1-1-2002

Functional Morphology and Developmental Biology of Zebrafish: Reciprocal Illumination from an Unlikely Couple

L. Patricia Hernández
Wesleyan University Middletown

Michael J.F. Barresi
Wesleyan University Middletown, mbarresi@smith.edu

Stephen H. Devoto
Wesleyan University Middletown

INTEG. AND COMP. BIOL., 42:222–231 (2002)

Functional Morphology and Developmental Biology of Zebrafish: Reciprocal Illumination from an Unlikely Couple[1]

L. PATRICIA HERNÁNDEZ,[2] MICHAEL J. F. BARRESI, AND STEPHEN H. DEVOTO
Biology Department, Wesleyan University, Middletown, Connecticut 06459

SYNOPSIS. Functional morphology has benefited greatly from the input of techniques and thinking from other disciplines. This has been especially productive in situations where each discipline has made significant contributions to a particular research topic. A combination of methodologies from functional morphology and developmental biology has allowed us to characterize feeding mechanics of first-feeding larval zebrafish (*Danio rerio*). Contrary to kinematic patterns commonly seen in adult teleosts, larval zebrafish showed no lateral abduction during the expansive phase of a suction-feeding event. Instead, dorsoventral expansion of the buccal chamber, more typical of patterns seen in primitive fishes, characterized the expansive phase. Moreover, a pronounced preparatory phase during which the buccal chamber is constricted by the protractor hyoideus was consistently seen in first-feeding larval kinematics. Key kinematic variables associated with first feeding correlated significantly with the hydrodynamic regime as measured by the Reynolds number. Using the tools of both functional morphology and developmental biology we have not only determined which cranial muscles are important for successful feeding but also uncovered important physiological differences in muscle structure. Muscles necessary for the rapid dorsoventral expansion of the head are composed primarily of fast-twitch fibers while those involved in more tonic contractions such as hyoid protraction have more slow-twitch muscle fibers. While most evolutionary developmental studies have examined mechanisms responsible for large evolutionary changes in morphology, we propose that the type of data uncovered in functional studies can lead to the generation of hypotheses concerning the developmental mechanisms responsible for smaller intra- and/or interspecific changes.

INTRODUCTION

Functional morphology and developmental biology are two disciplines across which a fruitful synergy could develop. However, while many functional morphologists understand the importance of developmental history, ontogenetic studies within this field are still relatively rare. Moreover, the notion of combining functional morphology with molecular developmental biology seems especially difficult, since researchers within these fields do not generally collaborate with each other. Functional morphologists often investigate the functional consequences of key evolutionary adaptations (Liem, 1974; Liem and Sanderson, 1986), while developmental biologists investigate the mechanisms generating morphological features. A union of these two fields can allow a deeper understanding of both the developmental origin and evolutionary importance of functional adaptations.

The union of two disparate fields is likely to be most productive when applied to a research topic in which both fields have made significant advances (Liem and Summers, 2000). The development of cranial form and function is one such topic. Functional morphologists have long recognized that structures involved in feeding are often under extreme selective pressures, leading to the evolution of a wide range of cranial morphologies (Liem, 1974; Liem and Osse, 1975). Developmental biologists, in turn, have made great progress in understanding the cellular and genetic basis of cranial development (Kimmel *et al.*, 2001; Noden, 1986; Noden *et al.*, 1999; Schilling, 1997; Schilling and Kimmel, 1997). However, functional morphologists have rarely characterized the developmental mechanisms involved in changes in feeding structures and developmental biologists have rarely focused on the function(s) of the structures whose development they are analyzing.

The zebrafish (*Danio rerio*) has become a popular model organism for developmental biologists. They are easy to maintain and breed in the lab, and their embryos are small, develop rapidly, and are optically transparent. These advantages, and the work of many labs, have led to the identification of several thousand mutations affecting development. The creation of a detailed genetic map, as well as the characterization of the movements and cell interactions that determine cell fate and tissue form have also led to great advances in understanding zebrafish development. Functional morphologists can exploit these same advantages. Recently, the functional morphology of feeding in early ontogenetic stages of zebrafish has been described (Hernández, 2000). Moreover, the development of zebrafish cranial musculoskeletal structures is being characterized, and the genetic mechanisms responsible for this development are being elaborated (Neuhauss *et al.*, 1996; Piotrowski *et al.*, 1996; Piotrowski and Nusslein-Volhard, 2000; Schilling and Kimmel, 1997).

We believe that the union of functional morphology and developmental biology can significantly impact the burgeoning field of evolutionary developmental biology, and we will first place our work within this

[1] From the Symposium *Molecules, Muscles, and Macroevolution: Integrative Functional Morphology* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 3–7 January 2001, at Chicago, Illinois.

[2] E-mail: phernandez@wesleyan.edu

context. Second we will present arguments for the importance of undertaking ontogenetic studies in functional morphology, and present as an example an ontogenetic study of feeding kinematics. Finally, we wish to illustrate the reciprocal illumination that can result from the union of molecular developmental biology with functional morphology by outlining our current and future work.

EVOLUTIONARY DEVELOPMENTAL BIOLOGY

Although the study of evolutionary developmental biology has a long, rich history (for reviews see Gilbert *et al.*, 1996; Hall, 1998; Raff, 1996), the field is undergoing a resurgence. Major reasons for this resurgence have been the synergistic use of multiple model organisms, the realization that developmental control genes are widely conserved across species, and increased use of a comparative approach. As a result, it is now possible to identify the developmental mechanisms that are responsible for morphological variations among different species (Arthur, 1997).

At least two approaches can be taken in evolutionary development studies, which mirror two classic approaches used to understand the mechanisms of evolutionary change. One approach examines the changes in developmental mechanisms that have led to such evolutionary novelties as the origin of the turtle shell (Burke, 1989; Gilbert *et al.*, 2001). This approach seeks to explain higher-level macroevolutionary changes—the origin of taxa. A second approach seeks to understand changes responsible for the modification of structures that have already evolved. This approach seeks to explain smaller evolutionary changes—morphological differences between populations or species. Both types of studies are essential in understanding the genetic mechanisms involved in all levels of morphological evolution. Evo-devo studies have focused on large-scale morphological changes and have largely ignored smaller scale evolutionary changes in morphology. We believe that it is in the examination of these smaller changes that the union of functional morphology and developmental biology will be most fruitful.

The degree of morphological change associated with speciation level events has been largely ignored by evolutionary developmental biologists. The ability of functional morphology to inform developmental studies depends largely on the structures being examined. If we are interested only in major changes in body plan, this will fall squarely within the purview of existing evo-devo studies, where functional morphology contributes little. Functional morphology can make a valuable contribution in evo-devo studies that examine much smaller morphological changes. Both types of studies are essential in establishing the types of developmental mechanisms associated with both lower and higher order levels of morphological differentiation across clades. Functional studies allow us to identify key heritable performance variables whose generation can then be examined using the tools of the developmental biologist.

