

Masculinization in a Hermaphroditic Female of the Mosquitofish *Heterandria formosa*

Rüdiger Riehl

Institut für Zoologie der Universität Düsseldorf, Lehrstuhl für Morphologie
und Zellbiologie, Universitätsstr. 1, D-4000 Düsseldorf,
Federal Republic of Germany

Abstract A masculinized female of *Heterandria formosa* is described for the first time. In size it ranges with 28 mm total length between adult males (17–20 mm) and adult females (35–40 mm). A masculinized anal fin of about 5 mm length is clearly evident. Rays I and II are reduced as in normal males. Rays III to V are elongated and show features of a developing gonopodium. The remaining rays VI to VIII show the expected shape of a “normal fin.” The genus-specific hook which normally originates in the anterior ramus of ray IV and the proximal serrae which derive from the posterior ramus of ray IV are absent in the masculinized analis. The gravid spots of pregnant normal females are absent. The causes for the formation of a masculinized anal fin in a female of *H. formosa* are still unknown. In the masculinized female a hermaphroditic gonad is found in which female and male sections are readily distinguishable. The female regions are dominant. The oocytes of the hermaphroditic gonad are in the stage of vitellogenesis showing a thin zona radiata. The male regions with spermatozoa and Sertoli cells are distributed throughout the ovarian tissue. This mode of hermaphroditism, in which both mature oocytes and mature spermatozoa simultaneously occur in the gonad, is unique among fishes.

The mosquitofish *Heterandria formosa* Agassiz, 1853 is a very small viviparous fish which belongs to the family Poeciliidae. In common with most poeciliids, *H. formosa* displays strong sexual dimorphism. Adult males are usually only one-half (20 mm) the size of mature females (up to 40 mm). The anal fin of males is modified for the transfer of sperm packets into the female genitalium during copulation. This analis modification is called gonopodium.

Gonopodia do not normally occur in female poeciliids, but they can be induced experimentally by androgenic hormone treatments (Turner, 1941, 1942; Takahashi, 1975) or plant sterol treatments (Denton et al., 1985; Howell and Denton, 1989). Strongly masculinized females were also found in a natural population of *Gambusia affinis holbrooki* inhabiting a stream which receives paper-mill effluents (Howell et al., 1980). Recently Rasotto and Zulian (1989) reported about abnormal hermaphroditic females of *Gambusia affinis holbrooki* from a hot spring in north-eastern Italy with similar masculinized characters.

Masculinization in females of *Heterandria formosa* has not been observed before. The presence of spermatozoa in the ovary of nulliparous adult females indicated the occurrence of an abnormal her-

maphroditism in this species (Riehl, 1980). This observation appears to be unique, because *H. formosa* is the only known species of fish in which mature spermatozoa and eggs are found *simultaneously* in the gonad.

These hermaphroditic females in *H. formosa* gave a first hint of masculinization in females in this species. During a study on the reproductive biology of *H. formosa* a single specimen with a hermaphroditic gonad was discovered which showed both physical secondary male and female sexual characteristics. The characteristics and the morphology of this hermaphroditic gonad are reported below.

Materials and methods

Heterandria formosa was kept in aquaria measuring 50×35×30 cm and reared with the usual methods. Juveniles were isolated after birth and kept solitary in small translucent plastic boxes during adolescence to prevent sperm storage.

For demonstration of fin rays by light microscopy a modified method of Spalteholz was applied as described in a previous paper (Riehl and Schmitt, 1981).

