



# Zinc isotope composition of enameloid, bone and muscle of gilt-head seabreams (*Sparus aurata*) raised in pisciculture and their relation to diet

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Received: 7 August 2023 / Accepted: 18 December 2023

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## Abstract

The isotope ratios of zinc ( $^{66}\text{Zn}/^{64}\text{Zn}$  expressed as  $\delta^{66}\text{Zn}$ ), a vital nutrient, increasingly demonstrate trophic discrimination among vertebrates, making  $\delta^{66}\text{Zn}$  a valuable dietary proxy for ecological, archaeological, and palaeontological studies. Given the novelty of the methodology, tissue-diet and tissue-tissue zinc isotope fractionation factors remain poorly understood and have so far only been studied in a few terrestrial mammals. Here, we investigate  $\delta^{66}\text{Zn}$  compositions of enameloid, bone, and white muscle of seven artificially-fed pisciculture gilt-head seabreams (*Sparus aurata*) from offshore Israel, in comparison to the Zn isotope composition of their diet. In addition, we also analysed  $\delta^{66}\text{Zn}$  values in the same tissues of wild-caught *S. aurata*, bluespotted seabream (*Pagrus caeruleostictus*) and grey triggerfish (*Balistes caprisiscus*) caught off the coast of Israel. We determine a tissue-diet  $\delta^{66}\text{Zn}$  offset for *Sparus aurata* of  $-0.04 \pm 0.09$  ‰ (2SD) for bone,  $-0.29 \pm 0.06$  ‰ (2SD) for enameloid, and  $-0.45 \pm 0.07$  ‰ (2SD) for white muscle. Wild-caught fish have much higher among-individual  $\delta^{66}\text{Zn}$  variability with values distinct from the pisciculture *S. aurata*, documenting a much more isotopically heterogeneous diet consumed by the wild individuals. Still, tissue-tissue  $\delta^{66}\text{Zn}$  differences in wild-caught individuals are close to those observed in the pisciculture ones with progressively lower  $\delta^{66}\text{Zn}$  values in the order bone > enameloid > white muscle. Our results demonstrate predictable tissue-diet and tissue-tissue  $\delta^{66}\text{Zn}$  differences among fish hard and soft tissues and can be applied to identify the  $\delta^{66}\text{Zn}$  values of dietary input, thereby informing trophic (palaeo)ecological reconstructions.

**Keywords** Zinc isotopes · Isotope fractionation factors · Trophic ecology · *Sparus aurata* · Pisciculture

Responsible Editor: K.Clements.

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## Introduction

Dietary zinc is a vital nutrient for animal health and has important functions in regulating gene expression and enzyme activity (Cousins 1998; Maret 2011; Maares and Haase 2020). Diets deficient in zinc can lead to high mortality rates, low growth rates, and other deficiency symptoms in fish (Ogino and Yang 1978). As such, zinc is of particular interest as a nutrient, especially for commercially relevant fish species, with research focussing on dietary zinc uptake and requirements (Serra et al. 1996; Isani et al. 2004; Nguyen et al. 2008), and bioaccumulation due to anthropogenic pollution (Clearwater et al. 2002; Reynders et al. 2008; Lozano-Bilbao et al. 2021). To our knowledge, however, zinc isotopes have not yet been used in vertebrate ecotoxicology or fisheries science context. Yet zinc isotope compositions ( $^{66}\text{Zn}/^{64}\text{Zn}$ ), reported as  $\delta^{66}\text{Zn}$  value, vary significantly within different tissues of mammals (Balter et al.

2010, 2013; Moynier et al. 2013; Mahan et al. 2018) and as such offer great potential to investigate in a tissue-specific approach Zn anthropogenic contamination, toxicity levels or dietary requirements in fish.

In addition,  $\delta^{66}\text{Zn}$  is increasingly used as a trophic level proxy in ecological, archaeological, and palaeontological studies (Jaouen et al. 2016a, 2016b, 2022; Bourgon et al. 2020, 2021; McCormack et al. 2021, 2022b). Because zinc is supplied to marine fish tissues through the diet, and branchial uptake via gills is negligible (Bury et al. 2003; Ranaldi and Gagnon 2008), zinc isotope ratios in fish tissues, as with mammals, document dietary zinc uptake and trophic levels (McCormack et al. 2022b, 2023). Zinc isotopes, typically measured in the mineral phase of vertebrate osseous tissues (i.e., bioapatite) for ecological purposes, demonstrate progressively lower values with an increase in the animal's trophic level (Jaouen et al. 2016a; McCormack et al. 2021). As such, zinc isotopes can be applied in a similar manner as the traditional trophic level tracer nitrogen isotopes ( $\delta^{15}\text{N}$ ), which in contrast to  $\delta^{66}\text{Zn}$ , generally increases in  $\delta^{15}\text{N}$  values higher up the food chain. Zinc isotopes can be analysed in archaeological or palaeontological remains (mainly teeth, i.e., enamel/enameloid) in the absence of organic matter (i.e., collagen) preservation necessary for traditional  $\delta^{15}\text{N}$  analysis (Bourgon et al. 2020, 2021; Jaouen et al. 2022; McCormack et al. 2022b). Furthermore, zinc isotopes have a great potential to enable more robust and refined trophic analyses of extant and extinct animals when combined with  $\delta^{15}\text{N}$  than possible by analysing  $\delta^{15}\text{N}$  alone (McCormack et al. 2021, 2022a, 2023; Leichliter et al. 2023). Accurately determining trophic levels is imperative for understanding foraging ecology, species interactions and effective management and conservation strategies (Horstmann-Dehn et al. 2012).

Despite the multi-disciplinary applicability of  $\delta^{66}\text{Zn}$ , its variability within and among tissues of an individual is still poorly understood. Only a few studies investigated tissue-diet fractionation factors, so far exclusively limited to terrestrial mammals, namely sheep, mice, and minipigs (Balter et al. 2010, 2013; Moynier et al. 2013; Mahan et al. 2018). Understanding tissue-diet  $\delta^{66}\text{Zn}$  fractionation factors is of importance for ecotoxicology to monitor Zn pollution in vertebrates and species-specific diet-borne Zn toxicity, for fisheries to optimise dietary Zn uptake and bioavailability, and in ecology for investigating dietary shifts within an individual or population (e.g., during ontogeny, migration).

Here we investigate tissue-diet zinc isotope fractionation within pisciculture gilt-head seabreams (*Sparus aurata*), a common Mediterranean aquaculture species, kept on a controlled pellet diet in cages offshore Central Israel. We compare the  $\delta^{66}\text{Zn}$  values of the pellets to those of enameloid, bone and white muscle from seven *S. aurata* individuals of similar size and weight. Further, we take

advantage of an ongoing marine life monitoring and protection program off the coast of Israel and compare tissue  $\delta^{66}\text{Zn}$  values among wild-caught *S. aurata*, bluespotted seabream (*Pagrus caeruleostictus*) and grey triggerfish (*Balistes caprisicus*) individuals. These taxa were chosen as all three species are of commercial relevance in the Mediterranean, live in a coastal habitat and generally feed on similar trophic levels and prey items (Tancioni et al. 2003; Hamida et al. 2009; Taieb et al. 2013; Goldman et al. 2016). Finally, because these wild specimens were caught in the same region in which the pisciculture is located, we can directly compare tissue  $\delta^{66}\text{Zn}$  values and variability among wild and control-fed individuals.

