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SHORT COMMUNICATION

Histological evidence and the existence of the cholesterol side-chain cleavage enzyme of the diminutive testis of the robust tonguefish *Cynoglossus robustus* (Pleuronectiformes: Cynoglossidae)

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Abstract Tonguefishes, commercially valuable marine flatfishes with a global distribution, are characterized by unusually small testes. Despite their economic importance, the detailed structure of these diminutive testes has not been adequately described. Mature testes of robust tonguefish (*Cynoglossus robustus*) were collected from the Seto Inland Sea, Japan. They were examined for gonadosomatic index (GSI) and histological characteristics. The localization of the cholesterol side-chain cleavage enzyme (P450scc) within the testis was also analyzed. The GSI of the sampled males was markedly lower than that of other bony fish. Histological analysis showed that the testes have a tubular structure, with spermatogenesis occurring in seminiferous tubules, which is less common among teleosts. The stage and structure of spermatogenesis varied depending on the location within the testis. In the cranial region of the testes, the spermatogonia and Sertoli cells were found only at the periphery. Meiotic spermatocytes, haploid spermatids and spermatozoa were primarily located in the inner part of the cranial region. Several seminiferous tubules containing only spermatids were observed in the caudal region. Strong positive signals for the P450scc antibody were detected in the interstitial Leydig cells surrounding the seminiferous tubules. This study provides a detailed description of the testicular structure in *Cynoglossus robustus* and contributes to understanding the relationship between testicular size, volume, and type in teleosts.

Keywords Tonguefish . Testis . Leydig cell . Sertoli cell . Spermatogenesis

Introduction

Flatfishes, members of *Pleuronectiformes*, are characterised by body asymmetry and are commercially important for fisheries worldwide (Friedman 2008; Gibson et al. 2014). In the Central Seto Inland Sea, Japan, three flatfish species belonging to the tonguesole family (Cynoglossidae) were caught using a small trawl net. These species include the robust tonguefish, *Cynoglossus robustus*, red tonguesole, *C. joyneri*, the and the threeline tonguefish, *C. abbreviates* (Baeck et al. 2011). These soles accounted for 9.7%–12.6% of the total fish caught per year in the local coastal area of the Seto Inland Sea (Mototani 2011). Due to the gradual decrease in the species' stocks (Nagai 2003) and their commercial value, they are considered candidates for farming in the Seto Inland Sea, Japan. However, their basic reproductive biology, particularly

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gametogenesis, has not been investigated despite information on their age, growth and spawning periods being limited. Consequently, little information is available on their breeding habits (Yamamoto et al. 2008; Yamamoto and Katayama 2013).

A typical reproductive characteristic of soles is their markedly lower volume and weight of testes than those of the ovaries. Thus, the male gonadosomatic index (GSI) is shallow throughout the year (0.0097 ± 0.004) in the Senegalese sole males (*Solea senegalensis*). Therefore, sole males produce poorer sperm volume and concentration (oligospermia) than other fish species (Ghaffari et al. 2015; Suquet et al. 1994). This characteristic leads to a considerable obstacle (low fertilization rate) in aquaculture (Agulleiro et al. 2006; Anguis and Cañavate 2005). Histological observation of the Senegalese sole testes revealed that their spermatogonia belong to the unrestricted tubular type (García-López et al. 2005). These tubular testes are quite different from those of most flatfish and Perciformes (García-López et al. 2005).

Sex steroid hormones such as androgens and oestrogens directly control the growth and function of the testes in teleosts (Schulz et al. 2010). Several steroidogenic enzymes synthesized steroid hormones from cholesterol, a common precursor. Cholesterol conversion to pregnenolone is the first step in the steroidogenic pathway (Simpson and Boyd 1966). This reaction is catalyzed by the cholesterol side-chain cleavage enzyme (P450scc), which expresses the cytochrome P450 family 11 subfamily A gene in the inner mitochondrial membrane of the steroid-producing cells (Winkel et al. 1980). Therefore, investigating this critical enzyme in the sole testes may lead to an improved understanding of the endocrinological mechanisms of the tubular-type spermatogenesis.

Tonguefish species hold significant ecological and economic importance, yet detailed knowledge of their testicular structure and function remains limited. This study investigates the testicular microarchitecture and steroidogenic capacity of the robust tonguefish (*Cynoglossus robustus*). We examine the fine structure of tonguefish testes, determine the spatial distribution of spermatogenesis, and localize steroidogenic cells using P450scc immunohistochemistry. Our findings contribute to the broader understanding of teleost reproductive diversity and provide insights into the adaptive significance of reduced testis size in flatfish. This research may inform future studies on flatfish reproduction and have implications for aquaculture practices and conservation strategies.

