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Original Article

Molecular discrimination of shelf-spawned eggs of two co-occurring *Trachurus* spp. (Carangidae) in southeastern Australia: a key step to future egg-based biomass estimates

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A molecular approach was successfully developed to discriminate between spawned eggs of the pelagic carangids *Trachurus declivis* and *Trachurus novaezelandiae* collected during ichthyoplankton surveys conducted in October 2002 and 2003 along shelf waters of Queensland (Qld) and New South Wales (NSW), in southeastern Australia (25°50′ – 37°30′S). Visually identified *Trachurus* eggs were subjected to mtDNA analysis by targeting specific fragments of the cytochrome oxidase subunit 1 (*CO1*) and cytochrome *b* (*Cyt b*) genes, with three diagnostic sites (single-nucleotide polymorphisms) within a 297 bp segment of *Cyt b* (558, 588, 825) providing the best approach to discriminate between species. Polymerase chain reaction amplification and sequencing of 608 suspected *Trachurus* eggs resulted in 586 (96.4%) high-quality sequences that unequivocally identified 315 and 207 eggs as *T. declivis* and *T. novaezelandiae*, respectively, as well as 18 "variant haplotype" eggs that exhibited a base substitution at one of the diagnostic sites; the remaining 46 sequences aligned to three different genera in GenBank including two carangids, thus highlighting the effectiveness of molecular methods for egg identification. Rehydrated, mtDNA-verified eggs of *T. declivis* were significantly larger (0.97 ± 0.01 mm) than those of *T. novaezelandiae* (0.82 ± 0.01 mm), though still proved problematic to identify to species when relying on morphology alone. Egg distributions showed main spawning areas of *T. declivis* and *T. novaezelandiae* confined mostly to southern NSW (>32°S) and northern NSW/southern Qld (<32°S), respectively, with *T. novaezelandiae* likely to continue spawning further south during summer with the gradual temperature increase associated with the south-flowing East Australian Current. Overall findings support the adoption of comparable molecular protocols to verify identification of wild spawned eggs to species level, especially eggs collected during the application of the daily egg production method to estimate spawning biomass of pelagic species, as well as biological fish studies.

Keywords: cytochrome *b*, mtDNA, pelagic fish eggs, *Trachurs* spp.

Introduction

Accurate identification of wild-spawned fish eggs to species level can be a difficult and time-consuming task when relying solely on morphological characters, and especially when dealing with plankton samples containing eggs of many species at various stages of development (Ahlstrom and Moser, 1980). Such difficulty extends to all fish that release eggs into the water column, regardless of spawning habitat (e.g. freshwater, coastal reefs, oceanic) and mode (e.g. demersal vs. pelagic). Main complications of species egg identifications include the fact that most distinguishing characters only become evident at late stages (Ahlstrom and Moser, 1980; Matarese and Sandknop, 1984) and that, in many instances, eggs of unrelated species may be morphologically indistinguishable (Shao *et al.*, 2002).

Sequencing of species-specific genetic markers (e.g. cytochrome *c* oxidase subunit 1 or *CO1*) from mitochondrial DNA (mtDNA) is becoming increasingly popular to validate fish egg identifications based on morphological characters (Taylor *et al.*, 2002; Fox *et al.*, 2005; Aranishi, 2006; Karaiskou *et al.*, 2007; Goodsir *et al.*, 2008;

Neira and Keane, 2008; Saitoh *et al.*, 2009; Kawakami *et al.*, 2010; Kim *et al.*, 2010), and has been applied in standard descriptive accounts of fish eggs as well as larvae (García-Vázquez *et al.*, 2006; Pegg *et al.*, 2006; Neira *et al.*, 2008). Molecular techniques have already been used to resolve taxonomy and systematic relationships in adult fish (e.g. Burridge and White, 2000; Karaiskou *et al.*, 2003a, b; Cárdenas *et al.*, 2005; Bektas and Belduz, 2008; Lara *et al.*, 2010), and comprise the main approach of an ongoing collaborative project to build a DNA barcode library of all the world's fish (Ward *et al.*, 2005, 2009). In terms of fish eggs, molecular approaches provide precise species identifications regardless of age/developmental stage (i.e. whole spectrum from newly spawned to late-stage eggs), and an independent means of confirming identifications based on morphological characters.

In this study we employ a molecular approach to discriminate between eggs of *Trachurus declivis* (jack mackerel) and *Trachurus novaezelandiae* (yellowtail scad), two small pelagic carangids (Family Carangidae) known to co-occur throughout temperate Australia where they currently support localized, low-volume commercial fisheries (Williams and Pullen, 1993; Woodhams *et al.*, 2012; André *et al.*, 2014). Eggs of both species in plankton samples taken along southeastern Australia in October 2002 and 2003 were initially identified based on morphological characters, thus posing the question of a possible mixed spawning area during that mid-spring period. The molecular approach employed herein involves standard polymerase chain reaction (PCR) amplification and sequencing of *CO1* and cytochrome *b* (*Cyt b*) fragments for species-specific single-nucleotide polymorphisms (SNPs), and aims to establish which of these mtDNA genes could be used to separate eggs of both species as well as to validate egg identifications based on morphology. Egg abundances estimated for each species following molecular-based validations were then used to characterize spawning habitat and described in terms of prevalent oceanographic conditions at the time of sampling.

The overarching rationale behind the study is to establish a protocol to accurately identify eggs of *T. declivis* and *T. novaezelandiae* as required in the application of the daily egg production method (DEPM) to estimate biomass of pelagic-spawning fish (Stratoudakis *et al.*, 2006; Bernal *et al.*, 2012). Such an approach was successfully applied for the first time to *Emmelichthys nitidus* off eastern Tasmania, whereby morphological identification of eggs was validated using quantitative PCR amplification of the mtDNA d-loop gene region unique to *E. nitidus* (Neira *et al.*, 2008) and the data subsequently used to estimate spawning biomass based on DEPM (Neira and Lyle, 2011). Since stock biomass levels are uncertain for either *Trachurus* spp. and there is commercial interest in developing a larger fishery for these and other small pelagic fish, efforts are currently underway to estimate biomass of *T. declivis* using DEPM to establish scientifically based harvest strategies for the fishery, and to incorporate these data into models being developed to better understand the role of this and other small fish in the pelagic ecosystem (Bulman *et al.*, 2010). Impetus to estimate stock biomass follows a 2-year interim ban by the Australian Federal Government on large-scale factory trawler operations to capture a specific annual quota of *T. declivis* and other small pelagic species across southeastern Australia (Tracey *et al.*, 2013). The use of the molecular approach adopted in this study to identify eggs is discussed in terms of its effectiveness in the application of DEPM, noting that incorrect egg identifications can result in biased assessments which may impact on the management and subsequent sustainability of exploited fish stocks (Fox *et al.*, 2005).

