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Running head: Swimming induced maturation in eel

19

Abstract

20

21 In this study, we subjected eels from Lake Balaton (Hungary) to a swimming
22 period of one, two or six weeks. Most eels were silver and were 13 - 21 years old. Time
23 dependent changes in morphometrical parameters and developmental characteristics of
24 the oocytes were determined. Already after one week of swimming, the gonadal mass
25 increased and oocytes became larger, filled with large numbers of lipid droplets. After
26 two and six weeks of swimming we found in addition a significant enlargement of the
27 eyes, which is a sign of sexual maturation. In contrast to the resting eels, that had oocytes
28 in the primary growth phase (stage 1 - 2); the swimming eels had oocytes in stage 3; the
29 cortical alveolus or lipid droplet stage. The results indicate that lipid mobilisation induced
30 by swimming is a requirement for the natural incorporation of lipid droplets in the
31 oocytes, a crucial step in oocyte maturation. As the Balaton eels responded stronger to
32 swimming than young farmed eels, it is suggested that older eels are more sensitive for
33 maturation triggers.

34

35 Keywords: *Anguilla anguilla* Linnaeus, reproductive migration, maturation, lipid
36 mobilisation, silvering, vitellogenesis

37

37

Introduction

38

39 European eels spend their feeding stage as immature yellow eels in the fresh and
40 brackish European waters. It appears that at the end of each growth season many eels
41 cease feeding and metamorphose (silvering) to prepare for oceanic migration. It is
42 suggested that fat content is a key factor in the onset of migration (Larsson et al., 1990;
43 Svedäng and Wickström, 1997) since reserves have to suffice to fuel migration (van
44 Ginneken and van den Thillart, 2000; van den Thillart et al., 2004; van Ginneken et al.,
45 2005) and are required for successful development of the oocytes. Drastic changes occur
46 during silvering, most apparent though is the enlargement of the eyes discriminating the
47 yellow and silver phase (Pankhurst, 1982). Durif (et al., 2005) demonstrated recently that
48 silvering and migration are closely related processes. As also the pectoral fins become
49 longer (Durif et al., 2005) and shape changes (Tesch, 2003), Durif et al. (2005) proposed
50 an index on basis of length, weight, eye diameter and pectoral fin length, which provides
51 an estimate of the proportion of silver eels that are true migrants. This was needed since
52 their abundance was overestimated as demonstrated by Svedäng and Wickström (1997)
53 and Feunteun et al. (2000).

54 The ovaries of silver eels contain oocytes in the first developmental stages
55 (Adachi et al., 2003) after transformation of the oogonia. Further progression requires
56 incorporation of lipids droplets (stage 3: cortical alveolus stage or lipid droplet stage) and
57 vitellogenin. Although separated in other fish species, these two processes occur
58 simultaneously in artificially matured Japanese eel (Adachi et al., 2003) and European eel
59 (Palstra et al., 2005), which suggests an unnatural situation. Untreated silver eels are in a

60 prepubertal stage, still far from sexual maturity (Larsen and Dufour, 1993; Dufour, 1994;
61 Dufour et al., 2003) and remain as such when kept resting in aquaria. Further sexual
62 development of silver eel appears to be blocked by dopaminergic inhibition of
63 hypothalamus and pituitary resulting in insufficient FSH and LH levels (Dufour et al.,
64 2003). This blockage is likely required in order to allow the long spawning migration.
65 Obviously there must be natural conditions that lead to release of this blockage. As
66 European eels have to swim about 5,500-km to reach their spawning site, we hypothesize
67 that swimming is the crucial trigger for releasing the dopaminergic inhibition.

68 Exercise has never been thoroughly investigated as a stimulating factor for
69 maturation in fish. Eels have to cover an enormous distance to their spawning grounds, it
70 is therefore likely that swimming is at least one of the factors that triggers natural
71 maturation. Recently, van Ginneken et al. (2007) observed increased oocyte diameters in
72 3 year old hatchery eels after swimming 5,500-km. Significantly higher levels of pituitary
73 LH were found in the exercised group as compared to the controls. Those results indicate
74 that long term swimming indeed has a stimulatory effect on maturation. Also in a recent
75 study on swim performance based on short swim trials, we found indications that
76 swimming triggered sexual maturation in Lake Balaton silver eels (Palstra et al., 2006d);
77 the GSI in the swim group was higher than in rest group. Apparently Balaton eels are
78 more sensitive to swimming than the farmed eels used by van Ginneken et al. (2007), as
79 they responded already within a week. Therefore we used Lake Balaton silver eels in this
80 study to investigate the effect of short swim trials (1-6 weeks) on oocyte development.

81

82

83

Materials & Methods

84

Experimental animals

86 At the end of August 2003 and end of September 2004, a total of 120 eels were
87 caught by electrofishing in Lake Balaton, Hungary, in the region of Keszthely and
88 Tihany. They were transported to the laboratory in oxygen-filled plastic bags and marked
89 individually by subcutaneous injection of PIT-TAGS (TROVAN, Aalten, The
90 Netherlands) just behind the head. Eels were then packed into large oxygen-inflated
91 nylon bags in Perspex and cardboard boxes after which they were sent to Leiden (The
92 Netherlands) in early September 2003 and early October 2004 respectively.

93

Swim-flumes & conditions

95 Swim experiments were performed in 22 Blazka-type calibrated swim-flumes
96 described in detail by van den Thillart et al. (2004). Swim-flumes were oriented towards
97 the Sargasso Sea (WNW) in a climatized room of about 100 m². The total water content
98 of about 7000 l was recirculated continuously over a bio-filter. The illumination in the
99 climatized room was switched to 670 nm light (bandwidth 20 nm). Based on eye pigment
100 changes during silvering, it can be assumed that this far-red light is invisible for eels
101 (Pankhurst and Lythgoe 1983). Indeed, the animals did not respond to movements of the
102 experimenter during red light illumination. Experiments were performed in air-saturated
103 (>75%) fresh water at 18 ± 0.5°C. The swim-flumes were 2 m long with a volume of 127
104 l, with an animal compartment of 110 cm suited for 1 individual eel.

105

106 *Protocol experiment 1*

107 Protocol 1 was performed in 2003 as part of a study on the influence of the swim-
108 bladder parasite *Anguillicola crassus* on the swim performance (Palstra et al., 2006d). As
109 part of the swim performance protocol, fish were exposed to up to 12 h swimming per
110 day over a 7 day period that cumulatively examined the effect of a week of heightened
111 and sustained but non-continuous swimming activity. The results are included here as
112 they provided the basis for longer term experiments described in the following protocol.

