ENDOCRINE CONTROL OF TERRITORIAL AGGRESSION IN THE EUROPEAN STONECHAT *(SAXICOLA TORQUATA RUBICOLA)*

DISSERTATION

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1. INTRODUCTION

1.1. Territoriality

In many bird species males establish a territory to defend resources such as food or nesting sites and consequently to increase survival and /or reproductive success. Females are attracted by males owning a territory of high quality during the breeding season (Krebs & Davies, 1993) for instance, one that offers abundant food or suitable perches for foraging and has few predators. Because it is essential for a male to maintain and defend a good territory against conspecific intruders, territorial defence is usually linked with aggressive behaviour (Marra, 2000).

Territorial aggression in males is composed of several diverse behaviours such as singing, display of colourful patches or other secondary sexual characteristics, and particular threat postures (e.g., Fig. 1.1) and may end with physical attacks (Harding, 1983; Wingfield et al., 1990a). Escalations during male-male interactions are not uncommon and severe injuries may result from aggressive encounters. However, in the long term, high levels of aggressiveness are limited by costs. A strong territory owner is able to establish and maintain a large territory of high quality; on the other hand, the expenditure of time, energy and hormones (especially androgens) required for aggressive behaviour may reduce the male's fitness (Wingfield et al., 1990a; Dufty Jr., 1989; Marler & Moore, 1988a; Marler & Moore, 1989; Runfeldt & Wingfield, 1985). Typical costs of intense territorial behaviour are reduction in foraging rates and parental care (Silverin, 1980), as well as an increase in predation risk due to conspicuous behaviour (Marler & Moore, 1988b).

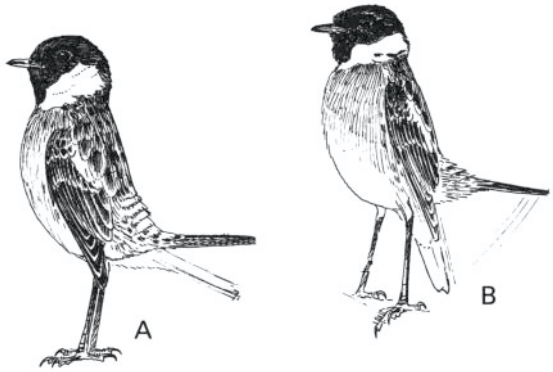

Fig. 1.1. Examples of threat postures of a male stonechat. ***A****. Presentation of white wing patches.* ***B****. Tail-flicking.*

1.2. The role of androgens

Many male-typical aggressive displays are androgen-dependent (Harding, 1983). The main active androgens are testosterone (T) and 5 α-dihydrotestosterone (DHT). Androstenedione (AE) is a biologically inactive androgen precursor of T. Androgens are produced in the gonads of both sexes. Males produce large amounts of androgens in the testes, whereas females secrete low concentrations from the ovaries. Moreover, small amounts of androgens are also produced by the adrenal gland (for example in humans see Table 1.1). The secretion of

Table 1.1. Relative contribution of the testes, adrenals and peripheral tissues to circulating levels of sex steroids in male humans (Braunstein, 1997).

	Testicular secretion %	Adrenal secretion %	Peripheral conversion of precursors %
testosterone	95	< 1	<5
dihydrotestosterone	20	< 1	80
estradiol	20	< 1	80
estrone	2	< 1	98
DHEA sulfate	< 10	90	...

androgens from the gonads is under the control of the hypothalamo-pituitary-gonadal (HPG) axis. The regulation of the feedback loop of the HPG axis is represented in Fig. 1.2. Briefly, the hypothalamus secretes Gonadotropin-Releasing Hormone (GnRH), which acts on the pituitary to induce the release of Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH). Increased levels of LH stimulate the secretion of oestrogens and progesterone from the ovaries (Fig. 1.2.a) and androgens from the male gonads (Fig. 1.2.b). Elevated plasma levels of sex steroids, in turn, have inhibitory effects on the hypothalamus and the pituitary (negative feedback loop).

