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Spencer F. Baird
First U.S. Commissioner
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Abstract—Gonad histology complements research on the life history of fish species and provides greater accuracy and precision than macroscopic characterization of the gonad for determining patterns of oogenesis at the cellular level and maturation at the individual level. In a fishery context, histology improves estimation of mature and spawning stock biomass, identification of spawning seasonality and grounds, and preselection of specimens for calculation of annual fecundity. However, in most studies, only a single staining method (hematoxylin and eosin) has been used. In this study on 3 taxonomically diverse species, using 4 different staining methods of varying complexity (degree of counterstaining), we compared confidence levels in identification of 8 stages of oogenesis, the presence and level of degradation for postovulatory follicles, and atretic (vitellogenic) germ cells. As anticipated, the method involving the least expensive, monochromatic stain provided the lowest level of confidence, whereas the most expensive and complex counterstaining method provided the highest confidence level, with hematoxylin and eosin staining and another simple counterstaining method in between them. The effect of staining method was most evident for identifying cortical alveoli, which can affect estimation of size or age at maturity, and for identifying postovulatory follicles, which can affect estimation of spawning frequency. These results are broadly applicable for determining best practices.

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A comparison of 4 histological staining methods for revealing oocyte development, atresia, and postovulatory follicles in 3 fish species

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The economic value of fish has long attracted interest in understanding how fish reproduce in the wild and through aquaculture. By the second half of the 19th century, a fundamental understanding of fish reproductive dynamics was emerging, specifically knowledge of how reproductive tissue in gonads generates cohorts of germ cells provisioned with yolk (Cunningham and Oxon, 1897; Fulton, 1898; Wallace, 1903). A powerful tool for this work was histology, in which gonad tissue was fixed, thinly sectioned, and stained for examination under a microscope. Early stains were adapted from the textile dyeing industry or from simple chemicals available in the laboratory (e.g., silver nitrate) (Titford, 2009; Riva et al., 2014). The use of even such basic stains yielded insight into major stages of oogenesis, such as the transition from mitotic oogonia to meiotic oocytes, the transition from unyolked to yolked oocytes, and the transition from oocytes to mature eggs. Recognition of how histochemical stains worked grew exponentially during the 20th century (Wick, 2012; Riva et al., 2014). Although many stains developed more than a century ago are still in use today, others have been replaced or discontinued because of their toxicity (Titford, 2009). Eventually, some stains became widely used because they were easy to formulate, were inexpensive, or provided improved resolution (e.g., hematoxylin and eosin [H&E]) (Javaeed et al., 2021), whereas others were more complex to execute but provided even finer resolution for examining subcellular features (e.g., trichrome stains) (Titford, 2009; Alturkistani et al., 2016).

Today, many different histological stains are available to highlight specific structures and features in cells and tissues of vertebrates, including fish species (Morrison, 1990; Grier et al., 2018; Smith et al., 2018; Uribe et al., 2019). Although different staining methods may yield different levels of identification and understanding, histological analysis of gonads with even

basic stains is considered superior to macroscopic methods in determining reproductive status (West, 1990; Ferreri et al., 2009; McBride et al., 2013). For these reasons, many fish biologists use gonad histology to increase the precision and accuracy of maturity estimates (McBride et al., 2013), spawning seasonality (Press et al., 2014; McElroy et al., 2022), and reproductive strategies for life history studies and stock assessments (Lowerre-Barbieri et al., 2023).

Gonad histology is, however, costly. In fishery science, we often characterize reproductive traits at the population level, in contrast to how, in medical science, doctors diagnose conditions of individuals. Consequently, even when subsampling, fishery biologists may examine the gonads of hundreds to thousands of individuals per species. They use gonad histology to examine specific features, such as the appearance of cytoplasmic inclusions or the migration of the nucleus, with the intent to estimate parameters, such as spawning seasonality, size at maturity, or the number of eggs produced per unit of time. Because of the large sample sizes required for fisheries population studies, it is necessary to optimize the cost-benefit ratio of different staining methods when using gonad histology. In other words, it is necessary to choose the most reliable method that can be used for the lowest cost. Such choices may be quite different between working in a research mode on species with yet unresolved patterns of oocyte development and reproduction and more routine monitoring of a familiar species. In a research mode, for example, for little additional cost, a complex stain can reduce the ambiguity of cytoplasmic features in the germ cells of an unfamiliar species or reduce the risk of biased or imprecise estimates of reproductive potential. Alternatively, in an operational mode for routine work, a simple stain suitable for a familiar species can allow more efficient processing of high numbers of slides, reducing the cost per slide.

Despite the diversity and utility of the different stains available, we determined that H&E is by far the most commonly used stain for most fish (and animal) studies (Riva et al., 2014), on the basis of a search in 2023 and 2024 of published papers on studies of fish reproduction in which gonad histology was used (number of articles=66) (Fig. 1). Only a few other stains, notably Mallory's trichrome, Masson's trichrome, periodic acid Schiff's (PAS) with counterstains of iron-hematoxylin and metanil yellow, and toluidine blue (TB) in sodium tetraborate, had been applied to fish species. The benefits of using H&E include a detailed view of intracellular and extracellular components and morphology of tissues, providing diagnostic information even when additional stains are applied for specific features. In clinical settings, H&E staining is used initially as the standard or routine method and additional stains (often referred to as *special stains*) are used only when further information is needed. The routine nature of H&E staining makes it amenable to use in fully automated systems to efficiently process large quantities. Although many complex stains provide increased contrast, clarity, and certainty in identifying key features, we postulate that they are too costly for routine monitoring programs. The cost of special stains can vary widely and can be equal or similar to the cost of H&E—or more than double the cost.

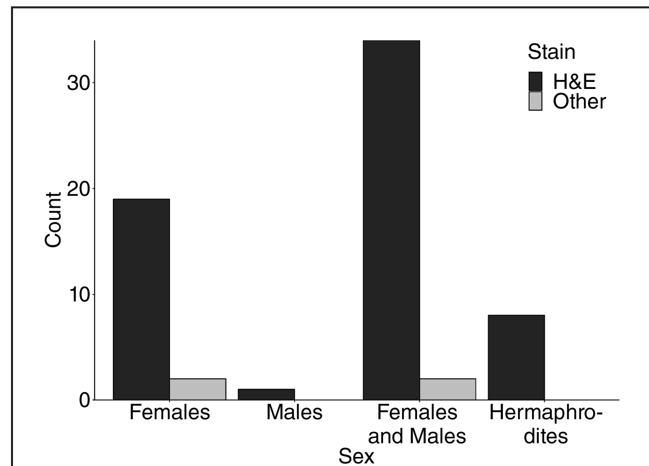

Figure 1

Number of research papers published in 2023 and 2024 on studies of teleost reproduction that involved the use of histological stains on gonad tissue, by sex of fish and category of stain. The 66 publications found in our literature search are classified on the basis of the sex of fish examined: females, males, both female and male individuals of gonochoristic species, or hermaphrodites. The papers are also separated into 2 categories based on the stains used in the studies: hematoxylin and eosin (H&E) or *Other*, which includes Mallory's trichrome, Masson's trichrome, a combination of periodic acid Schiff's, iron-hematoxylin, and metanil yellow, and a combination of toluidine blue and sodium tetraborate.

The objectives of this study were to evaluate the costs and benefits of 4 staining methods of varying complexity (levels of counterstaining) (Table 1) and to provide information that can help others determine the optimal stain considering the objective and resources available for their studies. The methods included one with a monochromatic stain and 3 techniques that involved counterstaining. Variation within each sample was minimized by applying all 4 staining methods to thin-tissue sections of the same sample (i.e., the same histology block). We chose 3 fish species that are economically important and phylogenetically diverse in the Northwest Atlantic Ocean: Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), and yellowtail flounder (*Myzopsetta ferruginea*). All 3 species have been previously reported to have group-synchronous oocyte development, with the larger, vitellogenic cohort of oocytes easily distinguishable leading up to spawning (Murua and Saborido-Rey, 2003). The spawning pattern for Atlantic herring differs from those of haddock and yellowtail flounder. Atlantic herring are total spawners, meaning they ripen, ovulate, and shed the whole annual clutch of eggs at once or over a short period of time (hours to days) (van Damme et al., 2009; Kennedy et al., 2011a; McPherson et al., 2011; Bucholtz et al., 2013). Haddock and yellowtail flounder are batch spawners,

Table 1

Type of oocyte development, spawning pattern, length ranges, stocks, collection times, sample sources, and gonadosomatic index (GSI) values for the female Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), and yellowtail flounder (*Myzopsetta ferruginea*) for which gonad tissue was analyzed by using 4 histological staining methods. Also provided are the numbers of fish from which tissue samples were taken, by species and stain. The 4 stains are toluidine blue, hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Price ranges for each stain, given in parentheses, are for the costs per slide of processing, embedding, and staining tissue samples at histology laboratories that we have used recently (in 2022–2024). Samples were taken from stocks in the Gulf of Maine (GOM), Georges Bank (GB), and Southern New England (SNE). Sample sources were bottom-trawl surveys (BTS) of the NOAA Northeast Fisheries Science Center (NEFSC), the Study Fleet program of the NEFSC Cooperative Research Branch (CRB) and fishery-dependent sampling of the Maine Department of Marine Resources (MEDMR). Additional information on the individual fish analyzed is provided in the Supplementary Table. FL=fork length; TL=total length.

	Species		
Characteristic	Atlantic herring	Haddock	Yellowtail flounder
Oocyte development	Group-synchronous	Group-synchronous	Group-synchronous
Spawning pattern	Total	Batch	Batch
Length	170–263 mm FL	408–676 mm FL	306–440 mm TL
Stock	GOM, GB	GOM, GB	GOM, GB, SNE
Years sampled	2015, 2016	2013, 2015	2010–2012, 2018, 2019
Months sampled	Sept.–Nov.	Jan.–March	March–June, Nov.
Sample sources	NEFSC BTS and CRB, MEDMR	NEFSC CRB	NEFSC CRB
GSI	<0.01–8.5	0.6–11.8	3.3–20.6
Stain (price per slide)	Number of fish used in tissue analysis		
Toluidine blue ($16.25–$17.25)	11	10	9
H&E ($11.00–$12.25)	11	10	9
PAS ($13.80–$17.25)	11	10	9
PAS-MT ($19.00–$23.00)	11	10	9

ripening, ovulating, and shedding multiple batches of eggs over a longer period of time (weeks to months) (Robb, 1982; Howell, 1983; Clay, 1989; Tronbøl et al., 2022).

We selected mature individuals of each species that best represented certain critical phases of oogenesis. Mature fish have the potential to have oocytes in multiple stages of development, including oogonia in nests, oocytes in various stages up to ovulation, and eggs, in addition to postovulatory follicles (POFs) and atresia, potentially present at one time (Table 2), in contrast to immature fish that have only oocytes in the primary growth stage. We included individuals in an early stage of maturing, with oocytes in the cortical alveolar stage; such oocytes can be difficult to detect with some histological stains. For example, Blazer (2002) notes that "[cortical] alveoli are produced within the oocyte and stain positively with a PAS (periodic-acid Schiff) stain," implying that these features may not show up without the use of a PAS stain. This reliance on the application of a PAS stain is important because some researchers do not consider cortical alveoli, which are produced endogenously within the oocyte (at the germ cell level), to be indicative of a mature female (at the individual level) whereas others do (see the "Discussion" section in Brown-Peterson et al., 2011; Prince et al., 2022). Another major step, vitellogenesis, according to Blazer (2002), "involves the sequestering (from the blood) and packaging of hepatically-derived [*sic*] vitellogenin," a step considered by some to be a threshold for maturity. The presence of oocytes in later additional stages, such as nucleus migration and ovulation, indicates that the fish is entering the second arrest phase (meiosis II), generally a sign that spawning is imminent for that individual. The presence of POFs indicates that the fish had already released at least one batch of eggs, if a batch spawner, or at least started to release its singular batch, if a total spawner. The presence and type of atretic germ cells was also recorded because these cells are often associated with past spawning activity, which may be particularly relevant when trying to distinguish an immature female from a mature female outside of the spawning season.

Because individual variation was minimized and fish in a range of maturity stages for 3 phylogenetically diverse

Table 2

Description of microstructural features recorded during readings of slides of stained gonad tissue from Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), and yellowtail flounder (*Myzopsetta ferruginea*) caught in the Northwest Atlantic Ocean during 2010–2019 (for details of collections, see Table 1). These features were used to identify the following 8 stages of oocyte development: perinucleolar, cortical alveolar, early vitellogenesis, late vitellogenesis, germinal vesicle migration, germinal vesicle breakdown, hydration, and ovulation. The stages can be categorized into 4 phases, primary growth, secondary growth, early maturation, and late maturation. Postovulatory follicles (POFs), follicular atresia, blood vessels, muscle bundles, and tunica are also described.

