Dear editor, here you will find a revised version of our manuscript in which we incorporated all the changes suggested by the reviewers. The most significant change is that we ran all analyses from scratch, including the hippopotamus and two other cetacean species (Vaquita and Narwhal). The new results remain the general trend we reported in our first submission. We hope you consider that the revised version is now in good shape to be recommended in PCI genomics.

lines 149 and 747. Change reference from 2020 by the updated one from 2023. https://pubmed.ncbi.nlm.nih.gov/36477806/ PMID: 36477806

The authors might consider the following in editing and improving their manuscript.
line 112. The authors note that, "Thus, given their pivotal role in different physiological axes, some of which have diverged extensively in cetaceans due to the conquest of the aquatic environment, it seems interesting to study their evolutionary trend in this mammalian group", but this has not been demonstrated yet in the paper, and a citation is not given for this assertion, so the text here should be adjusted. Perhaps change "some of which have diverged extensively in cetaceans some of which have diverged extensively in cetaceans" to "some of which may have diverged extensively in cetaceans"?

The reviewer correctly points out that we need to support this statement. To solve this problem, we provide references at the end of the statement. Now, the new statement reads as follows: "*Thus, given their pivotal role in different physiological axes, some of which have diverged extensively in cetaceans due to the conquest of the aquatic environment, it seems interesting to study their evolutionary trend in this mammalian group (Varró et al. 2021; Poole, 2022; Kashio & Tominaga, 2022)*"

line 125. Maybe let the reader know what 'TTX' is and why this change in sensitivity is of any interest evolutionarily here? I think few people will think this is of any interest unless add more text saying why here to set up the rest of the paper.

We understand the reviewer's concern. To solve this problem, we reworded this text, now it reads as follows: "*3) the $Na_V1.5$ ion channel of toothed whales (odontocetes), other than species of the genus Tursiops, seems to be sensitive to the potent neurotoxin tetrodotoxin (TTX), similar to $Na_V1.7$, given a replacement of cysteine for a tyrosine*"

The reader will see further details in the 1.5 pages we devoted to this discovery (lines 328 to 373).

Abstract - general. It might be better to frame the introduction in terms of testable hypotheses that were tested. As is, it reads as if the study is completely descriptive, which is fine, I guess. But, this might not be so compelling to a general reading audience.

We understand the reviewer´s concern but do not see a problem presenting a descriptive scientific work. In our way of thinking, descriptive studies also have a fundamental role in advancing science (Grimaldi & Engel 2007; Casadevall & Fang, 2008).

Grimaldi & Engel. 2007. BioScience (https://doi.org/10.1641/B570802)
Casadevall & Fang. 2008. Infection and Immunity (https://doi.org/10.1128/iai.00743-08)

line 131. It would have been of interest to sample a hippopotamid as these species are semi-aquatic and the extant sister group to Cetacea and have decent genome assemblies I think. Was there a reason that these were not sampled? Would it be possible to include these, or would that require doing everything over from the start?

The reviewer is correct. The new version of our manuscript incorporates the hippopotamus and two other cetaceans.

line 140. I do not know if this is the best approach to pulling out these genes. Has such an approach been used in other studies (or an analogous approach), or is the sequence of steps in this paragraph novel to this study. It might be good to perhaps justify each step a bit more, or the overall approach, to convince the reader that this is a decent pipeline for pulling out the desired set of coding sequences for ion channel genes from the genomes examined here.

We downloaded the protein-coding sequences from the world's largest public resources of biological sequence databases, with a long-term tradition (over 20 years, CDD, Wang et al. 2023) of genetic data curation and storage (https://www.insdc.org/). Further, figure 1 provides a graphical explanation of our bioinformatic pipeline with a reasonable way of detail. If the readers have questions regarding the databases used in our publication, they can check the papers we cite (Yates et al. 2022; Sayers et al. 2022).

