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EVOLUTION OF COMPLEXITY IN MOTOR PATTERNS AND JAW MUSCULATURE OF TETRAODONTIFORM FISHES

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Summary

The prey-processing behavior and jaw-adducting musculature of tetraodontiform fishes provide a novel system for studying the evolution of muscles and their function. The history of this clade has involved a pattern of repeated 'duplications' of jaw muscles by physical subdivision of pre-existing muscles. As a result, the number of adductor mandibulae muscles in different taxa varies from as few as two to as many as eight. We used electromyography (EMG) to quantify motor-pattern variation of adductor mandibulae muscles in four tetraodontiform species during feeding events on prey items that varied in durability and elusiveness. Statistical analyses of variation in EMG variables revealed significant differences in motor patterns between duplicated muscles derived from a common ancestral muscle in seven of nine cases examined. Overall individual EMG timing variables (e.g. relative onset or duration of bursts) were slightly less likely to diverge functionally than amplitude variables (e.g. relative intensity of bursts). Functional divergence was found in significant overall differences between muscles and twice as frequently in significant muscle-by-prey interaction terms. Such interactions represent an underappreciated way in which motor patterns can evolve and diversify. Regional variation was documented in undivided muscles in two species, indicating that it is possible for functional subdivision to precede anatomical subdivision. This study shows that phylogenetic increases in the number of tetraodontiform jaw adductor muscles have been associated with increases in the functional complexity of the jaws at the level of muscle activation patterns.

Key words: adductor mandibulae, evolution, feeding, motor pattern, muscle-by-prey interaction, Tetraodontiformes.

Introduction

A developing paradigm in the evolution of feeding systems of fishes posits that, while morphology and diets may diverge radically through evolution, the underlying neuromuscular control of prey capture and processing behaviors does not diverge to a similar degree. This idea is based upon repeated observations that the average motor patterns for a feeding behavior in different species are not significantly different from one another (Lauder, 1983a; Liem, 1978, 1979, 1980; Sanderson, 1988; Wainwright and Lauder, 1986; Wainwright, 1989; Westneat and Wainwright, 1989; Ralston and Wainwright, 1997). Overall, this abundance of evidence has led to the general perception that neuromuscular activity patterns are typically a conservative component of the feeding mechanism in anamniote vertebrates (Wainwright et al., 1989; Smith, 1994).

In spite of this trend for their historical conservation of average motor pattern, the muscle activation patterns of feeding fish have been shown to be remarkably flexible, as demonstrated by an apparently universal tendency for prey type to influence aspects of these patterns. Prey type has been shown to influence the patterns of muscular activity associated with prey capture by suction feeding (Liem, 1979; Wainwright and Lauder, 1986; Sanderson, 1988), prey capture by biting (Wainwright and Turingan, 1993; Ralston and Wainwright, 1997), prey processing by the pharyngeal jaws (Lauder, 1983a; Wainwright, 1989) and prey processing by the oral jaws (Wainwright and Turingan, 1993; Friel and Wainwright, 1998). In most cases, prey effects are similar across species examined and among synergistic muscles within an individual. For instance, highly mobile prey such as live fishes typically elicit earlier onsets of muscular activity, whereas hard armored prey such as snails elicit longer durations of muscular activity.

The idea of evolutionarily conservative muscle activation patterns may seem incompatible with the similarly widespread finding that motor patterns can be flexible, as indicated by the ability of fishes to modulate muscle activity in response to prey type. There is an essential distinction between these observations. When fishes of different species are fed the same prey, they often express muscle activation patterns that are

similar or statistically difficult to distinguish. Different species tend to utilize similar motor patterns when feeding on a common prey. Nevertheless, nearly all species appear to be able to alter muscle activity patterns in response to prey. Thus, across species undertaking a common activity, there is a tendency for motor patterns to be conserved, but within species motor patterns can be variable and altered in response to prey type.

In the light of this tendency for historical conservation of neuromuscular activity patterns that can readily be modulated by an individual fish in response to prey, we have been studying the evolution of muscle function in teleost fishes of the order Tetraodontiformes (Fig. 1). Our focus has been the A1 and A2 adductor mandibulae muscles that generate the biting forces applied by the jaws during prey processing. Repeatedly within this clade, singular A1 and A2 muscles have been effectively 'duplicated' by physical subdivision of pre-existing muscles to produce new descendant muscles (Friel and Wainwright, 1997, 1998). Such muscle duplication events have occurred on at least 10 separate occasions within this clade, and species vary in possessing 2–8 separate muscles from the complex of A1 and A2 muscles. We are interested in the functional consequences of this phylogenetic increase in structural complexity and its role in the evolution of feeding behaviors in this clade of fishes characterized by using their oral jaws both to capture and to process prey.

In this study of the prey-processing behavior of tetraodontiform fishes, we focused on four representative species and tested for modifications of the neuromuscular activity patterns in descendant muscles produced by six independent muscle-duplication events. Given the tendency for motor patterns to be conserved during the evolution of fish feeding behaviors, we expected few changes in activity patterns of new muscles produced by duplication events. We analyze activity patterns of muscle as one important aspect of their function, and interpret changes in activity pattern as changes in function. In addition to comparisons among descendant muscles, we also compare activation patterns in two different regions of undivided muscles in two species. We address four principal questions. (1) Are the evolutionary increases in the number of jaw muscles in tetraodontiform fishes associated with an increase in 'functional complexity', measured in this context as divergence in the activation patterns of descendant muscles? (2) Are different variables of muscle activation patterns (i.e. burst duration, burst onset and burst amplitude) evolving at similar rates and in similar ways? (3) Can regional variation in motor pattern within an undivided muscle occur prior to anatomical muscle subdivision? (4) What is the effect of prey type on feeding motor patterns in tetraodontiform fishes and how does the capacity of these fishes to modify muscle activity patterns during prey-processing behavior compare with previously reported patterns from other teleost fishes for suction feeding and pharyngeal processing.

Materials and methods

Experimental animals

Four representative species of the cosmopolitan order Tetraodontiformes were used in this electromyographic study: *Balistes capriscus*, the gray triggerfish (*N*=4, SL=240–270 mm, where SL is standard length); *Monacanthus hispidus*, the planehead filefish (*N*=4, SL=117–136 mm); *Sphoeroides nephelus*, the southern pufferfish (*N*=4, SL=110–160 mm); and *Chilomycterus schoepfi*, the striped burrfish (*N*=3, SL=110–170 mm). These species belong to four of the nine extant families of tetraodontiform fishes (Balistidae, Monacanthidae, Tetraodontidae and Diodontidae; Fig. 1) and possess jaw muscles produced by several muscle duplication events (Fig. 2). All specimens were collected in the northern Gulf of Mexico near the Florida State University Marine Laboratory,

