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1 Mechanisms regulating oocyte recruitment and skipped spawning in Northeast Arctic cod (*Gadus morhua* L.)

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Abstract

To examine mechanisms that affect fecundity, atresia and skipped spawning in Northeast Arctic cod (*Gadus morhua* L.), we conducted an experiment where wild-caught cod (>60 cm) kept under restricted food regimes were subjected to monthly biopsies, hormonal and physical measurements. The power of body weight as a fecundity proxy increased until the presumed end of follicle proliferation in early November, thereafter it remained stable. Atresia occurred in most females; but, for maturing females, mainly close to spawning. 18 % of the females had small gonads with predominantly previtellogenic oocytes at sacrifice in January. These females were past-spawners, verified by post ovulatory follicles in their gonadss. These 'skippers' had lower condition than maturing cod from December, smaller livers upon sacrifice and lower plasma 17-β estradiol values from early November. Until November, oocytes developed similarly for all females, but in November oocyte development was arrested at the early cortical alveoli stage and atresia occurred in all skippers. In sum, fecundity and skipped spawning seem highly influenced by energy reserves during early vitellogenesis and limited to females only. Finally, skippers were identifiable long before the predicted onset of spawning, which could have implications for forecasting of egg production and hence stock-recruitment relationships.

Keywords: cod, vitellogenesis, fecundity, skipped spawning

28 ## Introduction

29 The energy available for reproduction and growth in iteroparous spawners is surplus energy
30 after energetic requirements for basic metabolic needs have been fulfilled (Rijnsdorp 1990).
31 The energy state of each fish therefore influences its reproductive investment (Kjesbu and
32 Witthames 2007). Consequently, recent models of total egg production and recruitment have
33 also included various indices of individual condition (Marshall et al. 1998, 1999, 2000; Scott
34 et al. 2006). This has shown the potential merit in incorporating basic biological knowledge
35 for construction of realistic population dynamics models.

36 The major event responsible for energy transfer into developing oocytes and thereby
37 oocyte growth in marine fish is vitellogenesis (Tyler and Sumpter 1996). During
38 vitellogenesis, vitellogenin is sequestered into the developing oocytes, which thereby are
39 recruited to the maturing pool to become the year's potential egg production. Both the hepatic
40 synthesis of vitellogenin and its subsequent uptake by growing oocytes is under hormonal
41 control by the gonadal steroid, 17β-estradiol (Tyler and Sumpter 1996). Marine fish can be
42 broadly distinguished into two different categories depending on their reproductive mode;
43 indeterminate and determinate spawners (Hunter et al. 1992; Murua and Saborido-Rey 2003).
44 Indeterminate spawners, such as anchovies (*Engralius sp.*) (Motos 1996) and swordfish
45 (*Xiphias gladius*) (Arocha 2002), recruit new oocytes throughout the spawning season.
46 Determinante spawners on the other hand, e.g. Atlantic cod (*Gadus morhua*) recruit a finite
47 number of oocytes to the maturing pool prior to spawning. With this type of reproductive
48 strategy, the completion of oocyte recruitment marks the point of maximum potential
49 fecundity. Subsequent alteration of potential fecundity is only possible through 'down-
50 regulation' of oocytes through the process of atresia , i.e. reabsorbtion of vitellogenic oocytes
51 (Woodhead and Woodhead 1965; Kjesbu et al. 1991; Thorsen et al. 2006). Atresia seems to

52 take place within a specific oocyte diameter range, i.e., the atretic window (Witthames and
53 Greer Walker 1995; Óskarsson et al. 2002; Kurita et al. 2003)

54 Given that oocyte recruitment to the maturing pool, in principle occurs only during
55 early vitellogenesis in determinate spawners, their potential fecundity should be influenced by
56 individual energy reserves around the same time. A study on rainbow trout (*Oncorhynchus*
57 *mykiss*) found that fecundity was unaffected in fish that were fed on a low diet during the later
58 stages of vitellogenesis, while it was significantly reduced for females fed a reduced ration
59 during early vitellogenesis (Bromage et al. 1991). Conversely, cod fed on a low ration prior to
60 the start of vitellogenesis followed by a high ration during early vitellogenesis had similar
61 fecundities to fish fed on a high ration over the entire duration (Kjesbu and Holm 1994).
62 Skjæraasen et al. (2006) energy reserves during early vitellogenesis were significantly
63 correlated with potential fecundity at the time of spawning. In sum, these studies suggest that
64 fecundity is indeed correlated to individual energy reserves during early vitellogenesis.
65 However, in addition to its influence on fecundity, energy reserves at this time may also be
66 linked to the phenomenon of skipped spawning, which also can have considerable bearing on
67 stock reproductive potential and thereby the stock – recruitment relationship.

68 Skipped spawning can be defined as the failure of iteroparous spawners to spawn
69 every year following sexual maturity (Rideout et al. 2005). For cod this phenomenon and its
70 potential importance for spawning stock size assessment and stock-recruitment relationships
71 has received limited attention until recently (Rideout and Rose 2006; Morgan 2008), although
72 reported as early as the 1960s for cod captured in the Barents Sea, i.e. Northeast Arctic cod,
73 (Woodhead and Woodhead 1965) and again in the 1990s (Oganesyan (1993), and
74 experimentally for Norwegian Coastal cod in the early 1990s (Kjesbu et al. 1991). Jørgensen
75 et al. (2006) used a state-dependent-life history model (Jørgensen and Fiksen 2006) to model
76 the occurrence of skipped spawning in Northeast Arctic cod and predicted that as much as 30

% of the sexually mature population may skip spawning, which largely agreed with observations made by Rideout and Rose (2006) on 'Northern cod' following the stock collapse. However, skipped spawning may be common in many important commercial fish as reviewed by Nikolskii (1969) and Rideout et al. (2005).

It is possible that not only fecundity, but also the proportion of skipped spawners is linked to energy reserves of the spawning stock, in particular during early vitellogenesis. To target these questions we studied gonad investment and the incidence of skipped spawning in Northeast Arctic cod subjected to temporal changes in food availability under a controlled laboratory setting. This allowed us to examine how temporal changes in energy reserves during the maturation cycle affect fecundity and the incidence of atresia. Further, as skipped spawning in this species appears linked to individual energy reserves (Kjesbu et al. 1991; Rideout et al. 2005, Rideout and Rose 2006), we chose rations that would generate condition factors resembling that of wild cod in an attempt to induce skipped spawning under experimental conditions. Further, by following individuals for a prolonged period before spawning and continually assessing both oocyte development and sex steroids profiles we hoped to determine when skippers "separate" from maturing females. This knowledge could have considerable implications for forecasting stock reproductive potential.

Materials and methods

History of Fish

To target large cod with a high probability of having spawned at least once before, approximately 200 cod were caught by trawl on the main spawning grounds of the Northeast Arctic cod near Vesterålen, northern Norway (67° 38 N, 01° 30 E) on April 8, 2006 by the research vessel R/V 'Johan Hjort'. Aboard the vessel cod were kept in 1-3 m^3 aerated tank post capture and transported to Bergen (60° 23 N, 05° 20 E) where they arrived on April 17. Fish were then immediately transferred to two identical 30-m^3 tanks at the Institute of Marine

102 Research (IMR) research facility at Nordnes, Bergen. Fish health was monitored daily and
103 any fish showing signs of injury, stress or discoloration were removed. During this initial
104 acclimation period, cod were fed a mixture of shrimps (*Pandalus borealis*), herring (*Clupea*
105 *harengus*) and pellets *ad. lib.* with the goal of entraining fish to a pellet-only diet for the
106 upcoming experiment. On May 24, all cod were weighed (± 1 g), measured (± 1 cm) and PIT
107 tagged for individual identification. Fish were then allowed four more weeks of recovery
108 before the experiment started on June 22.

109 **The Experiment**

110 On June 22, all cod were sedated with benzocain (60 ppm), measured and weighed. In
111 addition a 2 mL blood sample was obtained from the caudal vein of every fish. After these
112 measurements fish < 70 cm in total length were transported to the High Technology Centre of
113 Bergen (HiB) and distributed randomly between two identical 7-m^3 tanks. The remaining fish,
114 between 70 and 99 cm, were distributed randomly between the two bigger 30-m^3 tanks at
115 IMR. Subsequently, one tank at IMR and one tank at HiB were subjected to a medium ration
116 food regime, hereafter denoted as the ML group, and the two remaining tanks to a low ration
117 feed regime, hereafter denoted as the LM group. The medium ration was calculated as a daily
118 portion of 0.25 % of the wet fish biomass in the tanks given as pellets (Skretting AS, www.
119 skretting.no/: Europa Marin 17) twice a week (Kjesbu et al. 1991), i.e., 0.875 % of the
120 biomass in the tank per feeding. The low feeding ration was calculated as a daily portion of
121 0.125% of the biomass given twice a week, i.e., 0.4375 % of the biomass in the tank per
122 feeding. The goal was to feed the cod a pellet-only diet during the entire experiment.
123 However, after the experimental start some fish showed a reduced appetite and did not partake
124 in the feeding sessions, leaving substantial amounts of pellets in the tanks. Therefore the food
125 regime was altered from August 7. From this date cod were given a mixed diet of herring and
126 dry pellets. The new feed ration was calculated so that herring would contribute 80 % of the

127 solids (mainly fat and protein) the cod received. Assuming that dry pellets contain
128 approximately twice the amount of solids (88%, cf. www.skretting.no) per unit weight
129 compared to fresh herring (20-40%) (Kent 1990), the new medium ration given twice per
130 week was 1.4 % of the biomass in the tank in herring wet weight and 0.175 % of the biomass
131 in pellets. The corresponding new low ration was 0.7 % of the biomass in herring wet weight
132 and 0.0875 % given as pellets. The fish were then subjected to monthly measurements and
133 blood collection (Table 1). After each measurement the food ration was adjusted according to
134 the new biomass in the tank. In all tanks, cod were kept under a natural photoperiod for
135 Bergen, while temperature was kept at 5-6 °C, which although at the upper limit is within the
136 natural range that Northeast Arctic cod experience in the Barents Sea (Godø and Michalsen
137 2000).

