Animal Biology, Vol. 57, No. 4, pp. 453-465 (2007)

Also available online - www.brill.nl/ab

Endocrine profiles during silvering of the European eel (*Anguilla anguilla* L.) living in saltwater

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Abstract—The transformation of yellow eel into silver eel is called 'silvering', and takes place prior to migration. This is the first study to provide hormonal profiles of European eel (*Anguilla anguilla* L.) during silvering. This transformation occurs in association with hormonal surges of testosterone (T) and estradiol (E2) but not with thyroid hormones (TH) and growth hormone (GH) which have a maximum activity in spring and a minimum activity in summer and autumn. It is therefore suggested THs and GH are not important for eel gonadal development in the autumn. Based on PCA analysis with physiological, morphological and endocrinological parameters it is concluded that the transition is gradual and that eels go through several stages.

Keywords: *Anguilla anguilla* L.; cortisol; estradiol; European eel; growth hormone; metamorphosis; seasonal changes; silvering; testosterone; thyroid hormone.

INTRODUCTION

The European eel (*Anguilla anguilla* L.) undergoes two metamorphoses during its life cycle. The first one corresponds to the transformation from *leptocephalus* larvae into glass eel during its oceanic migration from the supposed spawning grounds in the Sargasso Sea to the European coasts. The second metamorphosis occurs after the growth phase as a yellow eel, and marks the onset of puberty (silver eel). This

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transition phase, referred to as silvering, anticipates the long-distance migration back to oceanic waters. The drastic changes of habitat (from a freshwater or coastal habitat to an open-sea environment) and behavior (from sedentary to migratory) necessitate the modification of many systems (see Lokman et al., 2003 for review): visual (increase in eye-surface, and shift to shorter wave-lengths characteristic of deep-sea vision, Archer et al., 1995), osmoregulatory (hypo-osmoregulatory adaption), and metabolic (increase in muscle power output, and changes in location of fat stores). Silver or migratory eels have stopped feeding, and a regression of the alimentary tract can be observed. Although true sexual maturation only occurs during the oceanic migration, the gonadotropic axis is initiated before the eel starts its first downstream movements. A slight development of gonads can be observed in female silver European eels while they are still in fresh- or coastal waters.

Unlike smoltification in salmonids, silvering of eels is largely unpredictable. It occurs at various ages (5-20 years) and sizes (body length: 26-101 cm) (Tesch, 2003; Dekker et al., 1998). Because of the difficulty of getting individuals while they are in the process of metamorphosing, it is most common when studying eels, to separate individuals into two groups, yellow (resident) and silver (presumably migrant), and to compare the physiological profiles between the two. Basic knowledge was obtained in this way on the major differences in hormone levels between the two stages. Histology has shown that the thyroid gland of silver eels is more active than that of yellow eels (Callamand and Fontaine, 1942). High levels of total thyroxine (TT4) have been found in migratory eels (Marchelidon et al., 1999). The production of thyroid hormones is also thought to be responsible for the hyperactivity of eels at the onset of migration (Fontaine, 1975).

The most important changes however relate to the reproductive system. As in all vertebrates, the development and activity of the gonads are under the positive control of gonadotropic hormones (GTH), which are produced by the pituitary. In response to stimulation, gonads will produce gametes and sexual steroids, which in turn will stimulate other organs implicated in reproduction. Vitellogenin is secreted by the liver under the control of estradiol. In contrast to yellow eels, silver eels only, have cells with the necessary estradiol receptors (Burzawa-Gerard et al., 1994). Yellow eels are incapable of vitellogenesis, while silver eels also have the structures, which will allow endocytosis of vitellogenin. In the yellow eel, the gonadotropic function is totally inactive, while in silver eels plasma levels of gonadotropin are low (Dufour et al., 1983a; Dufour et al., 1983b). Although main differences between the two stages have been described, little is known about the dynamics of the silvering process, although it has been shown that the transition is gradual and that eels go through several stages (Durif et al., 2005). The objective of this study was to obtain a finer seasonal description of the silvering process (start and duration) as well as of the physiological mechanisms involved (triggers and endocrine control). Due to the difficulty to obtain early silvering eels or to predict eels that will start the metamorphosis, a monthly sampling schedule was carried out in which the biggest eels were collected as they were the most likely to start silvering (Durif

et al., 2005). Eels were sampled at regular intervals from April to November, and several endocrine and metabolic parameters were analyzed.

