Plasma sex steroid levels and steroidogenesis in the gonad of the self-fertilizing fish *Rivulus marmoratus*

Masako Minamimoto[1], Yoshitaka Sakakura[2,4], Kiyoshi Soyano[3], Yoshiaki Akaba[1] & Atsushi Hagiwara[1]

[1] *Graduate School of Science and Technology, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, JAPAN*
[2] *Faculty of Fisheries, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, JAPAN*
[3] *Institute for East China Sea Research, Nagasaki University, Taira 1551-7, Nagasaki 851-2213, JAPAN*
[4] *Corresponding author (e-mail: sakakura@net.nagasaki-u.ac.jp)*

Key words: mangrove killifish, steroid hormone, hermaphrodite, tissue culture

Synopsis

The mangrove killifish, *Rivulus marmoratus*, is the only known self-fertilizing vertebrate. This species is sexually dimorphic; sexually mature individuals are either hermaphrodite or primary and secondary males. Although the mangrove killifish has a unique reproductive strategy, there has been no study on the reproductive endocrinology of this species. Thus we investigated plasma sex steroid hormone levels and steroidogenesis in the gonads of *R. marmoratus* by enzyme linked immunosorbent assay (ELISA). Plasma 17β-estradiol (E2) and 11-ketotestosterone (11-KT) were detected both in hermaphrodite and in primary male. Ovarian follicles (follicle-enclosed oocytes) from hermaphrodites, which were categorized into early yolk stage and late yolk stage, and testis tissue of primary males were cultured with different concentrations of 17α-hydroxyprogesterone (OHP) or testosterone (T) for 24 hours. Production of T, E2, 11-KT and 17α-20β-dihydroxy-4-pregnen-3-one (17α,20β-P) in the medium from tissue culture were measured by ELISA. Early and late ovarian follicles of hermaphrodites and testis pieces of primary males synchronously secreted E2, 11-KT, and 17α,20β-P following incubation with OHP or T. We conclude that both hermaphrodite and primary male of the mangrove killifish secrete estrogen, androgen, and progestin synchronously.

Introduction

The mangrove killifish, *Rivulus marmoratus*, has a unique reproductive biology. This species is sexually dimorphic: sexually mature individuals are either hermaphrodite and male, with the hermaphroditic form the only known internal self-fertilizing vertebrate (Harrington 1961, 1967, 1971). Hermaphroditic mangrove killifish are self-fertilizing with both functional ovary and testis (ovotestis), while males have functional testis only (Soto et al. 1992). In males, there are two types: primary males which frequently arise from exposure to low temperatures during embryonic development and secondary males which arise from the hermaphrodite form by the digression of the ovary (Harrington 1967, 1971). Recent studies indicate that immature individuals (less than 60 days after hatching) are females and the frequency of hermaphrodites increases with increasing age; thus this species is considered to be a diandric protogyny (Cole & Noakes 1997, Sakakura & Noakes 2000). Tissue transplantation test (Kallman & Harrington 1964, Harrington & Kallman 1968) and DNA fingerprinting (Turner et al. 1990) confirmed that the genetically identical individuals within each self-fertilizing lineage are homozygous (sometimes referred to as clones). Therefore, the considerable recent interest in this species revolves around not only their reproductive biology but also the potential use as a unique experimental organism (Abel et al. 1987, Davis 1988, Lin & Dunson 1993, Park et al. 1994, Dunson & Dunson 1999, Frick & Wright 2002). However, little is known on the reproductive endocrinology of this species. One of the major questions surrounding the reproductive endocrinology of *R. marmoratus* is how spermatogenesis and oogenesis in the ovotestis occurs.

Many studies on sex steroids regulating gametogenesis of teleost fishes (e.g. Nagahama 1994, 1997) have been identifying estrogen (17β-estradiol, E2)□produced by the ovary as regulates oocyte growth, while androgen (11-ketotestosterone, 11-KT) produced by testis as regulating spermatogenesis (Nagahama 1994). In the euryhaline killifish (*Fundulus heteroclitus*, mummichog), which is in the same order as *R. marmoratus* (Cyprinodontiformes), the females secret E2 whereas males secret 11-KT, and the concentrations of these steroid hormones peak during the semilunar spawning cycles (Bradford & Taylor 1987, Cochran 1987). Progestin, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) and/or 17α,20β,21-trihydroxy-4-pregnen-3-one (20βs) are reported as maturation inducing hormones (MIH) regulating oocyte and sperm maturation in several species (Nagahama 1994, 1997). In the mummichog, 17α,20β-P is recognized as a MIH since it induced oocyte maturation and germinal vesicle break down (GVBD) by *in vitro*

