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Development and sexual dimorphism of the sonic system in three deep-sea neobythitine fishes and comparisons between upper mid and lower continental slope

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ABSTRACT

Based on morphology, NB Marshall identified cusk-eels (family Ophidiidae) as one of the chief sound-producing groups on the continental slope. Due to food scarcity, we hypothesized that sonic systems will be reduced at great depths despite their potential importance in sexual reproduction. We examined this hypothesis in the cusk-eel subfamily Neobythitinae by comparing sonic morphology in Atlantic species from the upper-mid (*Dicrolene intronigra*) and deeper continental slope (*Porogadus miles* and *Bathyonus pectoralis*) with three Taiwanese species previously described from the upper slope (*Hoplobrotula armatus*, *Neobythites longipes* and *N. unimaculatus*). In all six species, medial muscles are heavier in males than in females. *Dicrolene* has four pairs of sonic muscles similar to the shallow Pacific species, suggesting neobythitine sonic anatomy is conservative and sufficient food exists to maintain a well-developed system at depths exceeding 1 km. The sonic system in *Porogadus* and *Bathyonus* was reduced to a single pair of ventral medial muscles that connects to a smaller and thinner swimbladder via a long tendon. Small muscle fiber diameters, a likely indicator of rapid contraction, were present in males of five of the species. However, in *Bathyonus*, the deepest species (pale coloration, reduced eye size, shorter sonic muscles and longer tendons), muscle fibers were larger suggesting an adaptation to facilitate rapid bladder movement for sound production while using slower contractions and less metabolic energy. The six species separate into three groups in length-weight regressions: the three upper slope species have the greatest weights per unit length, *Dicrolene* is lower, and the two deep species are further reduced consistent with the hypothesis that food limitation affects sonic anatomy at great depths.

1. Introduction

Fish sounds are unknown at great depths (mid or deep continental slope to the abyss), and research in this field has been largely neglected since the pioneering work of NB Marshall in the 1960s (Marshall, 1962, 1967). A potential fish sound, localized at about 700 m (Mann and Jarvis, 2004), provides an indication of how little is known (Rountree et al., 2012; Wall et al., 2014) although numerous fish sounds have been recorded in shallow water (Fish and Mowbray, 1970; Lobel et al., 2010). Marshall focused on anatomy of fishes with muscles attached to the swimbladder and indicated that two families, the Macrouridae (grenadiers or rattails) and the Ophidiidae (cusk-eels), are the major sonic groups on the continental slope. The ubiquitous association of

swimbladder muscles with sound production (Ladich and Fine, 2006; Fine and Parmentier, 2015) justifies using these muscles as a proxy for the ability to produce sound, likely to aid in attracting mates in this dark, nutritionally dilute world.

Ophidiiform fishes include, in part, the families Carapidae and Ophidiidae (Nielsen et al., 1999), and unlike most sonic teleosts that produce swimbladder sounds with superfast muscles (Ladich and Fine, 2006; Parmentier and Diogo, 2006), carapids with slow sonic muscles stretch the swimbladder and produce sound during recoil (Parmentier et al., 2006a, 2006b, 2006c). Fishes in several families unrelated to ophidiiforms also use a recoil mechanism, which may form an intermediate condition in the evolution of superfast muscles (Mok et al., 2011; Parmentier et al., 2016). In most sonic fishes superfast muscles

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are necessary to move the swimbladder at high enough speeds to produce one sound cycle per contraction (Skoglund, 1961; Fine et al., 2001; Millot et al., 2011). Species with slow muscles produce a single pulse per contraction, and contraction rate does not determine frequency within a pulse (Fine et al., 2007; Parmentier et al., 2010, 2016; Mok et al., 2011; Parmentier and Fine 2016). For instance the cusk-eel *Ophidion marginatum* (subfamily Ophidiinae) produces sounds composed of one to 27 pulses with a peak frequency of about 1200 Hz (Mann et al., 1997; Sprague and Luczkovich, 2001; Rountree and Bowers-Altman, 2002; Mooney et al., 2016). This peak frequency is too high to be produced by individual contractions of superfast sonic muscles, suggesting a slow-muscle mechanism. This assumption is reinforced by a pulse period of about 23 Hz. Interestingly, females of *Ophidion rochei* have been shown to have fast muscles (Keever et al., 2014). Muscle-speed variation in the subfamily Ophidiinae is far from clarified.

Recently, we described sonic anatomy of three neobythitine cusk-eels, a subfamily of the Ophidiidae, from the upper slope of Southern Taiwan (Ali et al., 2016). Although there were sexual differences in swimbladder and sonic muscle development within species as well as interspecific differences between them, the sonic system was conservative with four sonic muscle pairs in all three species. Two medial muscles (ventral medial and intermediate medial), insert directly on the medial swimbladder, and two lateral muscles (ventral lateral and intermediate lateral) insert on specialized epineurals that attach to the lateral swimbladder. Sounds have never been recorded from a member of this subfamily. Sexually-dimorphic medial muscles (considerably larger in males) suggest an increased role in sound production that could be used in courtship and male-male competition. Additionally, the medial muscles have small fibers suggesting an adaptation for speed as in typical sonic swimbladder muscles (Ladich and Fine, 2006; Fine and Parmentier, 2015; Parmentier and Fine, 2016). Lateral muscles have larger fibers, equivalent to those of epaxial trunk muscles (Ali et al., 2016), suggesting that lateral muscles are more powerful and slower than medial ones (Rome and Linstedt, 1998). The function of lateral muscles is unclear, but they may modify the sound produced by contraction of the medial muscles.

In this paper we describe and quantify the sonic system of three Atlantic neobythitine species captured at respectively around 1 km and deeper (*Dicrolene intronigra*) and 1.6–5 km depths (*Porogadus miles* and *Bathyonus pectoralis*) for comparison with the upper slope Taiwanese species. The deeper two species live in an environment with great hydrostatic pressures (Angel, 1997; Bochus and Seibel, 2016) and little food (Lampitt et al., 1986; Collins et al., 2005; Sutton et al., 2010; Mindel et al., 2016). Owing to the food scarcity, we hypothesized that the sonic system would be reduced in deeper species. This hypothesis is supported by sonic muscles in *Barathodemus manatinus* (Carter and Musick, 1985); it occurs from 1800 to 2600 m and has a single sonic muscle pair that connects to the swimbladder in males. Unfortunately phylogenetic relationships in the Neobythitinae are not established, complicating consideration of an interaction of phylogeny and depth. Possible relationships of the six species will be considered in the discussion.

We find that the midslope *Dicrolene intronigra* has well developed muscles that are similar to the Taiwanese species (Ali et al., 2016), but the lower slope *Porogadus miles* and *Bathyonus pectoralis* have a single pair of ventral medial muscles (VM) that connect to a swimbladder with thinner and more pliable walls than the shallower species. The sonic muscles in the deeper species are short and end in an unusual long tendon that connects to the bladder. We therefore hypothesized that, as in carapids (Parmentier et al., 2006a, 2006b, 2006c), these muscles would be slow, and their contraction would put the tendons under strain that would likely drive the swimbladder for multiple cycles of sound production (Parmentier et al., 2006a, 2006b, 2006c, 2016; Mok et al., 2011).



Fig. 1. Photographs of A. *Dicrolene intronigra*, B. *Porogadus miles* and C. *Bathyonus pectoralis*. *Dicrolene* is in Howes' Group 1 (see discussion). Note the complex pectoral fins, dark coloring, large eyes and multiple lateral lines. The deeper *Porogadus* and *Bathyonus* are in Howes' Group 2 and also have multiple lateral lines. The eyes of *Bathyonus*, the deepest species are reduced, and it has pale coloration. All three species have elongated tails.

2. Materials and methods

Three species of Atlantic cusk-eels (subfamily Neobythitinae) (Fig. 1) from the Fish Museum of the Virginia Institute of Marine Science were collected by Jack Musick and colleagues (Musick et al., 1996). Fish were fixed in 10% formalin and preserved in 70% ethanol. *Dicrolene intronigra* (15 males and 16 females) were collected from Norfolk Canyon (Nov. 1974: 1403–1523 m, Sept. 1975: 1018 m, Nov. 1991: 1552–1670 m), *Porogadus miles* (8 males and 9 females) from the Mid-Atlantic Bight (Aug. 1990: 1900–2170 m, Nov. 1991: 1600–2960 m), and *Bathyonus pectoralis* (10 males and 10 females) from the Caribbean Sea off Venezuela (Nov. 1981: 3422–5000 m). In most cases these species will be referred to by their genus name in the text. These three species will be compared with data from the three upper-slope neobythitines from relatively shallow water (200–300 m) off Southern Taiwan (*Hoplobrotula armatus*, *Neobythites longipes* and *N. unimaculatus*) (Ali et al., 2016) to examine the relationship of depth on sonic development in the subfamily. We describe sonic anatomy (skeleton, sonic muscles and swimbladder), relative eye size and include some brief observations on external anatomy.

Fish were weighed to 0.1 g and measured for total length (TL) in mm. Unfortunately, stomachs from *Dicrolene* had been removed for gut content studies (Wenner, 1984), and therefore we recorded eviscerated weight. Based on measurements (Mok unpublished data) of *Hoplobrotula armata*, an upper slope neobythitine (Ali et al., 2016), we likely under represent the total weight by a factor of no more than 10%. We dissected fish to determine sex and expose the sonic muscles. Swimbladder and sonic muscle lengths were measured within the fish using calipers. Tissues were removed and soaked in 0.9% NaCl for 5 min to insure uniform hydration, blotted and weighed in milligrams. Swimbladder and muscle lengths were regressed against TL, and their weights were regressed against fish weight. Sexual dimorphism was determined by analysis of covariance on male and female regressions. To allow simple comparisons between sexes within species and to permit interspecific comparisons, adjusted means for 25 cm TL and 40 g fishes were calculated using the linear regressions. For smaller *Bathyonus* this size is a considerable extrapolation, and smaller fish (15 cm TL and 12.5 g) were used to compare male and female data.

