Regulation of fish fecundity types in changing environments:
the case of species of the genus *Alosa*

Doctoral dissertation

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Διδακτορική διατριβή

**Ρύθμιση του προτύπου γονιμότητας των ψαριών σε μεταβαλλόμενα περιβάλλοντα:
η περίπτωση των ειδών του γένους *Alosa***

ΦΟΙΒΟΣ ΑΛΕΞΑΝΔΡΟΣ ΜΟΥΧΛΙΑΝΙΤΗΣ

Βιολόγος

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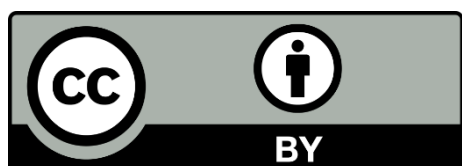
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Αφιερωμένο στον πατέρα μου,

τον συμβουλάτορα και καθοδηγητή

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List of abbreviations

AM = advanced mode in an oocyte size frequency distribution

AS = advanced oocyte developmental stage

BB = anadromous Alewife, *Alosa pseudoharengus*, population entering Bride Lake to spawn

CA = cortical alveolar oocyte developmental stages

CA-1 = primary cortical alveolar oocyte developmental stage

CA-2 = secondary cortical alveolar oocyte developmental stage

CA-3 = final cortical alveolar oocyte developmental stage

CF = condition factor

F_t = total fecundity

GSI = gonadosomatic index

GVBD = germinal vesicle break down oocyte developmental stage

GVM = germinal vesicle migration oocyte developmental stages

GVM-1 = early germinal vesicle migration oocyte developmental stage

GVM-2 = late germinal vesicle migration oocyte developmental stage

HYD = hydration oocyte developmental stage

INT = females in-between two ovulation/spawning events

L = total length

OD = oocyte diameter

OD_{AM} = mean oocyte diameter of the advanced mode in an oocyte size frequency distribution

OD_{PG} = mean diameter of oocytes at the primary growth phase

OD_{SG} = mean diameter of oocytes at the secondary growth phase

OG = oogonium/a

OSFD = oocyte size frequency distribution

PAT = landlocked Alewife, *Alosa pseudoharengus*, population inhabiting Pattagansett Lake

PG = primary growth

POF = postovulatory follicle

POF$_{XSA}$ = cross-sectional area of the biggest postovulatory follicle

POST = females immediately or closely after ovulation/spawning

PRE = females prior to ovulation

RF$_{AM}$ = relative fecundity of the advanced mode in an oocyte size frequency distribution

RF$_b$ = relative batch fecundity

RF$_{CA}$ = relative fecundity of oocytes at the cortical alveolar stages

RF$_{PG}$ = relative fecundity of oocytes at the primary growth phase

RF$_{Sb1}$ = relative fecundity of the oocytes at the second-most advanced developmental stage

RF$_{SM1}$ = relative fecundity of the first subsequent mode in an oocyte size frequency distribution

RF$_t$ = relative total fecundity

RF$_y$ = relative realized annual fecundity

RF$_{200-320}$ = relative fecundity of oocytes having diameters between 200 and 320 μm

RUN = females during ovulation/spawning

SG = secondary growth

SM1 = first subsequent mode in an oocyte size frequency distribution

SM2 = second subsequent mode in an oocyte size frequency distribution

SM3 = third subsequent mode in an oocyte size frequency distribution

VIT = vitellogenic oocyte developmental stages

VIT-1 = primary vitellogenic oocyte developmental stage

VIT-2 = secondary vitellogenic oocyte developmental stage

VIT-3 = tertiary vitellogenic oocyte developmental stage

W = total weight

W$_{ev}$ = eviscerated weight

W$_g$ = ovary weight

Glossary

Anadromous life-history form = fish are born in freshwater, then migrate to the ocean as juveniles where they grow into adults before migrating back into freshwater to spawn

Atresia = oocyte degeneration and resorption

Capital breeding = food resources are acquired in advance to offspring production

Determinate fecundity type = SG recruitment is completed before the onset of the spawning activity

Dynamic equilibrium = SG recruitment counterbalances the spawning of multiple oocyte batches, so that the released oocytes are replenished

Early active spawners = females that had spawned only once within the surveyed season

Fecundity type = how and when oocytes to be spawned are produced

Income breeding = food intake is adjusted concurrently with offspring production, without reliance on reserves

Indeterminate fecundity type = SG recruitment occurs also after the onset of the spawning activity

Iteroparous reproductive strategy = two or more reproductive cycles occur during lifetime

Landlocked life-history form = fish complete their life cycle in impounded freshwater bodies, such as reservoirs and lakes

Late active spawners = females that had spawned at least two times within the surveyed season

Massive atresia = generalized oocyte degeneration and resorption

Mopping-up = absorption of surplus of SG oocytes through massive atresia at the end of the spawning activity

Multiple/batch/partial/serial/heterochronal oocyte release strategy = oocytes are released in multiple sequential spawning events within the spawning season

Oceanodromous life-history form = migratory fish that spend their whole life in salt water

Oogenesis = the morphological and functional processes that lead to the production of fertilizable eggs

Oogonia = germ cells that may divide mitotically to maintain their population within the germinal epithelium or may enter meiosis and give rise to oocytes

Oogonial proliferation = mitotic division of an oogonium into two new oogonia

Ovulatory cycle = time interval between two sequential ovulation/spawning events

Postovulatory follicle = follicular layers that remain in the ovary after the release of the ovum during spawning

Postovulatory follicle cohort = all postovulatory follicles originated from a single spawning event

Relative batch fecundity = number of oocytes at the most advanced developmental stage per g of fish eviscerated weight

Relative realized annual fecundity = total number of oocytes that would have been released during the entire period of spawning activity per g of fish eviscerated weight

Relative total fecundity = total number of oocytes at the secondary growth phase per g of fish eviscerated weight

Reproductive potential = the ability of a fish stock to produce viable eggs and larvae that may eventually recruit into the adult population or fishery

Reproductive season = the period that extends from the onset of SG recruitment till the completion of spawning activity of a fish stock

Semelparous reproductive strategy = a single reproductive cycle occurs in lifetime

SG recruitment = recruitment of new oocytes from the primary growth to the secondary growth phase of oogenesis

Spawning capable fish = fish with oocytes at an advanced vitellogenic stage, capable of spawning during the current reproductive cycle

Spawning season = the period between the release of the first and the last egg by the females of a fish stock

Total oocyte release strategy = oocytes are released in a single spawning event within the spawning season

Vitellogenesis = accumulation of yolk protein in the ooplasm

Summary

Survival of a fish stock depends on its ability to replenish – through reproduction – any losses originated from natural or fishing mortality. Hence, the main parameter in applied fisheries reproductive biology is the reproductive potential, which is highly influenced by egg production. To understand egg production, the underlying mechanisms of oogenesis, i.e., the morphological and functional processes that lead to the production of fertilizable eggs, should be unveiled. Even though the course of oogenesis is known, many pieces of information remain elusive, such as the regulation of fish fecundity type, mainly due to the disproportional analysis of the different phases of oogenesis (i.e., oocyte production from oogonia, primary growth phase, secondary growth phase, ovulation). In that respect, the principal aim of the present study was to describe how the fecundity type is shaped by analyzing different phases of oogenesis at different temporal scales and specific time-frames, with special focus on the early SG recruitment process.

Different populations of species of the genus *Alosa* were analyzed in this study and the selection was based on their variability in reproductive strategy – life-history form combinations. In specific, a semelparous anadromous, two iteroparous anadromous and two iteroparous landlocked populations of four *Alosa* species were included in this study. Semelparity and anadromy enabled sampling designs that associated location and reproductive stage, and thus analyses at different temporal scales (lifetime, seasonal, ovulatory cycle) and time-frames (prior to, during and after the spawning activity). Moreover, the selected populations provided the opportunity to test whether life-history form influences the fecundity type.

The present study aimed to answer four main scientific questions and each one of them is analyzed at a different chapter. The chapters were organized based on the different temporal scales/time-frames; the main idea was to move from the broader (lifetime) to the narrower temporal scale (ovulatory cycle) and from the preceding (prior to spawning activity) to the latter time-frame (after spawning activity). Specifically, the role of oogonia and the oocyte recruitment process from the primary to the secondary growth phase of oogenesis (SG recruitment) prior to the onset of spawning activity were analyzed in a semelparous anadromous *A. alosa* population in chapter 3. Subsequently, in chapter 4, SG recruitment and the secondary growth phase of oogenesis were examined during the spawning activity in an iteroparous anadromous *A. aestivalis* population. In chapter 5, *A. pseudoharengus* was the model species and the analyses were focused on SG recruitment and the secondary growth phase of oogenesis during and after the spawning activity, while the influence of life-history form on fecundity type regulation was also tested. Finally, in chapter 6, the occurrence of SG recruitment at a specific time-frame within the ovulatory cycle was investigated by using *A. macedonica* as the model species.

In conclusion, this study managed to provide general conclusions for each one of the main scientific questions. More specifically, SG recruitment was proven to occur at a stepwise manner both prior and during the spawning activity and regardless of the fecundity type. In addition, SG recruitment was evinced to occur at a specific time-frame within the ovulatory cycle. Moreover, an untypical indeterminate fecundity type was revealed, and life-history form did not appear to be a decisive factor for regulating fish fecundity type.

Περίληψη

Η επιβίωση ενός ιχθυοπληθυσμού εξαρτάται από την ικανότητά του να αναπληρώνει – μέσω της αναπαραγωγής – πιθανές απώλειες οφειλόμενες σε φυσική ή αλιευτική θνησιμότητα. Συνεπώς, μία από τις κύριες παραμέτρους στην αναπαραγωγική βιολογία ιχθύων αποτελεί το αναπαραγωγικό δυναμικό, το οποίο επηρεάζεται σημαντικά από την παραγωγή αυγών. Η διαδικασία της ωογένεσης, δηλαδή η αλληλουχία των μορφολογικών και λειτουργικών διεργασιών που οδηγούν στην παραγωγή αυγών ικανών προς γονιμοποίηση, είναι επί πολλοίς γνωστή, ωστόσο αρκετά κομμάτια του παζλ λείπουν. Για παράδειγμα, η ρύθμιση του προτύπου γονιμότητας των ψαριών, δηλαδή ο μηχανισμός παραγωγής και ανάπτυξης των ωοκυττάρων που προορίζονται για απόθεση, αλλά και το χρονικό πλαίσιο εντός του οποίου διεξάγεται, δεν έχουν αποκαλυφθεί πλήρως. Βασικός λόγος της παρατηρούμενης έλλειψης γνώσης σχετικά με τη διαδικασία της ωογένεσης αποτελεί το γεγονός ότι οι διαφορετικές φάσης αυτής (η παραγωγή πρωτογενών ωοκυττάρων από ωογόνια, η πρωτογενής φάση ανάπτυξης ωοκυττάρων, η δευτερογενής φάση ανάπτυξης ωοκυττάρων, η ωορρηξία/ωοαπόθεση) έχουν μελετηθεί σε δυσανάλογο βαθμό. Στόχος της παρούσας διατριβής ήταν η περιγραφή του μηχανισμού ρύθμισης του προτύπου γονιμότητας ιχθύων αναλύοντας διαφορετικές φάσεις της ωογένεσης σε ποικίλες χρονικές κλίμακες.

Αναλύθηκαν διαφορετικοί πληθυσμοί τεσσάρων ειδών του γένους *Alosa*. Συγκεκριμένα, αναλύθηκαν: ένας πληθυσμός με ανάδρομο κύκλο ζωής τα άτομα του οποίου υλοποιούν έναν αναπαραγωγικό κύκλο στη διάρκεια της ζωής τους, δύο ανάδρομοι πληθυσμοί τα άτομα των οποίων αναπαράγονται πλέον της μίας φορές στη διάρκεια της ζωής τους, και δύο πληθυσμοί με ολοβιωτικό κύκλο ζωής τα άτομα των οποίων ολοκληρώνουν τουλάχιστον δύο αναπαραγωγικούς κύκλους στη διάρκεια της ζωής τους. Η επιλογή των εν λόγω πληθυσμών βασίστηκε στα πλεονεκτήματα που απορρέουν από τις διαφορετικές στρατηγικές ζωής και τους διαφορετικούς τύπους κύκλου ζωής που εμφανίζουν. Η ολοκλήρωση ενός μοναδικού κύκλου αναπαραγωγής εξασφαλίζει – σε μεγάλο βαθμό – ομοιογένεια ως προς την ανάπτυξη και δυναμική της ωοθήκης μεταξύ των αναπαραγωγικά ώριμων θηλυκών ατόμων, καθώς όλα ωοτοκούν για πρώτη φορά. Συνεπώς, πληθυσμοί με αυτή τη στρατηγική ζωής αποτελούν ιδανικά μοντέλα για την ανάλυση των πρώιμων φάσεων της ωογένεσης πριν την έναρξη της περιόδου ωοτοκίας. Αντιστοίχως, ο ανάδρομος κύκλος ζωής παρέχει τη μοναδική δυνατότητα για στοχευμένες δειγματοληψίες και συσχέτιση του πεδίου δειγματοληψίας και της χρονικής περιόδου δειγματοληψίας με την αναπαραγωγική κατάσταση των ψαριών. Οι στοχευμένες δειγματοληψίες σε διαφορετικά πεδία (π.χ. στις εκβολές του ποταμού, κατά μήκος του ποταμού, στα πεδία ωοτοκίας) μπορούν να υλοποιηθούν χάρη στην αρκετά προβλέψιμη χρονική περίοδο της αναπαραγωγικής μετανάστευσης ανάδρομων πληθυσμών προς τα πεδία ωοτοκίας. Ο ανάδρομος κύκλος ζωής δίνει συνεπώς τη δυνατότητα μελέτης της

δυναμικής της ωοθήκης σε συγκεκριμένες χρονικές κλίμακες, όπως πριν την έναρξη της περιόδου ωοτοκίας, κατά τη διάρκεια ή μετά το πέρας αυτής. Επιπροσθέτως, η εμφάνιση διαφορετικών τύπων κύκλου ζωής από διαφορετικά είδη του γένους *Alosa* ή ακόμη και από διαφορετικούς πληθυσμούς εντός του ίδιου είδους επιτρέπει την εξέταση της ενδεχόμενης ρύθμισης του προτύπου γονιμότητας από τον τύπο του κύκλου ζωής.

Τέσσερα βασικά ερευνητικά ερωτήματα τέθηκαν στην παρούσα διατριβή και κάθε ένα από αυτά εξετάζεται σε ξεχωριστό κεφάλαιο. Η σειρά των κεφαλαίων διαμορφώθηκε με βάση τη χρονική κλίμακα που εξετάζεται σε κάθε ένα από αυτά, με την πιο ευρεία ή/και χρονικά προηγούμενη κλίμακα να εξετάζεται πρώτη. Το πρώτο ερευνητικό ερώτημα αναφέρεται στο ρόλο των ωογονίων και στο μηχανισμό εισδοχής των πρωτογενών ωοκυττάρων στη δευτερογενή φάση ανάπτυξης πριν από την έναρξη της περιόδου ωοτοκίας. Ο μεν ρόλος των ωογονίων έχει εξεταστεί στο παρελθόν σχεδόν αποκλειστικά σε άτομα που έχουν ολοκληρώσει την περίοδο ωοτοκίας τους, ενώ ο μηχανισμός εισδοχής των πρωτογενών ωοκυττάρων στην επόμενη φάση της ωογένεσης παραμένει εν πολλοίς ένα μυστήριο. Το δεύτερο ερευνητικό ερώτημα αφορά τα δύο πρότυπα γονιμότητας ιχθύων, το καθορισμένο και το ακαθόριστο, και το ενδεχόμενο να υπάρχουν διαφοροποιήσεις στα – επί του παρόντος – τυπικά χαρακτηριστικά τους. Η πιθανότητα το πρότυπο γονιμότητας να επηρεάζεται από τον τύπο του κύκλου ζωής αποτελεί το τρίτο ερευνητικό ερώτημα. Το τελευταίο ερευνητικό ερώτημα αναφέρεται στην εισδοχή των πρωτογενών ωοκυττάρων στη δευτερογενή φάση ανάπτυξης και το ενδεχόμενο η διαδικασία αυτή να λαμβάνει χώρα σε συγκεκριμένη χρονική στιγμή εντός του κύκλου ωοτοκίας, δηλαδή, μεταξύ δύο διαδοχικών συμβάντων ωορρηξίας/ωοτοκίας.

Το κεφάλαιο 3 είναι αφιερωμένο στο πρώτο ερευνητικό ερώτημα. Συγκεκριμένα, αναλύονται θηλυκά άτομα ενός πληθυσμού του είδους *A. alosa* που βρίσκονται πριν από την έναρξη της περιόδου ωοτοκίας τους. Ο εν λόγω πληθυσμός εμφανίζει ανάδρομο κύκλο ζωής, εισέρχεται στον ποταμό Mondego της Πορτογαλίας για να αναπαραχθεί και τα άτομα αυτού υλοποιούν έναν αναπαραγωγικό κύκλο στη διάρκεια της ζωής τους. Παρά το γεγονός ότι στις ωοθήκες που αναλύθηκαν εντοπίστηκαν ωογόνια, δεν παρατηρήθηκε παραγωγή νέων ωοκυττάρων πρωτογενούς φάσης ανάπτυξης. Ταυτόχρονα, διαπιστώθηκε η εισδοχή του συνόλου των ωοκυττάρων πρωτογενούς φάσης στη δευτερογενή φάση ανάπτυξης, με την εν λόγω διαδικασία να υλοποιείται τμηματικά. Με βάση τα αποτελέσματα αυτά σχεδιάστηκε ένα μοντέλο που σχηματοποιεί τη διαδικασία εισδοχής και το οποίο παρουσιάζεται στο τελευταίο κεφάλαιο (7) της διατριβής.

Στο επόμενο κεφάλαιο (4) αναλύονται λεπτομερώς η διαδικασία εισδοχής των ωοκυττάρων πρωτογενούς φάσης ανάπτυξης και η δευτερογενής φάση της ωογένεσης εντός της περιόδου ωοτοκίας ενός πληθυσμού του είδους *A. aestivalis*. Τα άτομα του πληθυσμού αυτού εισέρχονται στον ποταμό Connecticut των ΗΠΑ για να

αναπαραχθούν και υλοποιούν πλέον του ενός αναπαραγωγικούς κύκλους στη διάρκεια της ζωής τους. Κύριο ζητούμενο ήταν να καθοριστεί το πρότυπο γονιμότητας του συγκεκριμένου πληθυσμού και να ελεγχθεί εάν τα χαρακτηριστικά του εναρμονίζονται με τα τυπικά χαρακτηριστικά του καθορισμένου ή του ακαθόριστου προτύπου. Τα άτομα του υπό εξέταση πληθυσμού αποδείχθηκε ότι ακολουθούν το ακαθόριστο πρότυπο γονιμότητας, αλλά με πρωτόγνωρες προσαρμογές. Συγκεκριμένα, η εισδοχή ωοκυττάρων από την πρωτογενή στη δευτερογενή φάση της ωογένεσης εντός της περιόδου ωοτοκίας δεν ισοστάθμιζε τις απώλειες από την ωοαπόθεση, με αποτέλεσμα ο αριθμός των ωοκυττάρων εντός της ωοθήκης να μειώνεται διαρκώς. Επίσης, άτομα που αλιεύτηκαν στο τέλος της περιόδου ωοτοκίας τους δεν εμφάνισαν μαζική ατρησία, δηλαδή, μαζική αποδιοργάνωση των εναπομεινάντων ωοκυττάρων και απορρόφησή τους. Τα δύο αυτά χαρακτηριστικά διαφοροποιούν το πρότυπο γονιμότητας του συγκεκριμένου πληθυσμού από το αναμενόμενο πρότυπο ιχθύων με ακαθόριστο πρότυπο και συνάδουν με αποτελέσματα πρόσφατων ερευνών που υποδεικνύουν αντίστοιχες διαφοροποιήσεις.

Η επίδραση του τύπου του κύκλου ζωής στη διαμόρφωση του προτύπου γονιμότητας των ιχθύων εξετάζεται στο κεφάλαιο 5. Δύο γειτονικοί πληθυσμοί του είδους *A. pseudoharengus* από την περιοχή East Lyme της Πολιτείας Connecticut των ΗΠΑ, με διαφορετικούς τύπους κύκλου ζωής αναλύθηκαν προκειμένου να καθοριστεί το πρότυπο γονιμότητάς τους και να συγκριθούν τα επί μέρους χαρακτηριστικά τους. Ο ένας πληθυσμός ολοκληρώνει τον κύκλο ζωής του στην Λίμνη Pattagansett, ενώ ο δεύτερος εισέρχεται στη Λίμνη Bride προκειμένου να αναπαραχθεί. Αμφότεροι πληθυσμοί αποδείχθηκε ότι εμφανίζουν το ακαθόριστο πρότυπο γονιμότητας και δεν εμφάνισαν διαφοροποιήσεις ως προς τα επί μέρους χαρακτηριστικά που εξετάστηκαν, γεγονός που δεν αναδεικνύει τον τύπο του κύκλου ζωής ως καθοριστικό παράγοντα διαμόρφωσης του προτύπου γονιμότητας.

Το κεφάλαιο 6 εστιάζει στο ενδημικό είδους *A. macedonica* της Λίμνης Βόλβης και επιχειρεί να απαντήσει στο τέταρτο ερευνητικό ερώτημα της παρούσας διατριβής· εάν η διαδικασία της εισδοχής των ωοκυττάρων από την πρωτογενή στη δευτερογενή φάση ανάπτυξης λαμβάνει χώρα σε συγκεκριμένη χρονική στιγμή εντός του κύκλου ωοτοκίας στα άτομα με ακαθόριστο πρότυπο γονιμότητας. Τα θηλυκά άτομα που αναλύθηκαν διαχωρίστηκαν σε τέσσερις κατηγορίες ανάλογα με τη φάση του κύκλου ωοτοκίας στην οποία βρίσκονταν όταν αλιεύθηκαν: αλιευμένα λίγο πριν την ωορρηξία/ωοαπόθεση, κατά την ωορρηξία/ωοαπόθεση, αμέσως μετά την ωοαπόθεση, και μεταξύ δύο διαδοχικών συμβάντων ωορρηξίας/ωοαπόθεσης. Με την κατηγοριοποίηση αυτή και τον έλεγχο διαφορετικών δεικτών ανάπτυξης της ωοθήκης εντοπίστηκε η χρονική στιγμή της διαδικασίας της εισδοχής. Συγκεκριμένα, μία νέα ομάδα ωοκυττάρων αποδείχθηκε ότι εισέρχεται στη δευτερογενή φάση ανάπτυξης παράλληλα με την ωορρηξία/ωοαπόθεση των ωοκυττάρων στο πιο

προηγμένο στάδιο. Με βάση τα αποτελέσματα αυτά σχεδιάστηκε ένα μοντέλο προσομοίωσης της δυναμικής της ωοθήκης και των διεργασιών που συμβαίνουν εντός του κύκλου ωοτοκίας ιχθύων με ακαθόριστο πρότυπο γονιμότητας, το οποίο παρουσιάζεται στο κεφάλαιο 7.

Συμπερασματικά, η παρούσα διατριβή παρέχει νέα δεδομένα αναφορικά με τη διαδικασία της ωογένεσης των ιχθύων και τη ρύθμιση του προτύπου γονιμότητάς τους μέσω της ανάλυσης της δυναμικής της ωοθήκης σε ποικίλες χρονικές κλίμακες και σε διαφορετικούς πληθυσμούς ειδών του γένους *Alosa* με διαφορετικές στρατηγικές ζωής και τύπους κύκλου ζωής. Η γνώση του προτύπου γονιμότητας είναι πρωταρχικής σημασίας για τη διαχείριση ιχθυοπληθυσμών και τα αποτελέσματα που παρουσιάζονται στη διατριβή αυτή θα συμβάλουν στην καλύτερη και ευρύτερη κατανόηση των διεργασιών της ωογένεσης και στην καλύτερη διαχείριση ιχθυοπληθυσμών ενδιαφέροντος.

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CHAPTER 1: Introduction

1.1 Genus *Alosa*

Shads, alosines or alosids are common "names" used to refer to fish species of the genus *Alosa*, none of which, however, is totally correct or exclusive to these fishes (Olney 2003). "Alosid" has no meaning, since the family name "Alosidae" has no standing in verified classification, while "shad" and "alosine" can be reference to any species of the clupeid subfamily Alosinae. The taxonomic classification of fish species of the genus *Alosa* is as follows:

Order: Clupeiformes
    Suborder: Clupeoidei
        Family: Clupeidae
            Subfamily: Alosinae
                Genus: ***Alosa***
                        Brevoortia
                        Ethmalosa
                        Ethmidium
                        Gudusia
                        Hilsa
                        Tenualosa

In the present study, only members of the genus *Alosa* were included, and thus any mention of the terms shad and alosine is an exclusive reference to species of this genus. The genus *Alosa* currently includes 24 living species (Froese and Pauly 2019), even though one of them has probably been extinct (*A. vistonica*; Bobori et al. 2015). Alosines are found in the Black Sea and Caspian Sea basins (Coad et al. 2003; Navodaru and Waldman 2003), in the Mediterranean Sea (Bobori et al. 2001), in the Baltic Sea (Aprahamian et al. 2003a), along the eastern Atlantic coast, from Norway to Morocco (Baglinière et al. 2003), in Gulf of Mexico and along the east coast of North America (Waldman 2003), along the eastern central and north Pacific coast and eastern Asia (Greene et al. 2009). Permanent freshwater alosine populations are also present in Greece, Italy, Ireland and US (Waldman 2003).

Alosines display various life-history forms, including anadromous (i.e., fish are born in freshwater, then migrate to the ocean as juveniles where they grow into adults before migrating back into freshwater to spawn), oceanodromous (i.e., migratory fish that spend their whole life in salt water) and landlocked (i.e., fish complete their life cycle in impounded freshwater bodies, such as reservoirs and lakes) populations (Fig. 1.1). Anadromy is the prevailing life-history form among alosines and is world-renowned, while the rest forms are regularly overlooked and remain understudied (Table 1.1).
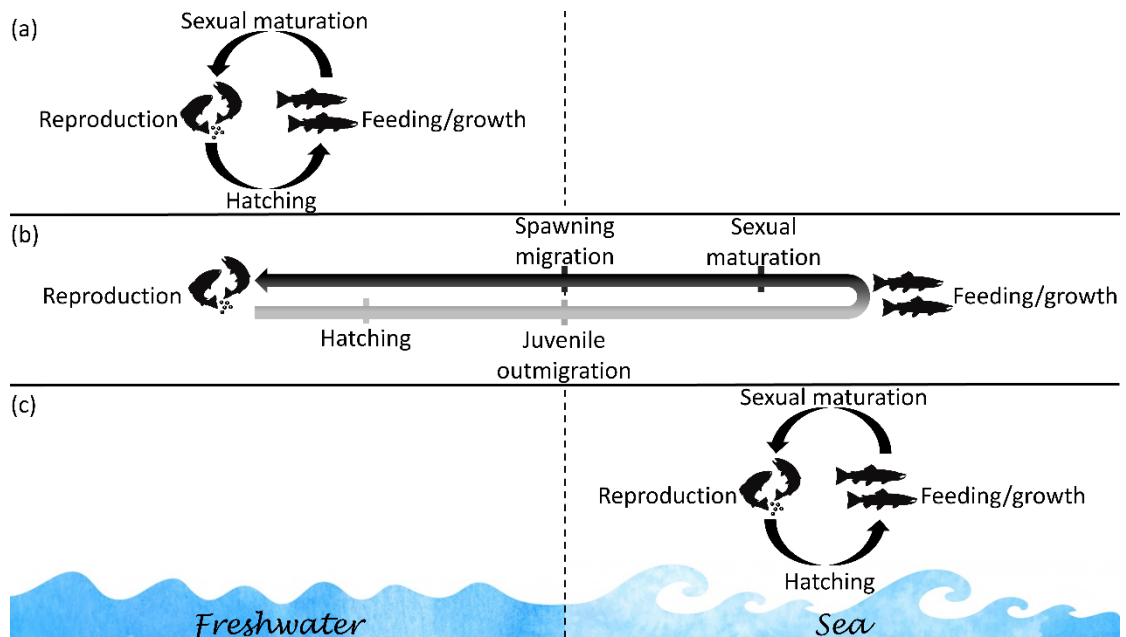
Figure 1.1. The life-history forms displayed by species of the genus *Alosa*: (a) landlocked, (b) anadromous, and (c) oceanodromous

Εικόνα 1.1. Διαφορετικοί τύποι κύκλου ζωής των ειδών του γένους *Alosa*: (a) ολοβιωτικός, (b) ανάδρομος, και (c) ωκεανόδρομος

Variability is also observed in reproductive strategy (or parity or repeat spawning, i.e., number of reproductive cycles during lifetime) among alosines. The degree of repeat spawning has been reported to range from obligatory semelparity (or uniparity, i.e., a single reproductive cycle occurs in lifetime of each fish) to obligatory iteroparity (i.e., each fish completes more than one reproductive cycles during its lifetime) (Fig. 1.2).
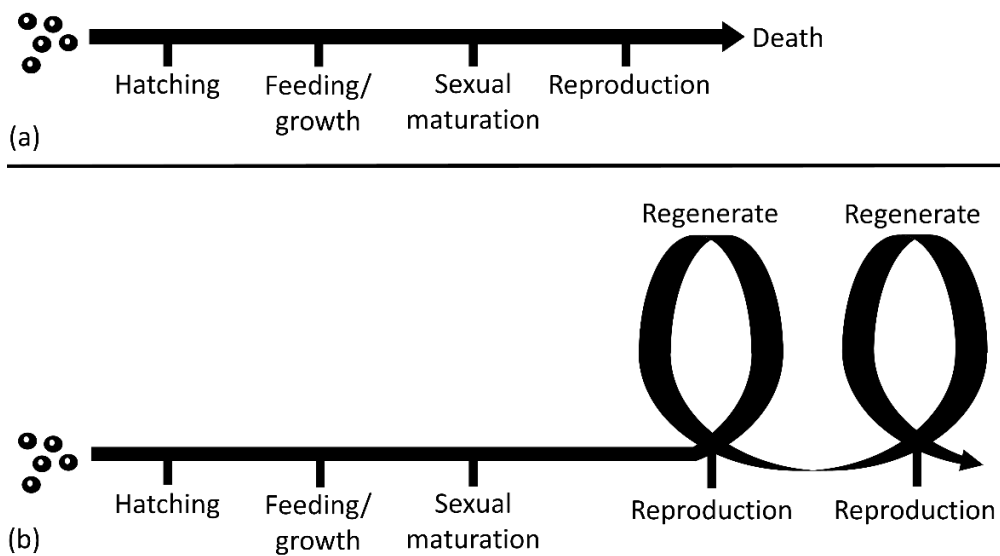
Figure 1.2. Representation of: (a) semelparous, and (b) iteroparous reproductive strategies

Εικόνα 1.2. Στρατηγική ζωής ψαριών που ολοκληρώνουν: (a) έναν αναπαραγωγικό κύκλο, και (b) περισσότερους του ενός αναπαραγωγικούς κύκλους, κατά τη διάρκεια της ζωής τους

Table 1.1. Life-history form of species of the genus *Alosa*. Note: *A. curensis*, *A. saposchnikowii*, *A. suworowi* are not presented due to data deficiency

Πίνακας 1.1. Τύποι κύκλου ζωής των ειδών του γένους *Alosa*. Σημείωση: για τα είδη *A. curensis, A. saposchnikowii, A. suworowi* δεν υπάρχουν επαρκή στοιχεία

Species	Life-history form		
	Anadromous	Landlocked	Oceanodromous
A. aestivalis	+	+[1]	
A. agone		+	
A. alabamae	+		
A. algeriensis	+		
A. alosa	+	+[2]	
A. braschnikowi			+
A. caspia	+		
A. chrysochloris	+		
A. fallax	+		
A. immaculata	+		
A. kessleri	+		
A. killarnensis		+	
A. macedonica		+	
A. maeotica	+		
A. mediocris	+		
A. pseudoharengus	+	+	
A. sapidissima	+	+[3]	
A. sphaerocephala			+
A. tanaica	+		
A. vistonica		+	
A. volgensis	+		

Many species of the genus *Alosa* maintain considerable social, economic and ecological merit. More specifically, many alosines sustain – or used to sustain – substantial fisheries in many regions due to their edibility and typically high abundances (Baglinière et al. 2003; Coad et al. 2003; Limburg et al. 2003; ASMFC 2007). Most of these fisheries have been focused on the spawning runs of anadromous alosines, since they offered high densities and efficient fishing at strategic locations due to the highly predictable timing of upstream migrations (Waldman 2003).

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[1] Very few populations (Prince and Barwick 1981; Limburg et al. 2001; Winkelman and Van Den Avyle 2002)

[2] Few populations (Collares-Pereira et al. 1999; Mennesson-Boisneau et al. 2000; Correia et al. 2001)

[3] Reports of a single successfully spawning landlocked population in Millerton Lake, California, US (Von Geldern 1965; Moyle 2002)

However, coastal fisheries have also been developed (Waldman 2003), as well as sea fisheries (Aprahamian et al. 2003a) and fisheries in lakes and reservoirs for landlocked alosines (Bobori et al. 2001). Recreational fishing is less widespread, but in some cases alosines receive intense angling interest, such as American shad, *A. sapidissima*, in North America and Allis shad, *A. alosa*, and Twaite shad, *A. fallax*, in Europe (Waldman 2003).

Alosines also support a remarkable range of human valuation. In the Northeastern US, the arrival of spring is celebrated by baking shad fillets, while shad roe constitutes a seasonal delicacy (Waldman 2003). Similarly, a multi-day festival is held in North Carolina to celebrate Hickory shad, *A. mediocris* (Waldman 2003), while in Cape Cod, the spawning run of Alewife, *A. pseudoharengus*, constitutes a local cultural event (McDowall 2003). Even Macedonian shad, *A. macedonica*, which is endemic to a small Greek lake, retains a cultural value, with festivities being organized annually (Giantsis et al. 2015).

Moreover, alosines play significant ecological role in the habitats in which they occur. Anadromous alosines exchange energy and nutrients among different habitats (ocean, estuaries, rivers and lakes) during their spawning migrations through gametes, excretion, and the carcasses of dead spawners (Durbin et al. 1979; Garman 1992; Post and Walters 2009; Walters et al. 2009), and thus can be labeled "ecosystem linkers". In addition, due to their predictable timing of upstream migrations and their homing behavior, alosines serve as marine-supported food for a variety of natural piscine and avian predators at specific time-frames each year (MacAvoy et al. 2000; Yako et al. 2000; Dalton et al. 2009; Davis et al. 2012). They can also apply significant top-down pressure on freshwater food webs during the riverine phase of their reproductive cycle as both adults and juveniles (Vigerstad and Cobb 1978; Haskell et al. 2013; Demi et al. 2015). On the other hand, landlocked alosines, especially after introduction to lakes and reservoirs, may cause several problems. More specifically, established landlocked alosines have forced changes in zooplankton communities (Brooks and Dodson 1965; Wells 1970; Harman et al. 2002; Palkovacs and Post 2008), outcompeted native fish species (Eck and Wells 1987; Porath et al. 2003; Madenjian et al. 2008), caused shifts in feeding habitats and prey consumption of native species (Crowder 1984; Bobori et al. 2001; Moring and Mink 2003), and thiamine deficiencies in their predator populations (Brown et al. 2005; Houde et al. 2015). Also, mass mortality incidences have been reported for several landlocked populations (Otto et al. 1976; O'Gorman and Schneider 1986; Kleanthidis 2002; Dunlop and Riley 2013) causing costly removals from fouled beaches and reduced lake-based tourist trade.

Despite their prominence, many alosines remain understudied and poorly understood, while others face considerable threats, such as degradation or loss of spawning habitat, water quality deterioration, overexploitation and by-catch mortality (Waldman and Limburg 2003). More specifically, "data deficiency" currently remains

for three alosines and six other species are categorized as "vulnerable", "endangered" or "critically endangered" at a global scale (IUCN 2019; Fig. 1.3).
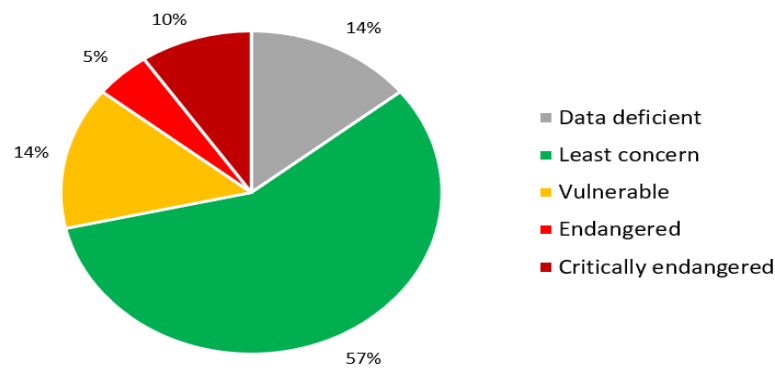
Figure 1.3. Status of species of the genus *Alosa* (IUCN 2019)
Εικόνα 1.3. Ποσοστό ειδών του γένους *Alosa* ανά κατηγορία κινδύνου (IUCN 2019)

1.2 Rationale behind *Alosa* sp. selection

All the above reasons would justify the selection of alosines as the species of interest in this study. However, alosines were chosen mainly due to their variant reproductive strategies and life-history forms that provide the opportunity for analyses on oogenesis (i.e., the morphological and functional processes that lead to the production of fertilizable eggs) and fecundity type (i.e., how and when oocytes to be spawned are produced) at different temporal scales and at specific time-frames, which was the goal of this study.

Oogenesis (Box 1) – as any fish reproductive trait – is commonly analyzed, at four temporal scales: (1) throughout lifetime, (2) during the reproductive season, i.e., the period that extends from the onset of oocyte recruitment from the primary growth (PG) to the secondary growth (SG) phase (SG recruitment) till the completion of spawning activity of a fish stock, (3) within the spawning season, i.e., the time period between the release of the first and the last egg of a fish stock, and (4) at the diel scale (Lowerre-Barbieri et al. 2011; Ganias and Lowerre-Barbieri 2018). However, the different phases of oogenesis (oocyte production from oogonia, PG phase, SG phase, ovulation) (Box 1) have been analyzed disproportionately and at different temporal scales. The dynamics of oogonia and PG oocytes, even though they play fundamental role in the SG oocyte production, remain largely unknown; the notorious "black box" in applied fisheries reproductive biology (Kjesbu 2009; Korta et al. 2010; Serrat et al. 2019a). More specifically, analyses of oocyte production from oogonia have rarely been analyzed at broad temporal scales (Wildner et al. 2013) and have mainly been confined to post-spawning females, suggesting that oogonial numbers in the ovary typically peak soon after spawning, in preparation for the next reproductive season (e.g., Billard 1987, 1992; Bromage and Cumaranatunga 1988; Khan and Thomas 1999;

Box 1 - Oogenesis

Oogenesis refers to the morphological and functional processes that lead to the production of fertilizable eggs. The unveil of its underlying mechanisms is paramount in applied fisheries reproductive biology for understanding the egg production process, which in turn influences the reproductive potential of a fish stock (i.e., the ability of a fish stock to produce viable eggs and larvae that may eventually recruit into the adult population or fishery; Trippel 1999), and thus its survival.

