nature portfolio

Peer Review File

Bluefin tuna sperm production is hastened by surrogacy in small Euthynnus

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The study reports initial success in surrogate reproduction of bluefin tuna sperm through germ cell transplantation into fertile small hybrid tuna (Euthynnus). While this advancement holds promise for artificial reproduction and breeding of bluefin tuna, a species of significant fisheries value, it falls short of presenting substantial scientific progress compared to prior research efforts from the same laboratory (Okutsu et al., PNAS, 2006; Takeuchi et al., Nature, 2004, and Okutsu et al., Science, 2007). The use of fertile hybrid host fish results in an exceptionally low efficiency in surrogate production of Pacific bluefin tuna (PBT) sperm, as evidenced by low rates of germ cell incorporation (only 2.5 germ cells/host), recipient fish survival rate (2%), PBT spermatozoa detection in recipient milt samples (13.2%), and the ratio of PBT spermatozoa in positive milt samples (even less than 1/2,500,000!). The exceedingly low efficiency of producing PBT sperm using the current surrogacy approach, coupled with the lack of scientific rationale in the study, raises concerns regarding its suitability for publication in Nature Communications.

Major:

1. The authors state that Pacific bluefin tuna (PBT) typically reach maturity between 3-5 years old, yet they isolated germ cells from 3-year-old male PBT and obtained "mature sperm" in surrogate hosts eight months later. Thus, this manipulation did not significantly accelerate the maturation period of donor PBT, which is a primary objective of the study. It is recommended to isolate germline stem cells from younger PBT, preferably before they reach one year of age, to truly shorten the maturation period of donor germ cells.

2. Despite repeatedly referring to the donor germ cells as "germline stem cells," the authors fail to demonstrate how these cells were isolated, purified, and identified. Instead, they isolated a large number of PBT testicular cells and transplanted them into larval fish hosts. Therefore, it is scientifically inaccurate to label these cells as germline stem cells. To accurately characterize the donor cells as germline stem cells, additional experiments such as immunofluorescence analysis using various antibodies (Nanos2, Piwil1, Ddx4, Pcna, Sycp3, etc.) should be conducted.

3. In the discussion (Line 306-307), it is discussed that “in this study, sterilized ELT could not be used as recipients due to very poor early survival after triploidization or dnd knockdown treatments”. However, the authors have never shown these results. Given that sterilized larvae should be a better host for germ cell transplantation, it is necessary to show all these results.

4. Throughout the manuscript, the authors repeatedly used some big words such as “groundbreaking advance”, “major breakthrough”, “revolutionize” etc, which are really inappropriate for a scientific manuscript.

Minor:

1. Line 26 (L26): Please provide the Latin name for Pacific bluefin tuna.

2. L32: Please clarify that the hybrid tuna hosts remain fertile and indicate the efficiency of Pacific bluefin tuna sperm surrogacy production.

3. L34: Replace "germline stem cells" with "germ cells".

4. L35: The authors did not demonstrate the complete process of spermatogenesis of transplanted PBT germ cells.

5. L37: Consider revising the use of terms like "groundbreaking advance."

6. L65-67: Provide a reference for germ cell transplantation technology.

7. L78: Clearly explain the genetic distance between Pacific bluefin tuna and the scombrid fish used as recipients.

8. At the end of the introduction, briefly summarize the major results and conclusions of the current study.

9. L100: Provide evidence supporting "multiple mortality-related factors."

10. L135-137: Suggest using genome sequencing instead of PCR for hybrid fish identification.

11. L165: Consider revising the use of "major breakthrough."

12. L181-182: Clarify the total number of germ cells in the host larval tuna recipients.

13. L188: Provide a reference for antibody No. 149.

14. L194, L202-203: Consider presenting all the efficiency parameters in a table format.

15. L199-201: Perform statistical analysis on sperm morphology between Fig. 5a and Fig. 4b.

16. L202-203: The efficiency is too low! So it is necessary to include strict negative control in this study. How to avoid any contamination of PBT sperm in the sample? How to prove that there is no non-specific staining of these two antibodies in HLT sperm sample?

17. L210-220: The calculation in this paragraph is more than speculation. Most conclusions are over-claimed.

18. L230-232: the detection of PBT-specific band was observed in 1 of 18,300 embryos, which is > 100 times higher than the detection rate of PBT sperm in the HLT sperm sample (less than 1/2,500,000). How could this happen?

19. L232-234: Suggest using DNA sequencing, instead of simple PCR analysis, to verify the genetic origin of No.8 sample.

20. L251: Consider revising the use of "groundbreaking advance."

21. L252: Consider revising the use of "revolutionize."

22. Fig. 1: Suggest presenting this figure as supplementary material.

23. Fig. 4: Only a test of the antibody. It is suggested to either merge it with Fig. 5 or move it to supplementary materials.