Since swimbladder and sonic muscle growth was isometric, comparisons were made between species using the sonic muscle somatic index, SMSI,

$$(\text{sonic muscle weight}/\text{fish weight}) \times 100.$$

Species were compared by analysis of variance and Tukey's test.

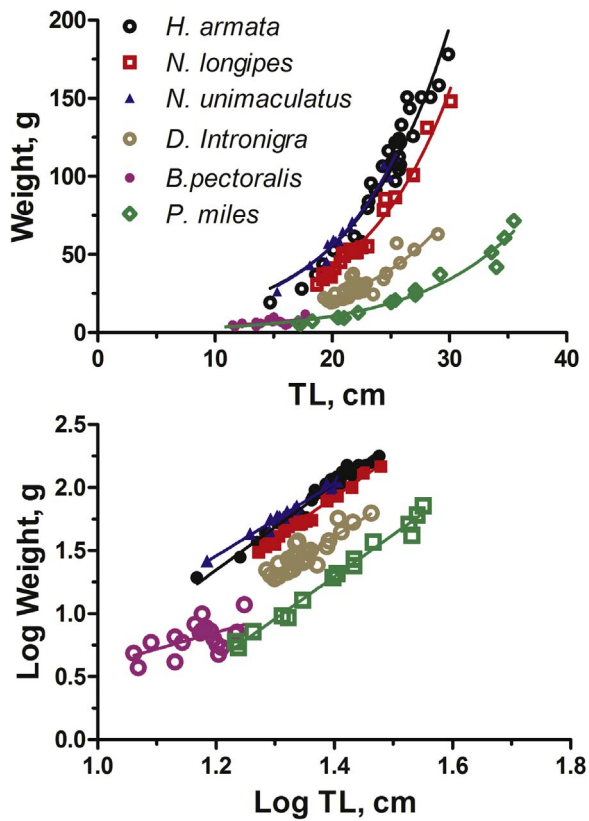


Fig. 2. Length-weight regressions (linear scale top and log scale bottom) of six neobythitids at depths from several hundred to five thousand meters. Upper-slope species (*H. armata*, *N. unimaculatus* and *N. longipes*) have the greatest weight per unit weight, *Dicrolene intronigra* from about a km and deeper is intermediate, and the two deepest species (*Porogadus miles* and *Bathyonus pectoralis*) have lower weights per unit length.

Similarly, length of the muscles was divided by TL. Eyes were dissected and weighed in the three species, and % eye weight was additionally compared with *Ophidion marginatum*, a nocturnal estuarine and shallow ocean species in the subfamily Ophidiinae. Sonic muscles from a representative male and female specimen from each species were cut in cross section at 11 μm on a cryostat and stained with methylene blue. We measured minimum fiber diameter at the widest point for 30 muscle fibers for each muscle and present median diameter.

3. Results

Length-weight regressions for the six species fell into three groups with no overlap: upper, mid, and deeper slope (Fig. 2). The three upper-slope species had the greatest weight per unit length with extensive overlap between *Hoplobrotula armata* and *Neobythites unimaculatus*. *Neobythites longipes* was lighter and did not overlap with the other two although the weight per unit length was relatively similar. *Dicrolene intronigra* occupied an intermediate position, and incrementing its weight by 10% due to evisceration would not change its relative position on the graph. *Porogadus miles* and *Bathyonus pectoralis* were considerably lighter than the shallower species. The sample of *Porogadus* included larger fish than for *Bathyonus* although the regressions were continuous on the linear graph. Log-transformation indicated a lower slope for *Bathyonus* (Fig. 2), due to smaller fish in the sample. Overall, weight per unit length decreased with depth in these six species. In comparison with a 25 cm TL *Porogadus*, the weight of *Dicrolene* was more than double, and the weights of the three shallow species were more than six fold greater. The deeper species had considerably longer tails (Fig. 1) than the three shallow species (see Fig. 1 in Ali et al., 2016).

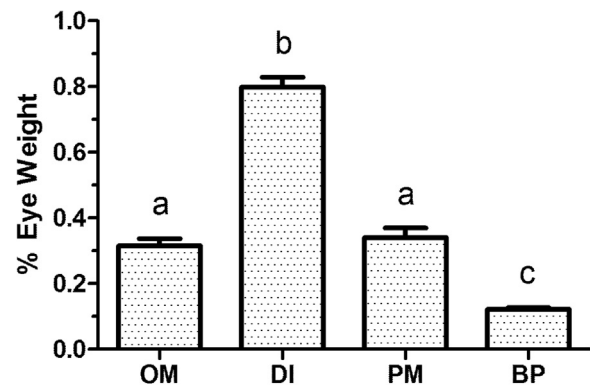


Fig. 3. % eye weight of *Dicrolene intronigra* (DI), *Porogadus miles* (PM) and *Bathyonus pectoralis* (BP) compared to the estuarine and shallow-ocean *Ophidion marginatum* (OM). Different letters indicate means that are significantly different.

Dicrolene has notably large eyes (Fig. 1) and melanin on the skin and peritoneum. Although considerably deeper, *Porogadus* has some black coloration on its outer surface and still has well-developed eyes. *Bathyonus*, the deepest species, is pale externally and has reduced eye diameter. Measurements of weight of dissected eyes as a percentage of body weight (Fig. 3) indicated differences between species ($F_{3,74} = 143.3$, $p < 0.0001$). The eyes of *Dicrolene* (0.80 ± 0.14) were significantly larger than in *Ophidion marginatum* (0.31 ± 0.02) an estuarine and shallow-ocean species in the subfamily Ophidiinae. *Porogadus* eyes (0.34 ± 0.03) were equivalent to those of *O. marginatum*, and the eyes of *Bathyonus* (0.12 ± 0.005) were reduced compared to the other species.

3.1. *Dicrolene intronigra*

The first three vertebrae in *Dicrolene* possess expanded epineurals that are connected by a thin sheet of connective tissue to form a united structure (Fig. 4). In males the first two epineurals bow sharply backward and unite with the third about $\frac{3}{4}$ from its distal end. Proximally, the first epineural is horizontal and slender, but it expands vertically to form a concave scoop on its anterior surface for attachment of the ventrolateral (VL) and intermediate lateral (IL) muscles. The third epineural is fused to the third and fourth vertebrae, and is extremely wide at its base. It tapers to a sharp point giving it almost the shape of an off-center equilateral triangle. In females the first epineural bows backward slightly, the second is relatively straight, and the two fuse at their distal tips with the third. The third epineural is again attached to the third and fourth vertebrae, but it is longer and thinner, tapering less sharply than in males. It attaches to the dorsolateral swimbladder in both males and females.

3.1.1. Swimbladder

The swimbladder is a single chamber covered with a thick white tunica externa (Fig. 5). It underlies the first to 11th vertebrae. The bladder has an anterior-neck region that is dorsoventrally compressed. Caudal to the neck, the bladder wall expands and then remains relatively straight posteriorly until curving bluntly at its caudal end. The posterior bladder rests against an unusual concave soft structure also found in the three Taiwanese species from the upper slope (Ali et al., 2016). The anterior lip of the bladder is rigid and attaches to the first epineural laterally. A stretchable orange swimbladder fenestra with no external tunic covering sits on the dorsal surface just caudal to the anterior lip. The fenestra cuts perpendicularly across the dorsal surface of the bladder in males but is absent in females (Fig. 5). Caudal to the fenestra, the dorsal surface of the swimbladder fuses rigidly to the ventral surfaces of the vertebrae and ribs, which are wide and vertically compressed proximally before tapering distally. Males, but not females, also have a slight round posterior tube surrounding a thin membrane on

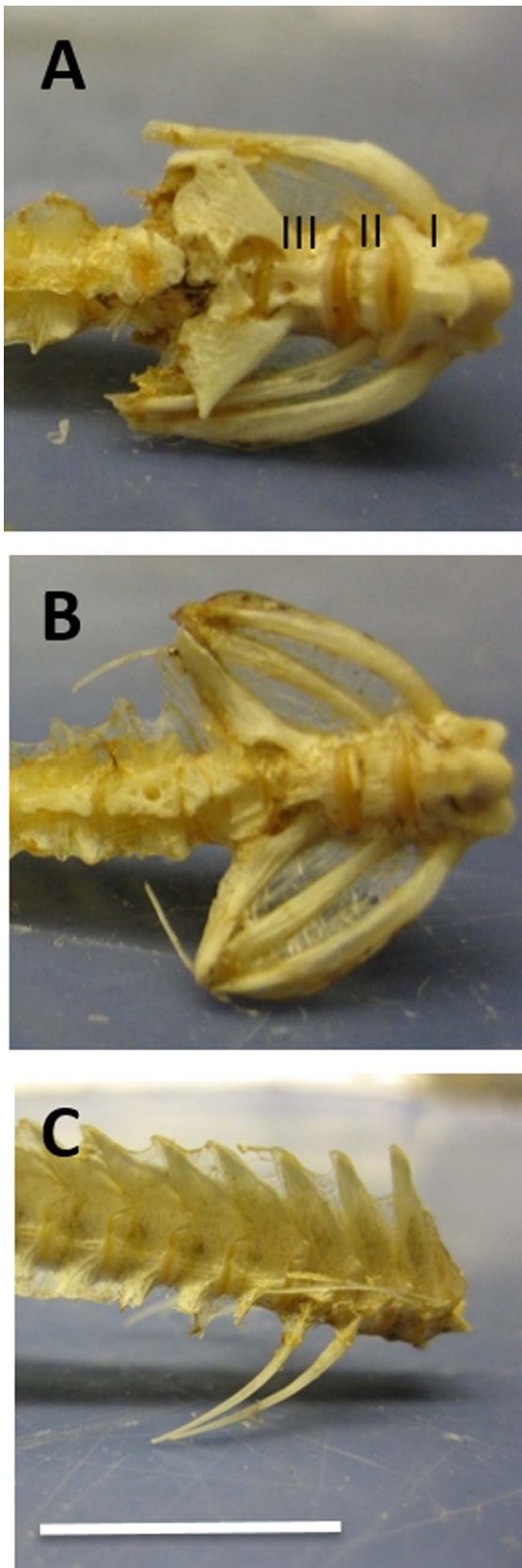


Fig. 4. Anterior vertebral skeletons of three deep neobythitines. A. Ventral view of male and B. female *Dicrolene intronigra*. Note sexual differences in epineural shape on vertebrae I, II and III. C. Lateral view of *Porogadus miles*. Scale bar = 1 cm.

