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REVIEW ARTICLE

The reproductive biology of small fishes and the clutch concept: Combining macroscopic and histological approaches

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Abstract

Accurate estimates of reproductive parameters important in understanding life history evolution and conservation of small fishes are dependent upon careful assignment of ovarian phases. Proper assignment is based upon the stages of propagule (oocyte) development, oocyte maturation and the location of any clutch or portion thereof within the ovaries. Macroscopic inspection and assignment of ovarian developmental phases have often been used for small freshwater fishes. By contrast, histological methods for assignment of reproductive phases have been developed and are widely used for marine fishes, but they have rarely been used for small freshwater fishes. We review oocyte development, ovum maturation, and the ovarian cycling process using both macroscopic and histological approaches which incorporate the clutch concept. New terminology, including three actively spawning phases based on macroscopic appearance of the clutch (actively spawning-maturation, actively spawning-hydration, actively spawning-ovulation), is proposed. We assert that combining histological and macroscopic methodologies, shown to be complementary, will allow a more accurate and precise understanding of the reproductive biology of small fishes—especially freshwater fishes experiencing effects of habitat change and loss of habitat.

KEYWORDS
batch, histology, oocyte, ovum, reproduction, terminology

1 | INTRODUCTION

Gaining an understanding of the reproductive output in animals is critical for understanding their life history and evaluating reproductive success. In fishes, clutch size, egg size, relative clutch mass, spawning interval, gonadosomatic index and length of the reproductive season are important parameters for understanding the reproductive life history of a species. The accurate determination of these reproductive parameters is of critical importance in the study of life history evolution and in understanding adaptiveness of traits to environmental conditions, which are becoming ever more important with environmental change and interests in conserving biodiversity (Krabbenhoft et al., 2014; Meador & Brown, 2015; Stevenson and Bryant, 2000; Tao et al., 2018). Moreover, accurate and precise techniques allow researchers to benefit from the collective efforts of the scientific community in making broad comparisons based on individual studies of limited scope.

Ovarian terminology, both macroscopic and histological, was first developed for commercially important marine fishes. Clark (1934) defined several macroscopic stages of oocytes in the Pacific Sardine (*Sardinops sagax caerulea*); these definitions were subsequently adopted

by a number of scientists working with freshwater fishes (reviewed in West 1990). Historically, biologists studying small freshwater fishes typically have adapted terminology developed for larger, commercially important species when developing macroscopic descriptions of ovaries. Varied approaches to the study of reproduction arose, based upon different views of the processes of oocyte development, ovum maturation and oviposition (Gale & Deutsch, 1985; Heins et al., 1992; Heins, 1990; Heins & Baker, 1993; Heins & Rabito, 1986; Hubbs, 1985; Page, 1983; Weddle & Burr, 1991). Similarly, terminology used to describe macroscopic development in small marine fishes has varied among studies (Hsiao et al., 1996; Privitera, 2002). As a result, the data generated by different authors to describe similar reproductive parameters varies considerably, resulting in confusion and unreliability across studies (Heins et al., 1992; Heins & Baker, 1993). A website (https://sse.tulane.edu/node/3384) with macroscopic images of ovarian development and associated terminology was developed (circa 1993) to help standardize the methodology and terminology used to describe reproduction in small freshwater fishes. Núñez and Duponchelle (2009) further attempted to alleviate this confusion by developing a new scale based on both macroscopic and histological terminology as it related to important African and Neotropical fauna, but this terminology has not been widely used in the fish literature. Terminology used to describe ovarian development and reproductive seasonality in marine fishes was similarly confusing until the development of a standardized terminology, based on histological descriptions (Brown-Peterson et al., 2011), became widely used in the literature.

Presented here is a review of macroscopic and histological methods for assessing ovarian phases of reproduction based upon staging of oocyte development and maturation. We show that macroscopic and histological approaches are complementary, and we propose a hybrid scheme combining both methods that can be used for macroscopic descriptions of small freshwater fishes. Included is a proposal for standard terminology to describe oocyte and ovarian processes, which are based upon the combination of recent studies of small freshwater fishes and marine fishes. These studies have important implications for different methods used for the study of life history traits, particularly for small freshwater fishes, and can also be applied to small marine fishes with similar reproductive strategies. The techniques presented here, based upon published results in a variety of fish species, represent an approach that will yield accurate and comparable data for future studies of reproduction, and in particular allow accurate and critically important descriptions and classifications of ovaries prior to ovulation and spawning. Because researchers typically work with preserved specimens, descriptions in this paper are based solely on preserved ovaries.

1.1 | Modes of reproduction

Fishes have a wide variety of reproductive strategies, varying from pelagic broadcast spawners with no parental care to viviparity with extensive parental care (Balon, 1975, 1984), and they can be semelparous (one reproductive cycle in the lifetime) or iteroparous (multiple spawning cycles in the lifetime; Lowerre-Barbieri et al., 2011a). Oocyte recruitment from oogonia can be continuous throughout the reproductive season (i.e., indeterminate fecundity; Hunter et al., 1985), or an individual can recruit all oocytes for a reproductive season at the beginning of the season (i.e., determinate fecundity; Hunter et al., 1992). A variety of reproductive strategies may employ a similar method of oocyte recruitment and development, despite variations in ovarian organization in various groups of fishes. Wallace and Selman (1981) defined three types of ovarian organization: synchronous, asynchronous and group synchronous. In synchronous oocyte development, all the oocytes to be spawned during that reproductive season recruit and develop together as a single batch and are spawned within a very short period of time. Fishes with synchronous oocyte development are total spawners with determinate fecundity (Pavlov et al., 2009), and can be semelparous (e.g., coho salmon *Oncorhynchus kisutch*) or iteroparous (e.g., striped mullet, *Mugil cephalus*; Lowerre-Barbieri et al., 2011a). Asynchronous oocyte development is characterized by oocytes of all sizes in the ovary with no definable mode until just before spawning, when oocyte maturation takes place (Murua & Saborido-Rey, 2003). Fishes displaying asynchronous oocyte development are iteroparous batch (clutch) spawners and have indeterminate fecundity (Murua & Saborido-Rey, 2003); this type of ovarian organization is most commonly seen in warm-water fishes. Group synchronous oocyte development is characterized by at least two modes of oocytes in the ovary; a group of small, primary growth oocytes, and one or more modes of larger, secondary growth oocytes (Murua & Saborido-Rey, 2003). Fishes with group synchronous oocyte development are iteroparous, can be total or batch spawners, can have determinate or indeterminate fecundity, and occur in a variety of water temperatures. Furthermore, there is variation in the developmental rate of secondary growth oocytes in batch spawners, such that a distinct batch (or clutch) can exhibit modal development, (i.e., develops relatively synchronously) or there is completely asynchronous development such that no distinct batch can be identified prior to oocyte maturation (Lowerre-Barbieri et al., 2011a). Batch spawning fishes that exhibit modal development within a batch can be considered 'group synchronous spawners' and often have indeterminate fecundity. Many small freshwater (i.e., darters and sticklebacks) and marine (i.e., killifishes and gobies) species exhibit this group synchronous spawning strategy, and it is this particular strategy that we will focus on here.

