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FACULTY OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES

**NEUROENDOCRIN REGULATION OF REPRODUCTION IN MALE
EUROPEAN STARLINGS**

Ph.D. Thesis

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1. PRELIMINARIES and OBJECTIVES

Birds use sophisticated reproductive strategies, enabling them to reproduce successfully under different environments and conditions. The hypothalamus is the main regulator of reproduction and courtship behavior is, which integrates environmental factors - primarily the daylength – with functions of the internal biological clock. The breeding period of birds, compared to mammals is generally shorter and more asymmetric.

Increasing daylength during spring stimulates release of gonadotropin-releasing hormone (GnRH) and gonadal maturation. Gonads produce sex steroids, which affect the behavior of animals and determine their secondary sexual characteristics. Beside of gonads, adrenals and probably the brain are also capable of producing sex steroids.

Thus, the high sex steroid concentration is crucially during breeding period. Although increased daylength in spring stimulates gonadal function, it may have an inhibitory effect in most birds from temperate and cold zones during summer. The latter is characterized by a decrease of hypothalamic GnRH (Dawson et al., 1985), which is at least in some species followed by an atrophy of the gonads and the adrenal cortex (Bhattacharyya, Ghosh 1965, Riddle 1923). Then also the concentration of sex steroids returns to levels of the non-reproductive period. This process leads to the non-breeding condition (photorefractory) and to the moult (Goldsmith, Nicholls 1984). Thus, the increasing daylength has a double role; first as stimulatory and later as inhibitory.

During early autumn, some bird species show partial sexual reactivation. The increased aggression and/or courtship behavior is characterized by regressed testes and low testosterone (T) plasma levels. However, the concentration of another androgen, dehydroepiandrosterone (DHEA), produced mainly by the adrenal, and the corticosterone (B) increases in this period (Dawson, Howe 1983, Soma et al. 2002). It is possible, that circulating small androgen molecules easily cross the blood-brain barrier and come into contact with hypothalamic preoptic area (POA) (include with medial preoptic nuclei (POM)) or telencephalic song nuclei. It seems that these androgens affect control of reproductive behavior in birds after being converted by aromatase enzyme (ARO) to estrogen.

The vast majority of published studies about the reproduction of birds is based on observations in captive and/or domesticated bird species. However, the trauma caused by captivity (the size of cages, the presence or absence of nests and mates, the different food quality and often the absence of ecological factors like natural light, temperature, humidity, etc.) may provide an explanation to the different results from laboratory experiments are sometimes different or even opposite from results of free-living/wild conditions studies (Calis, Bentley 2009, Kern 1979, Lambrechts et al., 1999).

In this thesis, we were interested in the neuroendocrine regulation of reproduction in free-living European starlings (*Sturnus vulgaris*). Starling is a widespread seasonal breeder, and shows highly predictable repeated changes in reproductive physiology and behavior in response to the annual cycle of the photoperiod. It is a very popular species among the avian endocrinologists and numerous studies have been published about the neuroendocrine control of the starling reproductive system. However, most of the reports are based on observations on captive starlings (Dawson, Goldsmith 1997, Foster et al. 1987, Parry et al. 1997, Riters et al. 2000, Stevenson, Ball 2009, Ubuka et al. 2008a).

Aim of study

The aim of our study was to define the width spectrum of regulation of seasonal breeding in wild male starlings: from GnRH expression through plasma steroid concentration to the courtship behavior in different phases of breeding period and during photorefractoriness. This included the specific topics:

- To examine the seasonal GnRH-I expression in the preoptic area (POA) and median eminence (EM) by immunocytochemistry.
- To describe the morphological characteristics of the testis and adrenals, and to perform their morphometric analysis in the annual cycle.
- To determine the plasma concentrations of two androgens (T, DHEA) and corticosterone (B) and to test for a possible correlation of these hormones with testicular and adrenal tissue data.
- To quantify the brain T → estrogen (E2) conversion by aromatase (ARO) enzyme in the POA/POM region.
- To analyze courtship song of males and to describe other courtship behaviors.

2. MATERIALS and METHODS

2.1. Animals

The studies were performed on adult male starlings. Birds were monitored in the natural environment and were collected by using shotgun. Collected starlings were quickly weighed and immediately decapitated. While behavioral observations were done in Budapest (XXI. district), tissue collection took place in Abony, Gyömrő and Etyek. According to their phenological state, birds were sorted in to five groups: second half of March (photosensitive, March II), first half April (pair formation, April I), second half of April (nesting, April II), first half of May (hatching of nestlings, May I), second half of August (photorefractory, August II).

2.2. Determination of the climatic factors

We studied the seasonal variation of the three climatic parameters that predominantly influence reproductive processes: daylength, temperature, and rainfall. In the case of daylength data from the European Meteorological Service were used. Data of temperature and rainfall were obtained from the National Weather Service.

