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The Origin and Evolution of the Surfactant System in Fish: Insights into the Evolution of Lungs and Swim Bladders*

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ABSTRACT

Several times throughout their radiation fish have evolved either lungs or swim bladders as gas-holding structures. Lungs and swim bladders have different ontogenetic origins and can be used either for buoyancy or as an accessory respiratory organ. Therefore, the presence of air-filled bladders or lungs in different groups of fishes is an example of convergent evolution. We propose that air breathing could not occur without the presence of a surfactant system and suggest that this system may have originated in epithelial cells lining the pharynx. Here we present new data on the surfactant system in swim bladders of three teleost fish (the air-breathing pirarucu Arapaima gigas and tarpon Megalops cyprinoides and the non-air-breathing New Zealand snapper Pagrus auratus). We determined the presence of surfactant using biochemical, biophysical, and morphological analyses and determined homology using immunohistochemical analysis of the surfactant proteins (SPs). We relate the presence and structure of the surfactant system to those previously described in the swim bladders of another teleost, the goldfish, and those of the air-breathing organs of the other members of the Osteichthyes, the more primitive air-breathing Actinopterygii and the Sarcopterygii. Snapper and tarpon swim bladders are lined with squamous and cuboidal epithelial cells, respectively, containing membrane-bound lamellar bodies. Phosphatidylcholine dominates the phospholipid (PL) profile of lavage material from all fish analyzed to date. The presence of the characteristic surfactant lipids in pirarucu and tarpon, lamellar bodies in tarpon and snapper, SP-B in tarpon and pirarucu lavage, and SPs (A, B, and D) in swim bladder tissue of the tarpon provide strong evidence that the surfactant system of teleosts is homologous with that of other fish and of tetrapods. This study is the first demonstration of the presence of SP-D in the air-breathing organs of nonmammalian species and SP-B in actinopterygian fishes. The extremely high cholesterol/disaturated PL and cholesterol/PL ratios of surfactant extracted from tarpon and pirarucu bladders and the poor surface activity of tarpon surfactant are characteristics of the surfactant system in other fishes. Despite the paraphyletic phylogeny of the Osteichthyes, their surfactant is uniform in composition and may represent the vertebrate protosurfactant.

Introduction

Many species of fishes can breathe air. Fish utilize a number of different structures for aerial gas exchange, including the skin, gills, mouth and buccal cavity, intestine, and other specifically evolved chambers. In particular, lungs appeared independently several times. Lung structure can vary from the simple, transparent, baglike structures of rope fish and bichirs (Polypterusformes, Cladista) to more complex compartmentalized structures in lungfish (Dipnoi). Lungs are likely to have
first appeared in the placoderms. Moreover, the possible presence of lungs in placoderm fossils indicates that these animals must also have had a breathing mechanism and possibly a respiratory oscillator for responding to changes in blood and lung gases. Recent evidence suggests that such mechanisms must have been in place before the lungs themselves evolved (Perry et al. 2001). However, placoderm lungs are not homologous with the original Ostechthyian lungs, because placoderm lungs were most likely derived from the anterior pharynx (Denison 1941; Perry et al. 2001). Moreover, placoderm lungs may have served a different function. They may have been important in promoting neutral buoyancy for these heavily armored fish. With the extinction of the placoderms, lungs disappeared in the stem group and are lacking in all cartilaginous fishes. Chondrichthyans are less dense than placoderms, because they lack armor plates, and they use squalene in their liver for buoyancy (Hickman et al. 2001). However, it is likely that lungs reappeared in the stem ancestor of the Ostechthyans. In both branches of the Ostechthyans (Sarcopterygii, i.e., lungfish and the most primitive Actinopterygii, i.e., Polypteriformes), the lungs appear as paired ventral structures derived from the posterior pharynx and posterior to the gills (reviewed in Perry et al. 2001). Primitive Devonian ostechthyian fish may have breathed air in response to low environmental O2 and used the neutral buoyancy that an air-filled lung provides to rest at the surface (Dehendra and Tripathi 1976; Fange 1983).

However, it is now accepted that while lungs occur in the most primitive fishes and tetrapods, their ontogenetic origin is different from that of swim bladders (Perry et al. 2001). The swim bladder is an unpaired air-holding structure arising dorsally from the posterior pharynx (Perry et al. 2001). Swim bladders are usually regarded as the primary buoyancy aid and also support hearing by amplifying sound in the Ostariophysi (Johansen 1968). They are, however, employed in breathing in gars (Ginglomodi) and bowfin (Halecomorphi), where they are termed “pulmonoid” because of the arterial supply from the sixth branchial arch. In some teleosts, including a basal teleost, the piranucu (Arapaima gigas), and a derived teleost, the tarpon or oxeye herring (Megalops cyprinoides), the swim bladder, which is supplied by a swim bladder artery, is respiratory (Perry et al. 2001).

Pulmonary surfactant plays a crucial role in the physical forces acting at the air-liquid interface in the lungs during the dynamic changes in surface area and volume that occur during inspiration and expiration. Surfactant consists of disaturated phospholipids (DSP), unsaturated phospholipids (USP), neutral lipids (predominately cholesterol), and surfactant proteins (SPs). Four SPs have been described in mammals: SP-A, SP-B, SP-C, and SP-D. Surfactants have also been located and described in fish swim bladders, including those that are used for buoyancy (e.g., in goldfish, eels, and carp; Daniels and Skinner 1994; Orgeig and Daniels 1995; Rubio et al. 1996; Sullivan et al. 1998; Prem et al. 2000; Bourbon and Chailey-Heu 2001) and gas exchange (gar; Smits et al. 1994), and also in lunged fishes (rope fish, bichirs, and the three species of sauropterygian lungfishes; Smits et al. 1994; Orgeig and Daniels 1995; Sullivan et al. 1998).

