

"Functional characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters."

Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, Tiziano Verri, and Elena Bossi

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The following individual(s) involved in review of this submission have agreed to reveal their identity: Stefan Broer (Referee #1); Chris Cheeseman (Referee #3)

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Reviewing Editor: Morag Mansley

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Professor Bossi,

Re: JP-RP-2021-282607X "Expression analysis and biophysical properties characterization of the two Atlantic salmon (*Salmo salar* L.) peptide transporters, PepT2a and PepT2b" by Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, Tiziano Verri, and Elena Bossi

Thank you for resubmitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 Referees and the reports are copied below.

I regret to say that the manuscript has not been accepted for publication.

Some positive comments were made on the manuscript. Unfortunately, they did not outweigh the more serious criticisms which led the Reviewing Editor to recommend rejection.

I am sorry to have to pass on this disappointing news, and hope it will not discourage you from making future submissions of new work to The Journal of Physiology.

However, we believe your manuscript is worthy of further consideration and suggest that you transfer your manuscript to Physiological Reports (<https://physoc.onlinelibrary.wiley.com/hub/journal/2051817X/aims-and-scope/read-full-aims-and-scope>), a peer-reviewed, open access, interdisciplinary journal, jointly owned by the American Physiological Society and The Physiological Society.

To transfer your manuscript to Physiological Reports, the corresponding author must send authorization within 120 days of receipt of this letter. Please use this link [Transfer to Physiological Reports](#) to send an authorization email to transfer your manuscript. If your manuscript does not require additional peer review, the editors of Physiological Reports will aim to give you an initial decision within 3 working days. In fact, >80% of transferred submissions are accepted for publication. Please note, of course, that we cannot guarantee final acceptance.

I hope you will take advantage of the opportunity to allow the editors of Physiological Reports to evaluate your manuscript.

You may be able to publish Open Access with no direct cost to yourself. You can check your eligibility here <https://secure.wiley.com/openaccess?>

Yours sincerely,

Dr Peiyong Fong
Senior Editor
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EDITOR COMMENTS

Reviewing Editor:

The authors have made notable changes to the manuscript, adequately addressing comments raised by Reviewer 1. However, Reviewer 1 also noted that the influence of this study is low. Reviewer 2 also notes low influence of this study and further raises major concern with a lack of molecular modelling carried out. Reviewer 2 has provided many more constructive points on how this could have been tackled and further notes a lack of care in proof-reading this special case resubmission. With the manuscript lacking depth with regards to focusing on the biophysical properties of these transporters, and both reviewers agreeing that the influence of the paper is likely to be low, I recommend this be rejected, but referred to Physiological Reports.

Senior Editor:

Two Expert Referees and the Reviewing Editor carefully evaluated this Special Case Resubmission. While the observations are interesting, as presented in this manuscript, they remain descriptive and not sufficiently quantitative. These points were

raised in the original submission and unfortunately were not addressed to the Referees' satisfaction in the present, Special Case Resubmission. There is continued concern regarding lack of focus, and consequently affecting the depth of insight gained within a chosen focal topic.

REFEREE COMMENTS

Referee #1:

The authors have addressed the concerns made by this assessor.

Referee #2:

Please see attached PDF file.

1st Confidential Review

27-Nov-2021

JP-RP-2021-282607X: "Expression analysis and biophysical properties characterization of the two Atlantic salmon (*Salmo salar* L.) peptide transporters, PepT2a and PepT2b" by Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, Tiziano Verri, and Elena Bossi.

Overview.

Some issues were appropriately addressed, e.g., Table 1 was reworked as suggested, and information about salmon rearing and handling, which is important for the field, was added. Likewise, the readability of some figures has improved. However, the revised manuscript falls short of expectations in most substantial subjects; particularly, the Authors appear disinclined, perhaps unequipped, to perform serious kinetic or molecular modeling, thereby dampening the enthusiasm of this Reviewer for the paper. In its present form, it continues to be mainly descriptive, and the findings incremental.

General concerns.

A figure was included with transport cycle cartoons, but no attempt was made at actual kinetic modeling, i.e. testing the goodness of fit of the data with published kinetic models for Pept1 or Pept2 transporters, using MATLAB or other simulation programs. In my review, I noted that one very interesting observation that could be made from the results is that, in salmon PepT2, presteady-state currents decrease in presence of substrate, as happens in vertebrate PepT1, but opposite to what happens in human PepT2. This appears to have gone unnoticed in the revised version, and would have been an interesting hypothesis to test by comparing simulations from both sets of kinetic constants, and perhaps ultimately producing a novel set, unique (intermediate?) to fish PepT2; this in turn could have tangible comparative and evolutionary implications. The Authors did include sequence alignments, but 1) they did not go beyond a cursory comparison of conserved domains, functional motifs and anecdotal amino acid changes, and 2) PepT1 was not considered.

Similarly, the ribbon cartoons in Fig. 2B, albeit produced from the latest available tridimensional structures, are not a substitute for molecular modeling. It is unsurprising that salmon Pept2 would superimpose well with its human or rat counterparts; beyond that: what is the meaning of the different comparisons made? What insights may be gained from the structure? For example, are there differences in the modeled tridimensional structures of P2a and P2b at the substrate-binding site that might predict differences in transport of substrates other than neutral dipeptides; if so, how could this hypothesis be tested experimentally? Are there structural differences in the proton-binding site, or in other relevant residues, that might explain the apparent differences in the Q/V shown in Fig. 10, or in the maximum transport rates? How does the substrate-binding site, and the transport pathway in general, look in presence of different substrates, and what are the potential implications for affinity or turnover?

In my previous review, I mentioned that, in the absence of comprehensive studies where a variety of compounds is tested (di- and tripeptides; differently-charged; natural substrates and drugs...),

the Authors might want to refrain from making claims about the physiological significance of the two paralogs. This seems to have gone somewhat unnoticed in the revised text. As such, the findings reported in this paper do not justify making the distinction between PepT2 isoforms in the title. For example, the kinetic and mechanistic behavior of PepT2a and PepT2b, for the one substrate used in all experiments is identical. On page 14, the Authors claim that “PepT2b has higher affinity in all the conditions tested for Gly-Gln if compared to PepT2a”, based on the $K_{0.5}$ values reported in Table 3; it does not: $37 \pm 9 \mu\text{M}$ versus $8 \pm 1 \mu\text{M}$, or $130 \pm 20 \mu\text{M}$ versus $35 \pm 6 \mu\text{M}$, are not different from a physiological point of view. The same applies to the statistically significant (but functionally irrelevant) differences in transport rate shown in Figure 6. There appear to be intriguing differences in charge movement at low pH between P2a and P2b (see above), but these are not brought up for discussion, beyond the mere description. Likewise, and also as previously mentioned, the presence or amount of RNA, on their own, do not necessarily translate into functionally meaningful protein expression; any differences derived from RNA levels may be meaningless when it comes to actual tissue distribution. While the Authors extensively discuss differences in tissue expression across species, they fail to mention the shortcomings of the approach.