Microstructural features	Histological criteria
Primary growth	
Perinucleolar	Perinuclear oocytes have multiple nucleoli oriented around the periphery of the germinal vesicle. Chromatin nucleoli are observed at the same time and classified with perinucleolar oocytes as primary growth, as per Blazer (2002). Chromatin-nucleolus oocytes have a single or a few prominent nucleoli in the germinal vesicle and are highly basophilic.
Secondary growth	
Cortical alveolar	Cortical alveoli first appear in the cytoplasm along the periphery of the oocyte as small, circular, white inclusions (Blazer, 2002). Cortical alveoli will then fill with dark dots and increase in number and, as vitellogenesis proceeds, the cortical alveoli are “pushed” to the periphery of the oocyte (Blazer, 2002).
Early vitellogenesis	Lipoprotein yolk globules begin to appear around the periphery of the germ cell and advance inward toward the germinal vesicle. Cortical alveoli are still present around the zona pellucida.
Late vitellogenesis	Lipoprotein yolk globules continue to fill the whole cytoplasm and grow in size. A small ring of cortical alveoli can still be seen near the zona pellucida.
Early oocyte maturation	
Germinal vesicle migration	After the lipoprotein yolk globules fill the cytoplasm, the germinal vesicle migrates toward the animal pole of the zona pellucida.
Late oocyte maturation	
Germinal vesicle breakdown	While the germ cell remains in the follicle, the germinal vesicle breaks down and the lipoprotein yolk globules fuse, eventually becoming a hyaline, homogeneous mass (Blazer, 2002).
Hydration	Remaining within the follicle, yolk globules become fused and water content is absorbed, increasing the size of the oocyte significantly.
Ovulation	The hydrated oocyte is released into the lumen from the follicle cells and overlying germinal epithelium, leaving behind the POF and becoming an egg.
Postovulatory follicle	
Recent	A complex structure consisting of granulosa cells (inner layer) and theca cells (outer layer). The columnar granulosa cells are typically separated from the theca. The follicle is loosely arranged and irregular in shape with a lumen larger than a perinucleolar oocyte.
Intermediate	The granulosa and theca cell layers remain distinguishable. The follicle is more compact and approximately the size of a perinucleolar oocyte. The lumen is still visible but much smaller in size than recent POFs.
Old	The 2-layer structure may be identifiable in some instances, but cell integrity is greatly deteriorated. The follicle is almost entirely collapsed without a distinguishable lumen and is smaller in size than a perinucleolar oocyte.
Follicular atresia	
Alpha	The germinal vesicle disintegrates, the zona pellucida breaks down, the basement membrane remains intact, and lipoprotein yolk globules are present in early and late vitellogenic oocytes.
Beta	Internal oocyte components (lipoprotein yolk globules and cortical alveoli) are digested through phagocytosis. Oocyte has a bubble-like appearance.
Other structures	
Blood vessels	Blood vessels or capillaries identified within the muscle tissue.
Muscle bundles and connective tissue	The presence of muscle bundle or connective tissue.
Tunica and tunica thickness	The presence of tunica and tunica thickness. The thickness of tunica varies between species and degree of ovarian development, and a measurement is recorded if present.

species were selected, the results of this study should be generally applicable to a wide range of species and, therefore, of interest to others considering the advantages and disadvantages of different staining methods for gonad histology.

Materials and methods

For this comparison study, we focused on ovarian (female) histology. As noted in the "Introduction" section, we selected 3 fish species of the Northwest Atlantic Ocean—Atlantic herring, haddock, and yellowtail flounder—to generalize phylogenetic diversity. We selected 8 stages of oogenesis, identifying major transitions of oocyte development (Table 2), and evaluated different methods of stain preparation by using samples of the same individuals from each species to minimize individual variation.

Individuals were selected from recent (Press et al., 2014; McElroy et al., 2016; Wuenschel and Deroba, 2019) and ongoing projects to best represent the variety of oocyte development stages in mature females within each species. These individuals were collected from fishery-independent (NOAA Northeast Fisheries Science Center [NEFSC] seasonal bottom-trawl surveys) and fishery-dependent (NEFSC Cooperative Research Branch Study Fleet) sources. The total length or fork length and the weight of fish were measured to the nearest millimeter and 0.1 g. The weight of gonads was measured to the nearest 0.001 g. The gonadosomatic index (GSI) for fish was calculated as follows:

$$GSI = 100(gonad\ weight\ /\ [total\ weight - gonad\ weight]).$$

Mature fish were chosen because they had oocytes in multiple stages of development present at one time (Table 2). The progression through the cortical alveolar and vitellogenic stages can be protracted in cold-water species, in extreme cases taking several years (Kennedy et al., 2011b; Press et al., 2014; McBride et al., 2022a, 2022b); therefore, these stages do not represent functional maturity if observed a year or more before a fish spawns for the first time (Lefebvre and Field, 2015; Pacicco et al., 2023). Once oocytes complete development, release from the follicle to become eggs, and are spawned, the presence of POFs helps in determining if a fish had successfully spawned (total) or had started to release some eggs (batch, partially spent).

Gonad samples were preserved in 10% buffered formalin for more than 1 month before the cutting of cross sections of 3–5 mm, and the cross sections were transferred into cassettes and sent to a histology lab. In the histology lab, tissue was dehydrated in an increasing series of ethyl alcohol concentrations, embedded in paraffin, and sectioned to 5 µm (Shaw et al., 2012). The 4 histological stains ranged from a simple monochromatic stain, TB, to the most complex stain, a combination of PAS and Mallory's trichrome (PAS-MT).

Toluidine blue is a basic thiazine metachromatic dye and binds to nuclear material of tissues with DNA and RNA content (Sridharan and Shankar, 2012). Slides stained with TB appear monochromatic in an array of blue hues with a nucleus of a violet-like color. Although TB is theoretically a simpler stain than H&E, the cost for TB-stained slides from production laboratories may be comparable to the cost for slides stained with H&E, unless a laboratory has an automated process for this stain. The standard TB protocol is very simple, with a single short dip in the stain solution followed by a rinse under running water; therefore, a less expensive option for some researchers may be to obtain sectioned, unstained slides that they can stain themselves. The method of staining with TB is suitable for circumstances in which laboratory resources are limited and is the standard technique applied in studies of coral-reef fishes carried out at remote field locations (Longenecker and Langston, 2018; Longenecker et al.[1]).

Hematoxylin and eosin was selected because of the familiarity that most histologists and fisheries scientists have with this stain as the standard or routine stain used in fisheries research (Shaw et al., 2012). In staining with H&E, slides were initially stained with the hematoxylin component, which has a deep blue-purple color that binds to nucleic acids. Next, the counterstain eosin was applied, staining the cytoplasm and extracellular matrix in varying degrees of pink. With the H&E staining method, tunica and muscle tissue appear light pink, the nucleus is pink, and the zona pellucida and lipoprotein yolk globules become a dark pink. Postovulatory follicles in all stages appear in a variety of pink hues.

A more complex stain, PAS can be used to discern the presence of carbohydrates and carbohydrate compounds. In the initial reaction, the periodic acid acts as an oxidizing agent, producing Schiff reactive aldehyde groups. In the second step, the tissue sections react with the Schiff's reagent to produce a bright magenta color. Hematoxylin is then used as a counterstain to reveal other tissue elements. With PAS staining, tunica and muscle tissue appear purple and oocytes have a light-purple nucleus, dark-purple cytoplasm, and purple lipoprotein yolk. The POF stages are stained from light to dark purple, with intermediate and old POFs having a definite magenta color.

The most complex stain we evaluated was PAS-MT. For this stain, the sample initially goes through the PAS staining protocol (as described in the previous paragraph), before application of the trichrome stain that consists of aniline blue, acid fuchsin, and orange *G* to help reveal more detail (Mallory, 1900; Titford, 2009). Although this stain is not widely used in fisheries research, per our literature searches (see Fig. 1), we have published comprehensive plates depicting oogenesis with this stain for several species: winter flounder (*Pseudopleuronectes americanus*) (Press et al., 2014), American shad (*Alosa sapidissima*) (McBride et al., 2016), Atlantic herring (Wuenschel and

[1] Longenecker, K., R. Langston, and E. C. Franklin. 2020. Standard operating procedure for histology-based rapid reproductive analysis of tropical fishes, 93 p. Report prepared by the University of Hawaii at Manoa for Tetra Tech's Supplemental Technical Assistance to the USAID SEA Project and Walton Family Foundation. Univ. Hawaii Manoa, Honolulu, Hawaii.

Deroba, 2019), Atlantic halibut (*Hippoglossus hippoglossus*) (McBride et al., 2022a), Atlantic wolffish (*Anarhichas lupus*) (McBride et al., 2022b), and cusk (*Brosme brosme*) (McElroy et al., 2022). We obtained the formula for this stain (Witthames[2]), which has been used to distinguish old POFs that have a basal membrane from atretic cells in Atlantic cod (*Gadus morhua*) (Witthames et al., 2010), and provide it in the Supplementary Materials because it is not widely published in histology handbooks. The PAS-MT stain reveals much more contrasting detail than the other stains used in this study, highlighting critical characteristics of oogenesis that help to determine the maturity and spawning phases of individual fish. With PAS-MT staining, the cytoplasm is light gray, the nucleus has a blue hue, and the lipoprotein yolk globules are red. The POFs are also very distinct, appearing dark purple, and can be easily identified and categorized into different stages.

Histology slides were scanned with an Ocus 40[3] digital microscope (model MGU-00003; Grundium, Tampere, Finland) and read by using the software Aperio ImageScope, vers. 12.4.6.5003 (Leica Biosystems, Nussloch, Germany). Three readers analyzed each histology slide independently. Expertise levels differed slightly among readers, but each of them had >10 years of experience reading histology of multiple species, primarily using PAS-MT, PAS, and some H&E preparations, but with exposure and familiarity to all stains through workshops and published literature. Readers recorded the most advanced stage of oocyte development and all other oocyte stages present, the presence of POFs and the stage of each POF (recent, intermediate, or old), the presence of atresia and the stage of atresia (alpha and beta), the presence of blood vessels and muscle bundles, and the presence of tunica and tunica thickness (Table 2). Each reader annotated representative stages of oocyte development and other features on the slides. In addition, the confidence level (high, medium, or low) was indicated for each recorded feature. High confidence was recorded if the reader had 100% certainty in identification of the microstructural feature, medium confidence was recorded if the reader was 50% certain in identification of the feature, and low confidence was recorded if the reader was unsure but tentatively noted the feature as present. In addition, to get a more general sense of the readability of each slide, each reader also recorded the overall confidence in the interpretation of each slide as high, medium, or low.

Results

For each of the 30 gonad samples, we evaluated 4 separate slides, each prepared with 1 of the 4 histological stains (Suppl. Table). A total of 3265 features were recorded from the 360 slide readings (3 readers×120 slides). Herein, results are summarized first by species, then confidence levels for identification of various features (i.e., oocyte development stages, POFs, and atresia) are described, and finally a comparison of stains and features across species is provided.

Oogenesis

Atlantic herring Across all 4 staining methods, oocytes in 7 of the 8 stages of oogenesis (all but ovulation) were identified in the slides for selected samples of Atlantic herring, with the presence of oocytes in stages from primary growth through hydration and other microstructural features, such as degradation of POFs and follicular atresia, recorded. Primary growth oocytes, which included those in chromatin nucleolar and perinucleolar stages, were present in every slide. Secondary growth stages, beginning with the presence of cortical alveoli, were detected with all stains (Fig. 2, A–D). Cortical alveoli were present initially around the periphery of the oocyte as clear circular structures, with darker staining inclusions evident in all slides but the ones made with the TB stain. The cortical alveoli increased in number and size, eventually being pushed to the outer edge of the cytoplasm. Early stage vitellogenesis (Fig. 2, E–H) began when lipoprotein yolk globules began to form around the periphery of the oocyte, eventually increasing in size and number and filling the cytoplasm with yolk. Once filled with lipoprotein yolk globules, the germinal vesicles migrated toward the zona pellucida of the oocyte, marking final oocyte maturation (Fig. 2, I–L). Hydration was the most advanced stage of oogenesis we recorded in Atlantic herring, identified when the lipoprotein globules were completely fused and when water uptake increased the size of the oocytes but they remained within the follicle.

Three stages of POFs were identified in Atlantic herring across all 4 methods of staining (Fig. 3). Recent POFs were irregular in shape and, in slides with some stains (PAS and PAS-MT), the theca and granulosa layers could be more easily distinguished (Fig. 3, G and H). Recent POFs were larger than a perinucleolar oocyte and had a large lumen (Fig. 3, A–D). The 2 layers were still visible in intermediate POFs (Fig. 3, E–H), which had a collapsed lumen and were smaller. Old POFs were significantly smaller than a perinuculeolar oocyte with a completely collapsed lumen, and the theca and granulosa layers were almost completely indistinguishable (Fig. 3, I–L).

Follicular atresia was categorized as alpha or beta atresia for Atlantic herring (Fig. 4). Alpha atresia was visible by the breakdown of the zona pellucida and the disintegration of the germinal vesicle (Fig. 4, A–D). Advanced (or late) alpha atresia was identified with the PAS-MT stain (Fig. 4H) because of the visible presence of lipoprotein yolk globules in a smaller, partially resorbed oocyte. Beta atresia, defined by the absence of lipoprotein yolk globules and cortical alveoli in the cytoplasm due to phagocytosis (Fig. 4, E–G), was rare in the samples selected and was present in 3 of the 11 individuals for

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[2] Witthames, P. R. 2009. Personal commun. Cent. Environ. Fish. Aquac. Sci., Lowestoft Lab., Pakefield Rd., Lowestoft, Suffolk NR33 0HT U.K.

[3] Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.