1.2 | The clutch concept

The 'clutch concept' is an important focus of ovarian function whether one assumes females produce only one clutch or multiple clutches of eggs each reproductive season. Accurate estimates of reproductive effort and fecundity require knowledge of variation in clutch size and spawning interval (i.e., the number of days between spawning episodes) throughout the reproductive season. For example, counting all gamete cells in an ovary, regardless of developmental stage, is likely to result in erroneous fecundity estimates (Ganias, 2013). This is particularly true in species with indeterminate fecundity, as oocyte recruitment

into secondary growth is an on-going process, and thus the number of oocytes in the ovary do not represent the total number that the female will produce during the reproductive season. Thus, counting the secondary growth oocytes in a fish with indeterminate fecundity will result in an underestimation of annual fecundity. Annual fecundity of fish with indeterminate fecundity can only be estimated when the number of oocytes in each clutch (i.e., the batch), the spawning interval and the duration of the spawning period are known (Hunter et al., 1985). Relatively accurate fecundity estimates can be made by counting all the secondary growth oocytes in the ovary of fish with determinate fecundity if these counts are made prior to first spawning even if the species releases more than one clutch during the spawning season, although this may result in a slight overestimation of annual fecundity since some oocytes will likely become atretic (Murua et al., 2003).

A clutch (or batch) is a group of synchronously developing oocytes that are spawned within a relatively short interval of time (Heins & Rabito, 1986). Results from a number of studies have shown that ripening oocytes in an ovary are ovulated as a group to produce a clutch of ripe eggs which is then spawned (oviposited) over a relatively short period of time, whether all at once (e.g., Threespine Stickleback, *Gasterosteus aculeatus*; Baker, 1994) or in successive bouts lasting varied times as in many darters (Percidae; Heins & Baker, 1993; Weddle & Burr, 1991) and minnows (Leucisidae; Heins & Rabito, 1986). Fish with this reproductive strategy are considered batch (clutch) spawners, and estimating the number of clutches (batches) a female will spawn during the reproductive season is critical for determination of annual fecundity. Histologically, recent spawning (i.e., within the past 12–48 h) can be inferred by the presence of the postovulatory follicle complex (POF, Hunter et al., 1985), and fish containing both fully grown vitellogenic oocytes and POF are considered batch spawners. Daily spawning fish can be identified histologically by the simultaneous presence of POF ≤ 24 h and oocytes undergoing oocyte maturation (Brown-Peterson et al., 2019; Lang et al., 2009).

1.3 | General description of ovaries, oocyte development and ovum maturation

During the spawning season, sexually mature females have ovaries containing gamete cells in distinct, identifiable stages that can be recognized either macroscopically or histologically. Any given female may present some or all of these cellular stages, depending on her reproductive phase.

Names used to describe the gamete cells in the ovaries of fishes have varied among authors. One common approach, particularly when using macroscopic assessment, has been to refer to all the cells as 'ova' or 'eggs' and to distinguish among them by using adjectives to describe the stage of formation, for example, immature ova' and 'mature ova' (Gale & Buynak, 1978; Heins & Clemmer, 1970; Page & Mayden, 1981). In contrast, female gametes are typically referred to as 'oocytes' in histological studies. To allow greater precision in studies of reproductive biology, modification of this terminology is needed to provide a more detailed, refined, and consistent nomenclature.

Macroscopic staging of oocyte development and maturation and assignment of ovarian phases have often been closely intertwined, and terminology for each phenomenon has been very similar based upon the stage of the most advanced oocytes (Table 1). Criteria are based on size, transparency, translucence, opacity and colour of ovaries and oocytes, as well as location in the follicles or lumens of the ovaries (Figure 1). The smallest, apparently latent, cells typically have transparent or slightly translucent cytoplasm with large nuclei easily visible. The active development of oocytes is discernible among cells that have increased in size with the nucleus obscured by cytoplasmic inclusions that cause the oocytes to become translucent and opaque white. Developing oocytes typically become white to cream and yellow in sequence as they increase in size (Figure 1a). The largest follicular cells will vary greatly in appearance from female to female, depending on whether they are completing oocyte development (i.e., vitellogenin loading) or undergoing oocyte maturation. They may be opaque yellow to dark yellow. In cases of advanced development transitioning to oocyte maturation, they may be translucent to transparent and yellow to amber or even orange (Figure 1b). Oocytes termed ripening, but still in follicles, will have elevated vitelline membranes and typically be translucent to transparent, in part as a result of hydration (Figure 1b). Ovulated oocytes, which may be referred to as ripe eggs, present the same characteristics as ripening oocytes; but they are found in the lumens of the ovaries (Figure 1c).