Materials and methods

Fish and sampling

Mature male robust tonguefish (n = 18) were collected from Bisan Strait of the Seto Inland Sea, Japan on July, 2014. Fish were captured using a bottom trawl net at depths of 8-20 m. Total length was measured to the nearest 0.1 cm using a measuring board, and body weight was recorded to the nearest 0.1 g using an electronic balance. Testes were carefully dissected and weighed to 0.01 g precision. They were weighed to calculate the gonadosomatic index (GSI), which was calculated as the ratio of gonad weight to body weight, expressed as a percentage (100%).

Testicular histology

The excised testes were immediately immersed in freshly prepared Bouin's solution at 4°C for 24 hours fixation. After fixation, the testicular specimens were washed in 70% ethanol to remove excess picric acid, then dehydrated using a graded series of ethanol (70%, 80%, 90%, 95%, and 100%, 30 minutes each step, repeated twice for 100%). The samples were then cleared in Lemosol (Wako, Inc., Osaka, Japan) for 30 minutes, repeated twice. The cleared samples were infiltrated with paraffin (Paraplast Plus®, Sigma-Aldrich) at 60°C for 2 hours, with one change of paraffin after the first hour. The infiltrated samples were then embedded in fresh paraffin. Serial sections were cut at 7 µm thickness using a rotary microtome. Sections were floated on a water bath at 42°C and collected on adhesive slides (Matsunami Glass Ind., Ltd., Osaka, Japan, catalog number MAS-GP). The slides were dried overnight at 37°C. For histological staining, sections were dewaxed in xylene (3 changes, 5 minutes each), rehydrated through a descending ethanol series (100%, 95%, 80%, 70%, 5 minutes each), and rinsed in distilled water. Sections were stained with Mayer's hematoxylin for 5 minutes, rinsed in running tap water for 5 minutes, counterstained with 1% aqueous eo-

sin Y for 3 minutes, and rinsed briefly in distilled water. The stained sections were dehydrated through an ascending ethanol series (70%, 80%, 95%, 100%, 2 minutes each), cleared in xylene (3 changes, 5 minutes each), and mounted with a xylene-based mounting medium.

Immunohistochemical analysis of P450scc localization

The sections were subjected to immunohistochemical staining using a polymerised reporter enzyme staining system (ImmPRESS™; Vector Laboratories, Burlingame, CA, USA) to ascertain P450scc spatial distribution. Dewaxed and rehydrated sections were first subjected to heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0) at 95°C for 20 minutes, then cooled to room temperature for 20 minutes. To suppress endogenous peroxidase activity, sections were incubated in 3% hydrogen peroxide (H_2O_2) in methanol for 10 minutes at 27°C. Nonspecific binding was blocked by incubating sections with 2.5% normal horse serum (provided in the ImmPRESS kit) for 30 minutes at room temperature.

Sections were then incubated with rabbit polyclonal antibody specific to rainbow trout P450scc (diluted 1:1000 in PBS with 1% BSA) overnight at 4°C. The primary antibody was previously characterized for specificity (Kobayashi et al. 1998). After washing in PBS (3 times, 5 minutes each), sections were incubated with ImmPRESS anti-rabbit IgG (ready-to-use) for 30 minutes at room temperature. The binding sites were visualized using a DAB substrate kit (Vector Laboratories, catalog number SK-4100). Sections were incubated in DAB solution (0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 50 mM Tris-HCl buffer, pH 7.4) for 5 minutes at room temperature. Negative controls were prepared by omitting the primary antibody and replacing it with normal rabbit IgG at the same concentration (Data not shown). The resulting sections were observed and imaged under an FSX-100 microscope (Olympus, Tokyo, Japan) The specificity of the primary antibody was meticulously characterised in a previous study (Kobayashi et al. 1998).

Results

General testicular structure of the mature robust tonguefish

The testis of robust tonguefish is located within the upper-posterior compartment of the body cavity. Similar to other sole species, the testis is considerably smaller than the ovary, a trend evident even when accounting for equivalent body dimensions. The gonadosomatic index was 0.064 ± 0.0023.