Reviewer #2 (Remarks to the Author):

This is another beautiful and rigorous germ cell transplantation study performed by Professor Yoshizaki's group. This reviewer has no questions about the experiment design, procedures, results, and discussion.

The only concerns this review has rested on the novelty of this research and whether it can reach Nature Communications' publication standard. The decision will be up to the editors.

These concerns are:

1. Although Bluefin tuna is a precious species and very difficult to work on in research and aquaculture, the germ transplantation that led to sperm production has been achieved in the recipients of different orders with more than 100 million years of phylogenetic distance (T. Saito, R. Goto, K. Arai, E. Yamaha, Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation, 2008) and 200 million years of phylogenetic distance (M.A. Silva, G.M.J. Costa, S.M.S.N. Lacerda, P.F.P. Brandão-Dias, E. Kalapothakis, A.F. Silva Júnior, et al. Successful xenogeneic germ cell transplantation from Jundia catfish into adult Nile tilapia).

2) The significance of increasing recipients' survival rate from less than 1% in ELT recipients (lines 103-104) to 2% in HLT recipients (line 194).

3) The significance of PBT sperm detection in only one (sample No. 8) of the 183 samples (18,300 embryos) obtained from four spawning days (lines 230-232).

Response to the reviewer

Please find below a detailed response (written in blue) to the reviewer's comments (written in **black**). The sentences written in red were copied from the revised manuscript.

Reviewer #1 (Remarks to the Author):

The study reports initial success in surrogate reproduction of bluefin tuna sperm through germ cell transplantation into fertile small hybrid tuna (Euthynnus). While this advancement holds promise for artificial reproduction and breeding of bluefin tuna, a species of significant fisheries value, it falls short of presenting substantial scientific progress compared to prior research efforts from the same laboratory (Okutsu et al., PNAS, 2006; Takeuchi et al., Nature, 2004, and Okutsu et al., Science, 2007). The use of fertile hybrid host fish results in an exceptionally low efficiency in surrogate production of Pacific bluefin tuna (PBT) sperm, as evidenced by low rates of germ cell incorporation (only 2.5 germ cells/host), recipient fish survival rate (2%), PBT spermatozoa detection in recipient milt samples (13.2%), and the ratio of PBT spermatozoa in positive milt samples (even less than 1/2,500,000!). The exceedingly low efficiency of producing PBT sperm using the current surrogacy approach, coupled with the lack of scientific rationale in the study, raises concerns regarding its suitability for publication in Nature Communications.

All of the concerns raised above are restated in the individual comments below and are addressed separately

Major:

1. The authors state that Pacific bluefin tuna (PBT) typically reach maturity between 3-5 years old, yet they isolated germ cells from 3-year-old male PBT and obtained "mature sperm" in surrogate hosts eight months later. Thus, this manipulation did not significantly accelerate the maturation period of donor PBT, which is a primary objective of the study. It is recommended to isolate germline stem cells from younger

PBT, preferably before they reach one year of age, to truly shorten the maturation period of donor germ cells.

As the aim of this study was to verify the possibility of efficiently producing PBT seedlings using germ cell transplantation technology, it was necessary to obtain a large number of fresh testes from PBT donors in a "stable" manner in order to optimize the transplantation procedures. Our study therefore used 3-year-old PBT donors, regularly shipped from commercial farms year round. To fulfill reviewer 1's request, we added a novel dataset showing that a large number of *ddx4*-positive type A spermatogonia are present in 1-year-old PBT testes (Supplementary Fig. 1a–d). Further, we confirmed that germ cells isolated from 1-year-old PBT testes can be incorporated into the gonads of eastern little tuna recipients (Supplementary Fig. 1e–g). These new sentences were therefore added:

(L103-105, Results) Moreover, we confirmed that ELT can also incorporate germ cells isolated from prepubertal 1-year-old PBT testes (Supplementary Fig. 1).

(L407-411, Methods)
In situ hybridization
Prepubertal testes of 1-year-old PBT were fixed with Bouin solution, cut into 4 μm sections under standard paraffin-embedding methods, and stained with hematoxylin and eosin. Localization of *ddx4* mRNA in the sections was analysed by *in situ* hybridization as previously described[36].

Moreover, our previous study assessed the transplantation efficiency of adult and prepubertal testes in rainbow trout (Sato et al., 2017), finding that the incorporation and colony-forming efficiencies of prepubertal type A spermatogonia are reproducibly higher than those of adult type A spermatogonia. We also confirmed a similar tendency in yellowtail amberjack (Morita et al., 2012). Although a comparison experiment with adult and prepubertal PBT donors could not be performed in the present study given the availability of hybrid recipients, we expect that transplantability is higher with prepubertal than with mature PBT donors.