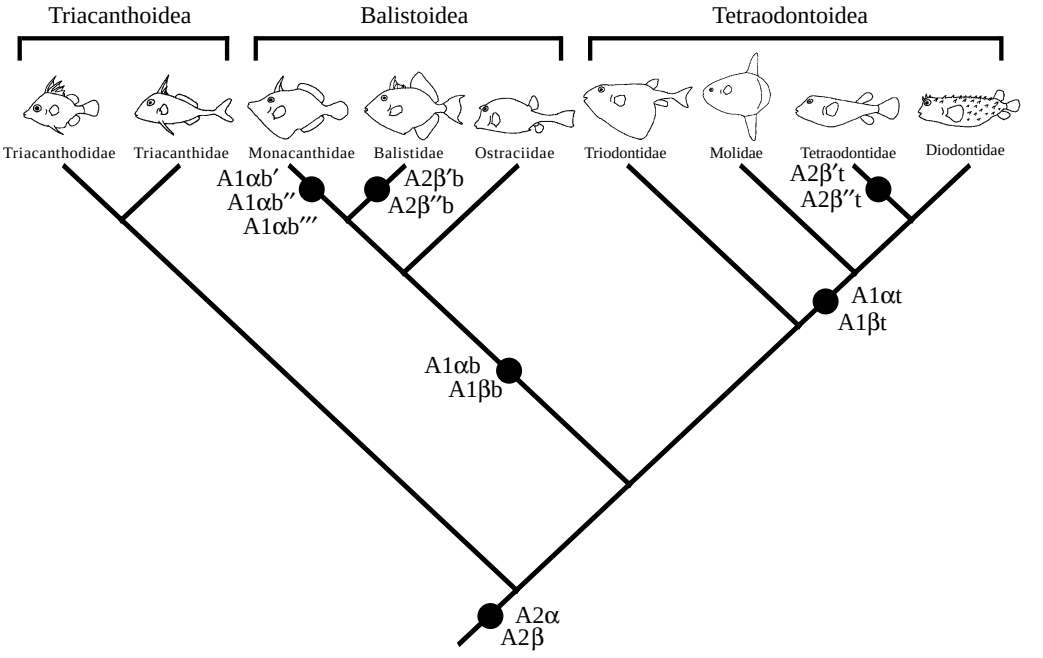

Fig. 1. Phylogeny of extant families of tetraodontiform fishes based on the research of Winterbottom (1974b), Matsuura (1979), Tyler (1980), Lauder and Liem (1983), Winterbottom and Tyler (1983) and Tyler and Sorbini (1996). Images represent the general body form of fishes in each family. Filled circles mark the six muscle duplication events examined in the present study. The names of new jaw muscles created by these events are listed next to the filled circles. See Friel and Wainwright (1997) for additional details.

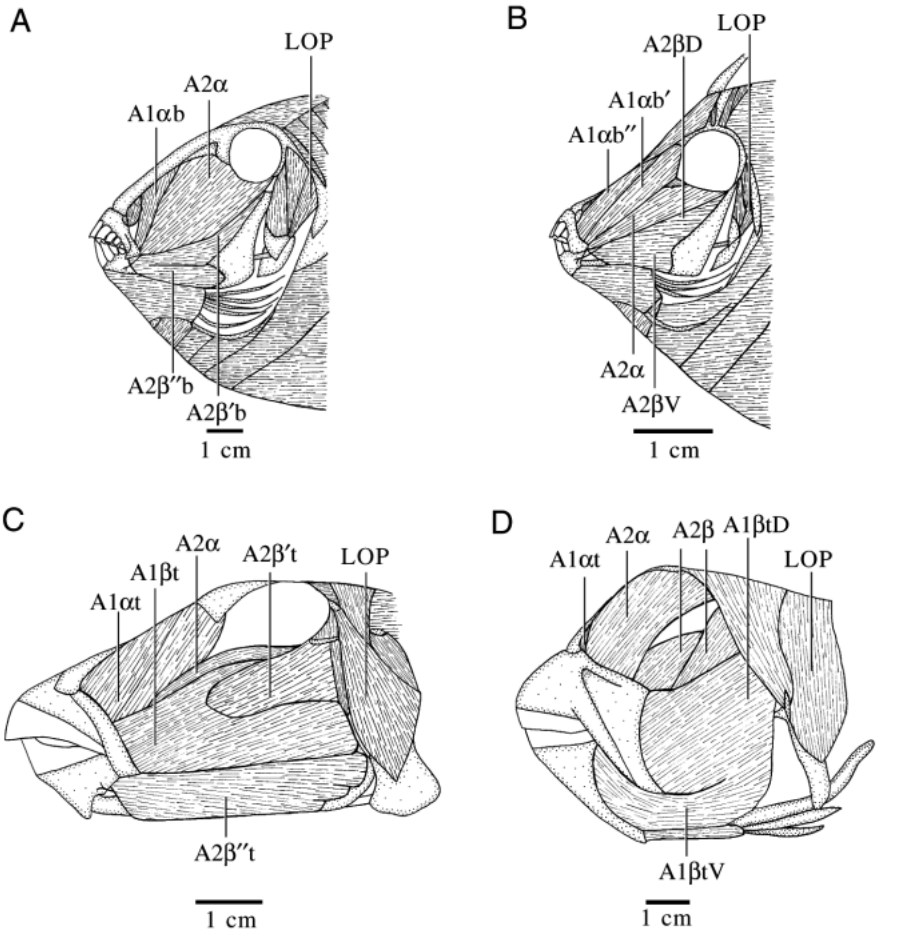

Fig. 2. Superficial adductor mandibulae muscles and levator operculi (LOP) of representative tetraodontiform fishes. (A) Gray triggerfish *Balistes capriscus*. A1 is subdivided into two muscles, A1αb and A1βb. A1βb lies deep to A1αb and is not shown. A2 is subdivided into three muscles, A2α, A2β′b and A2β″b. (B) Planehead filefish *Monacanthus hispidus*. A1 is subdivided into five muscles, A1αb′, A1αb″ A1αb‴ A1βb′m and A1βb″m. A1αb‴, A1βb′m and A1βb″m lie deep to the muscles and are not shown. A2 is subdivided into two muscles, A2α and A2β. Although A2β is undivided, the dorsal (A2βD) and ventral regions (A2βV) of this muscle are labeled separately. (C) Southern pufferfish *Sphoeroides nephelus*. A1 is subdivided into A1αt and A1βt. A2 is subdivided into three muscles, A2α, A2β′t and A2β″t. (D) Striped burrfish *Chilomycterus schoepfi*. A1 is subdivided into two muscles, a minute A1αt and a large A1βt, shown here with dorsal (A1βtD) and ventral (A1βtV) regions labeled separately. A2 is subdivided into two muscles, A2α and A2β.

Turkey Point, Florida, USA. Fishes were maintained in 100 l aquaria at 24±2 °C and fed a mixed diet of squid, shrimp and fiddler crabs for at least 1 week prior to electromyographic recording sessions. This research conforms to the guidelines of the Animal Care and Use Committee of Florida State University.

Myology

The activity patterns of selected sets of A1 and A2 adductor mandibulae muscles were quantified in multiple individuals of each species. As in other teleost fishes, A1 muscles insert on the upper jaw (or secondarily on the palatine bone as does the A1αβ‴ muscle of filefishes), whereas A2 muscles insert on the lower jaw (or secondarily on the upper jaw as does the A2α muscle of pufferfishes in the genus *Sphoeroides*). Details of the jaw-adducting musculature of tetraodontiforms have recently been reviewed and are briefly summarized here (Friel and Wainwright, 1997; and see Winterbottom, 1974a,b). Multiple times within this clade, singular A1 and A2 adductor mandibulae muscles have been effectively duplicated by physical subdivision to form new muscles. The complex history of these subdivision events is reflected in the names applied to the jaw musculature of tetraodontiform fishes (Figs 1, 2). The prefix of a muscle name reflects the hierarchical relationship of subdivided muscles to each other. For example, suppose a fish possessed four A1 muscles identified as A1α′, A1α″, A1β′ and A1β″. This would imply that these muscles were produced by subdivision of the common ancestral muscle (A1) into two descendant muscles (A1α and A1β) and that each of these muscles was subsequently further subdivided into two descendant muscles (e.g. A1α′ and A1α″).

All the jaw muscles examined in this study were produced by six separate subdivision events (Fig. 1). A1α and A1β muscles have arisen independently from a common singular A1 once in the Balistoidea (Ostraciidae, Balistidae and Monacanthidae; designated with the suffix ‘b’) and again in a subclade of the Tetraodontoidea (Molidae, Tetraodontidae and Diodontidae; designated with the suffix ‘t’). The A1αb muscle of filefishes (Monacanthidae) has been further subdivided once into three muscles: A1αb′, A1αb″ and A1αb‴. A2α and A2β muscles arose in the common ancestor of all tetraodontiform fishes from a singular A2 muscle. Finally, nonhomologous muscles A2β′ and A2β″ have arisen independently once in triggerfishes (Balistidae; designated with the suffix ‘b’) and again in one family of pufferfishes (Tetraodontidae; designated with the suffix ‘t’) from a common A2β.