analysis (Greeley et al. 1986, Petrino et al. 1993). In the belted sandfish (*Serranus subligarius*), a simultaneous hermaphrodite that does not self-fertilize, fish mate alternatively and secret E2, 11-KT, 17α,20β-P and 20βs during the spawning season (Cheek et al. 2000). Reproductive endocrinology of the mangrove killifish is presumably different from other teleosts because of the internal self-fertilization. Oogenesis and spermatogenesis of *R. marmoratus* presumably occur synchronously in hermaphrodites, while only spermatogenesis occurs in males. Therefore, we hypothesized that hermaphrodites of *R. marmoratus* secret estrogen, androgen and progestin synchronously while males secret androgen and progestin. On steroidogenesis in the gonad of the mangrove killifish, we further hypothesized that estrogen and progestin would be produced synchronously during *in vitro* steroid genesis by ovary, while androgen and progestin would be produced by the testis.

In this study, we investigated the secretion of the sex steroids in the mangrove killifish. First, we measured plasma concentration of E2 and 11-KT of hermaphrodites and primary males to determine circulating sex steroids titers. Secondly, we examined *in vitro* gonadal steroidogenesis of hermaphrodites and primary males. Ovarian follicles (follicle-enclosed oocytes) of hermaphrodites and testis tissue of males were cultured with different concentrations of the precursors of OHP or T and then, measured concentrations of T, E2, 11-KT, and 17α,20β-P in the culture medium.

Materials and Methods

Experimental Fish

A single clonal strain of *R. marmoratus* (PAN-RS) was used in this study. This strain was obtained from Dr. W. P. Davis of the U.S. Environmental Protection Agency, Gulf Breeze, Florida, U.S.A. All PAN-RS individuals were the descendants of a single hermaphrodite collected near Bocas del Toro, Republic of Panama, in 1994 (previously thought to have originated in Florida, U.S.A,. by Sakakura & Noakes 2000, Grageda et al. 2004) and has been successfully reared for over 5 generations in our laboratory (Grageda et al. 2005). All fish were kept individually in 1 litre of plastic containers with brackish water (17 ppt salinity, 25 ± 1 °C) under specially designed rearing chambers (Zebrafish rearing system, AQUA Inc., Tokyo, Japan) equipped with water circulating system (Grageda et al. 2005). Fish were maintained under a controlled photoperiod (14:10 hours light:dark) and fed commercial fish diet (TetraMin, Tetra, Germany) once daily until satiation.

Blood sampling and hormone assay

We used 65 adult fish (52 hermaphrodites and 13 primary males) in this experiment. We preliminarily identified hermaphrodite individuals as fish spawning fertilized eggs, while primary males were non-spawning individuals further identified by a distinct orange coloration (Soto & Noakes 1994). Further definitive identification of hermaphrodite and male forms was provided by gonadal histology as described later. The average of standard length and age of hermaphrodites were 25.6 mm and 788 days after hatching, 28.3 mm and 1076 days for primary males, respectively. All blood samples of hermaphrodites and primary males were collected from the aorta dorsalis using 3 or 5 μl of capillary (Microcaps, Drummond Scientific Company, PA, U.S.A.), immediately following the excision of the caudal peduncle by a razor blade. About 35 μl of blood from 6-14 hermaphrodites (6 groups), and of 5-7 primary males (3 groups) were pooled into heparinized (800 unit ml^{-1} sodium heparin) 0.2 ml micro tubes, respectively. They were centrifuged (1000 rpm, 5 min.) and plasma (2-15 μl for each pooled group) was collected. We extracted the steroid hormones by the use of a standard ethyl ether extraction method and measured plasma levels of E2 and 11-KT measured by enzyme-linked immunosorbent assay (ELISA). Estradiol EIA kit (582281, Cayman Chemical Company, Ann Arbor, U.S.A.) was used for E2 and 11-keto-testosterone EIA kit (582751, Cayman Chemical Company, Ann Arbor, U.S.A.) was used for the 11-KT assay, respectively.

After all fish were fixed with 10 % formalin after blood sampling, gonads were dissected and gonadosomatic index (GSI; gonad-weight body-$weight^{-1}$ × 100) was calculated. Then, gonads were embedded in paraffin and were sectioned at 5 μm thick and stained with hematoxylin and eosin. We observed gonads under the light microscopy to confirm whether they were hermaphrodite or primary male.