For scanning electron microscopy the specimen

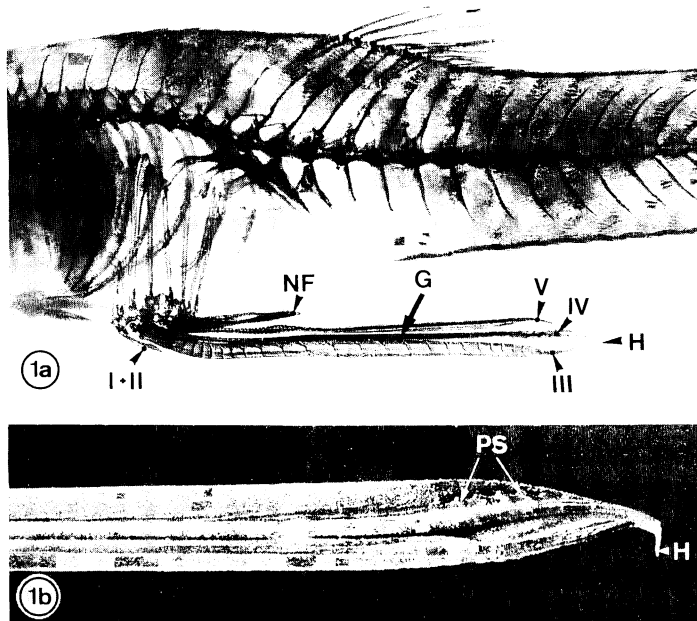


Fig. 1. Morphology of the gonopodium in a normal male of *Heterandria formosa*. a: The gonopodium consists of the rays III-IV. The bones are stained with a modified Spalteholz method. $\times 7$. b: SEM micrograph of the tip of gonopodium. Note the genus-specific hook of ray IV. The posterior ramus of ray IV shows proximal serrae. $\times 30$. G, gonopodium; H, hook; NF, normal fin; PS, proximal serrae; I + II, III, IV, V, rays of analis.

was fixed with 2.5% glutaraldehyde in veronal acetate buffer at pH 7.3 for 24 hours. After washing in the same buffer dehydration was carried out in a graded series of acetone. The specimen was dried in a Balzers critical point dryer, mounted on a brass support with conducting silver and then coated with a 30 nm thick layer of gold in an Emscope SC 500 sputter coater. The specimen was examined with a Leitz AMR 1000 scanning electron microscope at 30 kV.

For transmission electron microscopy the hermaphroditic ovary was fixed in 3% glutaraldehyde in cacodylate buffer with pH 7.4 for 4 hours. After washing with the same buffer sections of the ovary were postfixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in Araldite. Ultrathin sections were stained with lead citrate and examined with a Philips EM 301.

Results

The gonopodium of a normal male. In adult

males the anal fin measures up to 10 mm and the number of rays varies from 8 to 11. The rays are identified from cranial to caudal by roman numbers. The analis is subdivided into three distinct parts: 1) The anterior is considerably reduced in size and contains rays I and II. 2) The central section is the true gonopodium as it serves for the transfer of sperm. This function is achieved by an elongation and modification of the rays III to V. 3) The posterior part includes rays VI to VIII/IX/X/XI and is referred to as "normal fin" (Fig. 1a).

Ray III is the strongest and the main supporting element of the gonopodium. Rays IV and V are dorsal to ray III. They are composed of an anterior and a posterior ramus. All rays consist of bone segments. In some poeciliids ray III bears spines but in *H. formosa* these spines are strongly reduced. Ray IV is comparatively thin and its anterior ramus forms a hook that is genus-specific (Fig. 1b). The posterior ramus of ray IV shows proximal serrae and ray V has a canoe-like appearance (Riehl et al., 1978).

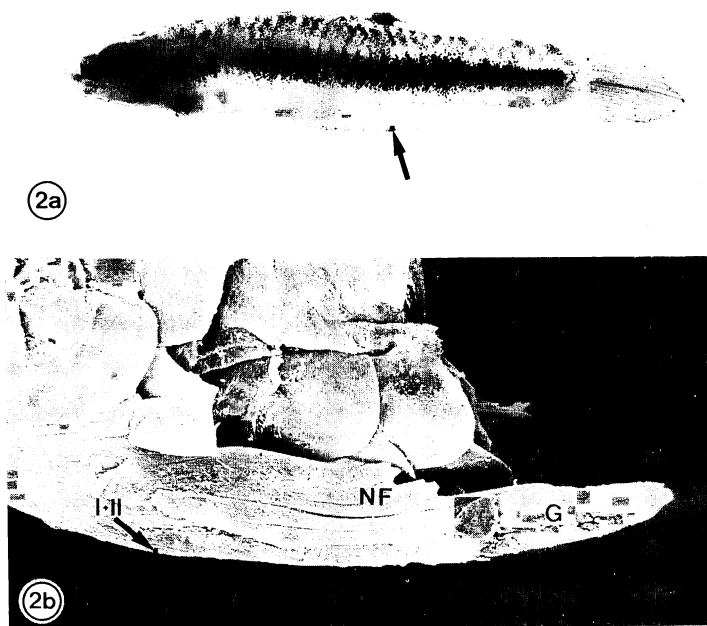


Fig. 2. Micrograph of the masculinized hermaphroditic female of *Heterandria formosa*. a: The arrow points to the gonopodium-like analis. Gravid spots are absent on either side of the abdomen. $\times 3$. b: SEM micrograph of the masculinized gonopodium-like analis in this female. $\times 19$. G, gonopodium-like analis; NF, normal fin; I+II, rays of analis.

