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Gonadal Development and Differentiation in Cultured Juvenile Winter Flounder, *Pseudopleuronectes americanus*

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Abstract

Winter flounder, *Pseudopleuronectes americanus*, is currently being evaluated as a stock enhancement candidate in New Hampshire, USA; however, little is known about the gonadal development or the sex ratio of cultured juveniles. To determine the size at gonadal differentiation, 327 cultured fish ranging from <20 to 110 mm total length (TL), in 10-mm-TL size classes, were examined histologically. Gonads had differentiated into testes and ovaries in fish ≥41 mm TL (98%), whereas the majority of fish (81%) smaller than 40 mm TL possessed undifferentiated gonads. A total of 313 cultured fish >40 mm TL were analyzed for sex ratio. In 2003, 67 females and 164 males were identified, yielding a sex ratio that was significantly skewed toward male ($\chi^2 = 40.7$, df = 1, $P < 0.001$). This trend held true when cultured fish were sorted by age and length, with the exception of those fish 61–70 mm TL. This aberration probably was because of a small sample size in this length category. However, in both the 2004 and the 2005 cultured populations, flounder sex did not deviate from a 1:1 ratio (2004 $\chi^2 = 0.12$, df = 1, $P = 0.724$ and 2005 $\chi^2 = 0.02$, df = 1, $P = 0.881$). The 2003 data suggest that environmental or genetic factors may affect winter flounder sex determination; rearing manipulation studies in the hatchery are needed to confirm this hypothesis.

Winter flounder, *Pseudopleuronectes americanus*, is currently being evaluated as a stock enhancement candidate in New Hampshire, USA. This species, found along the northwestern Atlantic coast from Georgia, USA, to Labrador, Canada, resides in depths from the shallow subtidal to 37 m (McCracken 1963). Estuaries serve as nursery grounds for winter flounder; adults typically undergo onshore migrations in early spring into estuaries where spawning occurs and the young-of-the-year remain for their first 2 yr before moving offshore (Pereira et al. 1999). Although some information exists on the adult reproductive cycle (Dunn and Tyler 1969; Dunn 1970; Burton and Idler 1984; Moulton and Burton 1999), there are no published studies on juvenile winter flounder gonad differentiation, or the sex ratio of juveniles, despite the frequent production of cultured populations in research laboratories (Howell and Litvak 2000).

Many fish exhibit a combination of genetic and environmental sex determination in which certain exogenous parameters (e.g., temperature, pH) interact with the genetic factors that influence sex determination, thereby influencing the male : female sex ratio (Nakamura et al. 1998). For example, in several flatfish, gonadal development is affected by juvenile incubation temperature (temperature-dependent sex determination [TSD]); either a decrease or an increase from a "normal" water temperature range during the period of gonadal differentiation yields a predominantly male population (Yamamoto 1999; Goto et al. 1999, 2000; Godwin et al. 2003; Luckenbach et al. 2003). For flounder used in stock enhancement programs, this phenomenon could pose significant problems by altering the expected 1:1 sex ratio and thus affecting the wild population. For this reason, it is imperative to know when

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stocked fish undergo gonadal differentiation and whether they exhibit a combination of genetic and environmental sex determination.

This study was undertaken because (1) gonadal differentiation has not been described for winter flounder and (2) the sex ratios of cultured fish need to be addressed prior to stockings. By histologically examining a size series of cultured fish, we determined the size and age when gonadal differentiation occurs in winter flounder. In addition, cultured juvenile fish from three different year classes were sexed to determine the sex ratio of each cultured population.