Tissue culture experiment

In this experiment, we used 24 fish (16 of hermaphrodites and 8 of primary males) different from the previous experiment. The average of standard length and age of fish were 30.4 mm and 404 days in hermaphrodites which had previously spawned fertilized eggs, while 32.6 mm and 764 days in primary males, respectively. Fish were anesthetized with 400 ppm of MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, USA). Then, they were dissected and their gonads were

transferred into L-15 medium (Leibovitz L-15, L-4386, Sigma Chemical Co., St. Louis, USA) kept at 20 °C containing 70 mg l^{-1} of penicillin G sodium and 100 mg ml^{-1} of streptomycin. Ovarian follicles (follicle-enclosed oocytes) were isolated from gonads of hermaphrodites with fine forceps and categorized into early yolk stage (Y1) and late yolk (Y2) stages according to Soto et al. (1992). Gonads of primary males (testis tissue: about 1 cm in length) were cut into a few fragments. These treatments were done within 2 hours for each fish. Dissected ovarian follicles and testis tissues were rinsed L-15 containing high dosage of antibiotics (350 mg l^{-1} of penicillin G sodium and 500 mg ml^{-1} of streptomycin) to prevent the bacterial contamination. Then, they were placed in 24-well culture plates, each containing 990 μl of L-15 medium containing 70 mg l^{-1} of penicillin G sodium and 100 mg ml^{-1} of streptomycin. A testis piece in each well was minced by dissecting scissors. 4-Pregnen-17α-ol-3,20-dione (OHP, 63H0426, Sigma Chemical Co., St. Louis, USA) and testosterone (T, APH4369, WAKO, Tokyo, Japan) was dissolved in ethanol at a concentration of 1000 mg ml^{-1}. Then, 10 μl of OHP and T were added in each well adjusting the concentrations of OHP or T in L-15 at 3 levels (10, 100, 1000 ng ml^{-1}). Tissue was cultured at 20 °C for 24 hours and all medium was collected in 1.5 ml micro tubes and frozen at –80 °C. For examination of cross-reactivity of OHP against antibody of E2, T, 11-KT, 17α,20β-P and 20βs, or T against anti-body of E2, 11-KT, 17α,20β-P and 20βs, plates including 990 μl of L-15 and 10 μl of OHP and T without gonads were also prepared. In order to confirm gonadal development, all cultured ovarian follicles and testis tissues were fixed in Bouin's fixative, then embedded in paraffin, sectioned at 5-7 μm thick and stained with hematoxylin and eosin. Ovarian follicles and testis were observed under a light microscopy and their developmental stages were examined.

We extracted the steroid hormones from the culture medium by the use of a standard ethyl ether extraction method. Then, the concentrations of E2, T, 11-KT, 17α,20β-P and 20βs in the L-15 culture medium were measured using ELISAs. ELISAs have been characterized and described previously (Asahina et al. 1995). The antigen and the horseradish peroxidase-conjugated antibody against these hormones were purchased from Cosmo Bio Co., Ltd (Tokyo, Japan).

We diluted the same sample at several different concentrations and measured the steroid titers and then corrected the value for the dilution. The results were the same regardless of the dilution employed. We also checked the cross-reactivity (%) of different concentrations of OHP against antibody of E2, T, 11-KT, 17α,20β-P and 20βs respectively, or T against antibody of E2, 11-KT, 17α,20β-P and 20βs respectively. Each cross-reactivity was less than 10 % and most of them were under the detection

limit (25 pg ml^{-1}), except for the samples cultured with 1000 ng ml^{-1} of T against 11-KT antibody (25.6 %). Thus, we did not include the results of it in this paper.

Statistical analysis

Data of hormone concentrations in plasma or culture medium were log_{10}-transformed before analysis. Between-group comparison of each hormone level was undertaken using Student's *t*-test for 2 groups, and using Fisher's Protected Least Significant Difference (PLSD) test after a one-way analysis of variance (ANOVA) treatment among 3 experimental groups. $P<0.05$ was accepted as indicating significant differences between means.

Results

Plasma concentrations of E2 and 11-KT

Both hermaphrodites and primary males secreted E2 and 11-KT (Figure 1). In hermaphrodites, plasma concentration of E2 (average ± standard error of means, 2453 ± 680 pg ml^{-1}) was significantly higher than that of 11-KT (346 ± 155 pg ml^{-1}; *t*-test, n=6, t=4.381, P=0.0014). In primary male, concentration of E2 (6104 ± 1122 pg ml^{-1}) showed higher trend than that of 11-KT (1180 ± 633 pg ml^{-1}). Plasma E2 level of hermaphrodite was significantly lower than that of primary male (*t*-test, n=3-6, t=2.707, P=0.03). GSI of hermaphrodites (7.9 ± 0.7; gonad weight 45.4 ± 9.9 mg) was significantly higher than that of primary males (3.6 ± 0.5; testis weight 23.3 ± 8.8 mg; *t*-test, t=3.277, P=0.003). Histological observation confirmed that hermaphrodites had both ovary with oocytes at different developmental stages (Y1 and Y2) and testis, while primary males had only testis with spermatocytes, spermatids and spermatozoa.