FUNCTIONAL MORPHOLOGY OF FEEDING IN ZEBRAFISH LARVAE

Functional morphology of early ontogenetic stages

Ontogenetic studies in functional morphology provide a powerful approach to understanding the relationship between changing form and function. Studies that encompass a wide range of developmental stages can examine varying degrees of morphological complexity and concomitant functional differences within genetically identical individuals. Such functional studies can highlight the functional consequences of anatomical differences as pronounced as those seen in comparative studies without the added logistical problem of adequately assessing phylogenetic relationship (Harvey and Pagel, 1991). Significant anatomical differences are often associated with ontogeny of feeding structures (Liem and Summers, 2000).

Feeding studies have played a major role within functional morphology (Lauder, 1980*a*; Lauder and Shaffer, 1993; Liem, 1974, 1979; Wainwright and Lauder, 1986; Westneat, 1990; Liem and Summers, 2000). Emphasis has been placed on feeding due to its importance not only for ensuring survival, but also for attaining a healthy reproductive state. Early life history stages consistently show greater mortality than later ontogenetic stages, and feeding success may play an important role in survival during these early stages (Houde and Schekter, 1980). Ontogenetic changes in feeding morphology provide an ideal scenario in which to examine the relationship between form and function.

The functional morphology of feeding by aquatic organisms has been extensively studied. However, since most research has been done on adults, the effects of viscous forces have been largely ignored. Due to their small size, larval fish perceive their fluid environment as significantly more sticky or viscous than do adults. The dimensionless Reynolds number (Re) represents the relative contributions of inertial forces versus viscous forces in a hydrodynamic situation:

$$\mathrm{Re} = \mathrm{UL}/\upsilon$$

Where, L = characteristic length, U = velocity, and υ = kinematic viscosity of the water (Vogel, 1981; Webb and Weihs, 1986; Weihs, 1980). Reynolds numbers, which vary over several orders of magnitude, significantly affect the lives of aquatic organisms (Vogel, 1981). By convention (Webb and Weihs, 1986), $\mathrm{Re} < 20$ indicate that viscous forces predominate, $\mathrm{Re} > 200$ indicate that inertial forces predominate, and Reynolds numbers intermediate to these extremes indicate that both forces must be taken into account (Fuiman, 1986). Zebrafish larvae are usually within the viscous and intermediate regimes (Fuiman and Webb, 1988).

Several studies have shown that fish larvae, trapped within a low Re environment, function in an aquatic world significantly different from that of adults (Fuiman and Webb, 1988; Hunter and Coyne, 1982). These basic differences in their physical environment should

be incorporated into models of feeding mode. For example, most models assume that during suction feeding, inertial forces play an important role in molding kinematics (Lauder, 1980*b*). However, due to their small size, inertial forces are relatively unimportant in the lives of larvae. Given that most anamniotes feed aquatically while at a very small size, the effects of low Re on feeding kinematics should be known for these important life history stages.

There are a number of important constructional issues that must be considered when dealing with fish larvae. Due to their small size, larval eyes take up a significant portion of the head. Indeed, larval zebrafish have eyes that are big enough to be unaffected by diffraction, a common problem encountered by small organisms (Easter and Nicola, 1996). Otten (1983), investigating ontogenetic changes in cichlid cranial anatomy, described the potential constructional constraint of the eye on muscles involved in larval feeding. The eye at first feeding is relatively large and often occludes the space that later in ontogeny will house lateral muscles of the cheek (unpublished data, L.P.H.). There are two ways larvae can ameliorate this problem. Rearrangement of key skeletal and ligamentous connections could offset this constraint (Otten, 1983). Alternatively, different muscles could be used during larval versus adult feeding. Either solution acts as a ''compensatory design'' to deal with space constraints imposed by a relatively large eye (Liem, 1991).

Evolutionary history plays an important role in molding present-day kinematic patterns (Liem, 1993), thus rigorous use of the comparative method is now commonplace in functional morphological studies (Harvey and Pagel, 1991; Lauder *et al.*, 1993). Developmental history may also play an important role in shaping adult feeding patterns. Both fish and amphibians must successfully compete in two markedly different arenas before reaching reproductive age. The dramatic morphological reconstruction that occurs during amphibian metamorphosis is accompanied by significant functional changes as these organisms switch from aquatic to terrestrial feeding. Given that the feeding apparatus changes completely during metamorphosis in most amphibians, the kinematic patterns established during aquatic feeding are mostly lost after metamorphosis. This is a potentially much more important issue in fish since they do not generally undergo radical morphological transformations which lead to the establishment of different feeding structures. Whether neuromuscular circuitry and associated musculoskeletal machinery established early in ontogeny affects adult feeding is unknown.

Limitations in experimental techniques have until recently slowed progress in understanding the functional morphology of feeding in early ontogenetic stages. However, although it remains difficult to directly measure muscle activity and buccal pressure in small larvae, advances in video recording technology now allow the filming of larval feeding at a very high frame rate (1,000 frames/sec). Careful quantification of kinematic patterns during larval feeding coupled with immunohistochemistry, to carefully assess larval feeding structures, will allow us to better understand larval feeding mechanics.

We will first describe feeding kinematics of larval zebrafish. By combining these kinematic profiles with an examination of muscular anatomy we then propose the likely sequence of muscle firing during feeding. Finally, examination of muscle fiber type composition based on immunohistochemical data allows us to illustrate how functional and developmental studies can lead to a novel approach within evo-devo studies.

Feeding kinematics

Given how much teleost larvae differ from adults in both morphology and size, and thus concomitant Reynolds number, feeding mechanics in these two stages are likely to be different (Sanderson and Kupferberg, 1999). While there is variation in kinematic profiles of suction feeding among adult teleosts, the typical feeding sequence entails an anterior to posterior abduction of bony elements to create a negative pressure within the buccal chamber (Lauder, 1985). Through the use of electromyography four distinct phases in feeding kinematics of fishes have been identified. These are a preparatory phase, during which the buccal chamber is constricted; an expansive phase, during which abduction of cranial elements effects reduced pressure within the buccal chamber; a compressive phase, during which water is moved out of the buccal chamber through adduction of cranial elements; and a recovery phase during which feeding morphology returns to its original configuration (Lauder, 1980*a*; Lauder and Lanyon, 1980; Liem, 1978; Liem and Osse, 1975). For many adult teleosts, pronounced lateral abduction of both opercula and suspensoria plays a key role in generating a drop in pressure during the expansive phase. A preparatory phase, during which the buccal cavity is constricted, has been identified predominantly in advanced percomorph teleosts (Lauder, 1985).