Materials and Methods

Cultured Fish

Cultured juvenile winter flounder from three different year classes (2003–2005) were used in this study. All fish were reared at the University of New Hampshire's Coastal Marine Laboratory (CML) in New Castle, NH. Mature winter flounder were caught locally on board commercial fishing vessels each March and returned live to the CML. Fish were distributed into 0.9-m-diameter, covered, flow-through ultraviolet-filtered (0.5 μm) seawater tanks containing one female and two males and were allowed to spawn naturally. Four females spawned in 2003, three females in 2004, and two in 2005. Once completely spawned out, adults and covers were removed from the tanks. Fertilized eggs were incubated in the tanks and provided with seawater at a rate of 1 L/min and gently aerated. During the incubation period, water temperature increased from 4 to 9 C and salinity fluctuated from 12 to 31 ppt (Table 1). Hatching occurred 9–21 d after fertilization, with the majority of all egg batches hatching on average at 14 ± 3 d (Table 1). Upon hatching, overhead fluorescent lights (24 L: 0 D) provided 30 μE/sec/m^2 at the water surface.

Yolk sac larvae were transferred from the incubation tanks into 2000-L grow-out tanks and provided with the same water system and aeration regime as the incubation tanks. A 24 L: 0 D photoperiod was maintained to promote survival and growth (Litvak 1994). In addition, culture water was enriched with 20 L of microalgae twice daily. Microalgae species used were *Nannochloropsis* sp. UTEX 2341, *Dunaliella tertiolecta* DE, *Isochrysis* sp., and *Tetraselmis suecica* Ply305, obtained as starter cultures from the National Marine Fisheries Service, Milford, CT, laboratory. From 3 to 8 d posthatch (dph), larvae were fed enriched cultured rotifers, *Brachionus* sp. (L strain), three times daily (0800, 1400, and 2000 h) at a density of 2000 prey/L. From 9 to 22 dph, larvae were fed rotifers enriched either with 3 L *Isochrysis* sp. or with DHA Selco (INVE, Salt Lake City, UT, USA) for at least 4 h before feeding. Newly hatched *Artemia* replaced the morning rotifer feed at 18 and 22 dph, while enriched *Artemia* (DC DHA Selco; INVE) completely replaced the rotifer diet.

TABLE 1. *Water quality of winter flounder culture tanks.*

	Year		
	2003	2004	2005
Egg incubation			
Temperature (C)	7–9	4–9	6–9
Salinity (ppt)	22–31	12–27	20–27
Hatching range (d)[a]	9–15	7–21	10–16
Larval period			
Temperature (C)	8–15	7–15	9–15
Salinity (ppt)	25–30	14–31	22–28

[a] Hatching range is reported as the days postfertilization.

Typically, fish were fed 1500–3000 enriched *Artemia*/L/tank/d. Weaning of fish from live prey to a formulated commercial diet began with cofeeding Biokyowa B400 diet (Biokyowa Inc., Chesterfield, MO, USA) 1 h prior to *Artemia* feedings from 37 to 41 dph. Starting at 42 dph, the ratio of commercial diet to live food gradually increased until the fish were only offered formulated food (55 dph). Size of the dry diet increased as fish size increased. Temperature increased from hatching through metamorphosis from 7 to 15 C, and salinity fluctuated between 14 and 30 ppt (Table 1). Juvenile fish densities, measured as the ratio of total fish area to tank bottom area, typically were maintained between 200 and 400% (Fairchild and Howell 2001).

Starting after metamorphosis, a total of 327 cultured fish were sampled from the grow-out tanks of the 2003 population at approximately

10-mm-total length (TL) intervals at 110, 124, 160, 231, and 323 dph (Table 2). A total of 32 (500 dph) and 45 (150 dph) fish were sampled from the 2004 and 2005 populations, respectively. On each sampling occasion, at least 30 randomly collected fish were euthanized by an overdose of anesthesia (MS-222), measured and weighed, and tissues were processed for histology.

