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Original Article

P450scc-like immunoreactivity throughout gonadal restructuring in the protogynous hermaphrodite *Thalassoma duperrey*

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ABSTRACT The source of steroid hormones, which potentially regulate gonadal restructuring throughout protogynous sex change in teleosts, remains largely unknown. To address this issue, immunocytochemical methods were employed to detect gonadal sites of steroidogenesis in the protogynous hermaphrodite wrasse *Thalassoma duperrey* at different stages in the sex change process. Steroidogenic cells were classified based on the presence of P450 cholesterol-side-chain-cleavage-like immunoreactivity (P450scc-ir). P450scc-ir cells were predominantly in the thecal layer of normal females. As females underwent sex change, P450scc-ir localization shifted from the thecal layer to the interstitium. P450scc-ir cells appeared to increase in number midway through sex change. In sex-changed males, P450scc-ir cells were found in small clusters interspersed among spermatogenic lobules. These results demonstrate for the first time the ability of the gonad to produce potential steroidal mediators of gonadal restructuring throughout the sex change process.

KEY WORDS: *sex change, P450scc immunoreactivity, gonadal restructuring, steroid production, wrasse*

Introduction

Sequential, protogynous hermaphroditism, a common phenomenon among teleosts (e.g., Ross, 1982; Warner, 1984), encompasses a broad range of biological reorganization: the underlying mechanisms of determining sex shift from female-specific to male-specific; behavioral repertoires become masculinized; secondary sexual characteristics (e.g., coloration and caudal fin filament length) become distinctly those of the male phenotype and ultimately, reproductive anatomy and physiology redifferentiate to form a functional testis.

Steroid sex hormones are potential mediators of the redifferentiation from female to male in hermaphrodites. Although steroid hormones are generally considered downstream mediators of sexual differentiation (for review, Adkins -Regan, 1987; Redding and Patino, 1993; Nagahama, 1994), the fate of teleost germ cells may be significantly influenced by hormonal profiles (Van den Hurk *et al.*, 1982; Kanamori *et al.*, 1985; Nakamura and Nagahama, 1989; Kobayashi *et al.*, 1991). Consequently, teleost sex-determining cascades may be post-maturationally labile and triggered by steroid hormones.

Significantly increased serum 11-ketotestosterone concentrations accompany sex change in *Thalassoma dupperrey* (Nakamura *et al.*, 1989), *Sparisoma viride* (Cardwell and Liley, 1991), and *Monopterus albus* (Yeung and Chan, 1987). Furthermore, androgen treatment can effectively induce sex reversal at all levels of biological complexity in protogynous hermaphrodites. Testosterone and/or 11-ketotestosterone treatment masculinizes behavior (M. Kobayashi, personal communication), brain morphology (Grober *et al.*, 1991), coloration (Kramer *et al.*, 1988; Grober *et al.*, 1991), gonad morphology and gonadal function (Reinboth, 1975).

Although steroid hormone treatment has been shown to elicit sex change under experimental conditions, it is unclear whether steroid hormones act as the natural, proximate trigger of sex change. Serum androgen concentrations do not become detectably elevated until the latter stages of sex change (Nakamura *et al.*, 1989; Cardwell and Liley, 1991). Serum levels may not accurately

Abbreviations used in this paper: P450scc, Cytochrome P450 cholesterol-side-chain-cleavage; P450scc-ir, Cytochrome P450 cholesterol-side-chain-cleavage-like immunoreactivity; SPC, Steroid-producing cell; YBC, Yellow-brown cell.

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reflect intra-gonadal differences. Nevertheless, alteration of steroid hormone concentrations within the gonad may be sufficient to induce testicular development. Consequently, identification of steroidogenic cells, steroidogenic capacity and metabolic pathways within the gonads throughout sex change is critical to understanding the role of steroid hormones in sexual redifferentiation.

Steroid-producing cells (SPC), as defined by the ability to convert cholesterol into pregnenolone (Miller, 1988), are the source of all steroidal precursors. In general, the predominant SPCs of the female and male are thecal cells and interstitial Leydig cells (for review, Nagahama, 1994), respectively. Throughout protogynous sex change there is a gradual shift within the gonad from female cell types to male cell types; however, the source of Leydig cells and steroid hormones during this transition remains unknown. Although thecal cells and Leydig cells are both derived from fibroblasts (Nicholls and Graham, 1972; Van den Hurk and Peute, 1979), it is unclear whether the initial "Leydig" cells in the transitional gonad result from redifferentiation of existing thecal cells or proliferation and differentiation of stem cells.