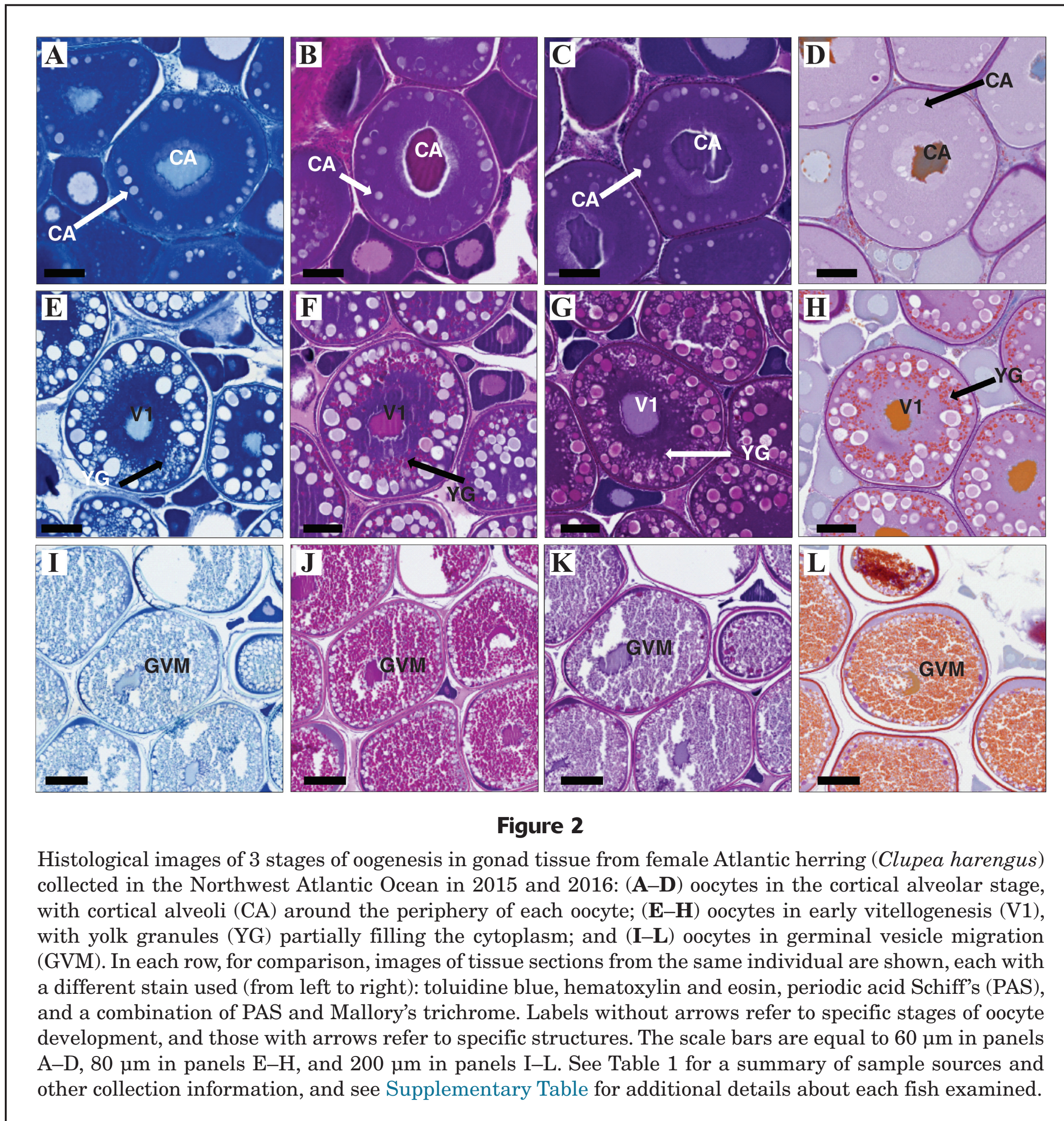

Figure 2

Histological images of 3 stages of oogenesis in gonad tissue from female Atlantic herring (*Clupea harengus*) collected in the Northwest Atlantic Ocean in 2015 and 2016: (**A–D**) oocytes in the cortical alveolar stage, with cortical alveoli (CA) around the periphery of each oocyte; (**E–H**) oocytes in early vitellogenesis (V1), with yolk granules (YG) partially filling the cytoplasm; and (**I–L**) oocytes in germinal vesicle migration (GVM). In each row, for comparison, images of tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. Labels without arrows refer to specific stages of oocyte development, and those with arrows refer to specific structures. The scale bars are equal to 60 µm in panels A–D, 80 µm in panels E–H, and 200 µm in panels I–L. See Table 1 for a summary of sample sources and other collection information, and see Supplementary Table for additional details about each fish examined.

which slides were examined. Sections from 1 fish had multiple examples of beta atresia, which was recorded by all readers for all stains. In the other 2 fish, beta atresia was rare (usually a single instance annotated) and was recorded with a low level of confidence by 1 or 2 readers, for one or more stains (for PAS-MT or for H&E, PAS, and PAS-MT).

Haddock Across all 4 staining methods, oocytes in 7 of the 8 stages of oogenesis (all but ovulation) were visible in the slides for selected samples of haddock. Primary growth oocytes were present in every histology slide. Cortical alveoli marked the secondary growth stage of oogenesis and first appeared in the periphery of the cytoplasm as small, circular, white inclusions (Fig. 5, A–D). Early vitellogenesis followed the cortical alveolar stage, with the presence of lipoprotein yolk globules forming around the germinal vesicle (Fig. 5, E–H). As oocytes reached final oocyte maturation, multiple stages of oogenesis (representing subsequent batches) were present in individual haddock (Fig. 5, I–L). The migration of the germinal vesicle toward the zona pellucida marked final oocyte maturation. Hydrated oocytes were also present and were identified as oocytes that remained within the follicle after the yolk globules completely fused together and water content was absorbed.

All 3 stages of POFs and both stages of follicular atresia were identified for haddock across all 4 methods of staining (Fig. 6). Recent POFs were irregular in shape, the theca and granulosa layers were distinctly visible, and the lumen was larger than a perinucleolar oocyte (Fig. 6, A–D). Intermediate POFs were slightly smaller, with a collapsed lumen (Fig 6, E–H), and in slides made with PAS and PAS-MT stains, the contrast in colors between the theca and granulosa layers was more pronounced. Old POFs were smaller than a perinucleolar oocyte, with an absent

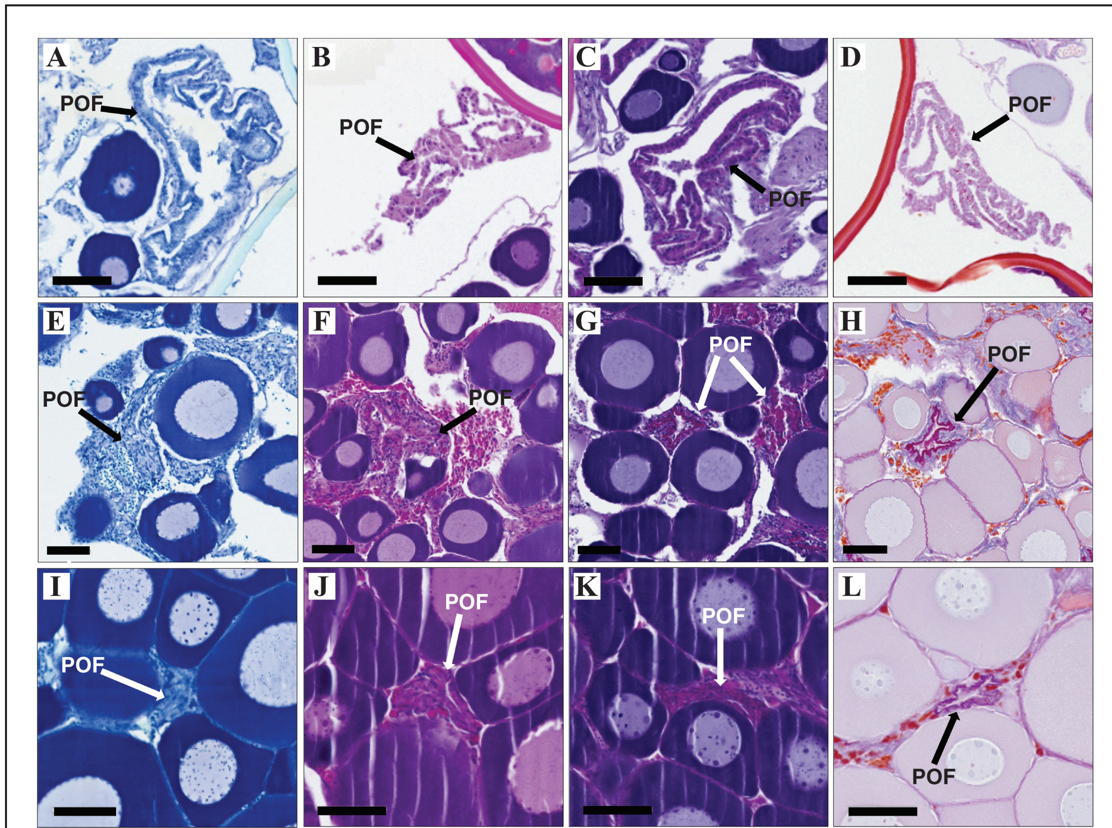

Figure 3

Histological images of 3 stages of postovulatory follicles (POFs) in gonad tissue from female Atlantic herring (*Clupea harengus*) collected in the Northwest Atlantic Ocean in 2015 and 2016: (**A**–**D**) recent, (**E**–**H**) intermediate, and (**I**–**L**) old. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 80 µm in panels A–D and 60 µm in panels E–L.

lumen and no distinction of the theca and granulosa layers (Fig. 6, I–L).

Follicular atresia was recorded as alpha or beta atresia (Fig. 7). Alpha atresia had a melted or liquefied appearance, with the zona pellucida breaking down but the contents within the cytoplasm sometimes still intact. Follicles in beta atresia were smaller in size than those with alpha atresia, and the internal components of the cytoplasm had been digested through phagocytosis.

Yellowtail flounder Across all 4 staining methods, oocytes in all 8 stages of oogenesis were identified in the slides for selected samples of yellowtail flounder. Primary growth oocytes were present in every histology slide. The cortical alveolar stage was identified with each stain used in this study, with oocytes slightly larger than a primary growth oocyte and faint circular, white inclusions near the periphery of the oocyte (Fig. 8, A–D). Early vitellogenesis was recorded when lipoprotein yolk globules began to appear as small dots close to the zona pellucida (Fig. 8, E–H). Similar to the haddock, the yellowtail flounder is a batch-spawning species, and multiple oocyte stages were identified in fish with oocytes in the late vitellogenesis stage and the late maturation phase (Fig. 8, I–L). Late vitellogenesis was recorded when the lipoprotein yolk globules filled more than half of the cytoplasm, followed by the migration of the germinal vesicle. Germinal vesicle migration marked the final phase of oocyte maturation. In addition to germinal vesicle migration, hydrated oocytes still within the follicle were identified (Fig. 8, I–L). Ovulation was also observed in yellowtail flounder, with fully hydrated oocytes released from follicles and becoming eggs (Fig. 8, M–P).

All 3 stages of POFs were identified in yellowtail flounder across all 4 methods of staining (Fig. 9). Recent POFs were irregular in shape, giving them a wispy appearance, with a visible lumen and the theca distinguished from the granulosa layer in slides with most stains (Fig. 9, A–D). Intermediate POFs (Fig. 9, E–H) were widely seen and were more compact in size, with a collapsed lumen and thicker layers. Old POFs (Fig. 9, I–L) were completely degraded with no visible lumen, and in some cases, these

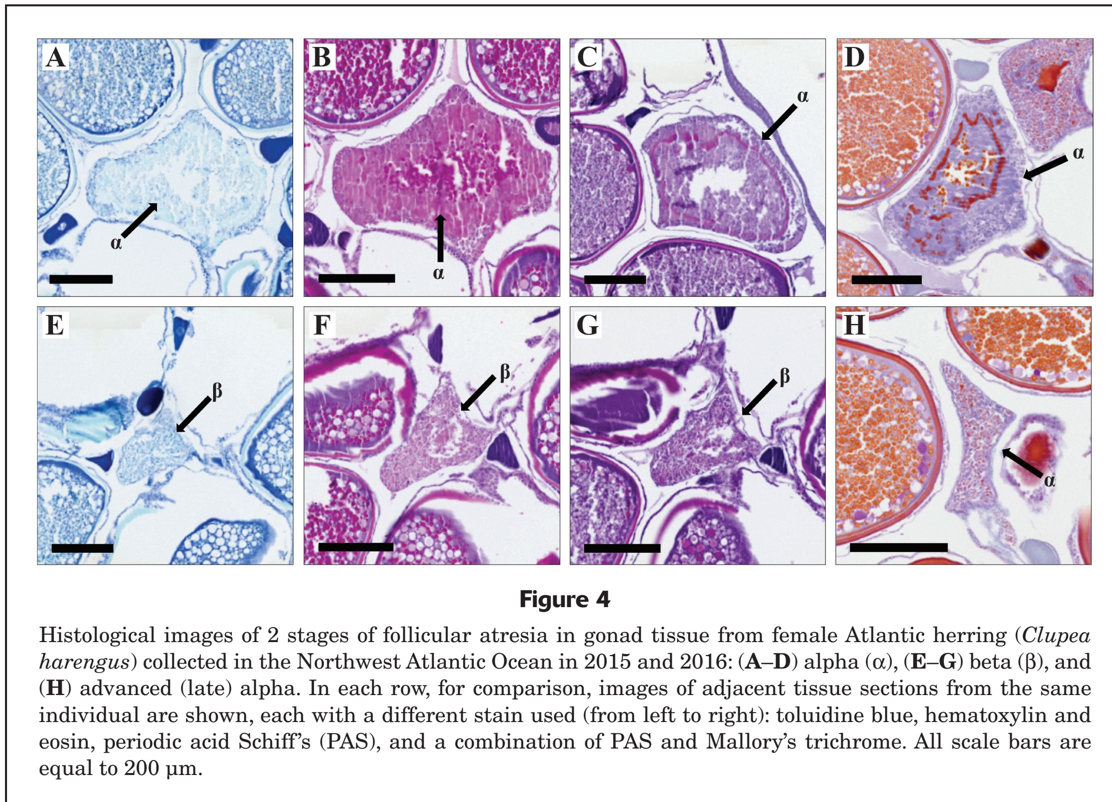

Figure 4

Histological images of 2 stages of follicular atresia in gonad tissue from female Atlantic herring (*Clupea harengus*) collected in the Northwest Atlantic Ocean in 2015 and 2016: (**A**–**D**) alpha (α), (**E**–**G**) beta (β), and (**H**) advanced (late) alpha. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. All scale bars are equal to 200 µm.