This complex musculature makes interspecific comparisons of activity patterns such as those in most other studies difficult because individual muscles may not have exact homologs in other species. However, the system provides a unique opportunity to study motor pattern evolution using intraspecific comparisons

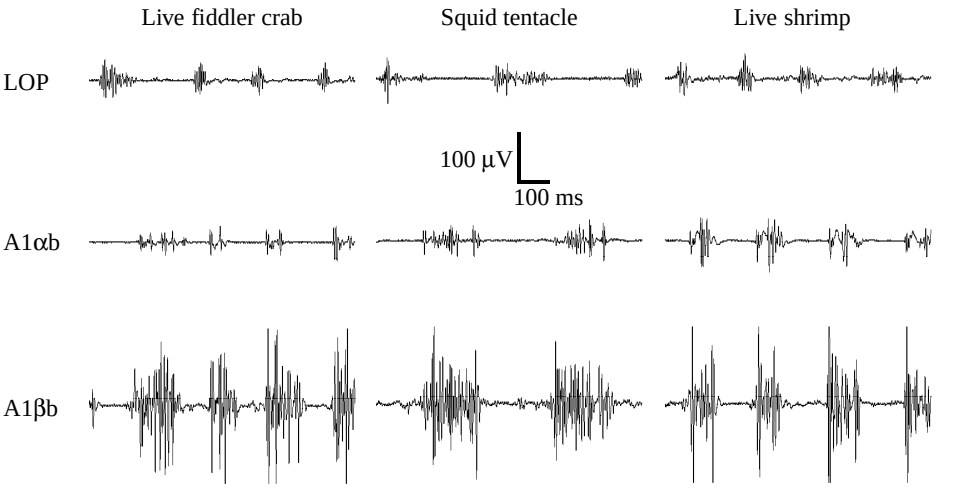

Fig. 3. Representative electromyograms from an individual gray triggerfish (*Balistes capriscus*) during prey processing of a live fiddler crab, a piece of squid tentacle and a live shrimp. Cycles of muscular activity are shown for the levator operculi (LOP) and two jaw-adducting muscles, A1αb and A1βb.

of descendant muscles. If motor patterns are highly conserved in fish feeding behaviors, we should find no significant differences between muscles derived from the same ancestral muscle. Any significant differences would be interpreted as evidence of divergence from the plesiomorphic motor pattern of the ancestral muscle. Furthermore, since this is a repeated phenomenon, we can examine sets of muscles produced by different subdivision events for recurring trends in motor pattern evolution associated with these gross morphological changes.

In each species, we compared the motor patterns of descendant muscles derived from one or more subdivision events from a common A1 or A2 muscle. In total, the four tetraodontiform species possess A1 and A2 muscles that illustrate nine cases of descendant muscles produced by the six historical muscle subdivision events included in this study (Fig. 1). Most of the historical subdivision events are represented by a single species in this study, but the splitting of A2 into A2α and A2β is represented by each of the four study species. Examining functional divergence between A2α and A2β in all four species allows some insight into how conserved any divergence in motor patterns is across this order of fishes. Since A2α was the only adductor muscle present in all four species, this muscle was examined for interspecific patterns of functional divergence.

We also compared different regions of large undivided muscles in two species to determine whether evolution of motor patterns can occur prior to physical subdivision. Specifically, in burrfishes, the dorsal and ventral regions of the large A1βt muscle (A1βtD and A1βtV) were examined. In filefishes, the dorsal and ventral regions of the A2β muscle (A2βD and A2βV) were compared. These regions of A2β in filefishes (Fig. 2B) occupy the same positions in the suspensorial fossa and physically resemble the separate A2β′b and A2β″b muscles studied here in their sister group, the triggerfishes (Fig. 2A). We hypothesize that the two regions of the filefish A2β are homologous with the A2β′b and A2β″b muscles of triggerfishes (see also Friel and Wainwright, 1997).

Electromyographic recordings were taken from the levator operculi muscle (LOP) and multiple A1 and A2 muscles in each species. The LOP muscle is the primary jaw depressor muscle in tetraodontiform fishes (Turingan and Wainwright, 1993) and was consistently active during jaw opening before any activity in the A1 and A2 muscles (Fig. 3). The LOP has been used as a standard reference muscle in similar EMG studies. The specific A1 muscles studied included: the A1αb and A1βb muscles of triggerfishes (Fig. 2A); the A1αb′, A1αb″ and A1αb‴ muscles of filefishes (Fig. 2B); the A1αt and A1βt muscles of pufferfishes (Fig. 2C); and the dorsal and ventral regions of the A1βt muscle of burrfishes (Fig. 2D). The specific A2 muscles studied included: the A2α, A2β′b and A2β″b muscles of triggerfishes (Fig. 2A); the A2α and the dorsal and ventral portions of the A2β muscle in filefishes (Fig. 2B); the A2α, A2β′t and A2β″t muscles of pufferfishes (Fig. 2C); and the A2α and A2β of burrfishes (Fig. 2D).

Feeding behavior

Most teleost fishes capture prey whole using either ram or suction feeding mechanisms (Norton and Brainerd, 1993; Lauder, 1985). During these feeding behaviors, the oral jaws are used to capture prey and reposition it within the buccal cavity if necessary before it can pass intact into the pharynx (i.e. buccal manipulation; Lauder, 1983b). Any subsequent processing of prey is typically performed with the pharyngeal jaws. In contrast to this generalized pattern, tetraodontiform fishes use their oral jaws for both prey capture and processing (Turingan and Wainwright, 1993; Wainwright and Turingan, 1993; Friel and Wainwright, 1998). Following capture by suction or direct grasping, prey are repeatedly bitten and reduced by the fish's powerful jaws before being transported into the pharynx. This distinctive prey-processing behavior is the focus of the present study as it was utilized by all species while feeding on all prey types. Unlike prey capture, which usually consists of a single cycle of muscular activity, a single bout of prey processing may contain up to 30 or more individual cycles of jaw opening and closing.

To investigate the effects of prey on the muscle activity patterns that drive prey processing, we selected three items to represent a spectrum of food types found in the natural diets of these fishes. Live fiddler crabs (*Uca* sp., length 20–40 mm) served as a mobile prey with a hard, brittle exoskeleton. Fishes had little difficulty capturing crabs and biting through their protective

armor. Live shrimps (*Paeneus* sp., length 40–100 mm) were used as an elusive prey that possessed a relatively weak exoskeleton. Fishes often required several attempts to capture shrimp and, even when captured, shrimp often escaped repeatedly during processing. For a completely non-elusive and unarmored prey, we used cut pieces of squid tentacle (*Loligo* sp., length 20–40 mm). While this prey lacked an exoskeleton, the firm muscular tissue was the toughest of the three experimental prey items, and considerable effort was required to reduce it into pieces small enough for the fishes to swallow. Decapod crabs and shrimp are common in the diets of these and other closely related tetraodontiform fishes (Randall, 1967; Frazer 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 h to ensure that the fishes would feed well during experimental recordings.

Experimental techniques

Electromyographic recordings 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 stripped of insulation and bent back against the shaft of the needle. This configuration formed a double hook that anchored each electrode after implantation.

Fishes were anesthetized in a saltwater solution of tricaine methanesulfonate (MS-222; $>1\,g\,l^{-1}$), and up to 10 color-coded electrodes were implanted pericutaneously 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. Once all electrodes had been implanted, the free ends of the 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 2–3 h following complete recovery from anesthesia. At the conclusion of each recording session, fishes were killed with an overdose of anesthesia, and the precise positions of the electrode tips were confirmed by dissection.

During recording sessions, voltage signals from implanted electrodes were amplified 10 000 times with Grass P511 preamplifiers and filtered with both 60 Hz notch and 100–3000 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 Graphtek Mark-11 thermal array recorder to produce hard copies of EMG data for visual reference (Fig. 3).

