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Evolution of Motor Patterns in Tetraodontiform Fishes: Does Muscle Duplication Lead to Functional Diversification?

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Key Words

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Abstract

Several times within the teleost fish order Tetraodontiformes singular jaw adducting muscles have been effectively 'duplicated' by physical subdivision to produce new muscles. This morphological system provides an opportunity to investigate how the functional complexity of muscular systems changes with evolutionary increases in the number of component muscles. In this study we asked if muscle duplication has led to functional diversification by comparing the motor patterns of muscles that result from subdivision events. The activity patterns of five different sets of duplicated muscles were quantified with electromyographic recordings (EMG) from four individuals in each of three species during processing of three prey types. Prey varied in durability and elusiveness (live fiddler crabs, pieces of squid tentacle and live paeneid shrimps). For each cycle of prey processing, measurements were made of the relative onset time of each adductor muscle, the duration of each burst of activity, and the relative intensity of each activity burst. Two types of functional divergence of muscles were observed in analyses of variance conducted on the EMG variables. In two of the 15 variables examined, the timing of activity of the descendant set of muscles differed. In another three of the 15 variables, there were significant interactions between muscle and prey type, indicating a prey effect which differed in the descendant muscles. Overall, evidence of motor divergence was found in three of five cases of muscle duplication. This indicates that muscle subdivision has led to increased functional complexity of the jaw-adductor muscle system in tetraodontiform fishes.

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Introduction

Recent interspecific studies of fish feeding behaviors have revealed a high degree of conservation of neuromuscular activity (i.e., motor) patterns in jaw muscles [Lauder, 1983a; Wainwright and Lauder, 1986; Sanderson, 1988;

Wainwright, 1989; Ralston and Wainwright, 1997]. In general, motor pattern differences between jaw muscles in one species have also been observed for homologous muscles examined in closely related taxa. For example, one jaw muscle may consistently have an earlier onset or longer duration of activity than another muscle in all taxa examined.

In addition, effects due to prey type and position have been shown to influence the neuromuscular activity patterns of both oral and pharyngeal jaw muscles in several groups of teleost fishes including the Centrarchidae [Lauder 1983a; Wainwright and Lauder, 1986], Characiformes [Lauder, 1981], Cyprinidae [Elshoud-Oldenhove and Osse, 1976; Sibbing et al., 1986], Cichlidae [Liem, 1978, 1979, 1980], Embiotocidae [Drucker and Jensen, 1991], Haemulidae [Wainwright, 1989], Labridae [Sanderson, 1988], and Tetraodontiformes [Turingan and Wainwright, 1993; Wainwright and Turingan, 1993; Ralston and Wainwright, 1997]. In general, when there is an effect of prey on motor patterns, this effect is similar on all synergistic muscles in the same fish or homologous muscles in other individuals of the same or related species.

While the conservation of motor patterns in vertebrate muscle systems has received much attention [see recent review by Smith, 1994], surprisingly little insight has been gained into how intermuscular differences in motor patterns within individuals originate or how these patterns may evolve in conjunction with gross morphological changes such as changes of muscle attachment or phylogenetic increases in muscle number. This study seeks to quantitatively examine the evolution of muscle function in a slightly different light. Rather than comparing homologous muscles in different taxa, we focus on homologous muscles in the same species: muscles that have developed from historical subdivision events. We address three general questions regarding the evolution of motor patterns following muscle duplication by subdivision: (1) As new muscles evolve by physical subdivision of a pre-existing muscle, do they retain a similar plesiomorphic motor pattern, or do their motor patterns diverge functionally? (2) Are effects of prey type on motor patterns always simple and relatively straightforward, or are there more complex interactions between prey type and muscles? (3) Are some features of motor patterns (e.g., onset time, burst duration, burst intensity) more conserved evolutionarily than others?

Tetraodontiform Jaw Muscles

The jaw adducting musculature of tetraodontiform fishes provides a model system for addressing such questions. Teleost fishes of the order Tetraodontiformes are a diverse group of primarily marine fishes that are distributed throughout the tropical and temperate regions of the world. This clade is represented today by nine families which are broadly divided into three large subclades (fig. 1). The relatively basal superfamily Triacanthoidea contains the Triacanthodidae (spikefishes) and Triacanthidae (triplespines). These triacanthoids are the sister taxon to the more familiar

tetraodontiform fishes placed in the other two superfamilies. The superfamily Balistoidea contains the Monacanthidae (filefishes), Balistidae (triggerfishes) and Ostraciidae (boxfishes, cowfishes; including the Aracanidae sensu Tyler and Sorbini [1996]). Their sister group, the superfamily Tetraodontoidea, contains the Triodontidae (pursefishes), Molidae (ocean sunfishes), Tetraodontidae (puffers), and Diodontidae (porcupinefishes).

One of the most distinctive features of most tetraodontiforms is their stalwart oral jaws with robust dentition. Unlike many bony fishes that engulf prey whole and subsequently process them with pharyngeal jaws, tetraodontiforms use their oral jaws to both capture and process prey [Turingan and Wainwright, 1993; Wainwright and Turingan, 1993; Turingan, 1994]. As in other fishes [Lauder, 1985], the muscles responsible for closing and generating the biting forces of the oral jaws are those of the adductor mandibulae complex [Turingan and Wainwright, 1993]. In the majority of teleost fishes, this complex consists of four separate muscles – A1, A2, A3, and A ω – all of which originate on the palatal arch and are innervated by branches of the fifth cranial nerve. The A1 uniquely inserts upon the upper jaw, whereas the other three muscles insert upon the lower jaw.

In contrast to the typical condition in teleosts most tetraodontiform fishes have a more complex set of A1 and A2 jaw muscles [Winterbottom, 1974a, b; Friel and Wainwright, 1997]. Within this clade, singular A1 and A2 muscles have been functionally duplicated by physical subdivision of pre-existing muscular tissue. This ‘muscle duplication’ phenomena has occurred at least 10 times within this clade [Friel and Wainwright, 1997]. As a result, most families of tetraodontiforms have unique combinations of muscles, and representative species may possess from two (Triacanthidae) to eight (some Monacanthidae) separate A1 and A2 muscles.