POFs were more easily identifiable because they appeared darker in color (Fig. 9, K and L).

In addition, follicular atresia was also recorded for yellowtail across all 4 staining methods (Fig. 10). Alpha atresia was identified as having a melted or liquefied appearance, with the zona pellucida broken down and becoming discontinuous and irregular in thickness (Fig. 10, A–D). Beta atretic oocytes were more completely degraded, without any remaining yolk or zona pellucida, and they were smaller in appearance because the contents of the cytoplasm were digested through phagocytosis (Fig. 10, E–H).

Confidence levels for features

Oocyte development stages Confidence levels for identification of stages of oocyte development varied across all species for each staining method (Fig. 11). For each species, primary growth oocytes were easily distinguished with all 4 stains.

The cortical alveolar stage was the most difficult stage to consistently identify, and lack of certainty is evident for each species for all 4 stains. The PAS-MT staining method provided the highest confidence level for identification of cortical alveoli, in comparison to use of the TB stain, the method that had the lowest level of confidence. Similar results were found when using the H&E and PAS stains to identify oocytes with cortical alveoli. A closer look at the defining stage of cortical alveoli revealed a slight advantage in recognizing small, circular inclusions for the H&E stain over the PAS stain (Fig. 12).

Confidence levels in identifying the early vitellogenesis stage differed among the species and stains (Fig. 11). The highest confidence levels were found for the PAS-MT staining method. Use of the H&E and PAS stains resulted in similar, slightly lower confidence levels, and distinguishing lipoprotein yolk globules was most difficult with TB staining.

The stages of late vitellogenesis and germinal vesicle migration were easy to identify for all 3 species, with a very low percentage of uncertainty, when the TB and PAS stains were used (Fig. 11). For the identification of the stage of germinal vesicle breakdown, confidence levels were high for the PAS-MT and PAS staining methods, and there is some ambiguity in results from the use of the H&E stain. The lowest level of confidence in identifying germinal vesicle breakdown was found for TB staining.

Hydrated oocytes were easily identified with all stains. There was minor variation in confidence levels for distinguishing these oocytes, with high levels for the PAS and PAS-MT staining methods (Fig. 11). For both H&E and TB staining, levels of confidence were slightly lower but still medium to high.

The confidence levels for recognizing ovulation were highest for the PAS-MT staining method and lowest for the TB staining protocol, but this stage was only observed in yellowtail flounder (the samples from individuals of other species did not have oocytes in this stage) (Fig. 11).

Postovulatory follicles The confidence levels of readers in identifying and staging POFs ranged from high with

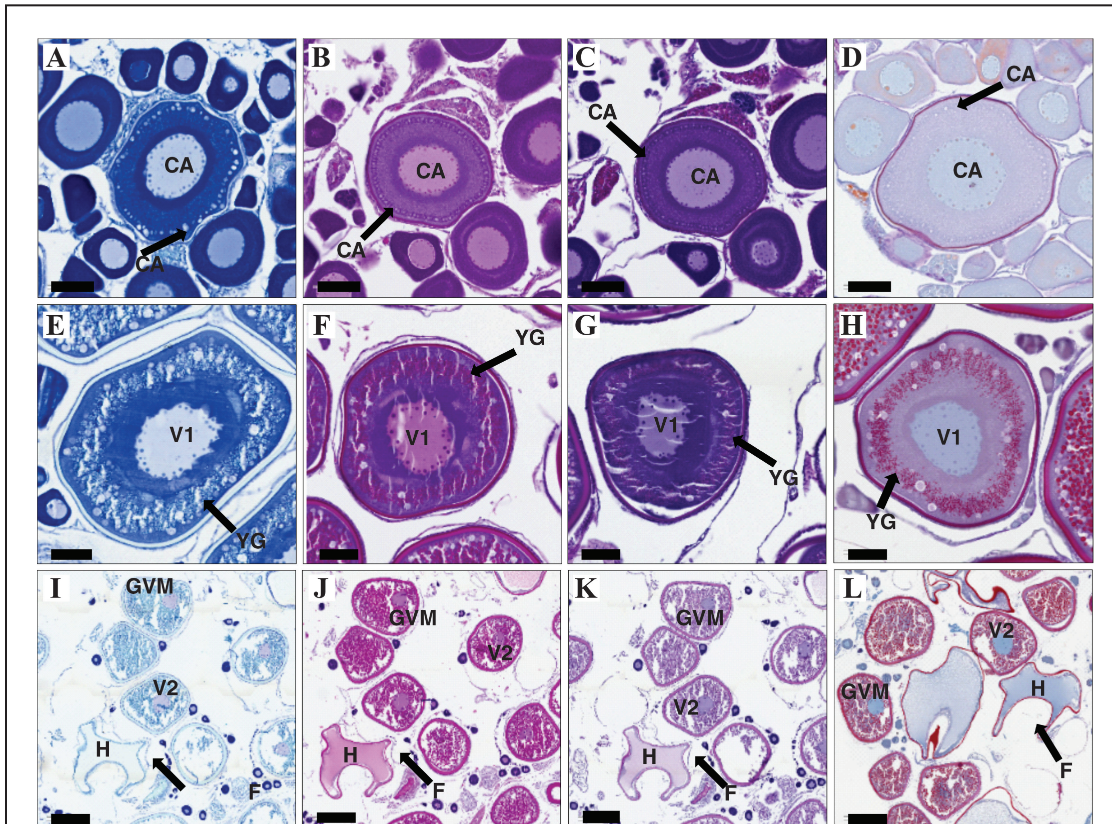

Figure 5

Histological images of 3 stages of oogenesis in gonad tissue from female haddock (*Melanogrammus aeglefinus*) collected in the Northwest Atlantic Ocean in 2013 and 2015: (**A**–**D**) oocytes in the cortical alveolar stage, with cortical alveoli (CA) around the periphery of the oocyte; (**E**–**H**) oocytes in early vitellogenesis (V1), with yolk granules (YG) partially filling the cytoplasm; and (**I**–**L**) oocytes representing multiple spawning batches—oocytes in late vitellogenesis (V2) and germinal vesicle migration (GVM) and a hydrated (H) oocyte within the follicle (F). In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. Labels without arrows refer to specific stages of oocyte development, and those with arrows refer to specific structures. The scale bars are equal to 60 µm in panels A–H and 400 µm in panels I–L. See Table 1 for a summary of sample sources and other collection information, and see Supplementary Table for additional details about each fish examined.

PAS-MT staining to low with TB staining (Fig. 13). Recent POFs were easily detectable for all species in slides with the PAS-MT stain and the least identifiable in slides with the TB stain. The H&E and PAS staining methods provided similar levels of confidence in identifying recent POFs, but results varied among species. For Atlantic herring, uncertainty in detection of recent POFs was greater with the PAS stain than with the H&E stain; however, the reverse occurred for haddock.

Intermediate POFs were most confidently identified by using the PAS-MT stain for all 3 species, with slightly lower certainty recorded by readers for haddock. As a POF collapses and becomes more compact and possibly confused with late-stage atresia, the concentric layers (theca and granulosa) of the follicle are brightly stained when using a PAS stain; therefore, in this study, use of the PAS stain resulted in greater confidence levels for identifying intermediate POFs than use of the H&E stain. Intermediate POFs were hardest to recognize when the TB stain was used.

Old POFs are arguably the hardest POF stage to identify for all 3 species. When the PAS-MT and the TB stains were used, the highest and lowest levels of confidence were recorded, respectively. Results from the use of the H&E and PAS stains were similar, but when readers directly compared magnified images of an old POF (Fig. 14), they observed more detail in slides with the PAS stain than in those with H&E.

Atresia Both alpha and beta atresia were identified for all species and staining methods. When using the PAS-MT stain, readers had the highest certainty in recognizing

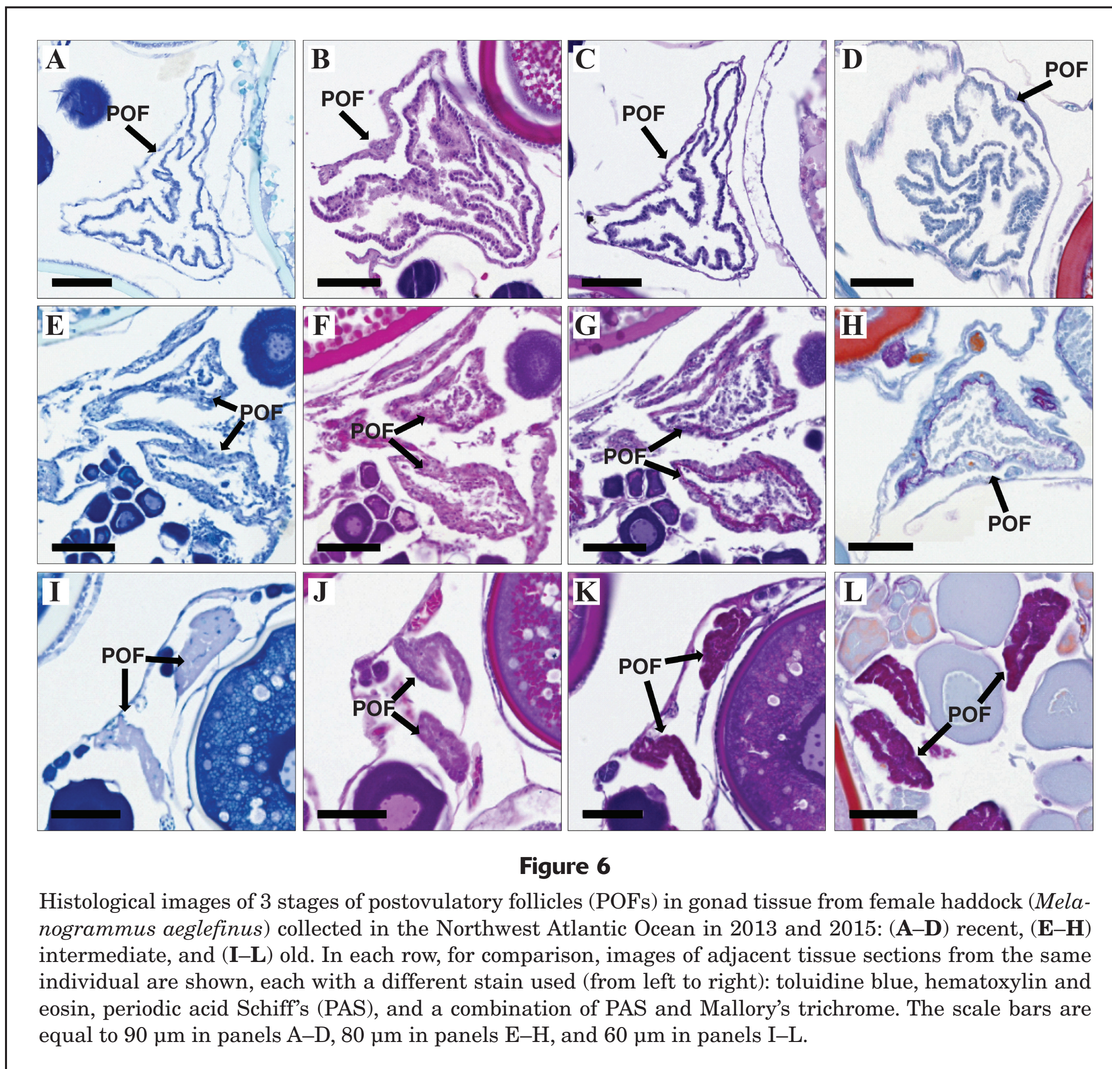

Figure 6

Histological images of 3 stages of postovulatory follicles (POFs) in gonad tissue from female haddock (*Melanogrammus aeglefinus*) collected in the Northwest Atlantic Ocean in 2013 and 2015: (**A**–**D**) recent, (**E**–**H**) intermediate, and (**I**–**L**) old. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 90 µm in panels A–D, 80 µm in panels E–H, and 60 µm in panels I–L.

alpha atresia for all 3 species, but identifying it with the TB stain proved to be difficult (Fig. 15). Use of the H&E and PAS stains resulted in similar confidence levels for recognition of alpha atresia. Beta atresia was a bit more difficult to identify because of the smaller size of oocytes in this stage and their similarity to an old POF. Readers were most confident in determining the presence of beta atresia when the PAS-MT stain was used, and beta atresia was much harder to confidently identify with the H&E, PAS, and TB stains.

Confidence levels across species

When all 3 species were compared, confidence levels across all 4 staining methods were the highest for yellowtail flounder, followed by Atlantic herring and then haddock (Fig. 11). For yellowtail flounder, a confidence level of nearly 100%, with minimal uncertainty, was found when the PAS-MT stain was used to identify the cortical alveolar stage. In slides with TB and PAS stains, identification of the cortical alveolar stage proved more difficult than in slides with the H&E stain. Confidence levels also were low for readings of the TB slides in identifying the final 2 stages of oocyte maturation (germinal vesicle breakdown and hydration) and ovulation.