Histological assessment assigns different terminology to oocyte stages and reproductive phases (Table 2). Terminology for oocyte stages is based on Brown-Peterson et al. (2011) and Lowerre-Barbieri et al. (2011b), and is divided into stages of oocyte growth and oocyte maturation (Figure 2). Mitosis occurs in oogonia, and the first stage of meiosis begins as oogonia transform into primary growth oocytes. Meiosis is arrested during the oocyte growth stages at the diplotene stage of primary growth, resumes upon initiation of oocyte maturation, and is again arrested following ovulation (Patiño & Sullivan, 2002). Oogonia and primary growth oocytes are present in the ovaries of all females (except for reproductively active semelparous fishes), and represent the 'stock' from which secondary growth oocytes are recruited. The appearance of cortical alveolar (CA) oocytes indicates the initiation of the reproductive season as fish move into gonadotrophin-dependent secondary growth and development (Brown-Peterson et al., 2011; Lowerre-Barbieri et al., 2011b; Figure 2, top), although fish containing only CA are not yet reproductively active. The appearance of primary and secondary vitellogenic oocytes (Vtg1 and Vtg2, respectively) signals fish are reproductively active, while the appearance of fully grown, tertiary vitellogenic (Vtg3) oocytes determines that the fish is now capable of but not actively spawning. The presence of fully grown oocytes undergoing oocyte maturation (OM) indicates spawning is imminent. There are several histologically distinct oocyte stages that follow the progression of OM, including lipid coalescence (LC, when the lipids in the cytoplasm of the Vtg3 oocyte begin to form several large lipid drops), germinal vesicle migration (GVM, the nucleus moves from the centre of the oocyte towards the animal pole), yolk coalescence (YC, yolk globules in the Vtg3 oocyte begin to coalesce into a liquid yolk), germinal

TABLE 1 An example of macroscopic assignment of ovarian (reproductive) phases based upon oocyte development and maturation of largest oocytes in the ovaries. The same terminology typically has been used for both the most-advanced oocyte stage and the associated reproductive phase

Oocyte stage & reproductive phase	Location of largest oocytes in ovary	Description of ovaries and oocytes
Latent (LA)	Follicles	Ovaries very small, thin and transparent to translucent. Latent oocytes very small and transparent to translucent, with visible nuclei.
Early maturing (EM)	Follicles	Ovaries small to moderate size and translucent to white. Early maturing oocytes small to moderate size, comprising unimodal group, and translucent to white with obscured nuclei.
Late maturing (LM)	Follicles	Ovaries moderate to greatly enlarged and white to cream or yellow. Late maturing oocytes moderate to large size and cream to yellow; size distribution typically bimodal or becoming bimodal.
Mature (MA)	Follicles	Ovaries moderate sized to greatly enlarged and cream to yellow. Two entirely separate size groups of oocytes: maturing and mature. The group of larger, mature oocytes light yellow to yellow without elevated vitelline membranes.
Ripening (MR)	Follicles	Ovaries moderate sized to greatly enlarged and cream to yellow. Two distinct groups of oocytes: maturing and ripening. The larger group of ripening oocytes translucent to transparent and yellow to amber with elevated vitelline membranes.
Ripe (RE)	Lumens	Ovaries moderate sized to greatly enlarged and cream to yellow or amber. Two distinct groups of oocytes: maturing (in follicles) and ripe (ovulated, in lumens). The group of larger, ripe oocytes (eggs) translucent to transparent and yellow to amber with elevated vitelline membranes.

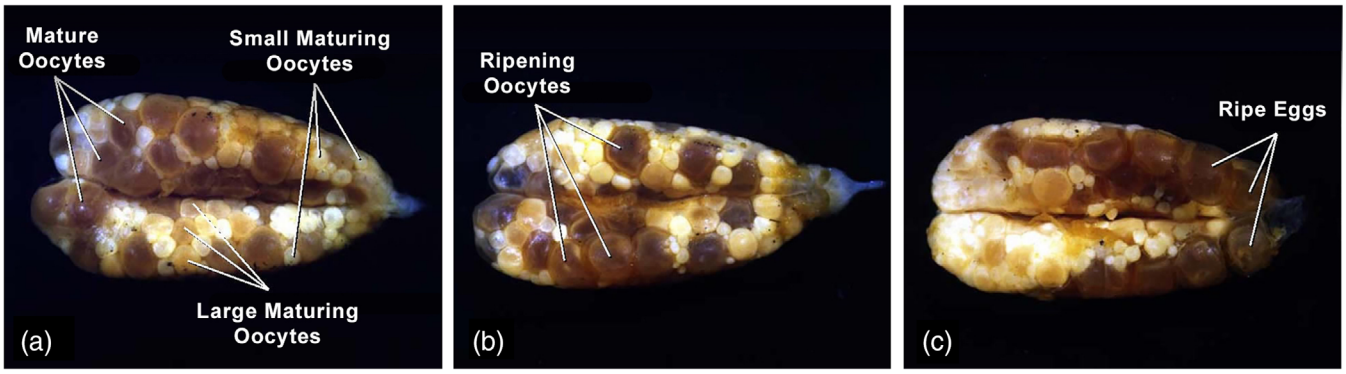

FIGURE 1 Macroscopic examples of oocyte stages during growth and development in the darter *Etheostoma caeruleum*. The largest oocytes represent a distinct clutch (cf. figure 3, panels b, c, and d, respectively): (a) Mature (MA) macroscopic phase. (b) Mature ripening (MR) macroscopic phase. (c) Ripe (RE) macroscopic phase. See Table 1 for oocyte and phase descriptions. Figure taken from https://sse.tulane.edu/node/3384

vesicle breakdown (GVBD, when the nucleus reaches the animal pole and the nuclear membrane dissolves, releasing the chromosomes into the oocyte cytoplasm) and hydration (H; the uptake of water by the oocyte; Figure 2, bottom). All steps of OM take place within the follicle, and hydrated oocytes are ovulated into the lumen. Upon ovulation, POFs are visible in the ovary and can be used as an indicator of recent spawning.

1.4 | Ovarian phases in annual cycles

Macroscopic assessments of ovarian phases are presented in Table 1, and are assigned based on the most advanced stage of oocyte development. As the spawning season of a species nears, sexually mature, adult females begin oocyte development. In doing so, individual females progress from the latent (LA) or sometimes very earliest early maturing (EM) phase through early maturing (EM) and late maturing (LM) phases of ovarian condition. Throughout this time only one group of oocytes, sometimes varying widely in size, colour and opacity is present in the ovaries, although this group typically shows a bimodal size distribution in late LM females. This group of oocytes, especially the larger ones in the bimodal distribution, is a recruitment stock from which the next clutch will be formed.