The course of oogenesis in fishes is known and is divided into four main phases. It begins with oocyte production from oogonia (i.e., germ cells that may divide mitotically to maintain their population within the germinal epithelium or may enter meiosis and give rise to oocytes during each reproductive season; Selman and Wallace 1989; Grier 2000). Subsequently, oocytes pass through several developmental stages during the primary growth (PG) and the secondary growth (SG) phase. Oogenesis ends with the ovulation of advanced SG oocytes capable of fertilization (Grier et al. 2009). However, many pieces of information remain elusive, impeding the full comprehension of oogenesis.

The SG phase of oogenesis has been the main focus in applied fisheries reproductive biology and its steps are summarized as follows (Fig. B1.1): Initially, cortical alveoli appear in the ooplasm of the newly recruited SG oocytes. As the number and size of these cortical alveoli increase, the SG oocytes develop through a series of stages named "cortical alveolar" (CA) stages. Subsequently, yolk protein starts to accumulate in the ooplasm (vitellogenesis), leading SG oocytes to enter a series of vitellogenic (VIT) stages. Afterwards, SG oocytes initiate their final maturation, passing from the germinal vesicle migration (GVM) to the germinal vesicle breakdown (GVBD) stage and finally to the hydration (HYD) stage, which is characterized by an intense increase in oocyte size due to rapid water intake.

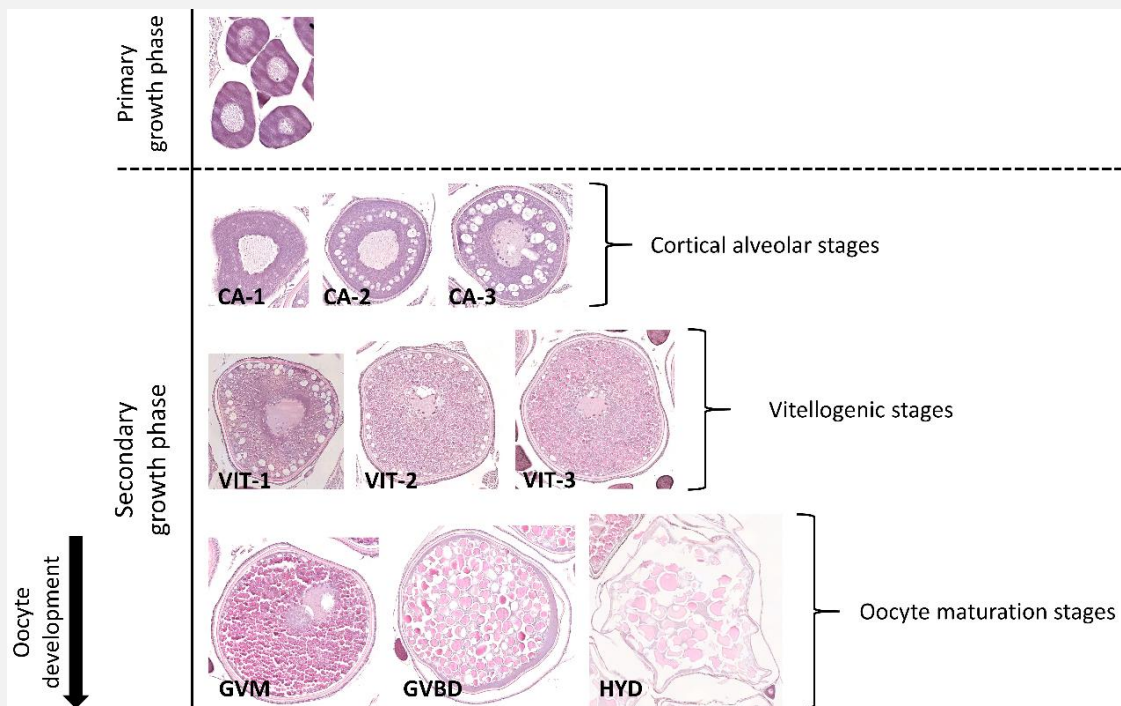**Figure B1.1.** Sequential oocyte developmental stages from the primary to the secondary growth phase. For explanation of the ovarian stage abbreviations refer to the text. Note: oocytes are not scaled

Εικόνα B1.1. Διαδοχικά στάδια ανάπτυξης ωοκυττάρων από στην πρωτογενή και δευτερογενή φάση. Οι συντομογραφίες επεξηγούνται εντός του κειμένου. Σημείωση: Τα ωοκύτταρα δεν παρουσιάζονται υπό κλίμακα

Grier et al. 2007). Similarly, the dynamics of PG oocytes have seldom been analyzed due to their small size, which impedes their enumeration and assessment (Kjesbu 2009; Kjesbu et al. 2011). Indeed, the early descriptions of small sized oocytes (e.g., Shirokova 1977) and attempts to estimate their numbers (e.g., Greer-Walker et al. 1994) have been followed by a limited number of qualitative (Grier et al. 2009; Kjesbu et al. 2011; Uribe et al. 2016; Grier et al. 2018) and quantitative studies (Korta et al. 2010; Kjesbu et al. 2011; Schismenou et al. 2012; Serrat et al. 2019a) during the last decade. The latter studies were very detailed, mainly due to methodological advancements, such as the development of the oocyte packing density theory (Kurita and Kjesbu 2009), but were almost exclusively focused on broad time-scales, like the reproductive season or the spawning season (Korta et al. 2010; Kjesbu et al. 2011; Schismenou et al. 2012; Serrat et al. 2019a), leaving the PG dynamics at narrow temporal scales clouded and slightly examined (Schismenou et al. 2012). On the contrary, the SG phase of oogenesis has been reviewed many times (e.g., Grier et al. 2009; Lowerre-Barbieri et al. 2011; Ganias and Lowerre-Barbieri 2018) and analyzed extensively at several temporal scales and in many different species (e.g., Ganias et al. 2004; Murua and Motos 2006; Ganias et al. 2017).

Considerable attention has been drawn to a specific process within oogenesis, the SG recruitment and its temporal association with spawning season; association that defines the fecundity type and classifies it as determinate or indeterminate (Hunter et al. 1985; Ganias 2013) (Box 2). The determinate fecundity type corresponds to fish stocks in which SG recruitment is completed before the onset of the spawning season. On the contrary, in fish stocks displaying indeterminate fecundity type, SG recruitment season and spawning season overlap at some extent (Fig. 1.4), meaning that new oocytes are recruited to the SG phase in parallel with spawning activity.
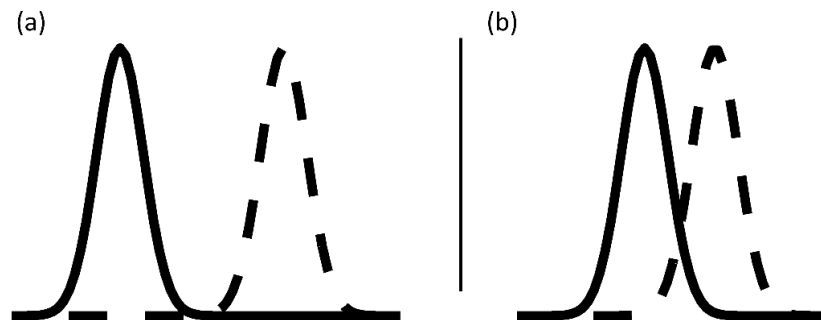
Figure 1.4. Temporal assossiation between recruitment period of oocytes from the primary to the secondary growth phase (solid lines) and spawning season (dashed lines); (a) clear temporal distinction defines the determinate fecundity type, and (b) temporal overlapping defines the indeterminate fecundity type. Adapted from Ganias (2013)

Εικόνα 1.4. Χρονική συσχέτιση μεταξύ της περιόδου εισδοχής ωοκυττάρων από την πρωτογενή στη δευτερογενή φάση ανάπτυξης (ενιαία γραμμή) και της αναπαραγωγικής περιόδου (διακεκομμένη γραμμή). (a) Πλήρης διαχωρισμός που ορίζει το καθορισμένο πρότυπο γονιμότητας, και (b) αλληλοεπικάλυψη που ορίζει το ακαθόριστο πρότυπο γονιμότητας. Σχήμα προσαρμοσμένο από Ganias (2013)

Box 2 – Fecundity type estimation

The operational knowledge of fecundity type is of great importance, since it defines the appropriate method for estimating total egg production and spawning biomass of a stock; a proxy of reproductive potential (Murua and Saborido-Rey 2003; Stratoudakis et al. 2006; Armstrong and Witthames 2012; Ganias 2013).

Since their first introduction by Hunter et al. (1985) and Hunter and Macewicz (1985a), the use of the terms determinate and indeterminate fecundity type has steadily increased in the scientific literature. A few years later, Hunter et al. (1989) reported the first set of criteria for distinguishing the two fecundity types in their paper on *Anoplompoma fimbria*, which were implemented, improved and enriched in subsequent studies (e.g., Hunter et al. 1992; Greer-Walker et al. 1994). Currently, the lines of evidence for identifying whether a fish stock displays determinate or indeterminate fecundity type are summarized as follows (Murua and Saborido-Rey 2003; Armstrong and Witthames 2012):

- The existence of a size gap, or hiatus as it is widely referred-to, between PG and SG oocytes in mature ovaries indicates determinacy; continuous distribution indicates indeterminacy
- Determinate spawners are expected to exhibit decline in the number of advanced vitellogenic SG oocytes during the spawning season; in indeterminate spawners the number of SG oocytes varies according to dynamic balance between spawning and recruitment
- An increase in the mean diameter of advanced vitellogenic oocytes over the spawning season points to determinate fecundity type; stable or declining diameter indicates indeterminacy
- Lower standing stock of advanced vitellogenic oocytes in females having postovulatory follicles (i.e., ovarian markers of recent spawning activity consisted of the follicular layers that remain in the ovary after the release of the ovum during spawning) than in females with no signs of recent spawning evinces determinacy
- Determinate spawners show continuous low level of atresia during spawning season; massive atresia by the end of the spawning season indicates indeterminacy

These "classic" criteria are not always construed in a straightforward manner, and their implementation occasionally leads to mixed results regarding the fecundity type (e.g., Greer-Walker et al. 1994; Ganias et al. 2017; Serrat et al. 2019b). One of the main requirements to implement these criteria is that the ovarian samples to be analyzed should cover the time period prior to the onset of spawning activity and throughout the spawning season of the surveyed stock. However, this prerequisite is not always met, leading to misinterpretations. For instance, a size hiatus could give the false impression of determinacy if the analysis is confined to a specific time-frame, since it may appear only at the latter parts of spawning season (Ganias and Lowerre-Barbieri 2018), like in Atlantic horse mackerel, *Trachurus trachurus* (Ganias et al. 2017), or due to a specific oocyte development pattern, such as in round herring, *Etrumeus teres*, where oocytes at the "cortical alveolar" stages continue their development after the hydration of the advanced oocyte batch (Plaza et al. 2007). Additionally, these "classic" criteria were developed to test for temporal patterns during the spawning season, based on the assumption that spawning activity is synchronized at the population level, meaning that all females are constantly at the same stage in their individual reproductive season. However, the latter assumption is commonly violated, since onset of spawning activity is regulated by several factors, such as demographics (e.g., Schultz et al. 1991; Jansen and Gislason 2011; Peer and Miller 2014). However, despite their limitations, the "classic" criteria have been regularly used in applied fisheries reproductive biology and for different stocks (e.g., Witthames and Greer-Walker 1995; Murua et al. 1998; White et al. 2003; Gordo et al. 2008).

In theory, the ovarian dynamics of a determinate spawner follows a seasonal pattern which can be divided into four phases (Macewicz and Hunter 1994; Kjesbu 2009; Ganias et al. 2015a): (i) pre-spawning phase, during which SG recruitment is completed, (ii) stabilization phase, during which the pool of SG oocytes remains constant, (iii) down-regulation phase, where atresia (i.e., oocyte degeneration and resorption) causes loss of developing SG oocytes, and (iv) spawning phase, during which developed SG oocytes are released in a single spawning event (total oocyte release strategy) or sequential events (multiple, batch, partial, serial or heterochronal oocyte release strategy; Holden and Raitt 1974; Hunter et al. 1985). Aspects of this conceptual model have been verified in different cases, like in North Atlantic Sole, *Solea solea* (Witthames and Greer-Walker 1995) and Atlantic cod, *Gadus morhua* (Kjesbu et al. 1991).

The theoretical seasonal pattern of an indeterminate spawner's ovarian dynamics is quite different and more complex (Hunter and Leong 1981; Hunter et al. 1985; Ganias et al. 2015a). SG recruitment occurs both prior and in parallel with spawning activity. The pool of SG oocytes remains in a "dynamic equilibrium" during spawning activity, wherein SG recruitment counterbalances the spawning of multiple oocyte batches, so that the released oocytes are replenished. Consequently, at the end of the spawning activity, a surplus of SG oocytes remains and is resorbed through massive atresia (i.e., generalized oocyte degeneration and resorption), a process known as "mopping-up" (Wallace and Selman 1981; Kjesbu 2009). Even though aspects of this theoretical pattern have been verified in some cases (e.g., Murua et al. 1998; Murua and Motos 2006; Schismenou et al. 2012; Ganias et al. 2014a), recent studies have challenged the generality of the "dynamic equilibrium" and "mopping-up" strategy in indeterminate spawners. Ganias et al. (2017) found cessation of SG recruitment and lack of massive atresia in late-season spawners in Atlantic horse mackerel, *Trachurus trachurus*. A similar pattern has been suggested in the clupeid Gulf menhaden, *Brevoortia patronus* by Brown-Peterson et al. (2017).

Even though fecundity type has been analyzed for more than three decades, and despite its major implication in fisheries management and the methodological and theoretical advances (Box 2), many pieces of information regarding its regulation remain elusive; from the type itself of many stocks, to the prevalence of each type, the universality of the theoretical patterns, and the mechanisms – biological and ecological – that promote the one type or the other (Box 3).

In that respect, semelparous alosines can serve as suitable models to analyze aspects of oogenesis at the lifetime temporal scale, like estimating the lifetime egg production. In parallel, semelparity ensures – to a great degree – homogeneity in ovarian development, since all the mature, spawning capable females (i.e, fish with oocytes at an advanced vitellogenic stage, capable of spawning during the current reproductive cycle; Brown-Peterson et al. 2011) are considered first time spawners.

Thus, semelparous alosines are convenient candidates for analyzing the dynamics of the early phases of oogenesis at the specific time-frame prior to the onset of spawning activity and test, for instance, whether the pool of oogonia and PG oocytes is depleted, as it is theoretically expected (Ganias and Lowerre-Barbieri 2018), or not.

Box 3 – Fecundity type regulation

Several theories have been stated, suggesting that fecundity type is a dynamic trait influenced by many factors. A widely accepted theory relates fecundity type of a fish stock to its latitudinal distribution (Ganias 2013). The latter idea was introduced by Hunter et al. (1985), who suggested that determinate fecundity type is displayed by species with boreal geographic distributions, while species inhabiting temperate and tropical environments are mostly indeterminate spawners. That theory was corroborated by other studies ever since; e.g., Witthames and Greer-Walker (1995), Wuenschel et al. (2013) and McBride et al. (2016) reported such a latitudinal gradient in fecundity type of the genus *Solea*, in Black sea bass, *Centropristis striata*, and American shad, *Alosa sapidissima*, respectively. Another theory links determinate fecundity type with narrow spawning seasons during winter, while indeterminacy is related with extended spawning activity during summer-time (Rijnsdorp and Witthames 2005; Kjesbu 2009; Ganias and Lowerre-Barbieri 2018). An alternative approach suggests fecundity type regulation by the strategy of energy allocation to reproduction (Rijnsdorp and Witthames 2005; Kjesbu and Witthames 2007; Kjesbu 2009; Armstrong and Witthames 2012). Specifically, capital breeders (i.e., food resources are acquired in advance to offspring production) tend to display determinate fecundity type, whereas income breeders (i.e., food intake is adjusted concurrently with offspring production, without reliance on reserves) are most likely indeterminate spawners. Even though none of the abovementioned theories has been tested thoroughly, it is generally accepted that fecundity type constitutes an ecophenotypic response to environmental factors. However, other factors may also contribute in fecundity type regulation, such as genetic drivers (Kjesbu and Witthames 2007; Ganias 2013), and this matter needs to be investigated in future studies.

On the other hand, anadromy provides unique opportunities in terms of analyses at the temporal scale of the spawning season and at different time-frames, prior to, during and after the spawning activity. Specifically, the predictable timing of upstream migration and spawning of anadromous alosines enables a sampling design that associates location and reproductive stage, giving the chance to sample females at specific sites *en route* to the spawning grounds (e.g., at the river mouth, along the river, at the spawning sites), and thus accurately distinguish fish at different spawning phases (i.e., stages in their individual reproductive season), such as females that had not commenced their spawning, females that had already spawned, and females that had completed their spawning activity.

Additionally, anadromous alosines incur substantial energetic losses during their upstream migration (Leggett and Carscadden 1978; Harris and McBride 2009; Murauskas and Rulifson 2011; Ganias et al. 2015b), and thus trade-offs between

mortality risk and reproductive output might force aspects of their fecundity types to deviate from typicality. For instance, continuous energy depletion during the upstream migration may lead to limited or continuously decreasing investment on reproduction or even premature spawning cessation. Hence, anadromous alosines are suitable models to test the universality of the characteristics of the two fecundity types; e.g., whether SG recruitment is curtailed prior or after the onset of spawning activity, whether the pace of SG recruitment remains constant or is subjected to tapering (i.e., progressive decrease), and whether atresia occurs as a means to replenish the energy reserves or not.

Moreover, alosines are perfect candidates to test whether life-history form is among the parameters that influence the fecundity type regulation; analysis that has never been conducted before. In fact, specific *Alosa* species (see Table 1.1) provide the unique opportunity of intraspecies comparisons between neighboring populations displaying different life-history forms.

Finally, the selection of alosines as models in this study was justified by the fact that analyses on their fecundity type are timely. Lately, information is accumulating, suggesting that various *Alosa* populations display the indeterminate fecundity type (Murauskas and Rulifson 2011; Hyle et al. 2014; Ganias et al. 2015b), contrasting the historical assumption that the species of the genus were determinate spawners (e.g., Carscadden and Leggett 1975; Loesch and Lund 1977; Jessop 1993).

1.3 Selected species

The selection of the *Alosa* species that were included in this study was based on the fulfillment of the following prerequisites: (a) spatial separation, (b) broad coverage of life-history form – reproductive strategy combinations, (c) display of more than one life-history forms, (d) existence of comparable data on aspects of reproductive biology, (e) significant social, economic and ecological merit, and (f) alarming conservation status and/or decreasing population trend. Specifically, five populations of four different species distributed in two continents (Fig. 1.6) were analyzed: (1) a semelparous anadromous Allis shad, (2) an iteroparous anadromous Blueback herring, *A. aestivalis*, (3) an iteroparous landlocked Macedonian shad, and (4) two iteroparous, an anadromous and a landlocked, Alewife populations.
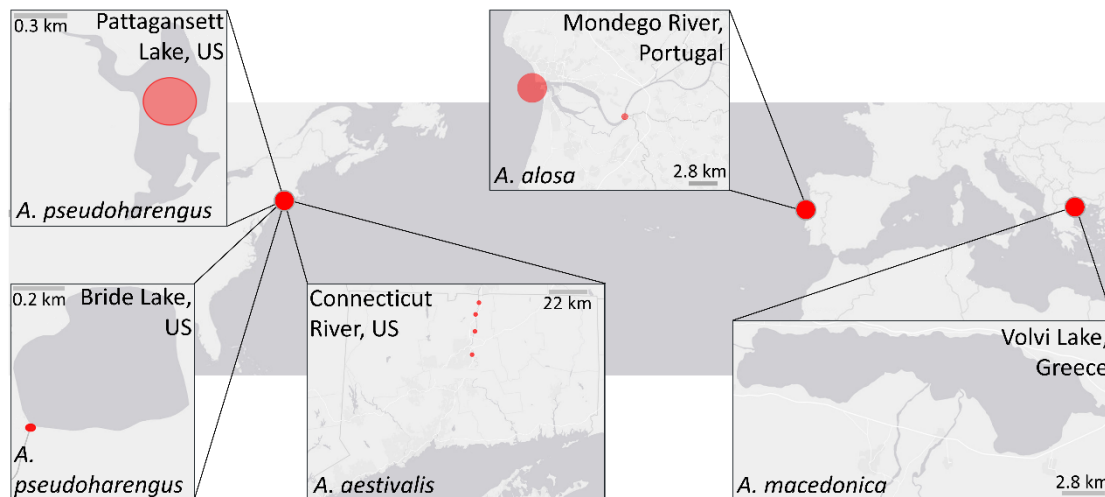
Figure 1.6. Sampling sites of the *Alosa* populations analyzed in this study

Εικόνα 1.6. Περιοχές δειγματοληψίας πληθυσμών ειδών του γένους *Alosa* που αναλύθηκαν στην παρούσα εργασία

1.3.1 Alosa alosa

Allis shad, *A. alosa*, is a highly semelparous (with very low proportions of repeat spawning; Mennesson-Boisneau et al. 2000; Mota et al. 2015), anadromous (with only few exceptions of landlocked populations; e.g., Collares-Pereira et al. 1999; Mennesson-Boisneau et al. 2000; Correia et al. 2001), economically and socially important fish (Mota and Antunes 2011; Pereira et al. 2013; Mota et al. 2015). Historically, Allis shad was distributed along the eastern Atlantic coast, from Norway to Morocco, in all large rivers draining to the North Sea and in the western Mediterranean Sea (Baglinière et al. 2003; Mota et al. 2015). According to IUCN, the species is globally considered of "least concern" (Freyhof and Kottelat 2008). However, its abundance and geographical distribution have declined since the middle of the twentieth century due to overfishing, dam construction, water quality degradation and deterioration or loss of spawning grounds (Baglinière et al. 2003; Rougier et al. 2012; Mota et al. 2015). Hence, Allis shad is included in European pieces of legislation (Bern Convention on the Conservation of European Wildlife and Natural Habitats, and European Union's Habitats Directive), is regarded as "vulnerable" at a more local level in some European countries (ICONA 1986; Vieitez and Rey 2005), and thus is protected by additional legislations, such as the OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic (Baglinière et al. 2003; IUCN-France, MNHM, SFI, and ONEMA 2010; Rougier et al. 2012; Mota et al. 2015; Stratoudakis et al. 2016). To reverse the decline in Allis shad abundancies and geographical distribution, measures have been taken, such as fisheries closures (Stratoudakis et al. 2016), and projects have been conducted, such as the LIFE-Projekt

Maifisch and the LIFE+ *Alosa alosa* Project that aimed to the conservation and re-introduction of the Allis shad to the Gironde and Rhine watersheds.

Reproductive biology of Allis shad has been studied at a significant degree. Age and size at sexual maturity, spawning season, spawning time, spawning habitat characteristics and mating behavior are among the reproductive traits that have been analyzed for the species (Mennesson-Boisneau and Boisneau 1990; Baglinière et al. 2003; Acolas et al. 2004; Lassalle et al. 2008; Mota et al. 2015; Langkau et al. 2016). However, its ovarian dynamics have never been studied in detail, and thus there is no available information on the species oogenesis and fecundity type.

1.3.2 Alosa aestivalis

Blueback herring, *A. aestivalis*, is distributed along the Atlantic coast of North America, from the southern Gulf of St. Lawrence in Canada southward to the St. Johns River in Florida, US (Schmidt et al. 2003). It is mainly an anadromous species, with only few exceptions of freshwater resident populations (Prince and Barwick 1981; Limburg et al. 2001; Winkelman and Van Den Avyle 2002), and different populations display variable degrees of repeat spawning. Historically comprised a valuable fishery for food, bait and fertilizer (Messieh 1977; ASMFC 2017). However, its populations have experienced dramatic declines throughout much of the species range due to damming, inadequate fish passage, overfishing in the ocean and in freshwaters, climate change, reduction in habitat quality and increased predation from recovering populations of other species, such as Striped Bass *Morone saxatilis* (Davis and Schultz 2009; Limburg and Waldman 2009; Bethoney et al. 2013; ASMFC 2017). As a result, Blueback herring is regarded as a species of concern (NOAA 2009) and many of its stocks have been characterized as overexploited (Schmidt et al. 2003; Limburg and Waldman 2009), while it is considered "vulnerable" by IUCN (NatureServe 2013a). To ameliorate the species abundances, several actions are in motion, such as moratoria, by-catch quota, spatial/temporal closure in fisheries, and reestablishment of river connectivity by dam removals (NOAA 2009; Gahagan et al. 2012; Bethoney et al. 2013; ASMFC 2017).

Several aspects of the species reproductive biology have been studied for various populations, including timing and distance of spawning run, spawning season, oocyte release strategy, spawning frequency, spawning habitat selection (e.g., Loesch and Lund 1977; Jessop 1993; Walsh et al. 2005; McBride et al. 2010). However, the ovarian dynamics has never been thoroughly studied for any Blueback herring population and information on the fecundity type is lacking.

1.3.3 Alosa pseudoharengus

Alewife, *A. pseudoharengus*, is found from the coast of Labrador and Newfoundland in Canada to southern Georgia, US (Schmidt et al. 2003). Its populations are mainly anadromous and the probability of surviving the first spawning season is variable. The species displays also the landlocked life-history form; the latter Alewife populations resulted from manmade or naturally constructed impoundments and introductions – intentional or involuntary – into lakes and reservoirs. Alewife introduction was encouraged and a common practice in the past aiming to augment resident forage fish species (Irwin and Bettoli 1995; Hendricks 2003). As a result, several permanent freshwater Alewife populations have been established (e.g., Brooks and Dodson 1965; Lackey 1970; Nigro and Ney 1982; Anderson and Neumann 2002; Dunlop and Riley 2013).

Alewife is a species of high socioeconomic value that once supported important fisheries for use as food, bait, and fertilizer (Messiah 1977; ASMFC 2017). Nowadays, its populations have experienced dramatic declines throughout the species distribution and have been reduced to historically low levels (NOAA 2009; Davis and Schultz 2009; Limburg and Waldman 2009). The most likely threats causing population declines include spawning habitat loss due to dam construction or inadequate fish passage, overexploitation, by-catch mortality and increased predatory pressure (Davis et al. 2009; Limburg and Waldman 2009; Bethoney et al. 2013; ASMFC 2017). Even though Alewife is characterized as of "least concern" by IUCN (NatureServe 2013b), it is regarded as a species of concern (NOAA 2009) and moratoria and closures in fisheries have instituted to invert the declining trend in the species abundances, as well as fishway installations to restore migratory pathways to historical spawning grounds (Davis and Schultz 2009; NOAA 2009; Gahagan et al. 2012; Bethoney et al. 2013; ASMFC 2017).

Some aspects of Alewife reproductive biology have often been examined and for various populations, such as timing of spawning run, spawning season and annual egg production (e.g., Norden 1967; Kissil 1974; Ellis and Vokoun 2009; Sullivan et al. 2019). On the contrary, ovarian dynamics and fecundity type of Alewife are virtually unexplored, since only a single study has analyzed these traits in detail (Ganias et al. 2015b). Moreover, a comparative study on reproductive traits between the anadromous and the landlocked form is – oddly enough – lacking, even though Alewife provides a suitable model species for answering questions pertaining to intraspecies diversion owed to the life-history form.

1.3.4 Alosa macedonica

Macedonian shad, *A. macedonica*, is endemic to Volvi Lake in northern Greece (Crivelli 2006) and one of the rare, naturally evolved, landlocked alosine species. It is an iteroparous species inhabiting a stable and well-studied reservoir (Bobori et al. 2001). During previous decades, Macedonian shad constituted one of the main fisheries of Volvi Lake, but, nowadays, it has low commercial value. Hence, it is no longer a targeted fishery, except in early summer, when small quantities are captured and cured with dry salt by local fishermen. Although it is currently reputed to be an undesired fish, it still forms a source of social and touristic importance (Giantsis et al. 2015). It is currently considered as "vulnerable" by IUCN and it is listed in Annexes II and V of the European Union Habitats Directive (Crivelli 2006).

Age and size at sexual maturity, spawning season and the characteristics of the spawning sites have been examined for Macedonian shad (Sinis 1981; Kleanthidis 2002). Ovarian dynamics have also been analyzed at some extent, including oocyte size and number estimations (Kleanthidis 2002). However, the fecundity type of the species remains undefined.

1.4 Aims and approach

Aim of this study was to unveil aspects of oogenesis that remain unknown or are insufficiently examined and see how they shape the fecundity type. Various aspects of oogenesis were analyzed at three different temporal scales (lifetime, spawning season and ovulatory cycle = time-lag between two sequential ovulation/spawning events) and at various time-frames (prior to, during and after spawning activity) in populations with different life-history form – reproductive strategy characteristics (Fig. 1.7). The main scientific questions are summarized as follows:

Q1. What is the role of oogonia and how SG recruitment occurs prior to the onset of spawning activity?

Q2. Does oogenesis prior, during and after spawning activity, in different congeneric species, conform with the theoretical temporal patterns that currently describe the ovarian dynamics of determinate and indeterminate spawners?

Q3. Is life-history form among the parameters that influence oogenesis and the fecundity type regulation?

Q4. Does SG recruitment occur at a specific time-frame within the ovulatory cycle?
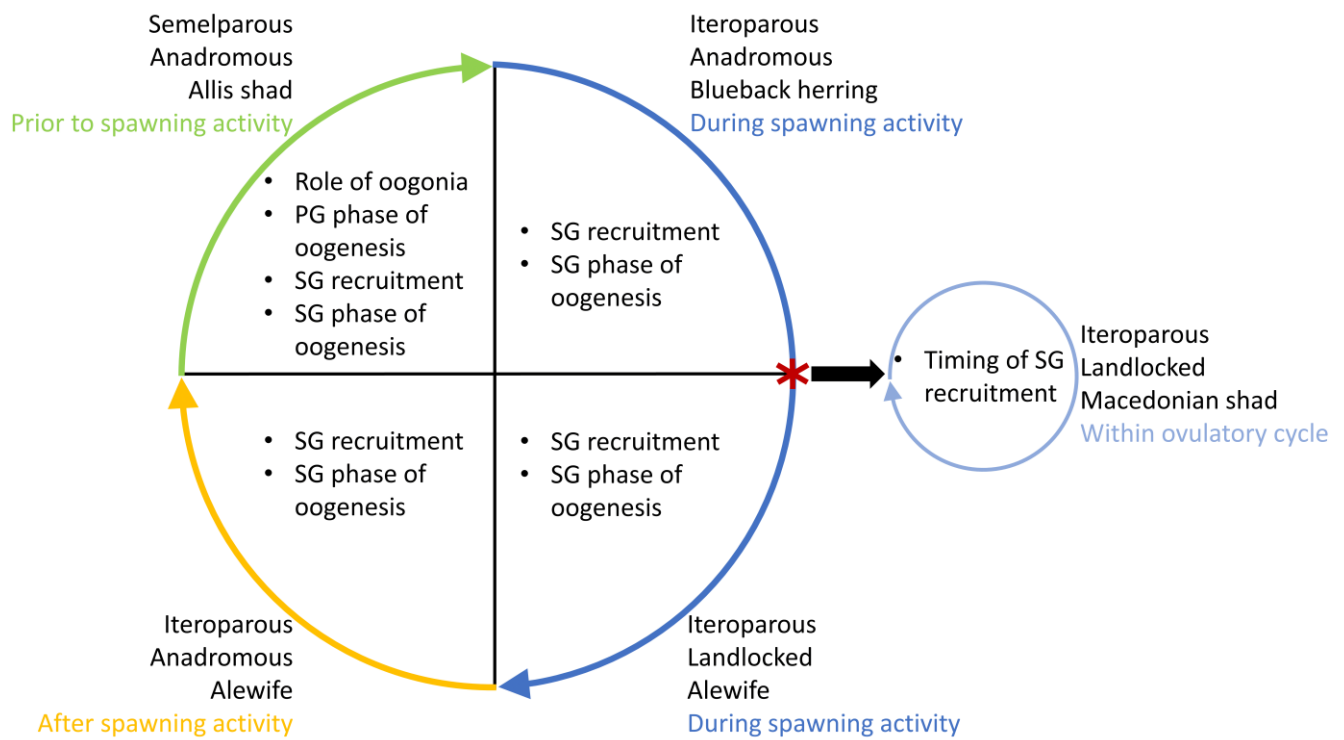
Figure 1.7. Aspects of oogenesis analyzed per species, life-history form – reproductive strategy combination and temporal scale/time-frame. Colors represent the different temporal scales/time-frames

Εικόνα 1.7. Φάσεις της διαδικασίας της ωογένεσης που αναλύθηκαν ανά είδος, τύπο κύκλου ζωής, στρατηγική ζωής και χρονική κλίμακα. Τα διαφορετικά χρώματα αντιστοιχούν στις διαφορετικές χρονικές κλίμακες

CHAPTER 2: General methodology

2.1 Sample processing

All fish were acquired through sampling or from local fish markets. Upon sampling, fish were stored on ice or 10% neutral buffered formalin and processed in less than 24 hours. Biometric measurements upon workup included total length (L, mm), total weight (W, g) and eviscerated weight (W_{ev}, g). The residuals of log(W_{ev}) on log(L) relationship were used as condition factor (*CF*; Jakob et al. 1996). Gonads were removed, weighed (W_g, g) and preserved in 10% neutral buffered formalin. Gonadosomatic index (*GSI* = 100 x W_g/W_{ev}) was estimated.

2.1.1 Histological process

Ovaries were processed histologically using standard procedures. Approximately a 2 mm thick hand-cut cross section was obtained from each ovary and embedded in a medium (paraffin or resin). Subsequently, 4-μm histological sections were cut and stained with toluidine blue or hematoxylin and eosin. All histological sections were scanned, resulting in high-resolution photomicrographs, using a NanoZoomer S60 slide scanner (Hamamatsu Photonics) or a BASLER acA1920-40uc camera fitted on a Zeiss Axio Lab.A1 microscope and the Microvisioneer software (Fig. 2.1).

Figure 2.1. (a) Photomicrograph of an ovarian histological section, (b) NanoZoomer S60 slide scanner, and (c) BASLER acA1920-40uc camera and Zeiss Axio Lab.A1 microscope image capturing system
Εικόνα 2.1. (a) Φωτομικρογραφία ιστολογικής τομής ωοθήκης, (b) NanoZoomer S60 σκάνερ ιστολογικών τομών, και (c) κάμερα BASLER acA1920-40uc και μικροσκόπιο Zeiss Axio Lab.A1

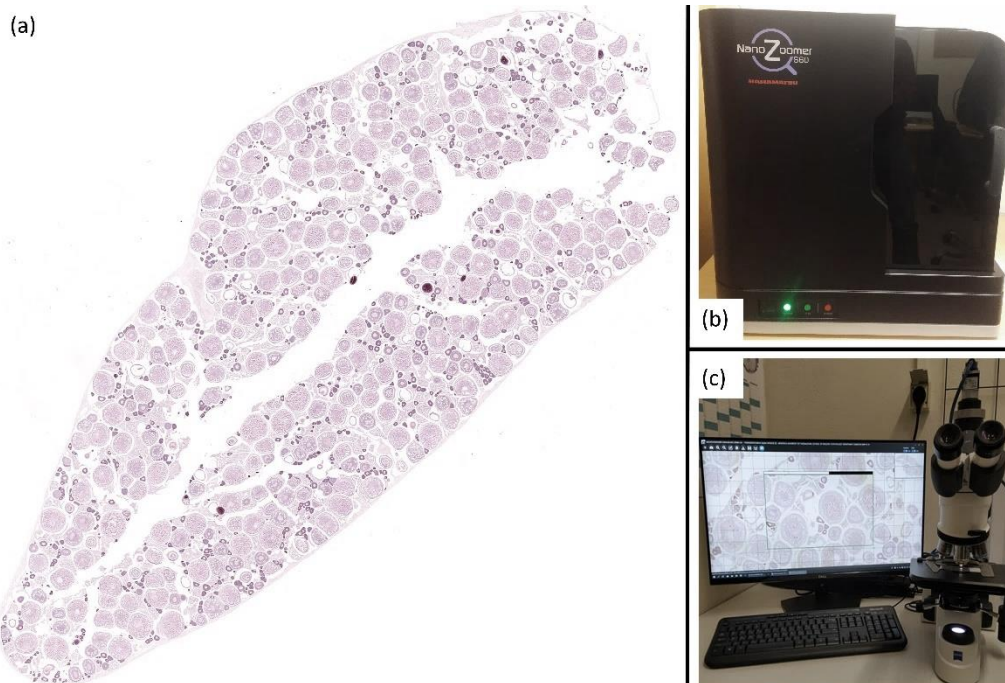Due to their great resolution, photomicrographs were inspected in high magnification, which yielded very detailed information for each female, including: (1) the presence or absence of oogonia, (2) identification of the distinct oocyte developmental stages at both PG and SG phase, (3) detection of postovulatory follicles (i.e., ovarian markers of recent spawning activity consisted of the follicular layers that remain in the ovary after the release of the ovum during spawning; POFs) and their classification as new or old based on their morphological characteristics (Annex 1), (4) detection of atresia and the stages of atretic oocytes and/or follicles (Annex 2), and (5) definition of the ovarian developmental stage as the stage of the most advanced oocyte batch (i.e., oocytes at the most advanced developmental stage; AS) in its histological section.

In addition, the high-resolution photomicrographs enabled the estimation of the cross-sectional area of the biggest POF situated along the epithelium of the lamellae of each female (POF_{XSA}) based on Ganias et al. (2007) and through the NanoZoomer Digital Pathology image viewing software (Hamamatsu Photonics) or ImageJ software (https://imagej.nih.gov/ij/). Relative batch fecundity, RF_b (i.e., number of the AS oocytes per g of W_{ev}), and relative fecundity of any other oocyte batch, RF_i (i.e., number of oocytes at the i developmental stage per g of W_{ev}), were estimated through the "stereological" method (Weibel et al. 1966) implemented on the photomicrographs and via the ImageJ software (Annex 3).

The pieces of information used and estimations made through the photomicrographs varied for each of the surveyed alosine populations and are specified at the corresponding chapters.