Since all new jaw muscles in tetraodontiforms are phylogenetically derived from preexisting muscles, the simplest assumption is that duplicated muscles will have inherited and possibly have retained the same plesiomorphic motor pattern as in their common ancestral muscle. This observation allows for a clear null hypothesis for each set of duplicated muscles examined in this study, even when the motor pattern of the ancestral muscle is unknown. Simply put: there should be no significant differences in the mean values of EMG variables used to quantify the motor patterns of duplicated muscles, unless one or more of them have diverged functionally.

Evolutionary duplication of muscles clearly provides an opportunity for increases in functional complexity through

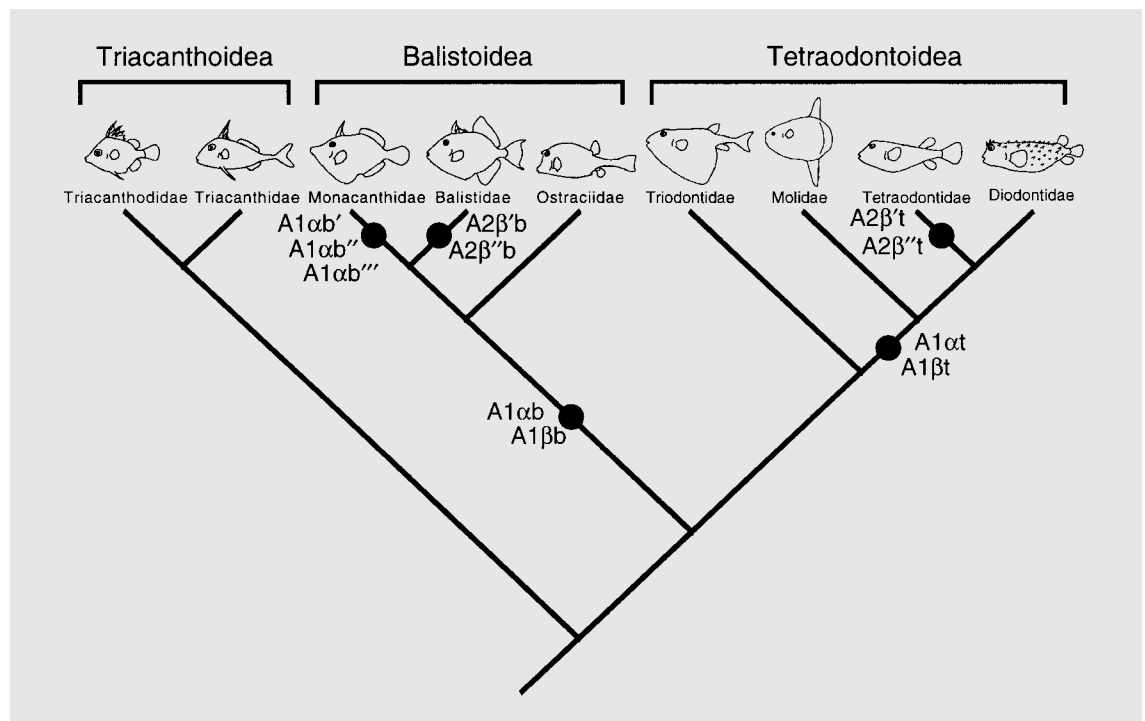


Fig. 1. Phylogeny of extant tetraodontiform families based on the work of Winterbottom [1974b], Matsuura [1979], Tyler [1980], Lauder and Liem [1983], Winterbottom and Tyler [1983] and Tyler and Sorbini [1996]. Images represent the general body form of fishes in these families. Solid circles mark the origins of the five cases of muscle duplication examined in this study. The specific names of muscles created by these events are listed next to the events.

the divergence of descendant muscles. The morphological redundancy of duplicated muscles could release functional constraints on one duplicated muscle, allowing it to diverge, while another member of the duplicated set maintains its plesiomorphic function. Such functional divergence may be reflected in the neuromuscular activity patterns of these muscles and could be detected in statistical analyses of variables used to quantify motor patterns. Additionally, we also investigate in this study whether or not such differences are consistently expressed for a variety of prey types.

Materials and Methods

The motor patterns of selected sets of duplicated muscles were quantified in individuals of three species of tetraodontiform fishes: *Balistes capriscus*, the gray triggerfish ($n=4$, SL=240–270 mm); *Monacanthus hispidus*, the planehead filefish ($n=4$, SL=117–136 mm); and *Sphoeroides nephelus*, the southern puffer ($n=4$, SL=110–160 mm). These species represent three of the nine extant families of tetraodontiform fishes (Balistidae, Monacanthidae and Tetraodontidae; fig. 1) and were selected because they were amenable

to laboratory study and available locally. All specimens were collected in the northern Gulf of Mexico near the Florida State University Marine Laboratory, Turkey Point, Florida.

Individuals were maintained in 100 liter aquaria at $24 \pm 2^\circ\text{C}$ and fed a mixed diet of squid, shrimp, and fiddler crabs for at least a week prior to electromyographic recording sessions. All guidelines used in this research were established by the Animal Care and Use Committee of Florida State University.

To investigate the effects of prey type on motor patterns, we selected three items to represent much of the spectrum of food types found in the natural diets of these fishes. Live fiddler crabs (*Uca* sp., 20–40 mm) served as an elusive prey with a hard, brittle exoskeleton. Fishes had little difficulty capturing crabs but were challenged by their armor. Live shrimps (*Paeneus* sp., 40–100 mm) were used as a highly elusive prey that possessed a relatively weak exoskeleton. Fishes often required several attempts to capture shrimp, and even when captured, shrimp often escaped before they could be consumed. For a completely non-elusive and unarmored prey, we used cut pieces of squid tentacle (*Loligo* sp., 20–40 mm). While this prey lacked an exoskeleton, the firm muscular tissue made this the toughest of the three experimental prey, and considerable effort was required to reduce it into pieces small enough for fishes to swallow. Decapod shrimp and crabs are common in the diets of these and other closely related tetraodontiform fishes [Randall, 1967; Frazier et al., 1991; Ralston and Wainwright, 1997]. Squid are not frequently eaten by these

species in the wild, but this prey was selected for its tough physical nature. Recording sessions were conducted after food had been withheld for at least 48 hours to ensure fishes would feed well during experimental recordings.