138 Originally, the plan was to obtain an ovarian biopsy sample (Kjesbu et al. 1996) at
139 every measurement date. However, in June and August, the pore of the urogenital papilla of
140 the initial 5 fish examined were greatly restricted in size and due to the risk of physical
141 damage further attempts to obtain a biopsy sample were aborted on these dates. From
142 September successful biopsies were completed (Table 1).

143 The ML group received the medium ration until the October measurement after which
144 they were switched to the low ration, whereas the LM group was given the reverse treatment.
145 In October a sub-sample of 7 males and 3 females from the ML group and 5 males and 4
146 females from LM group were sacrificed to compare gonadal development and energy reserves
147 at this stage.

148 Cod were monitored daily and any fish developing signs of injury or stress, i.e.
149 discoloration or loss of appetite were removed from the tanks. Any fish that were removed or
150 succumbed at any time during the experiment were omitted from all analyses, tables and
151 graphs. In total we obtained data from 16 females and 33 males in the ML group and 36 males

152 and 13 females in the LM group (Table 2). Examination of the female biopsies from January
153 10 and 11 2007 indicated that some females were only a few weeks from the start of
154 spawning (see **Oocyte measurements**). All cod were therefore sacrificed between January 18
155 and 23. All fish sacrificed at any date was subjected to the same protocol. Total weight, gonad
156 and liver weight were measured and a blood sample was collected from the caudal vein. A
157 section of the gonad was also fixed in 3.6% neutral – phosphate-buffered formaldehyde for
158 later histological analyses. For females, the tissue sample was taken from the middle part of
159 the right ovarian lobe for standardization. Finally, otoliths were removed from all fish for
160 ageing, classification of fish into Northeast Arctic and Coastal cod and examination of past
161 spawning history (Rollefsen 1933).

162 **Oocyte Measurements**

163 All biopsy samples (n = 150) and the final gonad samples (n= 22) were subjected to digital
164 image analyses (Thorsen and Kjesbu 2001). This method uses the contrast between
165 previtellogenic oocytes and vitellogenic oocytes in relation to the set background to
166 specifically select and measure the diameter of the last category of oocytes. However, oocytes
167 at the very beginning of vitellogenesis, i.e., the early cortical alveoli stage, may not be picked
168 up by this method. By combining the results of the histological (see **Histological analyses**
169 below) and digital analyses we were able to separate between various stages of previtellogenic
170 oocytes (PVO), early cortical alveoli (E-CA), late cortical alveoli (L-CA) and yolk granule
171 (YG) oocytes. For each sample containing vitellogenic oocytes that could be measured with
172 the digital image analyses, the size of 200 oocytes was measured. Also, from these data the
173 average size of the Leading Cohort (LC_{20}) (average of the largest 10% of the oocytes) was
174 calculated in each sample. From the final gonad sample obtained at the time of sacrifice we
175 calculated potential fecundity as:

176 $$F_p = 2.139 \times 10^{11} \times OD^{-2.7} \times OW \qquad (1)$$

177 based on Thorsen and Kjesbu (2001) and protocols therein, where F_p is potential fecundity,
178 OD is average vitellogenic oocyte diameter, estimated by the digital image analysis, and OW
179 is ovary weight (g) at the time of sacrifice.

180 **Hormonal samples**

181 Female blood plasma concentrations of the steroids testosterone (T) and 17-β estradiol (E2)
182 were measured by radioimmunoassay (RIA) according to Schultz (1985). In brief, steroids
183 were extracted from 200 µl plasma with 4 ml diethylether. The aqueous phase was frozen on
184 dry ice, where after the organic phase was transferred to a glass tube, evaporated in a water
185 bath, and then reconstituted with 600 µl assay buffer. Samples were assayed in duplicate.
186 Unfortunately, a technical problem with one of the freezers led to the loss of the samples
187 obtained on the final two sampling dates for the cod housed at HiB.

188 **Histological analyses**

189 Histology was only done for female ovaries, which were processed using standard protocols
190 for resin embedding (Technovit® 7100), producing 4-µm sections stained with 2 % toluidine
191 blue and 1 % sodium tetraborate. Oocytes were classified into stages as described above. In
192 addition the PVO stage was divided into the following three sub-stages based on the
193 classification of Shirokova (1977): phase 4A (indistinct circumnuclear ring (cnr) located
194 centrally in the cytoplasm), 4B (distinct cnr located centrally in the cytoplasm) and 4C (cnr
195 located in the periphery of the cytoplasm). The distinction between 4A and 4B was
196 considered in several cases to be ambiguous so the two phases were presently combined into
197 phase 4AB. Taken together 4A-C refers to the so-called perinucleuolus stage or advanced
198 PVO. In this line, the appearance of the cnr was considered to be an early indication of
199 intracellular preparation for further oocyte growth (see Kjesbu and Kryvi 1989, and citations

200 therein). All sections were carefully screened for post-ovulatory follicles (POF) produced by
201 past spawning females (Saborido-Rey and Junquera 1998; Witthames et al. 2009). The
202 prevalence of atresia was noted as the number of 'females with atresia' (see Table 3, #females
203 with atresia). The intensity of atresia was taken as the percentage of atretic oocytes within the
204 total number of oocytes taken from standard profile counts. In the case of vitellogenic (YG)
205 atresia the total number of YG and atretic YG oocytes examined was around 150.
206 Characterized atretic cells were either in the alpha-stage (i.e. containing yolk) or the beta-
207 stage (without yolk) (Hunter and Macewicz 1985). Ovaries showing only the latter stage were
208 specially noted. A similar type of estimation at the PVO and cortical alveoli stage included
209 examination of a significantly higher number of cells but, nevertheless, the estimate was
210 considered being less precise as it was based on an overall judgment of relative cell numbers
211 present. Consequently, all data were grouped into four atretic intensity classes resembling the
212 system introduced by Hunter and Macewicz (1985) for fully mature northern anchovy
213 *Engraulis mordax* : 0-5, 5-25, 25-50 and >50%. In all cases the intensity listed was oocyte-
214 stage specific, i.e., considering the stages 4AB/4C, cortical alveoli (CA) and YG separately.
215 For females containing atretic YG oocytes upon sacrifice and thereby used to estimate
216 fecundity, we applied a stereological correction factor to account for differences in size of
217 healthy and atretic cells which therefore have unequal chances of being hit in a two-
218 dimensional plane, i.e., during profile counting (Andersen 2003; Kurita et al. 2003; O.S.
219 Kjesbu (unpublished data, 2009)).

220 **Data analyses**

221 *Growth*

222 Length, weight and condition (condition defined as the residuals of a simple regression of ln
223 length against ln weight, e.g. Scott et al. 2006) development during the experiment was
224 examined by the following mixed effect model:

225 $y_{ijl} = \mu_0 + \beta_{0l} + b_{0i} + (\mu_1 + \beta_{1l} + b_{1i})t_j + e_{ijl}$ (2)

226 where y_{ijl} refers to the measurement, i.e., length, weight or condition, of the *i*'th fish at the *j*'th
227 time t_j. The subscript *l* denote feeding regimes (ML and LM), and the e_{ijl} is the unexplained
228 error. Subscript 1 refers to a slope and 0 to a constant (i.e. intercept). A Greek letter denotes a
229 fixed effect and a Latin letter denotes a random effect. The time intervals between
230 measurements were fairly equal except between the last two measurements (Table 1), and
231 were coded as 0, 1, 2, 3, 4, 5, 6, 7 and 7.3. Males and females and each feeding periods were
232 tested separately.

233 Hepatosomatic (*HSI*) (100×liver weigh×(total weight – gonad weight)$^{-1}$) and
234 gonadosomatic index (*GSI*) (100×gonad weight×total weight^{-1}) was compared between sexes
235 in the different feeding regimes by two-tailed t-tests. This was done both for fish sacrificed in
236 October and at the end of the experiment.

237 *Oocyte recruitment and atresia*

238 First, we wanted to compare the oocyte size of the leading cohort between females possessing
239 CA and YG oocytes, and, secondly, whether females possessing YG oocytes had finished
240 their oocyte recruitment to the maturing pool. To do this we first plotted all biopsy data to see,
241 if we could find a leading cohort threshold after which, all oocytes had reached the YG stage.
242 When females have finished recruiting oocytes to the maturing pool, there becomes a gap
243 between the vitellogenic sizes present and the smallest possible sizes of vitellogenic oocytes,
244 i.e., 250 μm for cod (Kjesbu 1991). Using the oocyte size frequency distribution curve
245 obtained from the image analyses we therefore further classified vitellogenic females as either
246 i) still recruiting oocytes to this years maturing pool, defined as > 5 % of the measured
247 oocytes being < 300 μm or ii) having finished recruitment, defined < 5 % of the oocytes being
248 < 300 μm (see Table 3 #fin).

