

Dear authors,

your manuscript has been reviewed by two colleagues who made a series of constructive comments to further improve it.

Dear editor,

Thank you very much for taking on the evaluation of our preprint, and for your constructive comments. We have now revised our paper addressing each of your and the two reviewers' points. Please find our detailed answers below, in green font.

Methodology is not always particularly well considered as worth publishing in ecology and evolution, a view I strongly disagree with because established methodologies are the base of the principle that science is based on replication. In that sense, your manuscript is very interesting.

Thank you!

I agree with one reviewer that it might however benefit from elaborating on key questions this approach can help studying and whether the methodology might be applied to a broader range of species.

We have now rephrased and expanded the end of the discussion, including one paragraph providing examples of research areas where this methodology could be helpful (L362-376).

One reviewer especially makes details suggestions about methodological aspects. I generally agree with them and I'm convinced that your manuscript could be improved by taking them into account, modifying some analyses or adding some words of discussion for aspects you cannot change (e.g. using a single tank per glue type).

This is now done – see answers below. The use of a single tank per glue type is now addressed in more detail L201 and L302-310. We have also analysed some earlier, preliminary data and added this supporting information as supplementary materials.

Extra minor comments:

(133) remove the unnecessary "to" before "2-octyl"

Done.

(342) I guess by "relies on fine motor skills" you mean that the experimenter needs to display some movement precision for the tagging to be performed adequately. Judging from the video, I guess it is likely possible to define a protocol that would ease this, e.g. by placing the dish on the table, using a magnifier... However, it's hard to determine if the precision needed is likely possessed by many individuals or only a few. Maybe a few words to precise what you mean by "relies on fine motor skills" would be useful.

This has been rephrased, see L362.

I look forward to reading your revised version to be considered for recommendation.

Best regards,

Nicolas

Review by Simon Blanchet, 12 Oct 2023 12:54

Dear Authors

I have now read your MS "Methods for tagging an ectoparasite, the salmon louse *Lepeophtheirus salmonis*". The MS is very well written and it describes a new method for tagging fish ectoparasite. I don't have major criticisms as most experiments have been seriously performed, as well as associated statistical tests.

Thank you for these comments!

I was a bit surprised that mortality and fecundity were not compared between a tagged and an untagged group (although authors provide an explanation that was not super convincing to me). This is the only methodological limitation I can see, and I suggest authors to discuss this briefly, or perhaps they have personal observation that may be included into the Discussion to convince readers that mortality (to a lesser extent fecundity as the test for this parameter is more solid) is not (strongly) different between tagged and untagged groups.

We did compare tagged / untagged in some preliminary experiments prior to the start of this project, albeit in two different ways for the two different kinds of glue – and did not find any indication of a difference between tagged and untagged. This is now reported as supplementary material, referenced in the methods (L201). We have expanded on this in the discussion (L302-310).

In addition, I was a bit frustrated not to read a bit more about the research avenues that are now opened thanks to this method. I would like you to elaborate a bit on what are the key scientific questions that can now be tackled (in salmon lice and other fish ectoparasites), and to which extent you think this tagging approach can be extended to other (fish or not) ectoparasite and other invertebrates.

We have now added a paragraph at the end of the discussion to elaborate on these points (see L368-376).

**Minor comments:**

l. 55-56: I think there are also good examples of individual tagging in butterflies (writing on wings). Please add references if you find some.

Done (L55-56). We also added references of studies in other insect groups (L54-59)

Figure 3: I would advice starting the y-axis to zero; as it is it seems like retention rate drop to 0 whereas it actually drops to 0.3

We have now changed both the analyses and the figures (see other replies below). The Y-axes now all start at 0. Thanks for the suggestion!

Discussion first paragraph: please indicate that a specific toxicity test would be required to tease apart the two hypotheses (tank effect or toxicity). If the agent is toxic this may be problematic for further studies.

The start of the discussion is now rephrased accordingly (L302-310), including a mention of preliminary data that are now reported in supplementary materials. In addition, the need for testing glue efficacy is mentioned at L334-336.

