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**Energetic requirements and environmental constraints of reproductive migration and maturation of European silver eel (*Anguilla anguilla* L.)**

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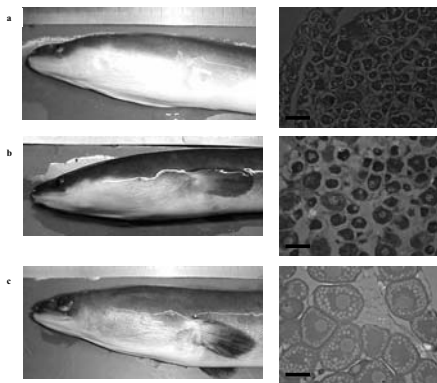
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**Figure 2** Paired observations showing eye size and oocyte stage with a) an eel of 58 cm weighing 239 g with EI= 4.87, oocyte developmental stage 1-2, b) an eel of 67 cm weighing 424 g with EI= 9.14, oocyte stage 2-3 and c) an eel of 72 cm weighing 626 g with EI= 16.6, oocyte stage 3. Scale bars are 100  $\mu$ m.

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### Swimming stimulates oocyte development of European eel (*Anguilla anguilla*)

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### ABSTRACT

European eel *Anguilla anguilla* is a primitive teleost with a semelparous life style and is one of the most extreme examples of reproductive homing. With this, eel is a perfect model to study the poorly understood relation between migration and maturation. Eels migrate downstream and leave the European coasts as silver eels in a prepubertal condition to arrive 4 to 5 months later in a mature condition at the spawning grounds in the Sargasso. As it is very likely that swimming triggers maturation during the 5,500-km migration, we hypothesized that swimming releases reproductive inhibition.

In this study, we subjected 55 old (>13 years) eels from Lake Balaton (Hungary) to swimming for durations of 1, 2 and 6 weeks. These eels were used since recent findings have suggested that older eels are more sensitive to mature. Changes in morphometry and oocyte development were determined to establish the silvering and maturation status. We found that swimming stimulates silvering, shown by enlargement of the eyes already within 2 weeks swimming. Furthermore, we found that swimming stimulates maturation. Already within 1 week swimming, the gonadal mass increased, oocytes became larger and large amounts of lipids were incorporated. However, vitellogenesis was not induced. Thus, it can be concluded that swimming plays a major role in release from reproductive inhibition and mobilisation of lipids to the oocytes. Findings support the hypothesis that older eels are more sensitive to mature.

### INTRODUCTION

European eel spend their feeding stage as immature yellow eels in the fresh and brackish European waters. It appears that at the end of each growth season certain eels cease feeding and metamorphose (silvering) apprehending oceanic preparation. Probably the fat content is a key factor in the onset of migration (Larsson et al., 1990; Svedäng & Wickström, 1997). Drastic changes occur during silvering. Most apparent is the enlargement of the eyes which is used to discriminate between the yellow and silver phase by an index developed by Pankhurst (1982). Durif (et al., 2005) recently demonstrated that silvering and migration are closely related processes. As also the pectoral fins become longer (Durif et al., 2005), shape changes (Tesch, 2003), Durif et al. (2005) proposed an index on basis of length, weight, eye diameter and pectoral fin length, which provides an estimate of the proportion of silver eels that are true migrants. This was needed since their abundance was overestimated as demonstrated by Svedäng & Wickström (1997) and Feunteun et al. (2000).

Silver eels show an early development of the oocytes (reviewed for fish in general by Wallace & Selman, 1981 and Tyler & Sumpter, 1996, and for *A. japonica* by Adachi et al., 2003) and transform from oögonia to growing oocytes. After deposition of lipid droplets, further development includes vitellogenesis, maturation and ovulation which, for European eel, has only been observed when artificially matured through hormonal injections (Adachi et al., 2003; Palstra et al., 2005). Untreated silver eels are still at a prepubertal stage and far from sexual maturity (Larsen & Dufour, 1993; Dufour, 1994; Dufour et al., 2003) and will remain as such if prevented from oceanic migration to the spawning grounds in the Sargasso Sea (Dufour et al., 2003).

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Prepubertal blockage of eel is due to a deficient GnRH stimulation and a simultaneous dopaminergic inhibition of the pituitary gonadotropes GTH-I (FSH-like) and GTH-II (LH-like). Severity of blockage is probably related to the migrational distance of the particular eel species (Todd, 1981). The question remains how and by what trigger or succeeding triggers eels are released from this blockage. Evidently, lipids play a major role in timing and thus triggering, since they are the ultimate requisite for successful reproduction. They are required for fuel as well as for incorporation in the oocytes (Palstra et al., 2005) thus forming a strong link between migration and maturation.

