Journal of the Marine Biological Association of the United Kingdom

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Marine Record

Cite this article: Ashida H, Tsukahara Y, Yasuike M, Nakamura Y, Maeta K, Tanaka Y (2024). Hermaphroditism in Pacific bluefin tuna *Thunnus orientalis* in the Sea of Japan. *Journal of the Marine Biological Association of the United Kingdom* **104**, e108, 1–5. https://doi.org/10.1017/S0025315424001012

Received: 25 February 2024
Revised: 6 October 2024
Accepted: 16 October 2024

Keywords:
disorders of sex development; intersexuality; maturity; tunas

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Hermaphroditism in Pacific bluefin tuna *Thunnus orientalis* in the Sea of Japan

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Abstract

A case of hermaphrodism in a wild Pacific bluefin tuna *Thunnus orientalis* from the Sea of Japan is reported. Morphologically and macroscopically, the gonad seemed to have both ovarian and testicular surfaces. Histological analysis showed that the gonad consisted of only sexually mature testis in all the sections examined, suggesting that the individual was male. Genetic analysis, however, indicated that the tissue samples from this individual had no male-specific genome region, resulting in it being inferred as a female. The observed inconsistency between genetic and histological analysis could help future understanding of the sex development of tunas.

Introduction

Tunas (tribe Thunnini) are gonochoristic and usually do not change sex throughout their life history (Schaefer, 2001). In tuna species (genus *Thunnus*), hermaphroditic gonads have been found in wild Atlantic bluefin tuna (BFT) *Thunnus thynnus* (Caprioli *et al.*, 2007) and reared Pacific bluefin tuna (PBF) (Sawada *et al.*, 2002). In reared PBF, it was reported that the frequency of gonads with a hermaphroditic appearance was less than 1% (Sawada *et al.*, 2002). Some tuna individuals with hermaphroditic gonads can mature sexually and some individuals can create gametes physiologically (e.g., Sawada *et al.*, 2002; Caprioli *et al.*, 2007; Macías *et al.*, 2014; Ashida *et al.*, 2018). For example, a specimen of BFT with hermaphroditic gonads had, both sexually mature ovary and testis (Caprioli *et al.*, 2007). In reared PBF, a part of the testis in a hermaphroditic gonad contained sperm (Sawada *et al.*, 2002). In skipjack tuna *Katsuwonus pelamis*, hydrated oocytes and postovulatory follicles were observed in a part of the ovaries in the gonads of hermaphroditic specimens (Ashida *et al.*, 2018).

In the skipjack tuna, the sequence pattern of the ovary and testis in the hermaphroditic gonads varied widely among different specimens (Raju, 1960; Ashida *et al.*, 2018). In some instances, the parts of the hermaphroditic gonad cannot release gametes because the ovarian cavity and main sperm ducts are structurally disconnected from the cloaca (Ashida *et al.*, 2018). The histological examination of the gonad structure can reveal the spawning capability of the fish with hermaphroditic gonads. In addition to the histological perspectives, a male-specific haploidal region (i.e., paternally-inherited region) carrying a putative oestrogen sulfotransferase gene (*sult1st6y*) has been recently found in the PBF genome (Nakamura *et al.*, 2021). The presence or absence of this region, or *sult1st6y* gene, is linked with the sex of PBF, hence applicable in genetic sex identification. The analysis in multiple ways could facilitate the understanding of the sex development mechanism in such hermaphroditic fish.

In this study, wild-caught specimens of PBF with evidence of hermaphroditism were examined, with morphological and histological characteristics of the gonads described. Genetic approaches were used to identify the original and genotypic gender of the specimens.