Figure 1 depicts the cross-sectional slices of the testes subjected to haematoxylin and eosin staining. The testis of the robust tonguefish prominently comprised orderly arrays of seminiferous tubules conspicuously devoid of discernible lobular luminal structures (Figure 1). This tubular arrangement, distinct from the lobular structure common in many teleosts, may facilitate efficient sperm production within the compact testicular volume characteristic of this species. The efferent ducts and spermatozoa were observed in the ventral domain of the cranial sector (Figure 1a). The cranial expanse hosted abundant spermatogenic germ cells, encompassing spermatogonia, spermatocytes, spermatids and spermatozoa interspersed with accompanying somatic entities (Figure 1b).

The testis of the robust tonguefish exhibited a distinct spatial organization of spermatogenesis. In the cranial region, spermatogonia and Sertoli cells were confined to the tubule periphery near the tunica albuginea (Figure 1c). Meiotic spermatocytes, haploid spermatids, and mature spermatozoa were predominantly found in the inner and intermediate parts of the tubules in this region (Figure 1b-d). Interstitial Leydig cells, responsible for hormonal function, were located in the interstitial spaces between seminiferous tubules in the cranial domain (Figure 1d). The caudal region showed a different pattern, with tubular tracts containing primarily spermatids (Figure 1e and f). The spatial distribution of spermatogenic cells observed in our study suggests a highly organized spermatogenesis process. This arrangement may contribute to the species' ability to maintain reproductive capacity despite having relatively small testes.

P450scc immunolocalization within the testis

The P450scc distribution within the testis of the robust tonguefish was examined using immunohistochemistry (Figure 2). Predominantly confined to the cranial region, distinct assemblages of robust positive signals

were conspicuously detected within the cytoplasm of the Leydig cells, occupying the interstitial expanse between the seminiferous tubules (Figure 2a and a'). Notably, the discerned immunopositive signals remained conspicuously absent within the distal precincts of the tubules where the spermatogonia and Sertoli cells reside. Similar to the observations from the cranial region, the regions encompassing the interstitial Leydig cells surrounding the tubules exhibited strong positive signals indicating P450scc activity (Figure 2b and b'). The localization of P450scc-positive cells in the interstitial spaces indicates concentrated areas of steroidogenesis. This distribution pattern may allow for efficient hormone production, potentially compensating for the reduced testicular volume in this species.

Discussion

Our findings on the testicular structure and steroidogenic cell distribution in robust tonguefish contribute significantly to the understanding of reproductive diversity in teleosts. This study advances our knowledge in three key areas: (1) structural adaptation of testes in species with reduced gonad size, (2) spatial organization of spermatogenesis in tubular testes, and (3) potential mechanisms for efficient steroidogenesis within a compact testicular volume. The unique tubular arrangement and the concentrated distribution of P450scc-positive cells provide insights into how testicular morphology and function can evolve to meet