Yates AD, et al. 2022. *Nucleic Acids Res.* (https://doi.org/10.1093/nar/gkac1096)
Sayers EW, et al. 2022. *Nucleic Acids Res. (https://doi.org/10.1093/nar/gkab1112)*
Wang J, *et al.* 2023. *Nucleic Acids Res. (https://doi.org/10.1093/nar/gkac1096)*

6) line 166. In this section, it should be noted whether dN/dS analyses were done in which different dN/dS was permitted on the stem and/or crown Cetacea branches. If not done, why not? It would seem that it would be good to test for significant shifts in selection intensity at the transition to aquatic envirnment and also within the crown Cetacea lineages which all represent evolution in obligately aquatic mammals, in contrast to the outgroups (terrestrial) and the stem Cetacea branch (transition to fully aquatic). Here again, I think it would be good to include one or both extant hippos in the analyses, since these are the closest extant relatives of Cetacea. For the models described in this section, it seems that what will be inferred is positive selection in a subset of sites, or not. But, is that the best or most interesting question?

We understand the reviewer's concern. Although the most obvious way of thinking is to test the stem and the crown, expecting most changes to occur in the stem, this pattern is only sometimes true. This also holds for other forms of genetic variability, not only dn/ds. For example, according to our results, the highest value of gene turnover rate was estimated for the crown group cetacea. Further, the value estimated for the stem was four times lower than for the non-cetacean species included in our sampling. In the case of dn/ds, although we did not report results, we also ran branch models, and in all of the instances in which we estimated separate omega values for the crown and the stem, the crown value was higher. We also ran branch-site tests labeling the stem cetacea as the foreground branch, not obtaining any gene with the signature of positive selection. In our way of thinking, all these results suggest that most of the "*evolutionary activity*" is happening in the crown group. For this reason, we ran site analyses, which are used to identify positively selected sites in a multiple sequence alignment in the group of interest. The statistical power of site-specific models has been demonstrated in the literature (Anisimova et al. 2001; Yang & Bielawski, 2000; Yang & Nielsen 2002). Interestingly, these results, i.e., that most of the "*evolutionary activity*" occurred in the

crown group cetacea, were very similar when we studied the evolution of tumor suppressor genes (Tejada et al. 2021).

Anisimova et al. 2001. Molecular Biology and Evolution. https://doi.org/10.1093/oxfordjournals.molbev.a003945
Tejada et al. 2021. Proceedings of the Royal Society B. https://doi.org/10.1098/rspb.2020.2592
Yang & Nielsen. 2002. Molecular Biology and Evolution. https://doi.org/10.1093/oxfordjournals.molbev.a004148
Yang & Bielawski. 2000. Trends in Ecology and Evolution. https://doi.org/10.1016/S0169-5347(00)01994-7

7) line 181. The breakdown of branches here might be useful to try for the dN/dS analyses (e.g., separating Cetacea from other mammals). However, note that 'stem Cetacea' as delimited in the current study includes also stem Cetancodonta. Because hippos are not included, some of this 'stem Cetacea' branch includes evolutionary history that is prior to the divergence of Cetacea from Hippopotamidae. As noted above, I think it would be useful to include hippo genomes in this study, for a variety of reasons.

The reviewer is correct. To solve this problem, we ran all the analyses again, including the hippo and two other cetacean species (Narwhal and Vaquita).