2.3. Determining the blood steroid content by ³H-RIA

After decapitation, aprox. 0,5-1 ml blood was sampled into heparinized polyethylene tubes and stored cooled. Blood samples were centrifuged (3500 rpm) and the plasma was stored at -20°C. Plasma samples of aliquots were extracted three times (sex hormones in diethyl ether and B in dichloromethane). The supernatants were collected in tubes, then the samples were dried and stored in -20 ° C and 100% ethanol.

Plasma steroids were analyzed by radioimmunoassay (RIA). Determination of plasma T was done according to the Jallageas method (Jallageas 1975), plasma DHEA according to the Fehér Tibor method (Fehér T. personal communication) and plasma B to the Mihály Katalin method (Péczely, Pethes 1981).

2.4. Histological examination of paraffin sections

Tissue samples (intra-abdominal fat, testes, adrenal gland) were fixed by immersion procedure in the 4% buffered formalin and dehydrated in ascending alcohol series then embedded in paraffin blocks. Five mm thick sections were cut using microtome (Reichert, Austria). From each block, representative

5-6 sections were mounted on glass slides treated with silane (3-aminopropyltriethoxy Silano (Sigma Aldrich Co., St Louis, MO, USA)). The sections were stained with hematoxylin and eosin (HE) (Reanal, Budapest). Digital images were taken by OLYMPUS DP70 microscope equipped with a 3-CCD camera (OLYMPUS BX51). In case of the testes, the volume was measured before the fixation. The morphology and the morphometry of the tissues were analyzed by the ImageJ program. The measured parameters are:

- the size of adipose cells (μm^2),
- the volume of testis (mm^3),
- the thickness of a tunica albuginea (μm),
- the area of the cross sections of the seminiferous tubules (μm^2),
- the thickness of interstitium (μm),
- the thickness of the germinal epithelium (μm),
- the type of spermatogen cells of germinal epithelium,
- the interrenal/adrenal ratio of the adrenal gland,
- the size of the nuclei in three zones of the interrenal tissue (μm^2).

2.5. Histological examination of the brain

The brains were rapidly removed and immediately immersed into a 4% paraformaldehyde fixative for 24h. After this they were transferred into cryoprotectant for 24h (10% sucrose in potassium phosphate buffer (KPBS)). Coronal sections (30 μm) of the fixed brains were cut with microtome (Reichert, Austria). Seasonal changes of gonadotropin-releasing hormone I (GnRH I) and the aromatase (ARO) enzyme expression were determined by immunocytochemistry. The measured parameters are:

- integrated density of the GnRH-I-ir perikarya and fibers in the POA,
- size of the GnRH-I-ir perikarya in the POA,
- integrated density of the GnRH-I-ir fibers in the EM,
- number of ARO-ir cells in the POA/POM.

2.6. Behavioral recording and measurements

Song recordings were obtained with a portable recorder (Sony MZ-R900) and a microphone (AV-JEFE AVL 600). Sonographic analysis was done using the *AviSoft-SASLab* Light software. The average song bout length, which was defined as a period of at least 5 sec of song with pauses no longer than 1,5 sec in duration was quantified.

In addition, the wing-waving (the tail is tucked downward, the beak pointed upward, the throat feathers ruffled and the wings rotated) and wing-flicking (fast and repetitive move to sidelong) behavior were also recorded.

2.7. Statistical analysis

Data were analysed by one-way ANOVA of the STATISTICA 6.0 software package (Tulsa, OK, USA). Post hoc test was done by the Newman–Keuls methods. Pearson’s correlation was performed to determine the correlation between blood androgen levels (T, DHEA) and ARO immunoreactivity in the POA/POM and behavior parameters. In the case of the correlation analysis, only mean values were correlated and not the individual ones, because different animals were used in histological and behavioral tests. Data are expressed as means \pm S.E.M. and the level of significance was set at $p < 0.05$.

3. RESULTS

3. 1. The climatic factors that affect reproduction and annual cycle

When starlings arrive to Hungary in spring the daylength is about 11 hours, but during the courtship and nesting period reaches higher values (12-13 hours per day). At hatching of the nestlings the daylight continues to grow and exceeds 14 hours. In late summer (August) the daylength declines to values between 13-14 hours.

In Hungary during courtship and nesting period of starlings, the average annual temperature is 10 °C and the average rainfall is around 40 mm. At hatching of nestlings the two parameters increase only slightly. At photorefractory period (August II), however, the average temperature reaches 20 °C, and the average rainfall is approx 60 mm.

3. 2. The general condition of animals

The season had a significant effect on body weight in male starlings [$F(4, 26)=5.56, p<0.01$]. It increased significantly in the first half of April (April I) compared to March II (from 76 ± 2.3 g to 85 ± 2.3 g, $p<0.05$). These birds retained increased body weight through the incubation period (April II), and lost the “overweight” only after hatching period (May I, 74 ± 1.2 g, $p<0.05$). We found tendency to increase before autumnal migration (August II, 79 ± 0.8 g).

