal portion of the urohyal, the A1 of

![Figure 3.1](image.png)

**Figure 3.1.** Location of sonic muscle used during sound production by the Pyramid Butterflyfish *Hemitaurichthys polyolepis*. Note that the sonic muscle is attached to the caudal neurocranium and rib of the 5th vertebra. Blue circles indicate approximate location of bipolar electromyography recording electrodes relative to the skeleton and body of experimental fish. Short dashed line indicates location of swim bladder. Long dashed line indicates location of pectoral fin rays. Inset shows exploded view of the sonic musculature with opercle, supracleithrum, and cleithrum bones removed. Abbreviations: AM=A1 of adductor mandibulae, C=cleithrum, EP=anterior epaxialis, CH=central hypaxialis, NC=neurocranium, OP=opercle, PH=posterior hypaxialis, R=rib, SC=supracleithrum, SH=sternohyoideus, SM=sonic muscle. Numbers indicate vertebra number.
the adductor mandibulae (fish 1), the hypaxial musculature (fish 2) at the level of the swim bladder and approximate middle of the body cavity (i.e. central hypaxial musculature), and the hypaxial musculature (fish 1 and 2) at the caudal end of the body cavity over the swim bladder (i.e. posterior hypaxial musculature). After observing kinematic activity over the anterior body cavity at the rostral end of the swim bladder (see Results) and recording from the above muscles, EMG electrodes in fish 2 were placed shallow (1-2 mm below the dermis) and bilaterally in the putative sonic muscle (see Results) in the hypaxial musculature at a location between the pleural ribs of the fourth and fifth vertebrae immediately caudal to, behind the pectoral girdle (Figure 3.1). Putative sonic muscle EMG electrodes were implanted in the left and right sides of fish 3 and in the left side only of fish 4. After recovery from anesthesia, fish were placed in the aquarium setup as described above.

**Sound and EMG analyses**

Sound waveforms were inspected in Cool Edit Pro 2.0 software visually to determine duration (relative to background noise). Pulses in trains with silent periods between them were considered as separate sounds. Sound spectrograms, power spectra, and intensity measurements were estimated using custom Matlab 7.0 scripts. Sound power spectra were determined from 1024-point fast Fourier transforms (FFT) with a Hanning window of zero-padded sounds. From power spectra, peak frequency (frequency with the highest intensity) and median 10 dB frequency (the median frequency value of all frequencies of the power spectrum that were within 10 dB intensity of the peak frequency) were determined. Sound pressure level (SPL) from sounds in laboratory experiments was
estimated from the root-mean-square pressure level of sound waveforms. Sounds were recorded from free-swimming fish, thus distance to the hydrophone was variable and could not be determined from our video. SPL in a shallow aquarium likely will decrease with distance between the theoretical extremes of cylindrical and spherical spreading (approximately 3 dB and 6 dB per doubling of distance) (Mann, 2006), thus SPL values should be considered estimates. Most sound events likely occurred within 20 cm of the hydrophone and low intra-individual variability was found (interquartile variability for each individual ranged from 4 to 7 dB).

Sound events sometimes included pulses in close succession with no silent interpulse intervals (see Results). In order to determine the timing and duration of these individual pulses and the timing relative to EMG events, full sample sounds were high-pass filtered at 20 Hz to remove low frequency noise which occurred on some events, rectified, and smoothed with a timing constant of 0.02 s with Spike 2 software. EMG data were rectified and smoothed with a time constant of 0.002 s. The timing onset and offset of individual pulses and EMG firings were then identified by the time at which the rectified waveform was 50% of the maximum rectified amplitude. Timing of body musculature movement (inward buckling) over the anterior swim bladder (see Results) relative to hydrophone and EMG data was determined from frame-by-frame examination of video in Quick Time 7.5.

**Statistical analyses**

Means and standard error were determined from averages of each individual for sound and EMG features. Differences between acoustic features from field and lab recordings
were tested with a two sample $t$-test or with a Mann-Whitney $U$-test when assumptions of normality and homogeneity of variance were not met. Inter-individual differences in acoustic parameters (sound duration, pulse duration, peak frequency, median 10 dB frequency, and SPL) from lab experiments failed assumptions of equal variance and thus were tested with Kruskal-Wallis one-way analysis of variance tests followed by Dunn’s post-hoc tests. EMG timing onset relative to pulse onset, buckling onset, and left sonic muscle vs. right sonic muscle onsets were tested with multiple regressions in which a subject factor (individual fish) and interaction term (fish X onset) were included in the models. Sonic muscles often fired multiply (usually twice, see Results) and thus the first firing and second firing associated with the nearest sound were tested separately. Differences between putative sonic muscles and non-sonic muscles in the absolute firing onset ($|\text{EMG onset} - \text{pulse onset}|$) were tested among 10 groups (sonic muscles from three fish, epaxial muscle from two fish, sternohyoideus from two fish, anterior hypaxial muscle from one fish, and posterior hypaxial muscle from two fish). The absolute firing onsets did not meet assumptions of equal variance and thus Kruskal-Wallis test followed by Dunn’s post-hoc test was used to assess differences. All statistical tests were conducted in Minitab v. 13.31. Multiple comparisons from the 20 conducted tests were corrected with a sequential Bonferroni procedure to an adjusted family-wide alpha of 0.05 (Rice, 1989). $P$-values for most of the 16 statistical tests used in this study were very low and a type I family-wide alpha level of 0.0125 was calculated after the sequential Bonferroni procedure.

Pulse emission frequency histograms in 2.5 pulses s$^{-1}$ bins were produced by determining the instantaneous pulse emission rate determined from two, three, four, and
five consecutive pulses ($1/([\text{duration of pulses} + \text{inter pulse intervals (s)}])$). A minimum estimate was calculated for the time each pulse rate was sustained ($([\# \text{ successive pulses}]/[\text{emission rate (pulses s}^{-1}]$. Similarly, sonic muscle EMG firing rate histograms in 10 events s$^{-1}$ bins were produced by determining the instantaneous EMG firing rate for two, three, four, five, and six successive EMG events.

**RESULTS**

**Acoustic behavior**

Fish were observed in the field producing pulse trains during late afternoon hours 15:00-19:00 at a period when most individuals were closer to the reef (i.e. not high in the water column feeding on plankton) and engaged in courtship behavior. Some fish appeared to be females with noticeably swollen abdomens (hydrated eggs). Putative male fish (distinguished by the lack of a swollen abdomen) occupied areas close to the substrate below reef ledges and were observed chasing conspecifics and the heterospecifics *Chaetodon miliaris* Quoy & Gaimard and *Acanthurus nigrofuscus* (Forsskål). A total of 114 sounds from six separate individuals were analyzed from field recordings. Pulse train sounds (Figure 3.2, Table 3.1) were emitted during courtship interactions between putative male and female fish in which the pair would carousel and engage in short chases, during agonistic interactions between adjacent putative males, agonistic interactions between heterospecifics that entered areas below reef ledges, and when divers approached fish beneath ledges. Sounds from each behavioral context were acoustically similar in terms of overall sound duration, pulse duration, peak frequency, and median 10 dB frequency (Table 3.1), but the small sample sizes of several contexts
Figure 3.2. Representative pulse train sound produced by a free swimming Pyramid Butterflyfish, *Hemitaurichthys polylepis* that approached a heterospecific under a coral ledge. (A) Oscillogram and (B) spectrogram show the repeated pulses (16) emitted over a 2.1 s period. The sound spectrum includes frequency components near 1000 Hz with strongest intensity near 100Hz. Spectrogram settings: 1024 point FFT, 2.5 % window length, 95% window overlap.

(2-4 individuals) precluded any statistical comparisons between different contexts or contexts and sounds recorded in the laboratory.

Fish held in the aquarium readily produced pulse sounds to conspecifics and solitary fish produced sounds when approached by observers. A total of 822 sounds were recorded and analyzed from four fish. Pulse sounds were produced singly or in trains (Figure 3.3). Sounds recorded in the laboratory were similar to field recorded sounds.
Table 3.1. Acoustic properties of sounds recorded in the field and during laboratory experiments

<table>
<thead>
<tr>
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<th>N</th>
<th>n</th>
<th>n Range</th>
<th>Mean ± s.e.m.</th>
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<tbody>
<tr>
<td>Sound duration (s)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Field (total)</td>
<td>6</td>
<td>114</td>
<td>8 - 40</td>
<td>0.083 ± 0.025</td>
</tr>
<tr>
<td>Field (agonistic courtship)</td>
<td>2</td>
<td>115</td>
<td>7 - 8</td>
<td>0.091 ± 0.074</td>
</tr>
<tr>
<td>Field (agonistic to heterospecifics)</td>
<td>2</td>
<td>33</td>
<td>9 - 24</td>
<td>0.113 ± 0.084</td>
</tr>
<tr>
<td>Field (reacting to diver presence)</td>
<td>4</td>
<td>66</td>
<td>9 - 27</td>
<td>0.106 ± 0.019</td>
</tr>
<tr>
<td>Lab</td>
<td>4</td>
<td>822</td>
<td>87 - 307</td>
<td>0.194 ± 0.031</td>
</tr>
<tr>
<td>Pulse duration (s)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Field (total)</td>
<td>6</td>
<td>142</td>
<td>8 - 50</td>
<td>0.038 ± 0.004</td>
</tr>
<tr>
<td>Field (agonistic courtship)</td>
<td>2</td>
<td>17</td>
<td>8 - 9</td>
<td>0.045 ± 0.010</td>
</tr>
<tr>
<td>Field (agonistic to heterospecifics)</td>
<td>2</td>
<td>41</td>
<td>9 - 32</td>
<td>0.034 ± &lt;0.001</td>
</tr>
<tr>
<td>Field (reacting to diver presence)</td>
<td>4</td>
<td>84</td>
<td>9 - 9</td>
<td>0.035 ± 0.002</td>
</tr>
<tr>
<td>Lab</td>
<td>4</td>
<td>1027</td>
<td>104 - 339</td>
<td>0.058 ± 0.006</td>
</tr>
<tr>
<td>Peak frequency (Hz)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field (total)</td>
<td>6</td>
<td>114</td>
<td>8 - 40</td>
<td>116 ± 21.1</td>
</tr>
<tr>
<td>Field (agonistic courtship)</td>
<td>2</td>
<td>115</td>
<td>7 - 8</td>
<td>155 ± 17.0</td>
</tr>
<tr>
<td>Field (agonistic to heterospecifics)</td>
<td>2</td>
<td>33</td>
<td>9 - 24</td>
<td>143 ± 13.4</td>
</tr>
<tr>
<td>Field (reacting to diver presence)</td>
<td>4</td>
<td>66</td>
<td>9 - 27</td>
<td>94 ± 24.2</td>
</tr>
<tr>
<td>Lab</td>
<td>4</td>
<td>822</td>
<td>8 - 307</td>
<td>97 ± 32.6</td>
</tr>
<tr>
<td>Median 10 dB frequency (Hz)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field (total)</td>
<td>6</td>
<td>114</td>
<td>8 - 40</td>
<td>182 ± 32.2</td>
</tr>
<tr>
<td>Field (agonistic courtship)</td>
<td>2</td>
<td>115</td>
<td>7 - 8</td>
<td>232 ± 94.9</td>
</tr>
<tr>
<td>Field (agonistic to heterospecifics)</td>
<td>2</td>
<td>33</td>
<td>9 - 24</td>
<td>176 ± 45.1</td>
</tr>
<tr>
<td>Field (reacting to diver presence)</td>
<td>4</td>
<td>66</td>
<td>9 - 27</td>
<td>138 ± 2.4</td>
</tr>
<tr>
<td>Lab</td>
<td>4</td>
<td>822</td>
<td>87 - 307</td>
<td>116 ± 26.2</td>
</tr>
<tr>
<td>SPL dB re: 1µmPa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lab</td>
<td>4</td>
<td>822</td>
<td>87 - 307</td>
<td>123 ± 3.7</td>
</tr>
</tbody>
</table>

N = number of individual fish observed for each category, n = number of events per category, n Range = range of n observed per individual fish
Data are means from individual fish averages.
Categories: Field = field observations with behavioral context in parentheses. Field (total) = average of all field sounds pooled per individual. Lab = lab experiments.

Sound waveforms of *H. polylepis* involved an initial low amplitude deflection of positive or negative polarity, followed by a series of larger amplitude cycles that decayed exponentially, and resulted in pulse durations that often lasted 40 ms. When pulses were emitted in rapid succession, the resulting sound blended together with no silent period. Power spectra indicated that most energy in an overall sound occurred below 500 Hz, with peak frequencies typically less than 150 Hz, and median 10 dB frequency below 200 Hz. Spectrograms indicated a general downward shift in frequency energy as the pulse proceeds and the waveform decays.
Figure 3.3. Acoustic features of individual sound pulses produced in the lab by the Pyramid Butterflyfish, *Hemitaurichthys polyplepis*. (A, B) Oscillograms, (C, D) spectrograms and (E, F) power spectra from a quadruple pulse train (A, C, E) and single pulse (B, D, F) sound event. Power spectra show peak frequency near 100 Hz (1024 point, zero padded, Hanning window, FFT). Spectrogram settings: 1024 point FFT, 2.5 % window length, 95% window overlap.
Sounds in the lab trended towards longer duration as would be expected from a quieter recording environment than the field, hydrophone placement closer towards the sound source, and potentially from reflections on tank walls, but neither overall sound duration nor rectified pulse duration were statistically different after sequential Bonferroni correction (two sample \(t\)-tests, d.f.=4, \(P=0.053\) and d.f.=6, \(P=0.031\), respectively). Both were of low frequency (peak frequency two sample \(t\)-test, d.f.=5, \(P=0.629\), median 10 dB frequency Mann-Whitney \(U\)-test, N=6 and 4, \(P>0.05\)) with most energy concentrated below 500 Hz (Figures 3.2, 3.3, Table 3.1). Sound pressure level approximations from the laboratory were high for small reef fishes, with a mean of 123 dB re: 1 \(\mu\)Pa, a maximum value of 148 dB and minimum value of 104 dB. Train sounds were produced in rapid succession and in some cases successive pulses occurred without any period of silence between pulses (Figure 3.3A). Thus, individual sounds were composed of varying numbers (from one to seven) of repeating, sometimes blended low frequency pulses, with single pulses produced most commonly (mean of field 91 \(\pm\) 5 s.e.m.% and 84 \(\pm\) 6% for laboratory sounds).

There were several acoustic differences between individual fish (Figure 3.4). Most individuals produced statistically different sound and pulse durations, peak frequency, median 10dB frequency, and SPL (Figure 3.4). Fish 4 was smaller than the other fish and produced substantially shorter sounds of weaker intensity (Figure 3.4). Spectral features (peak frequency and median 10 dB frequency) varied between individuals with larger fish producing higher frequency sounds (Figure 3.4). These
Figure 3.4. The relationship between features of sound production and body size in the Pyramid Butterflyfish, *Hemitaurichthys polylepis*. Boxplots (box bounds quartiles and median line, lines extend to 10th and 90th percentile and points indicate 5th and 95th percentile) show differences between individual fish in (A) overall sound duration, (B) rectified pulse duration, (C) peak frequency, median 10 dB frequency, and (D) sound pressure level (SPL). Kruskal-Wallis tests revealed overall differences between individuals for sound duration ($H=362.4$, d.f.=3, $P<0.0001$), pulse duration ($H=138.7$, d.f.=3, $P<0.0001$), peak frequency ($H=484.9$, d.f.=3 $P<0.0001$), median 10 dB frequency ($H=429.2$, d.f.=3 $P<0.0001$), and SPL $H=610.3$, d.f.=3 $P<0.0001$). Letter groups indicate statistically different groups after Dunn’s post-hoc test and sequential Bonferroni correction.
individual differences in sound features, however, did not correspond to differences in timing of muscle activity or body movements (see below).

**Muscle and motor buckling activity**

High-speed video revealed a unique buckling mechanism that involved a small area of dermal tissue and body musculature (ca. 0.5 cm diameter, 0.2 cm²) located lateral to the anterior swim bladder and immediately caudal to the dorsal pectoral girdle (Figure 3.5). Mean visible displacement during inward buckling occurred close to the start of sound emission: mean 0.026 ± 0.003 s.e.m. s after sound onset. The region of buckling included obliquus superioris hypaxial musculature below the dorsal midline, caudal to the supracleithrum and dorsal cleithrum. Muscle fibers in this area were packed loosely in gross dissection and less stiff than caudal and ventral hypaxial musculature in fresh and

![Image of kinematic buckling](A) Pre-acoustic condition of sonic buckling area from a video image. (B) Same fish (middle) 3.3 ms later shows buckling over region behind pectoral girdle. (C) Diagramatic representation of buckling location (right) highlighted by dashed line. Data taken from video at 300 fps.
preserved specimens. Based on these observations, EMG electrodes were placed in the center of this putative sonic musculature in the area of loose fibers between the pleural ribs of the 4th and 5th vertebrae in order to examine muscle activity in association with sound emission and buckling (Figure 3.1). The sonic muscle lies ventral to the midlateral horizontal septum, caudal to the supracleithrum, and ventral to the third visible epaxialis myocomma behind the skull. Fibers of this musculature originate on the pterotic of the neurocranium, on the posteromedial surface of the cleithrum, on the medial surface of the supracleithrum and Baudelot’s ligament. Fibers insert on the lateral faces of large laminae of the anterior ribs of vertebrae three to five (v3-v5), on a posterolateral myocomma at the level of the rib of vertebra five, and some on the tunica externa of the swim bladder between the enlarged space between ribs of v4 and 45. Anteriorly, the swim bladder extends to the rib of v3, which occurs in a narrow space between rib v3 and v4. Manipulation of muscle fibers of fish specimens lends support to the hypothesis that the large rib of v5 and the attached underlying swim bladder may be pulled anteriorly by contractions of this muscle, and allow for a buckling between the space between rib v4 and rib v5.

Sonic muscle firing estimated by EMGs was characterized by strong amplitude, short duration (Table 3.2) and occurrence before the onset of sounds (Figure 3.6). EMG waveforms recorded from local motor units resembled a single muscle action potential, similar to patterns shown in fishes with sonic muscle composed of a single fiber type and with fibers innervated by multiple axons, although this is not confirmed for this species. Each sound pulse occurred usually with one or two firings (singlets and doublets) (Table 3.3), and for train sounds often occurred as doublets followed by singlets (Figure 3.6).
Rectification and smoothing of EMG and sonic waveforms (Figure 3.6) allowed for subsequent timing comparisons between EMG, buckling, and individual pulses, the latter of which often appeared as a single complex sound with amplitude modulation prior to smoothing.

EMG initiation of sonic muscles, onset time of individual sound pulses measured from rectified and smoothed waveforms, and buckling were all highly correlated (Figure 3.7). Several multiple regression models were used to test correlations with sonic muscle firing onset (dependent variable) that included an individual fish subject factor and interaction term. In all models, individual fish and individual-firing time interactions did not contribute substantial variation to the models $P>0.05$. The onset of first firing of sonic muscles as determined by EMG was highly correlated with the onset of sound emission (Figure 3.7 X-axis vs. Y-axis), $R^2$=100.0%, $F=326835$, d.f.=3, 337, $P<0.0001$. A similar result was found for the second sonic firing, from instances when the sonic muscle fired two or more times per sound, $R^2$=100.0%, $F=1081000$, d.f.=3, 329, $P<0.0001$. Similarly, onset of first sonic EMG firing was highly correlated with the onset of visible buckling (Figure 3.7, Z-axis vs. Y-axis); $R^2$=100.0%, $F=195484$, d.f.=3, 293.

| Table 3.2. Sonic muscle EMG firing durations of first and second EMG firings |
|-----------------------------|--------|--------|----------------|----------------|
|                            | N  | n  | N Range | mean duration ± s.e.m. (s) |
| 1st sonic firing           | 3  | 442 | 41 – 237 | 0.007 ± 0.002 |
| 2nd sonic firing           | 3  | 332 | 31 – 187 | 0.005 ± 0.001 |

$1^{st}$ sonic firing = $1^{st}$ EMG event within a single sound pulse emission, $2^{nd}$ sonic firing = $2^{nd}$ EMG event within a single sound pulse emission, N = number of individual fish, n = number of EMGs, N Range = range of n recorded per individual fish.
Figure 3.6. Muscle activity during sound production by the Pyramid Butterflyfish, *Hemitaurichthys polylepis*. (A) EMG recording from the animal’s right and (C) left sides. (E) Associated sound waveform. Sound waveform consists of a single pulse sound followed by a triple pulse (single sound consisting of three pulses in rapid succession). Sonic muscles on both sides fired twice for the first sound and first pulse of the second sound and once for the remaining two pulses. (B, D, F) show the same waveforms after rectification and smoothing (see methods). Onset and offset times of muscle firing and individual pulses were estimated from the time at which the smoothed waveform was half of the maximum intensity for that event.
Table 3.3. Occurrence of single and multiple EMG firing types within pulse sound events (% of total)

<table>
<thead>
<tr>
<th>Occurrence (mean ± s.e.m. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

N = number of individual fish, n = number of EMGs

Figure 3.7. Relative onset timing of sonic muscle activity, pulse sound onset, and visible buckling over the anterior swim bladder during sound production in the Pyramid Butterflyfish, *Hemitaurichthys polylepis*. X-axis: sound onset, Y-axis: left sonic muscle EMG start time, and Z-axis: start of visible buckling. Data points from individual fish (N=3) outlined in different colors (yellow, red, and open). Note the strong correlation between sound onset, EMG activity, and visible swim bladder buckling.
Second sonic muscle firings in instances of doublets or more firings also were correlated highly with buckling, $R^2 \approx 100.0\%$, $F=203376$, d.f.=3, 226, $P<0.0001$. EMGs from sonic muscles were synchronized and bilateral. Sonic muscle firing onset from left muscles were correlated strongly; $R^2 \approx 99.6\%$, $F=21058$, d.f.=3, 230, $P<0.0001$. In all experiments, EMG and non-EMG, the start of visible buckling was correlated strongly with the start of rectified, smoothed sound waveforms (Figure 3.7, Z-axis vs. X-axis). A multiple regression with buckling onset (dependent variable) vs. sound pulse onset (independent variable) showed that the onset of visible buckling was highly correlated with the onset of sound emission, $R^2 \approx 100.0\%$, $F=958412$, d.f.=3, 568, $P<0.0001$.

Sound pulses produced in close succession by a stationary fish showed the same initial phase (positive or negative direction of zero-crossing) as would be expected from an acoustic swim bladder source that involves a consistent first deflection. However, the phase of onset of pulsed sound waveforms differed with location of the recording hydrophone relative to the fish body (Figure 3.8). An examination of 37 cases of successive pulses with high signal-to-noise and silence between pulses provided clear zero-crossing estimates revealed that 86% of positive phase pulses occurred while the head of the fish was oriented towards the hydrophone and 100% of the negative phase pulses occurred while the head of the fish was oriented away from the hydrophone (Fisher’s exact test $P<0.001$). This observation indicates that the sound source is more complex than a simple monopole, and is consistent with the hypothesis of rostral expansion of the anterior end of the swim bladder during lateral buckling.

EMGs were recorded from additional muscles of the body and head from two individuals during sound production: anterior epaxialis, A1 adductor mandibulae,
posterior hypaxial musculature over the swim bladder, central hypaxial musculature (between the sonic musculature and the posterior recording location), and the sternohyoideus. Unlike the putative sonic muscles, these muscles did not fire consistently during sound emission events and recordings from the A1 did not occur near sound emission. In the instances that the muscles did show activity near sound emission.
a comparison of the muscle firing time relative to sound emission onset (absolute value of muscle onset time – sound onset) was conducted which revealed that firing of putative sonic muscles occurred much closer to muscle onset time than these additional muscles. Sonic muscle recordings from all three fish had EMG onset times much closer to sound emission onset time than EMG onset times from anterior epaxialis, sternohyoideus, and posterior hypaxialis muscles (Table 3.4). These values were statistically different (Kruskal-Wallis H=652.2, d.f.=9, \( P<0.001 \)) for all non-sonic muscles tested (\( P<0.001 \) for each fish-muscle combination, Dunn’s post-hoc test) except central hypaxial muscle (\( P>0.05 \)), which had a low absolute onset difference, but for which there was a small sample size of 14 EMGs as this musculature only fired 5% of the time during sound emission.

| Table 3.4. Absolute EMG onset relative to sound start for sonic muscle, anterior epaxialis, sternohyoideus, central hypaxial musculature, and posterior hypaxial musculature |
|-----------------|--------|--------|-------------|-----------------|
|                 | N     | n      | n Range     | mean \( \pm \) s.e.m. (s) |
| Sonic muscle    | 3     | 837    | 100 - 463   | 0.021 \( \pm \) 0.005 |
| Epaxial onset   | 2     | 182    | 21 - 161    | 0.125 \( \pm \) 0.070 |
| Sternohyoideus  | 2     | 324    | 131 - 193   | 0.123 \( \pm \) 0.047 |
| Central hypaxial| 1     | 14     | 131         | 0.019            |
| Posterior hypaxial | 2  | 419    | 62 – 357   | 0.235 \( \pm \) 0.098 |

\( N = \) number of individual fish in which EMGs were recorded for that muscle, \( n = \) number of EMGs per muscle, \( n \) Range = range of \( n \) recorded per individual fish

Sound recordings from the laboratory indicated that Pyramid Butterflyfish were capable of high sound pulse emission rates, but these events were clustered in time and composed of a low number of pulses. Calculation of instantaneous pulse emission rate from two consecutive pulses showed that pulses sometimes occurred in rapid succession (maximum of 39 pulses s\(^{-1}\)), but were sustained briefly (0.051 s). The mode of all pulse
emission rates calculated from two successive pulses, however, indicated a lower typical pulse emission rate of 7.5 pulses s\(^{-1}\) (sustained for a longer duration of 0.27 s) (Figure 3.9A). Pulse emission rates sustained for five consecutive pulses were substantially lower (Figure 3.9D). Pulse emission rates recorded from the field (max. 59, mode 17.5 pulses s\(^{-1}\) for two consecutive pulses, and max. 19, mode 5.0 for five consecutive pulses) were somewhat higher than rates recorded in the lab, but showed a similar pattern. Thus pulses were emitted at a moderately high rate, but only sustained over short periods.