In this article, we use previously published biochemical and morphological analyses of the surfactant system of primitive fish and add new information on this system in teleosts (two freshwater-inhabiting air breathers, the piranucu A. gigas and the tarpon M. cyprinoides, and one marine fish, the snapper Pagrus auratus). This review (with new data) demonstrates that the surfactant system of fishes is most likely homologous with that of the tetrapods, despite the different ontogenetic origins and functional aspects of the air-breathing organs (Rubio et al. 1996; Sullivan et al. 1998; Prem et al. 2000). In addition, the high-cholesterol, low-saturated phospholipid composition of surfactant found in fishes and in the ancient sauropterygian, the Australian lungfish (Neoceratodus forsteri), represents a very primitive, poorly surface-active mixture, which we term a “proto-surfactant” (Daniels and Skinner 1994; Daniels et al. 1995a; Orgeig and Daniels 1995; Daniels and Orgeig 2001). A primitive, if not the original, function of fish surfactant is likely to be acting as an antiadhesive (Daniels and Skinner 1994), but surfactant may also prevent fluid from entering the bladder or lung, prevent oxidative damage to the epithelial lining, and act as an antiseptic/antibiotic (Daniels and Orgeig 2001). We also outline the evolution of the surfactant system in the fish, with particular emphasis on postulating a mechanism whereby a homologous system could appear in structures derived independently several times.

Material and Methods

Fish

The tarpon Megalops cyprinoides occurs in a wide range of marine and freshwater habitats, including East Africa, Southeast Asia, Japan, Tahiti, Australia’s tropical seas, and the freshwaters of far northern Australia. The fish can tolerate a wide range of habitats, including landlocked lagoons and oxbow lakes (termed billabongs in Australia), with waters ranging from pH 5.2 to 9.1 and temperatures between 23° and 34°C (Merrick and Schmida 1984). It has been reported that M. cyprinoides will die in aquaria if prevented from reaching the surface (Merrick and Schmida 1984), and the closely related Atlantic tarpon (Megalops atlanticus) will regularly ventilate the swim bladder by a characteristic surface roll, even in normoxic waters (Graham 1976). Therefore, air breathing may be essential for this species, particularly in water with low oxygen levels (Graham 1997).

The piranucu Arapaima gigas is one of the largest freshwater fishes in the world, reaching up to 4.5 m and 250 kg (Graham 1997). It is an obligate air-breathing teleost from the Amazon that will drown in 10–20 min without access to normoxic air.
The degree of aerial dependence is related to size. After hatching, *A. gigas* is a water breather but quickly becomes an air breather by the time it is about 18 mm long, 8–9 d posthatch (Graham 1997). To determine whether there is an effect of size on surfactant composition of the air-breathing organ, we collected two size classes, 30–70 g and 550–1,000 g. Unfortunately, these were the only sizes that could be obtained. Both sizes are capable of surviving anoxic water by securing all **O**$_2$ uptake from the air, and both groups secure approximately 80% of their metabolic **O**$_2$ requirement from air in normoxic water (C. J. Brauner and A. L. Val, personal observations; Stevens and Holeton 1978). Clearly, air breathing is essential for this species.

The snapper *Pagrus auratus* is a demersal marine species in the family *Sparidae* and is found in New Zealand, Australia, and Japan. Species of Sparid snappers comprise major commercial aquaculture and wild fisheries in many areas of the world. Sparids are Perciform fishes possessing a ephusoistous swim bladder, which is solely a hydrostatic organ and does not perform any respiratory function. The swim bladder develops as an extrusion of the gut wall and is first filled by swallowing air via the pneumatic duct at around 5 d after hatching. By around 30 d after hatching, the pneumatic duct has closed and the swim bladder loses its connection to the gut.

**Lavage Procedure**

*Tarpon* (Megalops cyprinoides). Four fish (body mass range: 236–540 g) were captured from the Mary River (Northern Territory, Australia) or the Brisbane River (southern Queensland, Australia). We killed the fish by bathing them in a solution of 1% MS-222 and removed the tissues overlaying the swim bladder. A tube 2 mm in diameter was passed into the swim bladder via the pharyngeal opening, and the air was sucked out with a 20 mL syringe. The bladder was lavaged with 5–6 mL of ice-cold 0.15 M NaCl. The saline was withdrawn, and the lavaging process was repeated twice. The lavage was centrifuged at 150 g for 10 min to remove cellular debris and lyophilized. Material for biochemical analysis, transmission electron microscopy (TEM), and immunohistochemistry was collected from this species.

*Pirarucu* (Arapaima gigas). Six small fish (33–66 g) and three larger fish (550, 655, and 1,006 g) were obtained from Boutique do Peixe Vivo and Amazonas Ecopixe S.A. and held at the National Institute for Research of the Amazon on a natural light cycle for 3 wk before sampling. During this time, fish were fed goldfish at a ration of 1% body weight per day. Fish were killed in a solution of 1% MS-222, a tube was inserted into the pharyngeal opening of the swim bladder, and lavage was obtained as described above for tarpon. The entire ventral lower half of the swim bladder was removed and weighed before and after lyophilization. Only lavage material was collected from this species.

**Snapper** (*Pagrus auratus*). Snapper (body mass = 500 g) were captured by line fishing in the Hauraki Gulf, New Zealand. Fish were killed by stunning and pithing, and a midventral incision was made from the pelvic girdle to the vent to expose the gut cavity. Both the internal and external surfaces of the swim bladder were fixed before excision to optimize tissue fixation and maintain the in situ arrangement of the delicate mucosal surface. We ligated the esophagus and removed all abdominal organs, taking care not to damage the swim bladder. The gut cavity was then filled with fixative (2% paraformaldehyde, 2% gluteraldehyde, 0.1 M sodium cacodylate, 375 mOsm, pH 7.8). In order to fix the inside of the swim bladder without altering its volume, the swim bladder gases were gradually replaced with fixative. A 20-mL syringe was half filled with fixative and the syringe needle introduced to the swim bladder lumen through the epaxial muscle. One milliliter of fixative was introduced, followed by the withdrawal of the same volume of swim bladder gas. This reciprocal replacement was repeated until the lumen was entirely filled. Only material for TEM was collected for this species.

**Electron Microscopy and Immunohistochemistry**

After in situ fixation of the snapper swim bladder (see above) for 1 h, the ventral swim bladder wall was carefully excised from the body and cut into small strips of approximately 2 × 5 mm and continued to fix for 24 h in 1% paraformaldehyde, 2% gluteraldehyde, 0.1 M sodium cacodylate, 375 mOsm, pH 7.8. In the case of the tarpon, sections of the bladder wall and associated respiratory tissue bands forming the accessory breathing organ were cut into small pieces and fixed for TEM, as previously described (Sullivan et al. 2001).