113 For the control group 10 eels were upon arrival randomly chosen, then
114 anaesthetized, measured, sacrificed and sampled. Before handling eels were anaesthetized
115 with 1 - 1.5 ml diluted clove oil per 1 water (oil of cloves: ethanol = 1:10). Forty eels in
116 two groups of 20 were subjected to a swim fitness test as described by Palstra et al.
117 (2006c). In short, eels were anaesthetized, measured and introduced into swim-flumes
118 two days before the experiment started.

119 The swim fitness protocol consisted of 7 daily experimental trials: 2 speed tests
120 and 5 endurance tests. On day 1, eels were subjected to a first speed test. Eels started to
121 swim at a speed (U) of 0.5 m s^{-1} for 2 h. After these 2 h at 0.5 m s^{-1} , U was raised with 0.1
122 m s^{-1} to 0.6 m s^{-1} for 2 h. Subsequently, this was repeated with steps of 0.1 m s^{-1} for a U
123 up to 1.0 m s^{-1} . On day 2 the eels were subjected to endurance tests starting at 0.5 m s^{-1}
124 for 12 h. On the following days the eels were swum at respectively 0.6, 0.7, 0.8, and 0.9
125 m s^{-1} . On day 7, the protocol was finished with a second speed test. When fish fatigued
126 during trials, the flow was lowered to 0.1 m s^{-1} .

127 A group of 10 eels were kept resting during the same period in a 1500 l tank
128 connected to a 2400 l recirculation system under dark conditions. PVC pipes were added

129 as shelter. At the end of the swim test both swimming and resting eels were
130 anaesthetized, measured, sacrificed and sampled.

131

132 *Protocol experiment 2*

133 After arrival, 10 randomly chosen eels were anaesthetized, measured, sacrificed
134 and sampled as control group. Twenty-one randomly chosen eels were measured and
135 introduced in the swim-flumes. They were allowed to swim continuously at a speed of
136 0.5 BL s^{-1} . Six eels showed problems with swimming during the experiment and were
137 stopped. We investigated the swim-bladder of these eels and found that three out of six
138 eels were infected, and that five out of six eels had heavily damaged swim-bladders
139 confirming the negative interference of the parasite with swimming (Palstra et al.,
140 2006d). Only one out of twenty-one eels showed problems with swimming that could not
141 be connected with swim bladder dysfunction. Data of these eels were not included for
142 analysis. Resting eels were kept as described above. After two weeks, 6 randomly chosen
143 swimming eels were stopped, anaesthetized, measured, sacrificed and sampled as well as
144 a group of 10 resting eels. After six weeks, the remaining 9 eels were stopped,
145 anaesthetized, measured, sacrificed and sampled as well as a group of 6 remaining resting
146 eels.

147

148 *Measurements & sampling*

149 Morphometric parameters included body-length (BL), body-weight (BW), eye
150 diameter horizontal (EDh), eye diameter vertical (EDv), pectoral fin length (PFL) and
151 pectoral fin width (PFW; see for abbreviations also Table 1). The following indices were

152 calculated according to the formulae below: condition factor (K), eye index (EI), pectoral
153 fin length index (PFLI) and pectoral fin width index (PFWI).

154

155 1. Condition factor (K) = $100 * BW BL^{-3}$

156 BW: body weight (g), BL: body length (cm)

157

158 2. Eye index (EI) = $100 * (((EDh + EDv) * 0.25)^2 p * (10 * BL)^{-1})$

159 EDh: eye diameter horizontal (mm), EDv: eye diameter vertical (mm)

160

161 3. Pectoral fin length index (PFLI) = $100 * PFL BL^{-1}$

162 PFL: pectoral fin length (cm)

163

164 4. Pectoral fin width index (PFWI) = $100 * PFW BL^{-1}$

165 PFW: pectoral fin width (cm)

166

167 The silver index (SI) was calculated according to the procedure described by Durif et al
168 (2005).

169 Blood samples were taken from the caudal vein with heparin flushed (10.000 IU
170 ml^{-1}) 1 ml syringes, which were immediately replaced on ice. The blood was centrifuged
171 for 5 min at 14,000 rpm and bloodplasm was stored at -80°C.

172 Liver, digestive tract and gonads were dissected and weighed. The following
173 indices were calculated according to the formulae below: the gonadosomatic index (GSI),

174 the digestive tract somatic index (DTSI) and the hepatosomatic index (HSI; see for
175 abbreviations also Table 1).

176

177 5. Gonadosomatic index (GSI) = $(GW BW^{-1}) * 100\%$

178 GW: gonad weight (g), BW: body weight (g)

179

180 6. Digestive tract somatic index (DTSI) = $(DTW BW^{-1}) * 100\%$

181 DTW: weight digestive tract (g)

182

183 7. Hepatosomatic index (HSI) = $(LW BW^{-1}) * 100\%$

184 LW: liver weight (g)

185

186 Gonads of all eels solely contained oocytes so all eels were females. From the gonad
187 at a standardised posterior location a piece of about 1 g was placed in Bouin solution at
188 room temperature. From all swimming eels, the otoliths (sagitta) were dissected for age
189 determination.

190

191 *Otolithometry*

192 Age estimation was carried out in the laboratory of Cemagref, Bordeaux, France
193 by otolithometry according to the method described by Daverat (2005). After extraction,
194 otoliths were cleaned of all organic matter in distilled water, dried with ethanol, and then
195 stored in eppendorf tubes. The otoliths were later embedded in synthetic resin (Synolith,
196 Euroresins Benelux BV, the Netherlands), then polished to the nucleus with a polishing

197 wheel (Streuers Rotopol-35, West Lake, Ohio, USA) using 2 different grits of sandpaper
198 (1200 and 2400). Fine polishing was done by hand with Al₂O₃ (1 µm grain) on a
199 polishing cloth. Etching was done using 5% EDTA. A drop of this solution was applied
200 on the mold for 3 min. The otoliths were then rinsed with distilled water and stored in dry
201 conditions. Year rings were visualised by staining with a drop of 5% Toluidine blue and
202 counted under a microscope. The age of each eel was determined by the number of
203 increments starting from the nucleus which was considered as year one of the eel's life.

