

Reply

We sincerely thank the reviewer for their thorough and constructive review of our manuscript. The comments raised were extremely helpful in identifying inconsistencies between the text, tables, and figures, as well as in improving the clarity and rigor of our interpretations.

In response to the reviewer's concerns, we have revised the manuscript to correct all inconsistencies in numerical values and sample ranges, and to ensure full consistency among the main text, figures, and supplementary tables. We have also revised the abstract and conclusions to avoid any potentially misleading emphasis on results derived from the selective exclusion of individuals.

Furthermore, we have substantially revised the Discussion to adopt a more cautious and balanced interpretation, explicitly acknowledging the limited sample size and inter-individual variability. Several statements have been toned down, and the conclusions are tuned not to be definitive. We believe that these revisions have significantly improved the transparency, robustness, and overall quality of the manuscript. We are grateful to the reviewer for their insightful comments, which have greatly strengthened this study.

Reviewer's Major Comments I: *My first main concern was the removal of two specimens (Mk-1 and Mk-5), after measurements were done, which produce a misleading narrow distribution. Either there is a strong incentive to remove these beforehand (e.g. sample compromised, biological justification), and it should be explicitly stated, or they should be included in the conclusion even if it adds more uncertainty. Moreover, the values resulting from the exclusion of these two specimens is mentioned as a key result in the abstract.*

Reply: We thank the reviewer for this important comment. We agree that post hoc exclusion of specimens requires clear justification, and that removing individuals without sufficient explanation may give a misleading impression of the results.

For Mk-1 and Mk-5, there was no strong a priori justification for exclusion. Therefore, in the revised manuscript, we have chosen to retain all individuals in the analysis. The relevant sections of the manuscript have been revised accordingly. In addition, we have modified the Abstract to remove statements based on results obtained after excluding these individuals, in order to avoid potential misinterpretation by readers.

In Section 4.1, we now present Δ values calculated using all individuals, based on the assumption of $\delta_{\text{Hm}} = -1.66\text{‰}$ as the reviewer noted below, and discuss the observed variability as biologically meaningful rather than excluding any specimens.

Correction:

[P.1, line15] $2.04 \pm 0.22\text{‰} \rightarrow 2.72 \pm 3.03\text{‰}$

[P.9, Line 188-199] has been replaced (Please see P.8)

Reviewer's Major Comments 2

Secondly, I have noticed several discrepancies between the text and the values in the tables or the figures:

- *Maybe there is some factor that I don't know about that is missing, but when I take the wet weight in table S1 and multiply it by the Fe concentration ($\mu\text{mol/gww}$) in Table S2, lots of the results are different from the one given in the column "Total Fe (mg)" of tableS2. "Total Fe (mg)" of tableS2. It is especially true for blood Fe content (see tables at the end of this review). The small differences can be explained by approximations of both values but as for the others I couldn't figure out the cause behind the discrepancies. Maybe it has something to do with the conversion from wet weight to dry weight?*

Reply: We appreciate the reviewer for pointing out this, and acknowledge that the explanation regarding the discrepancy between wet weight values in Table S1 and the "Total Fe (mg)" values in Table S2 was insufficient in the original manuscript.

We checked the discrepancy and found that the wet weights reported in Table S1 were measured immediately after dissection using a portable balance and include the weight of the sampling containers. However, the "Total Fe (mg)" values in Table S2 were obtained using precise tissue weights measured later with an analytical balance after subtracting the container weight. For blood samples, the wet weight in Table S1 corresponds to the amount of blood that could be physically collected at the time of dissection. However, this value underestimates the actual blood volume since the blood had partially coagulated and was difficult to collect completely. In the manuscript, total blood iron content was estimated using the blood volume conversion factor from body weight (around 30 mL/kg), rather than the collected mass. As a result, the blood iron values in Table S2 are not expected to match calculations based directly on the wet weights reported in Table S1. To avoid further confusion, we replaced Tables S1 and S2 with a single revised Table S1 based on the precise tissue weights. In addition, we revised Fig. S1 to clarify that the values represent weight proportions relative to the total body weight, rather than the combined weight of the eight tissues.