The PAS-MT staining method proved to be the best approach for identifying all stages of oogenesis in Atlantic herring. The overall confidence level for use of the H&E stain was slightly better than that for use of the PAS stain, and the most uncertainty occurred with the TB stain. For all 4 staining methods, readers had some hesitation in recognizing cortical alveoli in Atlantic herring. The highest level of confidence was found for use of the PAS-MT stain, followed by use of the H&E stain. With the PAS and TB stains, identification of cortical alveoli in oocytes was equally as difficult. Readers had slight uncertainty in distinguishing germinal vesicle migration when using the PAS and TB stains, and they lacked some confidence in identifying hydrated oocytes when using the H&E and TB stains.

The greatest difference in confidence levels across staining methods occurred for haddock. Use of the PAS-MT and

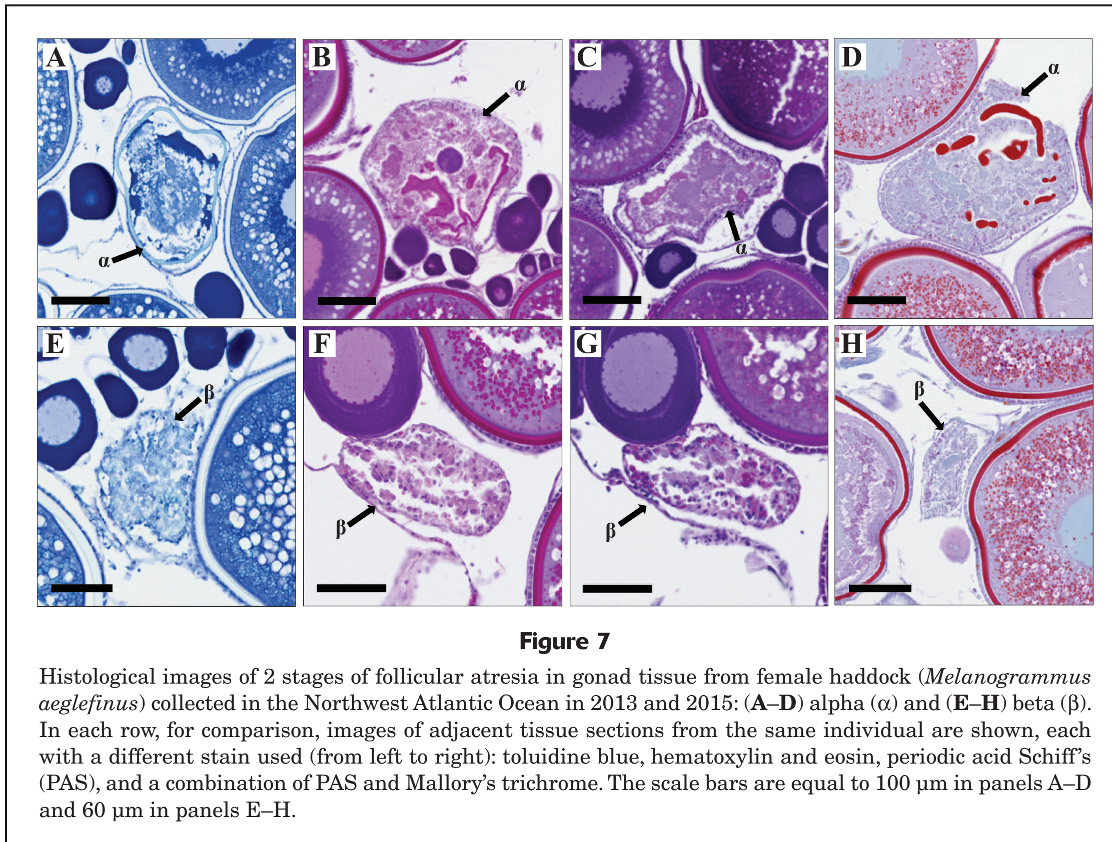

Figure 7

Histological images of 2 stages of follicular atresia in gonad tissue from female haddock (*Melanogrammus aeglefinus*) collected in the Northwest Atlantic Ocean in 2013 and 2015: (**A**–**D**) alpha (α) and (**E**–**H**) beta (β). In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 100 μm in panels A–D and 60 μm in panels E–H.

H&E stains resulted in higher confidence levels for identifying oocyte development stages than use of the other stains. For the cortical alveolar stage, the certainty of readers was slightly better with the PAS-MT stain than with the H&E stain. Distinguishing cortical alveoli in oocytes was difficult with both the PAS and TB stains. With all stains, readers had some uncertainty in recognizing early vitellogenesis, with the PAS-MT staining method being the best for identification of early lipoprotein yolk globules and TB staining being the technique with which it was most difficult. Germinal vesicle breakdown was sometimes difficult to consistently identify by using the PAS and TB stains.

Discussion

In this study, we took a comprehensive look at histological staining methods, comparing their effectiveness for identification of different stages of oogenesis and related gonad characteristics of fish across 3 phylogenetically different species. Although the same features should have been observed in slides with different stains for each individual, we found differences among stains in the accuracy in identifying certain structures. Studies in which tissue staining techniques have been compared by using samples for different individuals are useful (e.g., Alonso-Fernández et al., 2011); however, such assessments are less powerful than comparisons made by using samples from the same individuals, as was done in our study (and in Lowerre-Barbieri et al., 2023) to remove individual variation. Herein, we discuss the advantages and disadvantages of the staining methods we evaluated, highlight the potential implications of the different levels of information and confidence obtained by using these methods for fish reproduction studies, and suggest best practices for gonad histology of fish.

Staining methods

Ultimately, the method involving the most complex stain, PAS-MT, proved to be the best for confident identification of all stages of oogenesis, including early maturation of oocytes, postspawning indicators (POFs and thick tunica), and atresia. Although we acknowledge that the true development stages were not known, we equate high confidence levels with high precision (repeatability of measures) and high accuracy (reduced bias). Readers had more experience with the PAS-MT stain, but the higher confidence they had in using that stain reflects the greater detail they observed for specific features and structures and was not simply due to familiarity. A disadvantage of this complex stain, aside from its high cost, is that the pararosaniline in the PAS-MT formula is a carcinogen and requires special handling (Coghill, 1996).

The contrasting colors of the PAS-MT stain highlight important cellular features, in a way that those of the

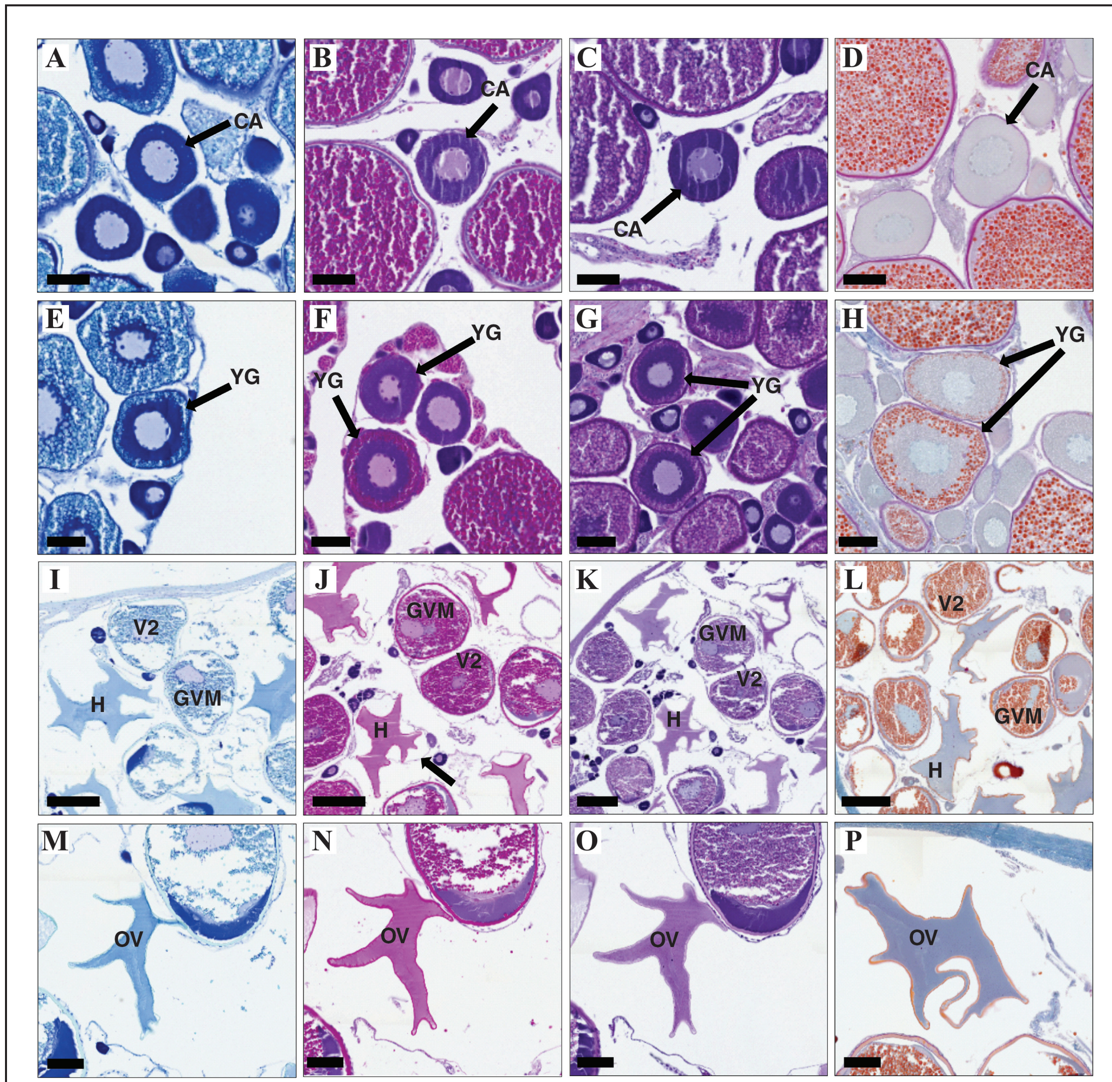

Figure 8

Histological images of 4 stages of oogenesis in gonad tissue from female yellowtail flounder (*Myzopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2012, 2018, and 2019: (**A**–**D**) oocytes in the cortical alveolar stage, with cortical alveoli (CA) around the periphery of the oocyte; (**E**–**H**) oocytes in early vitellogenesis (V1), with yolk granules (YG) partially filling the cytoplasm; (**I**–**L**) oocytes representing multiple spawning batches—oocytes in late vitellogenesis (V2) and germinal vesicle migration (GVM) and hydrated (H) oocytes within the follicle (F); and (**M**–**P**) ovulated (OV) eggs. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. Labels without arrows refer to specific stages of oocyte development, and those with arrows refer to specific structures. The scale bars are equal to 60 µm in panels A–H, 300 µm in panels I–L, and 100 µm in panels M–P. See Table 1 for a summary of sample sources and other collection information, and see Supplementary Table for additional details about each fish examined.

3 simpler, less expensive, and more commonly used stains—TB, H&E, and PAS—do not. Cytoplasm has a lighter color with the PAS-MT stain, allowing cortical alveoli and lipoprotein yolk globules to stand out. The presence of cortical alveoli is used to identify when a female has crossed a key physiological threshold of hormone and gonad development (Lowerre-Barbieri et al., 2023) toward maturation, even though some species may take several months or years to attain functional maturity (Kennedy et al., 2011b; Press et al., 2014; McBride et al., 2022b). In samples from fish that had spawned, confidence levels in identifying all stages of POFs were highest when the PAS-MT stain was used. As a POF degrades, the deep-purple color of this stain remains in the follicle layers,

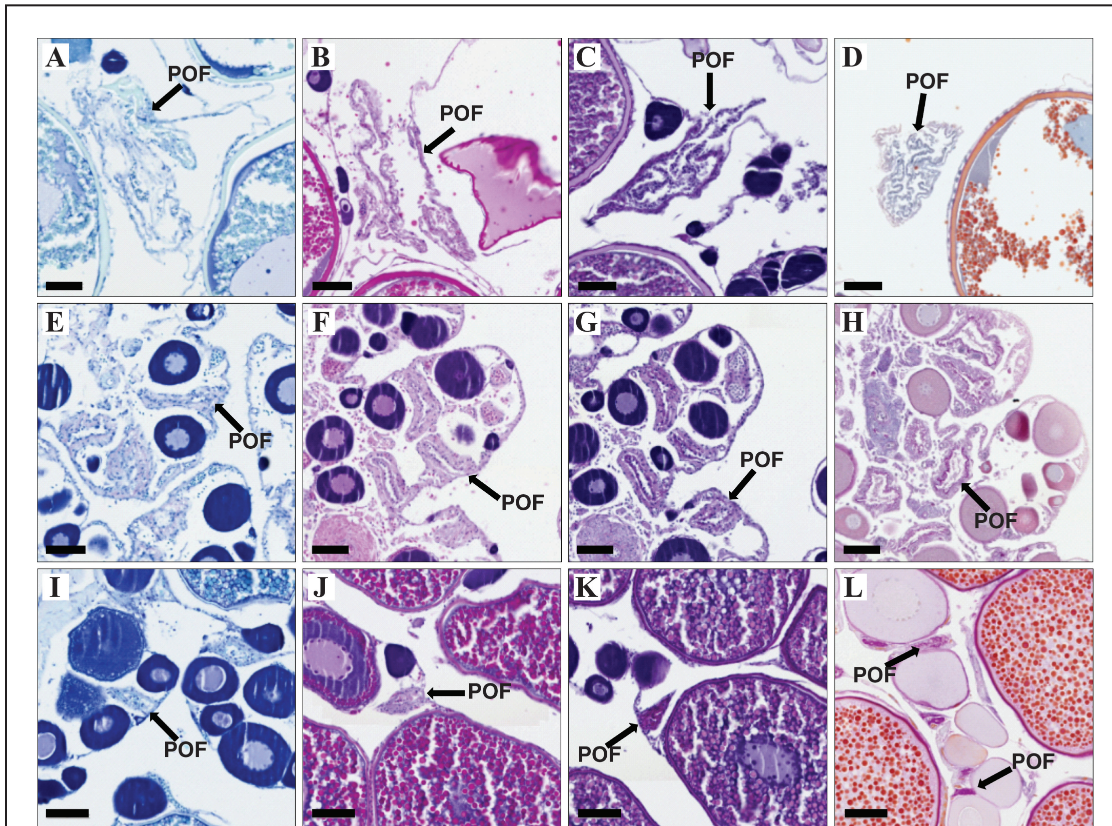

Figure 9

Histological images of 3 stages of postovulatory follicles (POFs) in gonad tissue from female yellowtail flounder (*Myzopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2012, 2018, and 2019: (**A**–**D**) recent, (**E**–**H**) intermediate, and (**I**–**L**) old. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. All scale bars are equal to 60 µm.

making the small structures easy to pick out. In addition, the deep-purple color also helps to differentiate between an oocyte in a very late atretic phase and a very old POF. Both alpha and beta atresia stood out and were easier to identify with the PAS-MT stain.