Sonic muscle firing rates measured across two consecutive EMG events had a maximum firing rate of 500 events s\(^{-1}\) and a bimodal distribution because of the presence of EMG doublets on many sounds. Measured across two consecutive firings, sonic muscle firing rate had a high mode of 120 events s\(^{-1}\) (sustained briefly, 0.017 s) and a low modes of 10 (sustained for 0.2 s) (Figure 3.10A). Firing rates measured across three firings were unimodal, with a high of 375 events s\(^{-1}\) (sustained for 0.008 s), and a mode of 20 (sustained for 0.15 s) (Figure 3.10B). Measured over six firings, there was a maximum firing rate of 13 events s\(^{-1}\) (sustained for 0.46 s), and a mode of 10 (sustained for 0.6 s) (Figure 3.10E). Thus sonic muscle firing occurs at high rates sustained very briefly during a doublet and at moderately high rates for short durations that correspond to multiple doublets fired during a pulse train sound. These high firing rates, however, are not sustained for long periods.
Figure 3.9. Variability of sound pulse emission rates in the Pyramid Butterflyfish *Hemitaurichthys polylepis*. (A) Instantaneous pulse sound emission rate measured over two, (B) three, (C) four, and (D) five consecutive pulses. Mean frequency of occurrence (% of total) and error bars ± s.e.m. of pulse emission rate (2.5 pulses s⁻¹) from N=4 fish is shown on the bottom x-axis and the minimum event duration for which this emission rate is sustained (s) is shown on the top x-axis (# pulses/duration of consecutive pulse events). Note that pulse emission rates can be moderately high for two consecutive pulses (up to 39 pulses s⁻¹), but are not sustained, indicated by lower emission rates when calculated across three or more consecutive pulses.
Figure 3.10. Variability of sonic muscle firing emission rates in the Pyramid Butterflyfish *Hemitaurichthys polylepis*. (A) Instantaneous firing rate measured over two, (B) three, (C) four, (D) five, and (E) six consecutive firings. Mean frequency of occurrence (% of total) and error bars ± s.e.m. of firing rate (10 events s⁻¹ bins) from N=3 fish is shown on the bottom x-axis and the minimum firing duration for which this rate is sustained (# firings/duration of successive firing events) is shown on the top x-axis (s). Note the skewed and bimodal distribution for instantaneous firing rates (the higher mode at 110 events s⁻¹) which results from the presence of EMG doublets for some sounds (top). Firing rate calculated across more firings drops to more typical skeletal muscle firing rates of 20 events s⁻¹ or less (bottom).
DISCUSSION

This study demonstrates sound production in the Pyramid Butterflyfish *H. polylepis* that involves hypaxial musculature and buckling over the anterior swim bladder in a manner that is similar to that reported in distantly related percomorph fishes but differs from the sonic mechanism in other butterflyfishes (genus *Forcipiger*). Pyramid Butterflyfish produce repeated, short duration, pulsed sounds with highly localized and previously unreported inward buckling over the rostral swim bladder. These experiments also show that sonic muscles fire at high rates, which correspond to moderately fast pulse train emissions. Pulse emission and rapid firing rates, however, are not sustained over long periods. These findings appear to be different from previously studied butterflyfishes, but bear resemblance to some members of distantly related percomorph taxa (e.g. Congiopodidae, Holocentridae, Sciaenidae) that produce pulse train sounds with fast extrinsic sonic muscles. The Pyramid Butterflyfish is a member of a monophyletic clade that includes the genera *Forcipiger*, *Heniochus* and *Johnrandallia* (Fessler and Westneat, 2007). *Forcipiger*, unlike *Hemitaureichthys*, produces sounds by rapid cranial elevation and body flexion that includes synchronous firing of the anterior epaxialis, sternohyoideus, and adductor mandibulae (Boyle and Tricas unpublished). These muscles fired only occasionally during *H. polylepis* sound production experiments from this study, when they did fire they were not associated as closely with sound emission, and no cranial elevation was observed.

The acoustic behaviors and ecologies differ in several ways among the Pyramid Butterflyfish and those reported for the more distant confamilial Pebbled (Multiband) Butterflyfish, *Chaetodon multicinctus*. The Pyramid Butterflyfish is a diurnal planktivore
that forms large social groups during the day. Use of closed circuit rebreathers permitted us to approach closely and record these fish in midwater but sound production was not detected among fish within these feeding groups. We identified production of the pulse sound only during dusk hours associated with apparent courtship behaviors and when approached by divers on the bottom. In comparison, the multiband butterflyfish forms long term monogamous pairs that defend permanent coral feeding territories from conspecifics during the day (Tricas 1989). This species produces at least six different acoustic behaviors during interactions with conspecific territory intruders that include clicks, pulses and pulse trains in the field (Tricas et al. 2006). Of these, the pulse grunt sound of *Chaetodon* appears most similar to the pulse of *Hemitaurichthys*. This sound was proposed to function as an alert or distress call to the pair mate, has slightly higher peak frequency of 163 Hz, similar pulse duration of 42 ms and lower pulse rate (~3 pulses s⁻¹). In addition, it was the only sound reported for *Chaetodon* that was not associated with overt body movement and was proposed to result from the action of internal musculature not directly associated with locomotion. More work is needed to determine whether the muscles that produce these similar sounds in *Chaetodon* and other genera are conserved or divergent from those of *Hemitaurichthys*.

Sound emission rates from Pyramid Butterflyfish in this study were moderately high (up to 38 Hz) for short durations. These sound emission rates are comparable to those reported for teleost fish species that produce similar pulse train sounds. Emission rates were higher than typical Southern Pigfish (Congiopodidae) repetition rates (8 Hz) (Packard, 1960), Haddock (Gadidae, 8 Hz) (Hawkins and Amorim, 2000), a pearlfish (Carapidae, 7 Hz) (Parmentier et al., 2003), but were comparable to sciaenid (Weakfish)
pulse emission rates (20 Hz) (Connaughton et al., 2000) and Atlantic croaker (~30 Hz) (Fine et al., 2004; Gannon, 2007), Striped Cusk-eel sounds (Ophidiidae, up to 25 Hz) (Mann et al., 1997), and jump sounds (20 Hz) of another chaetodontid, Chaetodon multicinctus (Tricas et al., 2006). Sound emission rates from this study, however, were not as high as those measured from holocentrids, Longspine Squirrelfish (85 Hz) (Winn and Marshall, 1963) and Bigscale Soldierfish (about 90 Hz) (Salmon, 1967) or from John Dory (Zeidae) (71 Hz) (Onuki and Somiya, 2004). Pyramid Butterflyfish pulse emission rates are higher than many distantly related ray-finned fishes, but still fall within the range known for teleosts.

EMG firing rates in this study were measured in free swimming fish and are within the range of those reported for other sonic species. Sonic muscles usually show highly synchronous, short duration contractions without tetany (Fine et al., 2001). Fishes with tonal swim bladder sounds have the highest firing rates and are capable of sustaining activity for long durations up to minutes in Porichthys notatus (Bass and McKibben, 2003). These tonal sounds show fundamental frequencies that correspond either directly to the rate of bilateral muscle contraction (e.g. batrachoidid toadfish and midshipman, Skoglund, 1961; Cohen and Winn, 1967; Fine et al., 2001) or twice the firing rate of alternating individual muscles (e.g. triglid sea robins and gurnards, Bass and Baker, 1991; Connaughton, 2004). The sustained sonic muscle firing rates for the Pyramid Butterflyfish were lower than tonal fish species, similar to those measured for sciaenid Weakfish (Connaughton et al., 2000) and congopodid Southern Pigfish (Packard, 1960), but less than the brief sustained levels of squirrelfishes (115 firings s⁻¹) (Gainer et al., 1965) and of Bigscale Soldierfish (~120 firing s⁻¹) (Salmon, 1967). These acoustically
similar, non-tonal, pulse train emitting species produce individual pulse sounds by single muscle contractions. Muscle firing in this study was highly synchronous between right and left sides of the body, as shown for other fishes examined (Packard, 1960; Skoglund, 1961; Cohen and Winn, 1967; Connaughton et al., 2000) except for triglids which have antiphase firing (Bass and Baker, 1991; Connaughton, 2004). Pyramid Butterflyfish muscle firing rates appear most similar to fish species that produce non-tonal, percussive swim bladder sounds, although available data are limited as EMGs are reported from relatively few soniferous fish species.

Sonic muscle activity measured in this study most often involved two muscle action potentials in rapid succession. This firing pattern is unusual and not reported in other sonic fishes. The rapid sequential firings did not produce sounds that were distinguishable from those produced by single firings, and thus the function remains unclear. Some video sequences showed subtle muscular movement during the buckling period, while the overall buckling area remained inward. This observation, however, was inconsistent and with no obvious association with single or double firings. The highest typical firing rates measured in this study (120 Hz) were associated with doublets. In Oyster Toadfish, which have highly apomorphic sonic muscles, a stimulation frequency of 60 Hz causes complete tetany in skeletal muscle but still produces, clear, distinct contractions on swim bladder musculature (Rome et al., 1999). Perhaps doublet firing produces a fused single contraction or allows the fish to recruit more motor units to ensure reliable pulse production, as EMGs reported for Southern Pigfish, which do not produce doublets, sometimes show unilateral contraction failure during a pulse train (Packard 1960).
Pyramid Butterflyfish sound waveforms recorded in this study have an unusual shape in which the initial component of the pulse waveform is a low amplitude deflection (half cycle) followed by several full cycles of large and variable amplitude, before an exponential decay. Swim bladder sounds are highly damped because of the tissues that surround the swim bladder (Fine et al. 2001) and thus the decay observed in the *H. polylepis* waveform may be explained by damping of the swim bladder. EMG data from this study indicate that muscles over the swim bladder produce at most two complete twitches (perhaps one if tetanic fusion occurs), yet the higher amplitude portion of the sound wave is sustained over several cycles. A single twitch sound that results in multiple cycles has been attributed to a sound production mechanism that involves excitation of the swim bladder via bones or tendons (Parmentier et al. 2006, Parmentier et al. 2010). The ribs of the fourth and fifth vertebrae, with wide laminae in close association with the tunica externa of the swim bladder are a potential candidate for such a system in *H. polylepis*.

Both single and doublet firing produced single pulse sounds and were also associated with a single buckling event. Connaughton et al. (2000) have proposed a single twitch sonic mechanism for sciaenids based on EMG measurements of weakfish calls. Weakfish single twitch mechanisms were modeled by Sprague (2000) as an impedance matching device between the gas in the swim bladder and surrounding water environment. The fundamental frequency of the sound produced is influenced both by the duration of muscle contraction and the resonance properties of the highly damped swim bladder. An observation in weakfish that is consistent with this model is that larger fish, which likely have longer contraction duration cycles because of their longer muscle
fibers, produce lower frequency sounds (Connaughton et al., 2002). Our data indicate a trend towards sounds of higher frequency, in terms of both peak and median 10 dB frequency, with larger body size, perhaps because larger fish are able to vibrate the swim bladder with more energy. The three larger fish did produce louder sounds than the smallest fish, however, as would be expected from fish with larger swim bladders, which increase the volume velocity of the sound source (Bradbury and Vehrencamp, 1998; Connaughton et al., 2000). Perhaps larger Pyramid Butterflyfish, with more extrinsic musculature mass, are able to deflect the swim bladder tunic at a higher velocity and thus higher frequency. Larger sample sizes are needed to confirm this body size-frequency relationship. In addition, further experiments are necessary to determine the relationship between body size and swim bladder motion.

The use of high-speed video to visualize movement of the musculature over the anterior swim bladder in this study demonstrated the strong association between inward buckling and sound emission. Recent studies (Parmentier et al., 2007; Longrie et al., 2009) used a functional morphological approach to examine kinematic patterns in order to determine how anatomical structures and motor patterns are related to sound production. The cichlid obliquus inferioris hypaxial musculature adjacent to the swim bladder is involved in sound generation (Longrie et al., 2009). In our study, sonic muscle consists of anterior hypaxial musculature behind the pectoral girdle, which contracts to produce an inward buckling. This musculature involved is consistent with the obliquus superioris hypaxial musculature sensu Winterbottom (Winterbottom 1974) and has fibers which insert on the back of the skull, run medial to the supracleithrum, and has attachments on the dorsal cleithrum, posterior myocomma, and ribs, which are medial to
the muscle and lateral to the swim bladder. The inward buckling of the dermis and underlying musculature occurs at the location between the second and third ribs (of v4 and v5). Our images from high-speed video do not show how the rest of the swim bladder responds during the buckling, but we expect that the internal bladder pressure should increase during muscle contraction in buckling, which would cause the swim bladder to expand outward at other surface locations. Our data indicate that positive phase sounds tend to occur when the fish is oriented towards the hydrophone and negative phase sounds when the fish oriented away from the hydrophone, which is consistent with the occurrence of initial displacement primarily at the rostral end of the swim bladder. Experiments that directly measure the displacement of the swim bladder, however, are necessary to confirm this prediction. Fine et al. (Fine et al., 2001) examined toadfish swim bladder displacement with a laser vibrometer during sound production and found that contraction of the intrinsic swim bladder muscles caused the swim bladder, which was exposed to air in their study, to move inward from the sides, and expand ventrally at the beginning of sound emission. Future comparative studies are needed on the motion of the swim bladder and surrounding tissues during sound production to determine if different spatial patterns are correlated with acoustic features of sounds.

Preliminary examination of the hypaxial sound production musculature in Pyramid Butterflyfish indicates features that are similar (but require confirmation) with fast twitch oxidative fibers described for other sonic fish muscles (Fine and Pennypacker 1988), such as lighter appearance of musculature and small muscle fibers. Additionally, the shape of the EMG waveforms observed is consistent with a strong, synchronous action potential of local motor units that would be expected from musculature composed
of a single fiber type. Sonic swim bladder muscles in other taxa have likely evolved independently in different lineages, but appear often to be derived from epaxialis and obliquus superioris trunk musculature (Winterbottom, 1974). Highly apomorphic, intrinsic musculature of batrachoidid fishes originally develops from anterior hypaxial musculature in the occipital region of the head, which migrates caudally in development (Tracy, 1961). Among fishes with known swim bladder muscle sonic mechanisms, several adult patterns of muscle attachment and associated innervation patterns exist (reviewed in Onuki and Somiya 2007). A common pattern seen among species with extrinsic sonic musculature is an insertion on the occipital region of the skull; this occurs in the Pempheridae, Terapontidae, Monocentridae, Holocentridae, and Scorpaenidae (Salmon, 1967; Onuki and Somiya, 2007), as well as in *H. polylepis* (this study). Cusk-eels (Ophidiidae) have multiple (3-4) pairs of extrinsic sonic muscles which originate on the back of the skull and are unusual among sound producing fishes in that they appear to operate antagonistically, as opposed to using internal swim bladder pressure as the antagonist (Parmentier et al., 2006; Fine et al., 2007). Both extrinsic sonic muscles of these fishes, as well as the intrinsic sonic muscles of batrachoidids and triglids, are innervated by motor nerves that exit occipital foraminae (reviewed in Onuki and Somiya 2007). Conversely, the intrinsic sonic musculature of John Dory (Zeidae) and Walleye Pollock (Gadidae) and extrinsic sonic muscles of piranhas (Characidae: Serrasalminae) and drums and croakers (Sciaenidae), are innervated entirely by spinal nerves (reviewed in Onuki and Somiya 2007). Based on developmental patterns of sonic motor neuron innervation in batrachoidid fishes compared with anurans, birds, and mammals, Bass et al. (Bass et al., 2008) proposed a homologous region of premotor-motor vocal circuitry in
the hindbrain (rhombomere 8) that is conserved among ray-finned fishes and tetrapods. Given the diversity of sonic muscle arrangements and innervation within derived teleosts, and a lack of information of sound production behavior and anatomy among sarcopterygian and basal actinopterygian fishes, this hypothesis is worthy of exploration in more fish taxa, including *H. polylepis* but may be difficult in species that develop as small planktonic larvae. Nonetheless, further comparisons of skeletal-muscular anatomy, neuroanatomical innervation patterns, and muscle ultrastructure in adults are needed to further understand homologies and other evolutionary relationships among species.

The location of sonic buckling and associated swim bladder musculature in the body of *H. polylepis* is near the site of several functional anatomical studies in the related genus *Chaetodon*. Members of the genus *Chaetodon* possess a laterophysic connection between paired anteriorly directed bullae of the swim bladder and a medial opening of the lateral line canal in the supracleithrum (Webb, 1998; Webb and Smith, 2000; Smith et al., 2003; Webb et al., 2006). This morphology is variable at the subgeneric level and has been hypothesized to impart sound pressure sensitivity to the mechanosensory lateral line (Webb et al., 2006). *H. polylepis* does not possess anterior swim bladder bullae (Webb et al., 2006), but the swim bladder extends anteriorly to the posteroventral edge of the supracleithrum and sonic muscle fibers run rostrocaudally, and medial to the supracleithrum (Boyle & Tricas unpublished). It is not clear if the presence of sonic musculature near the dorsal, posterior girdle would influence any putative function of the laterophysic connection or if the anterior swim bladder and bullae are involved in the similar short pulse sounds described by Tricas et al. (2006) for *Chaetodon multicinctus*. 
The propensity and motivation of the Pyramid Butterflyfish to produce loud pulse
train disturbance calls in the presence of human observers is distinct from that of several
other chaetodontids that we have observed in the laboratory and field (*F. flavissimus*, *F.
longirostris*, *Chaetodon auriga*, *C. kleinii*, *C. multicinctus*, *C. ornatissimus*, and *C.
unimaculatus*). As discussed above, this species usually occurs in large shoals over the
reef when feeding, but during courtship individuals are more solitary and often engage in
short chases of conspecifics and sometimes heterospecifics. Intense pulse trains were
also produced when divers approached fish in a manner similar to that reported for
Longspine Squirrelfish (Holocentridae) (Winn et al., 1964). The sounds from these
relatively small animals are of high intensity, may be important for mate selection and
defense of mating territories thus warrant further behavioral study.

Results from this study and ongoing work on chaetodontids indicate that sound
production mechanisms may be quite variable within the butterflyfish family. Further
studies on skeletal and muscle morphology, muscle ultrastructure, motor firing patterns,
innervation of musculature and sonic motor neuron location of these fishes will allow for
comparisons in the broader context of the evolution of sonic mechanisms within
butterflyfish and among teleosts.

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REFERENCES


CHAPTER IV:
ULTRASTRUCTURE, INNERVATION, AND DISTRIBUTION OF MOTOR NEURONS OF EXTRINSIC SWIM BLADDER MUSCLES IN THE SOUND PRODUCING PYRAMID BUTTERFLYFISH (*HEMITAURICHTHYS POLYLEPIS*)

ABSTRACT

Sound production mechanisms vary among teleost fishes and have evolved independently multiple times. Swim bladder muscles must contract quickly and synchronously in order to compress the swim bladder with enough velocity to emit sound. The common requirement of fast sonic muscles from various trunk muscle precursors has led to a variety of unusual muscle properties and different innervation patterns among teleost fishes. In this study, the sonic and non-sonic hypaxial muscle ultrastructure and distribution of sonic motor neurons were examined in *Hemitaurichthys polylepis*, a chaetodontid butterflyfish fish with extrinsic swim bladder muscle driven sound production. Sonic muscles in Pyramid Butterflyfish have an unusual loose arrangement of muscle fibers that are smaller than adjacent hypaxial trunk muscles. Unlike some unrelated sonic fishes, sound production muscles did not have cores of sarcoplasm and both sonic and non-sonic muscles have triads of the z-line type. Relative to non-sonic trunk muscle, sonic muscles had longer sarcomeres, a more elaborate sarcoplasmic reticulum, wide t-tubules, and more radially arranged myofibrils. Sonic muscle is innervated by spinal nerves (1-3) and retrograde biocytin labeling of sonic motor neurons indicates a motor column entirely within the spinal cord. Sonic motor neuron somata are located both centrally and ventrolateral within the spinal cord. Like distantly related vocal fishes, Pyramid Butterflyfish possess some, but not all known apomorphic features related to fast sonic contractions and an innervation pattern that likely reflects the embryological origin and location of their sonic musculature.
INTRODUCTION

Sound production organs have arisen repeatedly and independently in the course of teleost fish evolution. Unlike the relatively conserved pattern of vocal organ evolution in various tetrapod lineages, teleosts have evolved a variety of mechanisms for the production of sounds. Many disparate fish lineages have co-opted the swim bladder, which is principally a hydrostatic organ, as a sound radiator. Swim bladder muscles derived from trunk musculature have evolved independently in wide-array of teleosts and function to vibrate the gas filled bladder. Highly apomorphic intrinsic swim bladder muscles are found in some fishes (toadfishes and midshipmen, Batrachoididae; sea robins and gurnards, Triglidae; and Walleye Pollock, Theragra chalogramma, Gadidae), insert and originate entirely on the swim bladder, and are able to elongate after contraction because of the tension produced by pressure inside the swim bladder. Examples of extrinsic swim bladder muscles, which involve muscles that originate on skeletal features on the head, body, or both and insert on the outside of the swim bladder or on tendons or bones adjacent to the swim bladder, are well-known from a diverse array of fishes. Like intrinsic sonic muscles, extrinsic muscles typically relax from internal swim bladder pressure. Sound producing fishes that utilize extrinsic sonic muscle mechanisms include soldier and squirrelfishes (Holocentridae), tigerfishes (Terapontidae), and croakers (Sciaenidae).

Fishes with swim bladder sound production mechanisms have a common requirement of producing rapid synchronous contractions in order to deflect the swim bladder at a velocity great enough to generate sound. In most species studied, the muscle contraction rate determines the frequency of sound production. Species that produce
tonal sounds like toadfishes and sea robins must produce rapid, continuous contractions and Oyster Toadfish *Opsanus tau* produce the fastest known contraction rate of vertebrate muscle (Rome, 2006). Several features of sonic muscle evolved independently among sound producing fishes and are hypothesized to be adaptive for high contraction speeds. These include a smaller fiber diameter, more developed sarcoplasmic reticulum, cores of sarcoplasm surrounded by a radially arranged contractile cylinder, and T-tubules present at the A-I boundary (which results in two triads per sarcomere) rather than the Z-line (Parmentier and Diogo, 2006). However not all of these features are present in the limited set of taxa in which they have been examined. Other unusual ultrastructural properties of fish sonic muscles have been observed, such as widened Z-disc present in the Type I (most soniferous) male phenotype of the Plainfin Midshipman *Porichthys notatus* (Bass and Marchaterre, 1989). Additionally, sarcomere length has been shown to vary between sonic muscles and white trunk muscle within taxa, and sonic muscle sarcomere lengths are variable between sonic muscles of unrelated sound producing species. In pimelodid catfishes (*Pimelodus* spp.), the extrinsic sonic muscle sarcomere length is shorter than the adjacent tensor tripodis muscle that is involved in sound transmission via the Weberian apparatus (Ladich, 2001). Oyster Toadfish sonic muscle sarcomeres are relatively long (2 μm) (Fawcett and Revel, 1961), and type I male Plainfin Midshipman (*Porichthys notatus*) have very long (3.4 μm) sarcomeres relative to the much less vocal type II males and females (2.0 and 2.2 μm, respectively; Bass and Marchaterre, 1989). Sarcomeres are more moderate in the sciaenid Weakfish *Cynoscion regalis* (Ono and Poss, 1982), and short (1.2-1.4 μm) in piranhas (Characidae: Serrasalminae) (Eichelberg, 1977). The wide variability of muscle properties among
distantly related sonic fish groups likely reflects the independent evolution of apomorphic muscle characteristics to achieve similar goals.