In vitro steroidgenic capacities

In the presence of precursor (OHP or T), both Y1 and Y2 ovarian follicles and testis tissue secreted T, E2, 11-KT and 17α,20β-P synchronously (Figure 2, 3). 20βs level was less than one sixth of 17α,20β-P. Histological observation confirmed that ovarian follicles and testis were separated completely in the culture medium.

With increasing OHP concentration, Y1 and Y2 ovarian follicles produced higher concentrations of T and 17α,20β-P than those of E2 and 11-KT (ANOVA, df=2,

F=6.391, P=0.016; Fisher's PLSD test, P<0.05, Figure 2). Production of 17α,20β-P from testis tissue was by for the highest of the examined four hormones (ANOVA, df=3, F>3.835, P<0.04; Fisher's PLSD test, P<0.05, Figure 2 D).

When gonads were cultured with T, productions of E2, 11-KT and 17α,20β-P from Y1 showed the same level, while E2 level from Y2 was higher than that of 11-KT and 17α,20β-P (ANOVA, df=2, F=10.204, P=0.005; Fisher's PLSD test, P<0.05: df=2, F=16.847, P<0.001; Fisher's PLSD test, P<0.05, Figure 3). Testis tissue cultured with 10 and 100 ng ml^{-1} of T produced higher concentration of 11-KT than those of E2 and 17α,20β-P (ANOVA, df=2, F=97.519, P=0.002; Fisher's PLSD test, P<0.05: df=2, F=38.589, P=0.007; Fisher's PLSD test, P<0.05, Figure 3). Both Y1 and Y2 ovarian follicles cultured with 1000 ng ml^{-1} of T produced E2 higher than 17α,20β-P (t-test, df=18, t=4.722, P<0.001: df=10, t=2.682, P=0.023, Figure 3).

Discussion

This study is the first to examine the reproductive endocrinology of the internal self-fertilizing mangrove killifish. We measured blood circulating sex steroid levels and the ability of steroid production from the gonads in the matured hermaphrodites and primary males of this species. There were the variations in the plasma sex steroid levels and steroid production rates from gonads in this study, it may be due to the fact that this species does not have the clear diel rhythms in terms of spawning and ovulation (Grageda et al. 2005) and that the samples used in this study contain the various developmental stages of maturation. Even though considering the variations in the sex steroids levels, since these data were above the detection limits of ELISAs, we stress that the actual plasma sex steroid levels and the capacity of sex steroid productions in the gonads could be demonstrated.

E2 (estrogen) and 11-KT (androgen) were secreted synchronously in plasma of the hermaphrodites of this species, although these plasma steroid levels were about 10 times lower in comparison with those of a taxonomically close species, *Fundulus heteroclitus* (Cyprinodontiformes) (Bradford & Taylor 1987, Cochran 1987). Since the hermaphrodites of the mangrove killifish have both functional ovary and testis synchronously (Soto et al. 1992) and internal self-fertilization occurs, it is reasonable that hermaphrodite of *R. marmoratus* secreted E2 and 11-KT at the same time. On the contrary, we hypothesized that primary male secrete only 11-KT, because it had only functional testis. Surprisingly, primary male mangrove killifish secreted both E2 and 11-KT similar to the internal-self fertilizing hermaphrodite forms. Although we had relatively small sample size (n=3) for the plasma steroid hormone titers in the primary

males because of the low proportion of primary males in PAN-RS strain population used in this study (0.6 %, Grageda et al. 2005), the result may appropriately reflect the blood circulating steroid hormone levels. It is generally known that level of E2 in male is very low or undetectable in teleost fish. It has been reported that low concentration of E2 (10-1000 pg ml^{-1}) induces mitotic division of spermatogonia through Sertoli cells *in vitro* in the Japanese eel *Anguilla japonica* (Miura et al. 1999). However, plasma E2 titers of the primary male *R. marmoratus* showed titers as high as hermaphrodite form (ng ml^{-1} order), indicating that the testis of the primary male can secrete high levels of estrogen than previously reported.