Utilizing the protogynous hermaphrodite *T. duperrey* as a model, we sought to identify SPCs throughout sex change by immunolocalization of the cytochrome P450 cholesterol-side-chain-cleavage (P450scc) enzyme. Cleavage of the cholesterol-side-chain via the action of the P450scc is the first step in the biosynthesis of all steroid hormones, therefore, it serves an ideal marker of SPCs. Furthermore, immunolocalization of this key enzyme will lend insight into possible sources of SPCs as well as relative abundance within a redifferentiating gonad.

Results

Specificity of α-O. mykiss P450scc

Western blot analysis of *T. duperrey* gonadal proteins revealed that the antibody recognized a protein (approximately 54 kDa) that co-migrated with the positive control, *O. mykiss* P450scc (Fig. 1) (data not shown). Antibody pre-absorption with an excess of antigen (C-terminal region of *O. mykiss* P450scc) resulted in a loss of positive reactivity (data not shown).

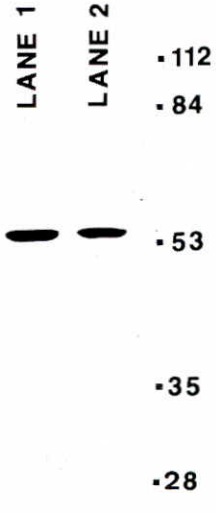

Fig. 1. Western blot analysis of *T. duperrey* gonadal proteins using the α-*O. mykiss* P450scc antibody. *An approximately 54 kDa protein was detected in T. duperrey gonadal extract (lane 2) which co-migrated with the positive control, O. mykiss gonadal extract (lane 1).*

General histology

Light microscopy confirmed the histological observations by Nakamura *et al.* (1989). Ovaries primarily consisted of previtellogenic and vitellogenic follicles. Follicles are comprised of the oocyte surrounded by a layer of granulosa cells. Although the oocyte is completely surrounded by the granulosa layer, the cells appear to be few in number and widely dispersed along the chorion. External to the granulosa layer lies the basement membrane and thecal cells. Thecal cells are few in number and appear to be randomly distributed along the surface of the follicle. Due to the relatively non-uniform distribution of follicular cell types, cell types were determined by location and the shape of the nucleus. Thecal cells contained flattened, irregular-shaped nuclei while granulosa cells had oval nuclei.

Histology of the transitional stages, described in detail below, was consistent with the previous report of Nakamura *et al.* (1989). Briefly, stage 2 is characterized by atresia of large, vitellogenic follicles. Stage 3 has evidence of proliferating somatic cells and atresia of immature follicles. Stage 4 is characterized by proliferation of putative spermatogonia. Stage 5 is identified by the onset of spermatogenesis in spermatogenic crypts. Stage 6 represents a fully-functional testis. Spermatogenesis and spermiation occur in synchronous crypts (lobules) which are associated with Sertoli cells. Interspersed among the lobules are clusters of interstitial Leydig cells.

P450scc immunocytochemistry

P450scc-like immunoreactivity was primarily detected in thecal cells of the follicular layer enclosing vitellogenic oocytes of females which had not begun to change sex (Fig. 2). P450scc-ir was never detected in the granulosa layer of the follicle. During stage 1, P450scc-ir cells also localized in the thecal layer of previtellogenic oocytes, although less frequently than vitellogenic oocytes. In rare cases, small clusters of interstitial cells showed positive reactivity. Similar patterns of P450scc-ir were seen in wild-caught, control females.

In stage 2, which is characterized by atresia of vitellogenic oocytes, P450scc-ir cells still localized in the thecal layer surrounding degenerating oocytes as well as immature follicles (Fig. 3). In contrast to stage 1, the follicular cell layers of many follicles appeared to have separated from the oocyte. Also during stage 2, small immunoreactive interstitial cell clusters were commonly observed (Fig. 4).

P450scc-ir cells localized primarily in large aggregations of interstitial cells during stage 3 (Fig. 5). Thecal cells associated with degenerating immature follicles also showed positive reactivity; however, these were few in number. Although not quantified, it appears that significantly more cells were immunoreactive for P450scc during stage 3 than previous stages.

