Bulk tissue and amino acid stable isotope analyses reveal global ontogenetic patterns in ocean sunfish trophic ecology and habitat use

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ABSTRACT: Ocean sunfish (*Mola* spp.) have largely been considered as obligate gelativores, but recent research has suggested that they undergo an unusual life history shift. In this study, analyses of bulk and amino acid nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) stable isotope ratios were employed to provide detailed insight into ontogenetic variation in sunfish trophic ecology and habitat use and to assess whether any observed patterns were common to populations around the world. Through this combined approach, a clear ontogenetic shift was identified in both trophic ecology and habitat use of ocean sunfish, indicating a complex trophic role that changes from more benthic to pelagic prey on a continuous scale as fish grow larger. The data also revealed strong population structuring with potential to assess connectivity between distinct groupings using isotopic analysis. When combined, these new insights into sunfish ecology may be of value to conservation management teams, indicating a broad ecological role, distinct population clustering and possible trans-Atlantic movements. These results suggest that the current mass bycatch of ocean sunfish may have far-reaching ecological implications and further highlights the growing need for conservation management of this vulnerable genus.

KEY WORDS: Diet · Pelagic · Benthic · Energy pathways · Ontogeny · *Mola* · Bycatch · Trophic position

1. INTRODUCTION

Anthropogenic pressures have reportedly reduced the biomass of marine megafaunal predators by 90% over the last 50 yr (Myers & Worm 2003, McCauley et al. 2015), resulting in pronounced ecological impacts (Cox et al. 2002, Heithaus et al. 2008). However, ecosystem studies examining the longer-term consequences of such mass removal of large fishes are currently limited by a paucity of data regarding the

complexity of marine food webs and the top-down role of consumers (Cox et al. 2002, Myers et al. 2007, Heithaus et al. 2008). In light of these growing concerns, here we provide new insights into the trophic ecology of ocean sunfish (genus *Mola*), a vulnerable group (Jing et al. 2015) subject to intensive bycatch globally (Petersen & McDonell 2007, Nyegaard et al. 2018, Mangel et al. 2019). This group is not a primary target for global fisheries (although smaller markets do occur across Asia), they are of low commercial value and remain unprotected by management regulations (Jing et al. 2015). However, as global fisheries effort increases, scientists and conservation bodies have noted an alarming rise in the bycatch of these fish (Silvani et al. 1999, Cartamil & Lowe 2004, Petersen & McDonell 2007, Nyegaard et al. 2018), highlighting a pressing need to understand the potential consequences of removing significant numbers from marine systems. Unwanted capture of sunfish is now estimated to remove hundreds of thousands of individuals across the global ocean each year (see review by Pope et al. 2010) and has resulted in the International Union for Conservation of Nature (IUCN) classifying ocean sunfish as 'Vulnerable' to extinction globally, but 'Data Deficient' on regional scales (Jing et al. 2015). This study was conceived to address some of these knowledge gaps to aid the conservation and management of this vulnerable taxon.

Ocean sunfish are unusual fish: they can grow to ~3 m in length and weigh up to ~2.3 t (Roach 2003). Smaller individuals form large schools in nearshore waters, then become more solitary and move into pelagic environments as they grow (Silvani et al. 1999, Cartamil & Lowe 2004, Pope et al. 2010). Along with their large body size, ocean sunfish are notable for their somewhat unusual ecological niche, with a diet largely (putatively) composed of gelatinous zooplankton (e.g. Fraser-Brunner 1951, MacGinitie & MacGinitie 1968, Hooper et al. 1973). Recent research has shown that the gelatinous portion of the sunfish diet extends beyond typical jellyfishes (scyphozoans), incorporating a wide range of gelatinous taxa including pyrosomes, siphonophores, salps and ctenophores (e.g. Potter & Howell 2011, Cardona et al. 2012, Nakamura et al. 2015, Thys et al. 2015). Although research over the last 50 yr (and in more recent times, the popular media) has largely focussed on their role as putative obligate gelativores (MacGinitie & MacGinitie 1968, Hooper et al. 1973), it is increasingly clear that smaller sunfish consume a more diverse diet (e.g. Fraser-Brunner 1951) containing both pelagic and benthic prey, with a shift towards a more gelatinous diet as they grow (e.g. Syvä-

ranta et al. 2012, Nakamura & Sato 2014, Sousa et al. 2016).

Smaller-bodied sunfish (<1 m total length) appear to feed relatively broadly within coastal food webs, with stomach contents noted to contain algae, crustaceans, ophiuroids, molluscs, hydroids and fish, as well as gelatinous prey (e.g. Norman & Fraser 1949, Fraser-Brunner 1951, Clemens & Wilby 1961). More recent studies based on stable isotope analysis (SIA) of bulk tissues (i.e. muscle or skin) in both the Mediterranean Sea (n = 8, size range: <100 cm) and the NW Pacific (n = 17, size range: 50–200 cm) showed that sunfish have higher nitrogen isotope ($\delta^{15}N$) values by ~6‰ relative to gelatinous prey (Syväranta et al. 2012, Harrod et al. 2013, Nakamura & Sato 2014), a difference too large to be explained by a single trophic step. Furthermore, smaller sunfish <1 m in total length are notably ^{13}C-enriched relative to pelagic taxa (and putative prey), indicating that they are likely feeding more in benthic and inshore food webs (Jennings et al. 1997), and have $\delta^{15}N$ and $\delta^{13}C$ values statistically indistinguishable from local benthic-feeding fish species. Overall, these patterns suggest that sunfish are not obligate gelativores (Syväranta et al. 2012, Harrod et al. 2013). However, it is not yet possible to confirm if such prey selection (and potential dietary shifts) can be applied to sunfish populations globally, as sample sizes and comparisons between populations in different regions remain limited. Additional data are required to explore the trophic position of sunfish throughout ontogeny to understand their changing ecosystem role. Without such information, there is a high risk that large schools of sunfish in coastal waters will continue to be removed en masse as bycatch, with little understanding of the potential consequences to ecosystem functioning.

In this study, we used both bulk tissue (skin) and amino acid (AA) $\delta^{15}N$ and $\delta^{13}C$ SIA to explore size-related shifts in diet and habitat use and to consider whether such patterns are ubiquitous among sunfish populations around the globe. The use of bulk tissue SIA in trophic ecology is largely based on the concept of predictable isotopic differences between consumers and their prey (Peterson & Fry 1987, Vanderklift & Ponsard 2003). $\delta^{15}N$ values have been used extensively to estimate consumer trophic level, since they typically increase by ~2–5‰ per trophic step due to preferential excretion of ^{14}N in nitrogenous waste products (e.g. ammonia) synthesized by consumers (DeNiro & Epstein 1981, Pinnegar & Polunin 1999, Vander Zanden & Rasmussen 2001). In contrast, $\delta^{13}C$ values vary less between trophic levels (~0–2‰), and

instead typically vary between different functional groups of primary producers and thus provide a means to identify use of habitats fuelled by different sources of production (France 1995, Perry et al. 1999), e.g. pelagic versus benthic (which can vary by ~5–10‰, with benthic endmembers having higher $\delta^{13}C$ values than pelagic sources). By combining these approaches and assessing the $\delta^{13}C$ and $\delta^{15}N$ values of consumer tissues, it becomes possible to simultaneously characterise habitat use and dietary strategy.

