Response to Reviewer 2

We sincerely thank the reviewer for the careful evaluation of our manuscript and for the constructive comments and suggestions. We are grateful for the time and effort the reviewer devoted to providing these insightful comments, which were very helpful to improve the clarity and quality of the manuscript. In response to the reviewer's suggestion, we carefully revised the manuscript to avoid speculative expression.

Major Comment 1: *I have two main comments. My first comment is I am slightly confused as to what the uncertainty you report in your tables & figures show. If I am understanding correctly, you only measured each tissue from each sample once. So, from sample Mk-1, you measured two splits of Red Muscle: 1) one split for Fe isotopes, which you report with a ± 2 S.E. in Table S2, and 2) Another split for Fe concentrations, which you report with no error for wet weights, dry weights, and calculated total iron in Table S2. What does the uncertainty on your Fe isotope data represent? Is this the analytical uncertainty? Or did you measure multiple Red Muscle tissues from Mk-1, and this is propagated error from those multiple tissue measurements? In addition, as you do not report error on the Fe concentration, I'm assuming that you did not measure multiple samples, and that you are assuming that the measurement uncertainty on this is zero. That is ok if so, just needs to be clarified. You could rectify this by having a brief statement in Section 2.2, like "Each tissue was measured once for both iron isotopes and iron concentrations; tissue replicates (i.e., multiple samples of red muscle) were not performed."*

Reply: Thank you for this important comment. Each sample was measured once for both iron isotope composition and iron concentration, and we did not conduct replicate measurements. Each iron isotope measurement consisted of 60 measurement cycles using MC-ICP-MS. The reported uncertainty for iron isotope ratios (±2 S.E.) represents the internal analytical precision of a single measurement based on these 60 cycles.

Revision:

[P. 3, Line 77] add sentence: Iron concentrations were determined from a single aliquot of each tissue sample.

[P. 3, Line 83] add sentence: Each tissue sample was measured once for iron isotope composition with 60 analytical cycles.

[SI Table S2] add notation: *2 S.E. in δ^{56}Fe represents the standard deviation during single analysis (60 analytical cycles)

Major Comment 2: *My second comment is about the discussion. Overall, I think the manuscript could be improved by interpreting the results more tightly and to refrain from making statements that perhaps extrapolate beyond the measured dataset.*

For example, your discussion starts with the statement, "The δ^{56}Fe values in the liver of chub mackerel were consistently higher than those in other tissues, indicating an enrichment in the heavier Fe isotopes." However, looking at your Figure 2, this only really appears to be true for Mk-3. The heaviest tissues for Mk-1 appear to be the liver, spleen, blood, and gill (I'm guessing that they are similar because their uncertainties overlap, but it would be helpful if you could calculate P values for significant differences). The heaviest tissues for Mk-2 and Mk-4 are both the Gonad and Liver. For Mk-5, they appear to all be similar, though again I cannot readily tell because there are no P values or other statistics plotted. For Mk-6, it's actually the Gonad that is heaviest. So, the opening statement of your discussion does not really appear to be supported by the evidence.

Reply: Thank you for this helpful comment. We agree that the liver does not always exhibit the highest δ^{56}Fe value among all tissues in every individual. Therefore, we revised the wording in the Discussion to avoid the overstatement. In the revised text, we instead compare liver δ^{56}Fe values with the calculated whole-body δ^{56}Fe values (from Eq.4). We also added the calculated whole-body δ^{56}Fe values for each individual as Table S3.

Regarding the reviewer's suggestion to calculate P values, we acknowledge that the limited sample size and the absence of replicate measurements constrain the statistical interpretation of the dataset. As also noted by Reviewer 1, statistical analysis would therefore be difficult to justify. Instead, we revised the Discussion to avoid subjective wording and to refrain from interpretations that extrapolate beyond the measured dataset.

Revision:

[P. 9, Line 174] The δ^{56}Fe values in the liver of chub mackerel were consistently higher than those in other tissues, indicating an enrichment in the heavier Fe isotopes.

⇒ First, we focused on the liver because it is a central tissue for iron storage in vertebrates and is expected to exhibit iron isotope fractionation through the synthesis of ferritin. All the chub mackerel individuals showed higher δ^{56}Fe values in the liver than whole-body δ^{56}Fe across eight tissues.

