**Abstract**

Marine heatwaves can drive large-scale shifts in marine ecosystems but studying their impacts on whole species assemblages can be difficult. Here, we leverage the taxonomic breadth and resolution of DNA sequences derived from environmental DNA (eDNA) in the ethanol preservative of a set of 23-year longitudinal ichthyoplankton samples, combining these with microscopy-derived ichthyoplankton identification to yield higher-resolution, species-specific quantitative abundance estimates of fish assemblages in the California Current Large Marine Ecosystem during and after the 2014–16 Pacific marine heatwave. This integrated dataset reveals patterns of tropicalization with increases in southern, mesopelagic species and associated declines in important temperate fisheries targets (e.g., North Pacific Hake (*Merluccius productus*) and Pacific Sardine (*Sardinops sagax*)). We observed novel assemblages of southern, mesopelagic fishes and temperate species (e.g., Northern Anchovy, *Engraulis mordax*) even after the return to average water temperatures, corroborating recent findings within the California Current Large Marine Ecosystem. Our innovative preservative derived eDNA metabarcoding and quantitative modeling approaches open the door to reconstructing the historical dynamics of assemblages from modern and archived samples worldwide.

**Introduction**

Climate-induced marine heatwaves are increasing in frequency and severity with far-reaching consequences in marine ecosystems (Oliver et al., 2018), ranging from severe organismal stress to cascading ecosystem effects (Frölicher & Laufkötter, 2018). Notable recent examples include repeated bleaching events across the Great Barrier Reef (2016, 2017, 2020) (Hughes et al., 2018) and near-total deforestation in Northern California, USA, kelp forests (2016-19) (Rogers-Bennett & Catton, 2019). These marine heatwaves precipitated drastic, unprecedented changes in dominant foundational species across hundreds of thousands of square kilometers of shallow, coastal ecosystems and also drove concurrent range shifts of marine species in response to these warming events (Thompson et al., 2022a; Walker et al., 2020).

The impacts of such large environmentally driven disturbances on coastal marine ecosystems have been ecologically and economically significant (Cheung & Frölicher, 2020; Nielsen et al., 2021; Pinsky et al., 2020). In the 1940s, the dramatic collapse of Pacific Sardine (*Sardinops sagax*) disrupted marine food webs, causing broad-scale, negative socio-economic impacts across the Northeast Pacific (Becker et al., 2019; Chavez et al., 2003; Checkley et al., 2017). To better understand the processes driving these complex marine ecosystem dynamics and to avert similar fisheries collapses within the California Current Large Marine Ecosystem (CCLME), the California Cooperative Oceanic Fisheries Investigations (CalCOFI) was formed in 1949. CalCOFI has continuously conducted systematic fisheries-independent surveys of the southern CCLME from 1951 until the present (Gallo et al., 2019; Lindegren et al., 2013; McClatchie, 2016) with a focus on monitoring larval fish assemblages, as larval fish dynamics are a key predictor of ecosystem health and function (Gallo et al., 2019; Nielsen et al., 2021; Smith & Moser, 2003).

Larval fish abundances help to characterize the state of marine ecosystems as they track spawning-stock biomass (Hsieh et al., 2006). Over 70 years, CalCOFI has documented decadal and annual changes in fish assemblages in response to environmental conditions, identifying major shifts in response to Pacific Decadal Oscillations and El Niño Southern Oscillations (Gallo et al., 2019; Moser P.E. Smith, and L.E. Eber, 1987; H. Moser et al., 2001; Thompson et al., 2012). These decadal and annual changes in ichthyoplankton dynamics are superimposed over the strong biogeographic assemblage associations with distinct water mass characteristics within the Southern California Bight (H. Moser et al., 2001). For example, ichthyoplankton assemblages differ among the colder and fresher California Current, warmer and saltier California Counter Current and Central Pacific water mass, and in upwelling conditions across the continental shelf (Asch, 2015; Lindegren et al., 2013; Smith & Moser, 2003; Snyder et al., 2003). Importantly, periods of elevated temperatures were historically associated with higher abundances of southern, mesopelagic species and Pacific Sardine while colder periods were associated with higher abundances of northern, mesopelagic species and Northern Anchovy (*Engraulis mordax*; Chavez et al., 2003; Thompson et al., 2022a). These insights into forage-fish community dynamics across decadal climatic regime shifts are vital to understanding the effects of climate change on the CCLME (Asch, 2015; Checkley et al., 2017; Lindegren et al., 2013).

Despite the value of previous CalCOFI ichthyoplankton data, such traditional manual identification of larvae is labor-intensive, and taxonomic resolution is often limited by a lack of discernible morphological characteristics (Thompson, Chen, et al., 2017). For example, the larvae of only 3 of the 66 species of rockfishes in the genus *Sebastes* that occurin the California Current can be identified morphologically, thus requiring genetic-based identification (Thompson, Chen, et al., 2017).

Here, we reconstruct ichthyoplankton assemblages over 23 years, using a novel “environmental DNA” (eDNA) approach: sequencing MiFish Universal Teleost 12S rRNA gene amplicons (Miya et al., 2015) derived from filtering the ethanol in which CalCOFI plankton samples were preserved, thereby maintaining the integrity of the historical larvae and egg samples, and pair these genetic data with morphological count observations in a joint Bayesian model to estimate species-specific larval abundance.

**Materials and Methods**

*Study Design*

To investigate decadal changes in the ichthyoplankton assemblages in the southern California Current vicinity, we identified ichthyoplankton from four stations during spring months over 2 decades (1996,1998-2019). Archived spring ichthyoplankton samples were collected across four biogeographically dissimilar stations (up to 370 km apart) with variable water properties (McClatchie, 2016; Nielsen et al., 2021; Thompson, Harvey, et al., 2019). The northernmost station was located offshore of Point Conception, CA within the California Current (34.14833˚N -121.1567˚W). The second station was located off San Nicholas Island, CA (33.32333 ˚N, -119.6667˚W) which experiences high variation in annual temperature depending on the respective strengths of the California Current and Southern California Counter Current. The third station was a southern coastal inshore station off San Diego, CA (32.84667˚N, -117.5383˚W) characterized by relatively warmer waters from the California Counter Current with seasonal (spring) upwelling of cool, nutrient-rich water. The fourth station was a southern offshore station (31.85000˚N, -119.5683˚W) characterized by sub-tropical oceanic waters (Figure 1).