The season had a significant effect on the size of fat cells [$F(4, 12)=3.33, p<0.05$], but the post hoc analysis failed to reveal significant differences among the groups, probable due to quite large variance. Maximum values were observed in the first half of April (April I, 1018 ± 152 μm^2), while the minimum values were measured in May (May I, 191 ± 55 μm^2). In late summer (August II), presumably due to increased food intake the size of fat cells showed an increased tendency (299 ± 54 μm^2).

Despite the fact that the fat cell size and body weight showed a similar change during the annual cycle, the two did not correlated significantly ($r_p=0.32, P>0.05$).

3.3. Seasonal changes of GnRH-I-ir expression

The staining of GnRH-I-ir perikarya and axon fibers in the POA significantly differed between the five observed timepoints of the year [$F(4, 15)=5.13, p<0.05$]. There was only a tendency for increased integrated density of GnRH-I-ir perikarya and fibers from March to May. In August only few cells and weakly stained fibers were observed. Despite the lack of statistical significance in the integrated

density, the staining of fibers dramatically enhanced during the active reproductive phase (from March to May). Technical difficulties of the measurement could have mask the obvious differences.

There were significant differences in the size of the immunoreactive cell bodies during the examined period [$F(4, 15)=20.06$, $p<0.0001$]. The cell size varied among the four groups of photosensitive and photostimulated phase without significant difference. Birds caught in spring had larger cells than birds caught in August ($p<0.001$). It is of note that the labeling of perikarya from the August II group was less intensive than perikarya from photostimulated birds.

Immunohistochemistry revealed the presence of GnRH-I-ir fibers in the internal and particularly in the external layer of the ME. The integrated density of GnRH-I-ir fibers in the ME changed significantly during the annual cycle of starling [$F(4, 13)=18.7$, $p<0.001$]. In March we observed relatively few GnRH-I-ir fibers in the ME. There was a tendency for increased quantity of GnRH-I-ir fibers in the ME till the end of May. Males caught in spring had a significantly higher amount of GnRH-I-ir fibers in the ME compared to the photorefractory males in August ($p<0.001$).

3.4. Morphological and morphometric changes of the testes

With the progress of the breeding season, the testes became more and more clear (yellowish-white), and then with the onset of the photorefractory status changed to black. Within the unique the left testis tended to be bigger in the most cases.

3.4.1. Testes volume

The differences in the volume of the testes were statistically significant between the five groups [left testis: $F(4, 13)=23.4$, $p<0.001$; right testis: $F(4, 11)=13.26$, $p<0.001$]. Volumetrical analysis of the testes showed a typical seasonal pattern: in March the volume of testes were relatively small (left testis: 47.2 ± 14.4 mm³; right testis: 35.1 ± 3.0 mm³). In the first half of April – time of pair formation and nest hole occupation – birds undergone rapid sexual maturation and the testes size increased dramatically (left testis: 524.8 ± 40.8 mm³, $p<0.001$; right testis: 455.5 ± 56.2 mm³, $p<0.05$). In the second half of April the size of testes were almost the same as in the previous group. Birds of the May I group showed full gonadal development with maximally enlarged testes (left testis: 698.5 ± 122.7 mm³; right testis: 590.5 ± 164.6 mm³). In the nonbreeding month (August II) the volume of testes significantly decreased (left testis: 3.4 ± 1.3 mm³, $p<0.001$; right testis: 2.4 ± 0.7 mm³, $p<0.001$). This reduction in size is approximately two hundredths compared to May.

There was a significant positive correlation between the integrated density of GnRH-I-ir in the POA and the volume of the left testis ($r_p=0.54$, $p<0.05$), but only tendency for correlation with right testis ($r_p=0.50$, $p=0.09$).

3.4.2. The thickness of the tunica albuginea

The thickness of the tunica albuginea showed seasonal changes during the examined period [F (4, 18)=2.97, $p<0.05$]. During the breeding period the values remained stable (34 ± 1.58 to 40 ± 6.78 mm); there were no significant differences among them. At the end of August, because of the formation of the new tunica albuginea and the remaining "old" one, the thickness of the tunica significantly increased (66 ± 14 μm , $p<0.05$). The color of the old outer layer was different from the newly formed layer, and its structure was also less homogeneous.

3.4.3. The thickness of the interstitium

The thickness of the interstitium continuously increased over the examined period, reaching the highest values in August II [F (4, 20)=3.07, $p<0.05$]. In spring we failed to detect significant changes among the groups (5.94 ± 0.3 to 6.8 ± 0.3 μm , $p>0.05$). However, March II and April I differed significantly ($p<0.05$), while the April II ($p=0.08$) and May I ($p=0.06$) groups showed only approximate significance compared to the August group (8.3 ± 0.5 mm). In the latter, the interstitium was heavily pigmented; the pigment cells represented a large proportion of the area.