As the larger oocytes in LM females undergo further development, they form an entirely separate group of larger oocytes which is distinct in appearance (Figure 3). At this time, the clutch is fully apparent for

TABLE 2 Histological stages of oocyte development and maturation and associated reproductive phases, including terminology, location within ovaries and description of typical cells. Reproductive phase is determined by the most advanced oocyte stage and is based on Brown-Peterson et al. (2011)

Oocyte stage	Location in ovary	Reproductive phase	Description
Oocyte growth			
Oogonia (OG)	Follicular nests	Immature	Small, transparent, little structure
Primary Growth (PG)	Follicle	Immature or Regenerating	Small, cytoplasm basophilic, large nucleus, single or multiple nucleoli
Cortical Alveolar (CA)	Follicle	Early Developing	Small, cytoplasm less basophilic, nucleoli surround nucleus, small lipid inclusions throughout cytoplasm
Primary Vitellogenic (Vtg1)	Follicle	Developing	Small acidophilic vitellogenin granules apparent, small lipid droplets visible
Secondary Vitellogenic (Vtg2)	Follicle	Developing	Both acidophilic vitellogenin granules and globules apparent throughout oocyte, lipid droplets visible
Tertiary Vitellogenic (Vtg3)	Follicle	Spawning Capable	Oocyte large, many acidophilic vitellogenin globules throughout oocyte, many lipid droplets visible, prominent follicular layers
Oocyte maturation			
Lipid Coalescence (LC)	Follicle	Actively Spawning	Many acidophilic vitellogenin globules throughout oocyte, lipids begin to coalesce into 1–5 large lipid droplets, nucleus in centre of oocyte
Germinal Vesicle Migration (GVM)	Follicle	Actively Spawning	Nucleus off centre, continued lipid coalescence, beginning of yolk coalescence
Yolk Coalescence (YC)	Follicle	Actively Spawning	Yolk coalesces into liquid sheet, single large lipid droplet, nucleus close to periphery
Germinal Vesicle Breakdown (GVBD)	Follicle	Actively Spawning	No visible nucleus, lipid and yolk coalescence prevalent
Hydration (H)	Follicle and Lumen	Actively Spawning	Oocyte increases in size, complete yolk coalescence with very acidophilic staining, single lipid droplet visible, oocyte often misshapen. No surrounding follicle if ovulated, but postovulatory follicle (POF) apparent in ovary

the first time. The entire group of larger oocytes progresses through three major stages of oocyte development and ovum maturation to complete the formation of the clutch that will be spawned. In a chronological sequence, mature oocytes are initially found in mature (MA) females. The fully grown oocytes are ready to undergo final maturation and are at that time referred to as ripening oocytes, which are found in ripening (MR, mature ripening) females. The ripening process involves the hydration of the follicles, which eventually will burst, releasing ripe eggs into the ovarian lumina of the ripe (RE) females. After oviposition, the female returns to the LM phase. This process is referred to as the clutch production cycle (Figure 3). Diameters of oocytes in a clutch are depicted in Figure 3 and show successive increases among MA, MR and RE females (Heins et al., 1992). Increases in dry mass occur into the MA phase as smaller oocytes in the clutch catch up with the larger ones, whereas dry mass should not increase among MR and RE stages as yolk loading is completed late in the MA phase (Heins et al., 1992).

Histologically, reproductive phases are assigned based on the most advanced stage of oocyte development (Table 2), and reproductive phase terminology follows Brown-Peterson et al. (2011). The immature phase is defined by the occurrence of oogonia and/or primary growth (PG) oocytes. Once fish first reach sexual maturity, they enter the reproductive cycle, with ovaries containing only CA and PG oocytes considered to be in the early developing subphase but not yet reproductively active. The appearance of vitellogenic oocytes signals movement into the developing phase of the reproductive season, where fish are considered reproductively active but not yet able to spawn. Fish enter the spawning capable phase with the appearance of Vtg3 oocytes. Batch spawning species often remain in the spawning capable phase for weeks or months, depending on the length of their spawning season. The early developing, developing and spawning capable phases are defined by oocyte growth and development (Figure 2, top). Fish enter the actively spawning subphase of the spawning capable phase upon the initiation of OM and spawning typically occurs within 12–48 h once fish enter this subphase, depending on water temperatures. Fish in the spawning capable and actively spawning phases can have ovaries containing POF, which indicates recently ovulated oocytes. Batch spawning species will cycle between the spawning capable and actively spawning phases throughout the spawning season. Once the spawning season is completed, fish move into the regressing phase, characterized by oocyte atresia, fewer vitellogenic oocytes and,

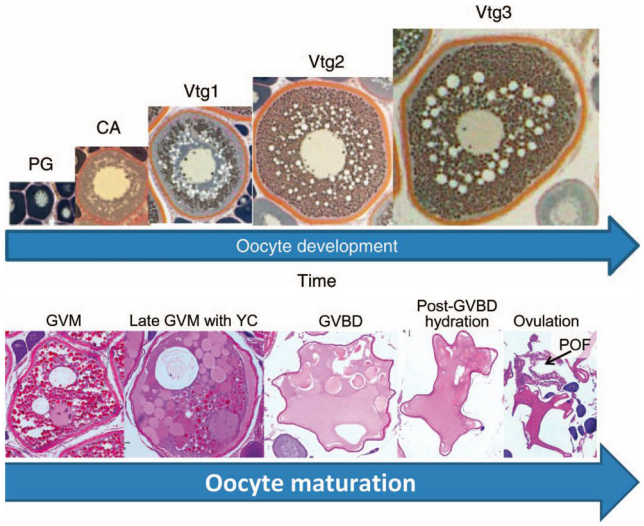

FIGURE 2 Histological example of oocyte stages during oocyte growth and development (top) and oocyte maturation (bottom): PG, primary growth; CA, cortical alveolar; Vtg1, primary vitellogenic; Vtg 2, secondary vitellogenic; Vtg 3, tertiary vitellogenic; GVM, germinal vesicle migration; YC, yolk coalescence; GVBD, germinal vesicle breakdown; POF, post-ovulatory follicle. Reproduced from Lowerre-Barbieri et al. (2011a). © 2011 Taylor and Francis Group, LLC, license number 5271501080943

in some species, POF; fish in this phase are considered reproductively inactive. Most species move relatively quickly from the regressing to the regenerating phase, which is characterized by PG oocytes, late-stage atresia, and evidence of previous spawning in the form of muscle bundles and enlarged blood vessels (Brown-Peterson et al., 2021). Fish remain in the regenerating phase undergoing active mitosis to replenish PG oocytes (Grier et al., 2009) prior to re-entry into the early developing subphase at the start of the subsequent reproductive season.

