Pufferfish Inflation: Functional Morphology of Postcranial Structures in *Diodon holocanthus* (Tetraodontiformes)

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ABSTRACT   For the purpose of defending itself against predators, the balloon-fish (*Diodon holocanthus*) can triple its body volume by pumping water into its stomach. *Diodon holocanthus* exhibits striking structural and functional specializations for inflation. The stomach of the balloonfish is highly extensible and has lost its digestive function; it is repeatedly folded upon itself and is lined with transitional epithelium. The peritoneal cavity of the balloonfish is also large and extensible. During inflation, the stomach expands into an existing peritoneal space surrounding the axial musculature and presses the folded peritoneum out into potential peritoneal spaces ventral to the head and surrounding the dorsal fin, anal fin, and caudal peduncle; only the dorsal and lateral surfaces of the head are unaffected. Balloonfish skin is also specialized for inflation. Because the collagen fibers in the dermis of unstretched skin are wavy, little force is required to extend the skin up to 40% of its rest length. At high strains, the collagen fibers are stressed directly and the skin suddenly becomes stiff. Stiff skin surrounding a ball of incompressible water provides a rigid framework for the support of the erectile spines, and the balloonfish becomes a formidable, spiny sphere. However, not all of the structural and functional features that contribute to the ability of balloonfish to inflate are evolutionary adaptations specifically for inflation. Absence of pleural ribs, absence of a pelvis, and the orthogonal arrangement of dermal collagen sheets in the skin were all present in the ancestor of pufferfishes, and thus, must be considered exaptations. Sagittal plane flexibility of the vertebral column may be an epiphenomenon of the lengthening of the body cavity for more spherical inflation. Together these structural and functional features—whether adaptations, exaptations, or epiphenomena—combine to produce a highly effective mechanical defense.   © 1994 Wiley-Liss, Inc.

Pufferfishes are named for their remarkable ability to inflate their bodies when attacked by predators (Fig. 1). Body inflation serves as a mechanical defense. Once in the grasp of a predator, a puffer may escape simply by becoming too large to swallow. Most puffers are able to inflate with either water or air. When captured by piscine predators, puffers inflate with water. When captured by avian (or human) predators and taken from the water, puffers inflate with air.

No direct evidence is available on the efficacy of inflation as a defense against piscine predators. The difficulty of observing large numbers of predation events underwater has prevented the collection of such data. However, useful indirect evidence has resulted from studies of the stomach contents of piscivorous fishes. As part of a note on estuarine flounder and flathead populations (*Pseudorhombus* sp. and *Platycephalus fuscus*), Hutchinson ('72) reported that, despite an abundance of puffers in the estuary, the predators rarely consume puffers. (Only two out of 210 examined fish contained a puffer.) A survey of the food habits of Caribbean reef fishes revealed that large sharks and groupers occasionally feed on puffers, but most piscivorous reef fishes do not (Randall, '67). In the Hawaiian Islands, no instances of pre-

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dation on puffers were reported by Hobson ('74), but his study did not include sharks.

Studies of shore bird predation on fishes benefit from the one-dimensionality of the shore line. In contrast with the three-dimensionality of the pelagic environment, this one-dimensionality allows observation of large numbers of predation events and, thus, direct assessment of the efficacy of puffing as a defense against avian predators. A study of herons preying on inshore fishes showed that inflation is an effective mechanism for escape from avian predators (Recher and Recher, '68). Of 2,592 observed captures of various minnow-like fishes with no specialized escape mechanisms, only 13 were observed to escape from the predator (0.5%). In contrast, of 11 puffers (*Spheroides*) captured by the herons, five were able to escape by inflating their bodies and causing the herons to drop them (45%). In the six cases of successful predation by herons on puffers, either the puffer was small relative to the size of the heron or the heron was able to deflate the puffer by spearing it with its beak. An analysis of the time from capture to swallowing (handling time) suggests that the puffer's defense is mechanical. The handling time per gram of prey was greater for puffers than for the other observed prey fishes, indicating that the predators had difficulty subduing and swallowing the puffers (Recher and Recher, '68).

Because puffers inflate by pumping water or air into their stomachs (Gabriel, '40; Jackson, 1848), much of the interest in pufferfish inflation has centered on the structure of the stomach. The stomach of puffers has been described as a large dilatable sac with robust esophageal and pyloric sphincters (Rosén, '12). In spiny puffers (Diodontidae), the stomach is a simple sac, whereas in smooth puffers (Tetraodontidae), the stomach is divided by a horizontal sphincter. In both families, the ventral portion of the stomach had been called "the inflatable sac" and the dorsal portion had been called "the stomach proper," thereby implying some division of stomach function (Breder and Clark, '47). In addition, Gabriel ('40) briefly described the buccal pump used for inflation, and modern techniques of functional morphology have recently been used to study the inflation pump (Wainwright, '92).

However, there are many other features of pufferfish skin, peritoneal cavity, axial skeleton, and axial musculature that allow these fishes to transfigure themselves into virtual spheres. Among the pufferfishes, this spherical shape is most pronounced in the balloonfish, *Diodon holocanthus* (Fig. 1). Herein I examine the postcranial specializations of the balloonfish that contribute to its ability to inflate, become spherical, and deflate.

MATERIALS AND METHODS
Animals

Balloonfish, *Diodon holocanthus*, were obtained from an aquarium fish collector (Noyes Marine Life, Ft. Lauderdale, FL). Specimens ranged from 5.5 to 14.4 cm standard length (SL, length not including the caudal fin). They were housed in 100-liter aquaria, filled with artificial seawater at 24–28°C. Specimens used for anatomical descriptions have been deposited in the Museum of Comparative Zoology, Harvard University (catalog number MCZ101614).

The first requirement for studying the inflation mechanism was to find a reliable
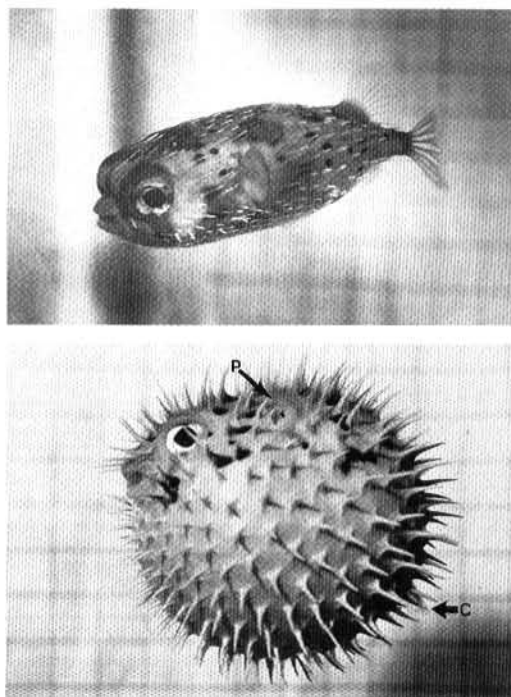

Fig. 1. *Diodon holocanthus*. Uninflated balloonfish (**top**) and balloonfish at maximal inflation (**bottom**). Note how completely spherical the animal becomes; the pectoral and caudal fins protrude, but the caudal peduncle, dorsal fin, and anal fin have been enveloped by the sphere. This individual was 10.2 cm SL. C, caudal fin; P, pectoral fin.

method for inducing the balloonfish to inflate. When the balloonfish first arrived in the laboratory, they inflated readily when held, but after a few days of acclimation, they became accustomed to being handled and became less responsive. However, I found that holding the balloonfish and applying gentle pressure on the sides of their heads was a reliable, noninjurious method for inducing inflation.

Observations of inflation behavior and pressure measurement

Four *Diodon holocanthus* individuals were used for pressure measurements and observations of inflation behavior (9.9, 10.2, 11.1, and 11.2 cm SL). Animals were anesthetized by immersion in an aqueous solution of tricaine methanesulfonate ($0.1–0.3$ g · liter^{-1}) for surgical implantation of pressure transducers. Pressure measurements from the stomach were obtained by passing a tip-sensitive pressure transducer (Millar SPR-249) into the opercular opening, through the esophagus, and into the stomach. A guide cannula (Intramedic o.d. 1.9 mm, i.d. 1.4 mm) was passed through the opercular cavity into the buccal cavity for buccal pressure measurements and a Millar SPR-249 pressure transducer was threaded down the cannula until the tip was flush with the opening of the cannula.

After surgery, the fish were placed in a small aquarium and allowed to recover for at least 1 hr. Buccal and stomach pressures for between three and eight inflations were recorded in each recording session. After three to eight inflations, the fish seemed to become fatigued and could no longer be induced to inflate. High-speed video recordings (NAC 200, 200 fields · sec^{-1}) were made along with the pressure measurements as an independent indicator of the number of buccal pump strokes (puffs) used by the fish per inflation.

To measure the total volume of water taken in during each inflation, I allowed the fish to inflate fully in an aquarium, and then quickly removed it to a beaker with a known amount of water. After it had fully deflated, the fish was removed from the beaker and the volume of water in the beaker was measured with a 500-ml graduated cylinder, marked in 5-ml increments.

Pressures were recorded on a Bell and Howell FM instrumentation tape recorder at a tape speed of 15 in · sec^{-1}, and played back on a Gould 260 chart recorder at ¼ speed for analysis.

Gross anatomy and radiography

Both dissection and frozen sections were used to observe the anatomy of uninflated *Diodon holocanthus*. The best sagittal and transverse sections came from specimens that had been fixed for about a year in 10% formalin, and then frozen and sectioned with a coping saw or hacksaw. Specimens that were frozen fresh, sectioned, and then fixed tended to disintegrate as they thawed in the formalin.