Application

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**Figure 1. Sea Surface Temperature Pre and Post Marine Heat Wave**

To visualize the dramatic shift in ecological conditions, we plotted average daily SSTs from A) pre (April 1 1996 to April 1, 2014) and B) post (April 1, 2014 to April 1, 2019) marine heatwave. Average SST were dramatically elevated during and after the marine heatwave. The four sites sampled are plotted in black.

Decades of research within the study region (Moser P.E. Smith, and L.E. Eber, 1987; H. Moser et al., 2001; H. G. Moser et al., 1993; Thompson et al., 2022a) indicate the majority of species spawn in spring and the resulting ichthyoplankton closely track adult biomass (Hsieh et al., 2005). Hence, we expect the spring ichthyoplankton to reflect underlying changes in the local fish assemblages.

At each station, oblique bongo net tows were conducted from 210 m depth to the surface using standard CalCOFI methods (Kramer et al., 1972; McClatchie, 2014; Thompson et al., 2012; Thompson, McClatchie, et al., 2017;). Cod-end contents of both bongo nets were preserved at sea. The starboard side was preserved in sodium borate-buffered 2% formaldehyde and the port side was preserved in Tris-buffered 95% ethanol. We expect morphological and molecular analyses to be independent, imperfect reflections of a common biological community because port- and starboard-side samples are not precisely identical (See Discussion). Ethanol samples were archived in the Pelagic Invertebrate Collection at Scripps Institution of Oceanography stored at room temperature and out of direct sunlight.

Microscopy was conducted by a team of globally recognized expert ichthyologists to identify species abundance from 84 formaldehyde-preserved samples following standardized CalCOFI techniques in which all larvae are sorted from the other invertebrate zooplankton assemblages within each cod end and then subsequently identified to the lowest possible taxonomic rank (McClatchie, 2016). We note that eggs of a few species are typically sorted from each sample, but were not included in our analyses (See Discussion).

DNA metabarcoding was conducted on the ethanol in which 84 paired port side samples were preserved. Given that liquid preservative was used as the target substrate (as opposed to water, soil, or air) and the mass of larvae and eggs in the jar were not directly disturbed, we refer to this process as eDNA metabarcoding herein. Up to 125 mL (mean = 121.7 mL, n=6 jars with < 125 mL, min. = 34 mL) of ethanol preservative was pipetted off of archived samples and filtered onto 0.2 µm PVDF filters. Filters were extracted using a modified Qiagen DNeasy Blood and Tissue kit (Curd et al., 2019) and amplified using the MiFish Universal Teleost (Miya et al., 2015) PCR primer set targeting the 12S rRNA mitochondrial gene region. Each DNA extraction was amplified in triplicate with each technical PCR replicate sequenced separately to capture stochastic variation within the amplification process. Eight technical PCR replicates with either low sequencing depth (n<30,000) or high dissimilarity (Bray Curtis dissimilarity > 0.7) were removed. See Supplemental methods for full description.

This resulted in a final data set of 84 jars across 4 stations and 23 years that were morphologically identified as well as 84 jars representing 90 unique DNA extractions and 244 unique PCR technical replicates that were sequenced.

*Estimating Abundance*

We estimated the abundance of ichthyoplankton in each jar using a novel joint Bayesian hierarchical model based on the quantitative metabarcoding framework described in Shelton et al. 2022, detailed here in the supplemental methods. Importantly, we model taxon sequence-read counts from metabarcoding to account for the PCR process in which each taxon is subject to a different amplification efficiency based on the primer set used. Briefly, we estimate that the number of sequenced amplicons, for any species *i,* is a nonlinear function of the species-specific fraction of DNA in the template (Kelly et al., 2019; McLaren et al., 2019; Silverman et al., 2021 ; we use *i* to represent species, but can be generalized to represent ASVs or other molecular targets). Our framework is built upon the premise that the amplicons produced during a PCR reaction are dictated by the amplicon efficiency parameter , which is characteristic of the interaction between the particular PCR reaction and each species being amplified. Thus, for any species *i*, the number of amplicons should be directly related to the efficiency of amplification and the starting concentration of DNA template such that

(1)

where is amplicon abundance, is the true number of DNA copies in the reaction attributable to species *i*, is the species-specific amplification efficiency (bounded on (0,1)), and is the number of PCR cycles used in the reaction (Lalam, 2006) (See supplemental methods for full description of the model and assumptions).

The above equation has three parameters making it impossible to determine (starting DNA concentrations) from metabarcoding data alone. To address this, Shelton et al. 2022 highlighted four general strategies to help estimate solutions to the above equation through the inclusion of additional independently derived sources of information. The first two strategies involve estimating amplification efficiencies alongside metabarcoding data, thereby providing estimates of 2 parameters ( and ) and allowing for estimation of input DNA concentrations, . These amplification efficiencies can be derived either by the sequencing of 1) mock communities of known DNA template composition or 2) samples of unknown composition across a range of PCR cycles. The third strategy employs the use of unique molecular identifiers to identify source molecules prior to amplification, functionally bypassing the need for the above equation. These above strategies have been successfully demonstrated in the literature (Hoshino et al., 2021; Hoshino & Inagaki, 2017; Shelton, Gold, Jensen, D’Agnese, Andruszkiewicz Allan, et al., 2022; Silverman et al., 2021).