Correction:

- Tables S1 and S2 were merged into a new Table S1.
- **[P.5, line118-121]** The hepatosomatic index (HSI) ranged from 1.05–2.14 in females and 0.74–1.26 in males, and the gonadosomatic index (GSI) ranged from 4.26–10.1 in females and 7.25–10.0 in males, consistent with spawning season in the East China Sea (Shiraishi et al., 2008). Among tissues, white muscle represented the largest biomass fraction (31–52% of total body weight; Fig. S1), followed by gonads (3.8–8.9%), red muscle (4.5–7.7%), gills (2.1–3.9%),
- **[P.5, line127-129]**
Mean iron concentrations (± 2 S.D.) were highest in the spleen ($3,100 \pm 860 \mu\text{g/g d.w.}$), followed

by blood ($1,300 \pm 480 \mu\text{g/g d.w.}$), heart ($720 \pm 580 \mu\text{g/g d.w.}$), liver ($630 \pm 510 \mu\text{g/g d.w.}$), gills ($290 \pm 90 \mu\text{g/g d.w.}$), red muscle ($250 \pm 84 \mu\text{g/g d.w.}$), gonads ($37 \pm 24 \mu\text{g/g d.w.}$), and white muscle ($15 \pm 10 \mu\text{g/g d.w.}$)

• **[P.5, line133-140]**

The total body iron inventory was therefore estimated to be approximately 17–26 mg/kg. Although the liver is the principal iron storage tissue, it accounted for only 4–8% of total body iron. In contrast, blood and red muscle together comprised 51–67% of the total iron burden. Mean iron isotope compositions ($\delta^{56}\text{Fe} \pm 2 \text{ S.D.}$) of each tissue across individuals were as follows: red muscle, $-1.54 \pm 0.20\%$; white muscle, $-1.46 \pm 0.21\%$; liver, $-1.19 \pm 0.16\%$; gonads, $-1.23 \pm 0.42\%$; spleen, $-1.37 \pm 0.15\%$; heart, $-1.43 \pm 0.19\%$; gills, $-1.37 \pm 0.22\%$; and blood, $-1.39 \pm 0.20\%$ (Fig. 2). **The net $\delta^{56}\text{Fe}$ in whole mackerel bodies weighted by tissue Fe contents ranged from -1.50% to -1.35% , with the liver in all individuals showing higher values than the net values.**

- *Page 5 line 131 “the total iron content in blood was estimated to be between 4.3 and 9.8mg (Fig. 1B)” these values are incoherent with the ones given in Table S2.*

Reply: As mentioned before, the discrepancy arose from the use of different approaches to estimate blood iron content in the main text and in Table S2. In addition, because reported conversion factors for fish blood volume per body weight vary widely, we initially applied multiple factors to estimate blood iron content, which resulted in inconsistencies among some values.

To resolve this issue, we recalculated all blood iron contents using a unified assumption of a blood volume of 30 mL/kg body weight and a specific gravity of fish blood of 1.05. The values in the text, Figure 1B, and Table S2 have now been updated accordingly and are consistent.

Correction:

[P.5, Line 131–132] Assuming a blood volume of 30 mL/kg and 1.05 as the specific gravity of fish blood (Davison, 2011), the total iron content in blood was estimated to be between 3.2 and 10.3 mg (Table S1).

- *Page 5 lines 132-133 again the ranges given are not the ones in Table S2 “gills (1.3-2.0mg)” should be (1.3-3.7 mg), and “liver (0.5-1.6 mg)” should be (0.5-2.0 mg). Or did you remove sample Mk-5 on purpose here?*

Reply: The reviewer is correct. The ranges reported in the main text were due to an error in reading the cell range, which resulted in the omission of the Mk-6 value during data compilation. The ranges for gills and liver have now been corrected in the text to match the values reported in Table S2.

Correction:

[P.5, Line 132] Red muscle contributed the next largest Fe pool (2.3–6.1 mg), followed by gills (1.3–3.7 mg), white muscle (0.9–2.0 mg), spleen (0.8–2.6 mg), liver (0.5–2.0 mg), gonads (0.3–1.0 mg), and heart (0.2–0.9 mg).

- *Page 7, l.155, section 3.3: Mk-1 and Mk-5 values do not seem to be included, otherwise the ranges given “17-29%” would be “8-31%” as it is the case on page 9 line 175.*

Reply: The reviewer is correct. The range reported on page 7 (17–29%) was the result of an inadvertent omission of Mk-1 and Mk-5 during data compilation. These samples were not intentionally excluded. The range has now been corrected to 8–31% to ensure consistency with the values reported on page 9 (line 175) and the underlying dataset.

Correction:

[P.7, line 155] whereas ferritin accounted for 8–31% of total Fe in the liver (Fig. 3B).