Material and methods

Egg surveys

Eggs used for this study were collected during ichthyoplankton surveys carried out during 12–20 October 2002 and 1–7 October 2003 along the shelf waters of southeastern Australia (25°50′S through to 37°30′S). Samples at fixed stations were collected vertically from a maximum depth of 200 m using a bongo sampler equipped with two 0.6 m diameter, 3 m long 300/500 μm mesh plankton nets, each fitted with a General Oceanics flowmeter to estimate total volume of water filtered during each haul; additional information on transects and stations as well as sampling methodology and collection of environmental variables (salinity and temperature by depth) are provided in Keane and Neira (2008). Oceanographic characteristics and boundaries of water masses where eggs originated are based on the study of Keane and Neira (2008). Samples from each station were fixed in 98% ethanol immediately after collection, and all eggs sorted from samples within 3 weeks of preservation.

Visual identification and selection of eggs

Trachurus spp. eggs were separated under a stereomicroscope from similar eggs of other carangid and non-carangid taxa using key morphological characters described by Ahlstrom and Ball (1954), Crossland (1981), and Cunha *et al.* (2008). These included: (i) spherical eggs between ~0.7 and 1.1 mm diameter; (ii) smooth (i.e. non-ornamented) chorion; (iii) narrow perivitelline space; (iv) prominent segmented yolk sac; (v) single pigmented oil globule located centrally on yolk sac (early to mid stage eggs); and (vi) a distinct single row of melanophores along dorsal surface of the trunk and tail in late-stage embryos. Eggs were further separated into 11 stages using the developmental key developed by Cunha *et al.* (2008) for *T. trachurus*.

The total number of eggs subjected to DNA testing varied among stations according to raw abundances of eggs visually identified as *Trachurus*. All such eggs were tested in stations with three or fewer eggs, three in stations with 4–6 eggs, 50% in stations with 7–40 eggs, and a minimum of 40 that were randomly selected for stations with >40 eggs. In all, a total of 608 visually identified *Trachurus* eggs were tested, as well as five late-stage eggs that were morphologically similar but assumed to be non-*Trachurus*. These were similar in diameter and had evidence of some yolk segmentation but differed from *Trachurus* eggs in the pigment pattern of the embryo. Testing was therefore necessary to exclude possible morphological variation in features such as degree of yolk segmentation and extent and positioning of embryo pigment.

Molecular protocols

DNA extraction

We employed high-throughput DNA extraction from eggs visually identified as *Trachurus* as well as muscle tissues samples from ethanol-preserved adult *T. declivis* and *T. novaezelandiae*. Adult muscle tissue samples were obtained from 10 *T. declivis* and 23 *T. novaezelandiae* stored at the Australian National Fish Collection (CSIRO, Hobart), and included one voucher specimen of *T. declivis* and 12 *T. novaezelandiae*. Additional muscle tissue samples were obtained from four *T. declivis* and two *T. novaezelandiae* vouchered

specimens housed at the Ichthyology Section of Museum Victoria (voucher numbers *T. declivis*: A 21544, A 25189-003, A 24884-001, and A 29361-021;*T. novaezelandiae*: A 24882-001 and A 29355-007). All adult DNA sequences are deposited in GenBank (Supplementary Appendix S1; accessions KM006708-KM006780, KM006497-KM006498; http://www.ncbi.nlm.nih.gov/nuccore/?term=trachurus%20declivis).

DNA extractions from eggs and muscle tissue samples (~10 mg) were carried out using the QIAamp® DNA Micro Kit (QIAGEN, USA) following the manufacturer's protocol for tissue extraction, with carrier RNA (supplied with the kit) used to improve DNA yield from eggs. Samples were cleansed in milli-Q water to remove ethanol before extraction, and DNA was eluted in a single 50 µl volume of Tris–EDTA (TE) buffer. Eggs required overnight digestion with 20 µl of proteinase K to remove the chorion and allow efficient cell lysis, whereas muscle tissues were digested with 5 µl of proteinase K for 4 h before DNA extraction.

PCR amplification and sequencing

Specific fragments of the mitochondrial genes *CO1* and *Cyt b* were amplified using standard PCR. The *CO1* gene was chosen for this study since it constitutes the marker being used in the Fish Barcode of Life Database (BOLD) being developed for animals including fish worldwide (Hebert *et al.*, 2003; Ward *et al.*, 2005), while *Cyt b* is widely employed in phylogenetic studies of fish (e.g. Burridge and White, 2000). A 652 base pair (bp) fragment of the 5′ region of *CO1* was amplified using primers FishF2 and FishR2 (Ward *et al.*, 2009), while a ~1000 bp fragment of the central region of *Cyt b* was amplified using primers CTB-F and CTB-R (Cárdenas *et al.*, 2005).

All PCRs were performed using MyTaq HS™ DNA Polymerase (Bioline) in a 25 µl reaction volume. The volume of DNA template used in PCRs varied between 2 µl for adult tissue samples and 3–10 µl for eggs. The thermocycling regime was consistent throughout all PCRs, and comprised initial denaturing at 95°C (3 min) followed by 34 cycles of 95°C (20 s), primer annealing at 50–55°C depending on primer combinations (20 s), and primer extension at 72°C (15 s). After cycling, a final extension at 72°C (1 min) was employed. PCR product purification and bi-directional sequencing was performed by Macrogen Inc. (Seoul, Republic of Korea).

To improve amplification success from low quantities of mtDNA, PCR primers were developed to amplify a smaller fragment of *Cyt b* containing species-diagnostic sites. One primer set was designed using Primer3 (Rozen and Skaletsky, 2000) to amplify a 385 bp region containing three SNPs in positions 558, 588, and 825 (F3: 5′-GTA GGA AAY ACC CTC GTC CA-3′, R3: 5′-ATT GAT CGG AGA ATG GCG TA-3′). Primer F3 required a degenerate nucleotide at one position to accommodate a thymine residue in most adult *T. declivis*, but cytosine in the corresponding position for all adult *T. novaezelandiae*. Primers were tested on adult DNA to determine whether they could successfully amplify and sequence the diagnostic polymorphisms. PCR and sequencing were performed as above, with sequencing in both directions.

