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Decoding comparable morphologies: Pigmentation validated for identifying southern California *Paralabrax* larvae

Erica T. Jarvis MASON[1,*], Lucille BULKELEY[1], William W. WATSON[2], Allyson C. Salazar SAWKINS[1], Matthew T. CRAIG[2], John R. HYDE[2], Andrew R. THOMPSON[2], Brice X. SEMMENS[1]
[1]*Scripps Institution of Oceanography, University of California San Diego, California, USA.*
[2]*Fisheries Resources Division, Southwest Fisheries Science Center, NOAA Fisheries, California, USA.*
Corresponding author: etmason@ucsd.edu

Abstract
The distribution and trends in larval fish abundance are often used to assess the status and trends of marine fish populations. However, for closely related species whose larvae are morphologically similar and whose genetic identities may be degraded by formalin preservation, unraveling species-specific larval abundances from long-term monitoring efforts presents a challenge. We used statistical methods and the molecular identities of 107 ethanol-preserved specimens to construct and test a taxonomic key based on pigmentation patterns observed in three species of *Paralabrax* (family Serranidae) from southern California. Previously, larvae of these species were not thought to be reliably distinguishable based on morphology or pigmentation. However, when using pigmentation characters paired with molecular identities, a Random Forest Classifier provided a tool for structuring and refining a taxonomic key to distinguish species. Following calibration and key refinement, the probabilities of achieving accurate and precise species classifications using our taxonomic key were ≥96%, indicating that ventral and pectoral fin pigmentation patterns can discriminate *Paralabrax* larvae. Importantly, we can now leverage existing and future ichthyoplankton survey collections to assess species-specific trends in larval abundance without requiring expensive and lab-intensive genetic analyses used with formalin-fixed specimens.

Keywords: Larvae, Pigmentation, Key, Random forest, Molecular validation.
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Introduction
Sea basses in the genus *Paralabrax* are targets of a popular recreational fishery in southern California (Jarvis et al. 2014a; Bellquist et al. 2017) and comprise important artisanal fisheries in Baja California, Mexico (Erisman et al. 2017; Cota-Nieto et al. 2018). Catches of two of the three species in southern California, *P. clathratus* (Kelp Bass) and *P. nebulifer* (Barred Sand Bass) have remained depressed since the mid-2000s, calling into question their population status and recovery potential (Erisman et al. 2010; Jarvis et al. 2014a). The third species, *P. maculatofasciatus* (Spotted Sand Bass), is primarily catch-and-release. Unfortunately, the long-term population dynamics of all three species are unknown due to a lack of species-specific fishery-dependent and -independent data. A potential source of fishery-independent time series data comes from the California Cooperative Oceanic Fisheries Investigations (CalCOFI), which has systematically sampled fish larvae, including *Paralabrax*, since 1951 (McClatchie 2014). Larval abundance can be used as a proxy of adult fish spawning stock biomass (Ralston and McFarlane 2010; He et al. 2015), but to date, *Paralabrax* larvae have not been able to be reliably identified to the species level. Here, we develop a novel technique to identify *Paralabrax* larvae and unlock a powerful data set to assist with managing these important species.

Paralabrax larvae (Family Serranidae) in southern California reside in shallow coastal waters and embayments during the summer and have a planktonic life duration of approximately one month (Findlay and Allen 2002; Allen and Block 2012). At settlement, *P. clathratus, P. maculatofasciatus,* and *P. nebulifer* are readily distinguished from one another by the presence and numbers of horizontal or vertical bars on the body and the relative height of the third dorsal fin spine (Butler et al. 1982; Love and Passerelli 2020). In contrast, their larval stages are morphologically similar, having overlapping meristics and morphometrics (Butler et al.

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1982; Watson 1998). Butler et al. (1982) documented the developmental larval stages of these three species for a small sample of larvae reared from eggs collected in the wild and identified a few distinguishing characters primarily relating to dorsal and ventral pigmentation patterns. However, diagnostic characters were not found for *P. nebulifer* and *P. maculatofasciatus* during notochord flexion, and the authors noted that pigmentation varied within larval stages. Owing to their similar morphologies and variability in pigmentation, larvae were not reliably identified to species and are only identified to genus in the CalCOFI database.

With additional *Paralabrax* larvae and genetic barcoding for validation, the potential exists to discern the extent of intraspecific pigment variation within each developmental stage and thus develop a reliable key for identifying *Paralabrax* larvae to species. A robust key would provide higher confidence in classifying *Paralabrax* larvae based on morphology. In addition, the key would provide a cost-effective means of identifying *Paralabrax* larvae in formalin-preserved ichthyoplankton collections and on-going ichthyoplankton monitoring surveys off southern California and Baja California, Mexico, including CalCOFI and Investigaciónes Méxicanas de la Corriente de California (IMECOCAL; Moser et al. 2001; Gaxiola-Castrol and Najera-Martinez 2002; Gallo et al. 2019). Herein, we use classical and modern taxonomic methods to construct, refine, and test a taxonomic key for distinguishing preflexion, flexion, and postflexion larval stages of *Paralabrax* spp. in southern California.

Materials and Methods

Taxon sorting: Since 1951, CalCOFI has collected and archived quarterly plankton samples from fixed stations offshore of California, USA, and Baja California, Mexico (Fig. 1; see McClatchie (2014) for an overview of the CalCOFI program). From 1951-1977, samples were collected using obliquely towed ring nets (Thompson et al. 2017). In 1978, ring nets were replaced with paired bongo nets (0.71 m diameter, 505 μm-mesh sizes) towed obliquely from a maximum depth of 210 m in deep waters and 5 m above the bottom in shallow water (McClatchie 2014). The starboard net contents are fixed and preserved in 5% neutrally buffered formalin while, since 1997, the contents of the port net have been preserved in 95% tris-buffered ethanol. Notably, ethanol preserves, while formalin degrades, DNA.

We sorted *Paralabrax* larvae from ethanol-preserved CalCOFI plankton samples collected in years with high larval counts in the starboard net (1998, 2004, 2006, 2012, 2013, and 2014). We selected samples inshore of CalCOFI station 60 (over the continental shelf; Fig. 1) because *Paralabrax* larvae occur relatively close to shore (Watson and Davis 1989). We also limited sorting to July cruises because *Paralabrax* spp. in southern California spawn almost exclusively in summer (Erisman and Allen 2005; Allen and Block 2012; Jarvis et al. 2014b; McKinzie et al. 2014). All ethanol-preserved larvae analyzed in this study are archived in the SWFSC larval fish collection; a select few were moved to the reference collection.

DNA extraction, PCR amplification, and sequencing: We developed and tested genus-specific primers to amplify a 282 base pair region of the mitochondrial (mt) cytochrome C oxidase subunit I (COI) gene for use in discriminating *Paralabrax* spp. This includes *P. auroguttatus* (Goldspotted Sand Bass), a southern species whose larvae may periodically enter the CalCOFI survey region during warm water periods. We removed the right eyeball or a small piece of muscle tissue from each larva and DNA was extracted using chelex 100 (Biorad laboratories) boiling protocol (Hyde et al. 2005) or the Qiagen DNeasy Blood & Tissue 96 extraction kit (Qiagen, Inc., Valencia, CA) following manufacturer's protocol.

