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Blackwater Diving: An Exciting Window into the Planktonic Arena and Its Potential to Enhance the Quality of Larval Fish Collections

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"Blackwater diving," or nighttime SCUBA diving in epipelagic environments, has become highly popular in recent years because lay participants encounter animals that are difficult and expensive to observe through other methods. These same observations can be priceless for researchers working with these species, so an interface between the scientific communities and recreational divers would be mutually beneficial. In this paper, we describe one such interface through the photography, collection, and DNA barcoding of larval fishes from the island of Hawaii. The images and videos from this activity provide an exciting window into the epipelagic environment and the way larval fishes appear and swim within it. Blackwater diving allows us to see the often-elaborate appendages and other specializations of these larvae as they appear *in situ*, prior to extensive net and fixation damage. However, blackwater diving remains an almost exclusively recreational pursuit, particularly popular among underwater photographers, who have little interest in (or object to) collecting specimens for scientists. Nonetheless, a logical next step is careful hand collection of specimens for scientific study. Growing numbers of recreational divers around the world have access to an otherwise expensive-to-research habitat. Here we present, for the first time, *in situ* and post-fixation photos of larval fishes that were hand collected and fixed in 95% ethanol by blackwater divers operating out of Kona, Hawaii, with DNA barcode identifications congruent with morphology and pigmentation where possible. With the right motivation, blackwater diving could augment research in the pelagic ocean and significantly enhance natural history collections and our knowledge of the larvae of marine fishes.

The two most engaging powers of a [photograph] are to make new things familiar and familiar things new.

—William Makepeace Thackeray

"BLACKWATER diving," which is diving in pelagic habitats at night, has roots in both scientific and recreational diving. The first documented scientific pelagic dives were conducted in France in 1962 for the purpose of collecting delicate organisms by hand (Ceccaldi, 1962; Totton, 1965). These techniques were later adopted by University of California researchers to study appendicularians and marine snow (Alldredge, 1972; Hamner, 1975; Silver et al., 1978; Trent et al., 1978). By 1986, scientific bluewater diving had become widespread enough to warrant a definitive set of published guidelines (Heine, 1986). Recreational blackwater diving began nearly 20 years after the pioneering work of Ceccaldi (1962) with the release of the book *Within a Rainbowed Sea* (Newbert, 1982). To make the photographs featured within, author Christopher Newbert described drifting miles offshore alone at night 150 feet deep while holding onto a line from his boat. By 1990, Newbert's book had inspired a recreational following for nighttime pelagic diving. Today, blackwater diving has spread to Florida, Palau, Philippines, Indonesia, South Africa, Japan, and French Polynesia. The images and videos (e.g., https://player.vimeo.com/video/283833597) from this activity offer much more information on how larval fishes, and other planktonic organisms, appear and swim in their natural habitat, in addition to the methods reviewed by Leis (2006, 2015). The often-elaborate appendages and other specializations of these larvae can be seen and documented as they appear *in situ*, prior to extensive net damage and fixation. However, blackwater diving remains an almost exclusively recreational pursuit, particularly popular among underwater photographers, who have little interest in (or object to) collecting specimens for scientific study. Nonetheless, a logical next step is careful hand collection of specimens for research.

Research on the early life history of marine teleosts has been an important part of ichthyology and fisheries biology for well over a century (Miller and Kendall, 2009). Planktonic fish eggs and larvae are usually collected using fine-mesh nets towed from vessels. The first systematic use of these plankton nets was by John Vaughan Thompson, British Royal Surgeon and amateur naturalist, beginning in 1816, to study the developmental morphology of crustaceans (Damkaer, 2016). Thus, the earliest plankton research focused on systematics, taxonomy, and morphology. Plankton was recognized early on as such an important component of the ocean's biodiversity that plankton tows were made a routine part of the sampling program in the first circumglobal oceanographic survey, the *HMS Challenger* expedition of 1872–1876 (Tizard et al., 1911). Research on those collections was on the taxonomy, systematics, morphology, and biogeography of the diverse species found during the expedition, most of which were new to science. Various modifications and improved versions of the plankton net became standard sampling gear for other major oceanographic expeditions.

The focus on the taxonomy, systematics, and morphology of planktonic species continued into the late 1800s, but the emphasis changed to ecological research thereafter. Research on fish eggs and larvae for fisheries studies began in the late 1800s when Victor Hensen (Fig. 1A), who coined the term plankton (Greek for "wandering") in 1887, investigated the use of egg surveys to assess the population sizes of

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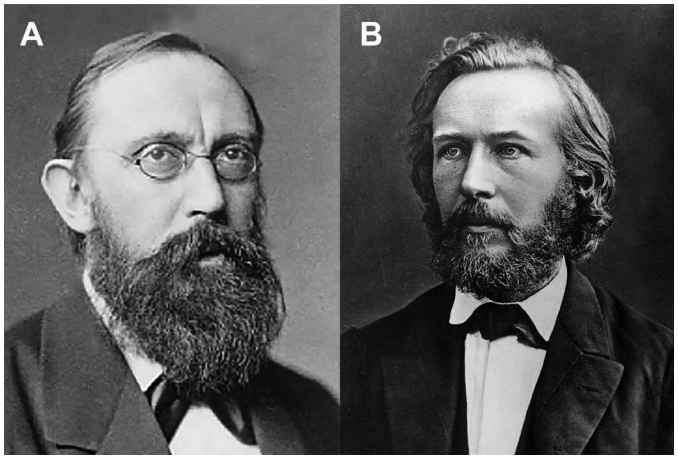

Fig. 1. (A) Victor Hensen (1835–1924) image from Wikipedia https://en.wikipedia.org/wiki/Victor_Hensen#/media/File:Victor_Hensen.jpg. (B) Ernst Haeckel (1834–1919) image from Wikipedia https://en.wikipedia.org/wiki/Ernst_Haeckel#/media/File:Ernst_Haeckel_1860.jpg.

commercially important fish species (Hensen and Apstein, 1897). Hensen (1887) wrote, "The material termed 'Auftrieb' [floating matter], which has been studied many times by zoologists and botanists, since the pioneering studies by Johannes Müller, and which is captured by a fine-mesh net has, in addition to its interest for systematics and anatomy, without doubt a great importance for the entire metabolism of the ocean" (translation by R. Britz). This dichotomy between systematic vs. fisheries research has been a fundamental part of the study of larval fishes ever since. On one side of the dichotomy was Hensen's goal to study the productivity of the ocean as a framework for maintaining and improving the fisheries. On the other side was Ernst Haeckel's (Fig. 1B) objection to Hensen's work: "How such an arithmetical Danaidae [sic] work can be carried through without ruin of mind and body I cannot conceive" (Haeckel, 1890 as translated by Field in 1893, p. 629, footnote). While discussing his research on the *HMS Challenger* plankton samples, Haeckel (1890, p. 636) wrote, "The farther the two great branches of biology, namely, morphology and physiology, have developed into higher planes during the last decade, so much further have the methods of investigation in both sciences diverged from one another." (Haeckel [1890] included ecology and population dynamics in his concept of physiology.)

The justification for almost all early life history research on marine teleosts changed in the early 20th century when Johann Hjort published his extraordinarily influential hypothesis that survival of early life history stages is what determines the annual changes in fish population numbers (Hjort, 1914). Larval fish collection and studies became largely the purview of fisheries biologists and, as funding for large oceanographic expeditions fell out of favor, the collection and study of larval fishes has primarily been justified for fisheries and environmental applications and funded accordingly. Research on early life history population dynamics to obtain an increased understanding of variations in fish recruitment has dominated fish egg and larval studies for about 100 years (Fuiman and Werner, 2002; Houde, 2008). It provided the impetus for quantitative plankton-sampling programs by fisheries organizations of many nations, including those of the United States National Marine Fisheries Service. Those programs collected, and still collect in some instances, large numbers of fish eggs and larvae, many of which are retained and some of which are archived in permanent museum collections.

Although most larval fish sampling in the 20th century was conducted for fisheries research, efforts continued to be given to basic taxonomic, morphological, and systematics studies using larvae collected in fisheries or oceanographic surveys. The 1910 British *Terra Nova* expedition to Antarctica was an early example. C. Tate Regan (1916) at the British Museum (Natural History) reported on the larvae collected by that survey. The Danish *Dana* Expeditions of 1920–1922 and 1928–1930 were the most important oceanographic surveys that collected fish larvae in the first half of the 20th century. The second *Dana* Expedition was a circumglobal program that emphasized the collection of pelagic samples, in contrast to the *Challenger* expedition's emphasis on benthic sampling (Schmidt, 1931). The samples collected by the *Dana* expeditions first became part of the permanent museum collection of the Marine Biological Laboratory at Charlottenlund Castle outside Copenhagen, but were moved in 1973 into the collections of the University of Copenhagen Zoological Museum when the Charlottenlund laboratory closed (Smith, 2008). The large collection of fish larvae from the *Dana* expeditions has been used for numerous publications on fish taxonomy, morphology, and systematics for many decades (e.g., Bertin, 1938; Johnson and Bertelsen, 1991). Today a number of other museums and laboratories have significant larval fish collections, including: the National Museum of Nature and Science (Japan), the Muséum national d'Histoire naturelle (France), the Australian Museum (Australia), the Natural History Museum of Los Angeles County (USA), the Museum of Comparative Zoology, Harvard University (USA), the University of Washington (USA), which houses the large larval fish collections of the National Marine Fisheries Service Alaska Fisheries Science Center, the Oregon State Ichthyology Collection (USA), the Nunnally Ichthyology Collection at the Virginia Institute of Marine Science (USA), and the National Museum of Natural History (USA). Some of the smaller fisheries-oriented collections have also been incorporated into these permanent museum collections. However, the numbers of lots of larval fishes in these collections are usually small compared to those of adult specimens. Some fisheries laboratories such as the NOAA NMFS Southwest Fisheries Science Center and the SeaMap program in Florida also maintain large collections of fish larvae, but these are not part of permanent museum collections.

The dichotomy noted by Haeckel (1890) between morphological and fisheries studies of larval fishes has persisted to the present, but a major bridge between the two approaches was established in the second part of the 20th century by the fisheries biologists Elbert H. Ahlstrom and H. Geoffrey Moser of the NMFS Southwest Fisheries Center. While conducting important fisheries research as part of the California Cooperative Oceanic Fisheries Investigation, Ahlstrom decided that all fish larvae collected in those surveys should be identified and enumerated to investigate the ecosystem interactions that influenced stocks of commercial species (Vlymen, 1989). Ahlstrom and Moser were strong advocates for the study of developmental stages to gain a better understanding of fish taxonomy and systematics

(Ahlstrom and Moser, 1981), publishing influential papers as examples (Ahlstrom, 1974; Moser and Ahlstrom, 1974). They also taught a course in fish egg and larval identification that trained a generation of ichthyoplankton biologists (e.g., GDJ), who passed that knowledge on to their students in turn (e.g., Sally Richardson to BCM). Elbert Ahlstrom's contributions to the use of early life history stages to study ichthyology were honored posthumously by a symposium organized by his colleagues in 1983, the proceedings of which were published as the first American Society of Ichthyologists and Herpetologists Special Publication (Moser et al., 1984). That volume was a milestone in increased understanding of fish development, morphology, and systematics. The symposium was an attempt to bring mainstream ichthyologists together with ichthyoplankton specialists to further investigations. It was successful in that regard. Another aspiration, unfortunately not fully realized, was that it would usher in a new era in systematics and prompt the greater development and use of museum collections of fish larvae for such research. The symposium volume was likely perceived to have accomplished its goals for its time, and most ichthyologists and funding agencies have not found the topic compelling. The difficulties in obtaining larval fishes, disputes about methods to incorporate developmental data into systematic research, and the high level of training and expertise required to identify fish larvae, which are morphologically very different from adults, are likely impediments to increased progress in this area of research (Leis et al., 1997).

