Temperature influence on postovulatory follicle degeneration in Atlantic menhaden, *Brevoortia tyrannus*

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The occurrence of postovulatory follicles within the ovaries of fish is a positive indication of spawning, because they are the evacuated follicles following ovulation. Therefore, their frequency of occurrence may be used to test factors influencing reproduction. For example, postovulatory follicles can be used to estimate spawning frequency, which in turn is used to estimate spawning biomass (Hunter and Macewicz, 1985). Their state of degeneration can be assessed from histological sections of the ovary to estimate the time elapsed since spawning for females sampled in the wild. In addition, the occurrence of postovulatory follicles might be useful to test hypotheses about events that may trigger spawning and benefit larval transport (see Taylor, 1984; Checkley et al., 1988).

A requisite for applying this histological method of determining spawning frequency is validation of the duration that postovulatory follicles can be detected in field samples. This has been done through closely timed observations of spawning in the field and by laboratory-induced spawning and subsequent sampling of female gonads (Hunter and Macewicz, 1985). Laboratory spawning offers more certainty in determining the time of spawning and facilitates more precise measurement of the degeneration time of postovulatory follicles. Uncertainty regarding the duration of postovulatory follicles has led to questions about estimates of spawning frequency (Brown-Peterson et al., 1988; Fitzhugh et al., 1993; and Goldberg et al., 1984). Variation in the duration of postovulatory follicles may be largely due to the particular species involved and its preferred spawning temperature (Hunter and Macewicz, 1985).

Atlantic menhaden spawn across a wide geographic range of U.S. coastal Atlantic waters (Cape Canaveral, Florida, to Martha's Vineyard, Massachusetts) and probably over a wide range of temperature as well (Judy and Lewis, 1983). Eggs have been collected between the 15° and 20°C surface isotherms in the mid-Atlantic Bight (Kendall and Reintjes, 1974). We wished to examine differences in postovulatory follicle duration and age at stage due to temperature in Atlantic menhaden. As a preliminary step to future estimation of spawning frequencies, our objective was to characterize postovulatory follicle degeneration and validate postovulatory follicle duration for Atlantic menhaden induced to spawn at different temperatures in the laboratory.

Materials and methods

Adult menhaden were collected near Harker's Island, North Carolina, between mid-August and mid-September 1988, 1990, 1991, and 1992. During each period, menhaden were maintained in outside tanks under ambient flow-through conditions until mid-October to mid-November when they were moved to inside tanks to begin conditioning for spawning. We induced spawning in either December (1990) or March (1988, 1991, 1992) with methods reported by Hettler (1981). Menhaden of both sexes were conditioned to spawn by means of intraperitoneal injections of human chorionic gonadotropin (HCG) and carp pituitary extract (CP). We gave injections between 0800 and 1000 hours on successive days, and spawning usually occurred the night following the second (CP) injection.

Spawning was induced under three temperature regimes; 14.8–15.7°C for one group in December 1990; 17.9–18.2°C for one group in March 1992; and 19–20°C for groups in March 1988, December 1990, and March 1991. Groups of menhaden ranged from 22 to 31

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individuals and the male to female ratio was about equal. In December 1990, only 12 individuals were induced to spawn at the coldest temperature regime (7 were subsequently identified as females). For each group, acclimation and holding temperatures were held constant prior to spawning and for the week following spawning.

The time of spawning was estimated the following morning when eggs were collected and identified to stage (Ferraro, 1980). Males were identified by the presence of free-flowing milt just prior to the CP injection and were marked by clipping the left pectoral fin. Females (identified as not having a fin clip) were sampled up to 4 days following spawning and killed in a solution of MS-222. We excised a tissue section from the middle right ovarian lobe from each female and fixed it in 10% neutral buffered formalin. Tissue samples were dehydrated, embedded in paraffin, sectioned, stained with Gill's hematoxylin and eosin for histological observation. Our interpretation of the histological states of the postovulatory follicles follows Hunter and Macewicz (1985), and the prominent features we observed are summarized in Table 1.