We used polymerase chain reaction (PCR) to amplify the primer-specific region of the mt COI gene in 10 μl reactions containing 67 mM Tris-HCl pH 8.8, 16.6 mM $[NH_4]^2SO_4$, 10 mM β- mercaptoethanol, 2 mM $MgCl_2$, 800 μM dNTPs, 0.5mg/ml Bovine Serum Albumin (BSA), 0.25μM of each primer (ParaF1 5' CCT TCT TAT

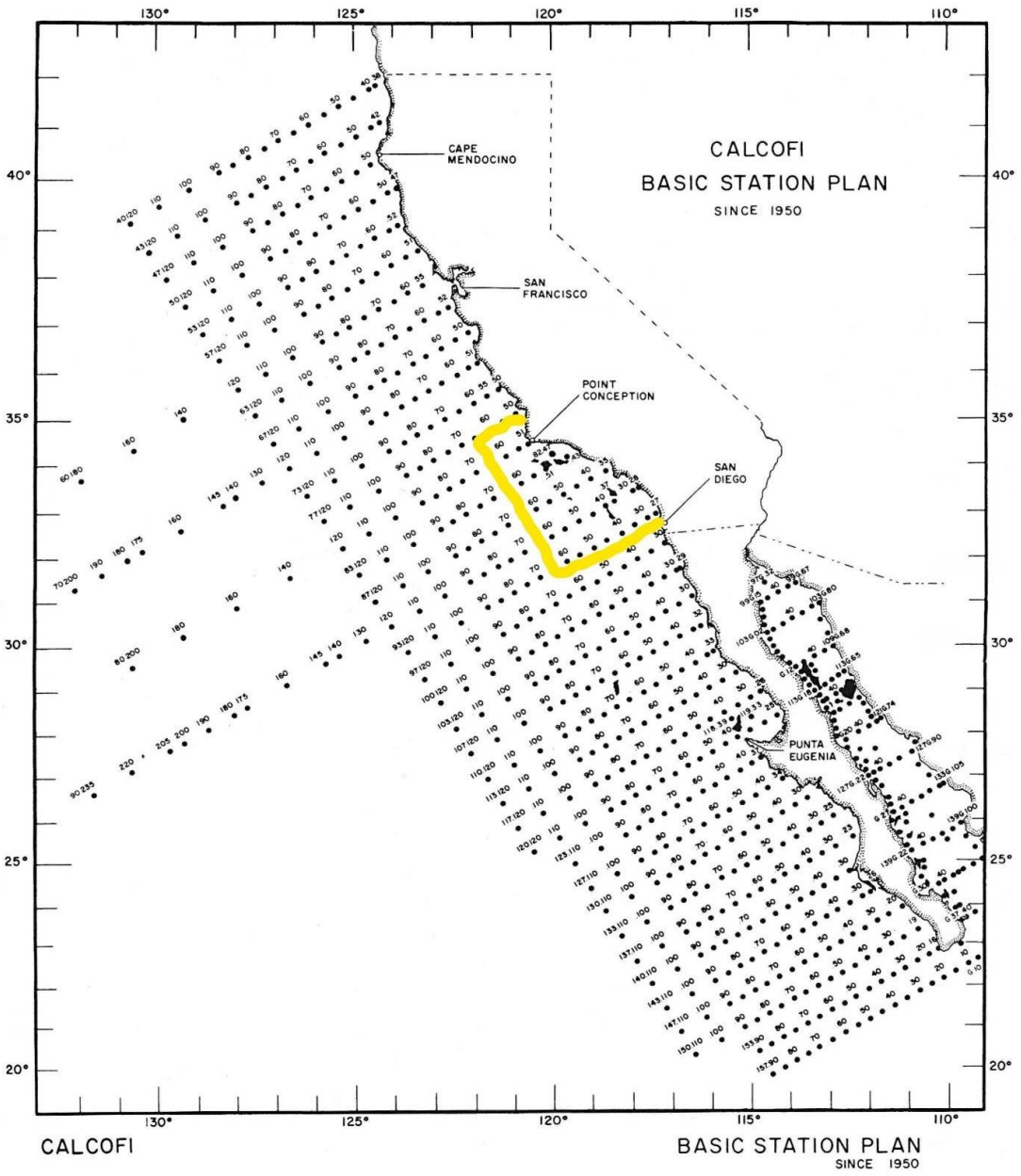

Figure 1. California Cooperative Fisheries Investigations (CalCOFI) hydrographic and planktonic sampling stations (since 1950 and 1951, respectively) off the coasts of California, USA, and Baja California, Mexico. Stations inside yellow lines indicate the region selected for sorting *Paralabrax* larvae from archived CalCOFI zooplankton samples, to construct and test a taxonomic key.

TCG AGC CGA GC-3'; ParaF2 5' GCA GGT ACA GGC TGA ACAG-3'), 0.25 units of *Taq* (*Thermus aquaticus*) DNA polymerase, and 1 µL of DNeasy extracted DNA or Chelex supernatant containing DNA template. Thermal cycling consisted of an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 3 min. For each set of PCRs, we included a no-template negative control. We visualized PCR products on a 2% agarose gel stained with ethidium bromide. We used ExoSap-IT (Affymetrix) to remove excess primers and unincorporated dNTPs, following manufacturer's protocol. Cycle sequence reactions were performed in one direction using BigDye v3.1 (Life Technologies) and the forward PCR primer 5' following manufacturer's protocol. We precipitated the sequence reactions using ethanol and sodium acetate and resuspended in HiDi formamide prior to being run on an ABI3730 Genetic Analyzer (Life Technologies). We used Sequencher v4.9 (GeneCodes) to edit the

sequences, which were then compared to reference sequences in GenBank® (http://www.ncbi.nlm.nih.gov/genbank/; accession numbers: MK029994.1, *P. clathratus*; MT311637.1, *P. nebulifer*; MG837969.1, *P. maculatofasciatus*).

Taxonomic key development and validation: We identified salient morphological characters described in Butler et al. (1982) across preflexion, flexion, and postflexion stages. These included the size, number, and location of dorsal and ventral melanophores and the presence or absence of other pigmentation (e.g., on the crown, pectoral fins, and mediolateral trunk). Yolk sac larvae were deemed visually indistinguishable and not included for analysis. We assigned the larval stage based on notochord development (Miller and Kendall 2019). For each larva, two taxonomists recorded larval stage and stage-specific morphological characters. Taxonomists were blind to the molecular identity of each larva and there was no attempt to assign species at this first step.

For every larva (i), we used the known molecular identity and data recorded in the morphological characters table to model each of j possible species classifications (stage-specific) using multinomial logistic regression in a Bayesian framework (McElreath 2019) with the R package r2jags, a wrapper for rjags (R-Core-Team 2020; Plummer 2021; Su and Yajima 2021):

$$logit(p[i,j] = \beta[1,j] + \beta[2,j] * k[1,i] + \beta[3,j] * k[2,i] \ldots \beta[n,j] * k[n,i].$$

We treated a larva's species classification as drawn from a multinomial probability distribution, where each classification is a linear function of k stage-specific morphological traits. We used normally distributed priors for the slope parameter and the beta parameters associated with each stage-specific morphological character. We used data recorded by the two taxonomists in separate models to examine posterior probability distributions for each larva's multinomial classification and to answer the following questions. For each larval stage, do the characters clearly distinguish a single species classification with high probability for most larvae? For each larval stage, do the characters accurately predict the true species for most larvae? Is there variability in model performance across larval stage and between taxonomists? Thus, this was an exploratory step used early on to determine which character traits required recalibration by taxonomists and thus which traits required clarification of the descriptions in the characters table; ultimately, the taxonomic key.