However, to date, no work has demonstrated the utility of the fourth strategy described in Shelton et al. 2022: employ the use of another independent set of observations of the same community to constrain the total possible parameter space of starting DNA concentrations and amplification efficiency values. In such an approach, observed DNA concentrations from a subset of all taxa observed by amplicon sequencing are used to constrain the parameter space of species-specific amplification efficiencies and thereby allow for the estimation of the starting DNA concentrations for all species observed from metabarcoding data. Thus, this study's objective is to demonstrate the validity of employing an independent set of abundance observations alongside metabarcoding data results to jointly estimate abundance for the entire amplicon observed biological community.

To achieve this, we link the sequencing data to the morphological ichthyoplankton counts from paired samples to constrain the species-specific starting concentrations of DNA in the ethanol jars, allowing for the estimation of abundance for all species observed in metabarcoding data (See supplemental methods). We highlight that the above modeling framework allows us to take advantage of the strengths of metabarcoding sampling, namely higher sensitivity and taxonomic resolution, as well as the strengths of morphological counts, namely quantitative abundance (larvae counts per standardized volume towed) estimates. The resulting integrated model leverages the taxonomic breadth and resolution (Gold et al., 2021; Miya et al., 2020) of amplicon sequencing, combining these with the power of morphological counts to yield species-specific quantitative abundance estimates. Together, the application of the integrated model provides a higher-resolution characterization of ichthyoplankton assemblages by providing abundance estimates for a broader diversity of species than observed by morphological counts alone.

*Data Analysis*

We first compared the results of metabarcoding and morphological surveys. We here focus our comparisons on the 59 species that were either observed in >9 technical PCR replicates within the metabarcoding data or enumerated >25 total times within the morphological data to ensure sufficient representation to achieve model convergence.

We then focused our analysis on the results of the joint model. Specifically, from the model output, we calculated mean abundance estimates per species per station per year. Mean abundance estimates were used as our response variable in the following analyses.

The resulting estimates capture major and sometimes highly unexpected changes to the fish assemblages during and after the 2014–2016 Pacific marine heatwave, the warmest 3-year period in the North Pacific in over 100 years of recorded history driven by a massive influx of warm, saline water from the central Pacific (Jacox et al., 2018). Given that similar ocean conditions persisted well beyond 2019 (Ren & Rudnick, 2021), we compare ichthyoplankton assemblages before and after the marine heatwave. A suite of environmental variables ­­– not just sea surface temperature (SST) ­­– changed dramatically during the event. Upwelling strength and location, dissolved oxygen, salinity, current strength, and other environmental covariates shifted during the climate-change influenced marine heatwave (Gentemann et al., 2017; Morgan et al., 2019; Ren & Rudnick, 2021; Schroeder et al., 2019). Here we use SST as a proxy for the onset and continuation of this suite of changes, documenting the resulting shift in community assemblage without attempting to identify any singular mechanistic driver responsible for this shift. To visualize the dramatic shift in ecological conditions, we plotted average SSTs from pre (April 1 1995 to April 1, 2014) and post (April 1, 2014 to April 1, 2019) marine heatwave using the *rerddapXtracto* package (Mendelssohn, 2020) in R to collect daily PathFinder Ver 5.3 remotely sensed composites (Figure 1).

We further visualized anchovy and sardine – key taxa of management interest – abundance over time by calculating the mean log (abundance) of each species per station per year. We then plotted the mean log (abundance) of each of the four stations while error bars represent the 95% confidence intervals (CI) observed for a given species at a given station in that year.

To evaluate the effect of the marine heatwave on CCLME fishes, we compared estimated species abundances before the marine heatwave (1996–2013) to that estimated both during and after the marine heatwave for each station (2014–2019). We first calculated the mean abundance for each species at each station for each model run. We then subtracted the pre-marine heatwave species-site abundance means from the post-marine heatwave species-site abundance means for each model run to evaluate changes in marine heatwave abundance per species per station per model run. We then calculated a 95% CI of change in marine heatwave abundance per species to identify which species were significantly different before vs. during and after the marine heatwave at each station. We further plotted the change in marine heatwave abundance for each “species grouping” by habitat associations derived from previous CalCOFI research (See Supplemental methods; Hsieh et al., 2005).

All data and code to conduct analyses and generate all figures are available on GitHub (https://github.com/zjgold/CalCOFI\_eDNA) and associated Google Drive link (https://drive.google.com/drive/folders/12cU9mY\_CWoro-x6Hgh\_pgv\_66zZEzm1h?usp=sharing) [will be replaced with a Dryad repository upon acceptance].

**Results**

eDNA metabarcoding of ethanol preservative with MiFish 12S(Miya et al., 2015) generated a total of 59.9 million sequence reads across 84 jars representing 90 unique DNA extractions and 244 unique PCR technical replicates. All sequence data were processed using the *Anacapa Toolkit* (Curd et al., 2019). After quality control, sequence-variant (ASV) dereplication, and decontamination processes (Curd et al., 2019; Gallego et al., 2020; Gold et al., 2021), we retained a total of 54.5 million reads (technical replicate range: 36,050–1.2 million reads) (See Supplement 1 Methods). From these data, we classified 130 unique taxa including 103 species-level assignments (79%), 15 genus-level assignments (12%), 11 family-level assignments (8.5%), and one class-level assignment.

Through these molecular taxonomic assignments, we identified two distinct morphologically indistinguishable lineages (ASVs) of the Northern Lanternfish (*Stennobrachius leucopsarus*). Although these two clusters of ASVs only differed by a single conserved base pair (99.5% sequence similarity), the two Norhtern Lanternfish lineages were repeatedly detected across samples and in high sequence read counts (variant 1: 6,145,100 total reads, 236 detections across technical replicates; variant 2: 259,989 total reads, 97 detections across technical replicates) and also exhibited dramatically different ecological patterns across the samples (Figures 2 and S1). Therefore, the two lineages were treated separately.