To observe the curvature of the vertebral column in an inflated balloonfish, I made X-ray plates of resting and inflated *Diodon holocanthus* (13.4 cm SL). Radiographs were taken with a Siemens radiographic unit in still mode with Industrex M film. To prevent loss of contrast as a result of the surrounding water, I removed the fish from water and placed it on wet paper towels. For radiographs of the animal in its inflated state, the balloonfish was allowed to inflate partially with water and then it was removed from the water and induced to complete its inflation with air. It was then positioned in front of the X-ray beam, perched on its ventral spines, and was prevented from rolling away by means of a string attached to a dorsal spine.

Histology

Specimens for histological examination were killed by immersion in a 1-g · liter^{-1} aqueous solution of tricaine methanesulfonate. For preparation of the skin, spines, and cutaneous musculature of an uninflated balloonfish, a small specimen (7.6 cm SL) was fixed whole in 10% neutral-buffered formalin. The fixed tissues then were removed for histological preparation. For comparison of the uninflated and the inflated states, skin and stomach samples were cut from an unfixed specimen (14.4 cm SL) and fixed in either the unstretched state or in the stretched state by pinning out the tissue in a dissecting tray. Tissue samples were washed and stored in 70% ethanol and skin samples containing spines were decalcified in formic acid. Samples were embedded in paraffin plastic polymer medium and sectioned on a rotary microtome at 6- or 10-mm thickness. Skin samples were sectioned at 45° to the longitudinal axis of the fish, in the longitudinal and transverse planes, and in a plane tangential to the slightly curved belly of the fish. Stomach samples were sectioned in the transverse plane.

Sequential sections were stained with either hematoxylin and eosin, van Gieson's collagen stain, Mallory's trichrome, or Verhoeff's elastin stain. Sections were photographed with Kodak TMAX 100 film in a Leitz Orthomate E automatic microscope camera attached to a Leitz Aristoplan microscope with a green filter in place.

Materials testing

Uniaxial mechanical tests were performed on strips of excised skin from three *Diodon holocanthus* individuals, (9.9–11.1 cm SL), one filefish (*Monacanthus ciliatus*, 7.1 cm SL), and one freshwater sunfish (*Lepomis gibbosus*, 12.5 cm SL). Fish skin is a composite material containing collagen fibers embedded in semifluid mucopolysaccharide ground substance (Roberts, '71; Hawkes, '74; Bullock and Roberts, '75; Whitear, '86). Such composite biomaterials typically exhibit viscoelastic mechanical behavior, which is a combination of solid and fluid behavior. Thus, unlike pure solids, biomaterials are sensitive not only to the total amount of deformation, but also to the rate at which deformation occurs (Wainwright et al., '76). Because of this sensitivity to strain rate, it is important to perform mechanical tests at physiologically relevant strain rates (Ker, '81).

The maximum inflation observed in this study was 3.3 times the resting body volume of the fish, resulting in a 50% skin strain in all directions. At the time the skin samples were tested, I had observed a mean inflation time of approximately 10 sec in two individuals. (The final mean after observing more individuals was 14.5 sec.) An inflation time of 10 sec, together with a total strain of 50%, corresponds to a strain rate of 5% sec^{-1}. Thus all skin samples, filefish and sunfish as well as balloonfish, were stretched and returned at 5% of rest length per second.

To obtain skin samples, fishes were killed by immersion in a 1-g $\cdot$ liter^{-1} aqueous solution of tricaine methanesulfonate, and strips of skin were removed from the abdominal area of the fishes and placed in physiological saline at 0°C. The skin samples were tested within 2 hr of removal from the fish. Because fish skin has a tendency to contract upon removal, it was necessary to mark the skin before excising it to determine the correct initial length when the strip of skin was placed in the testing apparatus. Two small loops of suture thread (4.0 silk) were tied into the outermost layer of the skin 1 cm apart.

Care was taken that the sutures did not penetrate the dermis (in the case of balloonfish skin) or were outside the working area (in the case if the other skins) so that they would not influence the mechanical properties of the tissues. Once the sutures were in place, 2×1-cm strips of skin were removed. When ready for testing, the ends of the strips were placed in clamps lined with emery paper and secured with screws. The resulting working length between the clamps was 1.1–1.5 cm. The initial length of the skin was set so that the distance between the sutures was the same as it had been in the animal, and rest length was measured as the distance between the clamps. The skin samples were tested uniaxially in the circumferential direction (hoop direction, sensu Wainwright et al., '78).

Because the cross-sectional area of the skin decreased as the skin was stretched, the stress was calculated as the force per cross-sectional area at each length. The volume of the strip was assumed to remain constant, thus allowing the instantaneous cross-sectional area of the sample to be calculated for any length. The volume of test pieces was determined by weighing the working area after cutting it from the clamps and calculating its volume on the basis of the skin's density (*Diodon holocanthus* 1.15 g $\cdot$ cm^{-2}, *Monacanthus ciliatus* 1.13 g $\cdot$ cm^{-2}, *Lepomis gibbosus* 1.09 g $\cdot$ cm^{-2}).

The skin samples were stretched by a rotating arm controlled by a servo motor (Cambridge Technology, Series 300, dual mode servo). Force was measured at the fixed end by a Kistler 9207 force transducer. The length output of the servo motor and the force output from the Kistler were recorded simultaneously on a Nicolet model 201 digital oscilloscope and down-loaded to a computer.

The length recorded represented the interclamp distance rather than a more accurate intermediate strain value. In mechanical tests of most soft tissues, such as tendon and ligament, the interclamp distance has been found to overestimate the actual strain as a result of slippage in the clamps (Ker, '81). However, when comparing the interclamp strain with intermediate strain obtained from video images, I observed the opposite result in balloonfish skin. The inter-clamp strain underestimated the actual strain by a mean of 7% (standard deviation ± 5%, 3 skin samples, 3 tests per sample). Video images showed that the clamps restricted the contraction of the tissue in the direction orthogonal to the test.

This restriction made the skin in the region of the clamps less extensible, and reduced the pparent extensibility of the tissue. Thus the extensions reported here for balloonfish skin somewhat underestimate the actual extensions. Nonetheless, these tests are sufficient to support the primary conclusion drawn from them—namely, that balloonfish skin is many times more extensible than other fish skin. The curves for filefish and sunfish skin are more accurate because the extensions, and therefore the orthogonal contractions, were smaller.

RESULTS
Observations of inflation behavior

During inflation, pufferfishes pump water into their stomachs with rhythmic buccal cavity expansions and compressions. In *Diodon holocanthus,* numerous mouthfuls or "puffs" of water are required to complete inflation. On buccal pressure traces, the individual puffs are visible as cycles of falling then rising pressure (Fig. 2). Individual peaks in stomach pressure, when plotted on the same scale as the buccal pressure, are barely visible during the early stages of inflation. When amplified, however, the pressure recordings reveal a small increase in stomach pressure corresponding to each puff. In the early stages of inflation, before the balloonfish nears its maximum volume and the pressure magnitudes increase, the magnitude of the buccal pressure peaks is in the range of 5–10 mm Hg, whereas the stomach pressure peaks are less than 2 mm Hg.

The number of individual puffs and the time required for a balloonfish to inflate completely can be read from buccal pressure traces (e.g., Fig. 2) or observed in video recordings of inflation. Within the small size range of *Diodon holocanthus* individuals observed, there was no significant effect of body size on the number of puffs or on the time required for a balloonfish to inflate completely (two factor ANOVA: number of puffs: $F_{(3,42)} = 1.44$, NS; inflation time: $F_{(3,34)} = 1.91$, NS). The mean number of puffs for the four individuals observed was 37 ± 8, n = 46 inflations (mean $\pm$ 1 standard deviation). The mean inflation time was 14.5 ± 4.1 sec, n = 38 inflations. When normalized for the initial volume of the animal, the volume change also was not significantly different for different-sized animals ($F_{(1,12)} = 0.40$, NS; initial volume not available for two of the four individuals). The mean volume change was 3.1 ± 0.2 times rest volume, n = 14 inflations, and the maximum observed volume increase was 3.3 times rest.

As long as the balloonfish were held in a tight grip, they remained inflated. When I loosened the grip, they usually deflated and attempted to swim away. If the fish had eaten immediately before inflation, food in the stomach often was regurgitated along with the expelled water. If 5 min was allowed to pass between feeding and inflation and if only a small amount of food was ingested, food was not regurgitated. If a large amount of food was ingested, food was still regurgitated 20 min after feeding. Deflation generally took about twice a long as inflation. The quickest deflation observed, however, was 12 sec, which was in the range of the most rapid inflations.
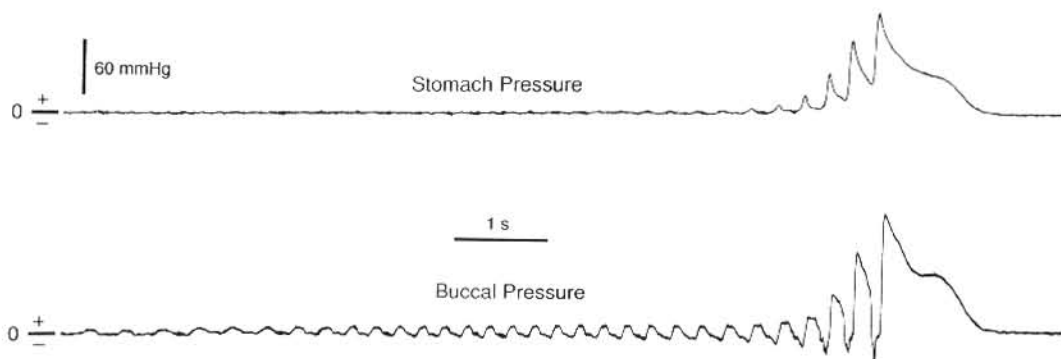

Fig. 2. *Diodon holocanthus.* Buccal cavity and stomach pressure during inflation. Each individual peak in the buccal cavity pressure reflects one mouthful of water being pumped into the stomach. Zero pressure is the ambient hydrostatic pressure at the level of the transducer.