## Material and methods

### Material

All specimens used in this study were collected legally and ethically, and most are housed in the Osteological Collection, Institute of Geosciences, Johannes Gutenberg-University, Mainz, Germany. We analysed enameloid, jaw bone, and white muscle from seven *Sparus aurata* individuals, harvested for food consumption, from a pisciculture (Lev-Yam Aquaculture Ltd.) located 3 km offshore of Central Israel (Michmoret). The pisciculture has four 3600-m<sup>3</sup> tension leg cages (TLCs, Refamed, Italy) floating approximately 19 m above the seafloor (water depth 40 m) to which they are moored (Korzen et al. 2016). All *S. aurata* were harvested (June 2019) at body lengths of 250 to 270 mm and weights of 350 to 500 g (Supplementary Data 1). While the sizes of extruded pellets (Zemach Feedmill, Ltd, Israel) provided as diet along the fish life cycle varied, compositionally, the main pellet ingredients do not vary and there is no compositional variation at all for extruded pellets provided to fish > 40 g. The *S. aurata* were seeded into the open ocean cages at weights of 5 to 10 g where they remained until reaching commercial weights (350–500 g) between 12 and 16 months. Based on studies considering *S. aurata* tooth replacement (Elgendy et al. 2016; Sisma-Ventura et al. 2018) and feeding studies examining Zn uptake into different fish tissues (Serra et al. 1996; Sun and Jeng 1998), we consider all here analysed tissues of the pisciculture *S. aurata* to be in equilibrium with the diet.

In addition to the pisciculture *S. aurata* individuals, we also analysed wild fish caught as part of an ongoing monitoring program (permit number 516192-4399), funded by the Israeli Ministry of Environmental Protection. These include two *S. aurata* caught in March of 2014 and 2017, one *Pagrus caeruleostictus* caught in March of 2017, four *P. caeruleostictus* and three *Balistes caprisicus* caught in March of 2020, two *P. caeruleostictus* caught in May of 2022 and

three *P. caeruleostictus* caught in December of 2022, all from Haifa Bay, Israel. Details on the fish individuals used in this study (including catalogue numbers) are reported in Supplementary Data 1 with all  $\delta^{66}\text{Zn}$  values.

## Tissue preparation

Fish specimens were dissected at the Israel Oceanographic & Limnological Research Institute, Haifa, Israel. White muscle tissue dissected from the dorsal musculature was rinsed with double distilled water, frozen, and lyophilised for 48 h. Freeze-dried samples were homogenised using a mortar and pestle, and then dried at 60 °C. Jaws were soaked in double distilled water for soft tissue removal. Four *P. caeruleostictus* and three *B. caprisicus* jaws of individuals caught in March of 2020 were soaked in water from the main tap of the Israel Oceanographic & Limnological Research Institute Chemistry department (referred to, from here on, as tap water). In contrast to the jaws soaked in double distilled water, the jaw bones soaked in tap water have taken up Zn from the tap water; thus, these bone values are excluded from data interpretations (Supplementary Discussion 2).

## Zinc isotope analysis

The here presented dataset also includes 13 enameloid and two bone  $\delta^{66}\text{Zn}$  values from 13 individuals already presented in McCormack et al. (2022b). Additional enameloid was sampled from different teeth for 11 of these 13 individuals to investigate within individual Zn isotope variability. In addition to the already published 15  $\delta^{66}\text{Zn}$  values of McCormack et al. (2022b), this study comprises a total of 114 combined enameloid, bone, white muscle, and fish pellet  $\delta^{66}\text{Zn}$  values (Supplementary Data 1).

Prior to sampling tooth enameloid, dentine had been drilled out of the teeth, except in the 2022 caught wild *P. caeruleostictus* individuals, for which enameloid powder was drilled off the cleaned tooth cap. In both cases, enameloid drilling was done with a rotary tool equipped a diamond-tipped burr. Bone chunks were cut using a diamond-tipped cutting wheel. All enameloid caps, bone pieces, and muscle tissues were cleaned with ultrapure water (Milli-Q water) prior to digestion. The enamel and bone were dissolved in closed perfluoroalkoxy vials with 1 ml 1 M HCl on a hotplate for 1 h at 120 °C, then evaporated and re-dissolved in 1 ml 1.5 M HBr. White muscle tissue and fish pellets were mineralised by microwave digestion (10 min at 100 °C, 10 min at 180 °C in a multiwave PRO microwave, Anton Paar) in 50 ml PTFE-TFM bombs filled with 6 ml concentrated  $\text{HNO}_3$  at the department of Geography of the Johannes Gutenberg-University Mainz.

All pisciculture enameloid samples were prepared and measured for their Zn isotope compositions at the Max

Planck Institute for Evolutionary Anthropology (MPI EVA). One set of bone and muscle (after microwave digestion) was prepared and measured at the MPI EVA, while the other was prepared and measured at the Frankfurt Isotope and Element Research Center (FIERCE) of the Goethe University Frankfurt, together with some of the wild *P. caeruleostictus* enameloid samples (Supplementary Data 1). Column chromatography and isotope measurements were performed as described in McCormack et al. (2022b). Zinc purification was performed in two steps, following the ion exchange method adapted from Moynier et al. (2006), described in Jaouen et al. (2016a), which included for each batch (13 samples) a chemistry blank and matrix-matched reference standard (NIST SRM 1400, bone ash) to monitor contamination and Zn elution. One ml of AG-1  $\times$  8 resin (100–200 mesh) was placed in 10 ml hydrophobic interaction columns (Macro-Prep<sup>®</sup> Methyl HIC) cleaned twice with 5 ml 3%  $\text{HNO}_3$  and 5 ml ultrapure water and then conditioned with 3 ml 1.5 M HBr. Following the sample loading, 2 ml HBr was added to elute the matrix, followed by Zn elution with 5 to 8 ml  $\text{HNO}_3$ . Following the second column step, the solution was evaporated overnight at 100 °C and re-dissolved in 1 ml 2 to 3%  $\text{HNO}_3$  ready for plasma mass spectrometry.