249 To clarify if atresia in maturing females was linked to individual energy reserves we
250 first divided females into three categories, i.e., i) females for which no atresia was detected in
251 any of the biopsy samples, ii) females for which atresia was confined to stages no more
252 advanced than the CA stage and iii) females that at one or more sampling points had atretic
253 YG oocytes. We first examined if *HSI* at sacrifice was different between these categories or if
254 intensity of YG atresia at this time was correlated to *HSI*. We then examined if atresia could
255 be related to short term changes in condition, by examining if there had been a decline in
256 residual condition in the past month for the female for which atresia was found in the sample.
257 Finally, we wanted to examine how temporal changes in energy reserves affected
258 fecundity. For these tests we employed simple regressions for each month separately. Total
259 weight was used as the independent variable and potential fecundity (F_p) and potential
260 fecundity adjusted for the atretic loss (F_{pa}) in the final sample (calculated from the
261 histological sections, i.e., $F_{pa} = F_p$*(1-intensity of YG atresia), were used as the dependent
262 variables. For these tests female weight and both measures of fecundity were ln-transformed
263 before applying the regression model.
264 *Skipping spawning in relation to hormonal values and energy reserves*
265 The values and distribution of hormonal samples for skipping and spawning females was
266 compared for each sampling date by two-tailed t-tests. On some dates, some females had
267 hormonal values so low that they were undetectable in the assays. These were given the same
268 value as the lowest detected value in all analyses and graphs, i.e., 0.17 ng•ml^{-1} for E2 and 0.3
269 ng•ml^{-1} for T. Since we had multiple comparisons significance was assigned at 0.0064,
270 according to Dunn-Sidak's method (Ury 1976). Similarly, we used our estimate of residual
271 condition to compare energy reserves through the experiment with a two-tailed t-test and
272 again assigned significance at 0.0064. At sacrifice we compared the *HSI* of skipping and
273 maturing fish.

274 ## Results

275 POF's were discovered in all biopsy samples from all female ovaries (e.g. Fig. 1). In total, 27
276 of the 69 males were deemed to be recruit spawners based on the absence of a spawning
277 check. Based on the otolith pattern, 44 of the 64 males were considered to be Northeast Arctic
278 cod, whereas 22 of the 28 females were deemed to be Northeast Arctic cod. The remaining
279 cod had otolith shapes typical of Norwegian coastal cod.

280 **Growth**

281 At the start of the experiment there was no difference in either length, weight or condition in
282 the LM and ML groups between either males or females (two-tailed t-tests, all p's > 0.17, Fig.
283 2). As expected the different feed regimes caused differences between the groups. During the
284 first feeding period, June 22 to October 3, females in the ML group increased significantly
285 more in weight than the LM group (eq (2), d.f. = 85, $p < 0.05$, Fig. 2) and nearly had
286 significantly larger increments in condition development ((eq (2), d.f. = 85, $p = 0.07$, Fig. 2).
287 There was no difference in length growth (Fig. 2). ML males had significantly larger
288 increments in condition, weight and length than LM males (eq (2), d.f. = 211, all p`s < 0.05,
289 Fig. 2). The following switch in food regimes caused a reverse situation and during the latter
290 period both male and female LM cod increased significantly more in weight and condition (eq
291 (2), all p`s < 0.05, Fig. 2), but not in length. Upon sacrifice there was no difference between
292 either sex in length, weight or condition (all p's > 0.05).

293 In October, *GSI* values were similar between sexes and groups (Fig. 3). Upon sacrifice
294 in January there was no differences between maturing males and females between groups, but
295 *GSI* values were generally higher in males (two-tailed t-test, sexes pooled across groups, d.f.
296 = 66, $p < 0.05$).

297 *HSI* values appeared to be somewhat higher in the ML group for fish sacrificed in
298 October, but this was not significant when pooling the results across sexes (two-tailed t-test,

299 d.f. = 18, p = 0.17, Fig. 3). Upon sacrifice there was no difference between maturing females
300 or males between groups, but females had significantly higher *HSI* values than males (two
301 tailed t-test, sexes pooled across groups, d.f. = 66, $p < 0.0001$, Fig. 3).

302 **Oocyte recruitment and atresia in maturing females**

303 *Oocyte recruitment*

304 The combined digital image and histology analyses showed that in September the ovaries of
305 only 4 females had reached the late CA stage, while 9 females were at the early CA stage and
306 5 females possessed only PVOs (Table 3). However, by November 13 females had reached
307 the YG stage, with the remaining fish possessing advanced CA oocytes (Table 3). Based on
308 our definition, fish had generally finished oocyte recruitment when they reached the YG stage
309 (Table 3). Hormonally, E2 and T values above 2 ng•ml^{-1} were associated with yolk granules
310 only, reaching a maximum level of approximately 7.5 and 6 ng•ml^{-1}. Lower values were
311 found for females possessing both PVO, CA or YG oocytes (Fig. 4). Comparing the leading
312 cohort size of females possessing YG and CA oocytes, there was great overlap < 400 µm, but,
313 with only one exception, once the leading cohort was > 400 µm, the oocytes had reached the
314 YG stage (Fig. 5).

315 *Atresia*

316 In the initial measurements very few maturing females showed any signs of atresia (Table 3).
317 However, from November onwards 5-7 of the maturing 18 females possessed atretic oocytes
318 at each monthly measurement. Only 5 out of the 18 females did not show any signs of atretic
319 loss at any stage. For the remaining 13 females, oocyte atresia was confined to the PV/CA
320 stage in 5 females, while atretic YG oocytes were observed in 8 females at one or more
321 samplings (Table 4).There was no indication of any link between female atresia and energy
322 (one –way ANOVA, $F_{2,15} = 0.041$, p = 0.96, data not shown), nor of any link between *HSI* at
323 sacrifice and intensity of YG atresia at sacrifice (data not shown, Simple regression, d.f. = 5, p

324 = 0.49). Further, short time changes in residual condition factor, were not associated with
325 atresia, i.e., females were as likely to have increased as decreased in condition just prior to the
326 sample where atresia was discovered (data not shown), nor was any particular form of atresia
327 more prevalent in ML than LM females. There was no indication of any of the maturing
328 females undergoing mass atresia and aborting spawning, as YG - atresia at sacrifice was never
329 above 22 % for any female at the final sampling. YG-atresia was confined to leading cohort
330 sizes of 415-640 μm.

331 *Proxies to fecundity*

332 Generally, female weight at all months was strongly correlated to potential fecundity (P_F) and
333 potential fecundity controlled for atresia (P_{FA}) (Table 5). However, even so, temporal patterns
334 were detectable. The explanatory power of weight generally increased from the June
335 measurements until November (Fig. 6) and from this date onwards the explanatory power
336 remained more or less the same (Table 5). Adjusting for atretic loss generally decreased the
337 variation explained for each month compared to non-adjusted values, but the between month
338 variation remained very similar to that of the unadjusted values (Table 4). The pattern of
339 explanatory power did not change if we only included cod that were deemed to be Northeast
340 Arctic cod based on their otolith shape.

341 **Skipped spawning**

342 Of the 22 females that were sacrificed in January four would have skipped spawning. These
343 females had ovaries containing oocytes no more advanced than the 4AB/C or early CA stages
344 (Table 3) and had lower *GSI* values at sacrifice (two-tailed t-test with unequal variances, $p <$
345 0.001, Fig. 3). These females also had significantly lower *HSI* values than the maturing
346 females at sacrifice (two-tailed t-test with unequal variances, $p < 0.001$, Fig. 3). Tracing the
347 residual condition of these females back to the experimental start, they were in significantly
348 lower condition than the maturing females from December onwards, all p values < 0.005 (Fig.

349 7). Skipping females were present in both the ML and LM group and had undergone a gradual
350 decline in condition through the experiment, whereas maturing females generally had
351 increased their condition (Fig. 7). Plasma E2 levels were significantly lower for these skippers
352 compared to the maturing females from November onwards (two-tailed t-test with unequal
353 variances, all p's < 0.006, Fig. 7). In fact E2 levels had decreased from October to November
354 in the skipping females (Fig. 7). T levels were only significantly lower for the skippers in the
355 final January samples ($p < 0.001$, Fig. 7). The results of the combined histological and image
356 analyses closely reciprocated the hormonal data. In October the skipping females possessed
357 oocytes at the early CA stage, and no atresia was evident. This was similar to the maturing
358 females (Table 3). However, in November none of the skipping females had advanced beyond
359 this stage, in fact one female now only possessed PV oocytes (Table 3), whereas 15 out of the
360 18 maturing females possessed yolk granules and the remaining three had reached the
361 advanced CA stage (Table 3). Further, at this time all skipping females showed signs of
362 atresia, whereas only 5 out of 18 maturing females possessed any atretic oocytes (Poisson test,
363 $m = 18$, $p < 0.05$). At the final sampling in January, 3 out of the 4 skipping females only had
364 PVO oocytes. One of the skipping females was deemed to be a coastal cod based on the
365 otolith shape. However, removing this female from the analyses did not change the results of
366 any of our analyses as there was great coherence between oocyte stage and hormonal values
367 in all skipping females (Table 3, Fig. 7). Even though males had on average lower energy
368 reserves than females (Fig. 3) only 1 out of 64 males did not mature. This male was however
369 deemed to be an immature cod based on the absence of a spawning check.