1.5 | Equivalent gamete stages and ovarian phases

The nomenclature of histology to describe oocyte stages can be equated with macroscopic classification, based upon the current understanding of ovarian processes (Table 3). Oocytes in the primary growth stage have been considered latent in macroscopic observations. Those oocytes in cortical alveolar and vitellogenic stages have been described as maturing oocytes, whereas ones in the early stages of OM (through GVM) have been considered mature oocytes while oocytes in later stages of OM (GVBD through hydration) have been recognized as ripening oocytes. Hydrated and ovulated oocytes have been identified as ripe eggs.

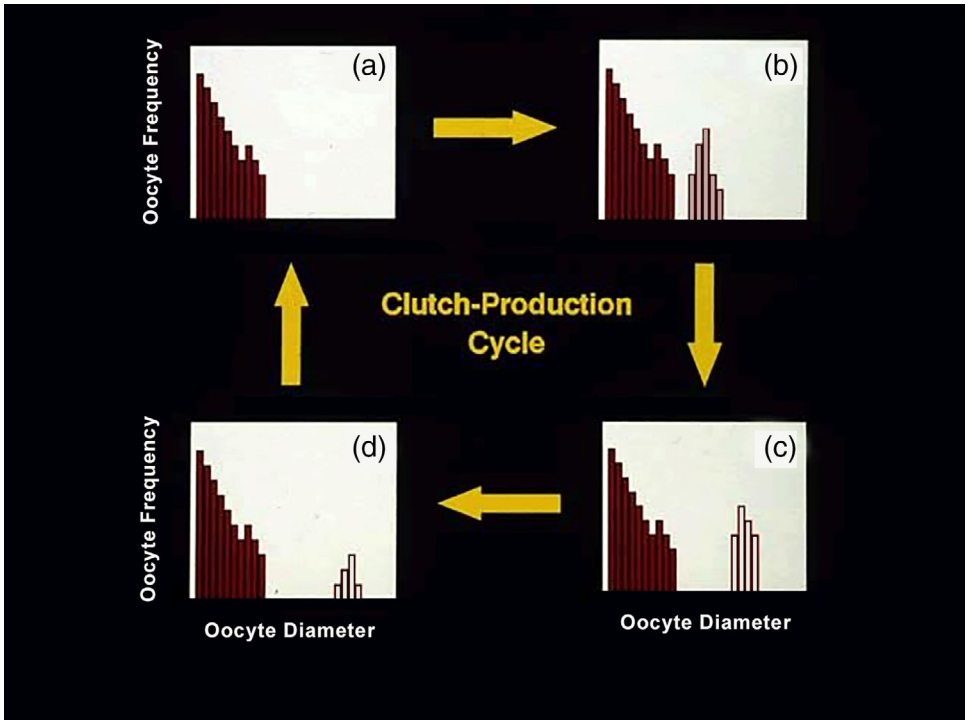

FIGURE 3 The clutch production cycle, showing the macroscopic development of and increase in size of oocytes in the next clutch to be spawned. (a) Late Mature (spawning capable) phase. (b) Mature (actively spawning-maturation) phase. The lighter-coloured bars represent the most advanced (clutch) oocytes in lipid coalescence through germinal vesicle migration. (c) Mature ripening (actively spawning-hydration) phase. (d) Ripe (actively spawning-ovulation) phase. Larger oocytes (open bars) in panels c and d are hydrated, with a partial clutch depicted in panel d, illustrating a female ovipositing small numbers of eggs during each spawning encounter over a short time. Figure modified from Heins and Baker (1993), with permission from the Fisheries Society of the British Isles, and from https://sse.tulane.edu/node/3384

TABLE 3 Equivalent stages of oocyte development and maturation drawn from macroscopic and histological techniques for the study of reproduction in freshwater and marine fishes

Macroscopic	Histological
Latent	Primary growth
Early maturing	Cortical alveolar to primary vitellogenic
Late maturing	Secondary to tertiary vitellogenic
Mature	Lipid coalescence to germinal vesicle migration
Ripening	Germinal vesicle breakdown to hydration
Ripe	Hydrated, ovulated

TABLE 4 Equivalent ovarian phases using macroscopic and histological techniques taken from the study of reproduction in freshwater and marine fishes. These phases are based upon the annual reproductive cycle

Macroscopic	Histological
Latent (LA)	Immature (IM) or regenerating (RN)
Early maturing (EM)	Developing (DV)
Late maturing (LM)	Spawning capable (SC)
Mature (MA)	Actively spawning (AS)
Ripening (MR)	Actively spawning (AS)
Ripe (RE)	Actively spawning (AS)

Females shown to be in the immature or regenerating phases histologically have been considered to be latent morphologically (Table 4). There is no universally used macroscopic equivalent for the regressing histological phase, although some authors have assigned a spent phase to fish at the end of the spawning season (Phillip, 1993; Williams and Bonner, 2006). Given the relatively ephemeral occurrence of the spent phase, regressing fish are also likely to be considered latent macroscopically. Developing females have been described macroscopically as early maturing, whereas late maturing females identified macroscopically are assigned to the spawning capable phase histologically. Females determined to be actively spawning using histology have been recognized as mature, ripening and ripe using macroscopic terminology.

1.6 | Integration of histological and macroscopic approaches

A unified taxonomy for macroscopic examinations and observations of ovarian phases, which is informed by histological research, is proposed (Table 5). The categories applied to ovarian phases incorporate processes of oocyte development based on the most developed oocytes. These phases distinguish between sexually immature and mature females. Among ovarian phases formerly considered macroscopically latent (LA), sexually immature females would now be categorized as immature (IMM), whereas sexually mature females are classified as regenerating (RGN) or regressing (RGS). Those macroscopic phases named early maturing (EM) and late maturing (LM) previously are now renamed developing (DEV) or spawning capable (SPC), respectively. Among clutch-bearing females, the three macroscopic phases formerly termed MA, MR and RE are now named actively-spawning-maturation (ASMA), actively-spawning-hydration (ASHY), and actively-spawning-ovulation (ASOV), respectively. The actively spawning phases are part of the clutch production cycle within the overall reproductive cycle (Figure 4). This terminology recognizes the oocyte stages that are easily identified macroscopically while incorporating standard terminology typically used in histological studies, thus achieving a single, standardized terminology for both macroscopic and histological assessments.