Material and methods

A specimen of PBF with hermaphroditic gonad was found in Sakai Port in Tottori Prefecture, a major tuna-landing port in Japan, on 21 June 2022. The fish was caught in a purse seine fishery in the Sea of Japan (37°38′N, 138°01′E) which is an important spawning area for the species, on 20 June 2022. Fork length (cm), gutted weight (kg), and gonad weight (g) of the specimens were measured. The whole gonad was fixed in 10% neutral-buffered formalin. Tissue samples from the heart, ovary, and testis were preserved in TNES-6M urea buffer (Asahida *et al.*, 1996) to determine sex using genetic analysis. Whole-genome sequencing of the genomic DNA extracted from the tissues was performed using paired-end (2 × 75 bp) sequencing on an Illumina NextSeq 500 (Illumina, San Diego, CA, USA). The read sequences were mapped to a male-specific scaffold using BWA-MEM (Li, 2013). The read depth (i.e., the number of mapped reads on that region) was computed according to Nakamura *et al.* (2021) to genetically identify their sex. Briefly, the relative read depth to the median depth among the whole genome sequences was computed, where it should ideally be one of three values: 1 for the diploid region, namely the average among autosomal regions, 0.5 for the haploid region,

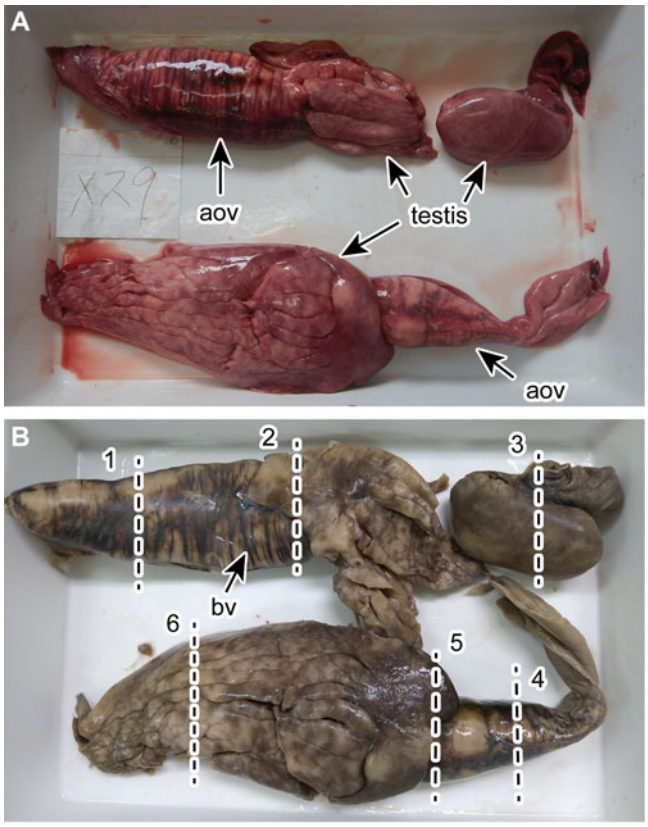

Figure 1. Morphological features of *Thunnus orientalis* (caught from the Sea of Japan) gonad with hermaphroditism. (A) Before fixation and (B) after fixation using 10% formalin. Dash lines and numbers represent the positions of transverse sections. aov, apparent ovary; bv, blood vessels.

such as Y chromosome in the XY sex-determination system, and 0 for the region absent in the sampled individual. Thus, the relative read depth for the PBF male-specific scaffold is expected to be nearly 0.5 if the sampled individual is genetically a male, otherwise zero. Transverse sections were made at six parts of the fixed gonad (Figure 1), and the morphological features of the inner structure in each transverse section were observed macroscopically. A small portion of tissue was extracted from transverse sections 1, 3, 4, and 6, dehydrated in a series of ethanol solutions, and embedded in a methacrylate polymer resin (Technovit 7100, Kulzer GmbH, Hanau, Germany). Histological sections (4 µm thickness) were cut and stained with 1% toluidine blue. The developmental stages of the germ cells were defined based on Schaefer (2001). The state of the germinal epithelium was classified as continuous or discontinuous based on Grier and Taylor (1998). The maturity status of the gonad was determined using the criteria of Brown-Peterson *et al.* (2011).