- *Page 7 lines 159-160: it is stated that “females exhibit lower ferritin-bound Fe proportions in the liver, red muscle and gonads than males ($p < 0.05$)”. However, Figure 3B shows a higher proportion of ferritin (Hs-Ft, red portions) in red muscle and gonads in the female specimens. And in the following text the authors explicitly say that “ferritin represented the predominant Fe form (70-80%)” in ovaries “whereas testes showed highly variable proportions (3-59%)” in agreement with Figure 3B but in contradiction with the previous sentence.*

Reply: We apologize for this oversight. The statement on page 7, lines 159–160 contains an error in the description of sex-specific differences in ferritin-bound Fe proportions. As correctly noted by the reviewer, Figure 3B shows higher ferritin-bound Fe proportions in red muscle and gonads of female specimens, and the subsequent text describing ferritin as the predominant Fe form in ovaries (70–80%) but highly variable in testes (3–59%) is consistent with the figure. We have therefore corrected the sentence to ensure consistency with Figure 3B and the accompanying text. In addition, we deleted p-values in these sentences because they are meaningless given the limited sample size.

Correction:

[P.7, line 159–161]

Sex-related differences were also apparent: females exhibited lower ferritin-bound Fe proportions in the liver, while higher proportions in the red muscle, and gonads than males (~~$p < 0.05$~~).

Reviewer’s Major Comments 3

Figure 1 (page 6) uses boxplots based on 3 values only. I am not convinced that this is the best

statistical analysis one can do on such a small sized sample and especially when comparing groups (p.5 1128-130, section 3.2). Boxplots summarize a distribution and thus need more observations to be robust.

Reply: We agree with the reviewer that boxplots are not appropriate for such a small sample size (n = 3 per group), as they are intended to summarize distributions and require a larger number of observations to be robust. We have therefore revised Figure 1 by replacing the boxplots with scatter plots showing individual data points. In addition, we have toned down the statistical interpretation in Section 3.2 to avoid overinterpretation of group differences based on a limited sample size.

Correction:

[P.5, line129–130] Male spleens ~~tended to have contained significantly~~ higher Fe concentrations than those of females (~~p=0.04~~), and liver and gonads also tended to be higher in males (~~p=0.06~~)

[P.6, line 139–140] No ~~significant~~ sex differences were detected, although ovarian $\delta^{56}\text{Fe}$ ~~tended to be~~ was lower than testicular values (~~p=0.06~~).

Reviewer's Major Comments 4

I would advise the authors to be more cautious with their conclusions as their sample size is small and present some variability.

Reply: We thank the reviewer for this important comment. We agree that the small sample size and the observed inter-individual variability require cautious interpretation of the results.

We have therefore revised the Conclusions and relevant parts of the Discussion to temper our statements and to emphasize that the observed patterns should be interpreted as preliminary trends rather than definitive conclusions. We now explicitly acknowledge the limitations associated with sample size and biological variability and highlight the need for future studies with larger sample sizes to confirm and extend our findings.

Correction:

[P.1, line15] no ~~significant~~ enrichment of heavy Fe isotope

[P.1, line18] Our results ~~suggested~~ that

[P.12, line262–266]

These results ~~suggest~~ that the high $\delta^{56}\text{Fe}$ of mackerel primarily reflects dietary iron sources ~~and intestinal uptake~~ rather than internal isotopic fractionation. The relative homogeneity of $\delta^{56}\text{Fe}$ 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. ~~Although the number of individuals analyzed in this study is limited, the consistency of $\delta^{56}\text{Fe}$ values across multiple tissues within individuals provides valuable constraints on iron isotope behavior in wild marine fish.~~

Reviewer's Major Comment 5

The comparison between values seems subjective as the authors state “the $\delta^{56}\text{Fe}$ values in the liver [...] were consistently higher than those in other tissues”, for a maximum difference of 0.35% between the liver and red muscle, and later say that the isotopic offset is “slightly higher but comparable to previous observation” for a difference of 0.46‰ at best. Either both are higher or both comparable in terms of differences. Or are there measurements for other organisms that show even larger isotopic offset? In that case I would understand the “comparable”.

Reply: We agree that the original phrasing may give the impression of a subjective comparison. We have therefore revised the text to harmonize the language used for isotopic differences. Specifically, we replaced “consistently” and “slightly” with a more cautious expression.

Correction:

[P.1, line13] In all the specimens, the liver $\delta^{56}\text{Fe}$ values were ~~consistently~~ higher than the average value ~~those~~ of all ~~other~~ tissues

[P9, Line172-173]

The $\delta^{56}\text{Fe}$ values in the liver of chub mackerel were consistently higher than those in other tissues, indicating depletion of lighter isotopes in this tissue. an enrichment in the heavier Fe isotopes.