Myology

Electromyographic recordings were taken from the levator operculi muscle and five sets of duplicated adductor mandibulae muscles. The levator operculi (LOP) was chosen as a reference muscle because it has been used as a standard reference muscle in similar EMG studies. The LOP is the primary jaw depressor muscle in tetraodontiform fishes [Turingan and Wainwright, 1993]. In this study the LOP was consistently active during jaw opening and always functioned before the adductor mandibulae muscles closed the jaw. Five sets of duplicated jaw adducting muscles were examined: the A1 α b and A1 β b muscles of triggerfishes (fig. 2c); the A2 β 'b and A2 β "b muscles of triggerfishes (fig. 2c); the A1 α b', A1 α b'', and A1 α b''' muscles of filefishes (fig. 2a, b); the A1 α t and A1 β t muscles of pufferfishes (fig. 2d), and the A2 β 't and A2 β "t muscles of pufferfishes (fig. 2d).

We recently reviewed the evolution of jaw adductor muscles in tetraodontiform fishes [Friel and Wainwright, 1997] and base our assumptions of muscle homologies on that study. Each of the five cases listed above represents a case in which new muscles have been formed by the physical subdivision of a single ancestral muscle. Thus, in each case, the descendant muscles are homologous to each other and to the original undivided muscle as paralogs.

The names of these jaw adductors refer to their insertion points and also reflect their complex phylogenetic history (fig. 1). As in other teleost fishes, the A1 muscles insert on the upper jaw (or secondarily on the palatine as does the A1 α b''' muscle of filefishes), and all A2 muscles insert on the lower jaw. Our previous work indicated that non-homologous A1 α and A1 β muscles have arisen independently from a single common A1 once in balistoid fishes (Ostraciidae, Balistidae, and Monacanthidae; designated with a 'b' suffix) and again in a sub-clade of tetraodontoid fishes (Molidae, Tetraodontidae, and Diodontidae; designated with a 't' suffix). The A1 α b muscle of filefishes (Monacanthidae) has been further subdivided into three muscles – A1 α b', A1 α b'', and A1 α b'''. Finally, nonhomologous A2 β ' and A2 β " have arisen independently once in triggerfishes (Balistidae; designated with a 'b' suffix) and again in puffers (Tetraodontidae; designated with a 't' suffix) from a common A2 β .

Feeding Behavior

Many teleost fishes capture prey whole using either ram or suction feeding mechanisms [Lauder, 1983b, 1985; Norton and Brainerd, 1993]. During these feeding behaviors, the oral jaws are used to capture prey and reposition it within the buccal cavity (i.e., buccal manipulation) if necessary before it can pass intact into the pharynx [Lauder, 1983c]. Any subsequent chewing or crushing of prey is done with the pharyngeal jaws. In contrast, tetraodontiform fishes use their oral jaws for both prey capture and processing [Turingan and Wainwright, 1993; Wainwright and Turingan, 1993]. Following capture by suction or direct grasping, prey are repeatedly bitten and reduced by the fish's powerful oral jaws before being transported into the pharynx. This distinctive prey processing behavior was utilized by the three species examined in this study while feeding on all prey types.

We focused on this prey processing behavior for several reasons. First, from an anatomical perspective, underlying morphological variation in the adductor mandibulae musculature should have its greatest functional consequences on this behavior. All but one of the adductor

mandibulae muscles examined here insert upon the oral jaws, and no other muscles are used directly for jaw closing. Second, motor patterns used during prey capture may be affected by prey position alone [Elshoud-Oldenhave and Osse, 1976]. When using elusive prey in a recording session, it is difficult to control the relative prey position during capture. This potential source of variation is minimized during prey processing because prey position is now limited to some extent by the fish's jaws and oral cavity. Finally, in a single feeding sequence of a tetraodontiform fish, prey capture may consist of only a single cycle of muscular activity, whereas prey processing often consists of up to 30 or more cycles. Thus, it is possible to generate much larger sample sizes of this behavior for statistical analyses.

Electromyography

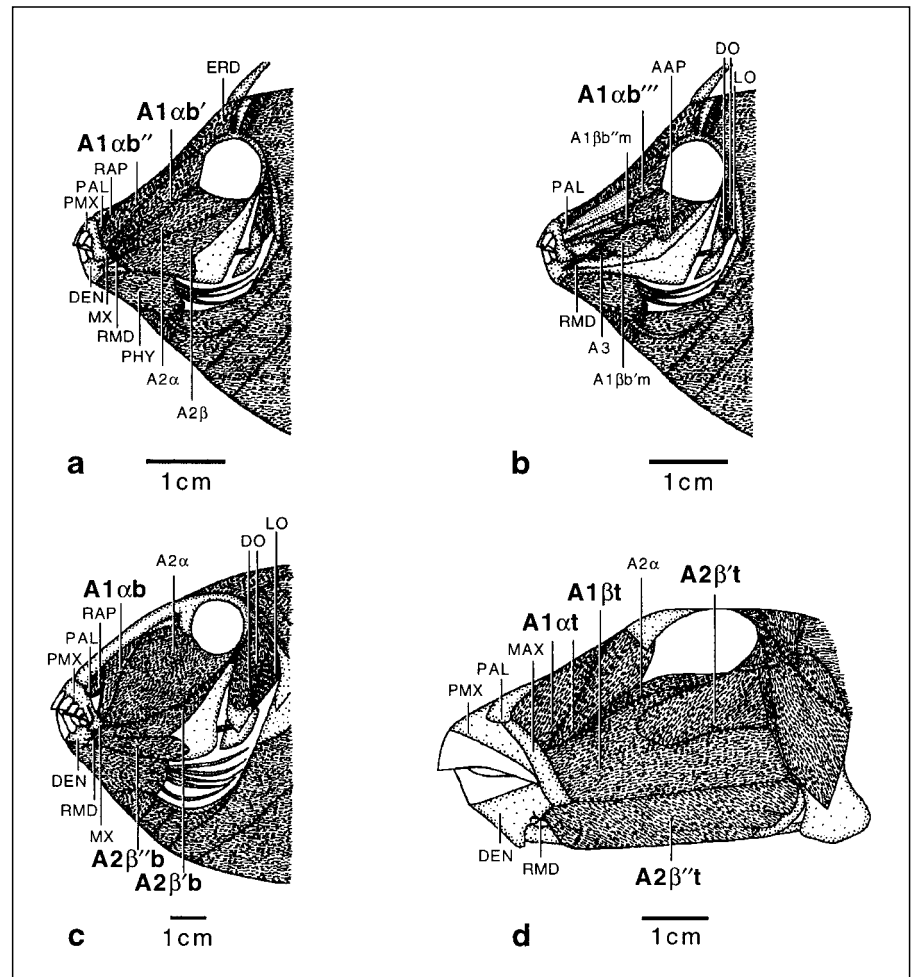
Electromyographic recordings of muscular activity during prey processing were made using bipolar electrodes constructed from paired and glued 120 cm sections of 0.002 gauge (0.051 mm diameter) insulated stainless steel wire (California Fine-Wire). This bipolar wire was threaded through a 26 gauge, 13 mm hypodermic needle before the tips of the wire were bared of insulation and bent back against the shaft of the needle. This configuration formed a double hook which anchored each electrode after implantation.