204

205 *Histology*

206 To remove the Bouin fixative, the gonads were washed in 0.1 M phosphate buffer
207 and 70% ethanol until the solution became transparent. After dehydrating through an
208 accumulating alcohol series the samples were embedded in air-free Technovit 7100
209 (Kulzer Histo-Technik) in Peel-A-way molds (Polysciences Inc.) covered with a layer of
210 air-free paraffin oil during polymerization to exclude oxygen (de Jonge et al., 2005).
211 Sections of 10 µm thick were cut using a sledge microtome (Jung Polycut E). Three
212 sections were put on a slide and five slides per sample were made and stained with
213 Mayers Haematoxylin-Eosin. The oocytes were studied visually under the microscope
214 (Nikon Eclipse E400) and overview pictures were taken (Nikon Coolpix 4500). Per
215 section, ten (protocol 1) or twenty (protocol 2) oocytes were randomly selected that were
216 cut through the nucleus. For each oocyte the developmental stage (OS; see for
217 abbreviations also Table 1) was determined. The diameter of the oocytes (OD) was
218 determined using UTHSCSA Image Tool 2.0. The number and diameter of lipid droplets
219 occurring in stage 3 oocytes were measured with the same method. In fact, what we call

220 lipid droplets are the empty lipid vesicles from which the lipids have been extracted by
221 washing in ethanol.

222

223 *Statistics*

224 Normality of the data and homogeneity of variances were checked by
225 Kolmogorov-Smirnov tests. Paired observations before and after swimming of
226 morphometric parameters were tested with student t-tests with one-tailed probabilities.
227 For SI, a Wilcoxon test with one-tailed probabilities was used.

228 With an univariate general linear model (GLM), analysis of covariance
229 (ANCOVA) with one-tailed probabilities was performed on log transformed unpaired
230 observations in search for group effects in swim parameters with either BL (for PFL,
231 PFW, ED) or BW (for GW, DTW, LW, OD, number lipid droplets, diameter lipid
232 droplets; for abbreviations see list) as cofactors. ANCOVA was similarly performed for
233 comparison between the two weeks and the six weeks swim groups. In case of occurrence
234 of significant group effects, ANOVA with a post-hoc Bonferroni test was performed to
235 specify the effects between particular groups. ANCOVA was especially required for the
236 scale difference between swim and rest groups in experiment 2 (Quinn and Keough,
237 2002).

238 Kruskal-Wallis tests with one-tailed probabilities were performed for comparison
239 of SI and oocyte stage. Spearman correlation tests with one-tailed probabilities were
240 performed between start parameters (BL*, BW*) vs. silvering parameters (EI*, SI*, HSI,
241 DTSI) vs. maturation parameters (GSI, OS, OD) for control groups, but also for pre-swim
242 groups for parameters marked with asterisks.

243 Pearson correlation tests with one-tailed probabilities were performed for
244 comparing between OD and lipid droplet number and size. OD between stages was tested
245 with student t-tests with one-tailed probabilities. All statistical tests were performed in
246 SPSS 10.0 for Windows. P-values < 0.05 were considered to indicate statistically
247 significant differences. Results were calculated and plotted as mean \pm standard deviation.

248

249

250

Results

251

Protocol 1

252

253

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255

256

257

In Table 1 the external measurements are given of the control and swim group. The pre-swim and post-swim data are from the same eels. Paired observations showed that after one week of swimming, experimental eels showed a significant weight loss of 15 g (Table 1). Also the K was significantly lower. There was a significant decline of the DTSI (compared to the controls). No increase was observed of silvering indicators.

258 Based on an eye index threshold of 6.5 (Pankhurst, 1982), 70% of all the eels was
259 scored as silver eels. No change in EI and silver index occurred as a result of one week
260 swimming. Still, histology data revealed significant increases of oocyte stage and oocyte
261 diameter. The GSI of eels that had swum was on average 39% higher than control and
262 rest groups but individual variation remained high and this difference was not significant
263 (Table 1). Percentages of eels with GSIs >1 were 11% in the rest group and 33% in the
264 swim group.

265 Eels of the control group in protocol 1 showed oocytes in stage 1, 2 and 3 with the
266 latter oocytes just having entered this stage containing only a few lipid droplets. The
267 average diameter of these oocytes was $103 \pm 36 \mu\text{m}$. After one week, eels from the swim
268 group showed a significant change in the average oocyte stage (Table 1). 85% of the eels
269 in the swim group had ovaries with exclusively stage 3 oocytes with a larger diameter of
270 $155 \pm 47 \mu\text{m}$ and containing much more lipid droplets (20 - 100).

271

272 *Protocol 2*

273 At the start of the two week protocol the eels were $62 \pm 4 \text{ cm}$, $347 \pm 76 \text{ g}$ (Table
274 2) and 83% of them were silver (EI > 6.5). At the start of the six week protocol the eels
275 were $63 \pm 5 \text{ cm}$ long, weighted $429 \pm 137 \text{ g}$ (Table 2) and 33% was silver. This
276 difference occurred despite the random procedure for assigning eels to the different trials.
277 Experimental eels were assigned to SI stages 2, 3 and 5, while not any fish was assigned
278 to stage 4. The experimental eels that had swum aged between 13 and 21 years (mean 16
279 years; Table 2). Paired observations showed that after two weeks of swimming,
280 experimental eels showed a significant weight loss of 14 g (Table 2). Also the K was

281 significantly lower. PFLI and PFWI did not show changes. Paired observations showed
282 that after six weeks of swimming, experimental eels showed a significant weight loss of
283 63 g (Table 2). Again the K was significantly lower. PFLI and PFWI did not show
284 changes in the swim group. The PFWI was however smaller in the rest group.

285 The EI increased significantly after 2 week swimming from 8.32 ± 2.20 to $10.31 \pm$
286 2.84 , also a significant increase in comparison with the rest group (Table 2). After two
287 weeks, all eels showed increases of the EI between 11 to 41% (Fig. 1), corresponding to a
288 rise in SI. The EI increased further after six weeks of swimming between 10 up to 66%
289 (Fig. 1) resulting in 100% silver eels. This change in eye diameter caused a significant
290 change in the SI.

291 The GSI was found on average 3 - 5 times higher in the two week swim group
292 than in the controls though not significantly (Table 2). The number of eels with a GSI > 1
293 was 50% in the swim group, which was significantly different from the control and rest
294 group. There were no eels at all in the control and rest group with GSI > 1. The GSI in
295 the six week swim group was significantly higher than in the control and rest group. Also
296 in comparison with two weeks of swimming, the GSI was significantly higher both in the
297 swim group and rest group.

298 DTSI and HSI values of the 2 and 6 week swim groups were lower than the
299 controls but not significantly. HSI was found significantly lower in the rest group both vs.
300 control and two week swim group. DTSI and HSI were lower in the six week swim group
301 but only significantly in the rest group. Also in comparison with two weeks of swimming,
302 the HSI was significantly lower (Table 2).