2 | DISCUSSION

Correct assessment of oocyte stages and assignment of ovarian phases is important in obtaining accurate, comparable data on reproduction of fishes. For instance, identifying the beginning of ovarian recrudescence in the developing phase is important for not only understanding when fishes are becoming reproductively active temporally, but is also important in identifying the size at sexual maturity. Similarly, identifying the regressing phase allows a better definition of the end of the reproductive season. Gaining a more complete understanding of the timing and duration of the reproductive season is important since fish reproduction can be impacted by habitat or climatic variation in environmental conditions influencing reproductive phenology (Divino & Tonn, 2007: Krabbenhoft et al., 2014; Martin, 2014a; Stevenson & Bryant, 2000). Moreover, using appropriate oocyte stages, as they are assigned to ovarian phases, is a prerequisite for quantifying clutch and ovum sizes with accuracy and precision.

Earlier systems for macroscopically categorizing ovarian phases usually did not recognize some of the phases presented here. For example, distinguishing between sexually mature and immature females is important for understanding the reproductive life history of small fishes. Sexually immature females were not distinguished from sexually mature females within the former 'latent' category, which could lead to confusion. Here there are separate phases for sexually immature (IMM) and mature, reproductively inactive females (RGS and RGN). Separating these phases during the reproductively inactive portion of the season may be a challenge macroscopically, but sexual immaturity or maturity can clearly be distinguished macroscopically during the reproductive season and be unambiguously classified. Distinguishing between these reproductively inactive phases macroscopically can be informed by knowing the size of the fish and time of the year of the collection.

Not everyone working with this schema will need to use it in detail. Combining phases presented in the schema proposed here may be appropriate under certain circumstances. For example, we define the reproductive season as that period of time during which females contain vitellogenic oocytes, which includes ovarian recrudescence. In contrast, the spawning season is only that period of time during which at least some females are actively spawning or capable of spawning.

TABLE 5 Proposed nomenclature for ovarian phases and oocyte stages for use in macroscopic investigations of reproductive biology in small fishes with group synchronous spawning. Histological research on processes of oocyte development has informed the terminology. Reproductive phases are determined using the stage of the most advanced oocytes in ovaries and the location in the ovaries. Those stages are given using histological taxonomy, but descriptions provide diagnostic characteristics for macroscopic inspections. Determining the reproductive phase is the most important aspect of morphological analyses for determining the reproductive period and for selecting phases to be used to estimate clutch parameters

Reproductive phase	Most advanced oocyte stage(s) (Histological)	Location in ovaries	Macroscopic description / comments
Sexually immature			
Immature (IMM)	Oogonia, primary growth	Follicular nests and follicles	Ovaries very small, very thin, flaccid, transparent to translucent; sometimes difficult to recognize. Oocytes very small, transparent and/or translucent with visible nuclei; oogonia not visible macroscopically
Sexually mature			
Regenerating (RGN)	Primary growth	Follicles	Ovaries small, somewhat flaccid, transparent to translucent with the proliferation of oocytes; easily recognized. Oocytes small, transparent and/or translucent with visible nuclei; often small percentage of oocytes translucent with obscured nuclei or white; reproductively inactive between reproductive seasons
Developing (DEV)	Cortical alveolar, primary–secondary vitellogenic	Follicles	Ovaries small to moderate size and white to cream in colour. Oocytes small to moderate size, white or cream colour, exhibiting early stages of oocyte development; prior to beginning of spawning season
Spawning Capable (SPC)	Tertiary vitellogenic, postovulatory follicles can be present	Follicles	Ovaries moderate to greatly enlarged and white to cream or yellow. Oocytes in many sizes (small to large), cream to yellow; size distribution easily discerned bimodal or becoming bimodal without separate clutch; seen throughout the spawning season in females not actively spawning
Actively Spawning–Maturation (ASMA)	Lipid coalescence (LC) through germinal vesicle migration (GVM)	Follicles	Ovaries moderate sized to greatly enlarged and cream to yellow. Oocytes cream to yellow; easily identifiable unovulated clutch
Actively Spawning–Hydration (ASHY)	Germinal vesicle breakdown (GVBD) through hydration	Follicles	Ovaries moderate sized to greatly enlarged and cream to yellow. Oocytes in distinct clutch translucent to transparent and yellow to amber with vitelline membranes elevated; unovulated clutch
Actively Spawning–Ovulation (ASOV)	Hydrated, ovulated	Lumens	Ovaries moderate sized to greatly enlarged and cream to yellow or amber. Clutch (distinct, complete or partial) of ovulated oocytes (eggs) translucent to transparent and yellow to amber with vitelline membranes elevated
Regressing (RGS)	Atretic vitellogenic oocytes, primary growth	Follicles	Ovaries small, transparent to translucent. Developing oocytes largely lacking; small numbers of oocytes in different stages dotting ovaries.

The developing phase cannot, therefore, be included when determining the spawning season. Combining the three actively spawning phases would allow one to determine when spawning is actively occurring. A combination of the actively spawning phases with the spawning capable phase would allow duration of the entire spawning season to be determined, while the addition of the developing phase includes the entire reproductive season, including recrudescence. In other words, when considering reproductive phenology, the reproductive season occurs during the time period in which developing, spawning capable or actively spawning females are present, whereas the spawning season occurs only when spawning capable and actively spawning females are observed. Overall, then, the actively spawning phases described here are most useful for obtaining an understanding of when spawning will occur.

