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An autofluorescence-based survey of late follicular atresia in ovaries of a teleost fish (*Thunnus thynnus*)

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Abstract

In this study, the authors examined late atretic follicles in the ovaries of Atlantic bluefin tuna, *Thunnus thynnus* (Linnæus 1758), at different times of the year using transmitted light and epifluorescence microscopy. Atresia (degeneration and resorption of developing ovarian follicles) is a natural process involved in fecundity downregulation in teleosts and is substantially enhanced in stressful conditions. Early (α and β) atretic stages of yolked oocytes have a relatively short duration in seasonally reproducing species, whereas later (γ and δ) atretic follicles (LAF) persist for longer time in the ovary, serving as a sign of previous vitellogenic activity. LAF can thus be used as reliable markers of maturity during non-reproductive periods. Lipofuscin granules accumulate in the cytoplasm of LAF cells as a result of lysosomal digestion of oocyte components. Taking advantage of the well-known autofluorescent properties of lipofuscins, LAF may be identified in unstained histological sections under fluorescence microscopy using appropriate excitation and emission wavelengths. The authors explore in this study the applicability of fluorescence microscopy to provide a fast and effective method to assess late atresia in fishes. This method may be particularly useful to determine sexual maturity in individuals sampled long after the spawning season, where LAF are difficult to detect in standard histological sections. Furthermore, LAF autofluorescence provides a rapid way to quantify late atresia in fishes using image analysis.

KEYWORDS
Atlantic bluefin tuna, autofluorescence, follicular atresia, maturity, ovary

1 | INTRODUCTION

The ovarian follicular atresia is a natural process that consists of degeneration and resorption of developing follicles. Although atresia may occur at any stage throughout the oocyte development in teleosts, atretic follicles are more prevalent during secondary growth, because vitellogenic follicles contain a greater amount of reusable energy than earlier oogenetic stages (Domínguez-Castanedo *et al*., 2019). Atresia is enhanced in stressful situations, such as panic (Corriero *et al*., 2011), starvation (Corriero *et al*., 2011; Hunter & Macewicz, 1985), exposure to contaminants (Johnson *et al*., 1999; Migliaccio *et al*., 2018) or captivity (Corriero *et al*., 2004, 2007, 2011; Passantino *et al*., 2020).

The physiological and regulatory pathways underlying follicular atresia are complex and involve apoptosis and autophagy mechanisms (Cassel *et al*., 2017; González-Kother *et al*., 2020; Krysko *et al*., 2008; Morais *et al*., 2012; Santos *et al*., 2008; Thomé *et al*., 2009). The dynamics of these processes has been described in detail in a variety of teleost species, both under light (Cassel *et al*., 2017; Domínguez-Castanedo *et al*., 2019; Ferrão *et al*., 2020; Grier *et al*., 2017; Hunter & Macewicz, 1985; Lambert, 1970; Morais *et al*., 2012; Pérez &

Figueiredo, 1992; Polder, 1971) and electron microscopy (Besseau & Faliex, 1994; Miranda *et al*., 1999; Moktar & Hussein, 2020; Qiang *et al*., 2021; Santos *et al*., 2008; Thomé *et al*., 2009). Overall, the course of fish follicular atresia has been staged into four sequential phases: α, β, γ and δ (Hunter & Macewicz, 1985; Lambert, 1970). Early (α) atretic follicles of yolked oocytes show disintegration of the oocyte nucleus and cytoplasmic organelles, fragmentation of the zona radiata and hypertrophy of follicle cells. The subsequent (β) atresia stage is characterized by complete resorption of the zona radiata and yolk granules by the phagocytic follicle cells. Late (γ and δ) atretic follicles (LAF) are less conspicuous structures that appear embedded in the ovarian stroma. LAF consist of small aggregates of follicle cells that show pigment granules containing lipofuscin.

Lipofuscin is a yellow-brown lipopigment that occurs in many animal organs, including fish ovaries, as a result of slow oxidation of lipids and lipoproteins. This pigment has been identified in LAF of various teleost species applying specific histochemical techniques (Besseau & Faliex, 1994; Kumar & Joy, 2015; Lambert, 1970; Lang, 1981). A remarkable feature of lipofuscins is the emission of blue or green fluorescence when excited with ultraviolet or blue radiations. Lipofuscin autofluorescence is because of the production of fluorophores that result from the phagocytic degradation of cell debris within secondary lysosomes (Brunk *et al*., 1992; Medina *et al*., 2000; Porta, 2002; Riga & Riga, 1995; Sohal & Wolfe, 1986; Vila *et al*., 2000).