MATERIAL AND METHODS

Animals

Every month from April until November 2002, local fishermen caught eels by fyke nets at the Grevelingen. This is the largest saltwater lake of Western Europe with a total area of 14 000 hectares. The lake is situated on the boundary between Zuid-Holland and Zeeland, The Netherlands. As they were most likely to start silvering, the largest 8 female eels were selected every month (body length 63-90 cm). The fish were rapidly anaesthetized with benzocaine (100 ppm). Blood was collected with a heparinized syringe and stored on dry ice for further analysis. The carcasses were taken to the laboratory to measure body length, body weight, vertical and horizontal eye diameters. Organs were collected to determine the weights of digestive tract, liver, and gonads.

Blood analysis

For hormones and vitellogenin (VTG) the plasma was divided in Eppendorf tubes (50 μl, 100 μl, 1 ml, 1 ml) for measurement of respectively VTG, growth hormone (GH) testosterone (T) and 17β-estradiol (E2), and stored at -80°C pending analysis. VTG was measured by immunoenzymatic assay according to the protocol of Burzawa-Gerard et al. (1991) and GH by radioimmunoassay according to Marchelidon et al. (1996). T and E2 measurements were performed by Radioimmunoassay. Total thyroxin (TT4), free-T4 and tri-iodothyronine (TT3) were determined with commercial Amerlite kits (Amersham International PLC, UK) modified for fish plasma.

Histology

After fixation in Bouin's fluid, gonads were dehydrated in a graded ethanol series and embedded in Historesin according to standard procedures (Romeis, 1968). They were sectioned at 5 μm and stained with haematoxylin and eosin. The length and width of thirty oocytes of one section were measured and then averaged.

Data analysis

The animals caught in the period from April until July were all 'yellow' while the animals in the period September until November were all 'silver'. Classification was performed according to external parameters (skin color, eye size and thickness skin). Comparison between 'yellow' and 'silver' was performed on the average value using a two sample T-test (table 3). Morphological and physiological descriptors were

Table 1.
Description of the transformed variables.

Variable	Transformation	Abbreviation
Body weight	Fulton's condition factor	K
Gonad weight	Log-transformed, standardized	GW
Alimentary tract weight	Log-transformed	AW
Liver weight	% of total body weight	HSI
Oocyte diameter	Log-transformed	OODIAM
Estradiol	None	E2
Testosterone	Log-transformed, standardized	T
Vitellogenin	Log-transformed	VTG
Free thyroxin	Log-transformed	FT4
Total thyroxin	Log-transformed	TT4
Tri-iodothyronine	Log-transformed	TT3
Growth hormone	Log-transformed	GH

tested for normality (Kolmogorov-Smirnov, Lilliefors probability). Those which differed significantly from the normal distribution ($p < 5\%$) were log-transformed (gonad weight, alimentary tract weight, FT4, TT4, TT3, oocyte diameter, testosterone, VTG, and GH). Hepato-somatic index (HSI) was calculated as HSI = (liver weight/body weight) $\times$ 100. Other common indices, such as gonado-somatic index for gonad weight, were not used as they were correlated to body length of eels and mean length of eels differed between monthly samples. Therefore, to remove any size effect, variables which were correlated to length of eels were standardized according to: Var_std = Var $-$ (M(L $-$ L_mean)) (MacCrimmon and Claytor, 1985).

Where Var_std is the corrected variable, Var is the original variable, M is the slope of the regression of the descriptor on total body length, and L_mean is the mean length of the eels in the sample. Three variables were standardized in this way: log-transformed gonad weight, liver weight, and log-transformed testosterone. Table 1 summarizes the different variables and transformations.

Pearson correlations (table 2) were calculated between the transformed variables, and their significance was determined with a Bonferroni test. $P \leqslant 0.05$ was considered statistically significant for all tests. Statistics were performed via Systat SPSS Version 10.