We used a machine learning algorithm, Random Forest Classifier (RFC, Cutler et al. 2007), in the R package, randomForest (Liaw and Wiener 2002a, b; R-Core-Team 2020), to identify the most important characters for accurate stage-specific species prediction ("variable importance"). We used a small subset of the data collected in the first step to train each stage-specific model. To test the models, the RFC generated a species classification for each new larva in the remaining testing data set, in which many random subsets of morphological traits were used to create a forest of decision trees that each resulted in a species classification. The ultimate species classification was based on the majority vote (classification) in the random forest and variable importance was provided as the mean decrease in accuracy across all trees in the forest when a specific variable (i.e., pigmentation character) was excluded.

As additional molecular identities became available, the taxonomists recalibrated and we refined the key where necessary. The RFC variable importance feature was useful for identifying which character(s) to include at the beginning of each stage-specific key. We evaluated the performance of the key before and after the final key refinement by calculating the probability of accurate and precise identifications with a binomial model using Bayesian methods and the R package r2jags (R-Core-Team 2020; Plummer 2021; Su and Yajima 2021). We also constructed multi-class, pre and post error "confusion" matrices to characterize additional measures of classification performance (overall and by species) using the R package caret (Kuhn 2022). These performance measures included sensitivity, specificity, precision, and balanced accuracy (see Jiao and Du (2016) for a review of performance measures).

Results

Molecular identities: We obtained DNA sequences for 119 *Paralabrax* larvae, of which preflexion larvae were dominant (64.7%), followed by flexion (21.8%) and postflexion larvae (10.1%); 3.4% could not be staged. By species, *P. clathratus* and *P. nebulifer* were the most common (47.9% and 46.2%, respectively). There were few *P. maculatofasciatus* (5.9%) and no *P. auroguttatus* (Table 1). Of the *P. maculatofasciatus* with molecular identities, six were preflexion and one was flexion (Table 1).

Important pigmentation characters: Dorsal and ventral pigmentation varied within and among species. However, the RFC identified aspects of ventral pigmentation as being the most important character in preflexion stages for species delineation (e.g., the presence and location of a large ventral pigment patch and the presence or absence of a series of closely spaced, uniform melanophores; Fig. 2a). Thus, we structured the preflexion key to begin with ventral pigmentation followed by other pigmentation. We found that preflexion *P. clathratus* and *P. maculatofasciatus* typically had few ventral melanophores (Fig. 3a, b), however they had a large ventral pigment patch midway between the anus and the end of the notochord (Fig. 3d). In *P. clathratus*, this patch is on the eighth or ninth post-anal myomere, while in *P. maculatofasciatus*, it is on the sixth or seventh post-anal myomere (Fig. 3a, b). In each case, the postanal dorsal melanophore, if present, was located directly above the postanal ventral patch. It was common for the postanal dorsal melanophore in preflexion *P. clathratus* and the middle dorsal melanophore in preflexion *P. maculatofasciatus* to be more prominent relative to the other dorsal melanophores (i.e., darker or larger). In contrast, preflexion *P. nebulifer* typically had a series of numerous small uniform melanophores along the ventral margin (~10-20), with usually more than one melanophore per myomere; it was also common for one of the ventral melanophores to be prominent relative to the other ventral pigment (Fig. 3c, e). Preflexion *P. clathratus* and *P. maculatofasciatus* occasionally had a series of small uniform melanophores along the ventral margin (in addition to the large ventral patch), but these were typically not as closely spaced as in *P. nebulifer*.

The RFC identified pectoral wfin pigmentation as the most important flexion stage character for species classification (Fig. 2b). Flexion *P. clathratus* commonly had pectoral fin pigment, but this pigmentation did not occur with any of the flexion *P. nebulifer*. While there was only one flexion *P. maculatofasciatus* for comparison, it had pectoral fin pigment but no crown, mediolateral trunk, or dorsal pigment. The large ventral pigment patch was typically present throughout the flexion stage in *P. clathratus* and may be retained to early flexion in *P. maculatofasciatus* (Fig. 4a, b). We found that ventral melanophores of flexion *P. nebulifer* were typically closer together and may become dense (Fig. 4c). In many flexion larvae of this species, the pigment along the ventral midline of each myomere appears to eventually coalesce into linear segments or a single continuous line of pigment.

Table 1. Numbers of ethanol-preserved *Paralabrax* larvae, identified by molecular methods for use in developing and testing a taxonomic key, by larval stage and species. Twelve of these specimens were in poor condition and could not be morphologically identified.

Species	Preflexion	Flexion	Postflexion	Not Staged	Total
P. clathratus	41	13	2	1	55
P. maculatofasciatus	6	1	0	0	7
P. nebulifer	30	13	9	3	57
Total	77	27	11	4	119

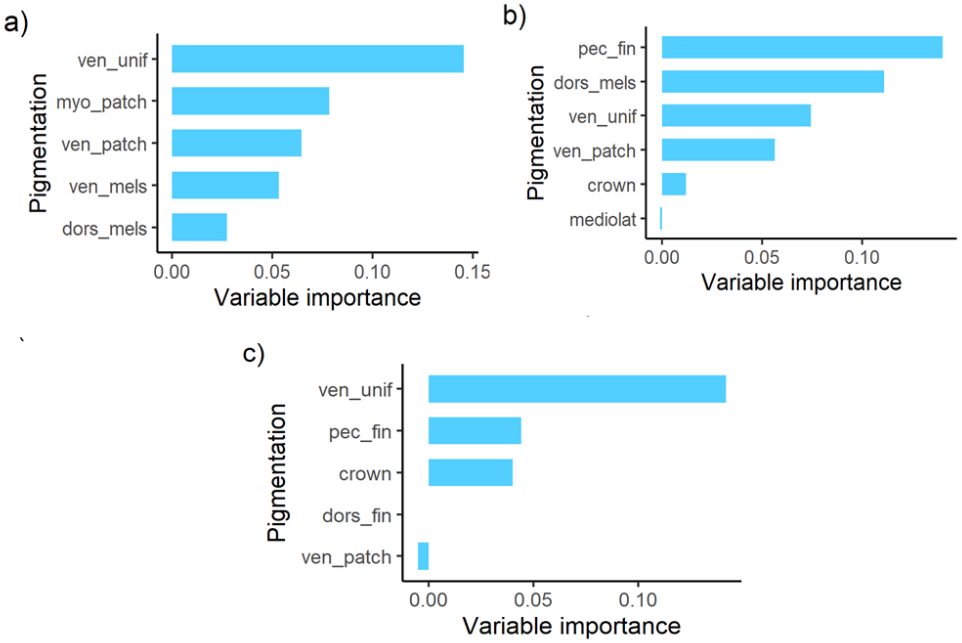

Figure 2. Random forest variable importance plots depicting the relative importance of pigmentation characters in contributing to accurate species delineation in *Paralabrax* spp. larvae for (a) preflexion, (b) flexion, and (c) postflexion developmental stages. Descriptions of pigmentation abbreviations include the following: crown = crown pigment, dors_fin = dorsal fin pigment, dors_mels = number and location (anterior, mid, posterior) of dorsal melanophores, mediolat = presence/absence of mediolateral pigment on trunk and/ozzr horizontal septum pigment, myo_patch = location of large postanal ventral pigment patch (myomere number), pec_fin = presence/absence of pectoral fin pigment on one or both pectoral fins, ven_mels = number of postanal ventral melanophores; ven_patch = presence/absence of large postanal ventral pigment patch, ven_unif = presence/absence of a postanal series of uniform ventral melanophores.