Stage 4 gonads, which are characterized by a marked proliferation of putative spermatogonia, also contained large aggregates of immunoreactive cells (Figs. 6 and 7). These aggregates were interspersed among somatic cells and loose connective tissue within the central region of the lamella.

During stage 5, several small clusters of immunoreactive cells were observed (Fig. 8). The clusters localized at the interface between the newly-formed spermatogenic crypts and the connective tissue of the central lamellar region. The central region of connective tissue was noticeably smaller in stage 5 gonads than all previous stages.

In wild-caught terminal phase males and sex-changed (stage 6) fish, P450scc immunolocalized in small clusters of interstitial Leydig cells (Fig. 9). The clusters were distributed throughout the interstitium of the entire lamella. The well-defined central region present in previous stages was no longer present in stage 6.

Discussion

We describe for the first time the immunolocalization of the cytochrome P450 cholesterol-side-chain-cleavage enzyme throughout gonadal restructuring in a protogynous hermaphrodite. P450scc which converts cholesterol to pregnenolone, the common precursor of all steroid hormones, serves as a marker for steroid-producing cells. Consequently, these results represent a critical step in the elucidation of the roles of steroid hormones in regulating gonadal redifferentiation by identifying the source of steroidal precursors. Furthermore, these findings demonstrate the potential for transitional gonads to actively produce steroid hormones throughout the sex change process.

Determination of specific cross-reactivity of the α-*O. mykiss* P450scc antibody for *T. duperrey* P450scc validated the use of the antibody for the present immunolocalization studies. Western blot analysis revealed that the antibody specifically recognized a protein from male and female *T. duperrey* that co-migrated with positive controls obtained from *O. mykiss* gonadal homogenates. The protein weighed approximately 54 kDa which is similar in size to other known fish P450scc proteins (Takahashi *et al.*, 1993). Absorption studies using an excess of antigen resulted in loss of positive reactions, suggesting the antibody specifically recognizes P450scc. Immunocytochemical absorption studies in *T. duperrey* also result in loss of positive reactivity, indicating that the α-*O. mykiss* antibody reliably detects the presence of *T. duperrey* P450scc. Phylogenetically, *T. duperrey* and *O. mykiss* are distant relatives. Therefore, the ability of the α-*O. mykiss* antibody to specifically detect *T. duperrey* P450scc indicates its potential application to teleosts in general.

As expected, SPC distribution, identified by immunolocalization of P450scc, appears to progress from female-specific patterns to male-specific patterns throughout sex change in *T. duperrey*. In females of strict gonochoristic species, cells typically found in the thecal layer are the primary SPCs (for review, Nagahama, 1994). Data presented in this study suggest that thecal cells in a healthy ovary of protogynous species are also the primary source of early steroid precursors. In mature ovaries obtained from wild-caught females or experimental females which have not begun sex change (stage 1), a P450scc-like protein is detected mainly in thecal cells. In both gonochoristic females and female *T. duperrey*, SPCs are sometimes found in the interstitial region of the ovigerous lamella; however, the significance of these rare clusters remains unknown. In gonochoristic males, SPCs form clusters which are distributed in the interstices among spermatogenic lobules (for review, Nagahama, 1983). Similarly, clusters of immunoreactive P450scc cells are found in the interstitial areas of completely sex-changed (stage 6) *T. duperrey*. Wild-caught terminal phase males, whether derived from initial phase males or females, also show male-typical distribution of SPCs. Therefore, the distribution of SPCs in both functional sexes of *T. duperrey* is not significantly different from gonochoristic species.