While collecting gear and imaging technologies for the study of plankton have greatly improved since the 19th century (Wiebe and Benfield, 2003; Cowen and Guigand, 2008), the knowledge of the early life history of marine fishes and their morphology to date is, with some exceptions (e.g., Baldwin, 2013; Greer et al., 2017), based on the study of net-collected, preserved specimens. In contrast, for most adult fish species we have images of live or fresh individuals, and many can readily be studied *in situ*. This brings us to the topic of this paper: blackwater diving provides a new window into the morphology and behavior of marine fish larvae when they are alive and *in situ*. As blackwater diving became popular, many divers/photographers (including JWM) began posting their images of gelatinous plankton and larval fishes on FaceBook and often asked for assistance in identifying them. BCM and GDJ were excited to see the larval fish images and delighted to help with their identification. In 2015, GDJ began corresponding with JWM and asked if he was willing to collect fish larvae for further research. He agreed to do this and was assisted by Sarah Mayte. This was the genesis of our paper.

Intact pelagic organisms for study are difficult to acquire. Logistically, working in the open ocean is expensive and often limited in the amount of time and resources that can be spent collecting specimens. Furthermore, many traditional methods of collection, including plankton nets, may produce damaged and unrecognizable specimens. The resulting illustrations and descriptions often lack delicate body parts that are otherwise pronounced structures on the live animals. Information on the animals' behavior and associations with other organisms can prove very useful, and yet is not available from traditional collection methods. Recreational diving could contribute much to studies of the pelagic ocean. Here, as examples, we present *in situ* and post-fixation photographs of larval fishes that were hand-collected and fixed in 95% ethanol while blackwater diving out of Kona, Hawaii, with DNA barcode (COI) identifications congruent with morphology where possible.

MATERIALS AND METHODS

Collecting and imaging.—The dives took paying guests to deep water near shore at night to observe vertically migrating organisms. Gelatinous plankton including salps, siphonophores, ctenophores, and cnidarians constituted the majority of animals encountered (Milisen et al., 2018), although the most popular organisms tended to be cephalopods and larval fishes. Prior to collection, intact specimens were photographed *in situ* (when possible) with a housed, digital SLR camera affixed with twin 2000-lumen focus lights, 60 mm macro lens, and two Ikelite external strobes. Collecting divers carried 30 ml scintillation vials filled with seawater in available pocket space on their buoyancy compensators. Once a specimen was located and, when possible, photographed, the animal was corralled into the jar and the lid closed behind it. The specimens usually survived the trip back to shore where the water was replaced with laboratory grade 95% ethanol. Labels were written on the lid to include the tentative specimen identification, date of collection, location, collector's name, and an ID number. Once fixed in ethanol, the specimen was stored with other specimens until shipping. Specimens were catalogued in the fish collection of the National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM). Larvae were measured to the nearest mm standard length after fixation and preservation for at least several months.

Larval fish specimens were collected by JWM. Many were photographed while alive when allowed by the dive conditions. All of the latter and the remaining specimens were photographed after fixation. Identification to various taxonomic levels was based on both DNA barcode data and pertinent morphological diagnostic characters. We were unable to assign species-level identities to some specimens through BOLD (http://www.barcodinglife.org) and GenBank; this is either because of the paucity of the available DNA barcodes/COI sequences in both data resources or the low quality of some tissue samples, resulting in bad sequences (including contamination). Consequently, these identifications were based solely on morphology.

Prior to tissue sampling, we photographed 44 specimens using a Zeiss SteREO Discovery.V12 stereomicroscope with an attached Zeiss Axio-Cam HRc digital camera. We preserved both the voucher specimen for subsequent morphological analyses and its tissue sample for genomic DNA extraction. For tissue sampling of each specimen, we removed only the right eye, so as to keep the left side intact for further morphological analyses.

DNA barcode protocol.—For genomic DNA extraction, the removed eyeballs were initially digested by immersion in M2 250 μL and M1 + prot K 250 μL. After the initial digestion, the extractions of genomic DNA from the digested samples were completed on the AutoGen Gene Prep Automated Isolation System (Kurabo, Japan) using the manufacturer's standard (default) phenol protocol, including elution of 100 μl of resuspension buffer (AutoGen DNA Resuspension Solution). The samples were divided into two batches that were

processed on two plates. For each individual sample in the first plate (USNM446983–USNM447030), the targeted DNA barcode marker–the cytochrome oxidase subunit I (COI) fragment–was amplified via polymerase chain reaction (PCR) using the primers FISH-BCL (5′–TCAACYAATCAYAAAGATATYGGCAC–3′) and FISH-BCH (5′–TAAACTTCAGGGTGACCAAAAAATCA–3′; Baldwin et al., 2009). Prior to this particular PCR, the genomic DNA dilution was initially prepared by diluting 1 μL of the genomic DNA sample with 9 μL nuclease-free water. For PCR, 3 μL of the genomic DNA dilution was used in a total 12 μL reaction, which also contained 0.1 μL BioLine (BioLine USA, Inc., Taunton, MA) Taq polymerase, 0.4 μL of 50 mM $MgCl_2$, 1 μL 10× PCR buffer (BioLine), 0.5 μL of 10 mM deoxyribonucleotide triphosphate (dNTP), 0.3 μL of 10 μM of each primer (FISH-BCL and FISH-BCH), and 6.4 μL nuclease-free water. The following thermal cycler program for PCR was performed: 1 cycle of initial denaturation at 95°C for 5 minutes; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 45 s; and ended by 1 cycle of final extension at 72°C for 5 minutes; and a hold at 10°C. The PCR products were purified with ExoSAP-IT (USB, Cleveland, OH) following the protocol of Weigt et al. (2012), using 2.75 μL 0.2× enzyme and incubated for 30 min at 37°C. The reaction was then inactivated for 20 min at 80°C.

For each individual sample in the second plate (USNM447031–USNM447058), PCR was performed with GoTaq® G2 Hot Start Colorless Master Mix (Promega Corporation, Madison, WI), which was conducted in a total 10 μL of reaction volume also containing 3.3 μL Hot Start Colorless Master Mix, 5 μL nuclease-free water, 0.3 μL of 10 μM of each primer (FISH-BCL and FISH-BCH), 0.1 μL Bovine Serum Albumin (BSA), and 1 μL of the genomic DNA. The thermal cycler program for PCR and the purification of the PCR products are the same as the ones used for the first plate (USNM446983–USNM447030).

Cycle sequencing reactions for both strands of amplified fragment were performed by adding 1 μL of the purified PCR product in a total 10 μL reaction, which also contained 0.5 μL of either the primer FISH-BCL or FISH-BCH , 1.75 μL BigDye buffer, 0.5 μL BigDye (ABI, Foster City, CA), and 6.25 μL nuclease-free water run using the thermal cycler for 30 cycles of 30 s at 95°C, 30 s at 50°C, 4 min at 60°C, and then held at 10°C. Products of cycle sequencing reactions were purified using Millipore Sephadex plates (MAHVN-4550; Millipore, Billerica, MA) following manufacturer's instructions and subsequently stored dry until analyzed. The purified cycle sequencing products were sequenced using an Automated ABI 3730 Sequencer (2011 Life Technologies). Raw chromatograms were edited using Sequencher® v5.1 (2012 Gene Codes Corp.), and sequence trace files were exported into Sequencher 5.4.6 (2012 Gene Codes Corp). Using the Sequencher program, both low-quality ends were trimmed from the raw sequences. After trimming, forward and reverse sequences for each specimen were assembled. Each assembled pair was examined and edited manually, and each sequence was checked for stop codons. Finally the consensus sequence (655 bp) from each contig was aligned and exported in a nexus format (*sensu* Swofford, 2002).

For species identification, we used the BOLD (http://www.barcodinglife.org) ID Engine to query barcode records within BOLD (Ratnasingham and Hebert, 2007).

RESULTS

For this study, 76 specimens were collected during blackwater dives, and all were sampled for DNA analysis to obtain COI barcodes (Table 1). Of those, 26 were photographed while alive and *in situ* before they were collected. Of the 76, 44 were identified to species or genus with a ≥99% match to barcodes available in GenBank and BOLD; nine were identified to species or genus with a <99% match, two had no match in the barcode databases, and seven had tissue samples that could not be analyzed because of various technical problems. The other 13 were identified by their barcodes only to family and one only to class, with 100% match.

DISCUSSION

The placement of our specimens in the permanently archived fish collection of the National Museum of Natural History (USNM) ensures that they will be available for research by ichthyologists on topics discussed below and others. Specimens of fish larvae and juveniles collected during blackwater dives can have additional value from photographs that document their live appearance and observations on their behavior, information not available from traditional collection methods. If properly preserved, tissue samples for genetic analysis can also be obtained, as with other sampling methods.

The west side of Hawaii Island, like many other oceanic island coasts, provides access to an unusual mixture of fish larvae of species that live as adults in a wide variety of marine habitats. Although Hawaii Island's slopes have an island-wide mean gradient of 4.84° at 1–300 m (Gove et al., 2016: supplementary table 1), the west side, including Kona, has very narrow insular shelves and gradients that are among the steepest of the island (Fletcher et al., 2008: fig. 11.1). Bathyal and abyssal depths are within only a few kilometers of the coast (Lipman and Coombs, 2006). Thus, populations of coral-reef, epipelagic, mesopelagic, bathypelagic, bathyal, and abyssal fishes are very near each other in this area. The complex oceanography of the area, with frequent eddies (Calil et al., 2008; Wren and Kobayashi, 2016; Wren et al., 2016), and the swimming capabilities of larger larvae (Leis, 2006) mix together larvae from populations in those habitats, so that they can be collected at the same sampling sites off Kona. Even our small number of 76 specimens exemplifies this unusual situation.

Several of the larvae in our study are of taxa that are so distinctive in their early life history stages that their morphological identifications matched their barcode identifications at ≥99%: *Dactyloptena orientalis*, *Phtheirichthys lineatus* (Fig. 2C, this larva was swimming in an inverted posture when observed, not as shown in the figure), *Polydactylus sexfilis*, *Pseudogramma brederi* (Fig. 3D), *Forcipiger longirostris* (Fig. 4A), *Coryphaena equiselis*, *Samariscus triocellatus* (Fig. 4C), *Acanthocybium solandri*, and *Ranzania laevis*. These are widespread circumglobal or Indo-Pacific species. Others were identified with confidence from morphology but were not identified by barcodes because of problems obtaining sufficient DNA of good quality: a *Zu cristatus* (Fig. 5B), a *Barbourisia rufa* (Fig. 6B), species of *Liopropoma*, species of *Decapterus*, two unidentified labrids, and a *Ranzania laevis*. Photographs of the live specimens of *Z. cristatus* and *B. rufa* show highly elongate fin rays that have frequently been broken or lost in specimens captured by

Table 1. Specimen data. Organized by % match categories. BOLD data acquired as of October 2019.