370
Discussion

371 To our knowledge this is the first study, examining the maturation cycle of Northeast Arctic
372 cod in a controlled laboratory setting with an emphasis on the underlying principles behind
373 the phenomenon of skipped spawning. Even though the Northeast Arctic cod is now the

374 largest and most important commercial cod stock in the world (ICES 2008), the large size of
375 sexually mature individuals (> 60 cm, Nash et al. 2008), coupled with the often long transport
376 routes to suitable tank facilities following capture, have made virtually all studies on adults
377 restricted to the field. Thus, even though we only had a total of 28 females and 69 males in
378 the present experiment, the results still represent a significant advance in the knowledge of
379 mechanisms affecting both oocyte recruitment, atresia and skipped spawning in this stock. We
380 further demonstrate the usefulness of POF's as a reliable long-lasting marker of past
381 spawning in Northeast Arctic cod, in agreement with the results of Saborido-Rey and
382 Junquera (1998) and Witthames et al (2009).

383 **Hormonal values, oocyte recruitment and atresia**

384 As expected there was an increase in hormonal values concurrent with oocyte development in
385 maturing females (Fig. 7). Overall these results closely mimics those found by Dahle et al.
386 (2003) for E2, whereas present T values are higher than those reported by both Dahle et al.
387 (2003) and Skjæraasen et al. (2004) working on captive Norwegian Coastal cod. While
388 female fish generally produce large amounts of T during sexual maturation, the exact
389 physiological role of this androgen is still uncertain (Borg 1994; Senthilkumaran et al. 2004).
390 Through its influence on GnRH release, T facilitates the massive release of LH prior to
391 ovulation. Also, T is the precursor for E2 biosynthesis, and it is believed that these two
392 steroids act in concert during oogenesis. Further, the rather steady increase in plasma T and
393 E2 levels prior to spawning, as also noticed presently for non-skippers, is known to be
394 replaced by highly cycling and significantly larger values during spawning, i.e., during the
395 process of final maturation and egg formation (Kjesbu et al. 1996), in agreement with studies
396 on other batch spawners such as halibut (*Hippoglossus hippoglossus*) (Methven et al. 1992).
397 Thus, the present actual levels were more of interest in the comparison between skippers and
398 non-skippers and clearly show that plasma levels of both steroids remained low (< 1 ng • ml^{-1})

399 in the former. Maturing females went from previtellogenic to the yolk granule (YG) stage in
400 the course of two or in some cases three months. When females had reached the YG stage,
401 oocyte recruitment seemed to be effectively finished (Table 3), i.e. the time window for
402 oocyte recruitment was in most cases two months in our experiment. The cortical alveoli
403 (CA) stage oocytes were, as expected, prevalent at the earlier stages and persisted at the most
404 until the leading cohort size had reached 400 μm. The smallest YG oocytes were found at a
405 leading cohort size of 350 μm and the range 350-400 μm therefore represented a transitional
406 zone between the CA and YG stage in our experiment, very much in agreement with field
407 results on the same stock (Kjesbu 1991).
408 For the maturing females, atresia was very limited (Table 3), until oocyte recruitment
409 had finished, when atresia became more frequent and was found in PV, CA and YG oocytes
410 (Table 3, 4). This is novel information as previous studies have mainly focused on
411 prespawning cod, i.e. the YG stage (Kjesbu et al. 1991; Kraus et al. 2008). However, in the
412 earlier study of Kjesbu et al. (1991) there were examples of poor-condition females arrested at
413 the interphase between the PV and CA stage. This type of arrest at an early stage of oogenesis
414 is known to take part also in other species such as winter flounder (*Pleuronectes americanus*)
415 (Burton 1994) and blue whiting (*Micromesistius poutassou*) (Kjesbu 2009). Somewhat
416 surprisingly we did not find any relationship between our condition proxies and atresia, as
417 atresia generally is negatively correlated to fish condition (e.g. Kjesbu et al. 1991; Kurita et
418 al. 2003; Kraus et al. 2008). However, this finding must be treated with some caution as 1)
419 this analysis referred to the maturing fraction only, 2) atresia levels might be highly
420 fluctuating over time (cf. atretic window) and 3) we actually did not undertake any proximate
421 chemical analyses as in Kjesbu et al. (1991) and Kurita et al. (2003). Although liver index is
422 generally considered a good proxy for liver energy content there is nevertheless large
423 variation in the specific energy content for a given liver size (Lambert and Dutil 1997).

424 The explanatory power of weight as fecundity proxy, showed a clear temporal pattern,
425 albeit the variation between measurement dates was quite low. This low variation could partly
426 be caused by differences in individual feeding rates, i.e., if some individuals constantly
427 acquired more food than others this may have masked the effect of the feeding rations at a
428 group level. Even so, explanatory power generally increased until November, where after it
429 remained similar (Figure 6, Table 5). In November all maturing females, had reached either
430 the late CA or YG stages and the majority of females were deemed to be close to the end or to
431 just have finished follicle proliferation (Table 3). In sum, the results indicate that energy
432 reserves during early vitellogenesis are influential for potential fecundity. This is in
433 agreement with studies on both Norwegian coastal cod (Skjæraasen et al. 2006) and plaice
434 (*Pleuronectes platessa*) (Kennedy et al. 2007).

435 **Skipped spawning**

436 Our study provides experimental evidence of skipped spawning for Northeast Arctic cod. For
437 this stock, this phenomenon has mainly been described from field samples in the Barents Sea
438 (Woodhead and Woodhead 1965; Oganesyan 1993; Marshall et al. 1998). Rideout et al.
439 (2005) partitioned skipped spawners into retaining, reabsorbing and resting females.
440 Retaining females do not shed their eggs during the spawning season due to factors such as
441 overcrowding, stress, pollution and lack of mates. Reabsorbing skippers reabsorb all
442 vitellogenic oocytes prior to spawning, and resting females do not start vitellogenesis at all
443 (*sensu* Rideout et al. (2005)). In our experiment all skipping females reached the early CA
444 stage, i.e., endogenous vitellogenesis (Wallace and Selman 1981) before further oocyte
445 development was arrested ahead of the main mobilisation of energy, i.e. the YG stage (Tyler
446 and Sumpter 1996), or true vitellogenesis (Wallace and Selman 1981). Thus based on the
447 histology, our females were resting-early reabsorbing skippers (Table 3); they only reached
448 the endogenous vitellogenic stage and were clearly on a different trajectory than the maturing

449 females, both hormonally and stage-wise, by early November, the same time the power of
450 weight as a fecundity proxy reached its maximum value for the spawning females (Table 5).
451 No indication of any cod undergoing mass atresia of yolk granules was found. Previous
452 reports have identified both resting and reabsorbing skippers in the Northeast Arctic cod
453 (Woodhead and Woodhead 1965; Oganesyan 1993). Although the data is limited, our results
454 indicate that for the majority of females, the "decision" to skip spawning is taken well ahead
455 of the spawning season. From a life-history perspective this makes sense, given the large
456 distance between spawning and feedings grounds for the Northeast Arctic cod. The cost of the
457 spawning migration may push the "decision" to spawn or not forward to a time before the
458 start of the main migration. This implies that for Northeast Arctic cod, skippers remain on the
459 feeding grounds, i.e. in the Barents Sea, and would therefore be unaccounted for in surveys at
460 the spawning grounds, i.e. Lofoten and Vesterålen, and elsewhere along the Norwegian coast
461 (Sundby and Nakken 2008). Tentatively agreeing with this, females with non-maturing
462 gonads are more or less absent from the long-term fecundity time series from Andenes,
463 Vesterålen (Kjesbu et al. 1996; Thorsen et al. 2006).

464 Skipped spawning has previously primarily been linked to insufficient energy reserves
465 (Rideout et al. 2005). However, from a life-history view point skipped spawning might also
466 be an adaptive trait, i.e. young females may trade off between investment in growth and,
467 potentially, enhanced future reproductive success, at the cost of the present spawning
468 opportunity (Rideout et al. 2005; Jørgensen et al. 2006). Our results clearly support the
469 contention that limited energy reserves cause spawning omission as skipping females had
470 smaller livers at sacrifice (Fig. 3) and lower condition from December onwards (Fig. 7) than
471 maturing cod. Similarly, for Canadian cod, liver energy is the best predictor of spawning
472 probability (Rideout et al. 2006). Due to the limited number of fish in our experiment we are
473 unable to evaluate if age did influence the likelihood of skipping, but there was no indication

474 of any increased investment in length growth in skipping females. However, very little
475 investment in length growth was observed for any cod through the experiment (Fig. 2), which
476 might relate to the present laboratory conditions, as adult Northeast Arctic cod normally grow
477 about 10 cm per year in the field (ICES 2008).

