EVOLUTION OF PUFFERFISH INFLATION BEHAVIOR

PETER C. WAINWRIGHT AND RALPH G. TURINGAN¹

Department of Biological Science, Florida State University, Tallahassee, Florida 32306-3050

E-mail: wainwrig@bio.fsu.edu

Abstract.-The evolution of the extraordinary inflation mechanism of pufferfishes was studied in the light of an independently derived phylogenetic hypothesis of tetraodontiform fishes. Inflation behavior is found in all members of the puffer sister taxa Tetraodontidae and Diodontidae. However, most other tetraodontiform fishes exhibit two functionally similar behaviors. All taxa exhibit a "coughing" behavior and, with the exception of the sister-group to all other tetraodontiforms, represented by the Triacanthidae, all lineages "blow" strong jets of water out of their mouth to excavate prey. Functional specializations associated with the three behaviors were identified from anatomical analyses and electromyographic recordings of muscle activity in representatives of the major lineages of the order. The phylogenetic distribution of the three buccal compression behaviors and their functional bases indicates the following: (1) the evolution of inflation behavior involved major structural modifications of the head that function in a novel mechanism that links depression of the floor of the mouth to posterior expansion of the buccal cavity; (2) the contraction patterns of four key head muscles used in the three behaviors are generally similar both across behaviors and taxa; (3) however, the distribution of the two significant modifications of muscle activity are consistent with the hypothesis that the three behaviors represent a transformation series from coughing to water blowing to inflation. The motor pattern for water blowing is a slightly modified version of that seen in coughing, and the inflation motor pattern retains the blowing specialization and adds a single additional modification. The convergent evolution of a poorly developed inflation behavior in at least one genus of filefish provides evidence that tetraodontiform fishes are predisposed to the evolution of this unusual defensive behavior. The presence of a well developed water-blowing behavior in most tetraodontiform lineages may represent an intermediate functional specialization that increased the probability of the evolution of inflation.

Key words.—Balistes, Chilomycterus, electromyography, inflation, Monacanthus, Sphoeroides, Tetraodontiformes, transformation series, Triacanthus.

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One of the central goals in functional morphology is to understand how major innovations come about during evolution (Liem and Wake 1985; Lauder and Liem 1989; Hanken and Hall 1993). Are major innovations forged by making changes at all or most levels of design of a system, or are changes at just one or a few levels the key modifications that permit the acquisition of substantially new functions (Lauder 1990; Reilly and Lauder 1992)? Does the acquisition of specific design features make a lineage more prone to some evolutionary directions than others (Mayr 1960; Gould 1980, 1988; McShea 1991)? In spite of the prominence of this class of questions in the field of evolutionary morphology, relatively few well-studied examples of major functional innovations have been developed (see review in Lauder 1990).

One of the major obstacles in understanding the origin of major innovations is that intermediate stages between the widespread generalized condition and the specialized state may not occur in extant taxa, may not be obvious, or may be difficult to envision. This problem may be overcome by the development of a robust phylogeny of the group in question because the resolution of historical relationships often permits the identification of cryptic intermediate states in closely related taxa. Thus, the origin of bird flight (Bock 1986; Ostrom 1986; Gatesy and Dial 1996), the origin of the tongue projection mechanism in chameleons (Schwenk and Bell 1988), and the origin of the highly protrusible jaw of the sling-jaw wrasse (Westneat 1991) are all examples of major functional innovations that frustrated attempts to resolve their evolutionary history until systematic research developed to the point that key relationships of the taxa in question were resolved, permitting the identification of prominent intermediate steps in the functional transformation series of each.

Inflation behavior of pufferfishes is an example of a major functional innovation whose origin has baffled biologists (Parr 1927; Winterbottom 1974a; Tyler 1980; Brainerd 1994). However, major anatomical and systematic revisions of the order of fishes to which puffers belong, the Tetraodontiformes (Tyler 1968, 1980; Winterbottom 1974a), provide an opportunity to study the functional morphology of inflation and related behaviors within the context of a phylogenetic hypothesis of the major lineages within this order of fishes (Winterbottom 1974a). In this paper, we identify a class of functionally similar behaviors related to inflation, buccal compression behaviors, and describe novel features of the muscle activation patterns and anatomy that are associated with each behavior. Using Winterbottom's (1974a) proposed phylogeny, we test the hypothesis that the three buccal compression behaviors represent a transformation series that culminated in the evolution of inflation.

Inflation and Related Behaviors

Within the teleost order Tetraodontiformes, the bizarre ability to inflate the body is seen in all members of the families Tetraodontidae (puffers and sharp-nosed puffers) and Diodontidae (spiny puffers). During inflation, the body is filled with water by repeatedly capturing mouthfuls of water that are pumped into an expandable stomach (Brainerd 1994; Wainwright et al. 1995). Puffers possess significant modifications of the pectoral girdle and head that function in the

¹ Present address: Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida 32901.



FIG. 1. Phylogeny of fishes in the teleost order Tetraodontiformes with the inferred origins of three buccal compression behaviors indicated. The phylogeny is based on Winterbottom (1974a) who used a parsimony analysis to infer cladogram topology from an analysis of 81 characters derived from muscle anatomy. This topology was supported by Lauder and Liem (1983).

pumping mechanism (Wainwright et al. 1995). The ventral and lateral skin of pufferfishes is highly extensible and ribs are absent, permitting the extreme shape change that accompanies inflation (Brainerd 1994). Diodontids are also equipped with bony spines, formed from modified dermal scales, that stand erect when the animal is inflated. Inflation is used as a defensive behavior. Individuals inflate when threatened or when captured, making it considerably more difficult for predators to swallow the enlarged body. The ubiquitous presence of this behavior in the puffer sister lineages, and its absence in the outgroups, implies that inflation evolved in the shared ancestor of smooth-skinned puffers and spiny puffers (Fig. 1).