Finally, the insights to be gained from the phylogenetic analyses remain unclear.

Specific concerns.

-Less care was taken than would have been advised for a Special Case Resubmission to proof-read the manuscript for typos, grammatical errors, inconsistencies and redundancy. Below is a (non-comprehensive) list of areas to improve.

-I suggest the title be further consolidated to “Functional characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters.”

-Indicating the figure number in the pages where the figures are, and the page number, will help with future reviews.

-There are several examples of subject-verb agreement discrepancies, in particular regarding the word “data”, which is plural and is sometimes followed by “is”.

-The Methods are excessively detailed, in particular regarding what are standard protocols, e.g., isolation and maintenance of *Xenopus* oocytes; or procedures stemming from commercially available kits, which the Authors claim to have followed exactly as set out. Conversely, information about sources is sometimes missing, or outdated; for example “Sigma-Aldrich” is now MilliporeSigma, Saint Louis, MO, USA. In the *Molecular Cloning* section, City, State, and/or Country are missing in many cases. Likewise, once a company has been fully identified, there is no need to repeat the affiliation again; for example, write (Invitrogen, Waltham, MA, USA) on first use, and (Invitrogen) subsequently.

-I brought this issue up in my previous review, but the manuscript continues to be peppered with excessive decimals; see for example Tables 3 and 4, and the text immediately below Table 3 in

page 11. In the Rebuttal letter, the Authors claim that this has been addressed; at least in the revised documents I had access to, it has not.

-The figure legends are too long, and in many cases reiterate what was described in the Methods. Some details are mentioned yet again in the discussion. Particularly, it is not necessary to repeat what is already shown in the figure. For example, the entire legend to Figure 5 could be reworded along these lines:

Figure 5. Transport of Gly-Gln in *Xenopus* oocytes expressing Atlantic salmon PepT2a (**A, B**) and PepT2b (**C, D**). **A, C**, representative traces. **B, D**, current/voltage relationships; insets, currents at pH 6.5 in the presence of 98 mM NaCl (Na, black squares) or tetraethylammonium chloride (TMA, white squares). Data are means \pm SD from 4-24 oocytes, obtained from 1-5 frogs.

Insets need not be numbered. All the remaining information is already written elsewhere, or is evident from the figure panels. Similar rewording should be attempted with all figure legends.

-Legends to figures 5, 7, 10, and elsewhere: it is unnecessary to list, and tedious to read, how many oocytes from how many frogs were used in each experiment. A sentence at the end of the legend, for example, “Data are means \pm SD from x-y oocytes from at least z frogs”, will suffice.

-There are discrepancies in data representation. For example, in some parts of the text, results are shown as mean (SD), and in others as mean \pm SD; please consolidate, according to the Journal guidelines. The legend to Table 4 does not state how data are represented, and neither in Table 3 nor in Table 4 is it mentioned how many oocytes were used.

-Figure 1A needs work. In particular, the circles with numbers in them are unreadable. The geometric symbols are too small. While all these are described in the legend, for ease of interpretation the panel could use a set of *in situ* descriptors, as done for example in figures 5B and 5D, 8 and 10.

-What are the x axes in Figure 4? It is not necessary to restate the rearing conditions in the legend.

-Figure 5: the baseline in panels A and C is barely visible.

-Figure 6: what is I_{tr} ? If the Authors represent I/I_{max} , do they still see these apparent significant differences between P2a and P2b?

-Figure 7, and elsewhere: use only solid lines, not dashes or points.

-Why is Figure 10 not followed by what is now Figure 10, if they are both part of the presteady-state analysis?

-Figure 13: Panel A is a more advanced representation of Panel B. Since the Authors did not include kinetic modeling in their study, for demonstrative purposes Panel B is sufficient. The Authors might want to consolidate Panel B and what is now Figure 11 in a fresh figure, where the general transport mechanism is described. That being said, the information contained in Figure 11 is unnecessary for a readership well-versed in biophysics, at which the Authors appear to aim.

P-RP-2021-282607X: "Expression analysis and biophysical properties characterization of the two Atlantic salmon (*Salmo salar* L.) peptide transporters, PepT2a and PepT2b" by Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, Tiziano Verri, and Elena Bossi.

Overview

Some issues were appropriately addressed, e.g., Table 1 was reworked as suggested, and information about salmon rearing and handling, which is important for the field, was added. Likewise, the readability of some figures has improved. However, the revised manuscript falls short of expectations in most substantial subjects; particularly, the Authors appear disinclined, perhaps unequipped, to perform serious kinetic or molecular modeling, thereby dampening the enthusiasm of this Reviewer for the paper. In its present form, it continues to be mainly descriptive, and the findings incremental.

A: We are regrated to know that our revised manuscript dampens the enthusiasm to the point of not recognizing the amount of work reported and the high quantity and quality of the presented data (which both Referees did recognize – at least we felt that – in their letters to the Authors).

We would like to highlight that this paper: a) assesses for the first time that two functional PepT2-type proteins coded by two different (duplicated) genes that operate in the same vertebrate (teleost fish) model such as the Atlantic salmon (*Salmo salar* L.); b) defines the presence of a PepT2-type transporter such as PepT2a at the gills level, which opens some intriguing scenarios in teleost fish physiology and in an applicative perspective; c) focuses on a detailed analysis by TEVC (although limited, for choice, to one substrate) of the transporter function d) report a full characterization of PepT2 I_{PSS} , that add an important pieces to the picture of the electrogenic activity and biophysical characterization of these transporters, opening the basis of unified model for Pept1 and PepT2 transporter. Taken together, our results go far beyond the observations already published on some teleost fish models such as the zebrafish (*Danio rerio*), the sea bass (*Dicentrarchus labrax*) and the Atlantic salmon itself, where the functional PepT1-type transporters coded by two different (duplicated) genes have been cloned and characterized (while to date a single PepT2-type transporter had been found to occur in the zebrafish genome) (see among others e.g. Verri et al. *FEBS Lett.* 549:115 2003; Romano et al. *Physiol. Genomics* 24:207 2006; Sangaletti et al. *Pflugers Arch.* 459:47 2009; Rønnestad et al. *J. Nutr.* 140:893 2010; Vacca et al. *Genes Nutr* 14:33 2019; Gomes et al. *Am J Physiol Cell Physiol.* 318:C191 2020). Moreover, to our knowledge, it is the first work that analyzed the I_{PSS} of the PepT2 in a non-mammalian ortholog. Studying such vertebrates is opening many new physiological questions, beyond the biophysical, that cannot directly be reconducted to the physiology of the mammalian systems given that the physiological 'set up' of a teleost fish largely diverges. Moreover, in this specific case, in order to reconduct the publication to the general aims of The Journal of Physiology [*i.e.* 'We are also keen to publish work on lower vertebrate or invertebrate models or preparation (e.g. *Drosophila*, *C. elegans* and zebrafish), as long as they further the understanding of the functioning of other organisms including mammals']; we need first to specifically 'characterize' in an 'almost descriptive' but possibly 'very rigorous' way the bases of the function in these – we said – alternative/non-conventional animal model systems. Then, other papers can and will come. The computational model is simply beyond the aims of this first paper on PepT2 transporters in the Atlantic salmon both because we are aware that a good and reliable computational model is a difficult task and because we are not fully equipped (in expertises and in machines) at the moment to perform these analyses in reasonable times requested in this second revision. More specifically on this point, Referee 2's says '... the Authors appear disinclined, perhaps unequipped, to perform serious kinetic or molecular modelling, thereby dampening the enthusiasm of this Reviewer for the paper. ...'. Thanks to the observation provided in the two revisions, we are sure that Referee 2's has enough experience and authority to say that if not disinclined (and we hope the Editors and the Referee believe we are not) 'the Authors appear ... perhaps unequipped ... to perform serious kinetic or molecular modelling, ...'. For sure, we agree with the Referee that our groups are not equipped with all the tools to answer Referee 2's major concerns by the indicated deadline (*i.e.* by the end of January).

