January 2021

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Ovarian dynamics and fecundity regulation in blueback herring, *Alosa aestivalis*, from the Connecticut River, US

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Running head: Fecundity of blueback herring in CT River

Abstract

We analyzed ovarian dynamics of anadromous blueback herring, *Alosa aestivalis*, in Connecticut River with the principal aim of exploring oocyte recruitment and how it shapes the fecundity pattern. We examined the oocyte release strategy and analyzed spawning cyclicity by linking oocyte growth to the degeneration of postovulatory follicles. Females were accordingly classified as pre-spawners, early and late active spawners, and oocyte recruitment intensity was compared among the different spawning phases. Oocyte recruitment occurred continuously and in parallel with spawning activity, a pattern which is diagnostic of indeterminate fecundity. However, both fecundity and oocyte recruitment intensity progressively decreased (tapered) throughout spawning, until the ovary was depleted of vitellogenic oocytes. There was no massive atresia of vitellogenic oocytes at the end of the spawning season, which is atypical of indeterminate spawners. We propose that tapering in oocyte recruitment and fecundity is an adaptation to the high energetic expenditure of the upstream spawning migration.

Keywords

Alosa aestivalis, fecundity pattern, multiple spawning, oocyte recruitment

Introduction

Indeterminate spawners recruit oocytes to the secondary growth phase (SG recruitment) in parallel with their spawning activity (Hunter *et al.*, 1989; Witthames *et al.*, 1995). This keeps the total number of SG oocytes in a "dynamic-equilibrium" state (Kjesbu, 2009), wherein SG recruitment counterbalances spawning so that the released oocytes from sequential batches are replenished. This state has been evinced both through extensive analyses of ovarian samples – e.g. Northern anchovy, *Engraulis mordax* (Hunter and Leong, 1981), European anchovy, *E. encrasicolus* (Schismenou *et al.*, 2012), and Macedonian shad, *Alosa macedonica* (Mouchlianitis *et al.*, 2020) – and through simulations of spawning dynamics (Ganias *et al.*, 2015a). Normally, continuous SG recruitment results in a surplus of SG oocytes at the end of spawning period which subsequently fall into massive atresia (Hunter and Macewicz, 1985a; Witthames *et al.*, 1995; Ganias *et al.*, 2014), a process known as "mopping-up" (Wallace *et al.*, 1981; Kjesbu, 2009).

Recent studies have challenged the generality of dynamic-equilibrium and mopping-up in indeterminate spawners. Ganias *et al.* (2017) report for the Atlantic horse mackerel (*Trachurus trachurus*) a cessation of SG recruitment and lack of massive atresia in late-season spawners. A similar pattern has been suggested in Gulf menhaden, *Brevoortia patronus* (Brown-Peterson *et al.*, 2017). Minimizing energetic expenditure by regulating oocyte production might be of adaptive value in fishes that are subject to high energetic costs of reproduction, such as capital breeders or anadromous fishes (McBride *et al.*, 2013).

Anadromous alosines (Clupeidae: *Alosa spp.*) incur substantial energetic losses during their upstream migration towards their spawning grounds (Glebe and Leggett, 1981; Murauskas and Rulifson, 2011; Ganias *et al.*, 2015b). Thus, indeterminate alosines would be suitable models to test for changes in SG recruitment. Broadly speaking, ~~two alternative possibilities are that~~ SG recruitment intensity could either remain~~s~~ constant, or ~~that~~ it could progressively decrease~~s~~ over the spawning season, a pattern we here call tapering. Herein we examine blueback herring *A. aestivalis* (BBH) in the Connecticut River. BBH

is an anadromous, economically important alosine that previously provided a valuable fishery for food, bait and fertilizer (Mullen *et al.*, 1986; Kocik, 1998). The species is now regarded as overexploited (Limburg and Waldman, 2009), vulnerable according to the IUCN (NatureServe, 2013) and is listed as a species of concern (NOAA National Marine Fisheries Service, 2009). BBH spawns in freshwater from mid spring to early summer (Loesch and Lund, 1977); the dynamics of its oocyte development remain undescribed.

Our main objective was to analyze ovarian dynamics of BBH in Connecticut River, with emphasis on how SG recruitment shapes the fecundity pattern. In particular, we focused on SG oocyte recruitment and tested whether intensity is affected by its upstream migration towards the spawning grounds. To do so, females were classified according to their spawning phase based on the characteristics of oocytes and postovulatory follicles. SG recruitment intensity was then compared amongst the different spawning phases and used to determine the equilibrium between oocyte recruitment and spawning. Our results will help improve future estimations of the species' reproductive potential.

Materials and methods

Fish collection and processing

Specimens for this study were collected as part of a project focused on adult BBH as prey during the spawning season (Davis et al. 2012). To capture BBH 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, USA (Table 1; Fig. 1). Sampling sites were located at a significant distance from the river mouth (approximately 60 to 85 km). 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. Measurements upon workup included total length, total weight, eviscerated weight (W_{ev}) and gonad weight. 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.