Sequence alignment

Resultant *CO1* and *Cyt b* sequences from adult tissue samples were imported into Sequencher®, and forward and reverse strands validated against each other and summarized as a consensus sequence. Egg sequences were then compared against these reference data in BOLD and via BLAST searches of GenBank.

Egg distribution and environmental relationships

Raw egg abundances were standardized to area (m^2) based on volume of water filtered by the sampler and maximum sampling depth of vertical tow (Keane and Neira, 2008), and plotted by station for each survey using Surfer®. For stations where subsampling for molecular analysis was carried out, the number of eggs by species was determined by the relative species mix in the subsample multiplied by the total number of visually identified *Trachurus* eggs at that station. Preserved eggs were rehydrated in distilled water (immersion time of ~5 min) to better reflect diameters of fresh eggs, and measured to 0.01 mm under a stereomicroscope fitted with an eyepiece micrometer. Diameter data obtained from 534 rehydrated eggs identified as *T. declivis*, *T. novaezelandiae*, *Decapterus macarellus*, and *Pseudocaranx dentex* using sequence information derived from PCR products were plotted using standard box and whisker plots.

A Welch two-sample *t*-test (assuming unequal variances) was used to compare diameters of rehydrated eggs of *T. declivis* and *T. novaezelandiae*. Linear regression models were used to examine relationships between egg diameters and water temperature (mean °C to a depth of 30 m), salinity (mean PSU to a depth of 30 m), developmental stage ($n = 11$), and year (2002, 2003) for both *Trachurus* species. Stepwise model selection by exact Akaike information criterion was undertaken using the StepAIC function in the MASS package (R core development team).

Results

Molecular analyses

Adult Trachurus

Sequencing of *CO1* from adult muscle tissue was successful for 36 of the 39 *T. declivis* ($n = 14$) and *T. novaezelandiae* ($n = 25$) tested during this study. However, comparisons of successful sequences against those stored in the BOLD matched 100% with *T. declivis* and *Trachurus japonicus*, and 99.54–99.85% with *T. declivis*, *T. japonicus*, as well as *T. novaezelandiae* (www.barcodinglife.org, last accessed January 2013). In addition, one *CO1* sequence from a vouchered *T. novaezelandiae* from Western Australia (CSIRO H 270 6343-06) matched *T. declivis* sequences. Excluding the latter, closer examination of the remaining 35 *CO1* sequences revealed two SNPs in positions 484 and 598, with all 14 *T. declivis* sequences containing adenine (A) and thymine (T), and 21 *T. novaezelandiae* sequences containing guanine (G) and cytosine (C).

Alignment of *Cyt b* sequences obtained from all 39 adult *Trachurus* spp. revealed three SNPs with species-specific variation in positions 558, 588, and 825. Adult *T. declivis* sequences contained G, C, and A in these three positions, respectively, and without exception, whereas *T. novaezelandiae* contained A, T, and G at the respective positions. GenBank sequences (National Centre for Biotechnology Information, NCBI) from two of the three *T. declivis* and two *T. novaezelandiae* matched expectations based on the adults sequenced during this study. The sequence from one *T. declivis* (accession number AB269697.1) aligned poorly with sequences from either *T. declivis* or *T. novaezelandiae* using the Basic Logical Alignment Search Tool (BLAST) algorithm, instead matching a sequence from *Seriola rivoliana*, suggesting that it may have been a case of misidentification.

Eggs

Sequencing *Cyt b* of 608 eggs visually identified as *Trachurus* resulted in 586 (96.4%) high-quality sequences leading to the validation of

540 eggs as *Trachurus* spp. along with those of three other genera including two species of the Family Carangidae (Table 1). A total of 315 and 207 eggs were unequivocally identified as *T. declivis* and *T. novaezelandiae*, respectively ($n = 522$). However, 18 eggs (3.1%) were regarded as *Trachurus* "variant haplotypes" as they exhibited a one base difference among the three nucleotides considered diagnostic based on sequencing of adult specimens, likely representing the retention of an ancestral polymorphism. Usually, all other eggs in stations containing a variant haplotype matched one of the two haplotypes identified from adults. Thus, eggs returning the variant haplotypes ACA ($n = 7$) and GCG ($n = 4$) were assumed to be *T. declivis*, whereas those returning the haplotypes ATA ($n = 4$) and GTG ($n = 3$) were assumed to be *T. novaezealandiae* (herein named Variant 1, 2, 3, and 4, respectively; Table 2).

Of the remaining 46 eggs tested, 29 and 16 were assigned to the carangids *P. dentex* and *D. macarellus*, respectively, while the sole remaining egg was identified as the non-carangid *Chanos chanos* (Family Chanidae). Eggs of these species were distinguished from those of *Trachurus* spp. by sequence similarities of 90% or less within the 385 bp region of *Cyt b*.

Successful PCR and sequencing obtained for each of the 586 eggs tested represented 93–100% success across all 11 developmental stages (Table 3). However, while most *Trachurus* eggs visually identified using morphological characters were confirmed by mtDNA sequencing for developmental stages 1 through to 4 (88–100%), matching between morphological and mtDNA identifications were least reliable for stages 5 through to 7 (47–64%). Such reduced accuracy in the morphological identification of mid-stage *Trachurus* eggs can be attributed to the presence of a relatively large proportion of developmental stages 4–7 eggs of *P. dentex* and *D. macarellus* in the tested samples.

Extrapolation of species ratios based on mtDNA sequencing to numbers morphologically identified as *Trachurus* eggs across all positive stations resulted in 2422 *T. declivis*, 501 *T. novaezelandiae*, 68 *P. dentex*, 23 *D. macarellus*, and five *C. chanos*. Eggs of the latter three species accounted for just over 3% of the total collected, while PCR failures, variants, and ambiguous molecular identifications collectively accounted for 4% of all eggs visually identified as *Trachurus* using morphological characters.

Table 1. *Cyt b*-based specific identifications of eggs collected during the October 2002 and 2003 surveys along southeastern Australia, and initially identified using morphological characters.