Said this, we have revised the manuscript by answering Referee 2's questions at the best of our actual capacities and based on the suggestions provided hitherto.

General concerns

A figure was included with transport cycle cartoons, but no attempt was made at actual kinetic modeling, i.e. testing the goodness of fit of the data with published kinetic models for Pept1 or Pept2 transporters, using MATLAB or other simulation programs. In my review, I noted that one very interesting observation that could be made from the results is that, in salmon PepT2, presteady-state currents decrease in presence of substrate, as happens in vertebrate PepT1, but opposite to what happens in human PepT2. This appears to have gone unnoticed in the revised version, and would have been an interesting hypothesis to test by comparing simulations from both sets of kinetic constants, and perhaps ultimately producing a novel set, unique (intermediate?) to fish PepT2; this in turn could have tangible comparative and evolutionary implications. The Authors did include sequence alignments, but 1) they did not go beyond a cursory comparison of conserved domains, functional motifs and anecdotal amino acid changes, and 2) PepT1 was not considered.

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A: We apologize to have neglected in our previous revision the reviewer observation about the I_{PSS} , we have underestimated the importance of this point. No, we have deeply discussed this aspect (see the last part of the discussion) and carefully evaluated the few results previously published. As the Reviewer says (Ref.) 'presteady-state currents decrease in presence of substrate, as happens in vertebrate PepT1, but opposite to what happens in human (and rat) PepT2'. The mammalian PepT2 I_{PSS} are clearly peculiar, not only if compared with Pept1 but also with most of the tested electrogenic transporters. Unfortunately, the data available in our knowledge in literature are only reported in two papers (for human-transporter and rat transporter) and this from one side limited the possibility to define the absence of I_{PSS} at acidic pH as distinctive of PepT2, on the other hand, add significance to our characterization because it can help to add an important finding necessary to understand the behaviour of peptide transporter. We also agree that "have been an interesting hypothesis to test by comparing simulations from both sets of kinetic constants, and perhaps ultimately producing a novel set, unique (intermediate?) to fish PepT2;" Indeed, it is possible to hypnotize, as reported in the discussion (see page21) and in the papers previously published that the different condition for visualizing the I_{PSS} is related to acidic pH effect on the affinity for substrates and probably also for protons. Moreover, the of mammalian PepT2 is quite interesting because between a set of mutant clones we have prepared from mammalian and piscine PepT1- and PepT2-type transporters some show similar responses. The modulation of such a 'phenotypic feature' appears associated with the presence of some charged amino acids in positions along the sequence of both Pept1 and pepT2. One of these interesting residues is suggested also by the Sala-Rabanal papers (E398-that is Y or N in fish, a neutral amino acid as in mammalian and piscine PepT1). Since we are deeply investigating this aspect (which goes beyond the scope of the manuscript) and a manuscript dealing with the different mutants is in preparation we will upgrade our capacity of work in generating a proper structure-to-function computational model and we will reserve a deep kinetic analysis in this future comparison greatly focused on I_{PSS} . However, we are open to discussing with the Reviewer these data confidentially.

(Ref.) The Authors did include sequence alignments, but 1) they did not go beyond a cursory comparison of conserved domains, functional motifs and anecdotal amino acid changes, and 2) PepT1 was not considered.

The lacking in the alignment with PepT1 as reference was to improve the clarity of Fig.1. Inserting the sequence of PepT1 would make the figure even more confusing and less readable, as also observed by the review (REF.)” *In particular, the circles with numbers in them are unreadable. The geometric symbols are too small*”. We have considered this revision the suggestion of comparison with PepT1 and we have added in the text a few comments (see page12).

Similarly, the ribbon cartoons in Fig. 2B, albeit produced from the latest available tridimensional structures, are not a substitute for molecular modeling. It is unsurprising that salmon Pept2 would superimpose well with its human or rat counterparts; beyond that: what is the meaning of the different comparisons made? What insights may be gained from the structure? For example, are there differences in the modeled tridimensional structures of P2a and P2b at the substrate-binding site that might predict differences in transport of substrates other than neutral dipeptides; if so, how could this hypothesis be tested experimentally? Are there structural differences in the proton binding site, or in other relevant residues, that might explain the apparent differences in the Q/V shown in Fig. 10, or in the maximum transport rates? How does the substrate-binding site, and the transport pathway in general, look in presence of different substrates, and what are the potential implications for affinity or turnover?

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This point was addressed in the answer to the overview, some information has been added to the results (see page 12).

(Ref.) What insights may be gained from the structure? For example, are there differences in the modeled tridimensional structures of P2a and P2b at the substrate-binding site that might predict differences in transport of substrates other than neutral dipeptides; if so, how could this hypothesis be tested experimentally?

As reported above and in our letter, many of these questions can be answered by a new manuscript.

(Ref.) Are there structural differences in the proton binding site, or in other relevant residues, that might explain the apparent differences in the Q/V shown in Fig. 10, or in the maximum transport rates?

As the reviewer probably know and can observe from the alignment proposed in our manuscript, and from the data reported in the two most recent structural papers, the protons binding site as well as the other determinants involved in the main functions of the transporters are well conserved in all the cloned slc15a1 and 2 family members. In this version of the manuscript, a detailed reference about this point was added in describing Fig.1 on page 12. Certainly, the structure of the protons binding site may be modified by the neighbours' residues. The differences of the behaviour between the two salmon transporters (but for mammalian transporter too) in pre-steady-state currents and consequently in Q/V relationship could be related to the small differences in amino acids sequence nearby the “conserved” determinants, and certainly, these could affect the transport rate as well as the I_{pss} as reported in papers dealing about the relation between the affinity, transport rate and transport efficiency.

(Ref.) How does the substrate-binding site, and the transport pathway in general, look in presence of different substrates, and what are the potential implications for affinity or turnover?