Histological analyses

Ovarian subsamples of all females (N = 164) were processed histologically using standard procedures (~2mm thick hand-cut cross sections, paraffin embedding, 4-µm sections, hematoxylin/eosin staining). Each ovarian subsample was taken from the middle of the ovary.; Bbased on previous results in several alosines (Loesch and Lund, 1977; Olney *et al.*, 2001; Olney and McBride, 2003; Grice *et al.*, 2014; Sullivan *et al.*, 2019), we considered there were no differences among the anterior, middle and posterior parts of both right and left lobes. Each histological section was digitized into a high-resolution photomicrograph using a slide scanner (NanoZoomer S60, Hamamatsu Photonics, Japan). The photomicrographs enabled identification of distinct developmental stages of SG oocytes (based on Brown-Peterson *et al.*, 2011) and detection of atretic and postovulatory follicles (POFs). We identified the cortical alveolar (CA) stage, the early, secondary and tertiary vitellogenic (VIT-1, VIT-2, VIT-3) stage, early and late germinal vesicle migration (GVM-1, GVM-2) stage, and the germinal vesicle break down (GVBD) stage. POFs were staged based on their histomorphological characteristics (Hunter and Macewicz, 1985b), and cross-sectional areas (POF_{XSA}; Ganias *et al.*, 2007).

The presence of POFs in imminent spawners and/or the co-occurrence of POFs from different daily cohorts (Fig. 2) served as proof of multiple spawning. POFs were also used to classify females into spawning phases:; Wwe 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 (1985b), 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.

Whole-mount analyses

Ovaries from 91 fish were further analyzed through whole-mount procedures. Weighed ovarian subsamples of ~ 0.1 g were digitally imaged using a Jenoptik Progress C3 camera mounted on a Euromex NZ 80 stereo microscope. The resulting images were subjected to particle analysis (Thorsen & Kjesbu, 2001) using ImageJ (https://imagej.nih.gov/ij/) yielding oocyte size frequency distributions (OSFD). By this means we subsequently identified and enumerated oocytes of the advanced mode (AM) and of the subsequent mode (SM), using methods described in Ganias *et al.* (2010).

We estimated the relative fecundity (i.e., number of oocytes g^{-1} W_{ev}) of the AM and the SM gravimetrically (Hunter *et al.*, 1985). In order to estimate relative total fecundity (i.e., total number of SG oocytes g^{-1} of W_{ev}; RF_T) it was necessary to distinguish primary growth oocytes (PG) from SG oocytes. Because PG oocytes are translucent and closely attached to each other (Anderson *et al*. 2000) the whole mount procedure that included oocytes smaller than 200μm needed some extra steps and was thus held in 10 out of the 91 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.

SG recruitment intensity

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 Weibel method is based on the Delesse principle stating that the fractional volume of a component (here, the CA oocytes) is proportional to its fractional cross-sectional area. 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.

Statistical analysis

All plots and statistical analyses were performed in R 3.5.2 (www.R-project.org). Plots were produced by use of R packages lattice, ggplot2 and ggridges. Cutpoint values for estimating size threshold between PG and SG oocytes were estimated using R package Shazam. Normality was tested by the Shapiro-Wilk test.

Results

Females were sampled during the spawning season and on the spawning ground. Of the 164 female blueback herrings that were analyzed histologically, 42 (26%) were identified as pre-spawners, 120 (73%) as active spawners and 2 (1%) as spent (Table 1). Active spawners were caught in almost all sampling dates and sites; two small samples taken early in the season consisted entirely of pre-spawners. Among active spawners, 16% had two different POF cohorts (Table 1, Fig. 2), indicating that POF degeneration lasted longer than the spawning interval. Most (87.5%) of the ovaries with two POF cohorts were at the VIT-2 stage and the remainder were at the VIT-3 stage. POFs occurred through the entire range of ovarian development, from early vitellogenesis (VIT-2 stage) to final oocyte maturation (GVBD stage, Fig. 3). There was a strong negative relationship between the cross-sectional area of the leading POF cohort, POF_{XSA}, and the mean diameter of oocytes in the AM ($P < 0.001$, $R^2 = 0.71$; Fig. 3).

Batches of oocytes to be spawned could be discerned in most females. The AM was clearly discernible in the OSFDs of females from the VIT-3 stage onwards (Fig. 4). The relative fecundity of the AM was considered equivalent to relative batch fecundity (RF_B), i.e. the relative number of oocytes that would have been released during the next spawning episode (see also Ganias *et al.*, 2015a). The subsequent mode (SM) representing the oocyte batch of a second

upcoming spawning episode was also discernible in >VIT-3 stage females (e.g., Fig. 5). However, in most cases the SM could not be resolved from smaller oocytes and its relative fecundity (RF_{SM}) 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) and we did not estimate RF_B and RF_{SM} for these females.

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. 6). 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 200μm threshold value previously used for other alosines such as the American shad, A*. sapidissima* (Hyle *et al.*, 2014), and Allis shad, *A. alosa* (Mouchlianitis *et al.*, 2019). Thus, for generality purposes we also used the 200μm threshold for distinguishing between PG and SG oocytes.