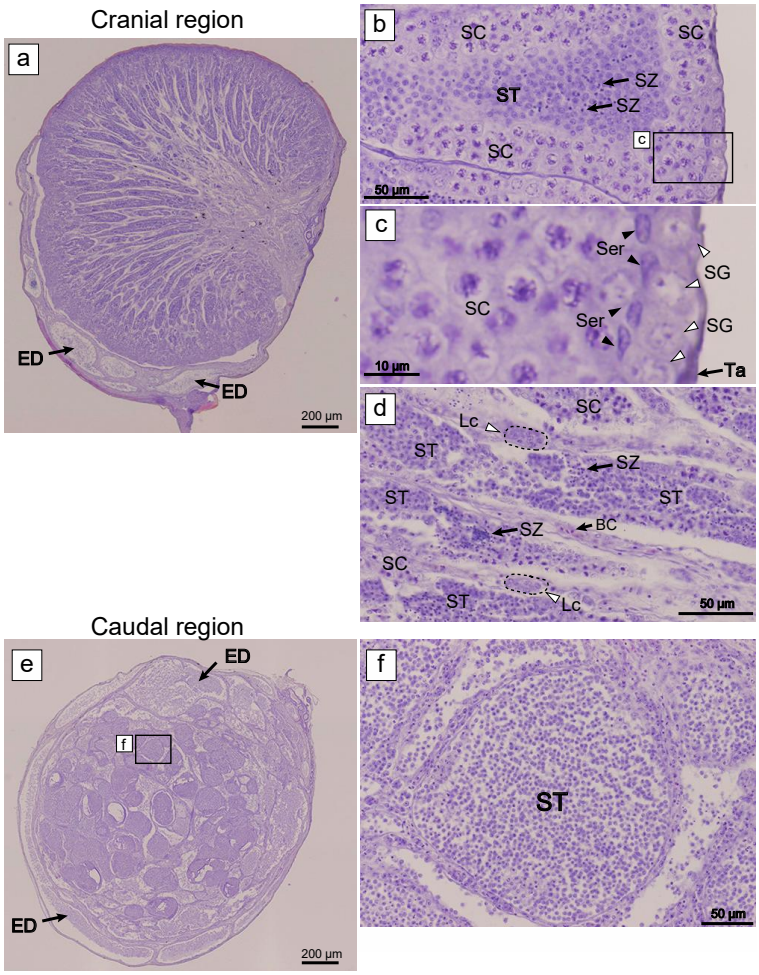

Fig. 1 Histological structure of the testis in the robust tonguefish showing cranial (a-d) and caudal regions (e-f). a Comprehensive overview of the cranial region at low magnification. Arrows indicate efferent ducts (ED) filled with spermatozoa. b Seminiferous tubule situated at the peripheral domain of the cranial region. c Higher magnification of the boxed area in b showing detailed structure of the seminiferous tubule. Spermatogonia (SG, white arrowheads), Sertoli cells (Ser, black arrowheads), and tunica albuginea (Ta, arrows) are indicated. d Seminiferous tubule positioned within the internal realm of the cranial region. Leydig cells (Lc) are outlined by dotted lines and indicated by white arrowheads. e Macroscopic depiction of the caudal region at low magnification. f Detailed examination of the inset in panel e at augmented magnification. Abbreviations: SG, spermatogonia; SC, spermatocyte; ST, spermatid; SZ, spermatozoon; Ta, tunica albuginea of the testis; Ser, Sertoli cell; Lc, Leydig cell; ED, efferent duct; BC, blood cell.

specific reproductive demands. These observations offer a comparative framework for understanding testicular adaptations across diverse teleost species, particularly those with small gonads. Our study underscores the importance of investigating species with unique reproductive characteristics to comprehend the full spectrum of reproductive adaptations in teleosts.

This study comprehensively investigated the intricate testicular architecture of the robust tonguefish. Meticulous histological analysis revealed a distinctive feature where the species' testes follow a tubular paradigm. Numerous actively steroidogenic cells displaying P450scc antibody reactivity within these compact testicular structures were conspicuously localized within the interstitial milieu.

Teleosts exhibit remarkable diversity in testicular morphology (Callard et al. 1978). The testicular organization in teleosts can be broadly categorized into two principal types: tubular and lobular, a classification framework elucidated by Nagahama. (1983). While the lobular-type testes predominate across various fish species, the tubular arrangement is a distinctive hallmark of the specific bony fish taxa, including *salmonids*, *cyprinids*, and *Lepisosteidae* (Uribe et al. 2014). Moreover, within the tubular classification, a subdivision emerges, namely the unrestricted and restricted types, defined by the spatial spermatogonia distribution, as elaborated by Grier et al. (1980). As described in the Introduction, the unrestricted spermatogonial type has been reported in the tubular testes of the Senegalese sole (*Sole senegalensis*) (García-López et al. 2005). However, the general histological observations in this study revealed that the tubular testes of the robust tonguefish were restricted to the spermatogonial type. Additionally, we observed that the developmental progression of spermatogenesis underlies zonation in the testes of the robust tonguefish; the early and advanced stages of spermatogenic germ cells were found in the periphery of and centrally in the tubules, respectively. This testis type is found in higher teleosts, such as *Atheriniformes*, *Cyprinodontiformes*, and *Beloniformes* (Parenti & Grier 2004; Sàbat et al. 2009). Thus, the testes of the red tonguesoles are rare among bony fishes.

While the intricate relationship between the testicular type and testis size in teleosts remains unclear, a general trend has emerged wherein promiscuous species tend to exhibit relatively larger testes than their monogamous counterparts (Stockley et al. 1997). A case in point can be found in the Dover sole (*Solea solea*), where the pairing observed during spawning (a manifestation of monogamy) entails a male positioned beneath a female (Baynes et al. 1994; Devauchelle et al. 1987).

The robust tonguefish distinguishes itself through a unique anatomical aspect. Its genital pores are in close proximity, a peculiarity attributed to the distinctive genital pore localization of the sole (dorsal and ventral surfaces in males and females, respectively), setting it apart from the arrangement observed in other teleosts. This distinctive feature led us to hypothesize that the diminutive and tubular-type testes observed in the robust tonguefish are adapted to support the spawning behavioral characteristic of monogamy despite the absence of documented investigations into such reproductive behavior.