Although functional gonadal phenotypes of *T. duperrey* are similar to their gonochoristic counterparts, the transitional stages between functional ovary and testis are less easily interpreted. In the protogynous eel, *Monopterus albus*, 3β-hydroxysteroid dehydrogenase, another SPC marker, is predominantly located in the thecal layer of developing follicles (Tang *et al.*, 1975). As sex change progresses in the eel, there appears to be a marked proliferation of interstitial SPCs. In contrast to *T. duperrey*, ovaries of *M. albus* contain distinct primordial testicular tissue. Within this primordial tissue, interstitial cells have 3β-hydroxysteroid dehydrogenase activity, suggesting that proliferation of this entire region may be sufficient for testis formation. The absence of any detectable testicular tissue in the ovary of *T. duperrey*, however, sug-

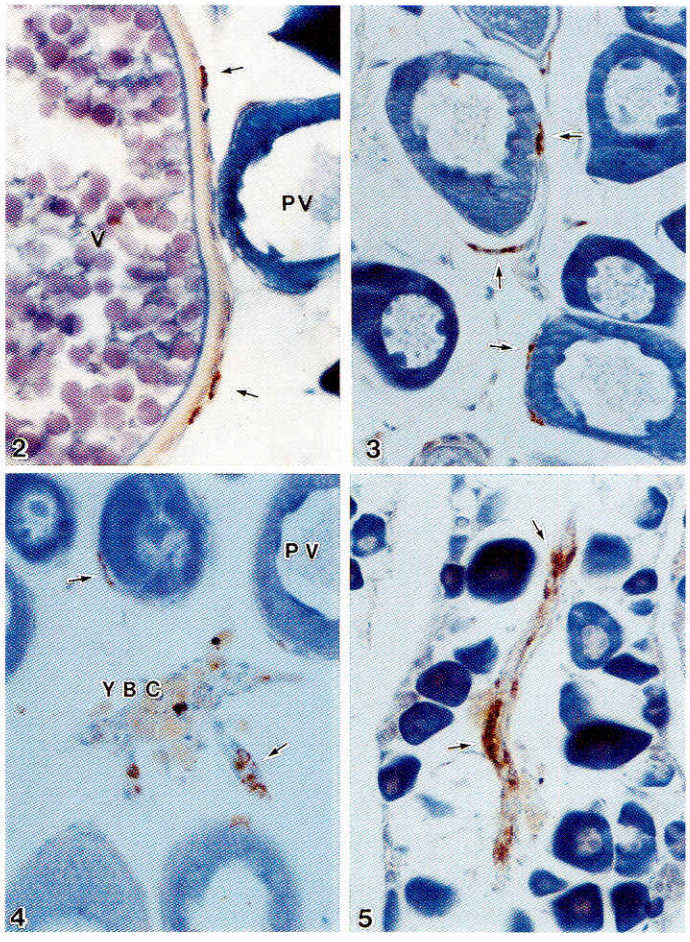

Fig. 2. Photomicrograph of a stage 1 ovary showing P450scc-ir cells (arrows) in the thecal layer of vitellogenic follicles (V). *Although not pictured, P450scc-ir cells also localized in the thecal layer of previtellogenic oocytes (PV). Magnification, x760.*

Fig. 3. Photomicrograph of a stage 2 gonad showing P450scc-ir (arrows) in the thecal layer of degenerating pre-vitellogenic follicles. *Separation of the thecal layer from the oocyte was a common observation. Magnification, x760.*

Fig. 4. Photomicrograph of a stage 2 gonad showing P450scc-ir (arrows) in small clusters of interstitial cells and thecal cells. *Immunoreactivity was easily distinguished from the yellow-brown cells (YBC) by the darker coloration. PV, previtellogenic oocyte. Magnification, x760.*

Fig. 5. Photomicrograph of a stage 3 gonad showing P450scc-ir cell aggregates (arrows) in the central region of the lamella. *Magnification, x370.*