Principal Component Analysis (PCA) was applied to examine the simultaneous variations of hormone levels and to detect common physiological profiles between individuals. Three internal morphological parameters were added as they are known to vary significantly during silvering (gonad weight, oocyte diameter, and alimentary tract weight). The analysis was carried out on the following standardized variables: E2, VTG, GW, TEST, TT4, TT3, OODIAM GW, and AW (fig. 1). The PCA was performed with ADE4 (Thioulouse et al., 1997). Missing values were most frequent for GH level, and its contribution to the axes was not significant. Its variations were presented separately (fig. 2).

Table 2.
Correlation matrix of the parameters sampled on the eels.

	K	E2	VTG	AW	FT4	TT4	TT3	OODIAM	GW	T	H.S.I.
K	1.00										
E2	0.29	1.00									
VTG	0.28	0.31	1.00								
AW	−0.10	−0.27	**−0.53**	1.00							
FT4	−0.17	−0.12	−0.26	0.09	1.00						
TT4	−0.01	0.00	−0.14	−0.07	**0.75**	1.00					
TT3	0.06	0.05	−0.05	0.04	0.25	0.45	1.00				
OODIAM	0.33	0.29	**0.68**	**−0.66**	−0.33	−0.20	−0.12	1.00			
GW	**0.71**	0.43	**0.57**	−0.44	−0.24	−0.07	0.02	**0.69**	1.00		
T	0.43	**0.56**	0.46	−0.39	−0.13	0.02	−0.03	0.32	0.48	1.00	
H.S.I.	0.17	0.08	0.01	0.32	−0.06	0.03	−0.07	−0.03	0.10	0.11	1.00

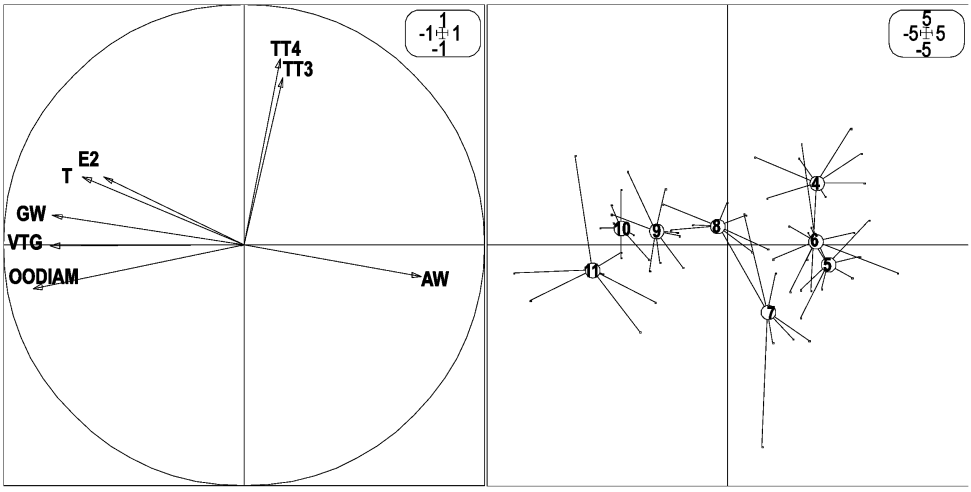

Figure 1. Seasonal evolution of physiological characteristics of eels sampled between April and November: testosterone (T), estradiol (E2), vitellogenin (VTG), total thyroxin (TT4), triiodothyronine (TT3), gonad weight (GW), alimentary tract weight (AW), and oocyte diameter (OODIAM). Left: Correlation circle of variables. Right: Factorial scores of individuals (eels). Clusters correspond to the months that were sampled (April to November).

RESULTS

In table 2 the Pearson correlations for the different sampled parameters are given in a Correlation matrix. In table 3 the mean ± SD of morphological and endocrine parameters examined over an 8-month period (from April until November) in female European eel are given.