Attainment of spherical shape

The peritoneal cavity and peritoneum of *Diodon holocanthus* are specialized to allow the balloonfish to become completely spherical. In an uninflated balloonfish, the peritoneal space extends into the posteroventral and posterodorsal quarters of the fish. The right and left sides of the peritoneal cavity meet at the middorsal line, but the peritoneal spaces do not connect. Thus, the peritoneal cavity completely surrounds the epaxial musculature (Fig. 3, dorsal peritoneal space).
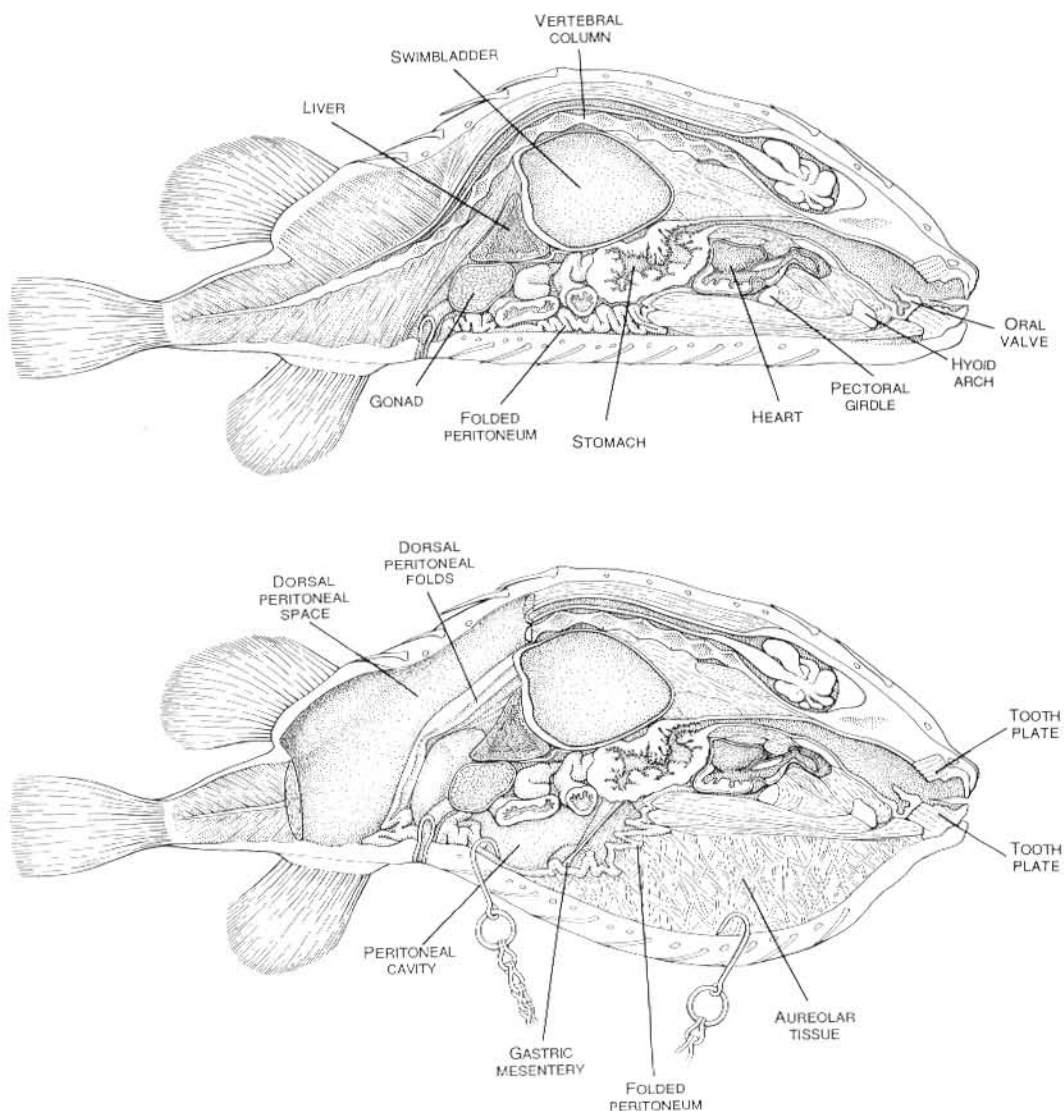

Fig. 3. *Diodon holocanthus*. Drawing of a sagittal section through an uninflated balloonfish (**top**) shows the folded peritoneal lining, the folded, papillated stomach, and the absence of median hemal processes on the abdominal vertebrae. Sagittal section with the ventral skin retracted and a portion of the dorsal musculature removed (**bottom**) reveals the potential expansion of the peritoneal cavity. During inflation, the stomach expands dorsad into the dorsal peritoneal space surrounding the body musculature and ventrad and craniad to the tip of the mandible. (This individual was preserved with an unusually kyphotic curvature of the vertebral column. In a live resting balloonfish, the vertebral column is straighter, and becomes more kyphotic during inflation.) This individual was 11.0 cm SL.

When the balloonfish inflates, the peritoneal cavity becomes even more extensive. Large folds in the peritoneum allow the stomach and peritoneal cavity to expand craniad to the tip of the mandible and caudad to engulf the unpaired fins (peritoneal folds, Fig. 3). Loose connective tissue (aureolar tissue) eases the expansion and unfolding of the peritoneum. Loose skin at the base of the unpaired fins allows the skin, peritoneum, and stomach to stretch over the dorsal and anal fins and the caudal peduncle, swallowing them into pockets of tissue (Fig. 4). Thus, when a balloonfish is completely inflated, the peritoneal cavity extends craniad to approach the tip of the mandible, dorsad to surround the body muscles, and caudad to engulf the unpaired fins.

The structure of the vertebral column also contributes to the balloonfish's ability to become spherical. When the fish is in the inflated state, the vertebral column is bent in a kyphotic curve (concave side toward the ventrum of the fish). This flexibility in the dorsoventral plane allows the balloonfish's body to curve around the ventral sack of fluid (Fig. 5). Balloonfish vertebrae have no ventral processes on the first to the 11th vertebrae (Fig. 3; Tyler, '80). The 12th vertebra has small ventrolateral flanges. The 13th to the 17th form a functional unit supporting the dorsal and anal fins. The intervertebral joints be-
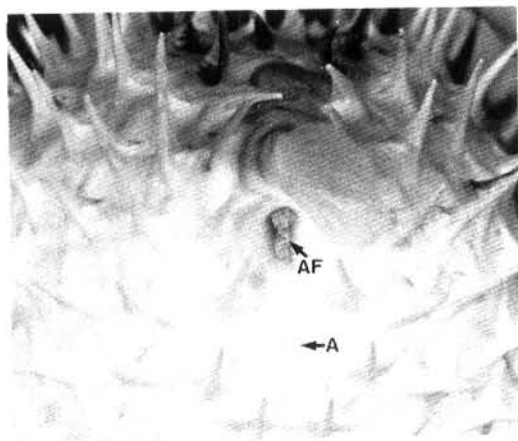

Fig. 5. *Diodon holocanthus.* X-ray plate of a live, uninflated (**top**) and inflated (**bottom**) balloonfish. The specimen was photographed in air. In the uninflated balloonfish, white tooth plates are visible on the far left and the dark area in the center of the fish is the gas bladder. The fish was allowed to inflate partially in a bucket of water and then removed to air and forced to inflate the rest of its volume with air. Thus, the ventral third of the sphere appears white because it is filled with water. Dorsal to the water is air, and dorsal to the air, the balloonfish's body is wrapped around the sphere. With careful examination, the vertebral column and anal fin base supports are visible in the inflated fish. This individual was 13.4 cm SL.

Fig. 4. *Diodon holocanthus.* Caudal view of a partially inflated balloonfish showing the anal fin in the process of being folded and enveloped by the expanding sphere. Loose tissue at the base of the fins expands and surrounds the anal fin, dorsal fin, and caudal peduncle, until the anal fin and caudal peduncle completely disappear and the dorsal fin is only slightly visible. This individual was 13.4 cm SL. A, anus; AF, anal fin.
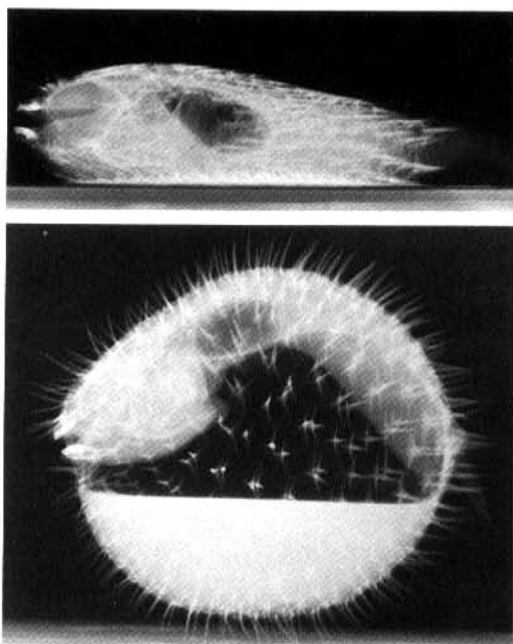
tween 13 and 17 are relatively inflexible, and the hemal spines and ventral fin supports (proximal anal fin pterygiophores) arising from these vertebrae prevent flexion of this region. Because this region does not flex during inflation, the vertebral column in the inflated animal is bent in a kyphotic curve in the abdominal region but changes direction to a slightly lordotic curve (concave side dorsad) in the caudal region (Figs. 3, 5).