In birds breeding in temperate zones, androgen levels undergo a seasonal cycle, which generally parallels the cycle of gonadal size: Androgen levels are high during the breeding season

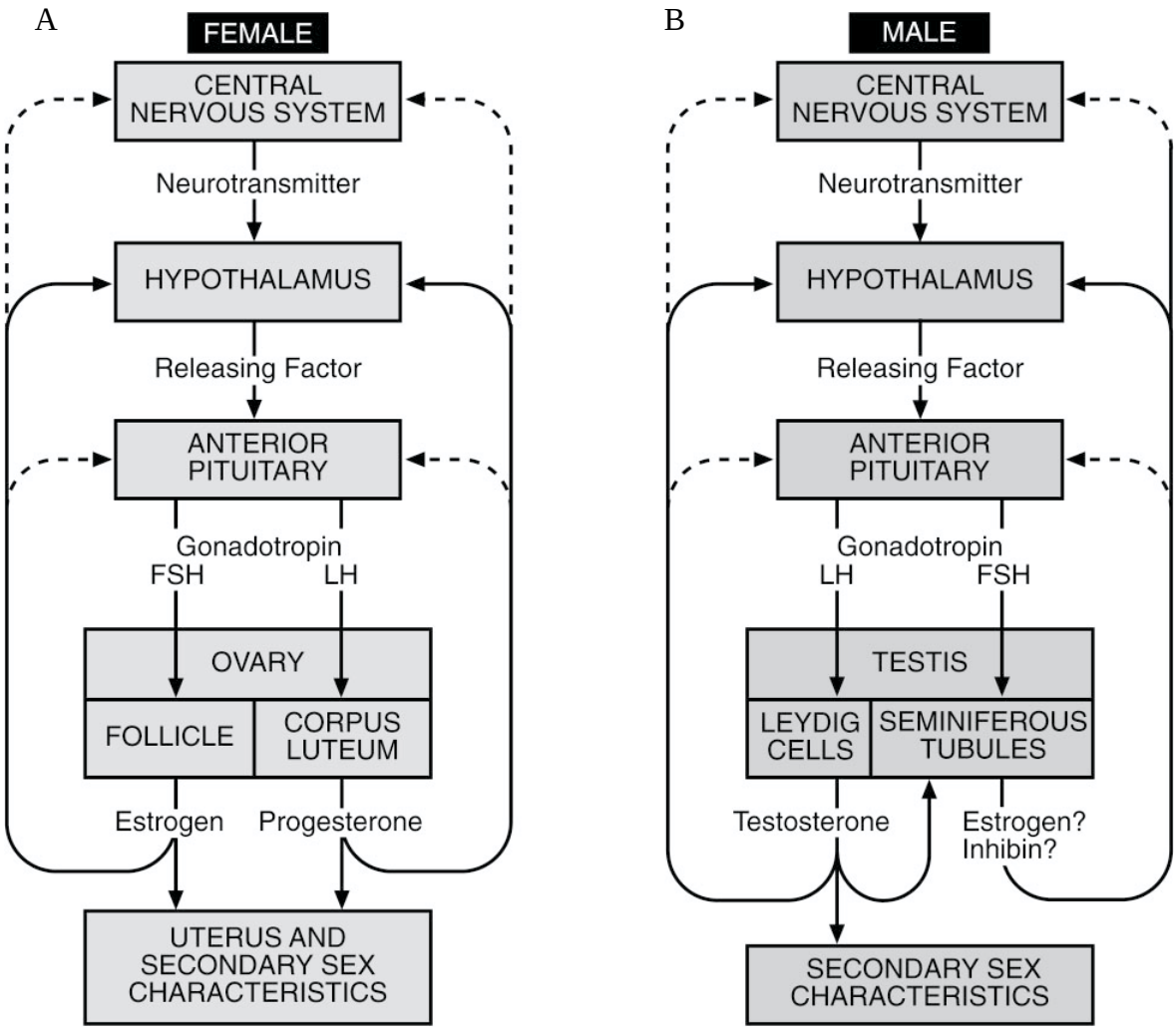

Fig. 1.2. Regulation of the hypothalamus-pituitary-gondal axis (HPG-axis) in females (a.) and males (b).

(particularly during the mate-guarding or egg-laying phase) and decline after breeding in midsummer. Seasonal fluctuations are controlled by an endogenous annual rhythm, which is synchronized by the annual photoperiodic cycle (Gwinner, 1986). The precise shape of the annual cycle is species-dependent and adjusted to the particular life history of a species (Gwinner, 1990).

Androgens have a variety of effects on reproduction, morphology, physiology and behaviour — for example, the development of secondary sexual traits (skin or feather coloration, ornaments) and the performance of song and courtship, all of which are essential for attracting a mate (Harding, 1983; Balthazart, 1983; Wingfield et al., 2000; Bentley, 1998; see Table

Table 1.2. Biological effects of testosterone (Modified from Wingfield et al., 2000).