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}). The RF_{SM} 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 $RF_{AM} \geq 396$ 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. Late spawners exhibited significantly lower $RF_{200\text{-}320}$ values than pre-spawners and early active spawners ($P < 0.05$; least significant difference, 95% confidence level; Fig. 7a). 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. 7b).

Discussion

Multiple spawning shown here for BBH is characteristic of anadromous alosines. Multiple spawning of BBH 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. Other anadromous alosines shown to be multiple spawners include Alewife *A. pseudoharengus* (Ganias *et al.*, 2015b), American Shad (Olney *et al.*, 2001; McBride *et al.*, 2016), Hickory Shad *A. mediocris* (Murauskas and Rulifson, 2011), Alabama Shad *A. alabamae* (Mettee and O'Neil, 2003) and Twaite Shad *A. fallax fallax* (Pina *et al.*, 2003), as well as the landlocked Macedonian Shad *A. macedonica* (Mouchlianitis *et al.*, 2020).

BBH 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 has been documented for several anadromous alosines, e.g., Hickory Shad (Murauskas and Rulifson, 2011), American Shad (Hyle *et al.*, 2014), and Alewife (Ganias *et al.*, 2015b) and the landlocked Macedonian Shad (Mouchlianitis *et al.*, 2020). We interpret indeterminate fecundity 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), we examined whether and how BBH 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, 1985b; 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, BBH 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, BBH 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 BBH collected from the same location in May 2018 (authors unpublished data) similarly lacked massive atresia. All females, including the spent ones, contained primary growth oocytes in their ovaries indicating the potential to migrate and reproduce in the next spawning season(s). Despite this potential, repeat spawners in Connecticut River female BBH 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 BBH (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 BBH in Connecticut River and thus, the smaller energetic cost of its spawning run we predict that other populations should deploy similar strategy of SG recruitment and fecundity regulation.

We conclude that BBH maximizes efficiency of investment in reproduction over the spawning season through tapering of SG recruitment, a pattern which – to our knowledge – has never been shown for a fish species. Our 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 paper, through tapering SG recruitment and fecundity until the ovary is depleted of its stock of vitellogenic oocytes.

Acknowledgments

The authors are grateful to the Connecticut Department of Energy and Environmental Protection, the Connecticut River Coordinator's Office of the US Fish and Wildlife Service, and the Center for Environmental Science and Engineering of the University of Connecticut for their financial, technical and logistical support during sampling. We are also grateful to J. Achilli, J. Divino, K. Gherard, S. Hovorka, S. Ruiz, B. Tate and A. Wasley for assistance with field and laboratory work. The histological sections were scanned at the Institute of Marine Research at Bergen, Norway under the supervision of O.S. Kjesbu and A. Thorsen. The research work was supported by the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and

334 Technology (GSRT), under the HFRI PhD Fellowship grant attributed to F.A.
335 Mouchlianitis with grant agreement No 493584.

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337 **Data Availability Statement**
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339 There is no data to share
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Figure legends

Figure 1 Sampling sites in the Connecticut River, State of Connecticut, USA: Wethersfield (41°43'31.31'' N, 72°38'58.73'' W), Farmington River (41°50'10.06'' N, 72°38'9.41'' W), Windsor Locks (41°53'49.60'' N, 72°37'17.53'' W), Enfield (41°59'29.72'' N, 72°36'13.15'' W).

Figure 2 Photomicrograph of an ovarian histological section (hematoxylin/eosin staining) showing two postovulatory follicles (POFs) originated from two sequential daily spawning events (newest and previous POF cohort).

Figure 3 Cross-sectional area of the biggest postovulatory follicle (POF_{XSA}) in the histological section of active spawners versus the mean diameter of oocytes at the most advanced developmental stage (OD_{AM}). Differing symbols represent ovarian developmental stage, i.e., developmental stage of the most advanced oocytes in the ovary. VIT-2 = secondary vitellogenic, VIT-3 = tertiary vitellogenic, GVM-1 = early germinal vesicle migration, GVM-2 = late germinal vesicle migration, GVBD = germinal vesicle breakdown stage. Line represents the fitted exponential regression.

Figure 4 Oocyte size frequency distributions (OSFD) of secondary growth oocytes in whole mount preparations of: (a) pre-spawners and (b) active spawners. Color of OSFD represents the ovarian developmental stage, i.e. the developmental stage of the most advanced oocytes in the ovary. OSFDs are displayed in order of increasing (bottom to top) mean oocyte diameter of the most advanced oocytes. Legend abbreviations for developmental stage are described in caption of Fig. 34.

Figure 5 Oocyte size frequency distribution of secondary growth oocytes of a single female with ovary in VIT-3 stage. Oocytes are divided into fine (25 µm) diameter classes to resolve modes. The advanced mode (AM) is distinguishable whilst the subsequent mode (SM1) is overlapping with remaining oocyte modes.

Figure 6 Combined oocyte size frequency distribution of all oocytes from the ten females that were used to estimate the mean size threshold value (solid red

546 line) between primary growth (PG) and secondary growth (SG) oocytes. Dotted
547 lines correcpond to 95% confidence intervals.