Hermaphrodite ovarian follicles and testis pieces of primary male *R. marmoratus* produced T, E2, 11-KT and 17α,20β-P simultaneously through T or OHP precursors. In general, it is known that estrogen (E2) is mainly produced by ovarian follicle and androgen (11-KT) is mostly produced by testis based on the study from amago salmon *Oncorhynchus rhodurus* (Nagahama 1987). In medaka *Oryzias latipes*, which is in the same series (Atherinomorpha) with the mangrove killifish, 17α,20β-P is confirmed as the progestin and is synthesized via OHP whereas E2 is synthesized via T from OHP (Kobayashi et al. 1996). Ovarian follicle of medaka cultured with OHP or T produced only E2 (Sakai et al. 1988). Also, in the mummichog, which is in the same order as *R. marmoratus* (Cyprinodontiformes), 17α,20β-P is recognized as progestin (Greeley et al. 1986, Petrino et al. 1993). Considering these evidences and the status of E2, 11-KT and 17α,20β-P production in the mangrove killifish, this species may have the same steroidogenic pathway as medaka and mummichog, and secret 17α,20β-P as progestin and MIH. As oocytes mature, a steroidogenic shift occurs in many teleost fishes (Nagahama 1994), where ovarian follicles 8 hours before spawning produced large amount of 17α,20β-P in medaka (Sakai et al. 1988). The study of follicle incubation with radiolabeled steroid precursor revealed that the steroidogenic pathway changes from synthesizing E2 to 17α,20β-P at final maturation in medaka (Kobayashi et al. 1996). However, early yolk stage oocytes in the mangrove killifish produced 17α,20β-P, while late yolk stage oocytes produced E2. Moreover, testis of primary males produced not only 11-KT and 17α,20β-P but also E2. Our results demonstrate that steroidogenesis in the mangrove killifish has two distinct features from other teleosts. One is that both ovary and testis can produce estrogen, androgen and progestin regardless of functional sex of gonads. The other feature is that at least ovary of hermaphrodite produces estrogen, androgen and progestin regardless of maturation stage of gametogenesis.

We conclude that it is not only the hermaphrodite forms but also primary male

secretes estrogen, androgen, and progestin synchronously. In one hermaphroditic individual of the mangrove killifish, many stages of oocyte and spermatocytes exist at the same time (Soto et al. 1992). Therefore, secreted estrogen, androgen and progestin in the hermaphrodite of this species regulate each stage of oogenesis or spermatogenesis specifically without any effect of other steroid hormones, which are not appropriate for oogenesis or spermatogenesis. In goldfish *Carassius auratus*, adult female developed testis tissue in the ovary after implantation of 11-KT capsules (Kobayashi et al. 1991). Female goldfish implanted with 11-KT and exposed to an ovulatory female experienced a typical male gonadotropin surge in goldfish, because of possession of sexual plasticity (Kobayashi et al. 1997). However, the mangrove killifish shows the sexual plasticity without artificial hormonal treatment; the hermaphrodite forms maintain both functional ovary and testis. This apparent paradox in the mangrove killifish can be explained by the regulation of the expression of sex steroid hormones receptors in the gonad and liver. It appears that the expression of the estrogen receptor, androgen receptor and progestin receptor are controlled in this species. On steroidogenic enzymes, there is a distinct shift between oocyte growth and maturation in activities of enzymes from aromatase to 20β-hydroxysteroid in ovarian follicle of medaka (Sakai et al. 1988, Kobayashi et al. 1996). However, in the mangrove killifish, estrogen and progestin were secreted regardless of maturation stage of oocyte, thus both enzymes are presumably active at the same time. These questions are to be resolved understanding the reproductive biology of the mangrove killifish.

Acknowledgements

We wish to thank Dr. David LG Noakes, University of Guelph, Canada for his helpful comments. We are also grateful to Dr. Masaki Nagae, Nagasaki University for providing facilities and technical advices. This study was financially supported by the Japanese Grants-in-Aid for Promotion of Scientific Research to Y.S. and Nagasaki Prefecture Collaboration of Regional Entities for Advancement of Technological Excellence, JST to Y.S., K.S. and A.H. We also thank two anonymous referees for their constructive comments and suggestions.

References

Abel, D. C., C. G. Koenig & W. P. Davis. 1987. Emersion in the mangrove forest fish *Rivulus marmoratus*: a unique response to hydrogen sulfide. Environmental Biology of Fishes 18: 67-72.

Asahina, K., A. Kanbegawa, & T. Higashi. 1995. Development of a microtiter plate enzyme-linked immunosorbent assay for 17α,20β-trihydroxy-4-pregnen-3-one, a teleost gonadal steroid. Fisheries Science 61: 491-494.

Bradford, C. S. & M. H. Taylor. 1987. Semilunar changes in estradiol and cortisol coincident with gonadal maturation and spawning in the killifish *Fundulus heteroclitus*. General and Comparative Endocrinology 66: 71-78.

Cheek, A. O., P. Thomas & C. V. Sullivan.2000. Sex steroids relative to alternative mating behaviors in the simultaneous hermaphrodite *Serranus subligarius* (Perciformes: Serranidae). Hormones and Behavior 37: 198-211.

Cochran, R. C. 1987. Serum androgens during the annual reproductive cycle of the male mummichog, *Fundulus heteroclitus*. General and Comparative Endocrinology 65: 141-148.

