EARLY LIFE STAGES OF THE SOUTHERN SEA GARFISH, *HYPORHAMPHUS MELANOCHIR* (VALENCIENNES, 1846), AND THEIR ASSOCIATION WITH SEAGRASS BEDS

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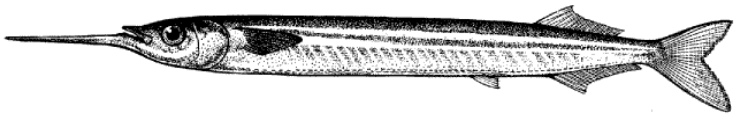

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Chapter 3 Molecular discrimination of *Hyporhamphus* larvae[§]

3.1 INTRODUCTION

Garfishes (order Beloniformes) are targeted in significant commercial and recreational fisheries in many parts of the world, including southern Australia (Collette, 1974). In this region, here defined as including southern W.A., S.A., Vic. and Tas., the garfish fishery is a multi-species fishery, with catch effort principally focused on two species, *H. melanochir* and the river garfish *H. regularis* (Günther, 1866).

Recent efforts to manage the commercial and recreational garfish fishery in southern Australia have focused on developing population models for predicting the strength of recruitment. Critical information for such models is the identification of spawning and recruitment locations and populations. This depends on being able to identify garfish species at all life-history stages. Whilst identifying adult garfish in southern Australia is not problematical (Collette, 1974), some uncertainty exists when allocating larvae to a particular species, based on traditionally used morphological characters such as pigmentation, meristic counts and body measurements. This uncertainty is due to intraspecies variation, growth and development related changes, and the occasional requirement for extrapolation between different-sized specimens. This is complicated by the fact that larvae of both species have been collected together in the same samples (pers. observ.).

The identification of closely related species of organisms is a frequent problem in marine biology, especially in the egg or larval stages of the life cycle (Burton, 1996). The advent of polymerase chain reaction (PCR)-based DNA analysis has provided a quick, often cheap, and potentially automatable method to solve these problems (Silberman & Walsh, 1992; Banks *et al.*, 1993; Medeiros-Bergen *et al.*, 1995; Grutter *et al.*, 2000; Rocha-Olivares *et al.*, 2000). Here we present a method, based on PCR of the mitochondrial *control region* (*CR*), that discriminates between life stages of *H. regularis* and *H. melanochir* or the eastern sea garfish *H. australis* (Steindachner, 1866) in southern Australia. Initially we partially sequenced the *CR* from a selection of adult garfish samples geographically representative of each species' range to phylogenetically identify nucleotide sites that distinguish between their mitochondrial lineages. We developed a multiplex PCR assay that distinguishes the species based on the

[§] The results presented in this chapter were derived from a collaboration with Dr Stephen Donnellan, Ralph Foster and Leanne Haigh from the South Australian Museum, North Tce, Adelaide 5000, and form the basis of the following publication:
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presence/absence of diagnostic bands and verified the assay on an extensive sample of adults from each species.

3.2 MATERIALS AND METHODS

3.2.1 SPECIMENS EXAMINED

Adult samples for DNA analysis were collected for the two *Hyporhamphus* species found in southern Australian waters (FIG. 3.1). A sample of *H. australis* found in New South Wales (N.S.W.) was included to ensure that our test could successfully discriminate this species from *H. regularis* of eastern Vic. in the event the distribution of *H. australis* extended to there. Our analysis will also provide a preliminary assessment of discrimination of *H. australis* and *H. melanochir* whose distributions overlap in southern N.S.W. A snub-nosed garfish *Arrhamphus sclerolepis* (Günther, 1866) was used for the outgroup (TABLE 3.1). Adults were identified using the keys and descriptions in Collette (1974). A sample of larval *H. melanochir* and *H. regularis* identified *a priori* was included to establish that this life stage could be successfully genotyped.

TABLE 3.1 Sample details of garfish examined for mitochondrial DNA variation. n_S indicates sample size for nucleotide sequencing; n_{PCR} indicates sample size for PCR assay. Locality codes are in parentheses.

Location	n_S	n_{PCR}	Life stage
Hyporhamphus melanochir			
Cockburn Sound, W.A.	1	3	Adult
Oyster Harbour, W.A. (OH)	1	3	Adult
Thevenard, S.A.	1	3	Adult
Tickera, S.A.	1	3	Adult
Arno Bay, S.A.	1	3	Adult
Port Gawler, S.A. (PG)	2	6	Adult
Western Port, Vic.	1	3	Adult
Corner Inlet, Vic.	1	3	Adult
Marion Bay, Tas. (MB)	1	3	Adult
Flinders Island, Tas. (FI)	1	3	Adult
Bay of Shoals, S.A.	1	19	Larval
Hyporhamphus regularis			
Port Adelaide, S.A.	1	20	Larval
Angas Inlet, S.A.	1	18	Adult
Onkaparinga River, S.A.	2	11	Adult
Peel Inlet, Mandurah, W.A. (PI)	1	8	Adult
Broken Bay, N.S.W.	-	1	Adult
Gippsland Lakes, Vic.	2	11	Adult
Hyporhamphus australis			
Broken Bay, N.S.W.	1	-	Adult
Arrhamphus sclerolepis			
N.S.W.	1	-	Adult

FIG. 3.1 Map of southern Australia showing the locations from which garfish were sampled for the examination of mitochondrial DNA variation. Details of sample sizes and life stages are given in TABLE 3.1.

3.2.2 DNA EXTRACTION, PCR AMPLIFICATION, AND NUCLEOTIDE SEQUENCING

DNA was extracted from either larvae preserved in 70% ethanol or frozen livers of adult fish using a salt extraction method (Miller *et al.*, 1988). A 2-4 mm length of tissue taken from the tail end of all larvae (n = 39; body length range 5.8-26.3 mm) was sufficient to obtain enough DNA for PCR analysis. An approximately 443-462 bp fragment from the mitochondrial *CR* was PCR-amplified using primers H16498 (designed by Meyer *et al.*, 1990) and L-M252 (TABLE 3.2). Verification that this product was of mitochondrial origin rather than a nuclear paralogue (Zhang & Hewitt, 1996) was done by Donnellan *et al.* (2002). Amplifications were carried out on a Hybaid Omn-E Thermal Cycler. Reactions volumes of 50 μL contained 50-100 ng of template DNA, 0.2 μM of each primer, 0.2 mM each of dATP, dTTP, dGTP and dCTP, 4 mM MgCl2, 1× GeneAmp® PCR Buffer II (PerkinElmer) and 1 U AmpliTaq Gold™ DNA polymerase (PerkinElmer). PCR cyclic conditions were: 95°C for 9 min, 50°C for 1 min, 72°C for 1 min for one cycle, 94°C for 45 s, 50°C for 45 s, 72°C for 1 min for 34 cycles and 72°C for 6 min, 30°C for 10 s for one cycle.

TABLE 3.2 Oligonucleotide sequences of primers used to discriminate garfish *Hyporhamphus* species found in southern Australian waters.

Primer	Sequence
L-M252	5'-ACCATCAGCACCCAAAGCTAGG-3'
L-M282	5'-GTGCTTCGCCATATAATCCAAC-3'
H16498 (Meyer *et al.*, 1990)	5'-CCTGAAGTAGGAACCAGATG-3'

PCR products were purified for sequencing with the UltraClean™ PCR Clean-Up DNA Purification Kit (Mo Bio Laboratories, Inc.). Both strands of the purified PCR product were cycle sequenced with the same primers used for PCR with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc.). Reaction volumes of 10 μL contained 50-100 ng of PCR product, 0.5 μM primer and 3 μL BigDye™. PCR cyclic conditions were: 94°C for 30 s, 50°C for 15 s, 60°C for 4 min for 25 cycles, and 60°C for 4 min, 30°C for 10 s for one cycle. Products were run on an ABI 373A automated DNA sequencer.

3.2.3 PHYLOGENETIC ANALYSIS

The sequence alignment, done initially with CLUSTAL X (Thompson *et al.*, 1997), was improved manually. Individual sequences of the alignment are deposited with GenBank under accession numbers AF368258-AF368268. Phylogenetic relationships among garfish haplotypes were reconstructed with the maximum parsimony (MP) criterion of optimality with branch and bound searches. Phylogenetic trees were tested for robustness with bootstrapping

(2000 pseudoreplicates done with branch and bound searches). All phylogenetic analyses were performed with PAUP* 4.0b4a (Swofford, 1999).

3.2.4 PCR TEST FOR SPECIES IDENTIFICATION

A species-specific primer for *H. regularis*, L-M282 (TABLE 3.2), was designed from the aligned garfish *CR* sequences once apomorphic sites had been identified from the phylogenetic analysis. This internal primer was used in conjunction with the external primers L-M252 and H16498 in a multiplex PCR with reaction volumes and cyclic conditions the same as those already described. Because of the presence of the external primer pair, unsuccessful amplifications could be detected for any of the species, i.e. the external primer pair acts as an amplification control. Amplified DNA fragments were electrophoresed for 1 h at 100 V in a 1.5% agarose gel, stained with ethidium bromide, and visualised by UV transillumination.