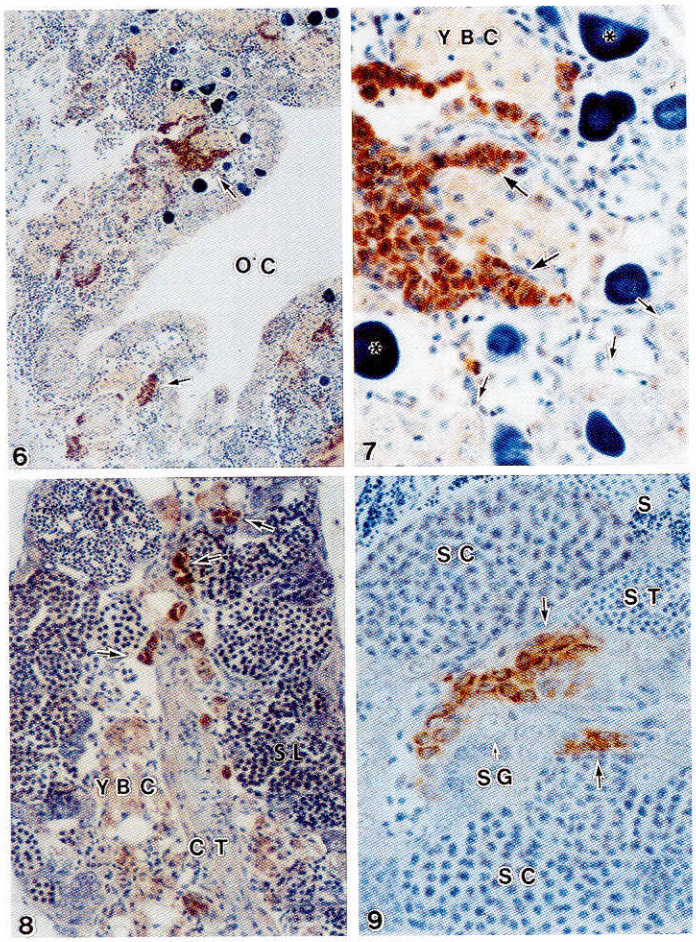

Fig. 6. Photomicrograph of a stage 4 gonad showing large clusters of P450scc-ir cells (arrows) in the interstitium and the remnant of the ovarian cavity (OC). *Immunoreactive cells appear to have increased in number. Magnification, x185.*

Fig. 7. Higher magnification of Figure 6 showing spermatogonia (small arrows), yellow-brown cells (YBC) and P450scc-ir cells (large arrows). *A few degenerating oocytes (*) are also seen. Magnification, x760.*

Fig. 8. Photomicrograph of a stage 5 gonad showing dispersal of small clusters of P450scc-ir cells (arrows) at the interface between the spermatogenic lobules (SL) and the central connective tissue (CT). *Yellow-brown cells (YBC) are still present; however, oocytes are completely absent. Magnification, x370.*

Fig. 9. Photomicrograph of a stage 6 terminal phase testis showing small clusters of P450scc-ir cells (arrows) interspersed among spermatogonia (SG), spermatocytes (SC), spermatids (ST), and mature sperm (S). *Magnification, x760.*

gests the source of testicular SPCs may be significantly different from that described for *M. albus*.

Throughout the early transitional stages of sex change in *T. duperrey*, the distribution of immunoreactive SPCs changes dramatically. During stage 2, P450scc immunoreactivity localizes in thecal cells. Many of the immunoreactive thecal cells are no longer intimately associated with the degenerating oocytes. Clusters of SPCs in the interstitial region are more common; however, the origin of these clusters is unknown. Based on the histological observations, these clusters of interstitial SPCs could be derived from convergence of existing thecal cells, proliferation of existing interstitial cells or newly-differentiated stem cells. Immunoreactive thecal cells are noticeably separated from the oocyte during stage 2. This separation may be the result of cellular migration (Nakamura *et al.*, 1993) towards the central region of the lamella or a passive by-product of gonadal and oocyte shrinkage. In either case, histological evidence supports the hypothesis of thecal cell convergence. Alternatively, stage 1 gonads contain a few clusters of interstitial SPCs. Proliferation of these existing clusters could also result in an increase of interstitial SPCs. Preliminary studies involving the incorporation of bromo-deoxyuridine as a marker of cellular proliferation, however, indicate very little proliferative activity during stage 2 (unpublished data). Finally, the developmental origins of SPCs in both male and female are fibroblasts (Nicholls and Graham, 1972; Van den Hurk and Peute, 1979). Therefore, differentiation of a fibroblastic stem cell cannot be excluded as a source for interstitial SPCs in the transitional gonad.