α and β atretic follicles (αAF and βAF) are easily recognizable in histological samples and prove useful in determining maturation stages throughout the reproductive cycle in fishes (Hunter & Macewicz, 1985; Schaefer, 1998, 2001). These atretic stages are short-lived and are thus absent for most of the year in fishes with brief spawning periods. In contrast, more advanced (γ and δ) atretic follicles remain for longer time in the ovary. Their presence during non-reproductive periods provides evidence of degradation of former yolked oocytes, indicating that the fish has already attained sexual maturity (Farley *et al*., 2013, 2014; Ganias *et al*., 2008; Hunter & Macewicz, 1985; Lowerre-Barbieri *et al*., 2011). These maturity markers are sometimes difficult to distinguish in standard histological samples because of their reduced size and number, and poor contrast on the connective tissue matrix. Nonetheless, the fluorescent properties of lipofuscin may be useful to localize LAF in fish ovarian sections. Using Atlantic bluefin tuna samples, the authors explored the applicability of lipofuscin autofluorescence to the identification and characterization of advanced follicular atresia in fish ovaries.

2 | MATERIALS AND METHODS

Ovaries of wild and captive Atlantic bluefin tuna *Thunnus thynnus* (Linnæus 1758) were obtained at different times of the year from commercial fisheries and aquaculture facilities. Wild individuals ranging from 116 to 254 cm in straight fork length (L_F) were caught in the Strait of Gibraltar area (southern Spain). Samples from captive individuals reared in offshore cages (size range: 163–215 cm L_F) were collected from a tuna farm located in l'Ametlla de Mar (Tarragona, NE Spain). Ovarian tissue was also collected from one juvenile (1.5-year old, L_F: 85 cm) raised in an experimental onshore aquaculture facility in Mazarrón (Murcia, SE Spain). With the exception of this young individual, all the tuna examined were estimated to be ≥4 years old (Cort *et al*., 2014; Luque *et al*., 2014). The present research involved no animal experimentation or harm, samples were collected from fish harvested for commercial purposes and permits for animal collections and animal welfare were not required.

Tissue samples were fixed in either 10% neutral buffered formalin solution or Bouin's fluid, dehydrated through graded ethanol solutions to xylene, and embedded in paraffin wax (Paraplast®). Following Bouin's fixation, the samples were thoroughly washed in 70% ethyl alcohol saturated with lithium carbonate for removal of picric acid from the tissue. Serial 7 μm sections were cut from the paraffin blocks. One of the sections was stained with haematoxylin and eosin (H&E), and the subsequent section was deparaffinized through two 10-min xylene changes and mounted without staining with Eukitt®. Some selected sections were treated for histochemical demonstration of advanced stages of lipofuscinogenesis applying the Sudan Black B (Bancroft & Gamble, 1990; Evangelou & Gorgoulis, 2017) and Schmorl's (Bancroft & Gamble, 1990) staining methods for lipofuscin.

Bright-field and fluorescence images were acquired with an AxioCam 503 Colour camera (Zeiss) coupled with a Zeiss Axio Imager. D2 epifluorescence microscope equipped with HBO 100 mercury vapour short-arc lamp and Zeiss filter sets 49 (exciter G 365, beamsplitter FT 395, emitter BP 445/50) and 38 (exciter BP 470/40, beamsplitter FT 495, emitter BP 525/50). The figures displayed in this paper were selected from a collection of digital captures saved using the Zeiss software (ZEN) without any modification except for light or contrast adjustments.

3 | RESULTS

Atretic stages of bluefin tuna yolked follicles are similar to those described for other teleosts. Atresia begins with disintegration of the oocyte nucleus, fragmentation and gradual dissolution of the zona radiata and yolk granules, and hyperplasia and hypertrophy of follicle cells, which phagocytize yolk granules and other oocyte components (α atresia, Figure 1a,d). This stage continues with complete resorption of the zona radiata and yolk granules, giving rise to an aggregate of follicle cells and intercellular cavities surrounded by the thin layer of thecal cells (β atresia, Figure 1b,e). More advanced atretic follicles (here referred to as LAF) are less conspicuous structures that eventually appear sunk in the connective stroma supporting the ovigerous lamellae. LAF are clusters of a few follicle cells showing finely granular cytoplasm (Figure 1c,f). Bluefin tuna LAF are morphologically similar to the δ atretic follicles of other fish species. The authors have not been able to distinguish between distinct γ and δ atresia stages because thecal cells, which typically surround γ atretic follicles, were no longer observed beyond the β stage.