Major Comment 3*: Next, the Albarede et al 2011 paper is cited as evidence that ferritin incorporates heavy iron – it should be specified by the authors that this study looked at variation in humans, not fish, and therefore an assumption is made that the same mechanism present in humans is also present in fish. I have no complaints about citing the Albarede paper – there are, I think, few papers on Fe isotopes in fish, but some additional detail is needed to justify this assumption. For example, how similar are human vs. fish ferritin proteins? This is an example where small changes will improve the manuscript from unintentional over-statements.*

Reply: Thank you for this helpful comment. We agree that the study by Albarède et al. (2011) was conducted on human samples, and that applying this interpretation to fish involves an implicit assumption that similar ferritin-related fractionation mechanisms operate in teleosts. To clarify this

point, we have revised the text to state that the interpretation is based on observation from human studies (Albarède et al., 2011). In addition, we added a brief explanation noting that the ferritin structure differs between mammals and teleost fish. Specifically, while mammalian ferritin consists of H and L subunits responsible for iron oxidation and mineralization, respectively, fish possess an M-type subunit with intermediate functional properties. We think that these differences in ferritin subunit may influence iron oxidation efficiency and potentially affect the magnitude of Fe isotope fractionation. Finally, we revised several sentences throughout the Discussion to avoid overinterpretation and to ensure that conclusions remain consistent with the limited sample size and the observed variability among individuals.

Revision:

[P.9, Line 177] Because ferritin preferentially incorporates heavier Fe isotopes during the oxidation in the storage process according to human studies

[P.9, Line 178] Insert the following sentences: It should be noted that human ferritin consists of H and L subunits responsible for iron oxidation and mineralization (Plays et al., 2021), while teleost fish possess an additional M-type subunit with intermediate functional properties (Dickey et al., 1987, Bury et al., 2012). Such differences in ferritin subunit composition may influence iron oxidation processes and potentially affect the magnitude of Fe isotope fractionation.

Major Comment 4: *Section 4.3 was interesting to read, as the authors tackle a question that affects any field that uses the stable isotope composition of organisms – are the isotopic fingerprints a result of differences in source (i.e., diet), or does it reflect isotopic fractionations (i.e., "internal redistribution;" fractionation due to ferritin)? This section would benefit from a figure, where you plot your data vs. the data from other studies that you mention in text (i.e., shipjack tuna, mammals) as well as their prey (i.e., zooplankton, squid, crustaceans). This, I think, would help strengthen the paper as you are adding to a larger body of data that shows an intriguing pattern – that mammals have more depleted Fe isotopes (-3.79 to -1.5‰) vs. fish (i.e., your data set ranges from about -1.6 to -0.8‰, in line with the -1.46 to -0.71‰ values that Hasegawa et al 2022 reports). (Although, it appears that sardine & herring, which you cite as having values of -2.64 to -1.73, are an exception and 'look' like mammals in this framework?) You then argue that since the only potentially fractionating component – ferritin – is a low fraction of total iron, your Fe isotope data likely reflect source instead. You then cite Fe isotope values of various prey to show that they have heavy values (-1.00 to -0.3‰) but note that these are a little too heavy for fish, particularly for fish like sardine and herring which are pretty depleted. So, perhaps this is because more data is needed of their prey (as you note), or maybe fish have a different model of ferritin cycling. For this latter point (starting Line 238), your argument would benefit from some numbers. For example, give the Delta values for humans, mice, and sheep and compare them to your values. Otherwise, this paragraph felt a little too speculative and not*

supported by data.

Reply: Thank you for this helpful suggestion. Following the reviewer's recommendation, we added a Figure 4 that compares the δ^{56}Fe values obtained in this study with previously reported values for marine fish, mammals, and representative prey organisms such as zooplankton and squid. This figure helps place our results within the broader context of Fe isotope variations in marine organisms and highlights the general pattern that some fish species tend to exhibit heavier δ^{56}Fe values than mammals.

As noted by the reviewer, some species such as sardine and herring show relatively low δ^{56}Fe values that overlap with the mammalian range. We have therefore revised the text to acknowledge this exception and discuss the possible influence of dietary sources and physiological processes.

In addition, we included representative Δ values reported for mammals (e.g., humans, mice, and sheep) and compared them with the values estimated in this study. These numerical comparisons have been added to the relevant paragraph in Section 4.3 in order to better constrain the discussion of ferritin-related isotope fractionation and reduce speculative interpretation.