Inflation behavior superficially seems to involve a radical innovation in the functional morphology of the head. However, at least two additional behaviors, "water blowing" and "coughing," are widespread in tetraodontiform taxa and show striking functional similarities with inflation. Like inflation, both water blowing and coughing involve a cyclical pattern of expansion of the oral cavity to draw water into the mouth followed by a compressive phase where the water is forced out of the mouth.

Several tetraodontiform taxa blow strong jets of water out of their mouth and use the flowing water to manipulate their environment. Water blowing is used by many taxa to manipulate prey, expose buried prey, or clean prey fouled by sediment (Fricke 1971, 1975; Frazer et al. 1991) and by some triggerfish in nest construction (Fricke 1980; Wainwright pers. obs.). We have observed water blowing in captive triggerfishes, filefishes, cowfishes, pufferfishes, and the ocean sunfish Mola mola. However, neither of the two species of triacanthids (Triacanthus biaculeatus and Tryxiphichthys weberi) that we studied in the laboratory exhibited this behavior, in spite of our attempts to induce the behavior. We have not had the opportunity to study living Triodon and have no evidence of the presence or absence of water-blowing behavior in this taxon. Based on our observations, we tentatively infer the evolution of this behavior at the base of the branch leading to all nontriacanthoid tetraodontiforms (Fig. 1).

Coughing behavior appears to be present in virtually all fishes. Coughing is used to forcefully expel unwanted material from the mouth (Hughes and Adeney 1977; Lauder and Lanyon 1980). The behavior commonly is used during feeding, when the digestible portion of a prey animal is separated from pieces of exoskeleton or material that was ingested when capturing the prey. Among teleosts, this behavior has been described in detail in trout, Salmo (Hughes and Adeney 1977), carp, Cyprinus carpio (Sibbing et al. 1986) and the bluegill sunfish, Lepomis macrochirus (Lauder and Lanyon 1980). We have noted coughing behavior in members of all tetraodontiform lineages that we have observed in captivity. This includes members of all terminal taxa shown in Figure 1 except the Triacanthodidae and Triodontidae. Because all of the taxa we observed exhibited the behavior and we have no reason to suspect it to be absent in any taxa (it is not known to be absent in any fish taxa), we tentatively assume this behavior is present in the Triacanthodidae and Triodon, and we indicate the ubiquitous distribution of this trait by placing the character at the base of the tetraodontiform tree (Fig. 1).

The key distinction that separates inflation from water blowing and coughing is that inflation involves the forceful pumping of water from the buccal cavity into the stomach, whereas water blowing and coughing are used to forcefully pump water out of the buccal cavity in an anterior direction. The similar functional underpinnings of the three behaviors and their phylogenetic distribution (Fig. 1) suggested to us that the behaviors may represent a transformation series of increasingly specialized behaviors, from coughing, to water blowing and finally to inflation. The principal aim of this study was to test this hypothesis in a comparative analysis of the morphological and functional bases of the three behaviors. We search for specializations of anatomy and muscle activation patterns that are associated with each behavior and ask if the pattern of character acquisition is consistent with the hypothesis that inflation evolved from water blowing and the latter evolved from coughing.

Although the phylogenetic distribution of the behaviors provides the first level of support for our working hypothesis, there are a number of alternative scenarios for the historical origin of the three behaviors that would be supported by particular findings. First, it is possible that each behavior uses a different mechanism to compress the buccal cavity or that the patterns of muscle activity are so different for the three behaviors that no strong functional link can be drawn between the behaviors. This would suggest that the similarities between the behaviors are superficial and that water blowing and inflation represent unrelated acquisition within tetraodontiform fishes. Second, because species that use water blowing also use coughing behavior, and hence the ancestor of puffers exhibited both behaviors, it is possible that inflation is a direct modification of coughing. This would be supported by a pattern in which the characters that distinguish coughing from inflation and coughing from water blowing are a nonoverlapping set. Evidence of a transformation series would be provided by a nested set of character state changes in which inflation could be most simply explained as a modification of the water-blowing mechanism, and the latter would be most simply interpreted as a modification of the coughing mechanism.

MATERIALS AND METHODS

We studied the cranial mechanisms used in the three behaviors by the major lineages of tetraodontiforms and one generalized outgroup. Two levels of design of this musculoskeletal system were analyzed; the structure of the muscles and bones that are used in the behaviors and the patterns of muscle contraction that are used to drive the behaviors. For the structural analysis, we relied heavily on previous work with tetraodontiform fishes (Tyler 1968, 1980; Winterbottom 1974a,b; Turingan and Wainwright 1993; Wainwright and Turingan 1993; Brainerd 1994; Wainwright et al. 1995).

The patterns of muscle contraction used during the various buccal compression behaviors were studied in six species, including five tetraodontiform taxa and one outgroup species. The warmouth sunfish Lepomis gulosus, a member of the endemic freshwater North American family Centrarchidae was studied as a representative generalized percomorph species. Warmouth were collected in Lake Jackson, Leon County, north Florida. Tripodfish, T. biaculeatus (Triacanthidae), were collected by ottertrawl in the mouth of the Ross River near Townsville, Queensland, Australia. The other four species were collected in the vicinity of the Florida State University Marine Laboratory in the northeastern Gulf of Mexico. These included the planehead filefish, Monacanthus hispidus (Monacanthidae); the gray triggerfish, Balistes capriscus (Balistidae); the southern puffer, Sphoeroides nephalus (Tetraodontidae); and the striped burrfish, Chilomycterus schoepfi (Diodontidae).