As sonic muscles have evolved independently from trunk muscle precursors in multiple fish groups, several patterns of innervation by peripheral nerves have occurred. Sonic muscles are innervated by occipital nerves in several distantly related sonic fish lineages with examples that include extrinsic sonic muscles that originate on the head, and entirely intrinsic muscles with no origin in the occipital region (Onuki and Somiya, 2007). Less common is a pattern of spinally innervated swim bladder muscles, in which several spinal nerves innervate sonic musculature and this pattern is seen in piranhas (Characidae), Walleye Pollock (Gadidae), John Dory (Zeidae), and croakers (Sciaenidae) (reviewed in Onuki and Somiya 2007). Mochokid catfishes (*Synodontis* spp.) are unusual in having a sound production system composed of both pectoral fin stridulation and an extrinsic swim bladder drumming muscle, in which the pectoral fin (as in other teleost fishes) by occipital nerve roots, but the swim bladder drumming muscle is innervated by both occipital and spinal (S1) nerve roots (Ladich and Bass, 1996). Variation also exists in the distribution of motorneurons rostrocaudally, as well as the lateral-medial and dorso-ventral extent within and among taxa with occipital and spinal nerve innervated muscles. Most sonic motor pathways described thus far in fish involve sonic motor nuclei that are present in the caudal medulla and rostral spinal cord and a pattern of either motor neurons clustered centrally, ventral to the central canal, or in ventral motor columns lateral to the medial longitudinal fasciculus (MLF) (Ladich and Bass, 2005). Piranhas, however, have spinal nerve innervated sonic muscles that are innervated by neurons not-clustered in distinct nuclei (Ladich and Bass, 2005; Onuki et al., 2006). This
pattern may be more widespread among teleosts with spinal nerve innervated sonic muscles, but such pathways have yet to be described.

Social sound production was described recently for several genera within the butterflyfish family Chaetodontidae (Tricas et al., 2006; Boyle and Tricas, 2009, 2010). This family includes approximately 122 species that are conspicuous members on coral reefs (Nelson, 2006). Production of rapid pulse train sounds that occur with buckling of the region surrounding the anterior swim bladder was recently described in the Pyramid Butterflyfish *Hemitauroichthys polylepis* (Boyle and Tricas, 2010). The purpose of this study is to further examine the sound production morphology of Pyramid Butterflyfish, to test for differences between the ultrastructure of sonic muscle and white trunk muscle, to determine sonic motor pathways of sonic muscle with retrograde tracing, and to compare muscle and innervation properties with sonic teleosts examined in previous studies.

**MATERIALS AND METHODS**

**Gross anatomy**

Live Pyramid Butterflyfish used in this study were obtained from commercial suppliers in the Hawaiian Islands. Gross dissections and examinations were conducted on fish that had been cleared and stained (n=1), first for bone with alizarin red-s (Taylor, 1967) and then later for nerves (Song and Parenti, 1995), and on formalin-fixed fish (n=4).
**Muscle histology**

Transmission electron microscopy

Two samples of muscle, one from sonic musculature in a region with very loosely packed muscle fibers described previously (Boyle and Tricas, 2010) and one from hypaxial trunk musculature were compared from each of four fish (two males, two females) 90-94 mm standard length (SL). Sonic muscle tissue was removed in the region over the swim bladder, between the fourth and fifth vertebra (Figure 4.1) in an area where sonic buckling is maximal. Trunk muscle tissue was removed in an area immediately ventral to the sonic buckling area, between the ribs of vertebra 4 and 5, and at a dorso-ventral level approximately equal to the pectoral fin radials (Figure 4.1).

For muscle extraction, fish were euthanized with an overdose of tricaine methanesulfonate (MS-222) and a small piece ($\leq 1 \text{ cm}^2$) of muscle was placed immediately in fixative (2% paraformaldehyde and 2% glutaraldehyde in 0.35 $M$ sucrose, 0.1 $M$ cacodylate buffer). Muscle tissue was further dissected while immersed in fixative into a smaller piece (approximately 1.5 mm by 4 mm) and kept overnight in fresh fixative. Tissue was washed in 0.1 $M$ cacodylate buffer with 0.35 $M$ sucrose, postfixed with 1% osmium tetroxide in 0.1 $M$ cacodylate buffer for 1 hr, dehydrated through a graded ethanol series, substituted with propylene oxide and embedded in LX112 resin. Ultrathin sections were stained with uranyl acetate, observed and photomicrographed with a Zeiss LEO 912 energy filtering transmission electron microscope.
Figure 4.1. Diagrammatic representation of fish (upper left) showing sonic muscle location and normal hypaxial trunk muscle. The supracleithrum, posttemporal, and operculum have been removed and the pectoral fin rays have been cut. Inset (top right), sonic muscle fibers are removed showing medial position of occipital and spinal nerves. Right middle, lateral view (upper pane) and dorsal view (lower pane) of brain and spinal cord in relative position as fish above (telencephalon not shown). Dots on dorsal spinal cord view indicate positions in which cell bodies where labeled in horizontal sections after application of biocytin to sonic muscle fibers in 3 fish. Bottom, three images of horizontal sections of the rostral spinal cord with biocitin labeled cell bodies and fibers. Abbreviations: 4v = 4th ventricle, bl = Baudelot’s ligament, cb = cerebellum, cc = corpus of the cerebellum, cl = cleithrum, epo = epioccipital, exo = exoccipital, hypax = hypaxial muscle, IL = inferior lobe of the hypothalamus, IX = glossopharyngeal nerve, hyp = hypothalamus, pto = pterotic, O = occipital nerve, r = rib, S1-4 = spinal nerves 1-4, sb = swim bladder, SM = sonic muscle, soc = supraoccipital, T = tectum, v1-5 = vertebrae 1-5, X = vagal nerve, XIII = eighth nerve.
Light microscopy

Sonic muscle fibers from one fish (female, 88 mm SL) on the non-reacted side of the sonic motor study were removed after transcardial perfusion (see below), post-fixed and stored in 4% paraformaldehyde overnight, rinsed in several changes of 70% ethanol until embedded in paraffin and sectioned at 10 µm and stained with hematoxylin and eosin. Light micrographs were observed and photographed at 400X on a Zeiss Axioskop microscope.

Sonic motor neurons

Retrograde biocytin labeling

Fish were anesthetized with 100 mg/L MS-222, a small 1-1.5 cm incision was made dorsoventrally across sonic muscle fibers that run rostrocaudally over the rib of vertebra 4 (Figure 4.1). An additional cut with small surgical scissors was used to sever muscle fibers anteriorly towards the supracleithrum, where sonic muscle fibers run medial. We then placed crystals of biocytin (Sigma, USA or Anaspec, USA) into the incision with a small insect pin. Incisions were sealed with a small piece of parafilm adhered to the fish with Vetbond (3M, USA). Animals were then returned to an aquarium for recovery and fed daily.

After survival periods of approximately four days, fish were deeply anesthetized in MS-222 and perfused transcardially with 0.9% heparanized saline followed by 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer (PB). The brain and rostral spinal cord was removed and postfixed in 4% paraformaldehyde/1% glutaraldehyde for 1 hr. Brains and spinal cords were stored in 0.1 M PB and
cryoprotected in 30% sucrose solution prior to sectioning. For sectioning, brains and spinal cords were embedded in Histoprep (Fisher Scientific) and transverse (five fish, three males 80-94 mm SL, and two females 88-94 mm SL) and horizontal sections (three females 90-92 mm SL) were cut at 40 μm.

Floating sections were quenched in 1% hydrogen peroxide in PB, incubated in 0.4% Triton-X in phosphate-buffered saline (PBS), then incubated for 3 h in avidin-biotin-horseradish peroxidase complex (ABC Elite kit, Vector Laboratories), rinsed in PB for 15 min, reacted with diaminobenzidine (DAB) chromogen substrate kit, and rinsed in distilled water for 10 minutes. Slides were counterstained with 0.5% cresyl violet or 0.1% methyl green, dehydrated and coverslipped. Some sections were also photographed before counterstaining.

Reference brain

A single *H. polylepis* was deeply anesthetized in MS-222, perfused transcardially with 0.9% heparinized saline, followed by alcohol formalin-acetic acid (AFA). The brain and rostral spinal cord was removed and stored in AFA overnight, rinsed in several changes of 70% ethanol, stored in 70% ethanol until embedded in paraffin. The brain and spinal cord were transverse sectioned at 10 μm and mounted on slides, deparaffinized and stained cresyl violet, dehydrated in an ethanol series, cleared in toluene and coverslipped with Cytoseal 60 (Richard Allen Scientific).
**Data analyses**

**Sarcomere lengths**

Sarcomere lengths were estimated from measurements of TEM micrographs. Fishes were deeply anesthetized, but not curarized prior to sacrifice, so these estimated values may be lower than actual sarcomere lengths, but should provide a low end estimate and allow for a comparison between sonic and non-sonic muscles which were prepared identically. Several measurements were made for each micrograph (1-9, total of 182) and values were averaged for each micrograph (a total of 33 micrographs, 17 sonic, and 16 non-sonic). Differences between sarcomere length were tested with a general linear model (GLM) with a random-subjects factor after log$_{10}$ transformation to meet assumptions of normality and homogeneity of variance.

**Transverse tubule spacing**

Initial observations (see Results) of the triads of sonic muscle TEM micrographs indicated unusual width differences between the membranes of transverse tubules (t-tubules). In order to examine this further, the spacing between the membrane of the transverse tubules of triads were measured with ImageJ software from 33 micrographs (17 sonic and 16 non-sonic). A total of 318 measurements were made (2-18 per micrograph) and values were averaged for each micrograph. Differences between t-tubule widths were tested with GLM with a random-subjects factor.
Muscle fiber diameters

Muscle fibers from sonic musculature and control hypaxial tissue were taken from one individual and embedded in paraffin, stained with hematoxylin and eosin. Muscle fiber area was measured with the outline tool in ImageJ software from micrographs from cross sections of 67 fibers chosen haphazardly among 8 sections (4 from sonic and 4 from non-sonic musculature). Muscle fiber diameters were estimated from area measurements using the formula for a circle \( \text{diameter} = 2 \times \frac{\text{area}}{\pi}^{1/2} \). Sonic and non-sonic muscle fiber areas were not normally distributed even after log transformation, so differences between sonic and non-sonic fiber area were tested with a Mann-Whitney \( U \)-test.

Cell-body sizes

Cell body sizes of biocytin labeled neurons were estimated with the outline tool in ImageJ software. Differences in cell size for the three fish that labeled both large-type and small-type cell bodies were tested with a GLM with a random-subjects factor after log10 transformation of data to meet assumptions of normality.

RESULTS

Pyramid Butterflyfish possess unusual, loosely arranged muscle fibers that were described previously (Boyle and Tricas, 2010). Fibers originate on the pterotic, epioccipital, and exoccipital of the neurocranium, run posteroventrally, and have insertions on the anterolateral faces of the ribs of vertebra 3, 4, and 5 (Figure 4.1). Additionally, some fibers attach to the connective tissue surrounding the swim bladder and to the medial face of the supracleithrum. Baudelot’s ligament passes through this
musculature (Figure 4.1). A large intercostal exists over the anterior swim bladder, at which point sonic musculature is widest (Figure 4.1). This area corresponds to the area of swim bladder buckling during pulse sound emission (Boyle and Tricas, 2010). The rib of vertebra 5 has a large anterolateral face for insertion and lies just anterior of a large aponeurosis that runs the length of the lower half of the body. Below the sonic musculature, stiffer hypaxial muscle fibers occur. These hypaxial muscle fibers are darker in appearance in preserved specimens than the sonic musculature.

**Muscle histology**

Sonic muscle fibers are cylindrical in cross section, highly variable in size, and surrounded by large extracellular space (Figure 4.2). Adjacent hypaxial trunk muscle fibers, by contrast, are polygonal in cross section, show less variation in size range and are packed tightly with little extracellular space (Figure 4.2). Fiber cross sectional areas measured from 39 sonic muscle fibers and 28 hypaxial muscle fibers from one individual were different (Mann Whitney, W=879.0, p<0.00001), with sonic muscle fibers of smaller area (median and quartiles 1083, 400, 1785 µm²) than ventral hypaxial fibers (3256, 2225, and 3842 µm²). Cores of sarcoplasm were not visible in any of the transverse sections of sonic or ventral hypaxial muscle fibers for light microscopy.

**Muscle ultrastructure**

TEM cross sections of sonic muscle fibers revealed some radial architecture (Figure 4.3A), similar to what was observed in Weakfish (Ono and Poss, 1982), in which long, spoke-like myofibrils occurred flanked by linear arrangements of sarcotubules. Unlike
contractile cylinder were observed. In addition, some fibers of sonic muscle did not possess an obvious radial arrangement (Figure 4.3B, C). Sonic muscle mitochondria were observed in sarcoplasm along the periphery (Figure 4.3A) and between myofibrils (Figure 4.3B, C). Ventral hypaxial musculature was not observed in a radial arrangement, with myofibrils of variable cross-section morphologies, and mitochondria located between myofibrils and along the sarcoplasm of the periphery of the fiber (Figure 4.3D, E). Mitochondria were not very densely concentrated in either muscle fibers, but were more abundant along the sonic muscle fiber peripheral sarcoplasm.

Longitudinal sections of sonic muscle fibers (Figure 4.4) and ventral hypaxial muscle fibers (Figure 4.5) revealed all triads to be located along the Z-line. Terminal cisternae of sonic muscle fibers tended to be flask-shaped, and enlarged relative to ventral
Figure 4.3. TEM cross sections of sonic muscle and white hypaxial trunk muscle of *Hemitaurichthys polylepis*. (A) Cross section of sonic muscle fiber showing spoke-like, radially arranged myofibrils (my). Unlike toadfish and weakfish sonic muscle, however, no cores of sarcoplasm were found in the center of the muscle fiber. (B) Small concentration of mitochondria (m) and sarcoplasm in the center of the contractile cylinder of sonic muscle. (C) Non-ribbon like myofibrils with interspersed mitochondria in sonic muscle. (D) Cross section of white trunk muscle fiber showing rounded, tightly packed myofibrils. (E) Cross section of white trunk muscle fiber showing irregular, tightly packed myofibrils with mitochondria along the fiber periphery. Scale bars = 1μm. Additional abbreviation: st = sarcotubules of the sarcoplasmic reticulum.
Figure 4.4. Longitudinal TEM micrographs of *Hemitarichthys polylepis* sonic muscle fibers. (A and B) Note position of T-system triads at the Z-line (Z), the widely spaced transverse tubules (t), and the enlarged flaring of the terminal cisternae (tc) of the sarcoplasmic reticulum (SR). (C) In panel C, note the well developed SR. (D) note the presence of numerous electron dense particles in the terminal cisternae (*). Scale bars = 1μm. Additional abbreviations: H = h-zone, M = M-line, st = sarcotubules of the sarcoplasmic reticulum.

hypaxial musculature (Figures 4.4 and 4.5). Electron dense glycogen granules were visible in terminal cisternae of both sonic and non-sonic muscles (Figures 4.4 D and 4.5 C). TEM micrographs of sonic muscle fibers revealed a complex sarcoplasmic reticulum with numerous envaginations of the membrane and a large surface area (Figure 4.4 C), that were not as developed in ventral hypaxial fibers. Additionally, the membranes of t-
Figure 4.5. Longitudinal TEM micrographs of *Hemitaurichthys polylepis* white trunk muscle fibers. Note position of T-system triads at the Z-line (Z), the narrowly spaced transverse tubules (t), and the smaller terminal cisternae (tc), and less developed sarcoplasmic reticulum surrounding myofibrils (relative to sonic muscle, previous figure) (A, B, C). Note visible electron dense particles particles (*), as in sonic muscle, present in terminal cisternae (C). Scale bars = 1µm. Additional abbreviations: H=h-zone, M=M-line.

tubules in sonic muscles were spaced more widely than the membranes of hypaxial t-tubules (Figures 4.4 and 4.5, GLM individual F_{44, 3} 11.10, p<0.001, muscle type F_{44, 1} 55.92, p<0.001, mean t-tubule width±s.e. 0.075±0.008 µm for sonic muscles and 0.051±0.005 for hypaxial musculature).
Sarcomere lengths estimated from micrographs indicated variability between individuals and longer sarcomeres for sonic muscles for each individual (GLM individual $F_{44,3} = 13.3$, $p<0.001$, muscle fiber type, $F_{44,1} = 6.49$, $p<0.015$). Sonic muscle sarcomeres were only modestly larger than ventral hypaxial fibers among individuals (backtransformed mean [95% CI]: sonic muscle 1.74 [1.66-1.83] $\mu$m, and hypaxial muscle 1.70 [1.64-1.76]). Sonic muscle sarcomeres within individuals, however, were $0.05 \pm 0.020 \mu$m (difference of backtransformed individual means $\pm$ S.E.) longer than hypaxial sarcomeres.

**Sonic muscle innervation and motor neurons**

Application of biocytin to sonic muscle fibers resulted in labeled neurons in the rostral spinal cord, caudal of the medulla and vagal motor nucleus. Sonic muscle fibers are innervated by spinal nerves (S1-S3), rather than occipital nerves and horizontal sections indicated that sonic motor neurons have a broad rostrocaudal distribution (Figure 4.1). No contralateral labeling of fibers or cell bodies were observed in any sections.

Sonic motor neurons were not labeled in a discrete nucleus and were represented by neurons with two different morphologies (Figures 4.6, 4.7). Large-type neurons were characterized by large ovoid cell bodies located ventrolaterally of the central canal and dorsolaterally of the Mauthner axon and MLF and border the neuropil that surrounds the central canal (Figure 4.6 A, B C). Few large-type cells were labeled on each transverse section (1-3 cells, median 1 per/section), but cells were present across a broad distribution of the spinal cord. Dendritic projections of these cells extend laterally, and to a lesser extent, dorsally and ventrally (Figure 4.6 A, B, C). Large-type cells tended to
Figure 4.6. Photomicrographs of counterstained transverse sections of *Hemitaurichthys polylepis* rostral spinal cord showing cell bodies and fibers labeled with retrograde application of biocytin in sonic muscle. (A, B) Larger, medially located cell bodies tended to be labeled near the central canal and contained dense, highly branched, laterally oriented dendritic fibers. (C) Fewer sections contained both large and small labeled cell bodies. Some sections contained small, ventro-laterally labeled cell bodies with dorsally oriented and moderately branched dendrites (D). Scale bars = 100 μm. Abbreviations: c = central canal, d = dorsal, FV = ventral fasciculus, MLF = medial longitudinal fasciculus.
Figure 4.7. Examples of photomicrographs of counterstained (A, B, C, E, G) and non-counterstained (D, F, H) transverse sections of *Hemitaurichthys polylepis* rostral spinal cord showing small-type, ventro-laterally located cell bodies labeled with biocytin. Note the extensive dendritic branches present dorsally in A, D, E, and G. Note prominent extension of dorso-laterally oriented dendrites in C, F, G, H. Arrows with closed heads in H point to axons that exit a ventro-lateral spinal nerve and arrows with open heads point to dorso-lateral oriented dendrites. Scale bars = 100 μm. Abbreviations: c = central canal, d = dorsal, FV = ventral fasciculus, MLF = medial longitudinal fasciculus.
have bifurcated dendritic projections and were somewhat stellate in appearance. Labeled cells were not distinct in cytoarchitecture, size, or location from adjacent non-labeled cells, which presumably innervated other muscle fibers or were not labeled by the biocytin application procedure.

By contrast, small-type neurons were positioned ventral and lateral to large-type neurons, and lateral to the MLF and FV (Figure 4.6 C,D, Figure 4.7). Cell bodies of small-type neurons were ovoid in shape. Similar with large type neurons, relatively few small-type cell bodies per transverse section were labeled (1-5, median 2 per section) and were present across a broad distribution of the spinal cord. Adjacent unlabeled neurons had a similar cytoarchitecture, size and location. Small-type cells typically displayed only one or two dendritic projections that extended dorsolaterally (Figure 4.7) and in several sections had faintly visible axonal projections to ventral spinal roots (Figure 4.7H).

Large-type neurons (n=45) were present on transverse sections from five fish, with the number of transverse sections with a range of 40 μm sections with labeled large-type neurons (5, 5, 6, 6, and 9 sections/fish). Small-type neurons (n=95) were found from three fish and much more variability in terms of sections present with labeled neurons (7, 8, and 24). Only two sections (1 section from two different fish) contained small-type neurons co-labeled with large-type (Figure 4.6C). Fibers were labeled on transverse sections from five fish with a range of sections with labeled fibers (5, 6, 10, 14, and 29). The extent of labeled neurons and fibers within the spinal cord had a wide range (10, 11, 17, 21, and 41 sections), which indicated an extent of at least 400 to 1640 μm. Somata of large-type neuron were of statistically greater area than small-type
neurons (back transformed mean 496 µm² vs. 273 µm², GLM individual F_{129,2} =0.06, p=0.943, neuron type F_{129,2}=27.68, p=0.026, Figure 4.8).

Figure 4.8. Histogram (A) of cell area and comparison of cell size between large (black bars) and small-type (white bars) cell bodies of three *Hemitaurichthys polylepis* individuals and bar graph (B) of mean cell area after back-transformation of log10 transformed data. (* denotes cell size difference, p<0.05, p>0.05 for individual and interaction, GLM with random subjects factor).
DISCUSSION

This study presents the first examination of sonic muscle histology and motor neuron innervation in a butterflyfish (Chaetodontidae). Features of the sonic muscles and the motor neurons that innervate them in Pyramid Butterflyfish share similarities with some, but not all fishes examined with swim bladder muscle driven sound production. These observations underscore the importance of independent evolutionary events that have shaped the diversity of fish sound production mechanisms.

Sonic musculature

Pyramid Butterflyfish possess apomorphic extrinsic muscle fibers that originate on the otic region of the neurocranium and the medial supracleithrum of the pectoral girdle and insert on the first three ribs and an aponeurosis of the hypaxial musculature. The location of this musculature and indirect association with the swim bladder bears similarity with a variety of distantly related sound producing percomorph fishes. Holocentrid fishes possess extrinsic sonic muscles that bear notable superficial similarity to *Hemitaurichthys polylepis* (Winn and Marshall, 1963; Salmon, 1967; Carlson and Bass, 2000). Winn and Marshall’s (1963) account of *Holocentrus rufus* sound production morphology describes muscles that originate on the ventral skull, run under the dorsal pectoral girdle and insert on the first two ribs and continues to end on ribbon-like fascia anterior of the third rib. Similarly, the Pineconefish (*Monocentrus japonica*) (Monocentridae) has extrinsic swim bladder muscles that originate on the back of the skull, but insert on the anterior swim bladder (Onuki and Somiya, 2007). Similar muscles have been described in scorpaeniform fishes such as the Southern Pigfish (*Congiopodidae: Congiopodus*
leucopaecilus) (Packard, 1960), and the Marbled Rockfish (Scorpaenidae: *Sebasticus marmoratus*), which has extrinsic muscles that originate on the neurocranium and insert on the anterior swim bladder (Miyagawa and Takemura, 1984; Suzuki et al., 2003). The perciform Tigerfish (Terapontidae: *Terapon jarbua*) has sonic muscles that originate on the caudoventral neurocranium and supracleithrum and insert on the anterior swim bladder (Eichelberg, 1976; Onuki and Somiya, 2007), and the perciform Silver Sweeper (Pempheridae: *Pempheris schwenkii*) has sonic muscles that originate on the pterotic of the neurocranium and insert on the anterior swim bladder (Takayama et al., 2003). Paracanthopterygian cusk-eels (Ophidiidae) also possess sonic muscles that originate on the caudal end of the neurocranium, course medial to the dorsal pectoral girdle, and insert on bony elements (epineurals and an ossification of the anterior swim bladder) in close association with the swim bladder (Parmentier et al., 2006; Fine et al., 2007; Parmentier et al., 2010). Though extrinsic sonic muscles of many distantly related fishes have similar anatomical location and functions, a variety of apomorphic histological features of the muscles and their motor neurons demonstrate the independent origins of these sound producing organs.

A trend towards smaller muscle fiber diameters is apparent among sonic fishes. Muscle fiber diameters of Pyramid Butterflyfish (interquartile range estimated from the cross-sectional area measurements of sonic muscle fibers, 11-24 µm) are similar to fiber diameters of other sound producing fish species (Batrachoididae, Carapidae, Dactylopteridae, Triglidae, Terapontidae, and Sciaenidae) (reviewed in Parmentier & Diogo 2006). White trunk muscle fiber diameters from Pyramid Butterflyfish were larger (27-35 µm), were at the low end of the more variable range of white trunk muscle fiber
diameters, but like other sound producing taxa were larger than their sonic counterparts (Parmentier and Diogo, 2006). Smaller sonic muscle fiber diameters are hypothesized to be an adaptation for more efficient transfer of metabolites, oxygen, and calcium ions from the increased surface to volume ratio provided by small fibers (Eichelberg, 1976; Parmentier and Diogo, 2006). Male Weakfish sonic muscle fibers enlarge during the spawning season when sounds are produced, but a central core of sarcoplasm also enlarges and fragments (Connaughton et al., 1997), which may reduce losses in surface to volume ratio of the myofibrils. Cores of sarcoplasm were not seen in Pyramid Butterflyfish in this study. It is not known at present, however, if the sonic musculature of Pyramid Butterflyfish changes with season or with sex. It is hypothesized that males produce sounds for courtship and agonistic competition among males (Boyle and Tricas, 2010), but both females and males produce distress calls in the laboratory (Boyle unpublished). Additional studies are required to determine if changes in sonic muscle fiber diameter and concentration of sarcoplasm occur seasonally, ontogenetically, or between sexes as has been found for several other fishes (Bass and Marchaterre, 1989; Connaughton et al., 1997; Loesser and Fine, 1997).