Studies on the interaction between migration and maturation are scarce which is surprising since especially migrant fish are often commercially interesting but difficult to reproduce in captivity. Exercise has never been thoroughly investigated as a stimulating factor of maturation of fish. Only recently, van Ginneken et al. (unpubl.) found increased oocyte diameters in 3 year old hatchery eels after swimming 5,500-km. Recently, we also found indications that older eels have higher capacity to incorporate fat from the muscle in the oocytes (Palstra et al., 2006). This finding was confirmed by Durif et al. (in press) who found positive correlations between age and condition factor, liver weight and vitellogenin. This implies that the advantage for eels having a greater age at reproduction results from higher energy stores and a more efficient vitellogenesis. Indeed, we found indications that older eels are more sensitive since they need less hormonal injections to mature (Palstra et al., unpubl.). We therefore hypothesize that older eels are more sensitive to induction of maturation.

In this study we investigated if eels are stimulated to silver and mature by swimming thereby releasing the prepubertal blockage. To exclude age as a limiting factor, we subjected eels from Lake Balaton (Hungary) to swim trials since these eels were at least 13 years old at the time of experimenting. Eels in Lake Balaton were stocked for the last time in 1990 and they cannot enter or escape since the lake is landlocked.

## MATERIALS & METHODS

### *Experimental animals*

At the end of August 2003 and at the end of September 2004, in total 120 eels were caught by electrofishing in Lake Balaton, Hungary, in the region of Keszthely and Tihany. They were transported to the laboratory in oxygen-filled foil bags and marked individually injecting PIT-TAGS (TROVAN) subcutaneously just behind the head. Eels were then packed into large oxygen-inflated nylon bags in perspex and cardboard boxes after which they were sent to Leiden in early September (2003) or early October (2004).

### *Swim tunnels & conditions*

Swim experiments were performed in 22 Blazka-type calibrated swimtunnels described in detail by Van den Thillart et al. (2004). Swim tunnels were placed in the direction of the Sargasso Sea (WNW) in a climatized room of about 100-m<sup>2</sup>. The total water content of about 7000-L was recirculated continuously over a bio-filter. The illumination in the climatized room was switched to 670-nm light (bandwidth 20-nm). Based on eye pigment changes during silvering, it was assumed that this far-red light is invisible for eels (Pankhurst & Lythgoe 1983). Indeed, the animals did not respond to

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movements of the experimentor during red light illumination. Experiments were performed in air-saturated (> 75%) fresh water at  $18 \pm 0.5^\circ\text{C}$ .

### *Protocol experiment 1*

In experiment 1, 10 randomly chosen eels were anaesthetized, measured, sacrificed and sampled at arrival as control group. Forty eels in two groups of 20 were subjected to a swim fitness test that was in detail described in *chapter 2*. In short, eels were anaesthetized, measured and introduced into swim tunnels two days before the experiment started. Experimental eels swam for about 1 week consisting of 7 daily trials of maximally 12 hours each, depending on individual fatigue times. A group of 10 resting eels were kept during the experimental period in a 1500-l tank connected to a 2400-l recirculation system under dark conditions. PVC pipes were added to serve as shelter. At the end of the experiment, swimming and resting eels were anaesthetized, measured, sacrificed and sampled.

### *Protocol experiment 2*

After arrival, 10 randomly chosen eels were anaesthetized, measured, sacrificed and sampled as control group. Fifteen randomly chosen eels were measured and introduced in the swim tunnels. They were allowed to swim at speeds of 0.5 BL/s. Resting eels were kept as described. After 2 weeks, 6 randomly chosen swimming eels were stopped, anaesthetized, measured, sacrificed and sampled as well as 10 resting eels. After 6 weeks, the remaining 9 eels were stopped, anaesthetized, measured, sacrificed and sampled as well as 6 remaining resting eels.

### *Measurements & sampling*

Morphometric parameters included bodylength (BL), bodyweight (BW), eye diameters horizontal and vertical (EDh, EDv) and pectoral fin length (PFL). With these measurements we determined:

- Fulton's condition factor K using formula 1 (Table 1)
- The eye index according to Pankhurst (1982) EI using formula 2 (Table 1)
- The pectoral fin length index according to Durif et al. (2005) PFLI using formula 3 (Table 1)
- The silver index (SI) according to Durif et al. (2005) based on BL, BW, ED and PFL.

**Table 1** Formulas for sampling parameters.

1.  $K = 100 \cdot BW / BL^3$
2.  $EI = 100 \cdot ((EDh + EDv) / 4)^2 \pi / 10 \cdot BL$
3.  $PFLI = 100 \cdot PFL / BL$
4. Gonadosomatic index (GSI): (Weight gonads / Body weight) \* 100%
5. Digestive tract somatic index (DTSI): (Weight digestive tract / Body weight) \* 100%
6. Hepatosomatic index (HSI): (Weight liver / Body weight) \* 100%

Withdrawal of blood (500 µl) was performed in the dorsal aorta in the tail with heparin flushed (10,000 IU/ml) 1 ml syringes which were immediately placed on ice. Hematocrit (Hct) values were measured in 9 µl whole blood samples in triplo using a micro-centrifuge

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(Bayer, F.R.G.). Haemoglobin (Hb) content in 10 µl was determined in duplo by a spectrophotometer (LS50B, Perkin Elmer) measuring the absorbance at a fixed  $\lambda$  of 550 nm using the MPR 3 kit (1 ml, Roche Diagnostics GmbH). The MCHC (Mean Cellular Haemoglobin Content) was calculated dividing Hb by Hc. It then was centrifuged for 5 min at 14,000 rpm and bloodplasm was stored at -80°C.