A random sample of 30 individuals is sufficient to detect at least one copy of a haplotype (i.e. a gel phenotype) that occurs at 10% frequency with 95% confidence (Schwager *et al.*, 1993). So, the PCR test was validated on 49 adult *H. regularis* (6 were also sequenced) and 33 *H. melanochir* samples (11 were sequenced) (TABLE 3.1). We also visually inspected for the L-M282 primer sequence in the *CR* sequences of a further 67 *H. melanochir*, sampled from across the species range, available from Donnellan *et al.* (2002). We subsequently PCR-tested larvae that we could unequivocally assign to species based on morphology from a much larger series of samples of each species (TABLE 3.1).

3.3 RESULTS

We initially sequenced part of the mitochondrial *CR* from 11 adult fish to survey nucleotide sequence variation in southern Australian *Hyporhamphus*. Haplotype diversity of *H. melanochir CR* was surveyed previously by Donnellan *et al.* (2002) with a denaturing gradient gel/nucleotide sequencing approach in which 39 haplotypes were identified among 273 fishes sampled from across the species range in southern Australia. We chose five haplotypes from this study to represent the haplotype lineages identified by phylogenetic analyses of these data. Donnellan *et al.* (2002) also tested whether the PCR primers amplified nuclear paralogues of the *CR* in *Hyporhamphus*. These tests based on titrations of enriched mtDNA did not show any evidence that the primers we used were capable of amplifying nuclear paralogues of the *CR* in either *H. melanochir* or *H. regularis*.

The final alignment of garfish *CR* haplotypes included 423 sites (APPENDIX B). For phylogenetic analyses, alignment gaps (indels), used to optimise the sequence alignment, were treated as a fifth state. Under the MP criterion of optimality, multi-site gaps were treated as a single ‘mutation.’ Under these conditions, 142 nucleotide sites were variable and 100 were

parsimony informative. The MP analysis recovered a single tree of 185 steps (FIG. 3.2). Two major lineages, strongly supported by bootstrapping (100%), are apparent among the *Hyporhamphus* haplotypes, one including both *H. australis* and *H. melanochir* and the second including *H. regularis*. Each lineage is characterised by a long basal branch (40 or more characters changing along these branches) and short branches among haplotypes reflecting the substantial nucleotide divergence between the two major lineages (21.6 to 25.6% uncorrected sequence divergence) and the small genetic distances among conspecific haplotypes (0 to 3.2% uncorrected sequence divergence). For both *H. melanochir* and *H. regularis*, sequences derived from larvae were identical in each case to a haplotype found among the adults (FIG. 3.2). Although both *H. australis* and *H. melanochir* are clearly genetically distant to *H. regularis*, the *CR* haplotypes of *H. australis* and *H. melanochir* are genetically much more closely related. Collette (1974) recognised the latter pair as separate species based on the lack of morphological intermediates in the region where their distributions overlap in southern New South Wales. A more thorough survey of *CR* haplotype diversity in these species in this region would be required before *CR* sequences could be used to discriminate between these taxa.

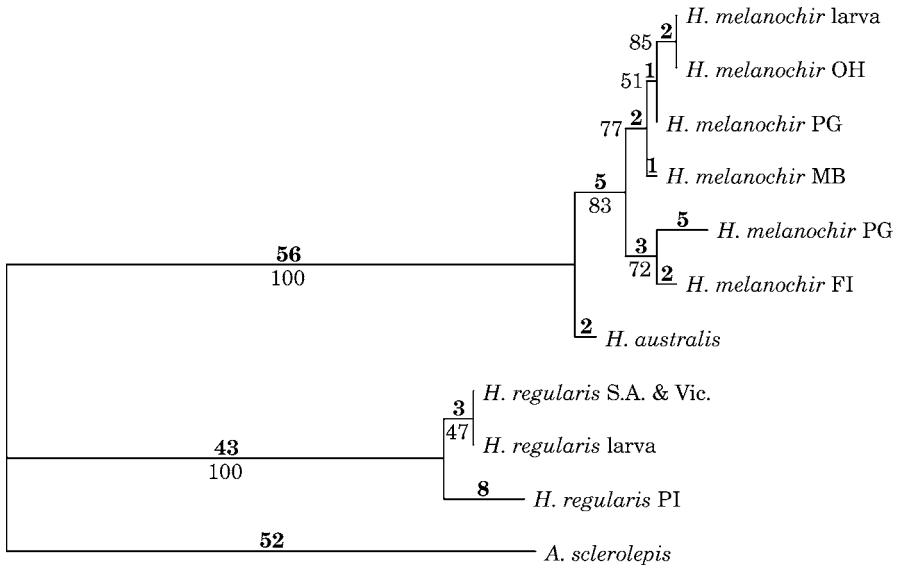

FIG. 3.2 Phylogenetic relationships among garfish *control region* haplotypes recovered with maximum parsimony. Unbolded numerals represent bootstrap proportions from 2000 pseudoreplicates; numerals in boldface are the number of sites that change along that branch. Refer to TABLE 3.1 for locality codes.

Examination of the aligned *CR* sequences revealed two multi-site indels of 6 and 12 bp starting at nucleotide positions 149 and 178 respectively of the alignment. The insertion character state for both indels is present in the six sequenced *H. regularis* specimens and the outgroup *A. sclerolepis*, while the deletion character state was present in both *H. melanochir* and *H. australis* (FIG. 3.3). Primer L-M282, located in the vicinity of the 12 bp indel (FIG. 3.3),

was designed to amplify in combination with primer H16498 only the *H. regularis CR*. The final PCR test used was a multiplex of the three primers (L-252/L-M282/H16498). While the predicted gel phenotypes for each species were the 443 bp product only for *H. melanochir* and both the 264 and 462 bp products for *H. regularis* (FIG. 3.4), a third outcome, the 264 bp product only, was observed in a minor proportion of *H. regularis* samples. The results of the PCR multiplex were 100% compatible with the *a priori* species identification of the 33 adult *H. melanochir* and 49 adult *H. regularis* tested. Inspection of a further 67 *H. melanochir* partial *CR* sequences along with the 11 that were also subjected to the multiplex PCR revealed that the 12 bp sequence required for annealing of the 5' end of L-M282 in *H. regularis* was deleted. We therefore inferred that the 264 bp product would not be amplified from the samples that had been sequenced only. These sample sizes for adults of known species identity (*H. melanochir*, n = 100; *H. regularis*, n = 49) represent the ability to detect a copy of the other species' gel phenotype if it were present at a frequency of <4% and <6% for *H. melanochir* and *H. regularis*, respectively, with 95% confidence. Larvae that had been unequivocally assigned to species *a priori* on a morphological basis (*H. melanochir*, n = 19; *H. regularis*, n = 20) were subsequently tested, and the gel phenotypes were 100% compatible with the predicted phenotype (FIG. 3.4).

H. melanochir larva	TTAAATAACTAAATTAAGACATA----------AAAA-TCCATCAATACATA-A
H. melanochir PG	.....C.....G..........----------G..............G-.
H. melanochir MB	.....C..............----------..............-.
H. melanochir OH	..........----------..............-.
H. melanochir PG	.....C..........----------..............-.
H. melanochir FI	.....C..........----------G..............G-.
H. australis	.....C.....T..........----------?..............-.
H. regularis S.A. & Vic.	AA.G.A..A.GTC.AT.A.....ATAATTCCCCAT.T.......A...AC....T.
H. regularis PI	GA.G.A..A.GTT.AT.A.....ATAACTCCCCAT.TT......A...AC-...T.
H. regularis larva	AA.G.A..A.GTC.AT.A.....ATAATTCCCCAT.T.......A...AC....T.
A. sclerolepis	.A..C.GGT.TCT..C.A......TAACTCCACAT...GA....CTCCA.A...A.
Primer L-M282	gTgcTTCgCCAT.T.......A.

FIG. 3.3 A section (nucleotide sites 155 to 210) of the complete sequence alignment (APPENDIX B) of mitochondrial *control region* haplotypes from adult and larval *Hyporhamphus melanochir* and *H. regularis*, *H. australis*, and the outgroup *Arrhamphus sclerolepis*. The oligonucleotide sequence of the PCR primer L-M282 (boxed), used to discriminate between *H. melanochir* and *H. regularis*, was designed from this section of the alignment; introduced mismatches in the sequence are given in lowercase. Dots (.) indicate identical nucleotides to *H. melanochir* larva; dashes (-) indicate alignment gaps; question mark (?) indicates unknown nucleotide. Refer to TABLE 3.1 for locality codes.

3.4 DISCUSSION

The results of this study demonstrate the impact that PCR technology using the mitochondrial *CR* has on resolving the discrimination of larvae of hemiramphid species from across southern Australia. The mitochondrial *CR* can have high haplotype diversity but low

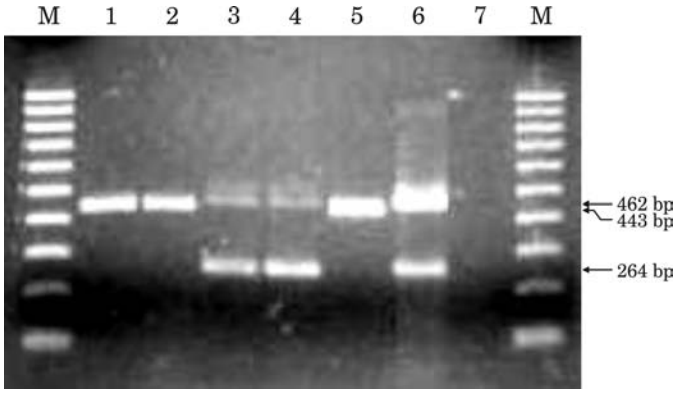

FIG. 3.4 Electrophoretic discrimination between mtDNA *control region* multiplex PCR products from *Hyporhamphus melanochir* (443 bp) and *H. regularis* (462 and 264 bp). Lanes 1 and 2, *H. melanochir* larvae; lanes 3 and 4, *H. regularis* larvae; lane 5, *H. melanochir* adult; lane 6, *H. regularis* adult; lane 7, no template PCR control. M indicates 100-bp ladder for molecular weight marker. The positions of the respective DNA products are indicated by arrows.

nucleotide diversity within fish taxa, such as is the case for garfish in this study, as well as for species of perches of the family Percidae (Faber & Stepien, 1997), in contrast with high nucleotide divergence between related taxa. Divergence often includes indels making the *CR* ideal for species-level discrimination tests. However, unlike some other mitochondrial genes where 'universal' PCR primers are available, e.g. *cytb* and *16S rRNA*, initial PCR-amplification of the *CR* can be problematic because of the limitations on the taxonomic scope of the homology of available *CR* primers.