To quantify motor patterns, analog EMG recordings were digitized with a Keithley 500A system using a sampling rate of 8 kHz, and a custom-designed computer program was used to measure three variables during individual cycles of prey processing (activity onset, burst duration and integrated rectified area of muscle activity). The absolute onset of activity in the levator operculi was used as a reference to calculate the relative onset times of A1 and A2 muscles in the same cycle of prey processing. From the original variables, the average amplitude of each cycle of muscular activity was calculated by dividing the integrated rectified area of a burst by the burst duration. Amplitude values, unlike timing values, are voltage measurements that are influenced by several sources of variation including the recording properties of individual electrodes, the number of motor units firing in the vicinity of the electrode tips and the individual amplifiers used during recording sessions (Gans and Gorniak, 1980; Loeb and Gans, 1986). This variation was apparent when we compared amplitudes recorded from different electrodes in the same region of a muscle. To normalize different electrodes to a common voltage scale, 'raw' amplitude values were standardized for each electrode by expressing them as a percentage of the maximum amplitude recorded from that electrode. We call this standardized variable relative intensity. By standardizing this amplitude variable, it can be compared directly between muscles irrespective of whether they are in the same or different individuals. We note that EMG burst duration and amplitude variables are typically strongly predictive of variation in force production among bouts of activity within a single muscle (Lawrence and De Luca, 1983; Basmajian and De Luca, 1985; Wainwright and Turingan, 1996).

Statistical analyses

We used separate analyses to explore intraspecific and interspecific variation in muscle function. Our primary analysis was aimed at testing within each species for motor pattern differences between descendant muscles produced by subdivision events. We began each of these analyses with a three-way mixed-model analysis of variance (ANOVA) that included all the A1 or A2 muscles examined in that species. In the two comparisons of muscle regions, the A1βt muscle of burrfish and the A2β muscle of filefish, we treated the regions statistically as though they were different muscles. In cases where there was a single subdivision event represented by the species (e.g. the A1 muscles of triggerfish), we used this overall ANOVA to test for muscle divergence. In these ANOVAs, we treated both 'muscle' and 'prey' as fixed effects and 'individual' as a random effect. In addition to these 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 these sources, but we focus here on just three effects (prey, muscle and muscle-by-prey). A prey effect meant that there was a significant effect of one or more prey items on a variable and that this effect was the same on all muscles or muscle regions. A muscle effect meant that there were fixed differences in a variable between muscles or muscle regions and that these difference were the same across all prey types. We also examined muscle-by-prey interactions. This kind of interaction meant that the effect of one or more prey types was not the same on all muscles or muscle regions.

Following Zar (1984), the *F*-ratio for the prey effect was constructed with the mean squares for the individual-by-prey effect in the denominator, 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 cases where two subdivision events were represented by the muscles from a single species (i.e. the three A2 muscles of triggerfish and pufferfish), we followed the overall ANOVA with two *post-hoc* contrasts, first comparing activity in the most recently evolved pair of muscles (e.g. the two A2β muscles of triggerfish) and then comparing activity in the A2α muscle with that in the A2β muscles together. The same design was also used for filefish A2 muscles, which in fact represent one real subdivision event and a second presumptive subdivision event (i.e. splitting of the dorsal and ventral regions of A2β). In these *post-hoc* contrasts, the *F*-ratios were constructed using the same denominator mean squares as in the overall ANOVA. Thus, all the A1 analyses and the burrfish A2 analysis were performed using the overall ANOVA, while the A2 muscles of triggerfish, filefish and pufferfish were analyzed using the sequential *post-hoc* contrasts following the overall ANOVAs.

To test for changes in activity of the A2α muscle between species, we analyzed the activity of this muscle using a two-way ANOVA with a nested level. Here 'individual' (a random factor) was nested within 'species' (a fixed factor), and these factors were both crossed with 'prey' (also a fixed factor). Our focus here was on the species and prey main effects and the interaction term between species and prey. We constructed *F*-ratios to test these effects following Zar (1984). The prey type mean squares were tested over the individual by prey mean squares, the species mean squares were tested over the individual mean squares, and the species-by-prey interaction was tested over the individual-by-prey mean squares. In this study, an average of 79 prey-processing cycles per prey type per individual fish were analyzed (minimum 31, maximum 132) for a total of 6837 cycles of muscle activity. All ANOVA calculations were run in SuperAnova version 1.11 for Mac OS, and other statistical calculations were performed using Systat version 5.1 for Mac OS.

Results

The ANOVA results for the nine cases of descendant muscles produced by subdivision and the two cases of regional variation are reported in Tables 1 and 2. Although not shown, significant individual effects were found for all EMG variables examined. This high level of inter-individual variation in motor patterns is consistent with the results of other published studies that have accounted for variance at this level (Wainwright and Lauder, 1986; Sanderson, 1988; Reilly and Lauder, 1989; Wainwright, 1989; Wainwright and Turingan, 1993; Ralston and Wainwright, 1997; Friel and Wainwright, 1998). The muscle, prey and

Table 1. *Summary of prey, muscle and muscle-by-prey effects on electromyographic variables of duplicated A1 adductor mandibulae muscles in four triggerfishes, four filefishes, four pufferfishes and three burrfishes*

	Prey effect			Muscle effect			Muscle-by-prey effect		
Comparison and EMG variable	*F*-ratio	d.f.	*P*	*F*-ratio	d.f.	*P*	*F*-ratio	d.f.	*P*
Triggerfish A1 muscles (A1αb *versus* A1βb)									
Relative onset	14.11	2,6	<0.01*	24.46	1,3	0.02*	1.61	2,908	0.20
Duration	3.39	2,6	0.10	47.85	1,3	<0.01*	1.46	2,908	0.23
Relative intensity	0.76	2,6	0.51	1.29	1,3	0.34	5.74	2,908	<0.01*
Filefish A1α muscles (A1αb′ *versus* A1αb″ *versus* A1αb‴)									
Relative onset	1.16	2,6	0.37	1.52	2,6	0.29	0.14	4,1413	0.97
Duration	13.66	2,6	<0.01*	0.97	2,6	0.43	0.81	4,1413	0.52
Relative intensity	6.27	2,6	0.03*	0.39	2,6	0.69	19.55	4,1413	<0.01*
Pufferfish A1 muscles (A1αt *versus* A1βt)									
Relative onset	10.03	2,6	0.01*	0.33	1,3	0.61	0.14	2,620	0.87
Duration	0.70	2,6	0.53	<0.01	1,3	0.97	0.28	2,620	0.76
Relative intensity	1.27	2,6	0.35	3.15	1,3	0.17	1.76	2,620	0.17
Burrfish A1 muscle (A1βtD dorsal *versus* A1βtV)									
Relative onset	2.11	2,4	0.24	2.90	1,2	0.23	0.03	2,430	0.97
Duration	1.18	2,4	0.39	1.89	1,2	0.30	0.67	2,430	0.51
Relative intensity	0.32	2,4	0.74	65.27	1,2	0.02*	3.93	2,430	0.02*

Effects are based on univariate ANOVAs of EMG data recorded during prey processing of live fiddler crabs, pieces of squid tentacle and live shrimps.

Onsets for muscle activity are relative to the activity of the levator operculi.

See Friel and Wainwright (1998) for related discussions.

*Significant effects at $P \leq 0.05$.

Table 2. *Summary of prey, muscle and muscle-by-prey effects on electromyographic variables of duplicated A2 adductor mandibulae muscles in four triggerfishes, four filefishes, four pufferfishes and three burrfishes*