USNM	GenBank accession number	Morphology-based ID		SL (mm)	BOLD ID Engine		%	Fig. no.	Comments in Discussion
447019	MN549731	Bramidae	*Brama*	8.5	*Brama*		100.00		X
447004	MN549734	Carapidae	Carapidae	110–120	Carapidae		100.00		X
447005	MN549735	Carapidae	Carapidae	ca 120	Carapidae		100.00	6	X
447026	MN549736	Carapidae	Carapidae	ca 80.0	Carapidae		100.00		X
447028	MN549732	Carapidae	Carapidae	ca 50.0	Carapidae		100.00		X
447040	MN549733	Carapidae	*Onuxodon*	damaged	Carapidae		100.00		
447048	MN549753	Melanocetidae	Melanocetidae	5.0	*Melanocetus*	*johnsonii*	100.00	3	
447009	MN549738	Coryphaenidae	*Coryphaena*	12.5	*Coryphaena*	*equiselis*	100.00		
447001	MN549740	Dactylopteridae	*Dactyloptena gilberti*	8.0	*Dactyloptena*	*orientalis*	100.00		
447031	MN549739	Dactylopteridae	*Dactyloptena*	8.0	*Dactyloptena*	*orientalis*	100.00		
447020	MN549741	Diodontidae	*Diodon*	4.5	*Diodon*	*hystrix*	100.00		
446989	MN549767	Echeneidae	Echeneidae	14.0	*Phtheirichthys*	*lineatus*	100.00		
447055	MN549768	Echeneidae	Echeneidae	17.0	*Phtheirichthys*	*lineatus*	100.00	2	
446996	MN549744	Gigantactidae	*Gigantactis*	4.5	*Gigantactis*	*vanhoeffeni*	100.00		
447000	MN549745	Gigantactidae	*Gigantactis*	5.0	*Gigantactis*	*vanhoeffeni*	100.00		
447045	MN549748	Gigantactidae	*Gigantactus*	4.0	*Himantolophus*	*albinares*	100.00	3	X
447056	MN549746	Gigantactidae	*Gigantactis*	7.0	*Gigantactis*	*vanhoeffeni*	100.00	3	
446985	MN549760	Holocentridae	Myripristinae	10.0	*Myripristis*	*kuntee*	100.00		
446987	MN549758	Holocentridae	Myripristinae	17.0	*Myripristis*	*berndti*	100.00		
446992	MN549754	Holocentridae	Myripristinae	15.0	*Myripristis*	*berndti*	100.00	7	
447007	MN549756	Holocentridae	Myripristinae	15.0	*Myripristis*	*berndti*	100.00		
447013	MN549755	Holocentridae	Myripristinae	16.5	*Myripristis*	*berndti*	100.00		
447015	MN549759	Holocentridae	Myripristinae	15.5	*Myripristis*	*kuntee*	100.00		
447037	MN549751	Istiophoridae	*Makaira*	17.0	*Makaira*	*nigricans*	100.00	7	
447043	MN549765	Labridae	*Cheilinus*	7.5	*Oxycheilinus*		100.00		
447033	MN549772	Molidae	*Ranzania*	8.0	*Ranzania*	*laevis*	100.00		
447044	MN549717	Monacanthidae	*Aluterus*	11.0	*Aluterus*	*monoceros*	100.00	4	
446988	MN549764	Ophidiidae	Ophidiidae	12.0	Ophidiidae		100.00		
446994	MN549763	Ophidiidae	*Barathrites*	23.0	Ophidiidae		100.00		
447016	MN549724	Ophidiidae	Ophidiidae	23.0	*Bassozetus*		100.00		
447052	MN549750	Ophidiidae	*Luciobrotula*	24.0	Actinopterygii		100.00	9	
446993	MN549770	Polynemidae	*Polydactylus sexfilis*	50.0	*Polydactylus*	*sexfilis*	100.00		
447010	MN549777	Scombridae	Scombridae	10.0	*Thunnus*	*albacares*	100.00	7	
447012	MN549715	Scombridae	*Acanthocybium*	13.0	*Acanthocybium*	*solandri*	100.00		
446983	MN549749	Epinephelidae	*Liopropoma*	10.5	*Liopropoma*	cf. *latifasciatum*	100.00	6	X
447003	MN549726	Stomiidae	*Bathophilus*	19.0	Stomiidae	possibly *Bathophilus*	100.00	5	
447014	MN549725	Stomiidae	Stomiidae	9.0	Stomiidae	possibly *Bathophilus*	100.00		
447050	MN549723	Stomiidae	Stomiidae	12.0	Stomiidae	possibly *Astronesthes*	100.00		
446991	MN549720	Congridae	*Ariosoma*	35.0	*Ariosoma*	*fasciatum*	99.85	2	X
446986	MN549757	Holocentridae	Myripristinae	14.5	*Myripristis*	*berndti*	99.83		
447053	MN549780	Apogonidae	*Apogon*	8.0	*Zapogon*	*evermanni*	99.82	7	
447039	MN549737	Oneirodidae	*Dolopichthys*	9.0	*Dolopichthys*		99.81	7	X
447057	MN549776	Scorpaenidae	Scorpaenidae	13.0	*Sebastapistes*	*fowleri*	99.81	7	

Table 1. Continued.

USNM	GenBank accession number	Morphology-based ID		SL (mm)	BOLD ID Engine		%	Fig. no.	Comments in Discussion
447023	MN549728	Bramidae	Bramidae	6.0	*Brama*	*orcini*	99.80	8	X
447024	MN549730	Bramidae	Bramidae	6.5	*Brama*	*orcini*	99.80	8	X
447025	MN549729	Bramidae	Bramidae	7.0	*Brama*	*orcini*	99.80	8	X
447011	MN549766	Paralepididae	Paralepididae	18.0	Paralepididae		99.79		
447036	MN549716	Acanthuridae	*Acanthurus*	6.0	*Acanthurus*	*thompsoni*	99.69	7	
446990	MN549747	Muraenidae	Muraenidae	65.0	*Gymnothorax*	*elegans*	99.68		X
446995	MN621852	Chaetodontidae	*Forcipiger*	12.0	*Forcipiger*	*longirostris*	99.67	4	
446984	MN549722	Soleidae	Soleidae	11.5	*Aseraggodes*	sp. 1 Moorea	99.65		X
447051	MN549721	Stomiidae	Stomiidae	24.0	*Aristostomias*		99.63	2	X
447002	MN549779	Muraenidae	Muraenidae	62.0	*Uropterygius*	*macrocephalus*	99.50		X
447038	MN549742	Gonostomatidae	*Diplophos*	35.0	*Diplophos*	*taenia*	99.46		X
447034	MN549718	Apogonidae	Apogonidae	13.0	Apogonidae	possibly *Ostorhinchus*	99.45		X
446999	MN549769	Holocentridae	Holocentridae	15.0	*Plectrypops*	*lima*	99.28	7	X
447030	MN549773	Samaridae	Samaridae	14.0	*Samariscus*	*triocellatus*	99.10	4	
446998	MN549771	Serranidae	*Pseudogramma*	9.0	*Pseudogramma*	*brederi*	99.08	3	
447035	MN549719	Apogonidae	*Apogon*	7.5	*Apogon*	*indicus* or *susanae*	98.81		X
446997	MN549778	Paralepididae	*Uncisudis*	14.0	*Uncisudis*	*advena*	98.61		X
447029	MN549775	Nettastomatidae	Nettastomatidae	105.0	*Saurenchelys*	*taiwanensis*	98.50	7	X
447006	MN621853	Stomiidae	*Eustomias*	19.0	*Eustomias*	possibly *bibulbosus*	97.92		X
447054	MN549727	Ipnopidae	Ipnopidae	19.0	*Bathymicrops*	cf. *regis*	97.84	9	X
447049	MN549743	Cetomimidae	*Eutaeniophorus*	44.0	*Eutaeniophorus*		97.67	9	X
447027	MN549761	Carapidae	Carapidae	ca 55.0	*Onuxodon*		96.00		X
447047	MN549774	Holocentridae	Holocentridae	11.0	cf. *Sargocentron*		94.15	7	
447022	MN549762	Carapidae	Carapidae	54.0	*Onuxodon*		89.00		X
447021	MN621851	Stomiidae	*Eustomias*	30.0	No match in BOLD			2	
447046	MN549752	Stephanoberycidae	*Malacosarcus macrostoma*	13.0	No match in BOLD			9	
447041		Barbourisiidae	*Barbourisia rufa*	8.5	Sequence quality low			6	
447008		Carangidae	*Decapterus*	9.0	Sequence quality low				
447017		Labridae	Labridae	11.0	Sequence quality low				
447042		Labridae	Labridae	12.0	Sequence quality low				
447032		Molidae	*Ranzania laevis*	5.0	Sequence quality low				
447058		Epinephelidae	*Liopropoma*	11.0	Sequence quality low				
447018		Trachipteridae	*Zu cristatus*	9.0	Sequence quality low			5	