References

Sato, M., Hayashi, M. & Yoshizaki, G. Stem cell activity of type A spermatogonia is seasonally regulated in rainbow trout. *Biol. Reprod.* **96**, 1303–1316 (2017).

Morita, T. et al. Production of donor-derived offspring by allogeneic transplantation of spermatogonia in the yellowtail (*Serioloa quinqueraduata*). *Biol. Reprod*. **86**, 1–11 (2012).

2. Despite repeatedly referring to the donor germ cells as "germline stem cells," the authors fail to demonstrate how these cells were isolated, purified, and identified. Instead, they isolated a large number of PBT testicular cells and transplanted them into larval fish hosts. Therefore, it is scientifically inaccurate to label these cells as germline stem cells. To accurately characterize the donor cells as germline stem cells, additional experiments such as immunofluorescence analysis using various antibodies (Nanos2, Piwil1, Ddx4, Pcna, Sycp3, etc.) should be conducted.

In our previous study using rainbow trout, we proved that germ cells incorporated into a recipient's gonads behaved as germline stem cells, which demonstrated self-renewal and differentiation activities for a long time (Okutsu et al., 2007). Thus, we consider that our germ cell transplantation system provides a functional assay to identify germline stem cells similar to the identification of germline stem cells in the mouse (Brinster, 2002). For that reason, we stated that PBT germ cells incorporated into the recipient's gonads and producing donor-derived sperm were germline stem cells. However, we also understand that an analysis from various perspectives (not only functional assay) is required to define germline stem cells. We therefore replaced the term "germline stem cells" with "germ cells" or softened the expression as needed. The revised sentences are shown below each minor comment (minor #3 and #17) to clarify the revision being addressed.

Moreover, based on the reviewer's comment, immunohistochemistry using No. 152 antibody, the only antibody available as a marker for type A spermatogonia in PBT (no molecular markers that can prospectively identify "functional" germline stem cells in PBT are yet available), was performed against a donor testis (Ichida et al., 2019). Immunohistochemistry using anti-Ddx4 antibody was also performed to confirm a germ

cell marker in PBT. Together, the results showed that the donor PBT testis used in this study contained a large number of Ddx4 and No. 152 double-positive type A spermatogonia, possibly containing germline stem cells. This result was added as Supplementary Fig. 5, and a sentence in the Results section was revised:

(L182-185, Results) PBT testicular germ cells containing a large number of type A spermatogonia (Fig. 3a, b; Supplementary Fig. 5) were intraperitoneally transplanted into a total of 6,300 HLT larvae at 8–10 DAH in three independent trials (Fig. 3c, Supplementary Table 2).

(L505-506, Methods) Type A spermatogonia in the donor PBT testis were detected using anti-Ddx4 antibody and No. 152 antibody as previously described[15].

References

Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y. & Yoshizaki, G. Production of trout offspring from triploid salmon parents. *Science* **317**, 1517 (2007).

Ichida, K. et al. Specific visualization of live type A spermatogonia of Pacific bluefin tuna using fluorescent dye-conjugated antibodies. *Biol. Reprod.* **100**, 1637–1647 (2019).

Brinster, R.L. Germline Stem Cell Transplantation and Transgenesis. *Science* **296**, 2174–2176 (2002).

3. In the discussion (Line 306-307), it is discussed that “in this study, sterilized ELT could not be used as recipients due to very poor early survival after triploidization or dnd knockdown treatments”. However, the authors have never shown these results. Given that sterilized larvae should be a better host for germ cell transplantation, it is necessary to show all these results.

We described the early survival of triploid ELT in a previous report (Yazawa et al. 2019). Mortality was observed to be much higher in triploid than in diploid fish two to four weeks after hatching because of selective cannibalism by diploid siblings. As

described in the previous report, we never succeeded in keeping a triploid ELT alive to maturation despite many trials. To emphasize that point, new sentences were added:

> (L113-115, Results) Although we had succeeded in inducing triploidy in ELT to be used as recipients, mortality was extremely high in triploid compared with diploid ELT in the juvenile stage because of selective cannibalism by diploid siblings[17].

The knockdown approach is required for the microinjection of antisense morpholino oligonucleotide (AMO) during the one-cell stage. Considering that the one-cell stage of ELT lasts approximately one hour and that the survival of ELT to maturation is less than 1%, 3,000 embryos must be treated within one hour to produce 30 adult recipients. Moreover, AMO treatment is known to further reduce the survival rate of embryos. Indeed, when we injected AMO against the dead end *(dnd)* gene into ELT embryos, we could inject only approximately 250 embryos during the one-cell stage. Further, the survival rates at hatching of the noninjected and injected embryos were 85 ± 7% and 30 ± 9% respectively. Consequently, no *dnd*-morphants survived beyond the juvenile stage. We therefore concluded that the knockdown approach is unrealistic in ELT.