Although SIA of bulk tissues collected from marine consumers can be highly informative, it must be noted that the baseline isotopic composition of primary producers can vary both spatially and temporally (Graham et al. 2010). Potential differences in localised baselines makes comparisons between differing populations challenging and often means that isotopic values cannot be interpreted without corresponding baseline values, which requires *a priori* understanding of species ecology. Recently, $\delta^{13}C$ and $\delta^{15}N$ analysis of individual AAs has been used to tease apart the relative influence of variation in baseline isotope values from variation in diet composition and trophic level (e.g. Bowes & Thorp 2015). For $\delta^{13}C$ analysis, AAs are divided into traditional 'essential' and 'non-essential' groups. Although plants, algae (Protoctista), fungi and bacteria can synthesize essential AAs (AA_{ESS}) de novo, animals typically cannot and are only able to obtain these from their diet (e.g. Hare et al. 1991, O'Brien et al. 2002, Fogel & Tuross 2003). Some animals have demonstrated symbiosis with the gut microbiome (Newsome et al. 2011), when hosts consume a protein-deficient diet. Consequently, the $\delta^{13}C$ values of AA_{ESS} generally show no significant isotopic discrimination between diet and consumer (the minimal isotopic alteration between diet and consumer), and thus can identify the primary source(s) of production at the base of the local food web (Howland et al. 2003, Larsen et al. 2013, McMahon et al. 2015a, Elliott Smith et al. 2018). Furthermore, patterns in AA $\delta^{13}C$ values can change in response to biosynthetic pathways and mode of carbon acquisition, which are different among plants, protists, fungi and bacteria (Scott et al. 2006, Larsen et al. 2009, 2013), which is likely because these synthesizers use unique biosynthetic pathways to synthesize AA_{ESS} that imprint on their $\delta^{13}C$ composition (Larsen et al. 2009, 2013). These unique AA_{ESS} $\delta^{13}C$ 'fingerprints' are potentially of great value to trace the ultimate source of primary production that contributes to the diet of consumers that occupy any trophic level of the food chain.

For $\delta^{15}N$, AAs are commonly grouped into 'source' and 'trophic' forms, a classification based on the rel- ative degree of isotopic discrimination with trophic transfer. Source AAs undergo minimal isotopic discrimination between trophic levels, because metabolic processing of these forms does not lead to deamination of the amine group, and thus there is minimal potential for ^{15}N isotopic fractionation (Popp et al. 2007, McMahon et al. 2015a, O'Connell 2017). Thus, source AAs can provide a measure of the $\delta^{15}N$ composition of the base of the food web(s) in which consumers forage. Alternatively, trophic AAs are heavily involved in nitrogen metabolism and undergo significant isotopic discrimination with increasing trophic level. Thus, comparison of $\delta^{15}N$ values among source and trophic AAs can provide a relative measure of a consumer's trophic position (e.g. Popp et al. 2007, McMahon 2015, McMahon et al. 2015a,b). This approach permits the estimation of baseline isotopic composition and trophic position from a single consumer tissue sample without the need for extensive sampling of basal sources of production (e.g. Chikaraishi et al. 2009), providing a powerful tool for simultaneously studying the diet and movements of elusive marine consumers (McMahon et al. 2015b).

As isotope-based approaches often use non-lethal sampling methods to unravel the trophic ecology and habitat use of wide-ranging consumers, such data have the potential to provide novel insights into the ecological role of ocean sunfish across their global range, without requiring the sacrifice of individual fish (as has been noted in previous dietary work; L. Kubicek unpubl.). In light of current fisheries pressures, including unregulated target fisheries and mass bycatch (e.g. Silvani et al. 1999, Cartamil & Lowe 2004, Petersen & McDonell 2007, Nyegaard et al. 2018), such insights are sorely needed. For this study, our goal was to use isotope data to examine sunfish trophic ecology. Firstly, we used bulk tissue SIA and mixing models to quantify prey taxa in the diet of sunfish throughout ontogeny at a regional scale within the Mediterranean Sea. Secondly, we extended our analysis of the Mediterranean sunfish population with AA_{ESS} $\delta^{13}C$ fingerprinting to assess pelagic versus benthic contributions to diet. Thirdly, we looked for global patterns in sunfish trophic ecology by comparing bulk tissue SIA, AA $\delta^{13}C$ fingerprinting and AA $\delta^{15}N$-derived trophic level estimates derived from samples collected from around the world. By providing a better understanding of the diet, trophic level and habitat use of ocean sunfish, our aim was to gain new data for species management and to facilitate prediction of the potential ecological consequences if the mass removal of sunfish continues.

2. MATERIALS AND METHODS

2.1. Sample collection

Samples of ocean sunfish (genus *Mola*) tissues were collected from multiple sites across their global range (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m633p127_supp.xlsx), including the Mediterranean Sea, northeast and northwest Atlantic and northeast and southeast Pacific. All sampled fish were morphologically identified as *M. mola*, but without genetic analyses to confirm species identification, for the purposes of this study we refer to our samples at the genus level. The primary sampling site for this study was in the northern Mediterranean off the northwest Italian coast, where there is a predictable annual bycatch of ocean sunfish in the Camogli tuna fishery ('tonnarella', Cappanera 2016). Regular sampling of bycatch at this site occurred during the summer fishing period (May–July) in 2015 and 2016, enabling collection of a large dataset.

During sampling, fish were maintained in the deep tuna nets or in a tethered keep-net until processing on a large tarpaulin on deck. The tuna nets were emptied 3 times daily and so it is not believed that the natural diet of the sunfish was affected by the brief period where they were held captive in the nets. Furthermore, by sampling tissues that turnover over a longer time frame than the captive setting, even if the sunfish were able to access non-typical food sources this should not be reflected in the data (Newsome et al. 2010, Madigan et al. 2012). Following consultations with the veterinary team at Queen's University Belfast, all sampling was carried out with the approval and support of the Portofino Area Marina Protetta and in accordance with Queen's University ethical guidelines. Processing time was kept to a minimum (2–7 min), where each specimen was measured for total length (TL, ±1 mm) and mass (±1 g) before a selection of tissues were sampled, after which fish were released. No mortalities occurred during processing, and each fish was observed to swim away or dive upon release; however, subsequent possible capture-related effects or mortalities are not known.

The samples collected for SIA consisted of a tissue punch from the dorsal flank of the fish behind the fin (2 mm ∅) including skin and subcutaneous capsule material (Davenport et al. 2018). Background samples of representative taxa of local putative prey and primary producers were collected by snorkel surveys from several locations along the Ligurian coastline (with permission from Area Marina Protteta di Portofino authorities), purchased from local markets or donated from unwanted discards by local fishermen; see Table S1 for additional details. Additional samples of stranded or bycaught ocean sunfish from other locations were collected opportunistically or donated by colleagues (Table S1). All samples were dried at 60°C for 12 h upon collection and later frozen for storage or frozen directly and shipped to the University of New Mexico Center for Stable Isotopes (UNM–CSI, Albuquerque, NM) for subsequent processing and stable isotope analysis.