The radial arrangement of myofibrils in some pyramid sonic muscle fibers is consistent with the hypothesis of increased transport efficiency of calcium ions and metabolic substrates. Radially arranged myofibrils are known from a variety of fish sonic muscles that include pimelodid and doradid catfishes (Ladich, 2001), Oyster Toadfish (Fawcett and Revel, 1961; Loesser and Fine, 1997), Plainfin Midshipman (Bass and Marchaterre, 1989), Weakfish (Ono and Poss, 1982), and Tigerfish (Eichelberg, 1976). Pyramid Butterflyfish sonic muscle, like the sound producing muscles of these other
fishes, possesses a well developed sarcoplasmic reticulum (SR), visible as a dense network of sarcotubules between myofibrils that may provide rapid exchange of calcium ions to the myoplasm for crossbridge formation and exchange back to the (SR) for cross-bridge detachment (Rome, 2006).

In addition to modifications of the SR, the t-system of some fish sonic muscles possess unusual location and morphology in some cases. T-tubules of typical fish muscles are located at the Z-line (Johnston, 1981), in the sonic muscles of some fishes, however, they are found at the A-I boundary like mammalian muscles in Oyster Toadfish (Fawcett and Revel, 1961), Plainfin Midshipmen and Northern Searobin *Prionotus carolinus* (Bass and Marchaterre, 1989), and tigerperch (Eichelberg, 1976). The sonic muscles of Pearl Fish (*Carapus acus*) are unusual in that triads are found at both the Z-line and A-I boundary (Parmentier et al., 2003). In Marbled Rockfish, triads are located at the A-I boundary in the middle portion of sonic muscle fibers, at the Z-line at the end points (neurocranium and swim bladder) and at both the Z-line and A-I boundary at an area adjacent to the middle of the fiber (Suzuki et al., 2003). Pyramid Butterflyfish sonic muscles possessed Z-line type triads in both trunk and sonic muscle. Several other sound producing fishes have Z-line type muscle fibers. These include piranha (Eichelberg, 1977), pimelodid and doradid catfishes (Ladich, 2001), and Weakfish (Ono and Poss, 1982). The position of triads among sonic muscle fibers thus does not follow an obvious phylogenetic pattern. The location of triads at the A-I boundary is hypothesized to be adaptive for fast contractions because this morphology results in two t-tubules per sarcomere rather than one (Ladich and Fine, 2006) and also should limit the distance of depolarization and diffusion to the site of contraction (Parmentier et al., 2003).
Eichelberg (Eichelberg, 1977) hypothesized that the short sarcomeres in sonic muscles (1.2 µm, vs. 1.4 µm in trunk muscle) may allow for similar conduction velocities to those found in muscles with longer sarcomeres and triads at the A-I boundary. This hypothesis gains some support from the observations that some of the fishes with A-I located triads tend to have long sarcomeres: Toadfish (2.0-2.22 µm) (Fawcett and Revel, 1961; Loesser and Fine, 1997), Plainfin Midshipmen (2.0-3.4 µm) (Bass and Marchaterre, 1989), Marbled Rockfish (2.2-2.3 µm) (Suzuki et al., 2003), and Tigerfish (2.2 µm) (Eichelberg, 1976). Pyramid Butterflyfish sonic muscles measured in this study had smaller sarcomere lengths (1.74 µm), similar to weakfish sarcomere lengths (1.5 µm) (Ono and Poss, 1982), but longer than piranha (Eichelberg, 1977). Two sound producing catfishes (Platydoras costatus and Pimelodus pictus), however, have long sarcomeres and Z-line (2.2-2.3 µm) (Ladich, 2001). Pyramid butterflyfish sonic muscles measured in this study had slightly longer sarcomeres than adjacent trunk muscle fibers. Tigerfish have longer sonic muscle sarcomeres than white trunk muscle fiber sarcomeres (Eichelberg, 1976), but piranha have shorter sonic muscle fiber sarcomeres than trunk muscle (Eichelberg, 1977).

The triads of Pyramid Butterflyfish sonic muscle examined in this study had unusual morphology. The terminal cisternae often were enlarged and flask shaped in sonic muscles but not in trunk muscle. Additionally, the t-tubule of sonic muscles are wider than their counterparts in trunk muscles. These features may be involved in the contractile properties of this muscle. T-tubules are involved in transmission of the action potential from the surface of the muscle fiber to deep inside the muscle cell where release of calcium ions from the sarcoplasmic reticulum is initiated (Rome, 2006). Diameter of
the t-tubule may impart differences to the speed or strength of depolarization. The enlarged terminal cisternae are the site of calcium ion release and may present a morphology with enhanced transmission of the action potential from the t-tubule to the sarcoplasmic reticulum, delivery and resequestering calcium ions to and from the myoplasm more efficiently, or both. Unusual t-system morphology is present in the sound producing Marbled Rockfish, which in addition to triads present at the Z-line and A-I boundary, has pentads and heptads composed of two or three t-tubules and three or five terminal cisternae, respectively (Suzuki et al., 2003).

**Sonic motor neurons-patterns among teleosts**

Phylogenetic and functional patterns of sonic motor pathways of a variety of fishes have received attention from a number of researchers (Bass and Baker, 1991; Ladich and Bass, 1998; Carlson and Bass, 2000; Ladich and Bass, 2005; Onuki and Somiya, 2007). The variety of distantly related teleost lineages with highly soniferous members that employ muscle-driven swim bladder mechanisms has led to interest in innervation patterns of musculature and identification of patterns of representation of motor neurons in the spinal cord and brain. There is evidence of possible deep homology for some aspects of vocal organ development among fish and tetrapods (Bass et al., 2008), but clearly many independent evolutionary events have occurred among taxa, as evidenced by the occurrence of different musculo-skeletal features involved in sound generation. Further, sound mechanisms are known currently from a diverse array, yet significant minority of fishes. Thus teleost-wide sound production homologies would require many independent evolutionary losses, which would seem unlikely (Parmentier and Diogo, 2006). Several
patterns of sonic motor neuron location and innervation exist among teleosts examined thus far.

Previous authors have examined the patterns of sonic motor innervation within a phylogenetic framework (Ladich and Bass, 1998; Carlson and Bass, 2000; Ladich and Bass, 2005; Onuki and Somiya, 2007) and some patterns of conserved innervation and motoneuron position are suggestive of homology. Recent revision of the phylogenetic hypothesis of teleostean relationships (Wiley and Johnson, 2010), casts doubt on the homology of some of the previously recognized patterns (Table 4.1). Sonic motor pathways described in fishes thus far, involve spinal nerves, occipital nerves, or both with the exception of mormyrids which appear to have vagal innervation of sonic muscle and a sonic motor nucleus contiguous with the vagal motor nucleus in the medulla and dorsal to the central canal (Bass, 1985; Onuki and Somiya, 2007). Sonic motor pathways are known from two ostariophysan orders (Table 4.1): Characiformes and Siluriformes. Piranhas (Characidae: Serrasalminae) possess extrinsic sonic muscles innervated entirely by spinal motor neurons in a non-discrete nucleus, centralized within the spinal cord (Ladich and Bass, 2005; Onuki et al., 2006). Within the catfish order Siluriformes, several innervation and motor nuclei pattern are known that differ at the family level (Table 4.1), all of which include occipital nerve innervation and motor nuclei in the caudal medulla and rostral spinal cord, but include one (Mochokidae) or two spinal nerves (Pimelodidae) and vary in location within the central nervous system CNS (centralized in Doradidae and Mochokidae, ventro-lateral in Ariidae, or a combination in Pimelodidae) (Ladich and Fine, 1994; Ladich and Bass, 1996, 1998). Most of the euteleost fishes examined for sonic motor pathways thus far (Table 4.1), possess occipital
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<th>Sonic Organ Innervation</th>
<th>Rostrocaudal Extent</th>
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Sonic Organ: Ex SM = extrinsic swim bladder muscles, In SM = intrinsic swim bladder muscles, Pect. Strid. = pectoral fin ray-tendon stridulation, Pect. Vib. = pectoral girdle vibration. Central = centralized, ventral to cc, dors. central = centralized but dorsal to cc, vent. lat. = ventral and lateral to MLF, vent. lat. & cent. = nuclei ventral and lateral to MLF and centralized ventral to cc.

rostral spinal cord, in ventral motor columns, lateral to the MLF (Table 4.1). The well-studied exception of batrachoidids have occipital nerve innervated apomorphic intrinsic sonic muscles with sonic motor nuclei that extend from the caudal medulla and rostral spinal cord, but are present in a centralized location, ventral to the central canal (Fine et al., 1982; Bass, 1985; Bass and Baker, 1991; Fine and Mosca, 1995). The holocentrid squirrelfishes have an extrinsic sonic mechanism, and a sonic motor nucleus positioned as a ventrolateral motor column that is distributed across the rostral spinal cord and caudal medulla (Carlson and Bass, 2000). Sonic pathways were described from other fishes of the Euteleostoeomorpha with uncertain relationship status among percomorph fishes (Table 4.1). Representatives of both scorpaeniform families examined thus far have occipital nerve innervated sonic muscles (extrinsic in Scorpaenidae and intrinsic in Triglidae) with sonic motor nuclei in the caudal medulla and rostral spinal cord in a ventrolateral motor column (Bass, 1985; Finger and Kalil, 1985; Bass and Baker, 1991; Yoshimoto et al., 1999). Sculpins (Cottidae) possess a similar sonic motor nucleus, but do not have a swimbladder and produce sounds with vibration of the pectoral girdle (Barber and Mowbray, 1956; Bass and Baker, 1991; Ladich and Bass, 1998). This similar arrangement was proposed as a potentially conserved motor nuclei arrangement despite divergent sound production mechanisms among scorpaeniform fishes (Ladich and Bass, 1998; Yoshimoto et al., 1999; Ladich and Bass, 2005). Recent phylogenetic hypotheses, however, have disputed the inclusion of cottoid fishes within the Scorpaeniformes (Wiley and Johnson, 2010). The Croaking Gourami (Trichopsis vittatus), which produces sounds by stridulation of pectoral fin ray elements across tendons (Kratochvil, 1978), has a similar ventro-lateral motor nucleus of the superficial
adductor muscle in the caudal hindbrain and rostral spinal cord (Ladich and Fine, 1992). The similar location of motor nuclei among pectoral girdle driven sound mechanisms is predicted in part by the conserved pattern of occipital nerve rami innervation of pectoral girdle muscles in teleost fishes (Parenti and Song, 1996), and a similar distribution of pectoral fin motorneurons is found in the mochokid catfish Synodus nigromaculatus which produces pectoral fin stridulation sounds in addition to swim bladder muscle sounds (Ladich and Bass, 1996).

The location of sonic motor neurons entirely within the spinal cord, not in distinct nuclei, and in ventrolateral and central locations of the spinal cord found in pyramid butterflyfish in this study is unusual relative to the other pathways described thus far (Table 4.1). This pattern is similar to what is described for the very distantly related piranhas, in which spinal nerve innervation has restricted motor nuclei to the spinal cord and motor neurons of sonic muscle lie adjacent to similar motor neurons that appear to innervate non-sonic areas (Ladich and Bass, 2005; Onuki et al., 2006). However, small-type motor neurons located along a ventral motor column were also labeled in pyramid butterflyfish in this study. Sonic motor neurons located entirely in the spinal cord are likely more widespread among acanthomorph fishes, as a variety of taxa with spinal nerve innervation of sonic muscle are known for which pathways of sonic motor neurons have yet to be identified (Onuki and Somiya, 2007). These taxa include the gadiform Walleye Pollock (Gadidae: Theragra chalcogramma; Onuki and Somiya, 2006), the zeiform John Dory (Zeidae: Zeus faber; Onuki and Somiya, 2004), and the highly soniferous perciform sciaenid fishes (Ono and Poss, 1982; Vance et al., 2002). Whether any taxa with spinally innervated sonic muscles possess discrete motor nuclei similar to
the pattern seen among occipital nerve innervated taxa remains to be determined. The diversity of sonic motor neuron patterns among distantly related teleosts indicates multiple independent evolutionary modifications of features associated with the occipital region of the CNS and anterior spinal nerves.

**Sonic motor neuron somata sizes**

Overall, the sizes of sonic motor neurons labeled by biocytin in this study were similar to sizes measured in other sonic fish taxa. Somata sizes in this study were similar to sizes reported from piranha: large-type sizes were comparable to ‘medium’ piranha motorneuron somata and small-type were intermediate to ‘medium’ and ‘small’ piranha motor neuron somata (Onuki et al., 2006). Sizes of small-type motor neuron somata from this study also were similar to the range reported for biocytin and BDA labeled cells in the catfish *Bagre marinus* (Ladich and Bass, 1998). Sizes of both large-type and small-type motor neurons in this study were similar to the range reported in biocytin and BDA labeled holocentrids (Carlson and Bass, 2000). Large-type motor neuron somata were similar in size to Type I male Plainfin Midshipman (Bass and Baker, 1990), but on average were substantially smaller (~3X) than male Oyster Toadfish sonic motor nucleus (SMN) somata (Fine and Mosca, 1995). Some exceptionally large somata found in this study, however, overlapped with size ranges reported for male Oyster Toadfish. Compared with Croaking Gourami, large-type soma were bigger than most Croaking Gourami dorsal motor column cells labeled from epaxial muscle and both small and large-type were bigger than most ventral motor column somata that innervate gourami pectoral fin musculature (Ladich and Fine, 1992). Large-type soma were smaller on
average, but both large-type and small-type soma overlap the distribution reported for scorpænid sonic motor somata (Yoshimoto et al., 1999). Large-type somata were smaller but overlapping in range with biocytin labeled non-sonic abductor superficialis (MAS) of the pectoral fin in the osteoglossiform Pantodon buchholzi, which utilizes MAS muscles for an escape response (Starosciak et al., 2008). Sizes of the somata identified from sonic muscle in this study also broadly overlapped with the range of trunk muscle motor neuron sizes reported for Goldfish Carassius auratus (Fetcho, 1986). Large motor neuron somata are hypothesized to be an adaptation for innervation of muscles with high contraction rates (Bass, 1985) and also are hypothesized to provide a metabolic advantage for increased call production (Fine and Mosca, 1995).

This study found distinctive small-type and large-type motor neurons based on location and cytoarchitecture. Small-type neurons resemble the ventral motor column neurons present in a variety of sonic taxa (Table 4.1), while large-type neurons labeled in this study resemble epaxial, non-sonic motor neurons labeled as control muscle in Croaking Gourami (Ladich and Fine, 1992) and Threespot Squirrelfish Sargocentron cornutum (Carlson and Bass, 2000). Labeled large type neurons in this study clearly did not come from epaxial myotomes, as all biocytin application took place well below the midlateral horizontal septum. The homology of sonic muscle in Pyramid Butterflyfish is not completely understood, but the location below the lateral septum and the insertion along an aponeurosis of hypaxial myotomes is consistent with hypaxial origin. Intrinsic sonic muscles of Oyster Toadfish migrate embryologically from occipital somites to a more caudal location around the swim bladder (Tracy, 1959, 1961). Though some sonic muscle fibers originate on the occipital region of the skull (Figure 4.1), the pattern of
origin is consistent with the obliquus superioris hypaxial musculature of many teleosts (Winterbottom, 1974).

In this study the large-type motor neurons were present in all fish labeled, while the small-type were more numerous, but labeled in only three of five fish. Based on differences in soma size between large motor neuron somata and small putative premotor neuron somata, and differential results with other neuronal tracers, biocytin was hypothesized to label premotor neurons via transneuronal transport in studies of mochokid catfish (Ladich and Bass, 1996) and squirrelfishes (Carlson and Bass, 2000). Other studies on sonic motor neurons with biocytin, however, have not found transneuronal transport (Yoshimoto et al., 1999; Ladich and Bass, 2005). The small-type neurons of this study, however, do not appear to be premotor neurons, as their size overlaps many sonic and non-sonic motor neurons found in other fishes, they appear to have axons that exit from ventral nerve roots, and they are similar in appearance to other ventral column motor neurons (e.g. the sonic motor neurons of squirrelfishes; Carlson and Bass, 2000). Thus the multiple neuron types may innervate different areas of sonic muscle fibers present in *Hemitaurichthys polylepis*, however, further experiments are necessary to determine the pattern of innervation within the overall sonic musculature.

**Aspects of fiber connections**

In this study, no evidence of contralateral dendritic connections from sonic motor neurons was observed. Sound production in Pyramid Butterflyfish involves highly synchronous motor activation in sonic musculature on the right and left side of fish (Boyle and Tricas, 2010). Highly synchronized muscle activation is evidenced by
electromyography (EMG) experiments that show waveforms that resemble two (doublet) or one (singlet) muscle action potentials indicative of synchronous activity within musculature, and the EMGs between right and left muscles are highly synchronized (Boyle and Tricas, 2010). Some other sonic fish taxa, which also may require synchronous contraction of muscles around the swim bladder to generate sound (Ladich and Fine, 2006), have connections between contralateral motorneurons that are hypothesized to maintain synchronous firing. Such sonic motor connections are found in batrachoidids (Pappas and Bennett, 1966; Bass and Baker, 1990), the scorpaenid *Sebasticus marmoratus* (Yoshimoto et al., 1999), but not in piranha (Ladich and Bass, 2005; Onuki et al., 2006) and Short-spined Sculpin *Myoxocephalus scorpius* (Bass and Baker, 1991). Motor neuron connections also are not found in Northern Searobin. The Northern Searobin produces antiphasic contractions of the right and left sonic muscles, and is hypothesized to possess a network of reciprocating pacemaker neurons (Bass and Baker, 1991). In a study on sonic motor nuclei in squirrelfish, contralateral projections from biocytin applications were interpreted by the authors as premotor neurons that were labeled transneuronally (Carlson and Bass, 2000). Thus premotor neurons may be important for the production of synchronized bilateral firing of sound production muscles in fish such as the Pyramid Butterflyfish that do not appear to have direct contralateral connections between motor neurons.

**Comparison with the butterflyfish genus Forcipiger**

Sound production is known from multiple genera within the butterflyfish family Chaetodontidae (Tricas et al., 2006; Boyle and Tricas, 2009, 2010). Experiments with
Forcipiger flavissimus, a species in the genus sister to a clade that comprises Hemitaurichthys, Heniochus, and Johnrandallia, demonstrate a dramatically different kinematic pattern associated with sound emission (Boyle and Tricas, unpublished). Sound production in *F. flavissimus* involves synchronous muscle activity in the anterior epaxial musculature behind the supraoccipital of the cranium, the sternohyoideus, and the adductor mandibulae that occurs at the initial onset of sound emission and results in a rapid elevation of the cranium without protrusion of the oral jaws, which are presumably kept shut by activity of the adductor mandibulae (Boyle and Tricas, unpublished). Unlike sonic muscle activity in *Hemitaurichthys polylepis*, muscle activity in *F. flavissimus* sound production events involves burst activity within each muscle group, representative of many unsynchronized muscle action potentials, rather than single synchronized events, and EMG experiments showed no activity of anterior hypaxial musculature in the same location of sound production muscle of *H. polylepis* (Boyle and Tricas, unpublished). Thus sonic motor kinematics in *F. flavissimus* likely involves diverse motor nuclei with muscles innervated by many different nerves that may include occipital and spinal nerves for epaxial myotomes (Westerfield et al., 1986; Thys, 1997; Carlson and Bass, 2000), occipital nerve rami for sternohyoideus (Parenti and Song, 1996; Boyle and Tricas, unpublished), and trigeminal for adductor mandibulae (Winterbottom, 1974; Nakae and Sasaki, 2004).

**Conclusions**

Results of this study indicate that differences in muscle fiber size and structure of sarcoplasmic reticulum and their associated transverse tubules may be related to features
necessary for call production in Pyramid Butterflyfish. Other features associated with sonic muscles in distantly related sound producing features, such as t-tubules at the A-I boundary and cores of sarcoplasm, however, were not detected. This study found sonic motor neurons entirely in the spinal cord in a pattern somewhat similar to what is known from the piranha. Future studies on ontogenetic and sexual differences related to sound production behavior and physiology and contraction properties and innervation of specific subregions within the sonic musculature of this species may answer remaining questions raised by this study.

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CHAPTER V:
SOCIAL SYSTEM, TERRITORIALITY, AND AGONISTIC SOUND PRODUCTION BEHAVIOR IN THE SYNTOPIC SISTER TAXA: FORCIPIGER FLAVISSIMUS AND F. LONGIROSTRIS (CHAETODONTIDAE)

ABSTRACT
Butterflyfishes (Chaetodontidae) exhibit a range of social behaviors, in which planktivorous species tend to shoal and be affiliative, while corallivorous species tend to occur in territorial monogamous pairs. This study examined the social dynamics and role of acoustic communication within two similar, syntopic, non-obligate corallivore butterflyfish species in Hawaii. Field observations and resurveys of marked individual Forcepsfish (Forcipiger flavissimus) and Longnose Butterflyfish (F. longirostris) provided evidence that Forcepsfish are socially haremic, Longnose Butterflyfish are socially monogamous, and both species defend feeding territories from conspecifics. Video and hydrophone recording experiments with caged conspecifics introduced within fish home ranges elicited territorial behavior and agonistic sound production. A subset of experiments was conducted in which caged intruders were presented along side caged mates in order to elicit sounds of resident fish to both mates and non-mates. Sounds were analyzed to test predictions of the hypothesis that sounds may signal territoriality, mate guarding, individual recognition, and species recognition. Discriminant analyses indicated that acoustic features (spectral features, sound duration, and amplitude) were relatively poor predictors of communication contexts (sex of the receiver, mate or non-mate identity of receivers, identity of the signaler) and species. Thus, acoustic discrimination of individuals is unlikely and sounds may function as greeting rituals during agonistic encounters produced before aggression escalates. Further, although Forcepsfish sounds trend towards lower frequencies and higher sound pressure levels compared to Longnose Butterflyfish sounds, high interspecific acoustic overlap indicates sounds may have a limited role for behavioral isolation of these sympatric species.
INTRODUCTION

Coral reef fishes display a wide range of social behaviors that are maintained through communication with multiple sensory modalities. Reef fish taxa include gregarious, affiliative school-forming species, such as some surgeonfishes (Barlow, 1974), damselfishes (Cole, 2008) and butterflyfishes (Hourigan, 1989), solitary-territorial damselfish species (Ebersole, 1977; Itzkowitz et al., 2000), socially monogamous species such as many butterflyfishes, anemonefishes, and some pipefishes (Roberts and Ormond, 1992; Whiteman and Côté, 2004), and socially haremic species that include some angelfishes (Hourigan et al., 1989; Allen et al., 1998) some butterflyfishes (Hourigan, 1989; Yabuta and Kawashima, 1997), and sex changing wrasses (Warner et al., 1975). Territoriality is a feature of some, but not all socially monogamous species (Whiteman and Côté, 2004) and several hypotheses have been proposed to explain the repeated evolution of monogamous and haremic social systems in marine fishes.

Socially monogamous reef fish maintain a pair-bond between breeding periods (Hourigan, 1989; Whiteman and Côté, 2004). Hypotheses for the ultimate evolutionary mechanisms that may favor social monogamy in reef fishes (reviewed by Whiteman and Côté, 2004, Strang 2005) include biparental care, resource limitation that accounts for low availability of females or low population density, increased reproductive success, mutual mate guarding, and territorial resource defense. Butterflyfish (Chaetodontidae) social behavior has generated research attention because many members of the family, which includes approximately 122 species (Nelson, 2006), exhibit pairing behavior and social monogamy (Hourigan, 1989; Roberts and Ormond, 1992; Whiteman and Côté, 2004; Yabuta, 2007). Butterflyfish are broadcast spawners that exhibit no parental care
(Lobel, 1989) and some species are corallivorous and defend feeding territories on the reef (Reese, 1975; Fricke, 1986; Tricas, 1989b; Righton et al., 1998). Thus two main hypotheses for the evolution of monogamy in these fishes have been favored: territorial defense of stable coral food resources (Hourigan, 1989; Roberts and Ormond, 1992) and mutual mate guarding (Fricke, 1986; Strang, 2005). These hypotheses are not mutually exclusive and some species exhibit behaviors consistent with both hypotheses (Fricke, 1986; Strang, 2005). The majority of butterflyfish species studies on social behavior with longer, repeated observations of individuals have focused on corallivorous species (Hourigan, 1989; Roberts and Ormond, 1992; Whiteman and Côté, 2004), which tend to occur in the genus *Chaetodon* (Bellwood et al., 2009), and the social behavior of many of the species from the long-snouted bannerfish clade is poorly known.

The butterflyfish genus *Forcipiger* includes two species, *F. flavissimus* and *F. longirostris* (Allen et al., 1998) with overlapping broad Indo-West Pacific distributions. These two species are similar in terms of color pattern and morphology, which led to taxonomic confusion until relatively recently (Randall and Caldwell, 1970) and the species are estimated to have separated 2.1 to 8.1 million years ago (Bellwood et al., 2009). The similarity in morphology and unusual jaw morphology of these fishes has prompted study on character displacement with respect to feeding ecology (Ludwig, 1984) and functional morphology of the jaws (Ferry-Graham et al., 2001b). Neither species is an obligate corallivore, and *F. flavissimus* is known to bite and remove pieces of benthic prey, while *F. longirostris* has highly prostrusible jaws that allow it to consume more mobile prey like caridean shrimps (Hobson, 1974; Ferry-Graham et al., 2001b). Detailed observations of the social behavior of these two species are lacking,
although accounts of pairing behavior for both species are reported (Hourigan, 1989) and some accounts of agonistic behavior (Ehrlich and Ehrlich, 1982; Ludwig, 1984). Despite differences in feeding ecology from other well-studied monogamous butterflyfishes, these species may exhibit social monogamy or polygyny as seen in related fishes and thus require complex behaviors to facilitate such sociality.