Feeding kinematics of first-feeding larval zebrafish (total length = 4 mm; 5 days post-fertilization) are markedly different from kinematic profiles of both adult zebrafish and other adult teleosts (Hernández, 2000 and unpublished data). Larval feeding kinematics were characterized by a distinct preparatory phase during which the hyoid was protracted (compare Fig. 1A and B). While the presence of a distinct preparatory phase has not been identified in all adult teleosts (including adult zebrafish), among zebrafish larvae it was a consistent part of the feeding attack. When present, electromyography has been required to identify a preparatory phase in adult teleosts (Lauder, 1980*b*). Given that this phase was readily identified in videos of larvae (Fig. 1B), such constriction of the buccal chamber prior to the expansive phase may be more important in larval fish. The synergistic effects of a small buccal chamber and viscous medium probably necessitate a preparatory phase. Constriction of the buccal chamber

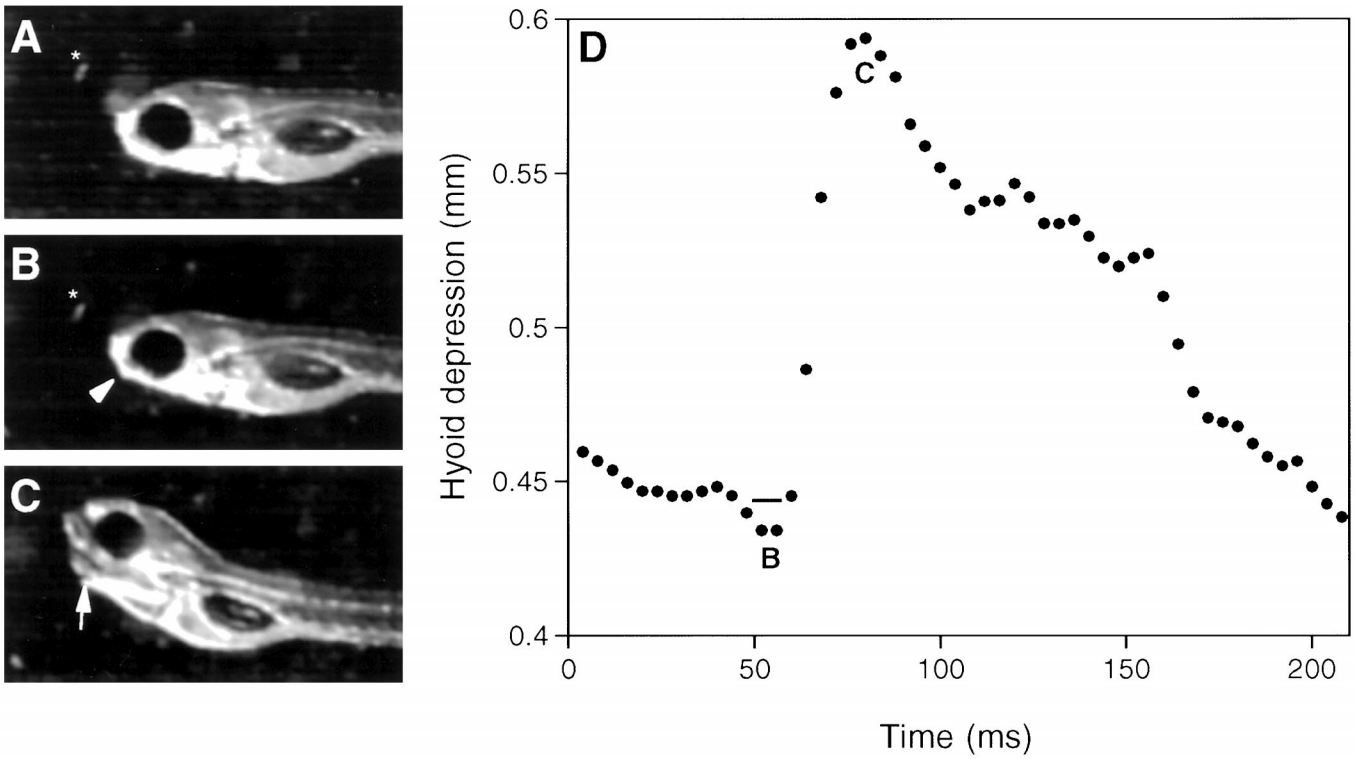

FIG. 1. Pronounced preparatory and expansive phases characterize larval feeding in zebrafish. Lateral views from video taken at 1,000 frames/sec showing: (A) Lateral profile of 5 day-old zebrafish before the start of the feeding strike; (B) Preparatory phase characterized by extreme protraction of the hyoid; and (C) Expansive phase characterized by extreme head elevation and hyoid depression. The asterisk reflects the position of the prey item, a paramecium. The arrowhead in B marks the posterior angle of Meckel's cartilage, indicating that the hyoid is extremely protracted. Also note the decreased white area under the eye in 1B as compared with 1A. D, Kinematic profile of hyoid movements in a first-feeding larva during a feeding strike. Time zero represents the time at which the hyoid begins to be protracted. The preparatory phase (B) and expansive phase (C) are indicated. Hyoid depression was measured as the distance between the bottom of the eye and the ventral-most point of the hyoid, thus increasing values indicate increasing hyoid depression. The line in D indicates the period immediately preceding hyoid depression during which the hyoid is maximally protracted and corresponds to the line in Figure 2C′.

serves to maximize the amount of fluid that can be brought into the buccal chamber, a task made more difficult by the viscous nature of buccal flow in larval fishes.

Unlike kinematic profiles seen in many adult teleosts, dorsal filming of feeding events revealed no opercular abduction at any stage of the strike (data not shown). Indeed, Cubbage and Mabee (1996) showed that ossification of these predominantly dermal elements is not complete until 9 days post-fertilization (at 6 mm TL). Thus lack of opercular abduction was likely due to both lack of ossification at this stage (Cubbage and Mabee, 1996) as well as to reduced size of lateral muscles responsible for such abduction. The size and orientation of these lateral abductors is likely influenced by the spatial constraints imposed by the eye (Otten, 1983).

Given this lack of lateral abduction, changes in buccal pressure are brought about primarily by dorsoventral movement of the neurocranium and hyoid (Figs. 1, 2). Head lift (Fig. 2A′) and mouth opening (Fig. 2B′) followed by rapid hyoid depression (Fig. 2C′) occurred after the preparatory phase. The extreme degree of hyoid depression (Fig. 1C), combined with one of the fastest and most pronounced cranial elevations ever reported (see Gibb, 1997 for comparisons), indicates that dorsal and ventral musculature is responsible for generating negative pressure within the buccal cavity. The degree and velocity of cranial elevation rivals that seen in many specialized adults (Gibb, 1997; Lauder and Liem, 1981). Such emphasis on hyoid depression and cranial elevation is characteristic of less derived fishes (Bemis and Lauder, 1986; Lauder, 1982), suggesting a possible relationship between ontogenetic and phylogenetic changes in feeding mechanics.