2.1.2 Whole-mount process

Whole-mount analysis was performed on ovarian subsamples (Annex 4). Two new steps were introduced to the standard procedure (except in case of Allis shad), both of which increased significantly the accuracy of oocyte number and size estimations. In specific, oocytes were separated ultrasonically (Anderson et al. 2020) and stained with hematoxylin (procedure developed during this study). The former procedure enabled the swift and complete separation of oocyte aggregations, while the latter enhanced the oocyte opacity regardless of the size or developmental stage (see Annex 4). All subsamples were digitized into high magnification to ensure high-resolution whole-mount photographs through a stereo microscope image capturing system (Fig. 2.2); either a Jenoptik Progress C3 camera fitted on a Euromex NZ 80 stereo microscope or a Leica DFC 280 camera fitted on a Leica MZ6 stereo microscope.
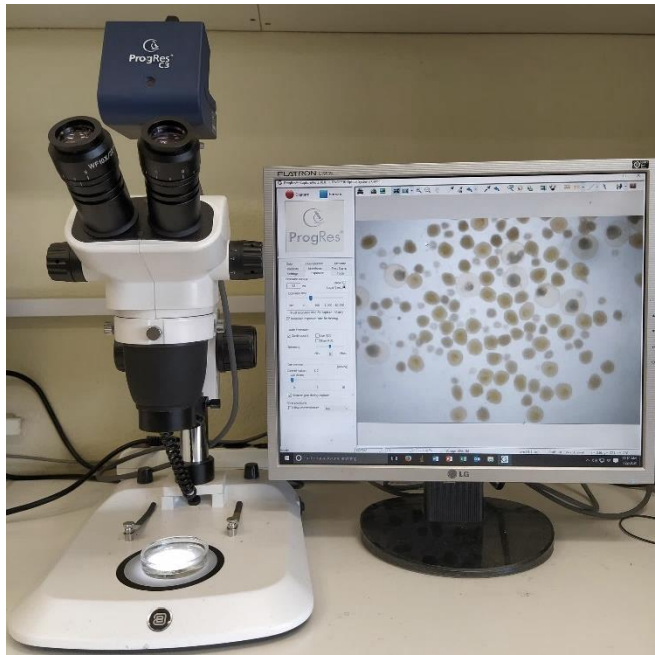
Figure 2.2. Stereo microscope image capturing system consisted of a Jenoptik Progress C3 camera and a Euromex NZ 80 stereo microscope

Εικόνα 2.2. Σύστημα λήψης φωτογραφιών αποτελούμενο από κάμερα Jenoptik Progress C3 και στερεοσκόπιο Euromex NZ 80

Image analysis was performed on the whole-mount photos via the ImageJ software and enabled for each female: (1) estimation of oocyte number and diameters (ODs) through the semiautomated particle analysis method (Thorsen and Kjesbu 2001; Ganias et al. 2010; Ganias et al. 2014b) and creation of an oocyte size frequency distribution (OSFD) (Annex 5), (2) definition of the advanced mode (AM) and the first subsequent mode (SM1) in the OSFD and their enumeration through the method of Bhattacharya (1967) and the FiSAT II software (http://www.fao.org/) (Annex 6), (3) identification of atretic oocytes based on their morphological characteristics, such as dark-orange color, presence of dark pigment spots and irregular shape (Bromage and Cumaranatunga 1988; Witthames et al. 2009), (4) estimation of dimensional features (e.g., circularity), for both healthy and atretic oocytes, through particle analysis, and (5) estimation of relative fecundity values through the gravimetric method (Hunter et al. 1985) (Annex 6). Among the fecundity values estimated in this study were: (i) relative fecundity of the AM, RF_{AM} (i.e., number of AM oocytes per g of W_{ev}), (ii) relative fecundity of the SM1, RF_{SM1} (i.e., number of SM1 oocytes per g of W_{ev}), (iii) relative total fecundity, RF_t (i.e., total number of SG oocytes per g of W_{ev}), (iv) relative fecundity of PG oocytes, RF_{PG} (i.e., total number of PG oocytes per g of W_{ev}), and (v) relative realized annual fecundity, RF_y (i.e., total number of oocytes that would have been released during the entire period of spawning activity per g of W_{ev}). The latter was estimated by summing RF_t and RF_{PG} and subtracting the atretic oocytes. The size threshold that distinguishes SG from PG oocytes was set at 200 μm based on statistical estimations (see Chapter 4) and previous values reported for American shad (Hyle et al. 2014).

The whole-mount process was used to acquire different estimations for each of the analyzed alosine populations; estimations that are specified in each of the following chapters.

2.3 Statistical analysis

All statistical analyses were performed by using R 3.5.2 (www.R-project.org). Most plots were produced also in R through the following packages: lattice (Sarkar 2008), ggplot2 (Wickham 2016), ggridges (Wilke 2018), ggpubr (Kassambara 2019) and SHaZam (Gupta et al. 2015). Normality was tested by the Shapiro test. $P < 0.05$ was considered a statistically significant result. The statistical tests varied among the surveyed populations; they are detailed in the respective chapters.

CHAPTER 3: Role of oogonia, SG recruitment and SG oocyte production prior to the onset of spawning activity in a semelparous anadromous fish

The results presented in this chapter have been published as:

3.1 Specific objectives

In this chapter, the ovarian dynamics of a semelparous anadromous Allis shad population were analyzed during the pre-spawning phase of its reproductive cycle and the seasonal reproductive output was estimated. To achieve these goals, PG, SG and atretic oocytes were enumerated and, subsequently, RF_b, RF_t and RF_y were estimated. The potential production of PG oocytes from oogonia and the SG recruitment pattern were analyzed in spawning capable females. Due to the semelparous reproductive strategy of the surveyed population, the working hypothesis was that mature, pre-spawning females would have nearly or even totally depleted PG reserves. This chapter provides the first multifaceted analysis focused on the ovarian dynamics of this species, and reports results that will enhance the effectiveness of management efforts across its distribution. The reported results will also assist in unveiling the early processes of oogenesis in fish.

3.2 Specific methodology

Female Allis shad were collected from late February to late March 2017 from the Mondego watershed in Portugal (Table 3.1). The sampling plan was designed to provide mature (ideally spawning capable) pre-spawning females. All samples (n = 57) were from local fishermen who operated mainly at coastal areas adjacent to the Mondego River mouth. Less fishing effort occurred along the river, and thus most of the samples analyzed were sea-caught (Table 3.1).

The fishing gear used were set and drift trammel nets in the sea and the river (ca. 10 km upstream from the river mouth), respectively (Fig. 3.1). The sampling dates were selected based on previous estimates of the upstream spawning migration period and the current regulations on the species management aiming to facilitate upstream recolonization (Mota and Antunes 2011; Mota et al. 2015; Stratoudakis et al. 2016).

The aim was to obtain samples from dates at wide intervals to test for population synchronization in upstream migration and ovarian development.

Table 3.1. Number (n) of female *Alosa alosa* analyzed histologically and through whole-mount analysis and total length (L in mm) range and mean (± SD) values per sampling date and site
Πίνακας 3.1. Αριθμός (n) θηλυκών ατόμων *Alosa alosa* που αναλύθηκαν ιστολογικά και με τη μέθοδο whole-mount ανά ημερομηνία και περιοχή δειγματοληψίας. Το μέσο ολικό μήκος σώματος (L σε mm) και η τυπική απόκλιση (± SD) παρουσιάζονται επίσης

Date	Site	Histological analysis (n)	Whole-mount analysis (n)	L range (mean ± SD)
24 February 2017	Sea	19	19	572 – 715 (653 ± 38)
10 March 2017	River	6	9	612 – 659 (632 ± 17)
28 March 2017	Sea	27	29	555 – 695 (639 ± 35)
Total		**52**	**57**	

Samples were stored on ice and processed in less than 24 hours at Marine and Environmental Sciences Centre in Lisbon. To ensure analysis was confined to Allis shad, gill rakers on the first gill arch were enumerated; gill rakers are <60 in Twaite shad, 60-115 in Allis-Twaite shad hybrids, and >115 in Allis shad (Alexandrino et al. 2006; Mota et al. 2015).
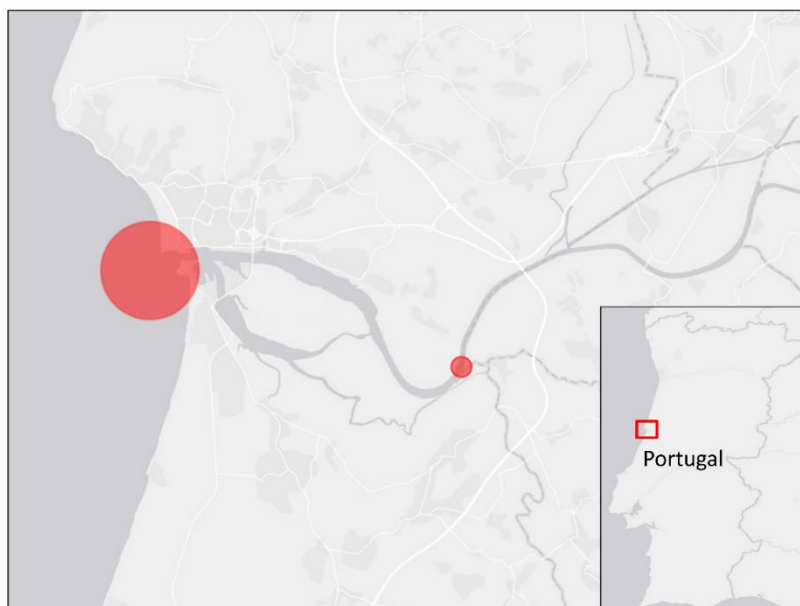
Figure 3.1. Sampling sites for *Alosa alosa* at the Mondego watershed in Portugal. Points on the map do not indicate the exact sampling sites, which were unknown, since all fish were obtained from local fishermen
Εικόνα 3.1. Περιοχές δειγματοληψίας για το είδος *Alosa alosa* στον ποταμό Mondego της Πορτογαλίας. Τα σημεία στον χάρτη απεικονίζουν κατά προσέγγιση τις περιοχές δειγματοληψίας, καθώς όλα τα ψάρια αποκτήθηκαν από επαγγελματίες ψαράδες

In total, 52 ovarian histological sections were prepared (embedding medium: resin, pigment: toluidine blue) and digitalized via a NanoZoomer S60 slide scanner. The resulting photomicrographs yielded detailed information for each female, including: (1) presence or absence of oogonia, (2) presence or absence of PG oocytes, (3) developmental stages of SG oocytes, (4) presence or absence of POFs, (5) occurrence of atresia and the stages of atretic oocytes and/or follicles, and (6) ovarian developmental stage.

In parallel, 57 whole-mount photos were captured using a Leica DFC 280 camera fitted on a Leica MZ6 stereo microscope, and their analysis enabled in each one: (1) identification of atretic oocytes, (2) evaluation of circularity values of all oocytes, healthy and atretic, (3) quantification of atretic oocytes through a threshold value in circularity, (4) estimation of ODs at both PG and SG phase, (5) creation of an OSFD, (6) definition and enumeration of the AM in the OSFD, and (7) fecundity estimations.

AM was composed of oocytes in a single developmental stage, based on previous results on Alewife (Ganias et al. 2015b) and on the gonadal development as depicted in the histological sections. Thus, the relative fecundity of the AM was considered equivalent to RF_b. RF_t and RF_{PG} were also estimated; the size threshold between PG and SG oocytes that was initially set at 200 μm, was confirmed by the minimum ODs estimated in the OSFDs of females with no PG oocytes in their histological sections. Finally, RF_y was estimated.

The SG recruitment pattern and the potential replenishment of the PG reserves from oogonia were also analyzed. Pearson's product-moment correlation analyses were conducted to test for relationships between ovarian development and each of the following parameters: mean size, number (i.e., RF_{PG}) and size range of the PG oocytes. Mean diameter of all SG oocytes (OD_{SG}) was used as an index of ovarian development. Only sea-caught females were used in the analyses of the SG recruitment pattern, since the river-caught females were expected to be at a more advanced ovarian developmental stage, and thus at a more progressed SG recruitment phase. The sea-caught females from the two sampling dates were analyzed together, since no differences were detected in their mean W or mean CF (t-tests: $P > 0.05$).

3.3 Results

All females were pre-spawning, since POFs were not detected in any histological section, while oogonia and oocytes, at several distinct developmental stages, were always present (Fig. 3.2). PG oocytes ranged in size (112 – 199 μm) and various SG developmental stages were defined; from the least to the most advanced they were: several CA stages, three vitellogenic (VIT-1 – VIT-3) stages and GVM stage. Oocytes of at least two CA and two VIT stages were simultaneously present in each ovary. All females were spawning capable; most sea-caught females (81%) were at the GVM

ovarian developmental stage and the remainder were at the tertiary vitellogenic stage (VIT-3), while all up-runners (i.e., river-caught females) were at the GVM stage.
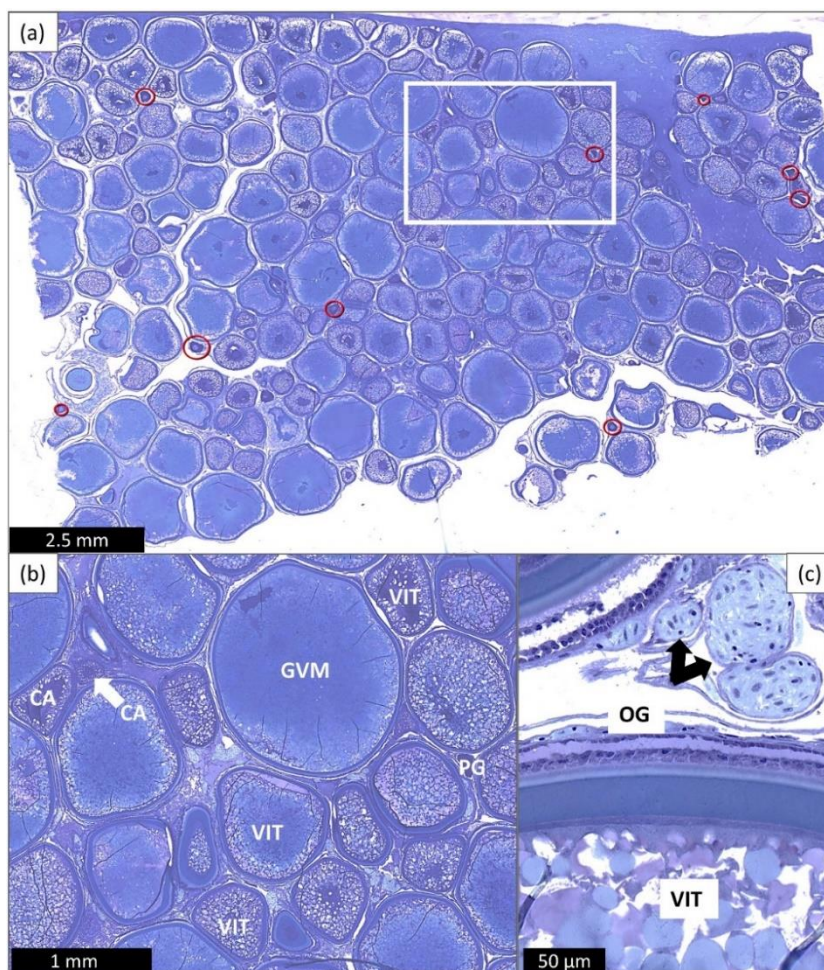
Figure 3.2. (a) Photomicrograph of an ovarian histological section of a pre-spawning *Alosa alosa* female containing oocytes at several developmental stages. Oocytes at the primary growth phase are indicated by the red circles. (b) The highlighted area in (a) magnified. (c) Oogonia (OG) and a vitellogenic (VIT) oocyte

Εικόνα 3.2. (a) Φωτομικρογραφία ιστολογικής τομής ωοθήκης ψαριού του είδους *Alosa alosa* πριν την έναρξη της περιόδου ωοτοκίας στην οποία απεικονίζονται ωοκύτταρα σε διάφορα στάδια ανάπτυξης. Τα ωοκύτταρα στην πρωτογενή φάση ανάπτυξης σημειώνονται με κόκκινους κύκλους. Το παραλληλόγραμμο υποδεικνύει τη θέση της μεγεθυμένης περιοχής στην εικόνα (b). (c) Ωογόνια (OG) και ένα ωοκύτταρο στο στάδιο της λεκιθογένεσης (VIT)

The PG reserves had been totally depleted in a proportion of the examined ovaries. PG oocytes were absent in 10% of the examined histological sections. Depleted PG reserves were observed also through whole-mount analysis, where 32% of the OSFDs lacked oocytes with ODs smaller than 200 μm (Fig. 3.3). The percentage of PG oocytes was very low in those ovarian whole-mount subsamples containing them (0.1 to 8% of all oocytes, except for one female where the PG oocytes consisted the 15% of the ovarian content; Figure 3.3). Females with and without PG oocytes were caught on all sampling sites and dates and at all ovarian developmental stages.
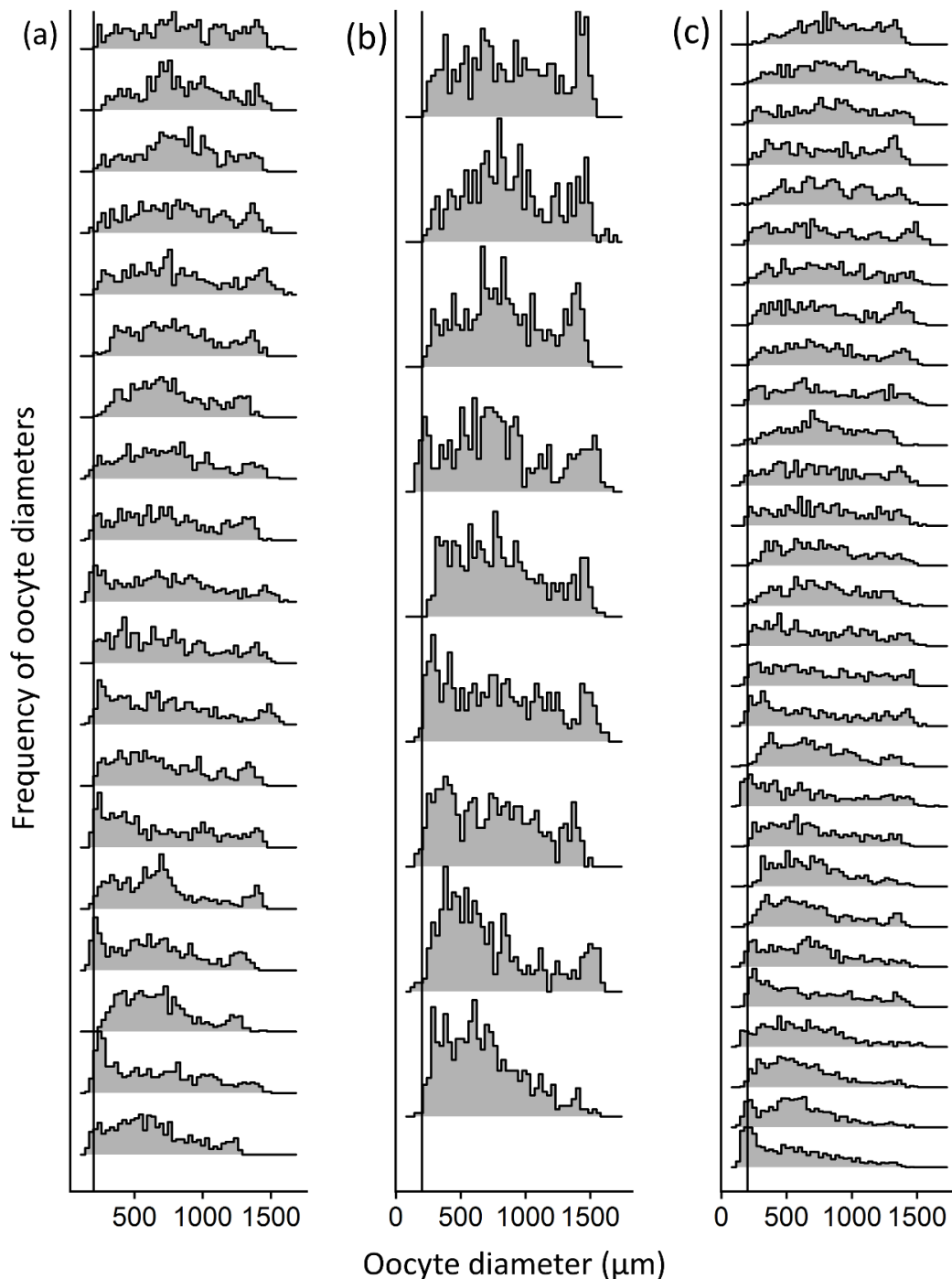
Figure 3.3. Size frequency distributions of primary and secondary growth phase oocytes of *Alosa alosa* sampled on: (a) 24 February, (b) 10 March, and (c) 28 March 2017. Within each panel, distributions are displayed in order of increasing (bottom to top) mean oocyte diameter of the secondary growth oocytes. The vertical lines indicate the size threshold differentiating primary from secondary growth oocytes (200 μm)

Εικόνα 3.3. Κατανομές συχνοτήτων μεγεθών των ωοκυττάρων στην πρωτογενή και δευτερογενή φάση ανάπτυξης ατόμων του είδους *Alosa alosa* συλλεγμένα στις: (a) 24 Φεβρουαρίου, (b) 10 Μαρτίου, και (c) 28 Μαρτίου 2017. Οι κατανομές εμφανίζονται με αυξανόμενη (από κάτω προς τα επάνω) μέση διάμετρο ωοκυττάρων στη δευτερογενή φάση ανάπτυξης. Οι κατακόρυφες γραμμές υποδεικνύουν τη διάμετρο που διαχωρίζει τα ωοκύτταρα της πρωτογενούς από τη δευτερογενή φάση ανάπτυξης (200 μm)

A clear SG recruitment pattern was evident in comparing females with PG oocytes in their ovaries by the time they were caught. Females at a more advanced ovarian stage – reflected by the larger SG oocytes – had PG oocytes that were aggregated at a narrower size range than females at a less advanced ovarian stage; there was a strong negative linear relationship between mean OD_{SG} and the CV (i.e., coefficient of variation) of PG oocyte diameters (Pearson correlation coefficient r = -0.64, confidence intervals: -0.83, -0.31; Fig. 3.4).
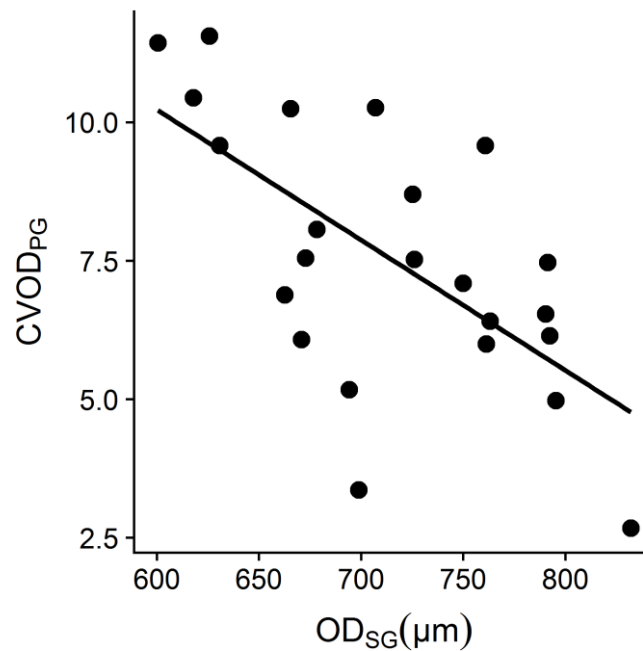
Figure 3.4. Linear relationship between coefficient of variation of the distribution of primary growth phase oocyte diameters ($CVOD_{PG}$) and mean diameter of the oocytes at the secondary growth phase (OD_{SG}) for sea-caught *Alosa alosa*

Εικόνα 3.4. Γραμμική σχέση μεταξύ του συντελεστή μεταβλητότητας της κατανομής των διαμέτρων των ωοκυττάρων στην πρωτογενή φάση ανάπτυξης ($CVOD_{PG}$) και της μέσης διαμέτρου των ωοκυττάρων στη δευτερογενή φάση ανάπτυξης (OD_{SG}) για άτομα του είδους *Alosa alosa* που συλλέχθηκαν στη θάλασσα

In addition, females at a more advanced ovarian stage had larger PG oocytes (Pearson correlation coefficient r = 0.47, confidence intervals: 0.15, 0.7; Fig. 3.5a). Finally, females at a more advanced ovarian stage had fewer PG oocytes (Pearson correlation coefficient r = −0.59, confidence intervals: −0.8 to −0.26; Fig 3.5b).
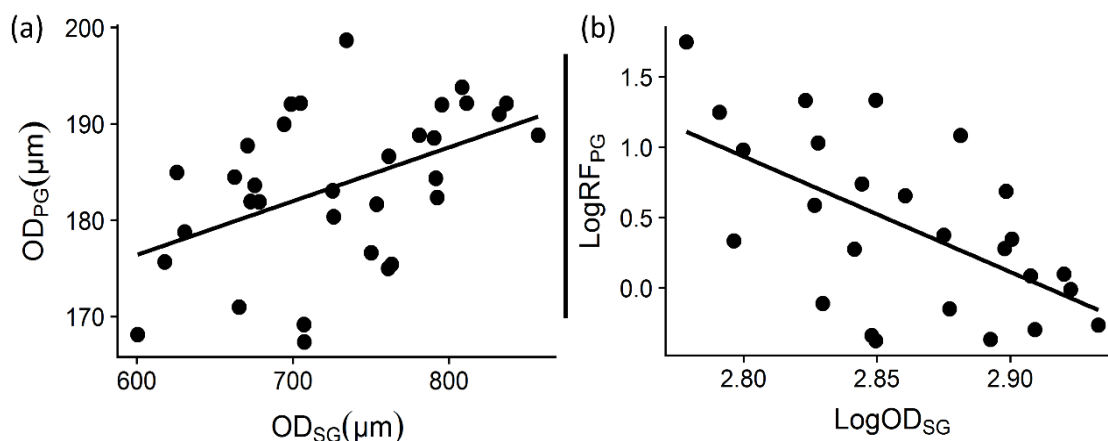
Figure 3.5. Linear relationships for sea-caught *Alosa alosa* between: (a) mean diameters of oocytes at the primary (OD$_{PG}$) and the secondary growth phase (OD$_{SG}$), and (b) between the log-transformed relative fecundity of oocytes at the primary growth phase (Log*RF$_{PG}$*) and log-transformed OD$_{SG}$ values

Εικόνα 3.5. Γραμμική σχέση: (a) μεταξύ της μέσης διαμέτρου των ωοκυττάρων στην πρωτογενή φάση ανάπτυξης (OD$_{PG}$) και στη δευτερογενή φάση ανάπτυξης (OD$_{SG}$), και (b) μεταξύ των λογαριθμημένων τιμών της σχετικής γονιμότητας των ωοκυττάρων στην πρωτογενή φάση ανάπτυξης (Log*RF$_{PG}$*) και των λογαριθμημένων OD$_{SG}$ τιμών, για άτομα του είδους *Alosa alosa* που συλλέχθηκαν στη θάλασσα

Large and deformed atretic oocytes at the alpha stage were identified in 73% of the histological sections (Figure 3.6a). Deformed large oocytes were easily detected also in the whole-mount subsamples of all females with atresia in their sections (Fig. 3.6b-c). The analysis of circularity of large oocytes (OD > 1000 μm) – spherical and deformed – in whole-mount photos revealed outliers and extreme outliers (values more than 1.5 times the interquartile range). These outliers were 6% of the values and were lower than 0.79 in a range from zero to one, where one represents a perfect circle. Thus, all values lower than this threshold were considered indicative of alpha atretic oocytes. Oocyte opacity and color were also taken into consideration, with the atretic oocytes being characterized by uneven transparency and/or dark yellowish color (Fig. 3.6c). These oocytes were excluded from any fecundity estimations. The intensity of alpha atresia ranged from 1.4% to 9.3% of the ovarian content.

RF$_t$ was estimated for all females and ranged from 136 to 368 oocytes per g of W$_{ev}$ (mean ± SD = 229 ± 51.4). *RF$_b$* ranged from 10.7 to 46.6 oocytes per g of W$_{ev}$ (mean ± SD = 26.8 ± 8.2) for those females with a single distinct AM in their OSFDs (41.4% of the females). *RF$_y$* ranged from 132 to 424 oocytes per g of W$_{ev}$ (mean ± SD = 228 ± 58.4); this is eightfold the size of *RF$_b$*, suggesting that each female contained a mean number of 8 oocyte batches in its ovary.
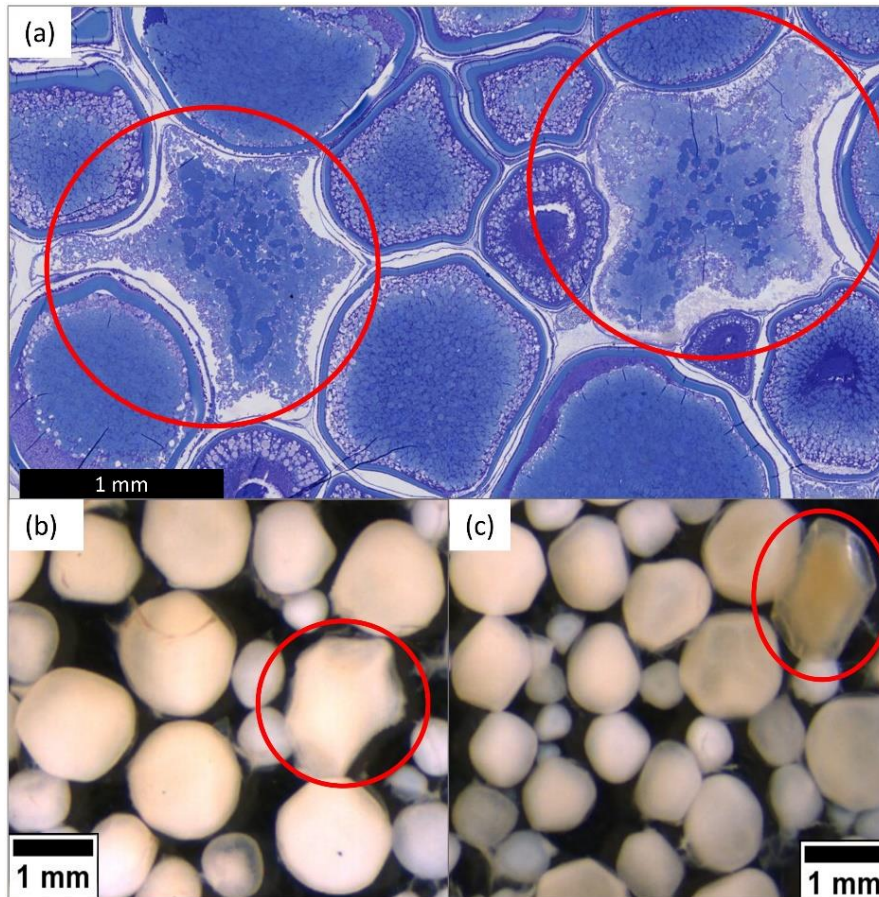
Figure 3.6. (a) Photomicrograph of an *Alosa alosa* ovarian histological section showing oocytes at the alpha stage of atresia (red circles). (b-c) ovarian whole-mount (unstained) subsamples showing atretic oocytes at the alpha atretic stage (red circles) characterized by the deformed shape and dark yellowish color

Εικόνα 3.6. (a) Φωτομικρογραφία ιστολογικής τομής ωοθήκης ατόμου του είδους *Alosa alosa* στην οποία απεικονίζονται ωοκύτταρα στο άλφα στάδιο ατρησίας (εντός των κόκκινων κύκλων). (b-c) φωτογραφίες υποδειγμάτων ιστού ωοθηκών (δίχως χρήση χρωστικής) που περιλαμβάνουν ωοκύτταρα στο άλφα στάδιο ατρησίας (εντός των κόκκινων κύκλων), τα οποία χαρακτηρίζονται από παραμορφωμένο σχήμα και σκούρο κιτρινωπό χρώμα

3.4 Discussion

The present chapter offers a detailed outline of the PG and SG oocyte growth dynamics of the anadromous, highly semelparous Allis shad in the Mondego River. Our working hypothesis was confirmed, since the pool of PG oocytes was depleted during a single reproductive period. The depletion of the PG reserves reflected a recruitment pattern to the SG phase, where females at a more advanced ovarian developmental stage had larger, fewer and distributed over a narrower size range PG oocytes. In parallel, replenishment of the PG reserves by oogonia did not occur. The latter two results evinced that the fecundity type is most probably determinate. In addition, the surveyed population was not synchronized regarding either its ovarian development

or the spawning migration. All females launched their upstream migration with mature ovaries (at the VIT-3 or GVM ovarian stage) containing oocytes at several distinct developmental stages, strong indication of multiple spawning. Ovarian development continued *en route* to the spawning grounds; all river-caught females were at the GVM ovarian stage. Most females had also a small proportion of atretic oocytes. Finally, mean relative realized annual fecundity exceeded the size of mean batch fecundity by a factor of eight.

The lack of PG reserves in females with the most developed ovaries indicated that all PG oocytes had been recruited to the SG phase. This result aligns with previous reports that Allis shad in the Mondego River is mostly semelparous (Alexandrino 1996), since complete recruitment of PG oocytes to the SG phase characterizes this reproductive strategy (Ganias and Lowerre-Barbieri 2018). All females lacking PG oocytes had mature ovaries at an advanced developmental stage, were in pre-spawning condition, and were caught away from their spawning grounds; indicative factors of a population tendency to deplete their PG reserves during the current reproductive period and, probably, before the onset of the spawning activity. To determine the degree of semelparity at a population level, the ovarian dynamics of females caught during and immediately after the spawning activity need also to be analyzed. Anadromous Allis shad populations display consistently low proportions of repeat spawning (<6.5% of repeat spawners; Eiras 1981; Alexandrino 1996; Mota and Antunes 2011; Mota et al. 2015), which might indicate a natural and obligatory tendency to semelparity, in contrast to the facultative reproductive strategies of other anadromous alosines (e.g., Twaite shad, American shad and Blueback herring), in which significant variability in the proportions of repeat spawners (0 to 98%) has been reported (Joseph and Davis 1965; Loesch and Lund 1977; Jessop et al. 1983; Creed 1985; Aprahamian et al. 2003a; Limburg et al. 2003; Davis et al. 2009).

The PG reserves were not replenished by the pool of oogonia. The presence of oogonia typically indicates the occurrence of an active germinal epithelium and ongoing folliculogenesis, that could lead to development of new PG oocytes during or after spawning activity, and thus to build-up of fecundity for a next spawning period (Brown-Peterson et al. 2011; Grier et al. 2007). Increase in PG oocytes' frequency has been reported, for example, in post-spawning downrunners of an iteroparous Alewife population (Ganias et al. 2015b), as well as in post-spawning females of the iteroparous Brown trout, *Salmo trutta fario* (Billard 1987, 1992), and Rainbow trout *Oncorhynchus mykiss* (Bromage and Cumaranatunga 1988; Grier et al. 2007). However, even though oogonia are considered as always present in teleost ovaries (Selman and Wallace 1989; Tyler and Sumpter 1996; Grier 2000; Grier et al. 2009), they are not necessarily indicative of ongoing folliculogenesis and production of new PG oocytes. Indeed, oogonia have been found also in the ovaries of true semelparous fish. Grier et al. (2007) (citing Zelennikov 2003) reports that: (1) in Pink salmon *O.*

gorbuscha the divisions of oogonia are blocked once the pool of oocytes is established in the 0+ age group, and (2) in Chum salmon *O. keta*, Salmon trout *O. masou*, Sockeye salmon *O. nerka* and Coho salmon *O. kisutch*, new germ cells resulting from divisions of oogonia fall into atresia or undergo resorption.

Complete SG recruitment in females with the most developed ovaries and lack of replenishment by the pool of oogonia rendered the production of new oocytes during the spawning season highly implausible. Even though all females were at pre-spawning condition and intercepted before they reach their spawning grounds, it is unlikely that additional energy would have been invested in the production and development of new oocytes from oogonia up to the final stages of maturation. The latter assumption is corroborated by the fact that Allis shad does not feed during the upstream migration (Baglinière et al. 2003; Mota et al. 2015). Hence, the fecundity type of the surveyed population is most probably determinate. However, to evidence the latter assumption, additional samples from spawning females should be analyzed in the future.

Clarification of the SG recruitment process and quantification of atresia enabled the estimation of the realized annual fecundity and demonstration of down-regulation due to atresia. Quantification of atretic oocytes through ovarian whole-mounts has been conducted for other species as well, such as in Norwegian Spring-spawning herring *Clupea harengus* (Óskarsson et al. 2002) and was suggested as a valid and useful methodology by Witthames et al. (2009). However, the reported estimate of atresia intensity may have been an underestimation, and thus the RF_y values might not be entirely accurate. As already mentioned, Allis shad does not feed during its upstream migrating riverine phase (Baglinière et al. 2003; Mota et al. 2015), and thus yolk oocytes may be used as an energetic resource to successfully complete migration and spawning (Pina et al. 2003). Hence, atresia intensity of the surveyed population may increase continuously *en route* to the spawning grounds. Indeed, Mota (2014) reported an increase in atresia intensity from the estuary to the spawning grounds of Allis shad in the Minho River in northern Portugal, a river with similar distance from river mouth to spawning grounds as in Mondego River (~80 km). Alternatively, maximum atresia intensity may occur during the most energetically demanding phase of oocyte growth (Kurita et al. 2003), i.e., soon after the cessation of feeding and at the onset of spawning migration in case of Allis shad, and subsequently may decrease from the estuary to the spawning grounds. Providing that atresia intensity is accurately estimated, the results reported in this chapter suggest that realized annual fecundity can be estimated at any phase of the reproductive period. The universality of the latter conclusion for semelparous fish needs to be tested, since annual fecundity is among the key parameters in quantifying the reproductive potential and in estimating the stock-recruitment relationship (Kjesbu 2009; Witthames et al. 2009). Finally, comparisons between the fecundity values presented herein and those

estimated for other Allis shad populations (RF_t: 77 – 236 oocytes per g; Aprahamian et al. 2003b; Baglinière et al. 2003; Mota 2014) should be made with caution, since the implemented methodology and/or the developmental stages of the oocytes enumerated to make these estimations were not clearly provided in many cases (Aprahamian et al. 2003b).

Finally, the lack of actively spawning females prevented the undoubted description of the oocyte release strategy. However, the co-occurrence of SG oocytes at several distinct developmental stages in all ovaries indicated display of multiple spawning; supposition supported by Mota (2014) who reported that Allis shad in the Minho River is a multiple spawner based on the presence of POFs in the ovaries of partially spawned females.

To summarize, this chapter unveiled a dynamic SG recruitment pattern and lack of replenishment from oogonia during the pre-spawning phase of the reproductive period in anadromous semelparous Allis shad. These results highlight the utility of the early phases of ovarian dynamics in applied fisheries reproductive biology. Anadromy offers the opportunity to sample at distinct sites during the spawning migration (e.g., at river mouth, along the river, at the spawning grounds) and to test for differences in ovarian dynamics. Furthermore, semelparity ensures homogeneity in ovarian development and provides the opportunity for hypothesis testing regarding the ovarian dynamics. The comprehensive analysis of both PG and SG oocyte growth dynamics of Allis shad will enhance the effectiveness of management efforts for this critically endangered and economically important species throughout its geographical distribution. Additional studies are strongly encouraged at the spawning grounds to estimate the atresia intensity just prior the onset of spawning that would lead to accurate estimates of realized annual fecundity, and to reveal aspects of the reproductive biology of the species that remain unknown, such as the timing of SG recruitment completion and the oocyte release strategy.