The ability of fish to maintain territorial social systems requires the recognition of habitat features and individual conspecifics and likely requires multiple sensory modalities. Butterflyfish are highly visual animals and their territorial behavior has been examined with respect to poster coloration (Ehrlich, 1977) and a large portion of territorial maintenance for these fishes may be visual advertisement (Tricas, 1985; Fricke, 1986). A recent study has demonstrated the use of agonistic sounds during territorial behavior in the Pebbled Butterflyfish (*Chaetodon multicinctus*) (Tricas et al., 2006). These observations indicate that social communication in butterflyfishes may be multimodal, and it is possible that communication at close range may involve olfactory systems as well. Some butterflyfish social groups may be stable over long periods of time (four to 10 years) (Fricke, 1986; Hourigan et al., 1988). Such social relationships require individual fish to recognize and discriminate mates, neighboring territory holders, and unfamiliar conspecifics. Periods of separation from mates beyond communication distances (visual or otherwise) requires quick discrimination of individuals in order to allow for adequate territory defense, maintenance of pair bonds, and to avoid injury to or from mates that would result from discrimination failure (Yabuta, 2002). Mated butterflyfish display brief periods of agonistic-like behavior that is consistent with a greeting ritual that may serve to increase the amount of time necessary for both fish to
recognize their partners (Yabuta, 2002), and it is possible that acoustic behaviors are part of such displays (Tricas et al., 2006). Experiments, such as model bottle experiments (Tricas, 1989b; Tricas et al., 2006), that test for the presence of territorial behavior by introduction of conspecifics into the home ranges of other individuals can provide insight into which behaviors and sensory modalities are used by reef fishes in interactions with conspecifics and similar heterospecifics.

The purpose of this study is, first, to examine the social behavior of two syntopic species of the genus *Forcipiger*: the Forcepsfish *F. flavissimus* Jordan & McGregor and the Longnose Butterflyfish *F. longirostris* (Broussonet) with respect to mating system. Second, territoriality is tested experimentally in both species by controlled introduction of conspecifics into other individual home ranges. Third, the use of acoustic signals in agonistic territorial behavior was tested with manipulated interactions in the field in which caged conspecifics were introduced within home ranges of free-swimming fish. Fourth, in order to test predictions of the hypothesis that acoustic signals are used to convey context-specific information with regard to sender and receiver identity, linear discriminant analyses were conducted to test if acoustic features (spectral features, duration, and sound pressure level) were predictors of behavioral context (sex of the receiver, mate or non-mate identity of receivers, identity of the signaler) or species identity (Forcepsfish or Longnose Butterflyfish). Results provide evidence that both Forcepsfish and Longnose Butterflyfish defend feeding territories from conspecifics and produce acoustically simple, pulsatile sounds during agonistic territorial interactions. High-similarity among individuals within each species regardless of context indicates that
acoustic discrimination of individuals is unlikely and sounds may be a function of greeting rituals during agonistic encounters that are produced before aggression escalates. Interspecific differences between agonistic sounds were relatively minor and thus agonistic sounds may have a limited role in behavioral isolation of these closely related species.

MATERIALS AND METHODS

Social group dynamics

Individuals of *F. flavissimus* and *F. longirostris* were observed by scuba divers before setup of agonistic sound production behavior experiments at field sites on the Kona and Kohala coasts of west Hawaii Island. Divers observed groups of individual fish within a common reef site to confirm social associations and to record interactions between individuals over time.

Preliminary observations indicated that *F. longirostris* individuals were highly paired, while *F. flavissimus* individuals were more likely to be encountered solitary, but were seen in foraging groups of three individuals (trios). Divers followed individual fish within a putative trio between 17 and 24 minutes (average 19.8 minutes). In one case three fish of a social group were identified but divers only followed two fish. Each diver carried a stopwatch with a countdown timer with an audible alarm that sounded once per minute. At the end of each minute, divers recorded when fish were within 1m to another member of their putative social group and if fish were within 1m, inter-individual distance was estimated by measuring a nearby position on the reef of each fish with a measuring (after the fish moved away to avoid disturbance). Divers recorded bites during
foraging and identified obvious benthic prey. Additionally, for eight *F. flavissimus* trios and six *F. longirostris* pairs, markers with plastic tape (~10 cm) were used to delineate the range of movements of individual fish as they moved during foraging. Two orthogonal dimensions were measured to provide a rough and likely conservative estimate of home-range use over the brief observation period.

Divers observed individual *F. longirostris* in the same manner as *F. flavissimus*, however, all *F. longirostris* observed occurred in pairs. Fourteen pairs of *F. longirostris* were observed between 13 and 22 minutes (average 19.1 minutes). Only one member of the pair was observed for eight of the pairs.

After observation, fish were collected with hand nets underwater, measured for standard length (SL) to the nearest mm, and sexed with a catheterization technique (Ross, 1984), and temporarily placed in mesh cages for agonistic sound production behavior experiments.

**Long-term dynamics of social groups**

In order to observe stability of site-attachment behavior and social group membership, several trios of *F. flavissimus* and pairs of *F. longirostris* were captured with handnets. Fish were held underwater briefly and measured (SL mm), sex determined by catheter, and marked with a colored bead tag, then released where they were captured. Colored beads (1-3 beads in different color combinations) provided a unique identifier for individuals within a study area and made it possible to determine if individuals associated with different individuals than members of the originally observed social group. Two tagging methods were employed. A suture thread tag that consisted of a loop of suture
thread inserted between the neural spines of vertebrae and dorsal spine pterygiophores tied between dorsal fin spines with beads attached. Suture thread tags were minimally invasive but were found to last on individual fish for no more than 30 days. Additional fish were tagged in a similar manner with a longer-lasting surgical wire tag. Surgical wire tags were found to last up to one year on fish and left a distinguishable scar on fish after tags were shed.

Agonistic sound production behavior experiments

Controlled interactions between different conspecific social groups were created by temporary removal of a trio (F. flavissimus) or pair (F. longirostris) and introduction into a conspecific’s territory. Fish were captured using hand and barrier nets, measured underwater (SL mm) and placed in individual mesh cages (25 x 25 x 25 cm) in home ranges of conspecifics. Two calibrated hydrophones (High Tech Industries, HTI Min 96: -163.7 and -163.8 dB re: 1V/μPa) were suspended at the center of a cage. The cages were placed approximately 50 cm and the hydrophones were thus separated by 75 cm (Figure 5.1). This arrangement with the hydrophones and cages separated spatially allowed all the cages to remain in view of the video camera (see below) but still aided interpretation of potential sound sources based on fish location, relative sound amplitude and arrival time at each hydrophone. For F. flavissimus, an additional caged fish (a trio of intruders) was used in all but one experiment and was placed 50 cm from the two cages with hydrophones in a triangular arrangement.
The two hydrophones were connected to two channels of a digital audio tape (DAT) recorder (Sony PCM-M1) in an underwater housing. A video camera connected to the lineout of the DAT recorder was placed on a small tripod in the field of view of both

Figure 5.1. Schematic of setup for agonistic sound production behavior experiments. A trio of *Forcipiger flavissimus* (pictured) or pair of *F. longirostris* held individually in 25x25x25 cm cages were introduced into a conspecific territory. A housed video camera synched to a two channel digital audio tape recorder was used to record agonistic interactions and sounds were recorded with two hydrophones placed in the center of two cages.

fish cages (in front of the two hydrophone cages for *F. flavissimus*) in order to record the behavior of the resident fish, captive intruder pair or trio. After the experiment began, divers left the area for 20 minutes to avoid adding SCUBA noise on the recording. At the end of the experiment, the fish were sexed underwater with a catheterization technique
(Ross, 1984) and released back in their home territories. Large adult fish of similar sizes were chosen for both species to avoid ontogenetic changes in sound production ability.

**Video and audio analysis**

Video and hydrophone recordings were uploaded digitally to a computer and synchronized within a single frame using Cinestream and Cool Edit Pro 2.0 software. Audio recording files were low-pass filtered and down sampled to 4000 kHz with the ‘high quality’ pre/post filter setting in Cool Edit in order to aid in the aural detection of fish sounds in a reef environment with high frequency noise from alpheid shrimps.

The presence, location, and behaviors of resident fish to caged intruder stimuli were determined from the video files. Video analysis was used to determine if agonistic behavior occurred more frequently to individuals of a particular sex. Behaviors from resident fish were analyzed from the segment of video when scuba bubble noise was not detectable from the setup and ending of the experiment. The time in seconds to the nearest frame (33 ms) was recorded in which a resident fish was: 1) present in the field of view and 2) within approximately two body lengths to an intruder cage. Any behavioral motor patterns discernable from the video were identified and tallied.

Sound production behavior was determined by monitoring video and audio segments and visualizing spectrographs in Cool Edit Pro. The source of sounds was determined from kinematic patterns visible on video and from the amplitude and time-of-arrival of sounds on each hydrophone. Only non-ambiguous sound events were analyzed, in which a sound occurred when a resident fish was visible and displayed body, fin movements or intention movements at the time of sound emission. Additionally, if the
suspected sound producing fish was within two body lengths of a hydrophone, a visible
time-of-arrival and amplitude difference between the two hydrophone recordings was
evident.

**Sound features analyzed**

Sound events in these two species consisted of emission of single pulses, often with large
interevent periods of seconds to minutes. Thus sound analyses were restricted to features
of the single pulses, as no pulse trains were observed. Pulse duration was estimated
visually from the sound waveform with Cool Edit Pro 2.0 software. Custom Matlab 7.0
programs were used to measure peak-to-peak sound pressure level (SPL dB re: 1μPa) and
to estimate power spectra with a zero-padded 1024 point fast Fourier transforms (FFT)
with a Hanning window. The following spectral features were determined from 512
frequency values and relative amplitudes values obtained from FFTs:

1) peak frequency, the frequency value with greatest relative amplitude

2) proportion of bandwidth within 10 dB of peak (BW)

\[ BW = \frac{\text{no. of frequencies } \geq (0.316 \times \text{maximum relative amplitude})}{512} \]

3) minimum frequency 10 dB from peak (10 dB min)

\[ 10 \text{ dB min} = \text{lowest frequency } \geq (0.316 \times \text{maximum relative amplitude}) \]

4) maximum frequency 10 dB from peak (10 dB max)

\[ 10 \text{ dB max} = \text{greatest frequency } \geq (0.316 \times \text{maximum relative amplitude}) \]

5) median frequency within 10 dB of peak (median frequency)

\[ \text{median frequency} = \text{median of frequencies } \geq (0.316 \times \text{maximum relative amplitude}) \]
6) 25\textsuperscript{th} percentile of frequencies within 10 dB of peak (10 dB 25\textsuperscript{th})

\[ 10 \text{ dB } 25\textsuperscript{th} = 25\textsuperscript{th} \text{ percentile of frequencies } \geq (0.316 \times \text{maximum relative amplitude}) \]

6) 75\textsuperscript{th} percentile of frequencies within 10 dB of peak (10 dB 75\textsuperscript{th})

\[ 10 \text{ dB } 75\textsuperscript{th} = 75\textsuperscript{th} \text{ percentile of frequencies } \geq (0.316 \times \text{maximum relative amplitude}) \]

All analyzed sounds occurred within approximately 1m of the hydrophones. No corrections for fish position within 1m to the hydrophones and signal loss from geometrical spreading or absorption were applied, so SPL levels should be considered approximations.

\section*{Data analysis and statistics}

A test for differences between the proportion of observations in which \textit{F. longirostris} pairs, \textit{F. flavissimus} males, and \textit{F. flavissimus} females were solitary was performed with a one-way ANOVA after arc-sine square root transformation. Post-hoc differences were tested with a Tukey-HSD test. A test for differences in the proportion of observations in which female \textit{F. flavissimus} individuals were paired with the other female trio member and the proportion of observations in which the female was paired with the male trio member was tested with a one-way repeated measures ANOVA after arc-sine square root transformation. Assortative mating in each species was assessed by linear least squares regression of female body length vs. male body length. Differences between the relative response rate of individual fish to caged intruders from each species (trio members or pair
members) were tested. The sex of resident fish was not confirmed, so for each pair or trio, the proportion of time each fish was present at the experimental arena was recorded. For each social group, resident 1-3 were assigned from the relative amount of time present (most to least) at the experimental arena. These data were highly non-normal, so a test for differences between the relative time spent at the experimental arena was tested with a non-parametric Kruskal-Wallis test and Dunn’s post-hoc test.

Differences in sound features (duration, peak frequency, or median frequency, and SPL) between species were tested with an unpaired t-test with individual fish as replicates and sound features averaged within fish. Differences in these sound features made towards male and female caged intruders were tested with a paired t-test with fish as replicates and sound features to each receiver type (male or female) averaged within fish. Multiple comparisons (8 initial comparisons) within species were corrected with a sequential Bonferroni correction (Rice, 1989). Parametric tests were conducted after testing for assumptions of normality and homogeneity of variance.

**Linear Discriminant analyses**

Classical linear discriminant analysis (LDA) (Engelman, 2000) was performed to determine the best subset of sound features to predict behavioral contexts and signaler identities (classification variables) of sound emission. Automatic backwards stepwise procedures were conducted with Systat 12.0. The procedure began with a saturated model and prediction variables were removed iteratively based on probability-to-remove values (>0.15) and probability-to-enter values (<0.15) in order to produce a final, simplified model with acoustic features that provided the best discrimination of
behavioral context. Several models with different classification variables were produced in order to test for the ability of acoustic features to distinguish sounds recorded from 1) different individual fish, tested separately with sounds from each species, 2) sounds made towards male or female caged conspecifics, tested separately for each species, 3) sounds made towards caged mates or caged non-mate intruders, tested separately for each species, and 4) agonistic sounds from both species (interspecific differences). The initial saturated models included the nine acoustic features (prediction variables): duration; peak frequency; BW; 10 dB min, 10 dB max, 10 dB 25th, 10 dB 75th, median frequency, and SPL. Adequacy of the discriminant analysis models to classify groups correctly was assessed with jackknife classification, in which each case is classified from the model without the case included.

**Time domain similarity**

Similarity between pulse sounds in the time domain was assessed with time-domain cross-correlation using the XCORR function in Matlab 7.0. Sound pulses were first normalized in Cool Edit to control for differences in SPL. Cross-correlation coefficients between sound pulses were scaled to the autocorrelation value and the absolute value of the coefficient was used (i.e. the phase of the waveform was ignored) so that correlation coefficients varied from zero (complete randomly dissimilar waveforms) to one (identical waveforms). Patterns in similarity between sounds of both species were visualized in two dimensions with classical multidimensional scaling (MDS) (Torgerson, 1952) of scaled cross-correlation coefficients. In addition, the average pattern of sound similarity between individual fish was assessed by averaging similarity coefficients between sounds
recorded from all individuals of both species. The average pattern of similarity between individuals was then visualized with a cluster dendrogram based on the average similarity coefficient between each individual with an unweighted pair group method of arithmetic averages (UPGMA) and in two dimensions with MDS.

RESULTS

Social group dynamics

*Forcipiger longirostris* individuals were observed on the reef in male-female pairs, while *F. flavissimus* individuals were observed in haremic trios (n=7 trios) in all but one case which was found to be a male female pair. *Forcipiger longirostris* individuals were observed separated from their mates the least proportion of time (Table 5.1) and a statistical difference in the association time of mates (ANOVA, $F_{31, 29, 2}=5.048$, $p=0.013$) between *F. longirostris* and *F. flavissimus* females ($p<0.05$), but not *F. flavissimus* males was found. When individuals of both species were observed associated (paired or in trios within 1m of each other), the average mean inter-individual distance (mean of multiple observations for each individual) and the average median inter-individual distance (median of multiple observations for each individual) was within three body lengths for each species (Table 5.1).

*Forcipiger flavissimus* individuals were observed in all association combinations: solitary, in a trio within 1m of each fish, in male-female pairs with either female, and in female-female pairs (Table 5.1). Females, however, were found paired with males for a greater proportion of time than with females (repeated measures ANOVA $F_{27, 13, 13, 1}=5.171$, $p=0.041$, Tukey HSD, $p=0.041$).
Table 5.1. Association habits of *Forcipiger longirostris* and *F. flavissimus*. **Top:** Mean and SE proportion of observations in which individuals were solitary (> 1m inter-individual distance), average of all fish mean inter-individual distances when associated with at least one additional fish from social group (i.e. in pairs or trios), and average of all fish median inter-individual distances. **Bottom:** Time budget dynamics within *F. flavissimus* trios. Proportion of time for each association category (rows), and average mean inter-individual distance and average median inter-individual distance for trio associations (all three fish ≤ 1m), male paired with either female but not the complete trio, and females paired without the male.

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Proportion of time solitary</th>
<th>SE</th>
<th>average mean distance when associated</th>
<th>SE</th>
<th>average median distance when associated</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. longirostris</em> all</td>
<td>14 pairs</td>
<td>0.19</td>
<td>0.49</td>
<td>30.8</td>
<td>3.5</td>
<td>26.4</td>
<td>3.6</td>
</tr>
<tr>
<td><em>F. flavissimus</em> all</td>
<td>7 trios</td>
<td>0.43</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>F. flavissimus</em> males</td>
<td>7 fish</td>
<td>0.37</td>
<td>0.09</td>
<td>34.6</td>
<td>2.9</td>
<td>30.6</td>
<td>2.9</td>
</tr>
<tr>
<td><em>F. flavissimus</em> females</td>
<td>21 fish</td>
<td>0.44</td>
<td>0.05</td>
<td>34.6</td>
<td>2.3</td>
<td>30.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>F. flavissimus</em> trio time budget</th>
<th>Proportion of time associated</th>
<th>SE</th>
<th>average mean distance when associated</th>
<th>SE</th>
<th>average median distance when associated</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>trio associations</td>
<td>0.23</td>
<td>0.09</td>
<td>34.2</td>
<td>12.9</td>
<td>28.8</td>
<td>10.9</td>
</tr>
<tr>
<td>male with either female</td>
<td>0.45</td>
<td>0.05</td>
<td>34.8</td>
<td>4.4</td>
<td>31.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Females paired</td>
<td>0.11</td>
<td>0.04</td>
<td>36.1</td>
<td>16.2</td>
<td>34.4</td>
<td>15.4</td>
</tr>
</tbody>
</table>

In addition to the association time differences between *F. flavissimus* and *F. longirostris*, *F. flavissimus* was observed foraging frequently on either benthic associated prey or sessile prey. Feeding rates were observed from 12 individuals and mean ± s.e. feeding rates (bites/minute) were highest for unknown prey (0.283±0.068), the coral *Pocillopora meandrina* (0.251±0.127), the echinoid *Echinothrix calamaris* (0.177±0.069), and radioles of the polychaete *Spirobranchus* spp. (0.109±0.042). In contrast, *F. longirostris* was not seen consuming any of these taxa.

Foraging territories of *F. flavissimus* observed over 20 minute observation (n=8) periods ranged from six to 17m (mean ± SE: 11±1m) in the shortest of two measured dimensions and 18 to 25 m (mean 22±1m) in the longest dimension, with ellipsoid area estimates that ranged from 452 to 1304 m² (mean 778±120m). Areas observed for *F.*
longirostris (n=6) ranged from six to 13m (mean 8±3m) for the shorter dimension and 7 to 21m for the longer dimension (mean 13±2m), with ellipsoid area estimates that ranged from 135 to 603 m² (mean 341 to 68 m²).

Long-term dynamics of social groups

Longer-term observations of *F. flavissimus* and *F. longirostris* social groups indicated some stability, at least for some individuals. *Forcipiger flavissimus*, when resighted, were always seen in the original home range area where they were tagged (Table 5.2). Fish were usually observed in close proximity with their trio social group members, or at least in the same home range during re-surveys within the first 31 days post-tagging (Table 5.2). One trio with a longer-lasting surgical wire tag was re-sighted in the same location 157 days post-tagging, while a male and female fish and third-non-tagged fish were seen up to 390 days post-tagging, and one male fish was seen with untagged fish in the original location of tagging 390 days later (Table 5.2). No tagged fish were resighted outside of the original home range. While the status of fish that disappeared is unknown, and the opportunities for resurvey of different fish varied, these data provide minimum estimates for how long a social group can remain intact.

*Forcipiger longirostris* socially monogamous pairings were largely stable (Table 5.3). Resighting rates of most tagged individuals were lower for *F. longirostris* than *F. flavissimus* (Table 5.2, 5.3), likely because of the deeper habitat and finger coral (*Porites compressa*) habitat occupied by most of the *F. longirostris* pairs observed. For most resightings, however, fish were seen in close association with their mates (Table 5.3) and longer-duration surgical wire tagged fish were seen together up to 605 days after initial
Table 5.2. Summary of resightings of tagged *F. flavissimus*. Tag type (T=short-term suture thread and beads, W=longer-term surgical wire and beads), Color phase (all *F. flavissimus* same yellow phase), Social system originally observed (T=harem trios, P=monogamous pairs), Sex (M=male, F1=female 1, F2=female2, N=not measured), Size (SL mm, N=not measured), Re-sightings (no. observed and attempted, % successful re-sightings, % of brief re-sightings in original social group or sub-combination: MF1=pair of male and female 1, F1F2=pair of female 1 and 2, MF1U=trio of male, female 1, and previously untagged fish, MU=male paired with untagged fish), last day observed in social group after initial tagging, last day observed after initial tagging, days re-sighted after initial tagging, and days attempted re-surveys were taken.

<table>
<thead>
<tr>
<th>Tag Type</th>
<th>Social System</th>
<th>Color phase</th>
<th>Sex</th>
<th>Size</th>
<th>Observed no.</th>
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<th>Rate (%) in original home range</th>
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Table 5.3. Summary of re-sightings of tagged *F. longirostris*. Tag type (T=short-term suture thread and beads, W=longer-term surgical wire and beads), Social system originally observed (P=monogamous pairs), Color phase (Black or Yellow), Sex (M=male, F1=female 1, F2=female2), Size (SL mm), Re-sightings (no. observed and attempted, % successful re-sightings, % of brief re-sightings in original pair), last day observed in social group after initial tagging, last day observed after initial tagging, days re-sighted after initial tagging, and days attempted re-surveys were taken. *This pair was seen ~100m north of the area where they were originally observed and tagged on three observations.

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<th>Attempted no.</th>
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<th>% in original home range</th>
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<td>605</td>
<td>105, 309</td>
<td>104, 105, 306, 309, 322, 363, 605</td>
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tagging (Table 5.3). Both chromatic phases (yellow and black) in both sexes were tagged as part of this study, however, no observations of switching from melanistic to xanthic or vice versa were observed. One tagged pair was observed paired, but approximately 100 m away from the location in which it was originally tagged on three occasions (13, 19, and 28 days after tagging), which indicates that the home range estimates from brief observation periods may well-underestimate actual home range size.

**Assortative Mating**

Measurements of *Forcipiger longirostris* pairs indicated evidence of assortative mating. Male length was correlated with female length ($r^2=72.3\%$, $p<0.001$) and males tended to be shorter than their female mate counterparts, however, the difference in male and female mate body size (y-intercept) was not statistically different ($p=0.08$). Male *F. flavissimus* body sizes, however, were not correlated with body sizes of female trio members ($p=0.852$) and female body size was not correlated with body size of other female trio members ($p=0.889$).

**Agonistic interactions and sound production experiments**

Responses to conspecific intruders from *F. flavissimus* and *F. longirostris* individuals were highly variable (Kruskal-Wallis, $H = 18.885$, d.f.=4, $p<0.001$, Figure 5.2). Both members of the *F. longirostris* did not differ in the proportion of time spent around caged conspecifics (Dunn’s post hoc test $p>0.05$), nor with the *F. flavissimus* individual that responded most, or second most ($p>0.05$, Figure 5.2). The third *F. flavissimus*
Figure 5.2. Differential response of residents to caged intruders. Boxplot (quartiles and median) of the percent of time around caged intruders by *Forcipiger flavissimus* and *F. longirostris* social group members. Residents were ranked within their social group (trios and pairs) by the proportion of time present at caged intruders. Statistical differences (p<0.05) of medians between residents after Kruskal-Wallis and Dunn’s post-hoc test denoted with letter groups. Abbreviations: Ff = *F. flavissimus*, Fl = *F. longirostris*, R1 = resident fish present for the longest time near intruders during an experiment, R2 = resident fish present for the second longest time near intruders, R3 = resident fish present for the least time near intruders.

individual, however, spent less time around the caged conspecifics than the most responsive *F. flavissimus* resident and the *F. longirostris* individual (p<0.05, Figure 5.2).

**Agonistic behavior and sound production**

Sound production in both species occurred when resident fish initially approached caged conspecifics and when agonistic behavior escalated after the initial encounter. Sound
production events typically co-occurred with erected dorsal and anal spines and displays in which individual fish swam with the head slightly downward. Sound pulse waveforms were similar to cranial elevation ‘headbob’ sounds (Boyle and Tricas unpublished), however, field video with low lighting and frame rate (33 fps) prevented direct confirmation of sound production kinematics. Sounds from both species were short duration, single pulse sounds with waveforms that typically consisted of several cycles of similar amplitude followed by an exponential decay (Figure 5.3). Both species produced broadband sounds with most energy occurring below 500 Hz (Figure 5.3, Table 5.4).