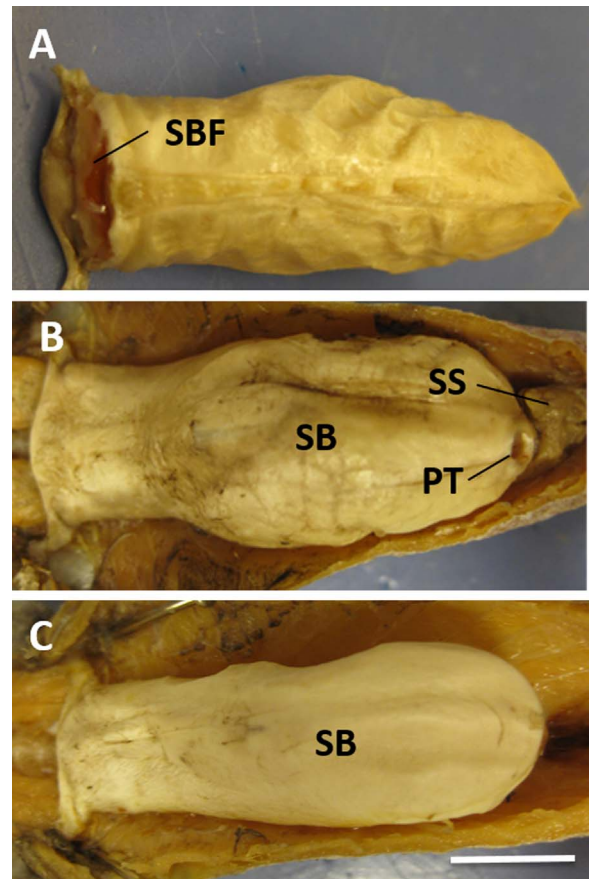


Fig. 5. Swimbladder of *Dicrolene intronigra*. A. Male dorsal surface illustrating swimbladder fenestra (SBF). Note the attachment scars from the vertebral column on the midline and the indentations of the ribs on the dorsolateral surface. B. ventral surface illustrating posterior tube (PT) and soft structure (SS) immediately caudal to the posterior swimbladder. C. Female dorsal surface with no fenestra. Note the dorsoventrally compressed neck region of the anterior bladder in both sexes. Scale bar = 1 cm.

the ventral surface near the posterior bladder (Fig. 5).

Swimbladder length increased linearly with TL in both sexes (Fig. 6), and regressions, although suggestive, were not significantly different in males and females (slope: $F_{1,26} = 3.20$, $p = 0.085$; intercepts: $F_{1,27} = 1.45$, $p = 0.240$). Adjusted means of swimbladder length calculated from linear regressions for a 25 cm TL fish were 38.1 and 35.4 mm respectively for males and females. Swimbladder weights increased linearly in both sexes but more rapidly in males (slope: $F_{1,27} = 5.79$, $p = 0.025$). Adjusted means for a 40 g fish were 1028 and 671 mg respectively. Heavier swimbladders in males appear to result primarily from thicker bladder walls.

3.1.2. Sonic muscles

Members of the Ophidiinae have ventral, intermediate and dorsal sonic muscles (Parmentier et al., 2006a, 2006b, 2006c; Fine et al., 2007). As in the shallower neobythitines (Ali et al., 2016), *Dicrolene* has four pairs of sonic muscles: two ventral muscles (ventral medial and ventral lateral) and two intermediate muscles (intermediate medial and intermediate lateral) (Fig. 7). Note this is a slight change from the nomenclature used in Ali et al. (2016). In males the ventral medial muscle (VM) originates on the prootic-intercalarium, travels below Baudelot's ligament and inserts broadly on the dorsal swimbladder just behind its anterior edge. The female VM is considerably thinner than in males. It originates laterally on the intercalarium, is penetrated by Baudelot's ligament and has a similar insertion as in males on the dorsal bladder. The intermediate medial muscle (IM) (Fig. 7) originates on the intercalarium caudal to VM, travels above Baudelot's ligament in both

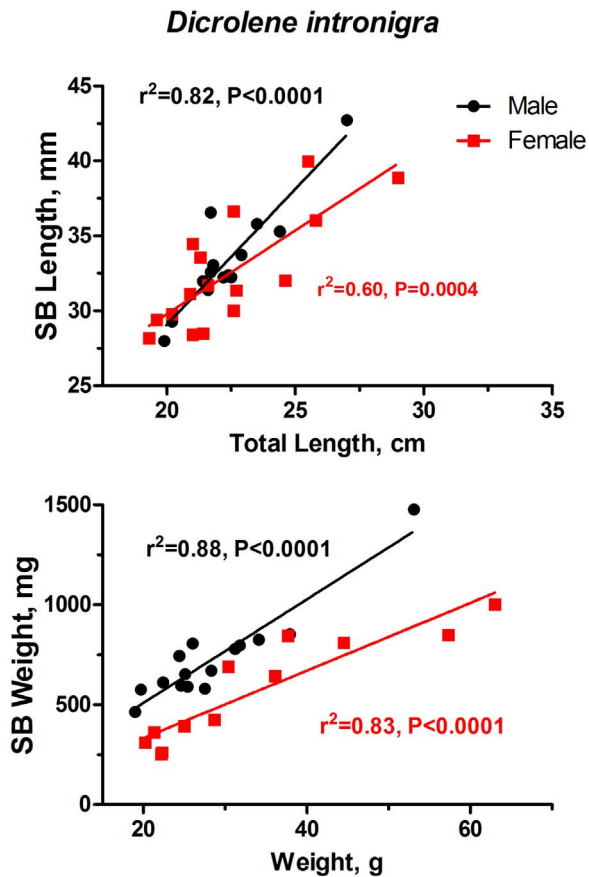


Fig. 6. Relationship of swimbladder length to fish total length (TL) (top) and swimbladder weight to fish weight (bottom), r^2 and p values for male (black) and female (red) *Dicrolene intronigra*.

sexes and inserts on the swimbladder more dorsally than the VM in males and on the lateral tip of the second epineural in females.

The ventrolateral muscle (VL) originates on the epiotic in both sexes and inserts on the lateral tip of the first epineural in males and the second in females. It surrounds Baudelot's ligament in males, and it travels above the ligament in females. The intermediate lateral muscle (IL) originates on the pterotic and inserts within the scoop on the first epineural. Unfortunately we discovered the small IL late in the study after most fish were dissected, and this muscle was not considered in regression analysis.

VM length increased linearly in both sexes (Fig. 8) and was longer in males than in females (slope: $F_{1,27}=2.91, p=0.099$; Intercept: $F_{1,28}=55.6, p<0.001$). Adjusted means for a 25 cm fish were 19.9 and 13.2 cm. The sex difference was dependent largely on elevation rather than the regression slope. VM weight was considerably greater in males than in females (slope: $F_{1,27}=63.6, p<0.0001$). Adjusted means for a 40 g fish were 244 and 3.6 mg respectively.

IM length increased with fish TL in females ($r^2=0.65, p=0.0002$) but not in males ($r^2=0.20, p=0.094$). IM was longer in males, again based on elevation rather than slope (slope: $F_{1,27}=2.16, p=0.15$; intercept: $F_{1,28}=55.6, p<0.0001$). Adjusted means for a 25 cm TL fish were 15.1 and 12.5 mm respectively. IM weight increased linearly in both sexes, and the muscle was heavier in males than in females ($F_{1,27}=14.5, p=0.0008$). Adjusted means for a 40 g fish were 28.5 and 11.7 mg. Summing the adjusted means for weight in both medial muscles (272.4 mg in males and 15.3 mg in females) indicates that the muscles will exert a greater force and cause higher amplitude swimbladder movement in males.

VL length increased with TL, and muscle length was equivalent in both sexes (slope $F_{1,27}=3.32, p=0.079$; intercept $F_{1,28}=0.17,$

$p=0.68$). Adjusted means were 14.3 and 12.8 mm in males and females respectively. VL weight increased with fish weight in females ($r^2=0.50, p=0.0021$) but was of borderline significance in males ($r^2=0.263, p=0.051$). VL was heavier in females (slope: $F_{1,27}=2.51, p=0.125$; intercept $F_{1,28}=15.84, p=0.0004$). For a 40 g fish adjusted weights were 5.4 and 12.3 mg for males and females. Unfortunately we do not have quantitative data on the IL.

3.2. *Porogadus miles*

The first and second vertebrae each possess extremely thin and fragile ribs that attach to the vertebral body dorsally and extend posteriorly (Fig. 4). These ribs do not attach to the swimbladder and appear to have no role in sound production. The base of the ribs on the third, fourth and fifth vertebrae attach lower down on the vertebrae. These ribs are stouter at the base and extend laterally before descending in a posterior-ventral direction. The fifth rib is thinner than the third and fourth. The remaining ribs surrounding the swimbladder are broad but thin at the base. They extend ventrolaterally, taper and continue as thin bones. The vertebral centra and ribs attach to the dorsal and dorso-lateral swimbladder.