Reference

Yazawa, R. et al. Production of triploid eastern little tuna, *Euthynnus affinis* (Cantor, 1849). *Aquac. Res.* **50**, 1422–1430 (2019).

4. Throughout the manuscript, the authors repeatedly used some big words such as “groundbreaking advance”, “major breakthrough”, “revolutionize” etc, which are really inappropriate for a scientific manuscript.

We agree with the reviewer’s comment. Those words were therefore revised throughout the manuscript. Each revised sentence is shown below each minor comment (minor #5, #11, #20, and #21) to clarify the revision.

Minor:

1. Line 26 (L26): Please provide the Latin name for Pacific bluefin tuna.

As suggested, the Latin name has been provided (L27).

2. L32: Please clarify that the hybrid tuna hosts remain fertile and indicate the efficiency of Pacific bluefin tuna sperm surrogacy production.

Given the limitation on words, this point could not be added in the abstract. Instead, it is described at L173-177 and L216-218 as follows:

> (L173-177) Additionally, the gonadal somatic index of 1-year-old HLT did not differ significantly from that of nonhybrid ELT of either sex, indicating that HLT of both sexes show fertility (Fig. 2g). Although HLT did not show hybrid sterility, they exhibited rapid growth and high survival during the larval stage.

> (L216-218) PBT sperm were detected in 9 of 68 (13.2%) milt samples of male HLT recipients (Supplementary Table 2). The ratio of PBT sperm to recipient sperm ranged from 1/5 million to 1/2.5 million (Supplementary Table 3).

3. L34: Replace "germline stem cells" with "germ cells".

As suggested, the term “germline stem cells” was replaced with “germ cells.” (L35)

4. L35: The authors did not demonstrate the complete process of spermatogenesis of transplanted PBT germ cells.

As suggested, this sentence was removed to avoid misleading readers.

5. L37: Consider revising the use of terms like "groundbreaking advance."

As suggested, the sentence was revised:

> (L37-39) This result represents an advance toward compact, low-cost, and time-efficient seedling production that will improve the efficiency of PBT aquaculture.

6. L65-67: Provide a reference for germ cell transplantation technology.

Review papers were provided as references in this sentence (L68).

References

Yoshizaki, G. & Yazawa, R. Application of surrogate broodstock technology in aquaculture. Fish. Sci. 85, 429–437 (2019).

Houston, R. D. et al. Harnessing genomics to fast-track genetic improvement in aquaculture. Nat. Rev. Genet. 21, 389–409 (2020).

7. L78: Clearly explain the genetic distance between Pacific bluefin tuna and the scombrid fish used as recipients.

The sentence in the Discussion section was revised to clarify this point:

> (L286-290) The first is the phylogenetic distance: The divergence times between the donor species, genus *Thunnus*, to which bluefin tuna belong, and each recipient species are 6.6–16.7 million years ago (Mya) for genus *Euthynnus*, to which ELT and ALT belong; 9.1–22.4 Mya for genus *Sarda*, to which striped bonito belong; and 25.5–49.0 Mya for genus *Scomber*, to which chub mackerel belong[23, 24, 25].

8. At the end of the introduction, briefly summarize the major results and conclusions of the current study.

Based on this suggestion, an entirely new sentence was added to the end of Introduction:

> (L80-84) Consequently, we revealed that xenogeneic germ cell transplantation facilitates surrogate production of functional PBT sperm in hybrid little tuna (genus: *Euthynnus*) at a mere eight months of age and 1 kg body size. Although the production efficiency of donor-derived PBT gametes still has to be improved, this study opens the possibility of highly efficient seed production of PBT.

9. L100: Provide evidence supporting "multiple mortality-related factors."

We described the survival characteristics of eastern little tuna in our previous studies (Yazawa et al. 2016, 2019). Appropriate references were added in this sentence (L107).