Liver, digestive tract and gonads were sampled and weighted. These were used to calculate the gonadosomatic index (GSI; Table 1, formula 4), digestive tract somatic index (DTSI; Table 1, formula 5) and the hepatosomatic index (HSI; Table 1, formula 6). All eels were females. Samples were taken including gonads (portion of tissue from a standardised posterior location in Bouin solution stored at room temperature) and otoliths (sagitta) for age estimation.

### *Otolithometry*

Age estimation was carried out in the laboratory of Cemagref, Bordeaux, France by otolithometry according to the method described by Daverat (2005a). After their extraction, otoliths were cleaned of all organic matter in distilled water, dried with ethanol, and then stored in eppendorf tubes. The otoliths were later embedded in synthetic resin (Synolithe), then polished to the nucleus with a polishing wheel (Streuers Rotopol-35) using 2 different grits of sandpaper (1200 and 2400). Fine polishing was done by hand with Al<sub>2</sub>O<sub>3</sub> (1µm grain) on a polishing cloth. Etching was done using 10% EDTA. A drop of this solution was applied on the mold for a duration of 15 minutes. The otoliths were then rinsed with distilled water and stored in dry conditions. Yearly increments were revealed by staining with a drop of 5% Toluidine blue on the otolith and letting it dry. Growth rings were then counted under a microscope. The age of each eel was determined by the number of increments starting from the nucleus which was considered as year 1 of the eel's life.

### *Histology*

To remove Bouin fixative the gonads were washed with 0.1M phosphate buffer for two days. Then the samples were put in a 70% alcohol solution and washed again for two days. To prepare them for embedding in Technovit 7100 (Kulzer Histo-Technik), the samples were taken through a series of accumulating alcohol percentage (70%, 80%, 90%, 96% and 100%, 1-1.5 h per step). Then they were stored in Technovit 7100 mix without the hardener for a few days to completely saturate them with the mixture (de Jonge et al., 2005). Then they were embedded in the plastic. Coupes of 10 µm thick were cut using the Leica microtome (Jung Polycut E). Three coupes were put on a slide and five slides per sample were made. Then they were dried on a heating plate. The slides were stained with Mayers Haematoxylin-Eosin for nuclei and cytoplasm. The oocytes were studied under the microscope (Nikon, Eclipse E400). For every oocyte a picture was taken (Nikon Coolpix 4500). Per coupe stage and diameter of ten oocytes was measured using UTHSCSA Image Tool 2.0 as well as the number and diameter of lipid droplets of stage 3 oocytes in experiment 2.

### *Statistics*

Normality of the data and homogeneity of variances were checked by Kolmogorov-Smirnov tests. Paired observations of morphometric parameters were tested

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before and after swimming with student t-tests with one-tailed probabilities. For SI, a Wilcoxon test with one-tailed probabilities was used.

With a univariate general linear model (GLM), analysis of covariance (ANCOVA) with one-tailed probabilities was performed on log transformed unpaired observations in search for group effects in swim parameters with either BL (for PFL, PFW, ED) or BW (for GW, DTW, LW, Hct, Hb, MCHC, OD, number lipid droplets, diameter lipid droplets) as cofactors. ANCOVA was similarly performed for comparison between 2 weeks and 6 weeks swim groups. In case of occurrence of significant group effects, ANOVA with a post-hoc Bonferroni test was performed to specify the effects between particular groups. ANCOVA was especially required for the scalings difficulty between swim and rest groups in experiment 2 (Quinn & Keough, 2002).

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

Pearson correlation tests with one-tailed probabilities were performed for comparing between paired swimming induced changes in EI after 2 and 6 wks swimming and for correlation between oocyte diameter and lipid droplet number and size. OD between stages was tested with student t-tests with one-tailed probabilities. All statistical tests were performed in SPSS 10.0 for Windows.

## RESULTS

### *Silvering status before swimming*

Eels before swimming in experiment 1 were  $69 \pm 6$  cm long, weighted  $525 \pm 142$  g (Table 2a) and 70% was defined as silver eels (EI >6.5; Pankhurst, 1982). GSIs of eels in the control group were  $0.59 \pm 0.34$  in a range 0.2-1. Eels before swimming 2 weeks in experiment 2 were  $62 \pm 4$  cm long, weighted  $347 \pm 76$  g (Table 2b) and 83% was defined as silver eels. Eels before swimming 6 weeks in experiment 2 were  $63 \pm 5$  cm long, weighted  $429 \pm 137$  g (Table 2b) and 33% was defined as silver eels. Eels in experiment 1 were larger and more silver. Experimental eels were assigned to SI stages 2, 3 and 5 (Durif et al., 2005). Not any fish was assigned to stage 4.