	October 2002		October 2003		Total	
Species	***n***	**%**	***n***	**%**	***n***	**%**
Chanos chanos	0	–	1	0.4	1	0.2
Decapterus macarellus	15	4.1	1	0.4	16	2.7
Pseudocaranx dentex	19	5.2	10	4.5	29	4.9
Trachurus declivis	255	70.4	60	26.8	315	53.8
Trachurus novaezelandiae	59	16.3	148	66.1	207	35.3
Trachurus variant	14	3.9	4	1.8	18	3.1
Total eggs sequenced	362		224		586	

Trachurus "variant" refers to haplotypes that exhibited a base substitution at one of the diagnostic sites for either *T. declivis* or *T. novaezelandiae*.

Table 2. Summary of haplotypes obtained in eggs of *T. declivis* (*T.d*) and *T. novaezelandiae* (*T.n*) found during this study. Information on the mean temperature at station where each egg was collected as well as diameter (mm) is also provided.

Latitude (°S)	Temperature (°C)	Diameter (mm)	Rehydrated	Haplotype	Species	Variant	Inferred species
26° 27′	22.2	0.76	N	ATA	–	3	*T.n*
27° 18′	21.8	0.80	Y	ATA	–	3	*T.n*
27° 30′	–	0.78	Y	GCA	*T.d*	–	–
27° 42′	21.0	0.88	Y	GCA	*T.d*	–	–
27° 42′	21.0	0.84	Y	GCA	*T.d*	–	–
28° 08′	20.4	0.82	Y	GTG	–	4	*T.n*
28° 08′	20.4	0.80	Y	GCA	*T.d*	–	–
28° 08′	20.4	0.80	Y	GCA	*T.d*	–	–
28° 20′	19.5	0.77	Y	GTG	–	4	*T.n*
29° 48′	19.8	0.81	Y	ATA	–	3	*T.n*
29° 48′	19.8	0.79	Y	ATA	–	3	*T.n*
32° 30′	19.6	0.87	Y	ACA	–	1	*T.d*
32° 30′	19.6	0.97	Y	ACA	–	1	*T.d*
32° 30′	19.6	0.87	N	ACA	–	1	*T.d*
32° 55′	19.5	0.97	Y	ACA	–	1	*T.d*
33° 20′	19.7	0.94	Y	ACA	–	1	*T.d*
34° 10′	17.3	0.92	Y	ATG	*T.n*	–	–
35° 00′	15.8	0.96	Y	ACA	–	1	*T.d*
35° 00′	15.8	1.02	Y	GCG	–	2	*T.d*
35° 00′	15.8	1.03	Y	GCG	–	2	*T.d*
35° 00′	17.1	0.91	N	ATG	*T.n*	–	–
35° 24′	17.4	1.02	Y	ACA	–	1	*T.d*
36° 40′	16.8	1.03	Y	GCG	–	2	*T.d*
36° 40′	16.8	0.97	Y	GCG	–	2	*T.d*
36° 40′	16.8	0.98	Y	ATG	*T.n*	–	–
36° 40′	16.8	0.98	Y	ATG	*T.n*	–	–
36° 40′	16.8	0.94	Y	ATG	*T.n*	–	–
37° 05′	16.4	0.95	Y	GTG	–	4	*T.n*

Inferred species correspond to 18 eggs labelled as variants (1–4).

Table 3. Sequencing results for 608 visually identified *Trachurus* eggs allocated by developmental stage (Cunha *et al.*, 2008).

Developmental stage	Total no. of eggs tested	Successful PCR	*Trachurus* spp.
1	32	32 (100)	32 (100.0)
2	71	71 (100.0)	71 (100.0)
3	36	34 (94.4)	34 (100.0)
4	26	25 (96.2)	22 (88.0)
5	38	36 (94.7)	23 (63.8)
6	20	19 (95.0)	9 (47.4)
7	15	14 (93.3)	7 (50.0)
8	46	45 (97.8)	41 (91.0)
9	154	145 (94.1)	137 (94.5)
10	108	107 (99.1)	107 (100.0)
11	62	58 (93.5)	58 (100.0)

Total numbers of eggs tested by stage and number of successful mtDNA sequences (%) are provided, as well as number (%) and of eggs correctly identified as *Trachurus*.

Egg morphology

Diameters of rehydrated eggs identified using the molecular approach were 0.78–1.10 mm for *T. declivis* ($n = 291$; mean $\pm$ 95% CI = 0.97 $\pm$ 0.01); 0.70–0.98 mm for *T. novaezelandiae* ($n = 199$; mean $\pm$ 95% CI = 0.82 $\pm$ 0.01); 0.75–0.99 mm for *P. dentex* ($n = 28$; mean $\pm$ 95% CI = 0.82 $\pm$ 0.02); and 0.75–0.85 mm for *D. macarellus* ($n = 16$; mean $\pm$ 95% CI = 0.81 $\pm$ 0.01; Figure 1). Eggs of *T. declivis* (Figure 2) were found to be significantly larger than those of *T. novaezelandiae* ($p < 0.05$) as well as those of the other two carangids. Co-occurring eggs of *P. dentex* and *D. macarellus* share similar morphological characters with those of the two *Trachurus*, including segmented yolk and a single, pigmented oil globule located centrally in the yolk sac. However, morphological features alone may be insufficient to reliably discriminate between late-stage eggs of *P. dentex* and *D. macarellus*, and between eggs of these species and those of *T. novaezelandiae*.

Final regression models for egg diameters included water temperature, salinity, and year for *T. novaezelandiae*, and water temperature and developmental stage for *T. declivis* (Table 4). Water temperature was highly significant for both species, implying a decrease in egg diameter with increasing water temperature. Salinity and year were also significant for *T. novaezelandiae*, whereas developmental stage was non-significant for *T. declivis*. Results of alternative models and their respective AICs are provided in Supplementary Appendix S2.

Spatial distribution of eggs

Eggs of *T. declivis* and *T. novaezelandiae* occurred along shelf waters between southern Queensland (Qld) and the southern end of New South Wales (NSW) during October 2002 and 2003, and were found to co-occur at six stations spread throughout the sampling area though in small concentrations (Figure 3). However, during both surveys, *T. declivis* eggs were notably more abundant along the mid to southern NSW area south of 32°S, while those of *T. novaezelandiae* were noticeably more prevalent north of 32°S. Of the 522 eggs unequivocally identified as *Trachurus* using mtDNA analysis, five *T. declivis* eggs were collected north of 28°S (GCA; 0.78–0.88 mm diameter), while five *T. novaezelandiae* eggs came from stations south of 34°S (ATG; 0.91–0.98 mm; Table 2). The few eggs of *D. macarellus* occurred only at the northern-most stations (25°50′S) during the two surveys, while eggs of *P. dentex* were sparsely distributed along the southern Qld–northern NSW coast, and mostly at inshore stations (not shown in Figure 3).