Revision

[P.10, Line 226] The range of δ^{56}Fe variation among tissues in chub mackerel was clearly narrower than that reported for mammals. In skipjack tuna, muscular δ^{56}Fe values are consistently high regardless of regional differences (−1.46‰ to −0.71‰; Hasegawa et al., 2022), whereas mammals generally show lower values (−3.79‰ to −1.5‰; Walczyk and von Blanckenburg, 2002; Balter et al., 2013).

⇒The range of δ^{56}Fe variation among tissues in chub mackerel was clearly narrower than that reported for mammals. For example, the differences in δ^{56}Fe values between muscle and liver range from 0.72‰ to 1.71‰ in mice (Balter et al., 2013), 1.64‰ to 2.72‰ in sheep (Balter et al., 2013), and 0.83‰ to 1.75‰ in humans (Walczyk and von Blanckenburg, 2002), whereas the maximum isotopic difference among tissues in the present chub mackerel specimens was 0.73‰. Despite this relatively small internal fractionation, δ^{56}Fe values in marine organisms often show species-specific ranges (Figure 4). For example, muscular δ^{56}Fe values in skipjack tuna are high regardless of regional differences (−1.46‰ to −0.71‰; Hasegawa et al., 2022), whereas mammals generally show lower values (−3.79‰ to −1.5‰; Walczyk and von Blanckenburg, 2002; Balter et al., 2013).

The Following Figure 4 was inserted:

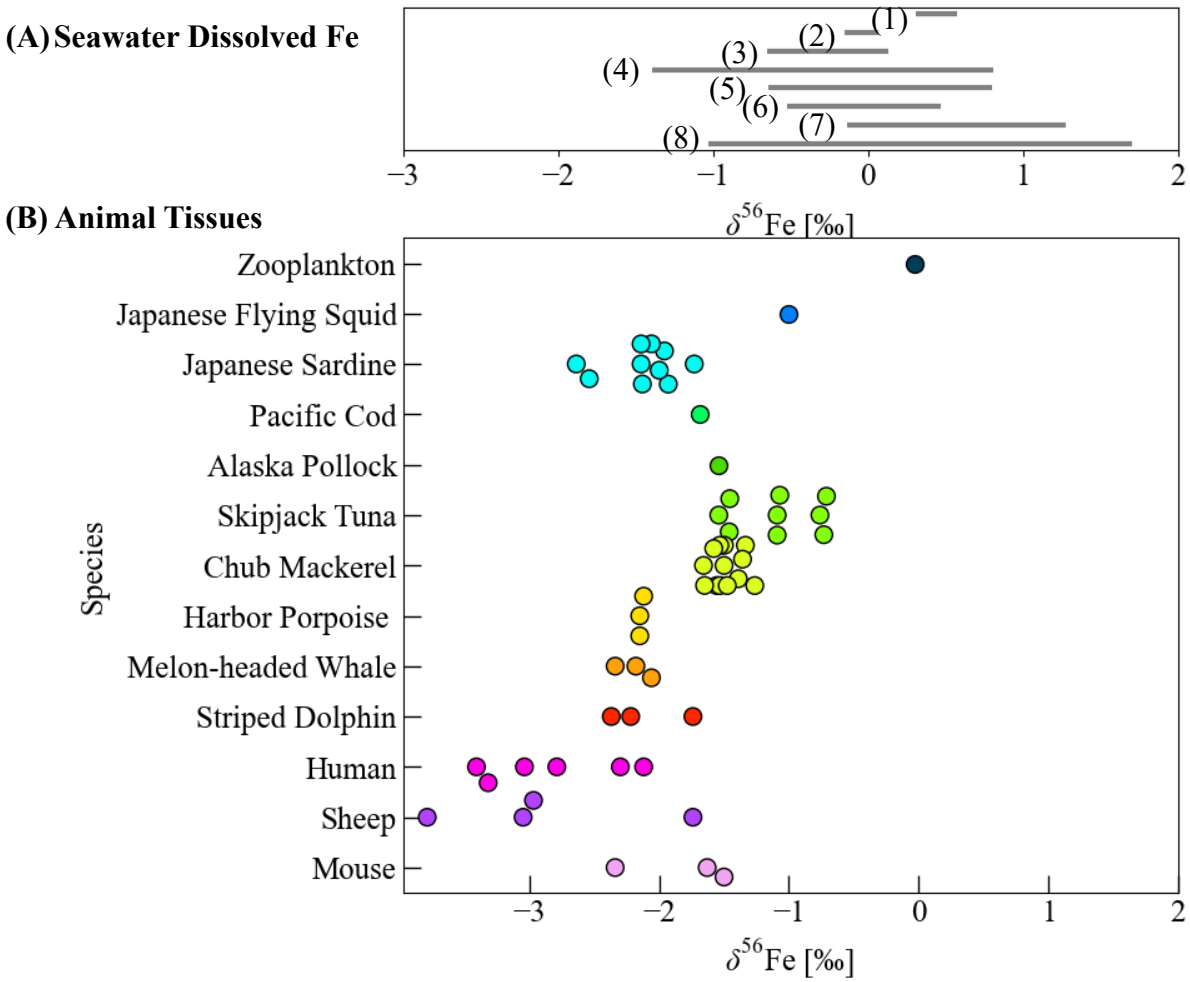

Figure 4. Comparison of δ^{56}Fe values in seawater (upper figure), and marine organisms and mammals (lower figure). Literature data in the upper figure are indicated by reference numbers: seawater from the Pacific Ocean (1–4: Radic et al., 2011; Conway and John, 2015; Pinedo-González et al., 2020; Kurisu et al., 2024), North Atlantic Ocean (5: Conway and John, 2014), and South Atlantic–Southern Ocean (6–8: Abadie et al., 2017; Ellwood et al., 2020; Sieber et al., 2021). The data of chub mackerel includes this study and Hasegawa et al. (2023), while values for zooplankton (whole body), squid (mantle), and mammals (harbor porpoise, melon-headed whale, striped dolphin, human, sheep, and mouse) are compiled from previous studies (Walczyk and von Blanckenburg, 2002; Balter et al., 2013; Hasegawa et al., 2022).

Minor Corrections:

Comment 5*: Lines 180-194: If I am understanding correctly, you are using Equation 6 to calculate the delta value of ferritin (d_{Ft}), and then using that value in your next equation ($D = d_{Ft} – d_{Hm}$) to get*

D. Could you report in the Supplemental the individual d_{Ft} and Dvalues you get for each fish, rather than just reporting the overall D in the main text?

Reply: Thank you for this helpful suggestion. We have now included the calculated δ_{Ft} and Δ ($\delta_{Ft} - \delta_{Hm}$) values for each individual specimen in the Supplemental Information. These values are provided in the newly added Table S3.

Revision:

Table S3, which describes the estimated value of δ_{Ft} (and δ_{Hm}) in each specimen was attached.

Table S3. Whole-body iron isotope ratio (δ_{WB}), and isotope ratios in liver ferritin (δ_{Ft}) and heme (δ_{Hm}) iron compartments in each chub mackerel specimen. The δ_{WB} values were calculated according to Eq.4. The δ_{Hm} value is assumed to be −1.66‰, the lowest value among mackerel samples. The δ_{Ft} values were estimated from Eq.6. The value Δrepresents isotope fractionation between δ_{Ft} and δ_{Hm} (i.e. $\Delta = \delta_{Ft} - \delta_{Hm}$).

Sample ID	δ_{WB}	δ_{Ft}	δ_{Hm}	Δ
Mk-1	−1.36	3.96	−1.66	5.62
Mk-2	−1.44	NA	−1.66	NA
Mk-3	−1.43	1.1	−1.66	2.76
Mk-4	−1.39	0.516	−1.66	2.18
Mk-5	−1.35	-0.513	−1.66	1.15
Mk-6	−1.50	0.281	−1.66	1.94

Whole-body δ^{56}Fe (δ_{WB}) was calculated as:

$$\delta_{WB} = \sum_i (\delta^{56}Fe_i \times T_i) = \sum_i (\delta^{56}Fe_i \times C_i \times M_i) \quad (4)$$

Comment 6: *Line 190: After excluding Mk-5, a D value of 2.04 ± 0.22 was derived for all five samples. It is then stated that this "slightly higher but comparable" to prior studies. Could you also give the uncertainties on the D values from those papers? For example, if the uncertainty for Katsuwomus pelamis was 1.52 ± 0.8, then that would be statistically similar to the value you derive. These additional statistics would enable you to make stronger statements overall.*

Reply: Thank you for this helpful suggestion. The Δ values reported in the previous studies were derived from single individuals and the associated uncertainties were not reported. Therefore, a direct statistical comparison with our value is difficult. We have clarified this point in the revised manuscript and adjusted the wording to avoid implying a strict statistical comparison.