Frequencies of failed PCRs, where gel bands did not appear, were generally low (i.e. <10%), perhaps due to poor preservation. It is expected that preservation in 95% alcohol in future testing, rather than 70% alcohol, would greatly reduce the rate of failed DNA extractions or PCRs. Importantly, the test never yielded any PCR products other than those species-specific products for which the assay was designed to amplify. These observations underline the necessity for each set of PCR reactions to include both positive controls for each species and a negative control (Hill *et al.*, 2001).

It is interesting to note and compare the multiplex-PCR assay with the several other molecular techniques that have the potential to solve the problem of species-level identification. Another PCR-based technique available for distinguishing species is the use of randomly amplified polymorphic DNAs (RAPD) (Crossland *et al.*, 1993; Coffroth & Mulawka, 1995). Although RAPD may be slightly less expensive than the multiplex-PCR technique developed in this study, it is substantially more difficult to analyse because it also involves the reading and relatively complex interpretation of gel bands. Other PCR-based techniques include DNA sequence comparisons (Litvaitis *et al.*, 1994), restriction enzyme digests, dot-blot hybridisations, and combinations of the above (Banks *et al.*, 1993; Geller *et al.*, 1993). Whilst these other techniques can be used to accurately identify species, the

procedures are generally time-consuming and labour-intensive, often requiring 2-3 days to obtain a result (Fell, 1993). Hu *et al.* (1992) used allozyme electrophoresis to identify mussel larvae. However, the preference to use alcohol as a preservative in this study precluded the use of this technique since it requires specimens to be fresh or snap-frozen. The comparative nature of the technique also means that, unlike analysis of mtDNA, reference specimens are constantly required (Wallman & Donnellan, 2001).

Primary advantages of the multiplex-PCR assay are reduced cost and increased efficiency. Most importantly, it is simple and accurate for discrimination of eggs and larvae of hemiramphids distributed along southern Australia. Because the discrimination is achieved during the course of the amplification reaction, no secondary step, such as sequencing or restriction fragment length polymorphism (RFLP) analysis (Bartlett & Davidson, 1992; Quinteiro *et al.*, 1998), is required in routine testing of specimens (Lockley & Bardsley, 2000). Furthermore, it avoids the expense and biochemical fragility of restriction enzymes (Hill *et al.*, 2001).

Morphological criteria that could be used to discriminate between southern Australian garfish species throughout their early life histories now can be independently verified by molecular techniques. The molecular method described allows partitioning of morphological variation, due to intraspecies variation and the morphological plasticity associated with larval growth and development, among the within-species and between-species components. A possible outcome of this analysis is that the morphological characters may still be unable to adequately discriminate between the larvae of these species, in which case the molecular approach could replace the morphological one entirely. Also, regardless of whether larval identification by morphology alone is achievable, morphological identification may require more work per specimen, making it relatively more efficient to use the molecular approach.

This study demonstrates a non-sequencing based method that is potentially automatable, permitting analysis of large numbers of specimens and thereby avoiding much of the labour-intensive identification work using morphological criteria. Furthermore, ecologists without detailed knowledge of taxonomy or molecular biology would require only a little molecular technical training for species discrimination.

Chapter 4 Larval development of *H. melanochir* and *H. regularis*[**]

4.1 INTRODUCTION

The beloniform family Hemiramphidae (garfishes or halfbeaks) are small to medium-size surface-dwelling marine, estuarine and freshwater fishes. The family contains 12 genera and 101 species worldwide, with more than one-third of the species belonging to the genus *Hyporhamphus* (Froese & Pauly, 2001). The Hemiramphidae are related to the Exocoetidae (flyingfishes) and, more distantly, the Scomberosocidae (sauries), Belonidae (needlefishes) and Adrianichthyidae (ricefishes) (Collette *et al.*, 1984). Six genera and 17 species of hemiramphids occur in Australian waters, where garfishes have long been considered valuable food and bait fish (Collette, 1974; Kailola *et al.*, 1993).

Two hemiramphid species inhabit the waters of S.A., namely *H. melanochir* and *H. regularis*. Adults of both are widely distributed along southern Australia from W.A. to N.S.W., although *H. regularis* have not been recorded in Tasmania. They support important commercial and recreational fisheries, particularly in S.A. (Kailola *et al.*, 1993). *Hyporhamphus melanochir* are commonly found in sheltered coastal waters while *H. regularis* are confined to estuaries (Jones *et al.*, 1996). Juveniles and adults of both species co-occur in some estuaries of southern Australia, e.g. Port River-Barker Inlet of S.A. (34°45’S, 138°31’E) (Jones et al., 1996) and Peel-Harvey Estuary of W.A. (32°32’S, 115°43’E) (pers. observ.).

Despite their widespread distribution and economic importance, the early life history of *H. melanochir* is only partially described (i.e. reproductive biology, Ling, 1958; egg development, Jordan *et al.*, 1998), whilst there is no published information for *H. regularis*. Furthermore, although adults are easily identified using keys and descriptions provided by Collette (1974), no such information exists for the larvae. A fundamental prerequisite for any larval fish study is, undoubtedly, their accurate identification (Neira *et al.*, 1998).

Thus far, at least some larval stages have been described for 19 hemiramphids worldwide (Sudarsan, 1966; Talwar, 1967; Hardy, 1978; Chen, 1988; Watson, 1996; Prince Jeyaseelan, 1998), eight of which belong to *Hyporhamphus*. The purposes of this paper are to describe the larval development of *H. melanochir* and *H. regularis* and to document distinguishing characters between larvae of these species.

[**] The results presented in this chapter form the basis of the following publication:
Noell, C. J. (2003). Larval development of *Hyporhamphus melanochir* and *H. regularis* (Beloniformes: Hemiramphidae) from South Australian waters. *Fishery Bulletin* **101,** 368-376. *{APPENDIX C}*

4.2 MATERIALS AND METHODS

Most larvae were collected in the Gulf St Vincent and the Bay of Shoals between November 1999 and March 2000 with a neuston net, the sampling methodology for which is described in Chapter 5. Additional larvae were hand-collected from beneath a wharf in Barker Inlet where they often school during daylight at mid-flood tide. Since transforming larvae and juveniles were rarely encountered in neuston samples, probably as a result of accumulated mortality and increased avoidance capacity (Sandknop *et al.*, 1984), these specimens were collected at Outer Harbor (34°46'S, 138°28'E) and Barker Inlet at night using a dab net and spotlight instead. The term 'transforming' is used here to describe the stage between the end of the larval phase and the start of the juvenile phase, i.e. after the attainment of all fin rays and before the formation of scales. Larvae were sorted from plankton samples immediately after collection based on reference larval specimens from the South Australian Museum fish collection that were identified to family. Larvae were fixed in 10% formalin buffered with sodium β-glycerophosphate (1 g L^{-1}) and later preserved in 70% ethanol.

A total of 47 *H. melanochir* (6.4-48.3 mm body length, L_B) and 49 *H. regularis* (7.0-46.9 mm L_B) larvae through juveniles were used to describe morphometrics, meristics and pigmentation. Larvae were identified as hemiramphids based on larval and adult characters reported in the literature (Collette, 1974; Hardy, 1978; Collette *et al.*, 1984; Chen, 1988; Watson, 1996; Trnski *et al.*, 2000). Developmental series were assembled using the series method (Neira *et al.*, 1998), the accuracy of which was verified by a molecular technique (Noell *et al.*, 2001; Chapter 3). Terminology of early life history stages follows Kendall *et al.* (1984). Representative series for both species are deposited with the I. S. R. Munro Fish Collection (CSIRO, Hobart, Tas.). {Registration numbers: *H. melanochir* (*n* = 13), CSIRO L 3072-01, 3073-01 to -08, 3074-01 to -02, 3075-01 to -02; *H. regularis* (*n* = 12), CSIRO L 3076-01 to -07, 3077-01 to -02, 3078-01 to -03.}

Larvae were examined with a Wild M3Z stereomicroscope at 6.5-40× magnifications using various combinations of incident and transmitted light. Body measurements were taken using SigmaScan Pro® 5.0 image measurement software (SPSS Inc., 1999*a*) and are accurate to less than 0.05 mm. This method was particularly useful for measuring cumulative distances of bent larvae. Definitions of routinely taken body measurements (FIG. 4.1) follow Leis & Carson-Ewart (2000). Lower jaw length (L_{LJ}) is defined as the horizontal distance from the tip of the lower jaw to the anterior margin of the pigmented region of the eye. Lower jaw extension (L_{LJx}) is defined as the horizontal distance from the tip of the lower jaw to the tip of the snout. Eye diameter was measured along both horizontal (D_{Eh}) and vertical midlines (D_{Ev}) of its pigmented region. Body depth was measured at two points, at the pectoral base (B_P) and at the anus (B_A). Other measurements taken were snout length (L_{Sn}), head length (L_H), pre

dorsal-fin length (L_{PD}) and preanal length (L_{PA}). All measurements are expressed as a percentage of body length. No attempt was made to adjust body measurements of hemiramphid larvae from preserved to live lengths as no significant difference was found for the morphologically similar saury larvae when preserved in ethanol (Oozeki *et al.*, 1991). Pigment refers to melanin. Drawings were prepared with the aid of a camera lucida.