Use of the simple monochromatic TB stain resulted in the lowest confidence in identifying structures for all 3 readers. Readers had a high degree of uncertainty when distinguishing stages of oocyte development, particularly the secondary growth stages. In the secondary growth stages, when cortical alveoli and lipoprotein yolk globules first appeared, these features were difficult to identify with confidence because of the subtle contrast in stain intensity throughout sections in slides. In addition, the presence of POFs and their stages were difficult to distinguish with this monochromatic stain. When identifying old POFs, readers almost never recorded their presence with a high confidence level. Similarly, when beta atresia was observed, it was rarely recorded with a high level of confidence. Importantly, low confidence levels in identification of old POFs (Fig. 14) and beta atresia (Figs. 4, 7, and 10) can lead to uncertainty in assignments of maturity stage that rely on information about these features. With the TB stain, both of these structures can look very similar; however, the presence of old POFs is a definite sign of spawning activity, and beta atresia is not.

Although we refer to it as a single stain, toluidine blue has metachromatic properties that make it well suited for a range of applications (Sridharan and Shankar, 2012; Longenecker and Langston, 2018; Vidal and Mello, 2019; Longenecker et al.[1]). Schemmel and Friedlander (2017) successfully identified oocyte and POF stages using a TB stain in histological characterization of the reproductive state of convict surgeonfish (*Acanthurus triostegus*). Kjesbu et al. (2011) used TB alone to study the shift of primary growth oocytes to cortical alveolar and vitellogenic stages. They revealed a seasonal progression of cytoplasmic features, such as a circumnuclear ring, cortical alveoli, 2 stages of vitellogenesis, and POFs in laboratory-reared Atlantic cod (Kjesbu et al., 2011, fig. 6). Familiarity with this species and the routine nature of sampling at specific times of year allowed

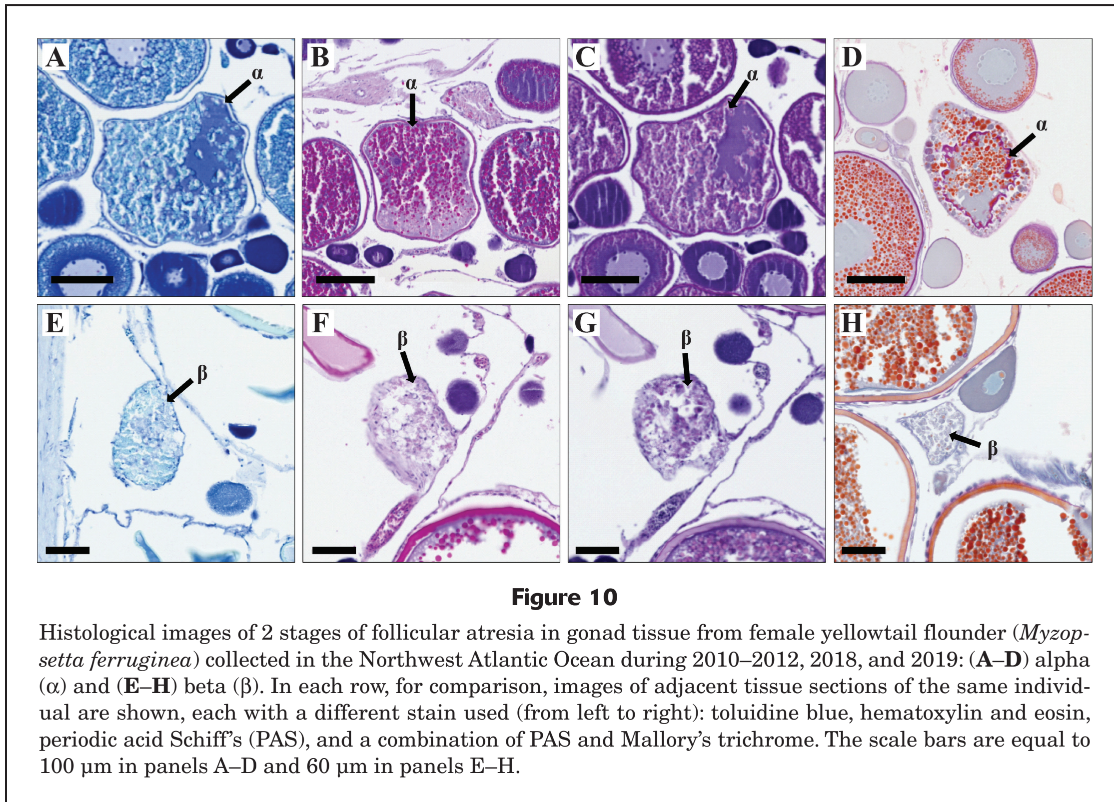

Figure 10

Histological images of 2 stages of follicular atresia in gonad tissue from female yellowtail flounder (*Myzopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2012, 2018, and 2019: (**A**–**D**) alpha (α) and (**E**–**H**) beta (β). In each row, for comparison, images of adjacent tissue sections of the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 100 µm in panels A–D and 60 µm in panels E–H.

Kjesbu[4] and his collaborators to use the inexpensive TB stain for a variety of purposes: to measure the size of the "leading cohort" of oocytes in a gonad (Anderson et al., 2020), to identify individual Atlantic cod for the presence of atresia (Witthames et al., 2013), or to check for POFs to screen for the suitability of an individual female for estimation of fecundity.

Use of the H&E and PAS stains resulted in similar levels of confidence in identification of features. Confidence levels in distinguishing oocyte development stages were higher with the H&E stain than for the TB stain but were still below the levels for oocyte staging with the PAS-MT stain. Interestingly, for differentiation between 2 early stages, cortical alveoli and early vitellogenesis, readers had slightly more confidence when using the H&E stain than when using the other stains. Cytoplasm has a light pinkish color when the H&E stain is applied but a dark-purple color when the PAS stain is used. The lighter hue of cytoplasm with the H&E stain makes cortical alveoli and lipoprotein yolk globules stand out more than they do with the PAS stain. For all of the 3 species evaluated, subtle differences in oocyte development were evident, leading to uneven ranking of staining methods across species.

Use of the H&E and PAS stains produced different results for identification of POFs. Readers had more confidence in identifying the presence and determining the stage of POFs with the PAS stain than with the H&E stain. Recent POFs were dark purple in slides with the PAS stain and light pink in slides with the H&E stain. As POFs progressed from the recent stage to the intermediate and old stages, little change in coloration occurred with H&E staining. In contrast, as POFs degraded, greater intensity and affinity of pigment was observed with the PAS stain, making them more obvious, and readers were able to identify intermediate and old POFs more easily. The dark-purple color remained as a POF broke down further, in addition to a magenta color becoming more noticeable in the granulosa layer. This magenta-like appearance remained in old POFs, making it possible to distinguish reliably between an old POF and very old atresia.

Among a plethora of staining methods we did not consider, some techniques are notable for their use in histological studies of fish gonads. Quintero-Hunter et al. (1991) modified a PAS stain with Weigert's iron-hematoxylin as a nuclear stain and metanil yellow as a counterstain. The PAS part of this stain highlights melano-macrophage centers in a bright pink hue, in contrast to the dull-brown centers ("brown bodies") that have been observed with H&E (McBride et al., 2002, 2012), and it reveals the PAS-positive nature of the yolk in certain fish orders (e.g., Beloniformes; McBride and Thurman, 2003). Stemming from an interest in the transition from oogonia to oocytes that occurs along basement membranes (i.e., folliculogenesis), Grier (2012, 2016) omitted the hematoxylin to better reveal basement membranes, and Grier et al. (2018) used

[4] Kjesbu, O. S. 2024. Personal commun. Inst. Mar. Res., P.O. Box 1870 Nordnes, 5817 Bergen, Norway.

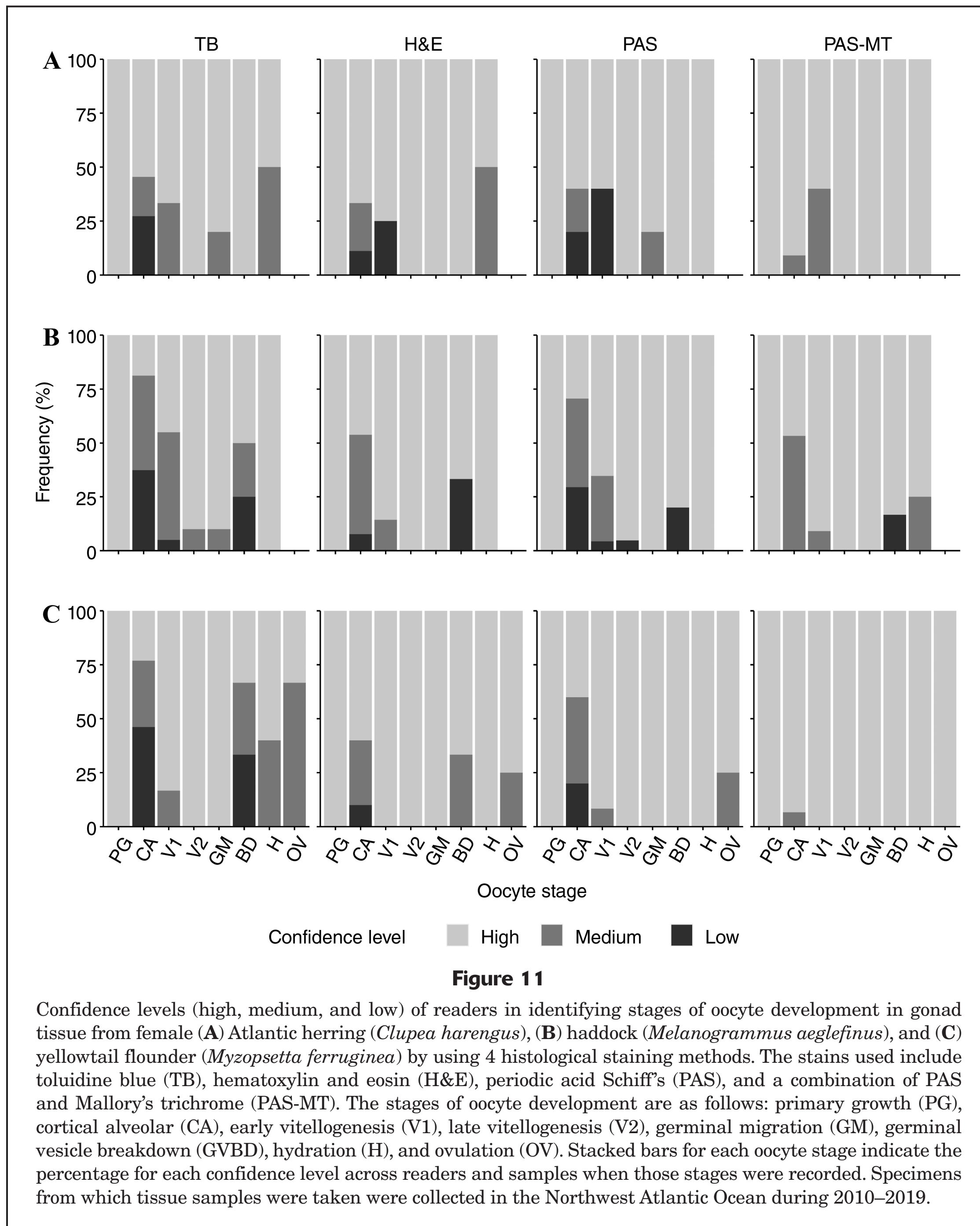

Figure 11

Confidence levels (high, medium, and low) of readers in identifying stages of oocyte development in gonad tissue from female (**A**) Atlantic herring (*Clupea harengus*), (**B**) haddock (*Melanogrammus aeglefinus*), and (**C**) yellowtail flounder (*Myzopsetta ferruginea*) by using 4 histological staining methods. The stains used include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). The stages of oocyte development are as follows: primary growth (PG), cortical alveolar (CA), early vitellogenesis (V1), late vitellogenesis (V2), germinal migration (GM), germinal vesicle breakdown (GVBD), hydration (H), and ovulation (OV). Stacked bars for each oocyte stage indicate the percentage for each confidence level across readers and samples when those stages were recorded. Specimens from which tissue samples were taken were collected in the Northwest Atlantic Ocean during 2010–2019.

a reticulin stain described by Mazzoni et al. (2014) to highlight basement membranes. Examples of the use of these stains and 2 others, alcian-blue as well as thionin and osmium, were depicted by Grier et al. (2016). Staining with Prenant–Gabe's trichrome involves application of a fast green stain after H&E, highlighting connective tissues, including the gonad wall as a light green, and it has recently been applied in histological studies of several fishes (Sauger et al., 2020; Dubroca et al., 2023; Heude-Berthelin et al., 2024).