Physiological effects	Morphological effects	Behavioural effects	Biological 'costs' of T
Negative feedback on gonadotropin secretion	Accessory organs	Sexual behaviour	Increased potential for predation
Miscellaneous secretions, e.g., in accessory organs, secretions of skin	Secondary sex characteristics	Aggressive behaviour in a reproductive context	Increased chance of injury
	Muscle hypertrophy		Energetic costs
	Spermatogenesis		Conflicts with pair formation and courtship
			Interference with parental care
			Suppression of the immune system
			Possible ontogenetic effects

1.2.). Many of the behaviours and morphological characteristics necessary for reproductive success during the breeding season are androgen-dependent (Eens et al., 2000; Harding, 1983). Androgens also regulate spermatogenesis (Bentley, 1998). In general the amount of circulating T is positively correlated with the intensity of the morphological or behavioural expression (Moore, 1984; Harding, 1983; Eens et al., 2000). Moreover, androgens appear to play a role in the control of aggressive behaviour, since castration reduces, and administration of exogenous androgens increases, aggressive forms of behaviour (Harding, 1983; Balthazart, 1983; see Chapter 5).

High levels of androgens for a long duration are thought to be 'costly'. Elevated T reduces reproductive success, since the rate of feeding the young is reduced (Wingfield, 1984a; Hegner & Wingfield, 1987a). T-implanted males remain longer at their breeding sites (Runfeldt & Wingfield, 1985) and/or experience a delayed moult (Schleussner et al., 1985). A few studies indicate that high levels of androgens have immuno-suppressive effects (Wedekind & Folstad, 1994; Folstad & Karter, 1992), but other studies do not support this hypothesis (Hasselquist et al., 1999; Ros et al., 1997). In addition, certain behavioural traits induced by high levels of androgens are necessary during certain phases of the breeding cycle (e.g. during the

establishment of the territory) but are inappropriate at other times (e.g. during parental care and moult). Therefore it is advantageous to have elevated androgen levels only when necessary.

1.3. The Challenge hypothesis

If high levels of androgens were costly, it would be beneficial to have increased androgen levels only when they are required, e.g. when an intrusion takes place. In fact, Wingfield and colleagues hypothesised that as soon as an intrusion occurs, androgen levels rise and facilitate aggressive behaviour (Wingfield et al., 1990b). In periods of high aggressiveness (unstable period; e. g. establishment of a territory) plasma levels of T remain elevated, whereas in periods of low aggressiveness (stable period; e. g. parental care period) T levels return to baseline (level *b*; Fig. 1.3.) and rise only when an interaction occurs. Accordingly, the seasonal pattern of plasma levels of androgens during the breeding season should depend on the mating system of the species. Species-dependent variations in the secretion pattern of T have been

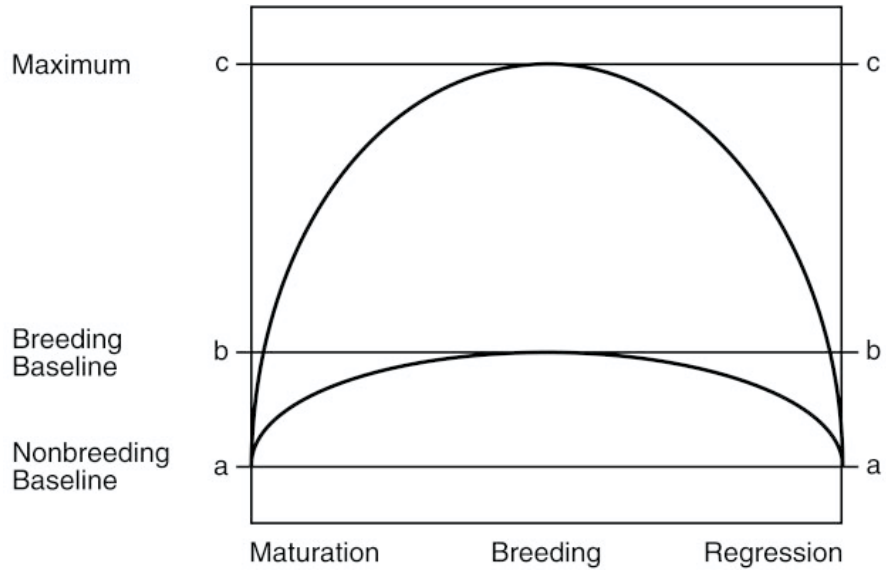

Fig. 1.3. General pattern of testosterone levels in male birds. During the nonbreeding season androgen levels are low or undetectable (level a). During gonadal maturation testosterone levels increase and reach the breeding baseline (level b), which is sufficient for reproduction. The physiological maximum (level c) can be reached, for instance, during a 'challenge'(Wingfield et al. 1990b).