In the tarpon, sections for immunohistochemistry were prepared by freeze substitution and immunolabelling, as previously described (Sullivan et al. 1998). The primary antibodies to human SP-A and SP-D and bovine SP-B were purchased (Chemicon Australia, Boronia, Australia).

**Surfactant Composition**

Methods for measuring total phospholipids, disaturated phospholipids, and phospholipid headgroups have been published previously (Daniels and Skinner 1994; Orgeig and Daniels 1995; Daniels et al. 1999). We measured cholesterol in the neutral lipid fraction (reconstituted with heptane) with a high-performance liquid chromatography (HPLC) system (LC1500 HPLC pump, LC1610 autosampler, DP800 data interface; GBC Instruments, Melbourne, Australia). We injected 30 µL onto a silica column (Alltech 4.6 × 250 mm with 5 µm spherical silica) equilibrated with a hexane : isopropanol (99 : 1 v/v) mobile phase (HPLC grade; APS Chemicals, Greenfield, Australia). Samples were eluted (1 mL/min) and measured at 206 nm (Model 2151 LKB Bronnma variable-wavelength UV detector).
A backpressure regulator (100 psi) was fitted to the detector outlet to minimize the formation of bubbles in the flow cell. Retention time for cholesterol was 10.2 min, and total run time per sample was 25 min.

SP-B in lavage samples from tarpon and pirarucu was quantified in a competitive ELISA based on a previously published method (Lesur et al. 1993). The primary antibody, rabbit antibovine SP-B polyclonal antibody (Chemicon Australia), and the secondary antibody, antirabbit IgG- HRP conjugate (Sigma, Sydney, Australia), were both used at a dilution of 1:1,000. The reaction was developed in Sigma Fast tablets (Sigma), and the absorbance at 492 nm was determined with a Titertek MCC Multiskan (Titertek, Huntsville, Ala.). The amount of SP-B in the lavage samples was compared to the amount of total protein, which was determined by the method of Lowry et al. (1951).

Surface Activity

Surface activity for an aliquot of lyophilized lavage from one tarpon was determined after the lavage was reconstituted in water and centrifuged at 40,000 g (Beckman Optima TLX Ultracentrifuge; Beckman, Sydney, Australia) for 30 min to pellet large aggregate material. A phosphorus assay of the pelleted material and the supernatant revealed that >90% of the phospholipids remained in the supernatant. The supernatant was lyophilized again and reconstituted in a buffered salt solution (140 mM NaCl, 10 mM HEPES, 2.5 mM CaCl₂, pH 6.9) to a concentration of 20 mg/mL of phospholipid.

We used a leakproof system, the captive bubble surfactometer (CBS), which enables the determination of surface tension, area, and volume of a bubble with a surfactant film at the air-water interface (Schürch et al. 1992). We used a previously published method for the preparation of small sample volumes (Codd et al. 2002). We measured the rate of adsorption for the first 5 min and then performed quasi-static compressions (Codd et al. 2002). From these we determined the minimum surface tension upon final compression (STₘᵣᵦ), the maximum surface tension before compression (STₘᵦ), and the percent surface area of compression (%SAᵦₘᵦ), which is a measure of the extent of compression required to achieve STₘᵦ. Measurements were performed both at room temperature (22°C) and at 37°C. We recorded the bubble continuously and calculated bubble volume, area, and surface tension from digitized bubble images, using height and diameter (Schoel et al. 1994). We compared these results to values obtained for other fish species using this and other techniques.

Results

Electron Microscopy and Immunohistochemistry

Tarpon. The respiratory surface of the swim bladder accessory breathing organ consisted of a thin layer of blood capillaries and an overlying layer of epithelial cells (Fig. 1). There were a
number of cuboidal epithelial cells containing a dark nucleus and numerous electron-lucent structures (Fig. 2A) that appear to represent one form of lamellar body (Table 1). Other lamellar bodies appeared as granular, electron-dense structures (Figs. 2B, 3A), and still others consisted of concentric rings of lamellae in a membrane-bound vesicle (Fig. 3B). Different structures for the lamellar bodies are also reported for the snapper (see below) and may represent a developmental sequence. At a higher magnification, SP-A, SP-B, and SP-D were located within the tarpon swim bladder. SP-A and SP-B were located both within the cells and in the airspaces. The location of SP-A appeared to be quite diffuse (Fig. 3A), whereas SP-B was found to be associated with lamellar bodies (Fig. 3B). A small amount of SP-D staining was found in the air spaces only (Fig. 3C).

Snapper. The lining of the swim bladder lumen was composed of squamous epithelial cells. These cells were generally in the region of 200 nm thick, except for a central thickening due to the presence of the nucleus and adjacent mitochondria. Cells were joined peripherally by tight junctions at the luminal surface and formed extensive interdigitations with adjoining cells that extended for 1–2 μm from the position of cellular abutment. The apical surface of the epithelium was interrupted by occasional microvilli that appeared more common toward the cell periphery. This cell type was characterized by the presence of numerous lamellar bodies or cytosomes. Cytosomes appeared to be more common in the region of central thickening close to the nucleus (Fig. 4A, 4B). The cytosomes of the luminal epithelium were of the form described by Creasey et al. (1974) as simian and common in human Type II alveolar cells, although their description of this structure as having concentric lamellae may be misleading and appears to depend on the plane of sectioning and possibly the developmental stage of the cytosome (Table 1). For example, a lamellar body with lamellae fused at the poles may appear concentric if sectioned equatorially. As with the tarpon, several different morphs of cytosome were recognized and may represent a developmental sequence based on the assumption that all eruptant cytosomes appeared to be of the concentric lamellate form. The proposed developmental sequence for these cytosomes is described in Figure 4C.

Cytosomes appear to form from membrane-bound, electron-dense aggregations of an amorphous material. Brooks (1970) reports formation of epithelial cytosomes in trout from similar electron-dense bodies and states that this material appeared to be composed of very tightly packed membranes. The lamellate structure of these bodies in the snapper could only be distinguished close to the site at which lamellae were separating from the electron-dense material (Fig. 4C). These osmiophilic lamellae begin to form and separate centrally but remain attached peripherally in an equatorial, or possibly polar, adhesion plaque (Fig. 4D). Total separation of the individual lamellae to form a truly concentric structure seems to occur just before eruption from the apical surface of the cell.