Cole, K. S. & D. L. G. Noakes. 1997. Gonadal development and sexual allocation in mangrove killifish , *Rivulus marmoratus* (Pisces: Atherinomorpha). Copeia : 596-600.

Davis, W. P. 1988. Reproductive and developmental responses in the self-fertilizing fish, *Rivulus marmoratus*, induced by the plasticizer, di-n-butylphthalate. Environmental Biology of Fishes 21: 81-90.

Dunson, W. A. & D. B. Dunson. 1999. Factors influencing growth and survival of the killifish, *Rivulus marmoratus*, held inside enclosures in mangrove swamps. Copeia : 661-668.

Frick, N. T. & P. A. Wright. 2002. Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus* I. The influence of environment salinity and external ammonia. The Journal of Experimental Biology 205: 79-89.

Grageda, M. V. C., Y. Sakakura & A. Hagiwara. 2004. Early development of the self-fertilizing mangrove killifish, *Rivulus marmoratus*, reared in laboratory. Ichthyological Research 51: 309-315.

Grageda, M. V. C., Y. Sakakura, M. Minamimoto, & A. Hagiwara (2005) Differences in life-history traits in two clonal strains of the self-fertilizing fish, *Rivulus marmoratus*. Environmental Biology of Fishes 73: 427-436.

Greeley, M. R., D. R. Jr. Calder, M. H. Taylor, H. Hols & R. A. Wallace. 1986. Oocyte maturation in the mummichog (*Fundulus heteroclitus*): effects of steroids on germinal vesicle breakdown of intact follicles *in vitro*. General and Comparative Endocrinology 62: 281-289.

Harrington, R. W. Jr. 1961. Oviparous hermaphroditic fish with internal

self-fertilization. Science 134: 1749-1750.

Harrington, R. W. Jr. 1967. Environmentally controlled induction of primary male gonochorists from eggs of the self-fertilizing hermaphroditic fish, *Rivulus marmoratus* Poey. Biol. Bull 132: 174-199.

Harrington, R. W. Jr. 1971. How ecological and genetic factors interact to determine when self-fertilizing hermaphrodites of *Rivulus marmoratus* change into functional secondary males, with a reappraisal of the modes of intersexuality among fishes. Copeia :389-432.

Harrington, R. W. Jr. & K. D. Kallman. 1968. The homozygosity of clones of the self-fertilizing hermaphroditic fish *Rivulus marmoratus* Poey (Cyprinodontidae, Atheriniformes) American Naturalist 102: 337-343.

Kallman, K. D. & Harrington R. W. Jr. 1964. Evidence for the existence of homozygous clones in the self-fertilizing hermaphroditic teleost *Rivulus marmoratus* (Poey). Biological. Bulletin 126: 101-114.

Kobayashi, D., M Tanaka, S. Fukuda & Y. Nagahama. 1996. Steroidogenesis in the ovarian follicles of the Medaka (*Oryzias latipes*) during vitellogenesis and oocyte maturation. Zoological Science 13: 921-927.

Kobayshi, M., K. Aida & N. E. Stacey. 1991. Induction of testis development by implantation of 11-ketotestosterone in female goldfish. Zoological Science 8: 389-393.

Kobayashi, M., K. Furukawa, M.-H. Kim & K. Aida. 1997. Induction of male-type gonadotropin secretion by implantation of 11-ketotetosterone in female goldfish. General and Comparative Endocrinology 108: 434-445.

Lin, H.-C. & W. A. Dunson. 1993. The effect of salinity on the acute toxicity of cadmium to the tropical, estuarine, hermaphroditic fish, *Rivulus marmoratus*: A comparison of Cd, Cu, and Zn tolerance with *Fundulus heteroclitus*. Arch. Environ. Contam. Toxicol 25: 41-47.

Miura, T., C. Miura, T. Ohta, M. R. Nader, T. Todo, K. Yamauchi. 1999. Estradiol-17□ stimulates the renewal of spermatogonial stem cells in males. Biochemical and Biophysical Research Communications 264: 230-234.

Nagahama, Y. 1987. Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. Zoological Science 4: 209-222.

Nagahama, Y. 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38: 217-229.

Nagahama, Y. 1997. 17α, 20β-Dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: Mechanisms of synthesis and action. Steroids. 62:

190-196.

Park, E.-H., H.-H. Chang, & W. N. Joo. 1994. Assessment of the estuarine hermaphroditic fish, *Rivulus marmoratus* as a useful euryhaline species for acute toxicity tests as shown using cadmium. Can. J. Fish. Aquat. Sci 51: 280-285.