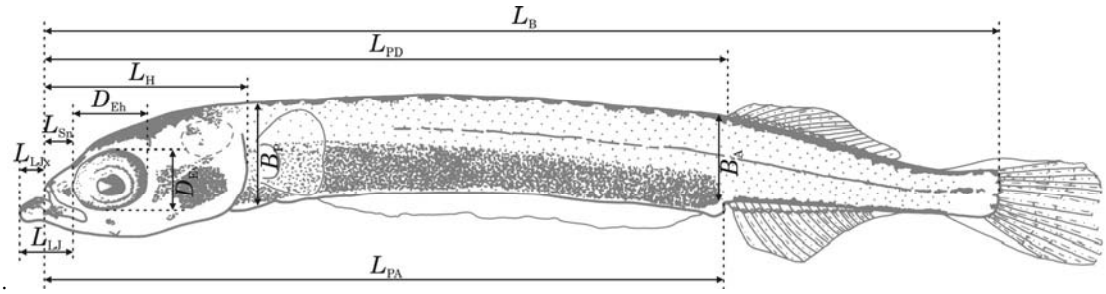

FIG. 4.1 Body measurements used to describe larval *Hyporhamphus melanochir* and *H. regularis*. Abbreviations: B_A, body depth at anus; B_P, body depth at pectoral base; D_{Eh}, horizontal eye diameter; D_{Ev}, vertical eye diameter; L_B, body length; L_H, head length; L_{LJ}, lower jaw length; L_{LJx}, lower jaw extension; L_{PA}, preanal length; L_{PD}, pre dorsal-fin length; L_{Sn}, snout length. Drawing of larva reproduced with permission from Bruce (1989).

Selected specimens were cleared and stained with alcian blue and alizarin red-S following the method of Potthoff (1984) (FIG. 4.2). These were used to count fin rays and vertebrae. Myomeres were difficult to count reliably at either end; thus, vertebral counts (which include the urostyle) of stained larvae were taken instead. For small larvae that had unformed centra, corresponding neural or haemal spines were counted to obtain the number of vertebrae.

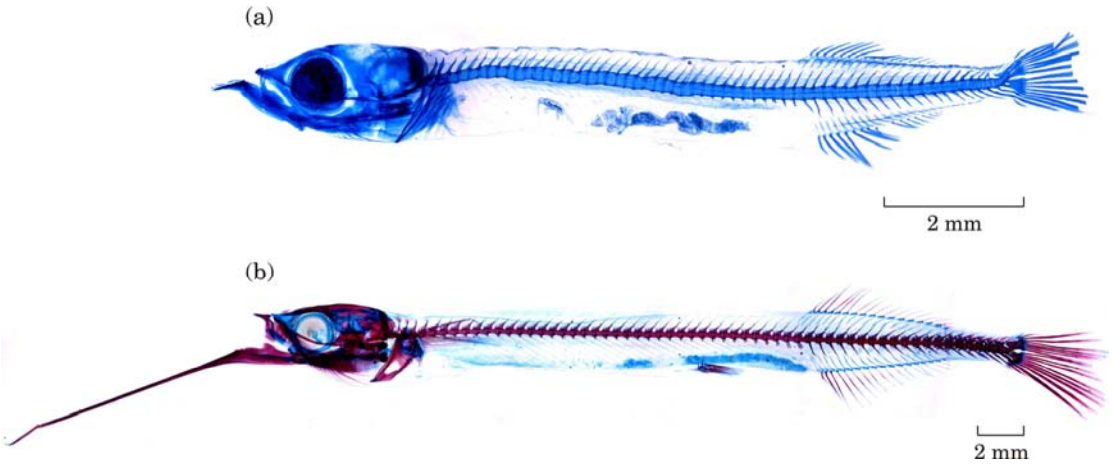

FIG. 4.2 Cleared and stained *Hyporhamphus melanochir* (a) larva (11.0 mm) and (b) juvenile (33.3 mm) for the purpose of counting fin rays and vertebrae.

Logistic regression analysis was performed to determine if differences in body measurements could distinguish between *H. melanochir* and *H. regularis* larvae. Logistic

regression analysis is used to determine the probability of a dichotomous outcome (dependent variable, i.e. *H. melanochir* or *H. regularis*) predicted by a set of independent variables (body measurements). In these circumstances logistic regression has the advantage of being a more robust classification technique than discriminant analysis, particularly when the assumption of multivariate normality is violated (Prager & Fabrizio, 1990). The analysis sample used to develop the logistic regression model consisted of the same larvae used to describe larval development, but excluding transforming larvae and juveniles (*H. melanochir*: 6.4-17.0 mm L_B, n = 41; *H. regularis*: 7.0-13.1 mm L_B, n = 44). Body measurements were firstly transformed, however, to reduce size effects caused by allometric growth following an equation taken from Thorpe (1975):

$$\hat{Y}_i = \log_{10} Y_i - b(\log_{10} X_i - \log_{10} \overline{\overline{X}}) \qquad [4.1]$$

where $\hat{Y}_i$ is the adjusted value of the *i*th specimen; Y_i is the raw value of the *i*th specimen; b is the pooled regression coefficient of $\log_{10}Y$ against $\log_{10}X$; X_i is the body length of the *i*th specimen; and $\overline{\overline{X}}$ is the grand mean of body lengths. Following transformation, each body measurement variable was regressed against body length. The regression coefficient of each transformed variable on L_B was close to zero and insignificant, which suggests negligible effects of allometry. The logistic regression model is described by the equation:

$$P = e^y (1 + e^y) \qquad [4.2]$$

where P is the probability that the larva is *H. melanochir*, and y is the linear regression:

$$y = B_0 + B_1 X_1 + B_2 X_2 + ... + B_n X_n \qquad [4.3]$$

where B_0 is the regression constant, and B_1, …, B_n are the coefficients associated with each morphometric predictor variable X_1, …, X_n. A probability of 0.5 is the cutting score for the prediction of species (i.e. *H. regularis* < 0.5 ≤ *H. melanochir*). The forward stepwise logistic regression procedure in SPSS® 10.0 (SPSS Inc., 1999*b*) was used to estimate the model by maximum likelihood. Independent variables were entered into the model at the 0.05 significance level and removed at the 0.10 level, with the greatest reduction in the log-likelihood value (-2LL) used to guide variable entry. The Wald statistic was used to assess the significance of the coefficients for the variables included in the model, while the overall model fit was assessed by the change in the -2LL value, estimates of $R^2_{\log it}$, accuracy of

classification tables, and the Hosmer and Lemeshow Test (Hair *et al.*, 1998). The estimated logistic regression model was cross-validated with another sample of larvae (*H. melanochir*: 6.0-13.4 mm L_B, n = 20; *H. regularis*: 7.1-15.5 mm L_B, n = 22) that were withheld from the sample used to develop the model (holdout sample).

4.3 RESULTS

4.3.1 *HYPORHAMPHUS MELANOCHIR* (FIG. 4.3)

4.3.1.1. Description of larvae

The smallest *H. melanochir* larva examined was a 6.4 mm newly-hatched postflexion-stage specimen from laboratory rearing. Some yolk remained, although yolk absorption was complete in the smallest field-collected larva (6.9 mm).

Larvae are elongate to very elongate (B_P = 7-13% L_B), with body depth slightly tapered towards the anus (B_A = 7-9% L_B). Relative body depth at the pectoral base decreases slightly during larval development (TABLE 4.1). Larvae have 58-61 vertebrae (TABLE 4.2). The gut is relatively thick, long, straight and non-striated. L_{PD} and L_{PA} remain in the ranges of 70-75 and 71-76% L_B, respectively (except for the 17.0 mm larva, which had a L_{PD} and L_{PA} of 62% L_B). The first dorsal-fin ray is slightly anterior to or directly above the corresponding anal-fin ray. There is no gap between the anus and the anal fin. A long preanal finfold, initially the same length as the gut, persists through to the transformation stage before it disappears. There is no head spination. The small to moderate head (L_H = 16-24% L_B) decreases in size relative to L_B with larval growth (TABLE 4.1). The longer lower jaw protrudes beyond the snout (L_{LJx}) by 4% L_B at 11.0-11.5 mm, increasing to a maximum of 34% L_B in the 29.3 mm juvenile. The mouth is oblique and reaches to the level of the center of the eye in the newly hatched larva. The maxilla subsequently moves forward relative to the eye and by 12.1-14.4 mm it does not reach the eye. Very small villiform teeth are present on both the premaxilla and dentary in newly hatched larvae. The moderate to large eye (D_{Eh} = 6-10% L_B or 33-42% L_H) is elongate (D_{Ev} = 78-88% D_{Eh}) and decreases in size relative to L_B. A single rudimentary nasal papilla first appears as a small fleshy lump in the olfactory pit by 17.0 mm. Scales first appear between 20.4 and 29.3 mm laterally on the tail, anterior to the caudal peduncle.