2.2. Bulk tissue $\delta^{13}C$ and $\delta^{15}N$ analysis

Once in the laboratory, a sub-set of sunfish skin samples was selected for analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope ratios to provide the broadest size range possible from each location sampled. This included 22 fish from Italy (40–97 cm TL), 6 from the northeastern USA (107–215 cm TL), 4 from Peru, (39–94 cm TL), 2 from the UK (72 and 76 cm TL) and 2 from the northwestern USA (each 41 cm TL). Since the majority of our sunfish samples were collected from Italy, locally collected background samples from the Italian site were also analysed for $\delta^{13}C$ and $\delta^{15}N$ (n = 59), to characterise the local ecosystem and consider putative prey (see Table S1).

Following bulk tissue analysis, selected sunfish skin samples (n = 36) were analysed for AA_{ESS} $\delta^{13}C$, and a subset of these (n = 12) was selected (across our measured size range: 41–215 cm TL) for AA $\delta^{15}N$ analyses. For both AA $\delta^{13}C$ and $\delta^{15}N$ analysis, ~7 mg of each *Mola* sample was hydrolysed to its constituent AAs in 1 ml of 6N hydrochloric acid (HCl) at 110°C for 20 h in tubes flushed with N_2 to prevent oxidation during hydrolysis. A selection of background primary producer samples from the Mediterranean Sea sites (n = 8, including representative green macroalgae and phytoplankton samples, see Table S1) were also hydrolysed in 1 ml of 6N HCL, with the exception of whole silica filters containing particulate organic matter (POM), which were hydrolysed with 1.5 ml of 6N HCl. Hydrolysed producer samples were passed through a cation exchange resin column (Dowex 50WX8, 100–200 mesh) to isolate AAs from other metabolites and filter silicates (for POM only) from our producer samples (Amelung & Zhang 2001). After hydrolysis/Dowex purification, AAs were derivatised to *N*-trifluoro-acetic acid isopropyl esters following established protocols (O'Brien et al. 2002, Newsome et al. 2011,

2014). Samples were derivatised in batches of 8–17 along with a house-made reference material containing all AAs for which we obtained δ^{13}C and δ^{15}N values from our samples.

δ^{13}C and δ^{15}N values of derivatised AAs were measured using a gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) system. Derivatised samples were injected into a 60 m BPx5 gas chromatograph column for AA separation (0.32 ID, 1.0 μm film thickness; SGE Analytical Science) in a Thermo Scientific Trace 1300, combusted into CO_2 gas via a Thermo Scientific GC Isolink II, and analysed with a Thermo Scientific Delta V Plus IRMS at UNM-CSI. Samples were run in duplicate and bracketed with our internal AA standard. For δ^{13}C analysis, samples were rerun if any AA$_{ESS}$ exhibited standard deviations >0.7‰ across injections. For the analytically more challenging δ^{15}N analysis, we set this threshold to 1.0‰. The within-run standard deviations of measured δ^{13}C values among AA$_{ESS}$ in the reference material ranged from 0.1‰ (leucine) to 2.1‰ (glycine). The within-run standard deviations of measured δ^{15}N values among AA$_{ESS}$ in the reference material ranged from 0.2‰ (serine) to 1.2‰ (glutamic acid).

We reliably measured AA δ^{13}C and δ^{15}N values of 11 AAs: alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), serine (Ser), isoleucine (Ile), leucine (Leu), lysine (Lys), phenylalanine (Phe), threonine (Thr) and valine (Val). Of these, we used the essential AAs Ile, Leu, Lys, Phe, Thr and Val for AA fingerprinting (as we can assume minimal isotopic alteration through trophic levels), and for AA δ^{15}N-based estimates of trophic level, we used data for Phe (source) and Glu (trophic). The reagents used during derivatisation add carbon to the side chains of AAs, and hence raw δ^{13}C values measured via GC-C-IRMS reflect a combination of the intrinsic AA carbon and reagent carbon (Silfer et al. 1991); see Text A1 in the Appendix for correction equation. AA δ^{15}N reflects only intrinsic molecular nitrogen, and for these analyses we corrected samples within each run using known δ^{15}N values of our internal AA standard. All data are available in Table S1.

2.3. Data analysis

Isotope data were plotted with 'ggplot2' (Wickham 2009), a coding package within R (R version 3.2.2; R Core Team 2015). Bulk tissue stable isotope values were used to (1) compare *Mola* values relative to putative prey and local fish species (see Fig. 1), (2) assess isotopic patterns against *Mola* size (see Fig. 2)

and (3) estimate consumption of different prey through mixing models (see Fig. 3; Stock & Semmens 2013). Putative prey were selected using published gut content analyses (e.g. Fraser-Brunner 1951, Sousa et al. 2016) and arranged *a priori* into 5 functional groups: pelagic zooplankton, benthic grazers, benthic scavengers, sessile filter feeders and pelagic fisheries discards; sources within each functional group exhibited equality of means (*t*-tests, p < 0.05). Once data were pooled and the mean isotope values and associated standard deviations were calculated, we used Stable Isotope Analysis in R (SIAR) (Parnell et al. 2008) to fit a Bayesian model to estimate the relative contribution of putative prey sources to sunfish diet. Mean (± SD) trophic discrimination factors (TDFs) were estimated as 2.0 ± 1.3‰ for δ^{13}C and 3.0 ± 1.2‰ for δ^{15}N based on fish muscle values published by McCutchan et al. (2003) and used by Syväranta et al. (2012) and Harrod et al. (2013). The models were then run following the methods of Inger et al. (2013). Models were run for sunfish grouped into 3 size classes (< 0.5, 0.5–0.75 and >0.75 m TL) to consider potential ontogenetic changes in dietary contributions.

Linear discriminatory analyses (LDAs) were used to classify sunfish samples collected globally by comparing their AA$_{ESS}$ δ^{13}C values to potential carbon sources (clustered into bacteria, macroalgae and microalgae groups). We selected primary producer and bacteria data from the dataset provided by Larsen et al. (2013), which included representative macroalgae (genera: *Cystoseira*, *Laminaria*, *Macrocystis*, *Pelvetiopsis*, *Petrospongium*, *Ralfsia*, *Scytosiphon*, *Silvetia*, *Calliarthon*, *Corallina*, *Endocladia*, *Mastocarpus* and *Odonthalia*) and pelagic microalgae (genera: *Adhnanthes*, *Emiliana*, *Isochyrsis*, *Prasinocladus* and *Stauroneis*). Additional primary producer samples collected from sites near Camogli, Italy, were added to the producer dataset, including filtered microalgae and macroalgae from the genera *Ulva* and *Dictyota*. The final primary producer AA$_{ESS}$ δ^{13}C data were used as a training dataset with cross validation reporting 84% classification success. Sunfish were then included in the LDA to be classified with potential producers; we assumed no δ^{13}C trophic discrimination for any AA$_{ESS}$. The LDA was first run to identify the producer groups associated with the Mediterranean sunfish (see Fig. 4A), and then using the same parameters we ran the global sunfish dataset to consider whether similar patterns were observed across broader geographic scales (see Fig. 4B).