Zinc isotopes were analysed using a Thermo Fisher Neptune Plus MC-ICP-MS at FIERCE and a Thermo Fisher Neptune MC-ICP-MS at MPI EVA. Instrumental mass bias is corrected by copper doping and standard bracketing after Maréchal et al. (1999). The reference material Zn Alfa Aesar-MPI was used for standard bracketing. All  $\delta^{66}\text{Zn}$  values are expressed relative to the JMC Lyon standard material (mass-dependent Alfa Aesar-MPI offset of +0.27 ‰ for  $\delta^{66}\text{Zn}$  (Jaouen et al. 2016a; McCoy-West et al. 2018)). Zn concentrations in the respective samples were estimated using a regression equation based on the Zn signal intensity (V) of three solutions with known Zn concentrations (150, 300 and 600 ppb). The  $\delta^{66}\text{Zn}$  measurement uncertainties per analytical session were determined from standard replicate analyses and ranged between 0.03 and 0.05 ‰ (2SD). Samples were typically measured in duplicate with mean analytical repeatability of 0.03 ‰ (2SD,  $n = 104$ ). Reference material NIST SRM 1400, prepared and analysed alongside the samples for each column chromatography batch, yielded inter-laboratory consistent  $\delta^{66}\text{Zn}$  values of  $+0.94 \pm 0.06$  ‰ (2SD,  $n = 19$ ) which compares favourably to mean values between +0.92 to +0.97 ‰ as reported elsewhere (Bourgon et al. 2020; Jaouen et al. 2020; Mahan et al. 2020; McCormack et al. 2021, 2023). Reference materials and samples show a typical Zn mass-dependent isotopic fractionation, i.e., the absence of interferences, as the  $\delta^{66}\text{Zn}$  vs.  $\delta^{67}\text{Zn}$  and  $\delta^{66}\text{Zn}$  vs.  $\delta^{68}\text{Zn}$  values fall onto lines with slopes close to the theoretic mass fractionation values of 1.5 and 2, respectively (Supplementary Data 1). Column

chemistry procedural blanks prepared alongside all samples document no relevant Zn contamination during sample dissolution and column chromatography as procedural blanks have average Zn signal intensities less than 0.1% compared to those from samples and in all cases less than 1.6%.

## Results and discussion

### Pisciculture *Sparus aurata* tissue-diet zinc isotope variability

All artificially pellet-fed pisciculture *Sparus aurata* individuals have homogenous mean  $\delta^{66}\text{Zn}$  values for bone ( $+0.24 \pm 0.09$  ‰ 2SD,  $n=7$ ), enameloid ( $0.00 \pm 0.06$  ‰ 2SD,  $n=7$ ), and white muscle ( $-0.16 \pm 0.07$  ‰ 2SD,  $n=5$ ; Table 1, Fig. 1). Pisciculture *S. aurata* zinc concentrations have tissue-specific ranges, decreasing from enameloid (545 to 1862  $\mu\text{g/g}$ ) to bone (65 to 99  $\mu\text{g/g}$ ) to white muscle (26 to 50  $\mu\text{g/g}$  dry wt, Fig. 1b, Supplementary Discussion 1). The intra- and inter-individual tissue-specific  $\delta^{66}\text{Zn}$  standard deviations are within the range of the standard deviation for replicate analysis of NIST SRM 1400 ( $+0.94 \pm 0.06$  ‰ 2SD,  $n=19$ ; Table 1, Fig. 1). The  $\delta^{66}\text{Zn}$  values of the 5 mm extruded pellets (Zemach Feedmill, Ltd, Israel) analysed from a single batch are also very homogenous with a mean of  $+0.29 \pm 0.02$  ‰ (2SD,  $n=6$ , Fig. 1a).

We assume that for the pisciculture *Sparus aurata* individuals, pellets are the main (and likely only) source of dietary zinc uptake, as these cages are floating too high above the seafloor for *S. aurata* individuals to reach their common benthic prey sources (Tancioni et al. 2003; Taieb

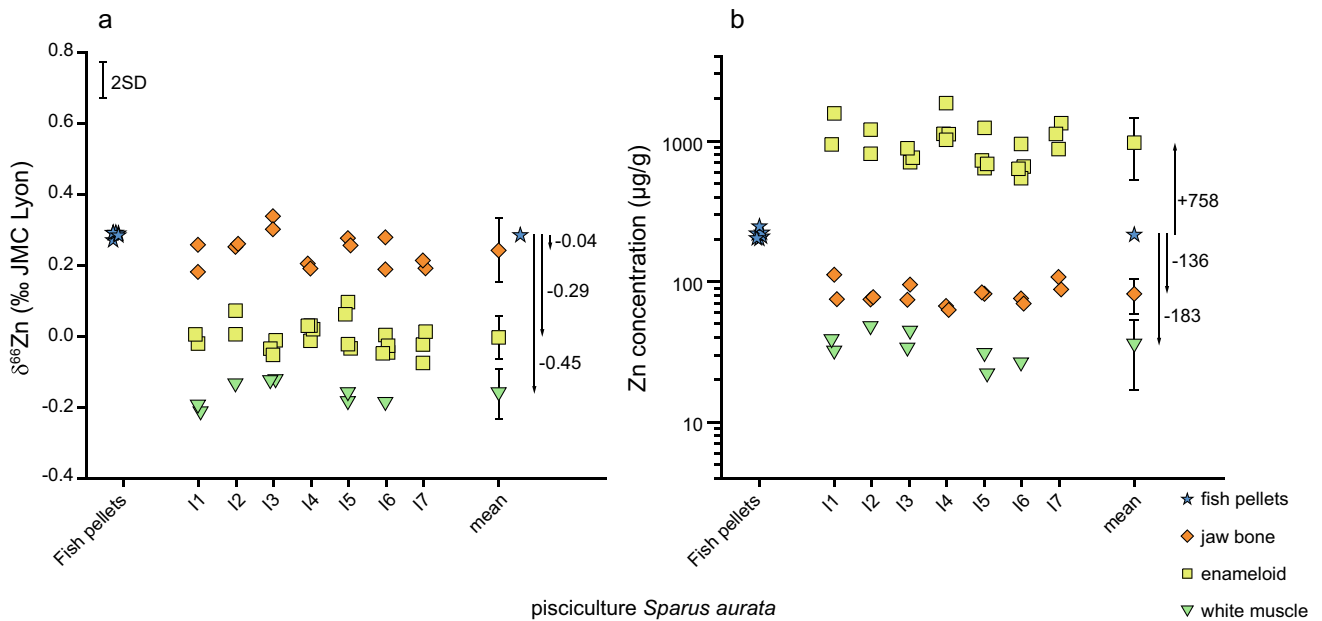
et al. 2013). In addition, the fish were fed twice a day with pellets weighing up to 2% of the total biomass in each cage (Korzen et al. 2016), making it unlikely that any diet other than the pellets could have contributed significantly to the dietary Zn uptake. Therefore, we consider the average *S. aurata*  $\delta^{66}\text{Zn}$  offsets between pellets and specific tissues ( $\Delta^{66}\text{Zn}_{\text{tissue-diet}} = \delta^{66}\text{Zn}_{\text{tissue}} - \delta^{66}\text{Zn}_{\text{diet}}$ ) as tissue-diet discrimination values. Mean tissue-diet  $\delta^{66}\text{Zn}$  discrimination for *S. aurata* are thus  $-0.04 \pm 0.09$  ‰ (2SD,  $n=7$ ) for bone ( $\Delta^{66}\text{Zn}_{\text{bone-diet}}$ ),  $-0.29 \pm 0.06$  ‰ (2SD,  $n=7$ ) for enameloid ( $\Delta^{66}\text{Zn}_{\text{enameloid-diet}}$ ), and  $-0.45 \pm 0.07$  ‰ (2SD,  $n=5$ ) for white muscle ( $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$ ) (Fig. 1a, Table 2).