Hyoid movements in larvae differed quantitatively and qualitatively from those at other ontogenetic stages. When corrected for differences in size, first-feeding larvae clearly produced the greatest hyoid depression of all ontogenetic stages considered (Fig. 3). Moreover, while many adult teleosts have kinematic profiles in which time-to-hyoid depression is equal to the time required for the hyoid to return to its resting position, the time it took for the hyoid to return to its starting position in zebrafish larvae was four times as long as time-to-hyoid depression (Fig. 2C). The prolonged period during which the hyoid was returned to its starting position may be due in part to the viscous regime in which these larvae are feeding (Re = 4.97 ± 0.54), since there is a significant correlation between time to

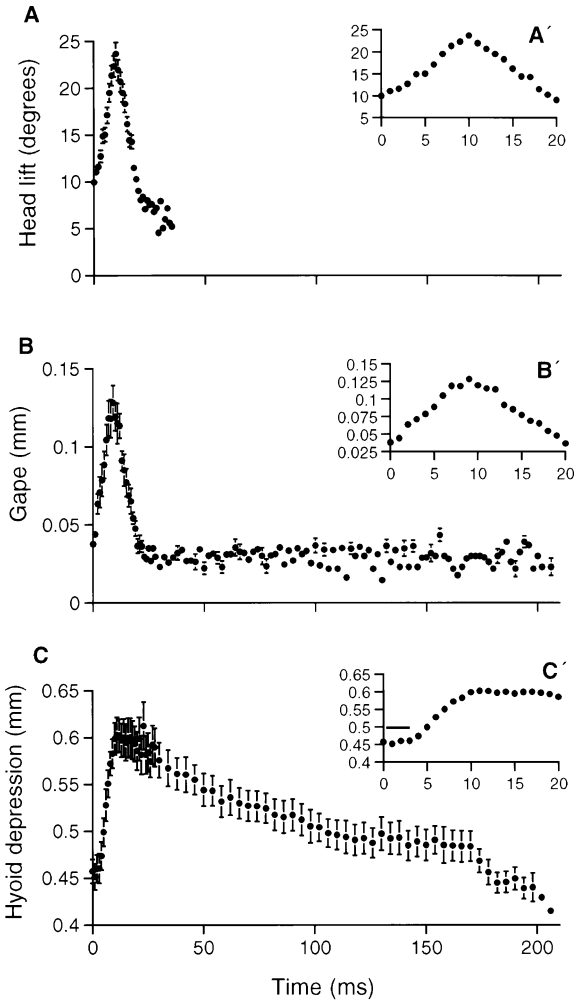

FIG. 2. Kinematic profiles of head, mouth (gape), and hyoid movements in a first-feeding larva during a feeding strike. Time zero represents the point at which mouth opening begins. Insets show a magnified view of the first part of the strike. Cranial elevation (A′) and mouth opening (B′) began while the hyoid was still maximally protracted. The line in C′ indicates the period during which the hyoid is maximally protracted during the preparatory phase preceding rapid depression of the hyoid. Compare line in 2C′ to the line in Figure 1D.

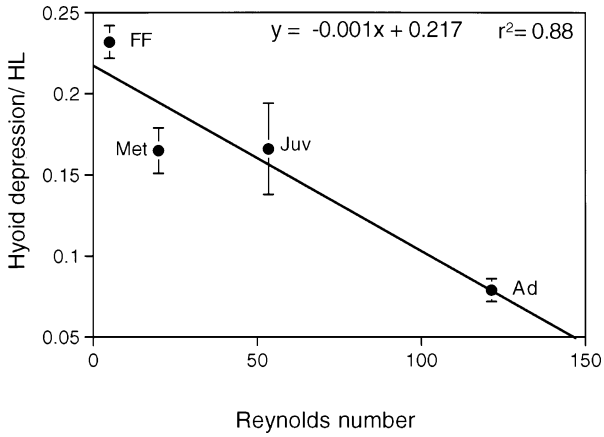

FIG. 3. Relative hyoid depression is correlated with Reynolds number regime and decreases with ontogeny. When corrected for size, hyoid depression is greatest in first-feeding larvae and decreases in older stages. Note that as Reynolds numbers increase relative hyoid depression decreases. FF = first-feeding larvae (3–4 mm total length); Met = larvae undergoing metamorphosis (6–7 mm total length); Juv = Juveniles (10–12 mm total length); Ad = Adults (25–27 mm total length).

hyoid elevation and Reynolds number ($r^2 = 0.560$; $P = 0.005$). Moreover, there was a significant correlation between degree of hyoid depression and Reynolds number (Fig. 3). This Reynolds number describes the hydrodynamic regime associated with suction feeding and uses the diameter of the mouth as the characteristic length and the mean velocity of prey entering the mouth as the measure of velocity.

Standard definitions of suction feeding assume that a rapid abduction of bony cranial elements results in reduced pressure within the buccal chamber, which brings in a bolus of water that will continue moving through the mouth and out the opercular openings. The typical kinematic profile seen in adult teleosts suggests that this is true for large fish. However such movement of incoming water is not possible within a viscous environment. In a sticky medium fluid will not continue to flow once skeletal elements have stopped moving. Moreover, since the opercular openings are quite small and the musculature responsible for abduction is not fully developed, fluid will tend to flow out of the mouth as the hyoid returns to its resting position. If the hyoid is elevated too quickly, water and adherent prey will be lost. Both lateral and dorsal filming of failed feeding events corroborated this (data not shown). Given the importance of feeding at these early ontogenetic stages, where efficient feeding can literally mean the difference between life and death, the effects of changing morphology and hydrodynamic regime should be incorporated into models of feeding behavior.

Feeding anatomy

Examination of 5 day-old, first-feeding zebrafish larvae revealed that while dermal elements of the skull had not yet developed, many cartilaginous elements were well formed (Fig. 4). The occipital arch at the posterior end of the neurocranium, the future site for development of exoccipitals and supraoccipitals, was well chondrified (Fig. 4). In first-feeding larvae the palatoquadrate, which suspends the lower jaw, was one undifferentiated block of cartilage. Contrary to the trapezoidal shape it bears in adult zebrafish (Cubbage and Mabee, 1996) the larval palatoquadrate (in lateral view) had a more triangular shape (Fig. 4). The ventral posterior edge of the palatoquadrate was attached to the long anterior process of the hyosymplectic. The cartilaginous lower jaw was well formed and the retroarticular process of Meckel's cartilage was already quite prominent (Figs. 4, 5B). Lack of dermal ossification accounted for lack of both the bony dentary in the lower jaw and the bony premaxilla within the upper jaw. The hyoid, interhyal and associated branchial

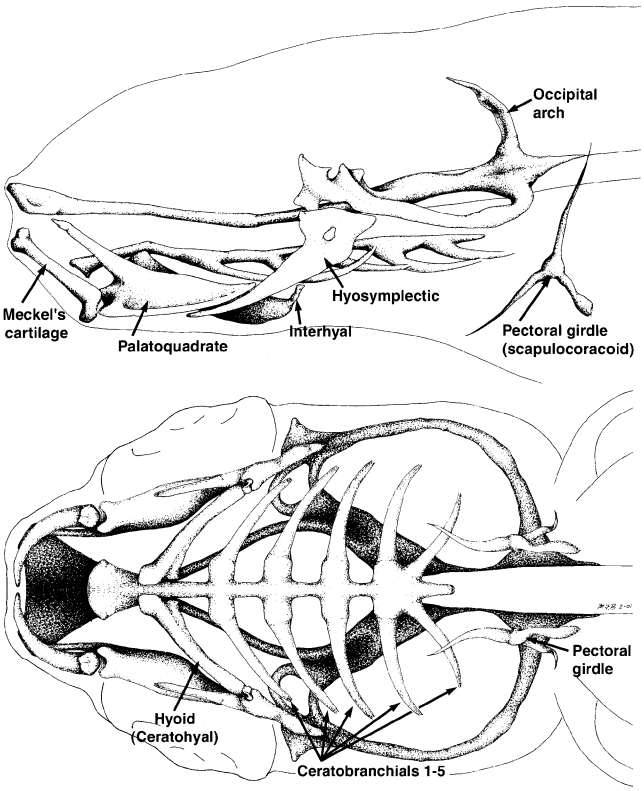

FIG. 4. Free hand drawing of cartilaginous elements of the skull of a 5 day-old larval zebrafish based on Alcian blue staining. Top, lateral view showing the hyoid fully protracted. Bottom, ventral view.

arches were quite large and well formed (Fig. 4). The hyoid, formed from the combined ceratohyal and epihyal, represented one of the most prominent elements in the head. The interhyal connected the hyoid to the hyosymplectic (Figs. 4, 5B). The hyoid and interhyal together constitute the hyoid apparatus (Barel *et al.*, 1976).