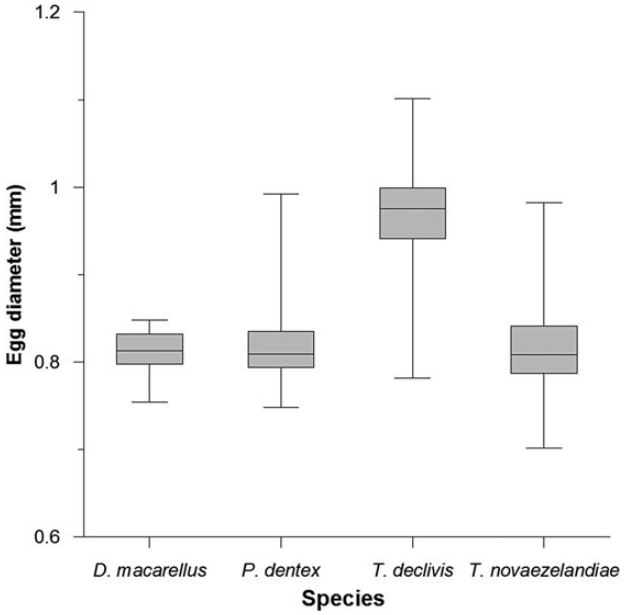

Figure 1. Diameters (mm) of rehydrated eggs of *D. macarellus* ($n = 16$), *P. dentex* ($n = 28$), *T. declivis* ($n = 276$), and *T. novaezelandiae* ($n = 193$) collected along southeastern Australia during October 2002 and 2003. Data on each box–whisker plot correspond to the median, 1st and 3rd quartiles, and range (minimum and maximum).

The seven Variant 1 eggs (0.87–1.02 mm diameter) and four Variant 2 eggs (0.97–1.03 mm diameter) allocated to *T. declivis* originated from stations located south of 32°S. In contrast, the four Variant 3 eggs (0.76–0.81 mm diameter) and two of the three Variant 4 eggs (0.77–0.95 mm diameter) allocated to *T. novaezelandiae* came from stations north of 29°S, while the remaining Variant 4 egg originated from a southern station at 37°05′S.

In the context of water masses, eggs of *T. declivis* collected both in October 2002 and 2003 were predominantly confined to mixed water (MIX; 18.5–19.8°C) comprising water of the warm, south-flowing East Australian Current (EAC) and Tasman Sea (TAS), as well as TAS water (16.0–17.0°C) further south along southern NSW (Figure 3). In contrast, eggs of *T. novaezelandiae* occurred mostly in the northern region dominated by the EAC (20.6–22.3°C) except for a few occurrences along the NSW region dominated by MIX and TAS waters during both October 2002 and 2003 (Figure 3). Despite the ca. 115 nautical mile (nm) difference between the latitudinal boundaries of the EAC/MIX water masses in October 2002 and 2003 (i.e. 31°48′S vs. 29°54′S, respectively), both the northern limit of the area where most *T. declivis* eggs originated as well as the southern limit where most *T. novaezelandiae* eggs were collected remained fairly consistent, and closely corresponded to the EAC/MIX boundaries across the surveys (Figure 3).

The greatest abundance of *T. declivis* eggs (991 eggs m^{-2}) was recorded at a shelf break station off mid-southern NSW (35°00′S) in October 2002, where the mean temperature to 30 m was 17.4°C. In contrast, the greatest abundance of *T. novaezelandiae* eggs (92 eggs m^{-2}) was obtained at a station some 9 nm shoreward from the shelf break (29°48′S) in October 2003, where the mean temperature was 19.8°C. The maximum seabed depths at these two stations were 177 and 68 m, respectively.

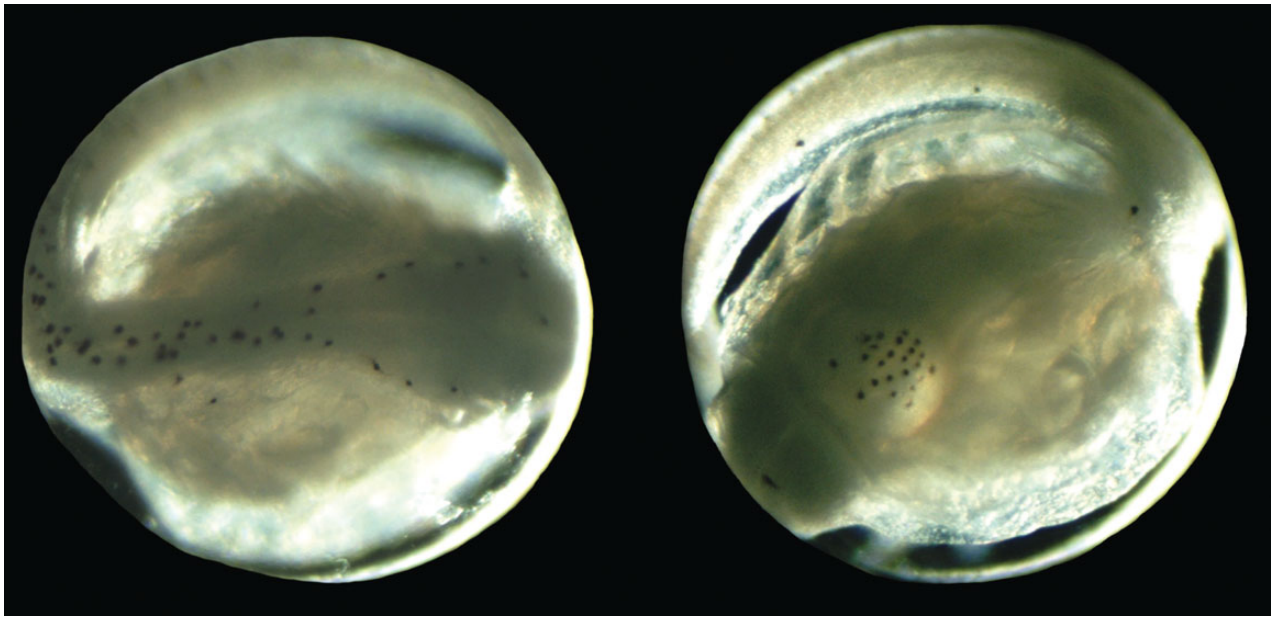

Figure 2. Late-stage egg of *T. declivis* (1.03 mm diameter; rehydrated) collected off southeastern Australia in October 2002. Key morphological characters include smooth chorion, narrow perivitelline space, and segmented yolk sac with centrally positioned pigmented, single oil globule. Photograph was obtained using a stereomicroscope fitted with Leica IM50 imaging software (R.A. Perry).