The gas gland epithelium contains multilamellar bodies, or cytosomes, of the form previously described for the toadfish gas gland (Morris and Albright 1975, 1977) and classified as submian by Creasey et al. (1974). This cytosome has lamellae that traverse from one side of the structure to the other (Fig.
Table 1: Morphological characteristics of surfactant from fishes (Osteichthyes)

<table>
<thead>
<tr>
<th>Species</th>
<th>Type II Cells (and Analogues)</th>
<th>Lamellar Bodies</th>
<th>Tubular Myelin</th>
<th>Other Forms and Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipnoi:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Protopterus annectens</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>Osmiophilic inclusion bodies in cells and in alveolar space</td>
<td>1</td>
</tr>
<tr>
<td><em>Protopterus sp.</em></td>
<td>+</td>
<td>+</td>
<td>?</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td><em>Lepidosiren paradoxa</em></td>
<td>+†</td>
<td>+</td>
<td>?</td>
<td>(*Only one cell type</td>
<td>3</td>
</tr>
<tr>
<td><em>L. paradoxa</em></td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Extracellular material also present</td>
<td>4</td>
</tr>
<tr>
<td><em>Neoceratodus forsteri</em></td>
<td>+†</td>
<td>+</td>
<td>?</td>
<td>(*Only one cell type</td>
<td>3</td>
</tr>
<tr>
<td><em>N. forsteri</em></td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Type II cells can be isolated; secrete LP</td>
<td>5</td>
</tr>
<tr>
<td>Teleostei:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmo gairdneri</em></td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Nonciliated (columnar or cuboidal?) cells that secrete mucus-like material into lumen of</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>swim bladder</td>
<td></td>
</tr>
<tr>
<td><em>Anguilla vulgaris</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td><em>Lota lota</em></td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Very isolated LBs in cells of the gas gland</td>
<td>8</td>
</tr>
<tr>
<td><em>Acerina cernua</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td><em>Coregonus lavaretus</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td><em>Fundulus heteroclitus</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td><em>Gadus callarias</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td><em>Opsanus tau</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td><em>Amphipnous cuchia</em></td>
<td>+†</td>
<td>–</td>
<td>–</td>
<td>(*Cuboidal cells; air sac instead of lung</td>
<td>11, 12</td>
</tr>
<tr>
<td><em>Anabas tetudineus</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Labyrinthine organ</td>
<td>11, 12</td>
</tr>
<tr>
<td><em>Channa punctatus</em></td>
<td>+†</td>
<td>–</td>
<td>–</td>
<td>(*Cuboidal cells; suprabranchial chamber</td>
<td>11, 12</td>
</tr>
<tr>
<td><em>Channa striatus</em></td>
<td>+†</td>
<td>–</td>
<td>–</td>
<td>(*Cuboidal cells; suprabranchial chamber</td>
<td>11, 12</td>
</tr>
<tr>
<td><em>Clarias battachius</em></td>
<td>+†</td>
<td>–</td>
<td>–</td>
<td>(*Cuboidal cells with vesicles; air sac</td>
<td>11, 12</td>
</tr>
<tr>
<td><em>Heteropneustes fossilis</em></td>
<td>+†</td>
<td>–</td>
<td>–</td>
<td>(*Cuboidal cells; air sac</td>
<td>12, 13</td>
</tr>
<tr>
<td><em>Misgurnus fossilis</em></td>
<td>+†</td>
<td>–</td>
<td>–</td>
<td>(*Goblet epithelial cell of respiratory intestine</td>
<td>14</td>
</tr>
<tr>
<td><em>Pogrus auratus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Different forms of LBs; they possibly represent a developmental sequence, i.e., only</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>eruptant LBs have concentric lamellae</td>
<td></td>
</tr>
<tr>
<td><em>Megalops cyprinoides</em></td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Different forms of LBs (electron-lucent, electron-dense, or with concentric lamellae);</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SPs A, B, D present in Type II cells and airspace</td>
<td></td>
</tr>
<tr>
<td>Halecomorphi:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Amia calva</em></td>
<td>?</td>
<td>+</td>
<td>?</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Polypteriformes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Polypterus senegalensis</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Electron-opaque granules in rare cells in secretory-opaque crypts</td>
<td>16</td>
</tr>
<tr>
<td><em>Polypterus ornatipinnis</em></td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>&quot;Classical-appearing&quot; Type II cells</td>
<td>13</td>
</tr>
</tbody>
</table>


* Asterisks are defined in the first entry in the "Other Forms and Comments" column.
Eruption of this form of cytosome through the apical cell surface was not observed. Simian or concentrically lamellate cytosomes of the form common to the general luminal epithelium were also observed and seen to occur in eruption from the epithelium. It was notable that eruption of this form of cytosome could occur either from the apical surface onto the surface of the lumen or through the basal surface into underlying circulatory sinuses. Extracellular material was occasionally observed on the luminal surface of the gas gland, trapped between apical microvilli (Fig. 4F). The transverse structure of this material (Fig. 4G) resembles the cytosomal tubular myelin of the vertebrate lung. As the crosshatcht structure of tubular myelin is highly characteristic of surfactant, it is likely that the material in the snapper gas gland is surfactant.

**Surfactant Composition**

**Tarpon.** The disaturated phospholipid (DSP) content of the lavage material was very low and was measurable in only one of the four tarpon fish samples (%DSP/PL = 4.04). The cholesterol (Chol) to DSP ratio (μg/μg) in this one sample was 5.65. The cholesterol to phospholipid (PL) ratio (μg/μg) in the lavage was 0.258 ± 0.069 (mean ± SE, n = 4; Table 2). Thin-layer chromatography (TLC) revealed that phosphatidylcholine (PC) was the predominant PL in lavage, accounting for 71% of total PL. The only other significant lipids were sphingomyelin (18.4%) and a combination of phosphatidylycerine and phosphatidylinositol (7.7%). Lysophosphatidylcholine (1%), phosphatidylglycerol (1%), and phosphatidylethanolamine (0.3%) were present only in trace amounts (Table 2). The ELISA demonstrated the presence of SP-B in lavage from two of the tarpon. There were 2 pg of SP-B per μg of total protein in one animal and 17.6 pg of SP-B per μg of total protein in the other.