Revision:

[P. 9, Line 196] These estimated values are higher than previous observations in skipjack tuna

(*Katsuwonus pelamis*, female, Δ = 1.52‰, n = 1; Hasegawa et al., 2023) and another chub mackerel specimen (Δ = 1.41‰, male, n = 1; Hasegawa et al., 2023)

Comment 7: *Line 221: You write, "As discussed in the previous section, hepatic ferritin iron likely undergoes isotopic fractionation on the order of approximately 1.5–2‰." Do you mean the D value you calculate? If so, how are you getting this 1.5-2‰ range, because your D values are either 1.99 ± 2.20‰ or 2.04 ± 0.22‰, depending on if you include or exclude Mk-5. Are you also including the Delta values from prior studies (K. pelamis = 1.52‰, another chub mackerel = 1.41‰)?*

Reply: Thank you for pointing this out. The fractionation range was intended to reflect both the Δ values obtained in this study and those previously reported for marine fish (e.g., *Katsuwonus pelamis* and chub mackerel). In addition, following the suggestion of Reviewer 1, the average Δ value was recalculated and is now 2.72 ± 3.03‰. To clarify this point, we have revised the sentence to explicitly state the basis of this estimate.

Revision:

[P.11, Line 238] As discussed in the previous section, hepatic ferritin iron likely undergoes isotopic fractionation on the order of approximately 1.5–3‰, based on the Δ values estimated in this study and Hasegawa et al. (2023).

Comment 8: *Line 241: You write, "In contrast to mammals that rely on ferritin-based iron recycling, fish appear to lack a long-term iron storage strategy based on ferritin, despite inhabiting chronically iron-limited environments." Are you making that inference based on your isotopic data alone, or has it been shown separately that fish lack a long-term iron storage strategy based on ferritin?*

Reply: Thank you for this comment. Our intention was not to conclude that fish lack ferritin-based long-term iron storage based solely on the isotopic data presented here. Rather, we intended to suggest that the relatively small inter-tissue δ^{56}Fe variation observed in chub mackerel may indicate a weaker influence of long-term ferritin-based iron storage compared with that reported for mammals. To avoid overinterpretation, we have revised the sentence to clarify that this point represents a possible interpretation of the isotopic patterns and remains to be confirmed by physiological studies.

Revision:

[P.11, Line 257] In contrast to mammals that rely on ferritin-based iron recycling, fish appear to lack a long-term iron storage strategy based on ferritin

⇒In contrast to mammals that rely on ferritin-based iron storage and recycling, the isotopic pattern observed in fish may indicate that long-term iron storage plays a less dominant role in iron homeostasis, despite inhabiting chronically iron-limited environments.

Comment 9: *Line 253: I agree that a Rayleigh-type mode would be interesting. Perhaps the authors*

could take it on in their next study! On that note, I recommend that the authors suggest somewhere in the manuscript that biochemical studies on the enzymatic fractionation of Fe isotope incorporation into ferritin or heme from fish is necessary for more detailed interpretations. For example, it would be great if someone could purify those proteins from fish and then perform the experiment in vitro.

Reply: Thank you for this constructive suggestion. We agree that biochemical studies investigating Fe isotope fractionation at the protein or enzymatic level would greatly improve the mechanistic understanding of Fe isotope behavior in fish. We have added a sentence in the Discussion highlighting the importance of future studies that directly analyze isotopic fractionation associated with ferritin and heme formation in fish.

Revision:

[P. 13, Line 284] Insert the following sentences:

In the future, applying a Rayleigh-type model may allow quantitative evaluation of the relative contributions of absorption and retention to isotopic fractionation in fish. Combined with XAFS analyses that provide species-level insights into ferritin- and heme-bound iron pools, these approaches could offer a powerful framework for linking biological iron metabolism to marine biogeochemical iron cycling. In addition, further biochemical studies examining Fe isotope fractionation during enzymatic incorporation into iron-binding proteins such as ferritin or heme in fish would help to better constrain the mechanisms controlling Fe isotope distribution in marine organisms.

Comment 10: *Line 260: You write, "Although isotopic fractionation of around 1.5-2‰ occurs in liver ferritin, its effect on whole-body $d^{56}Fe$ is minimal due to the limited ferritin fraction and overall liver iron content." Again, where is this 1.5-2‰ value coming from?*

Reply: Thank you for pointing this out. As noted in our response to Comment 7, the fractionation range refers to the Δ values estimated in this study as well as those reported in previous studies of marine fish. To clarify this point and maintain consistency with the revised discussion, we have modified the sentence accordingly.

