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GONADAL DIFFERENTIATION IN ATLANTIC COD, *GADUS MORHUA* L., AND HADDOCK, *MELANOGRAMMUS AEGLEFINUS* (L.)

Marcia *CHIASSON* [1, 2], Tillmann J. *BENFEY* [1*], and Deborah J. *MARTIN-ROBICHAUD* [3]

[1] *Department of Biology, University of New Brunswick, Fredericton NB, Canada*
[2] (*Present address*) *Department of Integrative Biology, University of Guelph, Canada*
[3] *St. Andrews Biological Station, Fisheries and Oceans Canada*

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Background. The purpose of this study was to determine the timing of gonadal differentiation in two gadoids: Atlantic cod, *Gadus morhua* L., and haddock, *Melanogrammus aeglefinus* (L.). This information is required to develop a practical protocol for the production of monosex populations of these species for aquaculture.
Materials and Methods. Cultured larvae and juveniles were collected weekly, measured (total length; TL), prepared histologically and then examined microscopically for the presence of characteristic stages of gonadal differentiation.
Results. In Atlantic cod, undifferentiated gonads were present by 18 mm TL, at 84 days post hatch (dph), and definitive germ cells by 19 mm TL (90 dph). Ovarian cavities were first observed at 27 mm TL (102 dph), and by 35 mm TL (112 dph) anatomical divergence into two types of gonads was clear. In haddock, undifferentiated gonads were observed at 21 mm TL (64 dph) and an ovarian cavity was evident at 29 mm TL (71 dph).
Conclusion. Gonadal differentiation in Atlantic cod and haddock occurs at roughly the same size in both species (27 and 29 mm TL, respectively). For successful sex reversal, the administration of steroids should therefore begin at approximately 25 mm TL, shortly after weaning onto dry feed.

Keywords: Atlantic cod, gadoid aquaculture, gonadal differentiation, haddock

INTRODUCTION

The aquaculture industry in North Atlantic nations has experienced rapid growth in recent decades. However, in both northern Europe and Canada this industry remains largely dependent on the production of Atlantic salmon, *Salmo salar* L., a species for which overproduction and global competition have decreased the market value. On Canada's east coast, the farming of Atlantic salmon is also limited by seawater site availability because of lethally low winter temperatures throughout most of the region. Salmon farmers in eastern Canada and northern Europe are currently looking to alternative coldwater species, such as the Atlantic cod, *Gadus morhua* L.; haddock, *Melanogrammus aeglefinus* (L.); and Atlantic halibut, *Hippoglossus hippoglossus* (L.), to maintain the value of their industry and to expand the range of possible farm sites. Due to the increased market demand for fish products and the diminishing supply available from traditional fisheries, these species remain good choices for commercial aquaculture in the North Atlantic. It has been suggested that in 15–20 years, cultured Atlantic cod may reach production levels similar to Atlantic salmon (Rosenlund and Skretting 2006). However, there are still some challenges, which need to be addressed if gadoid farming is to be a success.

One of the primary constraints to commercially viable production of farmed Atlantic cod and haddock is early (pre-harvest) sexual maturation (Kjesbu et al. 2006). The flesh quality of farmed fish is affected when sexual maturation begins, as protein and lipid energy stores are transferred from the muscle to the developing gonads. The fact that both male and female Atlantic cod and haddock mature before harvesting is of added concern because spawning can occur in sea cages, leading to the release of offspring from domesticated stocks into the environment where they may have negative effects on local populations (Bekkevold et al. 2006, Jørstad et al. 2008). The production of monosex stocks would eliminate successful fertilization of gametes in open cages. Furthermore, it can be combined with triploidy induction to produce sterile populations of female triploids (e.g., Felip et al. 2001), but to date only mixed-sex populations of triploid cod have been produced (Peruzzi et al. 2007, Trippel et al. 2008).