Fishes were anesthetized gradually in a saltwater solution of tricaine methanesulfonate (MS-222; $>1 \text{ g l}^{-1}$) and up to 10 color-coded electrodes were implanted percutaneously into the belly of target muscles. Since muscles were not visible externally, electrode placement was based on reference to dissections of preserved fishes and external landmarks. After all electrodes were implanted, the free ends of the electrode wires were glued together into a common cable. To allow fishes to swim without becoming entangled, this cable was secured with a loop of suture to the dorsal surface of the fish's head. EMG recording sessions did not begin until at least two to three hours following complete recovery from anesthesia. At the conclusion of every experiment, fishes were euthanized with an overdose of anesthesia, and the exact positions of electrode tips were confirmed by dissection.

During recording sessions, signals from implanted electrodes were amplified 10,000 times with Grass P511 preamplifiers and filtered with both a 60 Hz notch and 100 to 3,000 Hz bandpass filters. Electromyographic data were recorded along with a simultaneous voice description of fish behavior on high-grade VHS tapes using a TEAC XR-5000 analog recorder. Selected feeding sequences were replayed on a Western Graphtek Mark-11 thermal array recorder to produce hard copies of EMG data for visual reference.

To quantify motor patterns in this EMG data, analog recordings were digitized with a Keithley 500A system, using an effective sampling rate of 8 kHz, and a custom computer program [Updegraff, 1990] was used to measure three variables on individual cycles of prey processing (onset, duration, and integrated rectified area of muscle activity bursts). The absolute onset time of activity in the levator operculi was used as a reference to calculate the relative onset times of adductor mandibulae muscles in the same cycle of prey processing. From the original variables, the mean intensity (a measure of signal amplitude) of each cycle of muscular activity was calculated by dividing burst integrated rectified area by burst duration. Mean intensity values are voltage measurements which are greatly affected by variation in the recording properties of an electrode and the amplifier used during recording sessions [Gans and Gorniak, 1980; Loeb and Gans, 1986]. This variation was apparent when one compared mean intensities recorded from different electrodes in the same muscle. To remove

Fig. 2. Jaw musculature of representative tetraodontiform fishes. a Planehead filefish, *Monacanthus hispidus*, superficial view; A2 is subdivided into A2 α and A2 β , and these two muscles lie superficial to all A1 muscles except A1 α b' and A1 α b''. b *M. hispidus*, deep view with A1 α b', A1 α b'', A2 α , and A2 β removed. Note the three deep A1 muscles, A1 α b''', A1 β b'm, and A1 β b''m. c Gray triggerfish, *Balistes capriscus*, superficial view: A1 is subdivided into a superficial A1 α b and deep A1 β b (not shown). A2 is subdivided into A2 α , A2 β 'b, and A2 β 't. d Southern pufferfish, *Sphoeroides nephelus*, superficial view; A1 is subdivided into A1 α t and A1 β t. A1 β t lies superficial to some A2 muscles, and A2 is subdivided into A2 α , A2 β 't, and A2 β 't. Abbreviations: adductor arcus palatini (AAP), dentary (DEN), dilator operculi (DO), erectoris dorsalis (ERD), levator operculi (LO), maxilla (MX), palatine (PAL), premaxilla (PMX), protractor hyoidei (PHY), ramus mandibularis (RMD), and retractor arcus palatini (RAP).



this potential source of uninformative variation, intensity values were standardized for each electrode by expressing values of mean intensity as a percentage of the maximum mean intensity observed for a muscle across all prey types. This standardized variable – relative intensity – can be directly compared between any muscles, regardless if they are in the same or different individuals.

Statistical Analyses

To examine divergence between jaw muscles, we analyzed activity pattern variables (relative onset time, burst duration, and relative intensity) separately for each case of muscle duplication. The experimental design used in each instance was a three-way mixed model analysis of variance (ANOVA) with 'individual' as a random effect and both 'prey' and 'muscle' as fixed effects. In addition to the three main effects, this design also generated four interaction terms: individual-by-prey, individual-by-muscle, muscle-by-prey, and individual-by-muscle-by-prey. Variance in EMG variables was partitioned into all of these sources, but we focused on two factors as tests of the null hypothesis of conservation of muscle activity: the muscle main effect and the muscle-by-prey interaction term. The muscle main effect tested for differences in muscle activity that were consistent

across all prey types, while the muscle-by-prey interaction term tested for instances where the effect of prey type varied across muscles. Following Zar [1984], the F-ratio for the muscle effect was constructed with the Mean Squares for the individual-by-muscle effect in the denominator, and the F-ratio for the muscle-by-prey interaction was constructed with the error Mean Squares in the denominator. In this study, an average of 70 prey processing cycles/prey type/individual fish were analyzed (minimum = 51, maximum = 118) for a total of 4,182 cycles of muscle activity. All statistical procedures were run on untransformed data with both SuperAnova ver. 1.11 and Systat ver. 5.1 for the Macintosh.

Results

The results are reported in order: variation between individuals, variation between prey types, and variation between duplicated jaw muscles.