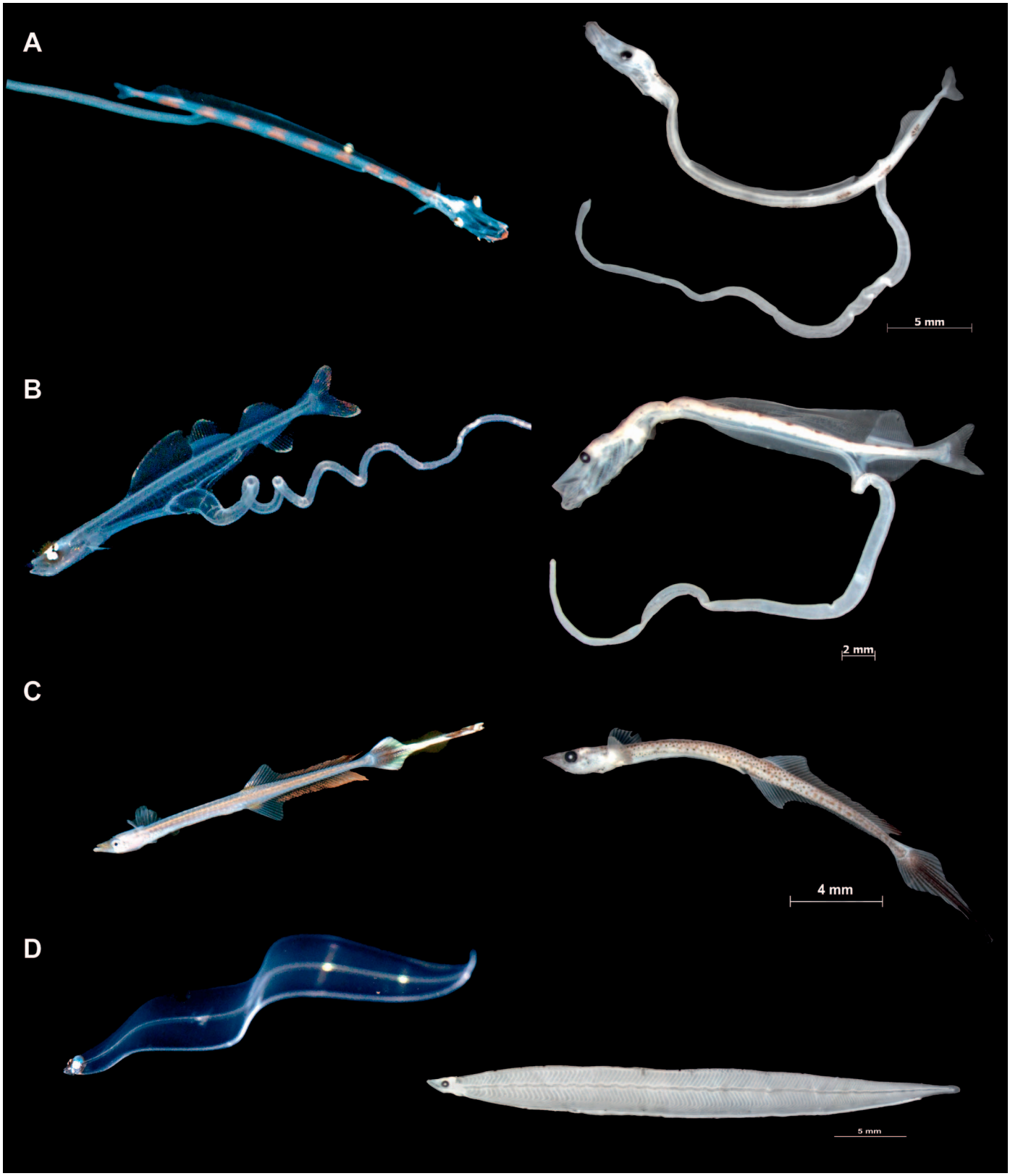

Fig. 2. Images of live (left column) and fixed (right column) larvae of: (A) *Eustomias*, USNM 447021, 30 mm. (B) *Aristostomias*, USNM 447051, 24 mm. (C) *Phtheirichthys lineatus*, USNM 447055, 17 mm. (D) *Ariosoma fasciatum*, USNM 446991, 35 mm.

nets. The careful collection of the specimen of *B. rufa* by hand allowed the newly-documented elongate pelvic-fin rays to be preserved (Fig. 6B). Even with that care, the elongate, ornamented fin rays of the *Z. cristatus* were detached during fixation because of the sudden dehydration in 95% ethanol (Fig. 5B).

A number of larvae in families whose early stages are difficult to identify beyond family or genus by external

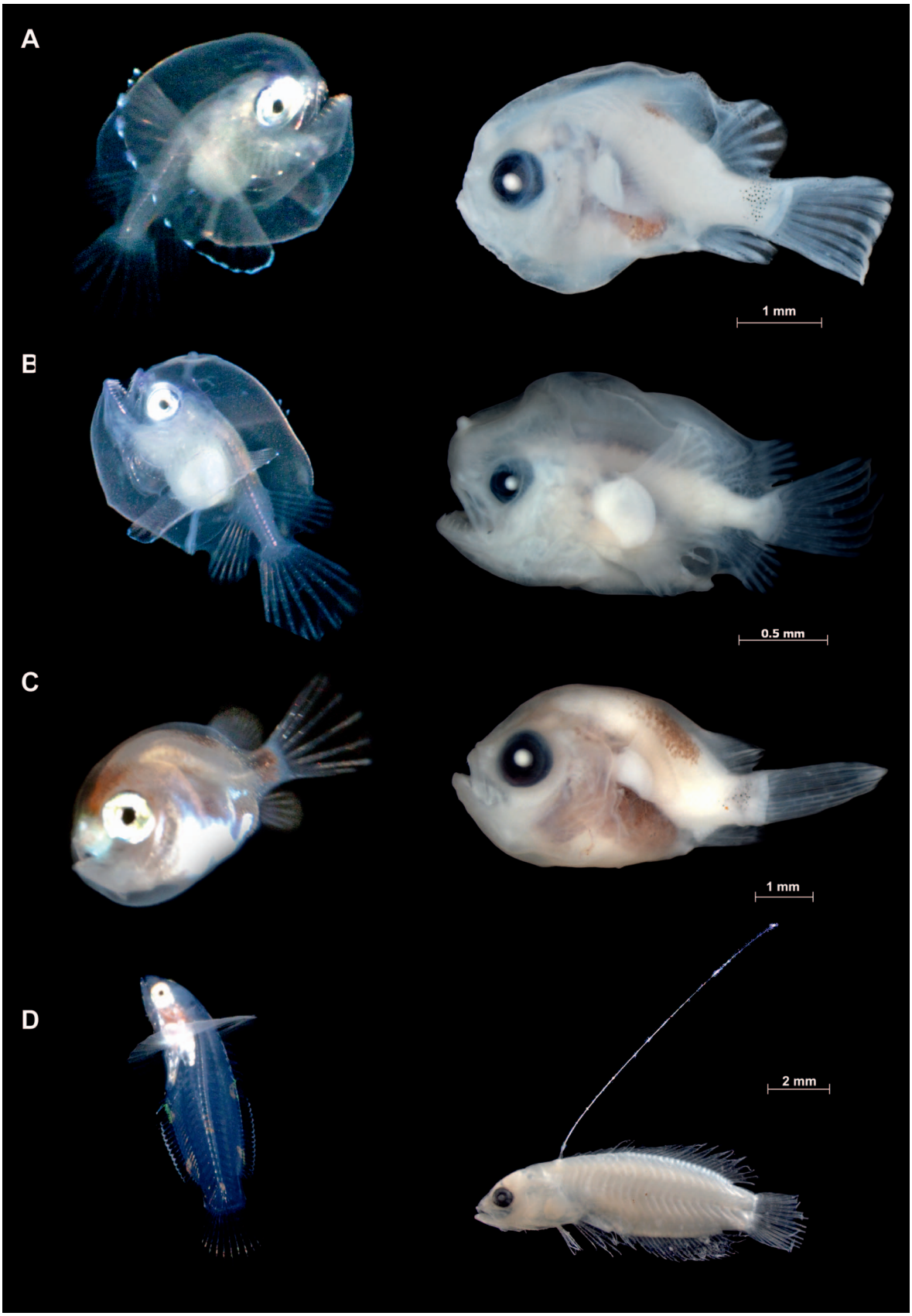

Fig. 3. Images of live (left column) and fixed (right column) larvae of: (A) *Himantolophus albinares*, USNM 447045, 4 mm. (B) *Gigantactis vanhoeffeni*, USNM 447056, 7 mm. (C) *Melanocetus johnsonii*, USNM 447048, 5 mm. (D) *Pseudogramma brederi*, USNM 446998, 9 mm.

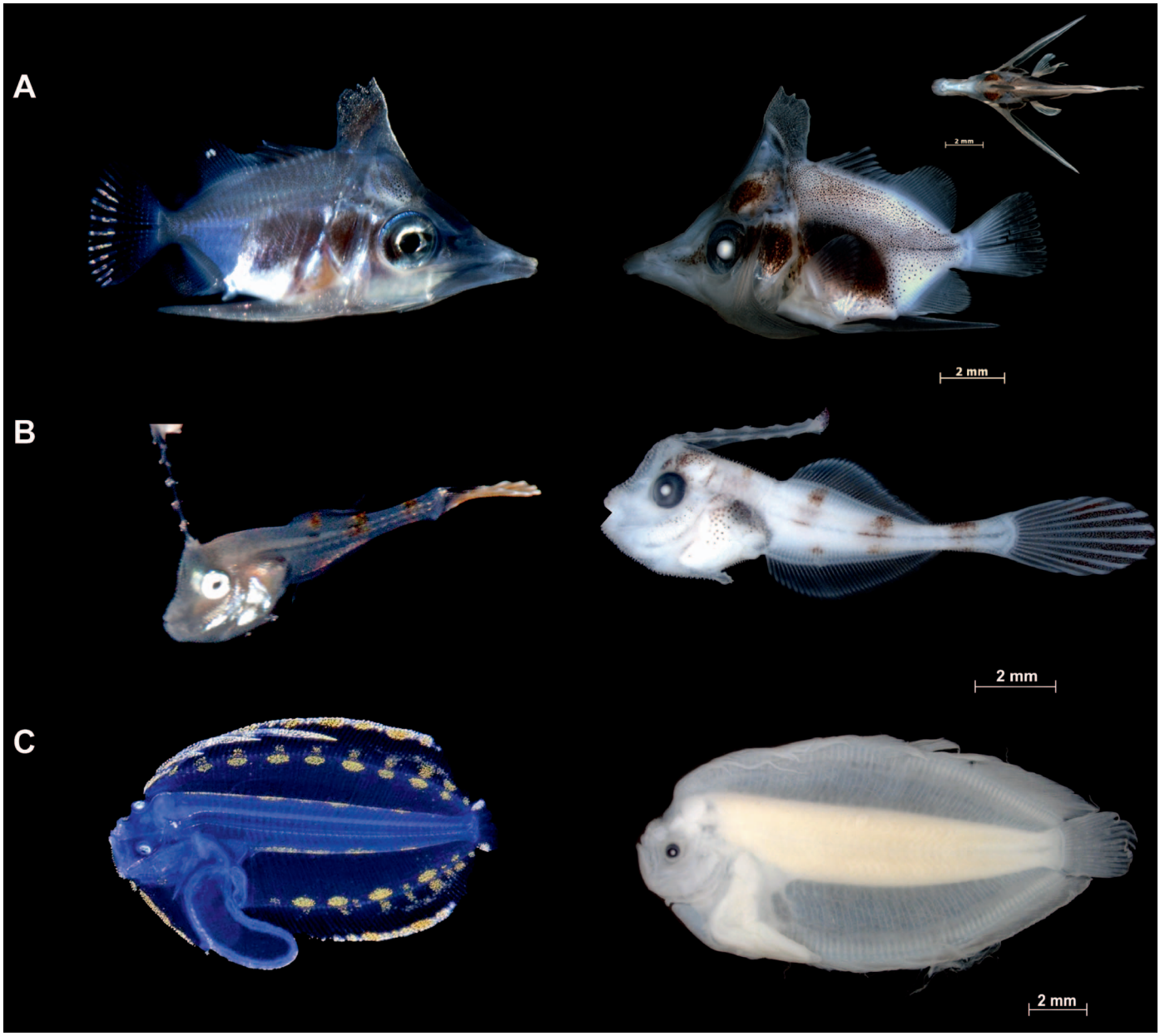

Fig. 4. Images of live (left column) and fixed (right column) larvae of: (A) *Forcipiger longirostris*, USNM 446995, 12 mm (with reduced dorsal view). (B) *Aluterus monoceros*, USNM 447044, 11 mm. (C) *Samariscus triocellatus*, USNM 447030, 14 mm.

morphology were identified to species at ≥99% confidence levels: a *Thunnus albacares* (Fig. 7F), a *Makaira nigricans* (Fig. 7E), an *Acanthurus thompsoni* (Fig. 7G), a monocanthid as *Aluterus monoceros* (Fig. 4B), two larvae of *Gigantactis* as *G. vanhoeffeni* (Fig. 3B), and a melanocetid as *Melanocetus johnsonii* (Fig. 3C). An unidentified labrid larva was confirmed by a 100% barcode match to be a species of *Oxycheilinus*. An apogonid unidentified beyond family by morphology was identified by its barcode as *Zapogon evermanni* (Fig. 7D) at a match of 99.82%, and a scorpaenid was identified as *Sebastapistes fowleri* (Fig. 7H) at 99.81%.