## Review by anonymous reviewer

### General appreciation

It is a very interesting method. Especially since this kind of methodology paper is not that common, while they are necessary to avoid multiple research teams wasting time trying to develop the same approach. It is also beneficial for a data-demanding field to answer questions that could not be answered before concerning individual ecology and evolution of small species. Moreover, since the tag loss rate has been estimated, it could also be used in further Mark-Release-Recapture analyses, correcting the apparent survival.

Thanks for your positive comments!

However, before being published, I think there are a few points that need to be addressed.

### Major critics

Even though I called them “major critics”, they do not have a huge impact on the main outcomes of this paper.

One of the major critics I have is concerning the variation of retention rate according to the glue batch. However, the explanation concerning shelf and opening dates seems logical and appropriate. It is reassuring that in 2023, with more care given towards the opening date, the results were closer to those of 2021. It is therefore a good point that you give recommendations for 2oc glue usage (best to prefer recent manufacturing dates and to avoid vials being opened for too long (more than 6 months)). One should however be careful with this unexpected variation that could still be due to the supplier; preliminary tests could be conducted to estimate the glue quality from different suppliers.

We share your concern, among others because glue quality directly impacts our workload, but have only been able to order 2oc from one brand so far, and could therefore not test variation in the quality of the glue from other suppliers. We added a suggestion for testing glue efficacy prior to research study start (L351-353).

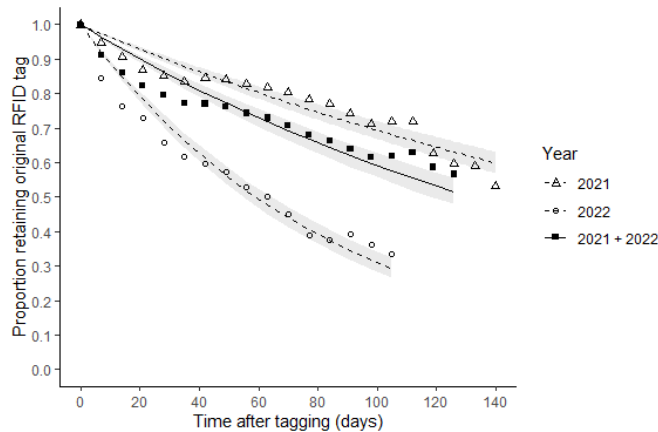
The other major critic I have concerns your analyses of retention time. For the analyses behind Figure 3, and Table 3, I would suggest setting the intercept as 1, since at day 0, 100% of the females had a tag.

Moreover, for this kind of loss rate data, an exponential fit ( $Y = a \cdot b^X$ , where  $a$  is the intercept and  $b$  is the retention rate between two consecutive days) would be best suited in contrast to a linear fit ( $Y = a + b \cdot X$ ), since it is not expected to follow a straight line, but rather a negative exponential curve tending asymptotically to 0, similarly to decay or survival rates; between each time interval you expect the same proportion of your Y axis being lost, not the same amount of Y. In this approach, you should fix  $a = 1$  since it will fix the intercept as 1, as mentioned above. You could test the goodness of fit for those two approaches.

Thank you for this suggestion. We have updated our analysis, now fitting an exponential rather than a linear decline, and also weighing the data by population size. We changed the

methods and results accordingly, as well as the summary and discussion (L152-155, L161-164, L184-187, L223-236, L240-249, L337-341 – see also the changes in figures, tables and their respective legends).

However, since our model also includes year and year \* day interactions; setting the model intercept is not straightforward. We did try, however, to fix the intercept at 1 using days since tagging as the sole explanatory variable for separate data subsets, in an attempt to have the curves intercept the Y-axis on Figure3 – as you can see below, the goodness of fit decreased. This seems to be due to a higher rate of tag loss in the first 7-day period than in the remaining duration of the study.



In the end, and for the sake of consistency, we show curves on Figure 3 that correspond to the fitted model reported in Table 3, because in this new version of the model they were satisfyingly close to 1 – but mention in the manuscript that tag loss was higher than predicted by this model in the first week after tagging (L340). We believe this is the best way to address the issue.