303

304 *Oocyte developmental status*

305 Eels of the control group contained oocytes representing stages 1, 2 and 3
306 according to Wallace and Selman (1981), Tyler and Sumpter (1996) and Adachi et al.
307 (2003).

308 Stage 1 oocytes represented small oocytes (30 - 90 μm), still in nest structure or
309 individually organised. An acellular zona radiata (zona pellucida, chorion vitelline
310 envelop) could not be identified yet. Oocytes were oval shaped and the cytoplasm was
311 darkly coloured (Fig. 2A). The nucleus was centred and round, contained 1 to 3 large
312 nucleoli and about 20 smaller ones dispersed throughout the nucleus. The stage 1 oocytes
313 that were found should be considered late stage 1 oocytes since size was already
314 considerable in comparison with Tyler and Sumpter (1996) stating that oogonia in
315 teleosts generally measure less than 10 μm in diameter.

316 Stage 2 oocytes represented larger oocytes (40 - 150 μm) with less darkly
317 coloured cytoplasm. In this phase a zona radiata could be identified. The number of
318 nucleoli was higher. Islands of lightly stained cytoplasm could be distinguished in the
319 oocyte. Lipid and connective tissue were surrounding the oocytes (Fig. 2A).

320 Stage 3 oocytes were characterised by the presence of lipid droplets. In eels from
321 control groups, stage 3 oocytes contained very few lipid droplets, generally dispersed in
322 the periphery of the ooplasm near the zona radiata (Fig. 2B). Mostly the nucleus was
323 roundly shaped and nucleoli were found in the periphery of the nucleus. Eels from swim
324 groups contained stage 3 oocytes with a large number of lipid droplets (Fig. 2C).

325 Yolk globuli, such as is characteristic for vitellogenesis, were not found. For
326 comparison, Fig. 2D shows an oocyte during final maturation in an artificially matured

327 silver eel (Palstra et al., 2005). Numerous yolk globuli could be observed. The number of
328 lipid droplets is decreased and the size increased indicating extensive fusion of the lipid
329 droplets. The oocyte showed considerable increase in size indicating a hydration
330 response. The nucleus (germinal vesicle) was migrating to the periphery (Germinal
331 Vesicle Migration – GVM).

332 Eels of the control group in experiment 2 contained oocytes in stage 1 and 2,
333 except for one eel that showed only stage 3 oocytes. The average diameter of the oocytes
334 was $83 \pm 39 \mu\text{m}$. After two weeks of swimming, eels showed oocytes in a more advanced
335 stage of development. Change in average stage was just not significant, but oocyte
336 diameter had significantly increased ($P < 0.01$; Table 2). Fifty percent of the eels had
337 exclusively stage 3 oocytes in the ovaries with large numbers of lipid droplets in contrast
338 to the eels in control and rest groups which only had stage 1 and 2 oocytes (Fig. 3).

339 After six weeks, eels from the swim group showed further oocyte development.
340 The oocyte stage in the swim group was significantly different vs. control and rest group
341 (Table 2). In the swim group, the oocytes were on average larger (not significantly). In
342 the rest group, eels had only oocytes representing stage 1 and 2. In the swim group, only
343 three eels showed stage 1 - 2 oocytes. The other six eels showed stage 2 - 3 or 3 oocytes
344 with large numbers of lipid droplets (Fig. 3). The change in the rest group was also
345 significantly different from the control group. Average stage and diameter were
346 significantly higher after six weeks of resting than after two weeks of resting (Table 2).

347

348 *Variation of number and size of lipid droplets in stage 3 oocytes*

349 In total 770 oocytes of 39 experimental eels in protocol 2 were histologically
350 analysed. 32 eels contained stage 1 and 2 oocytes (without any lipid droplets) with
351 diameters between 30 and 147 μm ($n = 615$). Stage 1 oocytes were on average 56 ± 14
352 μm ($n = 220$). Stage 2 oocytes were significantly larger ($P < 0.001$) and on average $87 \pm$
353 $23 \mu\text{m}$ ($n = 395$).

354 Ten of the 39 experimental eels also had stage 3 oocytes: nine swimmers and one
355 control eel. Stage 3 oocytes were on average $159 \pm 36 \mu\text{m}$ ($n = 165$) and again
356 significantly larger ($P < 0.001$). A large variation in the number and size of lipid droplets
357 in stage 3 oocytes was observed (Fig. 4). They contained on average 45 ± 30 lipid
358 droplets in a range between 6 and 102 which measured on average $11 \pm 2 \mu\text{m}$ in a range
359 between 5 and 17 μm . Significant positive correlations were found for these averages per
360 eel between oocyte diameter and the number of lipid droplets ($P < 0.001$) and between
361 oocyte diameter and the diameter of the lipid droplets ($P < 0.001$). Also when oocyte data
362 were pooled for all eels (Fig. 4), we observed a positive correlation between oocyte
363 diameter and the number of lipid droplets ($P < 0.01$) and between oocyte diameter and the
364 diameter of the lipid droplets ($P < 0.01$).

365

366 *Correlations between size, silvering and oocyte developmental indicators*

367 Data of experimental eels at the start (control and pre-swim measurements) were
368 used for correlation analyses. Correlations between size (BL, BW), silvering (externally
369 EI, SI and internally DTISI, HSI) and oocyte developmental indicators (GSI, OS, OD)
370 were analysed (Table 3). Significant positive correlations were found between size and
371 silvering indicators and between size and oocyte developmental indicators suggesting that

372 the silvering and maturation status of larger eels was more advanced. Between silvering
373 and oocyte developmental indicators, significant positive correlations were found only
374 between EI, SI and OS (Table 3). Positive correlation with GSI was just not significant.
375 No significant correlation was found between external indicators of the level of silvering
376 EI and SI vs. DTSI and HSI, which are often considered to change in relation to the
377 degree of silvering.

378

379

380

Discussion

381

Swimming triggers silvering

382
383 Continuous swimming at 0.5 BL s^{-1} resulted in an increase of the eye index (EI),
384 which was significant after after two and 6 weeks swimming. The observed changes were
385 even stronger after six weeks of swimming. In contrast no change could be observed in
386 the EI of the rest groups, indicating that the effect was not caused by time and/or
387 starvation. As increase of EI has been described with an external parameter for
388 progression of sexual maturation (Pankhurst, 1982; Tesch, 2003; Durif et al., 2005), these
389 observations suggest that swimming induces early maturation.