Results

The fork length, gutted weight, and gonad weight of the fish were 135 cm, 39 kg, and 1275 g, respectively. All of the sequences of the genome in the heart, testis, and ovary tissues were hardly mapped on the male-specific genome region (Figure 2). The relative read depths obtained from the three tissues, showing almost the same pattern, were different from that of the male reference with ~0.5 of relative depth, but similar to that of the female reference (i.e., relative depth was close to zero). Therefore, the individual was estimated as being genetically female.

The morphological features of both the ovary and testis were present in the two gonads (Figure 1). The morphological features of an ovary (i.e., rounded gonad shape, and blood vessels arranged like a network on the gonad surface) were observed in the tip of one gonad and the side of the cloaca in the other gonad (Figure 1). The morphological features more characteristic of the testis (i.e., flattened gonad shape, fine folds on the surface of the gonad, and absence of the blood vessels observed in the ovarian tissue) were observed in the right tip of one gonad and the side of the cloaca in the other gonad (Figure 1).

Thick connective tissue membranes, such as the ovarian wall, were observed in the outer region of the transverse section of the apparent ovary in the gonad (i.e., sections 1 and 4; Figures 1B, 3 & 4). The section colour and the apparent ovary shape were white and round, respectively, and the main sperm duct and ovarian lamellar structure were not observed macroscopically (Figure 3A, B, D, E). In the testis part of the gonad (sections 3 and 6), the section shape was bilobate, and the main sperm duct was located in the dent of the transverse section (Figure 3C, F). The state of the connection between the apparent ovary and testis was different in each section. The apparent ovary was attached to the outer region of the testis in section 2 (Figure 3B), whereas it was incorporated in the testis in section 5 (Figure 3E).

The histological structure of the apparent ovary in the gonad (sections 1 and 4) was the testis, and numerous lobules were observed in this tissue (Figures 1B & 4). A large vacant space was observed between the thick connective tissue membrane and lobules (Figure 4A). Furthermore, a structure similar to the testis duct system (Abascal *et al.*, 2004) was located between the lobules and thick connective tissue membranes, and sperm filled this structure (Figure 4A). In the lobules, all developmental stages of germ cells (i.e., spermatogonia, spermatocytes, spermatids, and

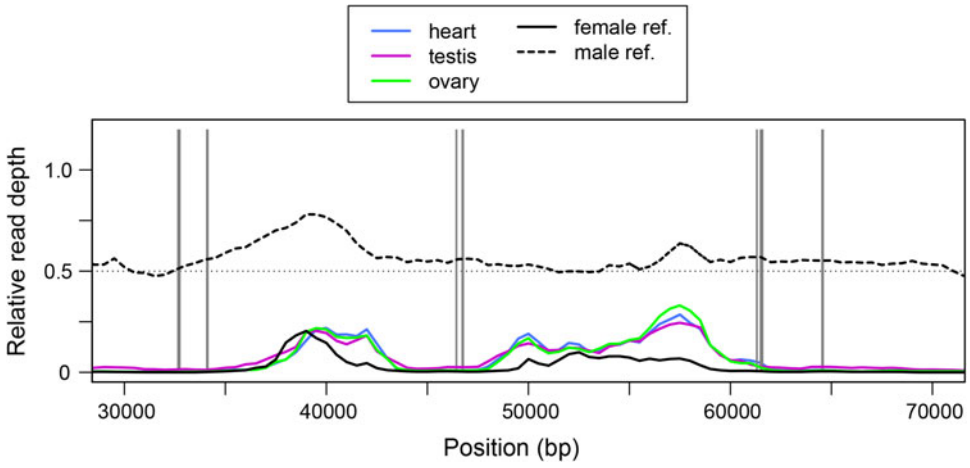

Figure 2. Relative read depth mapped to the median at the position from 30,000 to 70,000 base pair (bp) in the male-specific scaffold of *Thunnus orientalis*. The depths were computed using sliding-windows (window, 5000 bp; step, 500 bp). The reads of DDBJ Sequence Read Archive (accession numbers: DRR177382 and DRR177383) were used as the female and male references, respectively. The grey areas indicate the sex-associated marker gene, *sult1st6y*, consisting of seven exons.