CHAPTER 4: SG recruitment and SG phase of oogenesis during the spawning season of an iteroparous anadromous fish

The results presented in this chapter have been published as:

4.1 Specific objectives

The main objective of this chapter was to analyze the ovarian dynamics of the iteroparous (even with low, 15 – 30%, proportions of repeat female spawners; Davis et al. 2009) anadromous Blueback herring in the Connecticut River, particularly with respect to SG recruitment and fecundity type. Initially, spawning cyclicity was analyzed by linking oocyte growth to the degeneration of POFs. Females were accordingly classified as pre-spawners, early and late active spawners, and oocyte recruitment intensity was compared among the different spawning phases offering new insight into the species ovarian dynamics. The results reported in this chapter will contribute to the current trend of reassessing the fecundity type of anadromous alosines and will help improve future estimations of the species' reproductive potential.

4.2 Specific methodology

Specimens were collected as part of a project focused on adult Blueback herring as prey during the spawning season (Davis et al. 2012). To capture female fish on the spawning grounds over the duration of the season, sampling was conducted weekly via electrofishing (Smith-Root Model SR-18 electrofishing boat) from late April to late June 2006 from four sampling sites along the Connecticut River in northern Connecticut, US (Fig. 4.1; Table 4.1). Sampling sites were located at a significant distance from the river mouth (approximately 60 to 85 km). Surface water temperature was measured on each sampling date. To ensure a broad representation of size-classes, up to 5 fish per 5 mm size-class were selected, euthanized, placed on ice and processed within 24 hours. Sampling was conducted under the Connecticut State Scientific Collecting Permit SC-05012 and under University of Connecticut Institutional Animal Care and Use Council Protocol A05-013.

In total, 165 ovaries were processed histologically (embedding medium: paraffin, pigment: hematoxylin and eosin) and their sections were digitalized to photomicrographs via a NanoZoomer S60 slide scanner. The latter yielded detailed

information and enabled estimations for each female, including: (1) presence or absence of PG oocytes, (2) developmental stages of SG oocytes, (3) presence or absence of POFs, (4) estimation of POF_{XSA}, (5) occurrence of atresia, (6) ovarian developmental stage, and (7) estimation of relative fecundity values, including RF_b, relative fecundity of the second-most advanced oocyte developmental stage (RF_{Sb1}), and relative fecundity of the CA oocytes (RF_{CA}).

Figure 4.1. Sampling sites for *Alosa aestivalis* along the Connecticut River, Connecticut, US
Εικόνα 4.1. Περιοχές δειγματοληψίας για το είδος *Alosa aestivalis* κατά μήκος του Ποταμού Connecticut, στην Πολιτεία Connecticut των ΗΠΑ

POFs were used to test whether females were multiple or total spawners and to classify females into spawning phases: we identified pre-spawners (those that had not commenced their spawning activity), active spawners (spawning capable individuals that had spawned at least once during the surveyed season) and spent females (those that had completed their spawning activity). As indicated by Hunter and Macewicz (1985a), pre-spawners had vitellogenic oocytes and no POFs, active spawners had both vitellogenic oocytes and POFs, and spent females had POFs but no vitellogenic oocytes. To ensure that POF degeneration was sufficiently slow permitting accurate distinction between pre-spawners and active spawners, POF persistence or disappearance between two ovulation/spawning events (i.e., ovulatory cycle) was assessed. For this assessment, the persistence of POFs was tested with respect to temperature, which is expected to influence their degeneration rate (Fitzhugh and Hettler 1995; Ganias et al. 2007). Linear regression analysis was performed between water temperature and POF_{XSA} in each ovary, per ovarian developmental stage. Also, the relationship between POF_{XSA} and the mean oocyte diameter of the most-advanced oocytes (OD_{AM}; see next paragraph) was fitted to an exponential function.

In parallel, 91 whole-mount photos of ovarian subsamples were captured using a Jenoptik Progress C3 camera fitted on a Euromex NZ 80 stereo microscope. Particle analysis on these photos enabled for each female: (1) estimation of ODs at both PG

and SG phase, (2) creation of an OSFD, (3) definition of the AM and of the SM1 in the OSFD, and (4) estimation of relative fecundity values, including RF_t, RF_{AM}, RF_{SM1} and relative fecundity of oocytes having diameters between 200 and 320 μm ($RF_{200-320}$). Because PG oocytes are translucent and closely attached to each other (Anderson et al. 2020) the whole-mount procedure that included oocytes smaller than 200μm needed some extra steps and was thus held in 10 females. The size threshold between PG and SG oocytes was estimated statistically as the mean cutpoint value between the mode of very small oocytes and the SM in these 10 females.

Table 4.1. Number (n) of female *Alosa aestivalis* analyzed per sampling date, sampling site and method. WF = Wethersfield, FR = lower Farmington River, WL = Windsor Locks, EF = Enfield. The percentage of fishes per spawning phase is also shown (pre-spawners, active spawners, spent females)

Πίνακας 4.1. Αριθμός (n) θηλυκών ατόμων του είδους *Alosa aestivalis* που αναλύθηκαν ανά μέθοδο, ημερομηνία και περιοχή δειγματοληψίας. WF = Wethersfield, FR = lower Farmington River, WL = Windsor Locks, EF = Enfield. Παρουσιάζεται επίσης το ποσοστό ατόμων ανά φάση ωοτοκίας (πριν την έναρξη της περιόδου ωοτοκίας-pre-spawners, μετά την έναρξη της περιόδου ωοτοκίας-active spawners, μετά την ολοκλήρωση της περιόδου ωοτοκίας-spent)

Date	Site	Pre-spawners (%)	Active spawners (%)	Spent (%)	High atresia (%)	Histological analysis (n)	Whole-mount analysis (n)
27/4	FR	100				1	
28/4	WF	57.1	42.9			7	7
1/5	FR	25	75			4	4
2/5	WL	100				2	2
7/5	WF	11.1	88.9			17	12
8/5	FR	15.4	84.6			13	8
9/5	WL	43.8	56.2			16	9
10/5	EF	16.7	83.3			6	2
14/5	WF	38.5	61.5			13	4
24/5	EF	50	50			2	2
28/5	WF	20.7	75.9	3.4		29	13
29/5	FR	12.5	81.3		6.2	16	10
30/5	WL		100			5	4
31/5	EF		100			3	1
4/6	WF	33.3	66.7			6	1
5/6	FR	36.4	54.5	9.1		11	5
19/6	FR		100			2	1
20/6	WL		100			7	3
21/6	EF		100			1	1
27/6	WL	66.7	33.3			3	2
Total						**165**	**91**

To define fecundity type, variation of RF_t between pre-spawning and actively spawning females was tested; typically, constant RF_t indicates indeterminate fecundity, while decreasing values indicate the determinate fecundity type (see Box 2). Ongoing SG recruitment in actively spawning females was also tested. Specifically, oocytes at CA stages in the histological sections were interpreted as being newly recruited SG oocytes. Additionally, any oocytes with diameters between 200 and 320 μm in the whole-mount subsamples were regarded as an indication of recent SG recruitment. The latter approach was based on Greer-Walker et al. (1994).

SG recruitment intensity was estimated both through enumerating (a) the very early SG oocytes (i.e. CA stage) in histological specimens, and (b) oocytes with diameters between 200 and 320 μm in whole mounts, based on Greer-Walker et al. (1994). In particular, the relative fecundity of CA oocytes (RF_{CA}) was estimated through the Weibel method (Weibel et al. 1966), implemented on the histological photomicrographs, following the methodology of Emerson et al. (1990). The relative fecundity of oocytes between 200 and 320 μm ($RF_{200-320}$) was estimated gravimetrically from whole-mount analysis. To test whether SG recruitment intensity remained constant throughout spawning activity, the values of each of these two indices were compared among the different spawning phases.

4.3 Results

Each ovary contained co-existing oocytes in several SG developmental stages (Fig. 4.2). In specific, the stages identified were: CA-1, CA-2 and CA-3 (primary, secondary and final cortical alveolar stages), VIT-1, VIT-2 and VIT-3 (primary, secondary and tertiary vitellogenic stages), GVM-1 and GVM-2 (early and late germinal vesicle migration stages) and GVBD (germinal vesicle breakdown stage). Imminent spawners (i.e., at GVBD stage) were caught on all sampling sites, indicating that the entire region comprised a spawning ground.
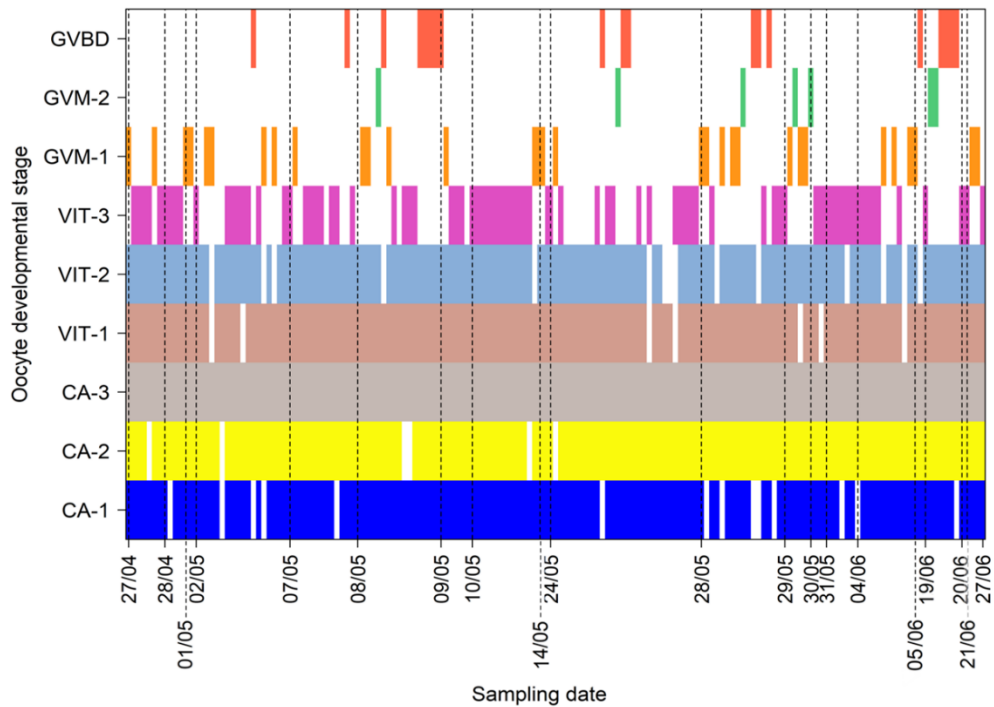
Figure 4.2. Oocyte developmental stages by female and sampling date for *Alosa aestivalis*. Each of 164 females is represented as a column; oocyte stages are indicated with filled rectangles of different colors. The sampling date of each female is indicated by the dashed line to its left. The single female identified with high levels of atresia is not presented

Εικόνα 4.2. Στάδια ανάπτυξης ωοκυττάρων για κάθε ένα από τα 164 θηλυκά άτομα του είδους *Alosa aestivalis* που αναλύθηκαν. Κάθε άτομο παρουσιάζεται ως μία στήλη και τα στάδια ανάπτυξης των ωοκυττάρων του ως παραλληλόγραμμα διαφόρων χρωμάτων. Η ημερομηνία δειγματοληψίας κάθε ατόμου σημειώνεται από την διακεκομμένη γραμμή στα αριστερά αυτού. Το άτομο που εντοπίστηκε με υψηλό ποσοστό ατρησίας δεν εμφανίζεται

The presence of POFs in imminent spawners and the co-occurrence of two POF cohorts (i.e., POF stages originated from different sequential daily spawning events) in several ovaries (Fig. 4.3) evinced multiple spawning. Most of these latter ovaries were at the VIT-2 ovarian developmental stage (Fig. 4.3).
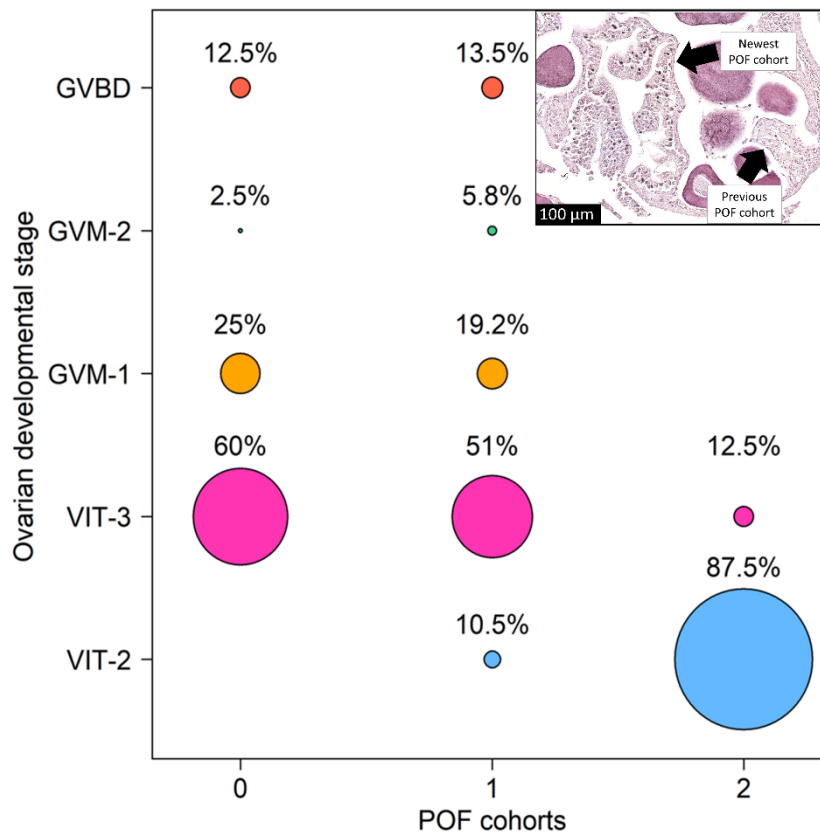
Figure 4.3. Percentage of *Alosa aestivalis* females in different stages of ovarian development versus number of postovulatory follicle (POF) cohorts, i.e., POF stages originated from sequential daily spawning events (none, one or two). The upper right panel shows co-occurring POFs from two cohorts

Εικόνα 4.3. Ποσοστό θηλυκών ατόμων του είδους *Alosa aestivalis* ανά στάδιο ανάπτυξης της ωοθήκης και ανά αριθμό κοορτών κενών ωοθυλακίων (POF cohorts), δηλαδή ανά αριθμό διαφορετικών ομάδων POF (καμία, μία, δύο) που κάθε μία προέρχεται από διαφορετικό συμβάν ωοτοκίας. Στην επάνω δεξιά γωνία απεικονίζονται δύο POF από δύο διαφορετικές κοόρτες

The co-existence of two POF cohorts in several ovaries on different sampling dates (Fig. 4.4a) showed that POF absorption lasted longer than a complete ovulatory cycle. This suggests that each active spawner would have POFs in its ovary from at least one cohort regardless of its ovarian developmental stage, and that the presence of POFs distinguishes active spawners from pre-spawners. Further evidence for this supposition is that the rate of POF degeneration was essentially invariant with respect to water temperature and throughout the sampling period. As the season progressed and temperature increased, the prevalence of POFs did not decline (Fig. 4.4a) and there was no relationship between POF_{XSA} values and temperature in any ovarian developmental stage (in all linear regressions: $P > 0.05$; Fig. 4.4b). The constant POF degeneration rate was further supported by the strong negative relationship that was found between POF_{XSA} values and the mean OD_{AM} ($P < 0.001$; Fig. 4.5).
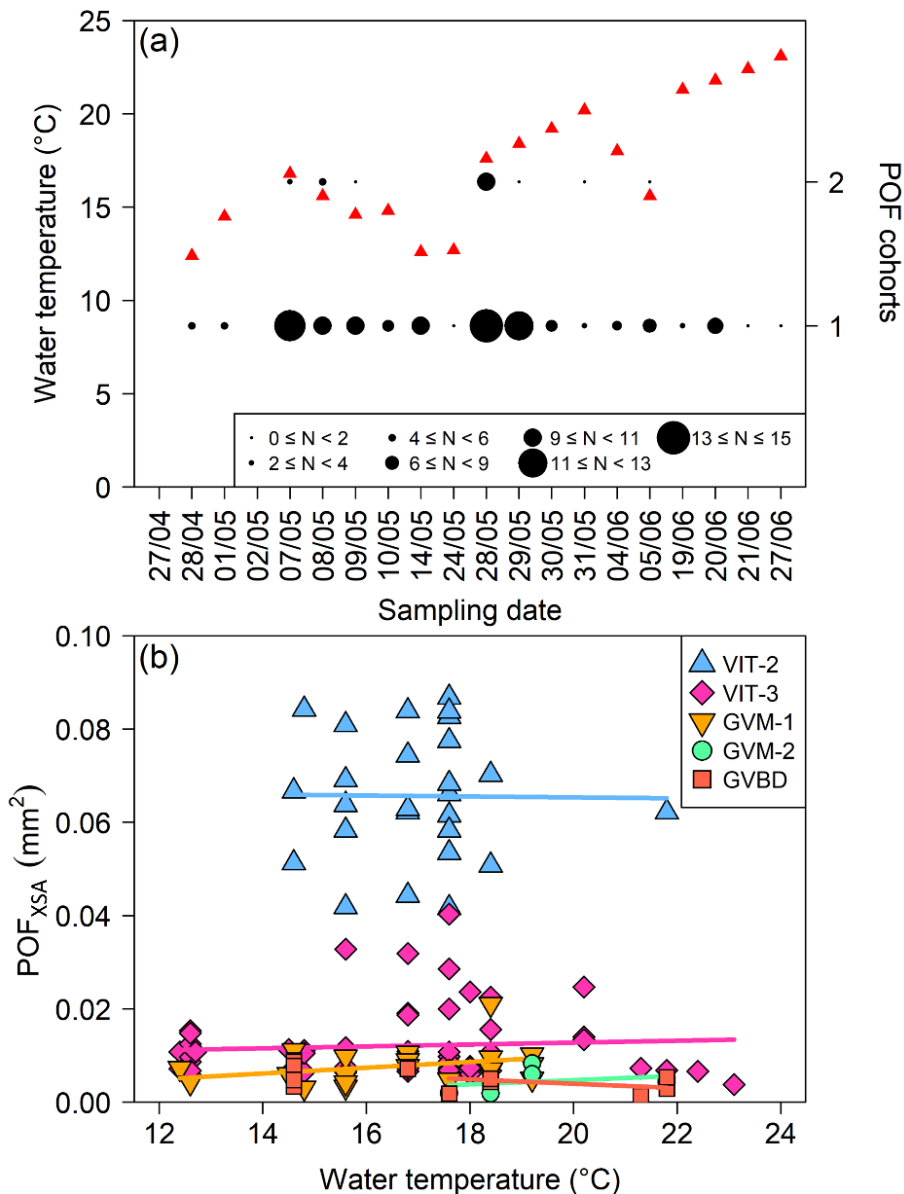
Figure 4.4. (a) Frequency (dots) of postovulatory follicle (POF) cohorts in *Alosa aestivalis* ovaries by sampling date. Surface water temperature (triangles) per date is also shown. Sampling date is plotted on an ordinal scale. (b) Distribution of the cross-sectional area of the biggest postovulatory follicle (POF$_{XSA}$) in each active spawner versus surface water temperature per ovarian developmental stage and the fitted linear regression lines. Colors and shapes represent the ovarian developmental stages

Εικόνα 4.4. (a) Πλήθος (μαύροι κύκλοι) κοορτών κενών ωοθυλακίων (POF cohorts) στις ωοθήκες ατόμων του είδους *Alosa aestivalis* ανά ημερομηνία δειγματοληψίας. Η επιφανειακή θερμοκρασία νερού σημειώνεται επίσης (τρίγωνα). (b) Κατανομή των τιμών εμβαδού του μεγαλύτερου κενού ωοθυλακίου (POF$_{XSA}$) στην ωοθήκη κάθε ατόμου ανά επιφανειακή θερμοκρασία νερού και στάδιο ανάπτυξης της ωοθήκης. Οι γραμμές τάσεις εμφανίζονται επίσης. Τα διαφορετικά χρώματα και σχήματα αντιστοιχούν στα στάδια ανάπτυξης της ωοθήκης

Three females were not pre-spawners or active spawners and were excluded from any further statistical analyses. Two of these females were identified as spent (Fig. 4.6). The third female displayed high levels of atresia but had vitellogenic oocytes; although it was not classified for this reason as spent, fecundity estimations were not possible.
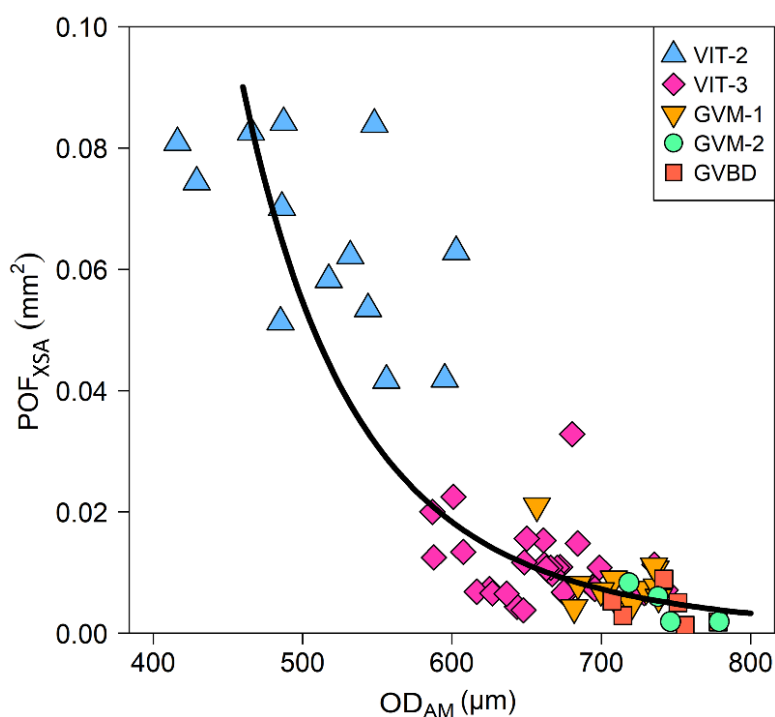
Figure 4.5. Relation between the cross-sectional area of the biggest postovulatory follicle (POF$_{XSA}$) and the mean oocyte diameter of the most advanced developmental stage (OD$_{AM}$) in active *Alosa aestivalis* spawners, by ovarian developmental stage, and the fitted exponential regression line. Colors and shapes represent the ovarian developmental stages

Εικόνα 4.5. Συσχέτιση μεταξύ του εμβαδού του μεγαλύτερου κενού ωοθυλακίου (POF$_{XSA}$) στην ωοθήκη κάθε ατόμου του είδους *Alosa aestivalis* και της μέσης διαμέτρου των ωοκυττάρων στο πιο ανεπτυγμένο στάδιο (OD$_{AM}$) ανά στάδιο ανάπτυξης της ωοθήκης. Η γραμμή τάσης εμφανίζεται επίσης. Τα διαφορετικά χρώματα και σχήματα αντιστοιχούν στα στάδια ανάπτυξης της ωοθήκης
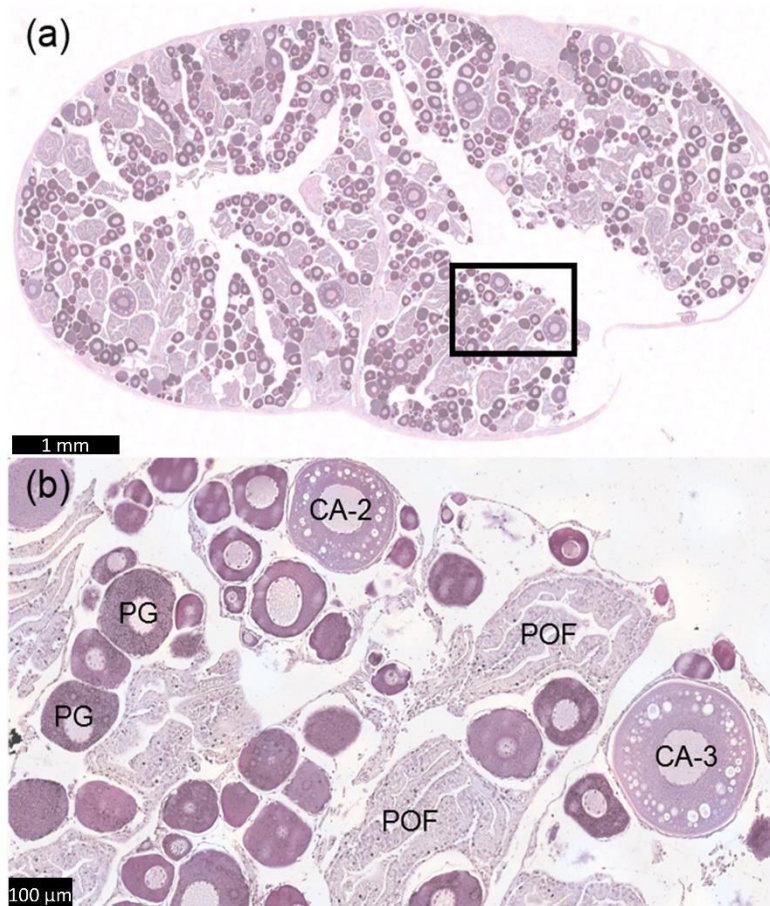
Figure 4.6. (a) Photomicrograph of the ovarian histological section of a spent *Alosa aestivalis*. Rectangle indicates the magnified area in (b). CA-2 = secondary cortical alveolar stage, CA-3 = final cortical alveolar stage, PG = oocytes at the primary growth phase, POF = postovulatory follicle

Εικόνα 4.6. (a) Φωτομικρογραφία ιστολογικής τομής ωοθήκης ατόμου του είδους *Alosa aestivalis* που έχει ολοκληρώσει την περίοδο ωοτοκίας του. Το παραλληλόγραμμο υποδεικνύει τη θέση της μεγεθυμένης εικόνας (b). CA-2 = ωοκύτταρο στο δεύτερο στάδιο κυστιδίων, CA-3 = ωοκύτταρο στο τελικό στάδιο κυστιδίων, PG = ωοκύτταρο στην πρωτογενή φάση ανάπτυξης, POF = κενό ωοθυλάκιο

The interpretation of whole-mount and histological analyses were in alignment, validating the estimates of fecundity and stage-specific oocyte size. The AM was clearly discernible in the OSFDs of females from the VIT-3 stage onwards (Fig. 4.7). The relative fecundity of the AM was considered equivalent to relative batch fecundity (RF_b). The subsequent mode (SM1) representing the oocyte batch of a second upcoming spawning episode was also discernible in >VIT-3 stage females (e.g., Fig. 4.8). However, in most cases the SM1 could not be resolved from smaller oocytes and its relative fecundity (RF_{SM1}) could only be estimated by statistically decomposing overlapping modes in the OSFD (see Ganias et al. 2010). The OSFDs of females at the VIT-2 ovarian stage were unimodal (Fig. 4.7) and we did not estimate RF_b and RF_{SM1} for these females.
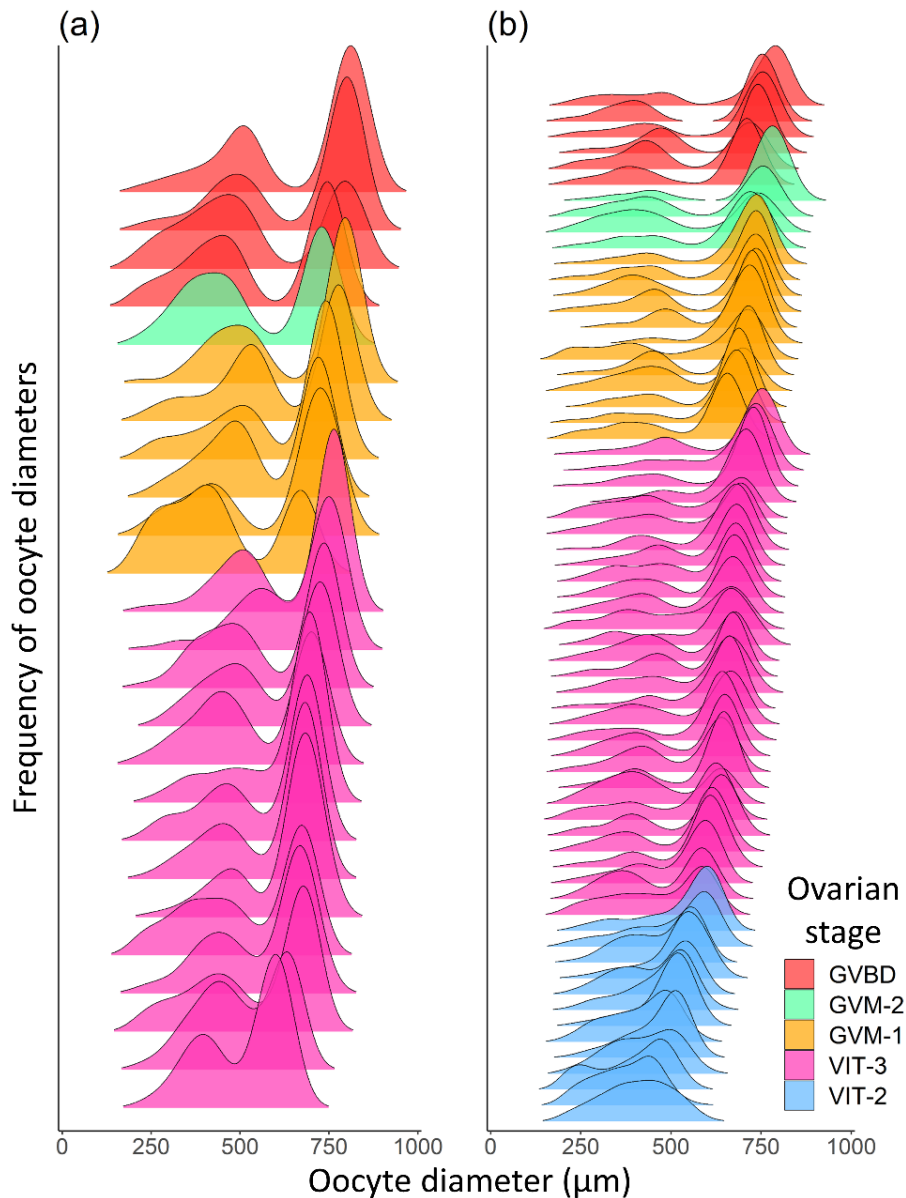
Figure 4.7. Oocyte size frequency distributions of secondary growth oocytes resulting from whole-mount analysis on *Alosa aestivalis* for: (a) pre-spawners and (b) active spawners. Distributions for each ovarian developmental stage are displayed in order of increasing (bottom to top) mean oocyte diameter of the secondary growth oocytes. Colors represent the ovarian developmental stage

Εικόνα 4.7. Κατανομές συχνοτήτων μεγεθών των ωοκυττάρων που βρίσκονται στη δευτερογενή φάση ανάπτυξης ατόμων του είδους *Alosa aestivalis.* (a) Άτομα των οποίων η περίοδος ωοτοκίας δεν έχει εκκινήσει, και (b) άτομα που έχουν εκκινήσει να ωοτοκούν. Οι κατανομές για κάθε μία από τις φάσεις ανάπτυξης της ωοθήκης εμφανίζονται με αυξανόμενη (από κάτω προς τα επάνω) μέση διάμετρο ωοκυττάρων που βρίσκονται στη δευτερογενή φάση ανάπτυξης. Τα διαφορετικά χρώματα αντιστοιχούν στις φάσεις ανάπτυξης της ωοθήκης
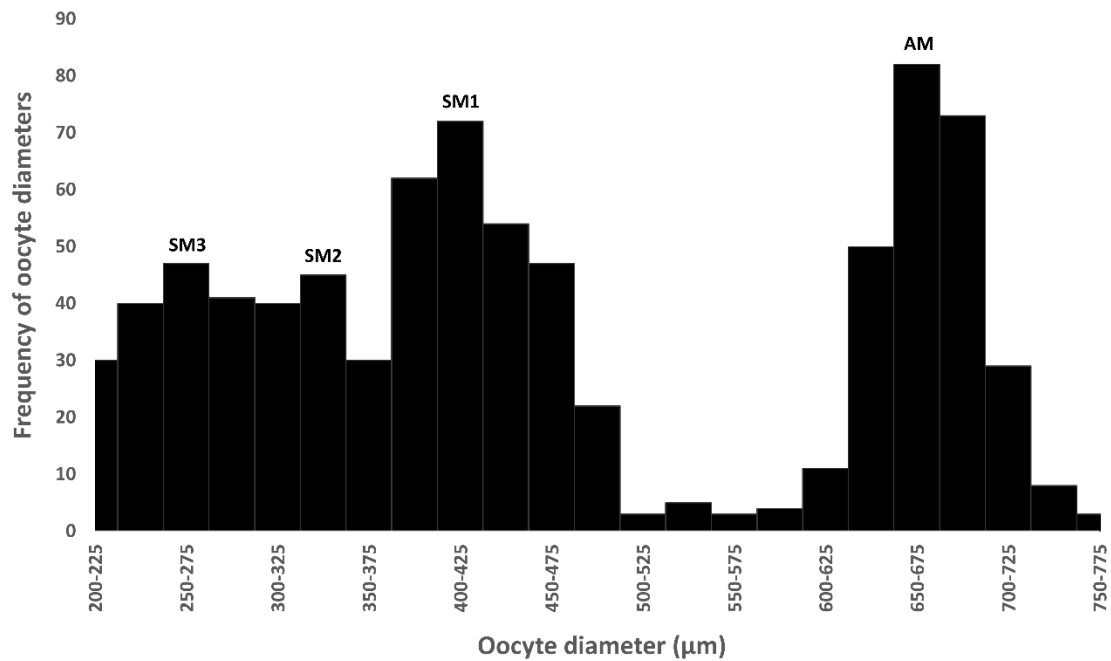
Figure 4.8. Oocyte size frequency distribution of a single *Alosa aestivalis* female with its ovary in VIT-3 developmental stage. Oocytes are divided into fine (25 μm) diameter classes to resolve modes. The advanced mode (AM) and the first subsequent mode (SM1) are distinguishable. The remaining modes (SM2, SM3) are overlapping

Εικόνα 4.8. Κατανομή συχνοτήτων μεγεθών των ωοκυττάρων ενός ατόμου του είδους *Alosa aestivalis* η ωοθήκη του οποίου είναι στο VIT-3 στάδιο ανάπτυξης. Τα ωοκύτταρα διαχωρίζονται ανά βήμα κλάσης μεγέθους 25 μm. Η πιο προηγμένη ομάδα (AM) και η δεύτερη πιο προηγμένη ομάδα (SM1) της κατανομής διαχωρίζονται. Οι υπόλοιπες ομάδες (SM2, SM3) αλληλεπικαλύπτονται

The OSFDs of the ten females used to estimate the size threshold between PG and SG oocytes showed a clear mode between 4 to 200 μm which corresponded to PG oocytes (Fig. 4.9). The mean cutpoint value between this mode and the SM was 197.3 μm (95% C.I.: ±40.9μm). This value was very close to the 200 μm threshold value previously used for other alosines such as the American shad (Hyle et al. 2014). Thus, for generality purposes we also used the 200 μm threshold for distinguishing between PG and SG oocytes.
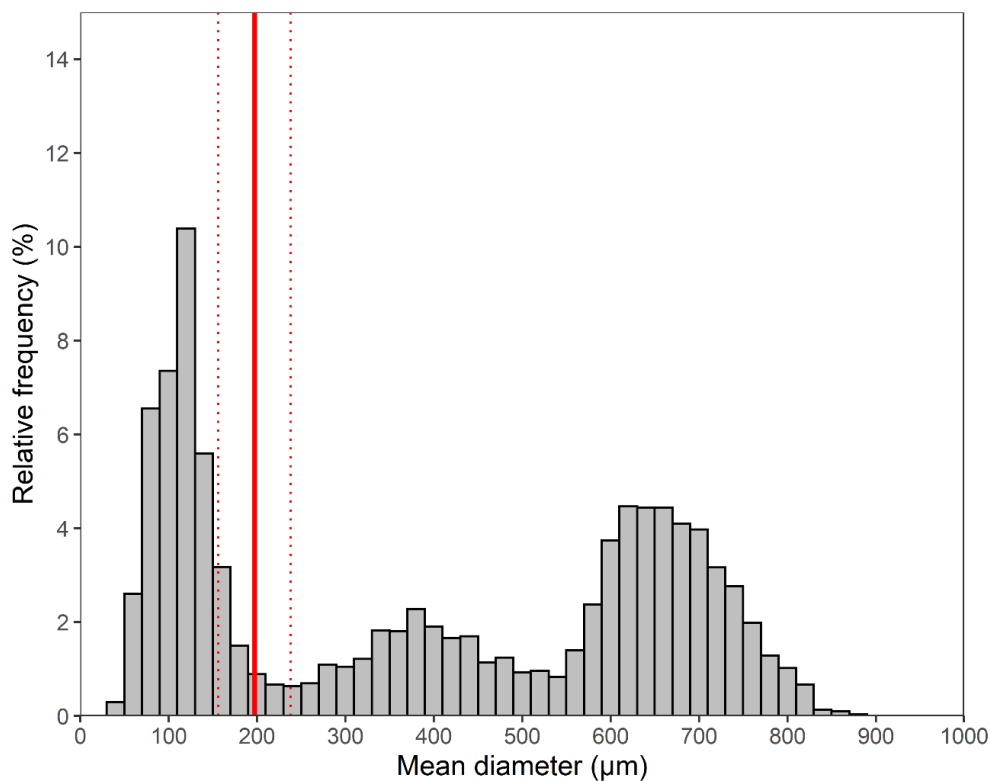
Figure 4.9. Combined oocyte size frequency distribution of all oocytes from the ten females that were used to estimate the mean size threshold value (solid red line) between primary growth and secondary growth oocytes. Dotted lines correspond to 95% confidence intervals

Εικόνα 4.9. Κατανομή συχνοτήτων μεγεθών όλων των ωοκυττάρων των δέκα ατόμων που χρησιμοποιήθηκαν για την εκτίμηση του μεγέθους διαχωρισμού (κόκκινη, συνεχής γραμμή) μεταξύ των ωοκυττάρων πρωτογενούς φάσης και των ωοκυττάρων δευτερογενούς φάσης ανάπτυξης. Οι διακεκομμένες γραμμές αντιστοιχούν στα 95% διαστήματα εμπιστοσύνης

The ovaries of all active spawners had newly recruited SG oocytes, indicative of an indeterminate fecundity type. Oocytes at different CA developmental stages were detected in all photomicrographs (cf. Fig. 4.2). In parallel, small-sized SG oocytes were found in the OSFDs of all actively spawning females (cf. Fig. 4.7b).