Fish Processing

For the 2003 samples, whole fish were processed, whereas for the 2004 and 2005 samples, the gonads were dissected from each fish. All samples were fixed and stored in modified Davidson's fixative for at least 48 h. Prior to histological processing, the samples were washed in freshwater and stored in 70% alcohol. Histological processing involved trimming and embedding the tissues in paraffin, sagittal sectioning (5 μm), and staining with hematoxylin and eosin. For each fish from 2003, a series of 20–30 slides was made by cutting sections from the paraffin block at a depth where we expected to find the gonads. For fish from 2004 and 2005, one slide was created for each gonad tissue sample. Slides were numerically coded and examined by three viewers in a blind test to determine sex based on structures and cells associated with gonadal tissue. Fish were scored male, female, or undifferentiated. Criteria for these sex categories are described in the Results. Sex ratio data were analyzed using chi-square goodness-of-fit tests ($P < 0.05$). Those fish that were scored undifferentiated were excluded from the statistical analyses. In addition, to determine if fish age or length affected the sex ratio, the 2003 data were sorted accordingly and reanalyzed using chi-square goodness-of-fit tests ($P < 0.05$).

Results

Of the 327 fish examined for this study from the 2003 cultured winter flounder population, 77 were determined as female, 171 as male, and 79 as undifferentiated (Table 2). Staging guidelines from Yamamoto (1956, 1995), Nagahama (1983), Tanaka (1987), and Luckenbach et al. (2003) were used to determine the sex of winter flounder by looking for structural and cellular ovarian- or testicular-identifying characteristics. Gross morphology characteristics typically were most helpful. Simply, females were recognized by an ovarian cavity, ovarian lamella, and oocytes in various stages of development (Fig. 1a), whereas males were recognized by compact, dense, highly vascular tissue organized into lobules separated by a layer of connective tissue containing clusters of spermatagonia (Fig. 2a, b). Most females ≥41 mm TL had oocytes in the perinucleolus stage (Fig. 1b); therefore, it was possible to determine sex for 94–100% of the larger fish size classes. For fish <41 mm TL, the proportion of sexually

TABLE 2. *Number and disposition of fish processed for sex determination from each size class of the 2003 cultured winter flounder population.*

Size class (mm)	Number of female	Number of male	Number of undetermined	Total	% Female[a]
<20	0	0	0	34	
21–30	3	2	24	29	
31–40	7	5	16	28	
41–50	13	33	3	49	**28**
51–60	18	46	1	65	**28**
61–70	8	8	1	17	50
71–80	7	17	0	24	**29**
81–90	4	19	0	23	**17**
91–100	14	30	0	44	**32**
101–110	3	11	0	14	**21**
Total	77	171	45	293	**29**

[a] Fish <41 mm TL were excluded from % female analyses because gonadal differentiation either had not occurred or was not clear histologically. Bolded numbers represent a significant ($P < 0.05$) deviation from a 50% female population.

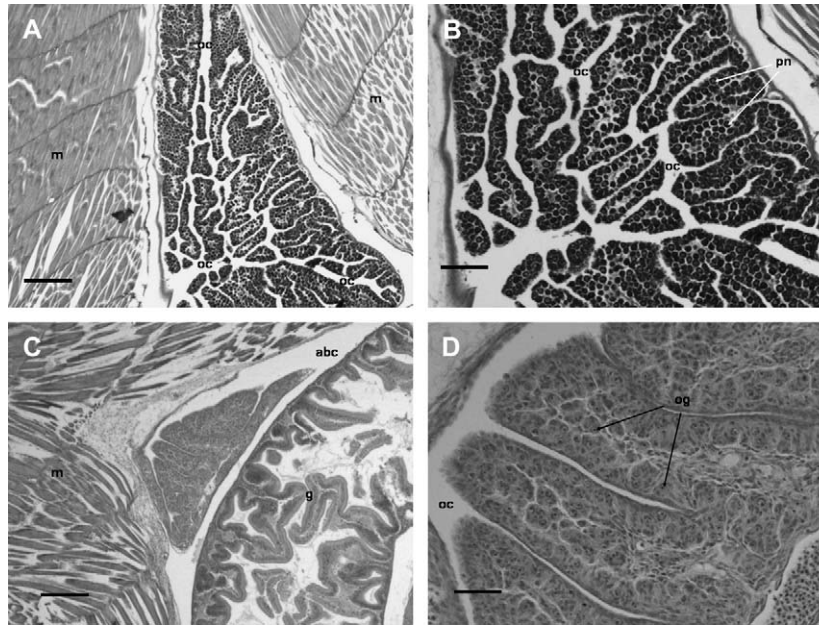

FIGURE 1. *Ovarian development in winter flounder. Sagittal section of a 160-dph 77-mm-total length fish at (A) low (bar = 400 μm) and (B) higher (bar = 200 μm) magnification. Sagittal section of a 110-dph 38-mm-total length fish at (C) low (bar = 200 μm) and (D) higher (bar = 40 μm) magnification. oc = ovarian cavity; abc = abdominal cavity; og = oogonium; pn = perinucleolus-stage oocytes; g = gut; and m = muscle.*