* Correspondence: Dr. T. Benfey, Department of Biology, University of New Brunswick, P.O. Box 4400, Fredericton, New Brunswick E3B 5A3, Canada, phone: 1-506-452-6293, fax: 1-506-453-3583, email: *benfey@unb.ca*

Growing all-female stocks has become common practice in the culture of salmonid fishes such as rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), and Chinook salmon, *O. tshawytscha* (Walbaum, 1792) (cf. Devlin and Nagahama 2002), and the commercial production of all-female Atlantic halibut populations has also recently begun (Melissa Rommens, Scotian Halibut Ltd., Canada, personal communication 2008). However, this approach has yet to be attempted in any gadoid species.

The development of successful sex reversal techniques requires knowledge of the timing of sexual differentiation of the gonads ("gonadal differentiation"), which can be determined through microscopic examination of the gonads. In gonochoristic teleosts, gonads can either develop directly into ovaries or testes (differentiated gonochorists) or both sexes can pass through a female or intersex phase prior to differentiating into ovaries or testes (undifferentiated gonochorists). Thorough reviews of the variety of mechanisms of gonadal differentiation in fishes can be found in Nakamura et al. (1998), Piferrer (2001), Devlin and Nagahama (2002), and Strüssmann and Nakamura (2002). From these reviews it is obvious that the timing of gonadal differentiation in fish is highly variable, even between closely related species. The time of gonadal differentiation is not known for any gadoid species.

The first microscopically detectable characteristic of gonadal differentiation is the migration of the primordial germ cell (PGC) along the peritoneal wall, resting just below the mesonephric ducts (Patiño and Takashima 1995). The primordial gonad forms the germinal ridge after the arrival of PGCs (Patiño and Takashima 1995). Initially, the number of PGCs in the gonad is small, but the number of germ cells then increases exponentially through mitotic divisions, particularly in female teleosts (Strüssmann and Nakamura 2002). The ovaries differentiate first in most gonochoristic species. The first morphological sign of ovarian differentiation is the formation of the ovarian cavity via the lengthening and folding of somatic tissue to form a wide, flat cavity (Strüssmann and Nakamura 2002). Testicular development is generally observed some time after ovarian development, as testes typically remain quiescent longer than ovaries (Patiño and Takashima 1995). Therefore, at the time of ovarian differentiation, the testes are filamentous with few, undifferentiated germ cells (Strüssmann and Nakamura 2002). Effective sex reversal can be achieved by steroid hormone administration just prior to and during early stages of gonadal differentiation (Piferrer 2001).

The objectives of this study were to describe normal gonadal development and to determine the timing of gonadal differentiation in Atlantic cod and haddock. This research is the foundation for subsequent hormonal sex reversal in these two gadoid species for the purpose of improved growth performance under culture conditions and to minimize genetic contribution to wild stocks.

MATERIALS AND METHODS

Fertilized Atlantic cod and haddock eggs were obtained from broodstock held at the Department of Fisheries and Oceans (DFO) St. Andrews Biological Station in 2005 and 2001, respectively. The eggs were incubated in the dark, in conical incubator tanks supplied with flow-through sea water (5.8 ± 0.1ºC) until yolk sac absorption was complete. Larvae were transferred to 5000-L tanks for the initiation of feeding. The water was greened with 100 000 algal cells per mL, a process known to improve the survival of marine fish larvae including cod (e.g., van der Meeren et al. 2007). Temperature was increased by 0.5ºC per day until 11ºC and 13ºC for cod and haddock, respectively. Fish were reared under continuous incandescent light (60 lx). The cod larvae were fed rotifers until 37 days post hatch (dph), then *Artemia* until 87 dph, followed by co-feeding with dry feed (0.5 mm Gemma^{TM}) and subsequent weaning. Haddock larvae were initially fed rotifers until 40 dph, and then weaned to a commercial dry feed (Biokyowa^{TM}) via co-feeding for 4 days. Excess feed was siphoned out daily and surface skimmers removed surface oil.