Compared to previous feeding experiments on terrestrial mammals (sheep and mice), tissue-diet fractionation factors for *Sparus aurata* are notably different and may not be directly comparable (Fig. 2). Mice bone  $\delta^{66}\text{Zn}$  values are higher than dietary values with mean  $\Delta^{66}\text{Zn}_{\text{bone-diet}}$  values of  $+0.47 \pm 0.26$  ‰ (2SD,  $n=16$ , (Moynier et al. 2013)) and  $+0.25 \pm 0.12$  ‰ (2SD,  $n=4$ , (Balter et al. 2013)). While mice muscle  $\delta^{66}\text{Zn}$  values are also  $^{66}\text{Zn}$  depleted compared to their diet, the  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  values are higher compared to the pisciculture *Sparus aurata*, with mice  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  values of  $-0.19 \pm 0.61$  ‰ (2SD,  $n=5$ , (Moynier et al. 2013)) and  $-0.14 \pm 0.12$  ‰ (2SD,  $n=3$ , (Balter et al. 2013)). For sheep,  $\Delta^{66}\text{Zn}_{\text{bone-diet}}$  values and  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  values were reported to be similar to each other,  $+0.17 \pm 0.15$  ‰ and  $+0.18 \pm 0.28$  ‰, respectively (2SD,  $n=4$ , (Balter et al. 2010)). The reasons for the observed  $\Delta^{66}\text{Zn}_{\text{tissue-diet}}$  and  $\Delta^{66}\text{Zn}_{\text{tissue-tissue}}$  differences among previously reported feeding experiments and *S. aurata* remain ambiguous (Fig. 2). Besides potential physiological factors, perhaps linked to taxonomy, digestive physiology, and potential differences in Zn bioavailability

**Table 1** Mean zinc isotope values and zinc concentrations with 2SD (per individual) of pisciculture *Sparus aurata* tissues for all seven individuals analysed

Individual	Bone			Enameloid			White muscle		
	$\delta^{66}\text{Zn}$ (‰ JMC Lyon $\pm$ 2SD)	[Zn] ( $\mu\text{g/g} \pm$ 2SD)	<i>n</i>	$\delta^{66}\text{Zn}$ (‰ JMC Lyon $\pm$ 2SD)	[Zn] ( $\mu\text{g/g} \pm$ 2SD)	<i>n</i>	$\delta^{66}\text{Zn}$ (‰ JMC Lyon $\pm$ 2SD)	[Zn] ( $\mu\text{g/g} \pm$ 2SD)	<i>n</i>
1	$+0.22 \pm 0.11$	$93 \pm 52$	2	$-0.01 \pm 0.04$	$1261 \pm 889$	2	$-0.20 \pm 0.03$	$35 \pm 9$	2
2	$+0.26 \pm 0.01$	$76 \pm 4$	2	$+0.04 \pm 0.09$	$1010 \pm 556$	2	$-0.13$	48	1
3	$+0.32 \pm 0.05$	$85 \pm 30$	2	$-0.03 \pm 0.04$	$787 \pm 187$	3	$-0.12 \pm 0.00$	$39 \pm 15$	2
4	$+0.20 \pm 0.02$	$65 \pm 6$	2	$+0.02 \pm 0.04$	$1282 \pm 779$	4	n.a	n.a	0
5	$+0.27 \pm 0.03$	$83 \pm 3$	2	$+0.03 \pm 0.13$	$825 \pm 561$	4	$-0.17 \pm 0.04$	$27 \pm 12$	2
6	$+0.23 \pm 0.13$	$73 \pm 9$	2	$-0.03 \pm 0.05$	$698 \pm 355$	4	$-0.19$	26	1
7	$+0.20 \pm 0.03$	$98 \pm 28$	2	$-0.03 \pm 0.09$	$1114 \pm 463$	3	n.a	n.a	0
Mean $\pm$ 2SD	$+0.24 \pm 0.09$	$82 \pm 23$	7	$0.00 \pm 0.06$	$997 \pm 467$	7	$-0.16 \pm 0.07$	$35 \pm 18$	5

For enameloid samples, *n* indicates the number of teeth analysed, while *n* for bone and white muscle indicates the number of samples prepared individually via column chromatography and analysed in different laboratories. The lowermost row gives the mean of all individuals, and *n* indicates the total number of individuals for which tissue data is available. Individuals for which no muscle tissue was available are indicated (n.a.). Each pisciculture *S. aurata* tissue sample was analysed in duplicate with a mean  $\delta^{66}\text{Zn}$  2SD of 0.02 ‰. See also Supplementary Data 1 for more details



**Fig. 1** Zinc isotope values (‰ JMC Lyon) in (a) and zinc concentrations (µg/g) in (b) from fish pellets and enameloid, bone, and white muscle of pisciculture *Sparus aurata*. Mean pisciculture *S. aurata*  $\delta^{66}\text{Zn}$  values, and Zn concentrations, are provided relative to the fish pellets with whiskers indicating 2SD and arrows and numbers depict-

ing mean relative isotopic differences ( $\Delta^{66}\text{Zn}_{\text{tissue-diet}}$ ) and mean relative concentration differences. Maximum measurement uncertainty is given in a) in 2SD. Zinc concentration is plotted on a logarithmic scale