α and β atresia were observed only in ovaries sampled from May through August; αAF were generally more abundant in May and June, whereas βAF predominated in July and August. These atretic stages

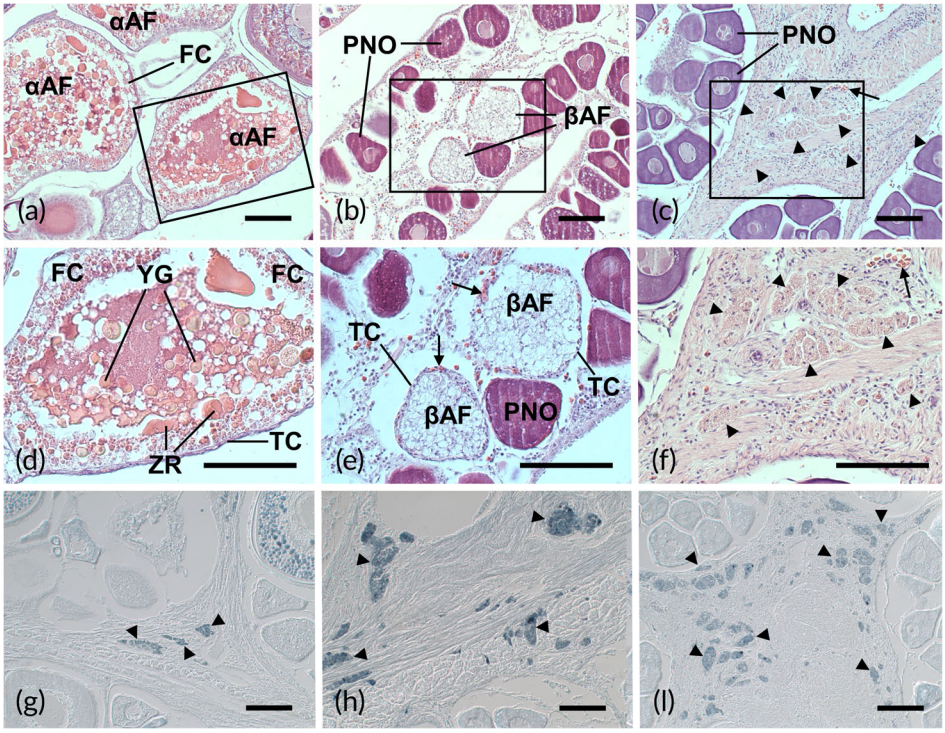

FIGURE 1 Different stages of atresia in ovaries of wild Atlantic bluefin tuna captured in the Strait of Gibraltar. (a, d) α atretic follicles of a 220 cm tuna (L_F) caught on 15 May 2019 (H&E staining). (b, e) β atretic follicles of a 183 cm tuna (L_F) caught on 7 February 2019 (H&E staining). (c, f) Ovary of fish (L_F: 144 cm) caught on 13 October 2020 showing late atretic follicles (H&E staining). (d–f) are higher magnifications of the square-boxed regions in (a–c), respectively. (g–i) Sudan Black B staining of LAF in ovary samples from individuals represented in a–c, respectively. Arrowheads: late atretic follicles (LAF), arrows: red blood cells in capillaries, αAF: α atretic follicles, βAF: β atretic follicles, FC: fagocytic hypertrophied follicle cells, PNO: perinucleolar (previtellogenic) oocytes, TC: thecal cells, YG: yolk granules, ZR: fragments of the dissolving zona radiata. Bars: 100 μm