Scatter chart

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**Figure 2. Co-Detection of Ichthyoplankton By Metabarcoding and Morphological Methods**

We plotted morphological and metabarcoding detections of the 59 species used within our joint model. Metabarcoding detected 11 species that were not detected by morphological identifications. In addition, metabarcoding more frequently detected 48 species than morphological identification whereas morphological identifications only detected one species more frequently. These results indicate that metabarcoding and morphological identifications sample distinct, but overlapping ichthyoplankton communities from the CalCOFI archived samples.

Independent microscopy-count data from paired, matching formalin-preserved samples consisted of 9,610 larvae sorted across 84 jars. From these data, we classified a total of 92 unique taxa including 76 species-level assignments (83%) and 16 genus-level assignments (17%).

For our integrated Bayesian model, we focused on the 59 taxa that had sufficient representation across either the metabarcoding data set or larval data set to achieve model convergence (observed in >9 technical PCR replicates and >25 total larvae counted across the dataset). We note that these 59 taxa represent 99.0% of all larvae identified and reads sequenced.

Of these 59 taxa, 48 taxa were detected by both methods. DNA metabarcoding efforts detected 11 additional taxa including six additional species (*Benthalbella dentata, Ceratoscopelus townsendi, Diaphus theta, Microstomus pacificus*, *Parophrys vetulus*, *Peprilus simillimus*), one additional family (Opisthoproctidae), one additional variant of *Stennobrachius leucopsarus*, and three distinct higher-level taxonomic groups that likely represent distinct taxa lacking reference barcodes (Bathylagidae and *Stenobrachius* sp.). eDNA approaches detected all species identified by morphological identifications. From these results we highlight the advantage of including DNA metabarcoding in the modeling efforts, particularly the inclusion of 11 taxa that were not detected by morphological methods, thus allowing us to track abundance changes across a broader taxonomic scope of larval fishes.

In addition, detection rates varied across species and survey methods (Figure 2). 58 species were more frequently detected with DNA metabarcoding while one species (*Argyropelecus sladeni*) was more frequently detected via morphological identifications. Only 8 taxa were more commonly jointly detected by both methods than by eDNA alone and only 1 taxawas detected as frequently by both methods as by eDNA. Of these 9 commonly detected taxa, 6 species were in the top 10 most abundant species in terms of total sequence reads and larvae counted, with 8 of 9 species in the top 20 most abundant species by reads and larvae counted. We also note that the ten most abundant taxa in terms of total morphological counts of larvae and sequence reads were identical across both methods.

Furthermore, non-detections across technical PCR replicates within a given jar were only observed for samples with fewer than 9 larvae, suggesting a metabarcoding limit of detection for rare larvae (Figure S2). We also observed a similar, albeit far weaker relationship between morphological non-detections and the mean proportion of sequence reads in a jar (Figure S3). In one instance, no larvae were identified despite that species comprising 89.5% of sequences in the jar.

*Quantitative Abundance Estimates*

The integrated Bayesian model had successful convergence for the 59 species described above (Figure 3). Model fits yielded station-, species-, and year-specific larval abundances for 59 fish species spanning a 23-year period. As expected, given the compositional nature of the metabarcoding dataset, we observed a poor correlation between (uncorrected) eDNA metabarcoding derived amplicon abundance and morphological larvae counts (Figure 3a). In contrast, model outputs (posterior means, given estimated amplification efficiency parameter values) of counts and sequence reads are much better reflections of observed data as visualized by goodness of fit (Figure 3b, c). Our model predicted larval abundance with high accuracy, particularly for larvae with abundant amplicon sequences and morphological counts. Thus our model provided reliable quantitative estimates even for the 11 taxa which were only detected by eDNA metabarcoding. Given the performance of the joint model, all subsequent analyses were conducted using the model estimated abundances.

Chart, scatter chart

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**Figure 3. Bayesian Joint Model Improves Quantitative Abundance Estimates**

Observed (uncorrected) eDNA metabarcoding derived sequencing reads and morphological counts do not follow a clear linear relationship (a). This non-linearity is unsurprising given that observed reads are a function of both DNA concentrations (here assumed proportional to morphological counts) as well as species-specific amplification efficiencies (here unknown). Thus, without accounting for species-specific amplification efficiencies within the compositional amplicon data set, we do not expect to observe a strong correlation between the two. Model predictions (posterior means, given estimated amplification efficiency parameter values) for counts (b) and sequence reads (c) are much better reflections of observed data, giving a visual indication of model goodness of fit. The one-to-one line is plotted in red and Pearson correlation coefficients (*r*) are reported. The strong correlation between predicted and observed counts and reads strongly suggests that our mechanistic model successfully accounted for amplification efficiency differences. Importantly, these strong model fits allow for model-derived quantitative estimates for the 11 taxa that were only observed by eDNA data alone. We note that variance is high at low observed morphological counts and observed sequence reads with non-detections between methods driving a large amount of the observed variance, particularly at low observed morphological counts (*n*<10) and sequences (*n*<3,176).

*Displacement of Target Fish Species and Tropicalization of Fish Assemblages Associated with the Marine Heatwave*

Marine ichthyoplankton assemblages transformed during the 2014–2016 marine heatwave where southern, mesopelagic species increased while several temperate species of ecological and economic importance declined. Such synchronous changes in the marine ichthyoplankton assemblages occurred during the marine heatwave despite the hundreds of kilometers between stations and unique biogeographic characteristics associated with each sampled geographic location (see Supplement 1 results). For example, the mesopelagic Mexican Lampfish (*Triphoturus mexicanus*) was at peak abundance during the marine heatwave and extended its typical range both poleward and into coastal shelf waters by hundreds of kilometers (Figure 4).

Ichthyoplankton assemblages shifted over time throughout the study region (Figures 4). Subtropical, mesopelagic species uniformly increased during and after the marine heatwave, while many coastal species typically seen in the region tended to decrease (Figure 4). In particular, the abundances of northern, mesopelagic species and fisheries targets such as Pacific Sardine (*Sardinops sagax*) and North Pacific Hake (*Merluccius productus*) were significantly lower after the onset of the marine heatwave and tended not to co-occur with warm associated southern, mesopelagic taxa (Figure 4).