In Florida, fish were transported immediately to the laboratory at Florida State University in Tallahassee and maintained separately in 100-liter aquaria at room temperature, about 22°C. In Australia, the tripodfish were transported to the laboratory at James Cook University, in Townsville, and maintained in 100-liter aquaria with flow-through seawater $(23 \pm 2^{\circ}C)$. All animals were fed a mixed diet of live and frozen invertebrates including squid, shrimp, decapod crabs, and earthworms.

All six species exhibited coughing behavior. Coughing was induced in each of the six species by feeding them large

earthworms or large pieces of squid mantle with skin (about 4 by 5 cm). With these prey, fish often completed a feeding sequence by clearing their buccal cavity of accumulated mucous and debris by executing several coughs. Water blowing was studied in the triggerfish and the southern puffer, whereas inflation was studied in the southern puffer and burrfish. Water blowing was induced in the triggerfish by burying pieces of squid mantle under a 40 to 50 mm bed of gravel in a 200 mm square glass dish and placing the dish in the aquarium with the fish. Triggerfish quickly began excavating for the squid by blowing strong jets of water into the gravel bed until the food was exposed. Puffers were induced to blow water by feeding them large pieces of squid and penaeid shrimp. Puffers would blow water at the squid to separate the flesh from the epidermis and blow water jets against partially eaten shrimp to separate flesh from the chitinous exoskeleton.

In Florida, electromyographic (EMG) recordings of muscle activation were made synchronously with pressure measured inside the buccal cavity using methods described in detail by Wainwright et al. (1995). Recordings were made from fishes during coughing, blowing, and inflation behaviors. Briefly, EMGs were recorded through 1.5 m stainless steel, bipolar electrodes from which about 0.5 mm tips were bared by removing insulation that covered the rest of the wire. Electrodes were threaded into hypodermic needles and the tips bent back against the needle shaft to form a hook that held the electrode in the muscle when inserted percutaneously into position. Color-coded electrodes were implanted into the left-side member of up to six muscles while fish were under anesthesia (tricaine methanesulfonate, < 0.7 g/liter). Once implanted, the electrodes were glued together into a single cable that was fastened to the dorsum of the fish behind the eyes with a loop of suture. EMGs were amplified 10,000 times with Grass P511 bioamplifiers using a bandpass of 100 to 3000 Hz (60 Hz notch filter always employed).

Buccal pressure was measured with a Millar SPR-407 catheter-tipped transducer that was threaded into the fish's mouth through a plastic canula that was mounted on or near the dorsal midline anterior to the eyes during the EMG implantation. Pressure and EMG were recorded simultaneously with a voice record on a 14 track TEAC XR-5000 FM recorder. In Australia, electromyograms were amplified 10,000 times with AM-Systems Model 1700 amplifiers and recorded with a voice track on an eight-channel TEAC RD-111T DAT recorder. Hard copies of all recorded data were made with a Graphtec thermal array recorder at a resolution of 200 mm of chart paper per second of recorded data. For further analysis, each recorded sequence was digitized with a Keithley analog-to-digital conversion system, and a custom computer program was used to measure the onset and offset times of muscle bursts. Onset and offset times were all measured relative to the onset of activity in the protractor pectoralis muscle, which we selected because of its propensity for discrete activity bursts. The voice record and pulses of positive pressure were used to confirm that a buccal compression behavior was taking place.

EMG recordings were made from four muscles (Fig. 2): the levator operculi (LO) is the primary depressor of the mandible in all tetraodontiforms (Turingan and Wainwright



FIG. 2. Diagrams of cranial anatomy in (A) the tripodfish, *Triacanthus biaculeatus*, (B) the gray triggerfish, *Balistes capriscus*, and (C and D) the striped burfish, *Chilomycterus schoepfi*. Noninflating tetraodontiforms, represented by *Triacanthus* and *Balistes*, primitively possess a small mouth aperture, small hyohyoideus abductor muscles and generalized hyoid apparatus. The evolution of inflation in puffers, represented by *Chilomycterus*, is associated with the origin of a highly mobile pectoral girdle, great reduction of the obliquus inferioris ventral body musculature, tremendously enlarged hyohyoideus abductor muscle and associated first branchiostegal ray. These modifications function in a novel mechanism of buccal expansion and compression that is used by puffers during inflation. Abbreviations: A1, section 1 of the adductor muscle; A2, section 2 of the adductor mandibulae muscle; BR1, first branchiostegal ray; EP, epaxialis muscle; HAB, first hyohyoideus abductor muscle; LO, levator operculi muscle; OBI, obliquus inferioris muscle; PG, pectoral girdle; PH, protractor hyoideus muscle; and PP, protractor pectoralis muscle.

1993); the protractor hyoideus (PH), protracts and elevates the hyoid apparatus, motions that are prominent during compression of the buccal cavity (Wainwright et al. 1995); the protractor pectoralis (PP), is a major protractor and stabilizer of the pectoral girdle; the adductor arcus palatini (AP), adducts the cheek elements (suspensorium) causing compression of the buccal cavity. The AP was not recorded in the burrfish because this species has a fixed suspensorium (fused to the neurocranium) and only a few fibers of this muscle. Thus, in all comparisons that did not include the burrfish, a total of seven electromyographic variables were examined: four burst durations (LODUR, PHDUR, PPDUR, and AP-DUR) and three relative onset variables (LOON, PHON, and APON).