Several muscles play a key role in both the preparatory and expansive phases of a suction feeding event. The protractor hyoideus is especially important during the preparatory phase. The posterior intermandibularis and interhyoideus (Schilling and Kimmel, 1997) together form the protractor hyoideus (Adriaens and Verraes, 1997; Winterbottom, 1974). The protractor hyoideus originates on the ventral surface of each side of the hyoid. It is composed of two, bilaterally symmetrical muscles, which merge in a midline aponeurosis and then once again diverge as two separate shorter straps that insert on the ventrolateral surfaces of both sides of the mandible, composed of Meckel's cartilages (Fig. 5A, B). The protractor hyoideus elevates the hyoid (Fig. 4) and keeps it protracted (Fig. 1B), as the head is elevating (Fig. 1B, C). The nomenclature of this muscle has been a contentious issue (Adriaens and Verraes, 1997), yet it is clear that the posterior intermandibularis and interhyoideus work together and should be considered one muscle in functional studies. While present as two muscles in the notopterids and mormyrids, in all other teleosts fusion of these muscles has led to the protractor hyoideus (Winterbottom, 1974).

Two muscles are key to dorsoventral expansion of the buccal cavity, which characterizes the expansive phase of a suction-feeding event in zebrafish larvae. The sternohyoideus (sternohyal, Schilling and Kimmel, 1997) originates on the pectoral girdle and inserts via a long tendon on the hyoid (Fig. 5A; Anker, 1978). This tendon later ossifies to form the urohyal (Arratia and Schultze, 1990). The epaxial muscles insert on the posterior aspect of the chondrocranium (Fig. 5A), which later ossifies into the exoccipitals and supraoccipital of the skull. Contraction of the sternohyoideus leads to depression of the hyoid while contraction of the epaxial musculature leads to cranial elevation.

While electromyography has not been used on first-feeding larvae, analysis of the sequence of skeletal movements combined with the anatomical analyses made possible by immunohistochemistry gives a good indication of the order of muscle firing. Protraction and elevation of the hyoid, clearly seen in Figure 1B indicates that the protractor hyoideus contracts first (compare Fig. 1A to 1B). As the hyoid is held in a protracted position (Fig. 2C′), larvae elevate the cranium through contraction of the epaxial musculature (Fig. 2A′). The final portion of the feeding strike entails extreme hyoid depression brought about by contraction of the sternohyoideus (Fig. 2C′). The rapid movements brought about by contractions of the epaxials and sternohyoideus suggest they might consist primarily of fast-twitch fibers, while the more tonic contractions of the protractor hyoideus suggest it might have more slow-twitch fibers (Fig. 5B). Thus, close examination of kinematic patterns, coupled with immunohistochemical data provides vital information regarding muscle physiology.

Given that the pectoral girdle at first feeding consists only of the small cartilaginous scapulocoracoid (Cubbage and Mabee, 1996) and that the sternohyoideus originates from the dermal elements of the girdle, which have not yet formed, it would not be surprising to see the origin of the sternohyoideus move anteriorly as the head is elevated. Such anterior displacement would deleteriously affect feeding since there would be a decrease in the maximum ventral excursion of the hyoid, and a concomitant decrease in buccal expansion. We believe that the inferior obliquus of the hypaxial musculature (Fig. 5C) prevents such anterior displacement. Posterior fibers of the sternohyoideus often merge with the anterior-most fibers of the inferior obliquus (Winterbottom, 1974). By retracting the dermal precursors of the pectoral girdle, excursion of the hyoid can be increased. Such pronounced depression of the hyoid would be unlikely without retraction of the origin of the sternohyoideus by this hypaxial muscle. Osse *et al.* (1997) suggested that *Cyprinus carpio* larvae might retract the pectoral girdle to maximize hyoid depression. Pectoral retraction through contraction of the inferior obliquus combined with slight head ele-