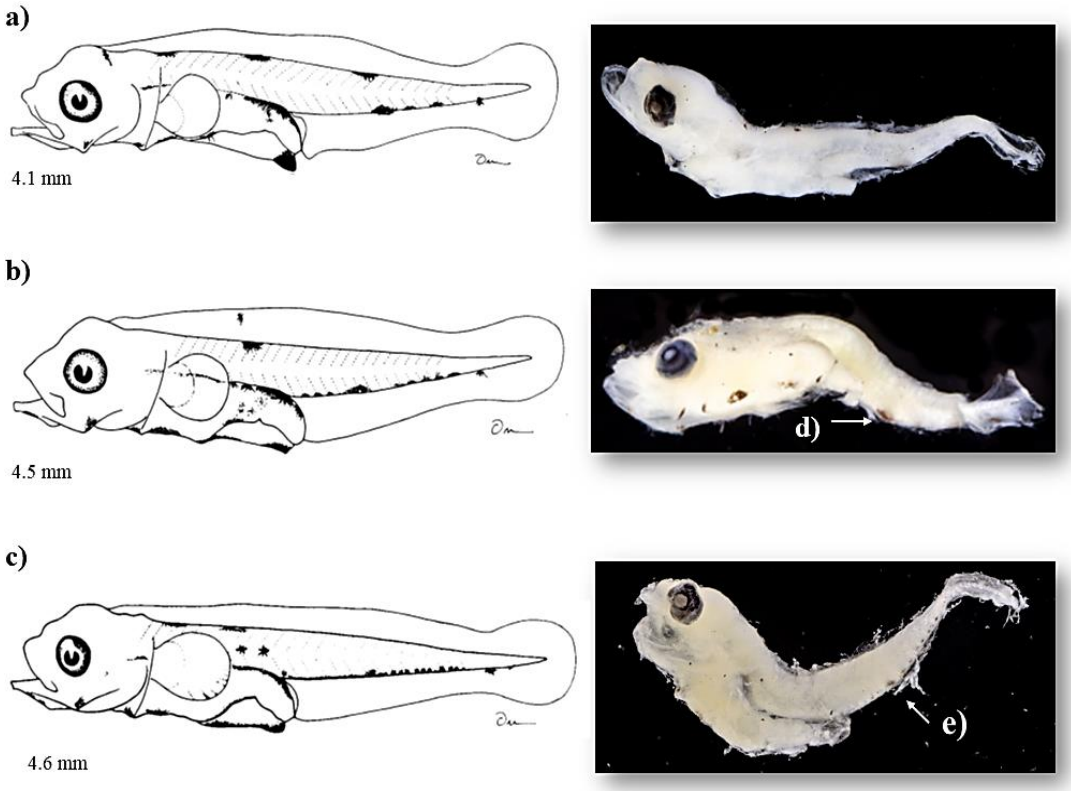

Figure 3. Illustrative and photographic comparison of preflexion stage larvae of (a) *Paralabrax clathratus*, (b) *P. maculatofasciatus*, (c) *P. nebulifer* (Butler et. al., 1982), and the (d) large postanal ventral midline melanophore versus (e) prominent postanal ventral midline melanophore typical of preflexion of both *P. clathratus* and *P. maculatofasciatus* and *P. nebulifer*, respectively. Representative photographs were taken of ethanol-preserved larvae used in constructing and validating the taxonomic key.

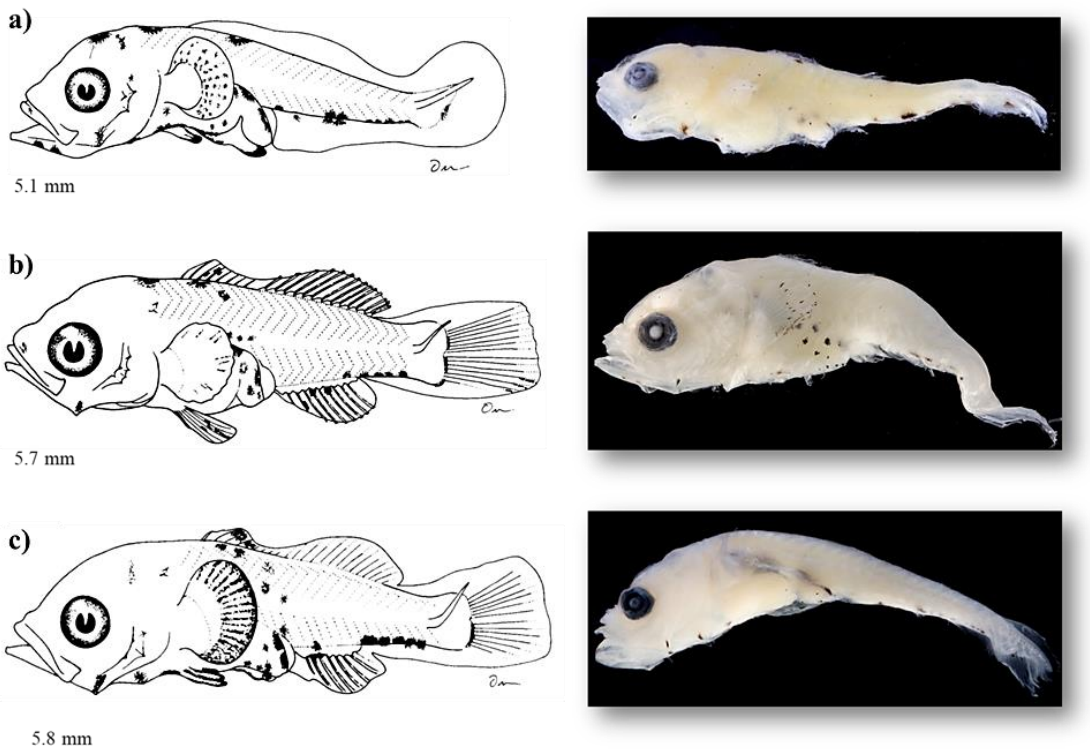

Figure 4. Illustrative and photographic comparison of flexion stage larvae of (a) *Paralabrax clathratus*, (b) *P. maculatofasciatus*, and (c) *P. nebulifer* (Butler et. al., 1982). Representative photographs were taken of ethanol-preserved larvae used in constructing and validating the taxonomic key.