Three eel larvae in our study are the first to be identified to their species. A leptocephalus misidentified from morphology as a species of Congridae matched the muraenid *Gymnothorax elegans* at 99.68%. A muraenid larva correctly identified by morphology to family was found by its barcode to be *Uropterygius macrocephalus* at 99.5%. A correctly identified congrid larva was found from its barcode to be *Ariosoma fasciatum* (Fig. 2D) at a 99.85% match. A new and surprising finding was that the photograph of the live specimen shows bright yellow blotches on its side, similar to those above the guts of ophichthid larvae that were photographed, but not collected, during previous blackwater dives by Matthew D'Avella off Kona (Miller et al., 2010). This pigment has not been reported before for congrid leptocephali.

Nine holocentrid larvae were identified to species and one other only to genus. Larvae of holocentrids are usually identified only to subfamily (e.g., Leis and Carson-Ewart, 2000; Okiyama, 2014; Johnson and Schnell, 2015). One of our specimens was identified from its barcode as *Plectrypops lima* (Fig. 7B), a genus whose larvae, to our knowledge, have not been identified previously.

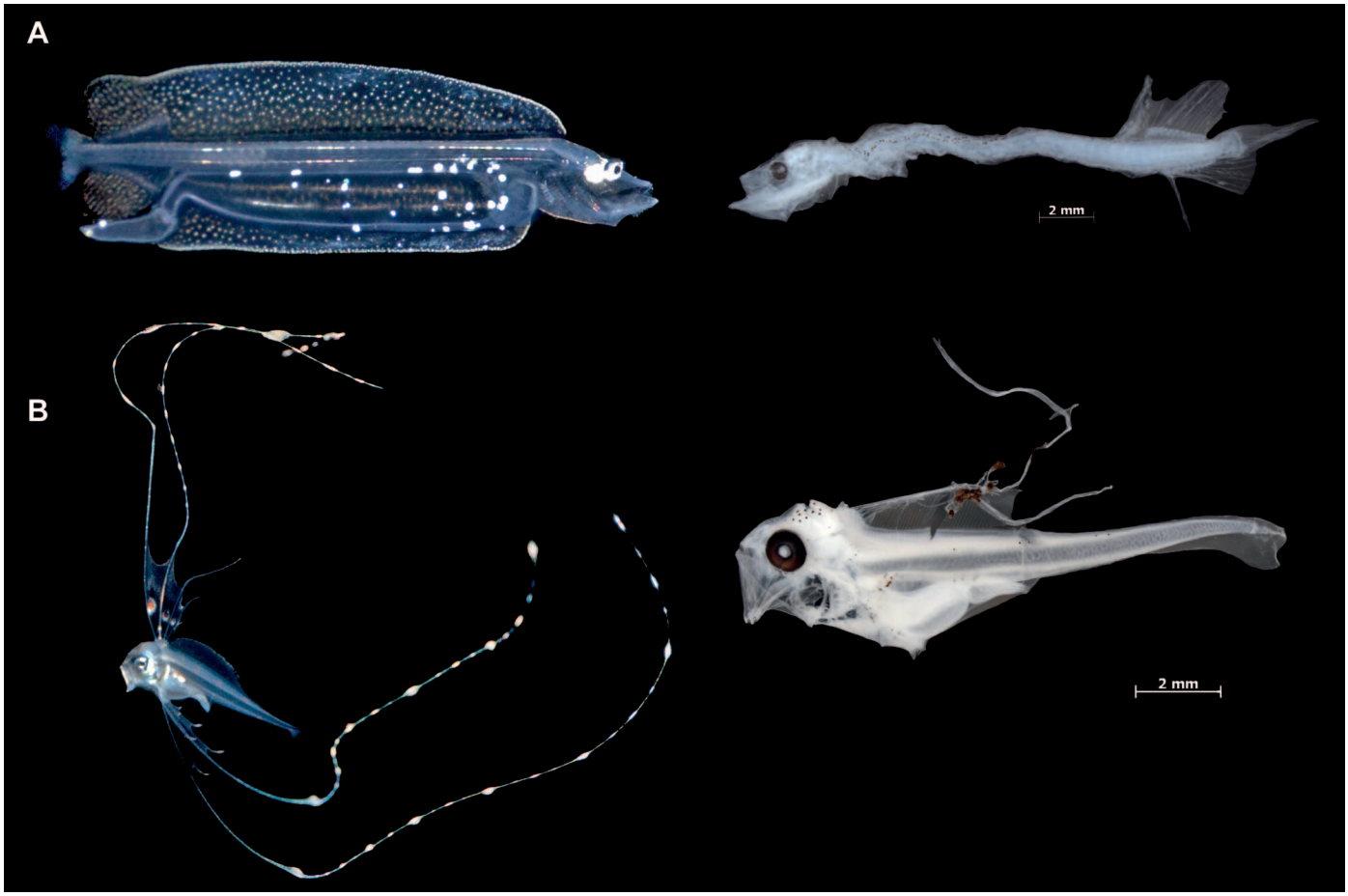

Fig. 5. Images of live (left) and fixed (right) larvae of: (A) *Bathophilus*, USNM 447003, 19 mm. (B) *Zu cristatus*, USNM 447018, 9 mm.

A contrasting result was seen for a specimen of *Liopropoma* with a barcode that matched *L.* cf. *latifasciatum* (Fig. 6A) at 98.15%. Three species of *Liopropoma* are known from the Hawaiian Islands, one of which (*L. aurora*) is endemic and two of which (*L. aurora* and *L. maculatum*) are mesophotic or rariphotic species (Randall, 2007). The third species is *L. collettei*. Of these three, only *L. maculatum* is represented in the barcode database. *Liopropoma latifasciatum* is a western Pacific species known from waters around Japan and Korea, eastward to Palau, but not the Hawaiian Islands (Randall and Taylor, 1988; Myers, 1999). All of the *L. latifasciatum* in the barcode database specimens (except for ours that was included with a low percent match) were collected within the known range of that species, supporting the correct identification of those specimens. The match of our specimen at 98.15% with a species that is not known to occur in Hawaiian waters suggests to us that the specimen is one of the Hawaiian Island species that is not yet in the barcode databases, either *L. aurora* or *L. collettei*. The photograph of our specimen when alive shows the distinctively ornamented, very elongate dorsal-fin spines of *Liopropoma*. The careful collection of our larvae enabled many of those elongate spines, together with their enlarged flaps, to be preserved. The rays and flaps are damaged or missing in specimens collected with towed nets. Kendall et al. (1984) presented an illustration of a well-preserved *Liopropoma* collected by G. R. Harbison while diving. This may have been the same larva in a photograph taken by Harbison that was published in Baldwin et al. (1991). Feeney et al. (2010) illustrated and discussed an exceptionally well-preserved *Liopropoma* that was collected with a dip net at the sea surface. It has been suggested that the unusual fin-spine morphology of larvae of *Liopropoma* is Batesian mimicry with noxious siphonophores, like that found in carapid larvae (Govoni et al., 1984), or even aggressive mimicry to attract as prey the hyperiid amphipods that associate with siphonophores (Baldwin et al., 1991). The latter hypothesis was based on a single anecdotal observation conveyed to Timothy E. Targett and has not been subsequently reported. Blackwater images (and even videos) of larvae of *Liopropoma* are not uncommon, and none has shown invertebrate association with the ornamented spines. Particular attention to this by blackwater divers could help elucidate the possible function of this remarkable morphological specialization. In any case, we maintain that Batesian mimicry must play an important role, because the burden of these elaborate structures clearly inhibits swimming mobility required for effective predator avoidance. Variation in the configuration of the ornamental bulbs is evident in *Liopoproma*, and with additional blackwater images and barcode data, we may be able to assign specific identity to some of these patterns.

A species of *Aseraggodes* matched an unidentified species in that genus from Moorea in French Polynesia at 99.69%. Three species of *Aseraggodes* are known from and considered endemic to the Hawaiian Islands (Randall, 2007). Three other species are known from the Society Islands (Randall, 2005).