A so specific and detailed analysis of structure-function and modelling it will be surely intriguing but for the authors, it is beyond the scope of this paper as we have reported in our comment of the overview. It is a very nice suggestion for further investigation together with the analysis and comparison between mammalian and piscine PepT2 transporters.

In my previous review, I mentioned that, in the absence of comprehensive studies where a variety of compounds is tested (di- and tripeptides; differently-charged; natural substrates and drugs...), the Authors might want to refrain from making claims about the physiological significance of the two paralogs. This seems to have gone somewhat unnoticed in the revised text. As such, the findings reported in this paper do not justify making the distinction between PepT2 isoforms in the title. For example, the kinetic and mechanistic behavior of PepT2a and PepT2b, for the one substrate used in all experiments is identical. On page 14, the Authors claim that “PepT2b has higher affinity in all the conditions tested for Gly-Gln if compared to PepT2a”, based on the $K_{0.5}$ values reported in Table 3; it does not: $37 \pm 9 \mu\text{M}$ versus $8 \pm 1 \mu\text{M}$, or $130 \pm 20 \mu\text{M}$ versus $35 \pm 6 \mu\text{M}$, are not different from a physiological point of view. The same applies to the statistically significant (but functionally irrelevant) differences in transport rate shown in Figure 6. There appear to be intriguing differences in charge movement at low pH between P2a and P2b (see above), but these are not brought up for discussion, beyond the mere description. Likewise, and also as previously mentioned, the presence or amount of RNA, on their own, do not necessarily translate into functionally meaningful protein expression; any differences derived from RNA levels may be meaningless when it comes to actual tissue distribution. While the Authors extensively discuss differences in tissue expression across species, they fail to mention the shortcomings of the approach.

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A: There is a biochemical difference between the two transporters based on the $K_{0.5}$ and I_{max} values. However, as suggested by the Reviewer, we have carefully revised our manuscript in order to avoid referring to any ‘physiological role(s)’ played by the two transporters.

(Ref.) There appear to be intriguing differences in charge movement at low pH between P2a and P2b (see above), but these are not brought up for discussion, beyond the mere description.

A: As requested by the Reviewer, a comment has been added to the Discussion (page 21) mainly relatively to the Q and rate constant.

Likewise, and also as previously mentioned, the presence or amount of RNA, on their own, do not necessarily translate into functionally meaningful protein expression; any differences derived from RNA levels may be meaningless when it comes to actual tissue distribution. While the Authors extensively discuss differences in tissue expression across species, they fail to mention the shortcomings of the approach.

The presence of mRNA is a common approach in investigating newly cloned transporter. We believe that it is sufficient for the aim of our manuscript. We have corrected the previous version of the manuscript unrelating the mRNA presence to the functional protein. We have now added a sentence to clarify the meaning of investigating the presence of the mRNA and the future investigative perspective. Nevertheless, the results here obtained by quantification of slc15a2 mRNA should be further validated using techniques such as Western blot or immunohistochemistry that can determine PepT2a and PepT2b protein expression in Atlantic tissues. This will be considered for our further work.

Finally, the insights to be gained from the phylogenetic analyses remain unclear.

A: The use of this phylogenetic-approach – jointly to the synteny-based approach – is meant to distinguish ‘what-is-what’ based on the relative similarity of the various sequences. By using this kind of approach, it is rather easy to establish which Atlantic salmon transporter is PepT2a and which transporter is PepT2b when more (than one) paralog(s) are present in an organism or a group of organisms. In this analysis, the use of an unrooted tree means that no specific temporal and/or evolutive meaning is given to the topology of the tree. A similar approach is currently used by us and other groups to assess these differences. Secondly, that two genes exist in the Atlantic salmon genome is assessed by the synteny analysis reported in Figure 3. As you can see the duplication event that occurred in salmonids is evident by the presence of the similar set of genes present in the Atlantic salmon chromosomes ssa21 and ssa25 vs. the zebrafish chromosome 9 and the Northern pike chromosome LG16. To clarify this point, the text has been modified in the sections: Results, Sequence and comparative analysis as follow: ‘Phylogenetic analysis of the putative Atlantic salmon PepT2a and PepT2b showed that these two proteins clustered into two distinct branches with their salmonid homologue sequences (Fig. 2). Northern pike PepT2 was the closest relative to the salmonid clade, and except for goldfish, which also underwent an additional WGD, only one PepT2-type protein was found in other teleost species’. In addition, the fact that the phylogenetic tree is ‘unrooted’ has been specified in the legend of Fig. 2.

Specific concerns

-Less care was taken than would have been advised for a Special Case Resubmission to proof-read the manuscript for typos, grammatical errors, inconsistencies and redundancy. Below is a (noncomprehensive) list of areas to improve.

A: We apologize for that. We have now carefully revised the text at our best and based on Referee 2’s indications (please see below).

-I suggest the title be further consolidated to “Functional characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters.”

A: The title has been consolidated as suggested by Referee 2.

-Indicating the figure number in the pages where the figures are, and the page number will help with future reviews.

We are very sorry for that. We did not consider that the merged file missed it, we thought that the system will insert the number or the name of the file as foot pages-we have inserted in the text where the figure must be placed but we understand that the absence of numbering in the figure could be irritating. We will check it carefully in the resubmission.

-There are several examples of subject-verb agreement discrepancies, in particular regarding the word “data”, which is plural and is sometimes followed by “is”.

A: We apologize for that. We have revised at our best the manuscript based on Referee 2’s, we hope to have eliminated all the typos errors.

-The Methods are excessively detailed, in particular regarding what are standard protocols, e.g., isolation and maintenance of *Xenopus* oocytes; or procedures stemming from commercially available kits, which the Authors claim to have followed exactly as set out. Conversely, information about sources is sometimes missing, or outdated; for example “Sigma-Aldrich” is now MilliporeSigma, Saint Louis, MO, USA. In the Molecular Cloning section, City, State, and/or Country are missing in many cases. Likewise, once a company has been fully identified, there is no need to repeat the affiliation again; for example, write (Invitrogen, Waltham, MA, USA) on first use, and (Invitrogen) subsequently.

(Ref.) *The Methods are excessively detailed, in particular regarding what are standard protocols, e.g., isolation and maintenance of Xenopus oocytes; or procedures stemming from commercially available kits, which the Authors claim to have followed exactly as set out.*

A: To write Materials and Methods we have followed the authors’ guidance (<https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>). In the revised version of the

manuscript, we have cut the various sections wherever possible, i.e. when other publications could be cited, we have also avoid the duplication of methods description in figures and text.

(Ref.) Conversely, information about sources is sometimes missing, or outdated; for example, “Sigma-Aldrich” is now MilliporeSigma, Saint Louis, MO, USA. In the Molecular Cloning section, City, State, and/or Country are missing in many cases. Likewise, once a company has been fully identified, there is no need to repeat the affiliation again; for example, write (Invitrogen, Waltham, MA, USA) on first use, and (Invitrogen) subsequently.