390 The mean eye index of all the eels that just arrived from Lake Balaton in
391 September was 8.2 ± 3.0 . As an EI of 6.5 is used as the threshold for silvering (Pankhurst,
392 1982), 59% of the group can be classified as silver eels. The degree of silvering and
393 oocyte development was positively correlated with body size (Table 3). This observation
394 of silver eels in Lake Balaton contradicts Bíró (1992), who stated that in Lake Balaton

395 eels never metamorphose into silver eels. However, we found even in the control group
396 some silver eels in migrant stage 5. These eels had a GSI > 1 and gonads with some stage
397 3 oocytes, although with few lipid droplets. Surprisingly, the migrant SI stage 4 (Durif et
398 al., 2005) was not represented at all, not before nor after the swim experiments. This
399 stage is characterised with elongated pectoral fins, however no changes of the pectoral
400 fins were observed due to swimming. In field studies it was found that downstream silver
401 eels have longer pectoral fins, from which it was concluded that swimming likely causes
402 the fins to grow (Tesch, 2003, Durif et al., 2005). As in our swim trials no change in fin
403 length occurred, we must conclude that the increase in fin size during downstream
404 migration must be due to other factors than swimming.

405

406 *Swimming triggers oocyte development*

407 After six weeks of swimming, changes were much more pronounced than after
408 two weeks of swimming, both GSI and oocyte diameter was significantly higher. More
409 than 50% of the eels that had swum for two and six weeks had oocytes predominantly in
410 stage 3, while in contrast resting eels had no stage 3 oocytes at all (Fig. 3). Oocytes in
411 stage 3 showed a high variation in numbers and diameter of lipid droplets, in other words
412 in total lipid content. The increase of lipid content is typical for stage 3. As resting eels
413 showed a slight oocyte development but not any lipid deposition in the oocytes, we
414 conclude that the deposition of lipids in oocytes occurs under conditions of increased
415 lipid mobilisation.

416 Recently, van Ginneken et al. (2007) simulated a complete migration of 5,500-km
417 using 3 year old silver eels from the farm. Those eels were 71 ± 4 cm long and weighed

418 792 ± 104 g, bigger than the ones used in this study. Significant higher levels of pituitary
419 LH and plasma estradiol were found in the swim group as compared to the controls, also
420 the oocyte diameter was increased. Those results indicate that long term swimming had
421 an effect on maturation of younger farmed eels. However, no changes in EI and GSI were
422 found. Thus, despite the increased hormone levels, the gonads did not develop.

423 The more explicit changes in this study, already after two weeks of swimming,
424 might be explained by the difference in age. The hatchery eels were young (3 years)
425 while the Lake Balaton eels in our study were much older (13 to 21 years). This finding
426 supports the hypothesis that older eels are more responsive and sensitive for maturation.
427 Durif et al. (2006) observed positive correlations between age vs. condition factor, liver
428 weight and vitellogenin level, suggesting that older eels are more suited for reproduction.
429 Indeed, we found recently that older eels require a shorter hormonal treatment to mature
430 (Palstra et al., 2006b), indicating a higher sensitivity for maturation.

431 In this study, we observed swimming induced oocyte development up to stage 3,
432 the lipid droplet stage. Stage 3 was found to be highly variable with respect to the
433 arrangement, number and size of the lipid droplets (Fig. 4). Oocytes of eels that had
434 swum contained more than 100 larger droplets. However, oocytes did not develop further
435 than stage 3. Most developed oocytes had lipid droplets that covered >50% of the
436 cytoplasm and formed a complete ring around the circumference of the oocyte (Couillard
437 et al., 1997), which is typical for pre-vitellogenic oocytes (Colombo et al., 1984). In
438 *Anguilla rostrata* this condition is shown to be the start of vitellogenesis (Cottril et al.
439 2001). However, we did not observe any yolk globuli in the oocytes of eels that had
440 swum, quite in contrast to artificially matured oocytes which are packed with yolk globuli

441 (Fig. 2D). Also the oocytes did not reach sizes that are characteristic for vitellogenesis.
442 Vitellogenesis is the major cause for oocyte growth in teleosts in general (Tyler, 1991),
443 including eel (Nagahama, 1994). Cottril et al. (2001) found oocytes of 200 μm for *A.*
444 *rostrata* and considered them already vitellogenic. Such values are, however, not typical
445 for vitellogenesis. Adachi et al. (2003) showed for *Anguilla japonica* that vitellogenesis
446 begins when oocytes are about 250 μm in diameter. In this study we found maximum
447 oocyte diameters of 236 μm , which are quite close to the onset of vitellogenesis.

448

449 *Perspectives*

450 The eels in this experiment swam for max 6 weeks, still all individuals showed
451 progression in sexual maturation. Earlier experiments with 3 year old farmed eels, that
452 swam for 5,500-km, showed hormonal changes but no increase in gonadal mass.
453 Therefore it is possible that maturation sensitivity depends on age, and that older eels
454 may develop further during continued swimming.

455

455 **List of abbreviations**

BL	bodylength
BW	bodyweight
DTSI	digestive tract somatic index
DTW	digestive tract weight
ED	eye diameter
EDh	eye diameter horizontal
EDv	eye diameter vertical
EI	eye index
GSI	gonadosomatic index
GW	gonad weight
HSI	hepatosomatic index
K	condition factor
LW	liver weight
OD	oocyte diameter
OS	oocyte stage
PFL	pectoral fin length
PFLI	pectoral fin length index
PFW	pectoral fin width
PFWI	pectoral fin width index
SI	silver index

456

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567

567 **Table 1** Effect of swimming (protocol 1) on morphometric and gonad parameters (mean
 568 \pm SD). Control eels (n = 10) were sampled at the start. Swimming eels (n = 40) were
 569 subjected to a swim fitness test (*see text for explanation*), corresponding to about a week
 570 of swimming, or kept resting (n = 9). Swimming eels were measured before (pre-swim)
 571 and after swimming (post-swim). Significant differences ($P < 0.05$) are indicated in bold.
 572 Symbols mark differences vs. the controls (*) or pre-swim values (&). (*For abbreviations*
 573 *see List*)

parameters	control	1 week		
		pre-swim	post-swim	rest
n (eels)	10	40	40	9
<u>external</u>				
BL (cm)	67 \pm 4	69 \pm 6	69 \pm 6	66 \pm 5
BW (g)	475 \pm 77	525 \pm 142	510\pm142 &	437 \pm 123
K	0.16 \pm 0.01	0.16 \pm 0.02	0.15\pm0.02 &	0.15 \pm 0.02
PFLI	4.68 \pm 0.39	4.96 \pm 0.43	4.88 \pm 0.42	4.68 \pm 0.49
EI	7.38 \pm 3.30	9.14 \pm 2.99	8.64 \pm 3.12	7.31 \pm 2.49
SI	3 \pm 1	4 \pm 1	4 \pm 1	3 \pm 1
<u>internal</u>				
GSI	0.59 \pm 0.34		0.82 \pm 0.43	0.59 \pm 0.32
DTSI	2.42 \pm 0.65		1.66\pm0.67 *	2.01 \pm 0.52
HSI	0.92 \pm 0.09		0.84 \pm 0.13	0.86 \pm 0.19
<u>oocytes</u>				
OS	2.7 \pm 0.3		2.8\pm0.4 *	2.9\pm0.2 *
OD (μ m)	103 \pm 36		155\pm47 *	128 \pm 48