	Prey effect			Muscle effect			Muscle-by-prey effect		
Comparison and EMG variable	*F*-ratio	d.f.	*P*	*F*-ratio	d.f.	*P*	*F*-ratio	d.f.	*P*
Triggerfish A2β muscles (A2β′b *versus* A2β″b)									
Relative onset	10.82	1,6	0.02*	0.42	1,6	0.54	4.96	1,924	0.03*
Duration	6.09	1,6	0.05*	0.37	1,6	0.56	20.80	1,924	<0.01*
Relative intensity	0.89	1,6	0.38	7.61	1,6	0.03*	45.31	1,924	<0.01*
Triggerfish A2 muscles (A2α *versus* A2β′b+A2β″b)									
Relative onset	3.47	1,6	0.11	2.49	1,6	0.17	29.69	1,924	<0.01*
Duration	4.23	1,6	0.09	0.49	1,6	0.51	27.59	1,924	<0.01*
Relative intensity	2.37	1,6	0.17	0.18	1,6	0.69	1.08	1,924	0.29
Filefish A2 muscles (A2βD *versus* A2βV)									
Relative onset	0.03	1,6	0.88	1.19	1,6	0.32	1.46	1,1428	0.23
Duration	1.91	1,6	0.22	0.01	1,6	0.94	0.08	1,1428	0.78
Relative intensity	6.36	1,6	0.05*	0.34	1,6	0.58	14.87	1,1428	<0.01*
Filefish A2 muscles (A2α *versus* A2βD+A2βV)									
Relative onset	1.03	1,6	0.35	10.69	1,6	0.02*	12.99	1,1428	<0.01*
Duration	1.64	1,6	0.25	7.99	1,6	0.03*	109.61	1,1428	<0.01*
Relative intensity	0.01	1,6	0.94	1.68	1,6	0.24	73.88	1,1428	<0.01*
Pufferfish A2β muscles (A2β′t *versus* A2β″t)									
Relative onset	10.88	1,6	0.02*	<0.01	1,6	0.97	<0.01	1,825	0.98
Duration	0.47	1,6	0.52	0.45	1,6	0.53	0.51	1,825	0.47
Relative intensity	0.24	1,6	0.64	0.02	1,6	0.89	0.23	1,825	0.63
Pufferfish A2 muscles (A2α *versus* A2β′t+A2β″t)									
Relative onset	11.84	1,6	0.01*	25.54	1,6	<0.01*	17.44	1,825	<0.01*
Duration	0.71	1,6	0.43	13.08	1,6	0.01*	15.01	1,825	<0.01*
Relative intensity	<0.01	1,6	0.97	2.76	1,6	0.15	31.73	1,825	<0.01*
Burrfish A2 muscles (A2α *versus* A2β)									
Relative onset	1.23	2,2	0.45	<0.01	1,1	0.94	0.39	2,282	0.67
Duration	0.69	2,2	0.59	<0.01	1,1	0.97	0.99	2,282	0.37
Relative intensity	5.08	2,2	0.16	44.68	1,1	0.09	6.30	2,282	<0.01*

Effects are based on univariate ANOVAs and contrasts of EMG data recorded during prey processing of live fiddler crabs, pieces of squid tentacle and live shrimps.

Onsets for muscle activity are relative to the activity of the levator operculi.

*Significant effects at $P \leq 0.05$.

muscle-by-prey effects detected in these statistical analyses can be illustrated graphically as interaction plots (Figs 4–6). These plots allow one to visualize which muscles, muscle regions or prey types are driving the effects found in the statistical analyses. In the absence of any effects, line segments for each muscle or muscle section should be parallel to the *x*-axis, and should overlie one another (Fig. 4A). When there is a simple muscle effect, line segments should remain parallel to each other, but should not overlie one another (Fig. 4B). In contrast, if there is a simple prey effect, line segments should also remain parallel to each other, but not with the *x*-axis, and these segments should overlie each other (Fig. 4C). Finally, when there is an interaction between muscle and prey, one or more line segments should no longer be parallel to the others (Fig. 4D).

Prey effects

Significant prey effects were found in six of nine analyses of duplicated muscles and one of two analyses on regional variation. Specifically, nine of 33 EMG variables showed significant prey effects (five onsets, two durations, two relative intensities; Tables 1, 2). In all instances, these overall prey effects were due mainly to a single prey item, pieces of squid tentacle. In general, squid elicited relatively later onsets and longer durations of muscular activity than did the two other

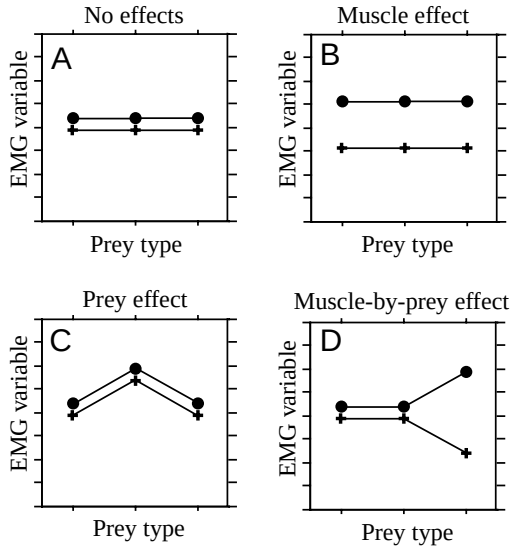

Fig. 4. Interaction plots for two hypothetical jaw muscles. Data points represent the mean values of a motor pattern variable during feeding events on three prey items and are connected for each muscle. (A) No effects on this variable due to either muscle or prey factors. (B) A muscle effect results when there are significant differences between muscles across all prey types. (C) A prey effect results when there are significant differences between prey types and the effect of prey type is the same on all muscles. (D) A muscle-by-prey effect results when there are significant differences between prey types and the effect of a prey type is not the same on all muscles.

prey types (Figs 5A,B,D,E,G,H,J,K, 6A,B,E,G,H,J). Despite this strong trend, no significant prey effects were detected in analyses of burrfish muscles (Tables 1–2).

Muscle effects

Significant muscle effects were found in four of nine analyses of muscle subdivision and in one of the two cases of regional variation. Overall, eight of 33 EMG variables showed a significant muscle main effect (three onsets, three durations, two relative intensities; Tables 1–2). The most striking muscle effects were seen for triggerfish A1 muscles and pufferfish A2 muscles. In triggerfishes, A1βb consistently had an earlier onset and longer duration of activity than A1αb (Table 1; Fig. 5A,B). Similarly, in pufferfishes, A2α had an earlier onset and longer duration of activity than A2β′t or A2β″t (Table 2; Fig. 6G,H). Muscle effects were also detected on the relative intensity of the dorsal and ventral regions of A1βt muscle in burrfishes (Table 1; Fig. 5L). In contrast, no significant muscle main effects were found on any variables of filefish A1α muscles, pufferfish A1 or A2β muscles, or burrfish A2 muscles (Tables 1, 2).

Muscle-by-prey effects

A significant muscle-by-prey interaction term indicated that the effect of prey was different on the two muscles or muscle regions. We interpret significant interaction terms as evidence of divergence in function between muscles or muscle regions. Significant muscle-by-prey interactions were found in seven of nine cases of muscle duplication and in both cases of regional variation within single muscles. In all, significant interactions were found in 16 of the 33 EMG variables (four onsets, four durations, eight relative intensities; Tables 1, 2). In most cases, the interactions involve effects of similar direction but different magnitude (e.g. Fig. 5L), but in some cases muscles exhibited responses in opposite directions (e.g. Fig. 5C).

One major difference between muscle-by-prey interactions and prey main effects was that the same prey type did not drive effects in all species. In triggerfish A1 muscles, the interaction was driven by the divergent responses of both A1αb and A1βb to fiddler crab (Fig. 5C). In filefish A1α muscles, the effect was apparently due to the response of A1αb″ to both squid tentacle and shrimp (Fig. 5F). In burrfishes, the dorsal and ventral regions of A1βt responded differently to fiddler crab (Fig. 5L). In triggerfish A2 muscles, the response of A2β′b to fiddler crab differed from the responses of either A2α or A2β″b (Fig. 6C). In filefish A2 muscles, the dorsal portion of A2β responded differently to both fiddler crab and shrimp (Fig. 6F). In pufferfishes, A2β″t responded differently from A2α or A2β′t to fiddler crab (Fig. 6I). Finally, in burrfish A2 muscles, the response of A2β to squid was much greater than that of A2α.

Phylogenetic changes in A2α activity pattern

Our single interspecific analysis found significant prey effects on the onset and duration of the activity of the A2α

Table 3. *Summary of prey, species and species-by-prey effects on electromyographic variables of the A2α adductor mandibulae muscle in four triggerfishes, four filefishes, four pufferfishes and three burrfishes*

	Prey effect			Species effect			Species-by-prey effect		
EMG variable	*F*-ratio	d.f.	*P*	*F*-ratio	d.f.	*P*	*F*-ratio	d.f.	*P*
Relative onset	7.39	2,20	<0.01*	4.24	3,10	0.04*	1.06	6,20	0.42
Duration	5.02	2,20	0.02*	7.96	3,10	<0.01*	0.21	6,20	0.97
Relative intensity	0.95	2,20	0.40	2.91	3,10	0.09	0.56	6,20	0.75

Effects are based on univariate ANOVAs of EMG data recorded during prey processing of live fiddler crabs, pieces of squid tentacle and live shrimps.