Batches diminished in size in subsequent spawnings and SG recruitment tapered as the season progressed. Active spawners had significantly ($P < 0.05$; two tailed t-test) lower mean RF_t (469.4 oocytes g^{-1} of W_{ev}) and RF_b (303.1 oocytes g^{-1}) than pre-spawners (RF_t = 917.2 g^{-1}; RF_b = 519.6 oocytes g^{-1}) (Fig. 4.10). The RF_{SM1} of imminent pre-spawners was intermediate (mean 396 oocytes g^{-1}). We accordingly classified active spawners as *early*, i.e. those that had only spawned once during the current season, and *late*, i.e. those that had spawned at least twice. Active spawners with RF_{AM} > 396 oocytes g^{-1} were classified as early spawners; all females with two POF cohorts or RF_{AM} < 396 oocytes g^{-1} were classified as late spawners.
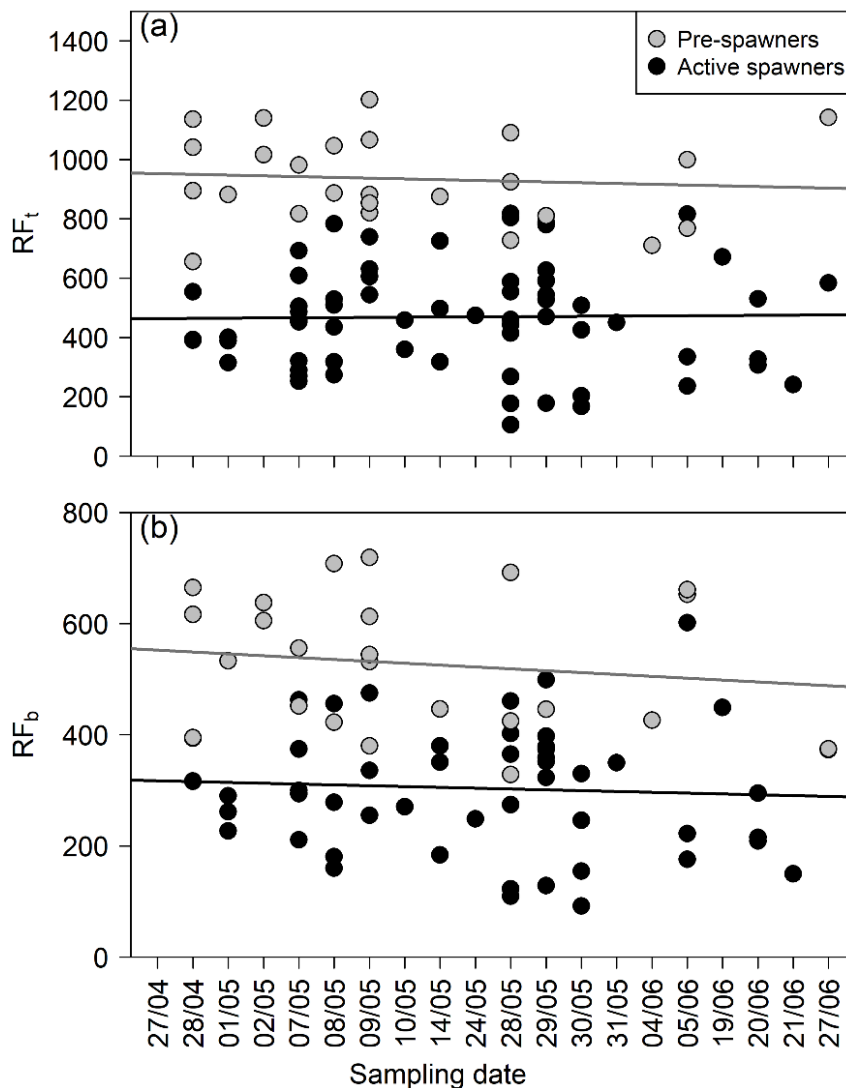
Figure 4.10. Temporal distribution of: (a) relative total fecundity (RF_t), and (b) relative batch fecundity (RF_b), in *Alosa aestivalis* pre-spawners and active spawners. Sampling date is plotted on an ordinal scale. The regression lines originated from the linear regression analyses where sampling date was used as a continuous variable

Εικόνα 4.10. Κατανομή της: (a) σχετικής ολικής γονιμότητας (RF_t), και (b) σχετικής γονιμότητας ομάδας (RF_b), ανά ημερομηνία δειγματοληψίας για άτομα του είδους *Alosa aestivalis* των οποίων η περίοδος ωοτοκίας δεν έχει εκκινήσει (pre-spawners) και άτομα που έχουν εκκινήσει να ωοτοκούν (active spawners)

Late spawners exhibited significantly lower $RF_{200-320}$ values than pre-spawners and early active spawners (P < 0.05; least significant difference, 95% confidence level; Fig. 4.11a). Similarly, late spawners displayed significantly lower RF_{CA} values than pre-spawners and early active spawners (P < 0.001; least significant difference, 95% confidence level; Fig. 4.11b).
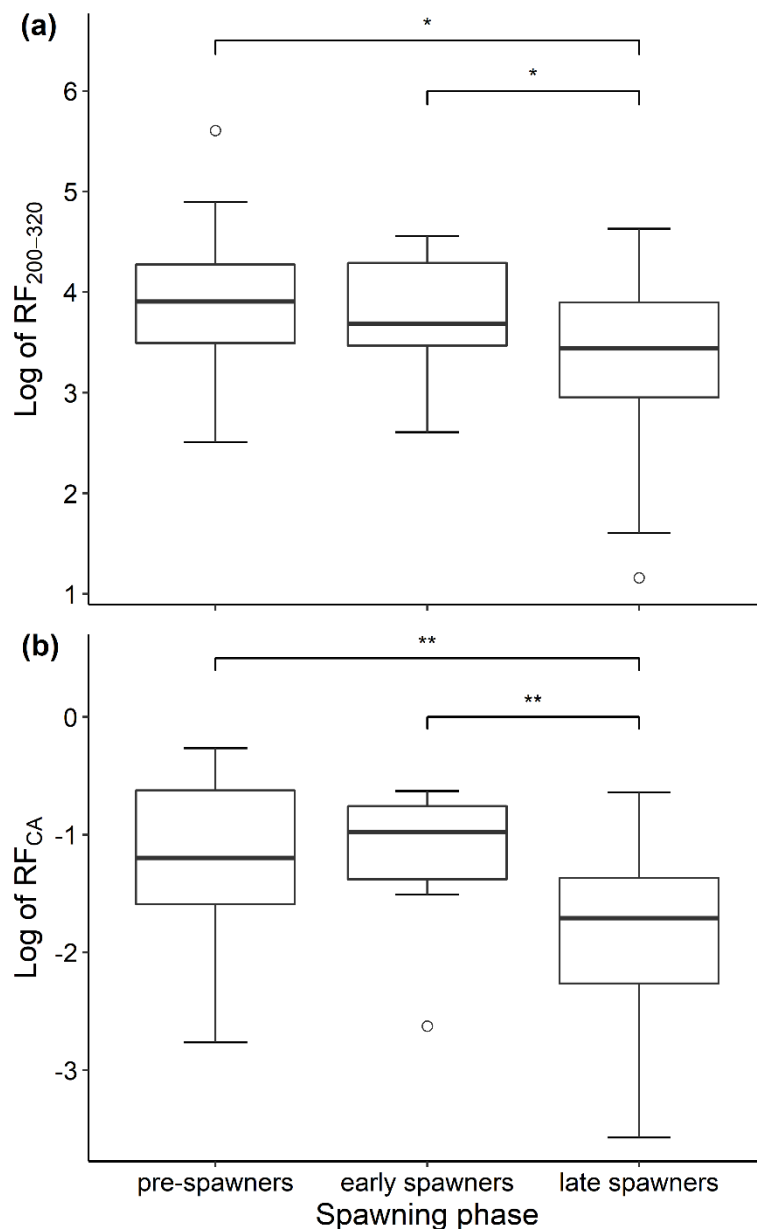
Figure 4.11. Box and whisker plots for *Alosa aestivalis* log transformed values of: (a) relative fecundity of the oocytes right above the size threshold differentiating primary and secondary growth oocytes ($RF_{200-320}$), and (b) relative fecundity of the cortical alveolar oocytes (RF_{CA}), by spawning phase. Statistically significant pairwise comparisons of mean values are shown with asterisks ("*" for $P < 0.05$, "**" for $P < 0.001$)

Εικόνα 4.11. Θηκόγραμμα των λογαριθμημένων τιμών της: (a) σχετικής γονιμότητας των ωοκυττάρων με διάμετρο μεταξύ 200 και 320 μm ($RF_{200-320}$), και (b) σχετικής γονιμότητας των ωοκυττάρων που βρίσκονται στα στάδια των κυστιδίων (RF_{CA}), ανά φάση ωοτοκίας των ατόμων του είδους *Alosa aestivalis* (άτομα που δεν έχουν ωοτοκήσει την τρέχουσα περίοδο-pre-spawners, άτομα που έχουν ωοτοκήσει μία φορά την τρέχουσα περίοδο-early spawners, άτομα που έχουν ωοτοκήσει τουλάχιστον δύο φορές την τρέχουσα περίοδο-late spawners). Οι στατιστικά σημαντικές διαφορές μεταξύ των μέσων τιμών σημειώνονται με αστερίσκο ("*" για $P < 0.05$, "**" για $P < 0.001$)

4.4 Discussion

The results reported in this chapter detail the ovarian dynamics of anadromous Blueback herring in Connecticut River and provide new insights into fish ovarian dynamics during the spawning season. Oocytes were spawned in multiple events and females displayed indeterminate fecundity type, given that SG recruitment occurred continuously and in parallel with spawning activity. However, SG recruitment intensity tapered as spawning progressed, and the paucity of atretic oocytes in spent females suggests that virtually all developed vitellogenic oocytes were spawned.

Display of multiple spawning by the surveyed population agrees with a previous report for the species. Specifically, McBride et al. (2010) identified Blueback herring in the St. Johns River in Florida, i.e., near the most-southern limit of the species distribution, as a multiple spawner. Multiple spawning of Blueback herring in the Connecticut River was indicated by the presence of POFs in imminent spawners and the co-occurrence of two different POF cohorts in several females. The production and the degeneration of POFs was in phase with the spawning cycle: very recent spawners with new and large POFs were at the beginning of vitellogenesis; then, POFs gradually shrunk as the ovary developed and they finally reached a very small size just before the next spawning episode. This pattern explained the co-existence of POFs from two different cohorts, especially in females at early vitellogenesis, i.e. the beginning of the new spawning cycle.

On the contrary, the fecundity type has never been defined for any population of the species. Thus, additional studies, covering the whole geographical range of Blueback herring, should be conducted in the future to test if the indeterminate fecundity is the predominant type for the species or not. Blueback herring in the Connecticut River displayed indeterminate fecundity. Oocyte recruitment continued to the end of the spawning period as indicated by the presence of early SG oocytes both in late active spawners and spent females. Indeterminate fecundity was interpreted as a strategy conferring flexibility of energy allocation in the face of migration costs; in contrast to determinate fecundity which designates the full complement of oocytes in advance, the indeterminate strategy commits energy to reproduction as spawning continues to be possible. Given that multiple spawning and indeterminate fecundity require an ongoing energetic investment into oocyte production (Lowerre-Barbieri et al. 1998; McBride et al. 2013), this study examined whether and how Blueback herring adjusts its fecundity pattern and SG recruitment strategy to counterbalance the costs of its spawning run.

In most indeterminate spawners, oocytes from each spawning are replenished by newly recruited SG oocytes in dynamic equilibrium (Kjesbu 2009). As a result, continuous SG recruitment leads to a surplus of SG oocytes that will not be spawned at the end of the individual spawning period; these oocytes fall into massive atresia

(Hunter and Macewicz 1985a; Greer-Walker et al. 1994; Ganias et al. 2014) a process known as mopping-up (Wallace et al. 1981; Kjesbu 2009). Mopping-up will only recover a portion of the energy that was invested in oocytes, representing a potentially expensive inefficiency. Some species, such as the Atlantic horse mackerel (Ganias et al. 2017) and the Gulf menhaden (Brown-Peterson et al. 2017) modulate or avoid mopping-up through a cessation of SG recruitment well before the end of spawning. In contrast, Blueback herring in the Connecticut River, even though they continue to recruit SG oocytes throughout the spawning period, avoid massive atresia by reducing the intensity of SG recruitment as spawning progresses.

By tapering SG recruitment, Blueback herring evidently maximize the efficiency of continued energetic investment in spawning through the season. Tapering in SG recruitment reduces batch fecundity in successive rounds of spawning; the advanced mode always had more oocytes than the subsequent batch(es), both in pre-spawners and in active spawners. Consequently, as batch fecundity decreased from one spawning event to the next, the total number of SG oocytes (i.e. total fecundity) also decreased, and finally, spent females showed no vitellogenic oocytes, avoided massive atresia, and only contained primary growth oocytes and a few CA oocytes. Ovaries of spent female Blueback herring collected from the same location in May 2018 (author's unpublished data) similarly lacked massive atresia. All females, including the spent ones, contained primary growth oocytes in their ovaries, organized in distinct batches (Fig. 4.12), indicating the potential to migrate and reproduce in the next spawning season(s). Despite this potential, repeat spawners in Connecticut River female Blueback herring are not common (15 – 30%: Davis et al. 2009), indicating low adult survivorship and potentially poor population resilience.

Tapering in SG recruitment and fecundity modulates energy allocated to oocyte production, perhaps to better meet the high energetic demands of the riverine phase of its reproductive cycle, which involves upstream migration, spawning and potentially prolonged residence on the spawning ground or repeated rounds of migratory return to the spawning ground. Other populations of Blueback herring (Simonin et al. 2007; McBride et al. 2010) incur substantial energetic losses during long upstream migrations up to ~400 km. The impact of such long migrations on energy available for reproduction has not yet been assessed. Given the significantly shorter upstream migration distance for the Blueback herring in Connecticut River and thus, the smaller energetic cost of its spawning run it can be predicted that other populations should deploy similar strategy of SG recruitment and fecundity regulation.

In conclusion, Blueback herring maximizes efficiency of investment in reproduction over the spawning season through tapering of SG recruitment, a pattern which has never been shown for a fish species. This study offers new insight into the ovarian dynamics of indeterminate spawners. A steady balance between oocyte recruitment and release, followed by mopping-up, was initially described for the Northern anchovy

Engraulis mordax (Hunter and Leong 1981) and has thereafter been adopted as a universal criterion of indeterminate fecundity in several fish reproduction studies and reviews (e.g. Murua and Saborido-Rey 2003; Armstrong and Witthames 2012). It is now clear that indeterminate spawners may deploy alternative strategies and avoid mopping-up, e.g. through ceasing SG recruitment at late-season spawners, or, as shown in this chapter, through tapering SG recruitment and fecundity until the ovary is depleted of its stock of vitellogenic oocytes.
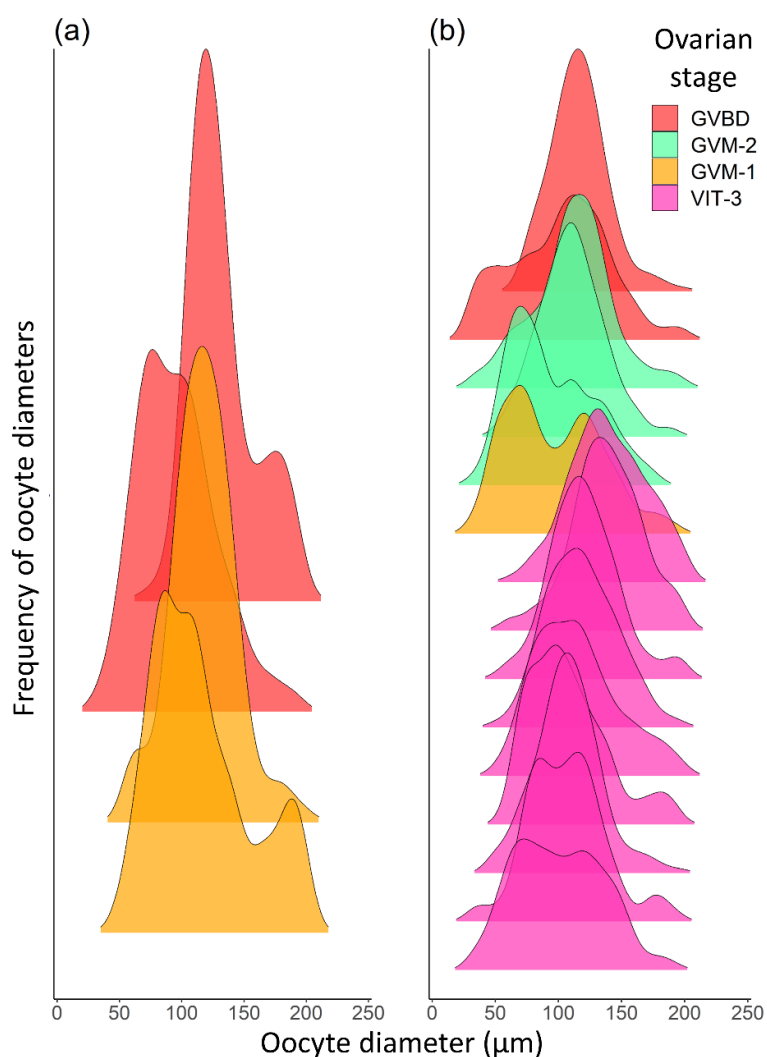
Figure 4.12. Oocyte size frequency distributions of primary growth oocytes resulting from whole-mount analysis on *Alosa aestivalis*: (a) pre-spawners, and (b) active spawners. Distributions for each ovarian developmental stage are displayed in order of increasing (bottom to top) mean oocyte diameter of the secondary growth oocytes. Colors represent the ovarian developmental stage

Εικόνα 4.12. Κατανομές συχνοτήτων μεγεθών των ωοκυττάρων που βρίσκονται στη πρωτογενή φάση ανάπτυξης ατόμων του είδους *Alosa aestivalis.* (a) Άτομα των οποίων η περίοδος ωοτοκίας δεν έχει εκκινήσει, και (b) άτομα που έχουν εκκινήσει να ωοτοκούν. Οι κατανομές για κάθε μία από τις φάσεις ανάπτυξης της ωοθήκης εμφανίζονται με αυξανόμενη (από κάτω προς τα επάνω) μέση διάμετρο ωοκυττάρων στη δευτερογενή φάση ανάπτυξης. Τα διαφορετικά χρώματα αντιστοιχούν στις φάσεις ανάπτυξης της ωοθήκης

CHAPTER 5: Influence of life-history form on SG recruitment, SG phase of oogenesis and aspects of the spawning season

Part of the samples used in this chapter had been collected, but not analyzed, during a previous project that led to the following publication:

Ganias K, Divino JN, Gherard KE, Davis JP, **Mouchlianitis F**, Schultz ET (2015). A reappraisal of reproduction in anadromous Alewives: Determinate versus indeterminate fecundity, batch size, and batch number. *Transactions of the American Fisheries Society* 144: 1143–1158

5.1 Specific objectives

The goal of this chapter was to test for intraspecies divergence in reproductive traits between anadromous and landlocked Alewife populations. Initially, fecundity type, oocyte release strategy and oocyte size were compared between two neighboring populations displaying different life-history form; the iteroparous (see 5.3 section), landlocked population in Pattagansett Lake (PAT) and the iteroparous anadromous population entering Bride Lake through Bride Brook to spawn (BB), in East Lyme, Connecticut, US. The ovarian dynamics of the former population has never been analyzed, while the latter has been suggested to display multiple spawning and indeterminate fecundity type (Ganias et al. 2015b). Additional BB samples were analyzed in this chapter to confirm indeterminacy following the methodology and the criteria implemented throughout the present study. Subsequently, aspects of the spawning season were compared among several anadromous and landlocked Alewife populations through a meta-analysis to detect patterns related to the life-history form. Differences were expected to be found, since landlocked populations are released from constrains associated with spawning migration.

5.2 Specific methodology

Female BB Alewives (n = 27) were sampled in late May and early July 2006, while they were leaving their spawning ground (i.e., Bride Lake) and commencing their downstream migration along Bride Brook (Fig. 5.1a). Upon sampling, fish were euthanized, placed on ice and processed within 24 hours.

Female PAT Alewives (n = 100) were fished from early June till early July 2018 at the deepest location of Pattagansett Lake (Fig. 5.1b). Sampling dates were selected based on previous estimations for the spawning season of the surveyed population and for neighboring landlocked Alewife populations (Littrell et al. 2018). Gill nets were set

from surface to approximately 12 m depth during late afternoon (~19:00 pm) and were retrieved before sunrise (~6:30 am) on the first sampling date, and at midnight (~00:30 am) for the next two sampling dates. Fishing time was decreased after the first sampling to lessen predation on Alewife; many fish were retrieved half-eaten probably by American eel, *Anguilla rostrata*, as has been reported in several Connecticut lakes (Anderson and Neumann 2002). Upon sampling, fish were placed on ice and processed within 12 hours. Samplings were conducted under the Collecting Permit SC-16022 issued by the CT Department of Energy and Environmental Protection and under the Institutional Animal Care and Use Protocol number is A18-021, issued by the University of Connecticut.
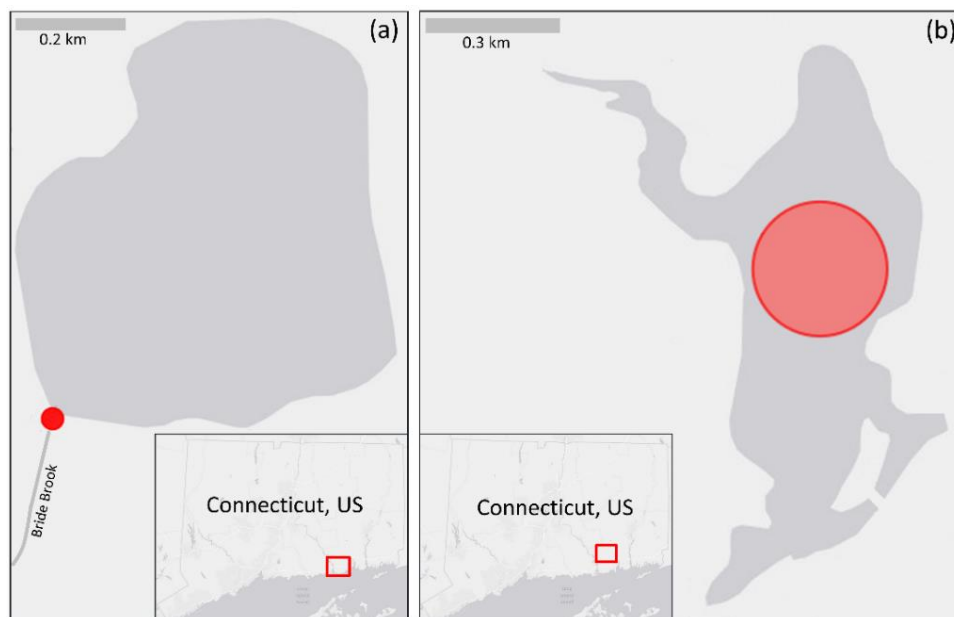
Figure 5.1. Sampling sites for *Alosa pseudoharengus* at: (a) Bride Lake, and (b) Pattagansett Lake, in East Lyme, Connecticut, US
Εικόνα 5.1. Περιοχές δειγματοληψίας για το είδος *Alosa pseudoharengus*: (a) Λίμνη Bride, και (b) Λίμνη Pattagansett, στην περιοχή East Lyme στην πολιτεία Connecticut των ΗΠΑ

Each PAT female was classified as mature or reproductively inactive (i.e., immature or spent), based on its ovarian morphology; fish with small, cordlike ovaries were considered inactive. *GSI* was also deployed in identifying the reproductive condition; values lower than 2 were considered indicative of inactive females, following previous reports on landlocked Alewife populations at Michigan and Claytor lakes (Hlavek and Norden 1978; Nigro and Ney 1982). All BB females were considered post-spawners, meaning that they had completed their spawning activity, since they were sampled while leaving their spawning ground.

In total, 54 mature PAT ovaries and 27 BB ovaries were processed histologically (embedding medium: paraffin, pigment: hematoxylin and eosin) (Table 5.1). PAT sections were scanned using a BASLER acA1920-40uc camera fitted on a Zeiss Axio

Lab.A1 microscope and the Microvisioneer software, while BB sections were digitalized using a NanoZoomer S60 slide scanner. Detailed information resulted from each photomicrograph, including: (1) presence or absence of PG oocytes, (2) developmental stages of SG oocytes, (3) presence or absence of POFs, (4) occurrence of atresia, and (5) ovarian developmental stage.

All 54 PAT ovaries processed histologically and 17 BB ovaries were subjected to whole-mount analysis (Table 5.1) and the subsamples were captured using a Jenoptik Progress C3 camera mounted on a Euromex NZ 80 stereo microscope. Particle analysis enabled, for each female: (1) estimation of ODs at both PG and SG phase, (2) creation of an OSFD, (3) definition of the AM in the OSFD, and (4) estimation of relative fecundity values.

Table 5.1. Number (n) and mean total length (L in mm) of female *Alosa pseudoharengus* analyzed per sampling date, site and method

Πίνακας 5.1. Αριθμός (n) και μέσο ολικό μήκος σώματος (L σε mm) θηλυκών ατόμων του είδους *Alosa pseudoharengus* που αναλύθηκαν ανά μέθοδο, ημερομηνία δειγματοληψίας και περιοχή δειγματοληψίας

Sampling date	Site	Whole-mount analysis: n (mean L)	Histological analysis: n (mean L)
19 May 2006	Bride Lake	11 (258)	19 (258)
30 May 2006	Bride Lake	5 (251)	7 (251)
6 July 2006	Bride Lake	1 (256)	1 (256)
7 June 2018	Pattagansett Lake	16 (139)	16 (139)
21 June 2018	Pattagansett Lake	19 (131)	19 (131)
2 July 2018	Pattagansett Lake	19 (130)	19 (130)

AM was discernible in all PAT and BB OSFDs (see 5.3 section) and was composed of oocytes in a single developmental stage; confirmation was provided by cross-checking OSFD modality with gonadal development as depicted in the histological sections. The latter assumption has been previously validated for BB population (Ganias et al. 2015b). Hence, RF_{AM} was considered equivalent to RF_b. The latter and RF_t were estimated only for PAT; BB females analyzed in this chapter were post-spawners, and thus any fecundity estimations would be meaningless (BB fecundity estimations are provided in Ganias et al. 2015b). OD_{AM} was estimated for both PAT and BB, and mean values of the two populations were compared between females that were at the same ovarian developmental stage by a t-test analysis.

Published information defining the onset and ending calendar dates of Alewife spawning seasons were collected for different anadromous and landlocked populations of the species. Instead of actual dates, the terms "early-", "mid-" and "late-" were commonly encountered; they were attributed to the 5th, 15th and 25th day of the month, respectively (e.g., early-May = 5th of May). Dates were then transformed

to Julian dates (e.g., 5th of March = 64th day of the year). For the latter transformation a non-leap-year calendar was used. Onset and ending Julian dates of the spawning season were estimated for each population. Duration of the spawning season was also estimated by subtracting the onset from the ending Julian date. The resulted estimations were plotted against latitude of the spawning site of each population. Onset timing and duration of the spawning season were compared between the anadromous and the landlocked populations through Mann-Whitney U test. Kolmogorov-Smirnov test was also implemented to compare the distributions of onset timing and duration of the spawning season for the two Alewife life-history forms.

5.3 Results

Based on *GSI* analysis, sampling period for PAT coincided with the main spawning activity, since the vast majority (51 out of 53; 96.2%) of females were mature on the first two sampling dates (Fig. 5.2). On the contrary, a significant proportion of females (20 out of 48; 41.7%) were reproductively inactive on the last sampling date, indicating that the spawning season was heading to completion (Fig. 5.2). All inactive females were considered spent, based on their L and date of capture. The significant proportion of spent females caught on the last sampling date suggested that PAT is an iteroparous population.
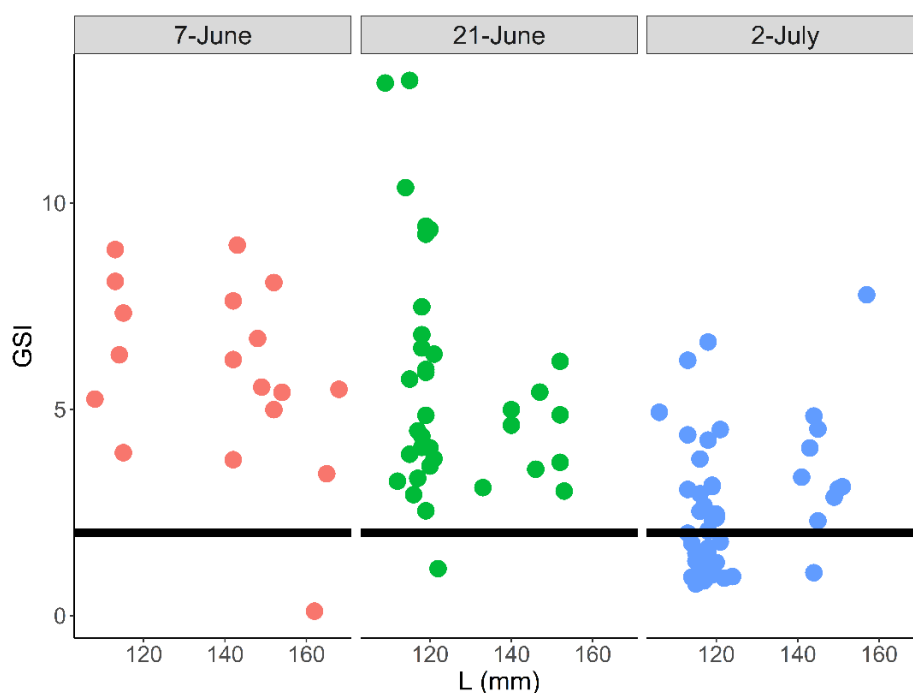

Figure 5.2. Scatter plot of gonadosomatic index (*GSI*) values per sampling date and total fish length (L) of *Alosa pseudoharengus* at Pattagansett Lake

Εικόνα 5.2. Διάγραμμα διασποράς τιμών του γοναδοσωματικού δείκτη (*GSI*) ανά ημερομηνία δειγματοληψίας και ολικό μήκος σώματος (L) για άτομα του είδους *Alosa pseudoharengus* στη Λίμνη Pattagansett

All PAT ovaries contained PG oocytes of varying sizes. SG oocytes were also present at several distinct developmental stages; CA-1 – CA-3, VIT-1 – VIT-3, GVM and HYD stages were identified. POFs were detected in ovaries at VIT-3 and GVM ovarian stages (i.e., imminent spawners), and thus PAT was categorized as a multiple spawner. Two PAT females were classified as spent, based on the lack of vitellogenic oocytes in their ovaries. Massive atresia occurred in the ovaries of both spent females, and thus were excluded from any further statistical analyses.

Oocytes of varying sizes at PG and distinct SG stages co-occurred also in all BB ovaries; the identified SG stages ranged from CA-1 to GVM. POFs were identified in 89% (24 out of 27) of the ovaries and were either newly formed or old. The latter were present in ovaries at VIT-3 or GVM ovarian stage, suggesting that BB displayed multiple spawning. Significant levels of alpha atresia occurred in few ovaries, while one ovary displayed massive atresia; the latter female was not included in any analyses.
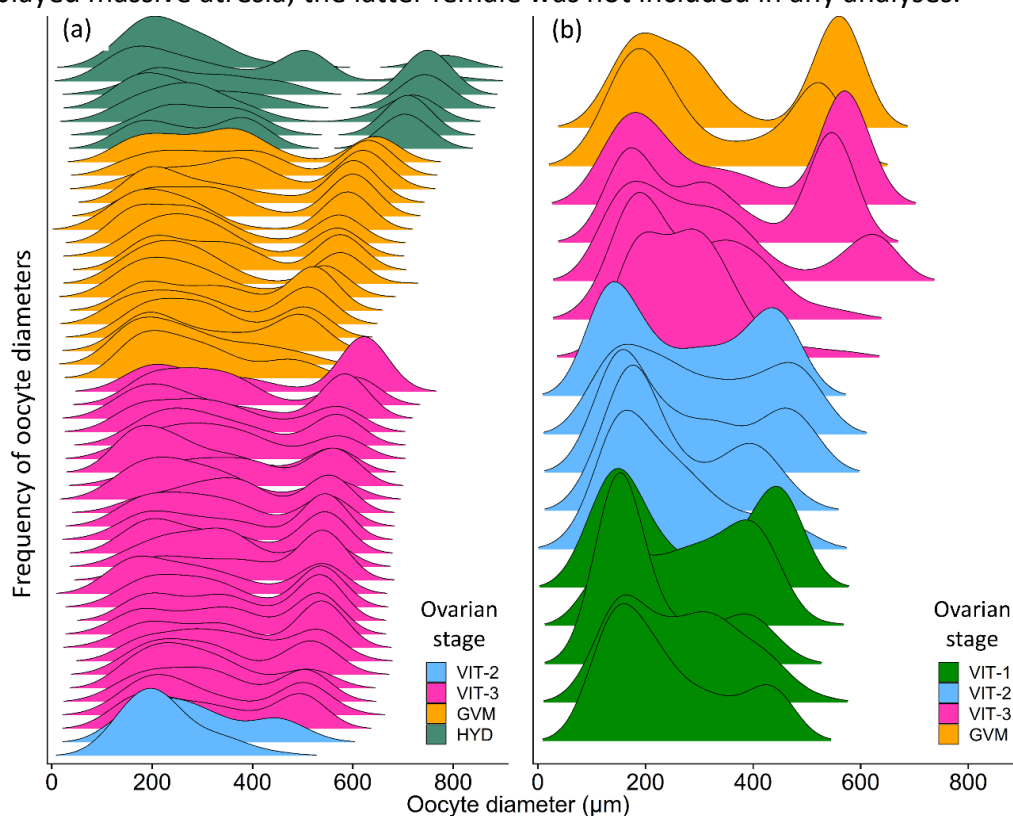
Figure 5.3. *Alosa pseudoharengus* oocyte size frequency distributions for: (a) the landlocked population at Pattagansett Lake, and (b) downrunners of the anadromous population at Bride Brook. Distributions for each ovarian developmental stage are displayed in order of increasing (bottom to top) mean oocyte diameter of the secondary growth oocytes. Color of each distribution represents the ovarian developmental stage

Εικόνα 5.3. Κατανομές συχνοτήτων μεγεθών ωοκυττάρων ατόμων του είδους *Alosa pseudoharengus* από: (a) τον ολοβιωτικό πληθυσμό της Λίμνης Pattagansett, και (b) τον ανάδρομο πληθυσμό του Ποταμού Bride Brook κατά την αποχώρηση από το πεδίο ωοτοκίας. Οι κατανομές για κάθε μία από τις φάσεις ανάπτυξης της ωοθήκης εμφανίζονται με αυξανόμενη (από κάτω προς τα επάνω) μέση διάμετρο ωοκυττάρων στη δευτερογενή φάση ανάπτυξης. Τα διαφορετικά χρώματα αντιστοιχούν στις φάσεις ανάπτυξης της ωοθήκης

Three lines of evidence indicated that both PAT and BB displayed the indeterminate fecundity type: (1) newly recruited SG oocytes (i.e., at CA-1 and CA-2 stage) were detected in the histological sections of all actively spawning, post-spawning and spent females, (2) OSFDs of all pre-spawning, actively spawning and post-spawning females were continuous in the size range between PG and SG oocytes (Fig. 5.3), and (3) high or even massive levels of atresia were detected in post-spawners and spent females.