The gonopodium of the hermaphroditic female.

Fig. 2a shows the hermaphroditic female of *Heterandria formosa*. Its 28 mm total length places it between adult males (17–20 mm) and adult females (35–40 mm). The masculinized anal fin of about 5 mm is clearly evident (Fig. 2).

Rays I and II are reduced as in normal males. Rays III to V are elongated and show the features of a developing gonopodium. The remaining rays VI to VIII are neither modified nor reduced and show the expected shape of the “normal fin” (Fig. 2b). The genus-specific hook originating in the anterior ramus of ray IV and the proximal serrae derived from the posterior ramus of ray IV are absent in this specimen as are the gravid spots which appear in normal pregnant females on both sides of the abdomen.

Ultrastructure of the hermaphroditic gonad. The ovaries of normal *Heterandria formosa* are paired, sac-like organs in the dorsal posterior of the body cavity and measure between 6 and 12 mm. The capsule of the ovary consists of several layers of smooth muscle which are covered externally by the

peritoneum.

The gonad of the hermaphroditic female has a length of 7 mm and does not visibly differ from those of normal females. It is not separated into male and female regions. In the electron microscope, however, the female and male sections of the gonad are readily distinguishable (Fig. 3). Serial sections showed considerably larger female regions. The male regions with the expected spermatozoa and Sertoli cells are distributed throughout the ovarian tissue. They occur without any topographic regularity in the ovary. Other germinal and somatic components, e.g. Leydig cells, were not found. The Sertoli cells were identified by means of their large nuclei, their amount of microtubuli, their processes and the fact that they rest on the basal lamina.

Vitellogenic oocytes (stage III according to Arndt, 1956) adjoin spermatozoa and Sertoli cells (Fig. 3). In common with other viviparous fishes, the oocyte is enveloped by a thin zona radiata ($1.5 \mu\text{m}$) which peripherally faces an intercellular space of variable width as known from many other teleost species.

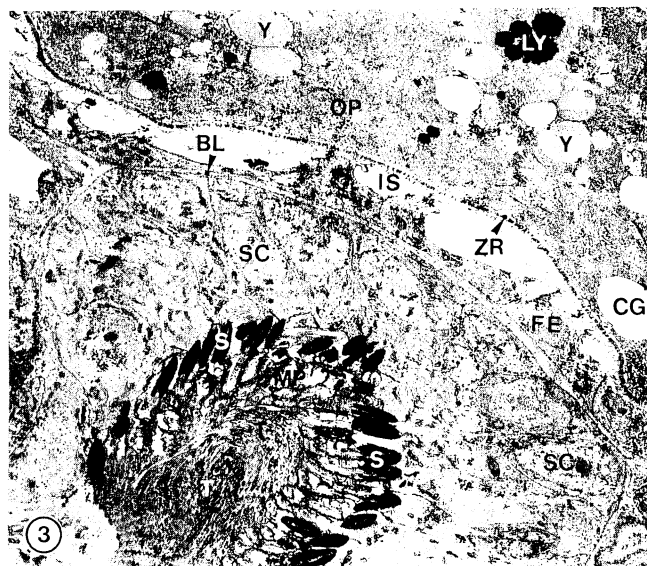


Fig. 3. TEM micrograph of the hermaphroditic gonad. Note oocyte in state of vitellogenesis and mature spermatozoa. The heads of spermatozoa are aligned in a horseshoe-shape and face the Sertoli cells. A vitellogenic oocyte adjoins spermatozoa and Sertoli cells. $\times 3,220$. BL, basal lamina; CG, cortical granule; FE, follicle epithelium; IS, intercellular space; LY, lipid yolk; MP, midpieces of spermatozoa; OP, oocyttoplasm; SC, Sertoli cells; S, sperm heads; T, tails of spermatozoa; Y, yolk; ZR, zona radiata.

Microvilli of the oolemma and the follicle epithelium project into this space. The oocytous microvilli pass the zona radiata. The follicle epithelium consists of elongated cells and is located next to the intercellular space. The follicle epithelium is surrounded by a well-developed basal lamina. A theca folliculi is apparently lacking, whereas a mono-layered theca folliculi occurs around the oocytes of normal females. The Sertoli cells which encircle the spermatozoa adjoin the described basal lamina. The sperm heads are aligned in a horseshoe-shape and face the Sertoli cells (Fig. 3).