Results and discussion

The postovulatory follicle, which is the evacuated follicle remaining in the ovary following ovulation of the hydrated oocyte, is characterized by an outer thecal layer and an inner epithelial granulosa layer (Fig. 1). Many species show similar features in postovulatory follicle degeneration (Hunter and Macewicz, 1985) to those that we noted for Atlantic menhaden. Onset of degeneration was evident when vacuoles and eosinophils were detected within the postovulatory follicle (Fig. 1A). The granulosa layer appeared contorted or folded but the granulosa cells and their nuclei imparted a linear or cordlike appearance (Fig. 1A). Initially, the lumen was apparent but narrowed and became less distinct with time (Fig. 1, B and C). We also noted that the linear arrangement of the granulosa cell nuclei became less distinct with time (Fig. 1B). Vacuolization of the follicle increased (Fig. 1C), and a point was reached when the lumen was no longer evident. The postovulatory follicle became reduced in size but the folded appearance of the granulosa layer remained evident and aided in identifying the postovulatory follicle (Fig. 1C). We used this point in degeneration to define the postovulatory follicle duration because as the postovulatory follicle aged further (e.g. Fig. 1D), there were no exact features that could distinguish it from old atretic follicles (preovulatory follicles) that remain after an intact oocyte undergoes atresia (Goldberg et al., 1984; Hunter and Macewicz, 1985). While atresia of oocytes occurs predominately at the end of a spawning season, it is common to observe some oocytes undergoing atresia throughout the spawning season (Hunter and Macewicz, 1985). We also observed some atresia of vitellogenic oocytes in our samples. Since preovulatory follicles can be present and at some point are not distinguishable from postovulatory follicles, we can only estimate the duration that the postovulatory follicles may be identified.

The 54 females sampled after spawning from the three temperature regimes ranged in size from 200 to 250 mm fork length which is typical of 2 to 3 year-olds (Nicholson and Higham, 1964). All females (n=35) from the warm regime (19–20°C) that were sampled within about 40 hours of spawning (determined from the collection and identification of stages of fertilized eggs) displayed detectable postovulatory follicles (100% spawned). Eleven of the twelve females sampled from the 17.9–18.2°C series displayed postovulatory follicles (92% spawned). Six females from the cold series (14.8–17.7°C) contained vitellogenic oocytes, indicating an advanced reproductive state; one female possessed cortical alveolar stage oocytes—an immediate precursor to the vitellogenic stage (deVlamming, 1983). However, only 4 of 7 females from the cold series possessed detectable postovulatory follicles (57% spawned). The reduced temperatures appeared to diminish our ability to induce spawning.

Table 1

Distinguishing features for the stages of postovulatory follicle degeneration[1] in Atlantic menhaden, *Brevoortia tyrannus*.

Stage	Characteristics of postovulatory follicles
1	Granulosa cells are aligned and granulosa-layer nuclei appear linear in orientation. Some lymphocytes and vacuoles may appear in the postovulatory follicle, signaling initial degeneration.
2	Loss of linear arrangement of granulosa layer nuclei; cell membranes and columnar/cuboidal shape of granulosa layer is no longer distinct. Lumen is still clearly visible.
3	Although irregular postovulatory follicle shape is still detectable, fewer folds are apparent as the lumen becomes reduced and is no longer distinct.
4	Linear appearance of the granulosa layer is no longer distinct; postovulatory follicle not readily distinguished from atretic oocytes.

[1] Adapted from Hunter and Macewicz, 1985.