548 **Figure 7** Box and whisker plots of log transformed values of: (a) relative
549 fecundity of oocytes with diameter between 200 and 320 µm ($RF_{200-320}$), and (b)
550 relative fecundity of the cortical alveolar oocytes (RF_{CA}), by spawning phase.
551 The heavy central line of each box represents the median, the top and bottom
552 lines extend from the lower to the upper quartile and the top and bottom
553 whiskers extend to the lowest and highest values within the 1.5x the
554 interquartile range. Observations outside the 1.5x the interquartile range
555 represent the extreme values and are represented as open dots. Statistically
556 significant pairwise comparisons of mean values are shown with asterisk(s) ("*"
557 for $P < 0.05$, "**" for $P < 0.001$).

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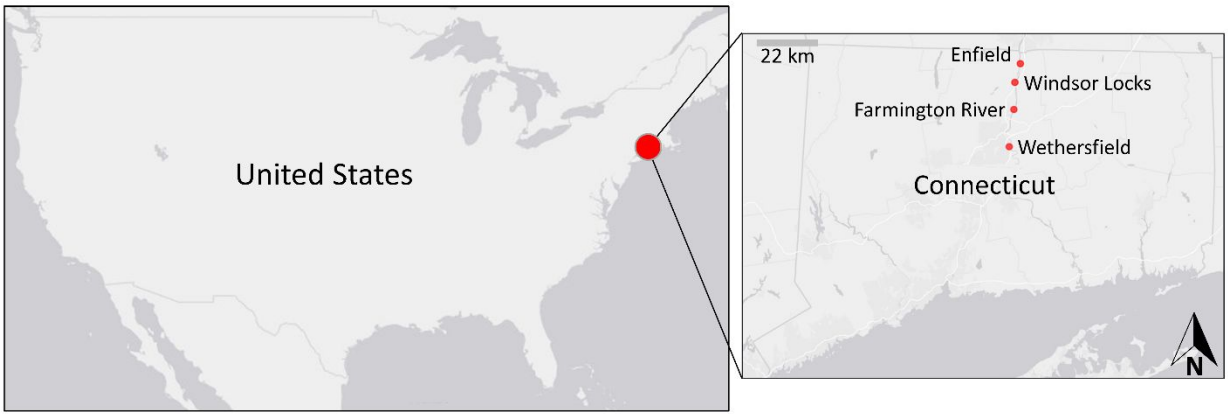

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562 **Figure 1**

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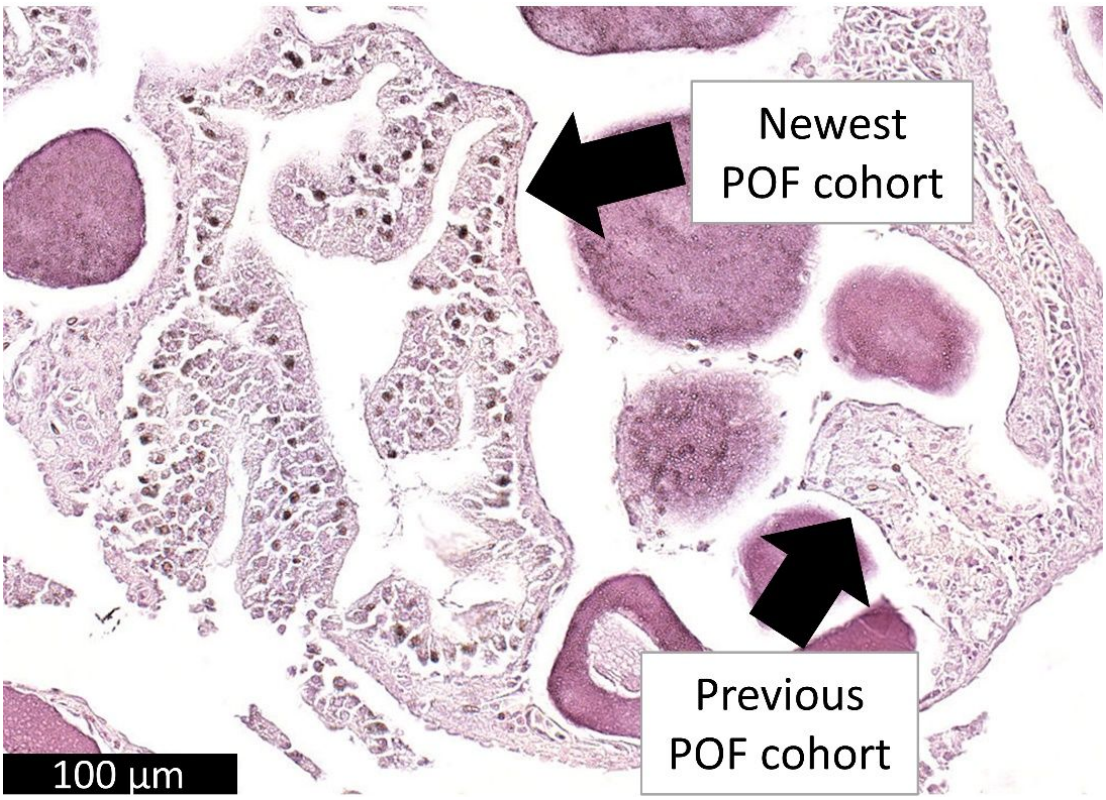