However, even if your analyses were not the best suited for this data in my opinion, the conclusions are expected to remain the same: this method is promising.

### Comments on the introduction

I have a few comments regarding the introduction to give more background to the reader.

You have used post-smolt Atlantic salmon in this study, but would the results presented here be transferable to the other life stages of the Atlantic salmon (since detachment rates could be different on other life stages)? I would suggest mentioning which salmon life stage *L. salmonis* infects preferably in the introduction, as I suppose it prefers post-smolt individuals. This would support your methodological choices.

*L. salmonis* is essentially an exclusively marine parasite; in particular the infective juvenile stage cannot survive in brackish or fresh water, and the adults survive for a short amount of time at low salinity only if they are attached to a host. Hence this parasite can only infect salmonids at sea (post-smolt Atlantic salmon and sea trout), not freshwater trout or juvenile salmon that during the first period of their lives, which they spend in freshwater. This is now clarified (L65-66).

In the same context, it would be interesting to know if (and how often) salmon lice change host in natural environments and if it occurred in your experiment.

We assume you mean how often salmon lice change the individual fish host, rather than host species. It is difficult to accurately assess this in a natural setting (as this would require tracking salmon out at sea in addition to tracking their parasites!) – but we did observe and quantify this in our lab experiments, which assumedly reflects densely-stocked marine aquaculture conditions. This is in fact one of the questions made possible to address by tagging individuals, and that we are currently investigating. We have added a few lines about this in the discussion (L356-358).

It could also be interesting to note how long these adult lice usually live to have a comparison point for the efficiency of the retention of the tags; how much of the adult lifetime could be covered by these tags?

In our longer-term experiment (still ongoing) we found a great variation in longevity, which is also sex-specific (with females living for longer than males on average, also reflected in Figure 2b on this paper), ranging from a few days after tagging up to over 300 days. We have added this information to the discussion (L354-361).

### **Comments on the methodology**

The methodology is generally very clear. Figure 1 and the video are very nicely appreciated.

Thank you!

In the video demonstration, the second black screen mentions “(Scanning chip and taking photo)”, even though (if I understood correctly) the video then shows the final process to set the glue (lines 114 to 117). If it is the case, it should be adapted. Or perhaps you meant that you scanned the chip and took a photo between the two parts of the video. If it is the case, it should be explained a bit more clearly.

We have now clarified this in the video and updated the link.

It would have been nice to have at least two tanks per glue type (and ideally per glue batch) to avoid pseudoreplication and be able to control for a tank (and glue batch) effect.

We are not sure what you mean by pseudoreplication, as salmon lice were individually tagged and different batches used did not overlap in time - but we agree that ideally there should have been more than one tank tested per glue type, to disentangle tank from batch effect. However, lab space or human resources are (sadly ☹️) not unlimited and these data represent the best we could achieve (collecting them already required a sustained team effort). We have added a few lines of explanation for why we did not carry out more comparisons in a greater number of tanks, and now also provide supplementary data as well as a reference, to support the interpretation for a likely tank effect (L302-310).

You could have added interactions between time and sex, as well as time and glue in the mortality test during the comparison of glue types. Even though no interaction seems to exist here, it could be best to test for it. Again, you should fix the intercept as 0.

We have now checked for interactions and changed the manuscript accordingly (L164-165 & L230-236 – see also the updated Table 1).

Why not take into consideration the replaced tags and add the individuals as a random variable for the analysis of retention time? The time=0 would be the day when the chip is replaced. It could provide more data and give insight to the variation of retention time due to individuals (some could be more adapted for glue applications).

Thanks for your valid suggestion, we had the same thought initially. In this time period, only 18 individuals were tagged more than twice, 14 of those in 2022 with the lower-quality batch. We could have included them in the analysis. However, as lice grow old so does their likelihood of dying prior to losing their tags, which could bias our estimates of tag retention. Since the initial tagging of lice occurred simultaneously in each tank (i.e., they were all at a similar age upon first tagging), we argue that retention time is more accurately estimated using such a cohort than by including also older individuals (i.e. that had a shorter residual life expectancy).

Line 216: For GLMs, you should check overdispersion, not normality and heteroscedasticity.