478 It might be argued that our strict food regime gave energy reserves that were
479 unnaturally low and that this comparatively high proportion is an artefact caused by the
480 experiment. However, we argue the opposite, as well-fed fish kept in the laboratory usually
481 have condition factors far exceeding that found in natural populations (e.g. Kjesbu 1989). The
482 females in our experiment had an average hepatosomatic index of approximately seven
483 percent (Fig. 6), which closely resembles the upper values seen in the field for the same stock
484 (Marshall et al. 1998). However, we would like to stress that we do not believe that our results
485 can be used to identify thresholds for maturation in the field. In an ongoing large – scale field
486 sampling program, numerous skippers have been identified with liver indexes similar to
487 spawning cod in our laboratory experiment (Skjæraasen et al. unpublished). We speculate that
488 this is caused by differential investment by fish in the laboratory and the field, where fish kept
489 under the low exercise and low, but reliable, food regime in the laboratory allocate relatively
490 more energy into reproduction and less to growth and maintenance than in the natural
491 situation. However, we do believe that our results represent general and true mechanism of
492 skipped spawning for Northeast Arctic cod in that i) the main body of skippers separate from
493 maturing females during early vitellogenesis and ii) that skipping is highly influenced by
494 individual energy reserves. Further, the complete absence of skipped spawning in males is
495 conspicuous, particularly when considering their overall lower energy reserves (Fig. 2).

496 In sum, both oocyte recruitment and skipped spawning seem to be highly influenced
497 by energy reserves during the critical period of early vitellogenesis for Northeast Arctic cod.
498 This closely mimics the results of Burton (1994) for winter flounder. Skipping was linked to

499 low energy reserves and clearly more common in females, presumably, because of the larger
500 cost associated with gonad maturation and spawning. Further, hormonally, skippers separated
501 from the maturing fraction long before spawning and oocyte development was arrested at the
502 early cortical alveoli stage. This implies that skippers i) can be identified early in the
503 maturation cycle, which is important for forecasting of egg production and in recruitment
504 studies and ii) remain on the feeding grounds in the Barents Sea when the spawning migration
505 starts. If so, estimates of the proportion of skippers at stock level would, if based on surveys at
506 the spawning grounds, underestimate the proportion of fish that are skipping spawning in any
507 given year.

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660 **Table 1**. Sampling dates and protocol on different dates

Date	Protocol
22 June 2006	Weight, length, blood sample
8 and 9 Aug 2006	Weight, length, blood sample
5 and 6 Sep 2006	Weight, length, blood sample and biopsy
3 and 4 Oct 2006	Weight, length, blood sample and biopsy, sub-sample of fish sacrificed, food rations switched
8 and 9 Nov 2006	Weight, length, blood sample and biopsy
5 and 12 Dec 2007	Weight, length, blood sample and biopsy
10 and 11 Jan 2007	Weight, length, blood sample and biopsy
18, 19 and 23 Jan 2007	Weight, length, blood sample and biopsy, all fish sacrificed

661 **Table 2.** Number and average length of males and females in the ML and LM group housed

662 at the Institute of Marine Research (IMR) and High Technology Centre of Bergen (HiB).

663 Number after IMR and HiB is the number of individuals in each sub-category. Weight and

664 length is the average values at the start of the experiment

	ML				**LM**		
	IMR		**HiB**		**IMR**		**HiB**
	Males (20)	**Females (11)**	**Males (13)**	**Females (5)**	**Males (20)**	**Females (13)**	**Males (16)**
Weight	3620	4373	1975	2056	3353	3720.5	1920
Length	77.6	83.4	62.0	63.2	77.6	79.4	61.7

665

666 **Table 3.** Maturation stage and number of samples containing atretic oocytes during the
667 experiment for females that i) were maturing, i.e. would have spawned, ii) were sacrificed in
668 October and iii) would have skipped spawning. The stage was decided mainly by histological
669 analyses, but we also used the results of image analyses to separate between early (E-CA) and
670 late (L-CA) cortical alveoli stages. YG denotes the yolk granule stage. # females with atresia
671 is the number of females for which at least on atretic oocyte was found in their biopsy sample
672 and # fin is the number of females deemed to have finished oocyte recruitment for a given
673 date.

Date	**Most advanced stage**					**# females with atresia**	**# fin**
	Pre-vitellogenic		**Vitellogenic**				
	3	**4AB/C**	**E-CA**	**L-CA**	**YG**		
Maturing							
Sep	4	1	9	4		1	4
Oct			8	5	5	1	8
Nov				3	15	5	15
Dec					18	7	17
Jan1					18	6	17
Jan2					18	5	18
Sacrificied							
Sep	1	1	2	2		0	
Oct			2	2	1	1	
Skipped							

Sep	2		1
Oct		3	0
Nov	1	2	3
Dec	2	1	2
Jan	3		2
Jan2	3	1	4

674

675 **Table 4**. The occurrence of atresia, divided into cell stage and intensity. Fish is the PIT-tag
676 code for individual females, codes given in italics identify a fish that would have skipped
677 spawning. Beta atresia signifies that the fish had atretic oocytes at the beta stage at the given
678 measurement date. Note that only dates and fish for which we found atresia is given in the
679 table.

		% Atresia stage 4AB/C				**% Atresia stage CA**				**% Atresia stage YG**				**Comment**
Fish	**Date**	**0-5**	**5-25**	**25-50**	**>50**	**0-5**	**5-25**	**25-50**	**>50**	**0-5**	**5-25**	**25-50**	**>50**	
3025	8 Nov 2006			x										
3025	5 Dec 2006				x	x								Few 4AB/C
3571	5 Dec 2006	x				x								
3571	10 Jan 2007									x				
8259	20 Jan 2007		x			x								
3e11	7 Nov 2006			x										
3e11	05 Dec 2006	x												
3e11	10 Jan 2007	x												

3e11	18 Jan 2007	x				
9e12	5 Dec 2006	x				
9e65	7 Nov 2006	x				
9e65	10 Nov 2006				x	
9e65	18 Jan 2007				x	
2dfb	3 Oct 2006	x				
5c14	3 Oct 2006	x				
5c14	18 Jan 2007				x	
605a	05 Dec 2006		x			
7cbd	8 Nov 2006			x		
9b99	10 Jan 2007				x	
9b99	18 Jan 2007					x
a3b7	7 Nov	x				

	2006								
a3b7	5 Dec 2006				x				
a3b7	10 Jan 2007						x		
adf8	7 Nov 2006	x							
adf8	18 Jan 2007							x	
af03	6 Sept 2006								Beta-atresia
af03	6 Dec 2006			x					Few 4AB/C
bea3	5 Sept 2006	x							
bea3	7 Nov 2006					x			
bea3	5 Dec 2006		x						Beta atresia
bea3	11 Jan 2007		x						
bea3	18 Jan 2007		x			x			Few CA
c0c7	10 Jan 2007						x		

cd1e	8 Nov 2006		x			
cd1e	20 Jan 2007					Beta-atresia
cdf3	5 Dec12 2006	x				Beta atresia
cdf3	10 Jan 2007			x		
cdf3	18 Jan 2007				x	

680

681 **Table 5.** The explanatory power of weight (W_F) as a proxy to potential (F_p) and atresia

682 adjusted values (F_{pa}) though the course of the experiment. R^2-adj. is the adjusted explanatory

683 value for the regression. The fecundity measurements and female weight were ln-transformed

684 before applying the regression.

Month	**Potential fecundity (F_p)**			**Potential fecundity adjusted for atresia (F_{pa})**		
	Regression formula	**R^2-adj.**	**p-val.**	**Regression formula**	**R^2-adj.**	**p-val.**
Jun	$F_p = 5.19+1.22*W_{FJ}$	0.77	$<1.0^{-5}$	$F_{pa} = 5.95+1.12*W_{FJ}$	0.62	$<1.0^{-4}$
Aug	$F_p = 6.64+1.03*W_{FA}$	0.85	$<1.0^{-7}$	$F_{pa} = 7.13+0.97*W_{FA}$	0.72	$<1.0^{-5}$
Sep	$F_p = 6.02+1.10*W_{FS}$	0.86	$<1.0^{-7}$	$F_{pa} = 6.45+1.04*W_{FS}$	0.74	$<1.0^{-5}$
Oct	$F_p = 5.97+1.11*W_{FO}$	0.86	$<1.0^{-7}$	$F_{pa} = 6.40+1.05*W_{FO}$	0.75	$<1.0^{-5}$
Nov	$F_p = 5.85+1.12*W_{FN}$	0.89	$<1.0^{-8}$	$F_{pa} = 6.17+1.07*W_{FN}$	0.80	$<1.0^{-6}$
Dec	$F_p = 5.69+1.13*W_{FD}$	0.89	$<1.0^{-8}$	$F_{pa} = 6.01+1.09*W_{FD}$	0.79	$<1.0^{-6}$
Jan	$F_p = 5.78+1.12*W_{FJa}$	0.88	$<1.0^{-7}$	$F_{pa} = 6.04+1.08*W_{FJa}$	0.80	$<1.0^{-6}$
Sacr.	$F_p = 5.58+1.14*W_{FSa}$	0.88	$<1.0^{-8}$	$F_{pa} = 5.82+1.11*W_{FSa}$	0.81	$<1.0^{-6}$

685

686 **Figure legends**

687 **Fig. 1** Picture of histological sections from a maturing female and a skipping female obtained
688 from gonad samples taken at sacrifice on January 19. Post-ovulatory follicles clearly show
689 that both females are past-spawners. However, whereas female cdfo (a) possesses large yolk
690 granule oocytes (YG), female cd1e's (b) most advanced stage is pre-vitellogenic 4A oocytes.
691 Scale bar is 100 µm.