10. L135-137: Suggest using genome sequencing instead of PCR for hybrid fish identification.

Based on this suggestion, we performed new genome-wide genotyping of HLT by genotyping using random amplicon sequencing–direct (GRAS-Di), which is a nontargeted PCR-based genotyping system. A genotype library was prepared for pooled DNA of ELT (n = 7), pooled DNA of ALT (n = 5), and each DNA of 12 HLT individuals. Trimmed reads were mapped onto a de novo assembled catalog sequence. To confirm that the HLT was truly an interspecific hybrid between ELT and ALT, SNPs that provided relevant evidence were extracted using these criteria: (1) SNPs that were homozygous in both parental species and had different alleles in each species, and (2) SNPs that had both alleles of the parent species in all HLT. Consequently, 4,238 SNPs in the HLT genome provided evidence of the interspecific hybrid. Typical examples of those SNPs are shown in Supplementary Fig. 4. These SNPs were distributed without bias on the catalog sequence—a result strongly indicating that the HLT are a true interspecific hybrid between ELT and ALT. All data are provided in supplementary datasets 1–3. To explain the results, entirely new sentences were added:

(L452-481, Methods)

Genome-wide genotyping

The genome-wide genotyping of HLT was performed using genotyping by random amplicon sequencing–direct (GRAS-Di)[38], which is a nontargeted PCR-based genotyping system. Library preparation, sequencing, and genotyping were performed by Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). A genotype library was prepared for pooled DNA of ELT (n = 7), pooled DNA of ALT (n = 5), and each DNA of 12 HLT individuals. The library was constructed in two sequential PCR steps. In the first PCR, 63 different primers, consisting of the Illumina Nextera adaptor sequence and 3 bp random oligomers were used. In the second PCR, primers consisting of the Illumina multiplexing 8 bp index and P7/P5 adapter sequence were used. PCR conditions matched those in a previous report[38]. The library was sequenced paired-end 2 × 150 bp on DNBSEQ-T7 (MGI Tech, Shenzhen, PRC) using the DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE150: MGI Tech).

Adaptor sequences were clipped using cutadapt (ver. 4.0), and low-quality reads were trimmed using Sickle (ver. 1.33). Trimmed reads shorter than 75 bp were removed. Moreover, sequences after 76 bases were deleted using fastx_trimmer (ver. 0.0.14). single nucleotide polymorphisms (SNP) calling for each individual used the denovo_map.pl program of Stacks (ver. 2.62) with the "−m 5" option to discard SNPs with read depth less than 5. The populations program of Stacks (ver. 2.62) was used with the "−R 0.1" option to extract SNPs found in more than 10% of the individuals in the overall sample.

Approximately 1,700,000 raw reads were obtained per sample (deposited in the DDBJ SRA database; accession number: PRJDB18157). After the foregoing analysis, 49,281 reference catalog sequences were obtained (Supplementary Data 2), and 36,727 SNP loci were found (Supplementary Data 3). To confirm that the hybrid F1 was truly an interspecific hybrid between ELT and ALT, SNPs that provided relevant evidence were extracted from Supplementary Data 3 based on these criteria: (1) SNPs that were homozygous in both parental species and had different alleles in each species, and (2) SNPs that had both alleles of the parent species in all analysed HLT except for undetected individuals.

(L147-150, Results) Additionally, the genome-wide SNP analysis found 4,238 SNPs carrying both parental species alleles in the HLT genome without biased distribution (Supplementary Data 1). Supplementary Fig. 4 presents typical examples of these SNPs.

(L153-154, Results) These results confirmed that the HLT is diploid hybrid inherited female ELT and male ALT genomes.

11. L165: Consider revising the use of "major breakthrough."

As suggested, the sentence was revised:

(L177-179) Because ELT demonstrated extremely low early survival, the improved survival of HLT was a meaningful advance in their use as recipients for PBT germ cell transplantation.

12. L181-182: Clarify the total number of germ cells in the host larval tuna recipients.
As suggested, a new sentence showing the number of endogenous germ cells in host larvae was added as below:

(L196-197) Note that host individuals were observed to retain an average of 10.8 ± 0.6 endogenous germ cells at this juncture (n = 5).

13. L188: Provide a reference for antibody No. 149.
Based on this suggestion, a reference was provided (L203).

References

Yazawa, R. et al. Establishment of a tracing technique for transplanted bluefin tuna germ cells in recipient's gonads using monoclonal antibodies specifically recognizing bluefin tuna spermatogenic cells. *Fish. Sci.* **87**, 105–112 (2021).

14. L194, L202-203: Consider presenting all the efficiency parameters in a table format.
The number of surviving recipients was already shown in Supplementary Table 2.
Based on this suggestion, a new table showing the production efficiency of donor-derived PBT sperm was provided as Supplementary Table 3. This table is cited at L206.

15. L199-201: Perform statistical analysis on sperm morphology between Fig. 5a and Fig. 4b.

In sperm smears, the full length of the sperm flagella is not always maintained because of damage caused by sample preparation. Thus, we analyzed the morphology of the sperm head. We measured the long and short diameters and the diameter ratios of the sperm heads in PBT, HLT, and donor-derived PBT sperm. No sign of deformation was detected in donor-derived PBT sperm from recipient semen, and those sperm were indistinguishable from the control PBT sperm. This result is added in Supplementary Fig. 7, and new sentences were added as follows:

(L214-216, Results) These PBT sperm detected in the recipient milt demonstrated typical sperm morphology and were indistinguishable from the control PBT sperm (Fig. 4c, Supplementary Fig. 7).