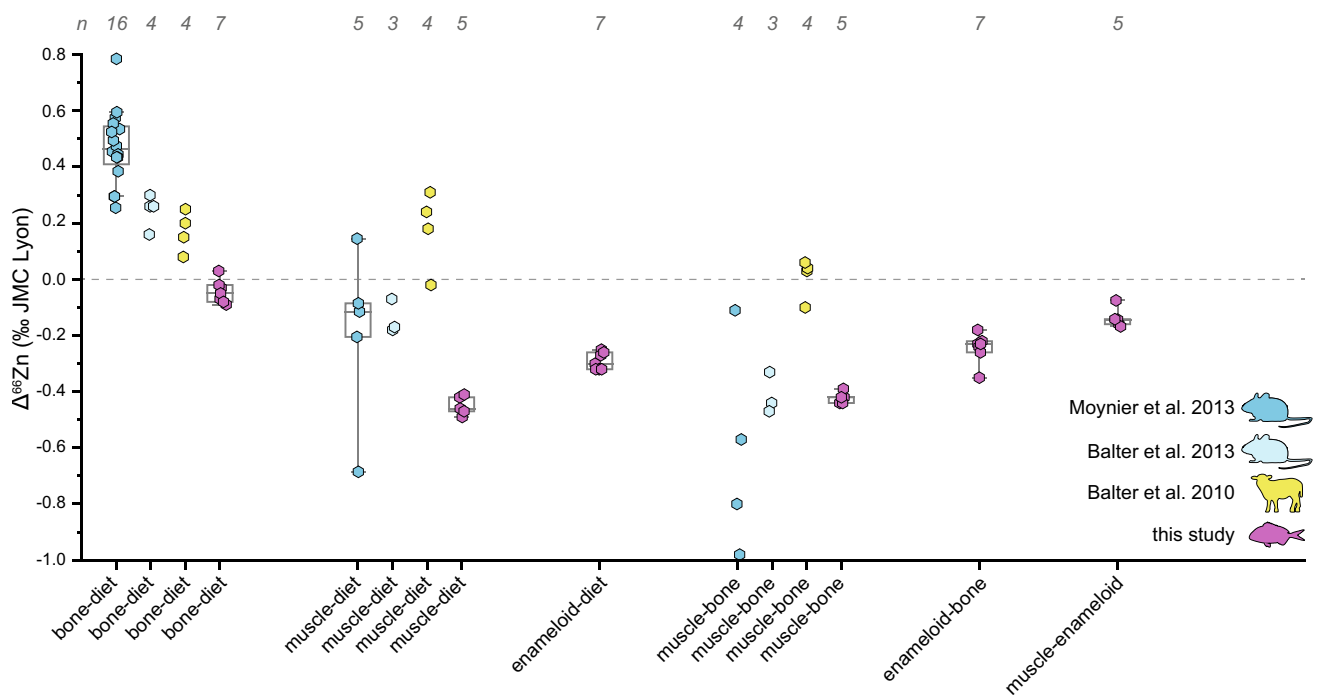
**Table 2** Offsets between mean tissue and mean diet  $\delta^{66}\text{Zn}$  values ( $\Delta^{66}\text{Zn}_{\text{tissue-diet}}$ ) and between mean tissues  $\delta^{66}\text{Zn}$  values ( $\Delta^{66}\text{Zn}_{\text{tissue-tissue}}$ ) for each pisciculture *Sparus aurata* individual

Individual	$\Delta^{66}\text{Zn}_{\text{bone-diet}}$	$\Delta^{66}\text{Zn}_{\text{enameloid-diet}}$	$\Delta^{66}\text{Zn}_{\text{muscle-diet}}$	$\Delta^{66}\text{Zn}_{\text{enameloid-bone}}$	$\Delta^{66}\text{Zn}_{\text{muscle-bone}}$	$\Delta^{66}\text{Zn}_{\text{muscle-enameloid}}$
1	-0.07	-0.29	-0.49	-0.23	-0.42	-0.16
2	-0.03	-0.25	-0.42	-0.22	-0.39	-0.14
3	+0.03	-0.32	-0.41	-0.35	-0.44	-0.08
4	-0.09	-0.27		-0.18	n.a	n.a
5	-0.02	-0.26	-0.46	-0.24	-0.44	-0.17
6	-0.05	-0.32	-0.47	-0.26	-0.42	-0.14
7	-0.08	-0.32		-0.23	n.a	n.a
Mean $\pm$ 2SD	$-0.04 \pm 0.09$	$-0.29 \pm 0.06$	$-0.45 \pm 0.07$	$-0.24 \pm 0.11$	$-0.42 \pm 0.04$	$-0.14 \pm 0.07$

All values are given in ‰ relative to JMC Lyon. Individuals for which no muscle tissue was available are indicated (n.a.)

of respective diets, differences might also relate to tissues not being in equilibrium with the diet. For example, the mice from Moynier et al. (2013) were euthanised at various intervals after birth, whereas the sheep in Balter et al. (2010) are significantly larger animals and were kept on the experimental diet for a limited time period only. Zinc metabolic turnover rates, which are largely unknown, differ among tissues perhaps also due to physiological factors such as body size and growth rate, as is the case for other dietary proxies such as nitrogen and calcium (Skulan and DePaolo 1999; Trueman et al. 2005).

Although the here reported  $\delta^{66}\text{Zn}$  tissue-diet fractionation factors differ from those reported for mice and sheep (Balter et al. 2010, 2013; Moynier et al. 2013), the tissue-tissue fractionation factors between bone and muscle of *S. aurata* and mice are similar. The pisciculture *S. aurata*  $\delta^{66}\text{Zn}$  muscle–bone fractionation factor ( $\Delta^{66}\text{Zn}_{\text{muscle-bone}}$ ) is  $-0.42 \pm 0.04$  ‰ (2SD,  $n = 5$ , Figs. 1, 2), while mice  $\Delta^{66}\text{Zn}_{\text{muscle-bone}}$  are  $-0.62 \pm 0.65$  ‰ (2SD,  $n = 4$ , (Moynier et al. 2013)) and  $-0.41 \pm 0.15$  ‰ (2SD,  $n = 3$ , (Balter et al. 2013)). The consistently lower  $\delta^{66}\text{Zn}$  values in muscle compared to bone may be tentatively explained by differences in the tissue-specific Zn coordination environment (Balter et al.



**Fig. 2** Tissue-diet and tissue-tissue fractionation factors within an individual approximated by the  $\Delta$  value (i.e.,  $\Delta^{66}\text{Zn}_{\text{tissue-diet}}$  and  $\Delta^{66}\text{Zn}_{\text{tissue-tissue}}$ ) for the pisciculture *Sparus aurata*, mice from Moynier et al. (2013) (blue) and Balter et al. (2013) (light blue), and sheep from Balter et al. (2010) (yellow). Each point represents a sin-

gle individual for which tissues and diet plotted on the x-axis could be analysed. The boxes, for  $n \geq 5$ , represent the 25th–75th percentiles (with the median as a horizontal line), and the whiskers show the 10th–90th percentiles. The number of individuals analysed is given by  $n$  in grey

2013; Moynier et al. 2013; Mahan et al. 2018). Heavier Zn preferentially binds to ligands with a stronger electronegativity ( $O > N > S$ ) (Balter et al. 2013; Moynier et al. 2013; Fujii et al. 2014). In bioapatite, Zn is bonded to oxygen atoms of one hydroxyl (OH) and three phosphate groups ( $\text{PO}_4$ ) (Tang et al. 2009), accordingly leading to an enrichment of heavy Zn. Conversely, Zn in muscle proteins binds to different amino acids, e.g., histidine in myosin (Ababou et al. 2008), where Zn is bonded to N (Maret 2012), which leads to a depletion of heavy Zn. However, ab initio calculations considering Zn coordination in muscle proteins for different muscle types and taxa, beyond the scope of our paper, would be necessary to quantify these results.