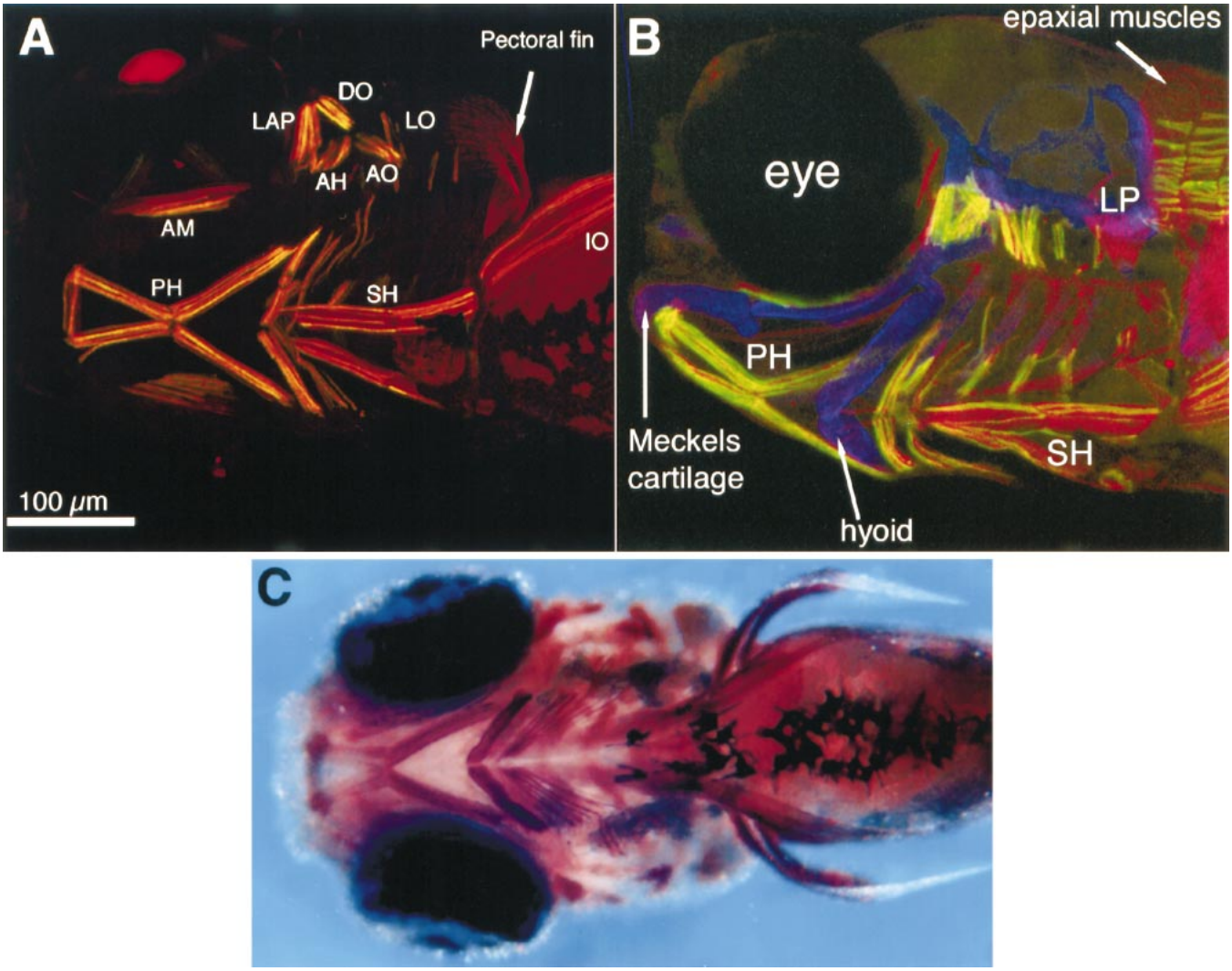

FIG. 5. Antibody labeling of zebrafish cranial muscles and cartilages. A, ventral view of a 5 day-old larva illustrating relative distribution of fast-twitch and slow-twitch fibers in cranial muscles. All muscle is stained red while slow fibers appear yellow. The protractor hyoideus clearly contains a greater proportion of slow-twitch fibers than either the sternohyoideus or inferior obliquus. B, lateral view of 5 day-old larva with all muscles stained red (MF20 antibody; Bader *et al.*, 1982), slow muscle fibers stained green (S58 antibody; Crow and Stockdale, 1986) and collagen stained blue (II-II6B3 antibody; Linsenmeyer and Hendrix, 1980). Note prominent Meckel's cartilage and hyoid apparatus. C, ventral view of a 5 day old zebrafish larva showing the relationship between the inferior obliquus and sternohyoideus (all muscles stained with MF20 antibody). AH—Adductor hyoideus; AM—Adductor mandibulae; AO—Adductor operculi; DO—Dilatator operculi; IO—Inferior obliquus; LAP—Levator arcus palatini; LO—Levator operculi; LP—Levator posterior; PH—Protractor hyoideus; SH—Sternohyoideus.

vation may allow for maximum change in buccal pressure.

Continuation of the hypaxial musculature into the sternohyoideus is seen in some adult teleosts (Winterbottom, 1974). If, however, due to lack of lateral abduction of suspensorial elements, pronounced hyoid depression commonly plays a key role in larval feeding, this morphological feature may be more important in larval fish. By contracting during a feeding strike this ventral musculature not only prevents the origin of the sternohyoideus from moving anteriorly, but may also facilitate a greater ventral displacement of the hyoid by moving the origin of the sternohyoideus posteriorly. Such movement could facilitate more pronounced volume changes within the buccal chamber. Thus we suggest that other small fish larvae, in which hyoid depression plays a key role in feeding, should all share this configuration of ventral musculature. Indeed, Patruna *et al.* (1998) and Mascarello *et al.* (1995), investigating muscle growth in marine larvae, made reference to the presence of such hypaxial musculature. Since marine larvae are typically smaller than freshwater larvae this adaptation of the hypaxial musculature may be more important in marine larvae.

FUTURE DIRECTIONS

Functional morphologists are not only interested in the way that morphological complexes function, they are deeply concerned with how such anatomical features have evolved over time. Functional morpholo-

gists add to the work of developmental biologists through their knowledge of muscle anatomy, remodeling, and fiber type. Developmental biologists contribute not only tools such as immunohistochemistry, but also their knowledge of what genes are involved in the proper development of assorted morphological features. Functional morphologists can generate hypotheses regarding the nature of morphological change within a clade. Developmental biologists can then examine which developmental mechanisms have led to morphological changes.

Using the tools of both functional morphology and developmental biology we have determined not only which cranial muscles are important for successful feeding but have uncovered important differences in the proportion of physiologically distinct muscle fibers in these muscles. Since the protractor hyoideus is used to hold the hyoid in a protracted position until rapid contraction of the sternohyoideus, we would expect to see differences in the relative degree of slow-twitch and fast-twitch fibers that make up these muscles. Indeed, there is a greater proportion of slow-twitch fibers in the protractor hyoideus than there is in the sternohyoideus, inferior obliquus, or anterior epaxial musculature (Fig. 5B).

Muscle fiber composition is hereditary in mice (Suwa *et al.*, 1996); thus this is an important trait upon which natural selection can act. If as stated by Liem (1991), ''functional demands dictate the precise timing, mechanically adaptive shape, and strategic kinematic connections of emerging structures during development'' in larval fish, strong selection may exist for the proportion of slow and fast fibers in each muscle. Understanding of the cellular and genetic mechanisms regulating muscle patterning may suggest which developmental mechanisms are responsible for minor interspecific differences in muscle fiber type differentiation.

The genes that regulate the development of slow and fast fiber type identity in cranial muscles are unknown. In the trunk, Hedgehog signaling from the notochord specifies slow muscle precursors very early in development (Barresi *et al.*, 2000; Blagden *et al.*, 1997; Currie and Ingham, 1996; Du *et al.*, 1997; Stickney *et al.*, 2000). Soon after specification these slow muscle precursors migrate radially away from the notochord to form a superficial layer of slow muscle; the remaining deep musculature is composed of fast fibers (Devoto *et al.*, 1996). Hedgehog genes (sonic and tiggy-winkle) are expressed in the notochord and floor plate (Chandrasekhar *et al.*, 1997; Ekker *et al.*, 1995; Krauss *et al.*, 1993) and may help specify cranial muscle fiber type identity. Future work entails documenting the precise distribution of slow and fast fibers in cranial muscles important in feeding. The developmental mechanisms by which muscle fiber type is specified in those functionally relevant muscles will then be investigated. We will also use zebrafish genetics to test several signaling pathways for their role in specifying muscle fiber type in cranial muscles.

Functional studies have allowed us to determine which muscles are important in feeding and characterize the relative contraction speed of these muscles. Identification of muscle fiber types using immunohistochemistry confirmed that muscles used for fast contractions contain more fast muscle fibers than muscle used for slow contraction. By examining fiber type distribution we have identified a heritable, functionally relevant performance trait. Fiber type distribution is a trait that may vary among species or even between populations that have diverged in feeding mode. Given that we are investigating the genetic mechanisms that lead to fiber type specification, future work entails identifying the genetic mechanisms involved in effecting interspecific differences in fiber type distribution within feeding musculature.