To identify the timing of gonadal differentiation, random samples of ten cod were collected weekly from 84 until 221 dph and random samples of five haddock were collected weekly from 64 until 110 dph. The total length (TL) of each fish was measured with a ruler to the nearest mm. All fish were killed with a lethal dose of the anaesthetic, tricane methanesulfonate (TMS AquaLife^{TM}). Whole fish were fixed for at least 48 h in 5% buffered formalin (cod) or Bouin's fixative (haddock) (Humason 1972). A cross-section of the fish was then cut from behind the dorsal fin to the anal pore for preparation using standard histological methods of dehydration and paraffin infiltration. Serial sections were cut to 5µm and stained with Azocarmine B and Hubbs II (Humason 1972). Cross-sections were compared to photomicrographs from the salmonid literature (Takashima et al. 1980, van den Hurk and Slof 1981, Nakamura 1982, Sacobie and Benfey 2005, Chiasson and Benfey 2007) and from the gadoid literature (Morrison 1990, Rommens 1997) to determine when gonadal differentiation occurred. The terminology of Patiño and Takashima (1995) is used to describe the cellular stages of differentiation.

The sampling of animals was approved by the UNB Animal Care Committee, following guidelines established by the Canadian Council on Animal Care.

RESULTS

Gonadal Differentiation in Atlantic Cod. Gonads from 18 mm TL fish (84 dph) were long, thread-like structures suspended by peritoneal membranes from the dorsal wall of the body cavity, located dorsal to the gut and ventral to the swim bladder. At this time gonads were composed of stromal cells and a few primordial germ cells (PGCs) (Fig. 1). At 19 mm TL (90 dph), the gonads were larger, taking on a pear shape, and PGCs had increased in number (Fig. 2). A lumen, suggestive of the ovarian cavity, was first evident in gonads from 27 mm TL fish (102 dph) (Fig. 3), but there was no other apparent distinction between ovaries and testes. The first clear sign of ovarian differentiation was in 35 mm TL fish (112 dph). By this

stage, gonads could be divided into 2 groups based on their morphology: those with large cavities, presumed to be the early ovarian cavity, and gonads with aggregations of dividing PGCs (Figs. 4, 5). Primary oocytes were observed in 79 mm TL fish (174 dph; Fig. 6).

At 90 mm TL (198 dph), ovaries containing primary oocytes were clearly differentiated (Fig. 7). Those gonads not containing oocytes had a distinctly different morphology and were classified as undifferentiated testes (Fig. 8). Some 94 mm TL cod (221 dph) had larger ovaries contain-