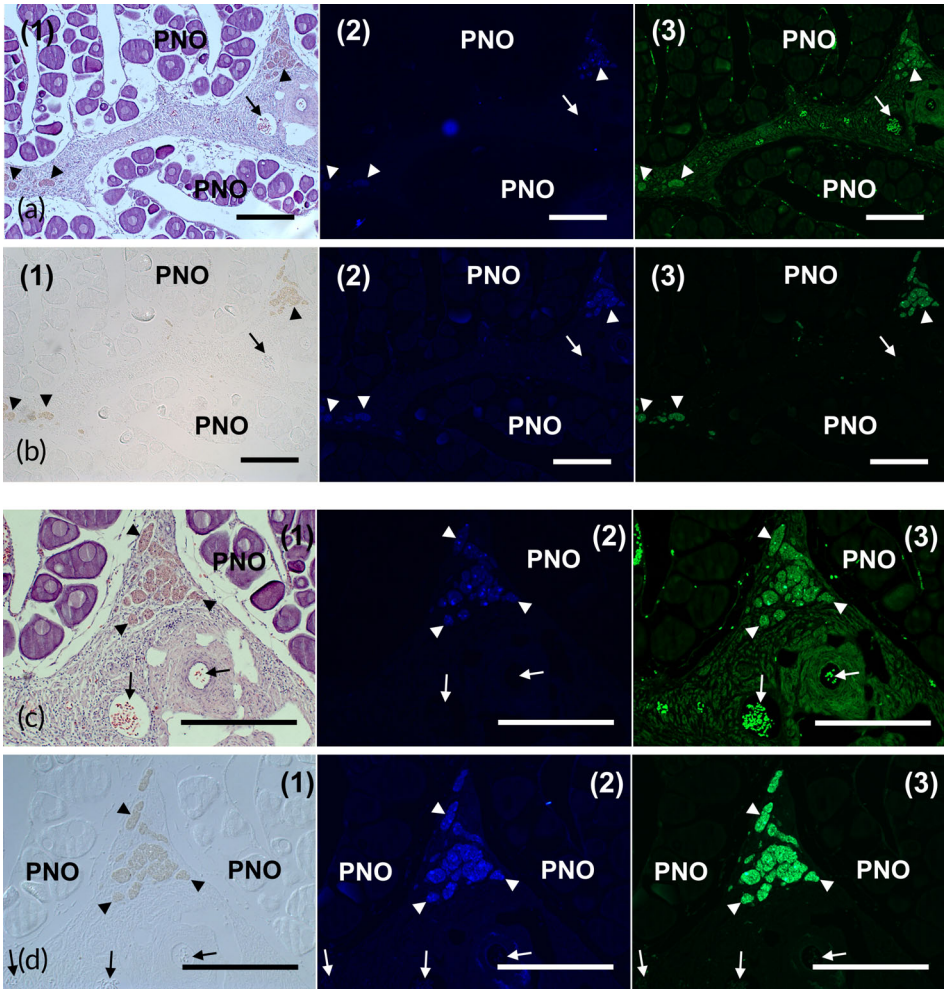

FIGURE 2 Ovary sections of wild bluefin tuna caught on 13 October 2020 (L_F: 148 cm). (a) (H&E-stained)/(b) (unstained) and (c) (H&E-stained)/(d) (unstained) are pairs of adjacent sections showing the same region of the ovary. (a–d) Include digital captures of a single micrographic field taken under (1) transmitted light microscopy, (2) epifluorescence microscopy (exc. 365 nm, em. 420–470 nm) and (3) epifluorescence microscopy (exc. 450–490 nm, em. 500–550 nm). Arrowheads: late atretic follicles (LAF), arrows: red blood cells in capillaries, PNO: perinucleolar oocytes. Bars: 250 μm

did not persist in reproductively quiescent females between September and April.

LAF were found throughout the year, but were more abundant during the months following the spawning period, *i.e.*, October–December. They usually appear in groups of differently sized cellular aggregates embedded in the ovarian stroma that supports the ovigerous lamellae (Figure 2). Under transmitted light, deparaffinized sections mounted without staining show LAF to contain a brownish granulation (Figure $2b_{(1)},d_{(1)}$) which is moderately positive to Sudan Black B staining for lipofuscin (Figure 1g–i) but stains very weakly with the Schmorl method (not shown). As the spawning season approaches, the number and size of LAF tend to decrease over time to eventually (July–August) become inconspicuous and mingled with the connective tissue (Figures $3a_{(1)},c_{(1)}$ and $4a_{(1)},c_{(1)}$). These old LAF are often only faintly pigmented, so that they may easily go undetected also in unstained sections (Figures $3b_{(1)},d_{(1)}$ and $4b_{(1)},d_{(1)}$). The difficulty to detect LAF during the reproductive period may be also because of the reduced volume of the connective stroma in comparison with the ovarian parenchymatic tissue, which increases as germ cells proliferate and grow.