### *Oocyte developmental status before swimming*

Before experiments, eels contained oocytes representing stages 1, 2 and 3 (Wallace & Selman, 1981). Stage 1 oocytes represented small oocytes organised as small oogonia in nests. They were oval shaped and the cytoplasm coloured dark. The nucleus was centred and round. It contained 1 to 3 larger nucleoli and about 20 smaller ones dispersed throughout the nucleus (Fig. 1a). Stage 2 oocytes represented larger oocytes with less dark coloured cytoplasm. In this phase a zona radiata was found starting to develop. The number of nucleoli had increased. Islands of lightly stained cytoplasm could be distinguished and connective tissue was present (Fig. 1b). Some stage 3 oocytes were found characterised by

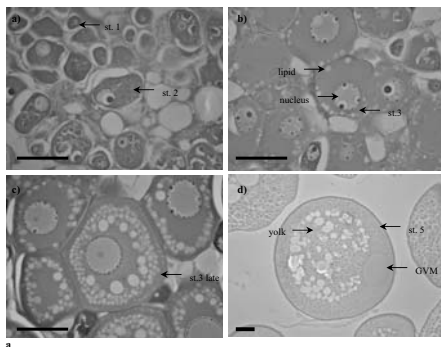
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**Table 2** Paired (pre-swim vs. post-swim) and unpaired (post-swim vs. rest, rest vs. control) measurements of a) experiment 1 (1 week swimming) and b) experiment 2 (2 and 6 weeks swimming). Expressed are number of countings n, the estimated age of swimmers (only experiment 2), morphometric parameters, blood parameters, internal parameters and oocyte parameters. Bold characters show significant differences (P<0.05) with \* vs. pre-swim or control and \*\* vs. pre-swim or control and rest. Control cells were sacrificed at the start of experiments while resting cells were sacrificed at similar time points as swimming cells (1, 2 and 6 weeks).

| a) experimental 1                                                  |           |            |           |            | b) experimental 2                                                  |           |           |              |             |
|--------------------------------------------------------------------|-----------|------------|-----------|------------|--------------------------------------------------------------------|-----------|-----------|--------------|-------------|
| parameters                                                         | 1 week    |            |           |            | parameters                                                         | 2 weeks   |           |              |             |
|                                                                    | control   | pre-swim   | post-swim | rest       |                                                                    | control   | pre-swim  | post-swim    | rest        |
| n                                                                  | 10        | 40         | 40        | 9          | n                                                                  | 10        | 6         | 6            | 10          |
| age (years)                                                        |           |            |           |            |                                                                    |           |           |              |             |
| <b>external</b>                                                    |           |            |           |            | <b>external</b>                                                    |           |           |              |             |
| HL (cm)                                                            | 67.14     | 69.66      | 69.66     | 66.43      | HL (cm)                                                            | 59.65     | 62.64     | 62.64        | 55.12       |
| BW (g)                                                             | 4752.77   | 5251.42    | 5161.42*  | 4711.21    | BW (g)                                                             | 267.68    | 343.76    | 333.66*      | 212.38      |
| SI                                                                 | 15.00     | 15.00      | 15.00     | 15.00      | SI                                                                 | 0.1470.19 | 0.1470.19 | 0.1470.19    | 0.1470.19   |
| PTL1                                                               | 4.68±0.29 | 4.96±0.43  | 4.83±0.42 | 4.66±0.49  | PTL1                                                               | 4.45±0.19 | 4.89±0.25 | 4.89±0.21    | 4.39±0.25   |
| PIV1                                                               |           |            |           |            | PIV1                                                               | 2.32±0.26 | 2.64±0.43 | 2.45±0.46    | 2.36±0.17   |
| SI                                                                 | 7.38±1.30 | 9.14±2.99  | 8.64±1.12 | 7.31±2.49  | SI                                                                 | 6.20±1.81 | 8.32±2.20 | 10.31±2.84** | 5.55±0.43   |
| SI                                                                 | 3.41      | 4.41       | 4.41      | 3.41       | SI                                                                 | 3.41      | 3.42      | 4.41         | 2.40        |
| <b>blood</b>                                                       |           |            |           |            | <b>blood</b>                                                       |           |           |              |             |
| Ht (cm)                                                            | 31.3±6.2  | 31.4±7.6   | 32.8±8.7  | 31.5±7.3   | Ht (cm)                                                            | 27.8±8.0  | 23.1±6.3  | 24.8±8.1     | 27.4±5.9    |
| Hb (cm)                                                            | 6.26±1.38 | 5.59±1.69  | 5.77±1.00 | 5.09±1.22* | Hb (cm)                                                            | 7.34±3.10 | 6.80±2.53 | 5.17±0.40    | 5.38±0.94   |
| MCPC (cm)                                                          | 19.1±4.1  | 18.9±2.9   | 17.8±3.2  | 15.3±2.3** | MCPC (cm)                                                          | 25.7±4.5  | 30.7±7.7  | 21.4±3.5*    | 20.1±3.9*   |
| <b>internal</b>                                                    |           |            |           |            | <b>internal</b>                                                    |           |           |              |             |
| GSI                                                                | 0.59±0.34 | 0.52±0.43  | 0.59±0.32 | 0.59±0.32  | GSI                                                                | 0.26±0.31 | 0.71±0.48 | 0.71±0.48    | 0.13±0.12   |
| DTSI                                                               | 2.42±0.65 | 1.66±0.67* | 2.01±0.52 | 2.01±0.52  | DTSI                                                               | 2.28±0.81 | 1.95±0.77 | 2.40±0.77    | 2.40±0.52   |
| HSI                                                                | 0.92±0.40 | 0.34±0.13  | 0.86±0.19 | 0.86±0.19  | HSI                                                                | 1.11±0.20 | 1.02±0.15 | 0.87±0.09**  | 0.87±0.09** |
| <b>oocytes</b>                                                     |           |            |           |            | <b>oocytes</b>                                                     |           |           |              |             |
| OD (µm)                                                            | 2.7±0.3   | 2.8±0.4*   | 1.54±0.7* | 1.28±0.48  | OD (µm)                                                            | 83±9      | 1.7±0.5   | 2.4±0.7      | 1.4±0.2     |
| OS                                                                 | 103.36    | 154.47*    | 128.48    |            | OS                                                                 | 83±9      | 1.7±0.5   | 1.36±0.6     | 61±14       |
|                                                                    |           |            |           |            |                                                                    |           |           |              |             |
| ** significant difference (P<0.05) vs. pre-swim / control and rest |           |            |           |            | ** significant difference (P<0.05) vs. pre-swim / control and rest |           |           |              |             |
| ** significant difference (P<0.05) vs. pre-swim / control          |           |            |           |            | ** significant difference (P<0.05) vs. pre-swim / control          |           |           |              |             |