Table 1. Results of univariate ANOVAs on EMG variables of duplicated jaw adducting muscles in *Balistes* (triggerfish), *Monacanthus* (filefish), and *Sphoeroides* (pufferfish) during prey processing of live fiddler crabs, pieces of squid tentacle and live shrimps

EMG variable	Individual effect			Prey effect			Muscle effect			Muscle-by-prey effect		
	F	df	P	F	df	P	F	df	P	F	df	P
<i>Balistes</i> A1 α b vs. A1 β b												
Relative onset	9.03	3,908	<0.01*	14.09	2,6	<0.01*	24.62	1,3	0.02*	1.61	2,908	0.20
Duration	3.75	3,908	0.01*	3.39	2,6	0.10	47.93	1,3	<0.01*	1.45	2,908	0.24
Relative intensity	35.94	3,908	<0.01*	0.76	2,6	0.51	1.29	1,3	0.34	5.74	2,908	<0.01*
<i>Balistes</i> A2 β 'b vs. A2 β "b												
Relative onset	50.44	3,616	<0.01*	5.26	2,6	<0.05*	0.33	1,3	0.61	0.27	2,616	0.76
Duration	59.21	3,616	<0.01*	6.32	2,6	0.03*	0.23	1,3	0.66	0.10	2,616	0.90
Relative intensity	67.83	3,616	<0.01*	3.67	2,6	0.09	2.56	1,3	0.21	2.69	2,616	0.07
<i>Monacanthus</i> A1 α b' vs. A1 α b'' vs. A1 α b'''												
Relative onset	43.25	3,1413	<0.01*	1.17	2,6	0.37	1.52	2,6	0.29	0.14	4,1413	0.97
Duration	47.26	3,1413	<0.01*	13.64	2,6	<0.01*	0.97	2,6	0.43	0.81	4,1413	0.52
Relative intensity	68.80	3,1413	<0.01*	6.27	2,6	0.03*	0.39	2,6	0.69	19.56	4,1413	<0.01*
<i>Sphoeroides</i> A1 α t vs. A1 β t												
Relative onset	5.45	3,620	<0.01*	10.03	2,6	0.01*	0.32	1,3	0.61	0.14	2,620	0.87
Duration	9.37	3,620	<0.01*	0.70	2,6	0.53	<0.01	1,3	0.97	0.28	2,620	0.76
Relative intensity	2.79	3,620	0.04*	1.27	2,6	0.35	3.15	1,3	0.17	1.75	2,620	0.17
<i>Sphoeroides</i> A2 β 't vs. A2 β "t												
Relative onset	3.85	3,550	<0.01*	12.27	2,6	<0.01*	<0.01	1,3	0.96	0.07	2,550	0.94
Duration	19.36	3,550	<0.01*	0.31	2,6	0.74	0.73	1,3	0.45	0.16	2,550	0.85
Relative intensity	11.68	3,550	<0.01*	0.17	2,6	0.85	0.25	1,3	0.89	8.71	2,550	<0.01*

Onsets for jaw adducting muscles are relative to onset of the levator operculi. * Significant effects at ≤ 0.05 level.

Variation between Individuals

Significant individual effects were found on all 15 EMG variables (table 1). A significant individual effect indicated that at least one individual had a different mean value for an EMG parameter than other conspecific fishes. Such inter-individual variation could be due to both biotic differences between individuals as well as abiotic variation between experimental preparations and recording sessions [reviewed in Wainwright, 1989]. Individual effects were not considered evidence for functional divergence between duplicated muscles, because these effects were the same on all muscles in an analysis.

Variation between Prey Types

Prey effects, while of general interest in other studies of motor pattern modulation, were also not considered evidence for functional divergence between duplicated muscles. A prey effect meant that at least one prey type elicited a significantly different mean value for an EMG variable and that this effect was the same on all duplicated muscles in an analysis. For example, one prey type could consistently elicit earlier onsets of activity for all duplicated muscles compared. Significant prey effects were found for

seven of the 15 EMG variables (four relative onsets, two durations, and one relative intensity) and in all five sets of duplicated muscles (table 1).

In general, all jaw-adducting muscles had their earliest onsets for fiddler crabs, had similar durations for fiddler crabs and shrimp, and had their latest onsets and longest durations while processing pieces of squid tentacle (fig. 3, 4). Prey only affected the mean relative onset of duplicated A1 muscles of the triggerfish (66 ms for fiddler crab, 123 ms for squid tentacle, 82 ms for shrimp), while it affected both the relative onset (107 ms for fiddler crab, 172 ms for squid tentacle, 100 ms for shrimp) and duration (123 ms for fiddler crab, 199 ms for squid tentacle, 138 ms for shrimp) of duplicated A2 β muscles in the same species. For the duplicated A1 muscles in the filefish *Monacanthus*, prey affected mean duration (89 ms for fiddler crab, 116 ms for squid tentacle, 87 ms for shrimp) and mean relative intensity (0.40 for fiddler crab, 0.51 for squid tentacle, 0.38 for shrimp). In the pufferfish *Sphoeroides*, prey affected the onset of both duplicated A1 muscles (94 ms for fiddler crab, 180 ms for squid tentacle, 122 ms for shrimp) and duplicated A2 β muscles (98 ms for fiddler crab, 189 ms for squid tentacle, 127 ms for shrimp).