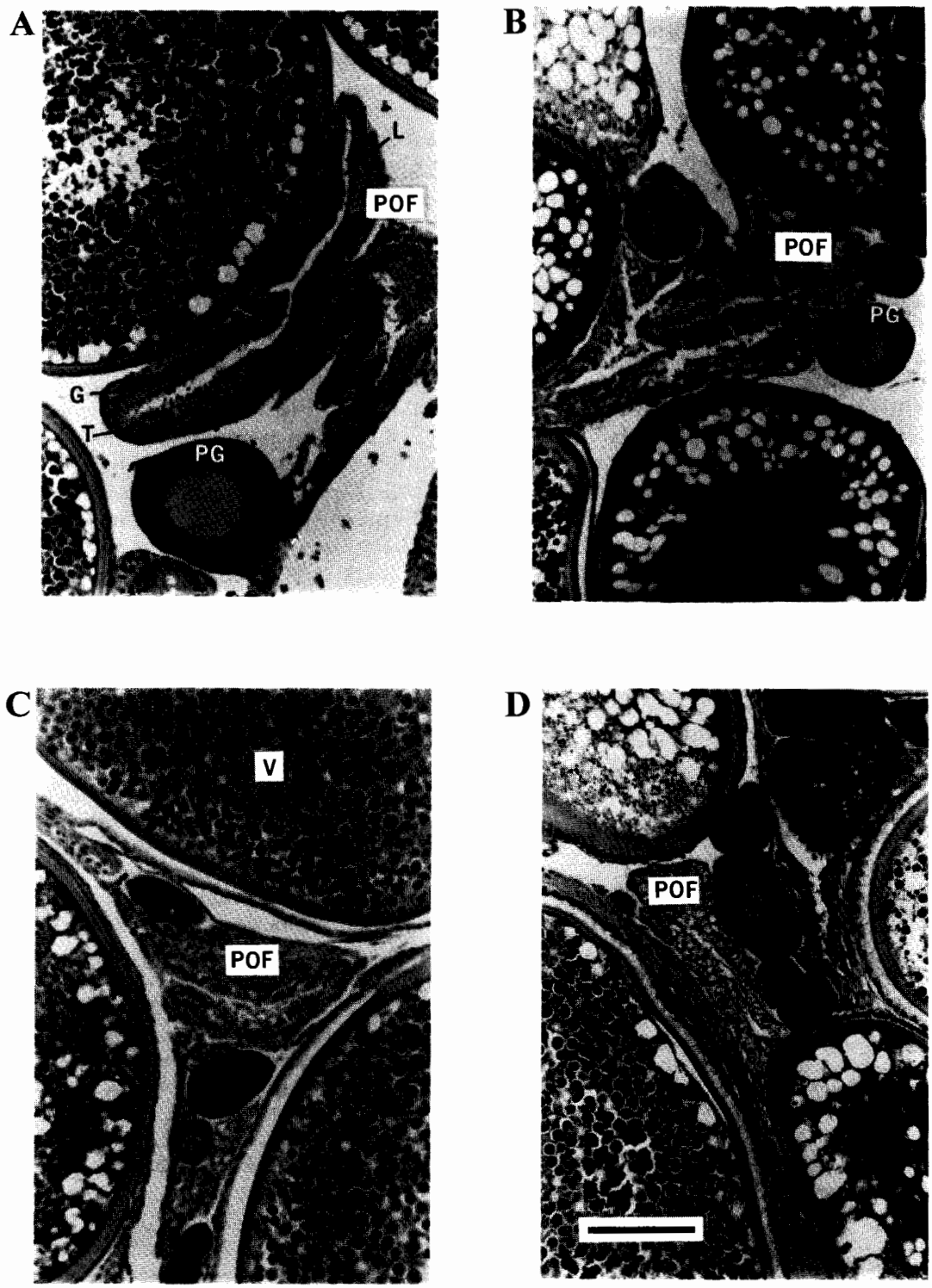

Figure 1

Postovulatory follicles (POF's) from female Atlantic menhaden held at 20°C. Elapsed time from estimated spawning is (**A**) 11 hours, (**B**) 21 hours, (**C**) 38 hours, and (**D**) 45 hours. Features characterizing postovulatory follicles are as follows: L = the lumen; G = the granulosa layer; and T = the outer thecal layer. Vitellogenic oocytes are denoted by (V) and primary growth oocytes are denoted as (PG). The scale bar represents 0.1 mm.

All spawning was estimated to have occurred between 2100 and 0200 hours and time from spawning was recorded at each sampling period. We compared histological states associated with these sampling periods by standardizing the time of spawning (midnight, hour-0; Fig. 2). In general, events associated with induction of spawning slowed down in the cold temperature regime. Spawning at 14.8–15.7°C occurred about 63 hours after HCG injection and about 39 hours after CP injection. However, within the warmest series, spawning occurred about 34 and 11 hours, respectively, after HCG and CP injections.

We saw characteristics of initial follicle degeneration about 6 to 12 hours after spawning for all three temperature regimes (Fig. 2, Table 1). There was an apparent difference in overall duration of postovulatory follicles between the high and low temperature regimes. Filling of the lumen (stage 3) was evident at the warm regime by about 21 to 46 hours (Fig. 2). At 19–20°C, postovulatory follicles were difficult to distinguish (stage 4) as early as 36 hours, but at the two colder regimes, postovulatory follicle degeneration was not as advanced even at 58 hours (e.g. stage 3, Fig. 2). Postovulatory follicle duration at 15°C is probably longer than 60 hours after spawning.

Transition to successive atretic stages was apparently faster at the warmest temperature and there was some overlap of atretic stages at a given time following spawning. For example, stages 1, 2, and 3 were observed at 20 hours from females held at 19–20°C (Fig. 2). This overlap may be due to variation in degeneration rates among females, the subjectivity of stage classification across a continuum of follicle degeneration, or to variation in the exact time of ovulation among females (because time from spawning was estimated for all the females in a group from a sample of fertilized eggs). A more precise analysis of the durations for the various stages of atresia would have been facilitated by sampling each group at constant intervals after the spawning of eggs and by using a larger sample size—particularly for the coldest treatment.