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the presence of lipid droplets. In eels from control groups, stage 3 oocytes contained very few fat droplets, generally dispersed in the periphery of the ooplasm near the zona radiata (Fig. 1c). Mostly the nucleus was roundly shaped and nucleoli were found in the periphery of the nucleus. Less connective tissue was found surrounding the oocytes. Yolk globuli, characteristic for vitellogenesis (Fig. 1d), were not found.



**Figure 1** HE-staining of couples of a) oocytes stage 1 and 2, b) oocytes early stage 3 with few lipid droplets and c) oocytes late stage 3 with a large number of fat droplets, d) for comparison: mature stage 5 oocytes displaying germinal vesicle migration (GVM) and with numerous yolk globuli of an artificially matured cel. Scale bars are 100 µm. Note that the mature stage 5 oocyte (d) is much larger than the oocytes in stage 1, 2 and 3 (a, b, c).

### *Correlations size, silvering and oocyte developmental indicators*

Correlations between size, silvering and oocyte developmental indicators were analysed for experimental eels at the start (control and pre-swim measurements; Table 3). Significant positive correlations were found between size and silvering indicators and between size and maturation indicators. Between silvering and maturation indicators, significant positive correlations were found only between EI, SI and oocyte stage (Table 3; Fig. 2). Positive correlation with GSI was just not significant. No significant correlation was found between external indicators of the level of silvering EI and SI vs. DTSI and HSI.

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**Table 3** Correlations and significance between size, silvering and oocyte developmental indicators. In bold are given significant correlations ( $P < 0.05$ ).

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

## Changes in silvering after 1 week swimming (experiment 1)

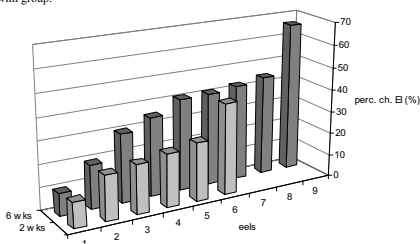
Paired observations showed that after 1 week swimming, experimental eels showed a significant weight loss of 15 g (Table 2a). Also the K was significantly lower. No increase was observed of silvering indicators. Paired observations of blood characteristics showed no changes in Hct, Hb and MCHC in the swim group (Table 2a). The rest group showed a significant lower Hb and MCHC. The GSI of eels that had swum was on average 39% higher than control and rest groups but individual variation remained high and this difference was not significant (Table 2a). Percentages of eels with GSIs >1 were 11% in the rest group and 33% in the swim group. The DTSI was significantly lower in the swim group. HSI was lower in the rest and swim group but not significantly.

## Changes in silvering after 2 and 6 weeks swimming (experiment 2)

Paired observations showed that after 2 weeks of swimming, experimental eels showed a significant weight loss of 14 g (Table 2b). Also the K was significantly lower. PFLI and PFWI did not show changes. The EI increased significantly from  $8.32 \pm 2.20$  to  $10.31 \pm 2.84$ , also in comparison with the rest group (Fig. 3a). After 2 weeks, all eels showed increase of the EI of 0.83 up to 3.36 which is between 11 to 41% (Fig. 3a). Accordingly, this caused a rise in SI. Paired observations of blood characteristics showed a slight, not significant increase in Hct and a slight, not significant decrease in Hb (Table 2b). The resulting MCHC became significantly lower which was also observed in the rest group. The GSI was found on average 3-5 times higher though not significantly (Table 2b). The

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number of eels with a GSI >1 was 50% in the swim group and significantly different from the control and rest group, as there were none in those groups. DTSI and HSI was lower but not significantly. HSI was found significantly lower in the rest group both vs control and swim group.