4.3.1.2. Development of fins

Completion of fin development in *H. melanochir* occurs in the sequence: C → D → A → P_1, P_2 (TABLE 4.2). All principal rays of the caudal fin (7 + 8) and several incipient dorsal- and anal-fin rays are present in newly hatched larvae. A full complement of 15-18 dorsal- and 17-20 anal-fin rays is attained at 11.4 and 12.1 mm, respectively. The pectoral base and finfold

form prior to hatch, with incipient rays appearing shortly after by 7.2 mm; all 11-13 rays are formed by 19.6 mm. The pelvic fin buds appear by 13.3 mm, and all six pelvic-fin rays are formed by 19.6 mm.

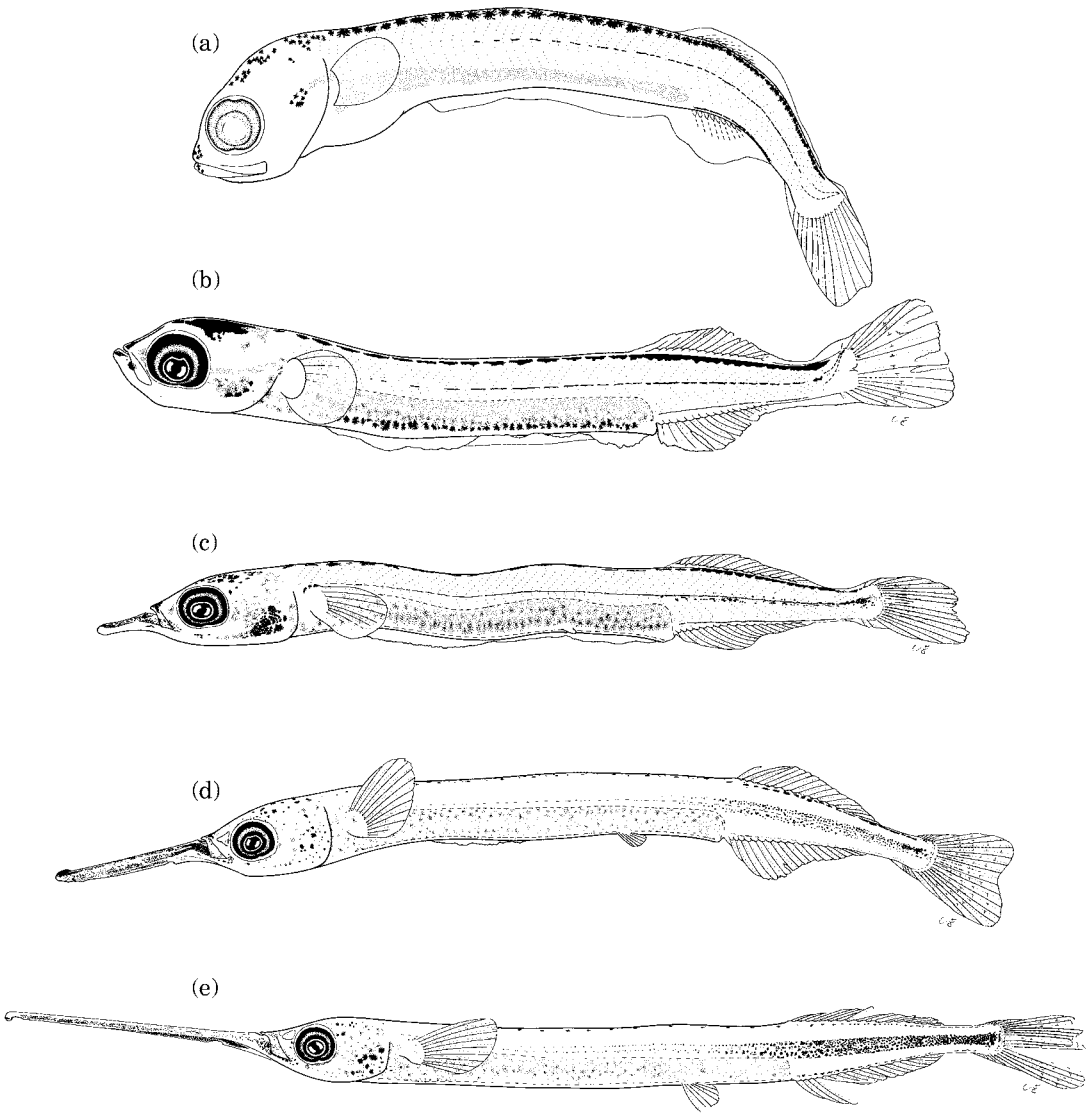

FIG. 4.3 Larval, transforming larval, and juvenile *Hyporhamphus melanochir*. (a) 6.4 mm reared yolk-sac larva; newly hatched (redrawn from Jordan *et al.*, 1998) (L 3072-01); (b) 9.3 mm larva (L 3073-01); (c) 13.3 mm larva (composite drawing of two damaged larvae of same L_B) (L 3073-02 and -03); (d) 20.4 mm transforming larva (L 3074-01), myomeres omitted; (e) 29.3 mm juvenile (L 3074-02), myomeres omitted.

4.3.1.3. Pigmentation

Hyporhamphus melanochir larvae are moderately to heavily pigmented. Head pigmentation consists of melanophores on the tip of the lower jaw, snout, olfactory pit and opercula, and a patch of several large melanophores on the midbrain. The extended lower jaw is heavily pigmented throughout development and melanophores extend laterally along the dentary. The eye is partially pigmented in the newly hatched larva, but fully pigmented by 6.9

TABLE 4.1 Morphometrics of larval, transforming larval, and juvenile *Hyporhamphus melanochir* (expressed as a percentage of L_B). Mean ± 1 S.D. is given when sample size $n > 1$. Dotted lines differentiate larvae, transforming larvae, and juveniles in descending order.

L_B (mm)	n	L_{Sn}	L_{LJ}	L_{LJx}	D_{Eh}	D_{Ev}	L_H	L_{PD}	L_{PA}	B_P	B_A
6.4*	1	2.1	2.7	0.6	9.9	8.7	24.4	74.6	75.5	16.3†	8.0
6.9	1	3.0	4.0	1.0	9.2	7.5	23.5	69.7	71.9	12.7	8.6
7.0-7.5	9	2.8 ± 0.8	3.9 ± 1.1	1.1 ± 0.4	9.1 ± 0.3	7.3 ± 0.3	22.0 ± 0.8	70.8 ± 0.9	72.6 ± 1.0	11.9 ± 0.3	8.9 ± 0.3
7.5-8.0	7	3.6 ± 0.9	4.6 ± 1.2	1.0 ± 0.4	9.1 ± 0.5	7.1 ± 0.3	22.8 ± 1.6	71.3 ± 1.3	73.0 ± 0.9	11.8 ± 0.6	8.9 ± 0.7
8.0-8.5	9	3.6 ± 0.5	4.9 ± 0.8	1.3 ± 0.4	8.8 ± 0.3	7.1 ± 0.3	21.9 ± 1.0	70.9 ± 0.7	72.7 ± 0.9	11.7 ± 0.7	8.9 ± 0.7
8.5-9.0	3	3.5 ± 0.8	5.0 ± 1.1	1.5 ± 0.3	8.6 ± 0.2	7.0 ± 0.3	21.1 ± 0.2	71.5 ± 0.2	72.5 ± 0.4	11.3 ± 0.5	9.1 ± 0.5
9.0-9.5	4	3.4 ± 0.5	5.0 ± 0.7	1.6 ± 0.3	8.0 ± 0.3	6.5 ± 0.2	20.9 ± 0.8	71.7 ± 0.7	72.8 ± 0.6	11.4 ± 0.7	8.4 ± 0.3
11.0-11.5	4	3.7 ± 0.7	7.2 ± 1.5	3.5 ± 1.2	7.5 ± 0.4	6.3 ± 0.2	20.3 ± 1.3	71.6 ± 0.6	72.3 ± 0.5	10.6 ± 0.7	8.5 ± 0.6
12.1	1	4.1	9.0	4.9	7.6	6.4	19.7	72.6	72.6	10.2	8.6
14.4	1	3.7	12.3	8.6	6.9	5.8	19.2	70.2	71.4	9.4	7.8
17.0	1	2.9	10.3	7.4	5.6	4.7	15.9	61.7	61.7	7.3	6.6
19.6	1	4.9	28.7	23.8	6.0	4.9	18.6	70.4	71.2	8.2	7.4
20.4	1	4.0	24.2	20.2	5.7	5.0	17.5	72.5	71.6	8.9	7.6
29.3	1	4.4	38.4	34.0	5.3	4.8	17.0	69.9	71.0	8.2	7.2
33.3	1	4.9	38.3	33.4	5.3	4.6	17.9	71.7	72.7	8.5	7.6
41.3	1	5.2	36.2	31.1	5.5	5.1	18.6	74.1	74.1	9.2	7.8
48.3	1	5.6	36.9	31.3	5.2	4.8	17.8	74.0	74.0	9.7	8.1

*Yolk-sac larva.
†Includes yolk sac.

mm. The gut is heavily and uniformly pigmented dorsally and laterally along the entire length, with melanophores often coalesced, but pigmentation becomes obscured as the overlying musculature develops. Dorsal pigmentation initially consists of 12-15 large melanophore pairs in longitudinal rows between the head and origin of the dorsal fin [FIG. 4.4(a)], and a continuous band along either side of the dorsal-fin base. Dorsal pigmentation gradually decreases in intensity thereafter. Three distinct lines of pigment appear along the dorsal margin in juveniles (by 29.3 mm) and remain to adult stage. A series of melanophores form a dashed, sometimes continuous, midlateral line. Melanophores appear laterally on the caudal peduncle by 14.4 mm, and then proliferate anteriorly to form a broad medial stripe that remains, forming a silver stripe from the caudal peduncle to the operculum of adults. Ventral pigmentation consists of continuous bands of melanophores either side of the anal-fin base [FIG. 4.4(b)]. Fins are unpigmented except the caudal fin, which has small melanophores on the ray bases.