Table 4. Coefficient estimates and significance of variables for linear regression models of egg diameters for *T. declivis* and *T. novaezelandiae*.

Model variable	Estimate	Standard error	*t* value	Significance
Trachurus declivis				
Intercept	1.089	0.036	30.433	***
Temperature	−0.007	0.002	−3.647	***
Developmental stage	0.002	0.001	1.901	n.s.
Trachurus novaezelandiae				
Intercept	−14.285	3.355	−4.258	***
Temperature	−0.018	0.003	−6.434	***
Salinity	0.435	0.095	4.594	***
Year (2003)	0.078	0.017	4.455	***

n.s. $p > 0.05$; ***$p < 0.001$.

Overall temperature ranges from sampling sites of *T. declivis* and *T. novaezelandiae* eggs during October 2002 and 2003 were 15.8–21.0 and 16.8–22.2°C, respectively (Figure 4; Table 5). Salinities were consistent across all positive stations and ranged between 35.3 and 35.7 during the two surveys.

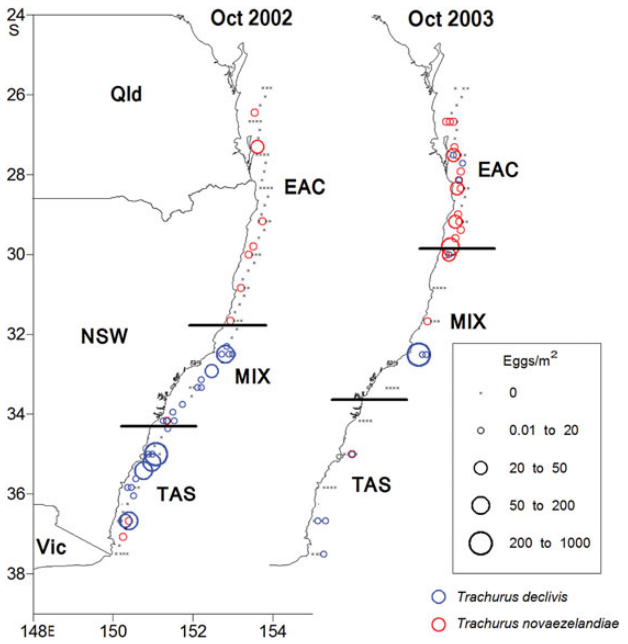

Figure 3. Spatial distribution of eggs of *T. declivis* and *T. novaezelandiae* along southeastern Australia during October 2002 and October 2003 (eggs m^{-2}). Horizontal lines correspond to boundaries of three water masses (EAC, MIX, and TAS) identified during the surveys [refer to Keane and Neira (2008) for further details]. EAC, East Australian Current; MIX, mixed EAC-TAS water mass; TAS, Tasman Sea.

Discussion

Morphology-based identifications

The primary purpose of this study was to develop and test a DNA-based technique to unequivocally identify the pelagic eggs of two co-occurring *Trachurus* species that had been tentatively identified using available species-specific morphological characters. Eggs tested originated from samples taken during ichthyoplankton surveys conducted in October 2002 and 2003 along shelf waters of southeastern Australia, most of which contained eggs of many species including *Trachurus* spp. While eggs of the latter are easily distinguished from those of other non-carangid genera using an array of specific morphological characters (Ahlstrom and Ball, 1954; Robertson, 1975; Ahlstrom and Moser, 1980; Cunha *et al.*, 2008), identifications to species level are problematic mainly due

to common features shared by *Trachurus* eggs and those of several other co-occurring carangid species, specifically size range, smooth chorion (cf. ornamented), and the distinctly segmented yolk-sac bearing a single, pigmented oil globule (Ahlstrom and Moser, 1980). This, for example, was the case with eggs of the carangids *P. dentex* and *D. macarellus*, which were present in samples and initially misidentified as *Trachurus* using morphology, as shown by the subsequent mtDNA analysis.

Size ranges of genetically identified rehydrated eggs of *T. declivis* and *T. novaezelandiae* examined for this study were 0.78–1.10 and 0.70–0.98 mm, respectively, corresponding closely with sizes reported for *T. symmetricus* from California (0.98–1.08 mm; Ahlstrom and Ball, 1954) and *T. trachurus* from South Africa (0.85–0.95 mm; King *et al.*, 1977). Before verifying identifications using mtDNA analysis (see below), eggs of both species had been tentatively identified to species level using available diameter information reported for populations of the two species from the Hauraki Gulf in New Zealand, i.e. 0.95–1.02 mm for *T. declivis* and 0.78–0.88 mm for *T. novaezelandiae* (Crossland, 1981). However, whereas the overall larger size of *T. declivis* eggs appears to be a valuable distinguishing character, and no eggs of either species were reported within the intermediate 0.88–0.95 mm range (Crossland, 1981), our results showed that egg diameters of both species overlap. While the discrepancy between the two studies may reflect known effects of fixing and/or lengthy ethanol preservation (Karaiskou *et al.*, 2007), it is also plausible that it may reflect different environmental conditions encountered by eastern Australian and New Zealand populations at the time of spawning. In this context, it is perhaps relevant that eastern Australian populations of *T. declivis* and *T. novaezelandiae* are considered as separate stocks from those around New Zealand (Lindholm and Maxwell, 1988).

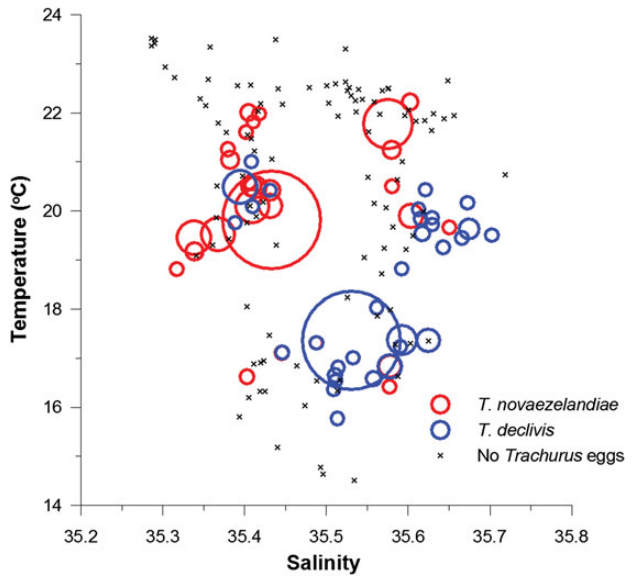

Figure 4. Abundances of *T. declivis* and *T. novaezelandiae* eggs (eggs m^{-2}) obtained at different combinations of temperatures (°C) and salinities (means to 30 m) along southeastern eastern Australia in October 2002 and 2003 (data combined). Bubbles sizes are proportional to abundances; black crosses correspond to stations with no *Trachurus* eggs.