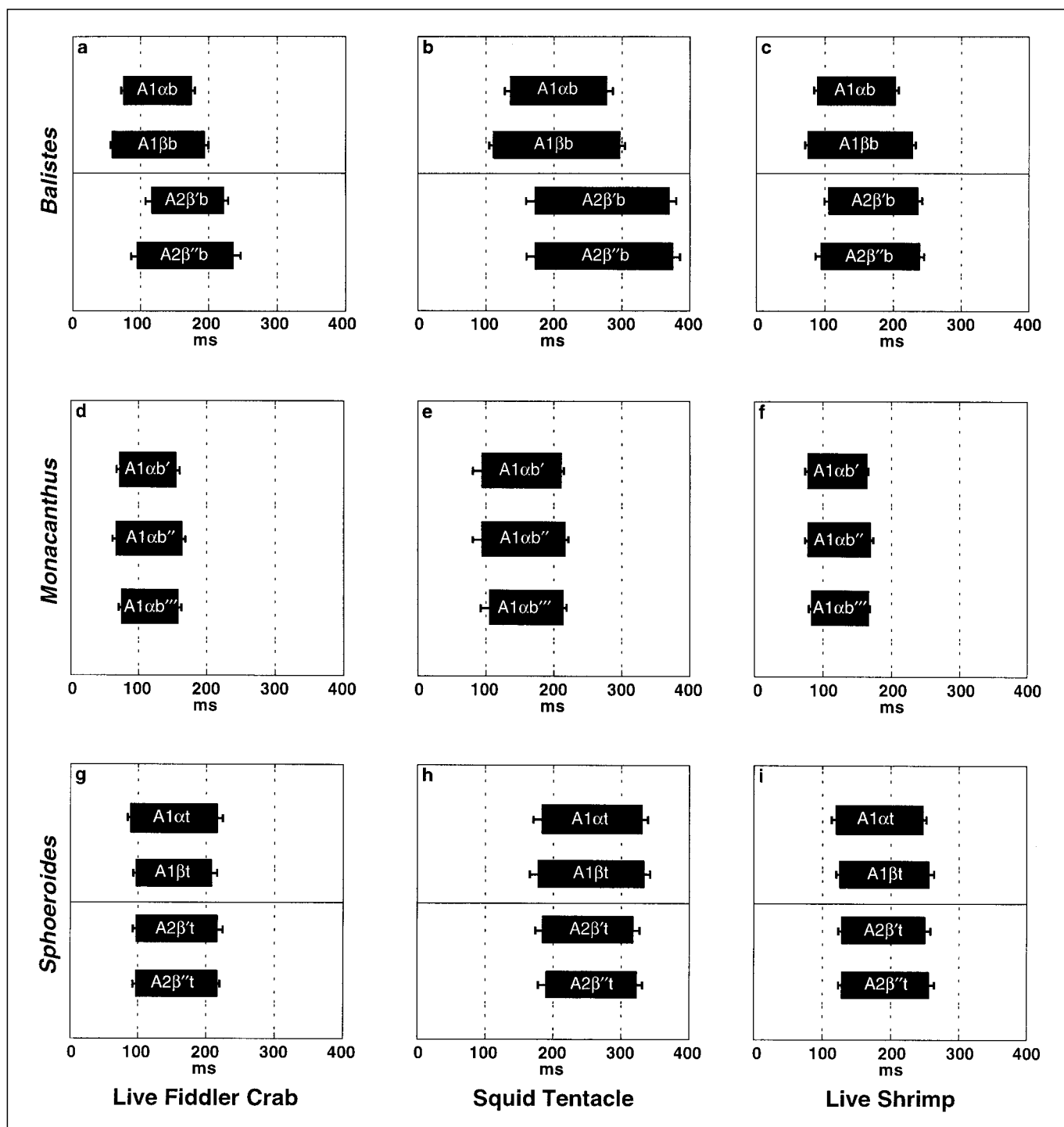


Fig. 3. EMG bar diagrams illustrating the average time course of activity in duplicated adductor mandibulae muscles in three taxa during prey processing of live fiddler crabs, pieces of squid tentacle, and live shrimps. Lengths of the labeled bars represent mean duration of activity, with the standard error indicated on the right; mean relative onset times are indicated by the distance of each bar from the y-axis, with standard error indicated on the left. These values are averages for four individuals of each species; see table 1 for quantitative analyses.

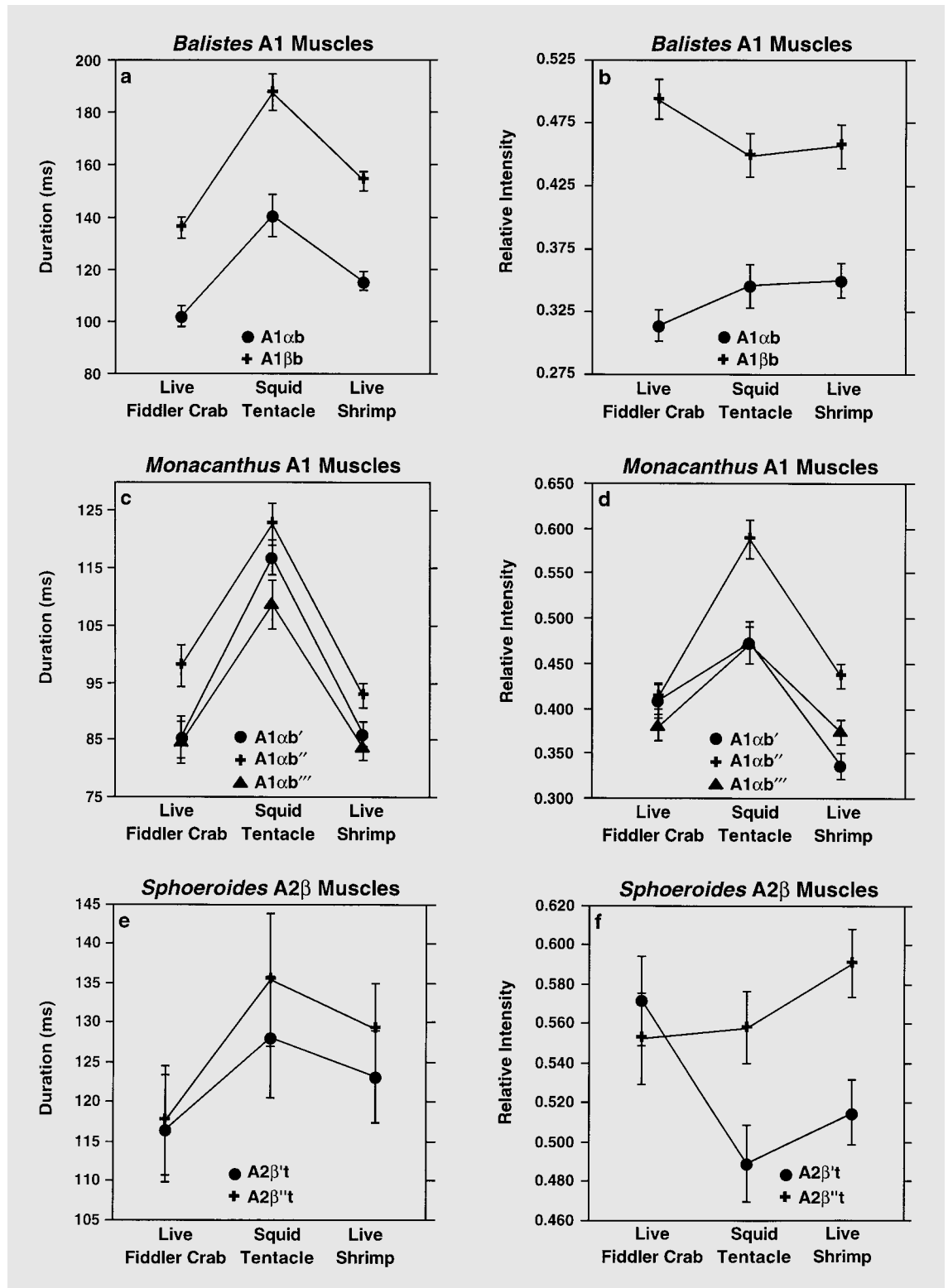


Fig. 4. Interaction plots illustrating the effect of prey on the durations and relative intensities of duplicated jaw muscles in the three study taxa during prey processing of live fiddler crab, pieces of squid tentacle, and live shrimp. Error bars represent one standard error. Note that simple prey effects are consistent across all muscles (e.g. panels a, c, and e), while muscle-by-prey interaction effects differ across muscles (e.g. panels b, d, and f).