Under fluorescence microscopy, H&E-stained LAF emit moderate blue and green light when excited with UV and blue radiations, respectively (Figures 2–4). Some large fluorescent granules are much brighter than the others. These bright granules are assumed to be larger (and older) lysosomes resulting from coalescence of smaller ones, therefore containing highly fluorescent end-products of lipofuscinogenesis (Figures $2c_{(2),(3)},d_{(2),(3)}$ and $4c_{(2),(3)}$, $d_{(2),(3)}$). The green fluorescence of H&E-stained LAF gives a lesser contrast than the blue fluorescence. This is due to interference with the connective matrix stained with eosin, because this dye emits fluorescence in the green channel when excited at the same wavelength. Thus, the highly eosinophilic red blood cells and yolk granules also emit intense green radiation in H&E-stained sections (Figures $2a_{(3)},c_{(3)}$ and $4a_{(3)},c_{(3)}$).

The pattern of LAF fluorescence in deparaffinized unstained sections is different from that observed in sections stained with H&E. Under UV excitation, the blue autofluorescence of LAF is hardly perceptible in many samples, making them less evident than they are in H&E-stained sections (*e.g.*, compare Figure $3a_{(2)}$ with Figure $3b_{(2)}$, Figure $3c_{(2)}$ with Figure $3d_{(2)}$, Figure $4a_{(2)}$ with Figure $4b_{(2)}$, and Figure $4c_{(2)}$ with Figure $4d_{(2)}$). Otherwise, when excited in the blue spectrum, a strong green emission clearly reveals the location of LAF, which appear distinctly highlighted against the dark background (Figures $2b_{(3)},d_{(3)}$, $3b_{(3)},d_{(3)}$ and $4b_{(3)},d_{(3)}$). As in H&E-stained sections, some large granular inclusions in LAF are strongly autofluorescent.