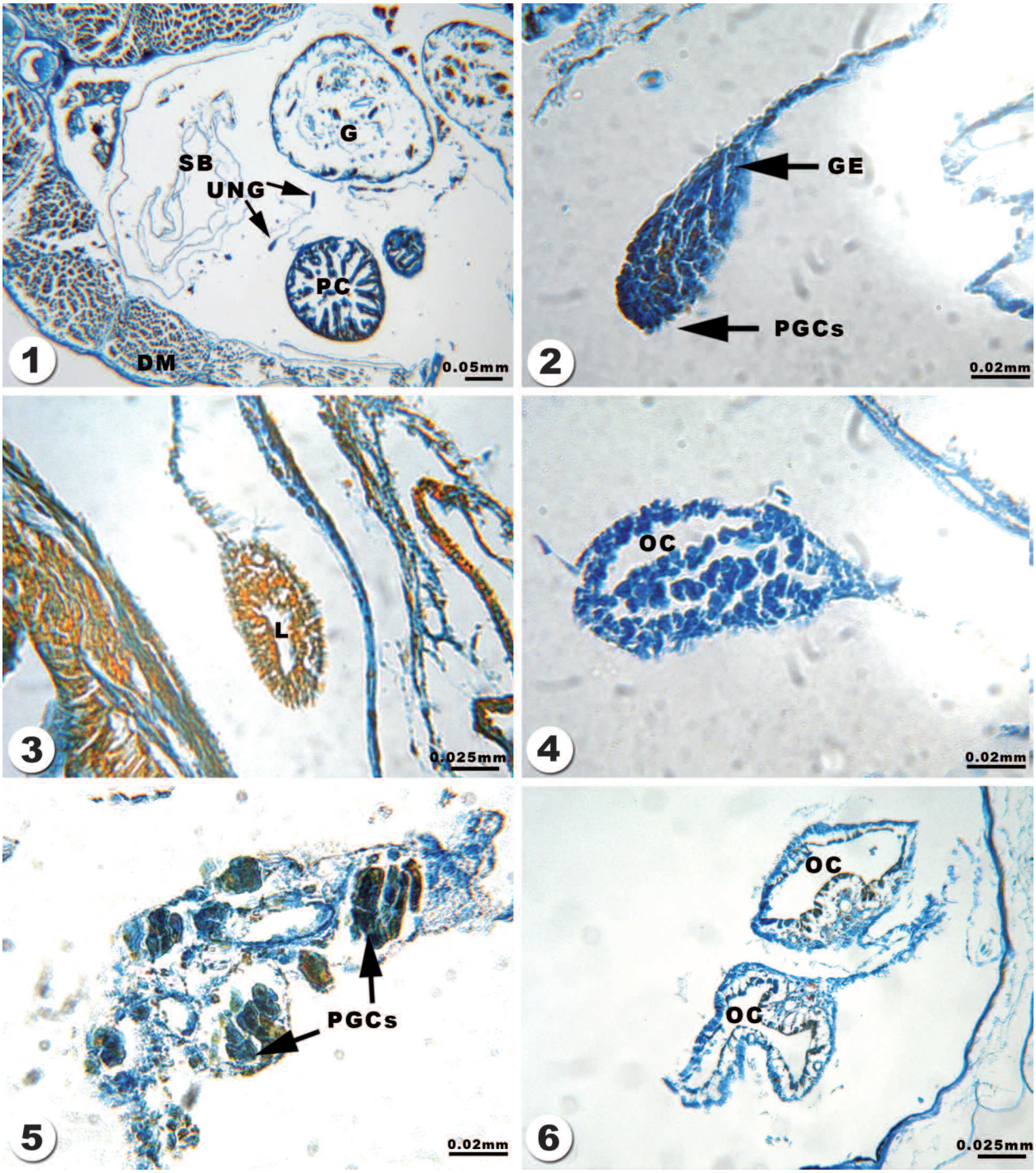

Fig. 1–6. Gonadal differentiation in Atlantic cod, *Gadus morhua* L., at varying days post hatch (dph); **Fig. 1.** Undifferentiated gonads (UNG) ventral to the swim bladder (SB) (TL = 18 mm, 84 dph; DM = dorsal muscle, G = gut, PC = pyloric caecae); **Fig. 2.** Undifferentiated gonad of increased size showing a nest of primary germ cells (PGCs) at distal end and germinal epithelium (GE) at proximal end (TL = 19 mm, 90 dph); **Fig. 3.** Undifferentiated gonad containing a lumen (L) suggestive of the ovarian cavity (TL = 27 mm, 102 dph); **Fig. 4.** Ovarian cavity (OC), the first sign of ovarian differentiation (TL = 35 mm, 112 dph); **Fig. 5.** Aggregations of primary germ cells (PGCs) (TL = 35 mm, 112 dph); **Fig. 6.** Ovaries with broad ovarian cavities (OC) and presumptive oogonia (TL = 79 mm, 174 dph)

ing lamellae, which extended into the ovarian cavity, and the oocytes continued to increase in size (Fig. 9). The testes in fish of the same size and age were pear-shaped vascularized organs containing primary spermatogonia, but no further development was apparent (Fig. 10). Macroscopically, the immature gonad at this size is a bilobed structure located in the posterior part of the body cavity that develops anteriorly from posterior to the anal pore.

Gonadal Differentiation in Haddock. Undifferentiated gonads were observed as thread-like structures between the gut and the swim bladder in 21 mm TL fish (64 dph) (Fig. 11). The gonads of some 29 mm TL fish (71 dph) had an ovarian cavity (Fig. 12). In 46 mm TL fish (83 dph), the gonads were larger, PGCs had increased in number, and vascularity was observed in presumptive testes (Fig. 13). At 51–55 mm TL (91 dph), gonads had increased in size. Ovarian cavities were observed in 3 out of 5 samples and other gonads were long and contained some small cavities (Figs. 14, 15). At the final sample (78 mm TL, 110 dph), gonads were paired structures located in the posterior part of the body cavity near the anal pore (Fig. 16).