We used AA δ^{15}N values to estimate the relative trophic position of *Mola*. Since species-specific AA

δ^{15}N TDFs are not available for sunfish, here we estimated relative trophic position using δ^{15}N data for the canonical trophic AA Glu minus that of the source AA Phe. This calculation considers geographical variation in isotopic baselines to provide a relative measure of sunfish trophic position with larger Glu – Phe δ^{15}N offsets indicative of feeding at a higher trophic level.

3. RESULTS

The Mediterranean ocean sunfish dataset provided the greatest size range of fish from a single location in this study, and as such, these data were used to consider our first 2 aims: (1) to unravel the trophic role of ocean sunfish and (2) to explore the potential for ecological ontogenetic shifts. To consider the trophic ecology of Mediterranean sunfish relative to the local ecosystem, their bulk δ^{13}C and δ^{15}N isotopic values (corrected for trophic discrimination) were plotted alongside the mean values of locally caught coastal species and offshore catches (from depths <1000 m) that were discarded in nearshore waters (Fig. 1). Overall, it was apparent that δ^{13}C and δ^{15}N values differed between taxa, with ^{13}C-depleted (mostly pelagic species) and ^{13}C-enriched (mostly benthic species) spread across ^{15}N-enriched (higher

trophic position) and ^{15}N-depleted (lower trophic position) spaces. Individual sunfish δ^{13}C and δ^{15}N values extended across these broad groups, with the bulk of sunfish samples appearing substantially ^{13}C- and ^{15}N-enriched beyond their putative gelatinous prey (although this excludes a small cluster of larger-bodied individuals which were more associated with gelatinous zooplankton).

When sunfish bulk δ^3C and δ^{15}N values were considered relative to total body length (TL; cm), the results indicated a strong ontogenetic shift, which was apparent between the 20 cm TL size increments. Although individual variation occurs, these data suggest there is a significant decline in δ^{13}C (R^2 = 0.67, p < 0.0001) and δ^{15}N (R^2 = 0.80, p < 0.0001) values ontogenetically by ca. −3‰ and −2.5‰, respectively, between body sizes of ~40 and ~90 cm. To visualise isotopic variability against fish length as a continuum, both δ^{13}C and δ^{15}N values were then plotted against individual TL (Fig. 2). These results reveal considerable individual variation, but with a strong overall decline in both δ^{13}C and δ^{15}N with TL.

Mixing models were then employed to estimate the contribution of different prey groups for Mediterranean sunfish ontogenetically, with individuals grouped into 3 size classes (<50, 50–75 and >75 cm TL; Table 1, Fig. 3). These results suggested that smaller sunfish (<50 cm TL) fed predominately on benthic taxa which formed ~57% of prey for this life stage (modal values; ~28% benthic grazers and ~29% sessile filter feeders). Although the majority of prey items were benthic in origin, the diet of smaller sunfish was also supplemented by a significant proportion of pelagic gelatinous zooplankton which formed ~27% of prey. As sunfish increased in size, mixing models indicated that their diet incorporated increasingly more gelatinous prey, from ~27 to ~72% over the size range noted here; however, it must be noted that even the largest Mediterranean individuals appear to supplement their gelatinous prey intake with other prey taxa.

To consider potential ontogenetic shifts in sunfish carbon sources, the Mediterranean sunfish AA_{ESS} δ^{13}C samples were run in an LDA with fish grouped into 20 cm size classes (Fig. 4A) alongside a broad producer dataset. The resulting LDA suggested
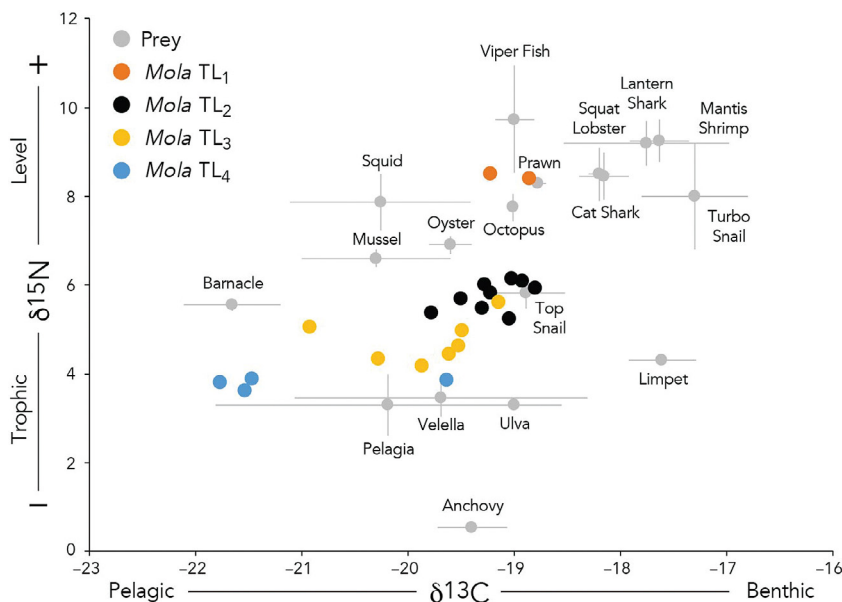

Fig. 1. Bulk tissue δ^{15}N and δ^{13}C values (‰) for Mediterranean ocean sunfish grouped into size classes by total length (TL) corrected using trophic discrimination factors estimated as 2 ± 1.3‰ for C and 3 ± 1.2‰ for N (McCutchan et al. 2003, Syväranta et al. 2012). Size classes were TL_1: 20–40 cm, TL_2: 40–60 cm, TL_3: 60–80 cm and TL_4: 80–100 cm. Also shown are mean (±SD) values for local taxa and putative prey (labelled with common names; also see legend of Fig. 3)
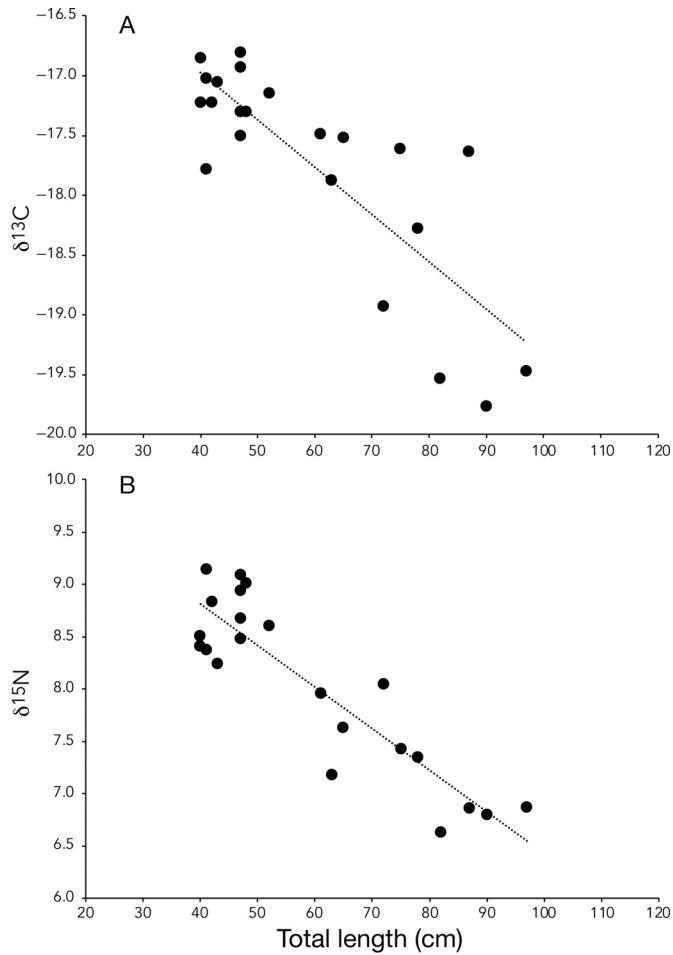
Fig. 2. Variation in bulk stable isotope values (‰) for Mediterranean sunfish, suggesting a strong decline in both (A) δ^{13}C ($R^2 = 0.67$, p < 0.0001) and (B) δ^{15}N with size ($R^2 = 0.80$, p < 0.0001)