explained by the 'challenge hypothesis' (Wingfield et al., 1990b), which states that at the beginning of the breeding season T levels rise from a nonbreeding level (*a*) to a breeding baseline (*b*) (Fig. 1.3.). This T baseline (*b*) is below the physiological maximum (*c*) but is sufficient for reproduction. T levels increase within a few minutes as soon as a male-male interaction occurs and, in turn, T increases the frequency and intensity of territorial aggression or mating behaviour. The increase of T has a physiological maximal level (level *c*). The consequence of this positive feedback loop is that in periods of social instability, e.g. during territory establishment or mating, when the levels of aggressiveness are highest, plasma levels of T remain high (level *c*). In socially stable periods during the breeding season, when the frequency and intensity of aggression are reduced, T levels decline to the breeding baseline (*level b)*. This decrease in the plasma levels of T is probably required to allow male parental care, because high plasma levels of T (*level c*) seem to be incompatible with parental care (Silverin, 1980). Species in which males provide no parental care will have high plasma levels of T (level *c*) throughout the breeding season, because they are more or less continuously engaged in interactions with other males trying to get access to more females.

Several studies have tested the 'challenge hypothesis', but almost all of them were conducted during the breeding season, when androgens also play an important role in control of reproductive physiology and behaviour. However, several bird species establish and aggressively defend a territory during the nonbreeding season, when androgen levels are expected to be low. Thus the question arises whether androgens facilitate aggressive behaviour even during the nonbreeding season.

1.4. Hormones other than androgens that might be involved in the control of aggression

During the nonbreeding season, androgen levels are low. Therefore it has been suggested that there may be seasonal differences in the control of aggressive behaviour (Schwabl & Kriner, 1991; Wingfield et al., 1990b), and other hormones such as oestrogens or glucocorticoids have been proposed to play a role in the endocrine control of aggressive behaviour.

1.4.1. Oestrogens

Oestradiol (E2) is the main oestrogen hormone produced in the ovaries of females and in the brain in males. The precursor of E2 is T and the enzyme responsible for the conversion of T into E2 is aromatase. Aromatase is present in high concentrations in the brain of all vertebrates (Callard et al., 1978). In the last 25 years it has been shown that in mammalian and avian species the action of T on male sexual behaviour depends partly on its conversion within the brain into E2 (Steimer & Hutchison, 1981; Balthazart et al., 1997; Lephart et al., 1996). Therefore it is possible that T-dependent behaviours, including territorial aggression, are in fact controlled by E2 produced in the brain from circulating T.

1.4.2. Glucocorticoids

Glucocorticoids are involved in many regulatory mechanisms listed in Fig. 1.4. and Table 1.3. A main function of glucocorticoids is the endocrine regulation of the stress-response. Adverse stimuli (stressors) activate the hypothalamo-pituitary-adrenal axis (HPA; see Fig. 1.4.) as follows. The hypothalamus secretes the Corticotrophin Releasing Factor (CRF), which acts on the pituitary to induce an immediate release of adrenocorticotrophic hormone (ACTH) into the bloodstream. Increased levels of ACTH result in an immediate enhancement of the secretion of glucocorticoids from the adrenal gland, which in turn exert inhibitory effects on the hypothalamus (negative feedback loop). In birds, the main biologically active glucocorticoid is corticosterone (CORT; Siegel, 1980; Harvey et al., 1984). The release of CORT is essential for an adequate physiological and behavioural response to acute unpredictable events. Increased concentrations of circulating CORT in response to stressors are thought to redirect physiology and behaviour away from ongoing activities such as reproduction towards immediate life-saving processes (Sapolsky et al., 2000; Wingfield & Ramenofsky, 1999). CORT mobilise energy (glucose), inhibit a variety of costly anabolic processes such as digestion, energy storage, growth or reproduction and are involved in the regulation of the immune response (Table 1.3.; Munck et al., 1984; Munck & Naray-Fejes-Toth, 1994; Wingfield et al., 2000). CORT also decrease the threshold of neuronal excitability,