A: We apologize for that. We have now revised and corrected at our best all the company were revised and reported according to Referee 2’s suggestions/indications. In particular, we have modified the text in the following points: a) section *Molecular cloning (Sigma Aldrich to Millipore and Life Technology to Thermo)* page 7 and correct the detail of city and state, b) section *Quantitative Real-Time PCR*, page 8- correcting inserting/deleting the cities and the states;

-I brought this issue up in my previous review, but the manuscript continues to be peppered with excessive decimals; see for example Tables 3 and 4, and the text immediately below Table 3 at page 11. In the Rebuttal letter, the authors claim that this has been addressed; at least in the revised documents I had access to, it has not.

A: We revised the text again as suggested by Referee 2. Now we have double-checked the manuscript, tables, legends, etc., the decimals have been adapted according to Referee 2’s requests for the table and the text.

The figure legends are too long, and in many cases reiterate what was described in the Methods. Some details are mentioned yet again in the discussion. Particularly, it is not necessary to repeat what is already shown in the figure. For example, the entire legend to Figure 5 could be reworded along these lines:

Figure 5. Transport of Gly-Gln in *Xenopus* oocytes expressing Atlantic salmon PepT2a (**A, B**) and PepT2b (**C, D**). **A, C**, representative traces. **B, D**, current/voltage relationships; insets, currents at pH 6.5 in the presence of 98 mM NaCl (Na, black squares) or tetraethylammonium chloride (TMA, white squares). Data are means \pm SD from 4-24 oocytes, obtained from 1-5 frogs.

Insets need not be numbered. All the remaining information is already written elsewhere, or is evident from the figure panels. Similar rewording should be attempted with all figure legends.

A: We have revised (shortened) all the figure legends and aligned them with the ‘Materials and Methods’ and ‘Discussion’ manuscript text and contents, according to the Reviewer’s suggestions. This has considerably shortened the text.

-Legends to figures 5, 7, 10, and elsewhere: it is unnecessary to list, and tedious to read, how many oocytes from how many frogs were used in each experiment. A sentence at the end of the legend, for example, “Data are means \pm SD from x-y oocytes from at least z frogs”, will suffice.

A: We have adjusted the figure captions considering the authors’ instructions and reviewer’s suggestions. We have reported here this information because the number of oocytes and frogs were different in the experiments reported in different figures. We have now summarized it in the ‘Materials and Methods’. This was the way we had presented this piece of information in the previous version of the manuscript, but in the previous revision process we were asked to detail the number of animals for each experiment.

-There are discrepancies in data representation. For example, in some parts of the text, results are shown as mean (SD), and in others as mean \pm SD; please consolidate, according to the Journal guidelines. The legend to Table 4 does not state how data are represented, and neither in Table 3 nor in Table 4 is it mentioned how many oocytes were used.

(Ref.) There are discrepancies in data representation. For example, in some parts of the text, results are shown as mean (SD), and in others as mean \pm SD; please consolidate, according to the Journal guidelines.

A: We apologize for that. We have consolidated the data representation according to the journal guidelines as suggested by Referee 2's. In particular changes have been provided in: a) Figure 5, in figure caption, page 19; b) Figure 7, in figure caption, page 21; c) Figure 10, in figure caption, page 25.; d) Table 3, page 23,.

(Ref.) *The legend to Table 4 does not state how data are represented,*

What is reported in the table 4 was in the previous version stated in the text after the figure 10 from which the data were referred.

Now we have detailed with the information requested (see Fig.10 and page 25, and table 4 page 26) also the description of the table as requested.

and neither in Table 3 nor in Table 4 is it mentioned how many oocytes were used.

A: For table 3 we did not understand the observation of the reviewer because the information was reported both in the figure 7 caption from which the data were collected (data are means (SD) from 10-19 oocytes, obtained from 2-3 frogs), and reported in the last column of the table and explained in the column header not only in this last version but also in the previous one.

-Figure 1A needs work. In particular, the circles with numbers in them are unreadable. The geometric symbols are too small. While all these are described in the legend, for ease of interpretation the panel could use a set of in situ descriptors, as done for example in figures 5B and 5D, 8 and 10.

A: This figure has been modified as suggested.

-What are the x axes in Figure 4? It is not necessary to restate the rearing conditions in the legend.

(Ref.) *What are the x axes in Figure 4?*

A: They are the different tissues tested, now we have called the x axes "**tissues**" and corrected also the figure caption .

(Ref.) *It is not necessary to restate the rearing conditions in the legend.*

A: What is reported are not the rearing conditions but the measures of the sampled fish (i.e. wet weight and total length) from which tissues have been extracted and used in the experiment. We have removed this information.

-Figure 5: the baseline in panels A and C is barely visible.

To correctly see the figure, it is necessary to download the high resolution figures and not only to look to the merged pdf.

-Figure 6: what is I_{tr} ? If the Authors represent I/I_{max} , do they still see these apparent significant differences between P2a and P2b?

A: We have preferred to use the same data as Figure 5 without normalization to better appreciate the statistical difference and single oocyte data. I_{tr} is the transport current obtained as described in Materials and Methods.

-Figure 7, and elsewhere: use only solid lines, not dashes or points.

We thought it is a problem of resolution of the merged file, To correctly see the figure and the line it is necessary to download the figure at high resolution -we have used solid line -

-Why is Figure 10 not followed by what is now Figure 10, if they are both part of the presteady state analysis?

Sorry we have not modify the figures because we do not understand what we have to do. Make only one figure with figure 9 and 10 ? or 10 and 11?

-Figure 13: Panel A is a more advanced representation of Panel B. Since the Authors did not include kinetic modeling in their study, for demonstrative purposes Panel B is sufficient. The Authors might want to consolidate Panel B and what is now Figure 11 in a fresh figure, where the general transport mechanism is described. That being said, the information contained in Figure 11 is unnecessary for a readership well-versed in biophysics, at which the Authors appear to aim.

(Ref.) -Figure 13: Panel A is a more advanced representation of Panel B. Since the Authors did not include kinetic modeling in their study, for demonstrative purposes Panel B is sufficient.

A: We agree with Referee 2, and according to the suggestion we include in Figure 13 (now Figure 12) Panel B only, and only for demonstrative purposes.

(Ref.) The Authors might want to consolidate Panel B and what is now Figure 11 in a fresh figure, where the general transport mechanism is described. That being said, the information contained in Figure 11 is unnecessary for a readership well-versed in biophysics, at which the Authors appear to aim.

A: The use of Figure 11 (now part of the new figure 11) was intended to represent the situation we want to analyze for a readership not well-versed in biophysics. However, we agree with Referee 2 and thus we have added the cartoon to the figure of that rate constant that is now the new Figure 11 in the new version of the manuscript. Manuscript, figure numbering, etc. have consequently been changed/adapted.

Dear Professor Bossi,

Re: JP-RP-2022-282781X "Revision to JP-RP-2021-282607X "Functional characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters." by Elena Bossi, Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, and Tiziano Verri

Thank you for resubmitting your revised Research Article to The Journal of Physiology. It has been assessed by the original Reviewing Editor and Referees and has been well received. Some final revisions have been requested.