that the *Mola* AA$_{ESS}$ δ^{13}C fingerprint most closely classified with microalgae, although it was noted that smaller individuals had a significant association with macroalgae (representing approximately a third of all carbon sourced by fish <40 cm), with a shift towards microalgae as fish TL increased. This evidence supports our proposed dietary shift from more ben-

thic to pelagic prey with increasing body size. For data tables of LDA classifications, see Table S2a in the Supplement.

To extend our analyses beyond a single population and consider sunfish isotopic patterns on a broader scale (the third aim of this study), bulk values of δ^{13}C and δ^{15}N were plotted from sunfish samples collected around the world (Fig. 5). The results indicated broad patterns of population clustering between Mediterranean, Atlantic and Pacific Ocean basins. Within these strong groupings, we noted that Pacific specimens sampled from Peru and the west coast of the USA clustered together, whereas samples collected from the east coast of the USA clustered with 1 sample collected from England. Aside from these groupings, there were several notable outliers, including 1 specimen from the east coast of the USA with a significantly lower δ^{15}N value (δ^{13}C: −17.3‰, δ^{15}N: 9.9‰), that was more closely associated with the Mediterranean fish. Also noted as an outlier was a sunfish sampled from Northern Ireland that was most closely grouped with samples from the Pacific. The bulk SIA values from each region have a relatively broad range, particularly of δ^{13}C, and in the Mediterranean data at least, we are confident this represents a shift in diet and habitat use within the population (range of isotopic values for each location: Mediterranean δ^{13}C: −19.7 to −17.0‰, δ^{15}N: −9.1 to −6.6‰; Atlantic δ^{13}C: −18.6 to −15.1‰, δ^{15}N: −12.6 to −9.8‰; Pacific δ^{13}C: −20.9 to −17.7‰, δ^{15}N: −13.6 to −11.3‰).

To consider the potential sources of primary production for sunfish over such broad scales, the AA$_{ESS}$ δ^{13}C of the global sunfish samples was then run in an LDA (Fig. 4B) with our collated producer dataset (data from Larsen et al. 2013 and from our samples of primary producers collected in Italy). The results suggested that most sunfish classified with microalgae sources (data detailing LDA classifications are shown in Table S2b). However, the data also suggest that a small proportion of smaller-

Table 1. Estimated contribution of different putative prey categories to the assimilated diet of Mediterranean ocean sunfish by size class, based on SIAR mixing models using bulk δ^{13}C and δ^{15}N values. TL: total length

| Prey functional group	<50 cm TL		50–75 cm TL		>75 cm TL	
	Prey proportion (mode) in diet	95% credibility interval	Prey proportion (mode) in diet	95% credibility interval	Prey proportion (mode) in diet	95% credibility interval
Pelagic jellies	0.27	0.27–0.28	0.46	0.45–0.46	0.72	0.71–0.72
Benthic grazers	0.28	0.28–0.29	0.18	0.17–0.23	<0.1	0.023–0.033
Benthic scavengers	<0.1	0.036–0.044	<0.1	0.12–0.017	<0.1	0.0086–0.011
Sessile filter feeders	0.29	0.29–0.3	0.22	0.21–0.23	<0.1	0.019–0.028
Fisheries discards	<0.1	0.047–0.054	<0.1	0.012–0.017	<0.1	0.0091–0.012
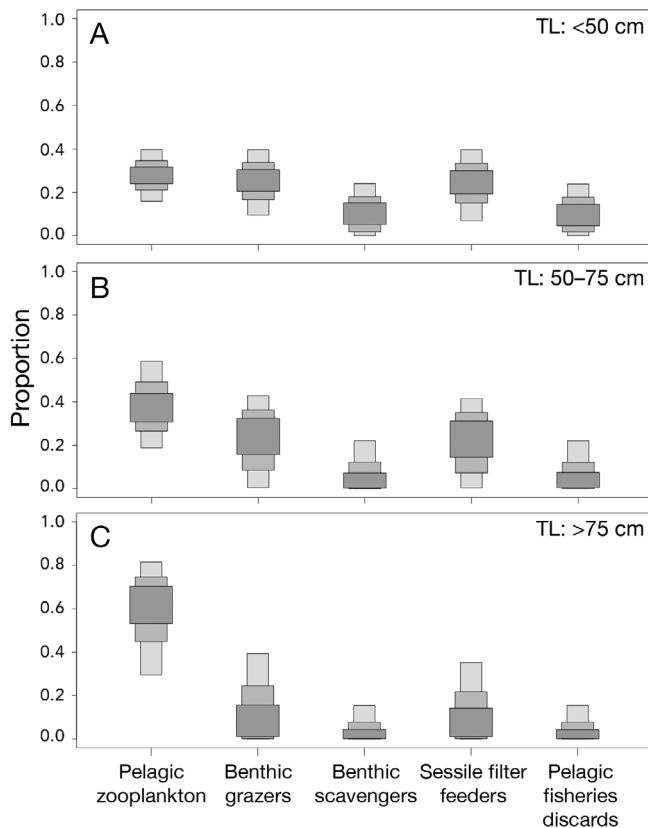
Fig. 3. Mean estimated proportions of putative prey using SIAR mixing models (grouped *a priori*) in the diet of sunfish from the Mediterranean Sea: (A) <50 cm, (B) 50–75 cm and (C) >75 cm total length (TL). The dietary proportions indicate credibility intervals at 25, 75 and 95%. Putative prey were categorised into the following functional groups: pelagic zooplankton, benthic grazers, benthic scavengers, sessile filter feeders and pelagic fisheries discards. For full data table, see Table 1