692 **Fig. 2** Length (a), weight (b) and condition (c) development during the course of the
693 experiment for ML (black) and LM (grey) males (squares) and females (circles).

694 **Fig. 3** Gonadosomatic index (a), i.e $GSI = 100 \times$ gonad weight $\times$ total weight^{-1} and
695 hepatosomatic index (b), i.e $HSI = 100 \times$ liver weight $\times$ (total weight – gonad weight)$^{-1}$ for
696 fish sacrificed in October and the end of January.

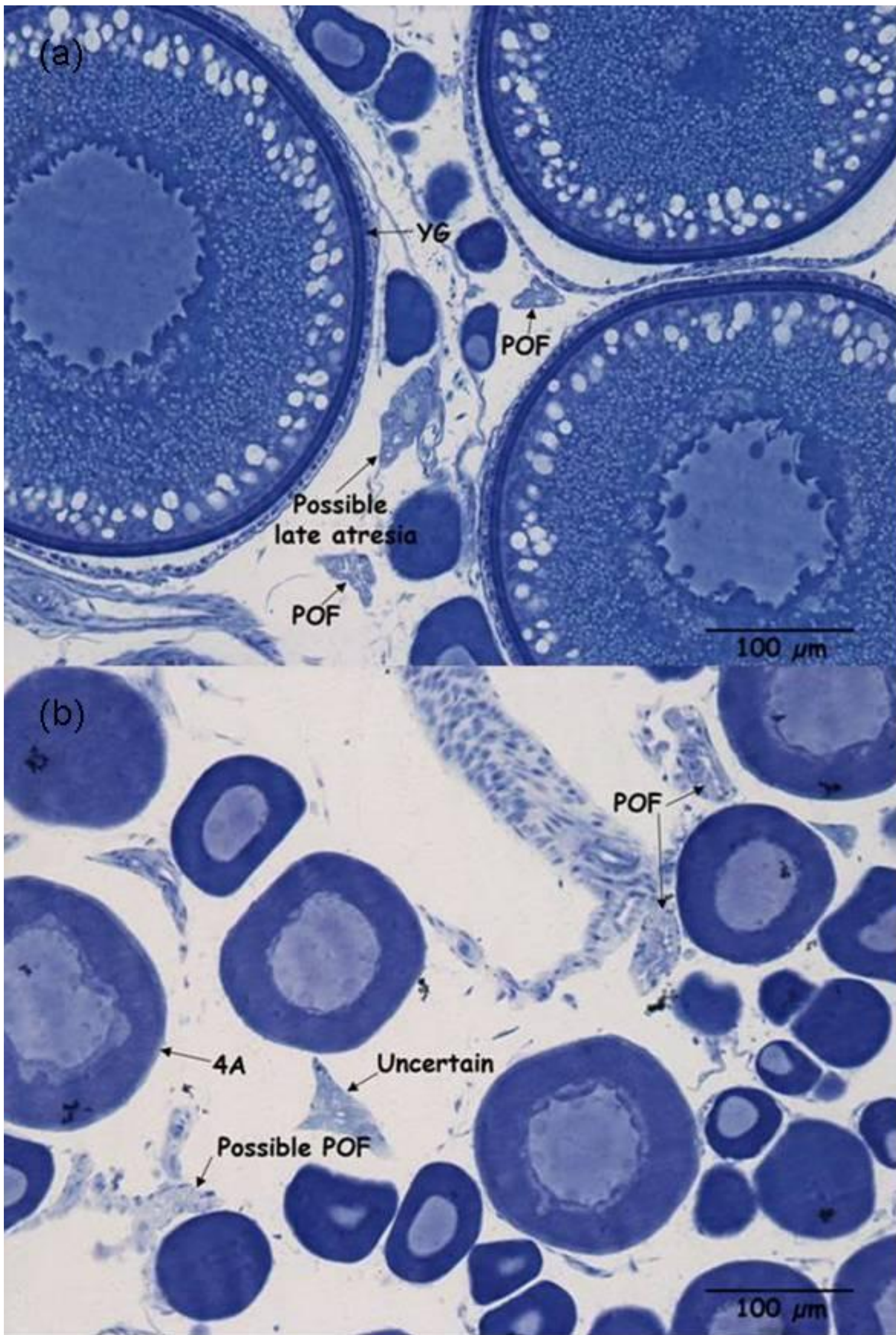
697 **Fig. 4** The relationship between leading oocyte cohort diameter (LC_{20}), grouped into
698 previtellogenic (PV), cortical alveoli (CA) and yolk granule (YG) oocytes, and hormonal
699 values, 17-β estradiol (a) and testosterone (b). The horisontal line depicts a sex steroid value
700 of 2 ng • ml^{-1}.

701 **Fig. 5** The relationship between leading cohort size (LC_{20}) and cortical alveoli (CA) or yolk
702 granule (YG) oocytes. The horisontal line depicts a leading cohort value of 400 µm.

703 **Fig. 6** Female weight in June (a) and November (b) plotted against potential fecundity.

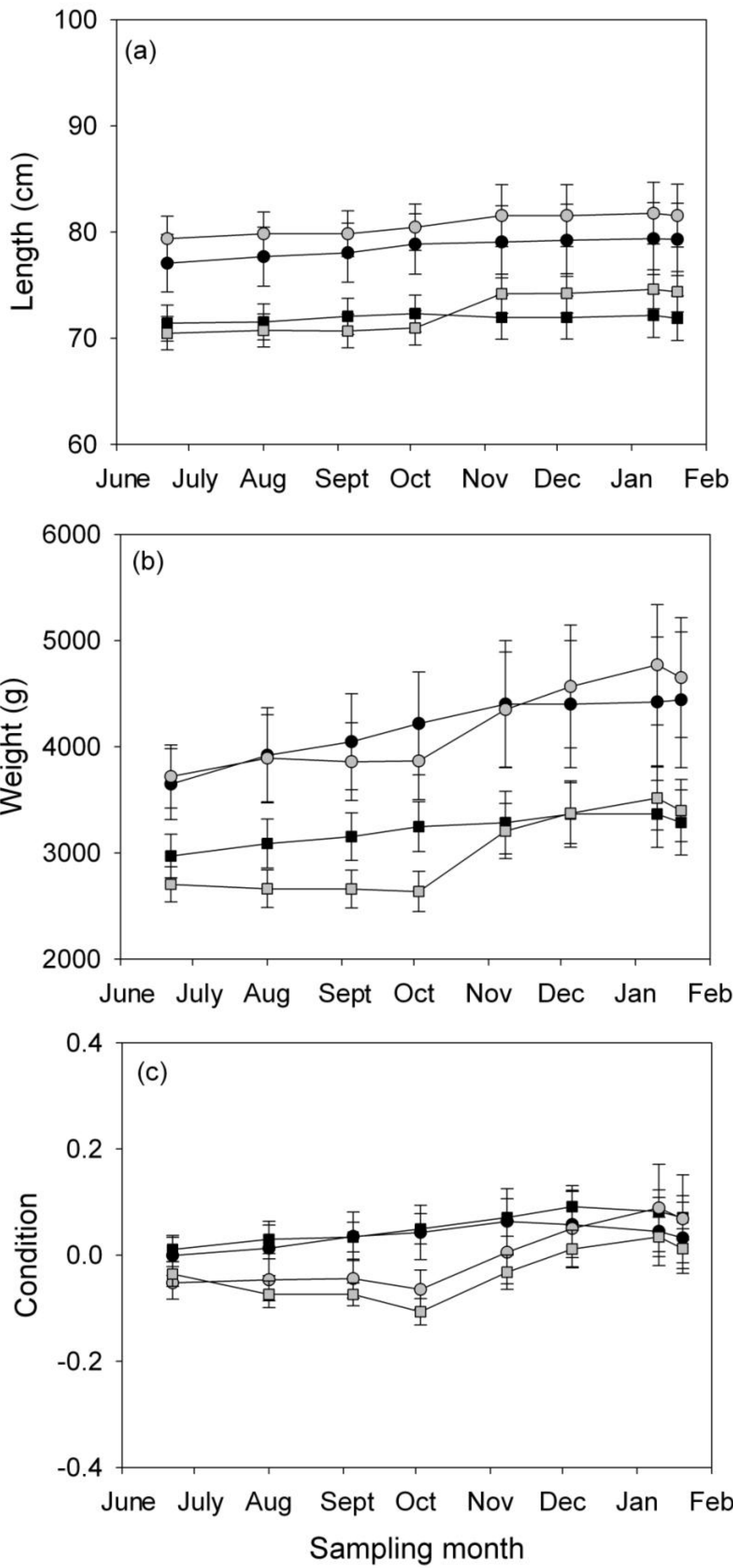
704 **Fig. 7** Residual condition (a), estradiol (b) and testosterone (c) values for females during the
705 experiment. Black circles indicate maturing females and white circles females that were
706 deemed to be skipping. Asterisks indicate significant differences between skipping and
707 maturing females at the date in question.