3.4.4. The area of the cross sections of the seminiferous tubules

The area of the cross sections of the seminiferous tubules changed significantly during the test [F (4, 19)=37.26, $p<0.001$]. The relatively low values in March (24195.8 ± 4117.9 μm^2), were followed by significant increase in the beginning of April (April I: 117301.2 ± 15014.7 μm^2 , $p<0.001$), and reached its maximum during the incubation period (April II: 169081.4 ± 13964.9 μm^2 , $p<0.05$). In early May, the area of the cross sections of the seminiferous tubules significantly decreased (128509.8 ± 12452.2 μm^2 , $p<0.05$). In the late summer (August II), the area of the cross sections decreased dramatically (4768.6 ± 689.6 μm^2 , $p<0.001$) and reached the minimum. The shape of the cross-sections changed during the cycle; rather elongated in spring, but being often circular in the late summer.

There was strong correlation between the area of the cross sections of the seminiferous and the integrated density of the GnRH-I-ir in the POA ($r_p=0.69$, $p<0.01$).

3.4.5. The thickness of the germinal epithelium

Due to the irregular shape of the cross sections of the seminiferous tubules, the thickness of the germinal epithelium varied within a given tubule. Therefore, four lengths per tubule were determined, and the same sampling scheme was applied on all analyzed tubules. The thickness of the germinal epithelium showed significant changes during the examined period [F (4, 19)=46.2, $p<0.001$]. In March we found relatively low values (45.1 ± 3.9), but significantly higher than in the August II group (21.4 ± 2.4 , $p<0.05$). Both April groups (113.1 ± 12.9 and 142.8 ± 6.3) showed significant increase compared to March ($p<0.001$). In the beginning of May (May I, 119.4 ± 5.5) the thickness of the germinal epithelium was significantly decreased to that to April II ($p<0.05$). The lowest value was reached in the late summer (August II, 21.4 ± 2.4 , $p<0.001$).

The thickness of the germinal epithelium positively correlated with the integrated density of GnRH-I-ir in the POA ($r_p=0.57$, $p<0.05$).

3.4.6. Identification and quantification of cell types

The testis of starlings showed not only quantitative but also qualitative changes in the studied periods. In March, a few cell layers were observed within the tubules; the primary spermatocyte was the most advanced spermatogen cell form. In the next three periods (April I, II, May I) each stage of spermatogenesis was found, however, in the beginning of May (May I) a major reduction in the total cell number was observed. In August, the cells in the lumen of the tubule showed a sharp decline, both in quantity and in quality. The advanced forms of cells have disappeared and only germ cells and spermatogonia were found, located in 1-2 cell layers.

3.5. The morphological and morphometric changes in the adrenal gland

As a result of HE staining, the red eosinophil interrenal (cortex) and the blue basophil adrenal (medulla) bundles have become easily distinguishable. The interrenal bundles of the outer zone showed mushroom-shaped or horseshoe-like tissue structure similar to the mammalian adrenal zona glomerulosa (ZG). Underneath most parts of the interrenal bundles had radial, elongated, columnar structures. This middle zone was reminiscent to the mammalian zona fasciculata (ZF). The inner interrenal tissue often form irregular, focal bands. We considered this part of the interrenal tissue - located mostly in the center of the gland along with the blood vessels - homologous to the mammalian zona reticularis (ZR).

3.5.1. Seasonal cycle of the interrenal/adrenal ratio

The effect of the season on the interrenal/adrenal ratio was not significant in the starlings [F (4, 16)=2.33, p=0.09]. Relatively high values were measured after the arrival in spring (March II, 2.83±0.10). Then the ratio decreased (April I, 1.93±0.16), which was followed by a sustained increasing tendency until the end of the nesting period (May II, 2.86±0.34). In the late summer period (August II, 2.26±0.48), the ratio dropped to around April I. In each slide, the area of the cortex was larger compared to the medulla.

3.5.2. The nucleus sizes in the three zones of the interrenal tissue

The nucleus size in the middle zone of the interrenal tissue changed significantly during the annual cycle [F (4, 15)=4.48, p<0.05], while the cell nuclei in the inner and outer zones only showed a tendency to significance [inner zone: F(4, 15)=2.44, p=0.09; outer zone: F(4, 15)=2.49, p=0.08]. Despite this, increasing tendency can be observed in the last two zones in the breeding season (mainly April I and II in April) and relatively low values in the non-reproductive August month (August II). The nucleus size in the middle zone significantly increased in the courtship period (April I, p<0.05) and May (p=0.05) but decreased in the end of August (p<0.05).