Petrino, T. R., Y.-W. P. Lin, J. C. Netherton, D. H. Powell & R. A. Wallace. 1993. Steroidogenesis in *Fundulus heteroclitus* V: Purification, characterization, and metabolism of 17α, 20β-dihydroxy-4-pregnen-3-one by intact follicles and its role in oocyte maturation. General and Comparative Endocrinology 92: 1-15.

Sakai, N., T. Iwamatsu, K. Yamauchi, N. Suzuki, Y. Nagahama. 1988. Influence of follicular development on steroid production in the medaka (*Oryzias latipes*) ovarian follicle in response to exogenous substrates. General and Comparative Endocrinology 71: 516-523.

Sakakura, Y. & D. L. G. Noakes. 2000. Age, growth, sexual development in the self-fertilizing hermaphroditic fish *Rivulus marmoratus*. Environmental Biology of Fishes 59: 309-317.

Soto, C. G., J. F. Leatherland, & D. L. G. Noakes. 1992. Gonadal histology in the self-fertilizing hermaphroditic fish *Rivulus marmoratus* (Pisces, Cyprinodontidae). Can. J. Zool 70: 2338-2347.

Soto, C.G. & D.L.G. Noakes. 1994. Colouration and gender in the hermaphroditic fish *Rivulus marmoratus* Poey (Teleostei: Rivulidae). Ichthyol. Explor. Freshwaters 5: 79-90.

Turner, B. J., J. F. Jr. Elder, T. F. Laughlin & W. P. Davis. 1990. Genetic variation in clonal vertebrates detected by simple-sequence DNA fingerprinting. Proc. Natl. Acad. Sci. 87: 5653-5657.

Figure Legends

Figure 1. Plasma concentration of estradiol-17β (E2) and 11-ketotestosterone (11-KT) in the hermaphrodite and male of the mangrove killifish. Column, vertical line and number in the columns indicate average, standard error of means and sample size, respectively. Asterisk shows statistical significance (*t*-test, $P<0.05$).

Figure 2. Production of (A) testosterone (T), (B) estradiol-17β (E2), (C) 11-ketotestosterone (11-KT) and (D) 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) from follicle-enclosed oocyte at different stages and testis cultured with 17α-hydroxyprogesterone at different concentrations 10 ng ml^{-1}, 100 ng ml^{-1}, 1000 ng ml^{-1} for 24 hours. Each column and number in parentheses indicates average and sample size, respectively. Vertical line and the number above the column indicate standard error of means. Alphabet above the vertical line shows statistical significance among each oocyte or testis (a<b, Fisher's PLSD test, $P<0.05$).

Figure 3. Production of (A) estradiol-17β (E2), (B) 11-ketotestosterone (11-KT) and (C) 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) from follicle-enclosed oocyte at different stages and testis cultured with testosterone at different concentrations 10 ng ml^{-1}, 100 ng ml^{-1} and 1000 ng ml^{-1} for 24 hours. Each column and number in parentheses indicates average and sample size, respectively. Vertical lines on the column indicate standard error of means. Alphabet above the vertical line and asterisk show statistical significance among each oocyte or testis (a<b, Fisher's PLSD test, $P<0.05$ or *t*-test, $P<0.05$).

Figure 1 (Minamimoto et al. 2004)