**Figure 3** Paired (pre vs. post-swim comparison of the same eels) individual percentual changes in EI after 2 and 6 wks swimming. All eels show increase after swimming. Changes are more pronounced after 6 weeks swimming.

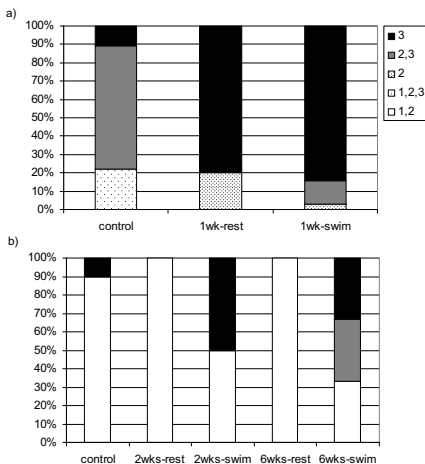
Paired observations showed that after 6 weeks of swimming, experimental eels showed a significant weight loss of 63 g (Table 2). Again the K was significantly lower. PFLI and PFWI did not show changes in the swim group. The PFWI was however smaller in the rest group. The EI increased in the swim group significantly with 1.40 up to 3.50 which is between 10 up to 66% (Fig. 3b) creating a rise in the percentage of silver eels up to a 100%. This resulted in a significant change in the SI. Paired observations of blood characteristics showed the same changes as after 2 weeks swimming resulting in significant decrease of MCHC in the swim but also in the rest group (Table 2b). The GSI in the swim group was found significantly higher than in the control and rest group. Also in comparison with 2 weeks swimming, the GSI was significantly higher both in the swim group and rest group. DTSI and HSI were lower in the swim group but only significantly in the rest group. Also in comparison with 2 weeks swimming HSI was significantly lower (Table 2b).

### *Changes in oocyte development after 1 week swimming (experiment 1)*

Eels of the control group in experiment 1 showed oocytes in stage 1, 2 and 3 with the latter oocytes just having entered this stage containing just a few lipid droplets. Eels showed heterogeneous ovaria mainly with stage 2 and 3 oocytes (Fig. 4a). The average diameter of these oocytes was  $103 \pm 36 \mu\text{m}$ . After 1 week, eels from both the swim group and rest group showed significant change in the average oocyte stage (Table 2a). Eels in the

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rest group had mainly oocytes with very few lipid droplets. For 85% of the eels in the swim group, ovaria had stage 3 oocytes with much more lipid droplets. The oocyte diameter was higher in the rest group ( $128 \pm 48 \mu\text{m}$ ) but only significant in the swim group ( $155 \pm 47 \mu\text{m}$ ).



**Figure 4** Distribution of oocyte stages 1-3 in eels of control, rest and swim groups a) after 1 week and b) after 2 weeks and 6 weeks. Oocyte categories (legend) represent combinations of oocyte stages represented in individual eels. Five categories were found: eels contained oocytes in stage 1 and 2, in all stages, only in stage 2, in stage 2 and 3 or only in stage 3 oocytes.

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### *Changes in oocyte development after 2 and 6 weeks swimming (experiment 2)*

Eels of the control group in experiment 2 showed oocytes in stage 1 and 2 except for one eel that showed only stage 3 oocytes, again with few lipid droplets. The average diameter of these oocytes was  $83 \pm 39 \mu\text{m}$ . After 2 weeks of swimming, eels showed oocyte development. Changes in average stage and oocyte diameter were just not significant (Table 2b). However, 50% of the eels had solely stage 3 oocytes in the ovaria with large numbers of lipid droplets in contrast to eels in control and rest groups which only had stage 1 and 2 oocytes (Fig. 4b). After 6 weeks, eels from the swim group showed further oocyte development. The change of average oocyte stage in the swim group was significant vs. control and rest group (Table 2b). In the swim group, oocytes were on average larger (not significantly). In the rest group, eels had only oocytes representing stage 1 and 2. In the swim group, only 3 eels showed stage 1-2 oocytes. The other 6 showed stage 2-3 or 3 oocytes with large numbers of fat droplets (Fig. 4b). The change in the rest group was also significantly different from the control group. Although values were similar, the eels in the 6 weeks rest group were smaller. Average stage and diameter were significantly higher after 6 weeks resting than after 2 weeks resting (Table 2b).

Large variation in number and size of lipid droplets in stage 3 oocytes was observed. Fig. 5 shows that both number and size of lipid droplets were positively correlated (for both  $P < 0.01$ ) to the size of the oocytes (pooled samples) indicating that lipids had been incorporated. Stage 1 oocytes were on average  $56 \pm 14 \mu\text{m}$  ( $n=220$ ). Stage 2 oocytes were significantly larger ( $P < 0.0001$ ) and on average  $87 \pm 23 \mu\text{m}$  ( $n=395$ ). Stage 3 oocytes were again significantly larger ( $P < 0.0001$ ) and on average  $159 \pm 36 \mu\text{m}$  ( $n=165$ ). They contained on average  $45 \pm 30$  lipid droplets in a range between 6 and 102 which measured on average  $11 \pm 2 \mu\text{m}$  in a range between 5 and 17.