Fig. 4. Linear discriminatory analyses (LDA) of (A) Mediterranean and (B) global sunfish essential amino acid $\delta^{13}C$ values alongside fingerprinting datasets of primary producers. In panel A, total length (TL) size classes are TL_1: 20–40 cm, TL_2: 40–60 cm, TL_3: 60–80 cm and TL_4: 80–100 cm. In panel B, size classes are TL_1: <50 cm, TL_2: 50–100 cm, TL_3: 100–150 cm, TL_4: 150–200 cm

bodied individuals use macroalgae carbon sources, an input that significantly declines with increasing body size. Given the distribution of data relative to the putative energy sources, there may also be a missing source producer that has not yet been identified, or more variation between producer values than illustrated by this dataset. The AA $\delta^{15}N$ dataset provided further insight into the trophic position of sunfish at a global level (Fig. 6) through the comparison of AA $\delta^{15}N$ of trophic (Glu) and source AAs (Phe). The difference between these values (having removed local baseline effects) varied substantially (Fig. 6), indicating that smaller sunfish (<1 m TL) had variable trophic positions, in contrast to our limited sample size of larger individuals with lower, and less variable Glu – Phe values, indicating a lower (and less variable) trophic position.
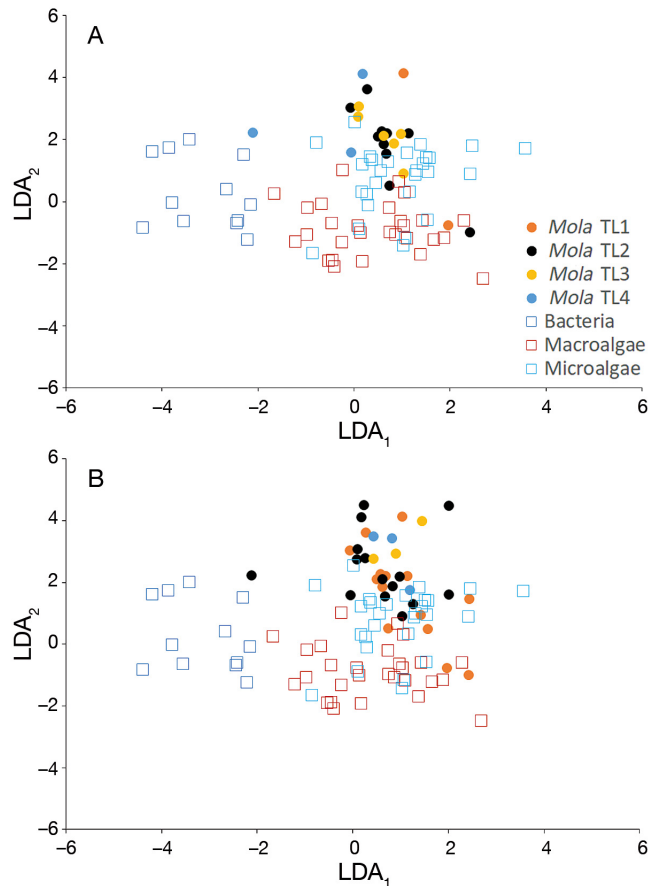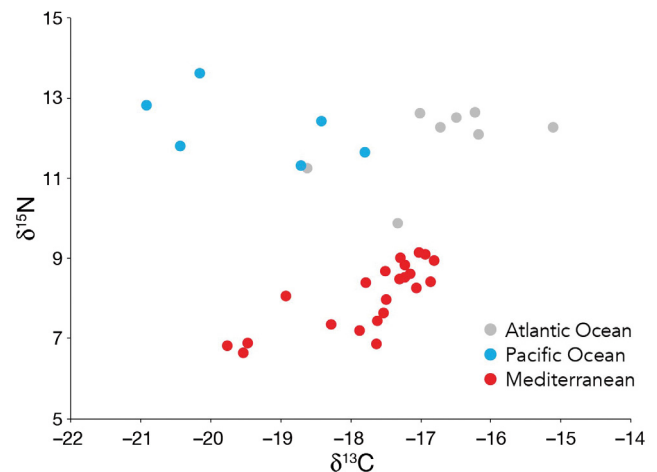

Fig. 5. Ocean sunfish bulk $\delta^{13}C$ and $\delta^{15}N$ values (‰) for individuals sampled globally
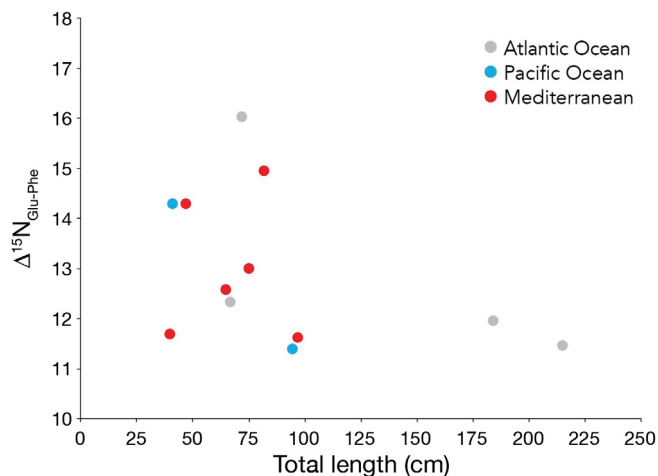
Fig. 6. Relative trophic position of ocean sunfish sampled globally based on amino acid $\delta^{15}N$ ($\Delta^{15}N = \delta^{15}N$ glutamic [Glu] acid – $\delta^{15}N$ phenylalanine [Phe]). For comparison, $\Delta^{15}N_{Glu-Phe}$ from other marine taxa has been estimated as $\Delta^{15}N_{Glu-Phe}$ ~2 for phytoplankton (trophic position 1), $\Delta^{15}N_{Glu-Phe}$ ~12 zooplankton (trophic position 2–3), $\Delta^{15}N_{Glu-Phe}$ ~15 for marine invertebrates (trophic position 2–4) and $\Delta^{15}N_{Glu-Phe}$ ~20 for marine fishes (trophic position 3–5) (Nielsen et al. 2015)

4. DISCUSSION

Our data provide novel insight into the complex ecology of ocean sunfish, with bulk tissue SIA and mixing models revealing evidence of a continuous ontogenetic shift from benthic to pelagic prey in the Mediterranean Sea. By extending this investigation using analysis of AA_{ESS} $\delta^{13}C$ 'fingerprints', and trophic-source AA $\delta^{15}N$ offsets, we were able to assess the baseline carbon contributions to diet in this population, and then consider the potential for such ecological patterns on a global scale. Given the current fisheries pressures on *Mola*, these data are highly valuable to understand their diet and habitat use, providing baseline information for conservation management and the inclusion of this widespread predator in ecosystem models.