Please advise your co-authors of this decision as soon as possible.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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I hope you will find the comments helpful and have no difficulty returning your revisions within 2 weeks.

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available.

Any image files uploaded with the previous version are retained on the system. Please ensure you replace or remove all files that have been revised.

REVISION CHECKLIST:

- Article file, including any tables and figure legends, must be in an editable format (eg Word)
- Abstract figure file (see above)
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- Upload each figure as a separate high quality file
- Upload a full Response to Referees, including a response to any Senior and Reviewing Editor Comments;
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To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Word, or similar, file and respond to each point in colour or CAPITALS and upload this when you submit your revision.

I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

Dr Peking Fong

Senior Editor
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REQUIRED ITEMS:

-You must start the Methods section with a paragraph headed [Ethical Approval](#). A detailed explanation of journal policy and regulations on animal experimentation is given in [Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology](#) by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: <https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

-If $n \leq 30$, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

-If $n > 30$, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

- n clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

-All relevant n values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

-The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

-Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

-Statistics Summary Document completed appropriately upon revision

-Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

EDITOR COMMENTS

Reviewing Editor:

The authors have responded satisfactorily to all points raised by reviewer 2 and referee 3 has provided a clear summary and review of both the manuscript and revisions made. Therefore, I recommend that the paper be accepted, pending the few minor revisions carefully noted by reviewer 2.

Senior Editor:

If the statistical summary document has errors please describe what is incorrect:

Definition of n should be clear on the statistical summary document. In this study, n likely refers to the numbers of individual oocytes, so this should be stated explicitly. The Authors might consider including the total number of oocyte isolation batches/frogs as well within the "Definitions of 'n'". Please refer to the example provided via the live link that can be accessed via Information for Authors.

Please incorporate all data that is reported in the manuscript onto one page/tab (rather than 3 pages/tab, as the Authors appear to have done). More specifically: the Statistical Summary Document seems to include only data presented in figure 6; please include data presented in all other figures reporting statistical analysis (figures 4, 5, 7-11) regardless of outcome of tests (that is, regardless of whether p is considered significant against a stated confidence limit).

Please also note information provided on column A ("Experimental question number) should state the questions being tested, rather than be descriptive of individual data points. These may appear within multiple lines under column C, titled "Experimental location/variable".

Comments to the Author:

Thank you for your responsiveness and thorough attention to the critiques raised in the previous reviews of your manuscript. We appreciate your persistence in addressing the points raised during the progression of your manuscript through the review process, and hope you will be pleased with the results of this last round.

You will see that Referee 1 was not available to review this last revision. Therefore, we engaged a third referee, who expressed enthusiasm for your study. Furthermore, Referee 2 overall was appreciative of your responsiveness in addressing their detailed, previous comments. There were some remaining, minor corrections in phrasing and presentation also offered. You likely will be able to address these readily.

We are also confident that you will be able to provide remaining details concerning statistical methodology and the Statistical Summary Document. In addition to necessary corrections to the Statistical Summary Document, please ensure that details pertinent to statistical tests performed are explicitly provided in the last paragraph of the manuscript; the present version describes solution compositions in the last paragraph. I suggest moving that section to the end of the preceding section pertaining to *Xenopus* oocyte expression and electrophysiology.

REFeree COMMENTS

Referee #2:

See my review attached.

Referee #3:

This is the third submission of this manuscript which is accompanied by a very thorough response to a reviewer's extensive comments and criticisms. The paper reports on the cloning and initial characterization of not one, but two, PepT2 transporters expressed in Pacific salmon. The second paralogue appears to have resulted from a gene duplication with some potentially significant differences in functionality and tissue distribution. All of the functional characterization using current measurements taken using the oocyte expression system suggests that the the second PepT2 protein has a significantly different affinity, at least for the test reference substrate Gly-Gly. This substrate was a good choice for this study as this is substrate which has been used in most of the previous studies with different species enabling appropriate comparisons to be made.

The manuscript has been significantly improved with regard to spelling (using English spelling) and grammar as well as reducing the duplication of methods and correcting manufacturers names. This has required a considerable effort for a group of researchers for whom English is not their first language and the manuscript benefits from that work.

As proposed by the reviewer the authors have changed the title.

The authors allude to ongoing further work in which they have substitution mutations to these proteins related to residues found in human PepT1. This work is yet to be submitted for publication but will be a second important study and is not required for this current paper under consideration, which is a very thorough study of 2 new proteins found in the Pacific salmon.

The authors have not included a sequence alignment for PepT1 to keep the figure manageable, but have now added some additional comments related to that comparison on page 12. This is a good resolution for keeping the paper more readable.

The inclusion of the ribbon structural models of the two proteins is an appropriate first step prior to working on more detailed molecular modelling for a subsequent manuscript. Similarly, detailed structure function analysis related to comparing the effect of residues close to the putative substrate binding site in PepT1 and 2 represent a very comprehensive future study not required for this paper.

The authors have removed the references to possible physiological roles of the 2 proteins, but they have added a comment which was requested in the discussion on page 21.

The authors correctly point out that the use of mRNA expression in different tissues represents possible levels of protein expression and is commonly used in the published literature. They do acknowledge that there are better methods, but those have significant technical challenges and again should be tried in future studies.

The use of an unrooted tree for phylogenetic studies is entirely appropriate and indicates where the gene duplication is likely to have occurred. The revised text in the legend of figure 2 helps to make that distinction.

The revisions to the language have greatly improved the manuscript as have the condensations to the methods sections. As requested the numerous corrections and changes have also been made regarding manufacturers names, addresses, the figure legends shortened and checked for omissions. Similarly, requests regarding combining some figures have been addressed.

The consequence of all of these edits is a significantly improved manuscript.

END OF COMMENTS

JP-RP-2022-282781X-CORRECTION: "Functional Characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters" by Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, Tiziano Verri, and Elena Bossi.

The Authors made a commendable effort to address this Reviewer's concerns, and now the scope, the conclusions drawn, and the general tone of the manuscript are in accordance with the data presented.

A few minor typos or inconsistencies were found when perusing the current version:

-Data in Table 3 appear to be presented as mean \pm SE, whereas elsewhere in the manuscript data are shown as mean \pm SD. Is there a reason for this (or is there a typo in the legend to Table 3)? Also, one style of data presentation should be chosen, i.e., 36.9 (9.2) or 36.9 \pm 9.2 and used for both Table 3 and Table 4 -perhaps the first one, as it is also used in the text (see second half of page 14)?

-Review the text for "*I_{pss}* currents", which is redundant, as *I* stands for *current*; in most cases it is correctly written, without the word "currents", but there are some examples of "*I_{pss}* currents" (e.g., page 16, lines 13-14; see also page 20). Also, review for some instances of *pre-steady-state* (instead of *presteady-state*).