Chart

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**Figure 4. Novel Marine Heatwave Assemblages**

Shifts in species modeled abundances with the onset of the marine heatwave (1996–2013 vs. 2014–2019). Synchronous increases in southern mesopelagic species and Northern Anchovy (*Engraulis mordax*) were observed across all stations. Stations are in rows, species in columns, and the joint model estimated change in abundance between the two ecological phases is shown as the response variable. Fisheries targets including Pacific Sardine (*Sardinops sagax*) and North Pacific Hake (*Merluccius productus*), as well as many other benthic and coastal species, had concurrent negative associations. Significant differences during and after the marine heatwave are marked with + or -.

*Abundance Changes in Forage Fishes*

We observed dramatic changes in anchovy and sardine abundance (larvae counts per standardized volume towed) across the 23-year time series (Figure 5). During and after the marine heatwave we observed high anchovy abundance (max: 3,548, mean ± sd: 397 ± 834), more than a five-fold increase in abundance than before the onset of the marine heatwave (62 ± 192). This observation was particularly dramatic given the low abundances immediately preceding the marine heatwave (1 ± 1.4). In contrast, on average, sardine abundances remained low before (31 ± 65) and during the marine heatwave (8 ± 19). However, there were regional variations in this pattern with relatively high sardine abundances at the San Diego Offshore station from 2005-2008 (119 ± 72) and an increase in Sardine abundance in nearshore coastal waters at the San Nicholas station after the marine heatwave (50 ± 6). These results align well with analyses of morphological identifications from the broader CalCOFI data set (Thompson et al., 2022a).

Graphical user interface, chart, application

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**Figure 5. Synchronous Increase in Anchovy Abundance During and After Marine Heatwave**

Model posterior estimates for larval fish abundances (larvae counts per standardized volume towed) over time at each of the four sampled stations. Joint modeling of metabarcoding and morphological counts reconstructed increases in Northern Anchovy (*Engraulis mordax*) [blue] during the recent Pacific Marine Heatwave and low spawning of Pacific Sardine (*Sardinops* sagax) [red] over the past decade (points are means and error bars are 95% credible intervals; the shaded region is during and after the marine heatwave). SST is plotted above the Northern Anchovy and Pacific Sardine abundances for reference.

**Discussion**

Application of eDNA metabarcoding on preserved ichthyoplankton samples reveals marked shifts in California Current Large Marine Ecosystem ichthyoplankton communities over a period of 23 years, including the tropicalization of these communities during the 2014–2016 marine heatwave. Although raw sequence abundance correlated poorly to manual ichthyoplankton counts, the application of a joint Bayesian hierarchical model (Shelton, Gold, Jensen, D’Agnese, Andruszkiewicz Allan, et al., 2022) resulted in a strong correlation between metabarcoding and ichthyoplankton counts, particularly for abundant species. Combined, this study demonstrates the feasibility of eDNA metabarcoding from ethanol used to preserve bulk samples, and that this data can provide quantitative abundance estimates that preclude the need for manual counting of ichthyoplankton, creating novel research opportunities from preserved sample collections (Gallo et al. 2019). Ultimately, our approach to studying historical fluctuations in ichthyoplankton assemblages corroborates observed climate-associated biological changes in the CCLME (Thompson et al., 2022a)and suggests ways in which these changes could alter the function and socio-economic benefits derived from marine ecosystems.

*Displacement of Target Fish Species and Tropicalization of Fish Assemblages Associated with the Marine Heatwave*

The tropicalization of ichthyoplankton communities during the 2014–16 marine heat wave is consistent with multiple recent studies that demonstrate the tropicalization of terrestrial and marine ecosystems in response to climate change (Chaudhary et al., 2021; Vergés et al., 2016). These shifts can induce novel species interactions, catalyzing changes in ecosystem function (Frölicher & Laufkötter, 2018; Morgan et al., 2019). In the current study, we observe the combination of high abundances of both Northern Anchovy and southern mesopelagic species (Nielsen et al., 2021; Thompson, Harvey, et al., 2019)– a pattern unique to the previous >70-year CalCOFI dataset (Moser  P.E. Smith, and L.E. Eber, 1987), our findings corroborated by Thompson et al., 2022a.

The collapse of specific fisheries such as sardines is well-documented (Chavez et al., 2003; Checkley et al., 2017). Results suggest that these and other coastal pelagic fisheries targets may continue to be scarce as environmental conditions that are similar to the 2014–2016 marine heatwave become more common (Nielsen et al., 2021; Santora et al., 2020; Thompson, Harvey, et al., 2019). Although the ecological implications of these novel assemblages are, by definition, unpredictable, our results suggest that if future assemblages resemble those seen in the marine heatwave, increases in Northern Anchovy and southern mesopelagic fishes are likely to be associated with decreases in Pacific Sardine and North Pacific Hake in the Southern CCLME (Piatt et al., 2020; Robinson et al., 2018), fundamentally changing ecosystems and fisheries relative to the recent past (Thompson et al., 2022a).

*Abundance Changes in Forage Fishes*

Sardine and anchovy fluctuations have been a major focus of fisheries research since the 1950s because of their commercial value and/or they are prey for other high-value fishery species and species of management concern (Checkley et al., 2017). Our model estimates are consistent with other studies (Sydeman et al., 2020; Thompson et al., 2022a) that documented a decline in both sardines and anchovy beginning in 2005. In the wake of the marine heatwave, anchovy continued to be abundant while sardine remained rare (Figure 5). Although anchovy larvae abundance was low in spring during the 2014–2016 marine heatwave, anchovy recruitment was high in the summer of 2015 (Thompson, Schroeder, et al., 2019). Anchovy mature in approximately one to two years (Parrish et al., 1986; Sydeman et al., 2020), and thus the 2015 class likely began spawning in mid-2016 (Thompson et al., 2022a), leading to high anchovy spawning stock biomass and larval abundances by 2016 and lasting into at least 2021 (Thompson et al., 2022b; Weber et al., 2021).