## DISCUSSION

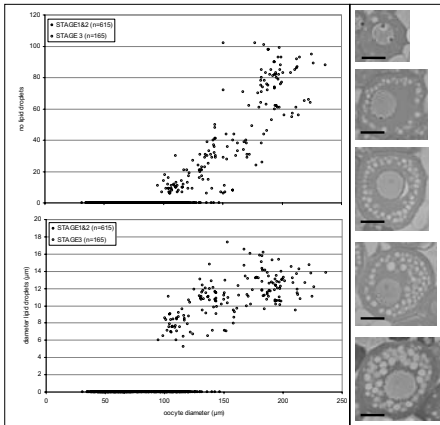
### *Swimming triggers silverying*

Swim exercise triggers increase of the eye index (EI) in all experimental eels already after 2 weeks of swimming. The EI was not increased in rest groups indicating that no time and/or starvation effect occurred. Swimming induced changes seemed even more apparent after 6 weeks swimming.

Already at the start, silver eels of the migrant SI stage 5 were present. They had GSIs  $>1$  and most had already some stage 3 oocytes but only with few lipid droplets. The presence of active migratory Lake Balaton silver eels is in contrast with Bíró (1992), who stated that Lake Balaton eels never become silver. Surprisingly, the migrant SI stage 4 (Durif et al., 2005) was not represented at all, before and after experiments. No changes of the pectoral fins were observed due to swimming. This does not agree with the findings that pectoral fins become larger and change shape during silverying (Tesch, 2003), changes that are supposed to correlate with migration (Durif et al., 2005).

DTSI and HSI were found not to be correlated with silverying level but with maturation level. Therefore, it does not seem surprising that decreases in DTSI and HSI also appeared as a starvation effect. They were found equally lower in all swim and rest groups and thus without additional effects of swimming.

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**Figure 5** Pooled oocyte samples of eels of experiment 2. Only swimming eels had stage 3 oocytes. A positive correlation existed for these oocytes between oocyte diameter and a) number of lipid droplets and b) diameter of the lipid droplets. The found diversity of number and size of fat droplets is illustrated by the oocytes in the pictures on the right. Oocytes have more and larger lipid droplets from top to bottom. Scale bars are 50  $\mu\text{m}$ .

### *Swimming triggers oocyte development*

Swim exercise triggers oocyte development. Significant increases were found in GSI, OS and OD in swimming eels. Swimming appears as a strong additional effect since also resting eels showed some development. After 6 weeks of swimming, changes were much more pronounced than after 2 weeks of swimming. GSI and oocyte diameter were significantly higher. Therefore, we can conclude that swim exercise not only triggers but also continues to stimulate oocyte development. We found that swimming induced shifting in oocyte development up to the lipid droplet stage that was characterised by high variation

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in numbers of lipid droplets. Developments occurred already after 1 week swimming and lasted up to swimming for 6 weeks. In this study we found that swimming caused a shift from stage 1-2 oocytes to stage 3 oocytes. Oocyte development also occurred to a smaller extent in rest groups. Oocyte stage was found significantly higher in rest groups already after 1 week. GSI and oocyte diameters were significantly higher in the 6 weeks rest group in comparison with the 2 weeks rest group.

Recently, van Ginneken et al. (unpublished) simulated a complete migration of 5,500-km for 3 year old hatchery eels. These eels were  $71 \pm 4$  cm long and weighed  $792 \pm 104$  g, larger than the experimental eels in this study. Significant changes were found in pituitary GTH-II and plasma estradiol compared to the controls. The oocyte diameter was the only change that was a significant effect of swimmers compared to controls and resting eels. Unlike this study, van Ginneken et al. (unpublished) did not find differences in EI and GSI. The more explicit changes in this study, already after 2 weeks swimming, might be explained by the difference in age. The hatchery eels were only 3 years old while the Lake Balaton eels in our study were between 13 to 21 years old. This finding supports our hypothesis that older eels are more sensitive for maturation. It may well be that the eels in this study, prevented from migration in their natural habitat, are more sensitive since they have regressed from silver to yellow, maybe even multiple times during successive years (Durif et al., 2005). The different results suggest that age might be a key factor for the start of migration, for silvering, for the onset of maturation and reproductive success at large.

### *Synchronous development of oocytes*

A point of discussion in literature concerns the question whether eels exhibit synchronous or asynchronous oocyte development. Eels are considered to have synchronous ovaries typical for semelparous teleosts (Wallace and Selman, 1981). When artificially matured, eels show however asynchronous development of oocytes maturing over several generations as shown by Palstra et al. (2005). In that study it is hypothesized that asynchronous development had an artificial rather than a natural origin. Oocytes were found to develop rather synchronized in this study, at least up to stage 3 (Fig. 4).