Data were collected from six warmouth (mean standard

length = 162 mm), and five each of the other species (mean SL: *T. biaculeatus* = 216 mm; *B. capriscus* = 217 mm; *Monacanthus hispidus* = 83 mm; *S. nephalus* = 179 mm; and *C. schoepfi* = 203 mm). The maximum number of individuals analyzed per species and behavior was five, the minimum number of individuals per species and behavior was three.

Data Analysis

Our strategy in analyzing the data on muscle activity was to first reduce the behaviors to characters that described the muscle-activity pattern (mean burst duration and relative onset time of each muscle), and then to compare the average values of these characters in different species performing the same behavior and the same species performing different behaviors. These comparisons were implemented in a series of four analyses of variance (ANOVA). The first analysis was a nested ANOVA (individual fishes nested within species) that contrasted the motor pattern used during coughing behavior by all six experimental species.

The second analysis was a two-way ANOVA that compared the pattern of muscle activity used during coughing and water-blowing behavior in the triggerfish and the southern puffer. In this analysis, the fixed species effect, and random individual factor (nested within species) were crossed with the fixed behavior factor. Following the guidelines in Scheffe (1959), the *F*-ratios used to test the species factor used the individual mean squares in the denominator, and the *F*-ratios used to test the behavior factor used the individual*behavior interaction mean squares in the denominator.

The third analysis was a two-way ANOVA designed to compare the motor pattern used in inflation, coughing and blowing by the southern puffer. This ANOVA included a fixed behavior factor and a random individual factor. Hence, the *F*-ratios constructed to test the behavior factor used the interaction mean squares in the denominator.

The fourth analysis was a two-way ANOVA with a nested level that compared the coughing and inflation motor patterns in the two puffer species. In this design, the species factor was fixed, the random individual factor was nested within species, and both were crossed with the fixed behavior factor. As with the second analysis, the *F*-ratios used to test the species factor used the individual mean squares in the denominator and the *F*-ratios used to test the behavior factor used the individual*behavior interaction mean squares in the denominator.

RESULTS

Phylogenetic Distribution of Buccal Compression Behaviors: Morphology

The skeletal and muscular morphology of tetraodontiform fishes have been described in comprehensive detail by Tyler (1968, 1980) and Winterbottom (1971, 1974a), respectively. Our aim in this section is to describe the anatomical modifications that our investigations have identified as significant components of the water-blowing and inflation mechanisms.

We have not identified any anatomical novelties associated with water-blowing behavior. Water blowing does not appear to require modifications of the primitive tetraodontiform morphology as seen in the triacanthids and triacanthodids. However, several generalized teleostean traits contribute to the ability of all tetraodontiforms to execute basic buccal compression behaviors. These traits include the following: (1) a laterally mobile suspensorium (cheek bones) which, when adducted, compresses the buccal cavity; (2) well-developed ventral body musculature, the obliquus inferioris, that attaches into the posterior margin of the pectoral girdle, thus stabilizing the latter as part of the mechanism of ventral buccal expansion; and (3) the primitive teleost mechanism of ventral buccal expansion via contraction of the sternohyoideus muscle. The sternohyoideus muscle connects the anterior margin of the fixed pectoral girdle to the central region of the depressible hyoid bar. Blowing and aiming well-formed water jets is greatly facilitated by having a small mouth open-

TABLE 1. Nested ANOVA results on seven EMG variables recorded during coughing behavior in five tetraodontiform species and one species of centrarchid, *Lepomis gulosus*. Species included in this analysis in addition to *L. gulosus* were *Triacanthus biaculeatus*, *Balistes capriscus*, *Monacanthus hispidus*, *Sphoeroides nephalus*, and *Chilomycterus schoepfi*. Column titled "Species effect (without *Lepomis*)" indicates the results when the ANOVAs were calculated omitting the data from *Lepomis*. Note that data were not collected from the greatly reduced AP muscle of *Chilomycterus*.

EMG variable	Species effect F (df)	Individual effect F (df)	Species effect (without <i>Lepomis</i>) F (df)	
LODUR	1.74 (5, 17)	5.93 (17, 81)**	0.98 (4, 15)	
LOON	3.02 (5, 17)*	3.15 (17, 81)**	1.21 (4, 15)	
PHDUR	1.96 (5, 13)	1.09 (13, 89)	1.66 (4, 11)	
PHON	4.98 (5, 13)**	2.38 (13, 89)**	1.07 (4, 11)	
PPDUR	6.23 (5, 20)**	1.80 (20, 119)**	0.73 (4, 18)	
APDUR	1.96 (4, 17)	6.66 (14, 81)**	1.88 (3, 15)	
APON	0.57 (4, 17)	0.89 (14, 81)	0.99 (3, 15)	

* P < 0.05; ** P < 0.01.

ing, a trait that appears to be primitive for living tetraodontiforms (Figs. 1, 2; Winterbottom 1974a; Tyler 1980), although it may be derived relative to some extinct, basal tetraodontiforms (Tyler and Sorbini 1996).

In contrast to water blowing, the evolution of inflation involved a suite of major anatomical modifications of the head that function during the cyclic buccal expansion and compression actions that characterize this behavior (Wainwright et al. 1995). These modifications include the following: (1) the presence of a kinetic joint between the cleithrum and supracleithrum that permits extensive anterior-posterior movement of the pectoral girdle; and (2) a greatly enlarged first branchiostegal ray and associated hyphyoideus abductor muscle that form a functional coupling with the hyoid bar (Fig. 2). This coupling results in a novel mechanism for hyoid depression and simultaneous pectoral-girdle retraction via the actions of the enlarged hyphyoideus abductor muscle. Additional modifications are found postcranially, including the extendible stomach and body wall that accommodate the great change in body volume that occurs during inflation (Brainerd 1994).