Discussion

Gonopodia do not normally occur in female poeciliids. Gonopodia and other secondary sex characters have been experimentally induced by the treatment of females with the androgenic hormone (ethynyl testosterone) (Turner, 1941, 1942). This hormone induced gonopodial development in young female *Gambusia affinis*. Similar experiments have been performed on the guppy (*Poecilia reticulata*) and the swordtail (*Xiphophorus helleri*) with testosterone propionate (Régnier, 1938) and on guppies

with methyltestosterone (Takahashi, 1975). These androgens induced gonopodial structures in the female anal fins of both species.

Apart from androgens a variety of other conditions or agents have been shown to induce masculinization in female poeciliids, such as treatment with chorionic gonadotropin (Baldwin and Li, 1942), X-rays (Vivien, 1952), incomplete hypophysectomy (Vivien, 1952), old age (Essenberg, 1926), parasites (Wurmbach, 1951) and paper-mill effluents (Howell et al., 1980; Howell and Denton, 1989).

In 1980 Howell et al. observed a puzzling situation in a population of *Gambusia affinis holbrooki* which inhabited a stream polluted by pulp wastes from a paper-mill. All females within this population were abnormal. They showed a well-developed, male-like gonopodium and displayed typical male reproductive behavior. This led Howell et al. (1980) to hypothesize that some chemical substances or combination of substances associated with the paper-mill effluents was exerting a strong androgenic effect upon this population. Histological data showed that the atypical females were not sex-reversed and did not have abnormal ovaries. The masculinization did not interfere with ovarian development or the ability to

copulate and to produce offspring. In 1981 Bortone and Drysdale discovered a second population of *Gambusia* with masculinized females which were collected also in a stream polluted by paper-mill wastes.

These reports prompted Denton et al. (1985) to look for compounds in paper-mill effluents with androgenic effects on female *Gambusia*. It was discovered that paper-mill wastes contained the plant sterols beta-sitosterol and stigmastanol. Furthermore, it was shown that a relationship between these plant sterols and added *Mycobacterium smegmatis* existed. Females exposed to the microbially degraded stigmastanol developed male-like gonopodia within six days.

In 1989 Rasotto and Zulian found a population of *Gambusia affinis holbrooki* inhabiting a small hot spring near Padova in north-eastern Italy. The females were masculinized and one showed a hermaphroditic gonad. This gonad contained a few degenerating vitellogenic oocytes and developed testicular tissue with many cysts of spermatids and spermatozoa. Despite the presence of spermatozoa in the gonad, this individual lacked a functional gonopodium. The reason for the masculinization remained unclear. Further investigations aim to identify possible environmental influences.

The hermaphroditic females of *H. formosa* which were described in a previous paper (Riehl, 1980) did not have the masculinized anal fin that has been pictured here. The absence of the gravid spots on each side of the abdomen is to be expected because these females were kept and reared isolated from males.

The causes for the formation of a masculinized anal fin in a female of *H. formosa* are still unknown. Masculinization due to environmental factors, e.g. plant sterols as reported for *Gambusia*, can be excluded. Furthermore, old age or parasites does not apply here either because the specimen studied in this paper was five months old when it was fixed and examined. The phycomycete parasite, *Ichthyophonus hoferi*, commonly infects a number of fishes and undoubtedly has caused masculinization in various live-bearing aquarium fishes (Atz, 1964). There was no evidence of such an infection in *H. formosa*. One can suggest a possible correlation between the occurrence of the hermaphroditic gonad and the appearance of the gonopodium-like analis in this female. Additional investigations will be necessary to confirm this supposition.

Hermaphroditism in fish can be expressed in a variety of ways, including normal and abnormal forms (e.g., Takahashi and Sugimoto, 1978; Bruslé, 1987). In normal hermaphrodites the condition of the gonads is the same in all specimens and ontogeny persists throughout in most if not all members of the species. Hermaphroditism is regarded as abnormal when these specimens occur rarely in a given species of fish. Abnormal hermaphroditism is generally distributed wider than the normal form. In synchronous hermaphrodites distinctly divided male and female parts of the gonad are active simultaneously and in some species self-fertilization has been suggested.