Extensibility of balloonfish stomach and skin

Although the increase in volume of the whole balloonfish is about three times initial volume, the stomach volume increases approximately 50- to 100-fold, because it starts out proportionately much smaller than the volume of the whole fish. (An 11-cm fish has

an initial stomach volume of approximately 3 cm^3, as estimated from the dimensions of the stomach lumen in frozen sections, and the maximum inflation volume recorded for an 11.1-cm individual was 270 ml.) In the uninflated state, the stomach wall of *Diodon holocanthus* is thick and the surface surrounding the lumen is repeatedly folded upon itself (Figs. 3, 6). In an 11-cm fish, folding on three length scales can be recognized: large folds approximately 3 mm wide (similar to the rugae seen in most fishes' stomachs); smaller folds approximately 1 mm wide; and covering the small folds are ridges approximately 50–100 μm wide. By stretching a piece of balloonfish stomach, I observed the 3-mm and 1-mm levels unfolding under a dissecting microscope. The 100-μm ridges also unfold during stomach stretching, as shown by their disappearance in histological sections of stretched stomach (Fig. 6).

The skin of *Diodon holocanthus* is not deeply folded on either its inner or outer surface. Small ridges are apparent on the outer surface, but these are too small and superficial to account for the skin's great extensibility. Despite the apparent absence of folds, the results of mechanical tests show that balloonfish skin behaves as though it were initially folded. Balloonfish skin can be extended through 40% of its initial length before it begins to stiffen (Fig. 7). In contrast, the force required to stretch the abdominal skin of a freshwater sunfish, *Lepomis gibbosus*, rises rapidly at small extensions (Fig. 7). Filefishes (e.g., *Monacanthus ciliatus*) have a rod-like pelvis, which can be depressed to increase body depth when threatened by a predator. The skin covering the filefish pelvis is more extensible than sunfish skin (Fig. 7), and its stress-strain curve shows the same general shape as the curve for balloonfish skin—the stress needed to produce extension rises very slowly at first and subsequently rises rapidly.

Balloonfish skin has three histologically distinct layers: the epidermis, the *stratum laxum* of the dermis, and the *stratum compactum* of the dermis. The epidermis is a thick, multilayered epithelium containing many mucous cells. Histological sections of unstretched skin show that the epidermis forms ridges that do not penetrate into the underlying *stratum laxum* (Fig. 8 top). The *stratum laxum* of unstretched skin contains wavy, disorganized collagen fibers, a few elastin fibers, and numerous pigment cells and blood

vessels. In sections of stretched skin, the epidermal ridges disappear, the epidermis becomes considerably thinner, and the wavy collagen fibers straighten and become more uniformly oriented (Fig. 8 bottom).

Three-fourths of the thickness of balloonfish skin consists of the *stratum compactum* of the dermis. This layer is composed entirely of thick bundles of densely packed collagen fibers. A tangential section along one layer of these fibers reveals that the fiber bundles are deeply folded when the skin is in its unstretched state (Fig. 9 top). When the skin is stretched, the folds pull out, and the underlying oblique orientation of the fibers is revealed (Fig. 9 bottom). Thus, although there are no macrofolds in balloonfish skin, the microfolds in the epidermis and in the collagen fibers of the dermis account for the skin's extensibility.

Modified scales in the form of long, erectile spines are embedded in the skin of puffers in the genus *Diodon*. When *Diodon holocanthus* is uninflated, the spines lie flat against the fish's body, but when inflated, the spines point at right angles to the body (Figs. 1, 5). The spines have triradiate bases: one short, axial process and two long, flattened, lateral processes orthogonal to the axis of the spine (Fig. 10). On a resting balloonfish, the spine points caudad, the axial process of the base points craniad, and the lateral processes point around the circumference of the fish. Histological sections through the long axis of a spine show that collagen fibers attach to the axial process (Fig. 10). In the uninflated state, the collagen fibers originate on the tip of the anterior pointing axial process and curve around toward the inside of the skin.

Deflation mechanism

During deflation, *Diodon holocanthus* exhibits two distinct behaviors. Early in deflation (phase 1) balloonfish simply open their mouths and water flows out. Later in deflation (phase 2) after most of the water has been expelled, the fish close their mouths, expand the buccal cavity, and arch their backs in an apparent attempt to remove the last of the water from their stomachs. One might expect that skin elasticity would contribute to the expulsion of water in phase 1. However, mechanical tests of skin elasticity show that the skin is almost perfectly inelastic. Upon release after stretching, stress in the skin drops rapidly to zero, indicating that the skin is exerting little force to help it return to its original length (Fig. 11).
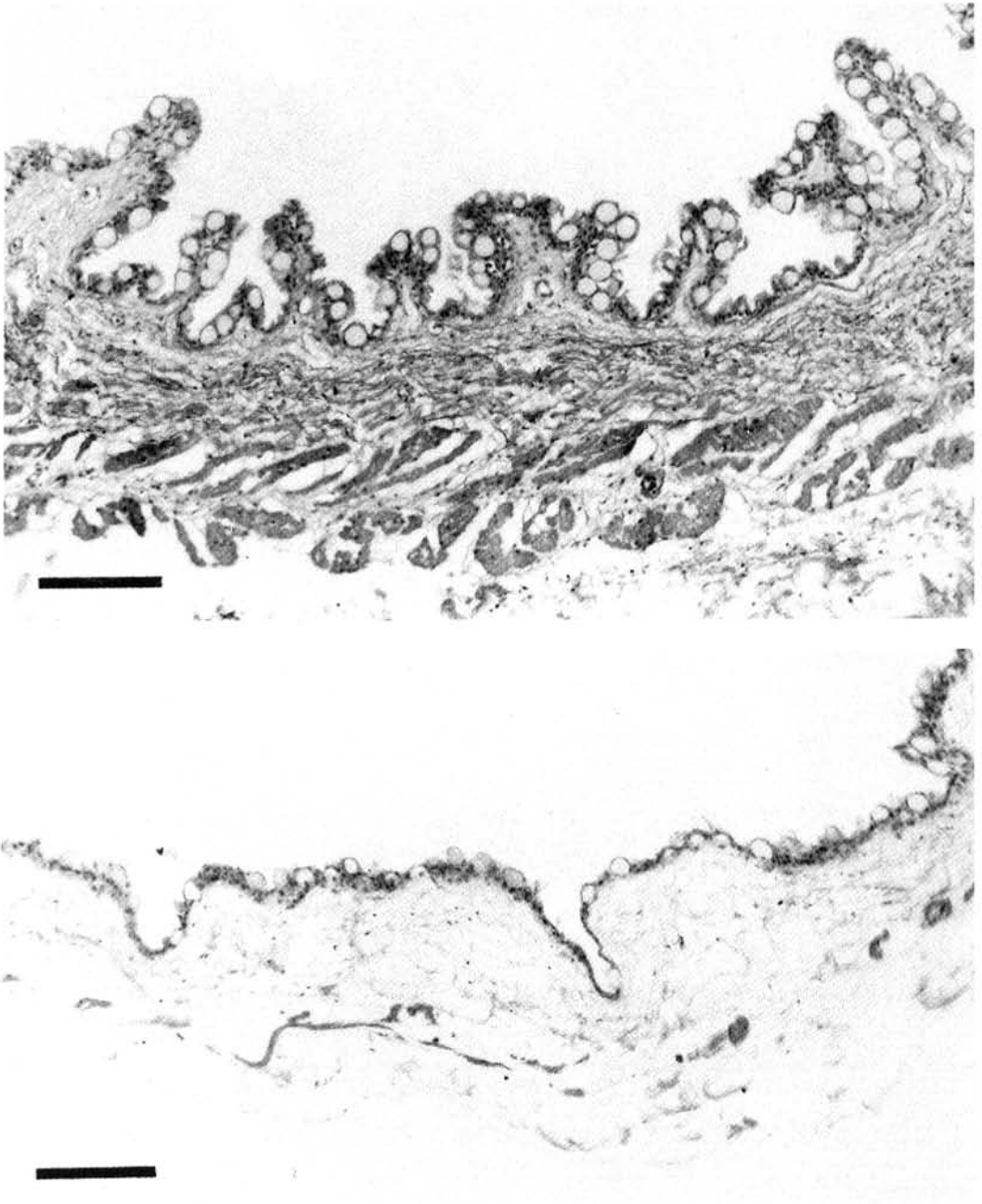
Fig. 6. *Diodon holocanthus*. Transverse sections through unstretched (**top**) and stretched (**bottom**) stomach. Sections were cut at 6-μm thickness, stained with hematoxylin and eosin, and photographed through a green filter. The lumen of the stomach is toward the top of both photos. The unstretched stomach shows 50- to 100-μm-diameter ridges and thick epithelial, collagenous, and smooth muscle layers. The stretched stomach shows the disappearance of the ridges and the thinning of the epithelial, collagenous, and smooth muscle layers. Scale bars = 100 μm.
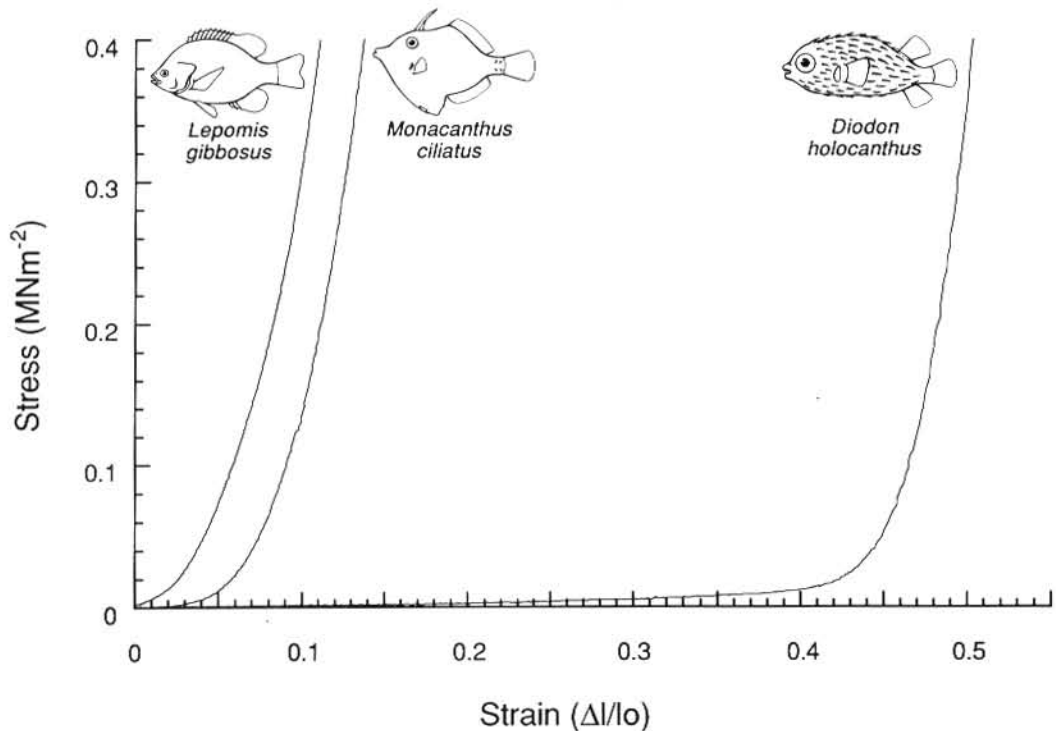
Fig. 7. *Diodon holocanthus, Monacanthus ciliatus,* and *Lepomis gibbosus* skin mechanics. Skin samples were taken from the abdominal area of each fish and tested uniaxially in the circumferential direction.