Muscle-Activity Patterns

The sequence of muscle contraction used during coughing did not differ among the five tetraodontiform species (Table 1). The LO initiated activity at least 120 msec prior to the PP and continued for at least 200 msec (Fig. 3–5). The PH, AP, and PP muscles were nearly synchronously active for about 100 msec. Thus, the LO was active throughout most of the activity period of the other three muscles. Coughing was associated with a pulse of positive buccal pressure that occurred during the period of activity in the compressive phase muscles (Figs. 3, 4).

The muscle-activity patterns seen in the warmouth sunfish differed from that seen in the tetraodontiforms (Fig. 5, Table 1). In the warmouth, the LO was active only for about 70 msec and overlapped completely with the PP muscle, thus lacking the period of activity prior to the contraction of compression muscles that characterized the tetraodontiforms.

The ANOVA on the seven activity duration and relative



Representative electromyograms and simultaneously re-FIG. 3. corded buccal pressure from one coughing event and one waterblowing event in a 177-mm gray triggerfish, Balistes capriscus. The two recordings were made from the same individual a few minutes apart. During the coughing event, unwanted pieces of a shrimp (Penaeus) skeleton were ejected from the mouth by compression of the buccal cavity, creating the pulse of superambient pressure. In the water-blowing sequence, jets of water were used to excavate pieces of squid that were buried in a bed of gravel at the bottom of the aquarium. Note that the activity of the AP muscle is reduced in coughing relative to water blowing. This was the only motor pattern difference between these behaviors that emerged from the quantitative analysis. Muscle abbreviates: LO, levator operculi; PH, protractor hyoideus; PP, protractor pectoralis; AP, adductor arcus palatini.

onset variables revealed significant species effects in three of the seven EMG variables (Table 1). However, a series of post hoc contrasts revealed that in each case it was the sunfish that differed from the group of five tetraodontiform species. When the ANOVAs were run omitting the warmouth data, none of the EMG variables showed a significant species effect (Table 1).

Water-blowing muscle activity patterns were similar in the southern puffer and triggerfish (Figs. 3, 4, 6; Table 2). Like coughing, blowing behavior was characterized by an initial, long burst of activity in the LO, which was followed by near synchronous activity in the three compressive phase muscles. In both species, there was a trend for the bursts of activity in the compressive phase muscles to be briefer during coughing behavior than water blowing (Fig. 5). However, the AN-OVAs on the seven EMG variables revealed only one significant difference between behaviors, the duration of activity of the AP (Table 2) was significantly longer during blowing behavior (Fig. 4).

Each cycle of inflation behavior in the puffer, *Sphoeroides*, was characterized by an initial burst of activity in the LO, which ceased prior to the onset of activity in the PP (Figs.

4, 7, 8). Thus, the expansive phase muscle (LO) was active out of phase with the three compressive phase muscles (PH, PP, and AP). This was distinct from coughing behavior which involved extensive overlap in activity of the LO and other muscles (Fig. 6). The ANOVAs on the seven EMG variables indicated two variables that differed across behaviors. First, the duration of activity in the LO was briefer during inflation than either of the other two behaviors (Table 3). Note that this variable did not differ between coughing and blowing behavior (Table 2); hence, the significant behavior effect is due to a smaller value during inflation (Fig. 8). The second motor difference among behaviors in the puffer was found for the AP (Table 3). This muscle had a longer burst of activity during inflation and blowing than during coughing (Table 3, Fig. 4). A post hoc contrast run for this variable comparing inflation and water blowing indicated no difference between the two behaviors. However, AP duration was shorter in coughing than blowing (Table 2).

The burrfish and puffer showed no differences between species in the inflation motor pattern (Fig. 7; Table 4). The ANOVAs on five EMG variables (the AP was not recorded in the burrfish, and therefore was omitted from this analysis) revealed one variable that differed between behaviors. The duration of activity of the LO was longer during coughing behavior than during inflation (Table 3).

DISCUSSION

Basic buccal compression behaviors such as coughing are found in virtually all fish species, but within the tetraodontiforms this behavior appears to have been modified and used in a variety of biological roles that are characteristic of this diverse group of fishes. Two buccal compression behaviors in particular, water blowing and inflation, form a nested set of increasingly specialized behaviors on the tetraodontiform phylogeny (Figs. 1, 9). Our comparisons of the morphological and motor pattern modifications that are associated with each behavior suggest that the three behaviors represent a transformation series with water blowing having evolved from coughing, and inflation evolving from blowing.

Underlying our interpretation that these behaviors represent a transformation series are three primary conclusions from our analysis. (1) The three behaviors show strong functional similarities. Each behavior involves a sequence of buccal expansion, when water is drawn into the buccal cavity, and buccal compression when water is forced out of the buccal cavity. With the exception of two key EMG variables that differ among behaviors, the patterns of muscle activity that drive the three behaviors are indistinguishable. Within each behavior, the motor patterns are conserved across taxa. (2) There is little evidence of a transformation series in the anatomical characters associated with the three behaviors. Specifically, there appear to be no specialized morphological characters associated with blowing behavior, though several major modifications are seen with the evolution of inflation. (3) Evidence from the motor pattern comparisons support the transformation series hypothesis. A single EMG character distinguishes both inflation and blowing from coughing; the duration of activity of the AP muscle is longer during blowing and inflation behavior. Inflation is further distinguished from



FIG. 4. Sample electromyograms and simultaneously recorded buccal pressure made during a coughing event, a water-blowing event, and an inflation event from a 154-mm southern puffer, *Sphoeroides nephalus*. All three behaviors were recorded a few minutes apart during a single experiment. Note the general similarity of the motor pattern associated with the three behaviors. Two features varied significantly among behaviors. AP duration is shorter in coughing than blowing or inflation, and LO duration was shorter in inflation than the other two behaviors. Muscle abbreviations are as in Figure 2.

blowing by a single EMG character; the duration of activity of the LO muscle is briefer during inflation.