Implications for reproduction studies

With a large array of stains to choose from (Riva et al., 2014) and each providing different levels of accuracy and precision for specific features, the optimal stain for any given study will differ depending on the objectives. As described herein, both oocyte development and spawning patterns differ across species. Analyzing and interpreting gonad histology can be complex and require identification of specific microstructural features; therefore, the choice of

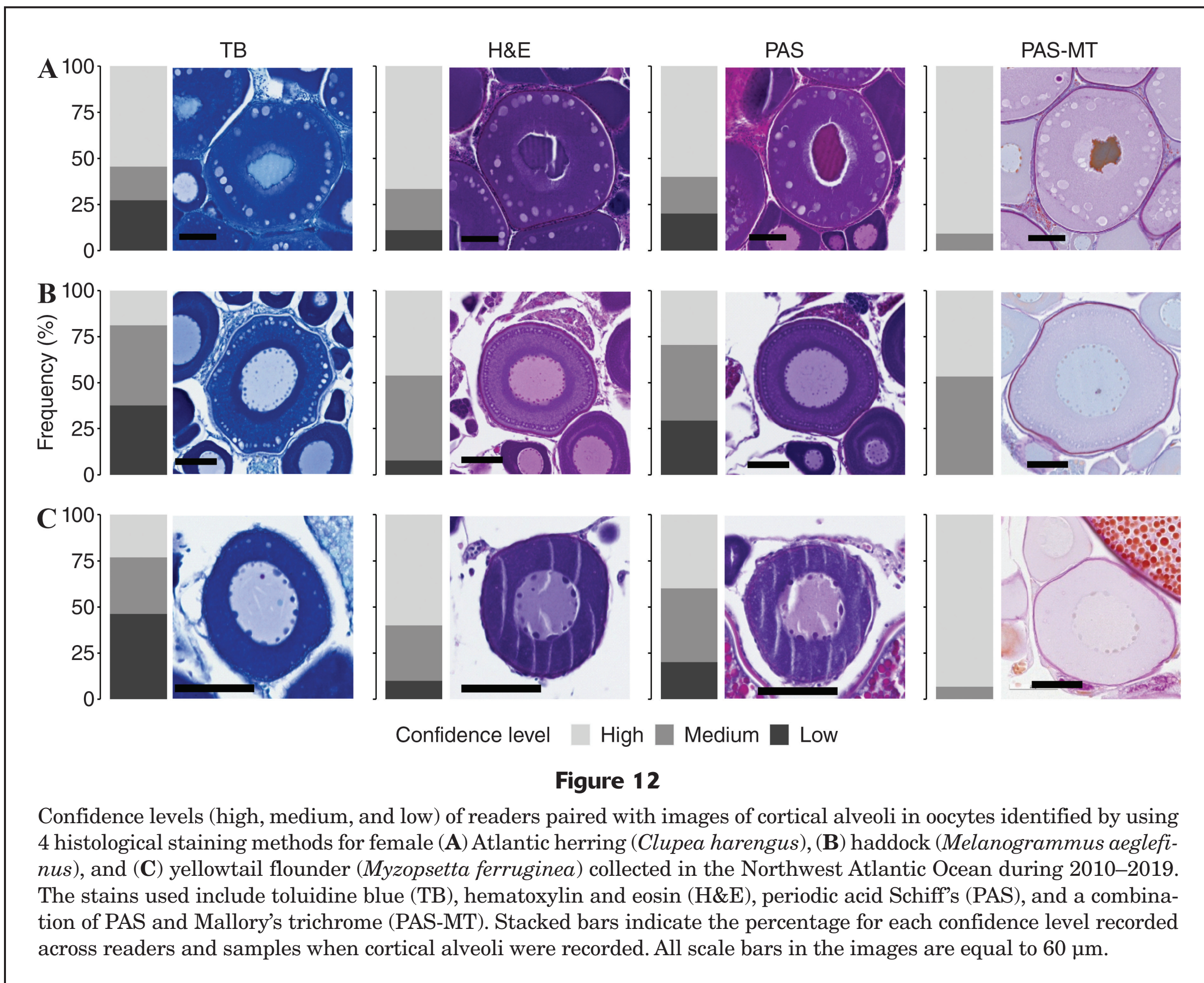

Figure 12

Confidence levels (high, medium, and low) of readers paired with images of cortical alveoli in oocytes identified by using 4 histological staining methods for female (**A**) Atlantic herring (*Clupea harengus*), (**B**) haddock (*Melanogrammus aeglefinus*), and (**C**) yellowtail flounder (*Myzopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2019. The stains used include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Stacked bars indicate the percentage for each confidence level recorded across readers and samples when cortical alveoli were recorded. All scale bars in the images are equal to 60 µm.

stain used in a study can have a large effect on the interpretation of (and level of confidence in) information obtained through gonad histology.

In fish reproduction studies, gonad histology is used to evaluate a variety of properties, including maturity, spawning seasonality, spawning frequency, and fecundity (e.g., atretic downregulation). The cellular features required to accurately assess each of these properties will vary, as will stain selection, consequently. In this section, for each of the reproductive properties typically evaluated in studies of fish, we expand on the advantages and disadvantages of various staining methods across the species evaluated in this study and in other studies.

Information on maturity plays an important role in managing sustainable fisheries. The size and age at maturity mark the critical point of transition when an individual becomes capable of reproducing, a transition occurring only once in an individual's lifetime that is most often defined in terms of physiological maturity. In other words, maturity is assumed once the critical developmental threshold of the brain-pituitary-gonad axis that regulates hormonal and gonadal development is passed (Brown-Peterson et al., 2011; Lowerre-Barbieri et al., 2023). However, recently, estimation of functional maturity, when fish are spawning or are going to spawn in the current or upcoming spawning season, is of greatest relevance as input to a fish stock assessment model, rather than when a fish passes some hormonal threshold (Prince et al., 2022). Moreover, in some species, individuals may initiate but abort a clutch of vitellogenic oocytes, thereby delaying functional maturity and misleading estimation of maturity (Kennedy et al., 2011a; Conrath, 2017; Lefebvre et al., 2018; McBride et al., 2022b). Although maturity classification can sometimes be fairly straightforward with simple macroscopic evaluations, accurate identification of functionally mature individuals outside of peak spawning periods may require the use of gonad histology.

Histology, regardless of the specific staining method used, provides several significant advantages over macroscopic methods, including an increased level of detail, subcellular stain affinities that aid identification, a means to assess tunica thickness, and a physical archival record that can be reevaluated. For these reasons, histology-based maturity estimates are often assumed to be more accurate than macroscopic determinations, without consideration of the nuances and potential biases of using different stains in identification of key features. For example, given that an individual with oocytes in the cortical

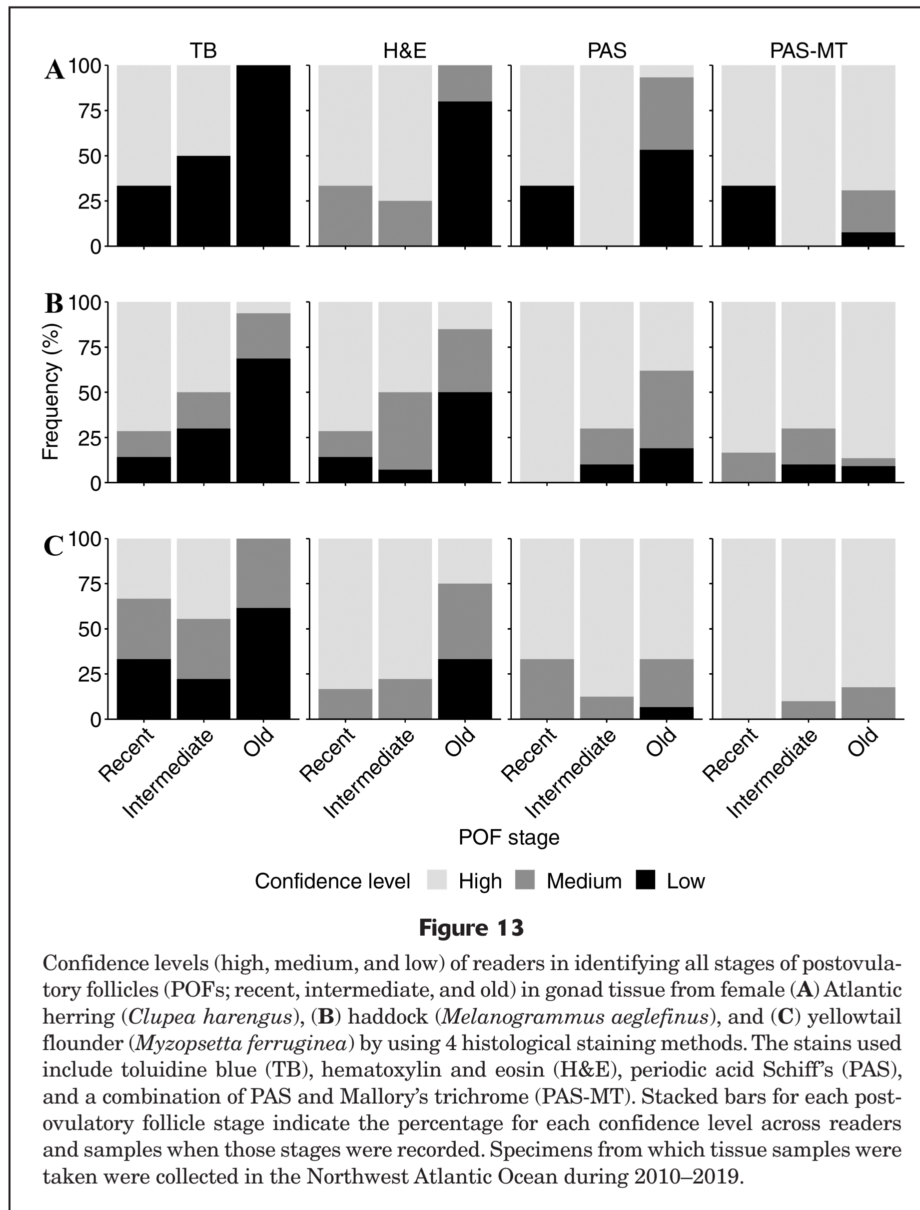

Figure 13

Confidence levels (high, medium, and low) of readers in identifying all stages of postovulatory follicles (POFs; recent, intermediate, and old) in gonad tissue from female (**A**) Atlantic herring (*Clupea harengus*), (**B**) haddock (*Melanogrammus aeglefinus*), and (**C**) yellowtail flounder (*Myzopsetta ferruginea*) by using 4 histological staining methods. The stains used include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Stacked bars for each postovulatory follicle stage indicate the percentage for each confidence level across readers and samples when those stages were recorded. Specimens from which tissue samples were taken were collected in the Northwest Atlantic Ocean during 2010–2019.

alveolar stage at a certain time of year may be considered mature, using a stain with which the presence of cortical alveoli is ambiguous can increase the risk of misidentifying the stage of maturity. For another case, consider that the accurate classification of skipped spawners may require successful identification of both tunica thickness and old POFs. Tunica thickness may be equally interpretable by using any number of stains, but old POFs, which may last more than 1 year in some cold-water species, may not be evident with all stains (e.g., Rideout et al., 2005, Skjæraasen et al., 2009, McBride et al., 2013, Press et al., 2014). Such false negatives (not seeing POFs because of the stain used) may bias estimates of spawning frequency. In most warm-water fishes, POFs degrade much faster than those in cold-water species and are rarely visible 48 h after spawning. Therefore, because old POFs do not persist long enough in warm waters to serve as seasonal markers of spawning, the use of special stains for distinguishing them is not warranted for these species.