TABLE 4.2 Meristic counts of larval, transforming larval, and juvenile *Hyporhamphus melanochir*. Numbers in bold indicate the L_B at which a full complement of rays is first attained. Dotted lines differentiate larvae, transforming larvae, and juveniles in descending order. D, dorsal; A, anal; P_1, pectoral; P_2, pelvic; C, caudal.

L_B (mm)	Fin rays					Branchi-ostegal rays	Vertebrae
	D	A	P_1	P_2	C		
6.4*	8	9	base		0+**7**+**8**+0	3	38+21
7.2	8	8	1		0+7+8+0	3	39+20
7.3	9	10	1		0+7+8+0	3	39+19
7.6	11	11	1		0+7+8+0	3	38+20
7.9	10	11	1		0+7+8+0	3	40+20
8.3	11	11	2		0+7+8+0	4	39+20
8.4	13	14	2		1+7+8+1	5	39+21
9.4	14	16	4		1+7+8+1	5	40+21
11.4	**15**	16	6		2+7+8+1	7	38+20
12.1	16	**17**	7		2+7+8+2	7	39+20
14.4	16	19	9	bud	2+7+8+2	9	39+20
19.6	17	19	**11**	**6**	**4**+7+8+**4**	**12**	38+20
20.4	16	17	12	6	4+7+8+4	12	39+19
29.3	17	18	11	6	4+7+8+4	13	38+20
33.3	17	18	12	6	5+7+8+5	12	38+20
41.3	16	19	11	6	4+7+8+5	12	40+19
48.3	16	19	11	6	4+7+8+5	12	39+19

*Yolk-sac larva.

4.3.2 *HYPORHAMPHUS REGULARIS* (FIG. 4.5)

4.3.2.1. Description of larvae

The smallest *H. regularis* larva examined (7.0 mm) had completed notochord flexion and had a yolk sac. Yolk absorption was complete by 7.6 mm.

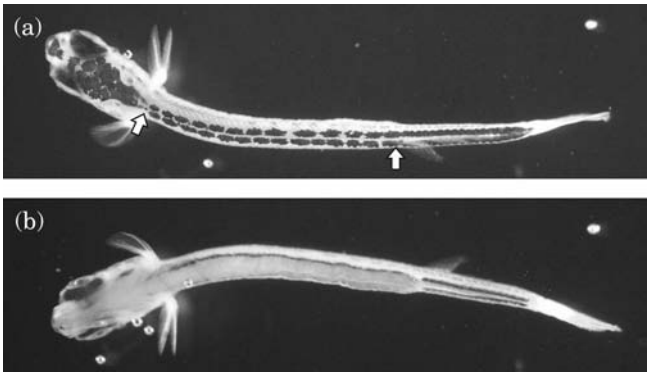

FIG. 4.4 Pigmentation of an 8.5 mm *Hyporhamphus melanochir* larva. (a) Dorsal view, arrows indicate the margins of the 12-15 melanophore pairs in longitudinal rows; (b) ventral view.

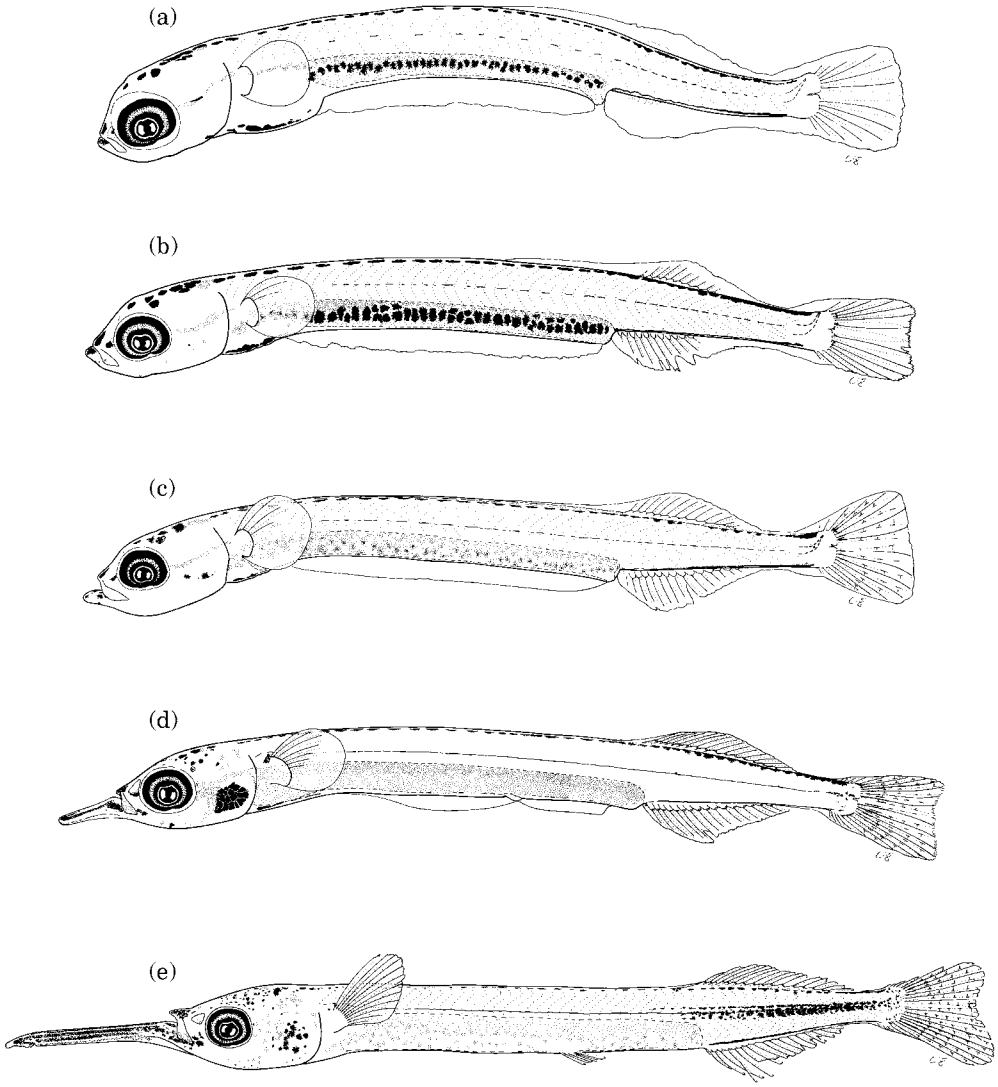

FIG. 4.5 Larval and juvenile *Hyporhamphus regularis*. (a) 7.1 mm yolk-sac larva (L 3076-01); (b) 9.4 mm larva (L 3076-02); (c) 12.3 mm larva (L 3076-03); (d) 15.5 mm larva (L 3076-04), myomeres omitted; (e) 24.7 mm juvenile (L 3077-01).

Larval *H. regularis* closely resemble larval *H. melanochir* morphologically (*cf.* TABLE 4.1 and TABLE 4.3), but differ somewhat in relative length of the lower jaw, relative positions of the dorsal- and anal-fin origins, and in number of vertebrae. The longer lower jaw protrudes beyond the snout (L_{LJx}) by 4% L_B at 13.1 mm and increases to a maximum of 24% L_B in the 31.5 mm juvenile. The first dorsal-fin ray is slightly posterior to or directly above the corresponding anal-fin ray. Larvae have 51-54 vertebrae. Scales first appear between 18.1 and 24.7 mm laterally on the tail, anterior to the caudal peduncle.

4.3.2.2. Development of fins

Completion of fin development in *H. regularis* occurs in the sequence: C → D → A → P_1, P_2 (TABLE 4.4). Development of the caudal fin is incomplete at birth, with 6 + 7 principal rays present in the 7.0 mm yolk-sac larva, and the full complement (7 + 8) shortly after by 7.7 mm. Distinct anal-fin bases are visible at 7.0 mm. A full complement of 14-17 dorsal and 15-19 anal-fin rays is attained at 10.1 and 10.5 mm, respectively. The pectoral base and finfold are present at birth, with incipient rays first appearing by 8.1 mm; all 11-12 rays are formed by 18.1 mm. The pelvic fin buds appear by 13.1 mm, and all six pelvic-fin rays are formed by 18.1 mm.