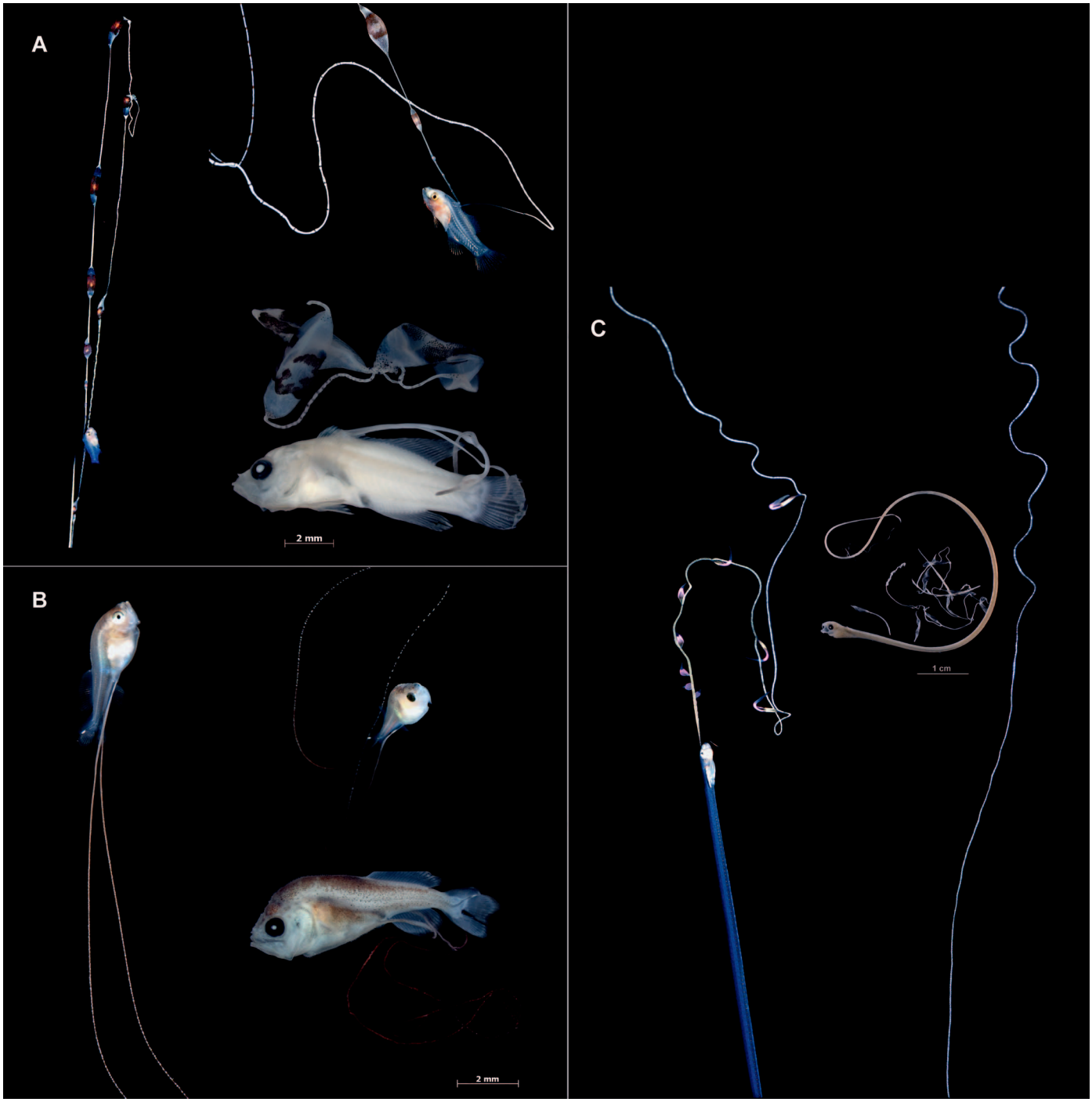

Fig. 6. Images of live (left and upper right) and fixed (lower right) larvae of: (A) *Liopropoma* cf. *latifasciatum*, USNM 446983, 10.5 mm. (B) *Barbourisia rufa*, USNM 447041, 8.5 mm. (C) Images of live and fixed (center) larva of: Carapidae, USNM 447005, ca 120 mm. Photo of fixed specimen by M. Montalvo.

The match of our larval specimen from Hawaii with an unidentified species of *Aseraggodes* from Moorea indicates that at least one unresolved taxonomic or biogeographic problem remains for this genus in Polynesia.

Four bramid larvae illustrated problems with the identifications of specimens in the BOLD database. Three of our specimens matched specimens identified as two species in the database with ≥99% agreement, *Brama orcini* and *B. japonica*. The group in the BOLD dendrogram of COI similarities (see Supplemental Information A, provided by BOLD systems [http://www.barcodinglife.org]; see Data Accessibility) that included these three specimens from this study had a few specimens identified as *B. japonica* and *B.* cf. *dussumieri*, but most of the specimens in that group were identified as *B. orcini*. Other specimens identified as *B. japonica* and *B. dussumieri* in the dendrogram were in separate groups, and no specimens in those groups were identified as *B. orcini*. We conclude that the group with most specimens identified as *B. orcini* contains specimens of that species, and that the putative specimens of *B. japonica* and *B.* cf.

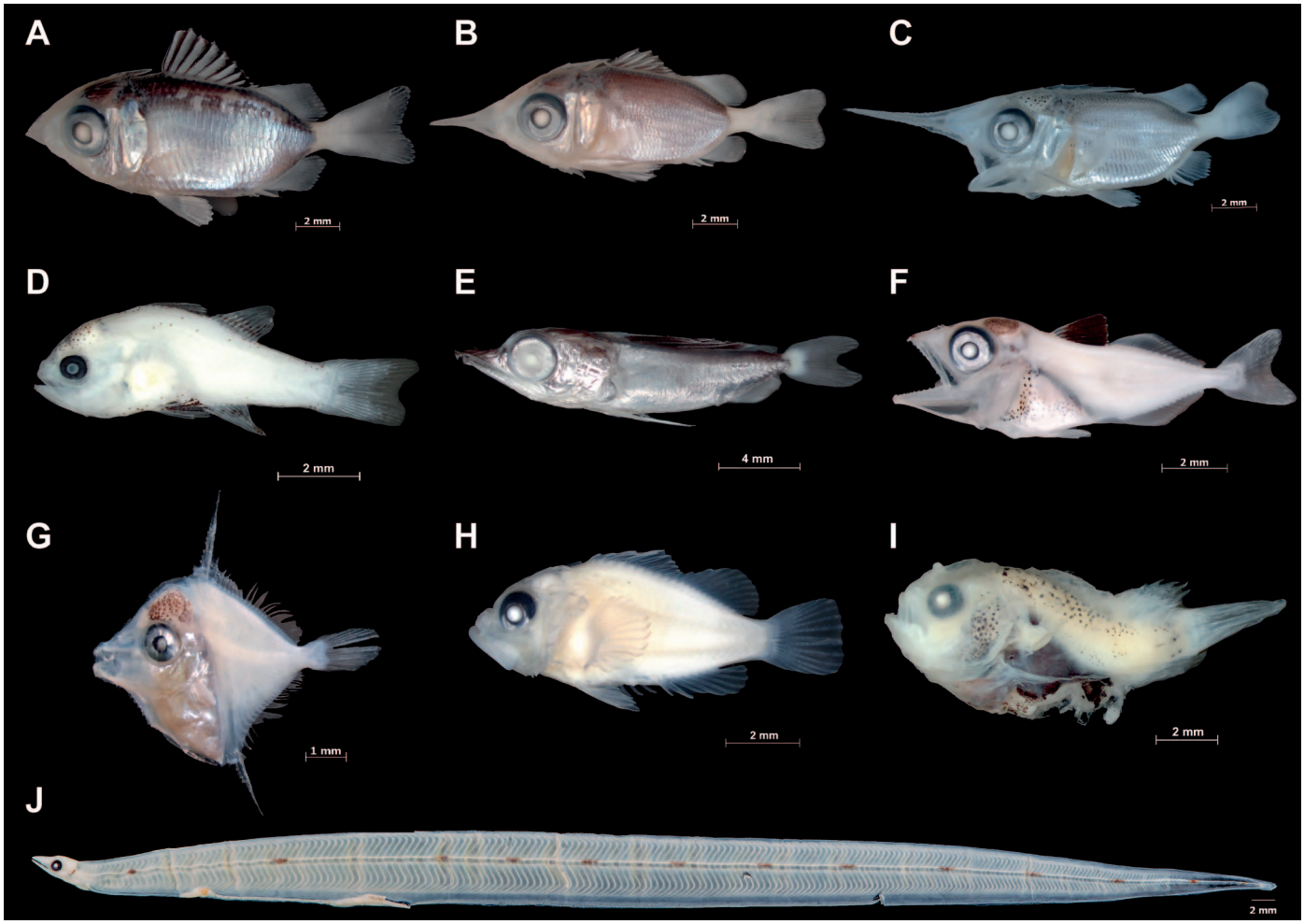

Fig. 7. Images of fixed larvae of: (A) *Myripristis berndti*, USNM 446992, 15 mm. (B) *Plectrypops lima*, USNM 446999, 15 mm. (C) cf. *Sargocentron*, USNM 447047, 11 mm. (D) *Zapogon evermanni*, USNM 447053, 8 mm. (E) *Makaira nigricans*, USNM 447037, 17 mm. (F) *Thunnus albacares*, USNM 447010, 10 mm. (G) *Acanthurus thompsoni*, USNM 447036, 6 mm. (H) *Sebastapistes fowleri*, USNM 447057, 13 mm. (I) *Dolopichthys*, USNM 447039, 9 mm. (J) *Saurenchelys taiwanensis*, USNM 447029, 110 mm. (J) Photo by S. Raredon.

dussumieri in that group were misidentified. Therefore, we identify our three specimens in that group as *B. orcini*, rejecting *B. japonica* for them. *Brama japonica* is a subarctic Pacific species known in the Hawaiian Archipelago southward only to Maro Reef, approximately 1,630 km northwest of Kona. It is thought to have its southern distributional limit at the 21°C isotherm (Seki and Bigelow, 1993), cooler than waters off Kona. Our fourth specimen of *Brama* was genetically distinct from any of the specimens of *Brama* identified to species in the BOLD database. *Brama myersi* is the species that occurs in the Hawaiian region (Mundy, 2005) that is not represented by identified specimens in the BOLD database. It is possible that our unidentified, genetically distinct specimen is *B. myersi*, but more research is also needed to resolve that question. The observations of our *Brama* larvae *in situ* revealed a previously unreported behavior, very close association (contact) with small medusae and other unidentified spherical organisms. Additional images posted on social media indicate that they are most often resting atop these organisms in a fashion similar to that reported for lobster phyllosomes (Greer et al., 2016). One of the bramids that we identify as *B. orcini* (Fig. 8A) appeared to be "riding" a corymorphid, possibly a species of *Euphysa* (Allen Collins, pers. comm.). Upon closer examination, we observed that it has notably thickened pelvic-fin rays, a previously undescribed morphology that would appear to facilitate this contact. Interestingly, the other two specimens of *Brama* also identified as *B. orcini* (Fig. 8B, C) do not have the thickened rays. The behavioral relationship and associated fin ray is under further investigation by Johnson, Milisen, and Nonaka.

Most shallow-water demersal and epipelagic fishes in the Hawaiian Islands are well-known taxonomically (Mundy, 2005; Randall, 2007), and enough of those species have been sampled for genetic barcode analysis to enable well-substantiated identifications of most of their larvae from barcode sequences. Species of Carapidae (Fig. 6C) are an exception in our study, which includes six specimens from that family. Four were identified only to family by both morphology and genetic barcodes. Two were identified as species of *Onuxodon* by their barcodes. *Onuxodon parvibrachium* and *O. fowleri* are found in Hawaii's waters (Randall, 2007). Both are represented in the barcode database, so the lack of a match by our specimens is another puzzle. Radiographs of USNM 447022, 447026, 447027, and 447028 show 4–6 vertebrae anterior to the insertion of the vexillum, which falls within the range of