574

575

575 **Table 2** Effect of swimming (protocol 2) for two weeks and six weeks on morphometric
 576 and gonad parameters (mean \pm SD). Control eels (n = 10) were sampled at the start. Eels
 577 swam two (n = 6) or six weeks (n = 9) at 0.5 BL s⁻¹ or were kept resting for two (n = 10)
 578 or six weeks (n = 6). Swimming eels were measured before (pre-swim) and after
 579 swimming (post-swim). Significant differences (P < 0.05) are indicated in bold. Symbols
 580 mark differences vs. the controls (*), pre-swim values (&) or the rest group (#). The 6
 581 weeks rest group had an average OS value of 1.73 and was found significantly different
 582 by a Kruskal-Wallis test from the control group. The control group consisted of 9 fish
 583 that together had an average value of 1.58 with one fish that solely had stage 3 oocytes.
 584 (*For abbreviations see List*)

parameters	control	2 weeks			6 weeks		
		pre-swim	post-swim	rest	pre-swim	post-swim	rest
n (eels)	10	6	6	10	9	9	6
age (years)			16 \pm 1			16 \pm 3	
<u>external</u>							
BL (cm)	59 \pm 5	62 \pm 4	62 \pm 4	55 \pm 2	63 \pm 5	63 \pm 5	53 \pm 2
BW (g)	267 \pm 68	347 \pm 76	333\pm63 ^{&}	212 \pm 26	429 \pm 137	366\pm114 ^{&}	174 \pm 23
K	0.13 \pm 0.01	0.14 \pm 0.02	0.14\pm0.01 ^{&}	0.13 \pm 0.02	0.16 \pm 0.03	0.14\pm0.02 ^{&}	0.12 \pm 0.02
PFLI	4.55 \pm 0.19	4.89 \pm 0.25	4.89 \pm 0.21	4.39 \pm 0.25	4.78 \pm 0.55	4.78 \pm 0.57	4.33 \pm 0.25
PFWI	2.32 \pm 0.26	2.64 \pm 0.43	2.45 \pm 0.46	2.30 \pm 0.17	2.52 \pm 0.18	2.52 \pm 0.16	2.03\pm0.21 [*]
EI	6.20 \pm 1.81	8.32 \pm 2.20	10.31\pm2.84 ^{&#}	5.55 \pm 0.43	6.89 \pm 3.16	9.09\pm2.91 ^{&#}	5.53 \pm 0.60
SI	3 \pm 1	3 \pm 2	4 \pm 1	2 \pm 0	2 \pm 2	3\pm1 ^{&}	2 \pm 0
<u>internal</u>							
GSI	0.26 \pm 0.31		0.74 \pm 0.48	0.13 \pm 0.12		0.80\pm0.35 ^{*#}	0.38 \pm 0.18
DTSI	2.58 \pm 0.81		1.95 \pm 0.77	2.40 \pm 0.52		2.14 \pm 0.54	2.18\pm0.20 [*]
HSI	1.13 \pm 0.20		1.02 \pm 0.15	0.87\pm0.09 [*]		0.77 \pm 0.13	0.72\pm0.12 [*]
<u>oocytes</u>							
OS	1.7 \pm 0.5		2.4 \pm 0.7	1.5 \pm 0.2		2.4\pm0.6 ^{*#}	1.7\pm0.1 [*]
OD (μ m)	83 \pm 39		136\pm46 [#]	61 \pm 14		109 \pm 41	94 \pm 17

585

585 **Table 3** Correlations between parameters of control eels (n = 19 - 20) and eels before
 586 swimming (n = 75). Significant correlations (P < 0.05) are given in bold. Significant
 587 correlations were found between size (BL, BW) with silvering (EI, SI) and oocyte
 588 developmental indicators (GSI, OS, OD). DTSI and HSI were correlated with oocyte
 589 developmental indicators rather than silvering parameters (*For abbreviations see List*)

		BW	EI	SI	DTSI	HSI	GSI	OS	OD
BL	corr.	0.944	0.740	0.680	-0.218	0.478	0.703	0.900	0.612
	P	0.000	0.000	0.000	0.178	0.017	0.000	0.000	0.003
	n	75	75	75	20	20	20	19	19
BW	corr.		0.729	0.658	-0.147	-0.522	0.734	0.884	0.582
	P		0.000	0.000	0.268	0.009	0.000	0.000	0.004
	n		75	75	20	20	20	19	19
EI	corr.			0.884	-0.211	-0.062	0.311	0.510	0.212
	P			0.000	0.186	0.398	0.091	0.013	0.191
	n			75	20	20	20	19	19
SI	corr.				-0.090	-0.020	0.351	0.603	0.269
	P				0.353	0.467	0.065	0.003	0.133
	n				20	20	20	19	19
DTSI	corr.					-0.013	-0.432	-0.323	-0.203
	P					0.479	0.028	0.088	0.203
	n					20	20	19	19
HSI	corr.						-0.342	-0.390	-0.229
	P						0.070	0.049	0.173
	n						20	19	19
GSI	corr.							0.721	0.730
	P							0.000	0.000
	n							19	19
OS	corr.								0.744
	P								0.000
	n								19

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600 **Captions to figures**

601

602 **Figure 1** Swimming induced changes of eye diameter. Eels were sampled after
603 swimming continuously at 0.5 BL s^{-1} for two and six weeks (also Table 2). The EI had
604 increased significantly after two and six weeks swimming. This bar graph shows that the
605 EI of each individual had increased. After two weeks swimming, the EI had increased by
606 $23.9 \pm 9.6\%$ (range 11 - 41%). After six swimming, the EI had increased by $36.6 \pm 16.0\%$
607 (range 10 - 66%). Individual increase was more pronounced after six weeks swimming.