Phase 1 deflation may be effected by contraction of transverse and longitudinal cutaneous musculature. The *m. cutaneus transversalis* lies just below the skin and consists of thin, short fascicles embedded in connective tissue (Fig. 12). During dissection, this layer often comes off with the skin. The transverse layer appears to have some connective tissue ties to the skin, but histological sections show no muscular connections to the spines. The *m. cutaneus longitudinalis* lies medial to the transverse layer. It consists of thicker fascicles running from the skin surrounding the mouth to a band of connective tissue surrounding the anus (Fig. 12).

When most of the water has been expelled, balloonfish begin phase 2 deflation. The fish closes its mouth and expands the buccal cavity, causing the pressure in the mouth cavity to drop and thus drawing water out of the stomach (Fig. 13). Water then is expelled from the mouth by buccal cavity compression; this cycle is repeated several times. Simultaneous with the reversal of the buccal pump, the fish also appear to arch their bodies in a lordotic curve. Whether this action forces water out, or simply provides a straight path for water to be expelled, is unknown.

DISCUSSION

The following structural and functional features contribute to the efficacy of inflation as a mechanical defense in *Diodon holocanthus:* 1) extensive peritoneal cavity, 2) folded and extensible peritoneum, 3) absence of pleural ribs, 4) absence of a pelvic girdle, 5) dorsoventral flexibility of the vertebral column, 6) multiply folded and thick-walled extensible stomach, 7) extensible skin containing microscopically folded collagen fibers, 8) underlying helical arrangement of dermal collagen fibers, 9) erectile spines, and 10) subcutaneous muscles for deflation. Although these features serve important functions in balloonfish inflation and defense, an analysis of their evolutionary history and anatomical structure is necessary to determine whether they may be true evolutionary adaptations for in-
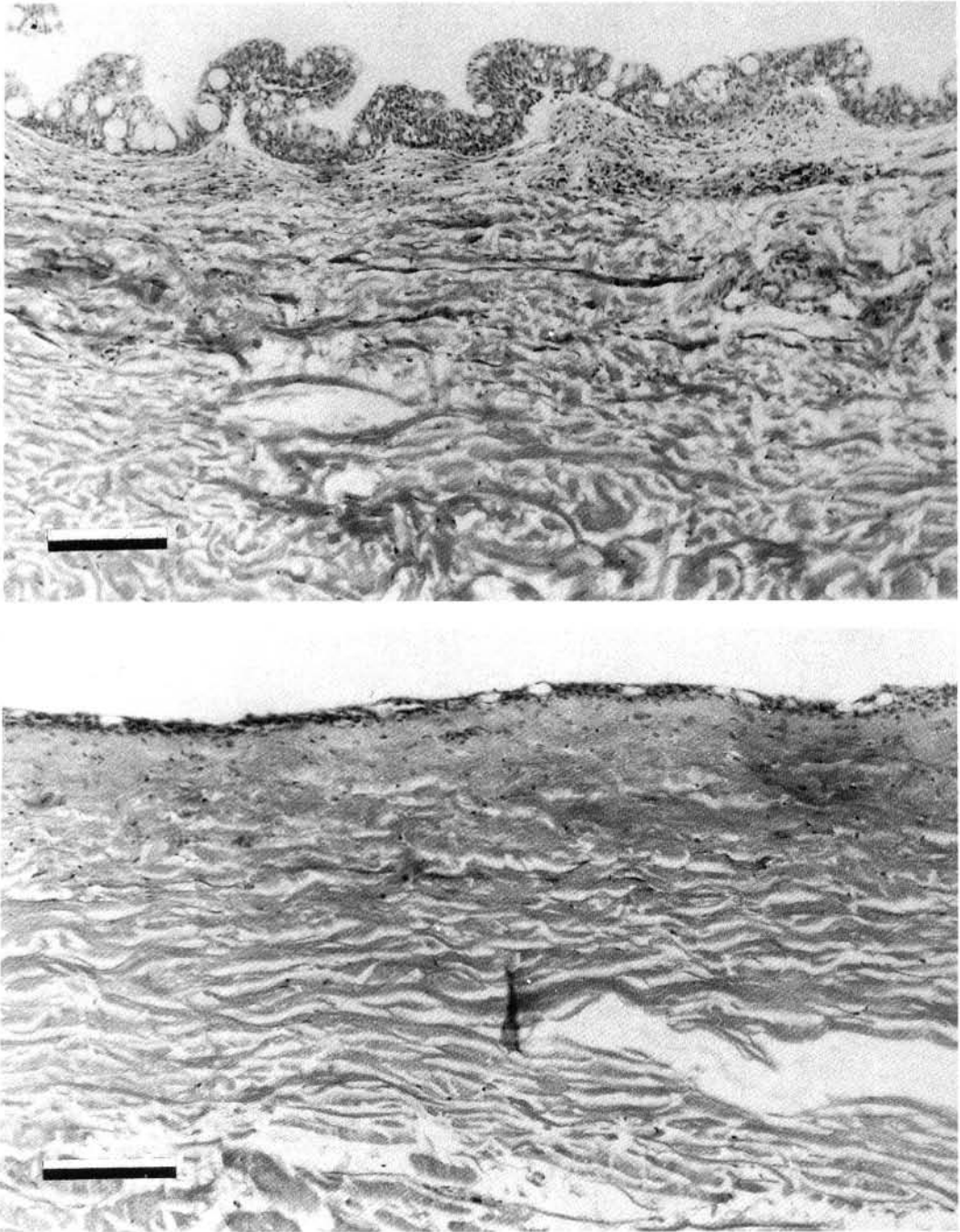
Fig. 8. *Diodon holocanthus*. Cross-sections through the outer layers of unstretched (**top**) and stretched (**bottom**) balloonfish skin. The skin was sectioned at approximately 45° to the long axis of the fish. Sections were cut at 6-μm thickness, stained with hematoxylin and eosin, and photographed through a green filter. Unstretched balloonfish skin shows superficial ridges in the epidermis and wavy, disorganized collagen fibers in the outer layer of the dermis, the *stratum laxum*. Stretched skin shows the disappearance of the epithelial folds, the thinning of the epidermis, and a decrease in the wavy appearance of the collagen fibers in the *stratum laxum*. Scale bars = 100 μm.
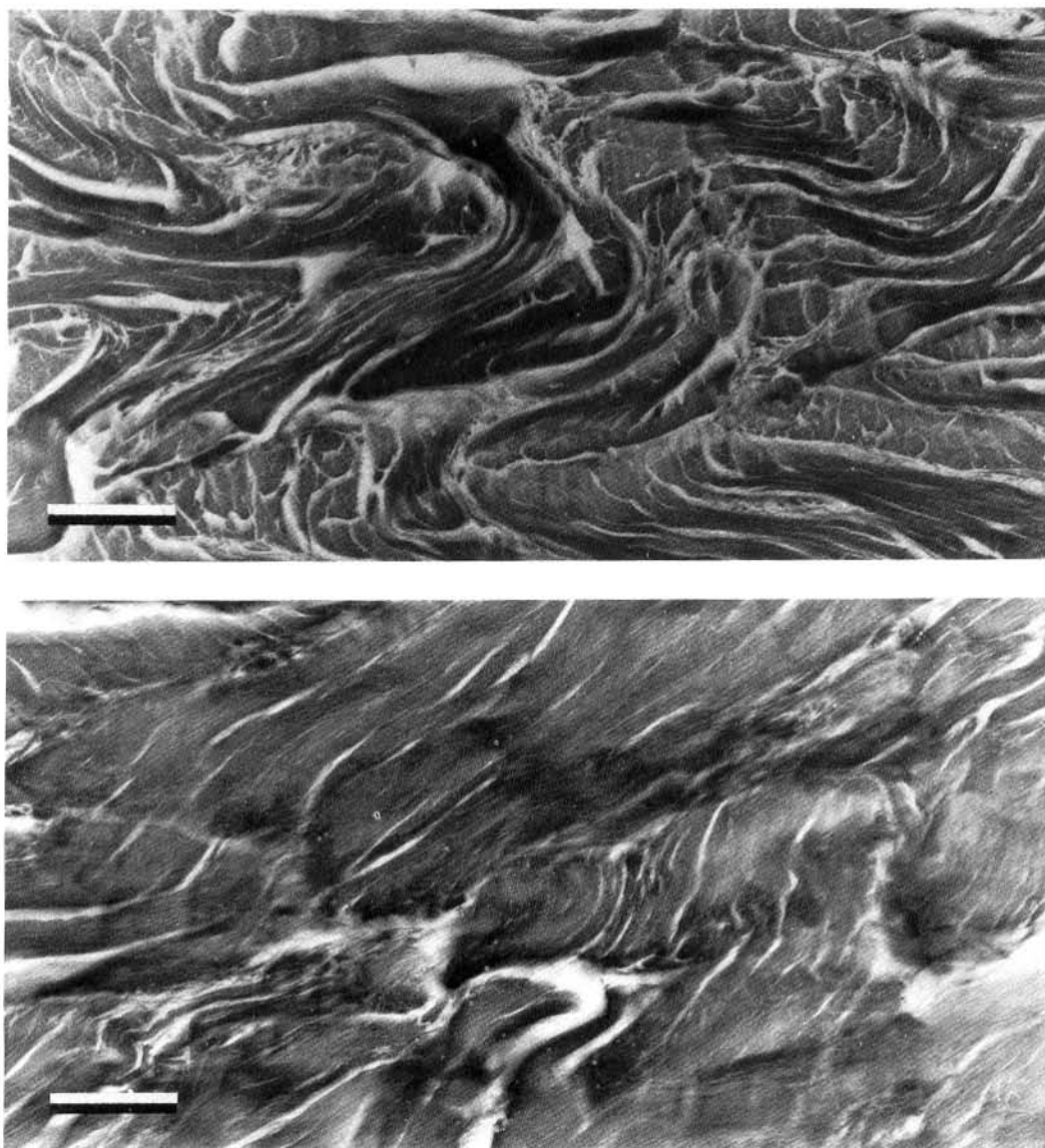
Fig. 9. *Diodon holocanthus*. Tangential sections through the inner layer of the dermis, the *stratum compactum*, of unstretched (**top**) and stretched (**bottom**) balloonfish skin. Sections were cut at 6-μm thickness, stained with Verhoeff's elastin and collagen stain, and photographed through a green filter. The long axis of the photographs corresponds to the anterior-posterior axis of the fish. The unstretched balloonfish skin shows folded bundles of collagen fibers, whereas the stretched skin shows the disappearance of the collagen folds and the oblique orientation of the unfolded fibers. Scale bars = 50 μm.