![Sound waveforms and power spectra](image)

**Figure 5.3.** Sound waveforms and power spectra (see Methods for details) from typical agonistic sounds recorded towards caged conspecifics in the field from *Forcipiger flavissimus* (top) and *F. longirostris* (bottom). Note both waveforms have rapid decay and similar peak frequency (273 Hz for *F. flavissimus* and 281 Hz for *F. longirostris*).
Table 5.4. Features of sounds produced towards caged conspecific receivers in field experiments from *Forcipiger flavissimus* and *F. longirostris*. Agonistic sounds were produced towards caged females and males. In a subset of experiments sounds were produced towards mates when cages were placed near non-mate conspecifics.

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<th>Duration (s)</th>
<th>Peak frequency (Hz)</th>
<th>3W</th>
<th>10 dB Min (Hz)</th>
<th>10 dB 25th (Hz)</th>
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<td>201±34</td>
<td>392±67</td>
<td>549±84</td>
<td>747±162</td>
</tr>
</tbody>
</table>

Exp. = experiments with available receiver, N = number of fish, n = number of sounds, Ave. n = average number of sounds per individual fish, n range = range of n/ per individual fish, BW = proportion of power spectrum ≤ 10 dB from peak. Values within 10 dB of peak: min = minimum, 25th= 25th percentile, median, 75th= 75th percentile, max = maximum value ≤ 10 dB from peak. SPL = sound pressure level (dB re: 1μPa).
Both species produced sounds towards non-resident caged fish of both sexes (Table 5.4). Experiments with caged mates also elicited sounds. Resident fish, however, appeared less aggressive and less motivated to remain near caged mates. Overall, only 21 *F. flavissimus* and 22 *F. longirostris* sounds were recorded within two body lengths of mate cages.

The average sound features of each individual fish on average did not differ between species in terms of duration, peak frequency, or median frequency (t-test on individual means between species, p>0.05). The estimated *F. flavissimus* mean SPL from individual averages was higher, but not statistically different after accounting for multiple comparisons (t = -2.523, d.f. 24, p= 0.019). No intraspecific differences of duration, peak frequency, median 10 dB frequency, or SPL between sounds made towards caged female fish or caged male fish were observed for either species (p>0.05).

**Interspecific interactions**

Caged intruder experiments elicited several interspecific interactions. Two occurrences of *F. longirostris* visits to caged *F. flavissimus* occurred. One involved a pair in which one fish approached within two body lengths of a caged *F. flavissimus* resident and the other occurrence was a solitary *F. longirostris* that approached a caged *F. flavissimus*. Both visits, however, were brief (12 seconds and 40 seconds). Six *F. longirostris* experiments had visits from *F. flavissimus* individuals. All involved approaches within two body lengths of caged *F. longirostris* and visit durations ranged from 16 seconds to 338 seconds, with three occurrences over 60 seconds. Two of the *F. longirostris*
experiments had visits from two *F. flavissimus* individuals, while the others involved a single fish.

Several sounds were produced by these fish to caged heterospecifics during these interactions. One *F. longirostris* produced three sounds: mean duration 0.034 ± s.d. 0.009 s, peak frequency 51 ± 88 Hz, median frequency 209 ± 66 Hz, and SPL 132 ± 4 dB re: 1µPa. Two *F. flavissimus* produced sounds towards caged *F. longirostris*: mean duration 0.035 ± SE 0.004 s, peak frequency 242 ± 10 Hz, median freuqency 222 ± 238 Hz, and SPL 131 ± 2 dB re: 1µPa. These sounds were very similar in duration, intensity and spectral sounds to many of the agonistic sounds recorded to conspecific caged intruders for both species (Table 5.4).

**Discrimination of signaler, context, and species from sounds**

Both species produced sounds that were similar among individuals and little evidence indicated that sounds have features necessary for discrimination of specific fish (Table 5.5). For both species only 17% of sounds were correctly classified to the proper individual and both models were based largely on SPL. Sound features also were relatively poor discriminators of receiver sex, with overall classification rates of 58 and 60% for *F. flavissimus* and *F. longirostris*, respectively from models based on different spectral features (Table 5.5). Stepwise LDA was not able to produce a model to discriminate intruder sounds from mate sounds in *F. flavissimus*, but for *F. longirostris* predicted 67% of sounds correctly with SPL contributing most to the model (Table 5.5). Agonistic sounds from both species also were similar, discriminated best by SPL and 10
dB 75th frequency, and the best LDA model classified 64% of the sounds to the correct species (Table 5.5, Figure 5.4).

**Interspecies pulse similarity in the time domain**

Pairwise cross-correlation of sound pulses from *F. flavissimus* and *F. longirostris* indicated a high level of similarity between sounds of each species (Figure 5.5). The pattern of sound similarity, however, resulted in some separation of *F. flavissimus* sounds.

### Table 5.5. Discriminant analysis models to test the predictive power of acoustic features for identification of behavioral context within each species and to test for the predictive power of agonistic sound features to discriminate species. Models were selected from a backwards stepwise LDA (see Methods). Note: An LDA model to discriminate *Forcipiger flavissimus* intruder sounds from mate sounds was not produced because all acoustic features had probability-to-remove values >0.15.

<table>
<thead>
<tr>
<th>Species and Discrimination Variables</th>
<th>d.f.</th>
<th>p-value</th>
<th>F-ratio</th>
<th>Wilk’s λ</th>
<th>Duration (s)</th>
<th>peak f (kHz)</th>
<th>BW (kHz)</th>
<th>10 dB  min</th>
<th>10 dB  max</th>
<th>median frequency</th>
<th>10 dB 25th</th>
<th>10 dB 75th</th>
<th>SPL</th>
<th>Jackknife classification rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Forcipiger flavissimus</em>: Discriminating individuals (n=14)</td>
<td>1, 13, 107</td>
<td>&lt;0.001</td>
<td>3.54</td>
<td>0.70</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.54 (range: 0-100, median 9)</td>
</tr>
<tr>
<td><em>Forcipiger flavissimus</em>: Discriminating Males vs. Females</td>
<td>2, 1, 98</td>
<td>0.130</td>
<td>2.01</td>
<td>0.96</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.85</td>
<td>3.75</td>
<td>-</td>
<td>75 ♀, 26 ♂</td>
<td></td>
</tr>
<tr>
<td><em>Forcipiger flavissimus</em>: Discriminating intruders vs. mates no stepwise model possible</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Forcipiger longirostris</em>: Discriminating individuals (n=14)</td>
<td>3, 13, 18</td>
<td>&lt;0.001</td>
<td>4.15</td>
<td>0.39</td>
<td>3.77</td>
<td>-</td>
<td>2.79</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.29 (range: 0-46, median 12.5)</td>
</tr>
<tr>
<td><em>Forcipiger longirostris</em>: Discriminating Males vs. Females</td>
<td>4, 1, 139</td>
<td>&lt;0.001</td>
<td>5.37</td>
<td>0.86</td>
<td>-</td>
<td>-</td>
<td>9.71</td>
<td>-</td>
<td>-</td>
<td>9.55</td>
<td>7.44</td>
<td>5.07</td>
<td>77 ♀, 52 ♂</td>
<td></td>
</tr>
<tr>
<td><em>Forcipiger longirostris</em>: Discriminating intruders vs. mates Overall 67</td>
<td>2, 1, 161</td>
<td>0.001</td>
<td>7.49</td>
<td>0.91</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.75</td>
<td>-</td>
<td>-</td>
<td>13.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both Species Combined: Between species Overall 64</td>
<td>2, 1, 282</td>
<td>&lt;0.001</td>
<td>18.72</td>
<td>0.88</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.74</td>
<td>36.27</td>
<td>63 Ff, 66 Fl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d.f., p-value, F-ratio, and Wilk’s λ of stepwise LDA models, F-values of predictive variables used in the final model, correct classification rates (jackknife classifications) for the final model showing the overall rate (percent of overall correctly classified sounds) and the classification rates within each group (range and median given for individual fish).
Figure 5.4. Biplot (10 dB 75th frequency vs. SPL) of features from individual agonistic sounds that best distinguish *Forcipiger flavissimus* agonistic sounds (black symbols) from *F. longirostris* (white symbols). Note that sounds from both species are not completely distinct in frequency spectra or SPL, but more *F. flavissimus* sounds tend to have lower frequency components (shown by 10 dB 75th frequency) and higher SPL from *F. longirostris* with more negative values on MDS axis I and positive values on axis II for *F. flavissimus* (Figure 5.5).

The pattern of pulse similarity among individual fish also showed a high level of overlap between species. The average pulse similarity (grand mean of similarity coefficients averaged across sounds between individuals) between individual *F. flavissimus* and *F. flavissimus* was 38%, with a maximum similarity of 52%. The average pulse similarity between *F. longirostris* individuals was 35%, with a range of 16% to 48%. Average between individuals of each-species was 36% with a range of 17% to 52%. These data indicate that on average, sounds from individual *F. longirostris* were more similar in the time domain to sounds from *F. flavissimus* individuals than other
conspecific sounds. This high level of similarity between typical sounds produced by each species is shown in the dendrogram in Figure 5.6. Average similarity values of 40% or greater exceeded the 75 percentile, yet two of three groupings of 40% similarity included individuals from both species (Figure 5.6). Multidimensional scaling showed a pattern of average similarity among individual fish that separated some *F. flavissimus* individuals from *F. longirostris* with a trend of *F. flavissimus* individuals having low values on MDS axis I and II (Figure 5.6).
Figure 5.6. Relationship of sound similarity between and within species (measured from time-domain cross correlation) among different individual fish represented in a UPGMA dendrogram (left) and in two dimensions from a classical MDS plot (black symbols = *F. flavissimus*, white = *F. longirostris*) (right). Similarity is the average of pairwise similarity values between sounds from each individual fish pairing. Clades drawn with dotted lines on the UPGMA dendrogram (left) indicate groupings of fish with sounds ≥ 40% similar (75th percentile of all similarity values). Note two of three clades with ≥ 40% similarity include individuals of both species. Four *F. flavissimus* and four *F. longirostris* had greater than 60% dissimilarity from all other individuals. On the MDS plot (right), although *F. flavissimus* individuals tend to be characterized by lower values on Axis I and II, there is broad overlap between individuals of both species.

**DISCUSSION**

Results from this study demonstrated a divergent social system between Forcepsfish and Longnose Butterflyfish that is maintained in part by agonistic interactions between social groups that involve visual and acoustic communication. Long-term observations were consistent with other butterflyfish species that maintain established home ranges within a
social group. Further, controlled experiments that introduced conspecifics demonstrated that individuals of both species exhibited agonistic behavior consistent with territorial resource defense. Based on observations from these experiments, it is hypothesized that males of the socially harem Forcepsfish exhibit more territorial behavior than females, whereas male and female Longnose Butterflyfish exhibit similar amounts of territorial aggression towards conspecifics within a home range. Production of agonistic sounds was a consistent feature of territorial defense for both species, which produced simple acoustic pulses similar to headbob sounds produced in the laboratory (Boyle and Tricas, unpublished). No differences were observed between sounds made to receivers of different sexes or to sounds made in the presence of captive mates. Intra-individual variability was also high, which indicates the likelihood of acoustic discrimination of individuals to aid in recognition is low. Even sounds made between species were largely similar, casting doubt on the efficacy of acoustic signals to prevent hybridization between syntopic species. Thus acoustic signals may function largely as greeting rituals produced during agonistic encounters that may warn conspecifics of potential escalated aggression and increase available time for mates to recognize each other after a period of separation. This study documents social monogamy in butterflyfish from the bannerfish clade (sensu Fessler and Westneat, 2007; Bellwood et al. 2003), which has received less research attention than the genus *Chaetodon*, and a different form of social haremy than the typical system described for other chaetodontids and related pomacanthids.

Social monogamy is widespread among chaetodontid fishes (Hourigan, 1989; Roberts and Ormond, 1992; Whiteman and Côté, 2004). Most accounts of social monogamy among butterflyfishes are known from members of the genus *Chaetodon*, a
genus for which corallivory is widespread (Reese, 1975; Hourigan et al., 1988; Bellwood et al., 2009), and defense of coral feeding territories is hypothesized to facilitate monogamous associations (Reese, 1975; Hourigan, 1989). Fricke (1986) observed long-term pair fidelity in Red Sea Bannerfish, *Heniochus intermedius*, with bonds lasting more than three years. Whiteman and Côté (2004) reported obligate social monogamy in *F. flavissimus* and *F. longirostris* based on Hourigan (Hourigan, 1987). Hourigan’s short term observations on *F. longirostris* that were consistent with the results of this study. Hourigan (Hourigan, 1987), however, was not certain about the social dynamics of *F. flavissimus* and cited Ludwig’s (1984) brief observations of solitary, paired, and small groups of individuals. The results of the current study are consistent with long-term social bonds and territory maintenance by both of these species. The harem trios of *F. flavissimus* observed in this study are consistent with the skewed sex ratio (2 females: 1 male) reported from populations of Hawaiian *F. flavissimus* (Ludwig 1984). Thus divergent social mechanisms exist between the syntopic members of the genus *Forcipger*.

The territorial arrangement of harem polygyny observed among *F. flavissimus* individuals in this study is different than what is known from other harem butterflyfishes and related angelfishes (Pomacanthidae). In Bluestripe Butterflyfish (*Chaetodon fremblii*) (Hourigan, 1987) and Chevron Butterflyfish (*Chaetodon trifascialis*) (Yabuta and Kawashima, 1997), females defend separate territories from female conspecifics and males have large territories that overlap with several female territories and are defended from male conspecifics. Similar harem systems exist in some species of pomacanthids (Hourigan et al., 1989; Sakai and Kohda, 1997). The harem trios of *F. flavissimus* observed in this study, differ in that all members of the
harem have an overlapping territory. Further, female members of the harem form temporary paired associations while foraging (Table 5.1).

Forcipsfish and Longnose Butterflyfish differ not only with respect to mating system type, harem polygyny vs. monogamy, but also with respect to association time within their social group. *Forcipiger longirostris* individuals were found to be paired (≤ 1m intra-individual distance) more frequently than *F. flavissimus* female trio members. Both species have similar home range sizes and thus individual encounter rates for *F. flavissimus* trios likely would be higher than for *F. longirostris* pairs. Pair-swimming is hypothesized to function as a visual advertisement of territorial occupation that may outweigh the cost of decreased territorial border vigilance (Fricke, 1986). The differences in foraging behavior and diet between these two species may affect some of the disparity in association time with mates. *Forcipiger flavissimus*, which consumes conspicuous benthic prey (e.g., sea urchin podia, tube-worm radioles, and coral polyps), appears to spend more time feeding than *F. longirostris*, which consumes more elusive prey like caridean shrimps (Hobson, 1974). Perhaps the time spent feeding on patchy benthic prey within territories adds more distraction from maintenance of close visual contact between trio members.

In addition to differences between *F. longirostris* and *F. flavissimus* association times, only *F. longirostris* displayed intra-social group size associations. Sizes of mated *F. longirostris* pairs were consistent with assortative mating. Sizes of another monogamous butterflyfish, *Chaetodon multicinctus*, also indicate assortative mating, but with a bias towards larger males (Hourigan, 1987; Tricas, 1989b; Strang, 2005). There was no size difference between sexes of *F. longirostris*, but there was a trend in the
opposite direction from *C. multicinctus*. *Forcipiger flavissimus*, however, did not show any size relationships associated with trio membership in this study. The mechanisms behind size assortative pairing in butterflyfish are hypothesized to be driven by pairing among cohorts (Strang, 2005). The reason for a lack of a size relationship observed among *F. flavissimus* trios is not known. Possible hypotheses, however, include less long-term social group stability (i.e. switching between mates from different social groups and cohorts) and variable and differential growth rates within a social group. No evidence for protogynous sex change has been observed in *F. flavissimus* (Ludwig, 1984) as is found in some haremic angelfishes (Lutnesky, 1996).

**Territorial behavior**

Introduction of caged conspecifics into the home ranges of both *F. flavissimus* and *F. longirostris* resulted in high levels of apparent motivation by residents to engage in agonistic behavior. Resident fish of both species typically ceased feeding or engaged in exaggerated motor patterns of feeding with the head down as part of a display and remained near caged conspecifics within territories for most of the duration of experiments. Further, displays with erected dorsal spines were a common feature of resident behavior for both species. These results are consistent with territorial defense displayed by both species and are consistent with model-bottle experiments with other territorial butterflyfish species (Tricas, 1989b; Kosaki, 1999). Previous observations on *Forcipiger* (not identified to species) in an experiment with a mirror on the reef did not elicit agonistic behavior, as would be expected from a visual stimulus on a reef territory (Ehrlich and Ehrlich, 1982), though it is possible that the mirror is not a sufficient
stimulus to incite aggression compared to the potential multimodal stimulus (visual, olfactory, auditory) of a live fish.

The presence of social monogamy and agonistic aggression towards conspecifics outside of a social group in butterflyfish has been hypothesized to be associated with food resource defense (Hourigan, 1989; Tricas, 1989b) and mutual mate guarding (Strang, 2005). These hypotheses, however, are not mutually exclusive and both may provide adaptive explanations for the evolution of these behaviors (Whiteman and Côté, 2004). In this study, responses by resident fish were most consistent with resource defense. Resident fish appeared to approach caged intruders of all sexes with similar frequency. *Forcipiger longirostris* males and females engaged in territorial behavior with the same apparent frequency, while *F. flavissimus* individuals had a skewed response within each harem. The skewed response within a trio could result from higher levels of territorial defense performed by male fish, however, fish responded to all caged conspecifics, not just caged males. The evolution of behavior that promotes long-term site fidelity and territorial defense is predicted for coral-feeding butterflyfishes that can defend a stable food resource (Hourigan, 1989). The more mobile prey that appear to constitute a significant portion of the diet of *F. flavissimus* (sea urchins) and *F. longirostris* (caridean shrimps) are likely less predictable in space and time. Nevertheless, both species appear to exhibit strong site fidelity over long periods and engage in defense of spatial areas from conspecifics.
Acoustic signals

Both species in this study produced acoustic pulses towards caged conspecifics that were similar to headbob sounds recorded in the laboratory (Boyle unpublished). These sounds were associated with agonistic behaviors that occurred shortly after approach by resident fish. During territorial intruder experiments, resident fish typically responded with fast directed swimming from a distance ≥ 5m from the experimental arena. These responses are consistent with visual recognition of conspecifics, or at least congeners as occasionally both species approached caged individuals. After close approach, a headbob-like sound often occurred along with head-down displays and dorsal spine erections. These behaviors often persisted throughout the experiment and increased in intensity, as the cage prevented escalated aggressive behaviors such as chases and fights with dorsal spines that have been observed (Boyle unpublished).

Ritualized behaviors may be an important feature of agonistic interactions among social butterflyfish. When monogamous or haremic butterflyfish are visually separated from mates and encounter a conspecific within a territory or along a border, ritualized displays may benefit both participants in order to facilitate recognition of mates or potential foes (Yabuta, 2002). Reese (1975) interpreted ritualized behaviors of mates after a period of separation as a greeting ceremony that may serve to reconfirm the pair bond. These hypotheses are not mutually exclusive. Results from this study indicate that acoustic signals may be part of the typical display rituals performed by both of these species as part of multimodal communication that involves at least visual and acoustic stimuli.
This study did not provide strong evidence that acoustic signals carry information about the behavioral context in which sounds were emitted. Sounds emitted towards male and female intruders were not predictably different in terms of spectral features, duration, or sound pressure level, nor were sounds emitted towards non-mates and mates. The interactions between free-swimming resident fish and caged fish did not appear as intense as interactions with non-familiar conspecifics. This result is consistent with recognition of mates and may explain the low rate of agonistic sound production around mate cages.

Sounds recorded during agonistic intruder experiments in this study also did not identify acoustic signatures that would facilitate recognition of mates and non-mates. Discriminant function analyses are often used to determine if acoustic features from animal sounds may be used to discriminate individuals (Sousa-Lima et al., 2002; Charrier and Harcourt, 2006; Melendez and Feng, 2010). In this study, LDA models for each species classified few sounds correctly and from only a fraction of individuals. One obvious reason for this is the relative simplicity of these single pulse sounds compared to other animals (Aubin and Jouventin, 2002; Sousa-Lima et al., 2002; Klump et al., 2004; Charrier and Harcourt, 2006; Melendez and Feng, 2010). Temporal patterning exists among many fish sounds (Zelick et al., 1999) and may provide additional complexity of sounds necessary for individual discrimination. In a playback study, Myrberg and Riggio (Myrberg and Riggio, 1985) demonstrated that Bicolor Damselfish increased dipping display behavior while exposed to sounds from non-familiar males. Other examples of individual discrimination based on acoustic signals among teleost fishes are lacking, though evidence for sufficient individual sound complexity exists in some mormyrid
fishes (Crawford et al., 1997). The single pulse sounds emitted by both *Forcipiger* species in this study, however, do not provide the temporal complexity of a pulse train. Forcepsfish were observed in the laboratory emitting low frequency pulses (<50 Hz peak frequency) that appear to be caused by rapid abduction of the anal fin spines, which occurs in synch with sound emission (Boyle unpublished). Such sounds occur in a train-like pattern as the anal fin spines are erected repeatedly and could potentially provide temporal pattern differences among individuals. These low frequency sounds, however, fall-off very rapidly and were not detected in the field. The best discriminator of individuals from sounds recorded in this study for both species was sound pressure level. It should be noted, however, that sound pressure level measurements from this study, were rough estimates from free-swimming fish in the field. Sounds analyzed were estimated to be made within two body lengths (up to ~30 cm) of a cage and hydrophone, which with geometric spreading, in this study predicted to vary from -15 dB (cylindrical spreading) to -30 dB (spherical spreading) (Mann, 2006), could add substantial variation.

Laboratory data from Forcepsfish indicates that headbob pulse sound pressure level is related strongly with body size, and to a lesser extent, cranial kinematic performance (Boyle unpublished). This relationship was not observed with Longnose Butterflyfish in the laboratory, but statistical power was likely hampered by small sample sizes (Boyle unpublished). Thus it is possible that under natural conditions, in which acoustic signals appear to be emitted at very close range (within a body length), sound pressure level may provide a strong indication of body size and to a lesser extent physical condition. Acoustic signals that vary in sound pressure level according to body size
would be of potential value for discrimination of individuals (mates, adjacent territory
holders, and unfamiliar conspecifics).

Sounds made from each species were also similar, but appear to provide more
potential cues for acoustic discrimination. LDA analyses indicated that spectral cues and
sound pressure level were the features most likely to separate sounds made from each
species. Though there was broad overlap among sounds recorded from each species,
Forcepsfish sounds tended towards lower frequency shifted power spectra and higher
sound pressure level sounds relative to Longnose Butterflyfish sounds (Figure 5.4).
Slight differences between sounds made from each species were also indicated in cross-
correlations in the time-domain (Figures 5.5, 5.6). It is not clear at this point, however, if
either species is able to utilize acoustic cues to aid in species discrimination or if there is
a sensory bias towards spectral features associated with either species. Studies have
found a good correlation between frequency spectra of social sounds and auditory ability
in some species (Myrberg and Spires, 1980; Kenyon, 1996; Maruska et al., 2007) but not
others (Ladich, 1999, 2000). It is not known if the auditory abilities of the two species in
the current study trend towards the differences between sounds of each species. Thus it is
possible that the slight differences in typical sounds emitted from each species are
important to reduce interspecific interactions and potential hybridization in sympatry
because of an acoustic preference or sensory system bias, consistent with the sensory-
drive hypothesis (Endler, 1992). Alternatively, these minor sound differences may result
from slight morphological (body size, swim bladder, and musculo-skeletal features)
differences that exist between these two similar species that resulted from selection
pressures largely unrelated to sound production.
Conclusions

This study provides evidence that *F. flavissimus* and *F. longirostris* both defend coral feeding territories from conspecifics and remain site-attached for long periods (many months to years). On Hawaiian reefs, *F. longirostris* is socially monogamous, while *F. flavissimus* is socially haremic and usually occurs in trios. Haremic trios of butterflyfish are unusual among haremic butterflyfish in that territories share joint occupation by female members. During territorial interactions, pulse sounds are emitted as part of a ritualized agonistic display that may provide a multimodal stimulus and increase time essential for mutual recognition of individuals. Simple acoustic pulses likely provide little information relative to behavioral context or individual identity, other than the signaler’s body size. Future studies should examine the behavioral response and sensitivities towards conspecific acoustic stimuli and the role of sound production in other behaviors such as spawning.