⇒ All chub mackerel individuals showed higher $\delta^{56}\text{Fe}$ values in the liver than average values across eight tissues (the red lines in Fig. 2), indicating depletion of lighter isotopes in this tissue.

Reviewer's Major Comment 6

p.10 1231-236: it seems the two sentences contradict each other. The part stating “are more likely attributed to variation in intestinal...” excludes the contribution of prey $\delta^{56}\text{Fe}$, which is not what you say before and after. Or can you demonstrate why the prey $\delta^{56}\text{Fe}$ cannot drive the variation in fish $\delta^{56}\text{Fe}$?

Reply: We thank the reviewer for this important comment. We agree that the original wording was overly exclusive and could give the impression that prey $\delta^{56}\text{Fe}$ does not contribute to the observed variability in fish $\delta^{56}\text{Fe}$, which was not our intention.

Our original interpretation was based on the observation that, for sardines and herrings, there were few reported prey candidates exhibiting $\delta^{56}\text{Fe}$ values as low as those observed in these fish, leading us to suggest that dietary isotopic composition alone was unlikely to fully explain their low $\delta^{56}\text{Fe}$ values. However, recent observations have identified some of zooplankton taxa exhibiting low $\delta^{56}\text{Fe}$ values (below -2‰), indicating that prey-derived $\delta^{56}\text{Fe}$ variability cannot be entirely excluded as a contributing factor. We therefore agree that prey isotopic composition may partially influence fish $\delta^{56}\text{Fe}$ values, particularly in species with selective feeding habits. Therefore, we have revised the text to clarify that differences in intestinal iron absorption processes are likely a major contributor, but not the sole controlling factor, and that both physiological regulation and prey-derived $\delta^{56}\text{Fe}$ variability

may jointly influence the isotopic composition of marine fish.

Correction:

[P.11 Line231-235]

~~Nevertheless, The $\delta^{56}\text{Fe}$ differences observed among fish groups such as tuna and mackerel (-1.58‰ to -0.71‰) versus sardine and herring (-2.64‰ to -1.73‰ ; Hasegawa et al., 2022) seemed difficult to explain ~~are unlikely to be explained~~ solely by isotopic differences in their prey since all prey species analyzed so far showed higher $\delta^{56}\text{Fe}$ values than sardine and herring(Hasegawa et al., 2022)., ~~but are more likely attributed to variation in intestinal iron absorption processes.~~~~

Minor Corrections:

[P.2, Line 39] efficient intestinal absorption mechanisms ~~that~~ specifically exist in marine fish

[P.2, line40-41] However, direct quantification of intestinal iron uptake has been largely restricted to laboratory animals using enriched isotopes (van den Heuvel et al., 1998, Fiorio et al., 2012), an approach that is not feasible for wild species especially marine fish.

[P.3, Line77] Iron purification was performed used anion-exchange chromatography following Maréchal et al., (1999).

[P. 4, Line103] following Di Iorio (1981) and Wilson et al. (2013) (delete comma)

[P.5, line 117] curve of Shiraishi et al., (2008)

[P.5, line127-130] spleen ($3,100 \pm 970 \mu\text{g/g d.w.}$), followed by blood ($1,300 \pm 530 \mu\text{g/g d.w.}$), heart ($720 \pm 640 \mu\text{g/g d.w.}$), liver ($630 \pm 560 \mu\text{g/g d.w.}$), gills ($290 \pm 99 \mu\text{g/g d.w.}$), red muscle ($250 \pm 92 \mu\text{g/g d.w.}$), gonads ($37 \pm 26 \mu\text{g/g d.w.}$), and white muscle ($15 \pm 10 \mu\text{g/g d.w.}$)

- p5 l.131 “the total iron content in blood was estimated to be between 4.3 and 9.8 mg(Fig. 1B4)”

Reply: Thank you for the reviewer’s comment. We revised the text because Fig. 1 does not explicitly present numerical values for blood iron content. Since the estimated values are summarized numerically in Table S1, we consider it more appropriate to refer to Table S1 rather than Fig. 1.

Accordingly, the sentence was revised as follows:

[P.5, line131-132] Assuming a blood volume of 30 mL/kg, the total iron content in blood was estimated to be between 3.2 and 10.3 mg (Table S1).

- p9 l.188: please check the value “ $1.99 \pm 2.20\text{‰}$ ”. Using formula 6 and 1.66‰ as δ_{Hm} (lowest $\delta^{56}\text{Fe}$ in red muscle here) I get a mean value of 2.7‰ with all samples and 2.24‰ excluding Mk-1 and Mk-5.