To our knowledge, no enamel(oid)-diet  $\delta^{66}\text{Zn}$  fractionation factors have been previously reported. Yet, the pisciculture *S. aurata* enameloid-bone  $\delta^{66}\text{Zn}$  offset ( $\Delta^{66}\text{Zn}_{\text{enameloid-bone}}$ ) of  $-0.24 \pm 0.11$  ‰ (2SD,  $n = 7$ ) is very similar to previously reported mean  $\Delta^{66}\text{Zn}_{\text{enamel-bone}}$  of  $-0.2$  ‰ in terrestrial mammals from Koobi Fora, Kenya (Jaouen et al. 2016a), the enamel-bone offset of  $-0.18$  ‰ in humans (Jaouen et al. 2017) and tooth enameloid-osteodentine offset in divers elasmobranch species of  $-0.21$  ‰ (McCormack et al. 2022b). Noteworthy, systematic isotopic offsets between enamel and bone/dentine were also observed for bioapatite carbonate–oxygen and –carbon

( $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ , (Webb et al. 2014)) and calcium isotopes ( $\delta^{44/40}\text{Ca}$ , (Heuser et al. 2011)) across different species, despite different metabolic functions of these elements. We thus assume that the  $\Delta^{66}\text{Zn}_{\text{enamel(oid)-bone}}$  offset is related to differences in enamel(oid)–bone mineralisation and maturation and that this offset might be relatively consistent among vertebrates in spite of differences in enamel and enameloid mineralisation (Sasagawa et al. 2009; Kawasaki 2013).

The lower body tissue  $\delta^{66}\text{Zn}$  values in pisciculture *Sparus aurata* compared to their diet are in good agreement with the decrease in bioapatite  $\delta^{66}\text{Zn}$  values within animals feeding at higher trophic levels (Jaouen et al. 2016a, 2016b; Bourgon et al. 2020, 2021; McCormack et al. 2021, 2022b). Indeed, *S. aurata*  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  values of  $-0.45 \pm 0.07$  ‰ (2SD,  $n = 5$ ) are very much in line with anticipated trophic level fractionation factors within the same tissue (e.g., enameloid) of a predator (or scavenger) and its prey. When assuming that tissue-tissue fractionation factors are constant among taxa and that muscle is the main digested tissue by a consumer, *S. aurata*  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  values suggest approximately 0.45 ‰ lower values within the same tissue of a predator compared to its prey. Previous trophic level  $\delta^{66}\text{Zn}$  spacing between predator and prey were reported to be between  $-0.32$  to  $-0.38$  ‰ (McCormack et al. 2021),  $-0.40$  to  $-0.50$  ‰

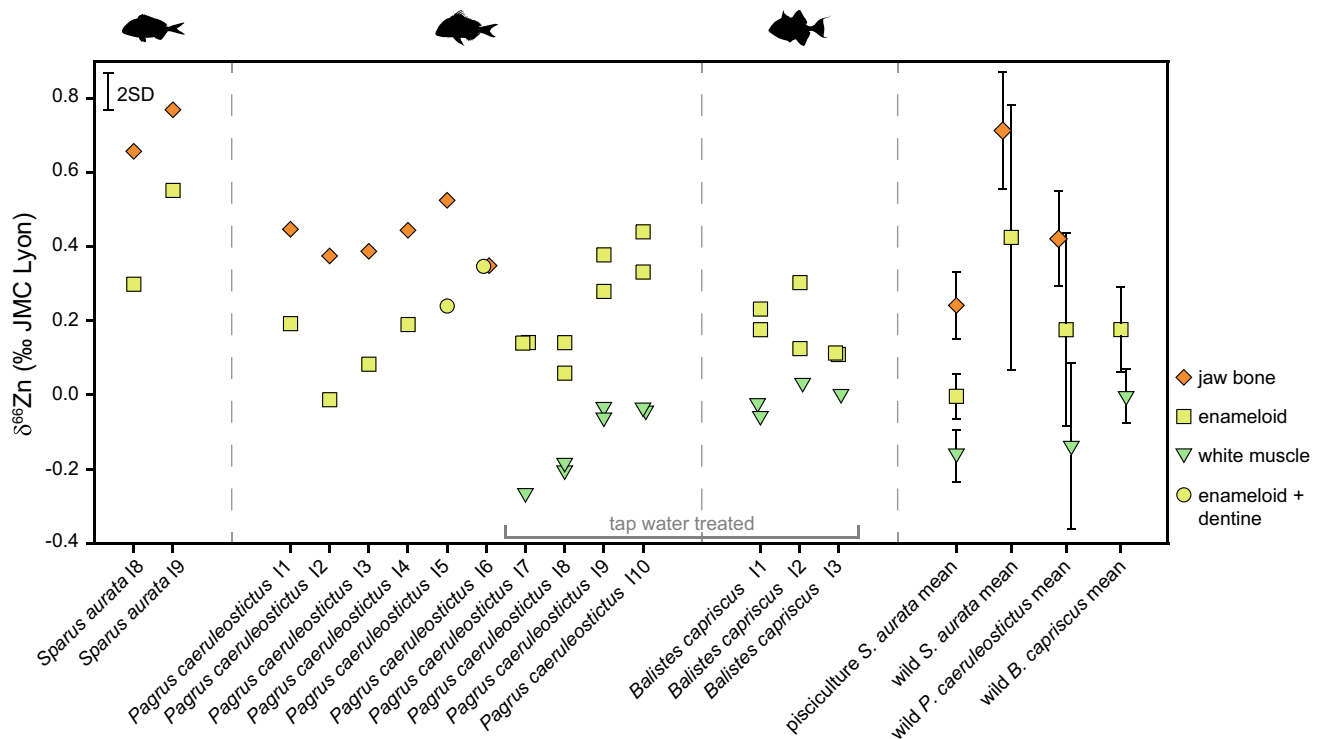
(Jaouen et al. 2016a) and  $-0.60\text{‰}$  (Bourgon et al. 2020) well within the wider range of the *S. aurata*  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  values.

All here examined pisciculture *Sparus aurata* tissues are  $^{66}\text{Zn}$  depleted compared to their diet (Fig. 1). Yet, we cannot exclude the possibility of other tissues being enriched in  $^{66}\text{Zn}$  compared to the dietary intake. Notably, Moynier et al. (2013) documented a constant enrichment of  $^{66}\text{Zn}$  in faeces and urine of mice relative to their diet. Although this remains to be tested, fish excretions may demonstrate a similar  $^{66}\text{Zn}$  enrichment. Besides faecal and urinal, these may even include Zn excretion via the gills, as, even though low seawater zinc concentrations lead to negligible Zn uptake via the gills, the gills are a possible excretion route for excess dietary Zn in fish (Hardy et al. 1987). All or most tissues being  $^{66}\text{Zn}$  depleted relative to the diet is in line with the observed lower  $\delta^{66}\text{Zn}$  values higher up the food chain, even though the actual trophic level offset will depend on which tissues (and their respective proportion) are consumed and the differences in Zn concentration and isotope composition in each tissue. For example, the constantly higher  $\delta^{66}\text{Zn}$  values in bone-consuming carnivores such as

hyenas compared to non-bone-consuming carnivores may be explained by the combined ingestion of higher  $\delta^{66}\text{Zn}$  bone and lower  $\delta^{66}\text{Zn}$  muscle-derived zinc (Jaouen et al. 2016a, 2022; Bourgon et al. 2020, 2021).