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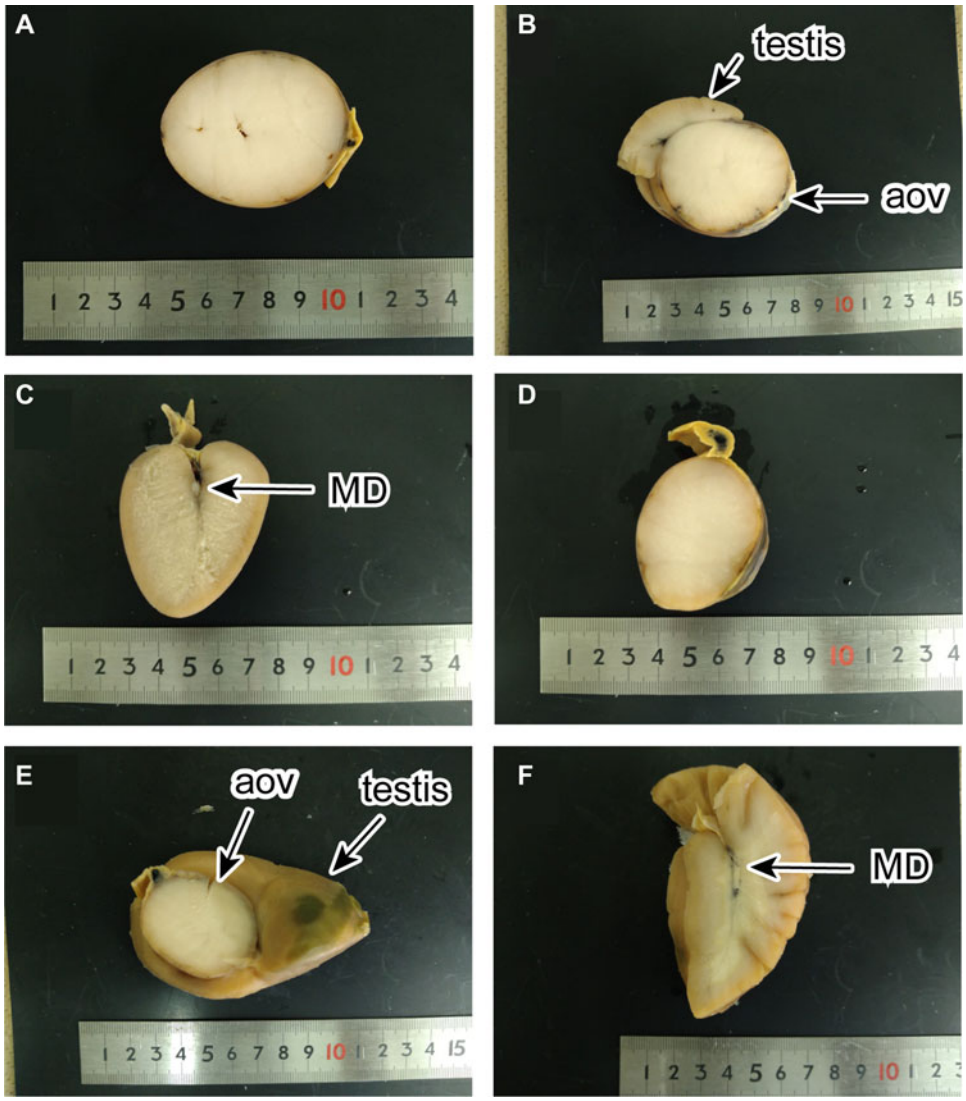

Figure 3. Photographs showing the transverse sections of each portion of *Thunnus orientalis* gonad with hermaphroditism. The positions of the transverse sections of the gonads with hermaphroditism are shown in Figure 1B. Scales indicate 1 cm. (A) Section 1, (B) Section 2, (C) Section 3, (D) Section 4, (E) Section 5, and (F) Section 6. aov, apparent ovary; MD, main sperm duct.

sperm) were observed, and the germinal epithelium was discontinuous throughout the histological section (Figure 4). Oocytes were not observed in the tissue of hermaphroditic gonad. Based on these histological observations, sections 1 and 4 were testis, and the maturity status of each section was classified as spawning capable phase.