-Page 16, lines 13-20. These statements are too convoluted and sometimes grammatically incorrect. At this stage, it is no longer necessary to identify the potential as *V*. Also, there is no reason to split the information contained in lines 8 to 20 in three paragraphs. May I suggest, starting at the end of line 12:

"...transport. The decay time constant τ , and the area underneath the *I_{pss}*, which corresponds to the amount of charge moved in the electrical membrane field *Q* (Peres *et al.*, 2004a; Bossi *et al.*, 2011; Renna *et al.*, 2011b), were measured at each potential; the τ/V and Q/V relationships obtained at each tested pH are plotted in **Fig. 10**. Like other peptide transporters..."

-Page 18, line 2: "...correlated to pH." (remove *the of*).

-Page 18, Discussion, line 7: "...goldfish (*Carassius auratus*), a cyprinid, the genome of which..."

-Page 18, Discussion, lines 8-11: The sentence is long and hard to understand. May I suggest:

"Of note, two genes encoding for PepT2-type transporters, namely *slc15a2a-1* and *slc15a2a-2*, have been recently described in another cyprinid, the common carp (*Cyprinus carpio*) (Dong *et al.*, 2020). In the Atlantic salmon..."

-Page 18, line 5 from the bottom: "Moreover, as previously reported in a variety of..."

-Page 20: See several examples of "*I_{pss}* currents". Also, at this point, it is no longer necessary to write "presteady-state currents (*I_{pss}*)", as is done in this page at least twice. Consolidate to "*I_{pss}*".

-Page 21, lines 10-11: "Together, these data confirm that [...] the polarity of *I_{pss}* kinetics and the magnitude of [...] **are** strictly dependent..." (instead of *is*).

-Page 21, line 19: "...transporters..." (plural).

-Page 22, line 7: "...piscine and mammalian transporters..." (plural).

Dear Professor Bossi,

Re: JP-RP-2022-282781X "Revision to JP-RP-2021-282607X "Functional characterization of Atlantic salmon (Salmo salar L.) PepT2 transporters." by Elena Bossi, Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, and Tiziano Verri

Thank you for resubmitting your revised Research Article to The Journal of Physiology. It has been assessed by the original Reviewing Editor and Referees and has been well received. Some final revisions have been requested.

Please advise your co-authors of this decision as soon as possible.

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If the statistical summary document has errors please describe what is incorrect:

[A: We have recognized some errors in the statistical summary document and corrected them accordingly.](#)

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[A: Sorry for the lapse, we did not look carefully at the documents. Now we have inserted the definition of n \(number of oocytes and N number of batches\). We have also inserted the table in a Word document.](#)

Please incorporate all data that is reported in the manuscript onto one page/tab (rather than 3 pages/tab, as the Authors appear to have done).

[A: Done](#)

More specifically: the Statistical Summary Document seems to include only data presented in figure 6; please include data presented in all other figures reporting statistical analysis (figures 4, 5, 7-11) regardless of outcome of tests (that is, regardless of whether p is considered significant against a stated confidence limit).

[A: Please consider that only Figure 6 shows a statistical analysis \(which is the analysis of the data of Figure 5\) for two selected voltages, for all the tested pH values. It compares the behavior at the different pH of PepT2a and of the PepT2b protein. It also provides a comparison of the current elicited by the two proteins at each tested pH. All the other figures report descriptive statistics or fitted data, which if we understand correctly, is not required to be reported in the statistical document.](#)

Please also note information provided on column A ("Experimental question number) should state the questions being tested, rather than be descriptive of individual data points. These may appear within multiple lines under column C, titled "Experimental location/variable".

[A: Sorry for our misunderstanding we have corrected it accordingly](#)

Comments to the Author:

Thank you for your responsiveness and thorough attention to the critiques raised in the previous reviews of your manuscript. We appreciate your persistence in addressing the points raised during the progression of your manuscript through the review process and hope you will be pleased with the results of this last round.

A: We are really pleased with this great opportunity to publish in the Journal of Physiology. We are also truly grateful to the editors for believing in our work and for giving us the chance to improve it significantly.

You will see that Referee 1 was not available to review this last revision. Therefore, we engaged a third referee, who expressed enthusiasm for your study. Furthermore, Referee 2 overall was appreciative of your responsiveness in addressing their detailed, previous comments. There were some remaining, minor corrections in phrasing and presentation also offered. You likely will be able to address these readily.

We are also confident that you will be able to provide the remaining details concerning statistical methodology and the Statistical Summary Document. In addition to necessary corrections to the Statistical Summary Document, please ensure that details pertinent to statistical tests performed are explicitly provided in the last paragraph of the manuscript; the present version describes solution compositions in the last paragraph. I suggest moving that section to the end of the preceding section pertaining to *Xenopus* oocyte expression and electrophysiology.

A: Thanks so much for the suggestion. We have now inserted the statistical analysis in the methods, and we have revised the statistical document wherever necessary.

REFEREE COMMENTS

Referee #2:

JP-RP-2022-282781X-CORRECTION: "Functional Characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters" by Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, Tiziano Verri, and Elena Bossi.

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Also, one style of data presentation should be chosen, i.e., 36.9 (9.2) or 36.9 \pm 9.2 and used for both Table 3 and Table 4 -perhaps the first one, as it is also used in the text (see second half of page 14)?

A: We apologize for the oversight of the \pm in Table 3. The data presented in Table 3 are reported with (SE) because it was determined by using the Origin fitting tools and the final data returned by the program are \pm SE. In addition, one style of presentation has been chosen throughout the manuscript, tables, and figures.

-Review the text for "Ipps currents", which is redundant, as I stands for current; in most cases it is

correctly written, without the word “currents”, but there are some examples of “Ipss currents” (e.g., page 16, lines 13-14; see also page 20). Also, review for some instances of pre-steady-state (instead of presteady-state).

A: Done

-Page 16, lines 13-20. These statements are too convoluted and sometimes grammatically incorrect. At this stage, it is no longer necessary to identify the potential as V. Also, there is no reason to split the information contained in lines 8 to 20 in three paragraphs. May I suggest, starting at the end of line 12:

“...transport. The decay time constant τ , and the area underneath the Ipss, which corresponds to the amount of charge moved in the electrical membrane field Q (Peres et al., 2004a; Bossi et al., 2011; Renna et al., 2011b), were measured at each potential; the τ/V and Q/V relationships obtained at each tested pH are plotted in Fig. 10. Like other peptide transporters...”

A: Done, thanks for the opportunity to use clearer language

-Page 18, line 2: “...correlated to pH.” (remove the of).

A: Done

-Page 18, Discussion, line 7: “...goldfish (*Carassius auratus*), a cyprinid, the genome of which...”

A: Done

-Page 18, Discussion, lines 8-11: The sentence is long and hard to understand. May I suggest: “Of note, two genes encoding for PepT2-type transporters, namely *slc15a2a-1* and *slc15a2a-2*, have been recently described in another cyprinid, the common carp (*Cyprinus carpio*) (Dong et al., 2020). In the Atlantic salmon...”