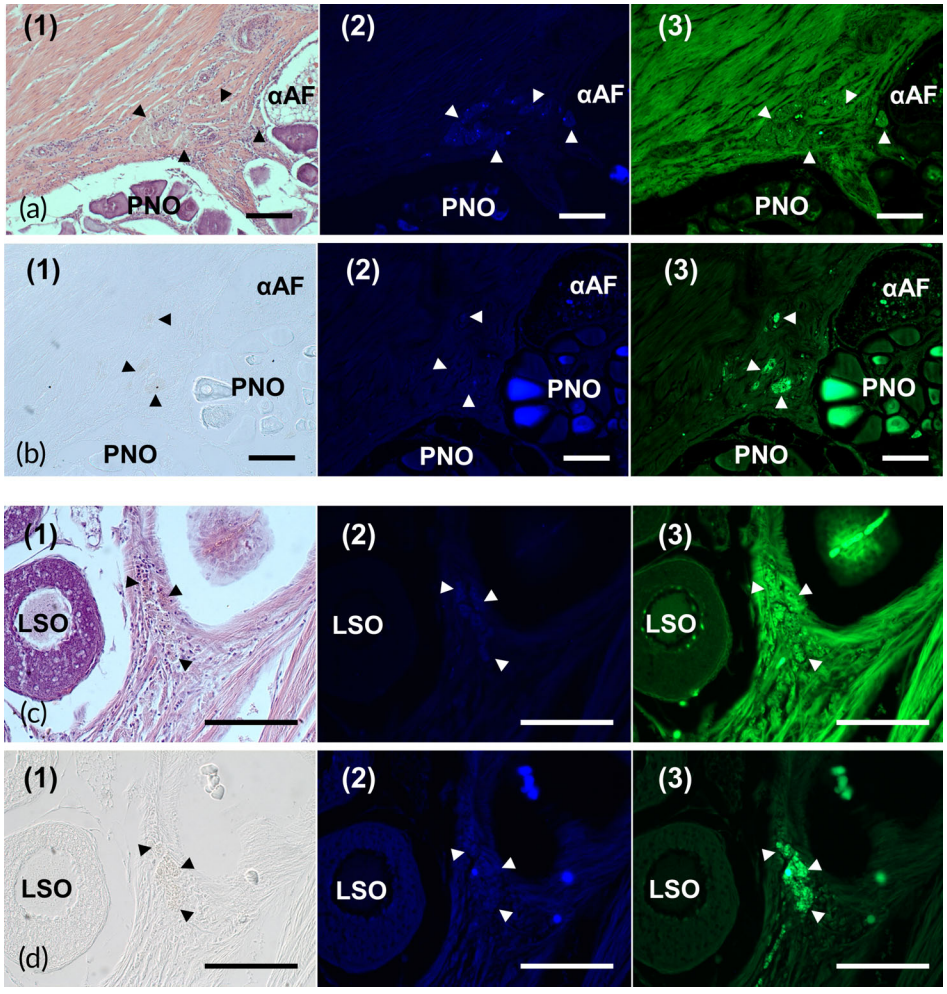

FIGURE 3 (a, b) Ovary sections of wild bluefin tuna caught on 25 June 2019 (L_F: 219 cm). (c, d) Ovary sections of wild bluefin tuna caught on 15 May 2019. (L_F: 220 cm). (a) (H&E-stained) and (b) (unstained) are close parallel sections showing the same area of the ovary. (c) (H&E-stained) and (d) (unstained) are adjacent sections. (a–d) Include digital captures of a single micrographic field taken under (1) transmitted light microscopy, (2) epifluorescence microscopy (exc. 365 nm, em. 420–470 nm) and (3) epifluorescence microscopy (exc. 450–490 nm, em. 500–550 nm). Arrowheads: Late atretic follicles (LAF), αAF: α atretic follicle, LSO: lipid-stage (previtellogenic) oocytes, PNO: perinucleolar (previtellogenic) oocytes. Bars: 100 μm