flation. In order to be a true adaptation for inflation, a feature must be a shared, derived character of the puffers. If the feature is found to have been present in a non-inflatable ancestor of the puffers, it must be considered an exaptation rather than an adaptation for inflation (Gould and Vrba, '82). However, even if a feature serves a function in inflation and is present only in the puffers, it still may not be a specific adaptation for inflation. Synapomorphy, while a necessary condition, is not a sufficient condition for determining
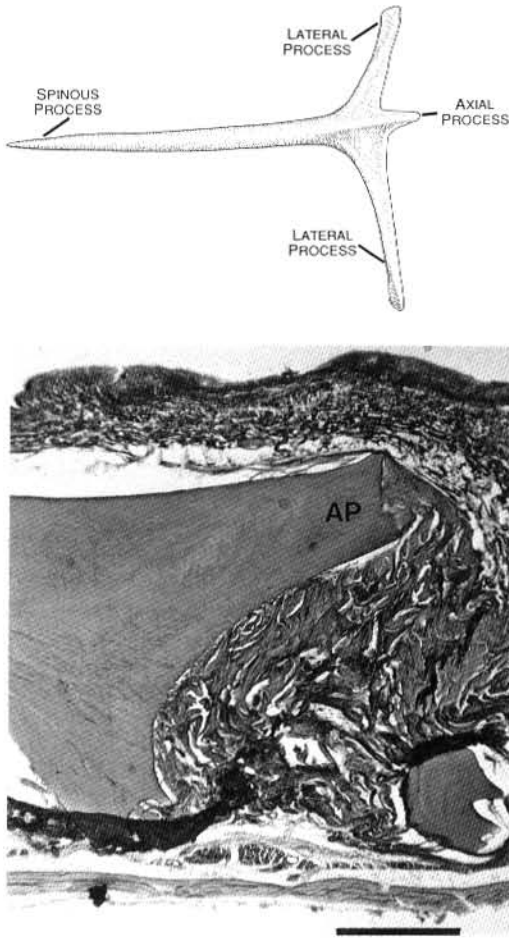
Fig. 11. *Diodon holocanthus.* Extension and return curves for balloonfish skin. Abdominal skin samples were tested uniaxially in the circumferential direction. The samples were stretched and returned at the same strain rate (5% of rest length per second). These curves show little potential for energy storage in the skin to power deflation because only a small force, and therefore little energy, is required to extend the tissue through most of its range. In the high range of extension, 40–50% strain, more energy is input to stretch the skin, but the return curve is well below the extension curve, suggesting that most of the input energy is lost.

Fig. 10. *Diodon holocanthus.* Spine erection mechanism. Drawing of one spine (**top**) and a longitudinal section through the base of a spine (**bottom**). Anterior is to the right, external layers of the skin are at the top, internal layers below. Collagen fibers originate from the axial process of the spine base and curve around medial to the spine. This section was cut at 10-μm thickness and stained with Verhoeff's elastin and collagen stain. AP, axial process. Scale bar = 500 μm.

adaptation, and further cladistic tests for adaptation would be required to confirm that a character is an adaptation for inflation (Coddington, '88). In addition, if a feature can be viewed as the necessary structural result of other anatomical changes, then it may be an epiphenomenon rather than an adaptation (Gould and Lewontin, '79).

The balloonfish abdominal cavity: adaptation, exaptation, and epiphenomena

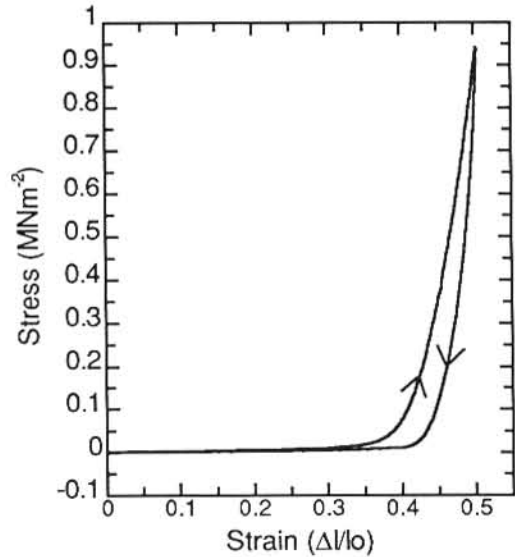The balloonfish's extensive peritoneal cavity and its folded and extensible peritoneum are features of the abdominal cavity that contribute to the animal's ability to become completely spherical. During inflation, the balloonfish stomach expands into the existing peritoneal space surrounding the axial musculature and presses the folded peritoneum out into the potential spaces ventral to the head and surrounding the dorsal fin, anal fin, and caudal peduncle (Figs. 3–5). When a balloonfish is completely inflated, the peritoneal cavity extends around its entire body— only the dorsal and lateral surfaces of the head are unaffected. In contrast, the peritoneal cavity of non-inflatable tetraodontiform fishes (e.g., Balistidae, Fig. 14) is limited to the anteroventral quarter of the body, with the anterodorsal quarter and posterior half devoted to axial musculature. In these non-inflatable fishes, firm attachments between the skin and the axial musculature prevent expansion of the peritoneal cavity. If the balloonfish were constructed in this way, the position of the peritoneal cavity and the dermal attachments would limit expansion to
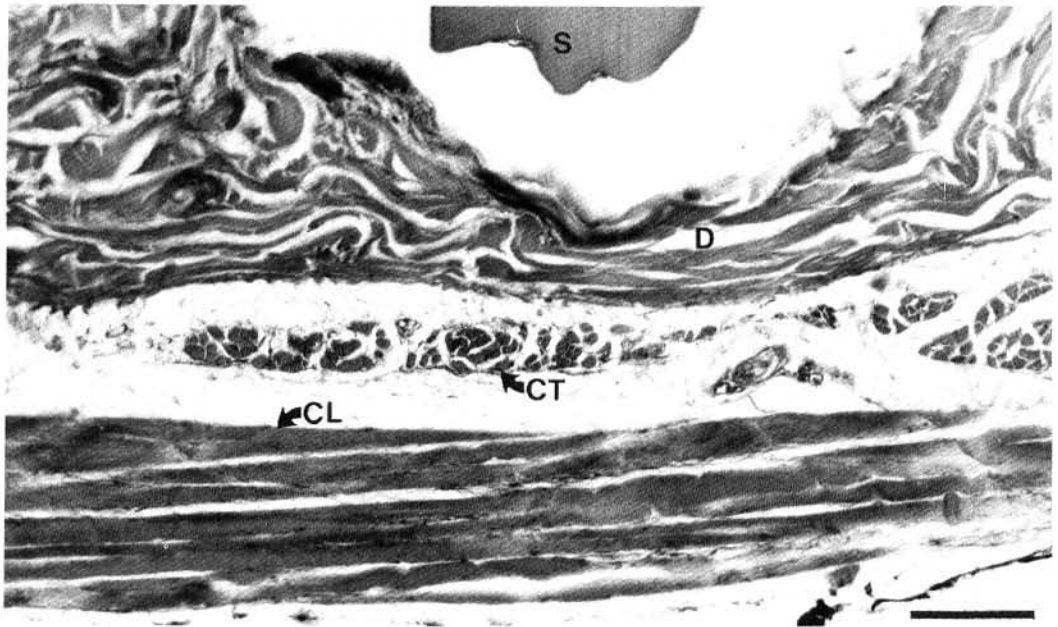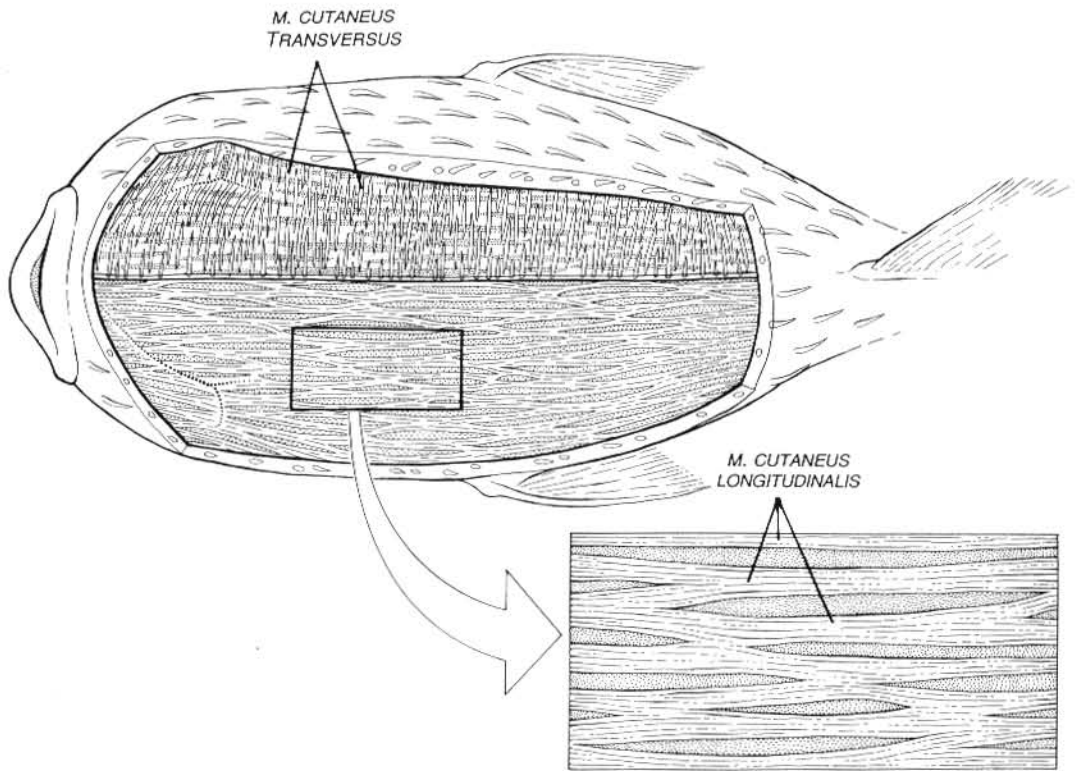
Fig. 12. *Diodon holocanthus* cutaneous musculature. Drawing of (**top**) and longitudinal section (**bottom**) through the two layers of balloonfish cutaneous musculature. The skin has been removed in the upper portion of the drawing to reveal the most superficial layer of the cutaneous musculature, *m. cutaneus transversalis*. In the lower portion of the drawing, the *m. cutaneus transversalis* has been removed to reveal the *m. cutaneus longitudinalis*. The longitudinal section is mounted to correspond to the dissection above—external is top and internal is bottom. Because this section was cut in the longitudinal direction, transverse cutaneous fiber bundles appear in transverse section and longitudinal cutaneous bundles appear in longitudinal section. This section was cut at 10-μm thickness, stained with Verhoeff's elastin and collagen stain, and photographed through a green filter. CL, *m. cutaneus longitudinalis;* CT, *m. cutaneus transversalis;* D, dermis; S, spine. Scale bar = 100 μm.
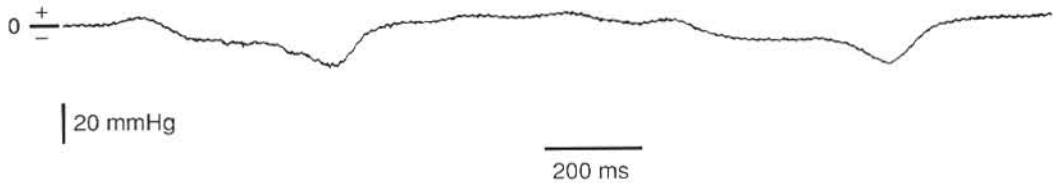
Fig. 13. *Diodon holocanthus*. Buccal cavity pressure during phase 2 deflation. Reversal of the mouth pump causes a reduction in buccal cavity pressure, thus drawing water out of the stomach. Two reversals appear in this trace. Zero pressure is the ambient hydrostatic pressure at the level of the transducer.