### Zinc isotope variability among wild fish

In contrast to the pisciculture individuals, wild-caught fish demonstrate a much larger Zn isotope variability among individuals (Fig. 3). For example, all enameloid  $\delta^{66}\text{Zn}$  values of the pisciculture *S. aurata* vary between  $-0.05$  and  $+0.10\text{‰}$  (mean of  $0.00 \pm 0.09\text{‰}$  2SD,  $n=22$ ), while wild *Pagrus caeruleostictus* enameloid varies between  $-0.01$  and  $+0.44\text{‰}$  (mean of  $+0.20 \pm 0.27\text{‰}$  2SD,  $n=12$ ). Wild-caught *S. aurata* individuals also have distinctly higher  $\delta^{66}\text{Zn}$  values than pisciculture ones, with bone values of  $+0.66$  and  $+0.77\text{‰}$  and enameloid values of  $+0.30$  and  $+0.55\text{‰}$  (Fig. 3). *Pagrus caeruleostictus* have bone  $\delta^{66}\text{Zn}$  value between  $+0.35$  and  $+0.53\text{‰}$  (mean of  $+0.42 \pm 0.13\text{‰}$  2SD,  $n=6$ ). *Balistes capriscus* have enameloid  $\delta^{66}\text{Zn}$  values between  $+0.11$  and  $+0.21\text{‰}$  (mean of  $+0.18 \pm 0.16\text{‰}$  2SD,  $n=6$ ). Mean white muscle values for *P. caeruleostictus*



**Fig. 3** Zinc isotope values (‰ JMC Lyon) in enameloid, bone and white muscle of pisciculture *Sparus aurata* (mean) and wild-caught fish from Haifa Bay. Mean pisciculture *S. aurata* ( $n=7$  for enameloid and bone, and 5 for white muscle) and wild fish individual Zn isotope values ( $n=2$  for enameloid and bone of *S. aurata*,  $n=3$  for *B. capriscus* white muscle and enameloid,  $n=4$ , 6 and 8 for *P. caeruleostictus* white muscle, bone and enameloid, respectively) are

depicted with whiskers indicating 2SD variability. Individuals caught in 2020, depicted by a grey bracket, were treated with tap water and their bones are contaminated by post-mortem Zn uptake. These bone values are thus not depicted here, see Supplementary Discussion 1, 2 and Supplementary Figs. 1, 2, and 3. Maximum measurement uncertainty is given in 2SD

individuals range between  $-0.27$  and  $-0.04$  ‰ (mean of  $-0.14 \pm 0.22$  ‰ 2SD,  $n=4$ ) and for *B. caprisicus* between  $-0.06$  and  $+0.03$  ‰ (mean of  $0.00 \pm 0.07$  ‰ 2SD,  $n=3$ , Supplementary Data 1, Fig. 3). The larger enameloid intra- and interspecific  $\delta^{66}\text{Zn}$  variability of wild-caught fish compared to the pisciculture individuals likely reflects a different and more variable diet for the former.

With the exception of the contaminated bone  $\delta^{66}\text{Zn}$  values of the 2020 caught *Pagrus caeruleostictus* and *Balistes caprisicus* (Supplementary Discussion 2), all wild-caught fish have similar  $\Delta^{66}\text{Zn}_{\text{tissue-tissue}}$  values as observed in the pisciculture *Sparus aurata*, with progressively lower  $\delta^{66}\text{Zn}$  values in the order bone > enameloid > white muscle (Fig. 1). For the two wild *S. aurata*,  $\Delta^{66}\text{Zn}_{\text{enameloid-bone}}$  values are  $-0.22$  and  $-0.36$  ‰. For four *P. caeruleostictus*,  $\Delta^{66}\text{Zn}_{\text{enameloid-bone}}$  values range between  $-0.25$  ‰ and  $-0.39$  ‰ (mean of  $-0.30 \pm 0.13$  ‰ 2SD,  $n=4$ ) and thus close to the mean pisciculture *S. aurata*  $\Delta^{66}\text{Zn}_{\text{enameloid-bone}}$  of  $-0.24 \pm 0.11$  ‰ (2SD,  $n=7$ ). For the three *B. caprisicus* mean  $\Delta^{66}\text{Zn}_{\text{muscle-enameloid}}$  values ( $-0.11$ ,  $-0.18$ ,  $-0.24$  ‰) are comparable to the mean pisciculture *S. aurata* value of  $-0.14 \pm 0.07$  ‰ (2SD,  $n=5$ ). The four *P. caeruleostictus* caught 2020, however, have generally lower  $\Delta^{66}\text{Zn}_{\text{muscle-enameloid}}$  values ( $-0.29$ ,  $-0.38$ ,  $-0.41$ ,  $-0.43$  ‰). In contrast to the pisciculture *S. aurata*, the wild fish species may have variable diets over time, both seasonally and through ontogeny (Tancioni et al. 2003; Hamida et al. 2009; Taieb et al. 2013; Goldman et al. 2016). Even though sparid teeth are replaced continuously throughout the animal's life cycle (Elgendy et al. 2016), perhaps even seasonally (Sisma-Ventura et al. 2018), zinc turnover rates for different tissues are still largely unknown and likely vary. In addition, once Zn is incorporated into enameloid it becomes chemically inert, whereas muscle Zn can still be exchanged. Thus zinc isotope values recorded in muscle may reflect diet consumed at a different time than those recorded in enameloid, perhaps explaining the different  $\Delta^{66}\text{Zn}_{\text{muscle-enameloid}}$  for wild *P. caeruleostictus* individuals. Still, in general, wild fish  $\Delta^{66}\text{Zn}_{\text{tissue-tissue}}$  values are in agreement with those from control-fed pisciculture *S. aurata*.

A detailed investigation into differences in diet among wild taxa is not possible with such a limited sample size and we do not observe any correlation for enameloid  $\delta^{66}\text{Zn}$  values with size (weight). All wild-caught individuals come from Haifa Bay, thus potential spatial variability in food web baseline  $\delta^{66}\text{Zn}$  values, as observed elsewhere in the Mediterranean (Chifflet et al. 2022), is most likely negligible among individuals and their  $\delta^{66}\text{Zn}$  variability relates to differences in dietary Zn intake as a result of their trophic ecology. Enameloid  $\delta^{66}\text{Zn}$  values for wild-caught fish range between  $-0.01$  and  $+0.55$  ‰ with overlap between species (Fig. 3). This indicates

a trophic range among these wild durophagous fish larger than one trophic level when using the pisciculture *S. aurata*  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  value of  $-0.45$  ‰ as an approximate trophic discrimination factor. Applying the  $\Delta^{66}\text{Zn}_{\text{enameloid-diet}}$  values from the pisciculture *S. aurata* to all analysed teleost wild fish indicates they have fed on a diet with average compositions between  $+0.28$  and  $+0.84$  ‰ (Supplementary Fig. 4). All three species feed on a variety of mainly hard-shelled prey items also depending on seasons, ontogeny and habitat, with bivalves, decapod crustaceans, gastropods, barnacles, echinoderms, annelids, and small teleosts generally considered the most important (Tancioni et al. 2003; Hamida et al. 2009; Taieb et al. 2013; Goldman et al. 2016). In the Levant, contrary to many other populations, smaller teleosts are likely a particularly important prey for *P. caeruleostictus* (Gilaad et al. 2017).