Variation between Duplicated Jaw Muscles

Muscle effects and muscle-by-prey interaction effects were interpreted as evidence of functional divergence between duplicated muscles. A significant muscle effect indicated that there was a difference in the mean value of an EMG variable for at least one duplicated muscle in the set examined and that these differences were the same across all individuals and prey types. Thus, a muscle effect represented a straight forward kind of divergence in motor patterns that would have been detected even if only a single prey type had been used in our experiments. Significant muscle effects were found for two of the 15 EMG variables (table 1). In both instances, these muscle effects were on timing variables of the duplicated A1 muscles of the triggerfish (fig. 3a–c, 4a). Across all prey types, A1 α b had a later mean onset (95 ms vs. 76 ms) and shorter mean duration (116 ms vs. 155 ms) than A1 β b. No other significant muscle effects were found in the four other cases of duplicated muscles.

A second type of functional divergence was reflected by significant muscle-by-prey interactions. This kind of effect indicated that the effect of prey was not the same on all duplicated muscles in an analysis. In other words, differences in the motor patterns of duplicated jaw muscles may be expressed for only some prey types and not others. Significant muscle-by-prey interactions were found for three of the 15 EMG variables (table 1, fig. 4b, d, f) in three of the five cases of duplicated muscles studied here (triggerfish A1 muscles, filefish A1 α muscles, and pufferfish A2 β muscles) and also approached significance in one other case (triggerfish A2 β muscles). In each case, muscle-by-prey interactions were found on the relative intensity of muscular activity, thus indicating that a least one prey type elicits different motor intensities from the descendant muscles.

The nature of muscle-by-prey interactions is readily visualized graphically (fig. 4b, d, f). In the absence of any interaction between muscle and prey, the line segments connecting data points for each muscle would be approximately parallel, since the effect of prey would be the same on all muscles (fig. 4a, c, e). For instance, if the intensity of one muscle changes for a particular prey type, the intensity of the other duplicated muscles should also change in the same direction and to the same degree. An interaction between prey and muscle is indicated when the slopes of these line segments diverge significantly (fig. 4b, d, f). For example, an interaction was seen for the duplicated A1 muscles of triggerfishes (fig. 4b), where a single prey type is responsible for the muscle-by-prey effect. Here, both duplicated muscles respond similarly when the animal is feeding on squid tentacle and shrimp; when the animal is feeding on

fiddler crab, however, A1 β b increases in relative intensity while A1 α b decreases. The most complex interaction between prey and muscle was seen for the duplicated A1 muscles of filefishes (fig. 4d). Although the shrimp appeared to have the most dramatic effect, all three prey types affected these duplicated muscles differently. Thus no one prey type drives this interaction. Finally, another relatively simple interaction is seen in the duplicated A2 β muscles of pufferfishes (fig. 4f). This set of duplicated muscles responds similarly to both fiddler crabs and pieces of squid tentacle. Yet, when the animal is feeding on shrimp, A2 β 't increases in relative intensity while A2 β 't decreases slightly. The interaction emerges from the fact that shrimp influences the intensity of the two A2 β muscles differently.

Discussion

Statistical analyses of EMG parameters revealed significant variation in the motor patterns of duplicated muscles at several levels. Variation at some levels (i.e., individual and prey effects), while not directly related to the specific questions addressed here, can be compared to motor pattern variation reported in other studies of fish feeding behaviors. Furthermore, individual and prey effects must be accounted for in analyses in order to properly detect significant variation at other meaningful levels (i.e., muscle and muscle-by-prey effects) that is associated with functional divergence between duplicated muscles.

Individual Effects

Inter-individual variation was the largest source of variation in our data set, as evidenced by the fact that every EMG variable examined had a significant individual effect, and these effects had the highest F-ratios in all analyses (table 1). This finding is consistent with other studies of fish feeding behaviors where researchers have statistically analyzed multiple individuals of the same species [Shaffer and Lauder, 1985; Bemis and Lauder, 1986; Wainwright and Lauder, 1986; Sanderson, 1988]. Together these findings stress the necessity of replicate individuals in electromyographic studies. Interpretations based on the motor patterns of single individuals are seldom representative of entire species and can be misleading. In fact, if we reanalyze our EMG data individual by individual, we find muscle effects on most EMG variables rather than just the few reported here.

Prey Effects

Effects of prey type or position on EMG variables of various oral and pharyngeal jaw muscles have been doc-

umented in several studies of fish feeding behaviors [Elshoud-Oldenhove and Osse, 1976; Lauder, 1981, 1983a; Liem, 1978, 1979, 1980; Sanderson, 1988; Sibbing et al., 1986; Wainwright and Lauder, 1986; Wainwright, 1989; Wainwright and Turingan, 1993]. Such effects have been found on several EMG variables, including onsets, durations, and integrated rectified areas of muscle bursts. We note that one of these variables – integrated rectified area – is the product of duration and mean intensity. Thus, it is not surprising that in past studies significant effects on duration are typically followed by concomitant effects on integrated rectified area [Wainwright, 1989; Wainwright et al., 1989]. These earlier studies confounded timing and intensity variables, thus obfuscating whether muscular intensity can be modulated independent of activity duration. To address this issue, we separated duration from activity amplitude by dividing integrated area by burst duration to get an average burst amplitude, which we then used to calculate relative intensity. This enabled us to discover several instances where duration and intensity were modulated independently (e.g., prey effects on triggerfish A1 muscles, and muscle-by-prey effects on triggerfish A1 muscles, filefish A1 α muscles, and pufferfish A2 β muscles). This independence is clearly evident when duration and intensity plots for these cases of duplicated muscles are viewed side by side (fig. 4).

In general, fishes feeding on elusive live prey have earlier onsets of jaw muscle activity during prey capture or prey processing [Liem, 1980; Wainwright and Lauder, 1986; Sanderson, 1988; Ralston and Wainwright, 1997]. Earlier onsets of muscular activity should produce faster strikes. Similarly, earlier onsets during prey processing should minimize the chance of live prey escaping after being captured. Escape of prey may be a potential problem for tetraodontiform fishes which have relatively small mouths and seldom are able to engulf prey whole.