3.6. The seasonal changes of hormone levels

3.6.1. Seasonal changes in the plasma T levels

One-way ANOVA revealed significant differences in the concentrations of T in males from different months [F(4, 17)=3.09, p<0.05]. The concentration of T started to increase from late March (5.88 ng/ml) and reached its peak in the end of April (12.4 ng/ml) and then decreased to 1.87 ng/ml in late August. Newman–Keuls post hoc test revealed significant changes in late August compared to early and late April (p<0.05).

Plasma T levels positively correlated with the integrated density of POA GnRH-I-ir ($r_p=0.58$, p<0.05).

3.6.2. Seasonal changes in the plasma DHEA levels

There were significant differences in the mean concentrations of DHEA in male starlings during the observed period [F(4, 18)=4.24, p<0.05]. It started to increase from late March and reached peak of 0.80 ng/ml in early April and then declined to its lowest level and remained low between 0.42 and 0.56 ng/ml during the rest of the time. Newman–Keuls post hoc test revealed significant changes between early April and late April (p<0.05).

The seasonal changes in the concentration of DHEA and interrenal/adrenal ratio showed no correlation ($r_p=-0.50$, $p<0.09$). In contrast, DHEA positively correlated with the size of cell nuclei in the ZG ($r_p=0.65$, $p<0.05$), ZF ($r_p=0.64$, $p<0.05$), but especially with the ZR ($r_p=0.76$, $p<0.01$).

3.6.3. Seasonal changes in the plasma B levels

B concentrations did not change significantly during the annual cycle, but showed a decreasing tendency during the whole period [$F(4, 24)=2.33$, $p=0.08$]. Maximum values were measured at the end of March (1.3 ± 0.3 ng/ml), which was followed by a decreasing tendency during courtship and incubation period (April I, April II). In our study plasma B levels reached its minimum at the beginning of May (May I), and in the late summer period (August II) (0.34 ± 0.1 to 0.29 ± 0.2 ng/ml). Values in the latter two periods were approximately the same.

Plasma B concentrations did not correlate with either the interrenal /adrenal ratio ($r_p=0.28$, $p>0.05$) or with the size of cell nuclei in the ZG ($r_p=-0.02$, $p>0.05$), ZF ($r_p=0.09$, $p>0.05$) or ZR ($r_p=-0.03$, $p>0.05$).

3.7. Seasonal changes of ARO-ir expression

The ARO staining was present in the entire perikarya of the cells, including dendrites, which were sometimes quite distant from the perikarya. The cell nucleus was always clear from any immunoreactive material. The ARO immunoreactivity in the POA/POM showed significant changes by one-way ANOVA [$F(4, 15)=2.97$, $p=0.05$]. The POA/POM of males from spring contained more stained cells than POA/POM of sexually inactive birds (August II).

The ARO enzyme activity in the hypothalamic POA/POM area positively correlated with plasma T concentrations ($r_p=0.53$, $p=0.05$), compared with DHEA, however we found no such correlation ($r_p=0.08$, $p>0.05$).

3.8. Courtship behavior

3.8.1. Bird song

The average song bout length increased from late March and reached the peak during the courtship period (early April) and then tended to decrease till the time of nesting [$F(4, 32)=19.72$, $p<0.001$]. At the beginning of May, the males stopped their singing. 10% of birds was singing at that time. The vocalization of sexually inactive birds (late August) should be termed as calls because it was short and non-melodic and used only when the birds descend into evening reed-bed roosts. It was impossible to analyze these calls due to the fact that more thousands of birds clamored simultaneously.

There was a significant positive correlation between the ARO activity in the POA/POM and average song bout length [$r_p=+0.88$, $p<0.05$] and a tendency for positive correlation between plasma T levels and average song bout length [$r_p=+0.85$, $p=0.06$]. Plasma DHEA levels and average song bout length did not show any correlation [$r_p=+0.42$, $p>0.05$].

3.8.2. *Wing movements*

Wing-flicking and wing-waving were performed by males singing on their song post close to their nest hole. Wing-waving occurred almost exclusively after the appearance of a female. Although both behaviors were observed during most of the breeding period (from late March till the end of April), wing-waving tended to be present just before mating. We did not observe wing-waving or wing-flicking behaviors during non-breeding August month.

4. CONCLUSIONS and RECOMMENDATIONS

4.1. The role of climatic factors in the regulation of the reproductive cycle in starlings

We used the 3 climatic factors (daylength, temperature and rainfall) to analyze the biology of European starlings. In Hungary all these parameters showed Gaussian distribution with a peak during summer. Former publications demonstrated that 12-13h (first half of April in Hungary) exposure to daylight significantly stimulated gonadal maturation, but then temperature and rainfall (as ultimate factors) determined the precise timing of nesting in starlings (Dawson 2008).