Common Functional Basis of the Three Behaviors

Coughing, water blowing, and inflation share a common functional basis, although the biological roles of these behaviors differ substantially. This conclusion is supported by the presence of a common activity cycle seen in all three behaviors, and from the broadly conservative nature of the muscle activation patterns used during the behaviors. The three behaviors are characterized by a fundamental cycle of activity involving a cyclic pattern of buccal expansion and compression. During expansion, water is drawn into the mouth to fill the enlarged buccal space. Frequently, a small pulse of subambient pressure is seen during expansion, immediately preceding the influx of water that eliminates the pressure gradient created by expansion (Turingan and Wainwright 1993; Wainwright et al. 1995; Wainwright and Turingan 1996). During all three behaviors, the compressive phase is characterized by a distinct pulse of superambient pressure (Figs. 3, 4). In coughing and water blowing, this superambient pressure pulse is associated with a strong flow of water out of the opened mouth. Inflation differs from the other two behaviors because the mouth is held closed during buccal compression, and the pulse of superambient pressure is instead relieved as water is forced through the esophagus into the stomach (Wainwright et al. 1995). Because the stomach and ventral body wall are so extendible, a considerable

volume of water is swallowed before the pressure inside the stomach rises significantly above ambient (Brainerd 1994).

Though two of the seven EMG variables studied differed significantly among the behaviors, the activity patterns of the four study muscles (the LO, PH, PP, and AP) were broadly similar across the three behaviors (Fig. 4). The expansive phase of all three behaviors is characterized by initial activity of the LO muscle, indicating that the mouth is opened by depression of the mandible (Figs. 5-7). During this phase, the cheek bones (suspensoria) are also spread laterally in most coughing and water-blowing taxa (Turingan and Wainwright 1993; Wainwright et al. 1995) contributing to expansion of the buccal cavity and subsequent filling with water. The compressive phase is characterized by simultaneous activity of the PH, PP, and AP muscles. Five of the seven EMG variables did not differ among behaviors (LOON, PHDUR, PHON, PPDUR, and APON). Although the other two EMG variables (LODUR and APDUR) did differ among behaviors, the general sequence of muscle activity in these four muscles was strikingly similar across behaviors and taxa.

Of the seven motor pattern characters surveyed in this study, none differed among the five tetraodontiform species during coughing behavior (Table 1, Fig. 5). Based on our selection of five species from various lineages, we therefore conclude that the motor pattern of the four study muscles used during coughing has been conserved through the tetraodontiform radiation. Differences in three of seven EMG variables between the warmouth sunfish and the five tetrao-





TIME (milliseconds)

FIG. 5. Bar diagrams representing the average myograms recorded during coughing in one centrarchid (*Lepomis gulosus*) and five tetraodontiform species (*Triacanthus biaculeatus*, *Balistes capriscus*, *Monacanthus hispidus*, *Sphoeroides nephalus*, and *Chilomycterus schoepfi*). The length of each bar represents the mean duration of activity of that muscle for all recordings made from that species, with the standard error of the mean indicated to the right of the bar. Mean onset of each muscle is shown relative to the onset of the PP, with the standard error of the mean to the left of the bar. Statistics are based on samples of six *Lepomis* and five individuals of each of the other species, with a minimum of 16 events and a maximum of 32 events summarized per behavior and muscle. See Table 1 for a quantitative analysis. Muscle abbreviations are as in Figure 2.





FIG. 6. Bar diagrams representing the average muscle activity pattern of four muscles during water-blowing behavior in five gray triggerfish, *Balistes capriscus* and six southern puffers, *Sphoeroides nephalus*. Bars indicate mean duration of activity and onset time relative to the onset of the PP muscle. Samples sizes are 64 events in the puffer and 25 events in the triggerfish. See Table 2 for a quantitative comparison between species of the muscle activity pattern shown here. Conventions as in Figure 4.

TABLE 2. F-ratios and degrees of freedom from two-way, nested ANOVAs contrasting EMG variables in coughing and water-blowing behavior in the triggerfish *Balistes capriscus* and the puffer *Sphoeroides nephalus*. ANOVAs were run with data from five *B. capriscus* and six *S. nephalus*. See Materials and Methods for a discussion of the experimental design.

.,	Factor from ANOVA model				
EMG variable	Species df = 1, 9	Individual (species) df = 9, 86	Behavior df = 1, 9	Species* behavior df = 1, 9	Individual* behavior df = 9, 86
LODUR LOON PHDUR PHON PPDUR APDUR	1.17 0.7 1.54 2.56 4.08 3.12	3.39** 4.14** 2.89* 0.89 4.22** 1.63	2.21 0.15 1.23 3.21 1.79 19.11**	1.10 0.23 2.78 4.61 2.49 1.67	2.01** 1.11 1.75 3.06** 2.34** 1.96**

* P < 0.05; ** P < 0.01.

dontiform species indicate that there has been some evolution of this behavior in the lineage leading to one or both of these groups (the Tetraodontiformes and the Centrarchidae). In the absence of a more detailed analysis of the coughing motor pattern in other tetraodontiform outgroups, we identify a tetraodontiform coughing motor pattern that is distinct from that seen in the sunfish, both quantitatively (Table 1) and qualitatively (Fig. 5). In other words, not only are there several motor pattern variables that differ between the warmouth sunfish and the tetraodontiforms, but the warmouth pattern also lacks the separate expansive and compressive phases that is characteristic of coughing and the other two behaviors in tetraodontiforms.