The most important postflexion stage character identified by the RFC was ventral pigment (Fig. 2c). However, this result was based on only 13 larvae that were mostly early postflexion stage (Fig. 5a-c). In contrast, our observations indicated that postflexion *Paralabrax* larvae are more easily distinguished by the dorsal, trunk, and head pigmentation characters, as described in Butler et al. (1982). For example, we observed a series of dorsal saddles in one *P. clathratus* larva, in contrast to the single dorsal saddle observed in one *P. nebulifer* larva (Fig. 5d, f). We also observed snout pigment in one *P. nebulifer* larva (Fig. 5f). Early postflexion *P. clathratus* typically retained the large postanal ventral patch and had a combination of the pectoral, crown, and dorsal fin pigment; while early postflexion *P. nebulifer* typically had a continuous, uniform series of ventral pigment and only occasionally had pigmentation on the crown and/or dorsal or pectoral fins. There were no *P. maculatofasciatus* postflexion larvae for comparison.

Some dorsal pigmentation patterns were more common in one species than in the others, and thus we placed this character later in the preflexion and flexion keys to provide additional species confirmation. With respect to the retention of all three dorsal pigment patches in preflexion *P. clathratus* and *P. maculatofasciatius*, Butler et al. (1982) reported a specific total length and snout:anus length cutoff to distinguish the two species. However, in our preflexion key, we chose not to use a specific length as a dichotomous pathway for this character, as larvae growth can vary temporally and spatially. Instead, we used "early" and "mid-to-late" preflexion to denote likely retention in both species and retention in only *P. clathratus*, respectively.

Comparisons to *P. auroguttatus*: As no *P. auroguttatus* were genetically identified in this study, we chose to exclude this species from the key. However, we found that ventral pigmentation patterns important for distinguishing southern California *Paralabrax* larvae appear distinct from the ventral pigmentation pattern of preflexion and flexion stage *P. auroguttatus* larvae described in Avendaño-Ibarra (2004). For example, in both preflexion and flexion *P. auroguttatus*, there is typically a prominent ventral patch located on the second

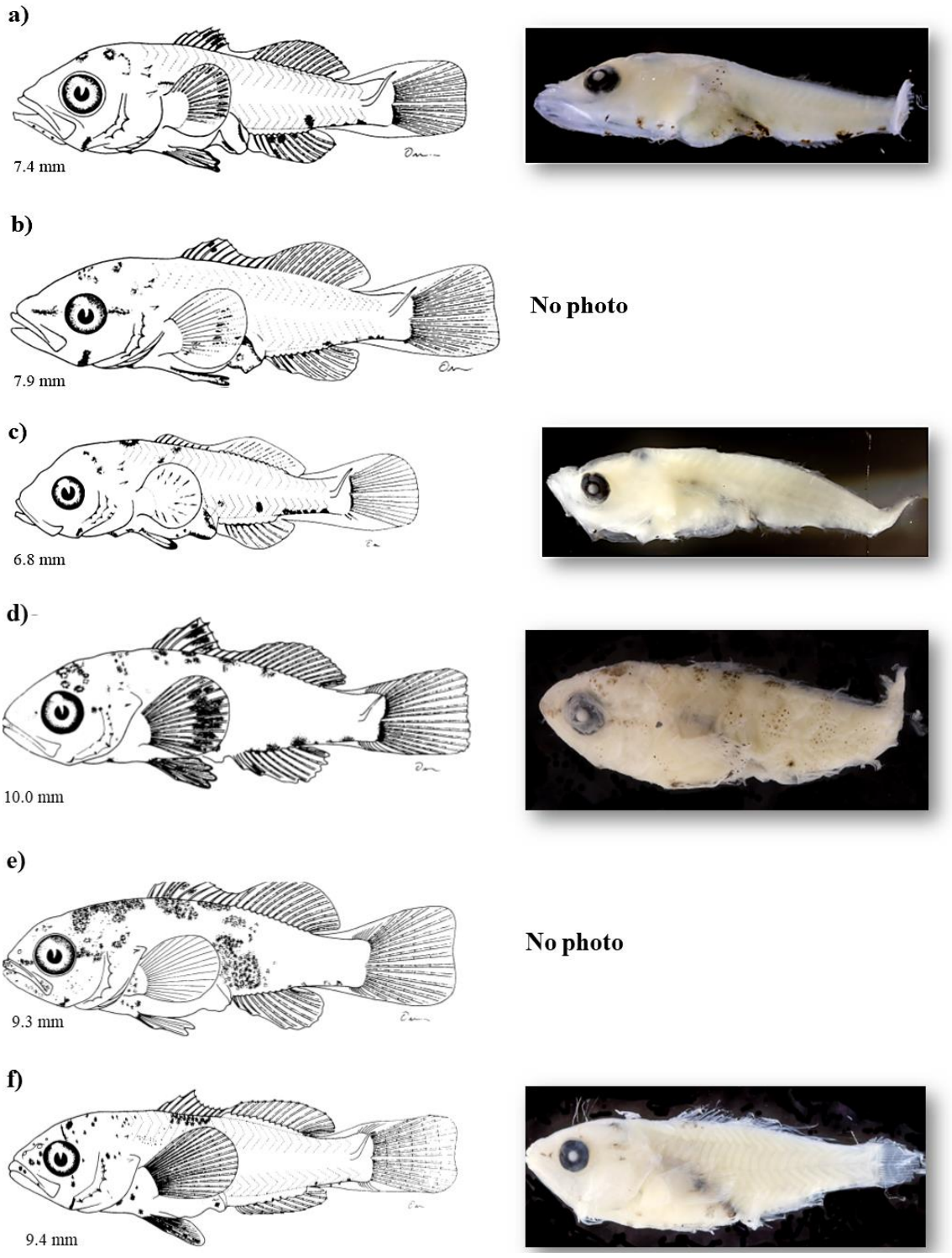

Figure 5. Illustrative and photographic comparison of early postflexion stage larvae of (a) *Paralabrax clathratus*, (b) *P. maculatofasciatus*, and (c) *P. nebulifer* (Butler et. al., 1982), and larger postflexion stage larvae of (d) *P. clathratus*, (e) *P. maculatofasciatus*, and (f) *P. nebulifer* (Butler et al. 1982). Representative photographs were taken of ethanol-preserved larvae used in constructing and validating the taxonomic key.

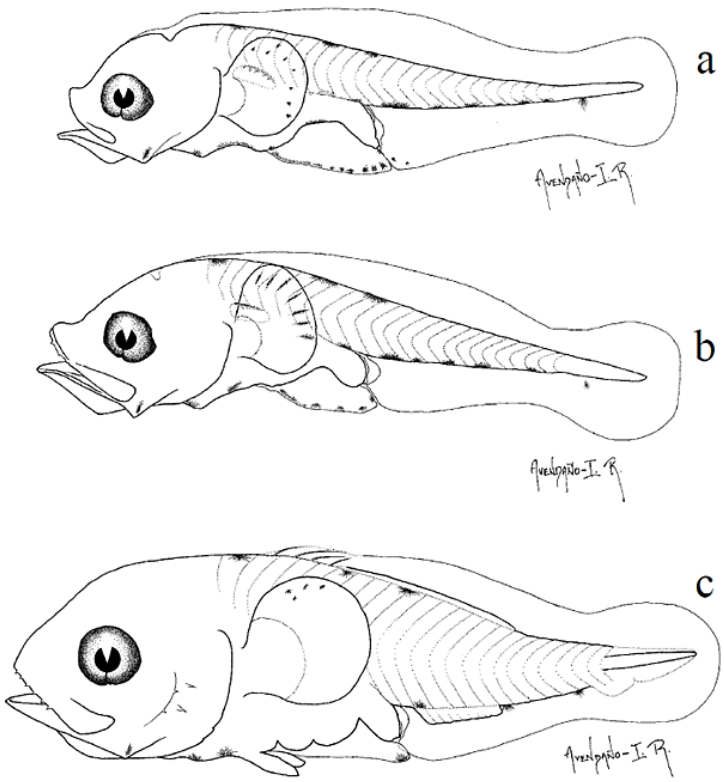

Figure 6. Illustrative comparison of larvae of *Paralabrax auroguttatus* representing (a) early preflexion (length 2.0 mm), (b) preflexion (3.0 mm), and (c) flexion (4.3 mm) developmental stages (Avendaño-Ibarra 2004).