In many fish species oocytes are reported from the testis, e.g. in catfishes (Srivastava and Sathyanesan, 1971), cobitids (Lodi, 1980), esocids (Dominguez et al., 1989), cichlids (Peters, 1975) and serranids (Smith, 1971). Testicular oocytes stop growing when they have reached a certain size, e.g. 200 μm in cichlids (Peters, 1975). Testicular oocytes never reveal vitellogenesis and remain "blocked" in the previtellogenic stage. Later they become atrophic (Reinboth, 1967; Bruslé and Bruslé, 1975; Lodi, 1980). In most accounts a zona radiata has been observed to be absent in testicular oocytes.

Compared with the above, oocytes of masculinized hermaphroditic *Heterandria formosa* attain the vitellogenic stages and these oocytes are surrounded by a thin zona radiata which is of similar appearance as that of normal oocytes. Mature spermatozoa are present at the same time in this hermaphroditic gonad. These observations agree with those reported by Riehl (1980) from two hermaphroditic, but not masculinized females of *H. formosa*. To date sex reversal has not been reported in *H. formosa* and more attention has to be paid to this observation before unequivocal results are available.

Acknowledgments

I am indebted to Dr. U. Santore, Düsseldorf, for linguistic checking of the manuscript. I thank Mrs. G. Reuter, Düsseldorf, for technical assistance in preparing the micrographs.

Literature cited

Arndt, E. A. 1956. Histologische und histochemische Untersuchungen über die Oogenese und bipolare Differenzierung von Süßwasser-Teleostern. Protoplasma,