**Pirarucu.** The %DSP/PL in the lavage of the small fish was 26.85 ± 3.35 (mean ± SE, n = 6). The Chol/PL and Chol/DSP ratios (μg/μg) were 0.266 ± 0.02 and 1.03 ± 0.08 (mean ± SE, n = 6), respectively (Table 2). The Chol/PL ratio of the lavage from the large fish was 0.34 ± 0.14 (mean ± SE, n = 3) and did not differ from that of the small fish. We did not measure DSP in the large pirarucu. SP-B was measurable by ELISA in the lavage of one of the large pirarucu. The lavage of this fish contained 44.6 pg SP-B per μg of total protein.

**Surface Activity**

The adsorption of surfactant from the tarpon swim bladder was extremely slow. At 22°C the surfactant adsorbed slightly

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Figure 3. Immunogold labeling of SP-A (A; scale bar = 0.2 μm), SP-B (B; scale bar = 0.5 μm), and SP-D (C; scale bar = 0.5 μm) in the respiratory swim bladder of the tarpon Megalops cyprinoides. SP-A was localized in the air spaces (filled arrows) and in the epithelial cells (open arrows), whereas SP-B appeared to be primarily associated with lamellar bodies (arrows). The presence of SP-D was restricted only to the airspaces (arrows).
Figure 4. Multilamellar bodies (cytosomes) and tubular myelin in the snapper (*Pagrus auratus*) swim bladder. A, Cytosome erupting from lumenal epithelium (scale bar = 1 μm). B, Semilamellate cytosome (scale bar = 1 μm). C, Progressive development of cytosomes from electron-dense amorphous granules to semilamellate form (scale bar = 1 μm). D, Semilamellate cytosome, lamellae fused equatorially (scale bar = 2 μm). E, Subsimian cytosome (scale bar = 250 nm). F, Lumenal surface material, tubular myelin (scale bar = 250 nm). G, Transverse structure of the material in F (scale bar = 100 nm).
<table>
<thead>
<tr>
<th>Species</th>
<th>$T_{amb}$</th>
<th>PL (mg/gWL)</th>
<th>Percent of PLs</th>
<th>Chol (mg/gWL)</th>
<th>DSP (mg/gWL)</th>
<th>DSP/PL (%)</th>
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<tr>
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<td>n</td>
<td></td>
<td>PC</td>
<td>PG</td>
<td>PE</td>
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<tr>
<td>A. gigas$^c$</td>
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Note. $T_{amb}$ = ambient or body temperature; PL = phospholipid; gWL = grams of wet lung mass; PC = phosphatidylycholine; PG = phosphatidylglycerol; PE = phosphatidyl-ethanolamine; SM = sphingomyelin; PI = phosphatidylinositol; PS = phosphatidylserine (a single value for PI and PS indicates that the bands could not be resolved); LPC = lysophosphatidylycholine; unknown = unknown PL; Chol = cholesterol; DSP = disaturated PL. RT = roomtemperature (22°C–25°C); ND = not determined.

$^a$ Orgeig and Daniels 1995.
$^b$ Phleger and Saunders 1978.
$^c$ This study.
$^e$ Smits et al. 1994.
Surfactant in Air-Breathing Organs of Fish

Figure 5. Rate of adsorption of tarpon (*Megalops cyprinoides*) surfactant (10 mg/mL PL) to the air-liquid interface of a bubble in the captive bubble surfactometer. The surface tension at the end of adsorption is the equilibrium surface tension ($ST_{eq}$).

faster than at 37°C (Fig. 5), and it reached a slightly lower $ST_{eq}$ (22°C: 38 mN/m; 37°C: 41 mN/m). Upon the first quasi-static compression cycle, tarpon surfactant demonstrated relatively high values for $ST_{max}$ (22°C: 41.0 mN/m; 37°C: 41.1 mN/m), $ST_{min}$ (22°C: 19.5 mN/m; 37°C: 21.7 mN/m), and %SA$_{comp}$ (22°C: 75.3%; 37°C: 75.8%; Fig. 6). Surface tension-lowering properties, including $ST_{min}$ and %SA$_{comp}$, were marginally better at 22°C than at 37°C (Fig. 6). Film stability was poor, as seen from the slight progressive increase in $ST_{min}$ following successive quasi-static cycles (Fig. 6; Table 3).

Discussion

Morphology

Cells containing lamellar bodies and surfactant lipids and proteins are easily observable in the swim bladder of the tarpon and the snapper. It is also clear that the function of the gas-holding structure (respiration or buoyancy) is irrelevant to the requirement for a surfactant system in fish (Table 1). The surfactant-secreting cells in the tarpon and the snapper do not differ structurally from those of other fishes and possess the same characteristics as the alveolar Type II epithelial cells found in tetrapod lungs (Daniels and Orgeig 2001). They demonstrate microvilli on the apical surface, and the lamellar bodies are membrane-bound and of similar size and appearance to those observed in all other vertebrate groups, including fishes (Table 1; Hughes 1970, 1973; Hughes and Weibel 1978; Prem et al. 2000; Wood et al. 2000; Daniels and Orgeig 2001).

Surfactant Proteins

The presence of the surfactant lipids and proteins (SP-A, SP-B, and SP-D) provides strong evidence that the surfactant system of all fishes is homologous with that of the tetrapods. Furthermore, the surfactant system located in the lungs or swim bladders of fish (lungfish, bichirs, gar, carp, eels, goldfish, snapper, pirarucu, and tarpon) is likely to be homologous (Daniels and Skinner 1994; Rubio et al. 1996; Sullivan et al. 1998; Prem...
Table 3: Surface activity of surfactant from fishes (Osteichthyes)

<table>
<thead>
<tr>
<th>Species and $T_a$ (°C)</th>
<th>Device</th>
<th>Min ST (mN/m)</th>
<th>%SA Comp</th>
<th>Stability</th>
<th>Bubble Clicking</th>
<th>Surpellic Property/ Surface Activity</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>RT</td>
<td></td>
<td></td>
<td>Stable$^a$</td>
<td>+$^c$</td>
<td>Fully surpellic$^b$</td>
</tr>
<tr>
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<tr>
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<td>+$^d$</td>
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<td>Fully surpellic$^b$</td>
</tr>
<tr>
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<td>WB$^d$</td>
<td>22</td>
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<tr>
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<td>WB$^d$</td>
<td>22</td>
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<td>37</td>
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<td>Calculated from bubble shape$^b$</td>
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<td>-$^c$</td>
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</tr>
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<tr>
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<td>-$^c$</td>
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</tr>
<tr>
<td>24</td>
<td>WB$^f$</td>
<td>17.0 ± 1.0</td>
<td>70</td>
<td></td>
<td></td>
<td>Very low$^a$</td>
</tr>
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</table>