4.1. Mediterranean sunfish bulk tissue $\delta^{13}C$ and $\delta^{15}N$

Our data revealed significant declines in Mediterranean sunfish $\delta^{13}C$ and $\delta^{15}N$ values as fish increased in size (Fig. 2). This was consistent with recent work proposing that sunfish show ontogenetic dietary and habitat shifts (Syväranta et al. 2012, Nakamura & Sato 2014, Sousa et al. 2016). Smaller individuals are suggested to target a mixed diet in nearshore waters be-

fore becoming increasingly dependent on gelatinous pelagic prey as body size increases (Nakamura & Sato 2014, Sousa et al. 2016). While previous studies have noted that such a shift may occur between sunfish grouped into broad size classes (e.g. Nakamura & Sato 2014), by incorporating smaller specimens, our results suggested that significant shifts in $\delta^{13}C$ and $\delta^{15}N$ values occur over a continuous scale, indicating a gradual dietary change to more pelagic prey (Fig. 2). Nakamura & Sato (2014) proposed that dietary shifts occurred once a given size threshold was reached (suggested to be ~1 m TL), but our data suggest that on a broader scale, the picture may be more nuanced. Although our data were collected from smaller fish, the results demonstrate a strong, gradual dietary shift rather than a significant change when a critical body size or other ecological threshold is attained. Although it is apparent that significant variability occurs (Fig. 2), possibly reflecting individual diet specialisation, the overall pattern from our bulk isotope data strongly indicated a gradual ontogenetic shift in diet and habitat in this population.

The suggestion of a continuous dietary shift from the bulk isotope data was further supported by isotope mixing model results, which indicated a gradual decreasing reliance on benthic prey, shifting towards greater proportions of gelatinous zooplankton with increasing body size (Fig. 3). Our results suggested that benthic grazers, filter feeders and fisheries discards are present in the diet of all size classes. The presence of benthic grazers (snails and limpets) alongside sessile filter feeders (mussels and oysters) may represent opportunistic feeding on naturally occurring molluscs; however, it is also possible that *Mola* sampled in the northern Mediterranean were consuming bivalves from local mariculture facilities, where rope-grown mussels are easily accessible in the water column. Our mixing model results indicated that fishery discards may also make a small contribution to assimilated diet, representing up to 5% of diet throughout ontogeny. Since many fishes scavenge on discards (e.g. Votier et al. 2010), it is perhaps unsurprising that sunfish, particularly those of smaller sizes which naturally target benthic prey, could opportunistically feed on fisheries waste, but this finding provides the first tentative evidence of scavenging in this taxon.

Nevertheless, the limitations of our isotope-based approach must also be considered, as mixing models will assign an estimated percentage contribution to all potential sources included in the models; therefore it is possible that the contribution of discards (~5%) or benthic scavengers (~4%) proposed in the

diet may have been exacerbated by model performance. While the relative importance of gelatinous prey (representing ~27–72% of diet in our sampled fish) is apparent in the mixing model results, the zooplankton species analysed here are by no means a complete evaluation of all taxa available in the Mediterranean. However, by including the dominant scyphozoan, *Pelagia noctiluca,* along with a seasonal surge of the hydrozoan *Velella velella*, we have incorporated typical prey and those opportunistically consumed under bloom conditions (Arai 2005, Hoving & Haddock 2017, Phillips et al. 2017).

4.2. Sunfish AA$_{ESS}$ δ^{13}C values

To expand our understanding of the potential basal sources of carbon that contribute to the diet of Mediterranean sunfish, we conducted AA$_{ESS}$ δ^{13}C fingerprinting analyses using an LDA with a global producer dataset (Larsen et al. 2013), together with samples of local producers (Fig. 4A). Given the observed patterns in bulk tissue SIA data, we expected to see evidence of a shift from benthic (e.g. macroalgae) to more pelagic (e.g. microalgae) derived carbon across ontogeny. Our AA$_{ESS}$ δ^{13}C data demonstrated a strong shift from mixed carbon sources (macro- and microalgae) to microalgae alone, with macroalgae featuring only in the diets of the smallest size classes (δ^{13}C source ~30% macroalgae: ~70% microalgae for fish ~20 cm TL, shifting to ~1% macroalgae: 91% microalgae for fish ~100 cm TL). However, the data also provided strong support for the use of microalgae-derived carbon throughout ontogeny. We suggest that this discrepancy was likely driven by consumption of prey fuelled by benthic (sessile) microalgae, which were expected to have relatively higher bulk tissue δ^{13}C values, but similar AA$_{ESS}$ δ^{13}C fingerprints as pelagic microalgae (phytoplankton). The potential contribution of benthic microalgae to ocean sunfish may be driven by the relatively deep euphotic depth in the Mediterranean Sea (50–200 m; Ferrari 2000, Marty et al. 2002, Mignot et al. 2011). It is worth noting that production of benthic microalgae can be high, contributing ~31% of total microalgal production in the Baltic Sea (Ask et al. 2016) and ~25% in the Mediterranean Sea (Riaux-Gobin et al. 1998), overall providing a substantial contribution to total primary production, particularly in coastal regions (Riera et al. 1996, Cahoon 1999, Glud et al. 2002, Yokoyama & Ishihi 2003, Ask et al. 2016). This finding highlights the importance of using a combination of bulk tissue and AA isotope

data from a wide range of functional groups to characterise habitat use by consumers in local food webs. Further sampling and analysis of both bulk tissue and AA isotope values will be required to assess whether microalgal sources can be differentiated into benthic/pelagic groupings and then used to determine localised carbon sources for sunfish in this region. There are some sunfish in the LDA not associated with any primary producer group, which may indicate that our AA$_{ESS}$ δ^{13}C dataset is missing a local carbon source. To fully investigate the carbon sources for sunfish ontogenetically, we suggest collecting a broader database of local producers for a more complete examination of benthic and pelagic carbon sources (Elliott Smith et al. 2018).

Following this assessment of the potential carbon sources for Mediterranean sunfish, we then pooled our AA$_{ESS}$ δ^{13}C samples collected from across the world, to consider patterns in basal carbon use on a global scale (Fig. 4B). These LDA data again highlighted the role of microalgae-derived carbon in fuelling the resources sunfish consume throughout ontogeny, with a small, declining classification with macroalgae as TL increased (from ~3 to 0% as fish TL increased from ~50 to 250 cm). It appeared that macroalgae-derived carbon was only used by the smallest individuals (~20 cm TL), and we suggest that the benthic microalgae pathway may also be important here. Further studies should seek to distinguish this from pelagic microalgae carbon sources, e.g. if AA$_{ESS}$ δ^{13}C values differ between benthic and pelagic microalgae (Vane et al. 2018). Assessing additional isotopes, such as bulk δ^{34}S may also help to disentangle benthic and pelagic sources in future studies (Hobson 1999). Such further work would provide vital new insight, to establish if AA$_{ESS}$ δ^{13}C data alone can be used to differentiate between benthic and pelagic sources and then to confirm if benthic microalgae are making important contributions to local ecosystems.

4.3. Global patterns in sunfish bulk tissue δ^{13}C and δ^{15}N values

Despite the need for further clarity on microalgae sources, the bulk SIA and mixing model results enabled us to answer the primary aims of this study. Firstly, that the trophic role of sunfish appears more varied than previously recognised, with evidence of highly opportunistic feeding and scavenging, particularly amongst smaller size classes. Secondly, we found evidence of a strong, continuous ontogenetic shift in our Mediterranean sunfish dataset, with

smaller sunfish likely functioning as part of the sub-tidal benthic food web, slowly incorporating more pelagic, gelatinous prey as body size increases.