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CHAPTER VI:
MULTIMODAL DISCRIMINATION OF MATES AND TERRITORIAL INTRUDERS:
VISUAL AND OLFACTORY STIMULI INFLUENCE TERRITORIAL
BEHAVIOR OF THE SOCIALLY MONOGAMOUS PEBBLED
BUTTERFLYFISH (CHAETODON MULTICINCTUS)

ABSTRACT
Recognition of conspecifics is essential for territorial and monogamous animals in order
to maintain pair bonds, mate guard, and defend territories. Multiple sensory modalities
may be used to distinguish individuals and these cues may have a strong influence on the
evolution of social communication. This study examined the relative role of visual and
olfactory cues in discrimination of familiar and non-familiar conspecifics in the
territorial, socially monogamous Pebbled Butterflyfish (Chaetodon multicinctus). A
series of model-bottle field experiments was conducted in which mates and non-mates
were presented simultaneously within butterflyfish feeding territories. When visual and
olfactory cues were intact at each bottle stimulus, free-swimming resident fish spent more
time near intruder bottle stimuli engaged in aggressive agonistic displays. When
olfactory cues of model bottle fish were pumped to opposite bottles, resident fish spent
equal time at each bottle stimulus. After reversal of scent cues to a matched-odor
condition (odor released next to bottled fish), resident fish spent more time with non-
mates. Experiments without odor cues resulted in near equal time spent by resident fish
with mates and non-mates. A set of experiments with two intruder fish and mate odor
released next to one of the intruders, however, did not result in a differential response by
resident fish to either bottle. Overall, results indicate that both visual and olfactory cues
may be necessary for butterflyfish to discriminate between mates and intruders that
attempt to forage within feeding territories.
INTRODUCTION

Mate recognition tasks may be of profound importance for social animals that maintain long-term pair bonds and such discriminatory ability is especially important for territorial species. When monogamous butterflyfishes (Chaetodontidae) encounter conspecifics after a period of separation from their mates, they need to recognize quickly whether the conspecific is a territorial threat or a mate. Failure to recognize a mate could weaken the pair bond and failure to recognize a territorial conspecific could result in an injurious attack or loss of territory. The family Chaetodontidae includes species that are known to maintain long (up to 10 years) socially monogamous pairings (Hourigan, 1989) and are able to discriminate between mates and non-mates that enter feeding territories.

Recognition of individuals based on previous experience can fall under two categories, condition-dependent, in which individuals discriminate between individuals on the basis of specific cues such as color and size, and condition-independent recognition, in which obvious cues are not present (Griffiths, 2003). Many fish species have the ability to discriminate kin from non-kin in the laboratory using odor stimuli (reviewed in Griffiths 2003). Several fish species have been shown to discriminate familiar conspecifics from unfamiliar conspecifics (see Griffiths 2003), including the Bluegill *Lepomis macrochirus* (Brown and Colgan, 1986), Three-spined Stickleback *Gasterosteus aculeatus* (VanHavre and FitzGerald, 1988), Guppy *Poecilia reticulata* (Magurran *et al.*, 1994), and Fathead Minnow *Pimephales promelas* (Brown and Smith, 1994). Studies have demonstrated behavioral mechanisms consistent with individual recognition in fishes based on multiple possible cues (Noble and Curtis, 1939; Hojesjo *et al.*, 1998; Sogabe, 2010), vision (Fricke, 1973), acoustic cues (Myrberg and Riggio,
1985), electrosensory cues (Painter and Kramer, 2003), and olfactory cues (Todd and Bardach, 1967; Carr and Carr, 1985; Ward et al., 2007). The ability of fishes to discriminate between individuals may vary widely and is expected to be affected by the social environment. Among reef fishes, Kolm et al. (Kolm et al., 2005) found that individual Banggai Cardinalfish (*Pterapogon kaudneri*) chose to join shoals in familiar locations rather than associate with familiar shoal members. Territorial butterflyfish, however, must discriminate between familiar and non-familiar conspecifics in order to prevent territory incursions and to avoid misdirected aggression towards mates and thus are an appropriate taxon to examine recognition cues.

The purpose of this study is to determine the relative roles of vision and olfaction in the recognition and discrimination of conspecifics. Butterflyfish may employ multiple senses (vision, olfaction, and acoustic/lateral line) to facilitate rapid recognition of conspecifics and even heterospecifics. A major prediction of this multiple sensory mediated recognition hypothesis is that removing one or more of these stimuli should reduce the ability of butterflyfish to distinguish mates vs. non-mates and conspecifics vs. heterospecifics. Predictions of this multimodal hypothesis were tested with a series of five two-choice experiments in the field with the Pebbled Butterflyfish (or Multiband Butterflyfish) *Chaetodon multicinctus* Garrett, a territorial Hawaiian endemic. Olfactory and visual stimuli of simultaneous presentations of a captive monogamous partner (mate) and captive intruder were altered and the response of a free-swimming resident fish was assessed. These experiments demonstrate that resident fish best discriminate mates from non-mate intruders when both visual and olfactory cues are present.
MATERIALS AND METHODS

A series of two-choice experiments were conducted in summers of 2007 and 2008 in order to examine the agonistic response of *C. multicinctus* to mates and unfamiliar, non-mate conspecifics. Experiments were conducted on reefs along the northwest shore of the island of Hawaii. Experiments utilized model bottle presentations of fish and measured the proportion of time spent by resident conspecifics to simultaneous presentations of mate and non-mate fish with different available cues for discrimination. For all experiments, paired fish were observed for approximately 10-15 minutes by scuba divers. Observations were used to confirm pair swimming and feeding behavior indicative territorial monogamous pairs (Tricas, 1989b). Experiments took place away from borders of conspecific territories and non-mate conspecific. After initial observation, one member of the resident pair was captured by scuba divers with hand nets and held captive outside the territory for approximately 10-20 minutes. A second fish, captured from a territory outside the study area was used as an intruder.

All experiments tested the discriminatory ability of resident fish to conspecific fishes in model bottles with various sensory cues. For all experiments, two model bottles, sealable 1 gallon jars (3.8 L) (22 cm wide), were placed two meters apart within the feeding territory of the remaining resident (focal animal) butterflyfish (Figure 6.1). Marked nails were placed in a square around each jar, 66 cm from the jar in order to create a square approximately 1.5 x 1.5 m² and a space 1.5 x 66 cm² between the squares. In each set of experiments divers ascended approximately five to 10 m above the experimental arena and recorded the time spent within either square. An effort was made to select unfamiliar non-mate conspecifics that were of similar size to focal fish. After
experiments, captured fish were measured for standard length (SL) to the nearest millimeter and an attempt was made to determine sex underwater with a catheterization method (Ross, 1984) that demonstrated unambiguous evidence of eggs but did not necessarily confirm male identity. Sex of resident fish, which were not captured, was inferred from the sex determination of the captured mate.

**Matched odor first experiments**

These experiments tested the discriminatory ability of resident fish to conspecific fishes in model bottles with all sensory cues available initially. One model bottle contained the mate and the other contained a non-familiar intruder (Figure 6.1 A). Two Atwood Water Buster submersible bilge pumps (750 liters/hour) were used to pump sea water through the fish jars (Figure 6.1 A). Pumps were placed approximately equidistant and outside of the square and connected to each jar with 13 mm clear vinyl tubing (3 m) to a connector on the jar lid. A short piece of tubing extended from the jar floor and connected to an additional excurrent length of tubing (3 m) to mix and carry water and scents away from the inside of the jar.

A paired experimental design was used in these experiments in which the odor delivery configuration was switched to crossed odor stimuli (Figure 6.1 B) after approximately seven and a half minutes of observation after the resident fish entered one of the two squares in the experimental arena. Divers descended to switch the scent delivery configuration without moving the position of the bottled fish (approximately three minute procedure) and then ascended five to 10 m to observe the resident for an additional
Figure 6.1. Design of *Chaetodon multicinctus* individual discrimination experiments. In all experiments, fish chambers are placed 2 m apart with a box perimeter (1.5 m per side) around each jar (delineated with nails/plastic tape). Divers recorded the time spent by resident fish in each square (indicated by dashed boxes) during the experiment. A) *Matched odor design*. Submersible pumps perfuse water through the jars via 3 m of tubing and release fish odorants from the fish bottle to the same location. B) *Crossed odor design*. Submersible pumps perfuse water through 3 m of tubing to the opposite bottle location: from mate to intruder and intruder to mate. C) *Intruder scent only design*. Submersible pump transfers water from intruder chamber (left), which is then split and
transferred to both the intruder bottle and mate bottle location. The bottled mate is ventilated with a pump, but odorants are transported to outside the experimental arena. D) *Mate odor-intruder visual design*. Two bottled intruders are placed in visual range. Intruder-B (left side) location has a matched odor stimulus while intruder-A location (right side) receives odorant from a mate held in a bottle covered with an opaque bag. A third pump ventilates intruder-A and transfers odorants outside the experimental arena (indicated by gray dashed lines). E) *No olfactory cue design*. Mate and intruder model bottles are ventilated with pumps, but tubing carries odorants outside of experimental arena to a common location. Large gray arrows indicate initial water source located away from the experimental arena.

Experiments lasted approximately 15 minutes. *Matched odor first experiments* began with setup in A for approximately 7.5 minutes, followed by setup B. *Crossed odor first experiments* began with setup B, followed by setup A. *Intruder scent only experiments* began with setup C and after 7.5 minutes, positions of the bottles were reversed with the same odorant setup, intruder scent at each bottle. *Mate odor-intruder visual experiments* began with setup D. After 7.5 minutes, mate scent was moved to intruder-B side, with intruder-B scent carried outside the arena and matched odor at intruder-A side. *No olfactory cue experiments* used setup E and were conducted for 15 minutes without alteration of the setup.

approximately seven and one half minutes. In this crossed odor configuration, water perfused through the jar that contained the mate was released next to the intruder jar.

**Crossed-order first experiments**

A set of experiments with a paired design was conducted in which the crossed the resident fish entered one of the two squares in the experimental arena. Divers then descended to change the scent configuration to a matched odor configuration (Figure 6.1 A). The position of the bottled fish remained the same as the first phase of the experiment and after divers ascended five to 10 m above the experimental arena, odor configuration (Figure 6.1 B) was used for the first seven and a half minutes after the resident was observed for an additional approximate seven and a half minutes.
**Intruder scent only experiments**

A set of experiments with a paired design was conducted in which the mate and intruder were presented in model bottles (as above). In these experiments, however, odor stimuli from the intruder jar was released at the intruder bottle and mate bottle (Figure 6.1 C). A single pump was used to perfuse water through the intruder jar and a t-connector was used to split the odor stimulus with two separate pieces of vinyl tubing (3 m). A second pump was used to ensure adequate ventilation for the mate bottle, in which the end of the excurrent tubing (3 m) was placed outside of the experimental square and placed within porous interstices of the reef substrate. The first phase of the experiment was observed (as above) for approximately seven and a half minutes after the resident fish entered one of the squares in the experimental arena.

For the second phase of the experiment, the intruder scent configuration was maintained, but the positions of the model bottles (mate and intruder) were reversed. After the first phase, divers descended to reverse the bottle positions (an approximately three minute procedure). Divers then ascended five to 10 m above the experimental arena and the second phase was observed for approximately seven and a half minutes. In this set of experiments the same intruder fish was used as a stimulus in two separate experiments and in all four intruders were used among seven experiments.

**Mate odor-intruder visual vs. intruder odor-intruder visual**

This set of paired experiments tested the response of resident fish to visual stimuli from two intruder fish in model bottles, intruder A and intruder B, with olfactory cues from a mate fish that was not visible at one of the intruder bottles. For these experiments,
intruder A and B were visible in the experimental arena, while the captured mate was kept in a one gallon jar placed within an opaque bag outside of the experimental arena (Figure 6.1 D). Intruder A and B in these experiments were not mated pairs, i.e. they were captured from separate territories that did not border the territory of the resident focal fish. In these experiments, three pumps were used with 3 m of incurrent and 3 m of excurrent vinyl tubing. For the first phase of the experiment, the excurrent tubing from the hidden mate odor stimulus was released at the intruder A bottle, while the intruder A excurrent tube was released away from the arena and directed into interstices of the reef. The excurrent tube of the intruder B bottle was released at the intruder B bottle (matched odor). During the first phase of the experiment, fish were observed for seven and a half minutes after the resident entered one of the squares of the experimental arena.

After the first phase, divers descended and reconfigured the tubing so that the hidden mate olfactory stimulus was released at the site of the intruder B bottle, the intruder B excurrent was released away from the experimental arena and into the interstices of the reef substrate, and intruder A excurrent was released at the intruder A bottle (matched odor). Reconfiguration of the odor delivery system took approximately three minutes. After reconfiguration, divers ascended five to 10 m above the experimental arena and observed the resident fish for approximately seven and a half minutes.

**No olfactory cue experiments**

A set of unpaired experiments in which the mate and an unfamiliar non-mate intruder were presented in the experimental arena without an odor stimulus was conducted.
Pumps were used to ventilate fish in both model bottles and excurrent tubing (3 m) from each bottle emptied into a common location away from the experimental arena and into the interstices of the reef (Figure 6.1 E). Divers observed the resident fish for approximately 15 minutes after the resident fish entered one of the squares in the experimental arena. In this set of experiments, one intruder was used in two experiments and one intruder was used for three experiments. In a total of six experiments, three intruder fish were used.

**Data analyses**

The proportion of time spent by resident fish within each square for each phase of the experiment was determined. Only experiments in which the resident fish visited both jars were included in the analysis, as in the few cases in which the resident visited only one bottle it appeared that the second bottle was not seen. One crossed odor first experiment, one intruder-scent only experiment, and two mate odor-intruder visual vs. intruder odor-intruder visual were not included for this reason. One no olfactory cue experiment was not finished because the resident fish did not show up to the model bottle arena.

Time proportions were arc-sine square root transformed and parametric statistics were performed after testing for normality and homogeneity of variance. For two phase experiments (matched odor first, crossed odor first, intruder scent only, and mate odor-intruder visual vs intruder odor-intruder visual), differences in proportion of time spent at either stimulus for each phase and interaction terms were tested with two-way repeated measures analysis of variance (RM ANOVA), with a factor for each phase of the experiment, and a stimulus factor (mate or non-mate bottle except in the case of mate-
odor intruder visual experiments which used intruder A or intruder B). Differences between groups within each RM ANOVA were assessed with Student Neuman Keuls (SNK) post-hoc tests. Differences in proportion of time spent at either bottle in no olfactory cue experiments was tested with a paired t-test. Alpha level for statistical comparisons was $P=0.05$. Statistical tests were conducted with Sigma Plot 11.0 software.

**RESULTS**

Resident fish were highly motivated by the visual presence of model bottle fish in all experiments. Once resident fish entered a square within the experimental arena, the percent of time spent near at least one of the stimulus jars was very high for at least the first phase of the experiment (between 23% and nearly 100%, median of all 32 experiments 81%). Fish were typically observed in close proximity to each of the stimulus jars engaged in agonistic-like behaviors that include tail slap displays, tail-up displays, exaggerated feeding, and sometimes darkening of coloration.

**Matched odor first experiments**

During matched odor first experiments, resident fish (n=6) visited mate and non-mate bottles with high frequency. During the first phase there were $0.578 \pm 0.361$ (average ± s.d.) visits to the mate bottle per minute and $0.907 \pm 0.260$ visits to the non-mate bottle per minute. In the second phase there were $0.822 \pm 0.355$ visits to the mate bottle per minute and $1.020 \pm 0.489$ visits to the non-mate bottle. One bottled mate from these experiments was female, but no eggs were removed via catheter from the other five bottled fish, which may have been male. One bottled intruder was female, but the other five bottled fish may
have been male. Responses of resident fish were similar, however, regardless of sex. Sizes of fish used in these experiments were similar (mate mean SL 80 mm, non-mate 78 mm). Relative size differences (mate SL - non-mate SL) ranged from -1 to 5 mm.

In these experiments, resident fish response was statistically different between mate and non-mate stimuli (Figure 6.2). There was a statistical effect of mate or non-mate ($F_{5,1}=9.324\ p=0.028$), but not experiment phase ($F_{5,1}=1.349\ p=0.298$) or stimulus X phase interaction ($F_{5,1}=0.134\ p=0.729$). SNK post-hoc tests revealed that resident fish spent more time at the non-mate bottle in the first phase of experiments when odor was matched ($p=0.029$), but not in the second phase after odor stimuli were reversed ($p=0.065$) (Figure 6.2). Thus fish displayed a differential response consistent with mate and non-mate discrimination with matched visual and odor stimuli, but not after crossed odor stimuli were presented.

**Crossed-order first experiments**

During matched crossed-odor first experiments, resident fish (n=5) visited mate and non-mate bottles with moderate frequency. During the first phase there were $0.340\pm0.114$ visits to the mate bottle per minute and $0.314\pm0.198$ visits to the non-mate bottle per minute. In the second phase there were $0.260\pm0.261$ visits to the mate bottle per minute and $0.260\pm0.153$ visits to the non-mate bottle. No eggs were removed via catheter from the bottled mate in these experiments, thus all residents may have been female. Eggs were removed in four of five bottled intruders, so only one bottled intruder may have been male. Responses of resident fish were similar in all cases, however, regardless of
sex. Sizes of fish used in these experiments were similar (mate mean SL 86 mm, non-mate 85 mm). Relative size differences (mate SL - non-mate SL) ranged from 0 to 4 mm.

In these experiments, resident fish responded differentially to mate and non-mate stimuli only after switching from crossed odor stimuli to matched odor stimuli (Figure 6.3). There was no statistical effect of mate or non-mate bottle (F_{4,1}=4.834 p=0.093) or experiment phase (F_{4,1}=0.000994 p=0.976), but there was a statistical interaction (bottle X phase) (F_{4,1}=13.399 p=0.022). SNK post-hoc tests revealed that after switching from
matched odor stimuli to crossed odor stimuli, resident fish spent less time at the mate bottle \((p=0.017)\) and more time at the non-mate bottle \((p=0.018)\) (Figure 6.3). In the second, matched odor phase of the experiment, resident fish spent more time at the non-mate bottle than the mate bottle \((p= 0.018)\) (Figure 6.3). Thus fish displayed a differential response consistent with mate and non-mate discrimination only after visual stimuli were matched with odor stimuli.

Figure 6.3. Proportion of time spent by resident fish near model bottle stimuli in \textit{crossed odor first experiments}. Bars and error bars are back-transformed (as in Figure 6.2). First \textit{crossed odor} phase, left two bars shows time spent near bottle of mate (black bars) in which mate visual (MV) and intruder odor (IO) cues were present and bottle of non-mate intruder (white bars) in which intruder visual (IV) and mate odor (MO) cues were present. Second \textit{matched odor} phase, right two bars shows time spent after odor cues were matched with visual cues. Statistically different groups (2-way RM ANOVA, after SNK post-hoc tests) shown by letter groups.
Intruder scent only experiments

During intruder scent only experiments, resident fish (n=7) visited mate and non-mate bottles with moderate frequency. During the first phase there were $0.650\pm 0.494$ visits to the mate bottle per minute and $0.697\pm 0.513$ visits to the non-mate bottle per minute. In the second phase, in which the position of the bottles in the experimental arena was reversed, there were $0.539\pm 0.595$ visits to the mate bottle per minute and $0.497\pm 0.284$ visits to the non-mate bottle. Eggs were removed via catheter from four of the seven bottled mates in these experiments, thus at least four bottled mates were female. No eggs were removed from any of the seven bottled intruders, all of which may have been male. Responses did not trend differently between resident fish of different putative sexes.

Sizes of fish used in these experiments were similar (mate mean SL 85 mm, non-mate 84 mm). Relative size differences (mate - non-mate SL) ranged from -2 to 8 mm.

In these experiments, resident fish did not respond differentially to mate and non-mate stimuli in either phase of the experiment (Figure 6.4). There was no statistical effect of mate or non-mate bottle ($F_{6,1}=2.143$ $p=0.194$), experiment phase ($F_{6,1}=0.064$ $p=0.809$), or statistical interaction (bottle X phase) ($F_{4,1}=0.00485$ $p=0.947$). Thus, when only intruder olfactory cues were present, resident fish did not display a differential response consistent with discrimination of mates and non-mate intruders. In addition, no evidence of a spatial response to the position of either model bottle was found, as resident fish did not change their association time with either bottle when the positions of the bottles were reversed.
Figure 6.4. Proportion of time spent by resident fish near model bottle stimuli in intruder scent only experiments. Bars and error bars are back-transformed (as in Figure 6.2). First phase, left two bars shows time spent near bottle of mate (black bars) in which mate visual (MV) and intruder odor cues (IO) were present and bottle of non-mate intruder (white bars) in which intruder visual (IV) and IO cues were present. Second phase, right two bars shows time spent after reversing the spatial presentation of each bottle continued to receive only IO stimuli (black bar and white bar represent mate and non-mate in opposite location). No statistical differences between groups (2-way RM ANOVA, P>0.05).

**Mate odor-intruder visual vs. intruder odor-intruder visual**

During mate odor-intruder visual experiments, resident fish (n=8) visited mate and non-mate bottles with moderate frequency. During the first phase there were $0.578\pm0.361$ visits to the intruder A bottle per minute and $0.907\pm0.260$ visits to the intruder B bottle per minute. In the second phase, in which the position of the bottles in the experimental arena was reversed, there were $0.822\pm0.355$ visits to the intruder A bottle per minute and
1.020±0.489 visits to the intruder B bottle. Eggs were removed via catheter from two of the eight hidden, bottled mates in these experiments, thus at least two bottled mates were female. Eggs were removed from one of the eight intruder A fish, and three intruder B fish, thus at least one intruder A and three intruder B fish were females. Responses did not trend differently between resident fish of different putative sexes. Hidden bottled mates, intruders A, and intruders B were all of similar size (81, 80, and 79 mm SL, respectively). Relative size differences between fish tested were similar (mate – intruder A SL, range -3 to 5 mm; mate-intruder B SL, range -4 to 5 mm; intruder A- intruder B SL, range -1 to 4 mm).

In these experiments, resident fish did not respond differentially to either intruder regardless of the odor cue (mate or matched odor) present (Figure 6.5). There was no statistical effect of intruder A bottle or intruder B bottle (F7,1=3.759 p=0.094), experiment phase (F7,1=0.565 p=0.477), or statistical interaction (bottle X phase) (F7,1=0.774 p=0.408). Thus resident fish did not display a differential response consistent with discrimination of intruders based on olfactory cues (mate or non-mate odors).

**No olfactory cue experiments**

During no olfactory cue experiments, resident fish (n=6) visited mate and non-mate bottles with high frequency. There were 0.797±0.452 visits to the mate bottle per minute and 0.692±0.496 visits to the intruder bottle per minute. The catheter was only used in three of six experiments and no eggs were removed from captured mates, thus at least three bottled mates may have been female. Eggs were removed from one intruder that
Figure 6.5. Proportion of time spent by resident fish near model bottle stimuli in *mate odor-intruder visual experiments*. Bars and error bars are back-transformed (as in Figure 6.2). First phase, left two bars shows time spent near bottle of intruder A (black bars), which is visible in a jar but has scent from mate released at site and intruder B (white bars) which has matched intruder odor released at jar. Second phase, right two bars shows time spent at each stimulus after release of matched odor stimulus at intruder A and mate odor at intruder B. Abbreviations below bars indicate cues present: IV = intruder visual, MO = mate odor. No statistical differences between groups (2-way RM ANOVA, P>0.05).

was used in two experiments. Sizes of fish used in these experiments were similar (mate mean SL 85 mm, non-mate 84 mm, length of one mate was not measured when the fish escaped). Relative size differences (mate - non-mate SL) of the five measured instances were minimal (range 0 to 3 mm).

In these experiments without either odor cue available, resident fish did not respond differentially to mates or intruders (Figure 6.6). There was no statistical effect of
Figure 6.6. Proportion of time spent by residential fish to model bottle stimuli in *no olfactory cue experiments*. Bars and error bars are back-transformed (as in Figure 6.2) with proportion of time spent at mate visual stimuli (black bars) and non-mate visual stimuli (white bars). Abbreviations below bars indicate cues present: IV = intruder visual, MV = mate visual. No statistical differences between groups (paired t-test, P>0.05).

mate bottle or intruder (t = -0.267, d.f. 5, p=0.800). Thus resident fish did not display a differential response consistent with discrimination mates from intruders when olfactory cues were not present.

**DISCUSSION**

These experiments demonstrated a differential response of butterflyfish towards mates and unfamiliar conspecifics. These results are consistent with the hypothesis that monogamous butterflyfish can discriminate mates from unfamiliar conspecifics, similar to results obtained recently in a pipefish (Syngnathidae: *Corythoichthys haemopterus*)
(Sogabe, 2010). These results indicate that multiple stimuli may be necessary for butterflyfish to discriminate between mates and potential competitors that attempt to forage within feeding territories. When visual and olfactory stimuli were both present, resident fish persisted around non-familiar fish and engaged in agonistic displays. When olfactory stimuli were mismatched first, resident fish spent equal time with mates and non-mates and when olfactory cues were restored, spent more time with non-mates. Experiments without odor cues resulted in nearly equal time spent by residents near mates and non-mates. Experiments with intruder odor cue only did not result in a differential response. Evidence for discrimination of fish by odor alone was not found, as resident fish did not associate with intruders differentially when mate odor was substituted for matched odor at one of the intruder bottles. As a whole, these results are consistent with a hypothesis of multimodal recognition of individuals that requires both vision and olfactory modalities.

Although it is clear that this species is attracted to the visual presence of conspecifics, no evidence for discrimination of mates and non-mates by vision alone was found in this study. Several studies in other fishes have demonstrated some degree of visual recognition of individuals. A species of anemonefish (Amphiprion bicinctus) was shown to attack non-mates more frequently than mates in field experiments, but mates were attacked with high frequency when their color was manipulated with dye or with plastic screens (Fricke, 1973). Evidence for discrimination of mates was found for female Jewel Cichlids Hemichromis bimaculatus (Noble and Curtis, 1939). Visual recognition of mates also was demonstrated in a cichlid fish from Lake Tanganyika with video playback experiments (Balshine-Earn and Lotem, 1998). Though, butterflyfish in
this study did not discriminate individuals based on vision alone, vision was required, along with olfactory cues to elicit differential associations between presented mates and non-mates.