Reply: We thank the reviewer for carefully checking this calculation. We agree on applying a single δ_{Hm} value of -1.66‰ (the lowest $\delta^{56}\text{Fe}$ observed in red muscle).

We have recalculated Δ using this definition of δ_{Hm} and revised the text accordingly. When all individuals are included, the estimated Δ values are $4.15 \pm 2.84\%$ (2 S.D.) for females and $1.76 \pm 0.88\%$ for males. Because the sample size is limited, we do not interpret these results as evidence for a definitive sex difference. Instead, we have revised the Discussion to emphasize that the higher Δ values observed in females may reflect transient physiological conditions, such as altered iron fluxes during the spawning season, rather than equilibrium isotope fractionation.

Correction:

[P.9, Line186–193] has been replaced as the following sentences.

By assuming that the lowest $\delta^{56}\text{Fe}$ observed here in red muscle represent δ_{Hm} (-1.66%), the isotopic offset between ferritin- and heme-bound Fe in the liver ($\Delta = \delta_{\text{Ft}} - \delta_{\text{Hm}}$) was estimated to be $2.72 \pm 3.03\%$ (2 S.D.) in all specimens, whereas $4.15 \pm 2.84\%$ (2 S.D.) in females and $1.76 \pm 0.88\%$ in males. These estimated values are higher than previous observations in skipjack tuna (*Katsuwonus pelamis*, female, $\Delta = 1.52\%$) and another chub mackerel specimen ($\Delta = 1.41\%$, male, Hasegawa et al., 2023), and, in females, appear higher than the equilibrium isotope fractionation between Fe(II) and Fe(III) ($\sim 2.8\%$, Johnson et al., 2002; Welch et al., 2003). The two females exhibited a lower ferritin-bound iron fraction than the three males, while their liver $\delta^{56}\text{Fe}$ values are comparable to those of the males, resulting in a tendency for their estimated Δ values to be higher. Because of the limited sample size, it cannot be definitively concluded that a sex difference exists. However, one possible explanation is that the release of isotopically lighter Fe from hepatic ferritin following Rayleigh fractionation leads to significant enrichment of heavy Fe in the residual ferritin and results in Δ values larger than those expected under isotopic equilibrium. As the sample consists of mature individuals during the spawning season, it cannot be ruled out that physiological changes relating to spawning may have temporarily altered iron metabolism, particularly in females.

- p11 l.243-245: “efficient uptake of iron” can be misleading. Fish have low intestinal absorption of Fe, no excretion processes for Fe, which can be toxic, and as you show low Fe storage so it would appear that they limit absorption and intensively recycle absorbed Fe.

Reply: We thank the reviewer for this important clarification. We agree that the phrase “efficient uptake of iron” was misleading in the context of fish iron physiology. We have therefore revised the text to replace “efficient uptake of iron” with wording that emphasizes efficient retention and recycling of absorbed iron rather than elevated intestinal absorption rates.

Correction:

[P.11, Line243-244]

They may instead have evolved a “~~recycle absorption~~ dominant” mode of iron homeostasis that relies on **intensive recycling of iron to sustain metabolic demands**.

Figures:

- Fig. 2: The Y-axis legend at the far left of the male plots was attached as well.

Reply: We thank the reviewer for pointing this out. The Y-axis legend position has been adjusted in the revised Figure 2 to avoid overlap.

- Fig. 3B: The proportions were put around of the pie charts.

Reply: The proportions have been repositioned around the pie charts in Figure 3B for improved readability.

- Fig. S5: Point Mk-2 is missing on panel Liver “Total Fe” (0.97 in Table S2)

Reply: We appreciate the reviewer’s comment. We carefully rechecked Figure S5 and found that the data point for Mk-2 in the liver “Total Fe” panel was not plotted because the ferritin proportion for this sample was unavailable. This is due to the fact that we did not obtain enough quality of XANES the liver sample from Mk-2. We have clarified this point in the revised manuscript and Table S2.

Correction:

[P.7, line153] Sentence inserted: We note that the XANES spectra of MK-2 liver was unavailable because the quality of spectrum was not good.

- Fig. S5: Y-axis seems wrong. Gonads “Total Fe” in mg is lower than 1 in Table S2 but you show data going up to 20 on the y-axis.

Reply: We thank the reviewer for pointing this out. The Y-axis scale of the gonads “Total Fe” panel in Figure S5 was incorrect and has been corrected to match the values reported in Table S2.

- Bibliography: “Von Blackenburg” and “Von Heghe” should be at Vs, “Di Iorio” at Ds.

Reply: We appreciate the comment. The bibliography has been corrected and the references are now properly ordered alphabetically.