Observations of postovulatory follicle duration vary for species that spawn at different temperatures. We observed durations ranging from about 36 to over 60 hours for Atlantic menhaden spawning from 15 to 20°C. At similar temperatures (13 to 19°C), northern anchovy, *Engraulis mordax*, Peruvian anchovy, *Engraulis ringens*, and Pacific sardine, *Sardinops sagax*, have postovulatory follicles that last about 48 hours (Hunter and Macewicz, 1985). Skipjack tuna, *Katsuwonus pelamis*, and Hawaiian anchovy, *Encrasicholina purpurea*, spawn at approximately 25°C and have postovulatory follicles that last about 24 hours (Hunter et al., 1986; Clarke, 1987). The dragonet *Callionymus enneactis* spawns at 28–30°C and has a postovulatory follicle duration less than 15 hours (Takita et al., 1983). Collectively, these observations confirm that increasing temperatures decrease the time that postovulatory follicles can be detected in fishes.

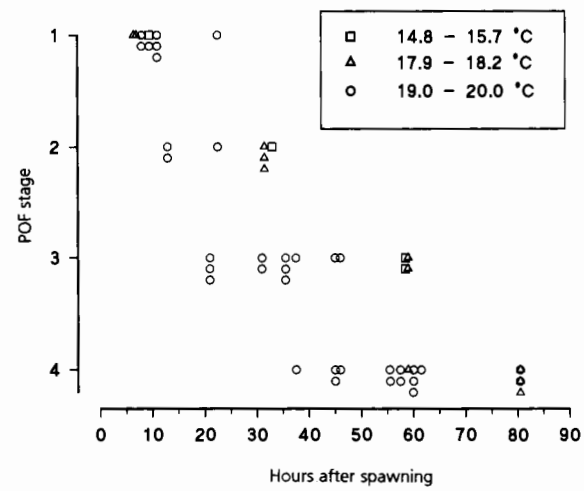

Figure 2

Chronology of spawning and postovulatory follicle (POF) degeneration for three temperature series (14.8–15.7°C, 17.9–18.2°C, and 19.0–20.0°C). Postovulatory follicle stages (1–4) are described in Table 1. All spawning times are set to midnight (hour-0) in order to make contrasts across temperature series (see text). Symbols slightly offset along the y-axis at each stage of degeneration correspond to multiple fish sampled at the same time.

The ability to identify the stage of postovulatory follicles in order to determine spawning frequencies becomes more important as their duration increases. If postovulatory follicles are known to last less than 24 hours, females possessing them can be defined with certainty as having day-0 postspawning ovaries for purposes of estimating spawning frequency. Hunter and Goldberg (1980) and Hunter and Macewicz (1985) developed this approach on the basis of the appearance of various features and assigned day-0 and day-1 classifications to fish known to have postovulatory follicle durations of about 48 hours. However, if durations are known to be longer, knowledge of the age of particular features of postovulatory follicles becomes more critical in classifying postspawning ovaries. We focused on the alignment of the granulosa layer (and nuclei), on the presence of vacuoles, and the appearance of the lumen for identifying stages because these features were easily recognizable in Atlantic menhaden postovulatory follicles and could be used to classify fairly discrete

stages (Table 1). In Atlantic menhaden spawning at about 20°C, disappearance of the lumen of the postovulatory follicle after loss of alignment of the granulosa layer (transition from stage 2 to 3) distinguished day-0 from day-1 postspawning ovaries. However, in several other species, features such as pycnotic nuclei, thickness of the thecal layer, and separation of the thecal and granulosa layers are diagnostic for classification of postspawning ovaries (Goldberg et al., 1984; Hunter and Macewicz, 1985; Agarwal et al., 1992).

Postovulatory follicle durations in *Engraulis* species spawning naturally or in those induced pharmacologically to spawn at similar temperatures are equivalent (Hunter and Goldberg, 1980; Goldberg et al., 1984) suggesting that laboratory observations can be used to access spawning frequencies of fish in the wild. Our estimates of the durations of particular features of postovulatory follicles must be considered preliminary on the basis of our small sample size, particularly for the 14.8–15.7°C treatment fish. However, our general finding that postovulatory follicle duration within Atlantic menhaden can vary from 36 to probably more than 60 hours corresponding to a 5°C range in temperature, supports the view that temperature should be an important consideration affecting spawning-frequency estimation in field surveys.

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