4.2. The seasonal variation of general condition in starlings

The general condition of birds was defined by their weights and the size of abdominal fat cells. Both parameters showed seasonal fluctuations, however the change of body weight was more markedly pronounced. Furthermore, we did not find a significant correlation between the fat cell size and body weight. In birds, the amount of accumulated fat is usually determined by measuring the size of the subcutaneous fat using different methods (Kaiser 1993, Rogers 1991). However, intra-abdominal fat cells are metabolically more active, namely they are more sensitive to lipolytic effects. Thus, the weight loss is more affected in the abdominal than in the subcutaneous fat (Ibrahim 2010). According to the above, the kinetics of the fat accumulation is probably more pronounced in the abdominal fat tissue and therefore it is possible that this resulted in a higher standard deviation. Despite the fact that in late summer (August II), the starlings are in premigratory phase, we found no significant growth of fat cells in this period. Since migratory songbirds can upload the adipose depots very fast (up to 6-10 days), and therefore, it is conceivable that sampling a week latter could show significant differences.

Our results about decreasing body weight during the breeding season (April I - May I) is consistent with other published data on starlings (Kordonowy et al. 2010, Meijer et al. 1994). Kordonowy et al (2010) found that female starlings significantly reduced body weight after laying and reached the minimum values at hatching. According to Freed's hypothesis (1981), this weight loss is not a disadvantage, but an advantage for parents, as higher weights during the time of the feeding of young may make it more difficult to fly.

4.3. The seasonal changes in GnRH-I expression

In our study the integrated density of GnRH-I cell bodies and fibers (full density) in the POA and integrated density of the GnRH-I fibers in the EM showed an increasing tendency in the spring (March

II - May I) and the respective periods differed significantly from the photorefractory group. These results are consistent with previously published data on captive European starlings (Dawson, Goldsmith 1997), House sparrows (Hahn, 1995 Ball, Stevenson, MacDougall-Shackleton 2005) and House finches (Cho et al., 1998). In addition, the size of GnRH-I-ir cell bodies did not change significantly in the spring, however, it was greatly reduced in the end of August.

Although we did not find significant differences the spring period, in the end of March (March II, photosensitive phase) many cell bodies, but only few fibers were observed. This may indicate that in the photosensitive phase the GnRH-I peptide synthesis increases, but the axonal transport and release are likely to be moderate only. It thus appears that in the photosensitive birds decreased GnRH-I release, and not the inadequate production of the peptid causes low gonadal activity (Stevenson et al. 2009b).

4.4. The seasonal changes of testicular morphology and function in starlings

Although we did not found significant differences in the annual cycle, left testis tended to be larger than right testis particularly during the sexually active period (April I – May II). Our findings support Bullough (1942) observations on starlings, namely that in the majority of cases the left testis showed to be larger. However, he did not find significant differences between the two sides.

Similar to other wild birds from the temperate zone, in starlings the size of the testes significantly changes through the season. In our study, both testes increased steadily during the breeding period, and then drastically decreased in the late summer (August II). Between March II and April I a considerable increase was observed, which coincides with the birds intensive courtship and copulation period. Maximum size was measured at the beginning of May, although this group did not differ significantly from April I and II groups. These results are roughly the same as those measured on wild starlings from Belgium, England and the U.S., the only difference was that in previous studies testes length and weight were determined. However, in May decreasing values were reported in all three sites regardless of whether the birds were wild or captive under laboratory conditions or natural light (Dawson 1983, Dawson 2005b, Dawson, Goldsmith 1984, Riters et al., 2001, Temple 1974).

In our study the thickness of tunica albuginea did not change during the spring period, but increased significantly in the end of August. This phenomenon has also been described in several bird species (Artoni Baraldi, et al. In 2007, Bullough 1942, Crouch 1939). The increased values measured in the photorefractory period are due to the newly formed layer of tunica albuginea. The role of the new layer is to substitute the less functional old layer. The two layers observed in this period can help to

distinguish adult specimens from yearlings, namely in the latter only one layer is present (Aire 2006b, Péczely 1987).

The thickness of the interstitium increased steadily during the spring period and reached its peak during late summer (August II). In spring, low values were measured in other seasonally breeding bird species too. This phenomenon is due to that the hypertrophy of the interstitium is "suppressed" by the more intensively developing seminiferous tubules. Although in starlings, the thickness of the interstitium did not change significantly in the breeding season, the size of the Leydig cells reached its maximum during this period (Temple 1974).

The area of the cross sections of the seminiferous tubules increased significantly during the spring, and then decreased drastically in the photorefractory period. Although in the literature the generally used parameter is the diameter of the seminiferous tubules, we found that measuring the area of the cross sections is more practical. In sexually active birds the cross section of the seminiferous tubules is often irregular in shape. However, the area of the cross section is not affected by its shape, while measuring the diameter can become more difficult. To our opinion, determination of the entire area of the cross section is more reliable and more feasible than the diameter method.