Note. $T_a$ = ambient temperature; Min ST = minimum surface tension; %SA Comp = percent surface area compression required to achieve min ST; RT = room temperature (22°-25°C). WB = Wilhelmy balance; PBS = pulsating bubble surfactometer; CBS = captive bubble surfactometer. The number of measurements performed regards whole lung extracts, not lavage. "Surpellic" is a term coined by Patte to describe the ability of lavage material to exist as stable bubbles and demonstrate bubble clicking. "Stability ratio" is a measure of a bubble's stability as determined by the change in surface area over a standard length of time after stabilisation. "Bubble clicking" is a phenomenon involving rapid changes in bubble shape and surface tension immediately after formation in water. It indicates that the fluid involved is capable of attaining very low surface tension.

$^a$ A plus sign indicates presence and a minus sign absence.

$^b$ Patte 1976.

$^c$ Hughes 1967.

$^d$ Fieger and Saunders 1978.

$^e$ Daniels et al. 1986.


$^g$ Daniels and Skinner 1994.

$^h$ This study.

e et al. 2000). Given that the lung and swim bladder have separate ontogenetic origins and arise from different pharyngeal regions (Perry et al. 2001), we can conclude that the surfactant system must predate the appearance of both lungs and swim bladders. Significant recent evidence has demonstrated surfactant-containing lamellar bodies secreted from epithelial cells in the mammalian gut (Engle and Alpers 2001). SP-A and SP-D co-localize with these structures (Rubio et al. 1995; Engle and Alpers 2001). Furthermore, SP-A antibodies also cross-react with material from both the intestine and swim bladder of the carp (Rubio et al. 1996). We therefore postulate that the surfactant system originated in the epithelial cells lining the pharynx. Here the SPs may have played an important role in the innate immune system of the organism.
The hydrophilic SPs, SP-A and SP-D, belong to the family of proteins known as collectins that are characterized by C-terminal carbohydrate recognition domains. These proteins are thought to function as molecules of the innate immune system by recognizing a broad spectrum of pathogens, including viruses, bacteria, and fungi, as well as allergens, such as pollen grains and mite allergens (Haagsman and Diemel 2001). In addition to their presence in gut epithelium, SPs have also been described in mucosal surfaces of other tissues, for example, the middle ear duct, esophagus, stomach, and peritoneal cavity (van Rozendaal et al. 2001), as well as in the genitourinary tract (Bourbon and Chailey-Heu 2001). Hence, these proteins may perform a generalized function in mucosal immunity, as these sites are directly exposed to the environment. Mucosal immunity involves both infectious defense and tolerance against environmental and dietary antigens (Haagsman and Diemel 2001). Furthermore, given that the epithelial lining of these structures arises from different regions of the pharynx, mucosal immunity may represent the ancient function of SPs in the pharynx.

However, it is not only the SPs that are expressed in gut mucosa. Surfactant-like lipid material enriched in phosphatidylcholine and demonstrating surface tension-lowering capabilities has also been reported as lining the gut epithelium (Engle and Alpers 2001). Although the function of these lipids is not clear, they are likely to have a role in protecting the underlying epithelial cells from both mechanical and chemical injury and may be involved in the binding of molecules for biochemical processing (Engle and Alpers 2001). It is also possible that the lipids may have a role in dealing with interfacial tension of liquids of different viscosities in the gastrointestinal tract and other mucosal surfaces (Sullivan et al. 1998).

Hence, it is possible that the surfactant system could have migrated with the epithelial cells during the out-pouching events associated with the generation of either lungs or swim bladders from different regions of the pharynx. To our knowledge, no analysis has been performed on the presence of SPs in other gas-exchange organs of fish, such as the gills, buccal cavity, or specialized respiratory regions of intestine. However, given that these structures also originate from the pharynx, it is likely that SPs are present in these structures.

This study is the first demonstration of the presence of SP-D in the lungs of nonmammalian species and the first to demonstrate SP-B in actinopterygian fishes. SP-B has been previously detected in chicken and salamander lungs (Zeng et al. 1998; Miller et al. 2001), and SP-A is well documented in fish (Rubio et al. 1996; Sullivan et al. 1998; Prem et al. 2000). The functions of the SPs in mammals are not yet fully understood. SP-A and SP-D are thought to function predominantly in immune defense by promoting the uptake of numerous pathogens by macrophages (Haagsman and Diemel 2001). SP-B, on the other hand, is intricately involved with the surfactant PLs and is important in regulating the biophysical activity of the surfactant film (Haagsman and Diemel 2001). It is likely that both the biophysical and immune functions of the SPs are important in the lungs and swim bladders of fish. Moreover, the cross-reactivity between the mammalian antibodies and the native, in situ fish proteins implies that the tertiary structures of all three proteins are highly conserved. This is remarkable, because in spite of major differences in the structure and function of gas-holding structures, both within a lineage and between distantly related animals, the structure of these proteins and their retention as part of the surfactant system have not been affected. We conclude that the proteins must be essential to both the function of the surfactant system and the operation of lungs and swim bladders. The genes for these proteins, therefore, may have extremely low mutation rates, or it may be that any mutation is extremely deleterious and is selected out. This evidence powerfully supports the hypothesis that a functional surfactant system is a crucial prerequisite for the evolution of aerial gas exchange using a lung or swim bladder.