- 47(1): 1-36.
- Atz, J. W. 1964. Intersexuality in fishes. Pages 145-232 in C. N. Armstrong and A. J. Marshall, eds. *Intersexuality in vertebrates including man*. Academic Press.
- Baldwin, F. M. and M. Li. 1942. Effects of gonadotropic hormone in the fish *Xiphophorus helleri* Heckel. *Proc. Soc. Exp. Biol. Med. N. Y.*, 49: 601-604.
- Bortone, S. A. and D. T. Drysdale. 1981. Additional evidence for environmentally induced intersexuality in poeciliid fishes. *Assoc. Southeast Biol. Bull.*, 28: 67.
- Bruslé, S. 1987. Sex-inversion of the hermaphroditic, protogynous teleost *Coris julis* L. (Labridae). *J. Fish Biol.*, 30: 605-616.
- Bruslé, J. and S. Bruslé. 1975. Ovarian and testicular intersexuality in two protogynous Mediterranean groupers, *Epinephelus aeneus* and *Epinephelus guaza*. Pages 222-227 in R. Reinboth, ed. *Intersexuality in the animal kingdom*. Springer-Verlag.
- Denton, T. E., W. M. Howell, J. J. Allison, J. McCollum and B. Marks. 1985. Masculinization of female mosquitofish by exposure to plant sterols and *Mycobacterium smegmatis*. *Bull. Env. Contam. Toxicol.*, 35: 627-632.
- Dominguez, J., J. C. Pena and G. Gonzáles. 1989. First occurrence of hermaphrodite pike, *Esox lucius* L., in the Esla basin (Spain). *J. Fish Biol.*, 34: 973-975.
- Essenberg, J. M. 1926. Complete sex-reversal in the viviparous teleost *Xiphophorus helleri*. *Biol. Bull.*, 51: 98-111.
- Howell, W. M. and T. E. Denton. 1989. Gonopodial morphogenesis in female mosquitofish, *Gambusia affinis affinis*, masculinized by exposure to degradation products from plant sterols. *Env. Biol. Fish.*, 24: 43-51.
- Howell, W. M., D. A. Black and S. A. Bortone. 1980. Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally-induced masculinization. *Copeia*, 1980(4): 676-681.
- Lodi, E. 1980. Hermaphroditic and gonochoric populations of *Cobitis taenia bilineata* Canestrini (Cobitidae, Osteichthyes). *Monitore Zool. Ital.* (N. S.), 14: 235-243.
- Peters, H. M. 1975. Hermaphroditism in cichlid fishes. Pages 228-235 in R. Reinboth, ed. *Intersexuality in the animal kingdom*. Springer-Verlag.
- Rasotto, M. B. and E. Zulian. 1989. Abnormal hermaphroditism in *Gambusia affinis holbrooki* from a hot spring of north-eastern Italy. *J. Fish Biol.*, 35: 593-595.
- Régnier, M. T. 1938. Contribution à l'étude de la sexualité des Cyprinodontes vivipares (*Xiphophorus helleri*, *Lebistes reticulatus*). *Bull. Biol. France Belg.*, 72: 385-493.
- Reinboth, R. 1967. Protogynie bei *Chelidoperca hirundinacea* (Cuv. & Val.) (Serranidae). Ein Diskussionsbeitrag zur Stammesgeschichte amphisexueller Fische. *Annot. Zool. Japon.*, 40: 181-186.
- Riehl, R. 1980. The occurrence of spermatozoa in the ovary of nulliparous females of *Heterandria formosa* Agassiz, 1853 (Pisces, Poeciliidae). *Cell Tissue Res.*, 205: 289-294.
- Riehl, R. and P. Schmitt. 1981. The skull of the mosquitofish *Heterandria formosa* Agassiz, 1853 (Pisces, Poeciliidae). A study using X-ray projection microscopy and a modified Spalteholz-method. *Mikroskopie (Wien)*, 38: 147-153.
- Riehl, R., A. Holl and E. Schulte, 1978. Morphologische und feinstrukturelle Untersuchungen an dem Gonopodium von *Heterandria formosa* Agassiz, 1853 (Pisces, Poeciliidae). *Zoomorphologie*, 91: 133-146.
- Smith, C. L. 1971. A revision of the American groupers: *Epinephelus* and allied genera. *Bull. Amer. Mus. Nat. Hist.*, 146: 67-242.
- Srivastava, S. S. and A. G. Sathyanesan. 1971. Gonadal changes in response to radiothyroidectomy and thiourea treatment in *Mystus vittatus*. *Anat. Anz.*, 129: 183-196.
- Takahashi, H. 1975. Functional masculinization of female guppies, *Poecilia reticulata*, influenced by methyltestosterone before birth. *Bull. Japan. Soc. Sci. Fish.*, 41: 499-506.
- Takahashi, H. and Y. Sugimoto. 1978. A spontaneous hermaphrodite of the Japanese eel, *Anguilla japonica*, and its artificial maturation. *Japan. J. Ichthyol.*, 24: 239-245.
- Turner, C. L. 1941. Gonopodial characteristics produced in the anal fins of females of *Gambusia affinis affinis* by treatment with ethynyl testosterone. *Biol. Bull.*, 80: 371-383.
- Turner, C. L. 1942. A quantitative study of the effects of different concentrations of ethynyl testosterone and methyl testosterone in the production of gonopodia in females of *Gambusia affinis*. *Physiol. Zool.*, 15: 263-380.
- Vivien, J. H. 1952. Role de l'hypophyse dans de détermination de l'involution ovarienne et de l'inversion sexuelle chez les xiphophores. *J. Physiol. Pathol. Gen.*, 44: 349-351.
- Wurbach, H. 1951. Geschlechtsumkehr bei Weibchen von *Lebistes reticulatus* bei Befall mit *Ichthyophonus hoferi* Plehn-Mulsow. *Arch. Entwickl. Mech.*, 145: 109-124.

(Received June 4, 1990; accepted September 12, 1990)

カダヤシ科の一種 *Heterandria formosa* の雌における雄化

Rüdiger Riehl

Heterandria formosa の雄化した雌1尾を初めて記載する。全長は28mmで、雄成魚(17-20mm)と雌成魚(35-40mm)の間である。長さ約5mmの雄化した臀鰭が明らかである。鱗条IとIIは正常雄にみられるように縮小している。鱗条III-Vは伸長して発達中の交接肢の特徴をみせる。残りの鱗条VI-VIIIは“正常鱗”に相当する形状をみせる。正常には鱗条IVの前枝から派生する属特異的な鈎状部と鱗条IVの後枝に生ずる基部鋸歯状突起は、雄化した臀鰭にはない。妊娠した正常雌にある妊娠斑点もみられない。*H. formosa* の雌における雄化臀鰭の形成の原因

はまだ不明である。雄化した雌は雌雄同体型の生殖腺を持っており、その生殖腺には卵巢組織域と精巢組織域が容易に識別される。卵巢組織域がより大きい。この生殖腺の卵母細胞は薄い卵膜を持ち卵黄形成の段階にある。精子とセルトリ細胞が存在す

る精巢組織域は卵巢組織全体に分布している。生殖腺内に成熟卵母細胞と成熟精子の双方が同時に出現するというこの様式の雌雄同体现象は魚類では注目に値するものである。