The rise in anchovy and continued low abundances of sardine during the marine heatwave is an ecological surprise. Correlative analyses between basin-scale environmental indices such as the Pacific Decadal Oscillation indicate that, for the latter half of the 20th century, anchovy thrived under cooler conditions and sardine under warmer conditions (Chavez et al., 2003). However, our findings and others (Checkley et al., 2017; McClatchie, 2012; Nielsen et al., 2021; Thompson, Harvey, et al., 2019) suggest that the mechanisms that govern the population dynamics of these species are not a mere function of temperature, but that more complex factors drive recruitment dynamics of these species (Thompson et al., 2022a). For example, despite largely synchronous responses of fish assemblages to the marine heatwave, sardine declines were not consistent across the CCLME (Figure 4), with refugia of localized abundance in nearshore waters potentially driven by distinct, favorable conditions (Checkley et al., 2017; Sydeman et al., 2020; Thompson et al., 2022a).

Further improving our mechanistic understanding of drivers of forage fish dynamics will better inform ecological predictions in the face of extreme ocean events such as marine heatwaves, which are likely to increase in frequency and duration under climate change (Deutsch et al., 2015; Frölicher et al., 2018; Howard et al., 2020; Oliver et al., 2021). As we demonstrate, a combination of metabarcoding and visual surveys can characterize and quantify species across trophic levels (Rose et al., 2015), and this has the potential to reveal ecological mechanisms. Here, we used metabarcoding to accurately characterize the abundance and composition of larval fishes in CalCOFI plankton samples, capturing forage fish recruitment patterns.

Several major hypotheses seeking to explain forage fish recruitment variability can be tested using quantitative metabarcoding approaches to evaluating the larval prey field and gut contents as recruitment is thought to be underpinned by the capacity of young larvae to consume appropriate prey that facilitates faster growth (Hare, 2014). Future research utilizing quantitative metabarcoding approaches can help us to finally understand the drivers of recruitment volatility in coastal pelagic and other fishes, allowing for improved prediction of forage fish population dynamics (Barbato et al., 2019; Erdozain et al., 2019; Garcia-Vazquez et al., 2021; Mariac et al., 2018; Nielsen et al., 2021; Pitz et al., 2020; Sydeman et al., 2020).

Such improvements in the characterization and prediction of forage fish abundances are critical for undersetting the ecology of this system as this unexpected rise in anchovy during and after the 2014- marine heatwave resonated throughout the CCLME (Santora et al., 2020). For example, California sea lion pups grew at anomalously high rates after their mothers consumed copious anchovy forage and produced ample milk (Robinson et al., 2018). High rates of almost exclusively anchovy consumption also seemingly induced thiamine deficiency in adult salmon resulting in poor condition of recruits (Thalmann et al., 2020). Birds capable of feeding on anchovy thrived (Thompson, Harvey, et al., 2019) while those unable to consume anchovy perished (Piatt et al., 2020). Given that conditions comparable to the 2014–2016 marine heatwave are predicted to be more common in the CCLME in the future (Oliver et al., 2018), our results suggest that continued biological responses to both anchovy-dominated forage-fish assemblages and marine heatwave-associated ocean warming conditions are likely to be without modern analog (Thompson et al., 2022a).

*Strengths and Limitations of the Our Mechanistic Quantitative Metabarcoding Framework*

Our results here provide strong empirical support for the mechanistic metabarcoding framework proposed by Shelton et al. 2022. Here we successfully developed a joint model to integrate morphological counts and compositional metabarcoding data to derive accurate quantitative estimates of larval fish abundances.

However, we recognize that the immediate application of this model as presented here is limited in utility to the broader metabarcoding community: few biological monitoring programs have access to expert taxonomists who painstakingly identified over 9,000 individual ichthyoplankton representing 92 taxa (Gallo et al., 2019). Yet, the true value of this work is in providing the foundation for the future development of molecular based quantitative abundance estimates that either ease the burden of current morphological work or lead to purely molecular based methods.

Our model suggests that not all species need to be identified by morphology to allow for abundance estimates of the entire community (here only 48 of 59 species were morphologically identified). This could allow for a reallocation of morphological identification efforts to focus on easily identifiable taxa (e.g. Northern Anchovy and Pacific Sardine), utilizing metabarcoding data and the application of the joint model presented here to both identify and quantify additional ichthyoplankton species. Such an approach has broad utility to allow for jointly derived quantitative estimates of biological communities. We also feel it is vital to mention that morphological data provides critical information on size, age, and condition that cannot be obtained from metabarcoding approaches. And thus while metabarcoding is a valuable tool, it cannot be viewed as a wholesale replacement for the full range of ecological and fisheries management questions.

Furthermore, this study lays the groundwork for the development of purely molecular based quantitative abundance estimates from metabarcoding approaches. As elaborated in Shelton et al. 2022, our joint modeling approach can utilize any estimate of abundance including qPCR or ddPCR derived absolute DNA concentration estimates. Therefore, the combination of multiple quantitative single-species molecular assays alongside metabarcoding data should be able to provide the information needed to for our joint model, providing a purely molecular approach to estimating abundance from metabarcoding data (M. McLaren et al., n.d.). Previous work from Pont et al., 2022 has demonstrated that combining metabarcoding and qPCR results of a target metabarcoding locus, essentially determining total DNA concentration of the observed locus in each sample, can improve metabarcoding abundance estimates. However, it is important to note that the study did not account for differential amplification bias across taxa and naively assumed that the observed proportions derived from metabarcoding data reflect the true underlying proportions of starting DNA concentrations (M. R. McLaren et al., 2019; Shelton, Gold, Jensen, D’Agnese, Andruszkiewicz Allan, et al., 2022; Silverman et al., 2021). Future work should focus on the application of such a joint molecular-based approach by utilizing locus specific or multiple single-species qPCR or ddPCR assays to estimate underlying DNA abundance and metabarcoding data to characterize the broader biological community. Our work here provides strong evidence that these purely molecular approaches can be used to derive accurate quantitative estimates from metabarcoding data.