**Surfactant Lipids**

The predominant lipid in all vertebrates examined is phosphatidylcholine (PC; Daniels and Orgeig 2001), and the disaturated form of this lipid, dipalmitoylphosphatidylcholine (DPPC), is usually regarded as responsible for the reduction in surface tension (Veldhuizen et al. 1993). However, the relative abundance of DSP, USP, and cholesterol varies greatly between different species (Daniels et al. 1995a). The surfactant of mammals, birds, and reptiles is rich in DSP. However, the surfactant in the lungs of the air-breathing, ray-finned fishes (Polypteriformes and Gymnodi) and the teleost swim bladders (Fig. 7; Table 2) have a Chol/DSP ratio that is up to 15 times higher than that of the tetrapods (Smits et al. 1994). Similarly, the most primitive of the lobe-finned fishes, the Australian lungfish Neoceratodus forsteri has DSP/PL, Chol/DSP, and Chol/PL ratios of the same order as those of the ray-finned, air-breathing fishes (Daniels et al. 1995a; Orgeig and Daniels 1995; Table 2). However, two derived species of lobe-finned fishes, the South American and African lungfishes Lepidosiren paradoxa and Protopterus Annectens, have a surfactant that is between two and three times richer in DSP than N. forsteri or the primitive, air-breathing, ray-finned fishes (Polypteriformes and Gymnodi; Orgeig and Daniels 1995; Table 2). The derived lungfish also have Chol/PL ratios that are between one-third and one-fifth those of N. forsteri, the Polypteriformes, and the Gymnodi. Furthermore, on the basis of their Chol/PL and Chol/DSP ratios, the surfactants of the teleost fishes (pirarucu, tarpon, and goldfish) group more closely with those of the primitive, lunged, air-breathing, ray-finned fishes and N. forsteri. Collectively, these species and groups (N. forsteri, Teleosts, Polypteriformes, Gymnodi) have Chol/PL ratios that are between three- and sevenfold greater and Chol/DSP ratios that are between four- and 25-fold greater than those in the derivedlung-
fish (*L. paradoxa* and *P. annectens*, Table 2). Therefore, on a holistic scale, it appears that the surfactant lipid composition of all the ray-finned and primitive lobe-finned fishes is relatively similar, in that they have a high-cholesterol, low-PL, low-DSP mixture. On the other hand, the derived lobe-finned fishes (*P. annectens* and *L. paradoxa*) have a surfactant composition much more similar to that of the tetrapods. However, upon closer examination it is apparent that among the ray-finned and primitive lobe-finned fishes there are some subtle differences in surfactant composition that may be related to the function of the gas-filled organ. For example, the goldfish (Table 2) is not an air breather, and it has the highest Chol/PL ratio of all the fish. Hence, it appears that the less the air-breathing organ is used for gas exchange, the more cholesterol its surfactant contains. This high-cholesterol, low-PL, low-DSP mixture may represent the protosurfactant (Daniels et al. 1995a).

The functions of this mixture will differ significantly from those of the surfactants of tetrapods, because it is poorly surface active and highly fluid (see below). We have previously argued that given the extremely smooth, nonseptated lungs of the polypertid fishes, the smooth swim bladder walls of the goldfish, and the comparatively large respiratory units of the gar, lungfish, and other teleosts, a highly surface-active mixture may not be required. It is possible that the larger size of the respiratory units themselves may confer stability by substantially reducing the collapse pressures that are present in the much smaller alveoli of mammals (Daniels et al. 1998a). In these lungs or swim bladders, a highly fluid (i.e., high-Chol, high-USP, lowDSP) mixture may assist in the easy spreading of the surfactant from (presumably isolated) clumps of secretory cells to cover the entire surface (Daniels et al. 1995b). Furthermore, such a mixture is adequate to perform the antiadhesive function that we have demonstrated for goldfish swim bladders, ray-finned, air-breathing fish (Ginglymodi and Polyperiforomides), and many reptiles and amphibians (Daniels et al. 1995a). It is also possible that the unusual combination of lipids, in particular the large amount of cholesterol, in fish swim bladder surfactant may serve as an antioxidant (Szebeni and Toth 1986), as the swim bladders of most physoclist teleosts are exposed to hyperbaric oxygen (Pelster 2001).

There are also differences in the relative abundance of the PL head groups between the fishes and the tetrapods. In all cases, PC is the dominant head group, but there is significant variation in the presence and abundance of the minor PLs. Surfactant isolated from mammals contains about 10% phosphatidylglycerol (PG) and only trace amounts of phosphatidylethanolamine (PE) and sphingomyelin (Daniels and Orgeig 2001). PG is relatively uncommon in vertebrate surfactants (restricted only to mammals, a few amphibians, the lungfishes, and one of three snake species that have been examined; Daniels and Orgeig 2001). The surfactant isolated from *N. forsteri* contains large amounts of sphingomyelin and PE and only traces of PG (Orgeig and Daniels 1995). The amount of sphingomyelin is even greater in the goldfish swim bladder than in the
Australian lungfish (Daniels and Skinner 1994). The most abundant minor PL in fish surfactant is also sphingomyelin, yet the surfactant essentially lacks PG. The significance of the variation in the minor PLs, in particular PG, is unknown.

In terms of surfactant lipid composition, the strongest evidence that the surfactant system had a single evolutionary origin comes from the similarity in composition between the Australian lungfish (N. forsteri), a primitive saccorhynctan closely related to the stem ancestor of the tetrapods, and the primitive actinopterygian air-breathing fish (Ginglymodi and Polypteroforms). Hence, the similarity in composition between primitive members of the two branches (Actinopterygii and Saccorhyncti) suggests that the surfactant system evolved once in a common ancestor. Furthermore, the difference in the surfactant between these primitive representatives of the two branches on the one hand and the more derived saccorhynctans and tetrapods on the other hand supports our suggestion of a protosurfactant.

**Surface Activity**

The surface activity of tarpon surfactant was marginally better at 22°C than at 37°C, as reflected by a slightly lower ST_{min} and a reduced %SA_{comp} (Table 3). This presumably reflects an adaptation to function at temperatures that are more similar to the body temperature experienced by the animal. It has previously been demonstrated that surfactant isolated from cold-acclimated animals, such as marsupials and bats, which are capable of undergoing torpor, functions more effectively at room temperature (22°C–24°C) than at 37°C. Conversely, if the surfactant is isolated from the active animals, it is more active at 37°C than at room temperature (Lopatko et al. 1998; Codd et al. 2002). Whether fish are capable of adjusting their surfactant composition and function to match changes in body temperatures is unknown. The activity of tarpon surfactant certainly appears sensitive to temperature.