postanal myomere, and the posterior dorsal melanophore, if present, is located directly above, such that the middle and posterior dorsal melanophores are closer together than in *P. clathratus* and *P. maculatofasciatus* (Fig. 6). In addition, preflexion *P. auroguttatus* typically have pectoral fin pigmentation, and we observed this to be less common in preflexion *P. maculatofasciatus* and *P. clathratus*.

Taxonomic key validation: Overall, 12 *Paralabrax* larvae with molecular identifications could not be visually identified to species level due to their poor condition. Thus, data on 107 larvae were used for validation. In the initial stage of key development, differences in the performance of the logistic regression model between taxonomists indicated that calibration was required in assigning larval stages and ventral pigmentation characters. For example, there was taxonomist bias in what constituted a large ventral pigment patch, which required the character description to be clarified within the key to limit subjectivity.

Post-calibration and key refinement, key performance increased overall (Fig. 7) and by species (Table 2). Overall, the final key achieved 97% accuracy and 96% precision (Fig. 7). By species, balanced accuracy was high for all three species (≥96%, Table 2). Precision was lowest for *P. maculatofasciatus* (70%), resulting from three false positives, in which three *P. clathratus* larvae were misclassified as *P. maculatofasciatus* (Table 2).

Key to the larvae of southern California species of *Paralabrax*

Larval *Paralabrax* are typical serranines, having 24-25 myomeres (usually 24), a moderate body shape (body depth typically ~ 15-30% body length) with preanal length about half of body length, an initially straight gut that coils during flexion stage, relatively few, small spines on the head, and pectoral girdle beginning late in preflexion stage (e.g., Watson 1996). Larvae that display these characters are not necessarily *Paralabrax*, but larvae that do are not *Paralabrax*.

Preflexion stage I
Flexion stage II
Postflexion stage III

I. Preflexion stage

1a. Ventral postanal midline melanophores arranged in a continuous, nearly uniform series[1], containing
  A. A single prominent postanal ventral melanophore[2] large/patch-like relative to other ventral midline pigment……...2
  B. A single prominent postanal ventral melanophore[2] not large/patch-like relative to other ventral midline pigment or not present 3
1b. Ventral postanal melanophores not arranged in a continuous, uniform series 2
2a. Prominent, postanal ventral melanophore located on 6th or 7th postanal myomere……*P. maculatofasciatus*[3]
2b. Prominent, postanal ventral melanophore located on 8th or 9th postanal myomere ……..*P. clathratus*[4]
2c. Prominent, postanal ventral melanophore location on postanal myomere(s) not discernable (postanal myomeres not countable) 4
3a. Ventral postanal melanophores numerous (>10, as many as 20)
  A. Pectoral fin and/or crown pigment 2
  B. No pectoral fin pigment and no crown pigment ..... *P. nebulife*[5]
3b. Ventral postanal melanophores few (<=10) 2
4a. One dorsal melanophore located mid dorsal ..... *P. maculatofasciatus*
4b. Two dorsal melanophores located anterior/mid ..... *P. maculatofasciatus*
4c. Two dorsal melanophores located mid/posterior ..... *P. clathratus*
4d. Three dorsal melanophores located anterior/mid/posterior
  A. Horizontal septum melanophore(s) present ..... *P. maculatofasciatus*
  B. No horizontal septum melanophore(s)
    a. Early preflexion ..... *P. clathratus*[6] or *P. maculatofasciatus*[7]
    b. Mid-to-late preflexion ..... *P. clathratus*

II. Flexion stage

1a. Pectoral fin pigment present on one or both fins 2
1b. No pectoral fin pigment 5
2a. Mediolateral trunk pigment present (not including lateral gut pigment) and/or horizontal septum pigment present ..... *P. maculatofasciatus*
2b. No mediolateral trunk pigment or horizontal septum pigment 3

[1] The postanal ventral melanophore series may originate at the first postanal myomere or near mid-tail and extends to the notochord tip.

[2] When present, the large postanal ventral patch is typically apparent from both the ventral and lateral views and may have a stellate or dendritic appearance. The large patch is also typically darker and generally more than 5-10x the size of other ventral midline pigment, whereas a prominent ventral melanophore is typically only 2-3x larger in size than other ventral midline pigment.

[3] Typically, has either one dorsal melanophore located mid dorsal or two located anterior/mid is also possible); the middle dorsal melanophore is typically more prominent relative to other dorsal melanophores and relative to the middle dorsal pigment of the other two species; all three dorsal melanophores may be large and dendritic in some larvae; may also have horizontal septum melanophores; may also have crown and pectoral fin pigmentation at this stage.

[4] Typically, has either 2 or 3 dorsal melanophores located mid/posterior or anterior/mid/posterior, however, other patterns are possible (e.g., anterior/mid in late pre-flexion; a single posterior melanophore); the posterior dorsal melanophore is commonly prominent relative to other dorsal melanophores and relative to the posterior dorsal pigment of the other two species; lacks horizontal septum melanophores at this stage; may also have pectoral fin pigmentation at this stage.

[5] Typically, has either one or two dorsal melanophores located anterior (or mid dorsal) or anterior/mid; the anterior dorsal melanophore is commonly prominent relative to the other dorsal melanophores and relative to the anterior dorsal pigment of the other two species; early preflexion stage retains three dorsal melanophores; may also have horizontal septum melanophores.