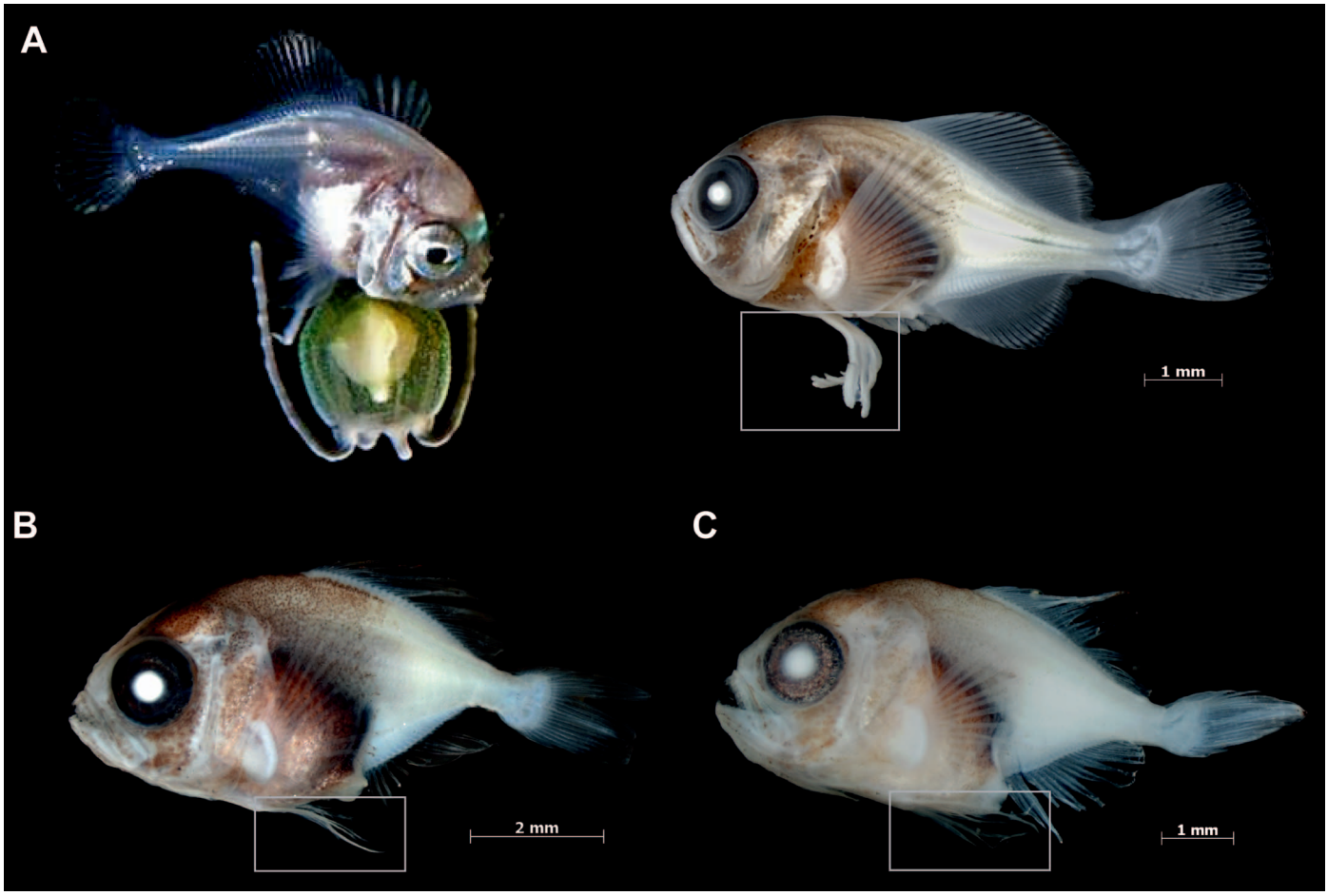

Fig. 8. (A) Images of live (left), fixed (right) larva of *Brama orcini*, USNM 447023, 6 mm. Images of fixed larvae of (B) *Brama orcini*, USNM 447025, 7 mm; (C) *Brama orcini*, USNM 447024, 6.5 mm.

Carapus, *Encheilophis*, or *Onuxodon* (Leis and Carson-Ewart, 2000). The elongate, ornamented dorsal-fin rays and filamentous tails seen when carapid specimens are alive have been suggested to mimic noxious siphonophores (Govoni et al., 1984). Sensory abilities and hydrodynamic stabilization have also been suggested as functions of the elongate fin rays of carapids (Govoni et al., 1984, which included black and white blackwater photographs of live larval Carapidae *in situ* by William Curtsinger). Numerous blackwater color images (and one video) of live carapid larvae have been posted on the internet. These, additional ones, and other behavioral observations will be critical to furthering our knowledge of the function of this extraordinary structure.

Two specimens of *Apogon* illustrate another problem sometimes encountered when trying to obtain barcode identifications of fish larvae from Hawaii's waters. One was identified as *Apogon indicus* or *A. susanae*, but with only 98.81% agreement. *Apogon indicus* is known from the western Indian Ocean to the Line Islands in the Pacific, and *A. susanae* occurs from the western Pacific to the Line Islands and French Polynesia. Neither is found in the Hawaiian Islands, where they are replaced by *A. erythrinus*, an endemic Hawaiian Islands and Johnston Atoll species (Greenfield, 2001). Specimens in the barcode databases identified as *A. erythrinus* are from localities where that species is not known to occur, indicating that the identifications were done without consulting the revision of the species group by Greenfield (2001). The second apogonid most closely matched species that are now placed in the genus *Ostorhinchus*, but also at ≤99%. Only one species of *Ostorhinchus* is known in the Hawaiian Islands (Randall, 2007), the endemic *O. maculiferus*, which is not included in the barcode database. Many endemic Hawaiian Islands species are not represented in the genetic barcode databases, causing the closest matches of barcodes for specimens to be with their more widely distributed Pacific or Indo-Pacific relatives.

Mesopelagic and bathypelagic fishes in the region are also relatively well known taxonomically (Mundy, 2005), but they are often difficult to identify correctly to species except by taxonomists familiar with them. Many of the common species are in the genetic barcode databases, but many of the rarer species are not. Archiving of voucher specimens in permanent museum collections is essential to allow verification or re-examination of identifications for those barcodes.

A morphologically identified species of *Diplophos* was identified from its barcode as *D. taenia* with at 99.46% match. This identification is noteworthy because two species of *Diplophos* can occur off Hawaii Island. *Diplophos taenia* is a cosmopolitan species that is found throughout the archipelago, but *D. proximus* is an eastern Pacific endemic species that can occur in the North Equatorial Current that sweeps past the southern end of Hawaii Island (Ozawa et al., 1990). Eddies that develop in part from instabilities of that current's flow (Calil et al., 2008) could entrain larvae and carry them

into the region off Kona. Thus, it cannot be assumed that all *Diplophos* off of the west side of Hawaii Island are *D. taenia*.

A species of *Eustomias* (USNM 447006) was found to be closest to *E. bibulbosus*, an Atlantic Ocean species, at a match of 97.92%. Two other larvae of *Eustomias* were not identified to species because their sequences did not sufficiently match specimens in the barcode database. The well over 100 species of *Eustomias*, 31 of which have been recorded from the Hawaiian Archipelago, are distinguished from one another primarily by pectoral fin-ray counts and sometimes-subtle differences in chin barbel morphology (Gibbs et al., 1983). Many species are known from only one or a few specimens, and most are not represented in the genetic barcode databases. Investigation of the congruence between the morphological characters now used to identify species of *Eustomias* and the barcodes of specimens would be an interesting research topic, to determine how well the taxonomy of this genus is actually known. Placement of voucher specimens in museum collections is essential for such research.

A different type of confusion applies to our oneirodid identified from morphology as a species of *Dolopichthys* (Fig. 7I). It was identified by its barcode as the same species as a specimen identified in the database as *Chaenophryne draco*, at a 99.81% match. The other specimens in the database in BOLD that were genetically most similar to our larva were identified as *Dolopichthys longicornis* and *D. pullatus*, two species known from Hawaiian waters (Pietsch, 2009). All other specimens of *Chaenophryne* in the barcode database appeared as a separate cluster in the similarity dendrogram for their sequences. Our larval specimen clearly has the separate groups of dorsal, lateral, and ventral melanophores on the caudal peduncle that are diagnostic for larvae of some species of *Dolopichthys* (Bertelsen, 1951; Pietsch, 2009). We suggest that the putative *Chaenophryne draco* that our larva matched in the database is a misidentified specimen of *Dolopichthys* (see Supplemental Information B, provided by BOLD systems [http://www.barcodinglife.org]; see Data Accessibility).

In another example of taxonomic uncertainty for deep-water pelagic species in the barcode databases, a morphologically identified *Himantolophus* matched *H. albinares* (Fig. 3A) at 100% with its barcode, but specimens in the database identified as *H. appelii*, *H. sagamius*, *H. stewarti*, and *H. groenlandicus* had nearly identical matches. In a manner analogous to the identification of species of *Eustomias* from chin barbel morphology, species of *Himantolophus* are identified primarily by sometimes-subtle differences in the morphology of their escae (Pietsch, 2009). Re-identification of voucher specimens in the databases and investigation of the congruence between the morphological characters now used to identify species of *Himantolophus* and the barcode identifications of specimens would be an interesting research topic.

Other deep-water pelagic taxa with barcode matches to genera or families, but not to species, are: a stomiid morphologically identified as an astronesthin species, a species of *Bathophilus* (Fig. 5A), a stomiid larva that matched the genus *Aristostomias* (Fig. 2B) in its barcode at 99.63%, a paralepidid (or lestidiid, depending on the classification used [see Gheddoti et al., 2014]) identified from morphology as a species of *Uncisudis* with a barcode sequence closest to *U. advena* at 98.61%, a second unidentified paralepidid (or lestidiid) species not identified to genus and a *Eutaeniophorus* (Fig. 9A; Cetomimidae). The tapetail larva of *Eutaeniophorus* was previously placed in the family Mirapinnidae, but adults were not identified. Johnson et al. (2009) demonstrated with morphology, transitional specimens, and mitogenomic DNA that mirapinnids are larvae of the whalefish family, Cetomimidae. The BOLD sequences of the COI barcode gene of our *Eutaeniophorus* and a specimen of *Gyrinomimus bruuni* (USNM 407647) from off Belize are very close (see Supplemental Information C, provided by BOLD systems [http://www.barcodinglife.org]; see Data Accessibility). Most species of whalefishes have broad distributions spanning the three oceans (Paxton, 1989). Female specimens of *G. bruuni* have been collected in Hawaiian waters (John Paxton, pers. comm.) as well as in the western North Atlantic. The search for other sequences in both GenBank with BLAST and BOLD to compare with these results yielded nothing close to this pair. However, numerous other sequences of both larvae and females form distinct groups in a preliminary tree that have the potential to link the two life stages of more species.

Demersal fishes at bathyal and abyssal depths of the central Pacific are poorly known taxonomically, unlike shallow-water and pelagic species (Mundy, 2005). Like the pelagic deep-water taxa, they are difficult to identify except by taxonomists who specialize in their families. In addition, specimens are lacking in the barcode databases to enable matches to be made with larval specimens of many bathyal and abyssal species. There are numerous images of adult deep-sea demersal fishes from drop cameras, manned submersible, autonomous underwater vehicles, and remotely operated vehicles in the Pacific Ocean (e.g., Yeh and Drazen, 2009; Kennedy et al., 2019), but there are few specimens from the central Pacific in museum collections to enable accurate identifications of many of the species in those images. Collection of specimens of Pacific Ocean deep-sea demersal fishes for museums is needed to understand the deep-sea biodiversity in the region.