In contrast to the onset of maturity, which occurs only once, annual patterns of oocyte development that repeat many times over a reproductive lifetime are useful in determining the optimal seasons for identification of spawning contingent, spawning frequency, fecundity, skipped spawning, and energetics and condition of fish before, during, and after spawning. For some species, components of a population spawn at different times of year (e.g., Atlantic herring; Wuenschel and Deroba, 2019), and oocyte development stage at a given time of year can be used to differentiate such contingents. We found little difference among staining methods in confidence level for distinguishing vitellogeneic and mature

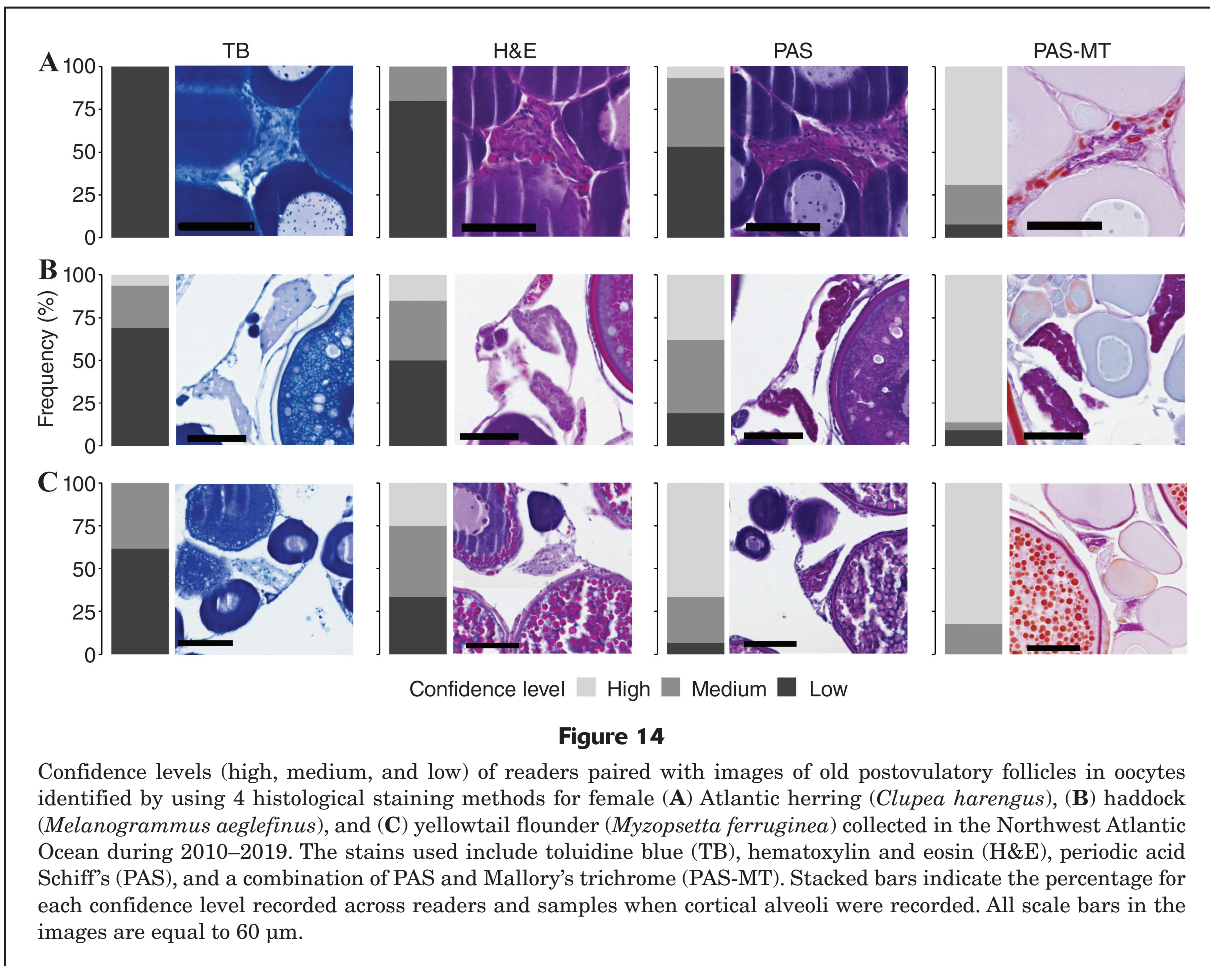

Figure 14

Confidence levels (high, medium, and low) of readers paired with images of old postovulatory follicles in oocytes identified by using 4 histological staining methods for female (**A**) Atlantic herring (*Clupea harengus*), (**B**) haddock (*Melanogrammus aeglefinus*), and (**C**) yellowtail flounder (*Myzopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2019. The stains used include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Stacked bars indicate the percentage for each confidence level recorded across readers and samples when cortical alveoli were recorded. All scale bars in the images are equal to 60 µm.

oocytes, indicating that all stains would perform equally in studies with objectives to relate other factors (e.g., feeding, location, and behavior) to these stages of oocyte development.

Microstructural features may also be used to identify fish that are actively spawning, an important component of defining the spawning fraction (or frequency) for estimation of indeterminate fecundity (McBride et al., 2012). Features that are used as markers for active spawning can be used to determine spawning locations (McBride et al., 2003; Lowerre-Barbieri et al., 2009) and to improve understanding of spawning behaviors, such as diel reproductive periodicity (McBride et al., 2002, 2003; Hyle et al., 2014). These features typically include oocytes in germinal vesicle breakdown, hydration, and ovulation and recent POFs; therefore, reliance on histological staining methods that bias the identification of these stages can lead to bias in estimates of spawning fraction or spawning areas. Although we found similar levels of confidence in identifying recent POFs across the 4 methods we evaluated, confidence levels declined for distinguishing old POFs with the TB and H&E stains.

Accurate identification of recent POFs is also a requirement when determining fecundity of group-synchronous batch spawners because their absence indicates that no batches were released yet that season (Witthames et al., 2009; McElroy et al., 2016). Because cold-water species have persistent POFs, studies of their reproduction require accurate identification of only recent POFs. As a result, stain selection will have little effect on studies of the fecundity of cold-water species. In contrast, the potential biases in detection of certain stages for a selected stain must be evaluated for histological studies that require accurate estimation of multiple stages of POFs, as is necessary for assessing indeterminate fecundity of warm-water fishes (Ganias et al., 2014).

Our level of confidence in identifying atresia (both alpha and beta) was variable across the species and staining methods evaluated. Accurate identification of atresia is important in fecundity studies, and biases could affect fecundity estimates in several ways. In stereological fecundity methods (Emerson et al., 1990; Medina et al., 2002; Aragón et al., 2010; Ganias et al., 2014), bias in detection of alpha atresia (including that due to handling and storage; Schemmel and Brown-Peterson, 2023) will affect what are counted as viable oocytes and, therefore, included in fecundity estimates. Additionally, studies in which atresia is quantified to refine fecundity estimates or determine the timing and rates of fecundity downregulation require accurate identification of both phases of

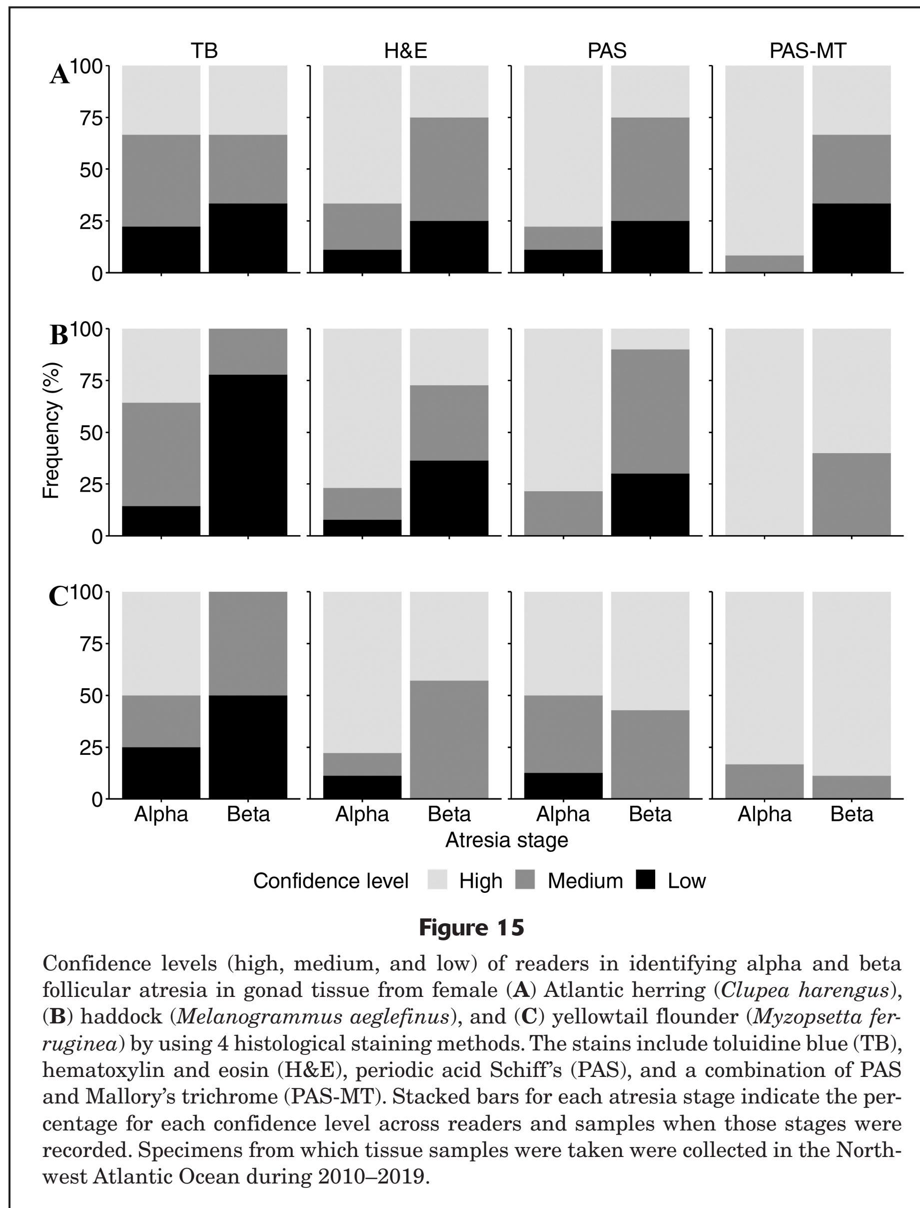

Figure 15

Confidence levels (high, medium, and low) of readers in identifying alpha and beta follicular atresia in gonad tissue from female (**A**) Atlantic herring (*Clupea harengus*), (**B**) haddock (*Melanogrammus aeglefinus*), and (**C**) yellowtail flounder (*Myzopsetta ferruginea*) by using 4 histological staining methods. The stains include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Stacked bars for each atresia stage indicate the percentage for each confidence level across readers and samples when those stages were recorded. Specimens from which tissue samples were taken were collected in the Northwest Atlantic Ocean during 2010–2019.

atresia (Andersen[5]; Kjesbu et al., 2010; McElroy et al., 2016). We documented variable confidence levels for identification of atresia across staining methods, and such variation could bias estimation of atresia, downregulation, and fecundity. On the basis of these results, investigators intending to quantify atresia should carefully consider which staining method they plan to use.

Although the sequence of oocyte development, from oogonia to mature egg, is similar across most fishes, the pace of oogenesis varies widely across species. Therefore, efforts to relate specific stages or thresholds of oocyte development to feeding, condition, or environmental factors will require accurate and unbiased determination of oocyte stages. The rate of oocyte development through specific stages (e.g., the cortical alveolar stage) may be fleeting in some warm-water fishes, and adjacent stages (e.g., early vitellogenesis) that are more easily identified with a given stain may be used to obtain an unbiased estimate of reproductive state. However, in cases in which oocyte development is protracted (as it is in many cold-water species) with stages (e.g., the cortical alveolar stage) that are difficult to identify persisting for long periods before a fish transitions to later stages that are easier to identify (e.g., early

[5] Andersen, T. E. 2003. Unbiased stereological estimation of cell numbers and volume fractions: the disector and the principles of point counting. *In* Report of the working group on modern approaches to assess maturity and fecundity of warm- and cold-water fish and squids, Bergen, Norway, 4–7 September 2001 (O. S. Kjesbu, J. R. Hunter, and P. R. Witthames, eds.), p. 11–18. Inst. Mar. Res. and Res. Council Norway, Bergen, Norway. [Available from website.]

vitellogenesis), such an approach may bias interpretations. Potential biases and misclassifications are not equally distributed across stages of oocyte development because the pace of oogenesis is highly variable across species (specific stages are not present for similar lengths of time in different species). Therefore, investigators will need to consider whether the specific oocyte development stages of interest warrant the more accurate classification that is possible by using special staining.

Conclusions

In practice, selection of histological stain must balance the benefits and drawbacks with the cost of identifying or misidentifying specific structures or stage of oocyte development discussed herein. It is not surprising that the advantages and disadvantages of using different staining methods vary depending on the objectives of a study. A single one-size-fits-all approach (e.g., one in which H&E is the only stain used) may provide logistical and pricing advantages but sacrifice precision or accuracy in the identification of certain features. In our review of recent literature, H&E was by far the most widely used stain. Standardization to H&E affords advantages in practice, such as automated slide processing, stable shelf life of solutions, lower toxicity than alternatives, and a simple protocol. Given that most fish biologists lack formal training or experience in histological techniques, there is likely a tendency to adopt H&E as the standard stain. Although we did not find a broad level of bias when using H&E for most applications, other staining methods provide greater accuracy and precision for identification of some important features. For example, PAS and PAS-MT staining can provide greater detail and accuracy in determining presence of cortical alveoli and late stage POFs, both of which can exist for long periods of time in cold-water species and are important markers for physiological and functional maturity. Researchers should consider using alternative staining methods to evaluate false positive and false negative detections of specific diagnostic features when their objectives require it, and we encourage experimentation with new techniques when appropriate.

Resumen

La histología de las gónadas complementa la investigación sobre la historia de vida de las especies de peces y proporciona mayor exactitud y precisión que la caracterización macroscópica de la gónada para determinar los patrones de oogénesis a nivel celular y de madurez a nivel individual. En pesquerías, la histología mejora el estimado de la biomasa de adultos y reproductores de la población, la identificación de la estacionalidad y sus zonas de desove, y la preselección de especímenes para el cálculo de la fecundidad anual. Sin embargo, en la mayoría de los estudios se han utilizado un único método de tinción (hematoxilina y eosina). En este estudio sobre 3 especies taxonómicamente diversas, utilizando 4 métodos de tinción diferentes de complejidad variable (grado de contratinción), comparamos los niveles de confianza en la identificación de 8 estadias de la oogénesis, la presencia y el nivel de degradación de los folículos postovulatorios y las células germinales atrésicas (vitelogénicas). Como se esperaba, el método de tinción monocromática menos costoso proporcionó el nivel de confianza más bajo, mientras que el método de contratinción más costoso y complejo proporcionó el nivel de confianza más alto, con la tinción de hematoxilina y eosina y otro método de contratinción simple entre ambos. El efecto del método de tinción fue más evidente en la identificación de los alvéolos corticales, que puede afectar a la estimación de la talla o la edad de madurez, y en la identificación de los folículos postovulatorios, que puede afectar a la estimación de la frecuencia de desove. Estos resultados son ampliamente aplicables para determinar las mejores prácticas.

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