The first two axes of the Principal Component Analysis (PCA) accounted for 57% of the total inertia (respectively 42 and 15% for axes 1 and 2). Axis 1 was positively correlated with variations in alimentary tract weight (AW), and negatively

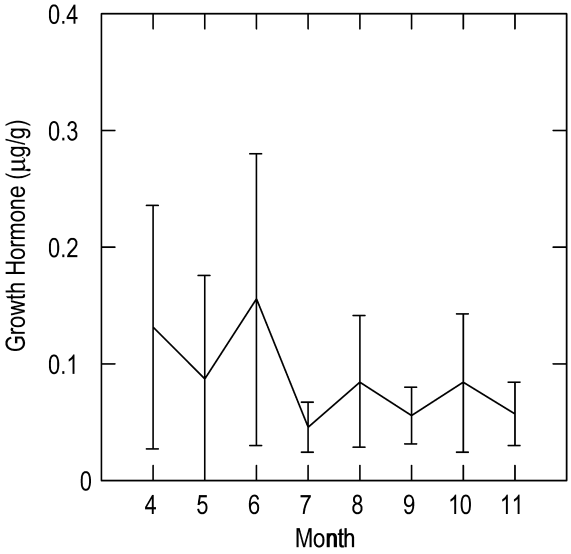

Figure 2. Monthly evolution of growth hormone (GH in μg/g) from April until November. Mean $\pm$ SD (n = 6-8 individuals).

correlated with estradiol (E2), testosterone (T), gonad weight (GW), vitellogenin (VTG) and oocyte diameter (OODIAM) (fig. 1, left panel). Axis 2 represented variations in tri-iodothyronine and total thyroxin (TT3 and TT4). Individuals formed tight clusters on this factorial plot, which corresponded to each monthly sample (fig. 1, right panel). Therefore eels caught during the same month displayed very similar physiological profiles. Monthly samples of eels were regularly distributed along the first axis indicating a seasonal evolution in which four major phases could be described. The first phase corresponded to the month of April, during which eels exhibited high levels of TT3 and TT4 (up to 29.4 and 75.4 nmol/l respectively). Samples from May and June were quite similar and displayed much lower levels of TT3 and TT4. They were mainly characterized by high AW, indicative of active feeding. The transition phase (to silvering individuals) seemed to occur during July and August, as noted by the regression of the alimentary tract (decrease of AW). It is visible through the decrease of the alimentary tract weight from spring to November (table 3).

While levels of TT3 and TT4 further decreased in July, they showed a slight increase in August. The last phase (September to November) corresponded to an overall increase of the parameters linked to the onset of puberty (E2, T, VTG, OODIAM). E2 stabilized between September and November, while VTG and OODIAM increased progressively during this period. TT3 and TT4 levels decreased again in November (table 3).

Levels of GH showed high within month variability especially between April and June (fig. 2). Certain individuals reached very high values (maximum of 0.325 μg/g in June, while the minimum was 0.015 (μg/g in May)). Variation in GH levels

Table 3.
Morphological and endocrine parameters examined over an 8-month period in female European eel (*Anguilla anguilla* L.). From April until July the animals are yellow (non-migratory phase) while from September until November the animals are silver (migratory phase). In August half of the animals is yellow and half of the animals is silver. The mean value of the yellow eel group was compared to the mean value of the silver eel group.