In all females, both PAT and BB, the application of the Bhattacharya method to the OSFDs resolved a distinct AM (Fig. 5.4). Mean PAT RF_b was estimated at 264 oocytes per g of W_{ev}, and mean PAT RF_t at 685 oocytes per g of W_{ev}.
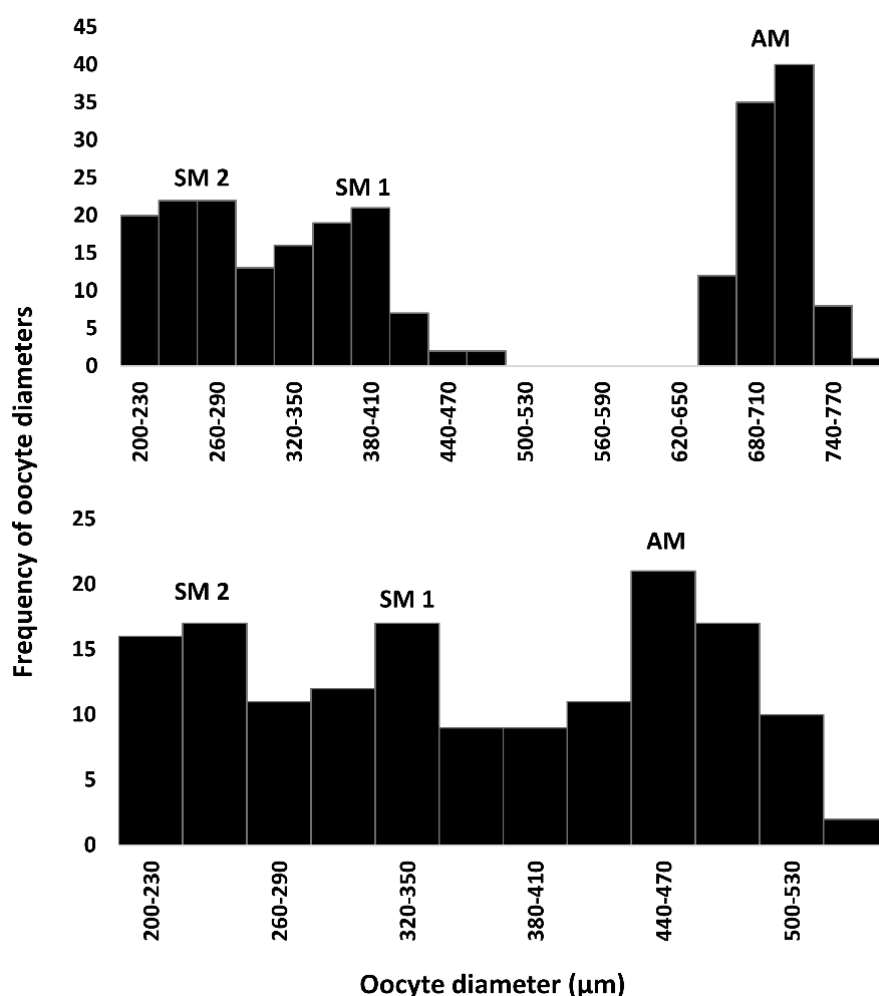
Figure 5.4. Oocyte size frequency distributions of *Alosa pseudoharengus* using a fine size-class of 30 μm of a: (a) landlocked female at Pattagansett Lake, and (b) downrunning anadromous female at Bride Brook. AM = advanced mode, SM1 = first subsequent mode, SM2 = second subsequent mode

Εικόνα 5.4. Κατανομή συχνοτήτων μεγεθών ωοκυττάρων για δύο άτομα του είδους *Alosa pseudoharengus* με βήμα κλάσης μεγέθους τα 30 μm από: (a) τον ολοβιωτικό πληθυσμό της Λίμνης Pattagansett, και (b) τον ανάδρομο πληθυσμό του Ποταμού Bride Brook κατά την αποχώρηση από το πεδίο ωοτοκίας. AM = η πιο προηγμένη ομάδα της κατανομής, SM1 = η δεύτερη πιο προηγμένη ομάδα, SM2 = η τρίτη πιο προηγμένη ομάδα

Mean OD_{AM} did not differ between the two surveyed populations. PAT females at the VIT-3 or GVM ovarian developmental stage had mean OD_{AM} at 553 μm and BB females at the same ovarian stages at 562 μm (t-test, $P > 0.05$; Fig. 5.5).
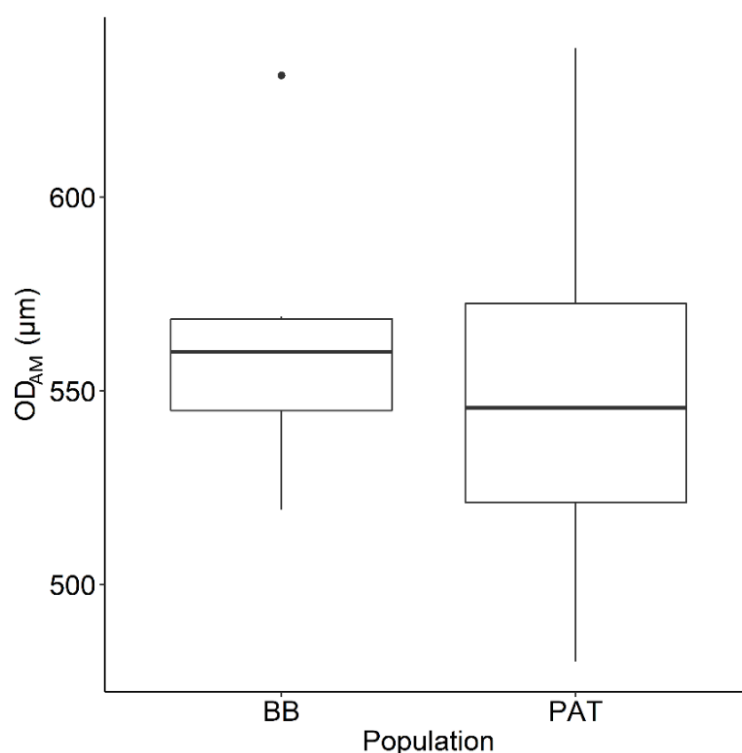
Figure 5.5. Box and whisker plot of diameters of the most advanced oocytes (OD_{AM}) for *Alosa pseudoharengus* at Bride Brook (BB) and Pattagansett Lake (PAT)
Εικόνα 5.5. Θηκόγραμμα των διαμέτρων των ωοκυττάρων στο πιο προηγμένο στάδιο ανάπτυξης (OD_{AM}) για άτομα του είδους *Alosa pseudoharengus* από τον ποταμό Bride Brook (BB) και τη Λίμνη Pattagansett (PAT)

Onset Julian date of spawning activity was significantly different between anadromous and landlocked Alewife populations (for both Mann-Whitney U and Kolmogorov-Smirnov tests: $P \ll 0.001$). Specifically, resident populations begun their spawning season later than the anadromous ones (Fig. 5.6). On the contrary, duration of spawning season did not differ between the two life-history forms (for both Mann-Whitney U and Kolmogorov-Smirnov tests: $P > 0.05$). Additionally, landlocked Alewives displayed greater variability in onset timing compared to anadromous populations. Finally, landlocked Alewife populations seemed to exhibit a latitudinal gradient regarding the onset of spawning activity; spawning started later at higher latitude (Fig 5.6).
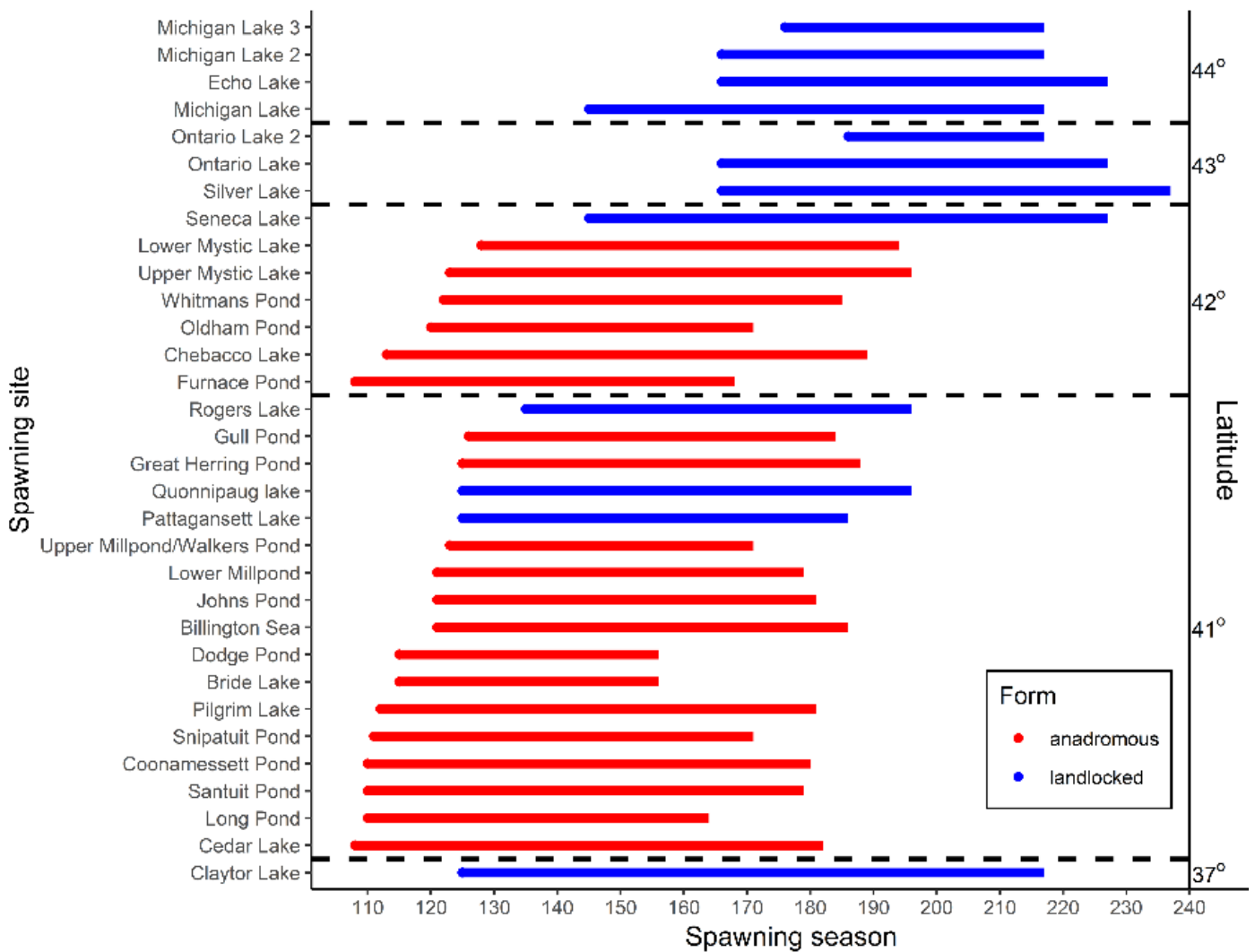
Figure 5.6. Onset and ending Julian dates of spawning season for anadromous and landlocked *Alosa pseudoharengus* populations. Latitude of the spawning site of each population is also shown. Data were collected from: Pritchard 1929; Odell 1934; Graham 1956; Norden 1967; Lackey 1970; Brown 1972; Hlavek and Norden 1978; Nigro and Ney 1982; Rosset et al. 2017; Littrell et al. 2018

Εικόνα 5.6. Ημερομηνία έναρξης και λήξης της περιόδου ωοτοκίας διαφόρων ανάδρομων και ολοβιωτικών πληθυσμών του είδους *Alosa pseudoharengus.* Το γεωγραφικό πλάτος του πεδίου ωοτοκίας κάθε πληθυσμού εμφανίζεται επίσης. Τα δεδομένα συλλέχθηκαν από: Pritchard 1929; Odell 1934; Graham 1956; Norden 1967; Lackey 1970; Brown 1972; Hlavek and Norden 1978; Nigro and Ney 1982; Rosset et al. 2017; Littrell et al. 2018

5.4 Discussion

In this chapter the ovarian dynamics of a landlocked and a neighboring anadromous Alewife population were analyzed in detail, and comparisons were conducted between the two life-history forms of the species. Both populations released their oocytes in multiple events and displayed indeterminate fecundity type. Life-history form seemed to affect the Alewife spawning behavior, since landlocked populations

displayed significant delay in commencing their spawning activity compared to the anadromous populations. A latitudinal gradient in onset timing of spawning activity was also observed among the landlocked Alewife populations.

Multiple spawning of PAT was confirmed by the occurrence of POFs in imminent spawners. The latter result is in accordance with the only previous report concerning the oocyte release strategy of a landlocked Alewife, in which Nigro and Ney (1982) implied multiple spawning in Claytor Lake. Multiple spawning could not be evidenced directly for BB in this chapter, since all females analyzed where downrunners. However, POF presence in ovaries at an advanced developmental stage, along with the co-occurrence of several SG stages in all ovaries provided strong indications that BB was a multiple spawner. Multiple spawning in BB was corroborated by the ongoing ovarian development after a spawning event during residency in the lake. Most downrunners, caught on the 19th of May, had left the lake immediately after their last spawning (as evinced by the presence of large, new POFs), were at an early vitellogenic ovarian stage and contained alpha atretic oocytes; indicators that their spawning activity had been completed. On the contrary, some downrunners, sampled on the 30th of May and 6th of July, had small POFs in their sections and were at an advanced vitellogenic ovarian stage with no signs of alpha atresia; indicative factors of their intension to spawn again during the surveyed season. However, spawning activity of the latter fish was most likely disrupted, and they were forced to exit the lake. Premature spawning cessation was probably caused by elevated water temperature, as has been previously reported for Alewife (Edsall 1970; Irwin and Bettoli 1995), Blueback herring (Loesch and Lund 1977) and other species (e.g., Graham and Orth 1986; Starzynski and Lauer 2015). An alternative hypothesis could be that these fish would have spawned again after they had left the lake. However, this scenario seems implausible, since most downrunners incurred atresia, from very low to massive levels; denotative factor of a population tendency to reabsorb the remainder SG oocytes sooner or later after egress from the lake. Multiple spawning in BB is supported by the results of Ganias et al. (2015b) stating that at least three oocyte batches were released inside the lake, and the report of Marjadi et al. (2019). Due to limited information, it is currently uncertain if multiple spawning is the predominant oocyte release strategy for the species. Additional studies are required to conclude if Alewife oocyte release strategy is invariant or not.

In parallel, information on the fecundity type of Alewife is also very scarce. Both PAT and BB populations displayed the indeterminate fecundity type, since newly recruited SG oocytes were present in ovaries regardless of their spawning phase (actively spawning, post-spawning or spent), no hiatus was formed in any OSFD and massive atresia was present in post-spawning and spent females. Indeterminacy in BB has been reported previously (Ganias et al. 2015b). These authors, even though they analyzed histologically a limited number of downrunners, also reported very early CA

oocytes in post-spawning females and massive atresia in a single ovary. However, their main argument in support of indeterminacy was provided via an inventory analysis; they showed that downrunners had only one oocyte batch less than uprunners despite their apparent expenditure of three batches. Thus, new oocytes had been recruited during their residency in the lake. Additionally, to further support their claim, they showed, through a numerical simulation, that newly recruited oocytes can grow and be spawned while in the lake. By combining the results reported in this chapter and those stated in Ganias et al. (2015b), it seems that fecundity type is unaffected by the life-history form of Alewife. However, additional studies should be conducted on other Alewife populations, both landlocked and anadromous, to determine the universality of indeterminacy for the species and to test for other possible influential factors, such as environmental conditions, latitude and breeding strategy.

Additionally to their similarities in egg release strategy and fecundity type, the two surveyed populations seemed to produce advanced vitellogenic oocytes of comparable size, around 550 μm. However, this result contradicts the estimations for BB uprunners made by Ganias et al. (2015b), who reported significantly larger mean ODs (between 620 and 780 μm) for females at advanced vitellogenic stages. The observed inconsistency was not an artifact owed to different methodological approach, since all measurements were made through the same software and by the author of this study. It is more likely that advanced vitellogenic oocytes of BB downrunners had not reached their maximum size due to spawning cessation. In fact, Ganias et al. (2015b) showed that VIT-3 oocytes in BB uprunners can be encountered at different sizes and that they continued to increase in size towards a maximum value of ca. 750-800 μm.

By comparing published information for several populations, a pattern immerged, where anadromous Alewives produce larger oocytes than the landlocked (Table 5.2); pattern that has been reported in other species as well, such as Sockeye salmon (Wood and Foote 1996). However, comparisons among Alewife populations should be made with caution. For instance, Jessop (1993) and Sullivan et al. (2019), prior to OD estimations, preserved the oocytes in Gilson's fluid, which is known to cause pronounced oocyte shrinkage (Joseph 1963; Klibansky and Juanes 2007). Additionally, the developmental stage of the measured oocytes was not clearly defined in most of the studies reported in Table 5.2.

Table 5.2. Range of diameter (ODs in μm) of the most-advanced oocytes reported in pre-spawning mature *Alosa pseudoharengus* landlocked and anadromous populations

Πίνακας 5.2. Εύρος τιμών διαμέτρου (ODs σε μm) ωοκυττάρων στο πιο προηγμένο στάδιο ανάπτυξης σε ολοβιωτικούς και ανάδρομους πληθυσμούς του είδους *Alosa pseudoharengus* πριν την έναρξη της περιόδου ωοτοκίας

ODs	Life-history form	Site	Reference
460 (maximum)	Landlocked	Lake Superior, US shore	Bronte et al. 1991
480 – 640	Landlocked	Pattagansett Lake, Connecticut	This chapter
560 – 700	Anadromous	Parker River, Massachusetts	Mayo 1974
580 – 720	Anadromous	Parker River, Massachusetts	Huber 1978
500 – 600[1]	Anadromous	Tusket Gaspereau and Margaree rivers, Nova Scotia, and Saint John River, New Brunswick	Jessop 1993
670 – 780	Anadromous	Bride Brook, Connecticut	Ganias et al. 2015b
750[1] (mean)	Anadromous	Lamprey River, New Hampshire	Sullivan et al. 2019

Differences between the two Alewife life-history forms were also observed in onset timing of the spawning season. Spawning activity of the resident populations has incurred a temporal shift towards summer months from the spring spawning strategy of their ancestral, anadromous form. Similar pattern and designation of life-history form as a major explanatory variable for the Alewife spawning time has been reported by Littrell et al. (2018), who compared five neighboring populations. Anadromous Alewives, that mature dozens or hundreds of kilometers away from their spawning grounds, display evolutionary fine-tuned timing of spawning migrations that ensures their survival during the upstream and downstream (in case of iteroparity) migration, and the survival of their offspring (Littrell et al. 2018). On the contrary, landlocked Alewives are liberated from strict time-frames associated with migration, since they reside in their spawning sites and can track environmental cues directly. Thus, spawning timing of landlocked Alewives may have shifted to coincide with the optimum conditions for breeding, incubation and larvae survival, such as higher temperatures during the summer. However, during the last decades, anadromous alosines have also incurred time shifts in their spawning phenology due to elevated water temperatures. Specifically, timing of spawning migration and activity have been advanced to earlier calendar dates in anadromous Alewife (Ellis and Vokoun 2009; Lombardo et al. 2019), American shad (Quinn and Adams 1996; Nack et al 2019) and Blueback herring (Lombardo et al. 2019), as well as in other fish, such as salmonids

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[1] Oocytes were preserved in Guilson's fluid

(Quinn and Adams 1996; Juanes et al. 2004; Kovach et al. 2013) and Striped bass (Peer and Miller 2014; Nack et al 2019).

The effects of changing environmental conditions, and of thermal shifts in particular, are highly significant for both anadromous and landlocked Alewives and should be analyzed further. The former exhibit natal homing (Jessop 1994; Bentzen and Paterson 2005), and thus may not be able to spatially account for detrimental effects caused by climate change. Moreover, environmental changes could alter the evolutionary fine-tuned reproductive phenology of anadromous Alewife and lead to mismatch between spawning activity and the optimal conditions for offspring survival, as has been suggested for other fishes (e.g., Edwards and Richardson 2004; Siddon et al. 2013; Bell et al. 2017; McQueen and Marshall 2017), or to increased residency time on spawning grounds, and thus to increased prey consumption and/or Alewife susceptibility to predators (Rosset et al. 2017). Similarly, landlocked populations have limited or zero capacity for distribution shifts in response to unfavorable conditions, and temperature upper limits of tolerance have been reported for Alewives (Graham 1956; Otto et al. 1976; McCauley and Binkowski 1982). If the differences among populations and years are explained, management of spawning runs, and stocking efforts would be improved. For instance, if temporal shifts in Alewife spawning activity occur, and continue to occur in the future, the current commercial and recreational seasons, as well as the operational seasons of fishways should be reevaluated (Ellis and Vokoun 2009).

Among landlocked Alewives, greater variability in onset timing of spawning activity was also observed in this chapter compared to the anadromous populations; result corroborated by Littrell et al. (2018). The detected interpopulation variability could be owed to local selection pressures at each impounded water body (Nigro and Ney 1982; Bronte et al. 1991; Littrell et al. 2018) or genetic differences (Littrell et al. 2018). On the other hand, the interannual variability that was also detected for a couple of landlocked Alewife populations can be explained by fluctuations in several parameters among years, such as thermal regimes (Marcy 1972; Kjesbu 1994; Irwin and Bettoli 1995), photoperiod (Bromage et al. 1992; Migaud et al. 2010), energy resources (Thorpe 1994) and condition (Kjesbu 1994; Yoneda and Wright 2005; Kjesbu 2009). Such interannual variability has been previously reported for Alewife and was attributed to natural selection or genetic drift, since no significant environmental differences were detected (Littrell et al. 2018).

A latitudinal gradient in the onset timing of the spawning season was also observed among landlocked Alewife populations; southern populations spawned earlier than the northern. This conclusion is supported by previous reports that spawning migration timing varied predictably with latitude, with southern populations migrating upstream earlier in American shad (Leggett and Whitney 1972), Allis shad (Baglinière et al. 2003) and Hickory shad (Harris et al. 2007).

In conclusion, in this chapter was evident that information on the ovarian dynamics of Alewife, both anadromous and landlocked, is scarce. It was evidenced that two neighboring populations with different life-history forms displayed multiple spawning and indeterminate fecundity type with similar aspects. In particular, the analysis on the anadromous population validated the results of a previous study on the same population. However, additional information for different populations are required to safely oppose the historical assumption that both landlocked and anadromous Alewife displayed total spawning and determinate fecundity type. Additionally, the Alewife life-history form seemed to affect aspects of the species spawning phenology, but the reasons for these intraspecies variations need further investigation and especially in the context of climate change.

CHAPTER 6: SG recruitment and SG phase of oogenesis within the ovulatory cycle of a landlocked iteroparous fish

The results presented in this chapter have been published as:

F. A. Mouchlianitis, G. Minos, K. Ganias (2020). Timing of oocyte recruitment within the ovulatory cycle of Macedonian shad, *Alosa macedonica*, a batch spawning fish with indeterminate fecundity. *Theriogenology* 146: 31–38.
https://doi.org/10.1016/j.theriogenology.2020.01.050

6.1 Specific objectives

The first objective of this chapter was to define the oocyte release strategy and the fecundity type of Macedonian shad, *Alosa macedonica*. Subsequently, SG recruitment and SG phase of oogenesis were analyzed within the narrow temporal scale of the ovulatory cycle, with the main goal to define the timing of SG recruitment. To achieve the latter goal, the ovulatory cycle was classified into four different phases, and then different ovarian developmental indices were compared among these phases to evince cyclicity in ovarian dynamics. Both the formation of the advanced oocyte batch and SG recruitment within the ovulatory cycle were analyzed in this chapter and a temporal association between these two ovarian processes was revealed.

6.2 Specific methodology

Macedonian shad females were collected from Volvi Lake (Fig. 6.1) on five sampling dates in June 2016 (Table 6.1), i.e., during the spawning season (Kleanthidis 2002). Samplings were conducted with vertical gill nets during the night. All fish were stored in 10% neutral buffered formalin immediately after each sampling.

Ovarian histological sections from 91 females were prepared (embedding medium: paraffin, pigment: hematoxylin and eosin) (Table 6.1) and scanned to photomicrographs using a BASLER acA1920-40uc camera fitted on a Zeiss Axio Lab.A1 microscope and the Microvisioneer software. Each photomicrograph provided detailed information regarding the ovarian dynamics and enabled estimations for each female, including: (1) presence or absence of PG oocytes, (2) developmental stages of SG oocytes, (3) presence or absence of POFs, (4) POF$_{XSA}$, and classification of each POF$_{XSA}$ as large or small based on its histomorphological characteristics and its value, and (5) ovarian developmental stage.
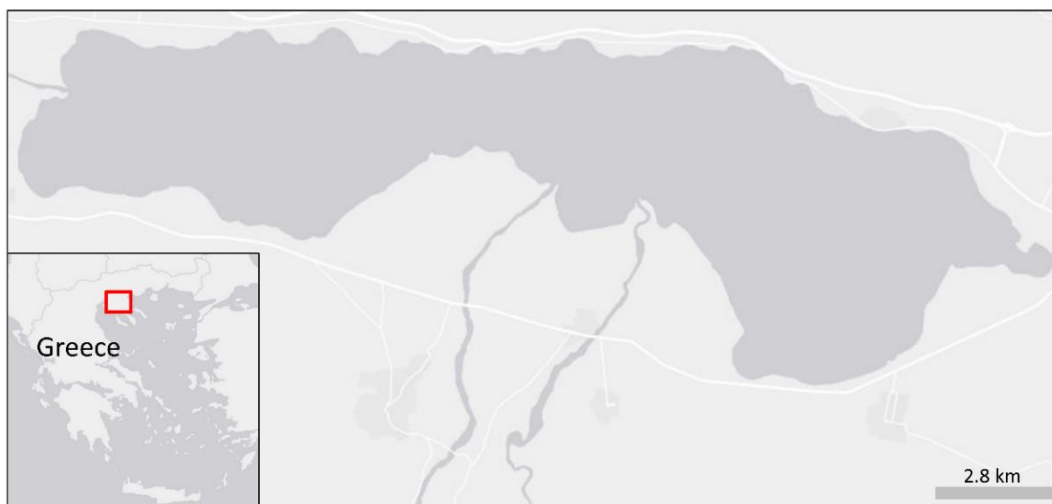
Figure 6.1. Volvi Lake in northern Greece; the habitat of *Alosa macedonica*. The exact sampling sites, were not known, since all fish were obtained from a local fisherman
Εικόνα 6.1. Λίμνη Βόλβη στη Βόρεια Ελλάδα· ενδιαίτημα του είδους *Alosa macedonica*. Οι ακριβείς περιοχές δειγματοληψίας των ψαριών που αναλύθηκαν στην παρούσα εργασία δεν είναι γνωστές, καθώς όλα τα άτομα αποκτήθηκαν από επαγγελματία ψαρά της περιοχής

In parallel, whole-mount analysis was implemented on the same 91 females (Table 6.1) and the subsamples were captured using a Jenoptik Progress C3 camera mounted on a Euromex NZ 80 stereo microscope. Particle analysis enabled for each female: (1) estimation of ODs at both PG and SG phase, (2) creation of an OSFD, (3) definition of the AM in the OSFD, and (4) estimation of relative fecundity values.

Table 6.1. Number (n) of female *Alosa macedonica* analyzed histologically and through whole-mount analysis, and total length (L in mm) range and mean (±SD) value, per sampling date
Πίνακας 6.1. Αριθμός (n) θηλυκών ατόμων του είδους *Alosa macedonica* που αναλύθηκαν ιστολογικά και μέσω της μεθόδου whole-mount, και το εύρος τιμών του ολικού μήκους σώματος (L σε mm) και η μέση τιμή και η τυπική απόκλιση (±SD) αυτού, ανά ημερομηνία δειγματοληψίας

Date	Histological analysis (n)	Whole-mount analysis (n)	L range (mean ± SD)
1 June 2016	21	21	152 - 184 (167 ± 8.8)
8 June 2016	29	29	155 – 209 (166 ± 10.6)
15 June 2016	15	15	151 – 179 (165 ± 8.5)
21 June 2016	12	12	152 – 184 (169 ± 10.9)
29 June 2016	14	14	157 – 183 (170 ± 8.6)
Total	**91**	**91**	

AM was separated in all OSFDs (see 6.3.2 section) and corresponded to the spawning batch, i.e., to the oocytes that are released during a single spawning episode. The latter was based on previous results on Alewife (Ganias et al. 2015b) and on the gonadal development as depicted in the histological sections. Thus, RF_{AM} was equivalent to RF_b. To avoid possible bias, RF_b was estimated only from hydrated females prior to an ovulation/spawning event, i.e., those classified as in pre-ovulatory phase (see 6.3.1 section). RF_t and relative fecundity of the small-sized SG oocytes, i.e., those having diameters between 200 and 320 μm ($RF_{200-320}$), were also estimated.

$RF_{200-320}$ was used as an index of SG recruitment intensity. The ratio of RF_t to mean RF_b (fecundity ratio) of females at the pre-ovulatory phase was used as a proxy of the number of co-occurring SG oocyte batches, as has been done previously by Ganias et al. (2017). In females caught while ovulating (running; see 6.3.1 section), the fecundity ratio was estimated by subtracting the remaining hydrated oocytes from RF_t and adding unity to the resulting RF_t/RF_b quotient to counterbalance the missing ovulating batch.

Cyclicity in ovarian dynamics was examined by comparing the numerical formation of the AM and the mean OD_{AM}, the ovarian developmental stage, the modality of OSFDs and GSI among the different ovulatory phases (see 6.3.1 section).

6.3 Results

6.3.1 Ovulatory phases classification

PG and multiple SG oocyte developmental stages were present in each ovary; CA stages, VIT-1 – VIT-3 stages, GVM stage and HYD stage were identified. All females were spawning capable.
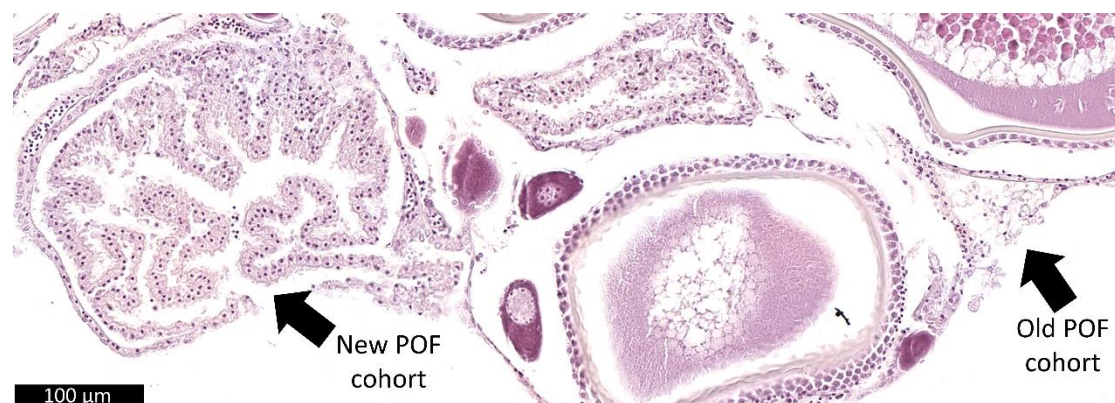
Figure 6.2. Photomicrograph of an *Alosa macedonica* ovarian histological section showing postovulatory follicles from two different ovulation/spawning events (old and new POF cohort)

Εικόνα 6.2. Φωτομικρογραφία ιστολογικής τομής ωοθήκης ατόμου του είδους *Alosa macedonica* όπου εντοπίζονται κενά ωοθυλάκια (POFs) από δύο διαφορετικά συμβάντα ωορρηξίας/ωοτοκίας (old και new POF cohort)

High proportion of females (87%) had POFs in their ovaries. Most of these ovaries (82.5%) had POFs from two different cohorts (new and old), which originated from two sequential daily spawning events (Fig. 6.2), evidencing that the surveyed population was a multiple spawner. The co-occurrence of two POF cohorts in such a high proportion of ovaries suggested also that POF degeneration period was constantly longer than the ovulatory cycle. POF_{XSA} of the newest cohort in each ovary, classified as large, had values > 0.05 mm^2, while small POF_{XSA} had values < 0.02 mm^2 (Fig. 6.3).
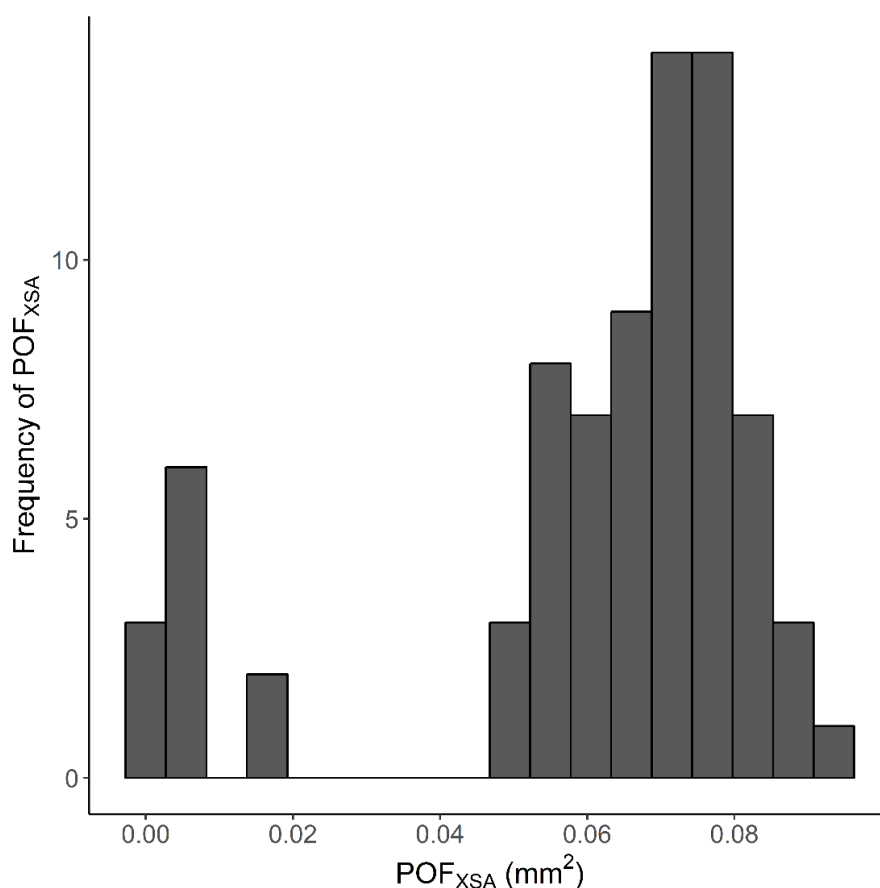
Figure 6.3. Frequency distribution of the cross-sectional area of the biggest postovulatory follicle (POF_{XSA}) in each of the 79 *Alosa macedonica* ovaries analyzed. Two POF_{XSA} size-classes were distinguished: small (area < 0.02 mm^2) and large (area > 0.05 mm^2)

Εικόνα 6.3. Κατανομή συχνοτήτων τιμών εμβαδού του μεγαλύτερου κενού ωοθυλακίου (POF_{XSA}) σε κάθε μία από τις 79 ωοθήκες ατόμων του είδους *Alosa macedonica* που αναλύθηκαν. Δύο κλάσεις μεγέθους POF_{XSA} αναγνωρίστηκαν: μικρή (εμβαδό < 0.02 mm^2) και μεγάλη (εμβαδό > 0.05 mm^2)

Four phases were identified within the ovulatory cycle based on the presence/absence of HYD oocytes and the presence of large or small POF_{XSA} (Table 6.2): (1) pre-ovulatory (PRE), (2) running (RUN), (3) post-ovulatory (POST), and (4) intermediate (INT). The criterion of POF_{XSA} of the newest cohort was clear-cut, especially for distinguishing PRE from RUN and POST from INT females (Fig. 6.4). The few females without POFs in their ovaries had HYD oocytes and were classified as PRE.

Table 6.2. Phases identified within the ovulatory cycle of *Alosa macedonica*, based on the presence/absence (+/-) of hydrated (HYD) oocytes, presence/absence (+/-) of postovulatory follicles (POFs) and the cross-sectional area of the biggest POF (POF_{XSA})

Πίνακας 6.2. Φάσεις του κύκλου ωοτοκίας ατόμων του είδους *Alosa macedonica*. Ο διαχωρισμός των φάσεων έγινε με βάση την παρουσία/απουσία (+/-) ενυδατωμένων ωοκυττάρων (HYD), την παρουσία/απουσία (+/-) κενών ωοθυλακίων (POFs) και το εμβαδό του μεγαλύτερου POF (POF_{XSA}) σε κάθε ιστολογική τομή ωοθήκης

Ovulatory phase	Hydrated oocytes	POFs	POF_{XSA} (mm²)	Description
Pre-ovulatory (PRE)	+ +	- +	- < 0.02	Prior to ovulation with HYD oocytes and no POFs or with small POF_{XSA}
Running (RUN)	+	+	> 0.05	During ovulation/spawning with HYD oocytes and large POF_{XSA}
Post-ovulatory (POST)	-	+	> 0.05	Immediately or closely after ovulation/spawning without HYD oocytes and with large POF_{XSA}
Intermediate (INT)	-	+	< 0.02	In-between two ovulation/spawning events without HYD oocytes and with small POF_{XSA}
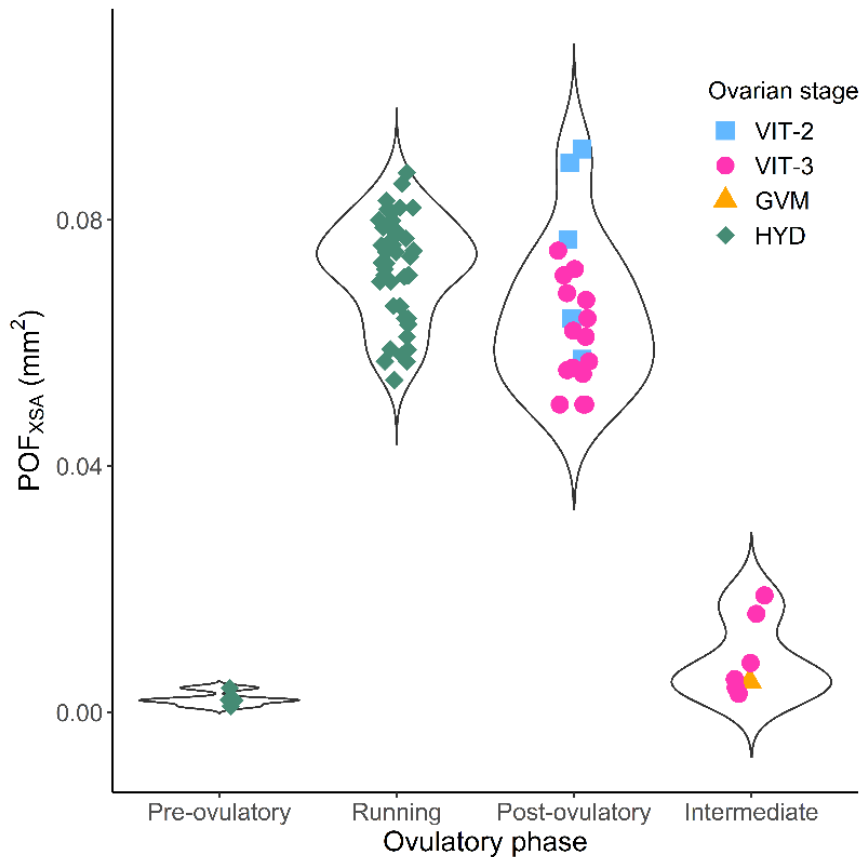
Figure 6.4. Violin plot of the cross-sectional area of the biggest postovulatory follicle (POF_{XSA}) in each ovarian histological section for *Alosa macedonica* females at different phases within the ovulatory cycle: prior to ovulation (pre-ovulatory), during ovulation/spawning (running), immediately or closely after ovulation/spawning (post-ovulatory) and in-between two ovulation/spawning events (intermediate). Colors and shapes represent the ovarian developmental stages

Εικόνα 6.4. Διάγραμμα τύπου βιολιού για την απεικόνιση της κατανομής των τιμών εμβαδού του μεγαλύτερου κενού ωοθυλακίου (POF_{XSA}) σε κάθε ιστολογική τομή ωοθήκης που αναλύθηκε ατόμων του είδους *Alosa macedonica*, ανά φάση του κύκλου ωοτοκίας: πριν την ωορρηξία (pre-ovulatory), κατά τη διάρκεια της ωορρηξίας/ωοτοκίας (running), αμέσως ή λίγο μετά την ωορρηξία/ωοτοκία (post-ovulatory) και μεταξύ δύο διαδοχικών συμβάντων ωορρηξίας/ωοτοκίας (intermediate). Τα διαφορετικά χρώματα και σχήματα αντιστοιχούν στις φάσεις ανάπτυξης της ωοθήκης