DISCUSSION

Atlantic cod and haddock exhibit differentiated gonochorism whereby the undifferentiated gonad develops directly into an ovary or a testis without an intermediate form (Devlin and Nagahama 2002). Gonadal development began in both species with the migration of primordial germ cells. This was followed by morphological differentiation of the gonads into two types: large, broad organs containing a large cavity enclosed by a thin wall (early ovaries) and smaller, pear-shaped organs containing small slits (presumptive testes), with both types containing primordial germ cells. These characteristics of gonadal differentiation are well described in the literature for teleost fishes, with ovarian differentiation characterized by intensive germ cell proliferation, evidence of meiosis, arrangement of somatic cells to form the ovarian cavity and the relative size of gonads and blood vessels (Strüssmann and Nakamura 2002), and differentiation of testes including the development of slit-like spaces (anlagen of efferent ducts) extending from the proximal region of the gonad into the central stromal region (Nakamura et al. 1998).

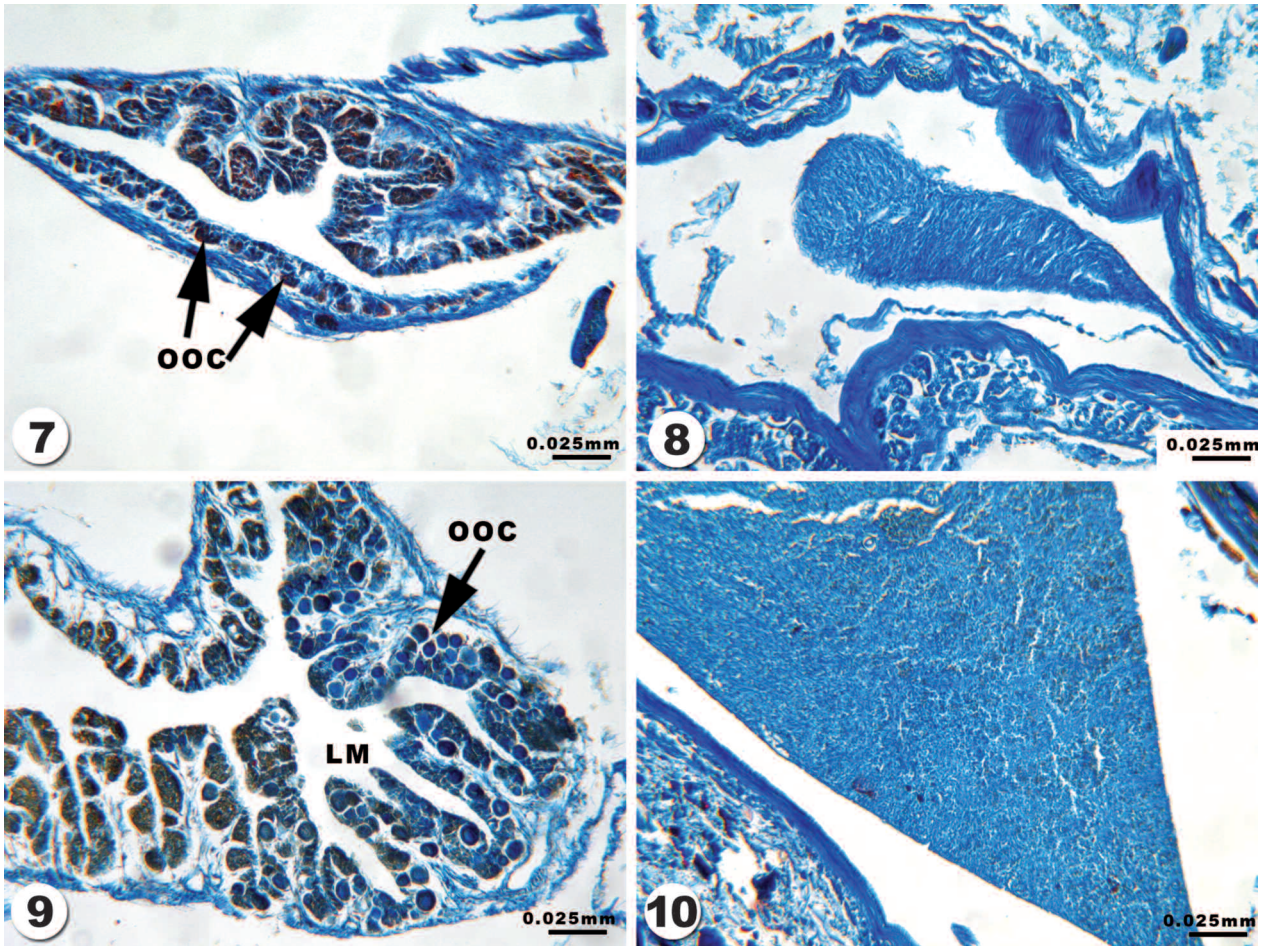

Fig. 7–10. Gonadal differentiation in Atlantic cod, *Gadus morhua* L., at varying days post-hatch (dph); **Fig. 7.** Differentiated ovary with an ovarian cavity containing primary oocytes (OOC) (TL = 90 mm, 198 dph); **Fig. 8.** Undifferentiated testes containing some small slits (TL = 90 mm, 198 dph); **Fig. 9.** Ovarian lamellae (LM) extending into the ovarian cavity and containing primary oocytes (OOC) (TL = 94 mm, 221 dph); **Fig. 10.** Testis containing primary spermatogonia (TL = 94 mm, 221 dph)