(L573-574, Methods) To compare the sperm morphology, the long and short diameters of the sperm head were measured using the Image J software application (ver. 1.54i).

16. L202-203: The efficiency is too low! So it is necessary to include strict negative control in this study. How to avoid any contamination of PBT sperm in the sample? How to prove that there is no non-specific staining of these two antibodies in HLT sperm sample?

To avoid any misjudgments, this study developed a new antibody No. 102B in addition to No. 149 (Yazawa et al. 2021) for double-checking. As Fig. 4a indicates, these PBT sperm–specific antibodies No. 102B and No. 149 can clearly distinguish between PBT sperm and HLT sperm without nonspecific staining. Further, to avoid contamination, disposable cytofunnels (Epredia Double Cytofunnel: Thermo Fisher Scientific) were always used with the Cytospin 4 Cytocentrifuge to smear sperm on glass slides. Moreover, we confirmed that not even a single positive sperm was detected in at least 200 million sperm from nontransplanted HLT controls. On the other hand, antibody-positive PBT sperm were reproducibly detected only from the semen of HLT recipients in three separate transplantation experiments (Supplementary Table 3). To emphasize those points, we added Supplementary Fig. 6 with low-magnification views of immunocytochemistry (ICC) against sperm smears from four different nontransplanted HLT individuals as negative controls (Supplementary Fig. 6a–d) and a low-magnification view of ICC against a sperm smear of nontransplanted HLT sperm mixed with PBT sperm at a ratio of 1,000,000:1 as a positive control (Supplementary Fig. 6e–h). The results clearly indicate that PBT sperm–specific antibody can specifically and strongly detected PBT sperm without nonspecific staining even in a low-magnification view. Moreover, low-magnification views of Fig. 4a were added in the source data. Supplementary Fig. 6 is cited in L206, and a new sentence was added:

(L557-560, Methods) Sperm were smeared on glass slides (Frontier FRC-05: Matsunami, Osaka, Japan) using disposable cytofunnels (Epredia Double Cytofunnel: Thermo Fisher Scientific) with a Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific) operated at 1,000 rpm for 5 min and air-dried for 30 min at room temperature.

17. L210-220: The calculation in this paragraph is more than speculation. Most conclusions are over-claimed.

This paper described a model that infers the number of germ cells in cysts from the number of germ cells observed in histologic sections, and it has been confirmed in many fish species that this model approximates actual measurements. Therefore, this method provides estimation for the mitotic division number in fish testicular germ cells. However, we also understand the reviewer's suggestion as already answered in major comment #2. Thus, we replaced the term "germline stem cells" with "germ cells" or softened the expression as follows:

(L234-237) Thus, although further detailed studies are needed to reveal whether colonized germ cells are truly germline stem cells, these data suggest that the colonized germ cells demonstrated stem cell activity capable of self-renewal and differentiation into sperm.

Original sentence

Although it was not possible to determine in this study why the HLTs used in this study were able to maintain PBT-derived germline stem cells and support the self-renewal and differentiation of these cells, two possibilities can be considered.

Revised sentence

Revised sentence (L284-286)

Although it was not possible to determine in this study why the HLTs used in this study were able to support spermatogenesis by donor-derived PBT germ cells, two possibilities can be considered.

Original sentence

Therefore, it is possible that PBT-derived germline stem cells transplanted into the recipient mackerels failed to proliferate and differentiate normally at approximately 20°C, which was used for mackerel larvae culture, but that they developed smoothly at approximately 25°C, which was used for HLT larvae culture.

Revised sentence (L297-301)

Therefore, it is possible that PBT-derived germ cells transplanted into the recipient mackerels failed to proliferate and differentiate normally at approximately 20°C, which was used for mackerel larvae culture, but that they developed smoothly at approximately 25°C, which was used for HLT larvae culture.

Original sentence

In this study, we found that the sperm-producing germ cells in the recipient testes behaved as germline stem cells. This fact implies that even larger amounts of sperm could be produced in the future if the somatic environment of the gonads is improved to facilitate their self-renewal and differentiation activities. Further detailed studies on these points will be an important future task.

Revised sentence (L302-306)

In this study, we have concluded that the sperm-producing germ cells in the recipient testes behaved as germline stem cells. This result implies that even larger amounts of sperm could be produced in the future if the somatic environment of the gonads is improved to facilitate their self-renewal and differentiation activities. Further detailed studies on these points will be an important future task.