SPC distribution in stage 3 is confined to large aggregates located in the central region of the lamella. Although not quantified, immunoreactive P450scc interstitial cells appear to increase in overall number during sex change in *T. duperrey*. Preliminary bromo-deoxyuridine studies indicating a rapid rate of proliferation of interstitial cells in stage 3 gonads support this observation (unpublished data). The increase in SPCs may indicate increased intra-gonadal steroidogenesis, presumably androgenic in nature. Stage 3 gonads appear relatively undifferentiated when compared to all other stages. More specifically, stage 2 and stage 4 gonads contain identifiable oogonia and spermatogonia, respectively. In contrast, visible germ cells in stage 3 gonads are few in number. Consequently, the increase in SPCs during stage 3 may be necessary to mediate, via androgen production, the differentiation of spermatogonia and a functional testis. Although the source of spermatogonia remains unknown, strong correlations between the appearance of SPCs and the proliferation of spermatogonia during testicular differentiation in gonochorists (Nakamura and Nagahama, 1989) support this hypothesis. Similarly, increased stromal aggregations and the appearance of loose connective tissue, additional characteristics of gonochoristic testicular development, also suggest that stage 3 may represent the onset of testicular differentiation in *T. duperrey*.

With the exception of a few remaining oocytes, stage 4 and 5 gonads appear similar to differentiating testes in gonochorists as described above. Stage 4 immunoreactive SPCs are localized in numerous large clusters within the central region of the lamella. At this stage immunoreactivity appears robust, which may signify an increase in steroid production. Although not determined in this study, serum androgen concentrations begin to increase during stage 4 of sex change (Nakamura *et al.*, 1989). Stage 5 marks the onset of spermatogenesis. As expected, SPC clusters begin to associate with spermatogenic lobules. Finally, immunolocalization patterns of SPCs at stage 6 are indistinguishable from a fully-functional testis. Small clusters of SPCs (putatively Leydig cells) are interspersed among spermatogenic lobules at all stages of sperm development.

Although further characterization of SPCs must be completed to understand the roles of SPCs more accurately during sex change, these results describe the localization of a critical steroidogenic enzyme, P450 cholesterol-side-chain-cleavage, during the transi-

tion from female to male in *T. duperrey*. Determining the ability of the gonad to produce steroid hormone precursors and identifying the sites of production provides the necessary foundation for future work investigating functions of steroid hormones in gonadal redifferentiation. Of particular interest are sources of testis-specific cells including Leydig cells, Sertoli cells and spermatogonia. Evidence in goldfish suggests that germ cells retain bi-potentiality in mature ovaries (Kobayashi *et al.*, 1991). Manipulation of the androgen to estrogen ratio results in differentiation into spermatogonia. Similarly, alteration of steroid hormone profiles during development in a number of fishes results in sexual differentiation opposite to the determined sex (e.g., Lone and Ridha, 1993). To address these ideas with regard to naturally-occurring sex change, future studies will focus on the characterization of aromatase and 11β-hydroxylase, the enzymes responsible for the production of estradiol-17β and 11-ketotestosterone, respectively. Utilizing the unique attributes of the protogynous *T. duperrey*, investigations of these enzymes should provide significant insight into these presumably potent mediators of sexual differentiation.

Materials and Methods

Animals

Thalassoma duperrey were collected from Kaneohe Bay (Oahu, Hawaii) throughout 1993 and 1994. Individuals were sexed by light abdominal pressure to elicit gamete release. Animals which did not release sperm were assumed to be female. Terminal phase males were distinguished from initial phase males on the basis of body size, coloration, and the relative amount of milt released. Subsequent histological analysis indicates this method of determining sex to be reliable (98% correct, C.E.M.).

Experimental conditions

Initial phase males were not used and were released back on the reef. Six terminal phase males and six females were sacrificed within 3 h of collection to serve as wild-caught controls. To obtain transitional animals, sex change was socially induced according to previously published methods (Ross, 1982; Nakamura *et al.*, 1989). Briefly, females differing in standard length by a minimum of 10 mm were paired in floating Vexar cages (1 m^3). Larger females are effectively stimulated to change sex by the presence of smaller females. Animals were able to forage on algae and invertebrates growing on the cages at all times. In addition, diet was supplemented with chopped squid to satiation 4 times per week. Large females were sacrificed at 1, 3, 5, and 7 weeks after pairing to obtain specimens at different stages in the sex change process.

At the start of the experiment, females were at different stages of ovarian development. The initial difference combined with individual rates of sex change makes quantification as a function of time exceptionally difficult. To account for this variation, the continuum of sex change has been divided into six stereotypical stages (see below) based on histological composition of germinal and somatic tissues (Nakamura *et al.*, 1989).

Histological analysis

All animals were euthanized in MS-222. Following measurements of standard length and body weight, gonads were removed and weighed. Gonads were fixed in Bouin's fixative for approximately 14 h and stored in 70% EtOH. After dehydration and paraffin embedding, gonads were sectioned at 5 μm.