The swimbladder is a single relatively straight chamber with rounded anterior and posterior surfaces (Fig. 9) beneath the 7th through 16th vertebrae. It is thinner (not bright white) and more pliable than in *Dicrolene* or in the upper-slope neobythitines and lacks a swimbladder fenestra and a posterior tube. Swimbladder lengths increased linearly with TL (Fig. 10), and there were no sexual differences (slope: $F_{1,13}=1.06, p=0.332$; intercept: $F_{1,14}=0.65, p=0.434$). Similarly, swimbladder weight increased linearly with fish weight without sexual differences ($F_{1,13}=3.90, p=0.070$, intercept: $F_{1,14}=2.74, p=0.120$) although the slope approached significance.

A single pair of ventral medial muscles (VM) originates on the lateral side of the intercalarium (Fig. 7). They surround Baudelot's ligament as they travel toward the swimbladder. The muscles are thicker in males. The caudal end of the muscle tapers to a point and terminates in a long slender tendon that extends caudally. The tendons of the right and left VM expand and fuse as they insert on the anterodorsal swimbladder.

VM length increased linearly with TL (Fig. 11), and the muscle was longer in males. The slopes of the regressions were parallel (Slope: $F_{1,13}=0.04, p=0.85$), but the elevations were different ($F_{1,14}=16.74, p=0.001$). Adjusted means for a 25 cm TL fish were 17.1 and 12.6 mm respectively. Tendon length increased linearly with TL, but tendons were longer in females (slope: $F_{1,13}=14.67, p=0.0021$): adjusted means were 6.9 and 11.3 mm respectively. The muscle was longer and the tendon shorter in males (17.1–6.9 mm), but the lengths of the muscle and tendon together were more similar in females (12.6 and 11.3 mm). Summing adjusted means for the muscle and tendon indicates that the combined lengths were similar in both sexes (24.0 and 23.9 mm). VM weight increased linearly with fish weight, and the muscle was heavier in males ($F_{1,13}=13.05, p=0.0032$). Adjusted means for a 40 g fish were 74.2 and 13.8 mg respectively.

3.3. *Bathyonus pectoralis*

The axial skeleton of *Bathyonus* is similar to that of *Porogadus* (Fig. 4) with a long slender rib in an elevated position on the first and second vertebrae, longer and stouter pointed ribs on the third through fifth vertebrae and remaining wide but short flattened ribs cradling the swimbladder. The single-chambered swimbladder is somewhat translucent and pliable. As in *Porogadus*, it resides further back in the body cavity beneath the 6th to 17th vertebrae. The bladder increases in width gradually for more than half its length and then tapers to a blunt point. There is no fenestra or posterior tube.

Females in our samples were longer and heavier than males (Fig. 10), which complicates sexual comparison. Swimbladder length

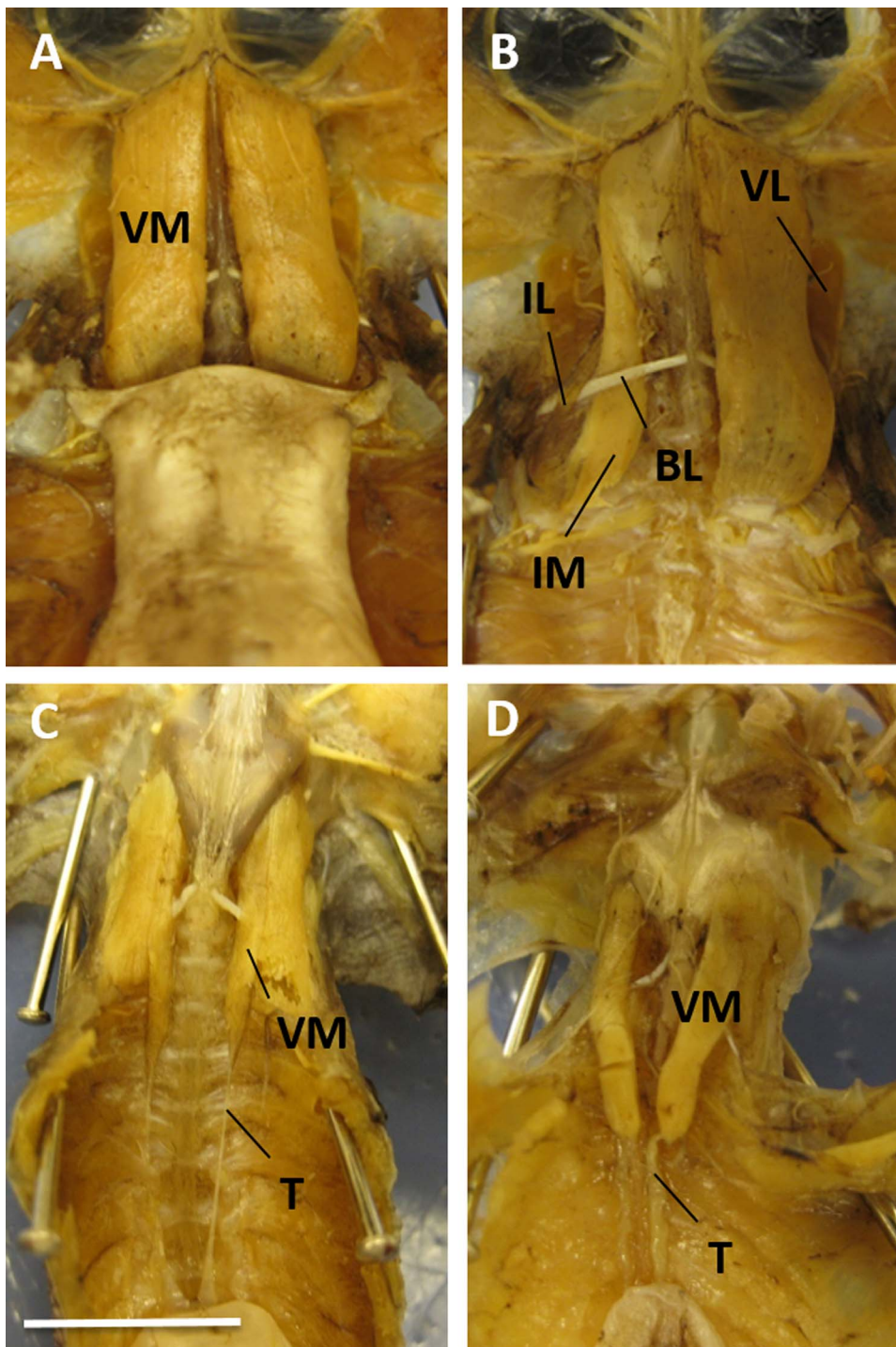


Fig. 7. A. Ventral view of exposed sonic muscles and anterior swimbladder in a male *Dicrolene intronigra*. B. Same view with the right ventromedial muscle (VM) removed to expose the deeper intermediate medial muscle (IM), intermediate lateral muscle (IL) and Baudelot's ligament (BL). The ventral lateral muscle (VL) is visible to the left of the intact VM. C. Ventral view of ventral muscle, tendon (T) and anterior swimbladder with anterior lip rolled back to expose the tendon insertions on the anterodorsal swimbladder. D. Ventral view of sonic system in *Bathyonus pectoralis*. The Bathyonus tendon is longer than it appears since its insertion is covered by the swimbladder. Scale bar = 1 cm.

did not increase with TL in males ($r^2=0.099$, $p=0.54$) but did in females ($r^2=0.668$, $p=0.0071$). Male and female regressions were not different (slope: $F_{1,11}=0.87$, $p=0.372$; intercept: $F_{1,12}=0.003$, $p=0.96$). Since the specimens were smaller than for *Porogadus*, adjusted means for *Bathyonus* were calculated for a 15 cm TL and 12.5 g fish and are not directly comparable to the other five species. Adjusted means for swimbladder length were 19.3 and 19.9 mm for males and females respectively. Bladder weights increased linearly with fish weight in both sexes. The difference in swimbladder weight approached significance based on elevation with females tending larger (slope: $F_{1,12}=1.25$, $p=0.285$; intercept: $F_{1,13}=3.95$, $p=0.068$). An extrapolation of adjusted means for a 40 g fish, the size used for the other species,

would be 47.1 mg in males and 68.6 mg in females.

VM (Fig. 7) originates on the intercalarium, surrounds Baudelot's ligament and continues posteriorly before tapering to a point and terminating in a slender tendon that extends to the swimbladder where it again fuses with the tendon of the opposite muscle as it enters the anterodorsal swimbladder. VM length increased linearly in females ($r^2=0.448$, $p=0.034$) but not in males ($r^2=0.261$, $p=0.137$), and regressions were not different (slope: $F_{1,16}=1.05$, $p=0.309$; intercept: $F_{1,17}=0.61$, $p=0.444$). Adjusted means for a 15 cm TL fish were 7.0 mm in males and 6.3 mm in females. Tendon lengths increased linearly in males ($r^2=0.84$, $P=0.010$), but not in females ($r^2=0.079$, $p=0.542$) and did not exhibit sexual differences (slope: $F_{1,9}=0.83$,