the anteroventral quarter of the body. Thus, the fish might develop a central bulge, but the head and most of the body would stick out as convenient protrusions for a predator to seize.

It is reasonable to hypothesize that the position and extensibility of the balloonfish peritoneal cavity evolved as adaptations for inflation. These features are shared, derived characters found only in the pufferfishes (characters 1 and 2, Fig. 14) and, thus, meet a primary criterion for determining whether a character is an adaptation (Coddington, '88; Gould and Vrba, '82).

In contrast, the absence of skeletal elements surrounding the abdominal cavity are not synapomorphic for pufferfishes, and thus are not adaptations for inflation (characters 3 and 4, Fig. 14). The absence of pleural ribs is a character shared by all fishes in the order Tetraodontiformes (with the exception of *Triodon*). Loss of the pelvic girdle is shared by both the pufferfishes and the non-inflatable Molidae. The phylogenetic distribution of these two characters (3 and 4 in Fig. 14) is evidence that the absence of ribs and a pelvis are characters that were already present in the non-inflatable ancestor of the pufferfishes. Thus, I conclude that these skeletal loss characters are exaptations rather than adaptations for inflation (Gould and Vrba, '82).

One feature of balloonfish, dorsoventral flexibility of the vertebral column (character 5, Fig. 14), may be an epiphenomenon of the lengthening of the body cavity rather than a specific adaptation for dorsoventral flexibility. The absence of ventral, median processes (hemal processes) on the anterior three quarters of its vertebral column allows the balloonfish to bend its body in the dorsoventral plane and curl around the ventral ball of fluid (Fig. 5). Because this feature contributes to a balloonfish's ability to become spherical, and is synapomorphic for pufferfishes, I initially

concluded that it might be an adaptation for inflation. However, the expression of this character is closely linked with the lengthening of the abdominal cavity (character 1). Because all fishes lack hemal processes on their abdominal vertebrae, lengthening the abdominal cavity necessarily results in an increased proportion of the vertebral column lacking hemal processes. Thus one might conclude that dorsoventral flexibility is an epiphenomenon of lengthening the body cavity. However, it is also true that lengthening the body cavity is one means of producing dorsoventral flexibility and, thus, lengthening the body cavity might be an epiphenomenon of selection for dorsoventral flexibility. This argument demonstrates a difficulty with applying the concept of an epiphenomenon to morphological systems. Without knowledge of the selection regime that produced these features, it is difficult to know a priori whether one of these characters was the primary target of selection or whether synergistic selection for both may have hastened the development of the character complex.

Thus I am reduced to speculation about the evolution of these characters. Lengthening the body cavity to engulf the tail during inflation is an extremely useful feature that prevents seizure of the tail by a predator. Although dorsoventral flexibility of the vertebral column contributes to the balloonfish's impressive sphericity, I do not believe that this feature contributes as much to the mechanical defense as does body cavity lengthening. Thus I conclude that dorsoventral flexibility of the vertebral column is more likely an epiphenomenon of body cavity lengthening than the converse.

Mechanical design of balloonfish stomach, skin, and spines

Extreme skin and stomach extensibility are synapomorphic for the pufferfishes (characters 6 and 7, Fig. 14). In histological sec-
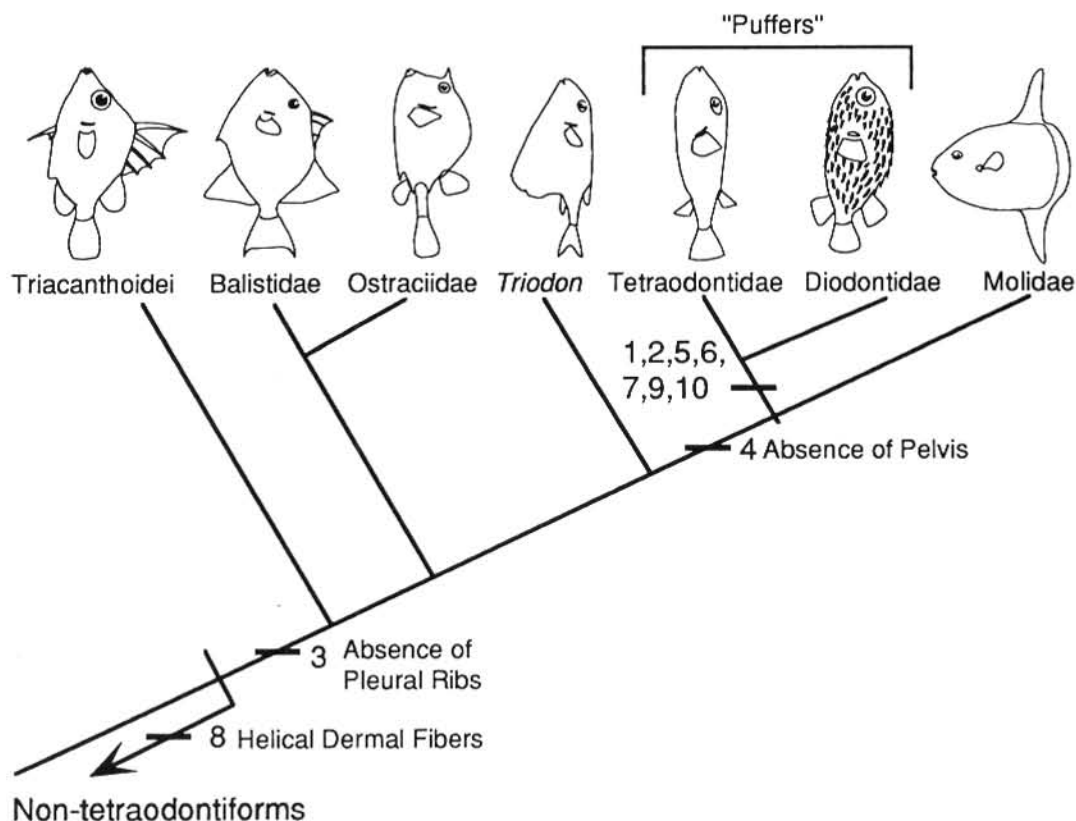
Fig. 14. Phylogenetic distribution of functional anatomical characters that contribute to body inflation in pufferfishes. Phylogenetic hypothesis of tetraodontiform familial relationships is based on work of Winterbottom ('74b) and Tyler ('80). Seven of these characters are shared, derived characters for pufferfishes: 1) extensive peritoneal cavity; 2) folded and extensible peritoneum; 5) dorsoventral flexibility of the vertebral column; 6) multiply folded and thick-walled extensible stomach; 7) extensible skin containing microscopically folded collagen fibers; 9) erectile spines; and 10) subcutaneous muscles for deflation. Character 5, dorsoventral flexibility of the vertebral column, is synapomorphic for puffers, but may be an epiphenomenon of lengthening the abdominal cavity (character 1). Character 4, absence of a pelvic girdle, is shared by the puffers and the ocean sunfishes, Molidae. Character 3, absence of pleural ribs, is synapomorphic for all Tetraodontiformes. Character 8, helical arrangement of dermal collagen fibers in the skin, is shared by all vertebrates and many invertebrates. (Line drawings of fishes after Tyler, '80; Nelson, '84.)