Molecular-based identifications

The molecular approach developed and tested on adult specimens during this study showed that pelagic eggs of *T. declivis* and *T. novaezelandiae* could be confidently identified using a fragment of the mtDNA *Cyt b*, but not with *CO1* which is becoming a popular marker in taxonomic and systematic fish studies (Ward *et al.*, 2005, 2009). Low levels of mtDNA sequence divergence have been reported among 11 species of *Trachurus*, with the mean interspecies divergences of 3.43 ± 1% for *Cyt b* and 3.50 ± 1% for D-loop described as among the lowest recorded in any marine fish genus (Cárdenas *et al.*, 2005). Despite such low mtDNA diversity among *Trachurus* species, diagnostic SNPs identified from adults in *Cyt b* at positions 558, 588, and 825 occurred in concert for the vast majority of *Trachurus* eggs tested, providing a robust discriminatory approach to separate between eggs of *T. declivis* and *T. novaezelandiae*. Where variant haplotypes existed for these three nucleotide positions, at least two of the positions were consistent with the dominant haplotype otherwise observed at that station or nearby, implying that species identification could be inferred on that basis.

Primers F3 and R3 designed during this study were targeted to carangids, and failed to amplify the target mtDNA *(CO1)* for the majority of the 31 non-*Trachurus* eggs tested. The 15 eggs tentatively assigned to *Trachurus* but which failed to amplify using these primers would have most likely had poor quality DNA, as indicated by the fact that they also failed to amplify for a smaller PCR product (not shown). Besides *T. declivis* and *T. novaezelandiae*, mtDNA amplified with primers F3 and R3 included eggs of *P. dentex*, *D. macarellus*, and *C. chanos* which inhabit coastal waters and co-occur within the range of the two *Trachurus* species (Gomon *et al.*, 2008). The only other member of *Trachurus* known to occur in Australian waters is the Chilean mackerel *Trachurus murphyi*. However, these are oceanic spawners (Cárdenas *et al.*, 2009) and,

Table 5. Total numbers of eggs of *D. macarellus*, *P. dentex*, *T. declivis*, and *T. novaezelandiae* collected during the October 2002 (1) and 2003 (2) surveys along southeastern Australia, and water temperatures and salinities of positive stations; temperatures and salinities correspond to the mean values obtained for each positive station to a depth of 30 m.

Species	Survey	Positive stations	Latitudinal range (°S)	Total no. of eggs per survey	Temperature (°C)	Salinity (PSU)
D. macarellus	1	1	25°50′	30	21.6	35.63
	2	1	25°50′	1	23.4	35.29
P. dentex	1	4	25°50′ – 32°30′	32	16.6 – 21.6	35.40 – 35.63
	2	3	28°20′ – 29°48′	38	19.5 – 21.0	35.37 – 35.43
T. declivis	1	26	32°18′ – 37°05′	2094	15.8 – 20.4	35.49 – 35.70
	2	11	27°30′ – 37°30′	327	17.1 – 20.5	35.39 – 35.45
T. novaezelandiae	1	10	26°27′ – 37°05′	137	16.6 – 22.2	35.40 – 35.65
	2	20	26°40′ – 35°00′	363	18.8 – 22.0	35.32 – 35.43

as such, their eggs are unlikely to co-occur with those of shelf spawning *T. declivis* and *T. novaezelandiae*.

Molecular-based identifications of early-stage *Trachurus* spp. eggs (1–4) as well as late-stage eggs (8–11) were highly successful (>88%) compared with mid-stage eggs (5–7), a finding primarily attributed to misidentifications resulting from the large proportion of *P. dentex* and *D. macarellus* eggs at stages 4–7 in the samples. Species misidentifications are expected during the initial visual sorting process, especially when dealing with eggs comparable in diameter and that also share similar internal characters. Despite the reduced identification accuracy with mid-stage eggs, our results suggest that most *Trachurus* spp. eggs could be readily staged and that characters used to define developmental stages are not affected by ethanol fixation and preservation. While ethanol has been reported to cause pelagic eggs of some gadoid fish to become opaque, thereby making it harder to distinguish between species and assign developmental stages (Fox *et al.*, 2008; Goodsir *et al.*, 2008), this does not appear to be the case for the comparatively smaller *Trachurus* eggs.

Potential species hybridization

Although eggs of both *T. declivis* and *T. novaezelandiae* co-occurred at six stations during the October 2002 and 2003 surveys, there was no evidence of frequent hybridization between the two species. Such observation can be drawn from the fact that eggs of each species inferred by mtDNA analysis were generally confined within latitudinal survey boundaries, i.e. very few *T. declivis* eggs were identified northward of 32°S and only six eggs matching the *T. novaezelandiae* haplotype, including Variant 4, were identified further south of the main egg distribution area, i.e. >32°S. Thus, if hybridization occurred regularly between *T. declivis* and *T. novaezelandiae*, it would be expected that haplotypes of each species would co-occur at more stations, although comparisons of nuclear DNA are necessary to exclude the possibility of hybridization entirely, particularly if it occurred only in one direction (Palumbi and Cipriano, 1998).

Based on *CO1* sequences alone, the presence of a *T. declivis* haplotype in an adult specimen of *T. novaezelandiae* from Western Australia examined during this study could be compatible with hybridization. However, if hybridization was apparent, then the sequence of all mitochondrial genes within the *T. novaezelandiae* specimen tested should resemble that of *T. declivis*, given that the complete mtDNA genome is inherited in a clonal, maternal manner (Rubinoff, 2006). The fact that this specimen possessed the diagnostic *T. novaezelandiae* nucleotides (ATG) within *Cyt b* confirms its identification as *T. novaezelandiae* and, as such, the proposition of hybridization must be rejected in favour of the retention of ancestral polymorphisms within Western Australian *T. novaezelandiae CO1*.