Dicrolene intronigra

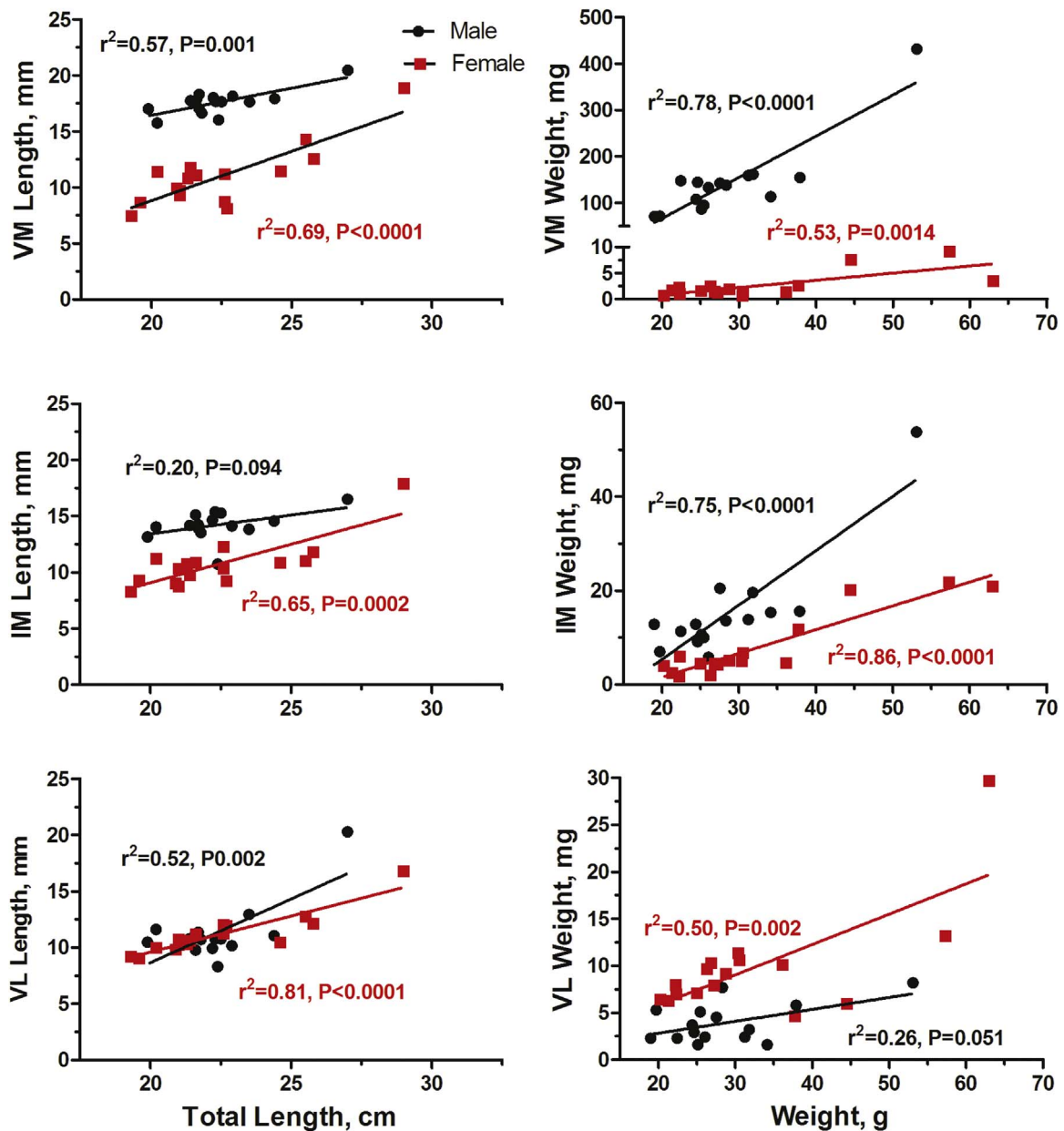


Fig. 8. Relationship of length of the ventromedial (VM), intermediate medial (IM) and ventrolateral (VL) muscles to total length (TL), r^2 and p values in male (black) and female (red) *Dicrolene intronigra* (left panel) and muscle weights to fish weight (right panel).

$p = 0.38$; intercept: $F_{1,10} = 1.53$, $p = 0.244$). Adjusted means for a 15 cm fish were 15.4 mm in males and 15.0 mm in females. Summed muscle and tendon lengths were 22.4 and 21.3 mm in males and females. In this case the muscle was relatively shorter than in *Porogadus*, and % tendon lengths in *Bathynus* were 69% and 70% respectively, suggesting increasing importance of the tendon and a relatively shorter sonic muscle. Muscle weights increased linearly with fish weight in males ($r^2 = 0.921$, $p < 0.0001$) but not in females ($r^2 = 0.207$, $p = 0.219$), and male muscle weights increased more rapidly than in females (slope: $F_{1,9} = 42.6$, $p < 0.0001$). Adjusted weights for a 12.5 g fish were 13.2 and 1.9 mg respectively. Extrapolating to a size comparable to the other species, although not particularly meaningful, would yield an adjusted mean of 60.9 mg in males and 6.1 mg in females.

3.4. Muscle fiber diameter

Fiber diameter was measured in one male and one female in each of the three species as a possible indication of fiber contraction speed (Fig. 12). *Dicrolene* medial muscle fibers were smaller than lateral ones, and fibers were larger in males than in females. Median diameter in VM was 23 μm in males and 14.5 μm in females, and the IM in males was 22 μm and 4 μm in females. Fibers in the lateral muscles were similar in males and females respectively for VL (42.5 and 42 μm) and IL (42 and 39 μm). Thus *Dicrolene* followed the pattern of the shallower species although the fibers of the medial muscles were somewhat larger. Median fiber diameter in the VM of males and females was respectively 10.5 and 5 μm in *Porogadus* and 30 and 9 μm in *Bathynus*.

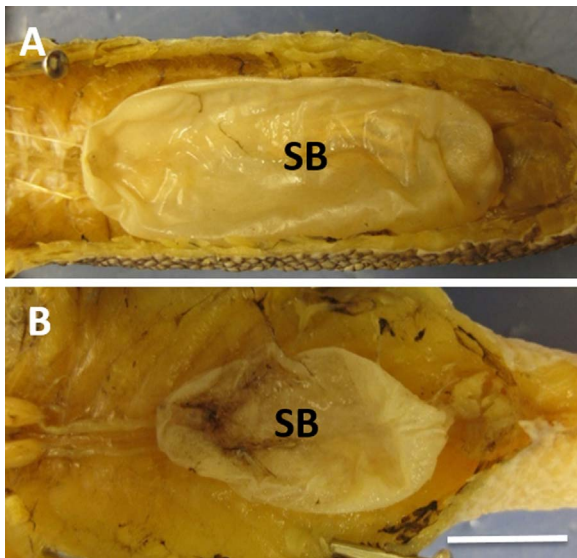


Fig. 9. Swimbladders of A. *Porogadus miles* and B. *Bathyonus pectoralis*. Scale bar = 1 cm.

3.5. Comparison of six species with depth

Mean swimbladder somatic index or SBSI (% swimbladder weight) varied among species ($F_{5,58}=138.1$, $P < 0.0001$ in males and $F_{5,63}=5.96$, $P < 0.0001$ in females) (Fig. 13). The index was similar in male *H. armata*, *N. longipes* and *N. unimaculatus* ranging from 1.45% to

1.72%, increased to 2.57% in *Dicrolene*, and dropped to 0.68% and 0.35% respectively in *Porogadus* and *Bathyonus*. In females the three shallow species and *Dicrolene* were similar varying from 1.27% to 1.35%. The index dropped nonsignificantly to 0.94% in *Porogadus* and significantly to 0.56% in *Bathyonus*. Relatively lighter swimbladders occurred in the deep species despite a much larger gas gland.

Mean sonic muscle somatic index or SMSI (% muscle weight) also varied considerably (males: $F_{5,61}=65.0$, $P < 0.0001$; females: $F_{5,61}=25.1$, $P < 0.0001$) (Fig. 14). In the upper slope species male *Hoplobrotula armata* had a heavier VM (0.16%) than the two *Neobythites* species (0.03% and 0.0068%), although lighter than in *Dicrolene* (0.48%). The index in the deepest species *Porogadus* and *Bathyonus* were 0.13% and 0.07% respectively, both of which were not significantly lighter than in *Hoplobrotula* or the two *Neobythites* species. In females VM was heaviest in *Hoplobrotula* (0.089%) compared to the two *Neobythites* species and *Dicrolene* (respectively 0.0017%, 0.0014% and 0.0073%). VM in *Porogadus* (0.046%) was heavier than in *Dicrolene* but not than in *Bathyonus* (0.029%). In summary there was a tendency of swimbladders to be smaller in the two deepest species (*Porogadus* and *Bathyonus*), which agrees with their having lighter bodies per unit length (Fig. 2). The ventral muscles exhibit differences in some cases and not others in species at similar depths. VM mass in the two deepest species was not relatively less than in all of shallow species although there was a decrease in sonic muscle pairs from four to one. Therefore the mass of the medial muscles attached to the swimbladder remained similar to depths greater than or equal to about a kilometer and dropped dramatically below that.