The surface activity of tarpon surfactant is extremely poor, compared with that of mammalian surfactant measured under the same conditions with the same device. Mammalian surfactant typically experiences an ST_{min} of <1 mN/m, an ST_{eq} of ~25 mN/m, and a %SA_{comp} of <20% (Schürch et al. 1992; Table 3). Moreover, the surface activity was also poor relative to that measured in lizards on the Wilhelmy balance (Daniels et al. 1998a). Lizard surfactant demonstrated intermediate surface activity with an ST_{min} of 13–14 mN/m, an ST_{eq} of 25 mN/m, and a %SA_{comp} of 30%. However, the surface activity of tarpon surfactant was very similar to that previously reported for other ray-finned fishes (Teleostei and Ginglymodi) and lungfishes (Daniels et al. 1998a; Table 3). The ST_{min} values measured with alternative instruments, that is, either the Wilhelmy balance or the Enhörning Bubble Surfactometer, ranged from 17 to 26 mN/m (Daniels et al. 1998a). This study is the first to use the modern, state-of-the-art CBS in combination with high PL concentrations (10 mg/mL, compared with 1 mg/mL PL) to measure the surface activity of fish surfactant. The CBS is currently regarded as the most sophisticated and accurate device for measuring the biophysical behavior of pulmonary surfactant mixtures (Veldhuizen et al. 1998; Schürch et al. 2001). The other methodologies are less reliable, particularly for highly unsaturated, highly fluid surfactant mixtures, as they tend to suffer from leakage of PLs out of the surfactant film (Veldhuizen et al. 1998; Schürch et al. 2001). Hence, the poor surface activity previously reported for fish surfactant appears to be a true reflection of its biophysical properties and is not an artifact of low PL concentrations or inferior (leaky) measurement devices (Table 3).

It is likely that the surface-active properties observed for fish surfactant directly reflect the differences in lipid and possibly protein composition that we have described. For example, the concentration of DSP in the tarpon swim bladder is only 4% or less of total PL, which is even lower than that of other air-breathing fishes (9%–14%; Table 2; Smits et al. 1994) and virtually nonexistent compared with that of reptiles, birds, and mammals (30%–50%; Veldhuizen et al. 1998; Daniels and Orgeig 2001). Given that DSP, and in particular DPPC, is the major PL responsible for achieving low surface tensions upon compression (Veldhuizen et al. 1998), it is not surprising that fish surfactant is unable to generate low surface tensions. A further characteristic of fish surfactant that mitigates against low surface tensions is the extremely high concentration of cholesterol (Chol/PL: 0.2–0.3; Chol/DPPC: 1.0–5.6), compared with that in mammals (Chol/PL: ~0.08; Chol/DPPC: ~0.12; Daniels et al. 1995a), reptiles (Chol/PL: ~0.03–0.08; Chol/DPPC: ~0.1–0.3; Daniels et al. 1996; Daniels and Orgeig 2001), and amphibians (Chol/PL: ~0.03–0.1; Chol/DPPC: ~0.2–0.3; Daniels et al. 1994; Daniels and Orgeig 2001). While cholesterol (of the order of 5%–10% of total PL) is essential in surfactant to promote the spreading of the saturated PL film upon inspiration (Nott et al. 1980; Fleming and Keough 1988), high concentrations of cholesterol tend to inhibit surfactant surface activity (Nott et al. 1980). Yet another characteristic of surfactant from the tarpon and pirarucu swim bladders is the very low concentration of SP-B (2, 17, and 44 pg/µg protein in the three fish tested), compared with 2 ng/µg protein in mice, determined by the same method (Tokieda et al. 1999). As SP-B is closely associated with the surfactant lipids and has a crucial role in promoting PL adsorption to the surfactant film (Haagsmann and Diemel 2001), the up to 100-fold lower concentration in lavage of this essential protein no doubt also contributes to the poor surface-active properties of fish surfactant.

We have previously argued that nonmammals, due to their larger respiratory units, less complex lungs, and greater natural lung distensibility, do not require a surfactant mixture capable of the extremely low surface tensions typical of mammalian surfactants. A more detergent-like surfactant (demonstrating higher ST_{min} and less variation) would be perfectly adequate...
Surfactant of nonmammals is not primarily responsible for maintaining alveolar stability and increasing lung compliance but rather is required as an anti-adhesive and an antiedema agent (Daniels et al. 1998a). Nevertheless, fish surfactant, due to its composition and function, appears to be in a class of its own—the protosurfactant. The extraordinary differences between fish surfactant and that of mammals, reptiles, and amphibians suggest that fish surfactant has functions that have not yet been elucidated. These may include functioning as an innate immune system or as an antioxidant system or even simply the provision of a medium for dissolving other antioxidant, antiviral, or antimicrobial agents.

Conclusion

It appears that the evolution of the surfactant system predated the evolution of lungs and swim bladders. However, the functional evolution of the surfactant system is closely coupled with that of lungs or swim bladders in the fish and lungs in the tetrapods. It also appears that there are two different types of surfactant, one in the actinopterygian fishes that is high in cholesterol and USPs and one in the advanced sarcopterygian fishes and tetrapods that is relatively low in Chol and USPs and high in DSPs. Fish surfactant is likely to be highly spreadable but is not very surface active. The tetrapod surfactant is much more surface active and may, therefore, allow the development of more complex lungs with smaller respiratory units and a greater total respiratory surface area, paving the way for the evolution of high-performance lungs in amniotes. It is possible that fish surfactant is an archaic or "proto-" surfactant that evolved into tetrapod surfactants but is also important as a lipid lining for the swim bladders in the modern fish.

Many reptilian, amphibian, and fish lungs are essentially bag-like, with a large central airspace and lacking a bronchial tree, and with few exceptions they can collapse completely (Bishop and Foxon 1968; Guimond and Hutchison 1976; Hughes and Vergara 1978; Martin and Hutchison 1979; Stark-Vancs et al. 1984; Brainerd et al. 1989; Frappell and Daniels 1991a, 1991b). A very important function of surfactant in these lung types is to prevent the epithelial surfaces from sticking together (Daniels et al. 1995a). Nonmammalian surfactant does not significantly influence inflation compliance. Given the highly derived nature of the mammalian lung, it is likely that regulation of surface tension during nonatelectatic breathing is a late development in the evolution of surfactant. Based on our studies of reptilian, amphibian, and piscine surfactants, we conclude that while acting as an antiadhesive may have been the original function of surfactant, it led naturally to the alveolar stability and compliance roles that dominate mammalian surfactant function.

The surfactant system has been highly conserved, morphologically and biochemically, throughout (and despite) the enormous radiation of air-breathing organs among the vertebrates.

The lipid composition is conserved, and homology of SP-A, SP-B, and SP-D demonstrates a single evolutionary origin for the system.

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