tions, balloonfish stomach differs from the stomachs of other fishes. The stomach wall of most fishes is bordered on the luminal side by a simple, unstratified cuboidal epithelium, and adjacent to the epithelium is a thick layer of tubular gastric glands (Groman, '82; Yasutake and Wales, '83). The epithelium of unstretched balloonfish stomach is thick and stratified, and is similar in appearance to the extensible transitional epithelium of mammalian urinary bladders (Andrew and Hickman, '74). When stretched, the epithelium becomes thin, and the individual cells change shape from cuboidal-columnar to discoid (Fig. 6). Balloonfish stomach lacks tubular gastric

glands and seems to have no digestive function. However, the stomach may sometimes serve for food storage. Large amounts of food remain in the stomach for some time, although small amounts are passed from the stomach within 5 min. Abundant epithelial mucous cells, also common in the gastrointestinal tracts of other fishes, most likely serve to lubricate the unfolding of the multiply folded stomach wall. These mucous cells also may contribute the balloonfish's ability to pass small amounts of food rapidly from the stomach to the intestine.

Balloonfish skin is dramatically more extensible than the skin of non-inflatable tetra-

odontiform fishes. Triggerfishes and file-fishes (Balistidae) exhibit modification of the skin covering their mobile pelvic girdles, but their skin is still more similar to the skin of a freshwater sunfish than to the skin of a balloonfish (Fig. 7). Balloonfish skin can be stretched through most of its working length before developing an appreciable amount of stress. Thus, little force is required to stretch the skin during inflation, a feature which may be important in the balloonfish's ability to inflate rapidly. If a muscle must generate large forces during contraction, the velocity at which that muscle can contract is reduced (McMahon, '84). Because the muscles of the buccal pump provide the force to stretch the skin during inflation, it is likely that high skin compliance permits the buccal pump to operate at higher speeds than if it were acting against stiff skin.

The stiffness of balloonfish skin increases suddenly at high extensions (Fig. 7). This increase in skin stiffness is reflected in pressure traces from the buccal cavity and stomach. As the skin becomes stiff toward the end of inflation, the pressure magnitude of the individual "puffs" becomes increasingly greater as more force is required to stretch the skin (Fig. 2). The structural basis for this sudden increase in skin stiffness can be seen in histological sections of unstretched and stretched skin. In most fishes, the dermal collagen fibers are arranged in long, flat sheets wrapped in cross-helices about the long axis of the body (Hebrank, '80; Hebrank and Hebrank, '86; Wainwright et al., '78). In unstretched balloonfish skin, the collagen fiber bundles are wavy in appearance (Fig. 9; Mittal and Banerjee, '76). As the skin is stretched at low strains, the unfolding of the waves and the deformation of the surrounding ground substance require little force. At high strains, the fibers are stressed directly and the skin becomes stiff.

Sections of stretched balloonfish dermis reveal that the underlying orientation of the collagen fibers, once the waves pull out, is oblique to the long axis of the fish (Fig. 9 bottom). This orientation is a remnant of the cross-helical wrapping observed in non-inflatable fishes (character 8, Fig. 14). As in the skin of other fishes, cross-helical wrapping results in orthogonal layers of collagen fibers in the balloonfish dermis. In non-inflatable fishes, these orthogonal layers provide a constant-volume, flexible covering, which is resistant to kinking (Wainwright et al., '78). In some fishes, the dermis may be important for force transmission during locomotion (Hebrank, '80; Hebrank and Hebrank, '86; Wainwright et al., '78). In balloonfish, by contrast, the skin is incapable of serving a locomotor function. The axial musculature has no dermal attachments, and the waves in the collagen fibers of the dermis render impossible any force transmission through the skin at low strains.

Although the orthogonal fiber layers are remnants of a lost function, the orientation of these layers may serve an important function in the balloonfish's defense. When a balloonfish is completely inflated, it becomes a hard, spiny ball. The stiff skin surrounding a ball of incompressible water provides a rigid framework for the support of the spines. When a predator attempts to bite the spiny ball, the orthogonal arrangement of collagen fibers resists herniation better than would purely longitudinally or circumferentially organized fibers.

Erectile spines are present in almost all pufferfishes, but the spines are longest in puffers in the genus *Diodon* (Character 9, Fig. 14; Tyler, '80). Spine erection in the balloonfish has two potential functions: as armor to prevent the teeth of a predator from piercing the inflated fish, and as added bulk to increase the effective diameter of the fish. The spine erection mechanism is a lever system—the lateral processes provide the fulcrum, and the collagen fibers attached to the axial process apply a caudally directed force to lever the spinous process away from the fish's flank (Fig. 10).

This observation explains the shape of the triradiate spine bases, but it does not explain the mechanism by which the collagen fibers attached to the axial process can apply a caudally directed force. The orientation of the fibers in Figure 10 suggests that they would be dragged in a caudal direction if the inner layers of the skin were to shear in a caudal direction relative to the outer layers. As yet it is unclear what mechanism might produce shear between the inner and outer layers during inflation. One hypothesis is that the outer layer of skin is stiffer anteriorly and more compliant caudally, and conversely, that the inner layer is more compliant anteriorly and stiffer caudally. As the skin expands, the inner layer would stretch more near the head of the animal and the outer later would stretch more near the tail,

thus producing shear between the layers in the caudal direction.

Deflation

Because microfolds in the collagen fibers cause the skin to exhibit high compliance at low strains, balloonfish skin is well designed for rapid inflation. This feature, however, makes it impossible for the skin to store sufficient strain energy to effect deflation (Fig. 11). Even excised strips of balloonfish skin do not behave elastically—once extended they do not return unassisted to rest length. This plasticity results from the small proportion of elastin fibers in balloonfish skin. If a larger proportion of elastin were present, however, the muscle power required to distend the skin would be greater and inflation performance might be compromised.

In contrast to the small number of elastin fibers in balloonfish skin, Mittal and Banerjee ('76) reported a large proportion of elastin in the *stratum laxum* of a smooth puffer, *Tetraodon fluviatilis*. It would be interesting to compare the material properties of smooth puffer skin with balloonfish skin and to determine whether differences in material properties affect inflation and deflation behaviors.

Despite the absence of skin elasticity, balloonfish can deflate as rapidly as they inflate. Deflation is produced by two separate mechanisms. First, through most of deflation, subcutaneous muscles contract to reduce the body cavity volume. Second, after most of the water has been removed, the buccal pump acts in the reverse of inflation, sucking water out of the stomach.

The subcutaneous musculature of pufferfishes is unlike any musculature observed in non-inflatable fishes (Winterbottom, '74a,b). Two layers, one circumferential and one longitudinal, form broad, unsegmented sheets covering the ventral and lateral sides of the balloonfish. The more superficial, circumferential layer is thought to be derived from the dorsal and anal fin inclinator muscles, and the more robust longitudinal layer from the anterior and medial infracarinals, which, in some fishes, connect the pectoral and pelvic girdles and the pelvic girdle and anal fin pterygiophores, respectively. The Antennariidae, which also exhibit some inflation ability, also possess a longitudinal cutaneous muscle thought to be derived from hypaxial musculature. The parallel appearance of subcutaneous musculature in these two distantly related clades of fishes is evidence that the possession of subcutaneous musculature is an important adaptation associated with the use of inflation as a defense mechanism.

CONCLUSIONS

Pufferfish inflation is an extraordinary phenomenon. Clearly it is a derived function for the puffers, but not all of the structural and functional features associated with the ability to inflate are adaptations specifically for inflation. Absence of pleural ribs, absence of a pelvis, and the orthogonal arrangement of dermal collagen sheets in the skin were all present in the ancestor of pufferfishes and, thus, must be considered exaptations (characters 3, 4, 8, Fig. 14). Sagittal plane flexibility of the vertebral column may be an epiphenomenon of the lengthening of the body cavity for more spherical inflation (character 5, Fig. 14). Numerous features of the peritoneal cavity, stomach, skin, scales, and musculature of the balloonfish are synapomorphic for puffers (characters 1, 2, 5, 6, 7, 9, 10, Fig. 14), and thus the phylogenetic distribution of these characters is consistent with the hypothesis of adaptation. These exaptations, epiphenomena, and possible adaptations combine their influence to enable the balloonfish to become more completely spherical than any other vertebrate.

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