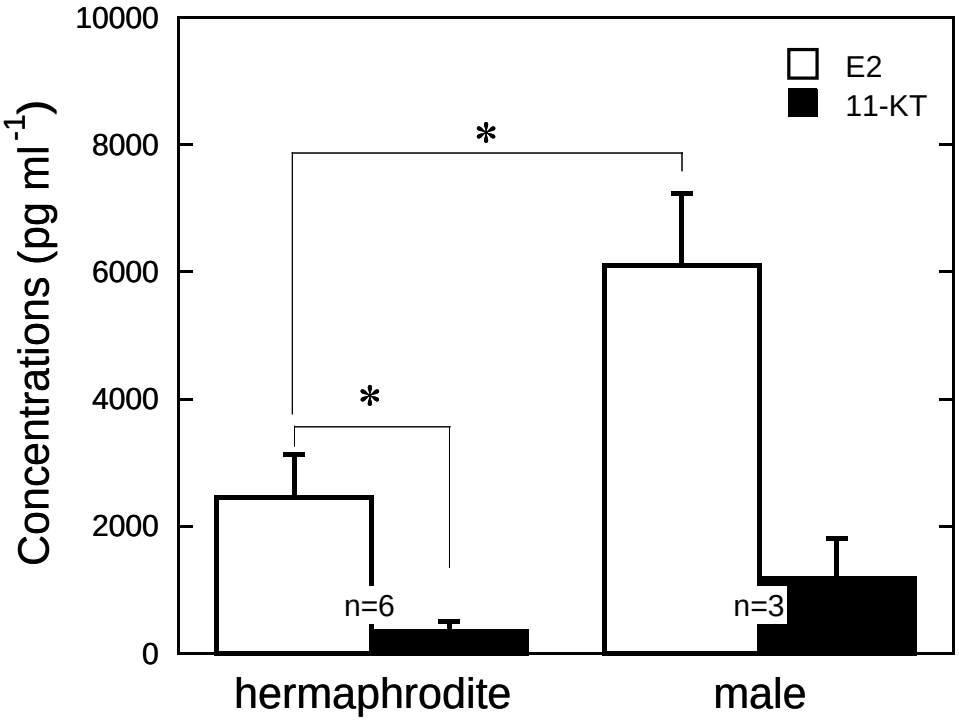

Figure 2 (Minamimoto et al. 2004)

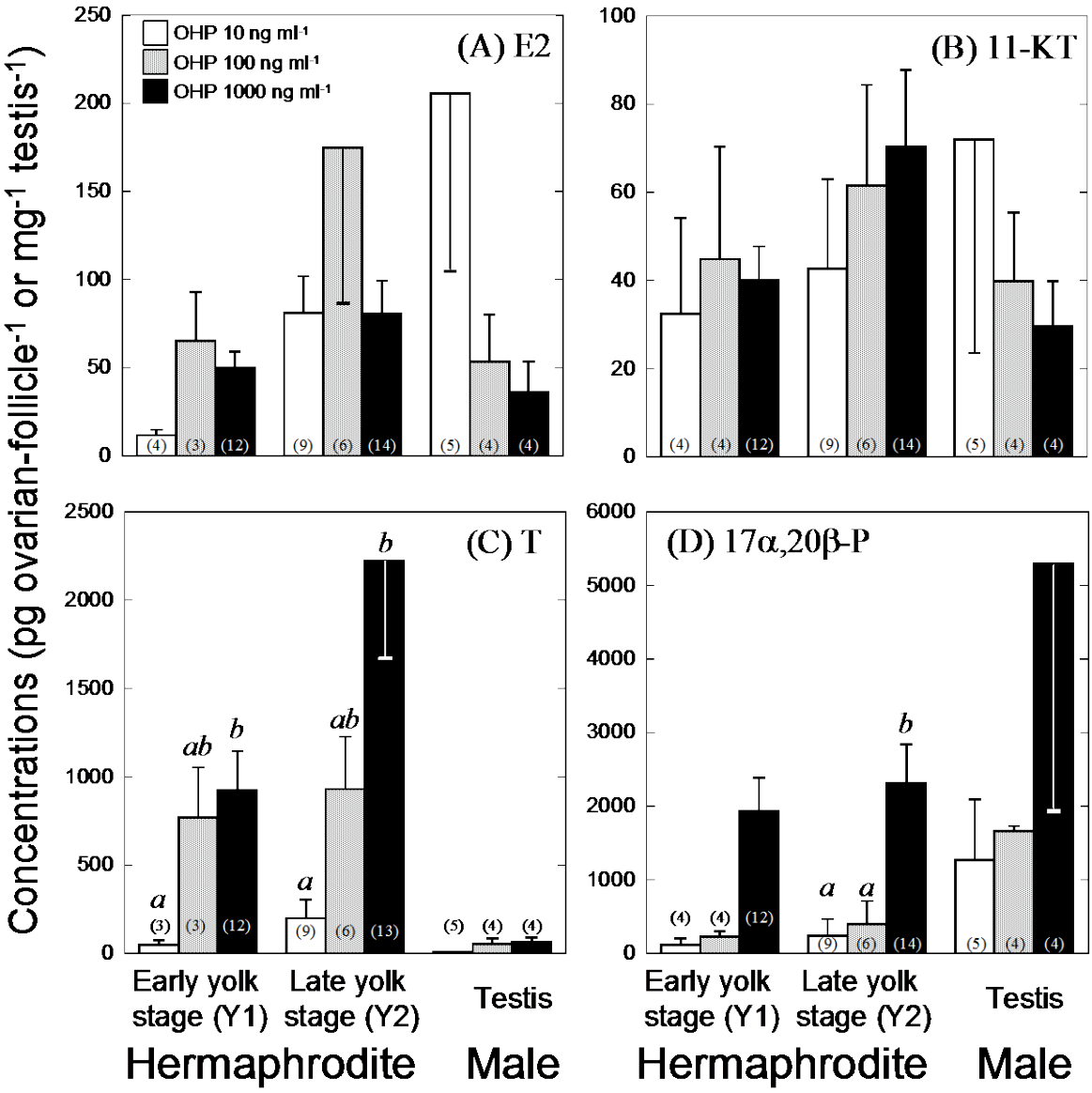

Figure 3 (Minamimoto et al. 2004)