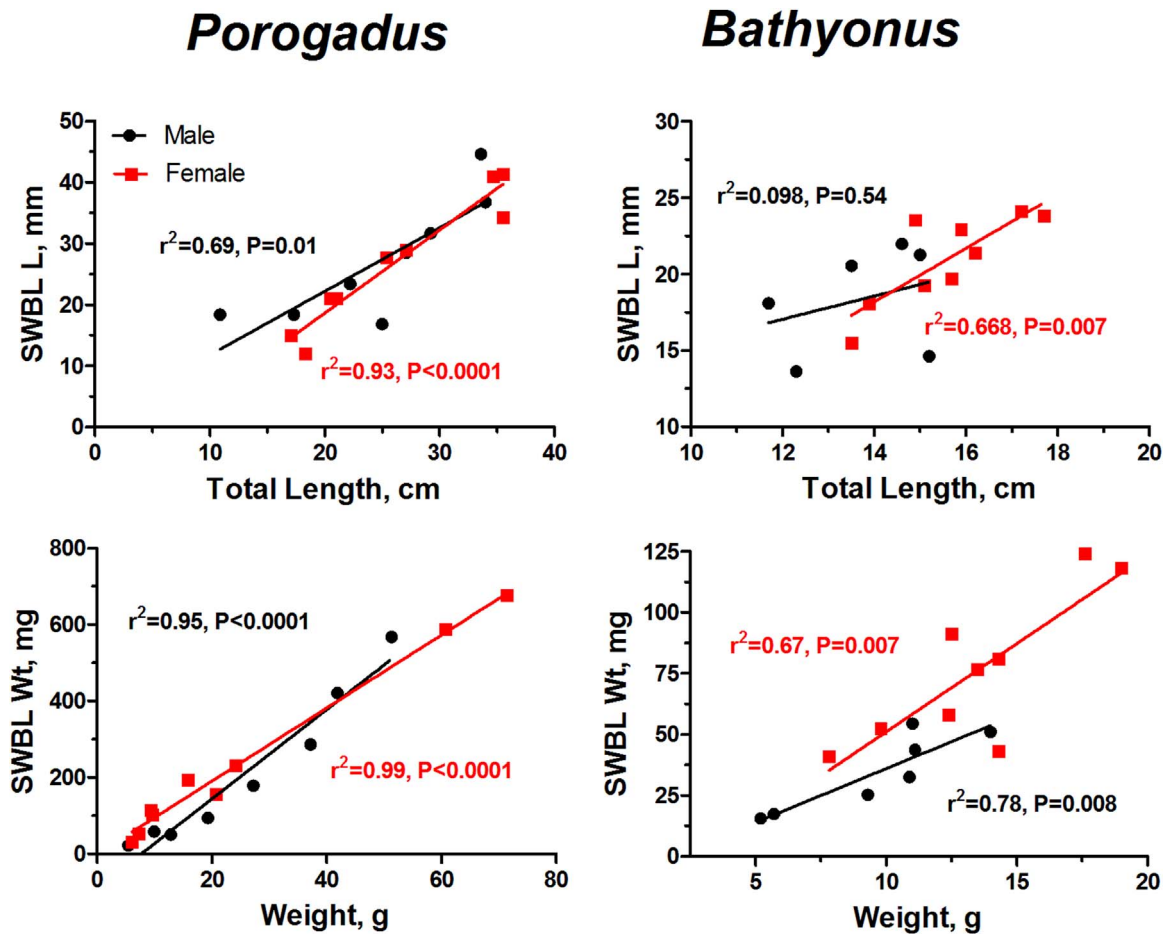


Fig. 10. Relationship of swimbladder length to total length, r^2 and p values and swimbladder weight to fish weight for male (black) and female *Porogadus miles* (red) in left panel and *Bathyonus pectoralis* in right panel.

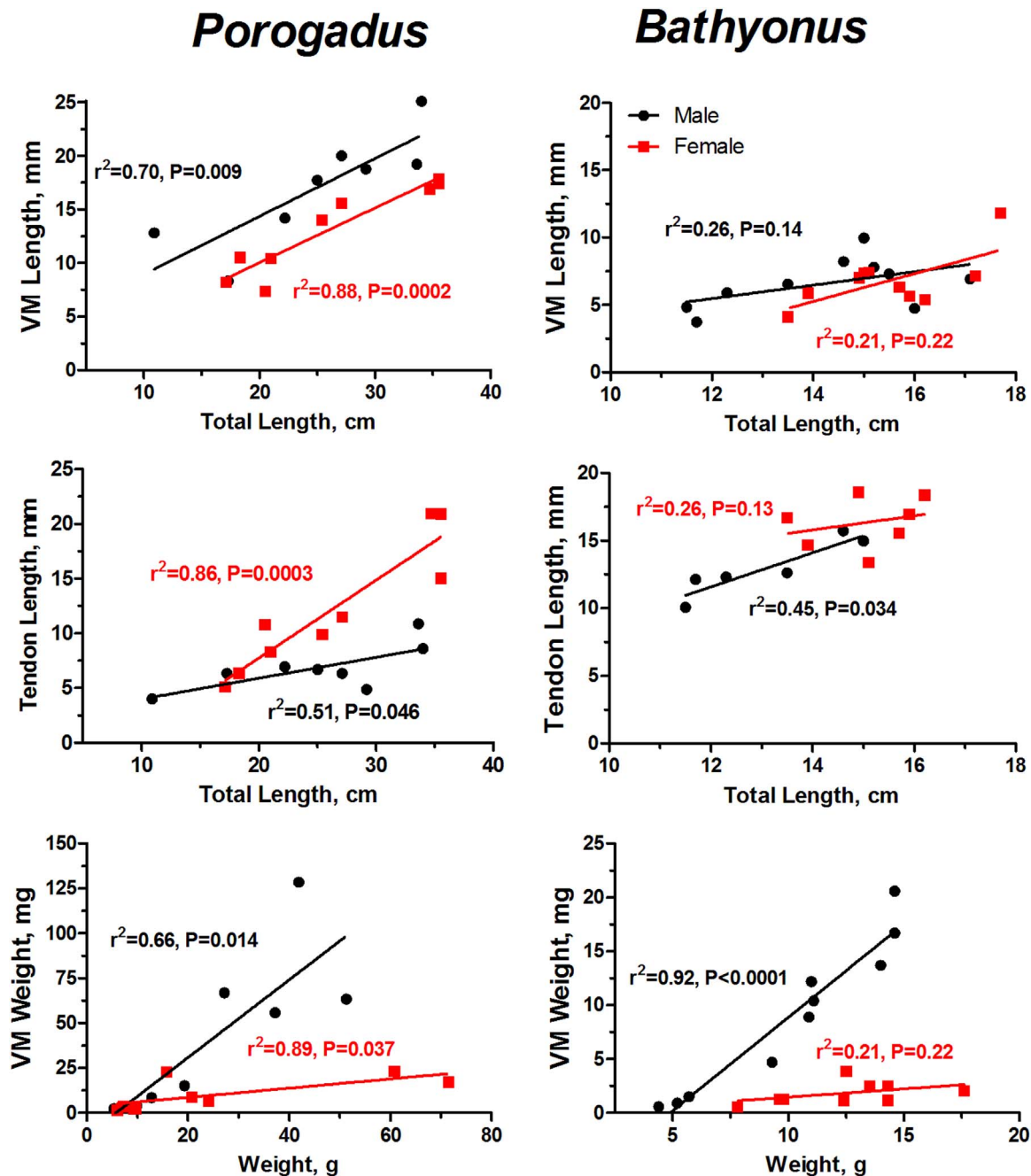


Fig. 11. Relationship of lengths of the ventral medial muscle and sonic tendon to total length, r^2 and p values and ventral medial muscle weight to fish weight for male (black) and female (red) *Porogadus miles* (left panel) and *Bathyonus pectoralis* (right panel).

4. Discussion

This study compares the morphology and development of the sonic system of six neobythitine cusk-eels from depths of two to three hundred to five thousand meters. In addition to phylogenetic changes (discussed later) there are potential twin effects on morphology from decreasing food supplies with depth (Lampitt et al., 1986; Collins et al., 2005; Sutton et al., 2010; Mindel et al., 2016) and increasing hydrostatic pressure (Angel, 1997; Bochus and Seibel, 2016). To our knowledge the effect of neither has been examined in terms of sonic morphology, a proxy for sound production since fish sounds have not been recorded from great depths (Fine and Parmentier, 2015). Although there tends to be a pattern of decreasing metabolism with depth in marine organisms, the decrease may not always result from resource limitation (Childress, 1995; Seibel and Drazen, 2007). Still, we

predicted that decreasing food with depth would correlate inversely with development of the sonic system of neobythitines. The effect of a limited food supply is supported by the decreasing weight per unit length of *Dicrolene* and still further reduction in *Porogadus* and *Bathyonus*. Another deep neobythitine *Acanthonus armatus* has apparently forgone sound production losing its swimbladder (Horn et al., 1978; Howes, 1992). It compensates by maintaining a high water content (almost 90%), a huge cranial cavity filled with dilute fluid and a small brain as well as numerous other adaptations to save energy and increase buoyancy without a swimbladder (Horn et al., 1978; Fine et al., 1987). One could argue that the trend in body-weight reduction is not as dramatic as it appears since the deep species have longer and more slender tails than the shallow species: compare Fig. 1 of this paper with Fig. 1 in Ali et al. (2016). Elongated tails are common in deep-water fishes (Neat and Campbell, 2013; Clarerie and Wainwright, 2014; Ferré

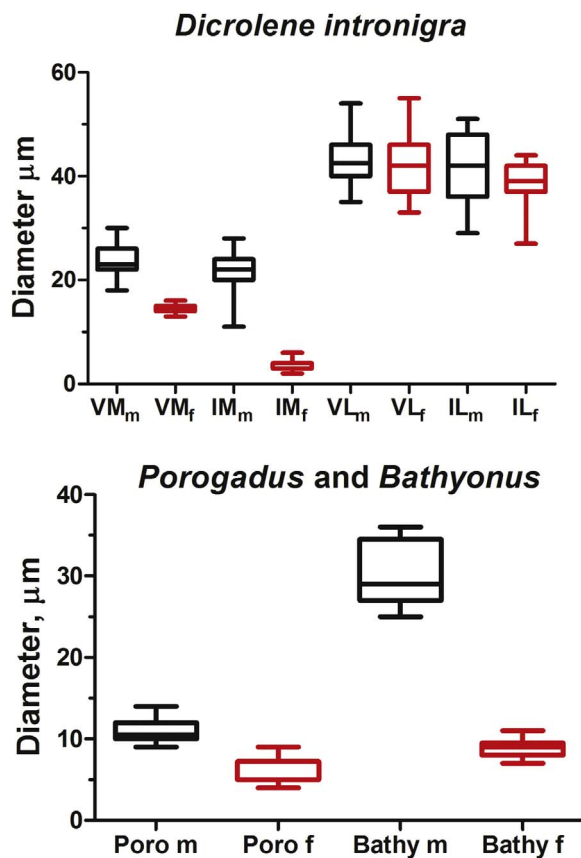


Fig. 12. Box and whiskers plot of fiber diameter (medians, quartiles and range) of ventromedial (VM), intermediate medial (IM), ventrolateral (VL) and intermediate lateral (IL) muscles of a representative male (m) and female (f) *Dicrolene intronigra* (top panel) and a male and female *Porogadus miles* and *Bathyoncus pectoralis* (bottom panel).

et al., 2016). Although there are likely multiple reasons for this adaptation, long-slender tails would increase the surface to volume ratio and could contribute to buoyancy. Further anguilliform motion associated with elongated tails is surprisingly efficient at slow speeds and puts less of a premium on the caudal fin for movement (Neat and Campbell, 2013).