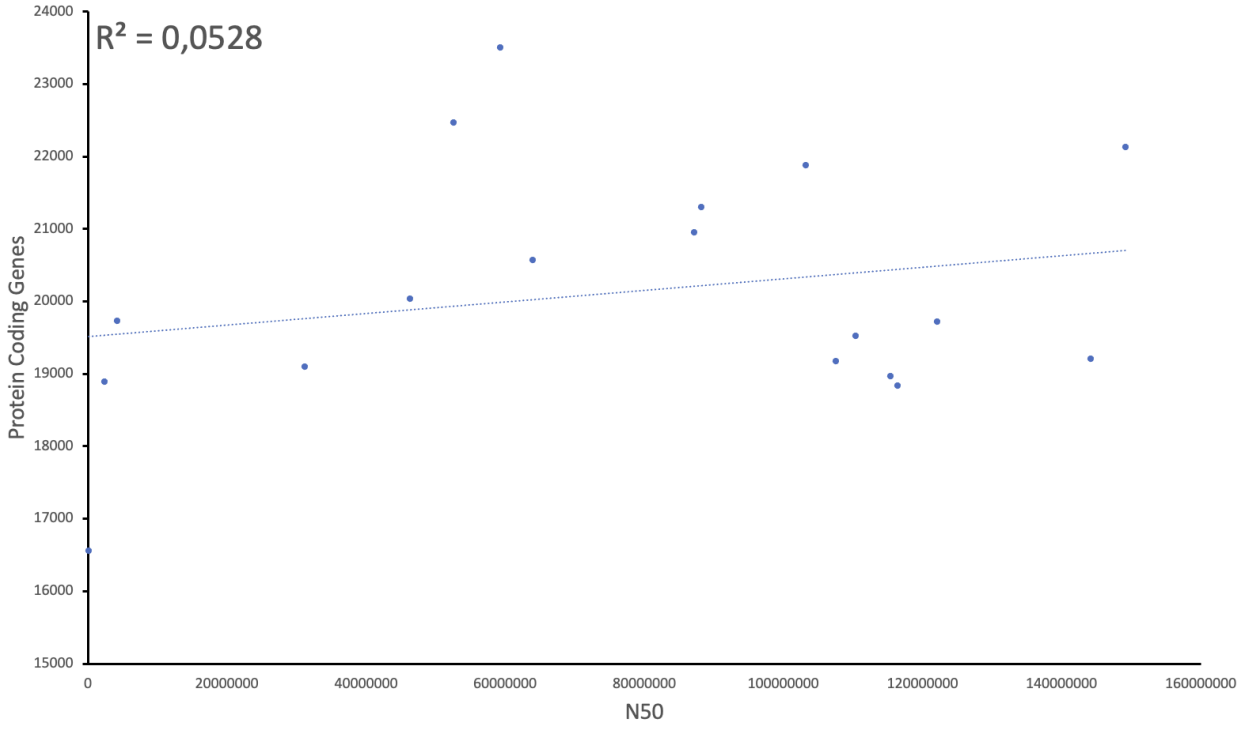
8) line 189. Clarify what 'adjusted' means here, presumably some sort of correction for multiple tests (or some other)?

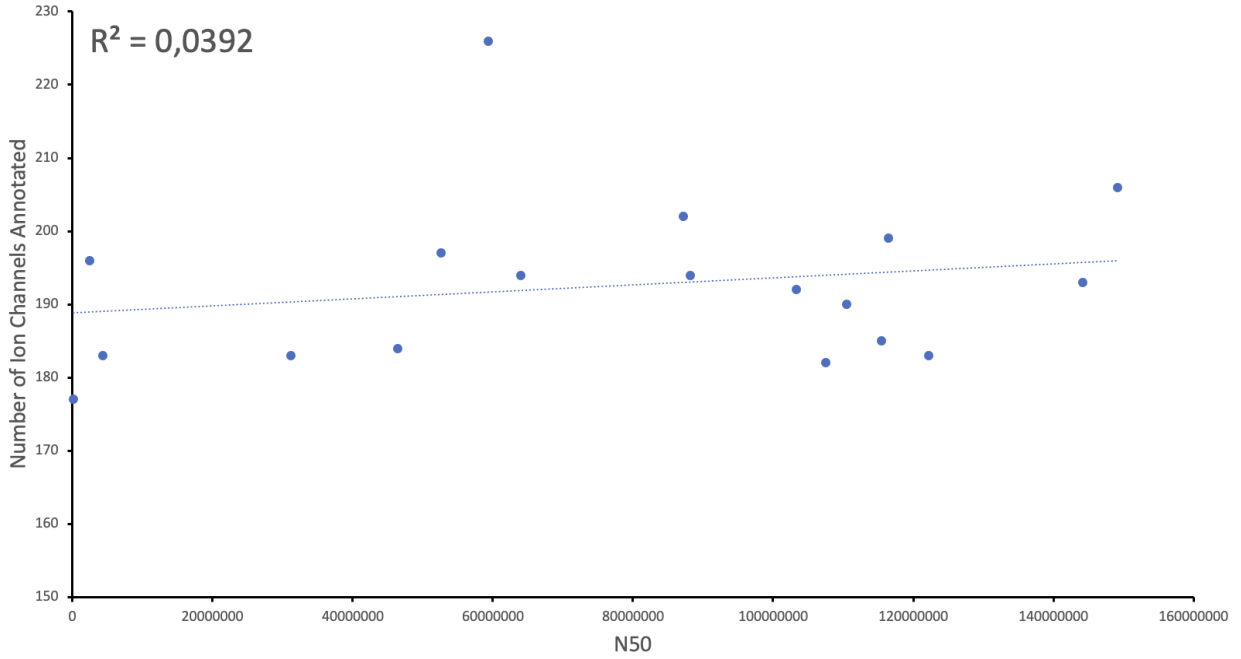
Thank you for noticing this. We have added an explanatory sentence with the original reference from Benjamini & Hochberg (1995). The new text reads as follows: "*The adjusted probability is calculated from the resulting list of categories with raw p-values equal or lower than 0.05, through the procedure of False Discovery Rate (chosen FDR is also 0.01) (PMID). The aim of FDR is to reduce the final number of false positive categories in the results.*"