### *Swimming does not trigger vitellogenesis*

In this study, we observed swimming induced oocyte development only up to stage 3: the lipid droplet stage. Stage 3 was found to be highly variable with respect to the arrangement, number and size of the lipid droplets. Oocytes were found with only few small droplets but especially oocytes of eels that had swum contained more than 100 larger droplets. This indicates that swimming induces the incorporation of fats. However, oocytes did not develop further than stage 3. Most developed oocytes had lipid droplets that covered >50% of the cytoplasm and formed a complete ring around the circumference of the developing oocyte (Couillard et al., 1997), clear stage 3 previtellogenic oocytes (Colombo et al., 1984). This should indicate the start of vitellogenesis in *A. rostrata* according to Cottiril et al. (2001). However, we did not observe any yolk globuli in the appropriate oocytes. In addition, the oocytes did not reach sizes that are characteristic for vitellogenesis. Vitellogenesis is the major cause for oocyte growth in teleosts in general (Tyler, 1991), including eel (Nagahama, 1994). Cottiril et al. (2001) found oocytes of 200  $\mu$ m for *A. rostrata* and considered them vitellogenic. Such values do, however, not correspond to the

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growth typical for vitellogenesis. Adachi et al. (2003) stated for *A. japonica* that vitellogenesis begins when oocytes are about 250  $\mu\text{m}$  in diameter. In this study we found maximum oocyte diameters of 236  $\mu\text{m}$ . Also wild (untreated) migratory Loire eels did not contain vitellogenic oocytes (no yolk globuli; < 250  $\mu\text{m}$ ; Palstra et al., unpublished data). Recently, Vtg in the blood plasma of all the experimental eels of this study was determined by ELISA following the method of Burzawa-Gerard et al. (1991) by the group of Sylvie Dufour (MNHN, Paris). Vtg content in all samples was under the detection limit of 0.5  $\mu\text{g/ml}$ . From this, we can conclude that already Vtg synthesis and secretion by the liver, hepatic vitellogenesis, is not triggered; a process which is under major control of E2. In general, Vtg levels remain very low for wild migratory silver eels. Van Ginneken & Dufour (unpublished) measured Vtg in 104 large female silver eels from Lake Grevelingen (The Netherlands), caught at the sluices at the Northsea side at 32 ppt about to start their oceanic migration. Of these eels, 96% showed similar low Vtg levels <0.5  $\mu\text{g/ml}$ . Thus, a similar situation is reflected in the wild. Also Lokman et al. (2003) reported on basis of unpublished data (Lokman, Okumura, Adachi, Yamauchi) that Vtg synthesis as evidenced by Northern blot analysis of hepatic RNA, is not apparent in silver female *A. anguilla*.

### Triggering vitellogenesis

We suppose that an extended swim period or another trigger is required for induction of vitellogenesis. Vitellogenesis might be induced only near or after arrival at the spawning grounds in the Sargasso. Since hepatic vitellogenesis is not induced by swimming, at least not within a swim period of 6 weeks, estradiol (E2) synthesis and release may thus be triggered but apparently not enough to induce vitellogenesis. Testosterone (T) synthesis and release, in contrast, is probably triggered by swimming resulting in the increase of the eye index. Recently, van Ginneken et al. (unpublished) found trends of increased 11-ketotestosterone and estradiol in the blood plasma of 3 year old hatchery eels after swimming 5,500-km. The reproductive axis in fish is closely related to fat metabolism since polyunsaturated fatty acids and their metabolites can have different modulatory effects on steroid metabolism and function (reviewed by Sorbera et al., 2001). Mobilisation of fats by fasting and, even more pronounced, by swimming may induce activation of the steroid metabolism. In the near future we will quantify the sex steroid levels in the blood plasma as well as expression of LH and FSH in the pituitary, expression of their receptors in the gonads and expression of vitellogenin 1 and 2, and estradiol receptors in the liver, to specify the mechanism behind the stimulation of maturation by swimming.

Thus, an extended swim period or some other factor is required to trigger the responsiveness of the liver to estrogen in the production of vitellogenins. Growth of oocytes is most pronounced during vitellogenesis and subsequent maturation. OD will increase 2-fold up to 400  $\mu\text{m}$  during vitellogenesis and again 2-fold up to 800  $\mu\text{m}$  due to hydration during final maturation (Palstra et al., 2005). These increases result in similar increases of gonadal mass and with that of the body diameter. This then causes increase of drag during swimming and with that increase of cost of transport. This situation is undesirable during migration.

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### *Future studies*

It is peculiar that the influence of swim exercise on maturation has never been thoroughly investigated, especially since migrant fish like tuna, salmon and eel are of major commercial interest but very difficult or even impossible to reproduce in captivity. Very recently, Patterson et al. (2004) recognized this issue also for salmon and stated that *'exercise associated with migration is presented as a potential obstacle to successful reproduction'* but that *'there has been no attempt...to reverse this paradigm and examine exercise as an integral part of normal reproductive development for long distance migrators'*. Indeed they found that non-exercised females had delayed maturity, lower egg deposition rates, and were more likely to die prior to egg ovulation than exercised females and natal spawners. In our study we found that swimming induces maturation of eel. Eel could serve as a model to elucidate the mechanism. Swim exercise may well be successfully included in future protocols for reproduction of eel in captivity. These are topics of our current investigations.

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