identifiable individuals dramatically decreased such that sex of only 43 and 17% of the fish was identifiable in the 31- to 40- and 21- to 30-mm-TL size classes, respectively. In these smaller size classes, the clearest indicator of ovarian tissue was the presence of an ovarian cavity (Fig. 1c, d). However, in most fish that lacked ovarian cavities, the gonadal tissue also lacked organization toward seminal lobules. Therefore, it was unclear if germ cells were developing into spermatocytes or oocytes (Fig. 3a, b); so, these individuals were scored as undifferentiated.

Because gonadal differentiation in the majority of fish <41 mm TL either had not occurred or was not clear to the viewers, these fish were excluded from the sex ratio analyses. A total of 67 females and 164 males were identified from the 41- to 110-mm-TL 2003 cultured flounder population, yielding a sex ratio that

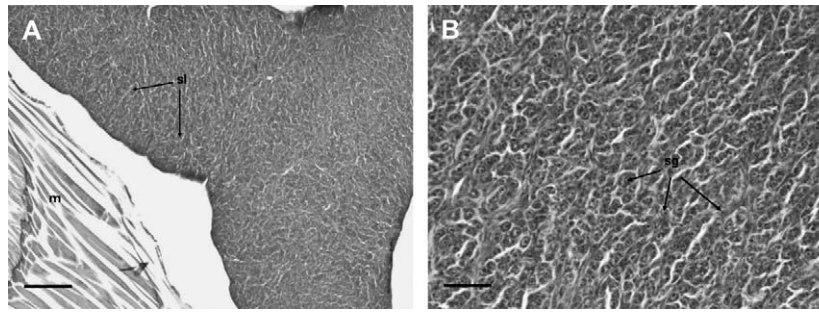

FIGURE 2. *Testicular development in winter flounder. Sagittal section of a 231-dph 94-mm-total length fish at (A) low (bar = 200 μm) and (B) higher (bar = 40 μm) magnification. sg = spermatagonium, sl = seminal lobule; and m = muscle.*

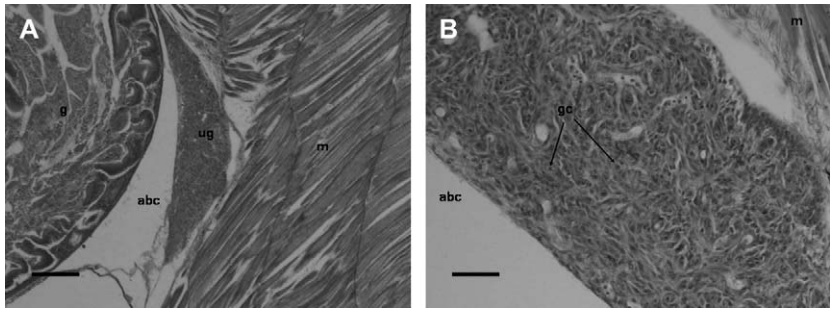

FIGURE 3. *Undifferentiated gonad of winter flounder. Sagittal section of a 110-dph 35-mm-total length fish at (A) low (bar = 400 μm) and (B) higher (bar = 40 μm) magnification. abc = abdominal cavity; g = gut; gc = germ cells; ug = undifferentiated gonad; and m = muscle.*