Figure 2

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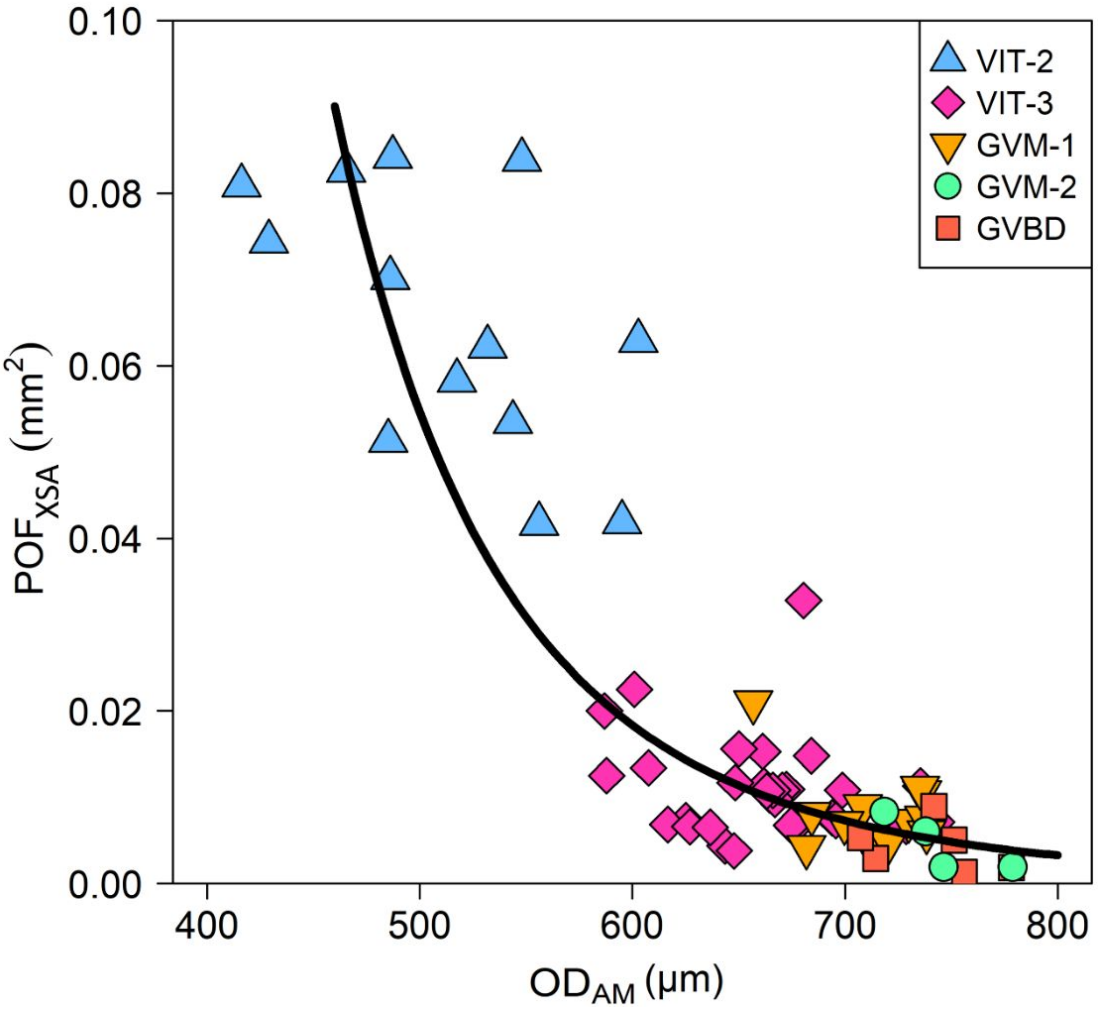