There is very little information on  $\delta^{66}\text{Zn}$  values for most of the prey species, but  $\delta^{66}\text{Zn}$  values of some filter-feeding bivalves from studies aimed at investigating anthropogenic Zn contamination have a wide range of bivalve soft tissue values from  $-0.11$  to  $+1.43$  ‰ worldwide (Shiel et al. 2012, 2013; Petit et al. 2015; Araújo et al. 2017, 2021; Ma et al. 2019; Jeong et al. 2021). A direct comparison with published bivalve soft tissue  $\delta^{66}\text{Zn}$  data is only tentative, as there is no data available from the Eastern Mediterranean, only a few taxa are represented, and reported  $\delta^{66}\text{Zn}$  values can vary significantly across localities in part also as most of these studies, by design, aimed to investigate anthropogenic metal contamination which varies geographically. Nevertheless, our anticipated dietary  $\delta^{66}\text{Zn}$  values are well within the range of values previously reported for filter-feeding bivalves. Our wild fish  $\delta^{66}\text{Zn}$  values are therefore in agreement with a trophic position as secondary to tertiary consumers, feeding on primary consumers (e.g., filter-feeding bivalves) and other secondary consumers (e.g., gastropods, echinoderms, teleosts) (Supplementary Fig. 4).

Because pisciculture and wild fish demonstrate constant and predictable  $\Delta^{66}\text{Zn}_{\text{tissue-tissue}}$  offsets, and in the case of controlled-fed pisciculture *S. aurata* also constant  $\Delta^{66}\text{Zn}_{\text{tissue-diet}}$  values,  $\delta^{66}\text{Zn}$  values of various tissues can be used to investigate marine trophic ecology and potentially identify sources of anthropogenic Zn contamination to marine vertebrates. In addition, because pristine biological  $\delta^{66}\text{Zn}$  values are highly resistant against diagenetic alteration in highly mineralised fish enameloid (McCormack et al. 2022b) and mammalian enamel (Weber et al. 2021), our  $\Delta^{66}\text{Zn}_{\text{enameloid-tissue}}$  and  $\Delta^{66}\text{Zn}_{\text{enameloid-diet}}$  values will allow identifying Zn dietary resources and reconstructing the trophic ecology of both extant as well as long-extinct fossil marine vertebrates.



## Conclusion

This study compares Eastern Mediterranean pisciculture *Sparus aurata* bone, enameloid, and white muscle  $\delta^{66}\text{Zn}$  values from seven individuals to the isotope composition of their pellet diet. Supplementing the pisciculture individuals, we also investigate the same tissue  $\delta^{66}\text{Zn}$  values of wild *S. aurata*, *Pagrus caeruleostictus* and *Balistes caprisicus* caught in Haifa Bay, close to the location of the pisciculture. Our results show:

1. All pisciculture *S. aurata* have constant diet-tissue discrimination factors ( $\Delta^{66}\text{Zn}_{\text{tissue-diet}}$ ) of  $-0.04 \pm 0.09$  ‰ (2SD,  $n = 7$ ) for bone,  $-0.29 \pm 0.06$  ‰ (2SD,  $n = 7$ ) for enameloid, and  $-0.45 \pm 0.07$  ‰ (2SD,  $n = 5$ ) for white muscle.
2. For both pisciculture and wild fish, we observe comparable  $\delta^{66}\text{Zn}$  tissue-tissue fractionation with progressively lower  $\delta^{66}\text{Zn}$  values from bone > enameloid > white muscle.
3. Wild-caught fish  $\delta^{66}\text{Zn}$  values are distinct from those of the pisciculture from the same area (i.e., Haifa Bay and Central Israel) and display a larger variability among individuals reflecting an isotopically more heterogenous diet in wild compared to controlled-fed specimen. Thus, tissue  $\delta^{66}\text{Zn}$  values are mainly related to Zn from the diet and not from ambient sea water.
4. The depletion in  $^{66}\text{Zn}$  in the here examined tissues compared to the diet is in line with the use of this proxy as a trophic level indicator, with lower  $\delta^{66}\text{Zn}$  values higher up the food chain. Our pisciculture  $\Delta^{66}\text{Zn}_{\text{muscle-diet}}$  value of  $-0.45 \pm 0.07$  ‰ (2SD) is close to values previously reported for trophic level spacing for both marine and terrestrial mammals.

In general, our study broadens the knowledge of tissue-diet and tissue-tissue Zn isotope fractionation factors to include the marine realm and non-mammalian vertebrates with multi-disciplinary applicability. Among others, our documented  $\Delta^{66}\text{Zn}_{\text{tissue-diet}}$  values in both controlled-fed and wild fish species could be used to monitor diet-borne Zn toxicity in marine vertebrates and to estimate individual and population dietary/trophic variability.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00227-023-04383-1>.

**Acknowledgements** This research was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) awarded to J.M. (Project Number 505905610), the Rhine-Main Universities (RMU) Initiative Funding for Research awarded to J.M., W.M. and T.T. and the Max Planck Society. FIERCE is financially supported by the Wilhelm and Else Heraeus Foundation and by the Deutsche Forschungsgemeinschaft (DFG: INST 161/921-1 FUGG, INST 161/923-1 FUGG and INST 161/1073-1 FUGG), which is

gratefully acknowledged. This is FIERCE contribution No. 149. Funding to T.T. and K.J. by the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Program (grant agreement numbers 681450 and 803676, respectively) is acknowledged. We thank A. Gerdes, A. Schmidt and L. Marko (FIERCE, Goethe University Frankfurt) and S. Steinbrenner and M. Trost (Department of Human Evolution, Max Planck Institute for Evolutionary Anthropology) for technical and analytical support. We also thank K. Grimm (Osteological Collection, Institute of Geosciences, Mainz, Germany) for the curation of sample material. Finally, we thank the two anonymous reviewers who helped improving the first version of the manuscript.

**Author contributions** J.M., T.T. and G.S.-V. designed the study. J.M. performed the  $\delta^{66}\text{Zn}$  analysis, J.M., G.S.-V., T.J.G.T. collected and/or prepared the samples for analysis. J.M., K.J., N.B., T.J.G.T., W.M., T.T. interpreted the data and contributed to writing the manuscript. All authors edited the final manuscript.

**Funding** Open Access funding enabled and organized by Projekt DEAL. This research was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) awarded to J.M. (Project Number 505905610), the Rhine-Main Universities (RMU) Initiative Funding for Research awarded to J.M., W.M., and T.T. and the Max Planck Society. Funding to T.T. and K.J. by the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Program (grant agreement numbers 681450 and 803676, respectively) is acknowledged.

**Data availability** All relevant data are provided within the paper and its Supporting Information files.

## Declarations

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** All specimens used in this study were collected legally and ethically. Analyses on fish farm *Sparus aurata* were performed on individuals that had been harvested for food consumption. All wild-caught individuals in this study had been caught as part of an ongoing monitoring program (permit number 516192-4399), funded by the Israeli Ministry of Environmental Protection.

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