The thickness of the germinal epithelium changed similarly as the area of cross sections. However, the qualitative changes showed less of such distribution. In the end of March, the primary spermatocytes were observed, which indicates that the testis is already active. During April I, II and May I periods sperms were observed in the lumen of the tubules, however the thickness of the germinal epithelium began to decline in May, suggesting that in the testes atretic processes had taken place. We found only spermatogonia and germ cells in the testes of the photorefractory birds. Cell types in the lumen of the seminiferous tubules showed a completely inactive reproductive state. Positive correlation was measured among the germinal epithelium, the number of meiotic cells and the diameter of the seminiferous tubules in different bird species (Baraldi-Artoni, et al. 2007, Tae et al., 2005).

In our study, plasma T concentrations increased steadily in the breeding season, peak values were measured in April month, when males showed typically high courtship activity. Then, the T levels decreased in late summer (August II) and reached its minimum. In the captive starlings the presence of nest holes and/or females significantly increased plasma T and LH levels, therefore these aspects must be considered in laboratory experiments (Ball, Wingfield, 1987, Gwinner et al. 2002, Pinxten et al., 2003).

These results suggest that it is not enough to determine the size of the testes, but also morphological/morphometric and hormonal measurements are desirable.

4.5. The seasonal changes of adrenal morphology and function in starlings

The relative proportion of the interrenal and the adrenal tissue and their seasonal variation was demonstrated by their ratio. The cortex was always bigger than the medulla throughout the examined period. The interrenal/adrenal ratio did not show significant changes in the studied periods. As the adrenal gland of starling is very small (1-1.5 mm long), after its cutting the sections may vary to some extent due to the different cutting angles. This may have resulted different interrenal/adrenal ratio in this study.

In contrast to the interrenal/adrenal ratio, the cell nuclei size in the three-zones showed maximum values in the first half of April (April I) and then declined steadily. Although seasonal changes were observed in all three zones, only the cell nuclei in the central zone (zona fasciculata) showed significant differences. In the reproductive period - using kariometric measurements - large nuclei have been described in the cortex of House sparrow (Moens, Coessens 1970) and Pied flycatcher (Silverin 1979), but then smallest values were measured in Ring-necked Parakeet (Kumar et al., 2008). During the spring the size of the nuclei in both zones started to grow. Nuclei from the inner zone exceeded those from periphery, despite the fact that outside of the breeding season the latter proved to be bigger (Moens, Coessens 1970).

We found that the plasma DHEA levels significantly changed during the annual season. It peaked in early April during courtship, but remained at a relatively low level during the rest of the examined period. Similar observations were made in Song sparrows (Newman et al. 2008). However, due to the relatively low amount of DHEA – compared to T levels – in starling, the probability that this molecule has a strong impact on courtship behavior is not yet proven.

Although B concentration did not show significant differences during the season, the same, decreasing tendency was observed in study on wild starlings in England (Dawson, Howe 1983). The probable reason could be that B shows not only annual, but a significant diurnal fluctuation too.

4.6. The seasonal changes in ARO expression

We showed here that the number of ARO-ir cells in the POA/POM changed seasonally. Namely it was the highest during the breeding and it was the lowest during the non-breeding season. In May, at hatching of the nestlings, male starlings completely ended the singing, despite the fact that the ARO activity (AA) was similar to March II, and plasma T concentration was relatively high. In May I the activity of 5 β -reductase enzyme (which forms inactive metabolites from T) showed maximum values, in contrast with AA (Riters et al., 2001). This latter or altered sensitivity of the brain (e.g.

downregulation of androgen receptor and/or estrogen α , β receptors) may have caused that the courting behavior ceased during this period (Gahr 2001).

4.7. The seasonal variation of courtship behavior in male starlings

Here we demonstrated that the average song bout length tended to increase during courtship period and, in most cases, song was accompanied with wing-waving/flicking. Although wing-flicking behavior was featured during almost the whole breeding season, wing-waving was more connected to female presence, especially during the time of copulation in April. At nesting and especially at hatching the song activity – together with wing-waving/flicking behavior – dropped dramatically. Eens and colleagues (1990) observed a similar pattern in captive starlings, namely that the wing-rotation and singing significantly decreased after the laying of last egg took place. The primary role of these behavior patterns (singing and wing movement) is to attract female's attention. In the non-breeding August month birds did not sing and the defined types of wing movement was absent. However, more thousands of starlings (mixed ages and genders) clamored as they descended into evening reed-bed roosts. Non-breeding context song in starlings is suggested to play a prominent role in flock cohesion and perhaps in the establishment and/or maintenance of dominance hierarchies within the flock (Hausberger et al. 1995, Wiley et al. 1993).