6.3.2 Ovarian dynamics cyclicity

The profiles of AM formation, as overviewed in Figure 6.5, revealed that AM was discernible in all four ovulatory phases. However, there were clear differences; the AM was gradually becoming completely separated from the smaller oocytes as ovulatory cycle progressed from POST to RUN females (Fig. 6.5). Cyclicity was evinced also through the ovarian developmental stage; both PRE and RUN females were at the HYD stage, POST females were at the VIT-2 or VIT-3 stage, and all INT females had progressed to VIT-3 or GVM ovarian stage (Fig. 6.5).
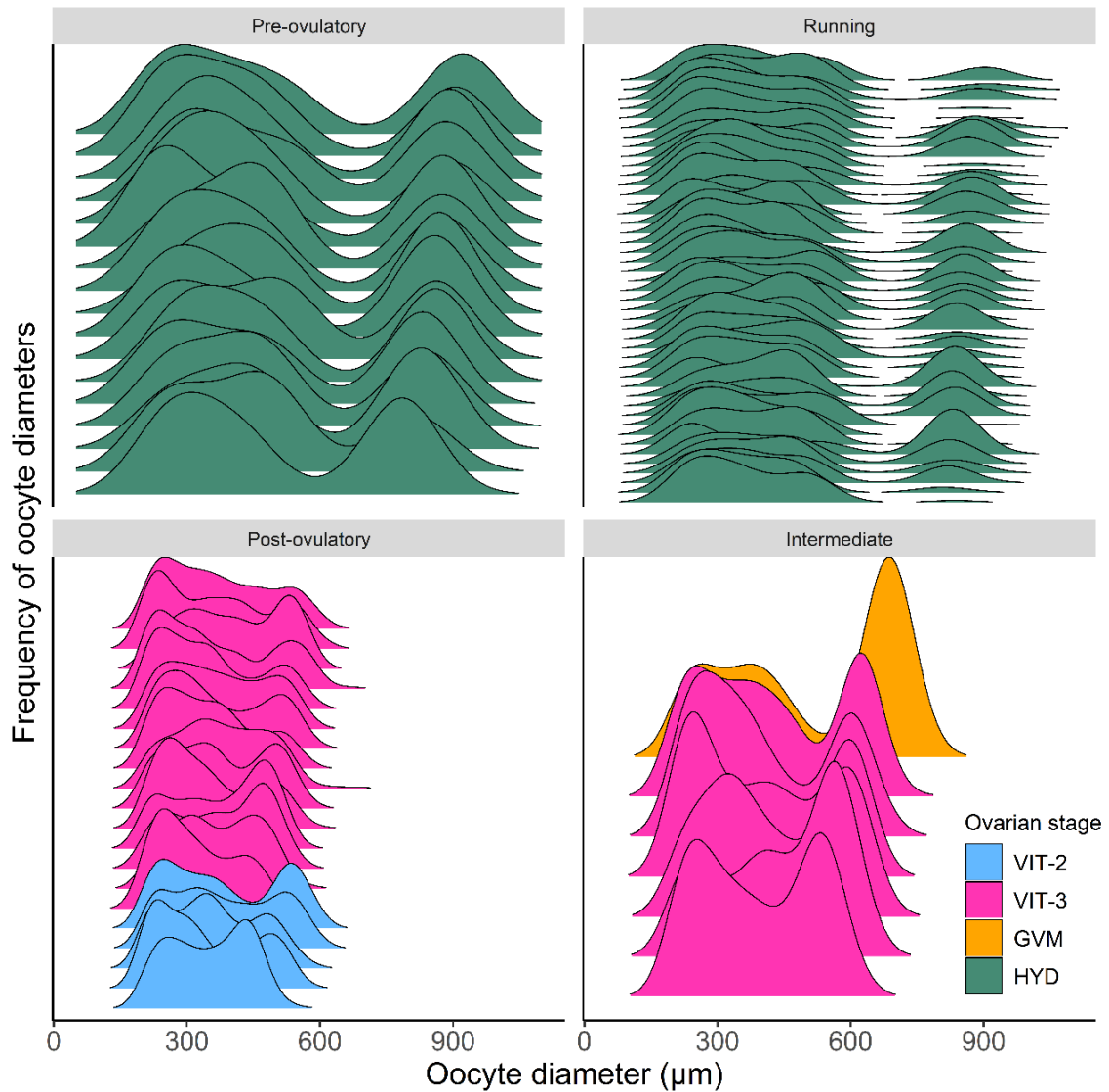
Figure 6.5. Size frequency distributions of oocytes at the secondary growth phase for *Alosa macedonica* females at different phases within the ovulatory cycle: prior to ovulation (pre-ovulatory), during ovulation/spawning (running), immediately or closely after ovulation/spawning (post-ovulatory) and in-between two ovulation/spawning events (intermediate). Within each panel, distributions of each ovarian stage are displayed in order of increasing (bottom to top) mean diameter of the secondary growth oocytes. Color of each distribution represents the ovarian developmental stage

Εικόνα 6.5. Κατανομές συχνοτήτων μεγεθών των ωοκυττάρων που βρίσκονται στη δευτερογενή φάση ανάπτυξης ατόμων του είδους *Alosa macedonica* ανά φάση του κύκλου ωοτοκίας: πριν την ωορρηξία (pre-ovulatory), κατά τη διάρκεια της ωορρηξίας/ωοτοκίας (running), αμέσως ή λίγο μετά την ωορρηξία/ωοτοκία (post-ovulatory) και μεταξύ δύο διαδοχικών συμβάντων ωορρηξίας/ωοτοκίας (intermediate). Οι κατανομές για κάθε μία από τις φάσεις ανάπτυξης της ωοθήκης εμφανίζονται με αυξανόμενη (από κάτω προς τα επάνω) μέση διάμετρο ωοκυττάρων στη δευτερογενή φάση ανάπτυξης. Τα διαφορετικά χρώματα αντιστοιχούν στις φάσεις ανάπτυξης της ωοθήκης

Detailed analysis of OSFDs revealed co-occurrence of various distinct modes within the smaller oocytes (SM1, SM2, etc.) (Fig. 6.6). In general, as females were approaching an ovulation event (PRE females), the different modes were becoming more distinct (Fig. 6.6).
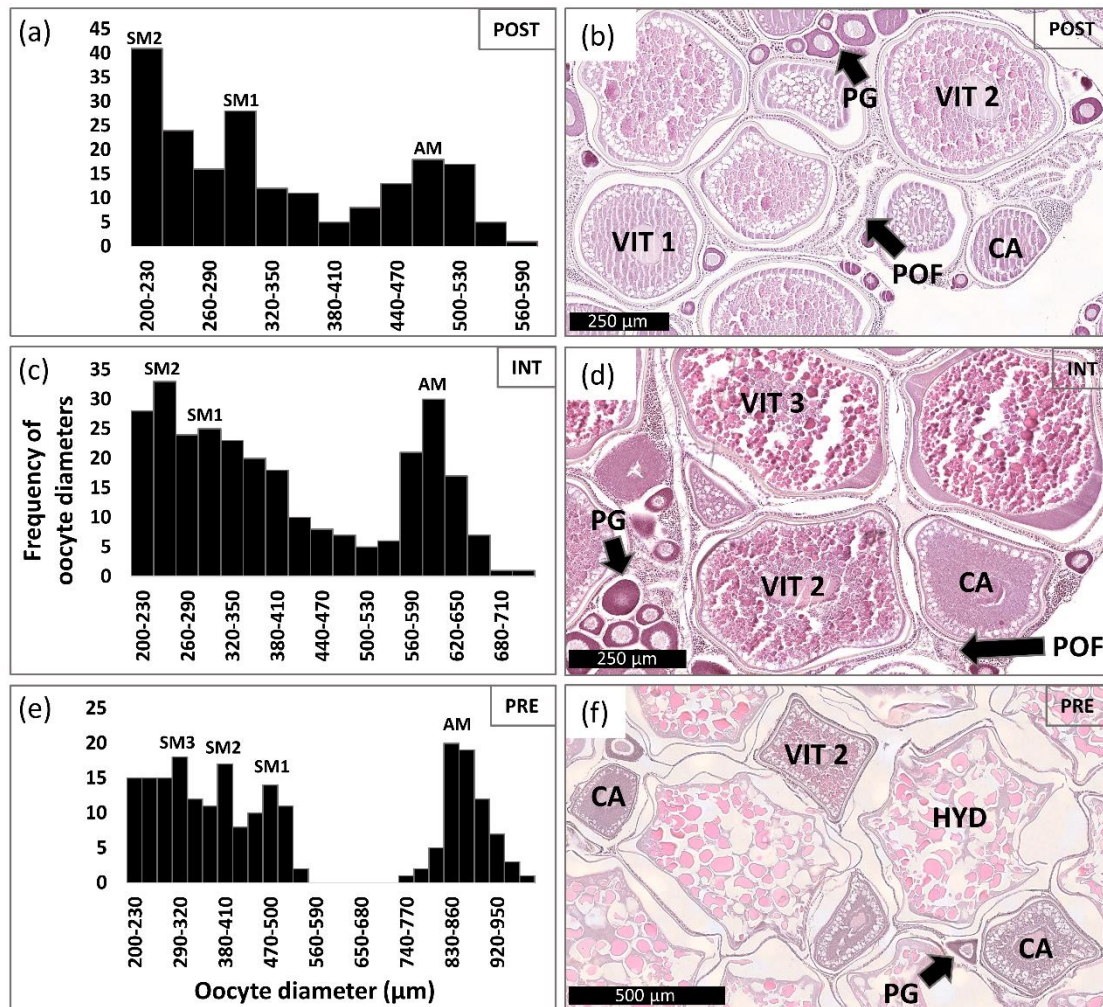
Figure 6.6. Size frequency distributions of oocytes at the secondary growth phase and the corresponding ovarian histological sections for *Alosa macedonica* females at different phases within the ovulatory cycle: (a–b) immediately or closely after ovulation/spawning (post-ovulatory; POST), (c–d) in-between two ovulation/spawning events (intermediate; INT), and (e–f) prior to ovulation (pre-ovulatory; PRE). AM = advanced mode, SM1 = first subsequent mode, SM2 = second subsequent mode, SM3 = third subsequent mode

Εικόνα 6.6. Κατανομή συχνοτήτων μεγεθών των ωοκυττάρων που βρίσκονται στη δευτερογενή φάση ανάπτυξης και η φωτομικρογραφία της αντίστοιχης ιστολογικής τομής της ωοθήκης για τρία άτομα του είδους *Alosa macedonica* που βρίσκονται σε διαφορετική φάση του κύκλου ωοτοκίας: (a-b) αμέσως ή λίγο μετά την ωορρηξία/ωοτοκία (post-ovulatory, POST), (c-d) μεταξύ δύο διαδοχικών συμβάντων ωορρηξίας/ωοτοκίας (intermediate, INT), και (e-f) πριν την ωορρηξία (pre-ovulatory, PRE). AM = η πιο προηγμένη ομάδα της κατανομής, SM1 = η δεύτερη πιο προηγμένη ομάδα, SM2 = η τρίτη πιο προηγμένη ομάδα, SM3 = η τέταρτη πιο προηγμένη ομάδα
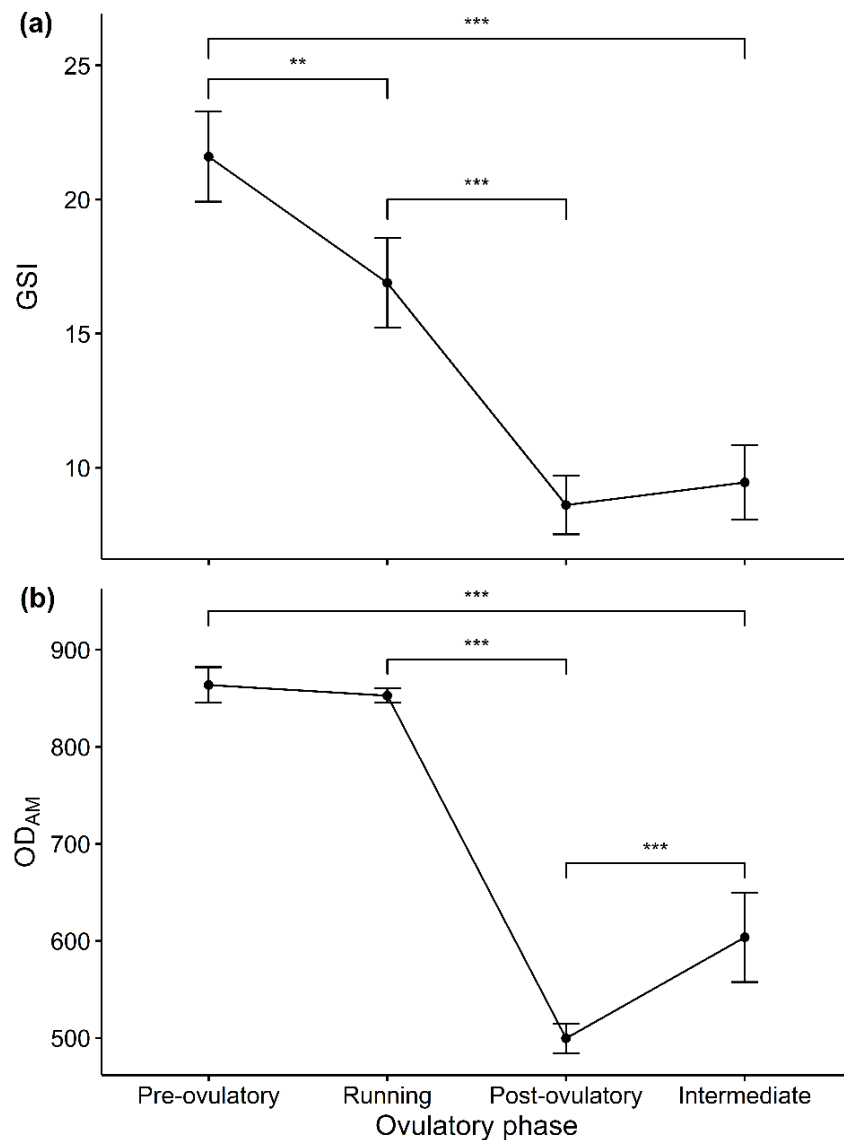
Figure 6.7. Line plot of mean values and confidence intervals of: (a) gonadosomatic index (*GSI*), and (b) diameter (in μm) of the advanced oocyte batch (OD_{AM}), for *Alosa macedonica* females at different phases within the ovulatory cycle: prior to ovulation (pre-ovulatory), during ovulation/spawning (running), immediately or closely after ovulation/spawning (post-ovulatory) and in-between two ovulation/spawning events (intermediate). Statistically significant pairwise comparisons of mean values are shown with asterisks ("**" for $P < 0.01$, "***" for $P < 0.001$)

Εικόνα 6.7. Μέσες τιμές και όρια εμπιστοσύνης για: (a) τον γοναδοσωματικό δείκτη (*GSI*), και (b) τη διάμετρο (σε μm) των ωοκυττάρων στο πιο προηγμένο στάδιο ανάπτυξης (OD_{AM}), για άτομα του είδους *Alosa macedonica* που βρίσκονται σε διαφορετική φάση του κύκλου ωοτοκίας: πριν την ωορρηξία (pre-ovulatory), κατά τη διάρκεια της ωορρηξίας/ωοτοκίας (running), αμέσως ή λίγο μετά την ωορρηξία/ωοτοκία (post-ovulatory) και μεταξύ δύο διαδοχικών συμβάντων ωορρηξίας/ωοτοκίας (intermediate). Οι στατιστικά σημαντικές διαφορές μεταξύ των μέσων τιμών σημειώνονται με αστερίσκο ("**" για $P < 0.01$, "***" για $P < 0.001$)

GSI and mean OD$_{AM}$ also exhibited a clear pattern, evidencing the continuity of ovarian dynamics within the ovulatory cycle (Fig. 6.7). Prior to ovulation (PRE females), *GSI* and OD$_{AM}$ were at their maximum values (mean *GSI* ± SD = 21.6 ± 3.3; mean OD$_{AM}$ ± SD = 864 ± 36 μm). Subsequently, both *GSI* and OD$_{AM}$ followed a decreasing trend and dropped to their minimum values (mean *GSI* ± SD = 8.6 ± 2.3; mean OD$_{AM}$ ± SD = 500 ± 33 μm) in POST females. As females approached readiness to ovulate again (INT females), both *GSI* and OD$_{AM}$ increased (mean *GSI* ± SD = 9.5 ± 1.5; mean OD$_{AM}$ ± SD = 604 ± 50 μm). Mean *GSI* and OD$_{AM}$ values were significantly different from one ovulatory phase to the next ($P < 0.05$; least significant difference, 95% confidence level). The only two exceptions were the lack of statistically significant difference in *GSI* between POST and INT females and in OD$_{AM}$ between PRE and RUN females ($P > 0.05$; least significant difference, 95% confidence level).

6.3.3 Fecundity type and timing of SG Recruitment

Macedonian shad displayed the indeterminate fecundity type, since SG recruitment occurred after the onset of spawning activity. The latter conclusion was supported by the lack of a hiatus in the OSFDs of all females, even those that had spawned at least twice by the time they were caught (c.f. Fig. 6.5). Additionally, early CA oocytes were observed in the ovarian histological sections of all actively spawning females.

SG recruitment occurred stepwise and in parallel with the ovulation/spawning of the advanced oocyte batch. RUN females had higher mean (± SD) *RF$_{200-320}$* values (411 ± 162 oocytes per g of W$_{ev}$) compared to PRE (323 ± 118 oocytes per g of W$_{ev}$) and INT (270 ± 99 oocytes per g of W$_{ev}$) females ($P < 0.05$; least significant difference, 95% confidence level; Fig. 6.8a). In addition, RUN females had higher mean (± SD) fecundity ratio (3.5 ± 0.9) than PRE (2.9 ± 0.6), POST (2.3 ± 0.8) and INT (2 ± 0.4) females ($P < 0.05$; least significant difference, 95% confidence level; Fig. 6.8b). The mean *RF$_b$* of PRE females used to estimate the fecundity ratio values was 415 oocytes per g of W$_{ev}$.
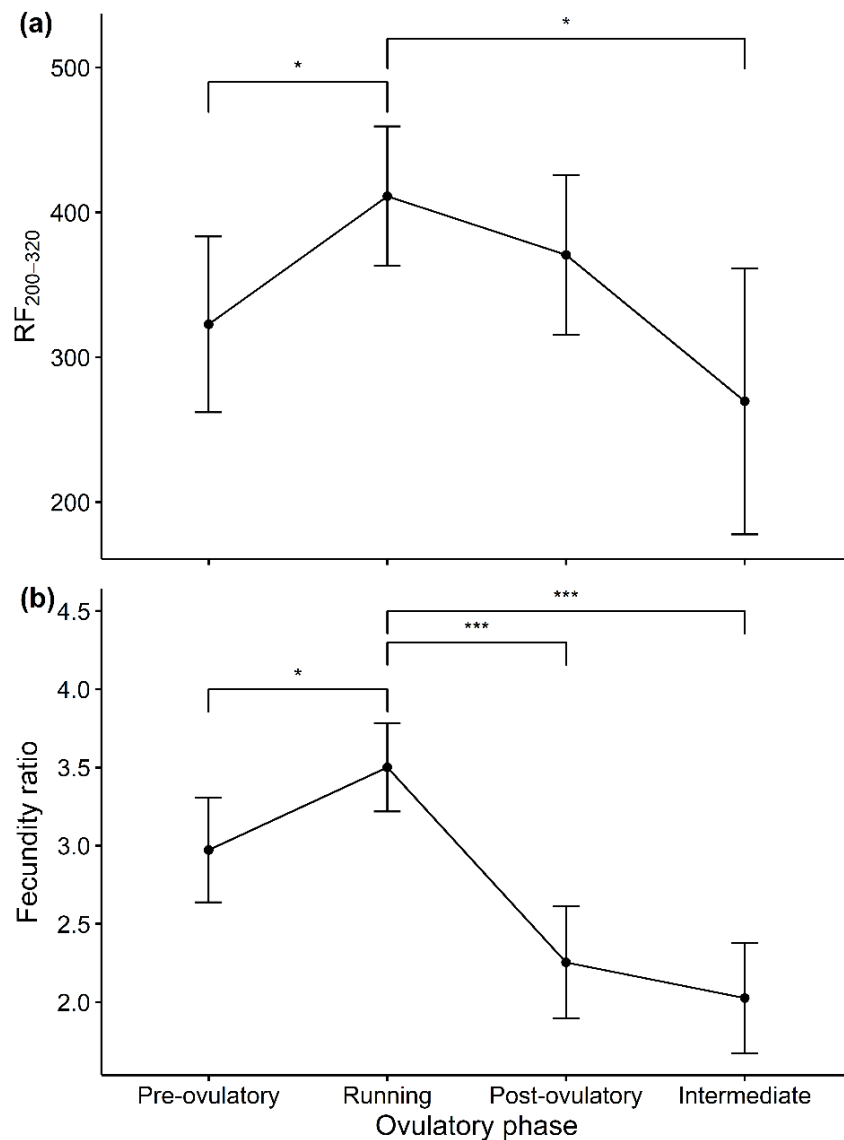
Figure 6.8. Line plot of mean values and confidence intervals of: (a) relative fecundity of small oocytes at the secondary growth phase, i.e., with diameter between 200 and 320 μm ($RF_{200-320}$), and (b) fecundity ratio (relative total fecundity/relative batch fecundity), for *Alosa macedonica* females at different phases within the ovulatory cycle: prior to ovulation (pre-ovulatory), during ovulation/spawning (running), immediately or closely after ovulation/spawning (post-ovulatory) and in-between two ovulation/spawning events (intermediate). Statistically significant pairwise comparisons of mean values are shown with asterisks ("*" for $P < 0.05$, "***" for $P < 0.001$)

Εικόνα 6.8. Μέσες τιμές και όρια εμπιστοσύνης για: (a) τη σχετική γονιμότητα των ωοκυττάρων με διάμετρο μεταξύ 200 και 320 μm ($RF_{200-320}$), και (b) το κλάσμα γονιμότητας (σχετική ολική γονιμότητα/σχετική γονιμότητα ομάδας), για άτομα του είδους *Alosa macedonica* που βρίσκονται σε διαφορετική φάση του κύκλου ωοτοκίας: πριν την ωορρηξία (pre-ovulatory), κατά τη διάρκεια της ωορρηξίας/ωοτοκίας (running), αμέσως ή λίγο μετά την ωορρηξία/ωοτοκία (post-ovulatory) και μεταξύ δύο διαδοχικών συμβάντων ωορρηξίας/ωοτοκίας (intermediate). Οι στατιστικά σημαντικές διαφορές μεταξύ των μέσων τιμών σημειώνονται με αστερίσκο ("*" για $P < 0.05$, "***" για $P < 0.001$)

6.4 Discussion

Batch spawning fishes with either determinate or indeterminate fecundity display ovulation cyclicity, i.e., processes occur within the ovulatory cycle to accomplish oocyte release in multiple sequential events during their spawning period. The ovarian processes that take place within the ovulatory cycle in indeterminate batch spawners include mainly the formation of the advanced oocyte batch to be spawned next and SG recruitment. The formation of the advanced batch has been frequently analyzed, whilst SG recruitment has often been overlooked and rarely examined in detail in a quantitative manner at the narrow temporal scale of the ovulatory cycle. In this chapter, cyclicity was evinced, and both the formation of the advanced batch and SG recruitment were analyzed within the ovulatory cycle in a multiple spawning fish with indeterminate fecundity type. The results reported herein enabled the detection of the timing of SG recruitment within the ovulatory cycle and revealed a temporal association between ovulation of the advanced oocyte batch and SG recruitment.

Ovulation cyclicity in multiple spawning fishes has been demonstrated in different ways, such as behavioral observations (Lowerre-Barbieri et al. 2008; McCartin et al. 2019) and analyses of hormone profiles (Bradford and Taylor 1987; Kobayashi et al. 1988; Murayama et al. 1994). Especially, cyclicity in ovarian processes has been evinced through different indices and in many species (Ganias et al. 2004; Murua and Motos 2006; Ferreri et al. 2016; Ganias et al. 2017). In case of Macedonian shad, it was shown in this chapter that ovarian processes were occurring within the ovulatory cycle by comparing several indices among four different ovulatory phases: the AM numerical formation and the OD_{AM}, the ovarian developmental stage, the modality of OSFDs and *GSI*. All these indices displayed specific patterns within the ovulatory cycle, and thus evinced the continuity of ovarian dynamics between sequential ovulation/spawning events. *GSI* and OD_{AM} followed an increasing trend from the completion of an ovulation/spawning event and prior to the next. The modality of OSFDs was getting more obvious and the AM was becoming more separated from the smaller oocytes as females were approaching ovulation. In addition, the developmental stage of the AM was more advanced in females that were prior to ovulation than in those that had just ovulated/spawned.

The two main ovarian processes that take place within the ovulatory cycle of indeterminate multiple spawners have been analyzed disproportionately. The formation of the advanced oocyte batch has been analyzed extensively (Murayama et al. 1994; Schaefer 1996; Ganias et al. 2004; Murua and Motos 2006; Ferreri et al. 2016; Ganias et al. 2017; Nyuii and Takasuka 2017), whilst detailed analyses of SG recruitment have been conducted at a far less extent. The studies focused on SG recruitment were either qualitative, resulting in detailed description of the sequential developmental oocyte stages prior and after the recruitment process (Shirokova 1977;

Uribe et al. 2016; Grier et al. 2018), or quantitative (Greer-Walker et al. 1994; Kjesbu et al. 2011; Schismenou et al. 2012; Serrat et al. 2019a). Most of these latter studies used advanced quantification techniques to analyze SG recruitment process mostly during broad temporal scales, such as the reproductive season and the spawning season (Kjesbu et al. 2011; Schismenou et al. 2012; Serrat et al. 2019a), or rarely during the narrower temporal scale of the ovulatory cycle (Schismenou et al. 2012). In this chapter, quantification of small-sized oocytes was conducted through a less informative – regarding the stages of oocytes – but equally effective approach.

Comparisons in recruitment intensity and fecundity ratio among females at different ovulatory phases revealed a clear pattern of SG recruitment. Females caught during ovulation/spawning had higher recruitment intensity and fecundity ratio compared to females at earlier or later ovulatory phases, revealing that a new batch of SG oocytes was recruited from the pool of PG reserves at a specific time-frame, during the ovulation of the advanced oocyte batch. The latter conclusion is corroborated by a previous report for another indeterminate batch spawning clupeid, European anchovy, *Engraulis encrasicolus* (Schismenou et al. 2012). More specifically, the latter study suggested that oocyte recruitment occurred in swift pulses triggered by the hydration of the spawning batch.

The temporal association between ovulation of the advanced oocyte batch and SG recruitment is in accordance with the concept of "dynamic equilibrium", which is one of the main features of the theoretical indeterminate fecundity type, where SG recruitment counterbalances the losses from spawning, and thus keeps the number of SG oocytes in the ovary stable (see Section 1.2). The concept of the theoretically continuous recruitment of small oocytes in parallel with spawning activity has been previously suggested for Northern anchovy *E. mordax* were mature ovaries were shown to contain a stable number of oocyte batches (Hunter and Leong 1981). Additionally, model simulations suggested that SG recruitment kept pace with spawning of the advanced oocyte batch, and thus sustained a balanced number of oocyte batches and total fecundity in indeterminate batch spawners (Ganias et al. 2015a). Finally, detailed analysis, through the oocyte packing density theory (Kurita and Kjesbu 2009), suggested that oocyte numbers in the ovaries were in a steady state, displaying an equilibrium also in European anchovy (Schismenou et al. 2012). In this chapter the concept of "dynamic equilibrium" was evinced within the ovulatory cycle and a temporal association between ovulation of the advanced oocyte batch and SG recruitment was reported. The latter association did not provide any justification of a cause-effect relationship between the two ovarian processes and future studies should focus on this matter.

CHAPTER 7: General discussion and conclusions

The different phases of oogenesis (oocyte production from oogonia, PG phase, SG phase, ovulation) have been analyzed disproportionately and at different temporal scales, leaving many of its aspects understudied or clouded, such as the regulation of fish fecundity type. In that respect, the principal aim of the present study was to describe how the fecundity type is shaped by analyzing different phases of oogenesis at different temporal scales and specific time-frames.

Species of the *Alosa* genus were analyzed in this study due to their variability in reproductive strategy – life-history form combinations. Semelparity and anadromy enabled sampling designs that associated location and reproductive stage, and thus analyses at different temporal scales (lifetime, seasonal) and specific time-frames (prior to, during and after the spawning activity). On the other hand, the existence of neighboring anadromous and landlocked populations of the same species provided the opportunity to test whether life-history form influences the fecundity type.

The present study aimed to answer four main scientific questions (Q1 – Q4) and managed to provide general conclusions for each one of them. Firstly, SG recruitment occurs stepwise both prior and during the spawning activity and regardless of the fecundity type (Q1). Particularly within the ovulatory cycle, SG recruitment occurs at a specific time-frame (Q4), in parallel with the ovulation of the advanced oocyte batch, as shown in the indeterminate multiple spawning Macedonian shad. Additionally, the universality of the theoretical indeterminate temporal pattern is challenged (Q2), since Blueback herring displayed an untypical indeterminate fecundity type. Finally, life-history form does not appear to be a decisive factor for regulating the fecundity type (Q3).

Q1. What is the role of oogonia and how SG recruitment occurs prior to the onset of spawning activity?

In chapter 3, pre-spawning females of the semelparous anadromous Allis shad population were analyzed to test the role of oogonia and the pattern of SG recruitment prior to the onset of spawning activity. Semelparity provided the opportunity to test whether oogonia and PG oocytes were depleted prior to spawning activity, as it is expected in fishes with such reproductive strategy (Ganias and Lowerre-Barbieri 2018). Even though oogonia were present in pre-spawning ovaries, new PG oocytes were not produced; result that is in accordance with the report of Wildner et al. (2013) that oogonial proliferation and entrance into meiosis decrease sharply during the spawning-capable phase. Additionally, PG reserves were depleted prior to spawning activity, and a stepwise SG recruitment pattern was revealed, where

females at a more advanced ovarian developmental stage had larger, fewer and distributed over a narrower size-range PG oocytes. Based on the latter results, a conceptual model describing the process of oogenesis in a semelparous fish, from the onset of SG recruitment (i.e., onset of reproductive period) till the final oocyte maturation, is provided in Figure 7.1.
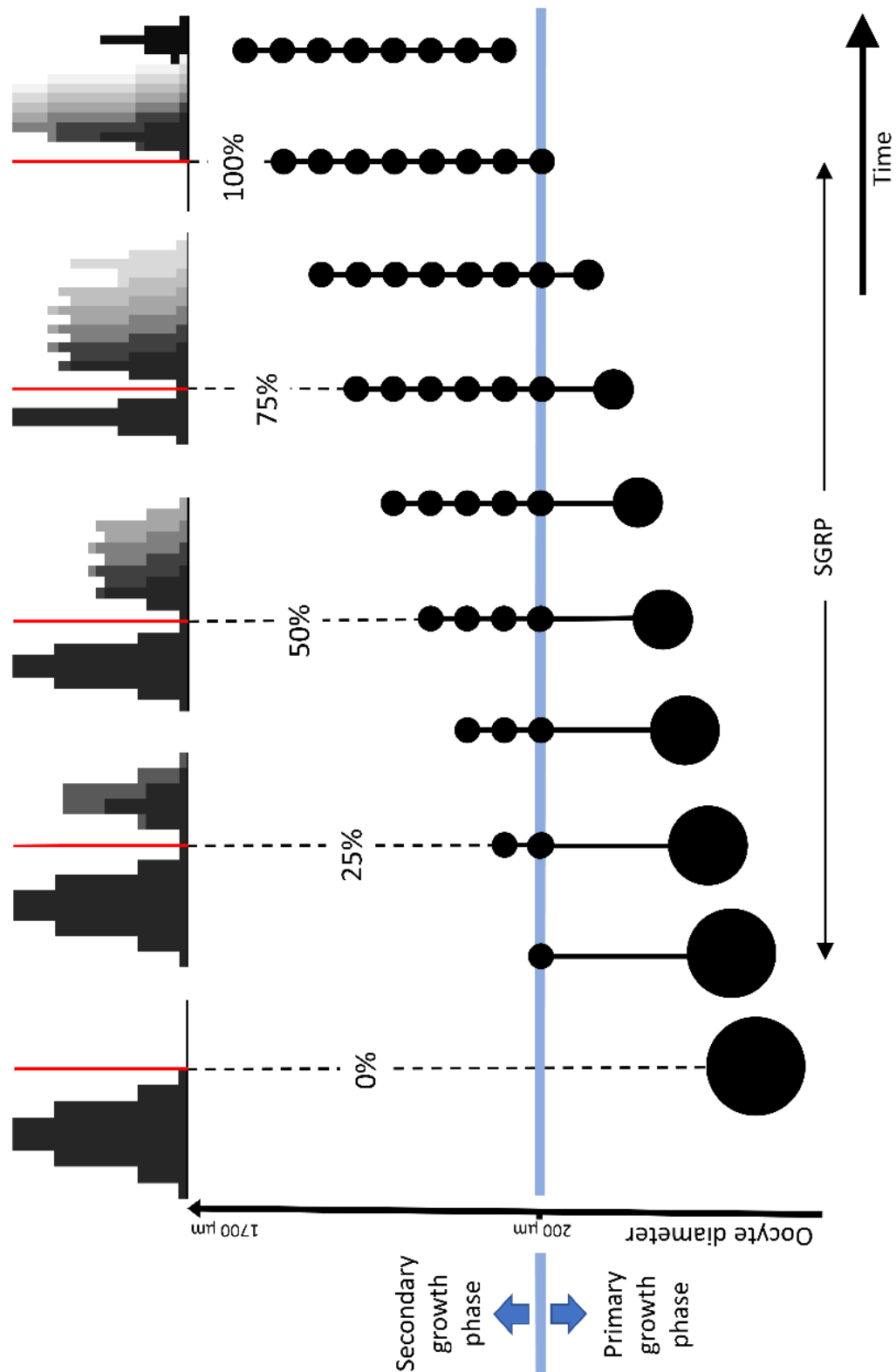
Figure 7.1. Conceptual representation – based on the analysis on *Alosa alosa* in chapter 3 – describing the oocyte recruitment pattern from the onset of the reproductive period and prior to spawning in a semelparous fish. Eight oocyte groups, each equivalent to the mean relative batch fecundity, are recruited from the pool of primary growth (PG) oocytes to the secondary growth (SG) phase. The recruitment to the SG phase occurs stepwise, until the PG reserves are depleted. With every oocyte batch recruited, the PG oocytes become fewer (indicated by the decreasing size of the PG circle), larger and distributed closer to the size threshold that differentiates the PG from the SG phase (PG circle progressively moves towards the 200 μm threshold). The PG reserves are depleted in imminent spawners in the final stages of oocyte maturation. The oocyte size frequency distributions in the upper panel are "snapshots" of ovarian development in different phases of the SG recruitment process (0, 25, 50, 75, 100% of completion of the SG recruitment). Note: PG reserves are not replenished by oogonia in the latter two distributions. SGRP = time period of SG recruitment; red vertical lines indicate the size threshold between PG and SG phase

Εικόνα 7.1. Απεικόνιση της διαδικασίας εισδοχής των ωοκυττάρων από την πρωτογενή (PG) στη δευτερογενή φάση (SG) ανάπτυξης και της ανάπτυξής τους, από την έναρξη της αναπαραγωγικής περιόδου και μέχρι πριν την έναρξη της περιόδου ωοτοκίας, σε ένα ψάρι που ολοκληρώνει έναν μοναδικό αναπαραγωγικό κύκλο κατά τη διάρκεια της ζωής του. Το μοντέλο βασίστηκε στα αποτελέσματα της ανάλυσης για το είδος *Alosa alosa* που περιγράφεται στο κεφάλαιο 3. Οκτώ ομάδες ωοκυττάρων, κάθε μία αποτελούμενη από τόσα ωοκύτταρα όσα η μέση σχετική γονιμότητα ομάδας, μεταβαίνουν από την PG στην SG φάση ανάπτυξης. Η διαδικασία της εισδοχής γίνεται τμηματικά, μέχρι να εξαντληθεί ο αριθμός των PG ωοκυττάρων. Με την εισδοχή κάθε ομάδας ωοκυττάρων στην SG φάση ανάπτυξης, τα PG ωοκύτταρα μειώνονται σε αριθμό (όπως υποδηλώνει το μειούμενο μέγεθος του μαύρου κύκλου), αλλά αυξάνουν σε μέγεθος και κατανέμονται πλησιέστερα στο όριο μεγέθους που διαχωρίζει την PG από την SG φάση (ο μαύρος κύκλος «κινείται» προς το όριο των 200 μm). Ο αριθμός των PG ωοκυττάρων μηδενίζεται σε άτομα που βρίσκονται στο τελικό στάδιο της ανάπτυξης των ωοκυττάρων τους και πριν την ωοτοκία. Οι κατανομές συχνοτήτων μεγεθών των ωοκυττάρων στο επάνω μέρος της εικόνας σχηματοποιούν διαφορετικά στάδια της πορείας ανάπτυξης των ωοκυττάρων (στο 0, 25, 50, 75 και 100% της ολοκλήρωσης της εισδοχής των PG ωοκυττάρων στην SG φάση ανάπτυξης). Σημείωση: Ο αριθμός των PG ωοκυττάρων δεν αναπληρώνεται από ωογόνια, όπως φαίνεται στις δύο τελευταίες κατανομές. SGRP = περίοδος εισδοχής των PG ωοκυττάρων στην SG φάση ανάπτυξης, οι κόκκινες κατακόρυφες γραμμές υποδεικνύουν το όριο των 200 μm

Q4. Does SG recruitment occur at a specific time-frame within the ovulatory cycle?

The occurrence of SG recruitment in a stepwise manner was proven also in chapter 6, where the analysis of ovarian dynamics was confined within the fine temporal scale of the ovulatory cycle of the indeterminate multiple spawning Macedonian shad. In fact, SG recruitment was shown to occur at a specific time-frame, in parallel with the ovulation of the advanced oocyte batch. Based on this conclusion, a conceptual model of the ovarian dynamics within the ovulatory cycle of an indeterminate spawner was designed (Fig. 7.2). In summary, GSI and OD_{AM} fluctuate between relatively constant minimum and maximum values within the ovulatory cycle, while the synchronization of SG recruitment and ovulation of the advanced oocyte batch keeps RF_t and number of SG oocyte batches in the ovary stable. The latter result confirms the existence of a "dynamic equilibrium" which is among the main features of the theoretical temporal pattern of indeterminate spawners. A similar temporal association, where SG recruitment was also shown to occur in a stepwise manner and to be triggered by the hydration process of the advanced oocyte batch, as well as the existence of a "dynamic equilibrium" during the spawning season, have been reported in another iteroparous indeterminate clupeid, European anchovy (Schismenou et al. 2012).

Q2. Does oogenesis, prior, during and after the spawning activity, in different congeneric species, conform with the theoretical temporal patterns that currently describe the ovarian dynamics of determinate and indeterminate spawners?

In chapters 4 and 5, the ovarian dynamics of three different *Alosa* populations were analyzed prior, during and after the spawning activity to test whether their fecundity types conform with the theoretical temporal pattern of either a determinate or an indeterminate spawner. In specific, an iteroparous anadromous Blueback herring, an iteroparous anadromous Alewife and an iteroparous landlocked Alewife population were analyzed. The latter two alosines displayed characteristics of a typical indeterminate spawner, while the analysis on the former revealed an untypical indeterminate fecundity type. Typically, in indeterminate spawners, SG recruitment counterbalances the spawning of sequential oocyte batches during the spawning activity, keeping SG oocyte number in a "dynamic equilibrium" (Hunter and Leong 1981; Hunter et al. 1985; Schismenou et al. 2012; Ganias et al. 2015a). Subsequently, at the end of spawning activity, the surplus of unspawned SG oocytes falls massively into atresia and be reabsorbed (Macewicz and Hunter 1994; Kjesbu 2009; Ganias et al. 2015a) in a process known as "mopping up" (Wallace and Selman 1981; Kjesbu 2009). On the contrary, chapter 4 and recent studies on Atlantic horse mackerel (Ganias et al. 2017) and Gulf menhaden (Brown-Peterson et al. 2017) showed that SG recruitment does not always keep pace with losses from spawning throughout the

spawning season, and lack of massive atresia at the end of the spawning activity. The accumulated information on divergence from typicality indicate that the currently accepted features of the indeterminate fecundity type might need to be reevaluated. Revision of the characteristics and redefinition of the indeterminate fecundity type seem timely. Recently, Serrat et al. (2019a) suggested that SG recruitment occurs at an earlier oocyte stage than it is currently believed in European hake, *Merluccius merluccius*, and thus definitions of PG and SG phases should probably be reassessed as well.