3a. Crown pigment present ..... *P. clathratus*
3b. No crown pigment 4
4a. Three dorsal melanophores located anterior/mid/posterior ..... *P. clathratus*
4b. Two dorsal melanophores located anterior/mid (mid is typically prominent) ..... *P. maculatofasciatus*
4c. Two dorsal melanophores located mid/posterior (posterior is typically prominent) ..... *P. clathratus*
4d. One dorsal melanophore located mid dorsal…….. .....*P. clathratus*[6] *or P.maculatofasciatus*[7] (latter case is more common)
4e. One dorsal melanophore located posterior dorsal..... *P. clathratus*
4f. No dorsal melanophores..... *P. maculatofasciatus*
5a. Mediolateral trunk pigment present (not including lateral gut pigment) and/or horizontal septum pigment present..... 6
5b. No mediolateral trunk pigment or horizontal septum pigment .....*P. maculatofasciatus*[8] *or P. nebulifer*[9]
6a. Crown pigment present..... *P. nebulifer*
6b. No crown pigment ..... *P. maculatofasciatus*[7] *or P. nebulifer*[8]

III. Postflexion stage

1a. Prominent postanal ventral melanophore large/patch-like; may be visible both ventrally and laterally .... *P. clathratus*
1b. Prominent postanal ventral melanophore not large/patch-like or not present 2
2a. Dorsolateral pigmentation below the base of the first dorsal fin 4
2b. No dorsolateral pigmentation 3
3a. Pectoral fin pigment, and
   A. Pigment forms horizontal stripe from snout through eye to operculum…….*P. maculatofasciatus*
   B. No pigmentation on snout.....*P. clathratus*
3b. Lacking pectoral fin pigmentation or not discernable 5
4a. Pigment on trunk of body, and
   A. Discrete dorsal saddles extend to trunk as vertical bars .....*P. clathratus*[10]
   B. Dorsolateral pigment lacks saddle pattern.....*P. maculatofasciatus*
4b. No trunk pigment, and
   A. Discrete dorsal saddles beginning to form vertical bars.....*P. clathratus*[11]
   B. A single broad dorsal saddle under dorsal fin or just forming.....*P. nebulifer*[12]
5a. Snout pigment appears to form horizontal stripe through eye to operculum .....*P. maculatofasciatus*
5b. Lacking pigment on snout .....*P. nebulifer*[13]

[6] Typically, retains large ventral patch throughout stage.
[7] May retain large ventral patch in early flexion. Ventral midline pigment may coalesce into linear segments.
[8] May retain large ventral patch in early flexion. Ventral midline pigment may coalesce into linear segments.
[9] Ventral midline pigment may coalesce into linear segments, becoming dense.
[10] Also typically with crown pigmentation; if discernable, first dorsal fin pigment heavy. Late postflexion typically with horizontal stripe from snout through eye to operculum.
[11] Typically with crown pigmentation; if discernable, first dorsal fin pigment heavy.
[12] Late postflexion stage typically has occipital pigment as well as snout pigment, which may extend horizontally through the eye to the operculum. May also have both crown and pectoral fin pigment in late postflexion.
[13] Early to mid postflexion stage may have crown pigment, but pectoral fin pigment found only in late postflexion.

Table 2. Confusion matrices of the numbers of larvae a) pre and b) post taxonomic key refinement, and c) associated classification performance metrics (in proportions) by species. PCLA = *P. clathratus*, PMAC = *P. maculatofasciatus*, PNEB = *P. nebulifer*, NA = not *Paralabrax* spp. For each species, sensitivity is the true positive rate (proportion correct in column), specificity is the true negative rate (proportion correct of all true non-positives), precision is the positive rate of the taxonomic key (proportion correct in row), and balanced accuracy is the mean of sensitivity and specificity.

a)

		True ID			
		PCLA	PMAC	PNEB	NA
Key ID	PCLA	44	1	1	0
	PMAC	0	2	0	0
	PNEB	6	3	47	0
	NA	2	1	0	9

b)

		True ID			
		PCLA	PMAC	PNEB	NA
Key ID	PCLA	48	0	0	0
	PMAC	3	7	0	0
	PNEB	1	0	48	0
	NA	0	0	0	9

c)

	PCLA		PMAC		PNEB	
	Pre	Post	Pre	Post	Pre	Post
Sensitivity	0.85	0.92	0.29	1.00	0.98	1.00
Specificity	0.97	1.00	1.00	0.97	0.87	0.99
Precision	0.96	1.00	1.00	0.70	0.84	0.98
Balanced Accuracy	0.91	0.96	0.64	0.99	0.92	0.99

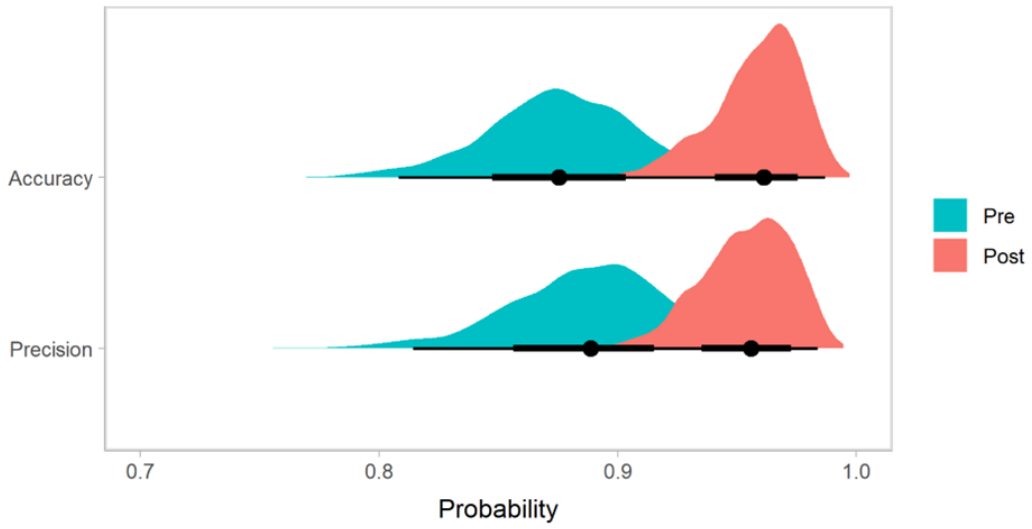

Figure 7. Probability density plots of accurate and precise species classification using the taxonomic key to identify *Paralabrax* larvae, pre and post key refinement. Point estimates and intervals depict Bayesian posterior medians and 66% and 95% credible intervals

Discussion

Using previous knowledge of pigmentation patterns in larval *Paralabrax* from southern California, coupled with machine learning and validation with DNA barcoding, we successfully constructed a robust taxonomic key for use with preflexion, flexion, and postflexion developmental stages. Until now, decades of formalin-preserved *Paralabrax* larvae collected in oceanographic monitoring surveys off California and Baja California could not be visually identified to species level with reasonable certainty due to similarities in their morphology (Moser et al. 2001). In addition, pigmentation patterns observed in a small number of laboratory-reared *Paralabrax*

larvae had never been validated on a larger sample size using molecular techniques. The ability to differentiate *Paralabrax* larvae in southern California using morphology alone, with a high degree of confidence, allows access to past, present, and future trends in larval abundance, a proxy commonly used in assessing adult fish spawning stock biomass.

Each larval stage key provides several pathways to distinguish larvae of the three *Paralabrax* species in southern California. One instance in both the preflexion and flexion keys leads to either *P. clathratus* or *P. maculatofasciatus* and two instances in the flexion key lead to either *P. nebulifer* or *P. maculatofasciatus*. In these cases, the location of collection may facilitate accurate identification. For example, adult *P. maculatofasciatus* spawn near the entrances of bays and estuaries (Allen et al. 1995), but the CalCOFI survey in southern California is limited to coastal and offshore waters. If *P. maculatofasciatus* larvae are typically retained within bays and estuaries, this would explain why very few were identified in the CalCOFI plankton samples analyzed in this study. In addition, further south off Baja California, Mexico, the larval abundances of *P. maculatofasciatus* and *P. nebulifer* are higher relative to *P. clathratus* (Avendaño-Ibarra 2004; Avendaño-Ibarra et al. 2004). Thus, depending on the habitat and geographic location of collection, it is likely that when arriving at one of these "either or" key endpoints, one could deduce the true species more often than not.