	April n = 8	May n = 8	June n = 8	July n = 8	August n = 8	September n = 8	October n = 8	November n = 8	Yellow n = 36	Silver n = 28	P-value
Morphology											
Body weight (g)	861 ± 194	969 ± 187	908 ± 187	751 ± 175	771 ± 184	1248 ± 302	1092 ± 132	1178 ± 293	855 ± 195	1132 ± 262	$P \leqslant 0.0001^{**}$
Length (cm)	74.9 ± 5.95	77.1 ± 4.76	75.8 ± 5.20	73.4 ± 3.47	70.1 ± 4.90	83.4 ± 5.78	78.4 ± 4.37	79.5 ± 5.81	74.7 ± 5.18	79.2 ± 6.36	$P \leqslant 0.003^{**}$
Eye-index	7.34 ± 0.98	7.28 ± 2.38	7.26 ± 0.72	6.69 ± 0.85	7.99 ± 0.93	10.42 ± 0.62	9.95 ± 0.92	10.91 ± 0.86	7.14 ± 1.31	10.17 ± 1.04	$P \leqslant 0.0001^{**}$
G.S.I.	0.65 ± 0.15	0.65 ± 0.20	0.76 ± 0.24	0.79 ± 0.28	0.87 ± 0.28	1.44 ± 0.17	1.54 ± 0.30	1.38 ± 0.26	0.71 ± 0.22	1.40 ± 0.28	$P \leqslant 0.0001^{**}$
H.S.I.	1.49 ± 0.45	1.28 ± 0.24	1.54 ± 0.50	1.23 ± 0.30	1.17 ± 0.20	1.32 ± 0.09	1.36 ± 0.28	1.37 ± 0.12	1.35 ± 0.39	1.34 ± 0.16	$P \leqslant 0.924$
Digestive tract (g)	23.9 ± 3.1	33.9 ± 12.4	32.5 ± 16.1	23.4 ± 7.8	13.9 ± 3.8	17.8 ± 4.7	13.4 ± 2.3	13.2 ± 4.5	27.1 ± 11.9	14.8 ± 4.4	$P \leqslant 0.0001^{**}$
Oocyte diameter (mm)	16.93 ± 1.41	19.26 ± 1.14	21.56 ± 1.79	24.54 ± 1.08	27.31 ± 1.33	29.01 ± 0.46	31.86 ± 1.29	34.51 ± 1.09	21.29 ± 3.57	30.76 ± 3.07	$P \leqslant 0.0001^{**}$
Endocrinology											
TT4 (nmol/l)	36.5 ± 24.6	16.6 ± 17.1	17.2 ± 27.4	7.4 ± 5.1	13.5 ± 5.4	13.5 ± 5.0	12.0 ± 5.9	7.6 ± 5.4	19.2 ± 21.3	11.1 ± 5.6	$P \leqslant 0.055$
Free T4 (pmol/l)	114.6 ± 143.9	28.3 ± 24.4	63.3 ± 138.6	17.7 ± 8.7	22.4 ± 8.5	21.8 ± 6.5	18.3 ± 12.4	12.0 ± 6.3	53.4 ± 98.9	17.7 ± 9.1	$P \leqslant 0.062$
TT3 (nmol/l)	12.4 ± 8.8	6.4 ± 2.8	18.3 ± 11.7	5.5 ± 5.1	10.3 ± 5.7	10.4 ± 5.9	6.8 ± 5.3	6.2 ± 5.0	10.8 ± 8.7	7.9 ± 5.6	$P \leqslant 0.137$
Growth-hormone (ug/g)	130.8 ± 97.1	86.9 ± 82.6	154.9 ± 109.6	45.3 ± 18.1	84.7 ± 52.1	55.3 ± 20.6	83.4 ± 49.7	56.9 ± 25.6	106.2 ± 86.4	66.3 ± 38.8	$P \leqslant 0.037^{*}$
Oestradiol (ng/ml)	1.83 ± 0.31	1.64 ± 0.69	1.11 ± 0.60	0.97 ± 0.37	1.83 ± 0.75	2.43 ± 0.60	2.49 ± 0.42	2.09 ± 0.63	1.38 ± 0.67	2.34 ± 0.58	$P \leqslant 0.0001^{**}$
Cortisol (ng/ml)	26.1 ± 10.3	31.5 ± 25.3	27.1 ± 15.5	70.2 ± 38.5	40.7 ± 24.7	108.1 ± 27.7	70.1 ± 55.0	85.8 ± 25.8	38.2 ± 29.1	80.7 ± 42.0	$P \leqslant 0.0001^{**}$
Testosteron (ng/ml)	0.75 ± 0.31	0.56 ± 0.25	0.47 ± 0.17	0.42 ± 0.22	0.41 ± 0.15	1.04 ± 0.25	1.04 ± 0.52	1.69 ± 1.27	0.53 ± 0.27	1.15 ± 0.81	$P \leqslant 0.0001^{**}$
Vitellogenin (μg/ml)	104.27± 85.31	84.03± 8.49	133.77± 62.45	159.48± 140.12	133.84± 34.10	1023.05± 1680.97	766.81± 1279.3	2438.99± 2626.92	122.01± 84.53	1189.05± 1883.15	$P \leqslant 0.001^{**}$