708 Fig. 1



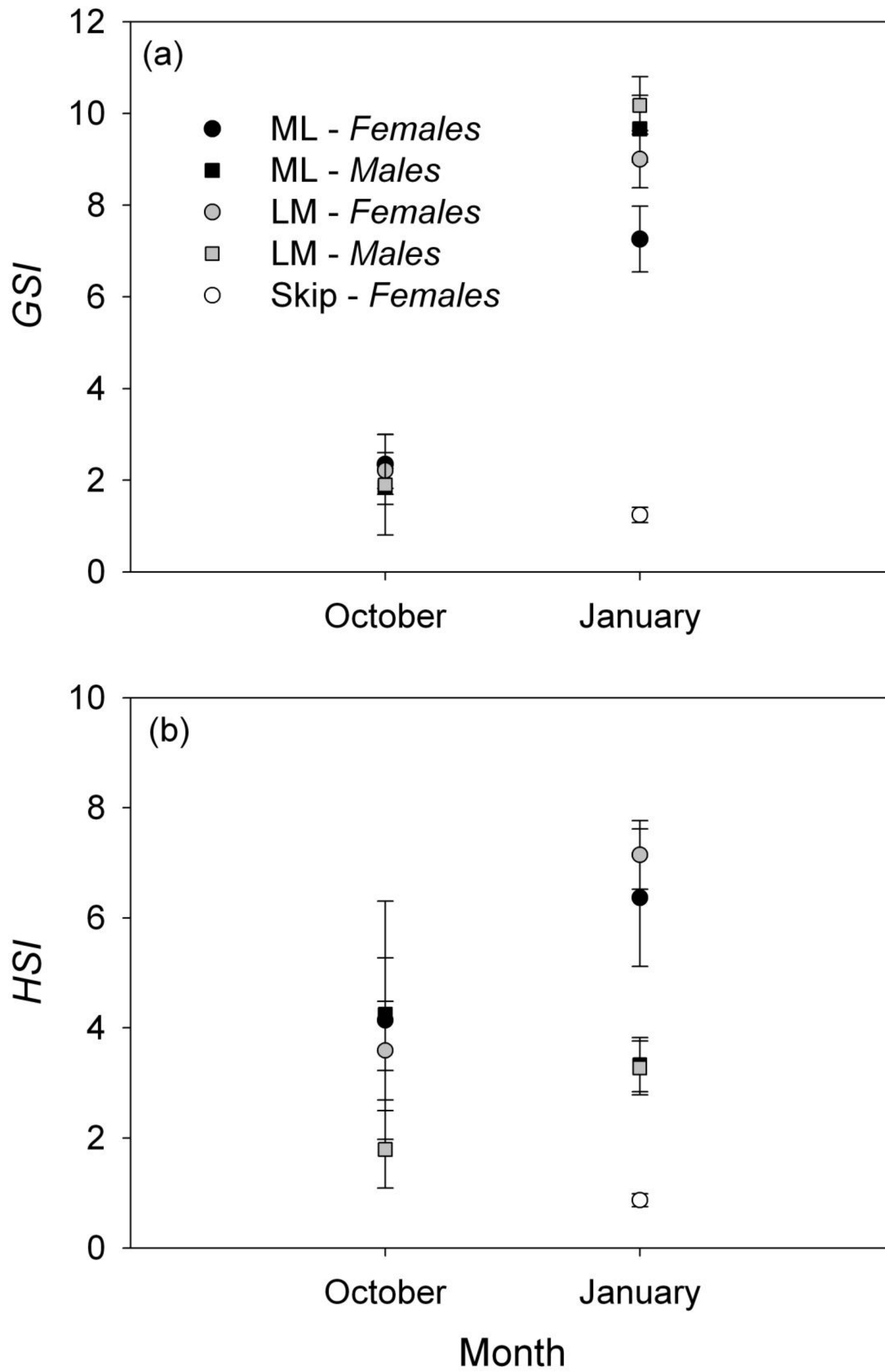
709

710 Fig. 2



711

712 Fig 3.



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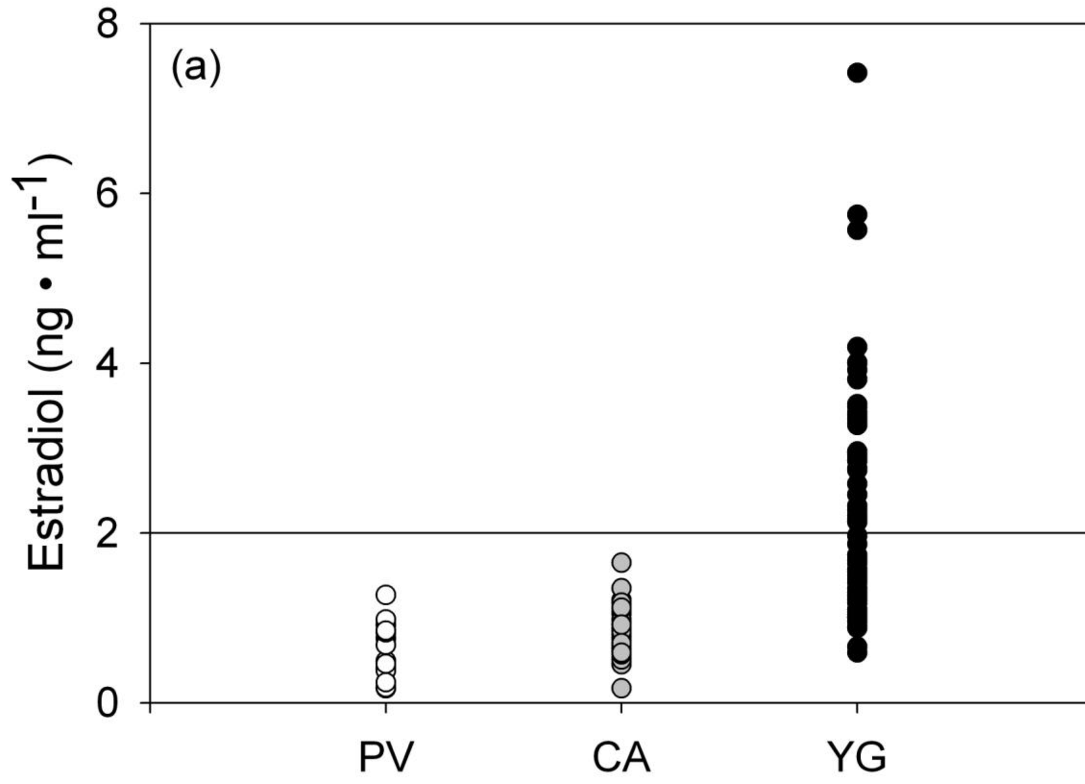
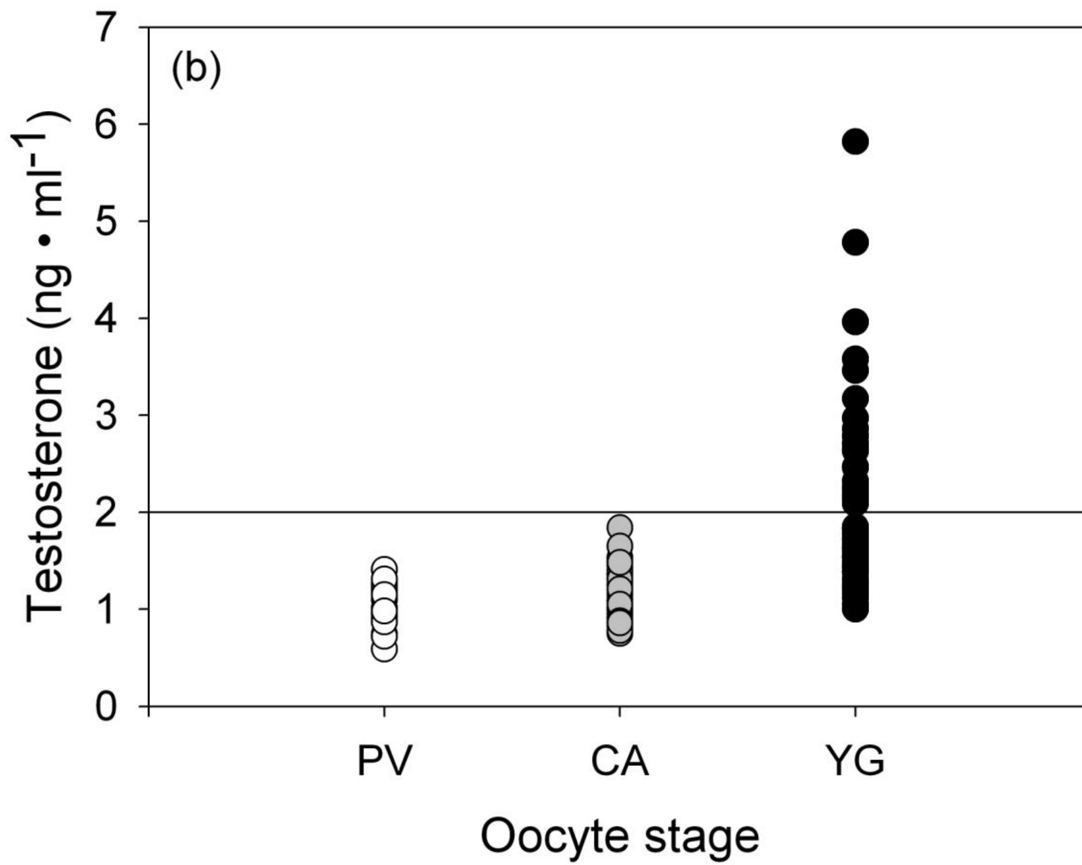
714 Fig. 4