Summarizing our results we can conclude that the methods applied here are suitable for studying the reproduction of free-living birds. Taking into account the data available in the literature showing that in birds taken out of their natural environment, significant changes of their endocrine milieu is likely to occur, we suggest the study of biology on free-living animals prior to experiments in the laboratory.

5. NEW SCIENTIFIC RESULTS

In this thesis a comprehensive reproductive biology study has been done on free-living male starlings.

The new results in our work are the follows:

1. We confirmed the seasonal GnRH-I expression in the POA and in the EM of free-living male starlings by immunocytochemistry. Although in spring we did not find significant differences among the groups there was a remarkable change in the distribution of GnRH-I cell bodies and fibers.
2. In starlings, morphological and morphometric techniques demonstrated structural fragmentation of the interrenal tissue with demarcation of three-zone.
3. We demonstrated that the size of nuclei in all three zones of the cortex - especially in the middle zone (ZF) - varies seasonally in starlings. Furthermore, the maximum values were measured in the breeding season.
4. We confirmed that plasma concentrations of DHEA changes seasonally in male starlings, and demonstrated its relationship with the adrenal cortex. In particular, the size of cell nuclei in the inner zone (homologue with the mammalian zona reticularis (ZR)) showed a strong correlation with DHEA levels.
5. In late summer, minimal ARO immunoreactivity was measured in the POA. This proved that ARO activity in the POA is not responsible for the vocalisation in the August month.
6. We described in detail the singing and accompanying wing movements (wing-flash, wing-rotation), and their seasonal occurrence in starlings.

7. PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Scientific articles:

- **Pintér Ottó**, *Péczely Péter* (2010) Seasonal changes in hypothalamic gonadotropin-releasing hormone-I immunoreactivity in relation with testicular volume in adult male free-living European starlings (*Sturnus vulgaris*). *Acta Biologica Hungarica*, 61(3):237-49. (IF: 0,793)
- **Pintér O.**, *Péczely P.*, *Zsebők S.*, *Zelena D.* (2011) Seasonal changes in courtship behavior, plasma androgen levels and in hypothalamic aromatase immunoreactivity in male free-living European starlings (*Sturnus vulgaris*). *Gen. Comp. Endocrinol.*, 172(1):151-157 (IF: 3,108)

Reffered paper:

- **Pintér Ottó**, *Péczely Péter* (2003) A seregély (*Sturnus vulgaris*) mellékvese szövettani jellemzői a szaporodási, valamint az őszi reaktivációs időszakban. *Állattenyésztés és Takarmányozás*, (52) 2:195-200.

Proceedings:

- **Pintér Ottó**, *Péczely, Péter* (2002) Quantitative histological study of seasonal functional zonation of adrenocortex in starling. Proc. 23rd International Ornithological Congress, Beijing, China.
- **Pintér Ottó**, *Biczó András*, *Péczely Péter* (2004) Seasonal variation of two androgens, dehydroepiandrosterone and testosterone in male European starling (*Sturnus vulgaris*). Proc. 8th. Intern. Symp. Avian Endocr., Phoenix, USA. p. 95. http://lweb.la.asu.edu/isae/isae_program%20.html
- *Péczely Péter*, *Ladjánszky Veronika*, *Biczó András*, *Szőke Zsuzsanna*, **Pintér Ottó**, *Kelemen Katalin*, *Végi Barbara* (2004) Dehydroepiandrosterone (DHEA): it's possible role in the avian

annual cycles. Proc. 8th International Symposium on Avian Endocrinology, Phoenix, USA. p. 94.

http://lweb.la.asu.edu/isae/isae_program%20.html

- **Pintér Ottó, Péczely Péter** (2008) Seasonal variation in neural GnRH-I immunoreactivity in adult male free-living European starlings (*Sturnus vulgaris*). Proc. MicroCAD 2008 Nemzetközi Tudományos Konferencia, Miskolc, Hungary. pp. 51-56.
- **Pintér Ottó, Péczely Péter** (2002) A seregély (*Sturnus vulgaris*) mellékvese szövettani jellemzői a szaporodási, valamint az őszi reaktivációs időszakban. 9. Szaporodásbiológiai Találkozó, Balatonfüred.
<http://www.szapbiol.hu/rendez/archiv/09/09.html>
- **Pintér Ottó, Péczely Péter** (2004) A tőkés réce mellékvese szövettani jellemzői az éves ciklus folyamán. X. Ifjúsági Tudományos Fórum, Keszthely – CD kiadvány.
- **Pintér Ottó** (2006) Az XX és XY agyi sejtek különbözőek? Endokrin Centrumok továbbképzései: TRENDS 4.: Gonádműködés, gonáthatás, inkább férfiaknál, December 20., Budapest.
<http://www.endokrinologia.hu/kongresszusok.aspx?oldcongress=1&>