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583 **Figure 3**

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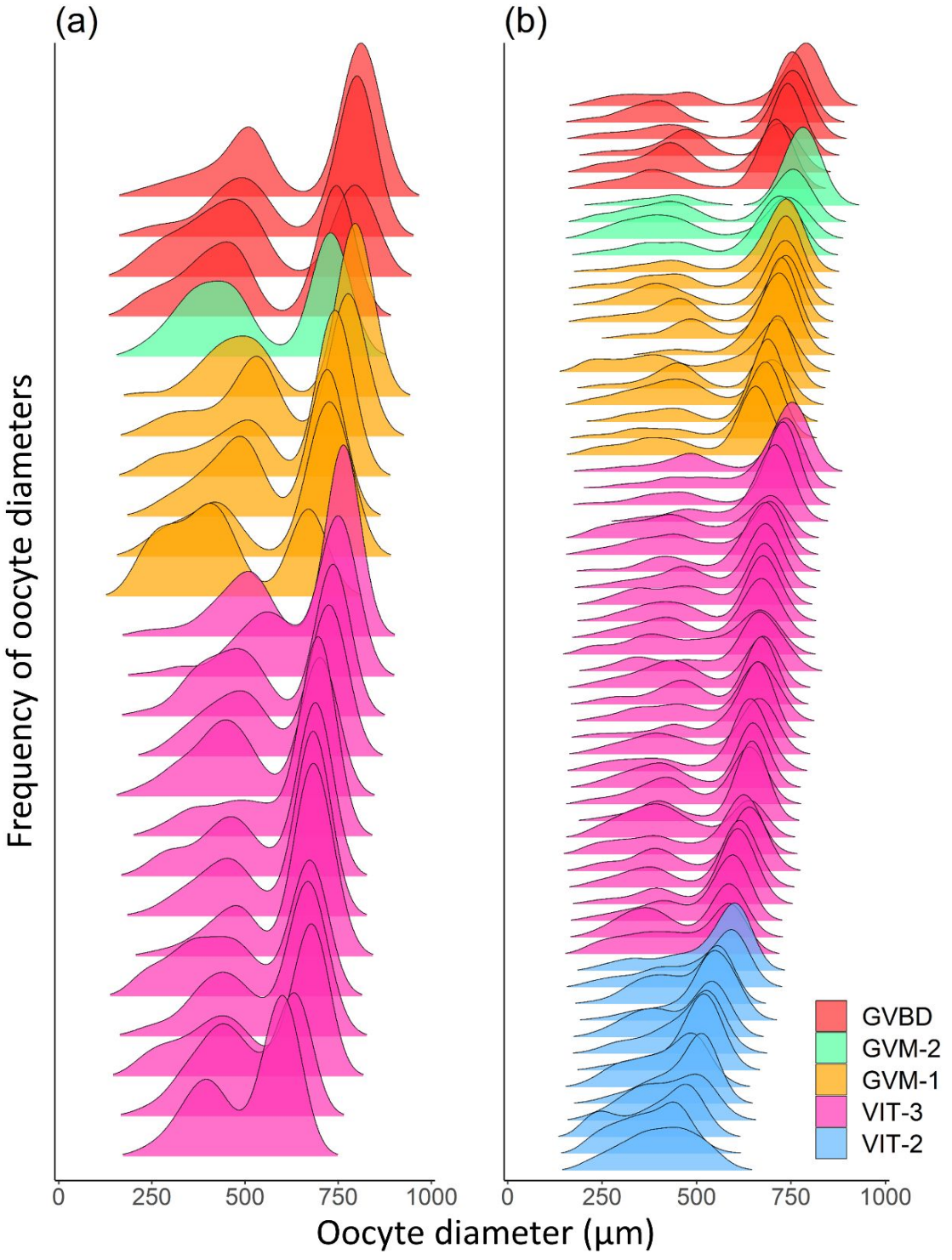

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588 **Figure 4**

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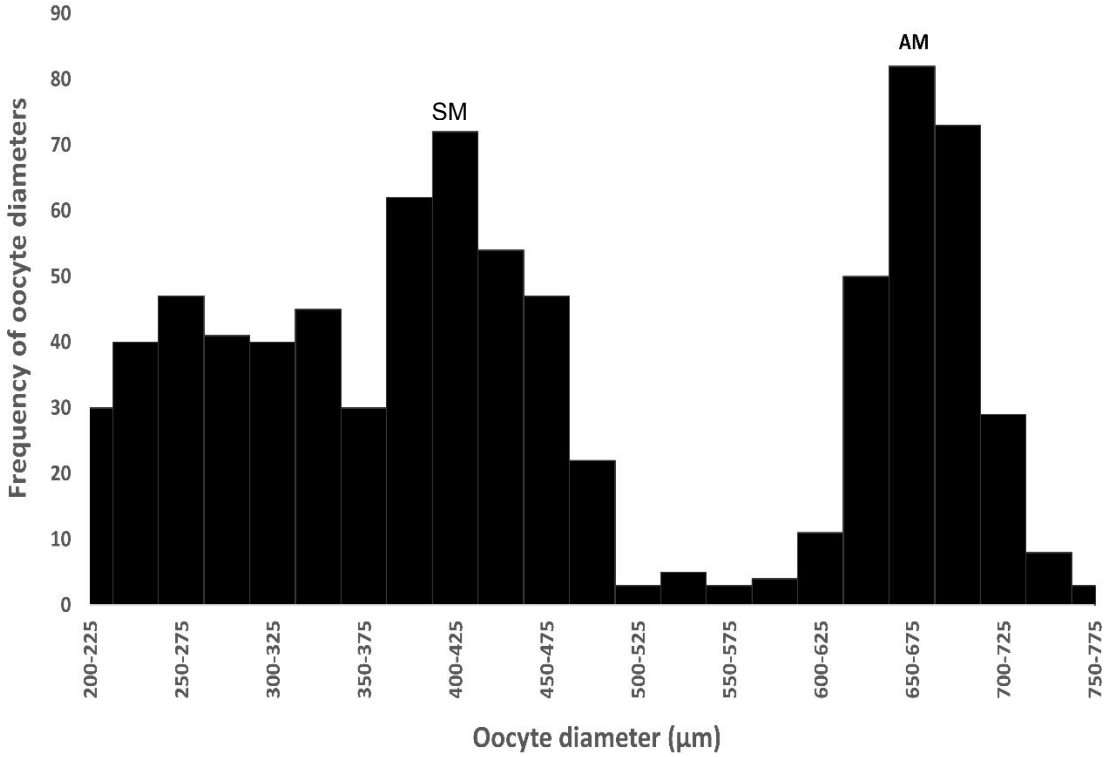