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-Page 21, lines 10-11: “Together, these data confirm that [...] the polarity of Ipss kinetics and the magnitude of [...] are strictly dependent...” (instead of is).

A: Done

-Page 21, line 19: “...transporters...” (plural).

-Page 22, line 7: “...piscine and mammalian transporters...” (plural).

A: Done

Referee #3:

This is the third submission of this manuscript which is accompanied by a very thorough response to a reviewer's extensive comments and criticisms. The paper reports on the cloning and initial characterization of not one, but two, PepT2 transporters expressed in Pacific salmon. The second paralogue appears to have resulted from a gene duplication with some potentially significant differences in functionality and tissue distribution. All of the functional characterization using current measurements taken using the oocyte expression system suggests that the second PepT2 protein has a significantly different affinity, at least for the test reference substrate Gly-Gly. This substrate was a good choice for this study as this is substrate which has been used in most of the previous studies with different species enabling appropriate comparisons to be made.

The manuscript has been significantly improved with regard to spelling (using English spelling) and grammar as well as reducing the duplication of methods and correcting manufacturers names. This has required a considerable effort for a group of researchers for whom English is not their first language and the manuscript benefits from that work.

As proposed by the reviewer the authors have changed the title.

The authors allude to ongoing further work in which they have substitution mutations to these proteins related to residues found in human PepT1. This work is yet to submitted for publication but will be a second important study and is not required for this current paper under consideration, which is a very thorough study of 2 new proteins found in the Pacific salmon.

The authors have not included a sequence alignment for PepT1 to keep the figure manageable, but have now added some additional comments related to that comparison on page 12. This is a good resolution for keeping the paper more readable.

The inclusion of the ribbon structural models of the the two proteins is an appropriate first step prior to working on more detailed molecular modelling for a subsequent manuscript. Similarly, detailed structure function analysis related to comparing the effect of residues close to the putative substrate binding site in PepT1 and 2 represent a very comprehensive future study not required for this paper.

The authors have removed the references to possible physiological roles of the 2 proteins, but they have added a comment which was requested in the discussion on page 21.

The authors correctly point out that the use of mRNA expression in different tissues represents possible levels of protein expression and is commonly used in the published literature. They do acknowledge that there are better methods, but those have significant technical challenges and again should be tried in future studies.

The use of an unrooted tree for phylogenetic studies is entirely appropriate and indicates where the gene duplication is likely to have occurred. The revised text in the legend of figure 2 helps to make that distinction.

The revisions to the language have greatly improved the manuscript as have the condensations to the methods sections. As requested numerous corrections and changes have also been made regarding manufacturers names, addresses, the figure legends were shortened and checked for omissions. Similarly, requests regarding combining some figures have been addressed.

The consequence of all of these edits is a significantly improved manuscript.

[A: We sincerely thank Referee #3 for carefully and deeply reading the last versions of the manuscript submitted, and for her/his efforts in detailing what we have revised in the submitted versions.](#)

Comments to the statistical documents.

E: More specifically: the Statistical Summary Document seems to include only data presented in figure 6; please include data presented in all other figures reporting statistical analysis (figures 4, 5, 7-11) regardless of outcome of tests (that is, regardless of whether p is considered significant against a stated confidence limit).

A: About the request to insert more statistical data in the statistical table, as we have reported in the responses to the referees' documents, we hope to have correctly interpreted the information that has to be reported in this document. In fact, Figure 6 reports a statistical analysis for two selected voltages, for all the tested pH values of the currents reported in Figure 5. It compares the behavior at the different pH of PepT2a and of the PepT2b protein. It also provides a comparison of the current elicited by the two proteins at each tested pH. All the other figures show instead descriptive statistics or fitted data, which if we understand correctly, is not required to be reported in the statistical document. In detail: Figure 4 is a box plot of data about the slc15a2(a or b) copy number per ng of total RNA normalized using β -actin copy number per ng of total RNA, collected for both transporters in the different tissues. It shows the expression, not the statistical differences between tissues. Figure 7 are the dose (Gly-Gln)/currents relationships at each voltage, the data were fitted to determine the affinity and I_{max} for each transporter and reported in Figure 8. In Figure 7, the statistic is descriptive statistics (mean and (SD)) and Figure 8 is the fitting of the value of Figure 7. This data is also reported in table 3. Figure 9 are representative traces of the currents recorded in 5 to 24 oocytes from several batches, analyzed respectively in Figure 5 for transport current (statistic in Figure 6) and Figure 10 and 11 for the biophysical parameters. These data are also reported in table 4. If it is necessary, we can add to statistical documents the value of each point reported in the figures and the SD, as the fitting value.

Dear Professor Bossi,

Re: JP-RP-2022-282781XR1 "Revision to JP-RP-2021-282607X "Functional characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters." by Francesca Vacca, Ana S. Gomes, Koji Murashita, Raffaella Cinquetti, Roseti Cristina, Amilcare Barca, Ivar Rønnestad, Tiziano Verri, and Elena Bossi

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EDITOR COMMENTS

Reviewing Editor:

Thank you to the authors for making the requested edits, everything appears to be in order.

Senior Editor:

Many thanks for addressing points raised in review of the previous version of your manuscript. The Statistical Summary document is vastly improved.

There are a few remaining minor points that require your attention.

1) Regarding figure 6: The following is a suggestion, and I apologize for not catching it when assessing the last version. This figure would align better with current journal statistical policy if the asterisk indicators of significance were replaced by actual p values obtained from statistical testing. The resultant figure would be more information-rich and moreover have a less busy appearance (although the latter might be simply a matter of personal preference). Substituting the exact p values for asterisks allows removal of the sentence regarding to the assignation of significance; this way, readers can judge for themselves what is or is not significant.

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Thanks, Correction Done

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The assumption of the editor is correct. The values reported in Figure 7 are mean (SD), as stated in the legend. The current/concentration curves of Figure 7 at each voltage were then fitted as indicated in Figure 8 legend and Table 3 with Origin. The results of Origin fitting returned as mean (SE) (not exchangeable vs to mean (SD)) were plotted in Figure 8 and inserted in the table.

Regardless of whether or not the plotted data need to be included in the Statistical Summary document, the legend for Figure 7 states that individual points are plotted as mean +/- SD. Please then state the values within the legend. It would be acceptable to provide the values at -60 and -120 mV for each condition, in alignment with the fit values presented in Table 3.

We have now reported all the mean values for each condition (concentration, voltage, and pH) in the statistical document and we are sharing the raw data and all the fitting parameters in the link indicated in the data availability statement.

We have decided to do not insert the values in the legend. In fact, even only for two voltages and one concentration of substrate, for the three pH conditions and for both transporters, were too many numbers to be inserted in the legend. We have inserted instead a sentence about their availability. Now all these data are accessible to all the readers in both places. We hope that the editor approves this solution.

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END OF COMMENTS

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