4.3.2.3. Pigmentation

Pigmentation of *H. regularis* larvae is similar to that of *H. melanochir* larvae except along the dorsal and ventral margins. Dorsal pigmentation consists of 19-22 melanophore pairs in longitudinal rows between the head and dorsal fin origin [FIG. 4.6(a)]. A large pigment blotch is present ventrally on the isthmus and anteriorly on the gut [FIG. 4.6(b)].

4.3.3 LOGISTIC REGRESSION ANALYSIS OF BODY MEASUREMENTS

Of the ten adjusted body measurements considered for logistic regression analysis, only the D_{Eh} and L_{PD} were selected as the dependent variables, the estimated coefficients of which were statistically significant (TABLE 4.5). Therefore, body measurements D_{Eh} and L_{PD} are significant and thus included in the model. Results of the logistic regression analysis emphasise the differences in certain body measurements that exist between *H. melanochir* and *H. regularis* that may otherwise not appear obvious. For example, the relationship between D_{Eh} and L_{PD} was subsequently examined and differences between species clearly demonstrated (FIG. 4.7).

TABLE 4.3 Morphometrics of larval, transforming larval, and juvenile *Hyporhamphus regularis* (expressed as a percentage of L_B). Mean ± 1 S.D. is given when sample size $n > 1$. Dotted lines differentiate larvae, transforming larva, and juveniles in descending order.

L_B (mm)	n	L_{Sn}	L_{LJ}	L_{LJx}	D_{Eh}	D_{Ev}	L_H	L_{PD}	L_{PA}	B_P	B_A
7.0*	1	3.2	4.4	1.2	8.2	6.5	20.4	73.2	71.6	11.6†	7.4
7.5-8.0	9	2.8 ± 0.3	4.4 ± 0.4	1.7 ± 0.3	7.6 ± 0.1	6.3 ± 0.1	19.9 ± 0.6	73.1 ± 0.6	71.8 ± 0.4	11.2 ± 0.2	8.2 ± 1.2
8.0-8.5	12	2.8 ± 0.4	4.3 ± 0.5	1.5 ± 0.3	7.5 ± 0.3	6.2 ± 0.2	19.6 ± 0.9	72.8 ± 0.7	71.6 ± 0.6	11.0 ± 0.5	7.7 ± 0.3
8.5-9.0	10	2.8 ± 0.2	4.2 ± 0.2	1.5 ± 0.2	7.2 ± 0.2	5.9 ± 0.1	19.2 ± 0.3	72.9 ± 0.9	71.8 ± 0.9	10.6 ± 0.2	7.4 ± 0.3
9.0-9.5	5	2.8 ± 0.1	4.4 ± 0.4	1.6 ± 0.4	7.0 ± 0.1	6.0 ± 0.2	19.1 ± 0.4	72.3 ± 0.7	71.5 ± 0.6	10.6 ± 0.2	7.6 ± 0.3
9.5-10.0	3	2.8 ± 0.1	4.4 ± 0.3	1.7 ± 0.2	6.9 ± 0.4	5.8 ± 0.2	18.7 ± 0.5	72.1 ± 1.6	71.2 ± 1.7	10.6 ± 0.4	7.3 ± 0.2
10.0-10.5	3	3.0 ± 0.3	4.8 ± 0.9	1.8 ± 0.6	6.8 ± 0.2	5.8 ± 0.2	18.9 ± 0.6	72.2 ± 0.6	71.3 ± 0.6	10.2 ± 0.3	7.6 ± 0.3
13.1	1	4.0	7.6	3.7	6.9	5.4	19.6	73.2	71.9	9.1	7.8
18.1	1	4.5	18.6	14.1	6.3	5.4	19.9	73.8	72.6	9.5	8.1
24.7	1	5.6	27.7	22.1	6.3	5.5	20.8	72.9	73.6	9.7	8.1
31.5	1	6.2	30.0	23.9	6.0	5.5	21.0	74.4	75.5	10.7	8.5
33.8	1	6.4	27.7	21.3	6.1	5.6	21.6	74.3	74.3	10.9	8.9
46.9	1	7.3	damaged	damaged	5.6	4.7	21.8	75.4	75.4	11.2	9.8

*Yolk-sac larva.
†Includes yolk sac.

TABLE 4.4 Meristic counts of larval, transforming larval, and juvenile *Hyporhamphus regularis*. Numbers in bold indicate the L_B at which a full complement of rays is first attained. Dotted lines differentiate larvae, transforming larva, and juveniles in descending order. D, dorsal; A, anal; P_1, pectoral; P_2, pelvic; C, caudal.

L_B (mm)	Fin rays					Branchi-ostegal rays	Vertebrae
	D	A	P_1	P_2	C		
7.0*	anlage	bases	base		0+6+7+0	2	35+19
7.7	4	6	base		0+**7**+**8**+0	3	34+19
7.8	6	7	base		0+7+8+0	4	34+18
8.1	5	7	1		0+7+8+0	4	34+19
8.3	8	9	2		0+7+8+0	4	33+20
8.6	9	11	2		0+7+8+0	5	34+19
8.9	11	11	2		0+7+8+1	5	34+20
9.3	11	12	3		1+7+8+1	5	33+18
9.6	10	11	3		0+7+8+1	5	35+19
10.1	**14**	14	4		1+7+8+1	6	33+20
10.5	13	**15**	5		1+7+8+1	6	35+19
13.1	14	16	7	bud	2+7+8+2	8	35+19
18.1	14	17	**11**	**6**	**4**+7+8+**4**	**12**	35+18
24.7	16	17	12	6	4+7+8+4	11	34+19
31.5	15	17	12	6	4+7+8+4	11	34+18
33.8	15	18	11	6	4+7+8+4	11	33+19
46.9	16	17	11	6	4+7+8+4	11	35+18

*Yolk-sac larva.

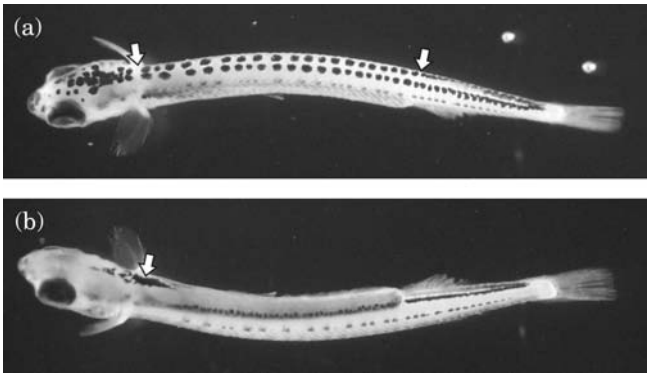

FIG. 4.6 Pigmentation of a 8.7 mm *Hyporhamphus regularis* larva. (a) Dorsal view, arrows indicate the margins of the 19-22 melanophore pairs in longitudinal rows; (b) Ventral view, arrow indicates the ventral pigment blotch.

Various measures were used to evaluate the final model fit, and all of these in combination provide support for acceptance of the two-variable model as a significant logistic regression model that is suitable for further examination. A chi-square test indicated that the reduction in the -2LL value from the base model was highly significant (χ^2 = 105.745, df = 2, P<<0.001). The high $R^2_{\log it}$ of 0.898 is further indicative of a good model fit. The classification matrix indicates that 97.6% of both analysis and holdout samples were correctly classified according to the two-variable model, with only three cases misclassified (TABLE 4.6,

FIG. 4.8). As expected, the Hosmer and Lemeshow Test revealed a non-significant difference between the observed and predicted classifications of species (χ^2 = 0.478, df = 7, P = 1.000).

TABLE 4.5 Summary of statistics for independent variables included in the logistic regression model used to distinguish between *Hyporhamphus melanochir* and *H. regularis* larvae. Regression coefficient (*B*) is given as maximum likelihood estimate ± 1 S.E.

Variable	*B*	S.E.	Wald
D_{Eh}	135.6	41.1	10.879*
L_{PD}	-316.7	135.1	5.499*
Constant	818.6	453.1	3.264

*P<0.05.

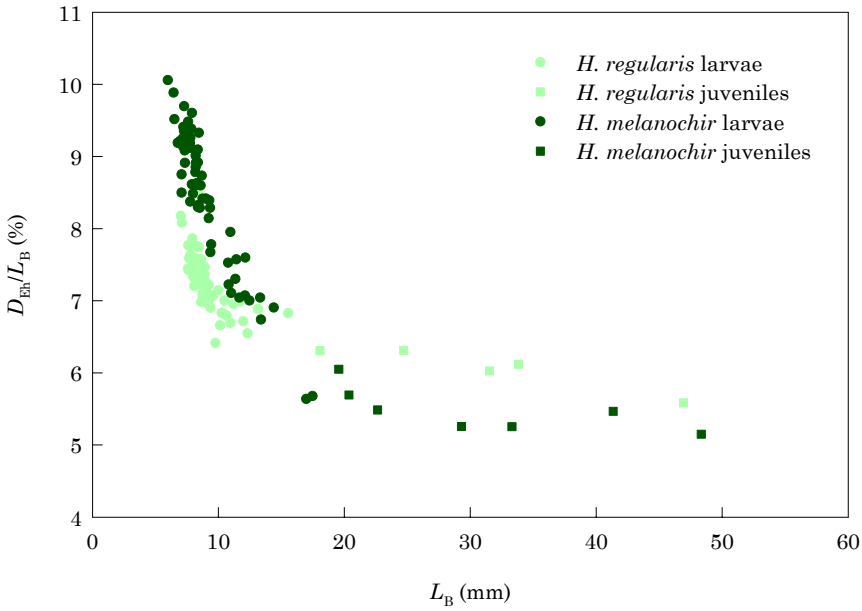

FIG. 4.7 Relationship between the unadjusted horizontal eye diameter to body length ratio (D_{Eh}/L_B, in percentage) and L_B (mm) for *Hyporhamphus melanochir* and *H. regularis* larvae and juveniles.