was significantly skewed toward male (χ^2 = 40.7, df = 1, $P < 0.001$). In addition, this trend held true for the five age groups (110 dph: χ^2 = 3.9, df = 1, $P = 0.047$; 124 dph: χ^2 = 15.8, df = 1, $P < 0.001$; 160 dph: χ^2 = 8.5, df = 1, $P = 0.003$; 231 dph: χ^2 = 9.3, df = 1, P = 0.002; and 323 dph: χ^2 = 5.1, df = 1, P = 0.023) and all but one length class (41–50 mm: χ^2 = 8.7, df = 1, $P = 0.003$; 51–60 mm: χ^2 = 12.2, df = 1, $P < 0.001$; 61–70 mm: χ^2 = 0, df = 1, $P = 1.0$; 71–80 mm: χ^2 = 4.2, df = 1, $P = 0.041$; 81–90 mm: χ^2 = 9.8, df = 1, P = 0.002; 91–100 mm: χ^2 = 5.8, df = 1, $P = 0.016$; and 101–110 mm: χ^2 = 4.6, df = 1, $P = 0.033$) sampled (Table 2). The sex ratio of fish 61–70 mm TL was not significantly different than 1:1 ($P = 1.0$), but because sexual differentiation of the 2003 population had already occurred, and was skewed toward males (Table 2), the most logical explanation for this is the small sample size ($n = 19$) in this length category.

For the 2004 cultured flounder population, a total of 17 females and 15 males were identified, yielding a sex ratio that did not deviate from a normal 1:1 ratio ($\chi^2 = 0.12$, df = 1, P = 0.724). This sex ratio was also true for the 2005 cultured population in which 23 females and 22 males were identified ($\chi^2 = 0.02$, df = 1, $P = 0.881$).

Discussion

The results from this study indicate that sex differentiation occurs in winter flounder prior to 41 mm TL because fish from the 41–50-mm-TL size class could be sexed via histology. Gonadal differentiation at such a small size occurs in other pleuornectiforms too. For example, in Japanese flounder, *Paralichthys olivaceus*, sex differentiation can be detected histologically between 27 and 37 mm TL based on morphological changes within the gonad but prior to this size range, fish sex is not identifiable (Tanaka 1987). Like winter flounder, both barfin flounder, *Verasper moseri*, and marbled sole, *Pseudopleuornectes yokohamae*, undergo gonadal differentiation when <41 mm TL (Suzuki et al. 1992; Goto et al. 1999). Other flounders, such as the warmer water summer flounder, *Paralichthys dentatus*, and southern flounder, *Paralichthys lethostigma*, undergo gonadal differentiation at larger sizes (King et al. 2001; Luckenbach et al. 2003).

Of the three year classes of cultured winter flounder examined for sex ratio, only one deviated from a normal 1:1 female to male ratio. The sex ratio of the 2003 population was predominantly male with a 1:2.5 female to male ratio. Although the cause of this skewed sex ratio is unknown, there are several possible explanations. First, sex could not be assessed in the majority of the small fish size classes; 24% of the population comprised fish with undifferentiated gonads. Had these smaller fish developed into females, then there would have been no difference in the sex ratio ($\chi^2 = 0.69$, df = 1, $P = 0.407$). Second, it is possible that winter flounder exhibit genetic and environmental sex determination and some exogenous factor affected gonadal development. Elevated rearing density (Lutnesky 1994; Roncarati et al. 1997), increased agonistic behavior (see

Godwin et al. 2003), poor water quality (Rubin 1985), and temperature (Yamamoto 1999; Goto et al. 1999, 2000; Godwin et al. 2003; Luckenbach et al. 2003) can affect sex determination in some species.

It is possible that a combination of high rearing density and aggressive behavior affected the 2003 population because fish production was highest that year. Although we tried to maintain density between 200 and 400%, there were periods when fish were denser prior to size grading and during tank shortages. Agonistic behavior was more than likely elevated at these times too (Fairchild and Howell 2001). In addition, the 24 L: 0 D photoperiod regime used to promote survival and growth may have exacerbated aggressiveness (Sakakura and Tsukamoto 2002). Because detailed density and behavioral measurements were not taken during the critical gonadal differentiation period, it is impossible to know if the skewed sex ratio in 2003 was a result of overcrowding and/or elevated stress. Density manipulation studies in the hatchery are necessary to conclude this.