All of the larvae of deep-sea species collected in this study provide examples of this need. A nettastomatid leptocephalus most closely matched *Saurenchelys taiwanensis* (Fig. 7J), but only with 98.5% agreement. *Saurenchelys taiwanensis* is known only from the Philippines and Taiwan (Lin et al., 2015). *Saurenchelys* from the Hawaiian Archipelago have been identified as *S. stylura* (e.g., Smith, 1989), a species described from a leptocephalus collected in the North Atlantic (Lin et al., 2015). The populations included in that nominal species globally are probably a complex of related species (Smith and Castle, 1982), which may account for the failure of our larva to match a known species in the barcode database. Likewise, an ipnopid identified from morphology as a species of *Bathymicrops* (Fig. 9D) had its best match with *B.* cf. *regis*, but only at 97.84%. *Bathymicrops regis* is the only species in its genus that has been recorded in the Hawaiian Archipelago (Nielsen and Merrett, 1992). The three other species, known only from one, four, and five specimens, have also been found in the Pacific. The incomplete match of our larval specimen to *B.* cf. *regis* indicates that more work on the taxonomy of this genus is needed when more specimens can be obtained for collections. Our specimen may be of one of the poorly known species previously unrecorded from the region, or there may be unrecognized genetic variability in the populations of *B. regis*, despite the observation by Nielsen and Merrett (1992, p. 153), who found "no indication of

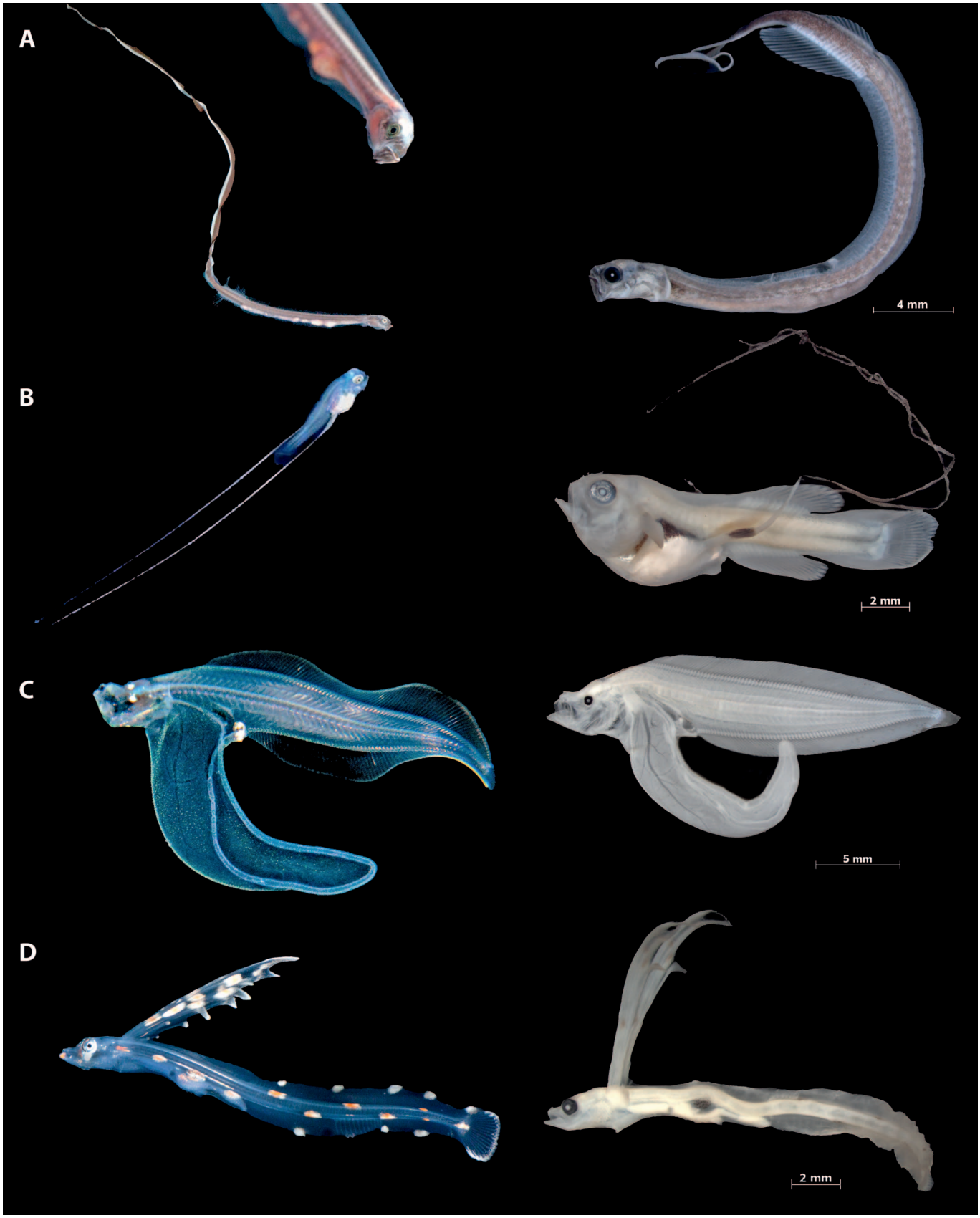

Fig. 9. Images of live (left column) and fixed (right column) larvae of: (A) *Eutaeniophorus*, USNM 447049, 44 mm (with head close-up image). (B) *Malacosarcus macrostoma*, USNM 447046, 13 mm. (C) *Luciobrotula*, USNM 447052, 24 mm. (D) *Bathymicrops* cf. *regis*, USNM 447054, 19 mm.

geographically induced variation" in *B. regis* from morphology. For a third example, an ophidiid larva identified only to family by morphology was identified as a species of *Bassozetus* at 98.49% match. *Bassozetus* is known in the Hawaiian Ridge from a specimen of *B. galatheae* collected in the northern part of the region (Iwai, 1976 as *B. elongatus*, but reidentified as *B. galatheae* by Nielsen and Merrett, 2000). Again, archived specimens of this genus from the central Pacific are needed to solve further problems with its taxonomy and the distribution of its species.

Finally, several larvae of bathyal and abyssal species in our collection failed to match any sequences in the genetic barcode databases. These were identified by morphology as an unidentified species of Ophidiidae, a species of *Barathrites* (Ophidiidae), a striking ophidiid larva with a greatly enlarged liver that was tentatively identified as a species of *Luciobrotula* (Fig. 9C) and a *Malacosarcus macrostoma* (Fig. 9B; Stephanoberycidae). *Barathrites* is known in the Hawaiian Archipelago only from individuals unidentified to species that were photographed from drop cameras (Yeh and Drazen, 2009). There are two species of *Luciobrotula* in the region: the Indo-Pacific *L. bartschi* and *L. lineata* which is known only from its holotype (Nielsen, 2009). The identification of our larva was based on illustrations and the identification hypothesis for this type of larva in Okiyama (2014). *Malacosarcus macrostoma* is known only from four over one-hundred-year-old adult specimens (two collected during the *HMS Challenger* expedition and two by the *USS Albatross*), a larva collected at the Hancock Seamounts north of the emergent Hawaiian Islands (Boehlert and Mundy, 1992), and several other larvae that have not been reported in the literature. Three of the adult specimens are disintegrated (including the holotype) and one is too fragile to be cleared and stained. Thus, the only specimens of *Malacosarcus* currently available for anatomical studies are larval ones. One of these from the National Museum of Nature and Science collections was cleared and stained by GDJ, who confirmed its possession of two characters (triangular process on fifth ceratobranchial and fusion of the third and fourth infraorbitals) that support its placement within the Stephanoberycidae (Kotlyar, 1990), despite the absence of head and fin spines characteristic of the family. The specimen of *Malacosarcus* collected in our study had very elongate pelvic-fin rays, not previously documented for larvae of this genus, that were retained by careful collection and fixation of the specimen (Fig. 9B). All of these examples illustrate once more that collection of specimens, both adult and larval, and placement of them in permanent museum collections is essential for the increase of knowledge about deep-sea fish diversity. The additional information and the images of larvae *in situ* obtained from blackwater divers when they collect specimens adds immensely to the value of those specimens.

Conclusions.—The larvae of most marine fishes occupy an evolutionary arena entirely different from that of the adults, and accordingly their morphology is often remarkably different as well. For two centuries, plankton and micronekton have been collected by pulling fine mesh nets through the water, and most of what we know about the larvae of marine fishes is based on specimens collected in this way. Valuable collections have been amassed since the late 19th century, and many of these are archived in major natural history museums where they continue to be studied from both a systematic and fisheries perspective, primarily by researchers with particular larval-fish expertise.

Since their earliest collection, the often-fanciful morphology of larval marine fishes has fascinated and captured the imagination of scientists and raised many questions about the biology of fish early life history stages. Despite the unquestionable value of net-collected specimens, elaborate, delicate appendages and other specializations are frequently damaged in the net or during subsequent, indiscriminate fixation. Color, except for melanophores, is lost during fixation and preservation (Baldwin, 2013). As we have demonstrated here, these structures can be retained with careful collection and fixation by blackwater divers. Carefully fixing individual larval specimens in 95% ethanol reduces major damage; however, delicate elongate ornamented filaments have sometimes been broken off due to the mechanical damage caused by sudden dehydration (Fig. 5). We hope to reduce this damage with further experience and may try different fixation solutions for such specimens that could help relax the specimen prior to final fixation (e.g., less concentrated ethanol, MS-222, etc.).

Furthermore, images of individuals *in situ* can offer valuable new insight into how these larvae actually appear when alive, how they swim within the plankton (Greer et al., 2017), and some of their unsuspected behaviors. Examples of such behaviors are the jelly riding of some species of *Brama* (this study), the inverted swimming of *Phtheirichthys lineatus*, the attachment to gelatinous organisms by monacanthids, probably species of *Aluterus*, with their teeth, and of a tight attachment of a specific gelatinous organism tightly around the chin of juvenile *Uraspis* (documented by other blackwater divers but not in our study). We acknowledge that observations by divers using bright lights at night may alter the natural behaviors of fish larvae, but all other observation and collection methods have effects as well, such as those mentioned by Leis (2006) and Leis et al. (2014). Blackwater diver observations of larval behavior, like observations by divers during the day (Leis, 2006), remain among the best that we have available. Blackwater diver observations are therefore a valid tool to generate hypotheses and obtain data about larval fish behavior *in situ*.

Important additional information can be obtained when blackwater divers collect the larvae that they have photographed and preserve the specimens in 95% ethanol. Identifications of the larvae by DNA barcoding can test morphological identifications and, in some instances, highlight taxonomic or barcode database problems that need more investigation. Preservation in 95% ethanol also allows for analysis of otolith daily increments to provide data on early growth and larval duration of the collected species. And perhaps of greatest importance, collection of the specimens allows them to be archived into permanent museum collections for further research. These specimens can significantly enhance natural history collections and complement existing juvenile and adult specimens of certain taxa, particularly of rare and otherwise difficult-to-collect species.

We hope that this paper will demonstrate to blackwater divers the scientific value of their efforts and inspire them to collect the specimens that they photograph (in compliance with permit and other legal requirements), and send them to museum collections, as part of a community scientist initiative. We also encourage more scientists to engage in blackwater diving, assist with specimen collection, and

observe behavior so that what is now an almost exclusively recreational activity can contribute to the increase of scientific knowledge of an under-explored part of the ocean ecosystem.

DATA ACCESSIBILITY

Supplemental material is available at https://www.ichthyologyandherpetology.org/i2019318. Unless otherwise indicated in the figure caption, the published images and illustrations in this article are licensed by the American Society of Ichthyologists and Herpetologists for use if the use includes a citation to the original source in accordance with the Creative Commons Attribution CC BY License.

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