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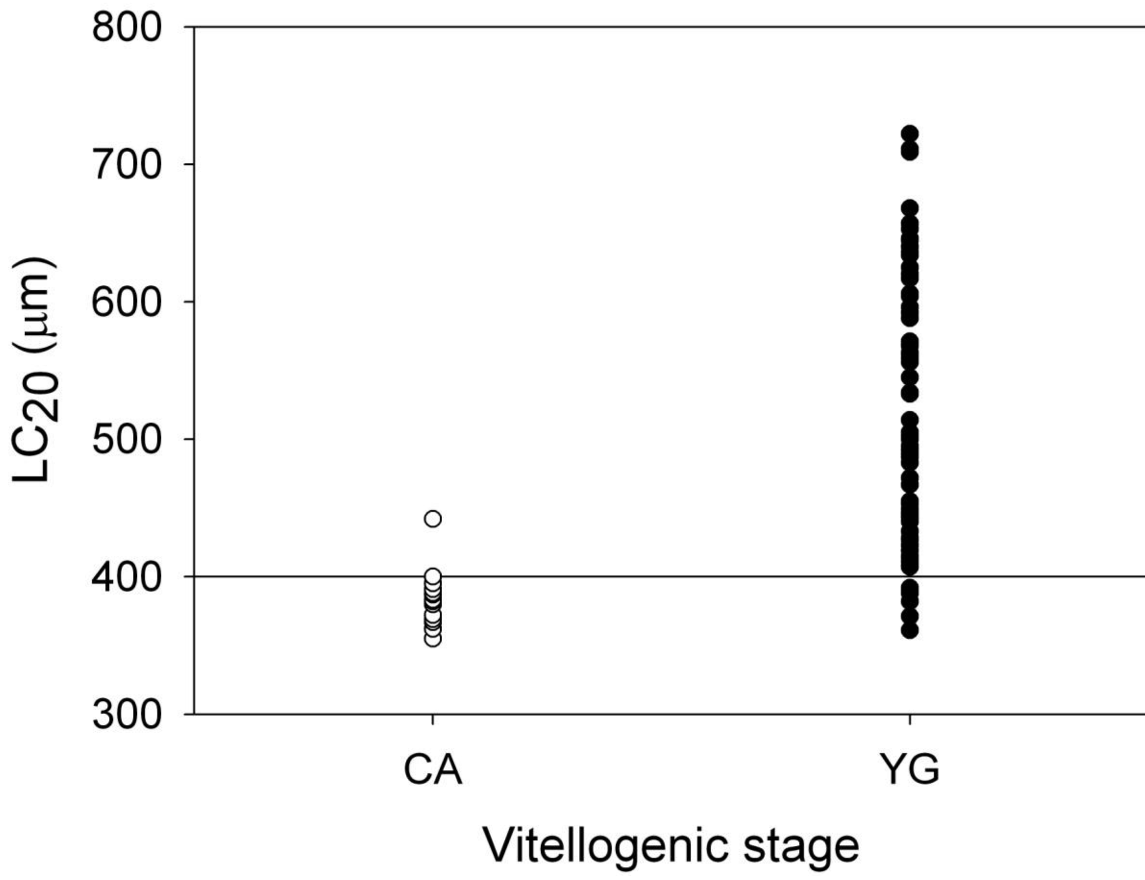
716 Fig. 5



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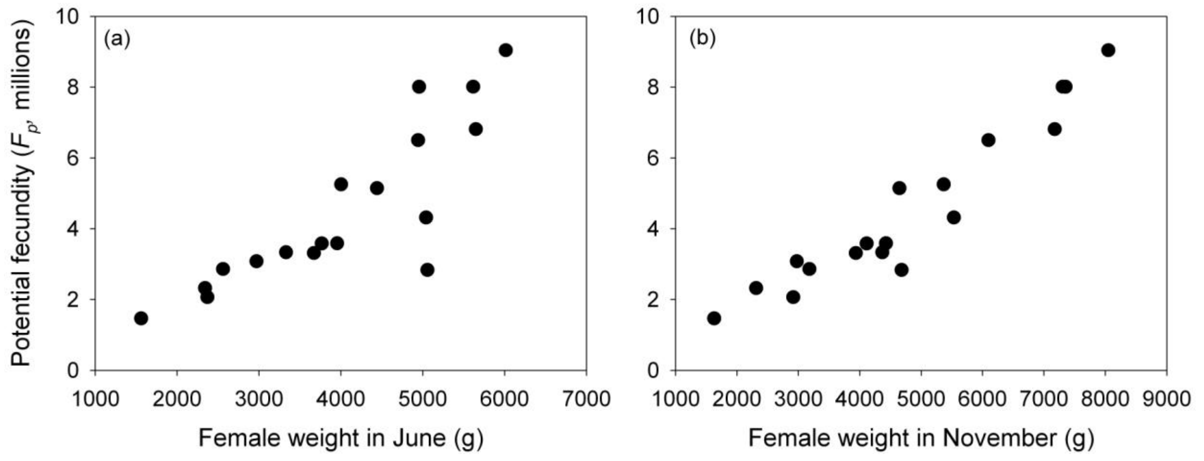
718 Fig. 6



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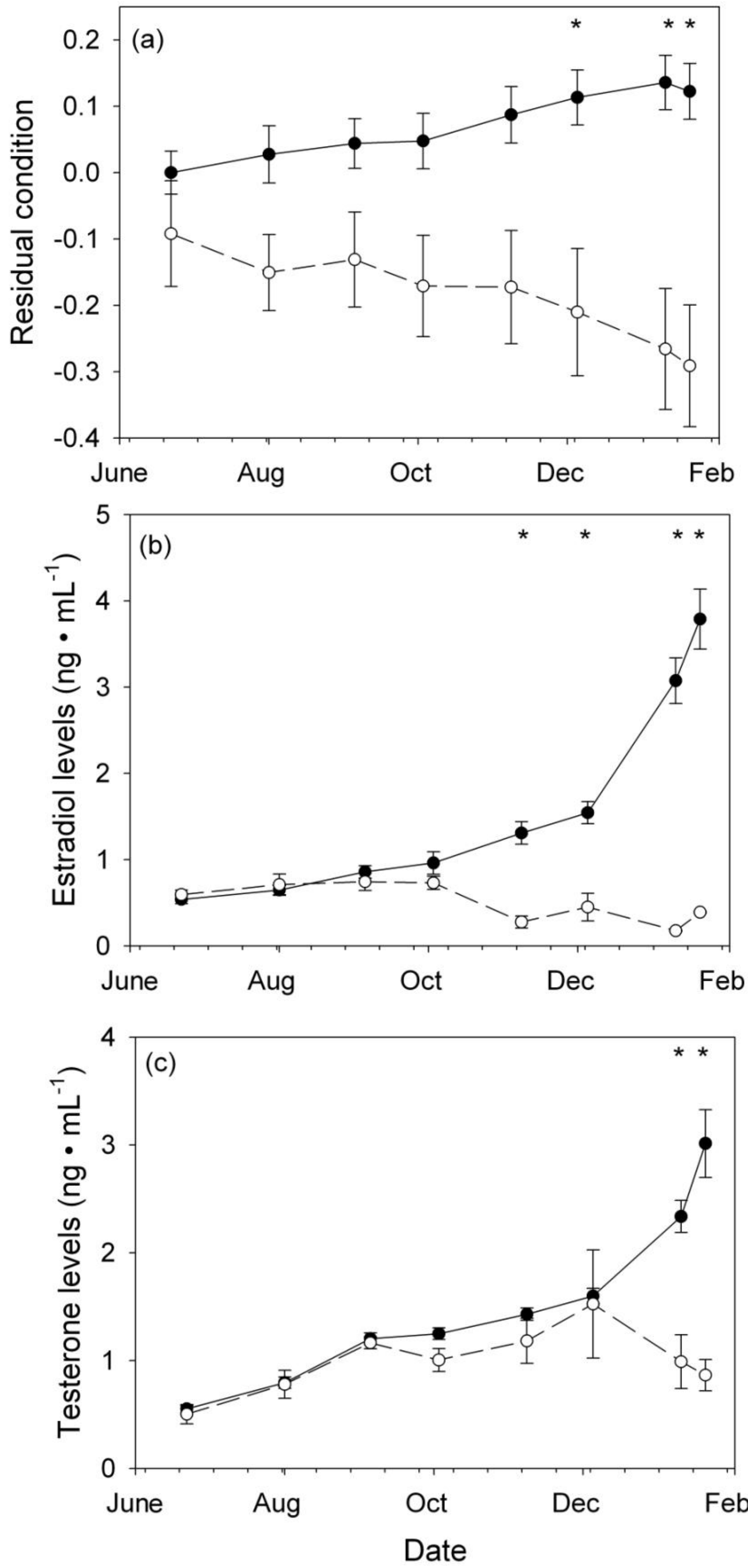
720 Fig. 7



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