It is unlikely that temperature affected sex determination. If winter flounder exhibit TSD, then temperature would influence phenotypic sex prior to, and during, the ontogenetic development of the gonads. Gonadal differentiation in winter flounder occurs in fish <41 mm TL, and for this New Hampshire population that developmental period corresponds to fish ≤110 dph, or from April to early August. Monthly rearing tank water temperatures for these critical months were compared for each flounder year class to determine if temperature affected the sex ratio of the 2003 population. Although significant differences were found in May, June, and July (Fig. 4), there was no clear pattern. The mean rearing temperature for the 2003 population only was significantly different from the other two populations in July when it was 1.3 C cooler on average (ANOVA: df = 2, $P < 0.001$). Slight deviations in temperature from year to year were expected at the CML because winter flounder are reared in a flow-through system using ambient temperature water, which fluctuates both tidally and seasonally. However, it may be possible that an extreme temperature

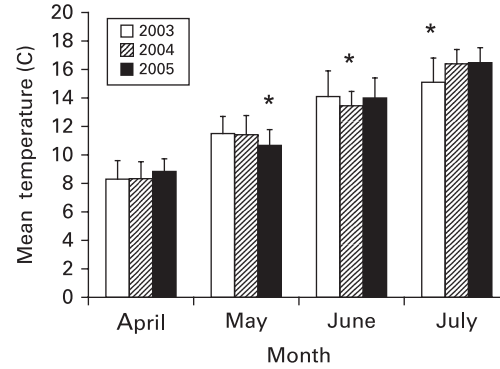

FIGURE 4. *Thermal history of cultured juvenile winter flounder from April through July 2003–2005. Monthly mean water temperatures (C ± 1 SD) are depicted only for the months which correspond to the critical period when gonadal differentiation occurred. Asterisks (*) denote significantly* ($P < 0.05$) *different temperatures between year classes within each month.*

event (hot or cold), however brief, could affect the sexual differentiation in fish that exhibit TSD if the thermal event coincided with some critical stage in the gonadal development. CML water temperature was never considered problematic if <18 C (Casterlin and Reynolds 1982) because winter flounder are eurythermal (Pereira et al. 1999).

There are at least two circumstances in which the sex ratio of cultured winter flounder could be important. One would be in a commercial aquaculture setting. Like many other flatfish species, female winter flounder grow larger and faster than males (Nash and Geffen 2005). Thus, there would be some economic advantage of an all-female hatchery population, and this could be achieved by manipulating environmental cues to influence sex determination. The other circumstance would be releasing hatchery-reared fish in an effort to enhance wild populations. Here, repeated releases of large numbers of fish with a skewed sex ratio could have ecological consequences. For example, changes could occur in sex-linked winter flounder population characteristics, such as growth rates (Howe and Coates 1975) and mortality rates (Witherell and Burnett 1993). Changes could also occur in mating systems because winter flounder males compete to spawn (Stoner et al. 1999), and the addition of proportionately more males could

intensify mating competition. Further, a change in the wild-fish sex ratio could lead to a reduction in reproductive potential. If the population was to rise to carrying capacity, and both sexes were equally fit, then the excess males could displace the females, leading to a reduction in the reproductive potential of the population (Kanaiwa and Harada 2000). Finally, altering the sex ratio of wild populations could result in a loss of genetic variability through genetic drift (Kanaiwa and Harada 2000). For example, if many hatchery males bred with relatively few wild females, the genes of the hatchery males would be overrepresented in subsequent generations and the genetics of the wild population could change.

Future work is needed to clarify if any exogenous variables affect sex determination in winter flounder by executing controlled experiments. To determine if there is an effect on the sex ratio of winter flounder, we have initiated temperature, rearing density, and photoperiod manipulation studies in the hatchery. This species should not be released in a large-scale stock enhancement effort prior to the full understanding of the sexual composition of the cultured population and that of the wild winter flounder population in the release area.

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