Revision:

[P.13, Line 290] Although isotopic fractionation of around 1.5-3‰ occurs in liver ferritin,

Comment 11: *Line 263: You write, "The relative homogeneity of $\delta^{56}Fe$ among major tissues such as muscle and liver suggests that iron isotope pools in fish remain relatively stable, providing new insights into iron transport and isotope systematics within marine food web." When you say this, do you mean that the isotopic fractionation of 1.5-2‰ is larger than the observed range you see in your samples, which is about 0.8‰? (Your data range from about -1.6 to -0.8‰; Figure 2). Stating actual numbers would help make this conclusion stronger. In addition, as far as that 'relative homogeneity' statement goes… you do spend some time pointing out how liver is very isotopically heavy, though see*

my disagreements above about it being universally isotopically heavy throughout all your samples. I'm assuming that your 'relative homogeneity' statement is saying that, despite variations among tissue types, the full range of variation (0.8‰) is smaller than the potential full isotopic fractionation of 1.5-2‰. Is that what you mean?

Reply：We thank the reviewer for this helpful comment. Yes, our intention was to indicate that the observed inter-tissue δ^{56}Fe variability (maximum 0.73‰ among all tissues) is smaller than the potential isotopic fractionation associated with ferritin synthesis (approximately 1.5–3‰ based on Δ values from this and previous studies). We have revised the sentences to clarify this point.

Revision;

[P.13, Line 292] The relative homogeneity of δ^{56}Fe among major tissues such as muscle and liver suggests that iron isotope pools in fish remain relatively stable, providing new insights into iron transport and isotope systematics within marine food web.

⇒The maximum δ^{56}Fe range among major tissues was 0.73‰, indicating relatively small tissue isotopic variability. The relative homogeneity of δ^{56}Fe among major tissues such as muscle and liver compared with the potential isotopic fractionation associated with ferritin formation, suggests that iron isotope pools in fish remain relatively stable, providing new insights into iron transport and isotope systematics within the marine food web.

Comment 12: Line 287: I appreciate and commend the authors on being forthcoming and transparent about using ChatGPT in improving the English and readability of the manuscript.

Reply: Thank you for your kind comment. We appreciate your positive feedback.

Comments on Figures:

Comment 13: *Figure 1: Could you give some more description in the figure caption? For example, for Panel A, I'm assuming that your boxplot is showing the typical interquartile range (IQR), with whiskers showing 1.5 times the IQR – for example, these are the default parameters when plotting boxplots using pandas in Python. But this should be explicitly specified. In addition, are you plotting or not plotting outliers? Right now, I'm assuming from Panel A that you have no outliers (i.e., no discrete points outside of your boxplots). For Panel B, it would be helpful to restate that percentages were calculated assuming a total blood volume of 30 mL/kg. Also – each proportion is shown with a solid line, but is there some uncertainty on that? If so, you could briefly state, for example, "Uncertainty on calculated iron content (%) is typically ±X% (not shown)." If not (as you do not report uncertainty on your iron concentrations in Table S2), you should state "No uncertainty is reported as multiple sample replicates were not measured, and measurement uncertainty is assumed to be zero."*

Reply: We thank the reviewer for the suggestions. We inserted additional sentences in the caption of

Figure 1. We also added a statement noting that uncertainty is not shown because replicate measurements were not performed. In addition, following Reviewer 1's suggestion, the boxplots in Panel A were replaced with scatter plots in the revised figure.

Revision:

Figure 1: (A) Iron concentration, and (B) Tissue iron burden of the chub mackerels (Mk-1 to Mk-3: female, Mk-4 to Mk-6: male)

⇒ Figure 2: (A) Iron concentration, and (B) Tissue iron burden of the chub mackerels (Mk-1 to Mk-3: female, Mk-4 to Mk-6: male). Total blood contents were assumed to be 30 mL/kg with a specific gravity of 1.05. No uncertainty is reported as multiple sample replicates were not measured. The measurement uncertainty was assumed to be zero.