We observed a trend of increasing and then decreasing eye size with depth. The large eyes of *Dicrolene* and melanin on the skin and peritoneum suggest bioluminescence may play a role in feeding and avoiding predation since surface light is negligible or absent at its depths of occurrence (Widder, 2010). *Porogadus* has some black coloration on its outer surface and has well-developed but smaller eyes. *Bathyoncus*, the deepest species, is pale externally and has reduced eye diameter suggesting adaptation to a darker environment. This pattern suggests that vision and coloration are still important at depths beyond surface-light penetration, but their importance may decrease at great depths.

Most work on ophidiid sonic anatomy and sound production has utilized shallow-water species of the subfamily Ophidiinae (Rose, 1961; Courtenay, 1971; Sprague and Luczkovich, 2001; Parmentier et al., 2006a, 2006b, 2006c, 2010; Fine et al., 2007; Nguyen et al., 2008; Kever et al., 2014, 2015; Mooney et al., 2016). Although conservative externally (Nielsen et al., 1999), various Ophidiinae species have a number of unusual morphological adaptations for sound production including antagonistic muscle pairs, a swimbladder that in some species secretes a lima-bean shaped rocker bone at its rostral end for sonic muscle attachment (Parmentier et al., 2008), and a neural arch above the first vertebra capable of pivoting in the rostral-caudal plane (Fine et al., 2007). By comparison the sonic systems of the Neobythitinae appear to be considerably more conservative. Although there is

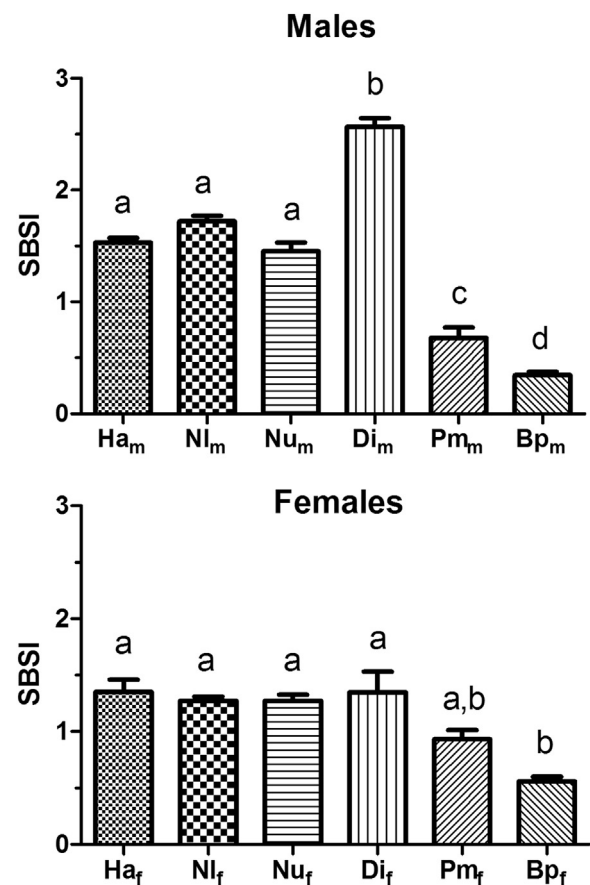


Fig. 13. Swimbladder somatic index (percent swimbladder weight) of male *Hoplobrotula armata* (Ha), *Neobythites longipes* (NI), *Neobythites unimaculatus* (Nu), *Dicrolene intronigra* (Di), *Porogadus miles* (Pm) and *Bathyoncus pectoralis* (Bp) (top panel) and females (bottom panel). Different letters indicate means that are significantly different. Compared to the upper slope species *Dicrolene* has a larger swimbladder in males but not in females, and the swimbladder in *Porogadus miles* and *Bathyoncus* males is reduced in relative size. Trends in females are not as clear, but the *Bathyoncus* swimbladder is smaller than in the shallower species.

quantitative variation in swimbladder and sonic muscle development in the shallow neobythitines, *Dicrolene intronigra* from the Atlantic shares the same four sonic muscles, including origins and most insertions, present in Pacific species from Taiwan. Additionally, *Dicrolene* and the Taiwanese species have expanded epineurals and share a concave soft structure behind the swimbladder that may function to stabilize its posterior end. To our knowledge this structure has not been described in other fishes. Males of both *Dicrolene* and *Hoplobrotula armata* have a swimbladder fenestra and a posterior tube (notably less protruding in *Dicrolene* than in *Hoplobrotula*) lined with a thin membrane. The swimbladder weight of *Dicrolene* is comparable to weights in the shallower species in females, but it is greater in males. A larger swimbladder coupled with larger sonic muscles should enable them to produce more intense sounds. The three shallower species have a swimbladder fenestra in both sexes, but the structure is only present in male *Dicrolene*. A fenestra is not present in either of the two deepest species, and specialized epineurals attached to the swimbladder are lacking. However, *Porogadus* and *Bathyoncus* both have smaller swimbladders with thinner more flexible walls, which would allow muscle contraction to pull the anterior bladder forward without a fenestra. Relative swimbladder weight (SBSI) in *Porogadus* and *Bathyoncus* males is lower than in any of the shallow species.

In the shallower neobythitine species, the two medial sonic muscles are considerably larger in males, and the two lateral muscles that insert on the epineurals are somewhat larger in females in some but not all

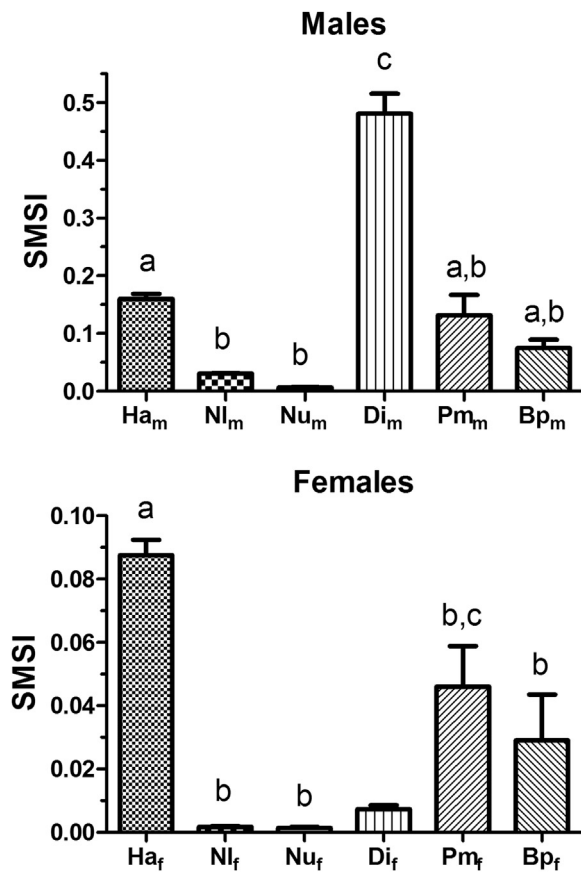


Fig. 14. Sonic muscle somatic index (% sonic muscle weight) of male *Hoplobrotula armata* (Ha), *Neobythites longipes* (NI), *Neobythites unimaculatus* (Nu), *Dicrolene intronigra* (Di), *Porogadus miles* (Pm) and *Bathyonus pectoralis* (Bp) (top panel) and females (bottom panel). Different letters indicate means that are significantly different. Note different y-axis scales for males and females.

cases (Ali et al., 2016). The size disparity between male and female muscles is greater for the medial than the lateral muscles. We associated the heavier medial muscles with rapid oscillations of the swimbladder for sound production, and the dramatically thinner muscles in females suggest they produce weak sounds or are mute. The reason for larger lateral muscles in females is unclear, but we hypothesized the lateral muscles would cause tonic extension of the bladder during sound production, which would put the swimbladder under tension, thus making it more efficient in sound production (Ali et al., 2016). Since the epineurals are relatively fixed however, it is also possible that the lateral muscles simply function to stabilize the flexible body or to prevent the anterior swimbladder from bowing during contraction of the medial muscles? *Dicrolene*, although an ocean apart from the Atlantic, exhibits a similar pattern with larger medial muscles in males and a larger lateral muscle in females. This finding suggests a conservative sonic system in shallow neobythitines.

The swimbladder in *Porogadus* and *Bathyonus* is located further caudally in the body cavity, and three of the four sonic muscles have been reduced to a single pair of ventral medial muscles to stimulate swimbladder movement. Again this muscle is considerably larger in males than in females, suggesting that male sound production, likely for mate attraction or male-male competition, is important at great depths. Notably the ventral medial muscles although well-developed terminate in the middle of the body cavity, giving rise to long tendons that extend to the swimbladder. The tendon is considerably longer than the muscle in *Bathyonus*, the deepest species. Long tendons are common in tetrapod limbs, e.g. there is little room for muscles in distal parts (fingers for instance) (Marieb and Mallatt, 2001). In *Porogadus* and *Bathyonus*

however, the tendons pass through the body cavity where there is ample room for longer muscles, and therefore these unusual tendons are likely to play an important role in sound production.

We hypothesized that the tendons would be coupled with slow muscles whose contraction would put the tendons and swimbladder under strain that could result in multiple bladder oscillations upon muscle relaxation, depending on vibratory properties of the tendon (Parmentier and Fine, 2016) and restorative forces of the stretched swimbladder. We note that swimbladders not associated with tendons typically experience rapid damping (Fine et al., 2001, 2009, 2016; Millot et al., 2011). Muscle contraction and tendon strain would pull the swimbladder forward and compress the ventral and lateral walls of the swimbladder. The tendon, stretched bladder wall and internal gas pressure would then cause swimbladder rebound.