Standard histological slides were prepared using hematoxylin and eosin to determine the stage of sex change (Nakamura *et al.*, 1989). Briefly, stage 1 indicates a reproductively-active female, indistinguishable from a wild-caught female. Stage 2 is characterized by atresia of large, vitellogenic follicles. Stage 3 has evidence of proliferating somatic cells and atresia of immature follicles. Stage 4 is characterized by proliferation of putative spermatogonia. Stage 5 is identified by the onset of spermatogenesis. Finally, stage 6 is a functional male, indistinguishable from a wild-caught TP male. Following standard histological analysis, immunocytochemical studies to determine the localization of P450scc were conducted on wild-caught controls and gonads representative of each of the six stages of sex change.

Somatic cell types were determined by standard classification parameters for light microscopy. Thecal cells containing flattened, irregular-shaped nuclei were found external to the basement membrane of the follicular layer. Granulosa cells with oval nuclei were located between the basement membrane and the chorion. Interstitial cells and/or Leydig cells were not directly associated with follicles or spermatogenic crypts.

Antibody production

A synthetic peptide representing the C-terminal amino acid sequence (ILLPEKPILLTLKPLKSGQ) of *Oncorhynchus mykiss* P450scc (Takahashi *et al.*, 1993) was produced (Beckman 990 peptide synthesizer) and purified by reverse-phase HPLC (high pressure liquid chromatography). After conjugation to bovine serum albumin (BSA), the peptide-BSA complex was utilized as an antigen in rabbit. The resulting polyclonal antibody, to be characterized elsewhere (T. Kobayashi, personal communication), specifically detects *O. mykiss* P450scc (Fig. 1).

Western blot analysis

Gonadal proteins of *T. duperrey* and *O. mykiss* (positive control) were isolated in Laemmli's SDS-Sample buffer containing 10% β-mercaptoethanol. Prior to electrophoresis (12% SDS-PAGE), samples were heated to 100°C for 3 min. Separated proteins were electroblotted onto Immobilon membranes (Millipore Inc., Bedford, MA. USA). Membranes were rinsed with Tris-buffered saline (TBS: 20 mM Tris-HCL, 150 mM NaCl, pH 7.5) and incubated for 1.5 h in TBS containing 0.1% Tween-20 and 10% non-fat dry milk (TTBS) to block non-specific binding. Membranes were then incubated for 2 h with serum containing *a-O. mykiss* P450ssc (1:2000 dilution in TBS). After washing with TTBS (3x5 min), membranes were incubated with alkaline phosphatase-conjugated goat a-rabbit IgG (1:1000; Tago, Burlingame, CA, USA). Membranes were again washed (TTBS; 3x5min). Phosphatase activity was visualized by treatment with 0.2 mM 5-bromo-4-chloro-3indolylphosphate p-toluidine salt and nitroblue tetrazolium (Sigma Co., St. Louis, MO, USA) dissolved in 100 mM diethanolamine buffer (5 mM $MgCl_2$, pH 9.5). All incubations were performed at room temperature.

Immunocytochemical detection

P450scc- 5 μm gonad sections from wild-caught and transitional animals were rehydrated and reacted with 10% hydrogen peroxide and normal goat serum to block non-specific reactivity. Sections were then incubated overnight at 4°C with a 1:200 or 1:400 dilution of rabbit α- *O. mykiss* P450scc in 0.1 M phosphate-buffered saline (pH 7.2-7.4). Detection and visualization of the primary antibody was completed using the Histofine α-Rabbit immunocytochemistry kit (Nichirei Corp., Tokyo, Japan) and diaminobenzidine.

Control slides for immunocytochemistry sections consisted of a substitution of BSA-PBS and normal rabbit serum for the primary antibody to determine non-specific reactivity of the secondary antibody to the tissue.

Nakamura *et al.* (1989) described the presence of yellow-brown cells (YBCs) within the gonads undergoing sex change. This color of YBCs is a natural phenomenon, not the result of the diaminobenzidine reaction used to visualize immunoreactivity. Therefore, YBCs should not be confused with immunopositive cells. Based on location and relative intensity of coloration, however, YBCs were easily distinguished from the positively immunoreactive cells described in this study.

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