TABLE 4.6 Classification matrix for the logistic regression model used to distinguish between *Hyporhamphus melanochir* and *H. regularis* larvae. For each cell, the number on the left denotes the analysis sample and the number on the right denotes the holdout sample. Hit ratio is the percentage correctly classified.

Actual species	*n*	Predicted species		Hit ratio (%)
		H. melanochir	*H. regularis*	
H. melanochir	41/20	40/20	1/0	97.6/100.0
H. regularis	44/22	1/1	43/21	97.7/95.5
Total				97.6/97.6

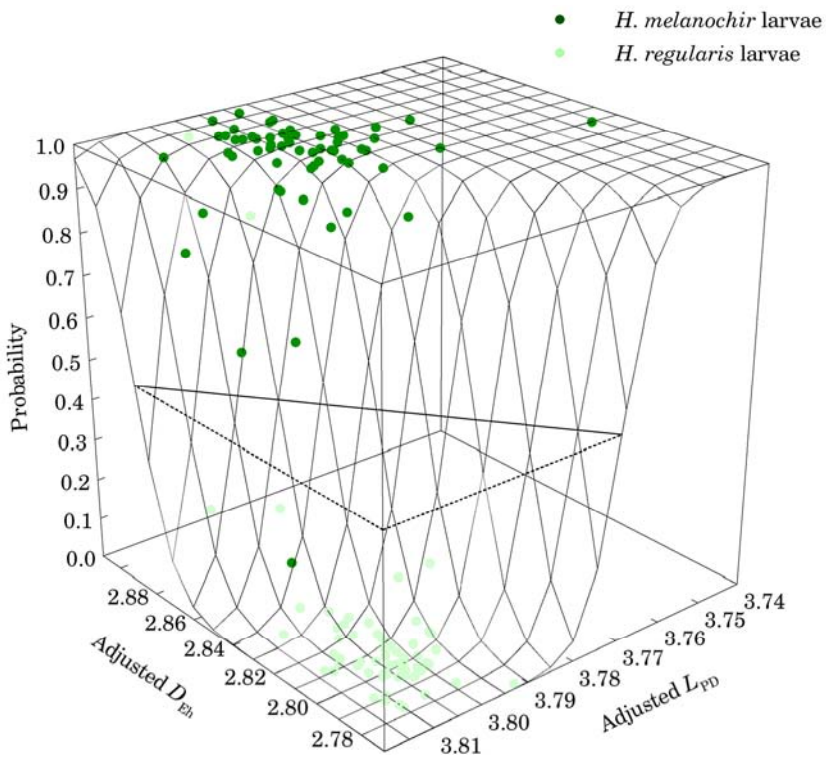

FIG. 4.8 Adjusted horizontal eye diameter (D_{Eh}) and pre-dorsal fin length (L_{PD}) of *Hyporhamphus melanochir* and *H. regularis* larvae. Data represent analysis and holdout samples and are superimposed on a 3D-mesh plot of the logistic regression model used to distinguish between *Hyporhamphus melanochir* and *H. regularis* larvae. The line bisecting the mesh plot indicates the 0.5 probability cutoff score for predicting species.

Although a marginal improvement in model fit was obtained with the entry of D_{Ev} (as indicated by the -2LL and R^2_{logit} values), the Wald statistic revealed a non-significant variable coefficient and so, D_{Ev} was subsequently omitted. Despite that, the two-variable model was clearly preferred because of its better classification accuracy of the holdout sample.

4.4 DISCUSSION

This study provides the first descriptions of larval development of hemiramphids endemic to marine (*H. melanochir*) and estuarine (*H. regularis*) waters of Australia.

Both *H. melanochir* and *H. regularis* share characters common to other described hemiramphid larvae. They are generally characterized by their lack of head or fin spines; elongate body; long straight gut; extended lower jaw; a main pigmentation pattern consisting of rows of melanophores on the dorsal, lateral and ventral surfaces of the body; and advanced state of development at hatching (Collette *et al.*, 1984; Watson, 1996; Trnski *et al.*, 2000). Although size varies slightly between *H. melanochir* and *H. regularis* at which fins develop, the sequence of development for both species is the same as most hemiramphids, i.e., C → D, A → P_1 → P_2 (Collette *et al.*, 1984).

Hyporhamphus melanochir larvae are distinguishable from *H. regularis* by: (i) having 58-61 vertebrae (*v.* 51-54 for *H. regularis*); (ii) having 12-15 melanophore pairs in longitudinal rows along the dorsal margin between the head and origin of the dorsal fin (*v.* 19-22 for *H. regularis*); and (iii) the absence of a large ventral pigment blotch anteriorly on the gut and isthmus which is present in *H. regularis*. Despite the difficulty in counting myomeres, either the number of vertebrae in cleared and stained specimens or the number of myomeres between the pectoral-fin base and anus (usually three less than the number of precaudal vertebrae; see TABLE 4.2 and TABLE 4.4) revealed a consistent difference between species. Further examination of body measurements by logistic regression analysis revealed a significant difference exists in the combined measurements of D_{Eh} and L_{PD} between *H. melanochir* and *H. regularis* larvae. This difference appeared to be mainly attributed to the consistently larger D_{Eh} of *H. melanochir* than *H. regularis* at a given body size throughout larval development.

The geographic distributions of larval *H. melanochir* and *H. regularis* were separate in most samples; only three *H. melanochir* were found amongst *H. regularis* from Barker Inlet, whilst no *H. regularis* were amongst *H. melanochir* from the Bay of Shoals or Gulf St Vincent. Larvae of other hemiramphid species may overlap in distribution with those of *H. melanochir* and *H. regularis* outside South Australian waters. Meristic characters (summarised in TABLE 4.7) can often distinguish *H. melanochir* and *H. regularis* from the other species, except the eastern sea garfish *H. australis*, which has overlapping meristic counts and currently undescribed larvae. The storm garfish *Hemiramphus robustus* has fewer anal-fin rays (11-14), and develops both a dark blotch below the dorsal fin and a pigmented pelvic fin in the juvenile stage (Collette, 1974; Collette *et al.*, 1984). The long-finned garfish *Euleptorhamphus viridis*, an oceanic species that rarely frequents nearshore waters, is strikingly different from other hemiramphids, being much more elongate and slender, and having divergent meristic counts including more dorsal- and anal-fin rays (21-25 and 20-24, respectively), more vertebrae (69-73), fewer pectoral-fin rays (7-9) and fewer gill rakers (25-33) (Collette, 1974; Hardy, 1978; Chen, 1988; Trnski *et al.*, 2000).

Larvae of the saury *Scomberosox saurus* (family Scomberosocidae) also occur in southern Australia and are the only other species that could be confused with hemiramphids. These are distinguishable from hemiramphid larvae by their higher myomere count (62-70), more principal caudal-fin rays (16-17), presence of dorsal and anal finlets, and much heavier pigmentation (Bruce & Sutton, 1998; Trnski *et al.*, 2000).

TABLE 4.7 Meristic counts of adult hemiramphids found in southern Australia. Data collated from Collette (1974) except where footnoted. A second range from another source is given if not in total agreement with Collette (1974). The distinguishing vertebral counts for *H. melanochir* and *H. regularis* in this study are also included. Vertebrae are given as precaudal + caudal; gill rakers are given as upper + lower. ? = no information available. D, dorsal; A, anal; P_1, pectoral; P_2, pelvic; C, caudal.

Species	Fin rays					Branchi-ostegal rays	Vertebrae	Gill rakers
	D	A	P_1	P_2	C			
Euleptorhamphus viridis	21-25	21-24	8-9	6†	?+7+8+?§	?	69-73	(5-9) + (18-23) = 25-33
		20-24†	7-9†				(44-46) + (26-29) = 70-75§	
Hemiramphus robustus	13-15	11-14	12-13	6¶	4+7+8+5¶	13¶	(35-37) + (17-19) = 52-55	(27-33) + (20-25) = ?
							(33-34) + (16-17) = 49-50*	
Hyporhamphus australis	15-17	17-20	11-13	6¶	4+7+8+4¶	12-13¶	(37-39) + (18-20) = 56-58	(31-39) + (23-33) = ?
			10-13*				(38-40) + ?*	
Hyporhamphus melanochir	15-18	17-20	11-13	6‡	4-5+7+8+4-5¶‖	12-13¶‖	(36-41) + (18-21) = 55-61	(27-35) + (21-29) = ?
							(38-40) + (19-21) = 58-61‖	
Hyporhamphus regularis	14-17	15-19	11-12	6‡	4+7+8+4¶‖	10-12¶‖	(33-38) + (18-20) = 51-58	(30-36) + (21-27) = 52-61
							(33-35) + (18-20) = 51-54‖	

*Parin *et al.* (1980).
†Chen (1988).
‡Gomon *et al.* (1994).
§Trnski *et al.* (2000).
¶Noell (unpubl. data).
‖This study.