Evidence of a Transformation Series

Our results provide positive evidence in favor of the hypothesis that coughing, water blowing, and inflation represent

a functional morphological transformation series. The three behaviors are nearly indistinguishable at the level of the motor pattern of the four muscles we studied. However, the evidence for a transformation series is seen in the distribution of the few motor pattern differences on the phylogeny. Among the seven EMG variables studied, two differed among behaviors.

The duration of activity of the AP muscle was shorter during coughing (mean APDUR across all five tetraodontiforms = 70.2 ms) than it was during water blowing or inflation (133.6 ms and 154.2 ms, respectively). Mean APDUR did not differ between blowing and inflation in the puffer. The AP muscle adducts the suspensorium (cheek bones) during the compressive phase of these behaviors. Increased duration of the AP burst indicates that the muscle is acting more forcefully during water blowing than coughing. Thus, the evolution of blowing behavior involved a single change in the coughing motor pattern within the group of muscles studied (Fig. 9).

The second EMG variables that differed among behaviors was the duration of activity of the LO. This muscle was active for a shorter time during inflation behavior (mean LODUR in the two puffers = 102.4 ms) than during coughing or water blowing (mean LODUR across all tetraodontiform taxa studied = 220.7 ms and 207.5 ms, respectively). This motor pattern difference appears to represent an important functional difference between inflation and the other two behaviors. The LO muscle acts through a coupling to depress the mandible and is the major mouth opening mechanism in these fishes. Associated with the longer LO burst in coughing and water blowing was a strong tendency for fishes to hold the mouth open during the compressive phase of these behaviors, facilitating the expulsion of water out of the mouth. In contrast, the mouth was always closed during the compressive phase of inflation, in association with the briefer burst of LO activity.



INFLATION BEHAVIOR

TIME (milliseconds)

FIG. 7. Bar diagrams representing the average muscle activity pattern of four muscles during inflation behavior in five southern puffers (Sphoeroides nephalus) and five burrfish (Chilomycterus schoepfi). Bars indicate mean duration of activity and onset time relative to the onset of the PP muscle. Samples sizes are 33 events in the puffer and 25 events in the burrfish. See Table 4 for a quantitative comparison between species of the muscle activity pattern shown here. Conventions are as in Figure 4.



FIG. 8. Bar diagram showing the average duration and timing of activity in four muscles during coughing (N = 17), water blowing (N = 19), and inflation behavior (N = 48) in four southern puffers, *Sphoeroides nephalus*. Bars indicate mean duration of activity and onset time relative to the onset of the PP muscle. See Table 3 for a quantitative analysis; duration of the LO and AP were the only two variables showing significant variations among behaviors. Conventions are as in Figure 4.

Given these two interbehavioral differences of the motor pattern (APDUR and LODUR), the simplest transformation scenario is that of inflation behavior evolving from waterblowing behavior through a single alteration in the motor pattern of the four muscles we studied, a decrease in the activity duration of the LO muscle. The alternative scenario, that inflation represents a modification of coughing behavior, implies two changes in the motor pattern, a lengthening of APDUR and shortening of LODUR.

Interestingly, although there are a number of functionally significant anatomical modifications of the head and pectoral girdle that accompany the origin of inflation (Fig. 9), we have not discovered any anatomical modifications that are derived for blowing taxa. Coughing and water blowing are therefore

TABLE 3. *F*-ratios from two-way ANOVAs comparing seven EMG variables across three buccal compression behaviors in the puffer *Sphoeroides nephalus*; inflation, water blowing, and coughing. Data analyzed were from five *Sphoeroides*.

Behavior df = 2, 8	Individual df = 4, 81	Behavior* individual df = 8, 81
21.7**	1.8	1.6
3.1	6.7**	7.7**
0.8	0.7	0.2
0.1	3.1**	1.9
1.6	5.7**	2.8**
17.5**	2.0	0.7
0.71	2.1*	3.2**
	Behavior df = 2, 8 21.7** 3.1 0.8 0.1 1.6 17.5** 0.71	Behavior df = 2, 8Individual df = 4, 81 $21.7**$ 1.8 3.1 $6.7**$ 0.8 0.7 0.1 $3.1**$ 1.6 $5.7**$ $17.5**$ 2.0 0.71 $2.1*$

* P < 0.05; ** P < 0.01.

nearly indistinguishable behaviors at the level of morphological and motor pattern specializations. The difference between the behaviors lies primarily in how the flow of water that exits the mouth is used by the fish. During coughing, material is ejected from the mouth, while during blowing, the jet of water is used to manipulate sediments and other objects in the environment.

The absence of morphological specializations associated with blowing and the minor motor pattern difference between coughing and blowing suggests that the tetraodontiform taxa that we have identified as lacking water blowing in their behavioral repertoire, the Triacanthidae and Triacanthodidae (Fig. 1), in fact possess the traits necessary for blowing behavior. The key anatomical feature of tetraodontiforms that allows them to effectively use water blowing appears to be the presence of a small, rounded mouth aperture (Fig. 2). A small mouth opening permits the jets of water to be directed and, given a constant volume of water in the jet, a smaller aperture will result in a higher velocity of water flow (Alexander 1967). Presence of a small, rounded mouth opening is primitive for the living members of the order Tetraodontiformes and is secondarily lost only in some puffer taxa (e.g., Diodontidae). It seems possible that although we did not observe water blowing in the two species of Triacanthus we studied, a more thorough survey of this clade may reveal that some members do use blowing behavior.