Despite uncertainties involving the identifications of the 18 eggs with low-frequency variant combinations based on *Cyt b* SNPs, each returned a sequence similarity ≥98% with *Trachurus* in GenBank, thereby eliminating the possibility that these belonged to another genus (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed September 2011). In addition, each variant was allocated to either *T. declivis* (Variant 1 and 2) or *T. novaezelandiae* (Variant 3 and 4) according to the protocol adopted in this study.

Trachurus spawning along southeastern Australia

The short temporal extent of the egg surveys underpinning this study precludes from providing conclusive insights on the spawning patterns of *T. declivis* and *T. novaezelandiae*. However, some important observations can be made from the data obtained. The presence of eggs unequivocally identified as *T. declivis* and *T. novaezelandiae* following mtDNA analysis provides evidence that these two pelagic carangids spawn along continental shelf waters of southeastern mainland Australia during the early southern hemisphere spring (October). Furthermore, close examination of egg distributions from the October 2002 and 2003 surveys indicates that the latitudinal extent of the main spawning areas differed between species, and that each species may spawn primarily within discrete water masses with specific temperature characteristics. Such conclusion is supported by the fact that *T. declivis* eggs occurred predominantly at offshore stations near and along the shelf break in the area south of 32°S, where the average seabed depth is 196 m, whereas *T. novaezelandiae* eggs occurred predominantly shoreward of the shelf break in the survey area north of 32°S, where the average seabed depth is 78 m. Moreover, following the classification of water masses provided by Keane and Neira (2008) based on the same surveys in the study area, most *T. declivis* eggs originated from MIX (EAC+TAS water) and TAS water masses identified along the southern region, whereas the bulk of *T. novaezelandiae* eggs came from EAC water identified along the northern region. Ten eggs that appeared to be mismatched in terms of their inferred haplotype and spatial distribution may reflect occasional spawning by each species outside their predominant spawning area.

The spring shelf distribution of *T. declivis* eggs described above is fairly consistent with that reported for this species along shelf waters further south off eastern Tasmania (40°30′–42°30′S), where spawning peaks between mid and late December and ceases by February (Jordan, 1994; Jordan *et al.*, 1995). While no information on peak spawning off southeastern mainland Australia is currently available for this species, spawning is likely to continue after October and throughout the ensuing summer, and to extend progressively southwards as a result of the gradual warming of waters of the Tasman Sea (TAS) triggered by the intrusion of the warm, south-flowing EAC at that time (Ridgway and Godfrey, 1997). A spawning pattern linked to the EAC intrusion has compellingly been put forward as a trigger mechanism for other abundant small pelagic shelf species in southeastern Australia, such as *Scomber australasicus* (Neira and Keane, 2008).

High concentrations of *T. declivis* eggs were obtained off an eastern seaboard headland at latitude 32°30′S both in October 2002 and 2003. The headland is located near a known topographically induced upwelling area at around 30–32°S, where cooler, nutrient-rich water is reported to surge from the narrowing of the continental shelf and the separation of the EAC from the coast and subsequent deflection offshore (Ridgway and Godfrey, 1997; Oke and Middleton, 2001; Roughan and Middleton, 2004). The eastward deflection of the EAC is in turn likely to trigger the offshore advection of both eggs and early larvae of a suite of shelf-spawning species like *Trachurus* spp., resulting in the entrainment of these early stage fish along the Tasman Front. This hypothesis has already been put forward for eggs and larvae of *S. australasicus* (Neira and Keane, 2008) and postflexion larvae of the sardine *Sardinops sagax* (Syahailatua *et al.*, 2011).

Compared with *T. declivis*, much less is currently known about the spawning dynamics of *T. novaezelandiae*. The few eggs of this species identified in samples from the southern half of NSW during this study indicate that spawning could take place further south than the main northern spawning area reported herein, likely triggered by the warming of the TAS water with the gradual southward advancement of the EAC. Such a finding is consistent with earlier reports of significant concentrations of larvae of this

species along shelf waters associated with the EAC separation zone in the mid-NSW coast during spring/summer (Syahailatua *et al.*, 2011), and off the mid-NSW shelf in early summer (Smith and Suthers, 1999; Smith, 2003). Furthermore, the shallower water column depth where most *T. novaezelandiae* eggs were found during this study is also consistent with high concentrations of preflexion larvae of this species reported within 3 km from shore in water 50 m deep (Smith, 2003).

Advantages of mtDNA identifications

This study has demonstrated the value and importance of mtDNA techniques to confidently identify fish eggs to species level, particularly in cases of samples with a mix of eggs (and early larvae) as is invariably the case with ichthyoplankton surveys (e.g. Neira and Sporcic, 2002). For example, eggs of *P. dentex* from plankton samples sorted during this study were practically indistinguishable from those of *Trachurus* spp. based on assessments of photographic evidence and direct morphological comparison. However, the mtDNA analysis identified these eggs down to species. In this case, the alignment of an unknown sequence against a consensus sequence derived from *T. declivis* and *T. novaezelandiae* resulted in a considerable number of nucleotide base mismatches (~16%), thus prompting a query of the GenBank database which revealed a closest match to *P. dentex*.

Molecular-based identifications of fish eggs and larvae are not new, and have already been applied to pelagic species such as *S. australasicus* and *E. nitidus* in southeastern Australia (Neira and Keane, 2008; Neira *et al.*, 2008), and other fish elsewhere (e.g. Taylor *et al.*, 2002; Fox *et al.*, 2005, 2008; Aranishi, 2006; Karaiskou *et al.*, 2007; Goodsir *et al.*, 2008; Saitoh *et al.*, 2009; Kawakami *et al.*, 2010). Results of this study support the application of such techniques when identifying eggs during the application of the DEPM to estimate spawning biomass of commercial species. While there is no need to test every egg to verify identifications based on morphological characters, a protocol can be established to test a subsample to add confidence to biomass estimates, noting that eggs are required to be staged/aged to develop the mortality model required by the DEPM, as well as computing spawning area (Stratoudakis *et al.*, 2006; Bernal *et al.*, 2012). The same protocol should be applied when dealing with early larvae (i.e. preflexion stage) of closely related species such as *T. declivis* and *T. novaezelandiae*, which are almost indistinguishable before the early postflexion stage except for a few specific pigment characters (Trnski, 1998).

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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