Benjamini & Hochberg. 1995. Journal of the Royal Statistical Society: https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

9) line 216. How much smaller? Is this a problem? Also, for Delphinidae, Tursiops is maybe not as complete a genome assembly as Orcinus (?). Was genome quality correlated with number of genes pulled out of these genomes? Using more species that are closely related might have helped to assess the effects of varying genome quality on the numbers of these genes in different genomes.

In the manuscript, we reported the values obtained according to our bioinformatic pipeline (human, 226; mouse,197) and the values reported in the literature (human, 235; mouse, 231). The difference is not a problem; it is just an update since the last estimation occurred 14 years ago. To avoid confusion, we modified the statement, and now it reads as follows: "*Our results are comparable to what is reported in the literature, 235 ion channels for humans (Homo sapiens) and 231 for the mouse (Mus musculus) (Jegla et al. 2009)*."
Regarding a possible correlation between genome quality and the number of protein-coding genes or annotated ion channels, we found no correlation (0.229 Pearson, p-value = 0.35, and 0.198 Pearson, p-value = 0.4707, respectively).





10) line 220. The "unpaired one-tailed t-test" is not appropriate when comparing different genomes within a phylogenetically coherent way as, for example, the different genomes within Cetacea are not independent data points due to shared common ancestry to varying degrees, so some other test should be utilized here (i.e., one that takes phylogenetic structure into account).

We agree with this comment. Although we reported this phylogeny-independent test in our manuscript, we also performed a test in which the phylogenetic relationships and divergence times of the species included in our sampling are considered, i.e., gene turnover rate estimation using the software CAFE.

11) line 221. I think the statement "This result is consistent with the hypothesis that gene loss can play a significant role in phenotypic evolution" needs more explanation here. All mammalian taxa analyzed here have unique traits and differ greatly in phenotype. For example, if there were a huge reduction in gene number in human, would this also be consistent with "the hypothesis that gene loss can play a significant role in phenotypic evolution"? Humans are highly derived, large brained primates that walk on two legs and have complex societies. At any rate, I think the statement here is fairly uncompelling; if cetaceans had way more gene copies than other taxa, would the exact same statement be made, or if highly derived flying bats had fewer copies (which is the case), etc., etc. In part, this relates back to the question regarding having prior hypotheses at the start of the study, rather than sort of just describing/documenting things and having to then consider plausible explanations as you go along.

We understand the reviewer's argument, and it is possible to fix this problem by rewriting this passage, stating that our result agrees with other studies in which fewer gene copies have been reported as a source of phenotypic innovation. The new passage reads: "*This result is consistent with other studies in which a reduction in gene copy number in cetaceans, and other taxonomic groups, are associated with evolutionary innovations (Feng et al. 2014; Nery et al. 2014; Sun et al. 2017; Huelsmann et al. 2019; Helsen et al. 2020; McGowen et al. 2020; Randall et al. 2022; Zheng et al. 2022; Osipova et al. 2023; Pinto et al. 2023).*"