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593 **Figure 5**

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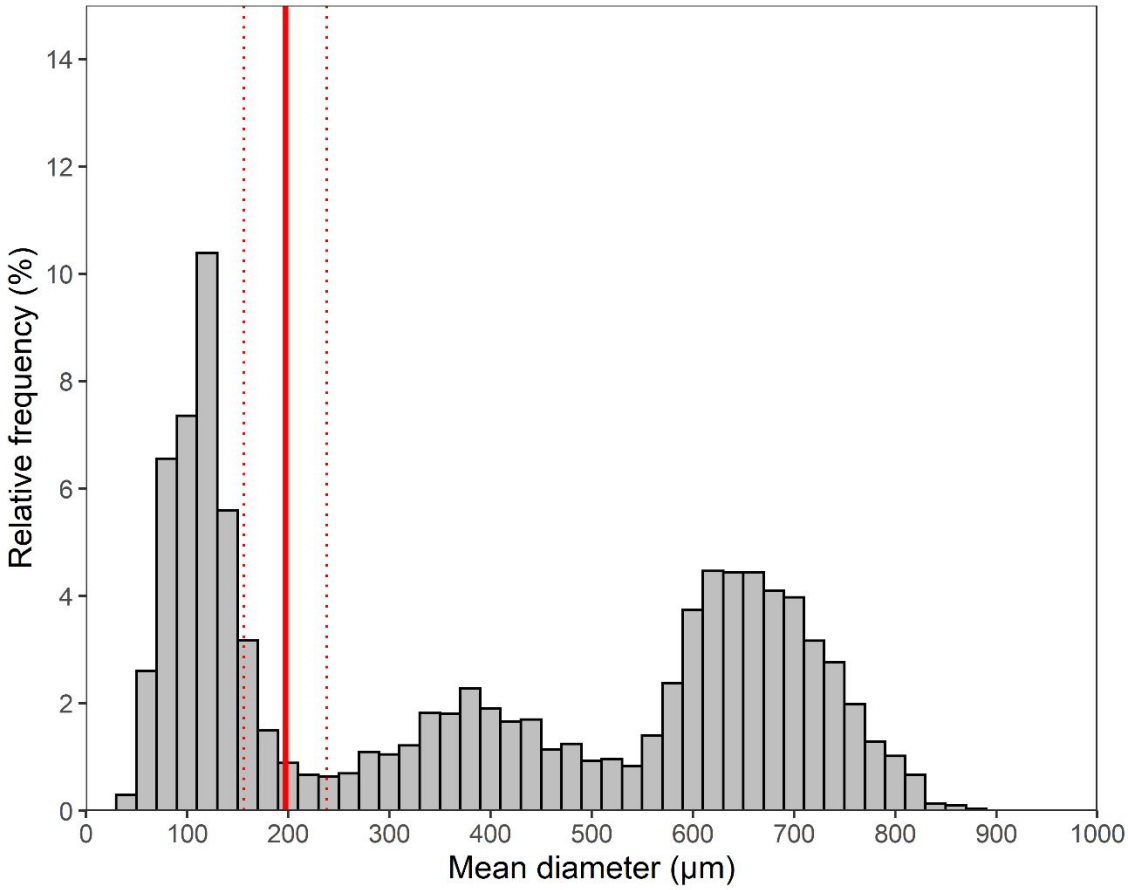