Other prey-effect patterns found here were less straight forward. For instance, durations of activity were almost twice as long for squid as compared to fiddler crab or shrimp (fig. 3, 4). Initially, this prey effect appears opposed to patterns found in previous studies. Wainwright and Turingan [1993] found that hard prey, such as live majid crabs, elicited significantly longer durations of activity in jaw adducting muscles of the queen triggerfish, *Balistes vetula*, than did soft prey like earthworms or pieces of squid mantle. Similarly, Ralston and Wainwright [1997] found that the legs of blue crabs elicited longer durations of jaw muscles in the southern pufferfish than did pieces of squid mantle. This paradoxical result is likely due to differences in the particular prey items used in each study. The cara-

pace of the fiddler crabs, while harder than that of the shrimps, is not as hard as the legs of blue crabs or the whole majid crabs used in these other studies. In addition, we used pieces of squid tentacles, which were much tougher to process into smaller pieces than the pieces of squid mantle used in these other studies. Thus, it appears that more effort was required to cut our squid prey than to crack the carapace of the armored fiddler crabs.

Muscle and Muscle-by-Prey Effects

Significant muscle or muscle-by-prey effects suggest that duplication of jaw adductor muscles by subdivision has given rise to functional divergence of the descendant muscles in three of five cases examined in this study of tetraodontiform fishes. Muscle function, as reflected by motor pattern, has evolved with the changes in muscle morphology. This pattern of divergence in motor pattern following muscle duplication may be viewed as a case of the more general phenomenon of increases in structural complexity giving rise to increases in functional complexity [Vermeij, 1973; Lauder and Liem, 1989; Lauder, 1990; Schaefer and Lauder, 1996]. This study suggests that muscle duplication has been one major historical mechanism for increasing the functional complexity of the tetraodontiform jaw system.

A second result we emphasize is that the majority of cases of divergence in muscle motor patterns involved instances where muscles differed in their response to the experimental prey, as reflected in the muscle-by-prey interaction terms from the ANOVAs. This interaction between muscle and prey has not, to our knowledge, been previously recognized as a significant route for divergence in muscle function.

Motor Pattern Differentiation of Adductor Mandibulae Subdivisions

Motor pattern variation between adductor mandibulae subdivisions has been reported by Ballintijn et al. [1972] in a cyprinid fish and by Smith [1982] in a varanid lizard. In these cases, differences were mainly qualitative and were not analyzed statistically to account for the effect of individuals on motor patterns. In an earlier study of another tetraodontiform fish, Wainwright and Turingan [1993] found timing differences between the A2 β subdivisions of the adductor mandibulae (A2 β 'b and A2 β "b) in several individuals of queen triggerfishes, *Balistes vetula*, during prey capture and water blowing behaviors.

Here, five out of 15 variables differed between duplicated jaw muscles. In three of the five sets of duplicated muscles studied there were either significant muscle effects

or muscle-by-prey interactions. We emphasize two aspects of these results. First, there was a difference in the types of EMG variables that showed overall muscle effects and muscle-by-prey interactions. The only significant overall divergence between descendant muscles was seen in timing variables (the duplicated A1 muscles of triggerfishes). In contrast, muscle-by-prey interactions were found only on relative intensity variables (triggerfish A1, filefish A1 α , and puffer A2 β muscles). Second, these significant interactions indicate a type of prey effect that is more subtle than the one described by the muscle effect, and that has generally not been explored in previous research. By showing that a particular effect of prey differs in homologous muscles, these results indicate that morphological subdivision has been followed by a functional divergence in which one of the descendant muscles has adopted a response to prey types that is not seen in homologs. In other words, duplicated muscles have diverged in how they modulate their intensities to prey type.

How do the levels of divergence observed among muscles in this study compare to levels of divergence that have previously been reported in comparisons among homologous muscles? After all, changes in muscle activity patterns did not occur in the majority of variables examined (only five of 15 variables showed divergence between muscles), and those changes that were found were not extreme in nature. We suggest that the frequency of divergence among homologous muscles found in this study was, in fact, considerable. Several previous studies have made quantitative comparisons of homologous muscles in different fish species [Wainwright and Lauder, 1986; Sanderson, 1988; Wainwright, 1989; Westneat and Wainwright, 1989]. In this body of work a total 42 electromyographic variables from 19 muscles were tested for species effects and species-by-prey interactions. Five of the 19 muscles exhibited muscle activity that differed significantly among species (i.e. five muscles indicated divergence of homologous muscles in different species by significant species effects or species-by-prey interactions). Hence, muscle activity patterns associated with various feeding behaviors in fishes tend to show a pattern of evolutionary conservation. This pattern of conserved motor patterns in homologous muscles across species contrasts with the results presented here, where three of five sets of muscles show evidence of divergence in comparisons of homologous muscles within the same species. Our point is that previous studies suggest that motor patterns are quite conservative in the evolution of fish feeding mechanisms, even in the face of substantial morphological changes [e.g., Sanderson, 1988; Westneat and Wainwright, 1989], and that the frequency of diver-

gence among homologous muscle pairs observed in the present study actually exceeds that typically observed in previous studies.

A number of features of tetraodontiform jaws may also lead one to expect that functional diversity of the adductor muscles of this group is likely to be modest relative to the divergence of homologous muscles in different species. First, comparisons of homologous muscles in different species generally capture interspecific morphological variation that may promote the divergence of muscle function, while the adductors studied here were in the same animals, acting on the same set of jaws. Second, the jaws of tetraodontiform fishes are relatively constrained in the range of potential movements they can make, because, in all but the most basal groups, these fishes possess fused maxillae and premaxillae, and the left and right side halves of the jaws show no kinesis. Further, upper jaw protrusion is lost in most tetraodontiforms [Tyler, 1980; Winterbottom, 1974b].

The pattern that one sees in tetraodontiform fishes is, therefore, one in which jaw mobility is reduced, while adductor muscles have diversified anatomically and to some extent functionally (as indicated by motor patterns). Tetraodontiform fishes typically feed by applying their jaws to the substrate to remove attached prey or by directly biting their prey. The reduction in jaw mobility results in a more robust, mechanically stable biting apparatus, while the duplication of adductor muscles may increase the fine motor control of the jaws. The significant interactions between muscle and prey found in our study, where homologous muscles differed in their response to some prey but not others, suggest examples of subtle, fine control of the jaws.

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