Developmental studies are the only ones that can establish how novel morphologies originate. Natural selection will cull failed experiments but only changes in developmental mechanisms can generate the diversity that natural selection acts on. Many evolutionary developmental biologists have embraced this reemerging field due to its great promise in uniting formerly disparate disciplines. We suggest that consideration of functional data when generating developmental mechanistic hypotheses may allow us to understand how functionally relevant traits under strong selection have evolved. Developmental genetic methods, combined with rigorous phylogenetic methodologies (Mabee, 2000; Wagner *et al.*, 2000), give promise that evolutionary developmental questions, posed from the time of Darwin, can finally be tackled.

ACKNOWLEDGMENTS

We wish to thank Ann Burke for providing helpful comments on the manuscript. LPH is indebted to Karel F. Liem for his constant support during completion of the functional morphological aspects of this study. LP also wishes to thank Beth Brainerd for use of video equipment and lab space as well as years of fruitful discussions. We are especially grateful to Lisa Zackowski, Ron Gordon, and members of the Wesleyan University Animal Care facility for excellent care of our zebrafish colony. This work was supported by NSF grant BSR-8818014 to Karel F. Liem and a Donoghue Foundation Investigator Award and NIH grant R01 HD37509 to SHD.

REFERENCES

Adriaens, D. and W. Verraes. 1997. Ontogeny of the hyoid musculature in the African catfish, *Clarias gariepinus* (Burchell, 1822) (Siluroidei: Clariidae). Zool. J. Linn. Soc. 121:105–128.

Anker, G. C. 1978. The morphology of the head muscles of a generalized *Haplochromis* species: *H. elegans*. Neth. J. Zool. 28: 234–271.

Arratia, G. and H. P. Schultze. 1990. The urohyal: Development and homology within osteichthyans. J. Morph. 203:247–282.

Arthur, W. 1997. *The origin of body plans: A study in evolutionary developmental biology*. Cambridge University Press, Cambridge.

Bader, D., T. Masaki, and D. A. Fischman. 1982. Immunochemical

analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J. Cell Biol. 95:763–770.
Barel, C. D. N., F. Witte, and M. J. P. van Oijen. 1976. The shape of the skeletal elements in the head of a generalized *Haplochromis* species: *H. elegans* Trewavas 1933 (Pisces, Cichlidae). Neth. J. Zool. 26:163.
Barresi, M. J., H. L. Stickney, and S. H. Devoto. 2000. The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. Development 127:2189–2199.
Bemis, W. E. and G. V. Lauder. 1986. Morphology and function of the feeding apparatus of the lungfish, *Lepidosiren paradoxa* (Dipnoi). J. Morph. 187:81–108.
Blagden, C. S., P. D. Currie, P. W. Ingham, and S. M. Hughes. 1997. Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. Genes Dev. 11:2163–2175.
Burke, A. C. 1989. Development of the turtle carapace—implications for the development of a novel bauplan. J. Morph. 199: 363–378.
Chandrasekhar, A., C. B. Moens, J. T. Warren, C. B. Kimmel, and J. Y. Kuwada. 1997. Development of branchiomotor neurons in zebrafish. Development 124:2633–2644.
Crow, M. T. and F. E. Stockdale. 1986. Myosin expression and specialization among the earliest muscle fibers of the developing avian limb. Dev. Biol. 113:238–254.
Cubbage, C. C. and P. M. Mabee. 1996. Development of the cranium and paired fins in the zebrafish *Danio rerio* (Ostariophysi, Cyprinidae). J. Morph. 229:121–160.
Currie, P. D. and P. W. Ingham. 1996. Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. Nature 382: 452–455.
Devoto, S. H., E. Melancon, J. S. Eisen, and M. Westerfield. 1996. Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development 122:3371–3380.
Du, S. J., S. H. Devoto, M. Westerfield, and R. T. Moon. 1997. Positive and negative regulation of muscle cell identity by members of the hedgehog and TGF-beta gene families. J. Cell. Biol. 139:145–156.
Easter, S. S. and G. N. Nicola. 1996. The development of vision in the zebrafish (*Danio rerio*). Dev. Biol. 180:646–663.
Ekker, S. C., A. R. Ungar, P. Greenstein, D. P. von Kessler, J. A. Porter, R. T. Moon, and P. A. Beachy. 1995. Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. Curr. Biol. 5:944–955.
Fuiman, L. A. 1986. Burst-swimming performance of larval zebra danios and the effects of diel temperature fluctuations. Trans. Am. Fish Soc. 115:143–148.
Fuiman, L. A. and P. A. Webb. 1988. Ontogeny of routine swimming activity and performance in zebra danios (Teleostei: Cyprinidae). Anim. Behav. 36:250–261.
Gibb, A. C. 1997. Do flatfish feed like other fishes? A comparative study of percomorph prey-capture kinematics. J. Exp. Biol. 200: 2841–2859.
Gilbert, S. F., G. A. Loredo, A. Brukman, and A. C. Burke. 2001. Morphogenesis of the turtle shell: The development of a novel structure in tetrapod evolution. Evol. Dev. 3:47–58.
Gilbert, S. F., J. M. Opitz, and R. A. Raff. 1996. Resynthesizing evolutionary and developmental biology. Dev. Biol. 173:357–372.
Hall, B. K. 1998. *Evolutionary developmental biology*. Chapman and Hall, New York.
Harvey, P. H. and M. D. Pagel. 1991. *The comparative method in evolutionary biology*. Oxford University Press, Oxford.
Hernández, L. P. 2000. Intraspecific scaling of feeding mechanics in an ontogenetic series of zebrafish, *Danio rerio*. J. Exp. Biol. 203:3033–3043.
Houde, E. D. and R. C. Schekter. 1980. Feeding by marine fish larvae: Developmental and functional responses. Env. Biol. Fish. 5:315–334.
Hunter, J. R. and K. M. Coyne. 1982. The onset of schooling in northern anchovy larvae *Engraulis mordax*. Calif. Coop. Oceanic Fish. Invest. Rept. 23:246–251.
Kimmel, C. B., C. T. Miller, and C. B. Moens. 2001. Specification and morphogenesis of the zebrafish larval head skeleton. Dev. Biol. 233:239–257.
Krauss, S., J. P. Concordet, and P. W. Ingham. 1993. A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. Cell 75:1431–1444.
Lauder, G. V. 1980*a*. Hydrodynamics of prey capture by teleost fishes. *Biofluid mechanics*, pp. 161–181. Plenum Press, New York.
Lauder, G. V. 1980*b*. The suction feeding mechanism in sunfishes (*Lepomis*): An experimental analysis. J. Exp. Biol. 88:49–72.
Lauder, G. V. 1982. Patterns of evolution in the feeding mechanism of actinopterygian fishes. Amer. Zool. 22:275–285.
Lauder, G. V. 1985. Aquatic feeding in lower vertebrates. *In* M. Hidebrand, D. M. Bramble, K. F. Liem, and D. B. Wake (eds.), *Functional vertebrate morphology*, pp. 230–261. Harvard University Press, Cambridge.
Lauder, G. V. and L. E. Lanyon. 1980. Functional anatomy of feeding in the bluegill sunfish, *Lepomis macrochirus*: *In vivo* measurement of bone strain. J. Exp. Biol. 84:33–55.
Lauder, G. V., A. M. Leroi, and M. R. Rose. 1993. Adaptations and history. TREE 8:294–297.
Lauder, G. V. and K. F. Liem. 1981. Prey capture by *Luciocephalus pulcher*: Implications for models of jaw protrusion in teleost fishes. Env. Biol. Fish. 6:257–268.
Lauder, G. V. and H. B. Shaffer. 1993. Design of feeding systems in aquatic vertebrates: Major patterns and their evolutionary interpretations. *In* J. Hanken and B. K. Hall (eds.), *The skull*, pp. 113–149. University of Chicago Press, Chicago.
Liem, K. F. 1974. Evolutionary strategies and morphological innovations: Cichlid pharyngeal jaws. Syst. Zool. 22:425–441.
Liem, K. F. 1978. Modulatory multiplicity in the functional repertoire of the feeding mechanism in cichlid fishes. I. Piscivores. J. Morph. 158:323–360.
Liem, K. F. 1979. Modulatory multiplicity in the feeding mechanism in cichlid fishes, as exemplified by the invertebrate pickers of Lake Tanganyika. J. Zool. 189:93–123.
Liem, K. F. 1991. A functional approach to the development of the head of teleosts: Implications on constructional morphology and constraints. *In* N. Scmidt-Kittler and K. Vogel (eds.), *Constructional morphology and evolution*, pp. 231–249. Springer-Verlag, Berlin.
Liem, K. F. 1993. Ecomorphology of the teleostean skull. *In* J. Hanken and B. K. Hall, (eds.), *The skull*, pp. 422–452. University of Chicago Press, Chicago.
Liem, K. F. and J. W. M. Osse. 1975. Biological versatility, evolution and food resource exploitation in African cichlid fishes. Amer. Zool. 15:427–454.
Liem, K. F. and S. L. Sanderson. 1986. The pharyngeal jaw apparatus of labrid fishes: A functional morphological perspective. J. Morph. 187:143–158.
Liem, K. F. and A. P. Summers. 2000. Integration of versatile functional design, population ecology, ontogeny and phylogeny. Neth. J. Zool. 50:245–259.
Linsenmeyer, T. F. and M. J. C. Hendrix. 1980. Monoclonal antibodies to connective tissue macromolecules: type II collagen. Biochem. Biophys. Res. Comm. 92:440–446.
Mabee, P. M. 2000. Developmental data and phylogenetic systematics: Evolution of the vertebrate limb. Amer. Zool. 40:789–800.
Mascarello, F., A. Rowlerson, G. Radaelli, P.-A. Scapolo, and A. Vegetti. 1995. Differentiation and growth of muscle in the fish *Sparus aurata* (L.): Myosin expression and organization of fibre types in lateral muscle from hatching to adult. J. Mus. Res. Cell Motility 16:213–222.
Neuhauss, S. C., L. Solnica-Krezel, A. F. Schier, F. Zwartkruis, D. L. Stemple, J. Malicki, S. Abdelilah, D. Y. Stainier, and W. Driever. 1996. Mutations affecting craniofacial development in zebrafish. Development 123:357–367.
Noden, D. M. 1986. Patterning of avian craniofacial muscles. Dev. Biol. 116:347–356.
Noden, D. M., R. Marcucio, A. G. Borycki, and C. P. Emerson. 1999. Differentiation of avian craniofacial muscles: I. Patterns