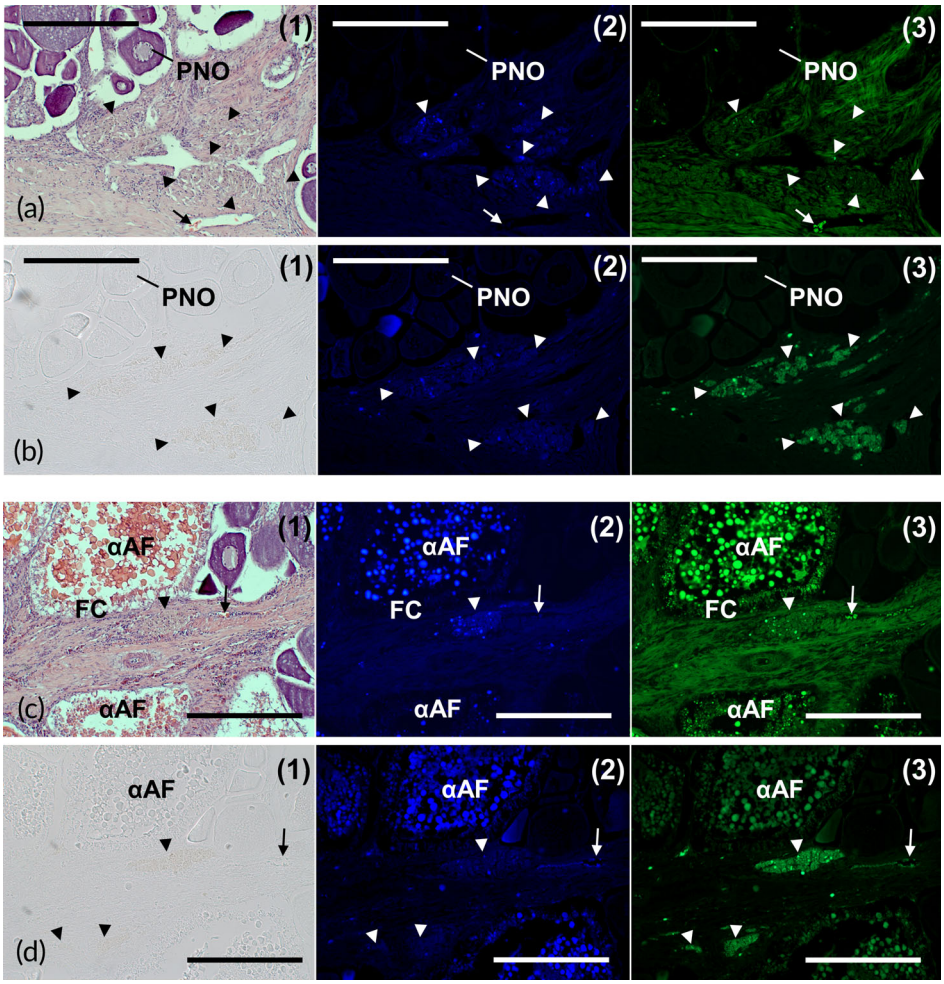

FIGURE 4 (a, b) Ovary sections of bluefin tuna caught by purse seine in the western Mediterranean Sea (June 2010) and reared in offshore cage until sampled on 8 June 2012 (L_F: 163 cm). (d, e) Ovary sections of bluefin tuna caught by purse seine in the western Mediterranean Sea (June 2010) and reared in offshore cage until sampled on 20 July 2012 (L_F: 169 cm). (a) (H&E-stained)/(b) (unstained) and (c) (H&E-stained)/(d) (unstained) are pairs of adjacent sections showing the same area of the ovary. (a–d) Include digital captures of a single micrographic field taken under (1) transmitted light microscopy, (2) epifluorescence microscopy (exc. 365 nm, em. 420–470 nm) and (3) epifluorescence microscopy (exc. 450–490 nm, em. 500–550 nm). Arrowheads: late atretic follicles (LAF), arrows: red blood cells in capillaries, αAF: α atretic follicles, FC: fagocytic hypertrophied follicle cells, LSO: lipid-stage (previtellogenic) oocytes, PNO: perinucleolar (previtellogenic) oocytes. Bars: 250 μm

The fluorescence of red blood cells and yolk granules is much weaker in unstained samples than it is in histological samples stained with H&E (*e.g.*, compare Figures $2a_{(3)}$,$c_{(3)}$ and $4c_{(3)}$ with Figures $2b_{(3)}$,$d_{(3)}$ and $4d_{(3)}$). Non-deparaffinized, unmounted sections are also suitable for fluorescence observations of LAF. They provide images of acceptable quality and allow the slides to be reused for further morphological or histochemical observations (Supporting Information Figure S1).

The smallest wild individual examined in this study (L_F: 116 cm, total mass: 35 kg, estimated age: *c.* 4 years) was captured on 8 November 2020 and possessed autofluorescent LAF (not shown in figures). Neither atretic follicles nor significant fluorescence of any ovarian structure, with the exception of red blood cells, was observed in both stained and unstained ovarian sections from the 1.5-year-old tuna reared in captivity (Supporting Information Figure S2).

4 | DISCUSSION

As in earlier studies (Corriero *et al.*, 2007; Medina *et al.*, 2016), αAF and βAF were found in bluefin tuna between May and August, then disappearing in reproductively quiescent females during September through April. Carnevali *et al.* (2019), however, observed early atretic follicles to persist until October–November in bluefin tuna maintained in a commercial farm off Malta. The occurrence of early atretic stages of yolked follicles (α and β atresia) is often used in fish maturity classifications and characterization of temporal reproductive patterns (*e.g.*, Lowerre-Barbieri *et al.*, 2011; Schaefer, 1998, 2001). Degenerated yolked follicles are helpful to distinguish reproductively inactive, but mature, individuals from young fish that have not yet attained maturity, which is a crucial issue in fisheries science (Arrizabalaga *et al.*, 2019; Medina, 2020).

When spawning takes place throughout the year, which is commonplace in species that reproduce in tropical waters (*e.g.*, Schaefer, 1998, 2001), no further stages of follicular degeneration are likely needed for maturity determinations, because α and β atretic yolked follicles usually coexist with subsequent batches of vitellogenic oocytes. Yet, in fishes with short spawning seasons, the relatively ephemeral αAF and βAF are not available to assess maturity during non-reproductive months. In such cases, other histological indicators of maturity are required (DeMartini, 2017). Past vitellogenic activity can be tracked back by investigating remnants of former yolked oocytes. In particular, late stages of atresia (γ and/or δ atretic follicles), which last further beyond the spawning season, may be used as maturity markers to separate quiescent females from virgin females with no previous reproductive history (Farley *et al.*, 2013, 2014; Ganias

et al., 2008; Hunter & Macewicz, 1985; Lowerre-Barbieri *et al*., 2011). This is particularly important at sizes/ages near the threshold between puberty and adulthood to draw reliable data informing about age at maturity. For instance, Farley *et al*. (2013, 2014) drew on LAF as direct proof of maturity in studies on reproductive dynamics of South Pacific albacore (*Thunnus alalunga*).