One has to be careful not to combine the actively spawning phases to quantify reproductive characteristics such as fecundity, egg size or clutch mass because the data may be inaccurate. For example, except in cases where females oviposit all clutch eggs in a single spawning effort (e.g., Threespine Stickleback), using the ASOV phase for fecundity determinations would lead to erroneously low numbers (see Figure 3d); moreover combining clutch counts of females in the ASOV phase with those in ASMA and ASHY phases would lead to

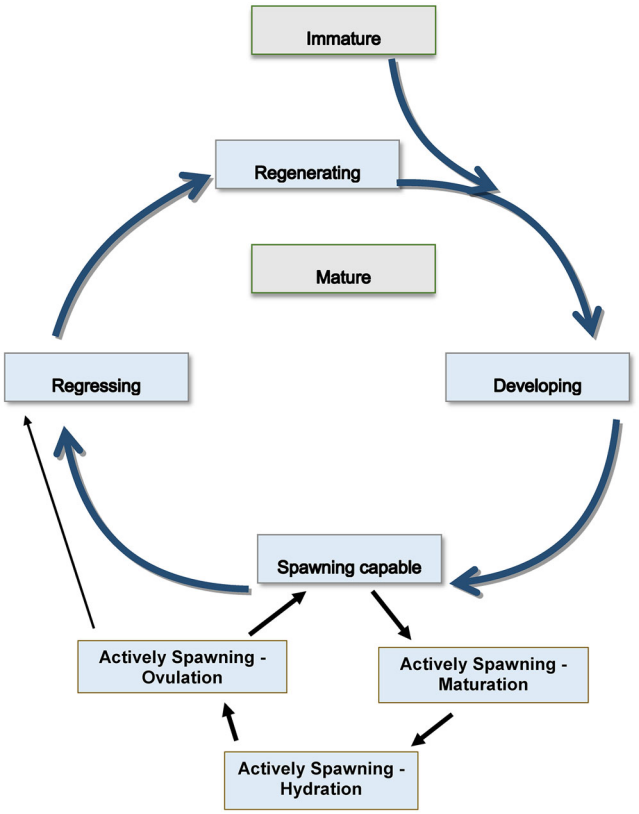

FIGURE 4 Diagram showing relationship of ovarian phases to reproductive cycle in proposed schema for staging small freshwater fishes. Figure adapted from Brown-Peterson et al. (2011)

greater variation in the data, inaccurate mean fecundity estimates, and inaccurate fecundity-body size relationships. In general, and with limited exceptions, the ASOV phase is best used for estimating ovum size, while the ASHY or ASMA phases should be used for fecundity estimates. In all cases, explicit indication and description of the phases used for obtaining estimates of reproductive parameters is advised.

Assigning the macroscopic reproductive phase to any one fish can be challenging since the descriptions of ovarian phases represent specific steps in a cyclic process and intermediate phases do occur. The difficulty is likely to involve distinguishing between some phases more than others. Thus, an informed decision may have to be made in some cases based on knowledge of time of collection, water temperature, or other variables. Such decisions should be conservative depending on the objectives of the investigation. For example, a decision involving data being gathered for clutch parameters should involve careful distinction between spawning capable and actively spawning fish so as not to include spawning capable females that do not have a fully developed, distinct clutch. Likewise, among actively spawning fish, one needs to be careful in decisions about which of the three stages to include in estimates of clutch size and egg mass or diameter. The most developed oocytes will provide the most accurate data, and therefore combining clutch size or mass data among the three actively spawning phases could introduce error into the measurements. Typically, the oocyte maturation stages are synchronous within a single female but not necessarily synchronous within the population, such that it may be possible to capture fish within all three of the actively spawning phases in a single collection. However, using either the ASMA or ASHY actively spawning phases to determine clutch size (batch fecundity) would likely result in fairly accurate estimates. Care should be taken estimating clutch size using the ASOV phase, as some eggs may have already been spawned.

Seasonal or annual fecundity is determined in part by the number of clutches produced within each spawning season. MacGregor (1970) summarized relationships between size frequency distributions of oocytes and the number of batches of ova produced in seasonal spawners. We know that females within a population can vary in the rate at which clutches are produced (Brown-Peterson & Heins, 2009). Within one sample there may be variation among individuals, and female spawning interval as well as clutch sizes may vary with time or age during the spawning season (Fitzhugh et al., 2012). Obtaining robust estimates of seasonal or annual fecundity remains a challenge and an area for future research. For individual females, determination of interclutch interval and clutch size, accounting for temporal variation, should allow accurate estimates. At the population level, the challenge increases considerably. Sophisticated modelling will be needed to account for the added influence of age/size distributions and differential entry into the spawning season, which may change seasonally or annually. We know that fecundity is positively correlated with fish size/age. Additionally, smaller/younger females often enter the spawning season later and exit it earlier than larger/older females, and they also tend to have a longer interclutch interval than larger/older females. Therefore, smaller females will contribute less to population fecundity than larger females.

We hasten to emphasize that within the ovaries of group synchronous fishes, oocytes in all stages of development are often present during the spawning season. Moreover, our schema is based upon the most developed oocytes in an ovary; therefore, less developed oocytes should not be considered when assigning phases despite their presence in the ovary. For example, females in the actively-spawning-maturation stage (ASMA) are expected to have Vtg3 oocytes as well as LC and GVM oocytes, but this phase is defined by either the LC or GVM oocytes (i.e., most advanced stage). There may be more Vtg3 oocytes present in the clutch early in ASMA, which is consistent with the catch-up phenomenon documented by Heins et al. (1992). In this respect, the oocyte stages in the proposed schema do not have the same terminology as the reproductive phase, unlike what was often the practice in previous macroscopic investigations.

The spawning interval, or number of days between successive spawns, can affect the percentages of different oocyte stages within the ovary. For example, Three-Spined Stickleback can be found in three different phases during the 6-week reproductive season, with varying percentages of oocyte stages apparent in the ovaries of each phase (Figure 5), although fish in the spawning capable phase were most commonly captured throughout the spawning season. The increase