Onsets for muscle activity are relative to the activity of the levator operculi.

*Significant effects at $P \leq 0.05$.

muscles of the four tetraodontiform species but not on relative intensity (Table 3). Significant species effects were also found for onset and duration, together with a nearly significant effect (P=0.09) on intensity. For both timing variables of A2α, burrfish and filefish lie at opposite extremes, with burrfish having the latest onset times and

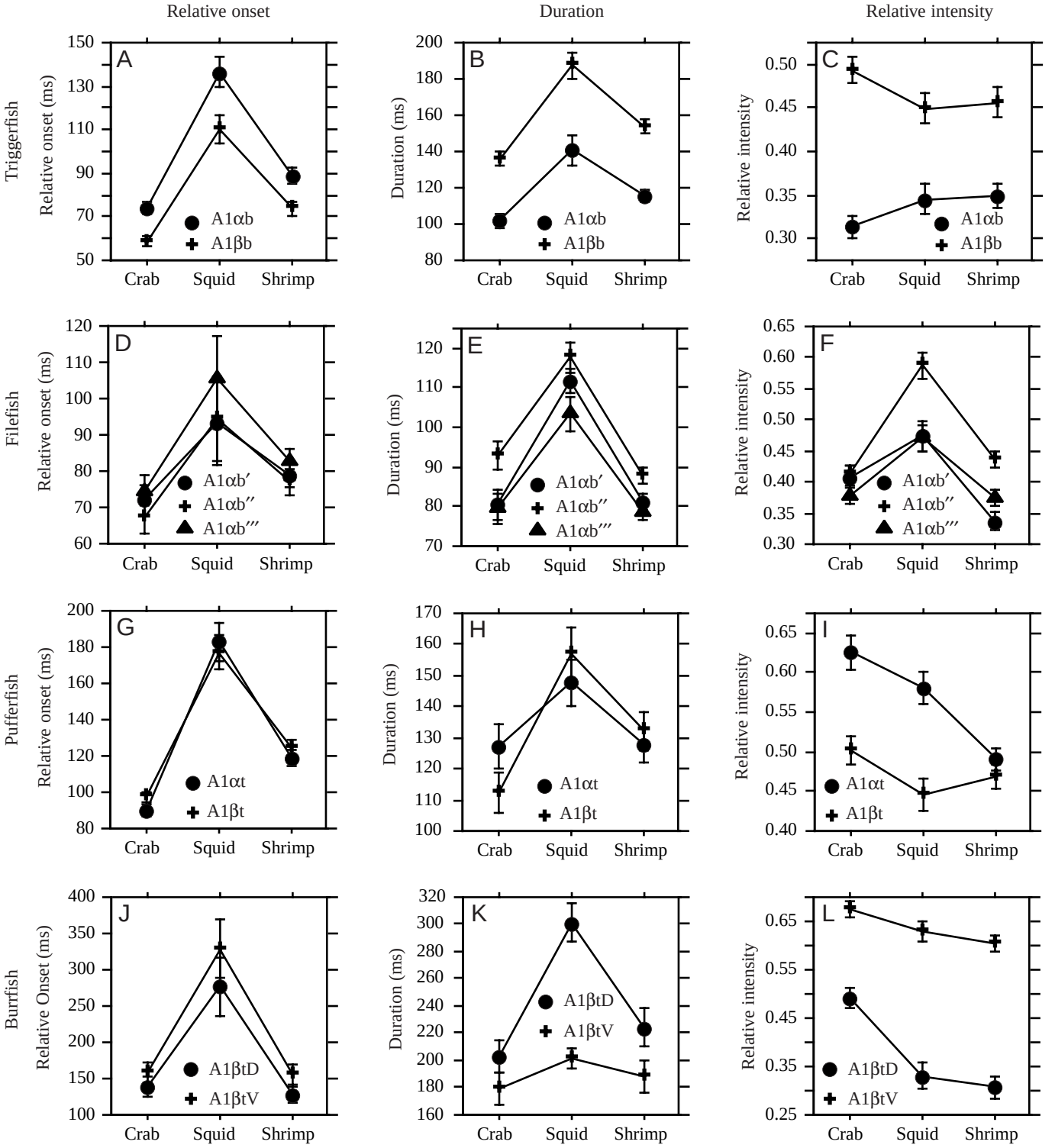

Fig. 5. Interaction plots illustrating the effect of prey on the onsets, durations and relative intensities of A1 muscles in four tetraodontiform species during prey processing of live fiddler crab, pieces of squid tentacle and live shrimp. Data points represent mean values ± 1 S.E.M. from four triggerfish, four filefish, four pufferfish and three burrfish. Note that simple prey effects on the onset and duration of activity are consistent across all muscles (e.g. A,B,D,E,G,H,J,K), while muscle-by-prey interaction effects differ across muscles (e.g. C,F,I,L). See Friel and Wainwright (1998) for related discussions of the data in B, C, E and F.

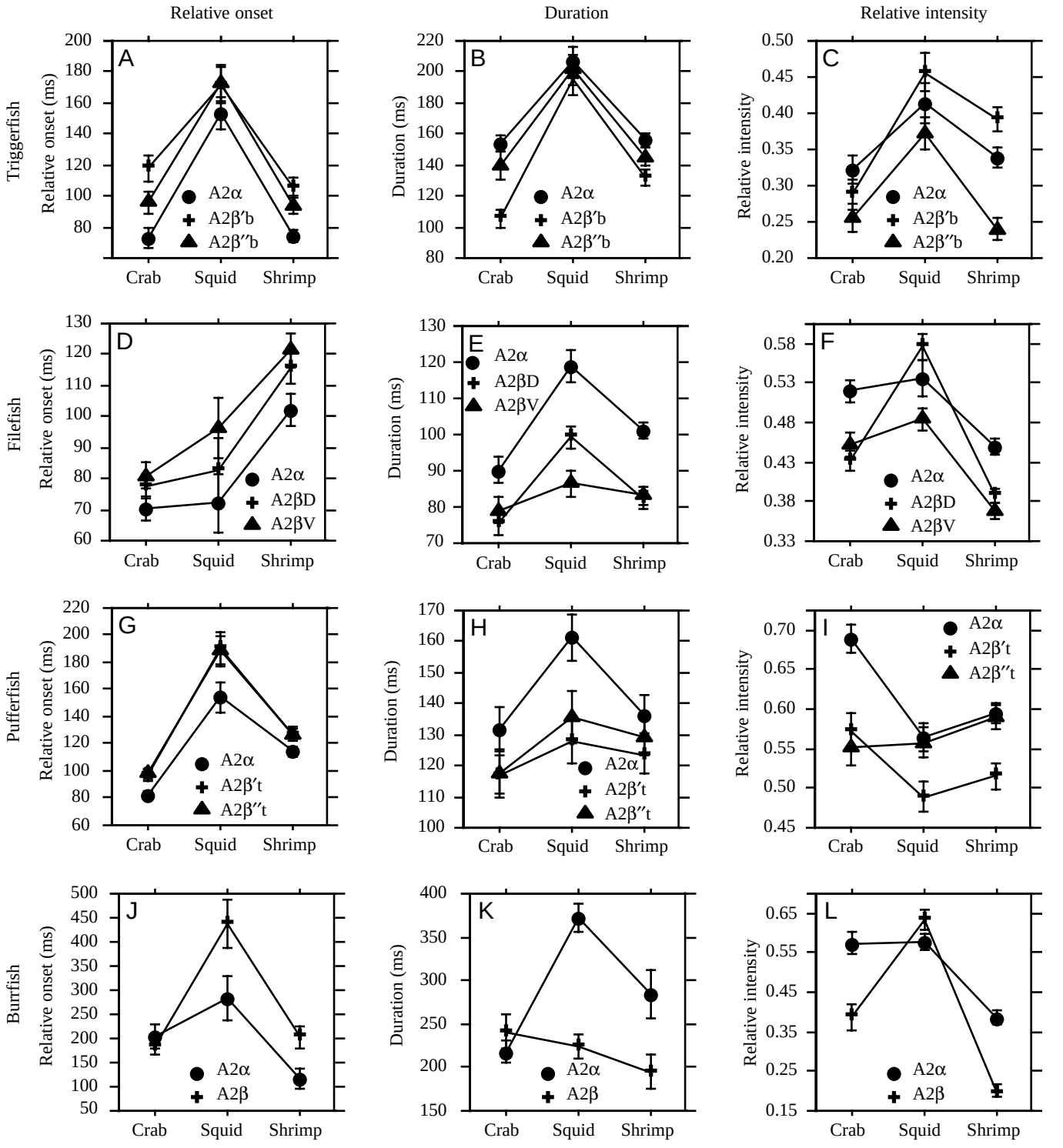

Fig. 6. Interaction plots illustrating the effect of prey on the onsets, durations and relative intensities of A2 muscles in four tetraodontiform species during prey processing of live fiddler crab, pieces of squid tentacle and live shrimp. Data points represent mean values ± 1 S.E.M. from four triggerfish, four filefish, four pufferfish and three burrfish. Note that simple prey effects are consistent across most muscles (e.g. A,B,E,G,H,J), while muscle-by-prey interaction effects differ across muscles (e.g. C,F,I,L).