All the developmental stages of germ cells were observed in the testis lobules (i.e., sections 3 and 6), and the lobules were filled with sperm (Figures 1B & 5). The morphological features of the germinal epithelium in the lobules near the main sperm duct and in the peripheral portion were discontinuous and continuous, respectively (Figure 5). Based on these histological observations, the maturity status of sections 3 and 6 was classified as the spawning capable phase.

Discussion

Generally, discovering tuna gonads with hermaphroditism is very rare. Sawada *et al.* (2002) reported that one gonad with hermaphroditism was found among 1680 gonads of reared PBF. Macías *et al.* (2014) reported that two hermaphroditic gonads were found among 449 little tunny *Euthynnus alletteratus*. In this study, we found the hermaphroditic gonad for the first time on 21 June 2022, even though approximately 1700 gonads had been sampled in Sakai Port between 2015 and 2022.

The morphological features of hermaphroditic gonads found in previous studies were consistent with the histological features (Sawada *et al.*, 2002; Caprioli *et al.*, 2007; Ashida *et al.*, 2018). Surprisingly, the histological analysis indicated that the hermaphroditic gonads in the present study were judged as testis, contrary to some morphological features. In contrast, according to the genetic analysis, this individual was likely female. This is the first report of inconsistency between morphological, histological, and genetic results in tunas.

Earlier studies had observed oocytes in the testis (Sawada *et al.*, 2002; Ashida *et al.*, 2018). In the present study, we observed the lobules and the developmental stage of germ cells in each section; but oocytes were not found. This suggests that the hermaphroditic gonad in the present study was histologically male; the morphological features of the gonads with hermaphroditism do not always correspond with the histological features; and that more information is required to understand sex development in fish with hermaphroditic gonads.

In some fish targeted for aquaculture, sex differentiation is controlled by various methods, such as hormonal and environmental manipulation (Budd *et al.*, 2015). In reared PBF, genotypic females can be artificially changed to phenotypic males using aromatase inhibitors before sex differentiation (Hayashida *et al.*, 2023). Sex differentiation in PBF occurs 40 to 70 days after hatching (Hayashida *et al.*, 2021). We did not directly detect the factors

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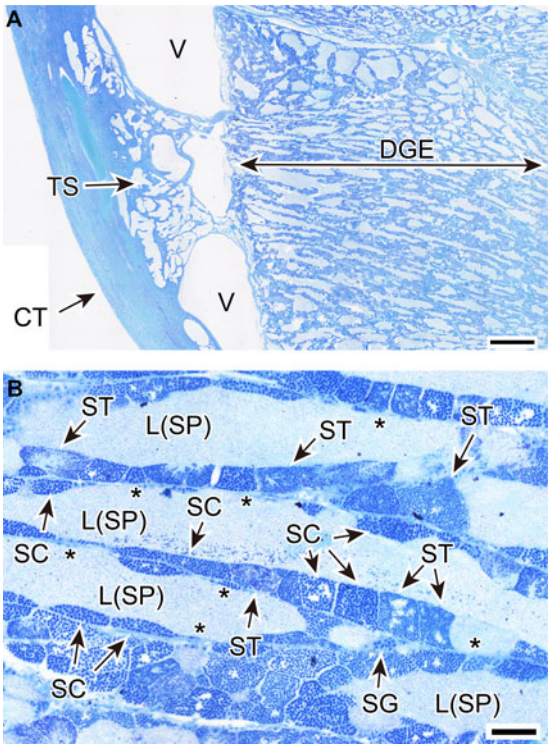

Figure 4. Photographs of apparent ovary histological section (i.e., section 1) of *Thunnus orientalis* gonad with hermaphroditism. [(A) Low resolution, scale bar = 2 mm and (B) high resolution in discontinuous germinal epithelium, scale bar = 50 μm]. CT, connective tissue membrane; DGE, discontinuous germinal epithelium; L(SP), lobules filled with sperm; SC, spermatocytes; SG, spermatogonia; ST, spermatids; TS, testis duct system; V, vacant space. Asterisks indicate the portions lacking the spermatocysts (i.e., DGE).