Comment 14: *Figure 2: Could you provide some additional information in the Figure caption? For example, are the error bars on each discrete point showing a 1 sigma or 2 sigma error? And, per my comments above, I'm assuming that this is analytical uncertainty on a single measurement, not propagated uncertainty over multiple tissue replicates, correct? In addition, you should re-label the y-axis on the lower three panels (i.e., Red Muscle, White Muscle, etc.) for improved readability. And the lower three panels are missing the x-axis label of delta 56 Fe, which should be centered as well.*

Reply: Thank you for this helpful comment. Error bars represent ±2 S.D. internal analytical uncertainty of a single MC-ICP-MS measurement (60 cycles). Because each tissue sample was measured once, the error bars reflect analytical precision rather than propagated uncertainty from multiple tissue replicates. In addition, following the suggestion of Reviewer 3, we modified the figures into a single combined figure to improve readability.

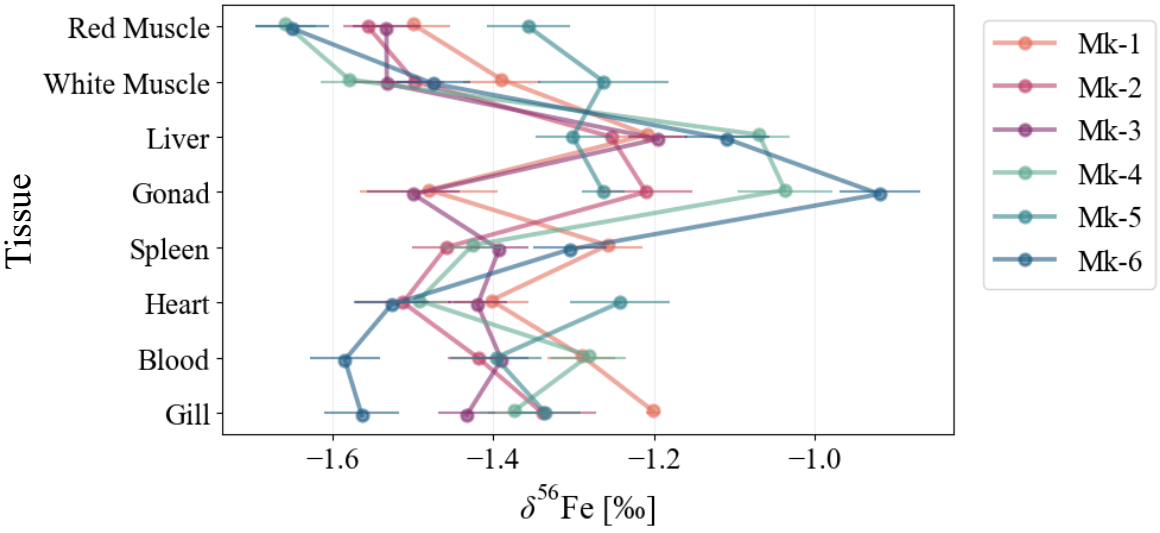

Figure 2: Iron stable isotope ratio in tissues of the six Scomber japonicus individuals (Mk1-3: females, Mk4-6: males). ~~The red line represents average δ^{56}Fe values across the eight tissues.~~

~~**The gray dotted lines represent 2 S.D. values from the average line.**~~

⇒Figure 2: Iron stable isotope ratio in tissues of the six *Scomber japonicus* individuals (Mk1-3: females, Mk4-6: males). Error bars represent 2 S.E. during a single measurement (60 analytical cycles).

Comment 15: *Figure 3: Overall, font size needs to be increased. Panel A is missing some description; it should also say "Black lines represent measured spectra while red lines are reconstructed spectra." And, in Panel B, could you move the percentages on the pie chart to the outside? This would help increase readability.*

Reply: Thank you for your helpful comments. We have revised Figure 3 to improve readability. The font size in the figure has been increased. In Panel A, we added the description "Black lines represent measured spectra while red lines are reconstructed spectra." In Panel B, the percentage labels in the pie chart have been moved outside the chart to improve clarity.

Revision:

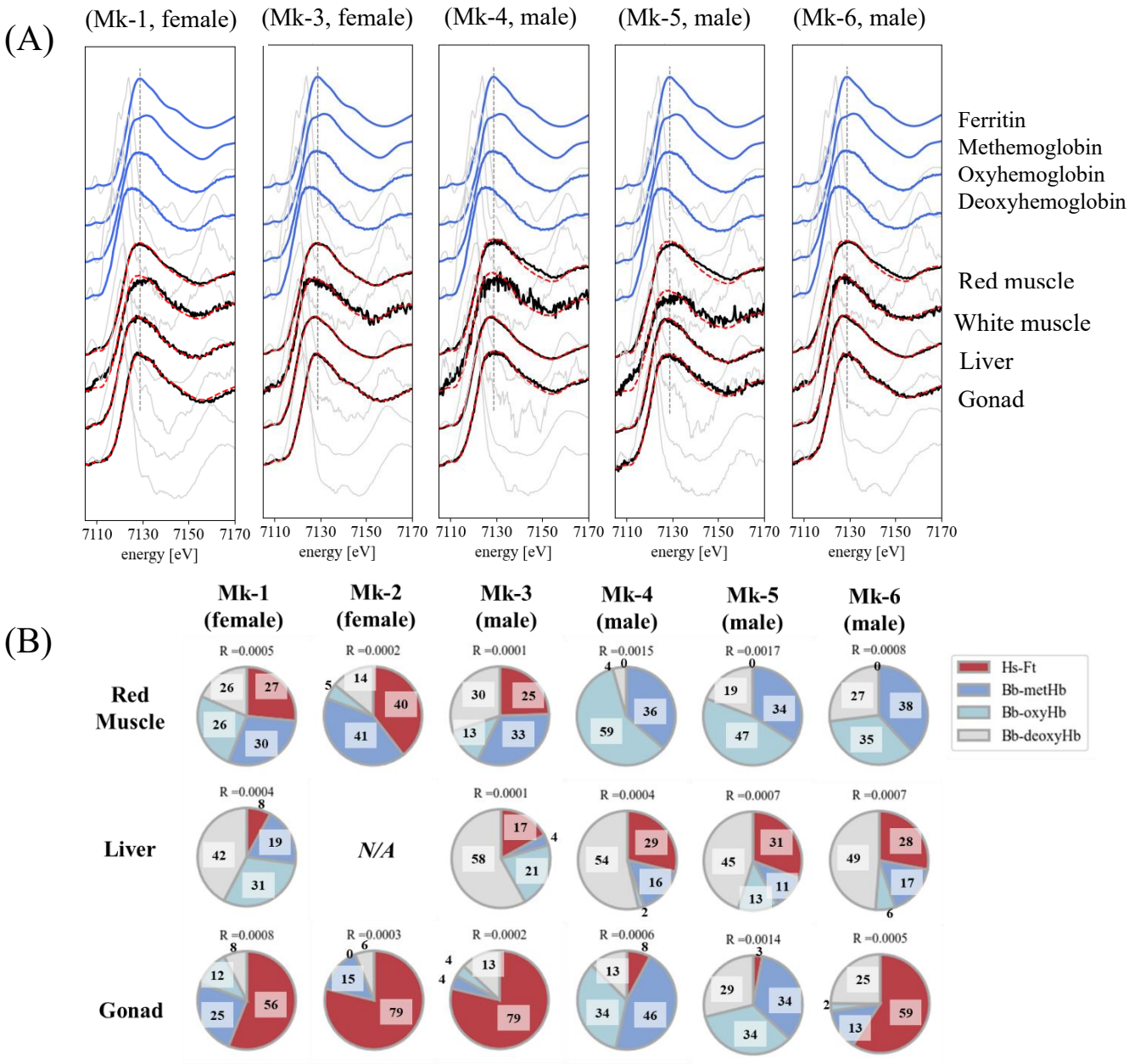

Figure 3: (A) Fe K-edge XANES spectra of *Scomber japonicus* tissues. Mk-1 and Mk-3 represent females, and Mk-4 to Mk-6 represent males. The standard spectra were obtained from ferritin from horse spleen and hemoglobin derivatives from bovine blood. Blue lines indicate the spectra of the standard materials, while black lines represent the tissue samples. Red dashed lines show the results of linear combination fitting (LCF) using the four standard spectra. Gray solid lines correspond to the first derivative of the normalized XANES spectra, which highlights the peak top energies, and gray dashed lines mark the peak top energy of Hs-Ft at 7129 eV. (B) Estimated proportions of each standard materials with linear combination fitting (LCF) of XANES spectra.

R value is a goodness-of-fit parameter described in Eq. 4. Each number on the pie charts represents proportions (%) of each component.

Comment 16: *Overall, I would recommend that you re-format the SI PDF because some of the figures are blurry to the point of being illegible. Figure S1 looks great, but then some (i.e., Fig. S2 and S8) are very blurry. Perhaps some issue with inserting images in the wrong format (i.e., PDF vs. JPG)?*
Reply: Thank you for pointing this out. We have re-formatted the Supporting Information to improve the figure quality.