A clear additional avenue of future research efforts is to unite the multiple amplification efficiency estimation strategies outlined in Shelton et al. 2022 to derive more accurate quantitative estimates. Here our model solves for amplification efficiencies by constraining the underlying starting DNA concentrations with observed morphological counts, allowing for a model estimate of amplification efficiencies and underlying starting DNA concentrations for all taxa observed through metabarcoding data. In contrast, previous work has utilized mock communities or variable PCR cycling efforts to estimate amplification efficiencies directly which when combined with observed metabarcoding data can be used to estimate underlying DNA concentrations (M. R. McLaren et al., 2019; Silverman et al., 2021). However, future effortd should leverage a fully integrated model that derives independent information for all three parameters (observed reads, amplification efficiencies, and underlying DNA concentrations) to better derive quantitative estimates for the entire observed community. Such an integrated mechanistic model would combine metabarcoding data, independent estimates of amplification efficiencies (derived from mock communities or variable PCR cycling efforts), and independent estimates of underlying DNA concentrations (e.g. morphological counts or qPCR/ddPCR assays) to derive accurate quantitative estimates of molecularly observed biological communities.

Key to the development of such an integrated approach is accounting for all the underlying mechanisms governing the observed metabarcoding reads. However, accounting for all such mechanisms remains difficult. Here, we note that the application of the Shelton et al. 2022 modeling framework to the ethanol-preserved samples likely failed to account for all such mechanisms by making two flawed assumptions: 1) an exponential PCR process across all 49 cycles and 2) that the observed morphological counts directly correlate with the starting DNA concentration in each jar.

Previous work has suggested that PCR reactions are not perfectly exponential but saturate over time as reagents are consumed and enzymatic activity declines in efficiency (Boggy & Woolf, 2010; Chatterjee et al., 2012; Kubista et al., 2006). If the employed PCR reaction were to indeed follow a saturating function, then the number of successful PCR cycles would be effectively lower than the full 49 cycles employed here. Thus, the resulting amplification efficiency coefficients generated by our model would be underestimated. However, given the function of the PCR equation, such a bias would be applied uniformly as a scalar across all estimated amplification efficiencies and thus not affect the broad interpretation of our model results. Fortunately, accounting for any impact of PCR saturation can be easily remedied with the incorporation of a decay coefficient in the function of the underlying PCR equation in future modeling efforts (Boggy & Woolf, 2010; Chatterjee et al., 2012; Kubista et al., 2006). Future work understanding the fundamental mechanisms of PCR amplification and bias, particularly whether PCR follows an exponential or saturating function, is warranted given the importance of metabarcoding approaches to biological observations (Gold et al., 2022; M. R. McLaren et al., 2019; Shelton, Gold, Jensen, D’Agnese, Andruszkiewicz Allan, et al., 2022; Silverman et al., 2021).

We also assumed that the observed morphological counts directly correlate with the starting DNA concentration in each jar. However, this assumption is likely invalid for a few reasons. First, our work did not incorporate morphological counts of egg abundance from the jars. Almost undoubtedly, our DNA metabarcoding approaches successfully detected DNA derived from eggs as well as any damaged larvae that could not be morphologically identified within the samples. Thus, a shortcoming of our model used here is the underestimation of the true concentration of DNA within the sampled preservative when morphological identifications are not possible, leading to overestimates of amplification efficiencies and underestimates of ichthyoplankton abundances (Figure 3b,c).

In addition, all morphological counts and eDNA data were conducted in paired bongo net tows which have subtle differences in ichthyoplankton abundance and composition, particularly for low abundant species (Thompson, McClatchie, et al., 2017). Thus, the stochastic nature of detecting rare ichthyoplankton across both cod ends may help explain the higher observed rates of non-detection across technical PCR replicates for rare (n<9 individuals) fish larvae.

Furthermore, we know that both metabarcoding and morphological taxonomic identification are imperfect, leading to mismatches between survey approaches that result in undercounting of larvae or DNA for specific taxa. Unsurprisingly, many taxa lack morphological characteristics, especially at early developmental life stages (H. G. Moser, 1996). Likewise, many taxa lack sufficient genetic differentiation to resolve species identification either because reference databases are incomplete despite extensive efforts (Gold et al., 2021) or because many adaptive radiations like the genus *Sebastes* have identical sequences for the employed marker sets (Min et al., 2021). These limitations for genetic identification can be overcome with additional markers and more comprehensive reference databases (Schenekar et al., 2020). Although, we took great efforts to develop a species mapping index that linked observations between both methods, all survey approaches will be limited by imperfect taxonomic identification between both sampling techniques.

A combination of the above limitations led to the observed non-detections in morphological data sets. For example, in one sample from 2014 nearly 90% of sequence reads were identified to *Citharichthys sordidus*, despite morphological methods failing to detect this species within the jar. Given that larvae from this species are regularly identified in the CalCOFI program, this non-detection was likely driven by a high occurrence of eggs, which are not morphologically identifiable from other congeners in the Family Pleuronectidae, resulting in a non-detection in the morphological data set while metabarcoding data was swamped by *Citharichthys sordidus* DNA.

Together, the above limitations impact our ability to both directly compare and jointly model genetic and morphological methods, resulting in the sampling of distinct, but overlapping portions of the sampled biological community (Figure 2) (Kelly et al., 2017). However, despite these limitations with our survey methods and model assumptions, we were still able to derive largely accurate quantitative estimates using our joint model, particularly for abundant taxa, suggesting that our framework captured much of the important variation in the observed dataset (Figure 3).