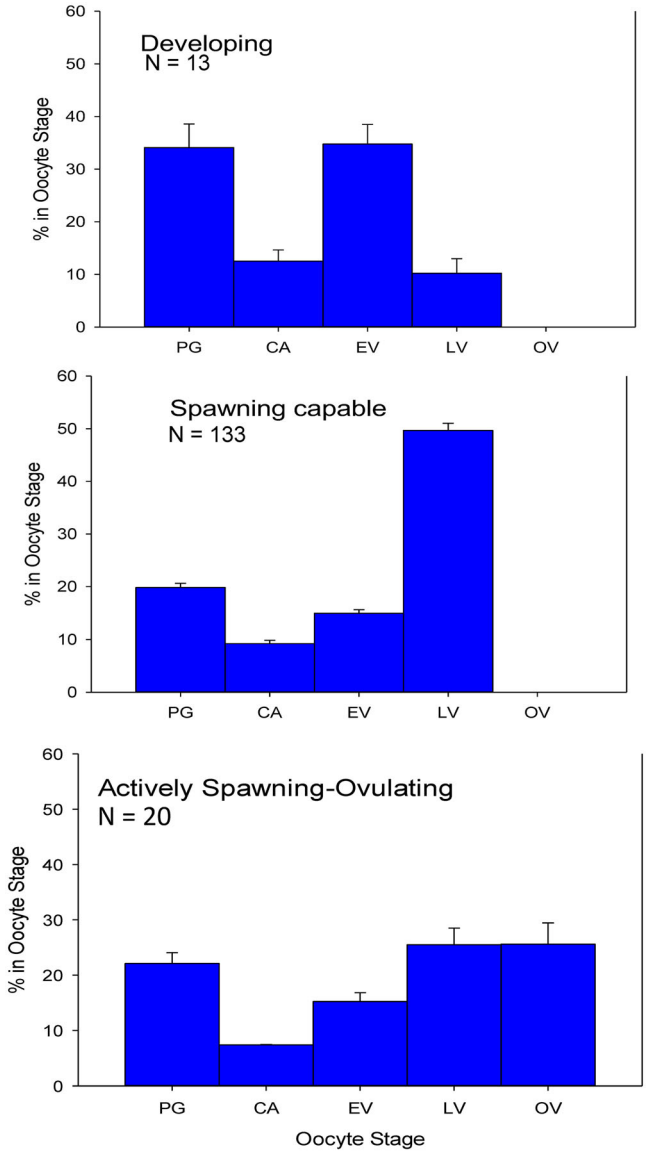

FIGURE 5 Oocyte frequency graphs of three reproductive phases of Three-Spined Stickleback during the 6-week spawning season in Alaska. *N* = number of fish in each phase. Oocyte stages defined as primary growth (PG), cortical alveolar (CA), early vitellogenic (EV; equivalent to primary vitellogenic), late vitellogenic (LV, equivalent to tertiary vitellogenic) and ovulated (OV). Data from Brown-Peterson and Heins (2009)

in percentage of late vitellogenic (e.g., Vtg3) oocytes between the developing and spawning capable phases is obvious both histologically and macroscopically. The largest oocytes, ovulated but still in the ovary, appear during the actively spawning-ovulated phase, which again is easily observed both histologically and macroscopically. The recruitment of late vitellogenic (LV) oocytes into oocyte maturation is evident by the decrease of the percentage of LV oocytes in the ovary between the spawning capable and actively spawning-ovulation phases (Figure 5). In this species, which has been shown to have a spawning interval of 2.2–7.8 days in Alaskan waters (Brown-Peterson & Heins, 2009), there is still a relatively high percentage of late vitellogenic oocytes present during ovulation which would be recruited into the subsequent clutch, described by Heins et al. (1992) as a yolk-loading phenomenon in darters. Similarly, *Fundulus jenkensii*, which has a semilunar spawning interval, have few (~4%) Vtg3 oocytes, no OM oocytes, and many (66%) Vtg1 oocytes present during neap tides (i.e., ~7 days post spawning; Lang et al., 2009) as most of the Vtg3 oocytes were recruited into OM during the three days of spawning around the spring tide. In contrast, during spring tides when the fish are spawning, 17–20% of oocytes are in OM, but there is still a high percentage of Vtg3 oocytes (25–27%; Lang et al., 2009) that will be recruited into OM; it is likely these fish show an oocyte size-frequency distribution with three overlapping modes (primary growth through Vtg1, Vtg2–Vtg3 and OM, respectively).

The term 'fractional spawner' has been used in the literature to refer to group synchronous fishes. Nikolsky (1963) introduced this term but did not define it. We strongly recommend against use of this term and propose that it be retired. The term may imply processes unrelated to what is now known about ovarian cycling, and its meaning is not specific. For instance, to what fraction does this term refer—release of part of an ovulated batch, or release of multiple batches during the spawning season? Thus, this term is a misnomer. Instead, investigators should refer to clutch-spawning or batch-spawning fishes and define the clutches following the terminology proposed here.

Although the terminology proposed here was developed primarily for macroscopic inspections of small freshwater fishes, it is also applicable to small marine fishes with a group synchronous spawning strategy that spawn demersal eggs. These marine fishes are dominated by semilunar spawners, typically releasing eggs during spring tides, and represent individuals from 12 orders and 21 families, including Atheriniformes (e.g., *Leuresthes* spp. and *Menidia* spp), Cyprinodontiformes (e.g., *Fundulus* spp.), and Perciformes (e.g., Blenniidae; Martin, 2014b). These species thus develop and spawn a clutch every two weeks, and macroscopic inspection of the ovaries during spring tides reveals oocytes in a distinct clutch, while this clutch of large oocytes is not apparent in fishes captured during neap tides (i.e., Lang et al., 2009). Some marine fishes with a shorter spawning interval, such as the tropical goby, *Asterropteryx semipunctata*, that spawns every 3–5 days, also show a bimodal oocyte size distribution, with the largest oocytes representing those that are translucent or nearing hydration (Privitera, 2002).

In conclusion, the schema presented here is a hypothesis reflecting the detailed processes of ovarian cycling in small freshwater fishes (Figure 4). This schema defines all phases of the reproductive cycle as it relates to oocyte growth and development. This proposed nomenclature (Table 5) is based on reproductive research spanning decades based on both marine and freshwater fishes and should provide a robust guide for future macroscopic reproductive research. Nonetheless, there may need to be minor changes in some details involving oocyte stages assigned to particular ovarian phases. A study involving macroscopic assessment of ovarian phases followed by histological examination of the same ovaries would be an appropriate test of the details.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

ETHICS STATEMENT

As a review article, no animal subjects were used for this report. The ideas expressed have been drawn from our own research and that of other authors who have been cited herein.

AUTHOR CONTRIBUTIONS

David C. Heins: Conceptualization, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, and Writing – review – editing; Nancy J. Brown-Peterson: Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, and Writing – review & editing

DATA AVAILABILITY STATEMENT

In so much as this is a review and a formulation of new perspectives on methodology, there are no new data to share.

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