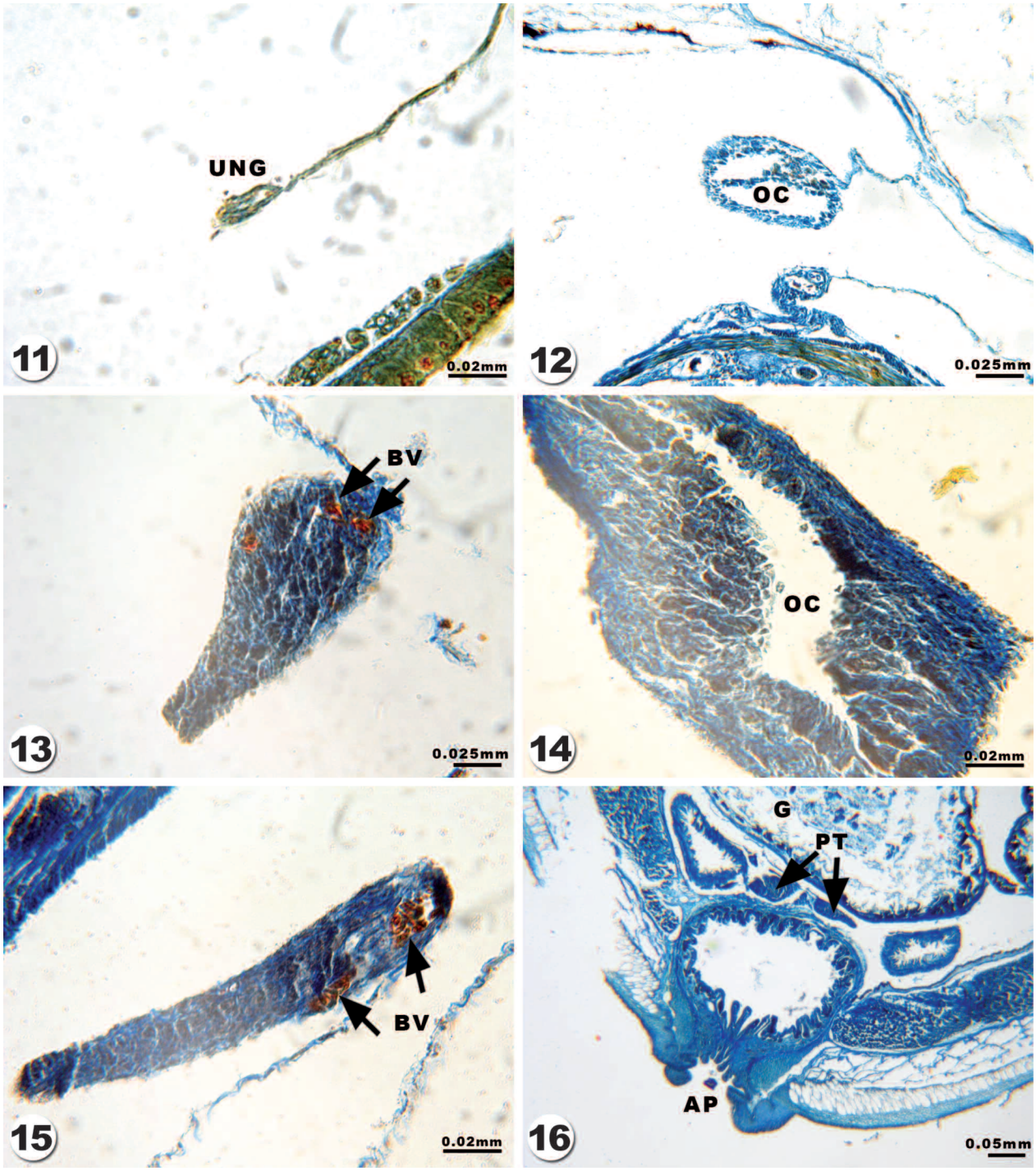

Fig. 11–16. Gonadal differentiation in haddock, *Melanogrammus aeglefinus* (L.), at varying days post-hatch (dph); **Fig. 11.** Thread-like, undifferentiated gonad (UNG) attached by a stock of stromal tissue (TL = 21 mm, 64 dph); **Fig. 12.** Early ovary with an ovarian cavity (OC) (TL = 29 mm, 71 dph); **Fig. 13.** Vascularization of a presumptive testis (TL = 46 mm, 83 dph; BV = blood vessel); **Fig. 14.** Ovary of increased size with ovarian cavity (OC) (TL = 51 mm, 91 dph); **Fig. 15.** Presumptive testis (TL = 55 mm, 91 dph; BV = blood vessel); **Fig. 16.** Presumptive testes (PT) observed near the anal pore (AP) (TL = 78 mm, 110 dph; G = gut)

Gonadal differentiation and the initiation of meiosis occurred earlier in ovaries than in testes in both species, as is typical of gonochoristic teleosts (Strüssmann and Nakamura 2002). Ovaries were first identified by the presence of the ovarian cavity and later easily distinguished in Atlantic cod by the presence of primary oocytes. Primary oocytes were not documented in haddock, for which fewer samples had been collected. The clear differentiation of testes was not observed in either species by the final sample, but presumptive assignment of testes was possible based on differences observed in the gonads of some fish compared to the differentiated ovaries in other fish of the same size.

Testicular differentiation, characterized by changes in germinal and somatic cells, generally comes later in development than ovarian differentiation (Nakamura et al. 1998), and would have been observed in older/larger fish.