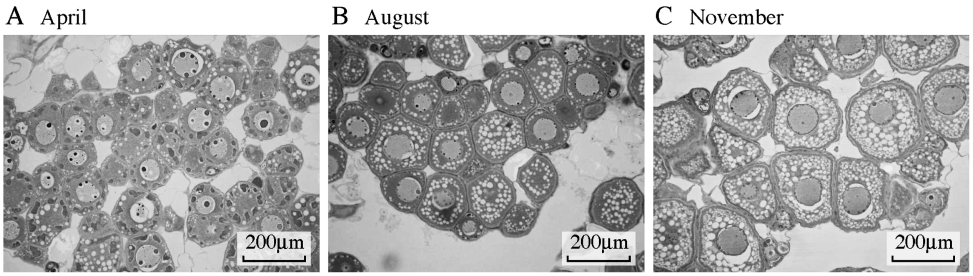

Figure 3. Example of the annual development of the oocytes of eels. Per month the 8 largest caught female animals were selected. The oocytes in april are in the previtellogenic or early vitellogenic stage containing peripheral yolk granules. The oocytes of the month August and November are in a more advanced stage called the lipid vesicle stage or cortical alveolair stage, which can be characterized by oocytes with oil droplets.

among individuals might be dependent on their size. Mean and standard deviation decreased there onwards (starting at the transition phase (July and August)). Levels remained low until November.

Histology showed that the ovaries of eels caught in April and August were characterized by previtellogenic oocytes, those of the November eels were dominated by oocytes in the lipid vesicle stage (fig. 3).

DISCUSSION

This study is the first to provide hormonal profiles of European eels during the silvering process. Sampling was not carried out randomly, as the 8 largest females were chosen. This was to ensure that some of the sampled individuals would be undergoing silvering. Surprisingly, there was little within-month variability in their hormonal profiles, indicating that the eels collected on the same month were all undergoing similar endocrine changes. This suggests that starting at a certain size (here 60 cm), eels may all start the first steps of the silvering process. Whether they all go through the final phases of the metamorphosis and actually start their spawning migration may be determined in a later phase and depending on the eel's fat stores (Tesch, 2003).

Growth hormone

Growth hormone (GH) is firstly implicated in body growth and development of organs and tissues in young animals. However, it has many roles in other physiological processes among which reproduction and osmoregulation by stimulating the production of thyroid hormones (TH) (Evans, 1993). For salmonids it is suggested that GH and TH's both may play an osmoregulatory role (Sakamoto et al., 1993). The close link to each other has been observed in one molecular study were it has been demonstrated that regulation of the growth hormone gene in salmon was mediated by all thyroid receptors (Sternberg and Moav, 1999). In salmonids elevated

GH is associated with developmental and osmoregulatory changes during processes like smoltification, migration and entry into seawater (Sakamoto et al., 1993). For Salmonids it is reported that decreasing temperatures may be the trigger for smoltification (Boeuf, 1994). For eel however, GH probably has no role in osmoregulation because hypophysectomized eel can survive in both fresh or seawater (Oliverau and Ball, 1970), while hypophysectomized salmonids only survive in freshwater (review: Sakamoto et al., 1993).

GH values in spring showed high variability. This suggests the fact that some of these eels (with low GH levels) would not have completed their metamorphosis, and would have waited another season.

Thyroid hormones

The peak in TT3 and TT4 observed in our study in April can be indicative for an increased thyroid activity at the onset of the silvering process. In general, for many fish species living in our latitudes, there is a maximum activity of the thyroid gland during the winter and spring, and minimum activity during the summer. This cycle has been observed in the trout, the minnow, cod and *Fundulus heteroclitus* (review: Swift, 1960). In the climbing perch, *Anabas testudineus*, it was observed that TT4 reaches its maximal concentration in spring at the beginning of the spawning season (Chakraborti and Bhattacharya, 1984). Studies on eel also showed increased thyroid and pituitary activity during metamorphosis (Callamand and Fontaine, 1942; Etienne, 1959; Knowles and Vollrath, 1966). Also in the study of Han et al. (2004), serum thyroxine levels increased in parallel with TSH β mRNA expression during silvering, supporting the hypothesis that the hypothalamus-pituitary-thyroid axis is correlated to silvering in the wild Japanese eels. Also in salmons during parr-smolt transformation thyroxin is involved and a peak was found in salmon plasma in April (Dickhoff et al., 1978).