608

609 **Figure 2** Gonad histology. Technovit 7100 sections of $10 \mu\text{m}$ after staining with Mayers
610 Haematoxilin-Eosin. A) Oocytes in stage 1 and 2 (small cells with dense cytoplasm and
611 nucleus), B) Oocytes early stage 3 (larger cells with light cytoplasm, few lipid droplets
612 swollen nucleus), C) Oocytes late stage 3 (larger cells with a large number of lipid
613 droplets, swollen nucleus with many nucleoli), D) Oocytes in late stage 5 displaying:
614 large cells with packed lipid droplets and yolk globuli, and very transparent nucleus in
615 stage of Germinal Vesicle Migration (GVM). This picture is from an artificially matured
616 eel, and included for comparison (Palstra et al., 2005). Scale bars are $100 \mu\text{m}$. Note that
617 the mature stage 5 oocyte (D) is much larger than the oocytes in stage 1, 2 and 3 (A, B,
618 C). Stages were assigned according to Wallace and Selman (1981), Tyler and Sumpter
619 (1996) and Adachi et al. (2003).

620

621 **Figure 3** Gonad developmental in relation to swimming. The % of silver eels with
622 oocytes in stage 1 + 2 (white bars), stage 2 + 3 (grey bars), and stage 3 (black bars) is

623 presented in control eels (initial situation), resting eels, and in eels after continuous
624 swimming at 0.5 BL s^{-1} for two and six weeks. In each eel the developmental stage of the
625 oocytes was found rather homogeneous. The difference in stage for two weeks swimming
626 was just not significant vs. the controls but significant vs. resting eels ($P < 0.01$). The
627 difference for 6 weeks swimming was significant vs. the controls ($P < 0.01$) vs. resting
628 eels ($P = 0.05$).

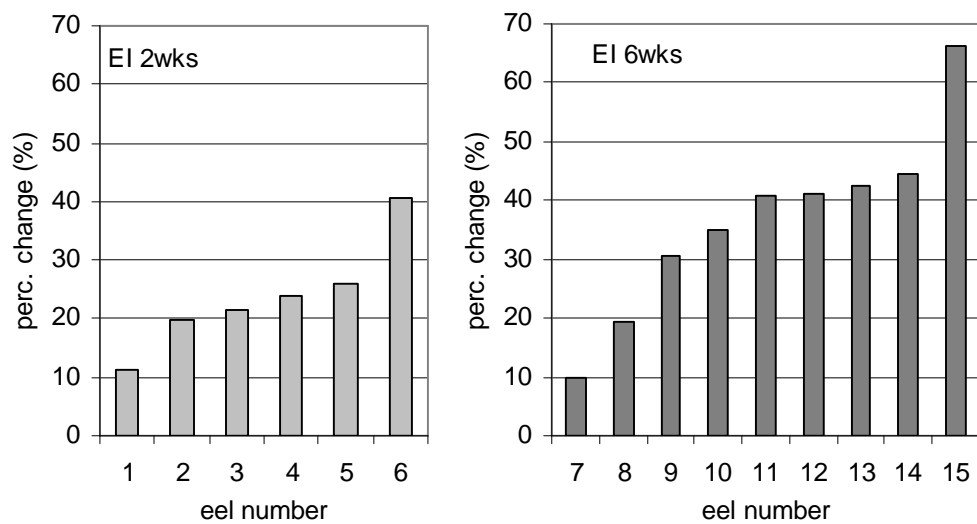
629

630 **Figure 4** Relationship between oocyte diameter and lipid deposition in stage 3 oocytes.
631 Depicted are the relation between the oocyte diameter and the number of lipid droplets
632 (upper panel) and the diameter of the lipid droplets (lower panel). All samples originate
633 from eels from experiment 2. Only eels that had swum had stage 3 oocytes. These eels
634 had stage 3 oocytes with an average diameter of $154 \pm 34 \mu\text{m}$ containing 38.4 ± 26.8 lipid
635 droplets with a diameter of $10.4 \pm 2.5 \mu\text{m}$. Significant positive correlations were found
636 for these averages per eel between oocyte diameter and the number of lipid droplets ($P <$
637 0.001) and between oocyte diameter and the diameter of the lipid droplets ($P < 0.001$).
638 Representative pictures showing the relation between number and size of lipid droplets
639 and oocyte diameter are given on the right side. Scale bars are $50 \mu\text{m}$.