Although the presence of LAF is indicative of previous production of yolked oocytes, it is not a conclusive evidence that spawning was accomplished. Postovulatory complexes (remnants of follicular and thecal cell layers left after ovulation of mature eggs) are also important markers of not only maturity but also spawning activity. Unfortunately, postovulatory complexes are quickly resorbed (within a few days) in fishes that, like tunas, spawn in warm waters. Nevertheless, in cold-water species such as cod, postovulatory complexes may remain for several months after spawning completion, thus allowing confirmation of past spawning (Kjesbu *et al*., 2010).

As in anchovy (Hunter & Macewicz, 1985), γ atretic follicles are not clearly distinguished in histological samples of bluefin tuna, because thecal cells are not observed around the atretic follicles after the β stage; therefore, the authors refer to atretic bodies (γ plus δ atresia) as LAF. LAF resemble the melanomacrophage centres containing lipofuscin described by Passantino *et al*. (2020) in the liver of captive-reared *Seriola dumerili*.

Bluefin tuna late atresia is sometimes difficult to detect in standard histological sections of the ovary, especially long after the end of the spawning season, owing to their smaller size and poor contrast. Ferrão *et al*. (2020) also reported low affinity of goldfish LAF to H&E staining. Under transmitted light microscopy, unstained histological sections show bluefin tuna LAF to be transparent or faintly brownish cell clusters (hence the term "brown bodies" commonly used in the literature). This colouration is likely to reflect the presence of lipofuscin, which was also demonstrated in LAF of a variety of teleost species using different specific histochemical techniques (Besseau & Faliex, 1994; Kumar & Joy, 2015; Lambert, 1970; Lang, 1981).

A universal property of lipofuscins is their autofluorescence when excited with ultraviolet and blue light. The fluorescence features of lipofuscins differ considerably among species and tissues, as a result of the large heterogeneity in their chemical composition. LAF in unstained bluefin tuna ovarian sections emit a distinct, though weak, blue fluorescence when excited at 365 nm, and a strong green fluorescence under 450–490 nm wavelength excitation. Although also present, this autofluorescence is less detectable in H&E-stained sections because of background noise. Specifically, the fluorescent background of the connective tissue masks the green fluorescence of LAF. In H&E-stained slides, the fluorescence of connective tissue fibres (and generally all eosinophilic components), under violet blue excitation, has been proved to depend on the fluorescent properties of eosin (Elston, 2002; Yamashita *et al*., 1986).

The present observations show that a simple and rapid way to localize LAF maturity markers in reproductively inactive fish would be to examine unstained sections under fluorescence microscopy with excitation at blue light and emission at green light spectra. This method may prove helpful in instances where LAF do not appear distinctly defined in standard histological preparations. Once spotted in unstained sections under fluorescence microscopy, LAF can be localized in adjacent or close H&E-stained sections. An additional advantage of the lipofuscin autofluorescence approach is the ease to quantify the contrasting highlighted LAF over the dark background by image analysis.

Autofluorescence-based localization of LAF is applicable to archival material routinely processed without any special handling and stored in paraffin blocks for years. Microscopic analysis of lipofuscin-containing cells does not require deparaffinized and mounted slides, but paraffin sections may be examined directly without significant quality loss, saving time in sample preparation and allowing for further processing of the histological specimens.

5 | CONCLUSION

Age at maturity is an important life-history trait in fish and fisheries biology. Fish are considered to be mature when they can produce yolked oocytes that are capable of progressing into mature eggs (Corriero *et al*., 2020). Fish maturity can be, therefore, determined from ovarian histological samples when yolked oocytes and/or degenerating yolked follicles (α and β atretic follicles) are present in the ovary. In many species with brief reproductive periods, these ovarian components disappear shortly after the spawning season, leaving only advanced atretic structures (LAF) as evidence of past vitellogenesis. In non-reproductive periods, LAF may be therefore used as maturity markers. Given that LAF cells accumulate lipofuscin, which is a well-known autofluorescent pigment, these structures can be detected under fluorescence microscopy. This technique takes little time and is simple to perform, allowing for rapid assessment of advanced atretic stages even when LAF are small, inconspicuous and widely scattered across the ovarian tissue.

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AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. Ana Magro collected the samples. Ana Magro, José Luis Varela and

Antonio Medina processed the samples for histological observation. José Antonio Paullada-Salmerón and Antonio Medina carried out fluorescence analysis. All the authors participated in the writing of the manuscript.

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SUPPORTING INFORMATION

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