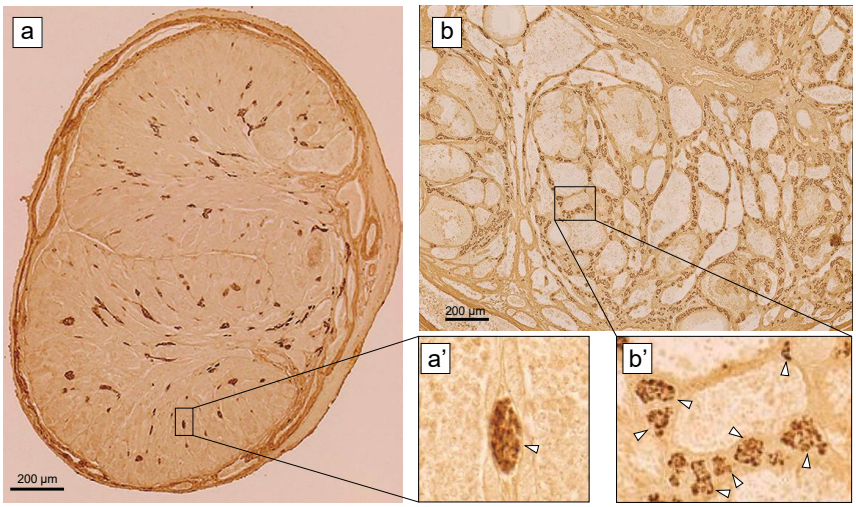

Fig. 2 Immunohistochemical localization of P450scc in the testis of the robust tonguefish. a Low magnification view of the cranial region. a' Higher magnification of the boxed area in a showing intense brown immunopositive signals indicated by white arrowheads. b Low magnification view of the caudal region. b' Higher magnification of the boxed area in b showing intense brown immunopositive signals indicated by white arrowheads. Abbreviations: P450scc, cholesterol side-chain cleavage enzyme.

The fact that gonads serve as the primary locus for producing sex steroid hormones inevitably evokes a pertinent question: Do the conspicuously diminutive testes of the sole fishes possess the inherent capability to yield a substantial quantum of steroid hormones? In the Senegalese sole, the plasma steroid hormone levels in males demonstrate consistent detectability across the annual cycle (García-López et al. 2006; García-López et al. 2007). Consequently, a working hypothesis assumes that the relatively modest dimensions of the sole testicular apparatus may harbor exceptional proficiency in synthesizing sex steroid hormones. This investigative endeavor entailed a meticulous survey of the P450scc immunolocalization, an elemental enzyme pivotal for orchestrating steroid hormonal biosynthesis (Miller 1988), within the testicular domain of the robust tonguefish. Conspicuously affirmative signals confined to the discrete agglomerations of the interstitial Leydig cells confirmed this finding. Intriguingly, the distribution density of these affirmative signals per unit area within the testis eclipses has been observed in analogous teleosts (Kobayashi et al. 2005; Miura et al. 2008), thereby cogently reinforcing hypothesis as mentioned above. This compelling corroboration prevails despite the notable absence of specific accounts detailing the aggregate steroid hormone yield per gonadal unit within this singular sole species.

In the contemporary realm of aquatic science, an effervescent surge exists in research dedicated to the enigmatic sole fish, primarily driven by their far-reaching significance in global aquatic ecosystems. Notably, within the domain of the half-smooth tongue sole (*Cynoglossus semilaevis*), a robust genomic research infrastructure has been meticulously cultivated, encompassing pivotal elements such as the draft genome databases and EST libraries (Cerdà et al. 2010; Cerdà et al. 2008; Chen et al. 2014). However, a conspicuous void persists in a foundational lack of fundamental reproductive insights on the sole fish despite their marked divergence from other piscine taxa, including the Pleuronectiformes. In our understanding, a glaring hiatus pertains to the intricate orchestration of reproductive processes in the sole fish, emphasizing on their testicular dimensions, which have remained largely uncharted because of their diminutive proportions.

Future research should focus on comparative studies across tonguefish species to elucidate evolutionary patterns of testicular structure and function. Additionally, investigating the relationship between testicular morphology and reproductive behavior, as well as quantitative analyses of steroid hormone production in relation to testis size, could provide further insights into the adaptive significance of these unique gonadal characteristics in teleosts.

Competing interests The authors declare no competing interests.

Author contributions Yasuhisa Kobayashi: conceptualisation, investigation and writing of the original draft. Tsuyoshi Mototani: Investigation and resources. Tatsuya Sakamoto: Writing, reviewing, and editing.

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Ethics approval All experiments and protocols were performed in strict accordance with the Guiding Principles for the Care and Use of Research Animals adopted by the Kindai University Committee on Animal Research and Bioethics (Approval ID: KAAG-30-0001).

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