longest burst durations and filefish have the earliest onsets and shortest durations (Fig. 7A,B). In fact, the onset of activity in burrfish A2α muscle was up to four times later than in filefishes, and burst durations of burrfish were up to three times longer than in filefish. In contrast, there were no significant species-by-prey interaction terms, indicating a common effect of prey type on activity of the A2α muscle in all four species.

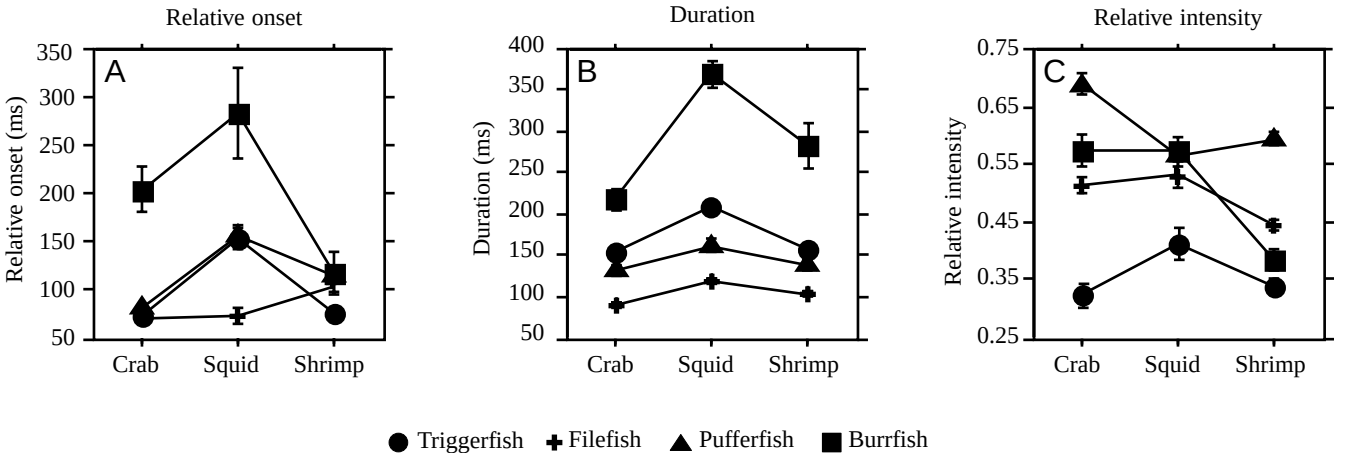

Fig. 7. Interaction plots illustrating the effect of prey on the mean onsets, durations and relative intensities of the A2α muscle in four tetraodontiform species during prey processing of live fiddler crab, pieces of squid tentacle and live shrimp. Data points represent the mean values ± 1 S.E.M from four triggerfish, four filefish, four pufferfish and three burrfish. Note that simple prey effects are consistent across most species (e.g. A,B).

Discussion

Four central results emerge from our analyses of the activity patterns of tetraodontiform jaw muscles. First, increases in the number of jaw adductor muscles due to muscle duplication by subdivision were associated with divergence of motor patterns in the descendant muscles in seven of nine cases. Second, the three EMG variables examined here diverged in subdivided muscles with slightly different frequencies, and divergence was seen in significant muscle-by-prey interaction terms twice as often as in significant muscle main effects. Third, we observed functional divergence within single muscles prior to morphological subdivision, indicating that it is possible that divergence of muscle function can predate anatomical divergence. Fourth, tetraodontiform fishes show patterns of modulation in response to prey attributes that are similar to those reported in other groups of teleost fishes. We discuss these results and their implications for a general understanding of the evolution of muscle function.

Does anatomical complexity beget functional complexity?

The role of structural complexity as a determinant of functional complexity has attracted considerable attention as a unifying concept in comparative biology (Vermeij, 1973; Liem, 1980; Lauder, 1981, 1990; Liem and Wake, 1985; Schaefer and Lauder, 1986, 1996; Emerson, 1988; Lauder and Liem, 1989). The basic hypothesis is that systems that are defined by fewer structural elements are restricted to a smaller range of possible designs and hence functional diversity (Schaefer and Lauder, 1986, 1996; Lauder and Liem, 1989). However, only a few systems have been developed sufficiently to provide tests of these predictions, and the breadth of significance of this phenomenon is uncertain (Vermeij, 1973; Emerson, 1988; Schaefer and Lauder, 1996).

The tetraodontiform jaw adductor muscle complex represents a clear example of varying morphological complexity, with taxa differing in the number of separate A1 and A2 adductor muscles that they possess. It is possible to ask whether the increase in morphological complexity (i.e. the number of separate adductor muscles) is associated with an increase in functional complexity or with the number of different patterns of activation seen in the adductor muscles. Electromyographic data provide one way of assessing functional divergence, and our results show a strong tendency for adductor muscles to diverge in activity pattern in association with the subdivision events. Divergence in motor patterns of descendant muscles increases the complexity of the functional output of the entire jaw system. Thus, in seven of nine cases of descendant muscles, we found the increase in morphological complexity to be associated with an increase in the complexity of adductor muscle activity patterns. This result indicates that tetraodontiform taxa with greater numbers of adductor muscles can exhibit more complex functional abilities in oral prey processing through differences in the timing and intensity of activity in individual jaw muscles during this behavior. One way in which the increased number of adductor muscles may enhance the functional abilities of the jaws is by providing finer control of the jaws as they are applied to prey. If two muscles attach onto the mandible where previously there had only been one, this may enhance the range of motion of the jaws and the subtlety of their control.

Extensive duplication of jaw muscles by subdivision is not commonly observed in teleost fishes (Friel and Wainwright, 1997), although there are a few other groups of fishes that present patterns similar to that in tetraodontiform fishes. Loricarioid catfishes (Schaefer and Lauder, 1986, 1996) and parrotfishes of the family Scaridae (Bellwood, 1994) are two clades of fishes that show a similar phylogenetic history of increased numbers of separate jaw muscles. Like tetraodontiforms, both these groups of fishes feed by directly applying the jaws to the prey or substratum and biting to remove or reduce the food item. As suggested above, the replication of independent muscles that adduct the jaws in

slightly different ways may enhance fine control of the jaws. Such fine control may contribute to feeding success in these fishes which apply their jaws directly to the substratum to remove prey, often adjusting the purchase and angle of attack of the jaws during repetitive feeding cycles. Future research aimed at examining the functional consequences of adductor muscle duplication in these other groups of fishes would provide valuable tests of the generality of the patterns described here for tetraodontiforms and would contribute to a broader understanding of how muscular systems evolve.

Are different features of muscle activity patterns evolving at similar rates and in similar ways?