Pigmentation patterns are useful for distinguishing bass larvae (across preservatives): Pigmentation patterns in fish larvae are largely transient and can vary within and among species. This may result from the ontogenetic migration of melanophores (e.g., dorsal trunk pigment onto dorsal fin) and/or melanophore expansion and contraction. However, we found pigmentation patterns useful for distinguishing among *Paralabrax* larvae from preflexion to postflexion stages. Reliance on pigmentation in the absence of morphometric or meristic characters has also been useful for identifying species of rockfish (subgenus *Pteropodus*) and engraulid larvae (Wang and Tzeng 1997; Taylor and Watson 2004).

Preservation methods can also affect pigmentation in fish larvae (Schnell et al. 2016), suggesting that this morphological feature is less reliable for identifying preserved specimens. In this study, the utility of the pigmentation patterns observed by Butler et al. (1982) in fresh, formalin-preserved *Paralabrax* larvae was tested with ethanol-preserved larvae. Despite our test specimens being preserved in ethanol, most pigmentation patterns were retained, and morphological identities determined using our key were achieved with high accuracy and precision. We have subsequently used our key on decades-old formalin-preserved *Paralabrax* larvae and found that specimens maintained for 50+ years still had reliable pigmentation patterns for classification (data not shown). We also found that melanophores visually identified in older formalin-preserved samples were typically lighter and smaller than those observed in ethanol-preserved, or younger, formalin-preserved samples, indicating that a careful eye is needed when using our key on specimens preserved in formalin for many decades.

Ventral and pectoral fin pigment most important: Prior to this study, we hypothesized that dorsal pigmentation in *Paralabrax* spp. would be the most useful character for delineating species. For example, the presence of three dorsal pigment patches was thought to be a typical pattern in early *P. clathratus* development, while *P. maculatofasciatus* and *P. nebulifer* were thought to have one and two dorsal melanophore(s), respectively (Butler et al. 1982; Watson, 1998). While we also observed these dorsal pigment patterns, there was sufficient interspecific variation such that this character alone was unreliable for species assignment. Contrary to our initial hypothesis, we found ventral pigmentation patterns were more important for accurately classifying preflexion larvae and pectoral fin pigment more important for accurate classification of flexion larvae. Thus, when identifying *Paralabrax* larvae, dorsal pigmentation in preflexion and flexion larvae is best used for final confirmation or corroboration of a species identity once the other pigmentation is already visually established.

Our key also has value for use with *Paralabrax* larvae collected off Baja California, Mexico, and the Gulf of

California. Ventral pigment characters that are important for distinguishing southern California *Paralabrax* larvae also appear distinct from *P. auroguttatus* at the preflexion and flexion stages. Larvae of *P. auroguttatus* can co-occur with the other three *Paralabrax* species along the Pacific Baja California coast but are more common in the Gulf of California (Avendaño-Ibarra et al. 2004, 2014). Larvae of *P. maculatofasciatus,* and *P. nebulifer* have also been documented in the Gulf of California (Avendaño-Ibarra et al. 2014); however, given their adult distributions (Heemstra 1995; Love and Passerelli 2020), larvae of *P. nebulifer* probably occur there relatively less frequently. With a possible northern latitudinal geographic range shift or range expansion of *P. auroguttatus* into southern California waters associated with ocean warming, the ability to distinguish *P. auroguttatus* from southern California *Paralabrax* larvae is likely to become more relevant in the not-too-distant future.

In general, the stage-specific distinguishing features described in Butler et al. (1982) were consistent with our observations; however, we note four major differences here. First, we observed numerous (>10) postanal, midventral melanophores in a few specimens of preflexion stage *P. clathratus* and *P. maculatofasciatus*, whereas Butler et al. (1982) reported 4-8 (mean = 6.2) and 6-11 (mean = 8.0) for each species, respectively. Second, Butler et al. (1982) noted that preflexion *P. clathratus* could be distinguished from the other two species by a lack of horizontal septum pigment, suggesting that this character was common in preflexion *P. nebulifer* and *P. maculatofasciatus*. In this study, horizontal septum pigment was rarely observed in preflexion larvae of the latter two species. However, we did occasionally observe some form of lateral pigment in flexion stage larvae. Thus, it may be that horizontal septum pigment in preflexion larvae is more easily lost during ethanol fixation and preservation. Nevertheless, we chose to include it in the preflexion key because when it is present, it is useful. Third, we found that preserved preflexion *P. clathratus* typically did not have a discernable prominent triangular ventral fin fold pigment patch as described in Butler et al. (1982). Given that net capture of larvae can damage the delicate fin fold, this character is likely more useful with fresh or freshly preserved *Paralabrax* larvae (Butler et al. 1982). Finally, on occasion, we observed pectoral fin pigment in preflexion *P. clathratus* and *P. maculatofasciatus* and in flexion stage *P. maculatofasciatus*, whereas Butler et al. (1982) only noted the formation of pectoral fin pigment in *P. clathratus* during flexion.

A multipronged approach is instrumental for robust key development: We have built upon the classical taxonomic approach for developing a morphological key by incorporating statistical methods and molecular identification as validation. Statistical methods provided an unbiased tool to aid in structuring and refining the key, and the molecular identifications allowed us to test the accuracy and precision of the key. We found multinomial logistic regression a useful exploratory tool for identifying taxonomist-specific subjectivity in assigning larval stages and morphological features. After recalibration, the key was improved to better define the character traits. The RFC was important for identifying which character(s) to include at the beginning of the key because it uncovered the most important characters contributing to high classification accuracy. This multipronged approach provided a reliable, accurate, and cost-effective means to visually identify southern California *Paralabrax* larvae based on pigmentation patterns alone.

The efficacy of the technique we developed, could be applied to other species whose identity thus far has been difficult to discern based on morphology. For example, rockfishes, *Sebastes* spp., were the fourth most common taxon sampled in CalCOFI surveys between 1951-1998 (Moser et al. 2001), but only a handful of rockfish larvae can currently be identified to species based on morphology (e.g., *Sebastes jordani*, *S. paucispinis*, and *S. levis*). Molecular identification of ethanol-preserved larvae from 2005 (Thompson et al. 2016) and 1998-2013 (Thompson et al. 2017) identified 39 species. Given that pigmentation patterns are consistent among species within at least some rockfish subgenera (Taylor and Watson 2004; Watson and Robertson 2004; Watson et al. 2016), this technique may help to identify species-specific characteristics for larval rockfishes.

For many years, *Paralabrax* larvae from southern California were deemed too morphologically similar for species identification. However, we have validated pigmentation patterns as reliable for identifying southern California *Paralabrax* larvae across preservation types. We can now leverage existing and future ichthyoplankton survey collections to evaluate trends in larval abundance of *Paralabrax* spp. from southern California. We have also shown that although pigmentation patterns in fish larvae can commonly show intra- and interspecific variability (Watson 1998; Gray et al. 2006), when they are considered collectively within a strategically arranged taxonomic key, they can be useful for discriminating closely related, morphologically similar species. When constructing a taxonomic key for use with other morphologically similar larvae, we recommend using machine-learning tools along with molecular validation to increase the accuracy and precision of the key's performance.

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