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597 **Figure 6**
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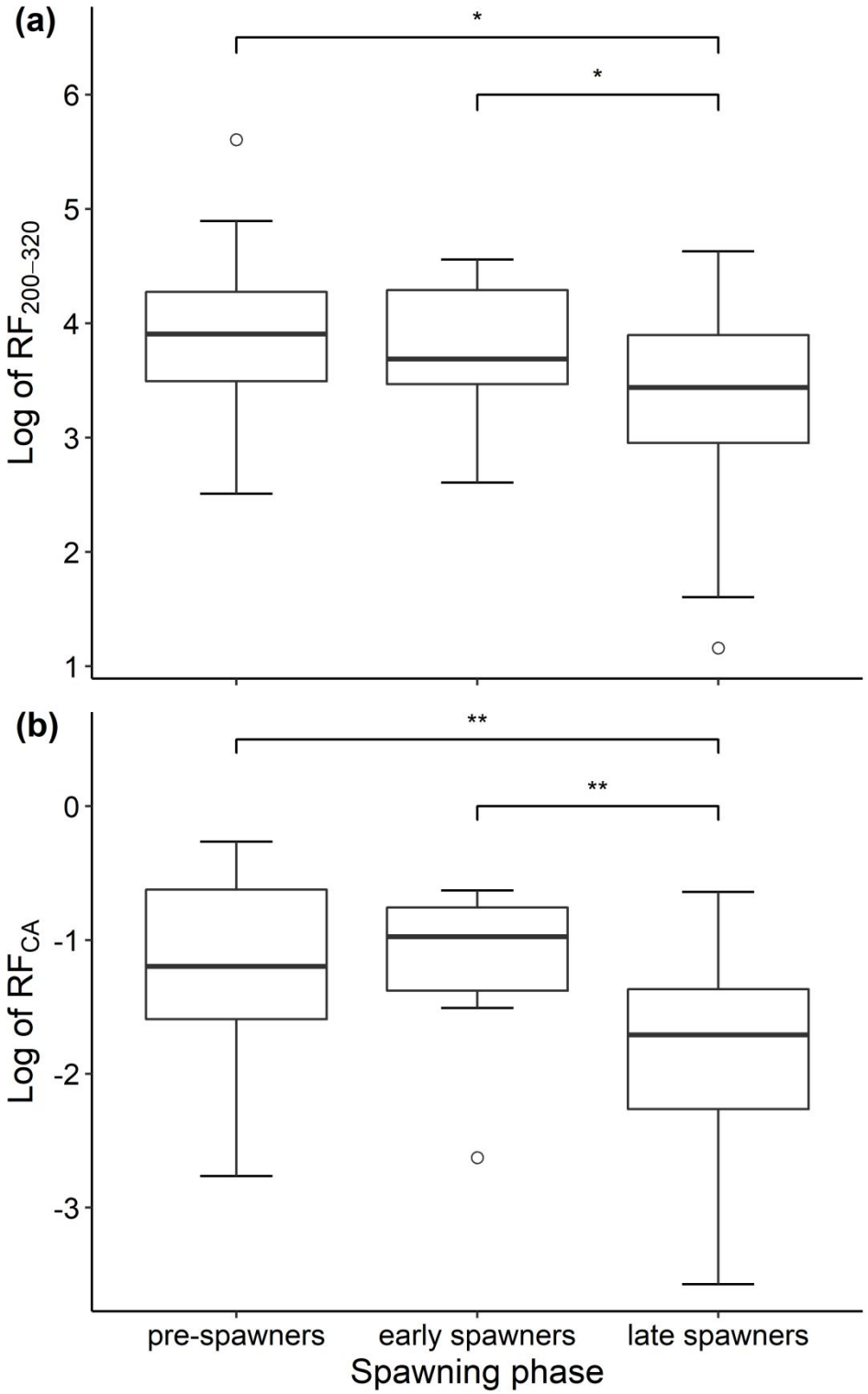

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603 **Figure 7**

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Table 1. Number of females analyzed histologically (N_{his}) and through whole-mount analysis (N_{wm}) and total length range (L in mm) per sampling date and sampling site. WF = Wethersfield, FR = Farmington River, WL = Windsor Locks, EF = Enfield. The percentage of fish per spawning phase (pre-spawners, active spawners, spent females) and the percentages of fish having one postovulatory follicle (POF) cohort and two POF cohorts are also shown.

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Sampling date	Sampling site	N_{his}	N_{wm}	L range	Pre-spawners (%)	Active spawners (%)	Spent (%)	1 POF cohort (%)	2 POF cohorts (%)
27/4	FR	1		282	100				
28/4	WF	7	7	256 - 279	57.1	42.9		42.9	
1/5	FR	4	4	233 – 278	25	75		75	
2/5	WL	2	2	271 – 284	100				
7/5	WF	17	12	231 – 288	11.1	88.9		77.8	11.1
8/5	FR	13	8	239 – 293	15.4	84.6		61.5	23.1
9/5	WL	16	9	235 – 289	43.8	56.2		50	6.2
10/5	EF	6	2	231 – 268	16.7	83.3		83.3	
14/5	WF	13	4	240 – 287	38.5	61.5		61.5	
24/5	EF	2	2	241 – 259	50	50		50	
28/5	WF	29	13	240 – 291	20.7	75.9	3.4	51.7	27.6
29/5	FR	15	10	242 – 290	13.3	86.7		80	6.7
30/5	WL	5	4	227 – 289		100		100	
31/5	EF	3	1	231 – 264		100		66.7	33.3
4/6	WF	6	1	236 – 245	33.3	66.7		66.7	
5/6	FR	11	5	234 – 274	36.4	54.5	9.1	54.5	9.1
19/6	FR	2	1	226 – 230		100		100	
20/6	WL	7	3	229 – 265		100		100	
21/6	EF	1	1	273		100		100	
27/6	WL	3	2	230 – 273	66.7	33.3		33.3	
Total		**164**	**91**						