One reason for the success of our abundance estimates efforts is likely due to the unique circumstances of our unique eDNA sampling - each larva in a bulk collection has experienced the same conditions in a constant volume of ethanol. As such, these samples may be more amenable to estimating abundance accurately because only a few processes are likely to affect the relationship between captured DNA and the amount larvae in the jar. For the ethanol-preserved samples here, we made the explicit assumption that all larvae share an identical DNA shedding rate into the ethanol preservative, ignoring any allometric or physiological effects on species-specific shedding rates. In contrast, metabarcoding efforts from more typical eDNA samples derived from water in the environment are impacted by not just specieis specific allometry and morphology but also life stage, behavior, temperature, state, origin, and fate and transport of the collected DNA (Andruszkiewicz Allan et al., 2021; Barnes & Turner, 2016; Harrison et al., 2019; Jo et al., 2019; Sassoubre et al., 2016; Shelton, Gold, Jensen, D’Agnese, Allan, et al., 2022; Thalinger et al., 2021; Yates et al., 2021). Thus, a current limitation of our model framework is that our mechanistic model only accounts for PCR and sequencing processes and does not account for the myriad of processes that may impact captured DNA sequence reads prior to the PCR reaction (Figure 6).

Graphical user interface

Description automatically generated

**Figure 6. Summary of Processes Affecting eDNA Metabarcoding**

We present a unified theory of environmental DNA, highlighting the distinct processes that affect observed sequence reads obtained from metabarcoding. Deterministic processes are shaded in navy and stochastic processes are shaded in teal. We note that the current modeling framework employed in this study only accounts for PCR processes through sequencing. Future joint modeling efforts should integrate additional processes to better derive quantitative estimates.

Recent concurrent work has begun to characterize the effects of additional mechanistic processes prior to amplification, particularly the effect of subsampling processes on observed read abundances and non-detections (Egozcue et al., 2020; Gold et al., 2022). Utilizing the dataset described in this manuscript in conjunction with additionally derived mock communities, Gold et al. 2022 demonstrates that species with lower amplification efficiencies and lower morphological counts had higher rates of non-detection. Thus, these results highlight the importance of not only modeling the deterministic PCR process as we have done here, but also modeling the stochastic subsampling process prior to amplification as both processes together drive observed biological signal (abundance of observed reads) and noise (the patterns of non-detections across technical replicates) in metabarcoding data sets (Gold et al., 2022).

Critically, these results demonstrate the need for a fully integrated modeling approach that accounts for the totality of mechanisms impacting observed metabarcoding reads. Such an integrated modeling effort must rely on a unified theory of eDNA metabarcoding pulling together our collective mechanistic understanding of shedding, degradation, and fate and transport processes (Harrison et al., 2019) alongside the subsampling and PCR amplification processes (Gold et al., 2022; Shelton, Gold, Jensen, D’Agnese, Andruszkiewicz Allan, et al., 2022) to ultimately derive accurate quantification and detection estimates (Figure 6).

However, despite all the current shortcomings of our modeling approach presented here, we demonstrate the value of the Shelton et al. 2022 framework to derive quantitative estimates of abundance from metabarcoding data (Figure 3). Our current approach is particularly well suited to acquiring abundance estimates from metabarcoding in controlled systems like the ethanol-preserved samples utilized here as well as microbiome and bulk metabarcoding approaches (Carew et al., 2018; M. R. McLaren et al., 2019). Addressing the above limitations by advancing mechanistic modeling efforts will further enhance the accuracy of quantitative estimates derived from metabarcoding data in the future, allowing for applications to aquatic eDNA.

*Novel Insights from Legacy Collections*

Ultimately, our novel approach of metabarcoding eDNA from ethanol used as a preservative combined with joint Bayesian hierarchical modeling provides quantitative estimates by non-destructively sampling legacy collections via metabarcoding, and at the same time provides a mechanistic framework for determining absolute abundance estimates from compositional amplicon sequencing data (Gloor et al., 2017; M. R. McLaren et al., 2019; Silverman et al., 2021). Importantly, the application of metabarcoding approaches allowed us to differentiate variants and species that were not morphologically identifiable in the ichthyoplankton (Thompson, Chen, et al., 2017). For example, metabarcoding identified unique variants of the Northern Lampfish *(Stennobrachius leucopsarus*) that are morphologically indistinguishable and combined as a complex exhibited little change before and after the marine heatwave. However, these two ASVs had markedly different responses to the marine heatwave with one variant largely disappearing after the marine heatwave onset (Figure 4). Thus, by illuminating such unseen variation, molecular methods reveal ecological dynamics otherwise hidden by shared larval morphology.

Beyond improved taxonomic resolution, the key advantage of our mechanistic framework is the ability to derive quantitative estimates from metabarcoding data, albeit with the many caveats described above. Determining abundance from eDNA metabarcoding has been challenging, with mixed results (Fonseca, 2018; Lacoursière-Roussel, Côté, et al., 2016; Yates et al., 2019). Unlocking such quantitative metabarcoding approaches expands the potential for linking ecological assemblages to environmental processes beyond just presence-absence analyses (Lacoursière-Roussel et al., 2016; Stoeckle et al., 2021; Yates et al., 2019). Such quantitative approaches may prove critical in modeling and predicting future ecosystem change, although directly linking assemblage dynamic responses to climate-driven forces remains inherently challenging. While the CalCOFI samples are specific to ichthyoplankton from the CCLME, bulk collection of community samples is commonly used to survey plankton, insects, pollen, gut contents, and microbiomes, among many other targets (Deiner et al., 2017). As such, the methodology we present here is broadly applicable to efficiently understand modern and historical changes in ecological communities.

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*Competing Interests*

The authors declare no competing interests.

*Data and Materials Availability*

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. All data and code to conduct analyses and generate all figures are available on GitHub (https://github.com/zjgold/CalCOFI\_eDNA) and associated Google Drive link (https://drive.google.com/drive/folders/12cU9mY\_CWoro-x6Hgh\_pgv\_66zZEzm1h?usp=sharing) [will be replaced with a Dryad repository upon acceptance].

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