Based on the results of this study, it is apparent that gonadal differentiation occurs between 27 and 35 mm TL (102–112 dph) in Atlantic cod and by 29 mm TL (71 dph) in haddock. Although haddock differentiated earlier than cod, they were about the same size since they grew faster than cod at this stage. This suggests that differentiation is determined by size rather than age, which is typical of poikilothermic animals that have their development largely regulated by temperature (Laurence and Rogers 1976). Successful sex reversal requires the introduction of hormones prior to gonadal differentiation, which is typically observed in females before males. Based on the results of this study, steroids should be administered to both species beginning at approximately 25 mm TL, shortly after weaning to dry feed, as is also the case for Atlantic halibut (Hendry et al. 2002, 2003). Steroids are easily incorporated into dry fish feeds, suggesting that sex reversal should be easy to achieve in these species once optimal dose and treatment duration are determined (Piferrer 2001).

What remains to be determined is the genetic basis of sex determination in gadoid fishes. If they have female homogamety (analogous to the mammalian XX-female/XY-male system), then androgen treatment can be used to create "neomales" (functional males that are genetic females) that are capable of generating all-female offspring when mated with normal females. If they have male homogamety (analogous to the avian WZ-female/ZZ-male system), then crossing neomales with normal females will yield populations containing some proportion of "super-females" (equivalent to WW-genotype) that can be crossed with normal males to generate all-female progeny. Such approaches have been used in numerous species of fish to create all-female populations of diploids and triploids (Devlin and Nagahama 2002) that have themselves not been treated with steroids and therefore can be sold for human consumption.

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