> **Q3.** Is life-history form among the parameters that influence oogenesis and the fecundity type regulation?

The results presented in chapters 3 to 5 corroborate previous statements that fecundity type is a dynamic trait (Ganias 2013). Several factors have been suggested to regulate the fecundity type, such as latitude (Hunter et al. 1985; Witthames and Greer-Walker 1995; McBride et al. 2016), timing of spawning (Rijnsdorp and Witthames 2005; Kjesbu 2009; Ganias and Lowerre-Barbieri 2018) and breeding strategy (Rijnsdorp and Witthames 2005; Kjesbu and Witthames 2007; Kjesbu 2009; Armstrong and Witthames 2012). In specific, indeterminacy has been linked with tropical and temperate environments, extending spawning during summer and income breeding, while determinacy is allegedly displayed by fishes inhabiting northern and boreal habitats, that spawn during narrow time-frames in winter-time and are capital breeders. Testing the validity of these theories was out of the scope of the present study. However, the untypical indeterminate fecundity type described in Blueback herring in chapter 4, where SG recruitment intensity and fecundity tapered as spawning progressed and massive atresia did not occur at the end of the spawning activity, may have been regulated by the energy reserves, and thus the breeding strategy of the surveyed population. Anadromous Blueback herring is known to expend substantial energy during its upstream migration and spawning activity (Crawford et al. 1986; Simonin et al. 2007; McBride et al. 2010), and thus the gradually decreasing oocyte production and release may follow the depletion of the energy reserves. Anadromous Blueback herring, Alewife and American shad are suitable models to test for influence of breeding strategy and energy management on fecundity type regulation, since they display varying feeding habits at different habitats and environmental conditions (Creed 1985; Walter and Olney 2003; Simonin et al. 2007), and such studies should be conducted in the future.
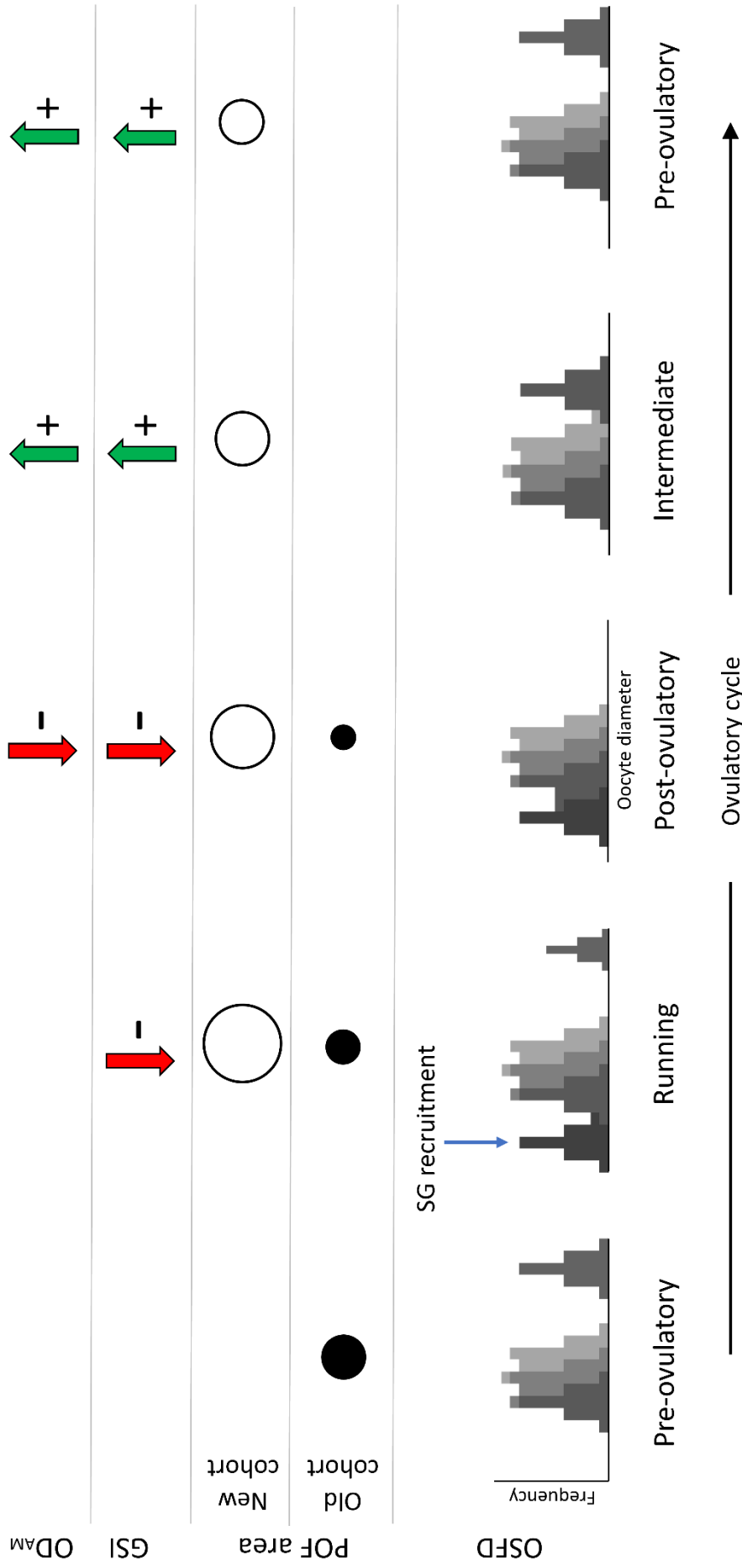
Figure 7.2. Conceptual model of the ovarian dynamics within the ovulatory cycle in an indeterminate batch spawning fish. Prior to ovulation (pre-ovulatory phase), gonadosomatic index (*GSI*) and diameter of the most advanced oocytes (OD_{AM}) are at their maximum values and any postovulatory follicles (POF) present originated from a previous spawning event (old POF cohort), are already partially absorbed, and thus have small area. In parallel with ovulation/spawning of the advanced batch (running phase), a new oocyte batch is recruited from the primary growth to the secondary growth phase (SG recruitment), resulting in an oocyte size frequency distribution (OSFD) with an additional mode. Concurrently, *GSI* begins to decrease and old POF continue to shrink, while a new POF cohort is forming. Immediately or closely after ovulation/spawning (post-ovulatory phase), the OSFD is comprised by several overlapping modes, *GSI* drops to its lowest values, old POF continue to decrease in size and new POF start to degenerate. As females approach readiness to ovulate/spawn again (intermediate phase), the advanced mode becomes gradually separated in the OSFD, *GSI* and OD_{AM} start to elevate again and old POF absorption is likely complete, while new POF degeneration continues. The OSFDs in the lower panel are "snapshots" of the secondary growth phase of the ovarian development for the different phases within the ovulatory cycle

Εικόνα 7.2. Απεικόνιση της δυναμικής της ωοθήκης εντός του κύκλου ωοτοκίας σε ένα ψάρι με ακαθόριστο πρότυπο γονιμότητας και στρατηγική πολλαπλής απόθεσης αυγών. Πριν από την ωοτοκία (pre-ovulatory phase) ο γοναδοσωματικός δείκτης (*GSI*) και η διάμετρος των ωοκυττάρων στο πιο προηγμένο στάδιο ανάπτυξης (OD_{AM}) εμφανίζουν τις μέγιστες τιμές τους και όσα κενά ωοθυλάκια (POF) είναι παρόντα έχουν προκύψει από προηγούμενη πρόσφατη ωοτοκία (old POF cohort), έχουν ήδη αποδιοργανωθεί μερικώς, και συνεπώς έχουν μικρό εμβαδό στην ιστολογική τομή της ωοθήκης. Παράλληλα με την ωορρηξία/ωοτοκία της πιο προηγμένης ομάδας ωοκυττάρων (running phase), μία νέα ομάδα ωοκυττάρων μεταβαίνει από την πρωτογενή στη δευτερογενή φάση ανάπτυξης (SG recruitment), με αποτέλεσμα η κατανομή συχνοτήτων μεγεθών των ωοκυττάρων (OSFD) να εμφανίζει μία επιπλέον ομάδα σε σχέση με την προηγούμενη φάση του κύκλου ωοτοκίας. Ταυτόχρονα, ο *GSI* αρχίζει να μειώνεται και τα POF από προηγούμενη ωοτοκία συνεχίζουν να συρρικνώνονται, ενώ νέα POF (old POF cohort) εμφανίζονται. Αμέσως ή λίγο μετά την ωορρηξία/ωοτοκία (post-ovulatory phase), η OSFD αποτελείται από αλληλεπικαλυπτόμενες ομάδες ωοκυττάρων, ο *GSI* πέφτει στις χαμηλότερες τιμές του, τα παλιά POF συνεχίζουν να αποδιοργανώνονται και τα νέα POF αρχίζουν επίσης να συρρικνώνονται. Καθώς πλησιάζει η επόμενη ωορρηξία/ωοτοκία (intermediate phase), η πιο προηγμένη ομάδα ωοκυττάρων στην OSFD αρχίζει να ξεχωρίζει από τις υπόλοιπες, οι τιμές του *GSI* και της OD_{AM} αρχίζουν να αυξάνονται και η απορρόφηση των παλιών POF έχει πιθανότατα ολοκληρωθεί, ενώ αυτή των νέων POF συνεχίζεται. Οι OSFDs στο κάτω μέρος της εικόνας σχηματοποιούν τη δυναμική της ωοθήκης σε διαφορετικές φάσης του κύκλου ωοτοκίας

In this study, the factor that was tested as a possible regulator of fecundity type was the life-history form. Three anadromous and two landlocked *Alosa* populations were analyzed and their fecundity type was defined, but no clear pattern was observed. In fact, the two neighboring Alewife populations analyzed in chapter 5 displayed many similarities in their indeterminate fecundity type. However, permanent residency in freshwater may lead to indeterminacy, since both landlocked alosines analyzed herein continued to produce new SG oocytes in parallel with their spawning activity (chapters 5 and 6). On the contrary, anadromy does not favor any of the two fecundity types, since both determinacy (chapter 3) and indeterminacy were identified (chapters 4 and 5). The latter conclusion is corroborated by recent studies that reported determinacy in some anadromous alosines (McBride et al. 2016), but indeterminacy in others (Murauskas and Rulifson 2011; Hyle et al. 2014; Ganias et al. 2015b). Thus, life-history form does not appear to be a decisive factor for regulating the fecundity type; but reproductive strategy might be. The only population that was defined as a determinate spawner was the semelparous Allis shad in Mondego River (chapter 3). In this river, apart from the semelparous anadromous Allis shad, an iteroparous landlocked population of the species exists in the Aguieira reservoir, above an unsurpassable dam (Collares-Pereira et al. 1999). Hence, a comparison of the fecundity type between these two populations could test the hypothesis that semelparity and iteroparity favor determinacy and indeterminacy, respectively.

Even though the analysis of the oocyte release strategy was not among the main scientific questions, the present study concluded that multiple spawning is – so far – universal in alosines, regardless of the life-history form or the reproductive strategy. It has been indicated for semelparous anadromous Allis shad (chapter 3; Mota 2014), and evidenced for semelparous and iteroparous anadromous American shad (Olney et al. 2001; Olney and McBride 2003; McBride et al. 2016) and iteroparous anadromous Blueback herring (chapter 4; McBride et al. 2010), Alewife (chapter 6; Ganias et al. 2015b; Marjadi et al. 2019), Hickory shad (Murauskas and Rulifson 2011), Alabama shad, *Alosa alabamae* (Mettee and O'Neil 2003; Grice et al. 2014), and Twaite Shad (Aprahamian et al. 2003b; Pina et al. 2003). Moreover, the landlocked iteroparous Alewife (chapter 5) and Macedonian shad (chapter 6) were shown to release their oocytes in multiple events. Display of multiple spawning by different life-history forms of the same fish species, such as in Alewife, has been reported in other genera as well. For instance, both landlocked and amphidromous Ayu *Plecoglossus altivelis* populations have been classified as multiple spawners (Matsuyama and Matsuura 1985; Shimizu et al. 2005, 2007). Several advantages may derive from multiple spawning that could explain its systematic display by alosines, such as release of more oocytes than would be permitted by the fish body cavity, lower risk of an unsuccessful spawning period due to labile environmental conditions, broader offspring distribution and spread risk of predation on eggs and larvae over a longer

period (Nikolsky 1969; Lambert and Ware 1984; Burt et al. 1988; McEvoy and McEvoy 1992; Murauskas and Rulifson 2011).

Finally, oogenesis and fecundity type analyses in this study were approached in a novel way. Commonly, analyses of fecundity type are depended on a sampling scheme that covers the time period before the onset of spawning activity and the whole spawning season of the surveyed stock. However, the latter prerequisite is usually overlooked, leading occasionally to mixed results and equivocal fecundity type assessments. Moreover, the "classic" criteria for defining the fecundity type were built to test for temporal patterns throughout the spawning season, such as hiatus in the OSFDs and massive atresia at the end of the spawning season. This strategy assumes spawning synchronicity at the population level, meaning that all females of the contextually population spawn in parallel, and thus are at the same spawning phase at any time. However, the latter assumption is commonly violated and leads to misinterpretations. On the contrary, in this study, the spawning phase of each female was accurately identified, and thus pre-spawning females were clearly distinguished from the actively spawning and the spent females. To do so, ovaries of all females included in this study were examined in detail for markers of recent spawning activity. Specifically, POFs were identified and classified into cohorts and their persistence was examined. In all cases, POF presence was a clear-cut criterion for distinguishing active spawners from pre-spawners and occasionally to separate those females that had spawned only once from those that had spawned at least twice. Upon classification of females in pre-spawners, active – occasionally early and late – spawners and spent females, the occurrence of newly recruited SG oocytes was tested for each spawning phase. Oocytes at early CA stages in the histological sections and oocytes with diameters between 200 and 320 μm in ovarian whole-mount subsamples were regarded as indications of recent SG recruitment, and thus their presence in active spawners and spent females would evince indeterminate fecundity type. The occurrence of massive atresia in spent females was used as a supplementary criterion of indeterminacy.

This alternative approach pursued herein does not require the assumption of spawning synchronization at the population level and each of the implemented criteria for defining fecundity type can be directed to females at the appropriate spawning phase (e.g., search for massive atresia only at the spent females). This methodology was proven valid and provided conclusive results for different populations, and thus could be introduced in the routine fecundity type assessments. However, its implementation may not be universally applicable, since it was heavily dependent on the persistence of POFs for at least the time interval between two sequential spawning events to distinguish active spawners from pre-spawners. POF degeneration period lasted longer that the spawning interval in this study, but POFs can be highly transitory and be absorbed in a few hours in populations inhabiting tropical environments (Takita et al. 1983; Hunter et al. 1986).

In summary, this study provided new insight into the oogenesis in fishes and the regulation of their fecundity type by analyzing – at various temporal scales and time-frames – the ovarian dynamics of several congeneric species displaying different reproductive strategies and life-history forms, and inhabiting variable and spatially unrelated environments. Firstly, the results presented herein show that the distinction between the two fecundity types is valid. However, some of the current criteria for making this distinction need to be reevaluated, such as the drop of relative total fecundity during the spawning activity. In addition, an alternative approach in analyzing the fecundity type was suggested to overcome current drawbacks and assumptions that occasionally lead to inconclusive results; it is proposed that the spawning phase of each female is accurately defined prior to the implementation of any criteria. The second major outcome of this study was that the indeterminate fecundity type does not display a universal pattern, but instead its aspects may vary among different populations. Another significant contribution of this study was the description of several steps of oogenesis; PG oocytes were not produced from oogonia and were recruited into the SG phase following a specific pattern in semelparous pre-spawning females, and SG recruitment occurred in parallel with the ovulation of the advanced batch in iteroparous multiple spawning females. Finally, fish fecundity type seemed to be regulated by several factors, such as reproductive strategy and energy reserves, but a clear influence of life-history form that promotes indeterminacy or indeterminacy was not observed.

The knowledge of fecundity type is imperative in applied fisheries reproductive biology, but the confinement of such analyses mainly on exploited marine stocks may have led to assumptions and generalizations. It is, however, clear that aspects of fecundity type are highly flexible and variant, especially in anadromous or landlocked populations that face more challenges than marine stocks, and that the analysis on the drivers that lead to the one type or the other should be investigated further. It is the author's impression that the current study contributed in unraveling the complexities of the process of oogenesis and fish fecundity type regulation, and, hopefully, that the results presented herein will assist in improving the utilization of this reproductive trait in applied fisheries reproductive biology.

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Annexes

Annex 1

Postovulatory follicles (POFs) are ovarian markers indicating recent spawning activity and they are consisted of the follicular layers that remain in the ovary after the release of the ovum during spawning. Upon their formation, POFs deteriorate gradually until their complete absorption (Fig. A1.1). They can be transitory and last from a few hours (Takita et al. 1983; Hunter et al. 1986) to a few days (Santos et al. 2005; Ganias et al. 2007) or persist for months (Zamarro et al. 1993; Saborido-Rey and Junquera 1998) in the ovaries; their degeneration rate is influenced by the ambient water temperature (Hunter and Macewicz 1985a; Fitzhugh and Hettler 1995; Ganias et al. 2007; Ganias 2012). New POFs share the following characteristics (Hunter and Goldberg 1980; Hunter and Macewicz 1985a; Ganias et al. 2007): (1) convoluted irregular shape with folds or loops, (2) a lumen containing some granular or particulate material, (3) a definite granulosa epithelial cell layer lining the lumen, (4) linearly arranged granulosa cells of cuboidal or columnar shape which contain a prominent nucleus, (5) a definite thecal connective tissue layer with blood capillaries, and (6) no signs of follicle degeneration. Older POFs display structural changes; they appear significantly shrunken having a semi-rectangular shape and the granulosa layer has lost its convoluted appearance and forms a single layer. As POF degeneration continues, their shape becomes triangular and the granulosa layer becomes thinner until only remnants are present as residual vacuoles.
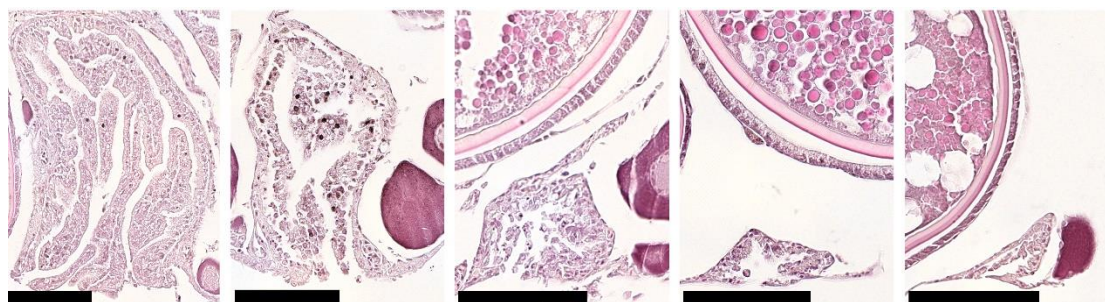

Figure A1.1. From left to right: sequential phases of postovulatory follicle degeneration in *Alosa aestivalis*. Scale bars: 100 μm

Εικόνα Α1.1. Από αριστερά προς τα δεξιά: διαδοχικά στάδια αποδιοργάνωσης κενών ωοθυλακίων στο είδος *Alosa aestivalis*. Γραμμές κλίμακας: 100 μm

Annex 2

Atresia is the process of oocyte degeneration and is divided into four stages (Lambert 1970; Hunter and Macewicz 1985b):

1) During the early phase of alpha (α) atresia (Fig. A2.1 a-b) the nucleus and the zona radiata start to disintegrate. Consequently, granulosa cells of the follicle enlarge and invade the degenerating oocyte. Yolk liquifies and becomes phagocytized by the invading granulosa cells. The cytoplasm is also resorbed by the granulosa cells. Numerous blood capillaries and vessels are observed in the thecal layer, which does not proliferate or invade the oocyte. The alpha stage ends when resorption of the oocyte is complete

2) In beta (β) atretic stage (Fig. A2.1 c) occurs the major degeneration and resorption of the follicle, which is a compact structure composed of numerous disorganized granulosa cells with pyknotic nuclei surrounded by a thin layer of thecal cells and blood vessels. Large intracellular cavities may also be observed

3) Atretic follicle in the gamma (γ) stage has decreased significantly in size. The granulosa cells contain flocculent material of yellow-brown shade and nuclei of irregular shape and are surrounded by fewer thecal cells and blood vessels than in the β atretic stage

4) Follicle in the delta (δ) stage of atresia has decreased even more in size. Dark yellow-brown pigments are present inside the remainder granulosa cells. Thecal cells and blood vessels no longer surround the granulosa cells
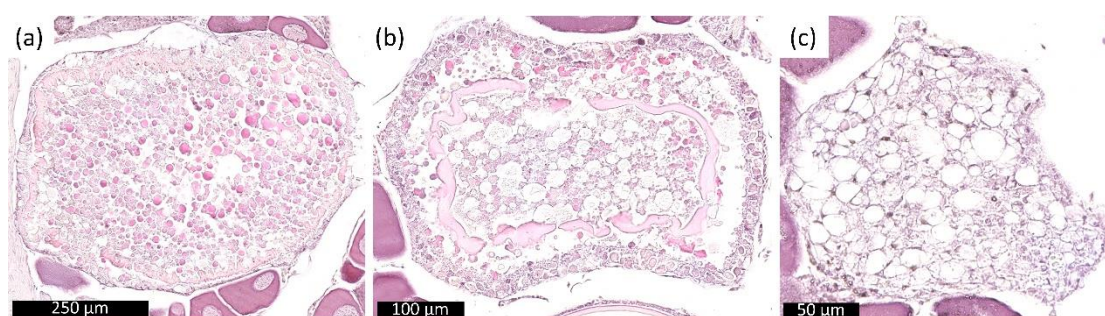

Figure A2.1 Ovarian photomicrographs of *Alosa aestivalis* showing: (a-b) atretic oocytes at the alpha stage, and (c) an atretic follicle at the beta stage
Εικόνα Α2.1. Φωτομικρογραφίες ιστολογικών τομών ωοθηκών ατόμων του είδους *Alosa aestivalis* που απεικονίζουν: (a-b) ωοκύτταρα στο άλφα στάδιο ατρησίας, και (c) ωοθυλάκιο στο βήτα στάδιο ατρησίας

Annex 3

The "stereological" or Weibel method (Weibel et al. 1966) is applied to estimate the size of each oocyte batch, i.e., the number of oocytes at each developmental stage. It is implemented on the photomicrographs and is based on the Delesse principle stating that the fractional volume (V_i) of a component (here, each oocyte batch) is proportional to its fractional cross-sectional area. The V_i of oocyte batch i is estimated by utilizing a standard grid that is overlaid to the photomicrograph; the number of points that hit any oocytes of batch i is counted and then divided by the total number of points within five counting fields of specified area (more details are provided by Emerson et al. 1990) (Fig. A3.1). The number of oocytes in batch i per volume (Nv_i) is estimated through the following formula:

$$Nv_i = \frac{K}{\beta} \times \frac{N\alpha_i^{\frac{3}{2}}}{V_i^{\frac{1}{2}}}$$

where β and K are coefficients reflecting oocyte shape and size distribution, respectively, and Na_i is the number of oocytes in batch i per unit area. The β_i of oocyte batch i is calculated from the mean diameter of 10 of its oocytes in which nuclei are visible. K_i is also estimated through the diameters of oocytes of batch i by using the formulas reported by Emerson et al. (1990).

Subsequently, fecundity of oocyte batch i (F_i) is estimated by multiplying Nv_i with the ovary volume. Total fecundity (i.e., total number of oocytes at the secondary growth phase; F_t) is calculated by summing the F_i values of all oocyte batches. Relative fecundity of oocyte batch i and relative total fecundity values are estimated by dividing F_i and F_t with eviscerated weight.
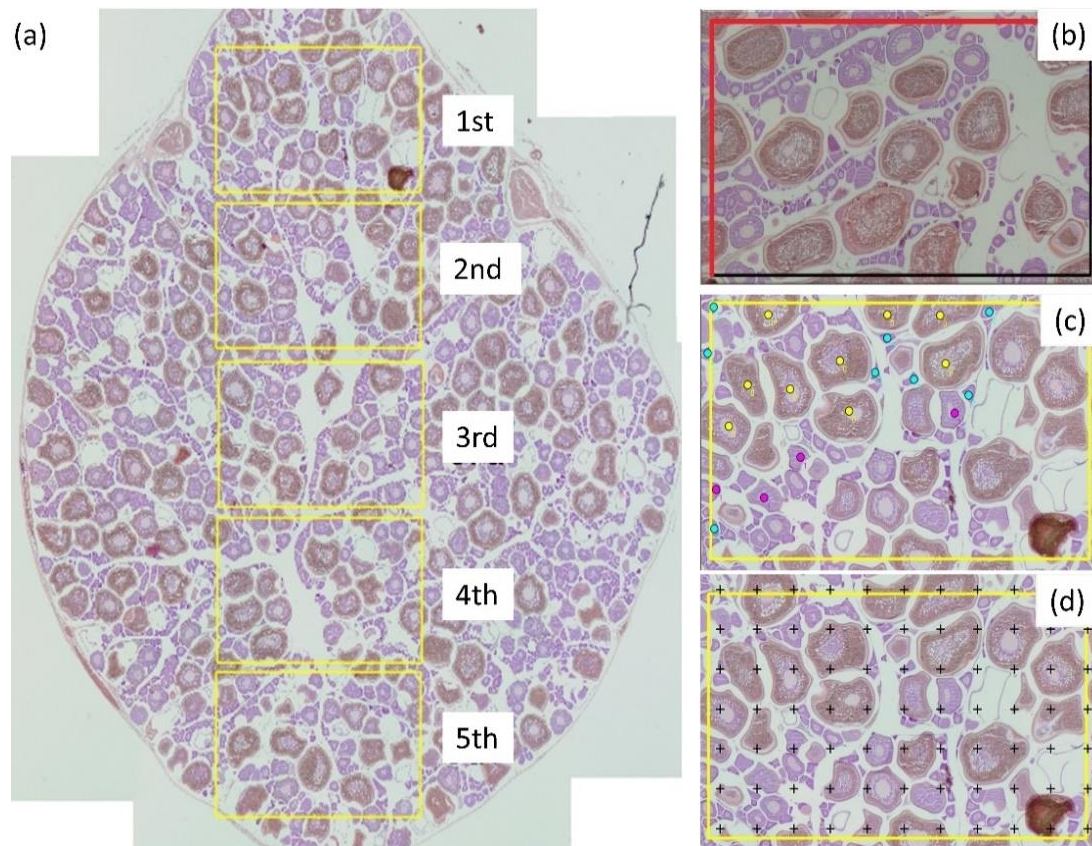
Figure A3.1 Sequential steps of the Weibel method: (a) superimposed frames on an ovarian photomicrograph, (b) determination of positive (red) and negative (black) sides of each frame, (c) identification of the oocyte developmental stages, and (d) superimposed grid

Εικόνα Α3.1. Διαδοχικά στάδια της μεθόδου Weibel: (a) τοποθέτηση πλαισίων μέτρησης επί μιας φωτομικρογραφίας ιστολογικής τομής ωοθήκης, (b) ορισμός θετικών (κόκκινο χρώμα) και αρνητικών (μαύρο χρώμα) πλευρών κάθε πλαισίου μέτρησης, (c) αναγνώριση των διαφορετικών σταδίων ανάπτυξης των ωοκυττάρων, και (d) τοποθέτηση πλέγματος σταυρών επί της φωτομικρογραφίας

Annex 4

The whole-mount process enables the preparation of ovarian subsamples of known weight for oocyte size and number estimations which can lead to approximations for the whole ovary (Ganias et al. 2010; Ganias et al. 2014b). Before any estimations, comparisons should be made among the anterior, middle and posterior parts of both right and left lobes of the ovary to determine if location within the ovary affects the size and number of oocytes[1].

The first step in whole-mount process includes cutting an ovarian subsample; the tissue weight may range (e.g., from 0.04 to 0.5 g) depending on the species, the ovarian developmental stage and the dimensions of the visual field during the microscopic processing (the rule of thumb is to have ~200 oocytes of the most-advanced stage; Hunter et al. 1985). Afterwards, the subsample is weighed to the nearest 0.001 g, and oocytes are separated ultrasonically and washed into a sieve (e.g., 150 μm) to discard the small-sized oocytes. The remainder oocytes are stained with hematoxylin to enhance the opacity of oocytes, spread on a monolayer and any membranes or other non-oocyte material are removed to maximize oocyte visibility.

The idea of increasing oocyte opacity through staining is not new, since several pigments (rose bengal, periodic acid-schiff, eosin, toluidine blue) have been tested in previous studies (Kennedy et al. 2007; Witthames et al. 2009). However, the staining process using these pigments is time-consuming (up to 24 hours), and/or the colored oocytes need to be rinsed frequently. In addition, the effectiveness of these pigments is biased by the species analyzed and/or the oocyte developmental stage. On the contrary, hematoxylin has been proven to enhance the opacity of oocytes in all developmental stages during routine whole mount procedures (Mouchlianitis and Ganias 2018). Staining with hematoxylin is almost instantaneous and all the histological structures (oocytes, membranes, tissue remnants) acquire similar color shade (purple). The addition of hematoxylin does not alter the follicular size measurements of the oocytes, while leaching out does not occur after the staining. This inexpensive and labor-efficient process could improve significantly the total fecundity estimations and the analyses of previtellogenic ovarian dynamics for assessment purposes within applied fisheries reproductive biology.

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[1] This step was skipped in this study, since such differences have not been detected in several alosines (Mayo 1974; Loesch and Lund 1977; Olney et al. 2001; Olney and McBride 2003; Grice et al. 2014; Sullivan et al. 2019) and other clupeids (Hunter et al. 1985; Sanz and Uriarte 1989; Pérez et al. 1992; Ganias et al. 2004)

Annex 5

The semiautomated, image-based particle analysis enables oocyte counting and estimates of oocyte diameters and shape descriptors, such as cyclicity (Thorsen and Kjesbu 2001; Ganias et al. 2010; Ganias et al. 2014b). These data lead to creation of oocyte size frequency distributions (OSFDs), which are valuable tools in assessing where a female resides in its reproductive season (West 1990; Murua and Motos 2006; Kjesbu 2009). A very popular software for performing particle analysis is ImageJ, an open source image analysis program that can be downloaded for free and offers a great variety of plugins and macros for specialized analyses (https://imagej.nih.gov/ij/).

The first step in particle analysis through ImageJ software is to convert the whole-mount photo to an 8-bit gray-scale format. Brightness and contrast are then adjusted to increase oocyte visibility and a watershed filter is applied to separate oocyte aggregations. Oocytes are consequently outlined using a segmentation algorithm providing oocyte area estimations. The resulting mask image of outlines is then overlaid on the original image, permitting manual addition of any missing measurements or correction of any false outlines. Finally, oocyte areas are converted to diameters, which are grouped into size-classes to create the OSFD. The major steps of particle analysis are shown in Figure A5.1.
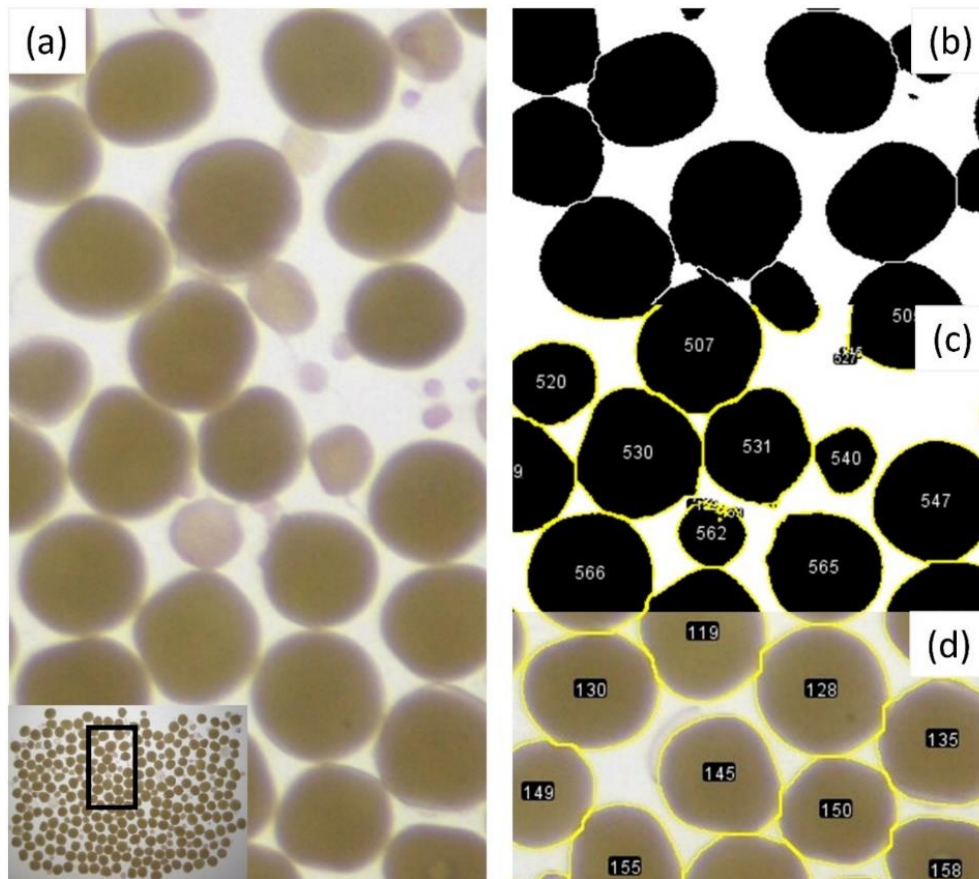
Figure A5.1. Major steps of the semiautomated, image-based particle analysis of a whole-mount photo through ImageJ software: (a) magnified area of the original photo shown in the panel on the left-down corner, (b) brightness and contrast adjustment, (c) oocyte outlines, and (d) mask image of outlines overlaid on the original photo

Εικόνα Α5.1. Κύρια βήματα της ημι-αυτοματοποιημένης μεθόδου ανάλυσης φωτογραφιών ιστού ωοθηκών μέσω του λογισμικού ImageJ: (a) Μεγεθυμένο τμήμα της αρχικής φωτογραφίας που απεικονίζεται στην κάτω αριστερή γωνία της εικόνας, (b) ρύθμιση φωτεινότητας και αντίθεσης της φωτογραφίας, (c) σχεδίαση περιγραμμάτων των ωοκυττάρων, και (d) τοποθέτηση των περιγραμμάτων επί της αρχικής φωτογραφίας

Annex 6

The Bhattacharya (1967) method, applied within the FiSAT II software (http://www.fao.org/), is a modal progression analysis normally used to infer growth from the shift of the modes or means in a time series of length frequency samples (Gayanillo et al. 1996). It practically resolves separate normally distributed groups within composite multimodal frequency distributions. In applied fisheries reproductive biology this methodology is utilized to separate identifiable modes (which correspond to distinct oocyte batches or groups of batches) within the oocyte size frequency distributions (OSFDs) (Fig. A6.1).

Initially, oocytes are binned into fine size classes (e.g., 30 μm). Then, in a semiautomated manner, the distinguishable modes are separated. Upon separation, the oocyte number and the mean oocyte diameter of each mode is estimated.
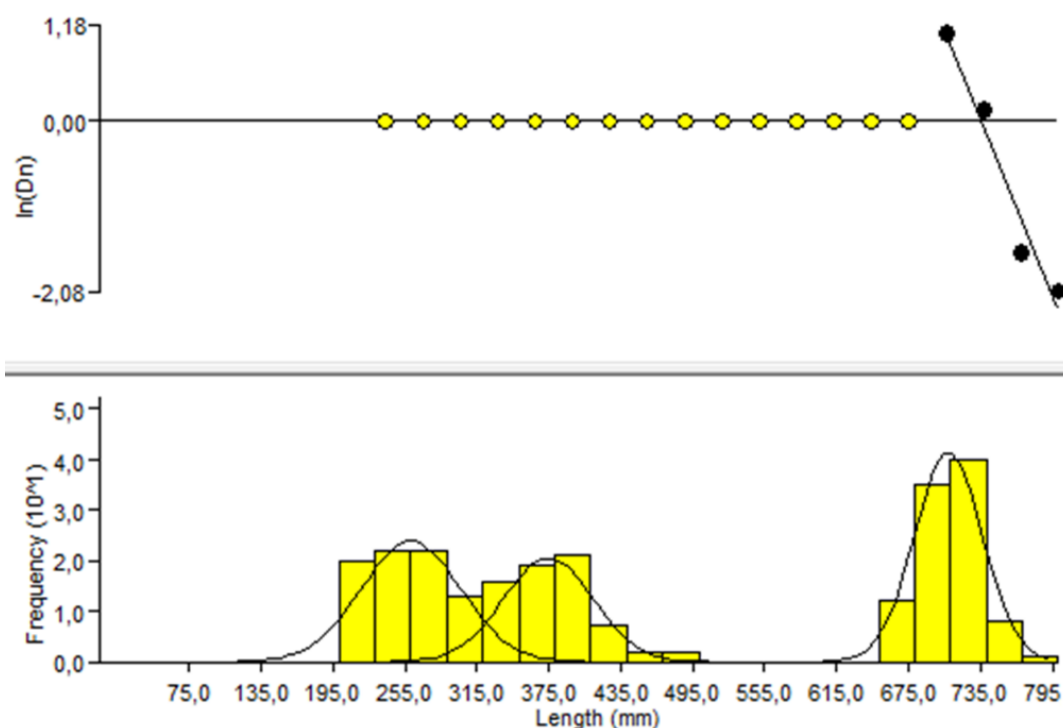

Figure A6.1. Semiautomated resolution of distinct modes within a multimodal oocyte size frequency distribution using the FiSAT II software and the Bhattacharya method

Εικόνα Α6.1. Ημι-αυτοματοποιημένος διαχωρισμός των διαφορετικών ομάδων ωοκυττάρων εντός μιας κατανομής συχνοτήτων μεγεθών ωοκυττάρων με τη χρήση του λογισμικού FiSAT II και της μεθόδου Bhattacharya

Relative fecundity values are then feasible for each female through the gravimetric method, which is based on counting oocytes in weighed ovarian subsamples and then project these counts to the entire ovary (Hunter et al. 1985). For each mode *j* in an OSFD, relative fecundity *RF$_j$* (i.e., number of oocytes in mode *j* per g of eviscerated weight) is estimated by using the formula:

$$RF_j = \frac{n_j \times \dfrac{W_g}{W_{ss}}}{W_{ev}}$$

where *n$_j$* is the number of oocytes of mode *j*, W_g the ovarian weight, W_{ss} the weight of the whole-mount subsample, and W_{ev} the eviscerated weight.