of early regulatory gene expression and myosin heavy chain synthesis. Dev. Dyn. 216:96–112.

Osse, J. W. M., J. G. M. van der Boogart, G. M. J. van Snik, L. van der Sluys. 1997. Priorities during early growth of fish larvae. Aquaculture. 155:249–258.

Otten, E. 1983. The jaw mechanism during growth of a generalized *Haplochromis* species: *Haplochromis elegans* Trewavas 1933 (Pisces, Cichlidae). Neth. J. Zool. 33:55–98.

Patruna, M., G. Radaelli, F. Mascarello, and C. M. D. Carnevali. 1998. Muscle growth in response to changing demands of functions in the teleost *Sparus aurata* (L.) during development from hatching to juvenile. Anat. Embryol. 198:487–504.

Piotrowski, T. and C. Nusslein-Volhard. 2000. The endoderm plays an important role in patterning the segmented pharyngeal region in zebrafish (*Danio rerio*). Dev. Biol. 225:339–356.

Piotrowski, T., T. F. Schilling, M. Brand, Y. J. Jiang, C. P. Heisenberg, D. Beuchle, H. Grandel, F. J. van Eeden, M. Furutani-Seiki, M. Granato, P. Haffter, M. Hammerschmidt, D. A. Kane, R. N. Kelsh, M. C. Mullins, J. Odenthal, R. M. Warga, and C. Nusslein-Volhard. 1996. Jaw and branchial arch mutants in zebrafish II: Anterior arches and cartilage differentiation. Development 123:345–356.

Raff, R. A. 1996. *The shape of life: Genes, development and the evolution of animal form.* University of Chicago Press, Chicago.

Sanderson, S. L. and S. J. Kupferberg. 1999. Development and evolution of aquatic larval feeding mechanisms. *In* B. K. Hall and M. H. Wake (eds.), *The origin and evolution of larval forms,* pp. 301–377. Academic Press, San Diego.

Schilling, T. F. 1997. Genetic analysis of craniofacial development in the vertebrate embryo. Bioessays 19:459–468.

Schilling, T. F. and C. B. Kimmel. 1997. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. Development 124:2945–2960.

Stickney, H. L., M. J. Barresi, and S. H. Devoto. 2000. Somite development in zebrafish. Dev. Dyn. 219:287–303.

Suwa, M., T. Nakamura, and S. Katsuta. 1996. Heredity of muscle fiber composition and correlated response of the synergistic muscle in rats. Amer. J. Physiol. 271:R432–436.

Vogel, S. 1981. *Life in moving fluids: The physical biology of flow.* Princeton University Press, Princeton.

Wagner, G. P., C. Chiu, and M. Laubichler. 2000. Developmental evolution as a mechanistic science: The inference from developmental mechanisms to evolutionary processes. Amer. Zool. 40:819–831.

Wainwright, P. C. and G. V. Lauder. 1986. Feeding biology of sunfishes: patterns of variation in the feeding mechanism. Zool. J. Linn. Soc. 88:217–228.

Webb, P. W. and D. Weihs. 1986. Functional locomotor morphology of early life history stages of fishes. Trans. Am. Fish. Soc. 115: 115–127.

Weihs, D. 1980. Energetic significance of changes in swimming modes during growth of anchovy larvae, *Engraulis mordax*. Fish. Bull. 77:597–604.

Westneat, M. W. 1990. Feeding mechanics of teleost fishes: A test of four-bar linkage models. J. Morph. 205:269–295.

Winterbottom, R. 1974. A descriptive synonymy of the striated muscles of the Teleostei. Proc. Acad. Nat. Sci. Phil. 125:225–317.