Our third aim was to identify patterns in sunfish trophic role on a global scale, which we assessed via bulk tissue $\delta^{13}C$ and $\delta^{15}N$ values, as well as AA $\delta^{15}N$ analysis of sunfish collected in different ocean basins (Figs. 5 & 6). These data provided new insight into sunfish ecology, revealing significant variation in baseline values between oceans, as noted in similar studies comparing differing oceanic water masses (St. John Glew et al. 2019). Our results suggest that bulk tissue isotope values can identify population structuring of sunfish and have the potential to assess individual long-distance movements. Outside of the distinct regional groupings, a few individuals were noted to have isotope values that appear as outliers, which may represent recent migrants which have not yet reached isotopic equilibrium with local isotopic baselines. For example, the outlier specimen with relatively low carbon and nitrogen isotope values that was sampled off the coast of New England, USA, ($\delta^{13}C$: −17.3‰, $\delta^{15}N$: 9.9‰) likely travelled from an area with low baseline $\delta^{15}N$ values such as the Mediterranean, eastern Atlantic or Sargasso Sea (it has been suggested that sunfish spawn in the Sargasso Sea; T. Thys pers. comm.). In a similar vein, the outlier specimen sampled off the coast of Cornwall, England, ($\delta^{13}C$: −16.2‰, $\delta^{15}N$: 12.6‰) that clustered with samples collected in New England, USA, may have made the opposite journey; isotope values of this specimen place it well outside of the existing isoscapes of the capture region in the northeast Atlantic (St. John Glew et al. 2019). However, further detailed study of sunfish movements would be required to fully explore this concept.

The isotopic differences shown by these 'outlier' sunfish are notably similar to those of migratory Mediterranean and North Atlantic fin whale *Balaenoptera physalus* populations, which have been successfully tracked across oceans using SIA (Giménez et al. 2013). The potential for such movements will remain speculative pending further detailed analysis of individual ranges via satellite telemetry and population structuring via genetic analysis to explore the potential for sunfish to undertake trans-Atlantic crossings and attempts to locate potential spawning grounds. Even without precise information on fish movements, however, the new insights into sunfish distribution patterns provided by our results are of great interest, as the potential for isotopic data to identify population structure (Harrod et al. 2005) and long-distance movements is highly significant

for stock management (Hoffman 2016). In light of the current mass bycatch of sunfish around the world, this finding may facilitate identification of discreet stocks and prediction of fisheries bycatch impacts, along with the potential for the replenishment of depleted populations by external migrants. This is of particular importance given the recent realisation that the systematics of fishes in the genus *Mola* is far more complicated than previously recognised (Nyegaard et al. 2017, Sawai et al. 2018). As such, it is likely that a combination of genetic and isotopic research will be required to classify populations into their respective species and identify individual stocks (Nyegaard et al. 2017, Sawai et al. 2018).

4.4. Sunfish AA $\delta^{15}N$ values

We used the $\delta^{15}N$ of individuals' AAs to estimate relative trophic position of ocean sunfish globally. Since precise $\delta^{15}N$ TDFs for specific trophic AAs are unavailable for sunfish, we calculated the difference ($\Delta^{15}N$) in the $\delta^{15}N$ values of the canonical trophic (Glu) and source (Phe) AAs to estimate relative trophic level (Fig. 6). This enabled direct comparison of each fish's trophic AA value minus local baselines, which likely vary significantly as suggested by our global bulk tissue dataset. The results indicated that individual smaller ocean sunfish may occupy a range of trophic levels, perhaps due to differing localised availability of prey taxa or higher competition for resources in coastal environments that drives diversification of individual diet. $\Delta^{15}N_{Glu-Phe}$ values generally decline with increasing body size, suggesting that the trophic position of larger sunfish (>100 cm TL) is lower than the average trophic level of smaller individuals (Fig. 6). Interestingly, smaller individuals (<100 cm TL) appear to have more trophic plasticity than larger ones. Overall, these patterns suggest that smaller fish have a more diverse diet relative to larger fish and that the trophic niche width of sunfish decreases ontogenetically as they specialise on pelagic gelatinous prey as they grow. This interpretation is supported by the bulk isotope data from the Mediterranean sunfish, illustrating a strong, continuous ontogenetic shift in diet (Fig. 2), with the proportion of gelatinous zooplankton prey increasing from 27 to 72% as fish grow from <50 to >75 cm TL. Since the sunfish for this study were sampled across different ocean basins, they are likely to feed on differing local species, but the data suggest they may operate at similar functional levels as indicated by similar $\Delta^{15}N_{Glu-Phe}$, narrowing their trophic niche as they grow.

4.5. Conclusions

We identified a clear, continuous ontogenetic shift in the diet and habitat use of Mediterranean ocean sunfish, with the possibility of trophic niche narrowing with increasing fish size. These data confirmed that ocean sunfish are not obligate gelativores throughout their life history, but have a complex trophic role with differing feeding modes ontogenetically. The primary producer data indicated an intriguing relationship between ocean sunfish size and carbon source in the Mediterranean Sea, and here we recommend further research to consider additional local producers and if possible, to differentiate between benthic and pelagic microalgae using AA_{ESS} $\delta^{13}C$ fingerprinting. Such research would enable us to confirm the basal carbon sources used by sunfish and other taxa throughout ontogeny and over broader spatial scales.

This study has also highlighted the potential for SIA to identify individual sunfish movements, which may be of considerable use to assess population structure and connectivity. Since ocean sunfish are subject to unregulated target fisheries and high bycatch globally that removes 100 000s of individuals per year, our data are important by providing evidence of population structure at the basin scale, potential trans-Atlantic movements and a broader trophic role than previously recognised. When combined, these elements suggest there may be broad ecological consequences if the mass removal of this genus continues, highlighting an urgent need for active conservation management.

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Appendix. Equations for correcting amino acid $\delta^{13}C$ data.

Text A1. The reagents used during derivatization add carbon to the side chains of amino acids, and so the raw $\delta^{13}C$ measured via GC-C-IRMS is a combination of original amino acid carbon and reagent carbon (O'Brien et al. 2002, Newsome et al. 2014). However, because amino acid standards of known $\delta^{13}C$ composition were derivatized and run with each batch of samples, we were able to correct for this carbon addition for each amino acid using the following equation (from O'Brien et al. 2002, Newsome et al. 2011, 2014):

$$\delta^{13}C_{sample} = \frac{\delta^{13}C_{dsa} - \delta^{13}C_{dst} + \left(\delta^{13}C_{std} \times p_{std}\right)}{p_{std}}$$

Here, $\delta^{13}C_{dsa}$ refers to the measured value of the derivatized amino acid within the sample and $\delta^{13}C_{dst}$ refers to the measured value of the same derivatized amino acid within the internal standard. $\delta^{13}C_{std}$ is the known un-derivatized value of that amino acid in the standard (measured via EA-IRMS), and p_{std} is the proportion of carbon in the measured derivative that was originally sourced from the amino acid, which varies quite substantially among amino acids.