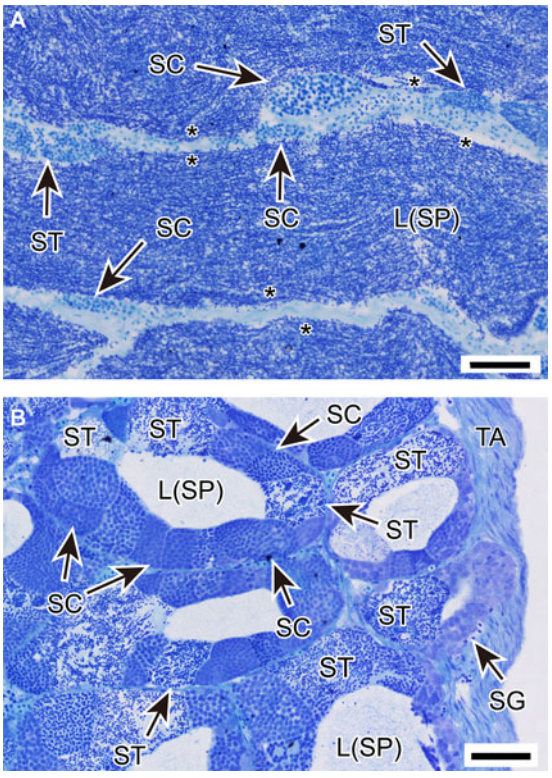

Figure 5. Photographs of testis histological section (i.e., section 6) of *Thunnus orientalis* gonad with hermaphroditism [(A) Discontinuous germinal epithelium, scale bar = 50 μm and (B) continuous germinal epithelium, scale bar = 50 μm]. L(SP), lobules filled with sperm; SC, spermatocytes; SG, spermatogonia; ST, spermatids; TA, tunica albuginea. Asterisks indicate the portion lacking the spermatocysts (i.e., discontinuous germinal epithelium).

that caused the hermaphroditism; however, it may have been caused by some factors experienced by fish in their early life history before sex differentiation.

Histological observations revealed that the hermaphroditic gonad found in the present study was in the spawning capable phase (physiologically able to spawn; Brown-Peterson *et al.*, 2011). A previous study related to the reproduction of PBF in the Sea of Japan showed that the fishing position and date which the fish with hermaphroditic gonad was caught in the present study correspond to a known spawning ground and season of PBF (Ashida *et al.*, 2021). These results suggest that the fish with hermaphroditic gonad found in this study was sexually mature and may have acquired the ability to spawn as a male histologically, although it is unclear whether wild individuals with hermaphroditic gonads have the ability to spawn successfully as a male.

Data. The data that support the findings of this study are available from the corresponding author, HA, upon reasonable request.

Acknowledgements. This study was conducted under the Research and Assessment Program for Fisheries Resources of the Fisheries Agency of Japan.

Authors' contributions. H. A.: Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization. Y. T.: Conceptualization, Methodology, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization. M. Y.: Methodology, Validation, Investigation, Data curation, Resources, Data curation, Writing – Review & Editing, Visualization. Y. N.: Methodology, Validation, Investigation, Data curation, Resources, Data curation, Writing – Review & Editing, Visualization. K. M.: Investigation, Data curation, Writing – Review & Editing. Y. T.: Writing – Review & Editing, Supervision, Funding acquisition.

Financial support. This work was supported by the Research and Assessment Program for Fisheries Resources of the Fisheries Agency of Japan.

Competing interests. None.

Ethical standards. This work followed the guidelines for animal experiments at the Fisheries Resources Institute, Japan Fisheries Research and Education Agency.

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