Butterflyfish in this study showed a strong visually mediated reaction to model bottle stimuli, evidenced in some cases by a cessation of feeding activity and rapid swimming from distances of five to 10 m from bottles. This behavior is similar to reactions from fish after a period of visual separation from mates and provides evidence of at least species recognition, as individual fish do not react in a similar manner to other reef fishes (Tricas, 1985). Visual discrimination of closely related species has been demonstrated in Guppy fish separated from chemical cues with Perspex dividers (Warburton and Lees, 1996) and the pomacentrid Dascyllus marginatus which demonstrated a higher association with video images of conspecifics than to some (but not all) congeners (Shashar et al., 2005).

Olfactory modalities are important for social communication in a variety of fishes. Scent cues for conspecific attraction and recognition were shown in Fathead Minnow (Cole and Smith, 1992), Crucian Carp Carassius carassius (Lastein et al., 2008), Delicate Swordtail Xiphophorus cortezi (McLennan and Ryan, 1997; Hankison and Morris, 2003; Fisher et al., 2005; Wong et al., 2005; McLennan and Ryan, 2007), and Banded Killifish Fundulus diaphanus (Ward et al., 2008). Olfactory cues were shown to be used for discrimination of kin in European Perch Perca fluviatilis (Behrmann-Godel et al., 2006), Zebrafish Danio rerio (Mann et al., 2003), some populations of Three-spined Stickleback (Mehlis et al., 2008) (but not all populations, see Steck et al., 1999), and Arctic Charr Salvelinus alpinus (Olsén et al., 1997; Olsén, 1999; Olsén et al., 2002, 2003). Olfaction
was implicated in recognition of individuals in bullhead catfishes (*Ameiurus* spp.) (Todd and Bardach, 1967; Carr and Carr, 1985), recognition of fry by Midas Cichlid *Amphilophus citrinellus* parents (Noble and Curtis, 1939; McKaye and Barlow, 1976), self recognition in the cichlid *Pelvicachromis taeniatus* (Thünken *et al.*, 2009), and Three-spined Stickleback (Ward *et al.*, 2007). In this study, however, fish did not respond as would be expected if odor alone were sufficient to discriminate between mate and non-mate fish. When mate visual stimuli were hidden, and mate odor was released at a non-mate visual stimulus, no difference in time spent near a matched odor non-mate visual stimulus relative to the non-mate paired with mate odor. Thus it appears that both odor and visual cues are required for discrimination.

Communication in fishes often employs multiple sensory modalities. Mate attraction and courtship in another group of reef fishes (Pomacentridae) is known to include multiple sensory modalities, sound and vision, in which male fish produce a strong visual signal (termed the ‘signal jump’) in association with a courtship sound (Mann and Lobel, 1998). Males of at least some mormyrid fishes produce electric organ discharges with acoustic displays simultaneously during social communication (Crawford and Huang, 1999). Social mediated sex change in a protogynous goby (*Coryphopterus glaucofrenum*) is mediated by chemical and visual cues (Cole and Shapiro, 1995). In a poeciliid, *Xiphophorus pygmaeus*, multimodal stimuli of vision and olfaction are required to overcome a visual bias in female fish that results in a preference of heterospecific (*X. cortezi*) males when olfactory cues are not present (Hankison and Morris, 2003). In the current study, integration of at least visual and olfactory cues may allow fish more cues for individual recognition.
This study did not examine acoustic communication between individuals. Pebbled Butterflyfish are known to produce sounds during agonistic interactions with conspecifics and during distress (Tricas et al., 2006). Individual recognition based on sounds is known from a variety of animals, e.g. penguins (Lengagne et al., 1999; Aubin and Jouventin, 2002), sea lions (Charrier and Harcourt, 2006), manatees (Sousa-Lima et al., 2002), and possibly bats (Melendez and Feng, 2010). Very few fish are known to discriminate between individuals on auditory cues alone. Myrberg & Riggio (1985) demonstrated that male Bicolor Damselfish *Stegastes partitus* produce more sounds when sounds of unfamiliar males are played from a familiar male’s territory. There is evidence of sufficient inter-individual variability of acoustic signals to permit individual recognition in two species of mormyrids (Crawford et al., 1997). The sounds of Pebbled Butterflyfish include low frequency pulses with highly variable and long interpulse intervals (Tricas et al., 2006, Boyle unpublished), unlike, Bicolor Damselfish sounds which have multiple pulses emitted rapidly with a short interpulse interval but temporal patterning that is hypothesized to allow for individual recognition (Myrberg and Riggio, 1985). Thus acoustic recognition of individuals seems unlikely in Pebbled Butterflyfish. Pebbled Butterflyfish also communicate with strong tailslap behaviors that transmit both sound pressure stimuli and shorter range hydrodynamic stimuli (Tricas et al., 2006). The bottles used in this study, would not permit hydrodynamic stimuli from the tail-slap to or from captive fish.

Several additional factors may have influenced results of the matched odor experiments and mate odor-intruder visual experiments. First, the behavior of the captive butterflyfish within the bottles may have been affected by visual cues of the reef habitat.
Butterflyfishes forage on predictable routes within territories and display behavior consistent with complex spatial learning (Reese, 1989). Intruders may react differently than bottled mates if reef landmarks are recognized and initiate different behaviors, such as increased stress. Recognition of the reef by either the bottled mate or bottled intruder during the duration of the experiment, however, seems unlikely given that the fish are confined to the bottle and not able to visit a broad area to provide visual references. Second, the olfactory cue of the bottled mate could be influenced by the experimental treatment. In matched odor and crossed odor experiments, the bottled mate maintains visual contact with the free-swimming resident. If the odor cues, which are unknown at this time, are released under control by the bottled fish (e.g. through urine, skin, or gills), it may be beneficial for fish to advertise their odor identity to avoid misrecognition by their mates. When fish are separated from visual contact and potentially under stress, as was the case for bottled mates during mate odor-intruder visual experiments, it may benefit fish to minimize odor release which could be a cue for potential predators. The hypothesis of modulation of odor release by fish warrants further investigation.

The odor cues available for discrimination in Pebbled Butterflyfish are not known at this time. Potential candidates include major histocompatibility complex (MHC) proteins, prostaglandins, bile, and amino acids (Sorensen and Caprio, 1998; Hansen and Reutter, 2001; Zielinski and Hara, 2007). Genetic variation may provide olfactory diversity in terms of MHC, while physiological state may provide cues in terms of prostaglandins, bile salts, and amino acids. Differences in territory quality and food resources may further affect the physiological state and suite of chemicals released by individual fish.
Experiments in this study did not demonstrate discrimination of mates and non-mates by vision alone, however, vision was necessary (along with chemical cues) for discrimination. Many butterflyfishes have complex and variable color patterns, which are used by researchers to identify individuals over study periods (Tricas, 1989a). Variation in eyespot (ocellus) color pattern, measured in Four-spot Butterflyfish *Chaetodon capistratus*, was the basis of a hypothesis by which many butterflyfish could utilize visual recognition of mates (Meadows, 1993). Such a mechanism may be important for Pebbled Butterflyfish, which do not possess an ocellus, but do have a black bar over the eye, a melanistic crown over the supraoccipital crest, a dark band on the caudal peduncle, and several highly variable brown bars and melanistic scales over a light background. Variable color patterns may provide visual cues in at least some circumstances. In addition, fish size, which was purposely controlled for in this study may provide a cue for some individuals.

Sex was not controlled for in this study and may account for differences in resident behavior, as well as affect the stimulation properties of the bottled fish. Both male and female resident fish engage in territorial behavior and chase conspecifics out of feeding territories, but males typically produce more chases (Tricas, 1989b). Sex specific visual stimuli based on morphology or chromatic cues seems unlikely, at least within the biases of human observers that have not identified sexual dimorphism in *C. multicinctus*. It is possible, however, that sex-specific behaviors of bottled fish (e.g. activity level) would affect the behavior of resident fish. Sex-specific olfactory cues are used by some fishes for reproduction (Cole and Smith, 1987, 1992). Whether sex specific cues play a role in chemical communication by butterflyfish remains to be tested.
A recent experiment on Pebbled Butterflyfish, which as a whole display strongly size assortative mating, has demonstrated that females which were experimentally separated from their mates show no difference in time spent near larger or smaller males, while males under the same conditions spend more time with larger females (Strang, 2005). It is not known which stimuli, visual, acoustic, or olfactory are responsible for the behavior of male fish. In light of the results of this study, and recent studies on acoustic communication in butterflyfishes (Tricas et al., 2006, Boyle & Tricas in press, Boyle unpublished), multimodal communication may provide cues for mate assessment, choice, and recognition. Individual recognition experienced by social butterflyfishes is a complex task that is undoubtedly affected by the behavior of both members after pair separation. Future research is needed to shed light on the role of potential chemical cues to facilitate discriminatory behaviors.

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CHAPTER VII:

CONCLUSIONS, SIGNIFICANCE, AND FURTHER RESEARCH

Conclusions

This dissertation provides significant advances in the understanding of acoustic communication and other aspects of broader, multimodal communication in several species of butterflyfishes. These results bear relevance to patterns of social sound production evolution in teleost fishes more generally; a taxon important for study because the lack of homologous vocal mechanism makes them a model for behavioral evolution.

A variety of conclusions can be drawn from this study.

Sound production in the butterflyfish genus *Forcipiger* involves a novel mechanism in which opposing muscles of the head contract synchronously. These contractions are hypothesized to rotate the ventral pectoral girdle anteriorly and indirectly expand the swim bladder rostrally (Chapter II).

During *Forcipiger* headbob sounds, loading of epaxial musculature at sound emission onset results in rapid cranial rotation that lags behind sound emission and occurs after release of tension from the sternohyoideus and adductor mandibulae (Chapter II).

Acoustic signals of *F. flavissimus* are correlated with body size. Sound pressure level and sound duration, both measures associated with the overall loudness of the sound. These features may factor in to the honesty of the signal as an indicator of body size (Chapter II).

Cranial kinematic performance of *F. flavissimus* is correlated with sound signal features. Sound pressure, duration, and to a lesser extent, frequency spectra are
positively correlated with the angular acceleration of the head that occurs after sound emission. Further, epaxial muscle activity is correlated positively with sound duration and pressure level. Thus, acoustic signals are potentially honest signals of 1) body size, and 2) cranial performance (Chapter II).

The peak and median frequency of *F. flavissimus* sound power spectra were not correlated with body size. This finding is consistent with the hypothesis that the swim bladder is highly damped by surrounding tissues. Swim bladder resonance should scale negatively with body size. Thus it appears resonance properties are not the main factor that contributes to the frequency of sounds. This finding is consistent with studies of toadfish swim bladders and would prevent large depth related sound frequency changes (resonance of gas filled bubbles increases with depth/pressure). *Forcipiger flavissimus* is found in shallow water (1-2 m) to depths of at least 50-70m in Hawaii, and large frequency shifts in acoustic signals as a side-effect of ambient pressure would potentially make acoustic communication less effective (Chapter II).

*Forcipiger* is capable of producing cranial elevations, velocities, and accelerations greater than what has been measured during feeding in other studies. The values measured during sound emission are faster than some rapid ram-suction feeders (e.g. Largemouth Bass) and slower, but with overlapping values, than extremely rapid syngnathid fishes (Chapter II).

Sound production by Pyramid Butterflyfish *Hemitaurichthys polylepis* involves emission of rapid pulse trains. Each pulse is acoustically similar to single pulses emitted by *Forcipiger*, but can be emitted at much higher rates (up to 40 pulses s⁻¹). The ability
of Pyramid Butterflyfish to produce pulses in rapid succession trains allows for temporal patterning of sounds (Chapter III).

Pyramid Butterflyfish *Hemitaurichthys polylepis* produce pulse train sounds with a different mechanism than the headbob possessed by the related genus *Forcipiger*. This mechanism allows for rapid pulse trains that have the potential to produce temporal patterning and a modulated amplitude envelope (Chapter III).

Pyramid Butterflyfish pulse train sounds are produced during courtship-like behavior near sunset by fish that are hypothesized to be males. Sounds are produced while putative males defend small reef areas from other putative males and during interactions with putative female fish in the water column. Sounds also are produced during distress when heterospecific fish approach or divers approach fish during courtship activity. Distress sounds are also readily emitted in the laboratory. Both sexes produce sounds in the laboratory (Chapter III).

Contractions of extrinsic swim bladder muscles produce pulse sounds in Pyramid Butterflyfish. Contractions produces buckling between the ribs of the 4th and 5th vertebra at the anterior end of the swim bladder (Chapter III).

Sonic muscle activity in *H. polylepis* involves synchronous action potentials, rather than burst firing patterns as seen in the head muscles involved in *F. flavissimus* sound production. Doublet firing (two muscle action potentials) occurs during the production of most pulses, however, distinct contractions and sound pulses do not appear to occur when doublets are produced (Chapter III).

Pulse waveforms of Pyramid Butterflyfish sounds indicate that the swim bladder likely does not behave like a simple monopole, but rather expands rostrally with each
contraction. Thus the phase of sounds varies according to the position relative to the fish body (Chapter III).

Pyramid Butterflyfish sonic muscle fibers are smaller in cross-sectional area and more round than adjacent hypaxial muscle fibers. Pyramid Butterflyfish sonic and non-sonic hypaxial muscles have triads of the z-line type. Some myofibrils of pyramid sonic muscle are arranged radially, however, no cores of sarcoplasm are present at the center. Sonic muscles have well developed sarcoplasmic reticula. Transverse tubules of sonic muscles are characterized by widely-set membranes. Sonic muscles have sarcomere lengths that are greater than non-sonic muscles (Chapter IV).

Sonic muscles in Pyramid Butterflyfish are innervated by spinal nerves 1-3, not occipital nerves. This pattern is similar to several distantly related teleosts with extrinsic sonic muscles (piranhas, John Dory, and croakers). Sonic motor neurons occur along a ventro-lateral and medial-lateral motor column. Medial-lateral motor neurons have more finely branched dendrites and are larger than ventro-lateral motor neurons, which have less densely branched dendrites. Sonic motor neurons are not arranged in a distinct motor nucleus. Their position entirely within the spinal cord is similar to the distribution present in piranhas and may be typical of fishes that have sonic muscles innervated entirely by spinal nerves (Chapter IV).

Both *Forcipiger flavissimus* and *F. longirostris* are site-attached, home ranging, and territorial on reefs of west Hawaii. Fish of both sexes engage in agonistic territorial behavior with intruders of either sex, consistent with the hypothesis of territorial defense of feeding territories. Mate-guarding may also be important in both species, a hypothesis which is not mutually exclusive (Chapter V).
Forcipiger flavissimus is socially harem and usually occurs in territorial trios composed of two females and one male. The harem polygyny of F. flavissimus is unusual among studied butterflyfishes and the sister family angelfishes because a common territory is occupied by all members of the harem, rather than the more common arrangement of separate female territories that overlap with a larger male territory. This mating system allows for an unusual group dynamic in which females pair up when separated from the male during foraging, and pairings of all combinations as well as trio groupings occur throughout the day (Chapter V).

Forcipiger longirostris is socially monogamous and strongly paired. Unlike F. flavissimus trios, pairs of F. longirostris are mated assortatively. A similar pattern was found in a previous study on the monogamous Chaetodon multicinctus, however, males tended to be larger than their female mates (Chapter V).

During agonistic territorial encounters, both F. flavissimus and F. longirostris produce similar pulsatile sounds. No evidence was found for different sound types emitted from territorial fish to different receivers (intruders of different sexes, captive mates). Further, sounds between species were largely similar and likely provide little information for species identification, though this remains to be tested. Sounds recorded between individuals in the field were remarkably similar. Laboratory studies, however, indicate that the overall sound pressure level and sound duration is likely to vary with body size and would potentially provide information on the size of an individual fish. Broad overlap between sound pressure level in the field may result from similar sized individuals in the study, a broader range of distance between the signaling fish and hydrophone that would cause acoustic decay, or both (Chapter V).
Pebbled Butterflyfish *Chaetodon multicinctus* show an agonistic response towards simultaneously presented mates and intruders, in which resident fish spend more time engaged in aggressive behavior around intruders. This response demonstrates behavioral discrimination consistent with individual recognition, a predicted requirement of pair-bonding and territory maintenance. The response by Pebbled Butterflyfish, a small abundant species with small territory sizes make them an appropriate butterflyfish model for examining the sensory requirements of discrimination and multimodal communication (Chapter VI).

Visual and olfactory cues are required to elicit discrimination of mates from non-mate intruders in *Chaetodon multicinctus*. Visual stimuli alone will elicit an aggressive response from resident fish to mates and non-mates. When odor cues of captive fish are present, resident fish spend more time with the non-mate intruder. When odor cues are manipulated by reversal of spatial associations, fish do not discriminate between mates and non-mates. Resident fish do not appear to track odor plumes from conspecifics released away from visual stimuli. Thus, visual stimuli are extremely important and probably allow for conspecific recognition. Additional chemical cues, however, assist in discrimination of individual fish (Chapter VI).

Agonistic behaviors and sound production that occur in several social butterflyfishes when solitary fish encounter a conspecific after a period of visual separation from mates may 1) serve to quickly advertise territorial ownership, 2) bring fish in close visual, chemical, and acoustic contact to facilitate aggression and threat potential of either fish, 3) benefit both sender and receiver by providing additional time for recognition and assessment of the other fish, and 4) prevent injury to mates from early
recognition errors. These greeting ritual-like behaviors facilitate multimodal communication and may employ acoustic signals specifically to assess body size, which is a cue for both individual identity (mate, similar sized non-mate, or dissimilar sized non-mate) and threat potential (Chapter (VI).

Recommendations for future research

The recent discovery of acoustic communication in several butterflyfish taxa during the course of this study has provided an area ripe for further research. The logistical detail and correlative nature of some of the aspects of field study has left some questions unanswered and generated many more. Sound production in toadfishes, sea robins, and sciaenids has a history in scientific publication greater than 100 years and these animals continue to be important subjects for neuroethology, physiology, behavioral ecology, and passive fisheries acoustics. Discoveries from this dissertation may provide a platform for promising future research on acoustic communication in a reef fish model.

The axial and appendicular skeletal kinematics and swim bladder deformation during *Forcipiger flavissimus* sound production could be examined with high-speed cineradiography. This dissertation developed a hypothesis of anterior rotation of the pectoral girdle based on muscle activity patterns, muscle manipulation (inactivation and transaction) and high speed kinematic patterns. It was not possible to visualize the swim bladder or bones without obvious external landmarks. Movement of the pectoral girdle and ribs in association with swim bladder could potentially be visualized in a sound producing fish with a high speed camera and x-ray fluoroscope.
The dynamics of swim bladder motion could be tested with sonomicrometry techniques. Sonomicrometric crystals allow for the analysis of small scale movements of internal landmarks in physiology studies. Ultrasonic sounds are used to measure time-of-arrival differences in order to determine changes in position. Sonomicrometry crystals could be placed along various landmarks of the tunica externa of the swim bladder to measure motion of the swim bladder as sound emission occurs.

Contraction rates of Pyramid Butterflyfish sonic muscles could be measured \textit{in vivo} and in physiological preps. Sonic muscle contraction rates were inferred indirectly from the sound wave and EMG measurements in this dissertation. These data indicated moderately fast contractions sustained over brief periods. The actual contraction rate could be even faster, but not sustained (i.e. fast contractions with periods of no activity between pulses). The actual contraction rate may be important for the frequency spectra of the sounds. Contraction rates in free-swimming fish could be measured with sonomicrometry crystals. Contraction rates could also be measured physiologically by extracting living muscle tissue, connecting the muscle to a strain gauge and stimulating with different frequency square waves. The physiological prep experiment would allow for determination of maximal contraction rates, rates in which contractions begin to fuse, and when complete tetany occurs.

The fiber-type of \textit{H. polylepis} sonic muscles should be determined with histochemical methods. These muscles are hypothesized to be adapted for fast contraction rates and to be composed predominately of type II fast twitch oxidative fibers. ATPase and NAD diaphorase fiber typing could be used to confirm this hypothesis.
The concentration of metabolic substrates (glycogen, lipid) in *H. polylepis* sonic muscles should be compared to non-sonic muscles. Sonic muscles are hypothesized to have abundant glycogen stores. Comparisons between sexes and across seasonality could be used to test hypotheses related to courtship function of sound production.

The axonal innervation pattern of individual muscle fibers of *H. polylepis* should be determined with silver staining or acetylcholinesterase techniques. Many fast contracting fish muscle fibers, especially in sound production muscles are polyaxonally innervated. The EMGs measured in *H. polylepis* do not show a compound action potential and indicate there may be high synchronization of firing between muscle fibers.

Neuroanatomical pathways between *H. polylepis* motorneurons and higher levels in the CNS should be determined. Premotor pacemaker neurons have yet to be identified. Of particular interest is whether premotor neurons provide connections allowing for the bilateral symmetry of motor neuron firing observed with EMGs during sound production. Other pathways of interest are efferent pathways from motor neurons to auditory nuclei and premotor pathways from midbrain and forebrain centers that may be important for behavioral initiation of sound emission. Pathway tracing of *Forcipiger* sound production motorneuron nuclei may also reveal unusual connections. Sound production motor patterns of *Forcipiger* are unusual in requiring simultaneous activity from muscles innervated by diverse sets of cranial (adductor mandibulae-trigeminal, sternohyoideus-occipital) and spinal nerves epaxial (spinal nerves and possibly occipital nerves for anterior myomeres).

Audiometric studies on vocal species from this study should be conducted in order to determine the ability of receivers to perceive acoustic signals. Auditory evoked
potentials (AEP) were tested in *F. flavissimus* (Tricas and Boyle unpublished) and indicate sensitivity to low frequencies (100-200 Hz) that are well represented by conspecific signals. Tests with thresholds to particle acceleration, arguably the more relevant auditory stimulus for most fishes should also be conducted. AEPs could also be conducted with facsimiles of conspecific signals. Of particular interest is whether temporal pulse train patterns of *H. polylepis* calls are preserved by eighth nerve afferents.

Are acoustic signals important for spawning butterflyfishes? Results from this dissertation indicated that sounds are probably an important feature of courtship for *H. polylepis*. Spawning behavior is reported from several species of butterflyfishes but no sounds during spawning have been recorded. Spawning behavior may be somewhat difficult to observe because of the time of probable spawning (dusk), because species that have been observed engage in one spawning rush a night, and because in some species observed spawning occurs between pairs or few individuals, rather than large aggregations. Accounts of sound production during spawning are important, however, because of the obvious potential selection pressure spawning behavior may have on acoustic communication.

What chemical compounds provide potential discrimination cues for butterflyfish mates? Several non-mutually exclusive chemical cues could be released by fish in proximity to conspecifics. The presence of these compounds should be identified in a chemical ecology study in which water of fish held in captive could be screened as well as extracts from the urogenital opening. Potential compounds of interest include bile acids, prostaglandins, steroid hormones, and MHC compounds. Physiological sensitivity to these compounds could be tested with electro-olfactogram techniques.
Laboratory tests could be conducted with combinations of different extracts to assess the behavioral importance of potential olfactory chemicals. Choice tests with conspecific fish (mate and non-mate) or mirrors could be used to assess if there are innate attractive or avoidance responses to particular biogenic compounds.

Several studies have had recent success with video visual stimuli with other fish subjects. This paradigm could be tested with butterflyfishes and has promise to provide a controlled visual stimulus. Video stimuli allow for presentation of two potentially realistic stimuli that are identical in choice tests. Further, these stimuli can be manipulated easily with computer software to control for color patterns and body size.

The Pyramid Butterflyfish is a species with passive acoustic monitoring potential. This species is part of the Hawaii aquarium fishery, though not currently a large component. As a locally abundant planktivore, it likely provides an important ecological service that links nutrients from more oceanic food webs to coral reefs. The relatively loud and long duration pulse trains associated with reproductive behaviors make them a candidate species for coral reef passive acoustic monitors.

A diversity of sound production mechanisms within chaetodontid fishes is beginning to emerge. Sound production is likely widespread throughout the family Chaetodontidae. Observations of acoustic behaviors, however, are often difficult to obtain, arise opportunistically because many species may need proper motivation to vocalize, and lack of sound production from a particular species does not preclude the possibility that vocal behavior exists. The recent discovery of distress sound production in *H. polylepis* indicates that sounds may be elicited more easily for at least some butterflyfish taxa. Distress sounds in many fishes appear to exist with sound production
in broader contexts. Their discovery, however, can provide insights into potential sound production mechanisms and indicate taxa that are likely to use sounds in communication in other contexts. Chaetodontids have robust phylogenetic hypotheses supported by osteology, swim bladder morphology, mtDNA and nuclear DNA. Determination of acoustic communication in additional genera may ultimately allow for comparative analyses within the family. Two apparently dramatically different mechanisms exist for *Forcipiger* and *Hemitaurichthys*. Additional studies on terminal taxa within their clade (*Heniochus* and *Johnrandallia*) if these mechanisms are autapomorphic at the generic level for either taxon. Sound production should be examined in the adjacent coral fish clade. Lastly, *Prognathodes*, the non-laterophysic outgroup to *Chaetodon*, should be examined.