In addition, we deleted the following statement: "*This result is consistent with the hypothesis that gene loss can play a significant role in phenotypic evolution (Olson 1999; Albalat and Cañestro 2016; Helsen et al. 2020).”*

12) line 252 and following paragraphs. Are the positive selection signals for 'heart genes' on the cetacean lineages or across the whole tree? If there is no specific evidence of positive selection just on the cetacean 'stem lineage' and in crown Cetacea, why infer that that adaptation in cetaceans is driving the high dN/dS in these genes. According to the methods, it does not appear that cetacean and 'background' branches (non-cetacean branches) were partitioned such that different dN/dS are permitted for these different categories. Unless I am not understanding something, I do not see how the authors can make the inferences they are trying to make given the results that they have presented.

The confusion comes from the misunderstanding regarding the site analyses. This type of analysis, used to identify positively selected sites in a multiple sequence alignment in the group of interest, includes only sequences (and phylogenetic relationships) from cetaceans. This is why we can make generalizations for the cetacean group. To avoid misunderstandings, we added the names of the models we used.

13) line 299 and following paragraphs. This section is quite speculative and rambling. Why is the mutation not in mysticetes? There is a further reversal in Tursiops with a speculative explanation for that as well. As the authors note, all of this needs to be tested experimentally, and I am not sure that the amount of text here is warranted given the speculative nature of all of this. But this is potentially interesting.

We understand the reviewer's concern. It is not possible to answer the question of why the mutation is not present in mysticetes. We can show when the mutation occurred based on how the species in our sampling are related. A similar situation occurs regarding the reversal in the ancestor of the genus *Tursiops*. As we mentioned in the manuscript and noticed by the reviewer, the ideal would be to test the protein with the mutation experimentally. However, in this case, we feel lucky as, in the literature, the sensitivity for TTX has been extensively studied. The main conclusion is that "*this residue is the structural determinant that differentiates the TTX-insensitive sodium channels (Nav1.5 and Nav1.8–Nav9) with a Cys or Ser from the TTX-sensitive channels (Nav1.1–Nav1.4, Nav1.6, and Nav1.7) with a Tyr or Phe*" (Jiang et al. 2020). So, based on the argument exposed, we feel that our writing is not very speculative.

Jian et al. 2020. Cell. 180: 122. (https://doi.org/10.1016/j.cell.2019.11.041)

14) line 447. I have worked on cetaceans for over 30 years, I am not convinced that "Hearing is undoubtedly the most critical sense for life underwater", and I am not sure that this statement is even true, no less "undoubtably" true. I would go with sight probably, and the importance of sight vs. hearing varies considerably among different lineages of cetaceans that are specialized in different ways.

We understand the reviewer's concern. Based on the new analyses, including more species, the hearing was not among the top categories, so this text was removed.

15) lines 579-581. I do not think this statement is supported by the results of the analysis. This is possible, of course, but is a leap in logic certainly.

We understand the reviewer's concern. To fix this problem, we removed that statement.

Reviewer #2
This study employs a bioinformatics pipeline to investigate the evolutionary dynamics of ion channels in cetaceans. The findings reveal a reduction in the repertoire of ion channels in cetaceans compared to their terrestrial mammalian counterparts. Notably, the NaV1.5 ion channel in most toothed whales exhibits specific amino acid variations deemed pathological in humans. Particularly, a significant proportion of these whales possess a tyrosine residue at a precise position within the NaV1.5 channel, potentially rendering them more susceptible to certain toxins. These discoveries offer profound insights into the mechanisms underpinning cetacean adaptations to their aquatic habitat. This research not only presents intriguing implications but also holds substantial scientific significance. The study encompasses a variety of functionalities related to ion channels, including cardiac and skeletal muscle contraction, echolocation, and polycystic kidney syndrome. However, experimental validation of these bioinformatic analyzes is necessary and requires in-depth investigation of the specific functions of ion channels.

We appreciate all the positive comments. We agree that experimental validation is necessary for further understanding the genomic bases of the conquest of the aquatic way of life of cetaceans. However, it is out of the scope of our work. In the future, scientists who do experiments will take some of our results to the bench.