18. L230-232: the detection of PBT-specific band was observed in 1 of 18,300 embryos, which is > 100 times higher than the detection rate of PBT sperm in the HLT sperm sample (less than 1/2,500,000). How could this happen?

Currently, we lack data sufficient to draw a definitive conclusion, and we consider this issue to be stochastic. However, our study validated whether the PBT-positive no. 8 sample contained an embryo derived from donor PBT sperm, while excluding the possibility of any PBT DNA contamination. As Fig. 4f demonstrates, PCR analysis using several primers targeting PBT-specific sequences in nuclear DNA indicated that the No. 8 sample was PBT-positive. Furthermore, as described in the next response,

sequencing analysis showed that the sequences of the amplicons fully matched PBT sequences (Supplementary Fig. 8). Importantly, if these amplicons were derived from PBT DNA contamination, mitochondrial DNA from PBT would also have been detected in the No. 8 sample. (Unlike nuclear DNA, mitochondrial DNA is present in multiple copies per cell.) However, despite repeated tests, no amplification of PBT mitochondrial DNA was found in the No. 8 sample (Fig. 4f). We therefore concluded that the No. 8 sample contained at least one embryo derived from fertilization between an HLT egg and donor-derived PBT sperm. Again, the detection rate of PBT-positive embryos is a stochastic issue, and discussing this in detail is currently difficult. In future research, we will address this problem by improving the production efficiency of donor-derived PBT gametes.

19. L232-234: Suggest using DNA sequencing, instead of simple PCR analysis, to verify the genetic origin of No.8 sample.

Based on this suggestion, we performed a sequencing analysis of the PCR products, confirming that the sequences of PCR products amplified from sample No. 8 using PBT-specific primers fully matched the PBT sequence (Supplementary Fig. 8). As already mentioned, the lack of amplification of PBT mitochondrial DNA is crucial evidence indicating that these amplicons were truly derived from an embryo fertilized with donor-derived PBT sperm. This sequencing data was therefore added as Supplementary Fig. 8:

> (L251-253, Results) Moreover, sequencing analysis confirmed that sequences of the PCR products amplified from sample No. 8 fully matched the PBT sequence (Supplementary Fig. 8).

20. L251: Consider revising the use of "groundbreaking advance."

21. L252: Consider revising the use of "revolutionize."

As suggested, the sentence was revised:

> (L269-272) Although we still have to overcome issues concerning the production of donor-derived PBT eggs, this research represents an advance toward compact, low-

cost, and time-efficient seedling production that will improve the efficiency of PBT aquaculture and stock enhancement.

22. Fig. 1: Suggest presenting this figure as supplementary material.

These data are important for showing the necessity of crossing eastern little tuna with Atlantic little tuna. Given that the display items in this manuscript are within the journal's limitation, we would like to display these data as Fig. 1.

23. Fig. 4: Only a test of the antibody. It is suggested to either merge it with Fig. 5 or move it to supplementary materials.
Based on this suggestion, Fig. 4 was merged with Fig. 5.

Reviewer #2 (Remarks to the Author):

This is another beautiful and rigorous germ cell transplantation study performed by Professor Yoshizaki's group. This reviewer has no questions about the experiment design, procedures, results, and discussion.
The only concerns this review has rested on the novelty of this research and whether it can reach Nature Communications' publication standard. The decision will be up to the editors.

These concerns are:

1. Although Bluefin tuna is a precious species and very difficult to work on in research and aquaculture, the germ transplantation that led to sperm production has been achieved in the recipients of different orders with more than 100 million years of phylogenetic distance (T. Saito, R. Goto, K. Arai, E. Yamaha, Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation, 2008) and 200 million years of phylogenetic distance (M.A. Silva, G.M.J. Costa, S.M.S.N. Lacerda, P.F.P. Brandão-Dias, E. Kalapothakis, A.F. Silva Júnior, et al. Successful xenogeneic germ cell transplantation from Jundia catfish into adult Nile tilapia).

The significance of this study lies not in donor-derived gamete production by genetically distant recipients, but in successfully applying xenogeneic germ cell transplantation to PBT, a commercially valuable species whose stocks are declining globally. Saito et al. 2008 used zebrafish as the recipient. Like the application of data obtained from mouse research to medical research, many technical breakthroughs are required to apply germ cell transplantation to nonmodel fish species that are commercially valuable and facing extinction. For example, one required technical breakthrough is the improvement of survival and growth in recipients. This study used heterosis to successfully overcome that obstacle. Moreover, donor-derived germ cells can easily be visualized in model species using the *vasa-gfp* transgene. However, the application of that method to PBT is unrealistic given their huge body size and high mortality during the larval stage. Instead, this study established an immunocytochemistry assay using novel monoclonal antibodies that specifically recognize PBT sperm. Using those antibodies, we reproducibly proved that HLT recipients can produce donor-derived PBT sperm. The strategies developed in this study are applicable to various fish species that are commercially valuable or facing extinction.