Overall, the present study finds that the motor patterns of jaw muscles are evolving in a mosaic fashion within the Tetraodontiformes. We conducted tests of the muscle main effect and the muscle-by-prey interaction term on two timing variables, onset and duration, and one amplitude variable, relative intensity, in nine cases of duplicated muscles and two cases of muscle regions. Significant divergence in muscle function was found with onset in five cases, with burst duration in five cases and with relative intensity in eight of these cases (Tables 1, 2). Thus, there was a slight bias towards conservation of individual timing variables relative to the amplitude variable we examined. A stronger pattern that emerges from comparing the different variable types is the observation that the vast majority of intensity changes were revealed by muscle-by-prey interactions, whereas changes in burst timing were revealed equally by significant main effects and interactions. We note that the much greater number of degrees of freedom in our tests of the muscle-by-prey interactions term suggests that these tests are considerably more likely to yield significant results than are tests of the muscle main effect (see Tables 1, 2).

The muscle-by-prey interaction term revealed twice as many positive tests of functional divergence as the muscle main effect (16/22 tests *versus* 8/22 tests; Tables 1, 2). This interaction term identifies a form of functional divergence different from the muscle main effect, in which muscles differ in how they respond to some prey, not necessarily in their overall action pattern. The use of muscle-by-prey interactions in intraspecific studies and species-by-prey interactions in interspecific studies has not previously been developed as a method for identifying modes of evolution of muscle function. The numerous significant interaction terms found in this study indicate that muscles can differ and diverge functionally in ways that are only manifest during feeding on certain prey.

The interspecific analysis of activity in the A2α muscle indicated that tetraodontiform species could differ considerably in the activity pattern of this homologous muscle (Table 3; Fig. 7). This result contrasts with numerous previous analyses of homologous muscles in multiple species that have often found a strong tendency for conservation of muscle activation patterns (summarized above). Only rarely have other studies described differences in activation patterns of homologous muscles as great as those described here between the filefish and the burrfish (but see Wainwright et al., 1989).

One factor that should be considered when comparing muscle activity patterns is body size. Previous research has shown that body size can influence patterns of muscle activity in feeding fishes (Wainwright and Richard, 1995). In the present study, the focus was on variation in activity of different muscles within species and, since the range of body size within species was narrow, any possible effects of size in these analyses was probably minimal. However, the analysis of A2α activity did cross the four species, which varied in average size. The findings of Wainwright and Richard (1995) suggest that relative onset times of the adductor muscle tend to be longer in larger fish. Triggerfish were larger than the other three species in our study, but A2α activity in this species showed intermediate values (Fig. 7), suggesting that body size was not a major factor determining activity of this muscle.

We note that past EMG studies have typically used the rectified integrated area of a burst cycle to quantify the amplitude of muscular activity. This EMG variable is the product of duration and mean amplitude, and will therefore covary with any modulation of duration. Furthermore, most studies have not standardized amplitude variables to account for differences between individual electrodes. Here, we have attempted to avoid these difficulties by using a new intensity variable (i.e. relative intensity) that could vary independently of duration and was standardized for each electrode. We found several instances where duration and relative intensity variables were modulated independently not only to different degrees but also in divergent directions (e.g. triggerfish A1 muscles (Fig. 5B,C), pufferfish A1 muscles (5H,I), burrfish A1 muscles (Fig. 5K,L) and pufferfish A2 muscles (Fig. 6H,I). These individual findings are consistent with our overall observation that timing and intensity variables of motor patterns need not be coupled and can evolve independently.

Can functional divergence occur prior to anatomical subdivision?

Our observation of widespread functional divergence in tetraodontiform adductor muscles raises the issue of whether such functional diversification can predate the anatomical subdivision. In most of the cases of muscle subdivision being studied, we have no estimate of whether there was regional variation in the primitively undivided muscle, and it is therefore unclear which modification occurred first. However, we did compare regional activity patterns of physically continuous muscles in two species. The two cases we studied were selected for different reasons. The A1βt muscle of burrfishes is large and shows regional modifications along its broad attachment to the maxilla. We recorded from the far dorsal and ventral regions of this muscle, anticipating that the different organization of the muscle in these regions might be associated with differences in activity patterns. Thus, we selected this case as a likely candidate for regional functional variation. The second example was the A2β muscle of the filefish, which has anatomical regions that correspond to the

separate A2β muscles of triggerfishes, the sister group to filefishes (Fig. 2) (Friel and Wainwright, 1998). Thus, in this case, we appear to have recorded from regions of the undivided filefish muscle that correspond directly to the separate muscles of triggerfish. Such a condition was probably found in the common ancestor of filefishes and triggerfishes. In both the A1βt muscle of the burrfish and the A2β muscle of the filefish, we found evidence of functional divergence. The regional differences we observed were similar to differences observed between separate muscles.

The case of the undivided A2β muscle of filefishes, in particular, strongly suggests that it is possible that functional separation occurred prior to the physical subdivision of this muscle in the ancestor of triggerfishes. It would be particularly illuminating in future studies to obtain recordings from outgroups to filefishes plus triggerfishes, such as ostraciids, which possess an A2β muscle that shows no evidence of anatomical subdivision. A detailed study of the history of regional specialization within this muscle and its descendants could be repeated on other examples of muscle subdivision to test whether evidence of functional subdivision appears prior to anatomical subdivision. Such a line of research may shed light on the factors that underlie the phenomenon of muscle subdivision and whether an initial functional subdivision is typical.

As a counterpoint, it is interesting to note that subdivision of muscles can also occur without divergence of motor patterns in some tetraodontiforms. Evidence for this comes for our analyses of pufferfish A1 and A2β muscles. In both cases, there were no significant muscle or muscle-by-prey effects for any EMG variables. Thus, the motor patterns of these muscles have not diverged prior to or following subdivision events.

The finding that regions of a muscle may differ in activity pattern suggests the need for a caveat in our interpretation of divergence in muscle function. In this study, we assumed that the ancestral undivided and unregionalized adductor muscles were uniform in activity pattern throughout the muscle. Unfortunately, the specific taxa included in this analysis did not allow us to examine regional variation in completely unsubdivided muscles that would have represented ancestral conditions of the subdivision events we studied. Thus, we cannot be certain that the variation that we describe between subdivided muscles did not exist between the corresponding regions of the ancestral single muscle. Some of our future research is designed to address this issue in an attempt to determine the sequence of morphological and motor pattern changes that occurred in the evolution of tetraodontiform adductor muscles.

Are patterns of jaw muscle modulation conserved in teleost fishes?

During prey processing, tetraodontiform fishes modulate muscle activity much like other fishes do during suction feeding and pharyngeal prey-processing behaviors. Here, we found that mobile prey, such as live shrimp and fiddler crabs, elicited earlier onsets of activity, while tougher prey, such as pieces of squid tentacle, elicited longer bursts of activity. These patterns of modulation manifested themselves in the four species examined and in most A1 and A2 muscles as significant prey effects or at least as non-significant trends in interaction plots. The only notable exceptions were for two variables, the onsets for filefish A2 muscle activity (Fig. 6D) and the durations of A2β activity in burrfish (Fig. 6K), which each had unique response profiles. These results are generally consistent with other studies that reported that mobile prey elicited later onsets and tougher prey elicited longer durations of activity (Liem, 1978, 1979, 1980; Lauder, 1981, 1983a,b; Sibbing et al., 1986; Wainwright and Lauder, 1986; Sanderson, 1988; Wainwright, 1989; Wainwright et al., 1989; Wainwright and Turingan, 1993; Ralston and Wainwright, 1997; Friel and Wainwright, 1998). Thus, prey effects on timing variables such as onset and duration are a generally conserved feature of motor patterns in teleost fish feeding systems.

The muscle-by-prey type interaction terms that we emphasize in the present study have received little attention from workers interested in the response of animals to various feeding stimuli. In studies that compare activity patterns in homologous muscles across species, the analogous variable would be the species-by-prey type interaction term. In the present study, the muscle-by-prey interaction revealed considerable evidence of muscle and prey-type effects that were not apparent from the main factors of the ANOVAs. Further, by their nature, the interaction terms reflect a more subtle aspect of fish behavior, the ability of different muscles to respond differently to various prey. Indeed, in some cases, we found muscles responding to certain prey with opposite trends (e.g. Fig. 5C). This type of modulation, in which various prey may elicit different response patterns from different muscles, illustrates a level of complexity in the motor basis of feeding behavior that has not previously been recognized and underscores the need to use ecologically relevant prey types in studies that aim to characterize the natural feeding behavior of animals (Smith, 1994).

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