640

640 **Figure 1**

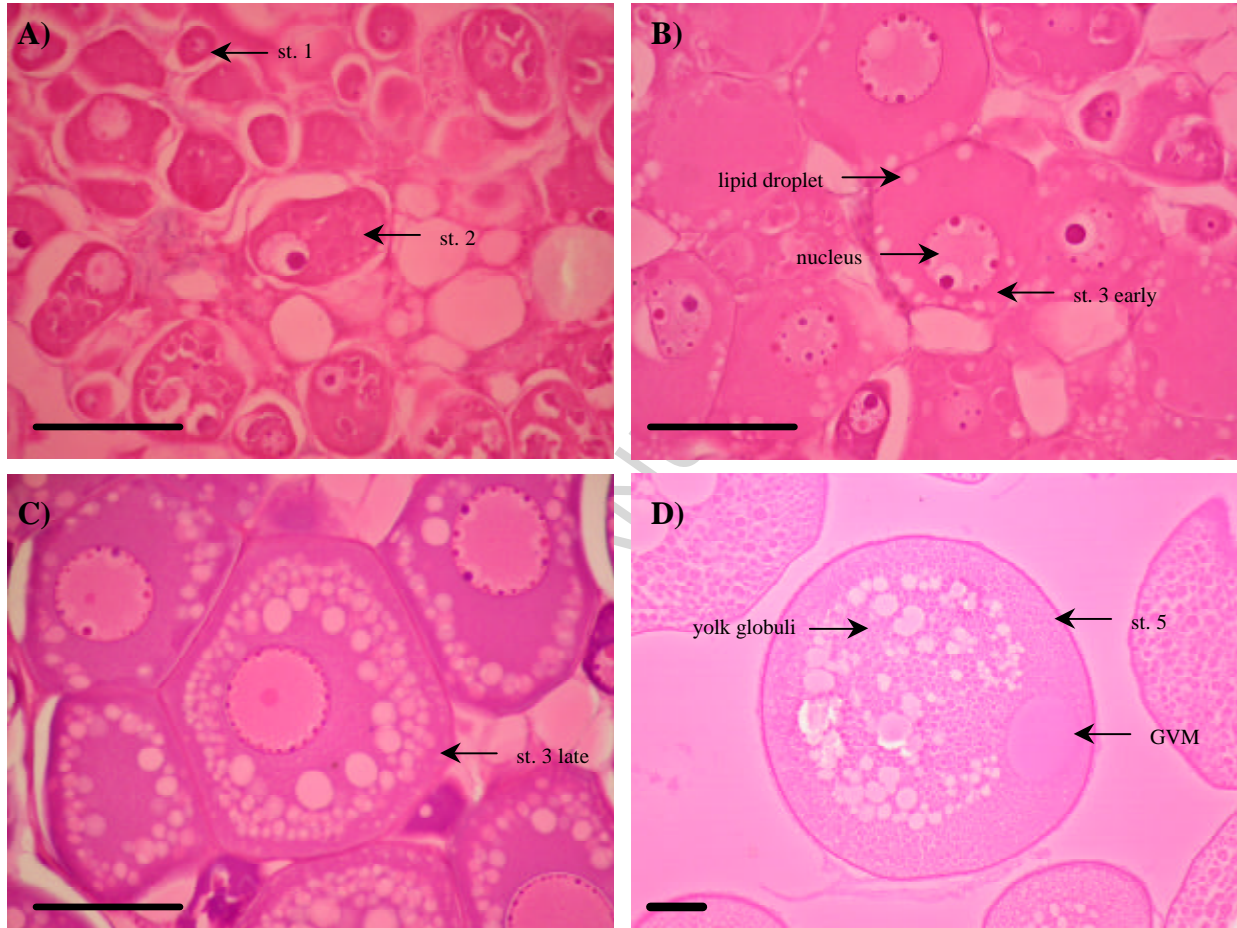
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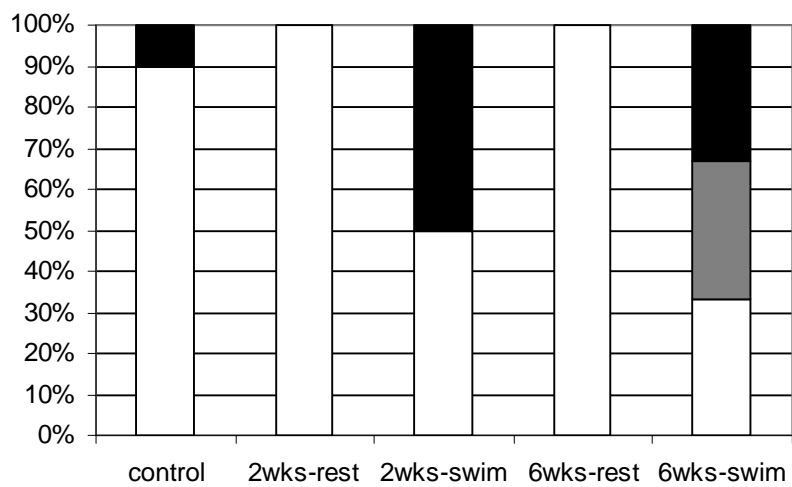
641 **Figure 2**

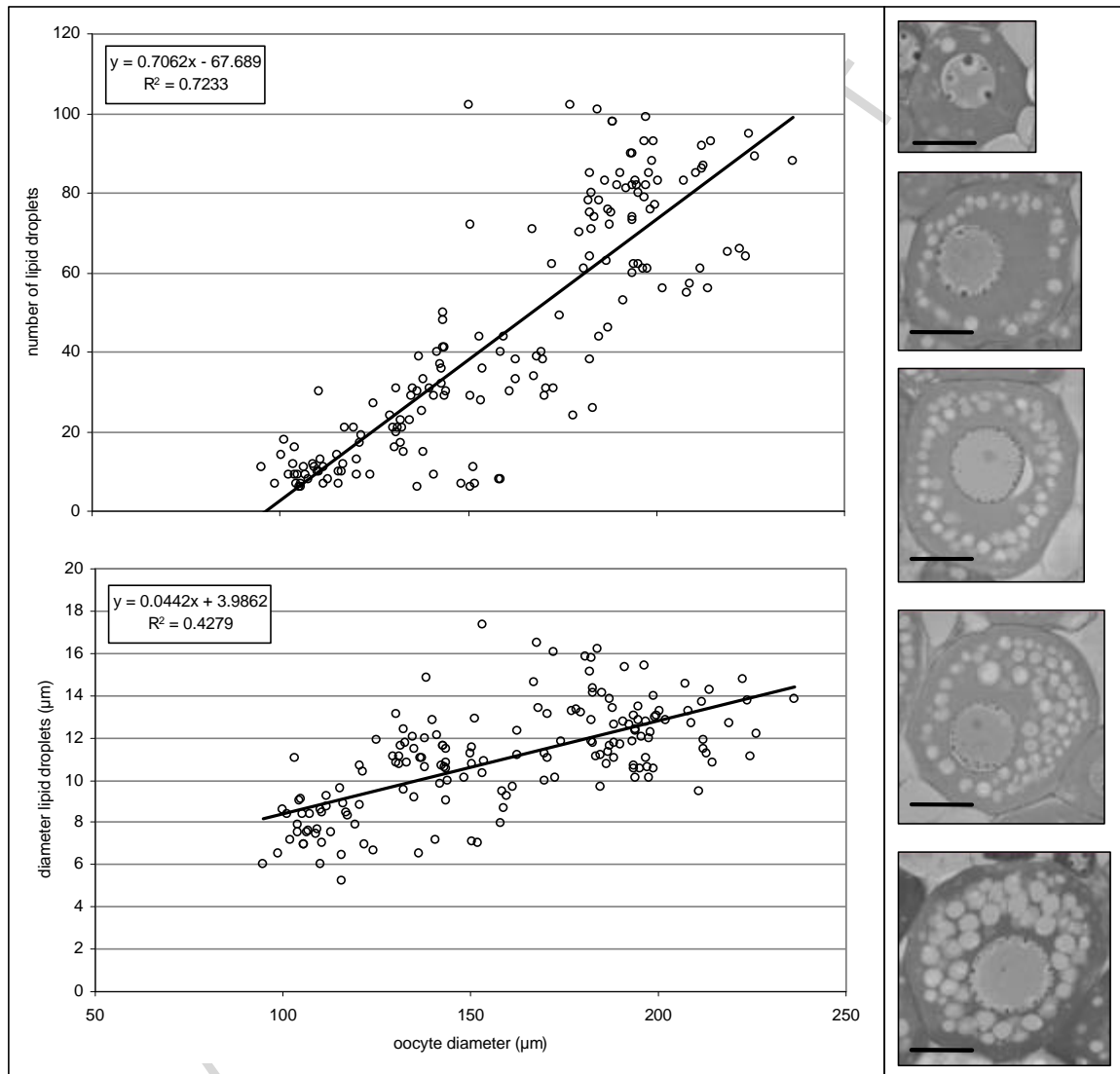
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642 **Figure 3**

643



643 **Figure 4**

644

645