Moreover, although we understand the importance of a publication by Silva et al. 2016, those authors confirmed the existence of donor-derived cells by PCR analysis with a primer set specific to the donor species by using template DNA prepared from recipient testis containing various stages of germ cells. However, our study confirmed donor-derived PBT sperm production by directly visualizing the PBT sperm in the recipient semen using our PBT sperm–specific antibodies. This method can clearly prove the existence of PBT sperm in the recipient semen. Furthermore, Silva et al. 2016 did not confirm the functionality of the donor-derived sperm. We consider the functional assay of donor-derived gametes to be the crucial experiment to demonstrate successful production of xenogeneic gametes. Our study used F1 embryos to confirm the functionality of donor-derived PBT sperm by PCR analysis with several specific primers. The results strongly suggested that donor-derived PBT sperm can fertilize and initiate embryonic development.

2) The significance of increasing recipients' survival rate from less than 1% in ELT recipients (lines 103-104) to 2% in HLT recipients (line 194).

If the survival rate of ELT had improved from 10% to 20%, we would also consider that the improvement was not truly significant. However, doubling the survival rate of ELT with an extremely low survival rate (less than 1%) is meaningful progress. As Supplementary Table 2 reveals, the transplantation experiment in this species can be performed only 1–2 times annually in a laboratory-scale facility. When we used ELT recipients, all transplanted recipients sometimes died before the maturation period. This problem significantly hindered the optimization of the transplantation procedures. However, the survival rate of HLT is stably 2% as Fig. 2 demonstrates, and HLT recipients reproducibly reached the maturation period. Accordingly, HLT recipients enabled a reproducible analysis of the production of donor-derived PBT sperm using many matured recipients. For that reason, developing the HLT recipient exhibiting heterosis was a breakthrough for establishing germ cell transplantation technology in PBT.

3) The significance of PBT sperm detection in only one (sample No. 8) of the 183 samples (18,300 embryos) obtained from four spawning days (lines 230-232).

As mentioned earlier, the functional assay of donor-derived gametes is one of the most crucial experiments to demonstrate successful transplantation. Although the progeny test in this study detected only one positive sample containing the donor-derived embryo, this single donor-derived embryo provided undoubted evidence that donor-derived PBT sperm can fertilize and induce embryo development. It is noteworthy that the PCR amplification pattern in sample No. 8 (PBT nuclear DNA positive and PBT mitochondria DNA negative) cannot be explained by contamination with PBT DNA, which always includes both nuclear and mitochondria DNA. Thus, this result strongly convinced us of the successful production of functional donor-derived PBT sperm.

As the reviewer commented, a concern connected to this study is the lower production efficiency of donor-derived PBT sperm. However, we consider that the

significant progress in this study lies in “proving the feasibility” of the surrogate production of PBT sperm in small-bodied recipients within a short generation time. As the history of most life sciences research shows, proof-of-concept constitutes a bigger hurdle than improving efficiency. Indeed, the HLT recipient and fine-tuned immunocytochemistry and PCR analysis developed in this study make it possible to reproducibly analyze the production efficiency of donor-derived gametes using many matured recipients, presumably accelerating the evaluation of various methods to improve efficiency. We sincerely hope that reviewers will understand that this research is a significant work that has moved the needle from zero to one.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Given the challenges involved in the sampling process of bluefin tuna, the reviewer is satisfied with the current revisions in terms of technical challenges.

Reviewer #2 (Remarks to the Author):

The revised manuscript is a better version of this study. As stated in the first report, this reviewer's concern was how significant this study has accomplished in granting publication with Nature Communication since the fish testicular germ cell transplantation technology has been successfully developed and established in many species.

Bluefin tuna is a precious and high-value species that is very difficult to work on in research and aquaculture settings, which highlights the complexity of this study. However, the considerably low efficiency of producing Bluefin tuna sperm in HLT recipients [2% survival rate and only one positive detection from 183 samples (18,300 embryos)] achieved in this study raises concerns regarding its application in the actual field.

Transferring the existing technology to this highly economically valuable fish would need to demonstrate the possibility of its cost-effective real-world application. Even if the one-year-old males were used as testicular germ cell donors, it would take almost two years to harvest very few Bluefin tuna sperm from the recipients (1-year-old Bluefin tuna donors plus 8-10 months in HLT recipients). In this reviewer's opinion, Letting these 1-year-old male Bluefin tuna donors grow for two more years (reaching sexual maturity at 3 to 5 years old, as stated by the authors) would make more economic sense.