An important question is the selective advantage and function of long tendons in the nutritionally dilute lower-slope environment. Tendon function in movement has been examined mostly in leg muscles in a variety of tetrapods - frogs, turkeys, kangaroos, wallabies and primates including humans (Alexander, 2002; Biewener and Baudinette, 1995; Lichtwark and Barclay, 2010; Vereecke and Channon, 2013; Sawicki et al., 2015; Mayfield et al., 2016). Leg movement generally involves muscle contractions over a range of 0.2–11 Hz (Alexander, 2002), which would result in a volume velocity too low to produce audible sound. Volume velocity, the product of the surface area of a sound radiator (in this case a swimbladder) and the velocity of its movement, determines sound amplitude (Bradbury and Vehrencamp, 1998; Fine et al., 2001). Therefore rapid movement is required for effective sound production with a small swimbladder, explaining the evolution of superfast muscles in fish sound production (Fine et al., 2001; Millot et al., 2011). Alternately, slow systems in fish sound production utilize a tendon or bone, which can be activated by a slower contraction, and then drive the swimbladder into vibration although such tendons can also be coupled with fast muscles (Parmentier and Fine, 2016). We apply several generalizations about muscle-tendon physiology from Alexander's insightful review (Alexander, 2002): 1) metabolic energy should be saved when tendons stretch and recoil storing and losing kinetic energy; 2) tendons recoil elastically faster than muscles shorten, which would enable faster swimbladder motion and volume velocity; 3) tendons have low-energy dissipation returning approximately 93% of the energy used in stretching them. Heat development is unlikely a problem for a slender marine fish surrounded by cold water at great depths. Further Alexander notes that faster contraction causes lower force and that power output (force x shortening speed) is greatest when shortening at moderate speed (ca 0.3 v_{max}). Therefore a shortened muscle with a long tendon should cause more rapid swimbladder movement and require less metabolic energy than a longer muscle attaching to its effector with a typical short tendon.

How do these principles align with morphological measurements from the upper slope species (Ali et al., 2016) and three mid to lower-slope species, *Dicrolene*, *Porogadus* and *Bathyonus*? The shallow species from 200 to 300 m and *Dicrolene*, from about 1 km depth and greater (Wenner, 1984), have typical short tendons that connect sonic muscles to the swimbladder and expanded epineurals. However, in male *Porogadus* and *Bathyonus* the tendons take up respectively 29% and 70% of the distance between the muscle and the bladder, e.g. a relatively shorter muscle, particularly in *Bathyonus*. Alexander's generalizations predict a slower muscle in species with long tendons. Since live neobythitines are not available for experimentation, we use muscle fiber diameter as a potential marker for speed. The medial muscles (VM and IM) in upper-slope species have small fibers, generally > 10 μ m in males and a little more than half that size in females, suggesting fast muscles (Ali et al., 2016). The two lateral muscles have larger fibers, generally with medians between about 40–60 μ m, equivalent to epaxial trunk muscle used in slow swimming motions. The typically small-fibered intermediate medial muscle in female *Neobythites unimaculatus*

was an exception with large fibers although it had typical small diameter fibers in males. Medial muscle fibers in male *Dicrolene* were approximately twice as large as in the three shallow species ($> 20 \mu\text{m}$), with those of females again about half the size of fibers in males. Fibers of the two lateral muscles were about twice as large as in the medial muscles, echoing the pattern in shallower species. Small fibers in the medial muscles suggest rapid contraction, and medial fibers in *Dicrolene* are about the size of toadfish sonic fibers (Fine et al., 1990, 1993), a classic example of a superfast muscle.

Fiber diameter in *Porogadus* was surprisingly small (a little greater than $10 \mu\text{m}$ in males and half of that in females), suggesting fast muscles, contrary to our prediction based on the long tendons. We note that the tendon in *Porogadus* is considerably shorter than in *Bathyonus*. *Porogadus* has a larger eye and black pigment unlike the pale *Bathyonus*. *Porogadus* may therefore represent an intermediate situation, adapting to a deeper habitat while still maintaining fast fibers? The external morphology of *Bathyonus* (pale coloration, small eyes) is suggestive of living in a darker habitat. Fibers in the male ventral medial muscle of *Bathyonus* are considerably larger (ca $30 \mu\text{m}$) than in *Porogadus*. Presuming slower muscles, the long tendons in this species would suggest a major role in causing rapid swimbladder movements and sound production. Both *Porogadus* and *Bathyonus* have thinner more flexible bladders, which would obviate the need for a swimbladder fenestra to facilitate forward motion of the anterior swimbladder. Differences in fiber diameter among species suggest that muscle speed is a plastic character open to selection. Sexual selection would favor larger muscles in males (Wells and Taigen, 1989) who presumably produce greater amplitude sounds, whereas depth and decreasing food supply presumably selected for a reduced sonic system still putatively capable of producing effective courtship and agonistic sounds.

4.1. Phylogenetic implications

The phylogeny of the neobythitines is poorly understood, and there are few characters that unequivocally separate different clades (Ken Sulak personal communication). In a large study Howes (Howes, 1992) divided ophidiids into two groups based on morphology. In Group 1 the swimbladder is anteriorly situated and intimately associated with the expanded first three epineurals. The swimbladder often has a modified anterior cap for muscle attachment, and the first neural arch is short. In Group 2 the swimbladder is posteriorly situated and not associated with anterior ribs, which are unexpanded, the swimbladder is not modified although there is muscle attachment, and the first neural arch is reduced and autogenous. As recognized by Howes, group 1, which includes the neobythitines *Hoplobrotula*, *Neobythites* and *Dicrolene* from this study, also includes members of the subfamily Ophidiinae such as the genera *Ophidion* and *Lepophidium*, which have been the subject of studies on sound production (*Ophidion* only) and sonic morphology (see earlier references). The deeper species included in this study *Porogadus* and *Bathyonus* as well as *Barathodemus* (Carter and Musick, 1985) fall into group 2. In on-going molecular studies Møller, Knudsen, Schwarzhans and Nielsen (personal communication) determined the Ophidiinae is basal to the Neobythitinae. Although many ophidiid characters appear to be plastic, Howes' Group 1 suggests an affinity between the two subfamilies, which would suggest that the shallow neobythitines formed from the more basal subfamily before the group entered great depths? Note the epineurals look quite different between members of the two subfamilies: they are more rigid in the Neobythitinae and have less ability to deform the swimbladder. Additionally dorsal sonic muscles are so far unknown in neobythitines. These findings suggest invasion of the deep slope involved reduction in number of muscles attaching to the swimbladder, the loss of a swimbladder fenestra and posterior tube, and the loss of expanded epineurals.

Barathodemus, *Porogadus* and *Bathyonus*, although united in Howes' Group 2, have a number of differences. *Barathodemus* has a rounded, inflated snout (not flattened-depressed), small eyes, no lateral line

specializations, small body scales, no specialized rays in the pectoral fins (Ken Sulak, personal communication) and a typical short tendon connecting the sonic muscle to the swimbladder (Carter and Musick, 1985). *Porogadus* (Carter and Sulak, 1984) and *Bathyonus* have a more attenuate body and multiple lateral lines (Fig. 1 and Sulak personal communication). The presence of a shortened ventral medial sonic muscle with a long tendon connecting to the swimbladder is an unusual character that unites the two species and separates them from the deep *Barathodemus*.

We see a potential chain in modification of the sonic system in neobythitines from the upper slope to several thousand meters. Given the small number of species examined, we present our ideas as a hypothesis subject to change as more species are examined. Sonic morphology, equivalent to shallower species, is present in *Dicrolene* males although females have lost the swimbladder fenestra, suggesting sufficient food at depths of a kilometer and greater to maintain a robust sonic system. *Dicrolene* was lighter per unit length than the surface species, but its swimbladder was larger in males than females. Swimbladder size in females was comparable to the upper-slope species. Similarly the sonic muscle in males was relatively heavier than in *Hoplobrotula armata*, the surface species with the greatest SMSI. *Barathodemus manatinus* provides a potential next step in reduction with a single muscle pair attaching to the swimbladder in males (Carter and Musick, 1985). The third step, present in *Porogadus* and *Bathyonus*, is to again have a single well-developed but short ventral medial muscle with a long tendon. We consider the long tendon of the two deep species an important character uniting them, but it is premature to indicate whether long tendons are a common feature of deep-slope species or a feature characteristic of a smaller clade of related deep-water neobythitine species.

5. Conclusions

Compared to upper slope neobythitine cusk-eels, deeper species in this study have a longer tail and a lower weight per unit length suggesting an environment with less food. Swimbladders of the deepest species are thinner and more pliable than in shallower species, and potential acoustic specializations such as a thick white tunica externa, a swimbladder fenestra and a posterior tube, and modified epineurals are not present. In all species, medial muscles are larger in males than in females, suggesting male sound production in agonistic and courtship behaviors from shallow to deep-slope bottoms. Sonic muscles in *Dicrolene*, with four pairs of muscles, are similar to more shallow species from a different ocean. These observations suggest sufficient food at depths of 1 km and greater to maintain a robust sonic system and a conservative one compared to that in the sister subfamily Ophidiinae. The increased size of the swimbladder and sonic muscles in male *Dicrolene* as well as its enlarged eyes, suggest that vision and sound production are important to this species. The sonic systems of deeper species *Porogadus* and *Bathyonus* (1600–5000 m) are reduced to a single pair of muscles. These muscles do not extend to the swimbladder, as in shallower species, but terminate in long tendons, interpreted as an adaptation to produce rapid movement and sound with slower muscle contractions and decreased-energy expenditure. Findings suggest some sonic-morphological similarities between shallow Neobythitinae and Ophidiinae with a subsequent loss in neobythitines with invasion of deeper-slope habitats.

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Competing interests

The authors declare no competing financial interests.

Author contributions

MLF conceived the study, HQM collected and identified the shallow species, TKN and HA, HQM performed the experimental work, HA, TKN, HQM, EP and MLF analyzed the data and wrote the manuscript. The results were discussed by all authors, and all authors reviewed and approved the final manuscript.

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