The modest motor modifications associated with inflation are in contrast to the extensive suite of morphological

TABLE 4. *F*-ratios from two-way, nested ANOVAs comparing five EMG variables across two behaviors, coughing and inflation, in two puffer species, *Sphoeroides nephalus* and *Chilomycterus schoepfi*. Experiments were performed with five *S. nephalus* and five *C. schoepfi*.

EMG variable					
	Species df = 1, 8	Individual (species) df = 8, 87	Behavior df = 2, 8	Species* behavior df = 1, 8	Individual* behavior df = 8, 87
LODUR	7.2*	2.01	20.2**	0.17	0.97
LOON	2.6	4.6**	0.15	0.81	2.33*
PHDUR	3.1	3.1**	1.73	1.24	1.12
PHON	0.7	2.7**	0.63	2.81	1.2
PPDUR	1.1	1.6	0.97	3.87	2.43*

* P < 0.05; ** P < 0.01.



FIG. 9. Summary of motor pattern characteristics of the three buccal compression behaviors studied in five tetraodontiform fishes and one outgroup. The evolution of muscle activity patterns in compression behaviors is most simply explained as a transformation series with coughing giving rise to water blowing by a lengthening in the duration of activity of the AP muscle, and water blowing giving rise to inflation through a second modification, shortening of the LO burst. Many anatomical changes accompany the origin of inflation but there are no morphological changes associated with the introduction of water-blowing behavior.

changes that characterize puffers. The prominent postcranial modifications revolve around the ability of the puffer body to expand as the stomach is filled with water (Brainerd 1994). There is a general reduction in ventral structures (hypaxial body musculature is lost and the pelvic girdle is absent), and the skin possesses modifications of collagen fiber organization that allow it to be extraordinarily stretchy. The key specializations in the head are all related to the ability of puffers to expand and compress the buccal cavity (Wainwright et al. 1995). The pectoral girdle has a kinetic joint with the neurocranium that permits extensive posterior and ventral expansion of the buccal space as the cleithrum is retracted. The first branchiostegal ray and associated hyphyoideus abductor muscle are greatly hypertrophied and function as a novel mechanism of hyoid depression. This mechanism is linked to the pectoral girdle such that hyoid depression and pectoral girdle retraction are coupled and caused by contraction of the hyohyoideus abductor muscle.

The loss of hypaxial musculature and presence of a kinetic pectoral girdle render the primitive mechanism of hyoid depression ineffective. In generalized teleosts, the pectoral girdle has limited motion and acts as a stable attachment site for the sternohyoideus muscle that inserts on and functions to depress the hyoid (Lauder 1983, 1985). Stabilization of the pectoral girdle is enhanced by the hypaxial musculature that is activated during prey capture when the sternohyoideus plays a major role in hyoid depression, and hence buccal expansion (Lauder 1985). In puffers, the sternohyoideus muscle is reduced in size and hyoid depression is instead brought about by contraction of the enlarged hyohyoideus abductor muscle (Wainwright et al. 1995).

Two of the muscles that we studied with electromyography function primitively to protract the pectoral girdle (PP) and the hyoid apparatus (PH). The functions of the muscles are retained throughout coughing, blowing and inflation, even in the face of the substantial change in pectoral girdle movement and the mechanism of hyoid depression in puffers. It is interesting to note that neither of these muscles showed modifications in motor pattern across the three behaviors.

Convergent Evolution of Inflation

Although puffers appear to exhibit the most specialized system yet described for inflation in fishes, limited inflation is seen in a small number of other fish taxa. These include a single species of goby, Sufflogobius bibartatus (Smith 1956) and the swell shark, Cephaloscyllium vetrosum (Compagno 1988). In addition, a limited ability to inflate the abdomen is also seen in a few species of filefish, most prominently the four species in the genus Brachaluteres (Hutchins and Swainston 1985). In Brachaluteres, the abdomen is not extensible as seen in puffers, but these fish do appear to swallow water when threatened, causing their abdomen to become somewhat inflated and turgid (Hutchins and Swainston 1985). Fish in this genus lack the morphological specializations seen in puffers for inflation. They possess a typical balistoid (Balistidae, Monacanthidae, and the Ostraciidae) hyoid arch with no hypertrophy of the first branchiostegal ray or the associated hyphyoideus abductor muscle, and the pectoral girdle is unmodified relative to other filefishes (Matsuura 1979; Tyler 1980).

Because Brachaluteres, within the filefish lineage, is separated from the puffers by several noninflating lineages (Fig. 1, Ostraciidae, Triodon, and the Molidae) the presence of a limited inflation behavior in Brachaluteres clearly represents a second, convergent case of the origin of inflation behavior in tetraodontiform fishes. We note that Brachaluteres lies within a lineage in which water blowing is well developed. Because inflation has only rarely evolved within fishes, and yet has evolved in at least two cases within the Tetraodontiformes, we suggest that some feature of this group of fishes may have predisposed them to the origin of inflation behavior. The general reliance among tetraodontiform fishes on structural defenses (e.g., spines, poisons, and boxlike exoskeletons), and the presence of an already well-developed repertoire of buccal compression behaviors may have increased the probability that these fishes would give rise to inflation behaviors.

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