This is now corrected (L217). There was overdispersion for the models in the glue comparison, but the issue was solved after switching to an exponential fit (while not impacting the main results).

### **Comments on the results and discussion**

You show promising results. However, the analysis of retention time should be reconsidered as mentioned above.

Done (see our replies above).

For Figure 2b, I would represent the data as a proportion of dead lice rather than the number of dead lice, since the total number of lice in the different tanks were not the same.

Done, although we chose instead to show the proportion of living lice to reflect the model.

Very interesting absence of effect of tagging on reproduction. The very anecdotal amount of potential blocked oviduct is very promising. Interesting apparent absence of effect of tagging on mortality as well. However, I am quite surprised with the latter since in the first experiment, lice had a higher mortality with 2oc. Nonetheless, as you mentioned, this result is probably a tank effect since even males were affected even though you used very small amounts of glue in tanks of 500L, making the toxicity hypothesis very unlikely, since females, which were directly exposed to the compound, were dying less than males.



We fully agree. We were also surprised to find out that males in the tank where females were tagged using 2oc also had a higher mortality, but as you say glue toxicity is unlikely the primary reason for it (as fresh glue was only applied to the females outside of the tanks, and the males were therefore not in direct contact with it at any point). This is supported by data from an earlier pilot study that we have now analysed. We have expanded on this a bit in the discussion (L301-310), and added the report from the preliminary study as supplementary materials. We also found prior documentation of unexplained variation in survival even when all other aspects are kept the same, and now reference this L306-308 (Hamre et al. 2009).

About the death of lice, are there any reports of salmon eating lice? Could it explain why sometimes you have individuals disappearing? If it is the case, having a different number of salmon in the tanks could produce a bias in the number of deaths.

Salmon are not known to feed on lice and we did not observe this in our lab setting either. What we did observe is that lice, occasionally and temporarily, can sit on surfaces (e.g. tank walls) before attaching to their host again. They would usually eventually be tallied on a fish or found in the filter, but not always (here, 6 lice out of 141 were unaccounted for). What we believe may have caused the “disappearance” of a few lice is the outlet water system, where water first runs at a somehow slower pace in about 1m of pipe that is not easily accessible for inspection before falling through our filters. Some lice having died might have occasionally been “stuck” into this piece of pipe and decomposed before we had a chance of collecting them.

### Specific comments

Lines 43-44: A more recent source than that dating from 2002 would be preferable to talk about the lack of information on a topic.

Done (L42-43).

Line 135: “[...] the most effective [...]” instead of “[...] the more effective [...]” would be more appropriate.

Using “more” as a superlative when there are only two alternatives to compare is appropriate.

Lines 142-143: “All lice at every step were recorded as being either male or female, and as being either tagged or untagged for the females.” would maybe a better phrasing.

We changed the phrasing slightly, from “and if female” to “and for the females”.

Lines 175-176: You could maybe make it a bit more explicit that there were 216 instances of missing + nonfunctional tags. I first thought that the “/” separated the number of instances of both events, meaning that there would have been 4 missing tags and 216 nonfunctional tags. Maybe just write “[...] the date of the first check when the p-Chip was observed to be missing (212 instances) or nonfunctional (4 instances).”

Done (L176).

Lines 201-206: I had problems understanding this section. I eventually managed to understand what you have done thanks to the discussion. I would suggest rephrasing these sentences.

This was indeed unclear. We have now rephrased, hopefully improving clarity (L200-213).

Lines 217-218: I am not sure that “[...] to test prediction proportionality [...]” are the correct terms to use.

This part of the sentence has now been removed (L211).

Line 231: I think that you forgot the minus sign in front of 0.19.

This part of the results was updated, and we double-checked the signs for all estimates.

Lines 308-309: The number of weeks does not seem to match with what was mentioned before in the manuscript.

To improve clarity we removed the “weeks of” from “two consecutive weeks of exposure(s)” to limit the number of weeks being mentioned to only those that were tested (now L316-318).

And finally, a very small detail: remove lines 37, 88, 124, 212, 234, 241, as they are empty, and replace them with a spacing.

Done.