Steroids

In the study reported here, we found elevated cortisol levels in silver eel during the migration period. Cortisol is released from the interrenal tissue when an animal is exposed to a stressor (Wendelaar Bonga, 1997) but changes can also be attributed to a daily rhytm and to sexual maturity and season (Pickering and Christie, 1981). We observed a strong significant difference of $P \leqslant 0.001$ in cortisol levels between yellow and silver animals (table 3). The high cortisol levels during the silvering period may play a role in mobilization of energy stores during migration (van Ginneken et al., 2007). However within the tested period (April-½ August for 'yellow') and (½ August-November for 'silver') there were large fluctuations for cortisol between months. For the 'yellow' period especially in the month July a high value of around 70 ng/ml was found which is comparable to the level reached during the 'silver' period. However the high cortisol value reached in July can be attributed to two individuals (see large Standard Deviation). Within the 'yellow period' no

significant difference was observed between April and July ($P \leqslant 0.24$) but only between June and July ($P \leqslant 0.008$). Months that are significantly different at a 5% level are mainly between the 'yellow' and 'silver' period: April and September ($P \leqslant 0.001$), April and October ($P \leqslant 0.014$), April and November ($P \leqslant 0.001$); May and October ($P \leqslant 0.028$), May and November ($P \leqslant 0.001$). June is different from September ($P \leqslant 0.001$), October ($P \leqslant 0.005$), November ($P \leqslant 0.001$). August is different from September ($P \leqslant 0.002$) and November ($P \leqslant 0.03$).

So despite the large fluctuations of plasma cortisol within months, major significant differences can be observed between the 'yellow' and 'silver' period. So changes in cortisol levels for eel can possibly be attributed to sexual maturity and season in order to mobilize energy stores during migration. The high value of 70 ng/ml found in July may be a release of this hormone in anticipation of the 'silvering' period.

From the well-marked seasonal pattern in female T, which lags behind but follows female E2, we can conclude there is a correlation between the two ($R = 0.56$, table 2). This supports the possibility that testosterone may act as a precursor for E2 synthesis during the vitellogenic season.

The increased E2 profile in the period September-November suggests that in the period of gonad development the aromatizing enzymes are partially stimulated. In several fish species aromatizing activity have been observed in the ovary (review: Nagahama, 1994).

For eels in general T and 11-Ketotestosterone (11-KT) may play a more prominent role in females during silvering and/or maturation. Lokman et al. (1998) found high values of 11-KT in females of *Anguilla dieffenbachi*, and suggested that this steroid may play a role in preparing maturing animals for their spawning migration. Indeed, Rohr et al. (2001) demonstrated in immature short-finned eels (*A. australis*) implanted with a vehicle containing 11-KT, that this steroid was involved in the process of silvering. Observed changes were: a) a change in head shape and pectoral fin appearance, b) structural changes of the skin, and c) an increase in eye size and ventricular, liver and gonad mass (Rohr et al., 2001). Also Han et al. (2003) found that androgen, but not estrogen, plays a major role in silvering process of the eels in both sexes.

E2 is the 'trigger' for VTG synthesis (Burzawa-Gerard and Dumas-Vidal, 1991). Although we found a low correlation coefficient of ($R = 0.31$, table 2) between plasma E2 and VTG it is generally assumed that there is a causal relation between those two components in the vitellogenesis which is at the basis for the growth of the gonad by incorporation of yolk proteins in the oocytes (review: Nagahama, 1994). The low correlation coefficient can be explained by the substantial increased levels of VTG in fall, in comparison with the stabilization of plasma E2 from September to November. The progressively larger vitellogenic response can be ascribed the so called 'memory effect', an increased sensitivity of the liver to E2 via its receptor mechanism (sensitivity and density) and also possibly by an enhanced

post-transcriptional mechanism of hepatic vitellogenesis (Jackson and Sullivan, 1995).

In conclusion, we described the hormonal profile of European eel during the 'silvering' process, the onset of puberty and seaward migration. Especially levels testosterone and estradiol were high in autumn during the migration season while thyroid hormones and growth hormone were high in spring and are therefore not involved in the 'silvering' process.

ACKNOWLEDGEMENTS

We thank the fishermen Wim and Piet Bout (Brunisse, The Netherlands) for supplying Grevelingen eels every month. This study was supported by the Netherlands Organisation for Scientific Research (STW-project no. LBI66.4199) and by the European Commission (EELREP-Q5RS-2001-01836).

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