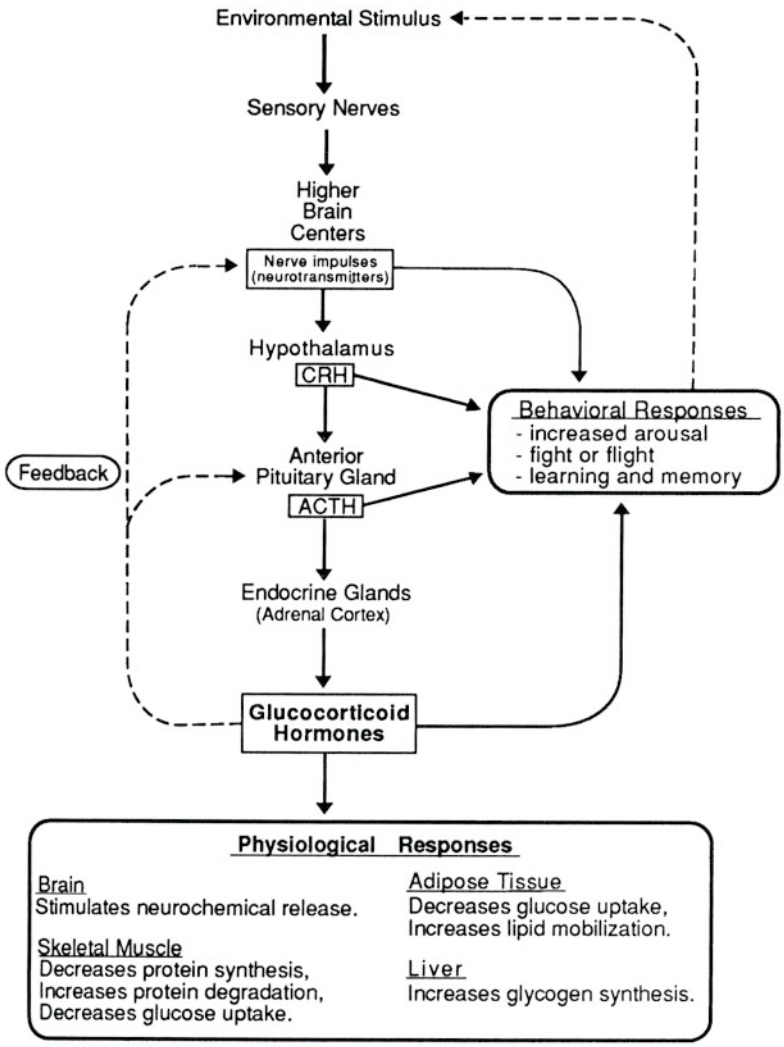

Fig. 1.4. Regulation of the hypothalamo-pituitary-adrenal axis (HPA-axis; from Brown, 1994).

to increase awareness and promote memory (Saldanha et al., 2000; McEwen & Sapolsky, 1995). However, chronic high levels of CORT are deleterious and can induce irreversible damages such as neuronal cell death (Sapolsky, 1987; Table 1.3.). Therefore, the short-term nature of the stress response is important: it lasts just long enough to induce behavioural or physiological reactions sufficient to prevent the stress from becoming chronic.

The physiological or behavioural outcome of an aggressive encounter depends on the experience and developmental history of an individual. For instance, the behaviour chosen during a male-male interaction may involve aggression or submission. Individuals may also adapt the sensitivity of their HPA axis to their life-history stage (Wingfield et al., 1995). Thus

Table 1.3. Effects of corticosterone (from Wingfield et al., 2000).

Short-term stress response	Chronic (long-term) stress response
Suppresses reproductive behaviour	Inhibits reproductive system
Regulates immune system	Suppresses immune system
Increases gluconeogenesis	Promotes severe protein loss
Increases foraging behaviour	Disrupts second-messenger systems
Promotes escape (irruptive) behaviour during day	Neuronal cell death
	Suppresses growth and metamorphosis
Promotes night restfulness by lowering standard metabolic rate	
Promotes recovery on return to normal life history stage	

baseline levels of CORT can vary within and between individuals during different life-history stages. This variability is also modulated by sex steroids. In rats, it has been shown that sex differences in the sensitivity and responsiveness of the HPA axis depend on circulating sex-steroids (Almeida et al., 1997; Handa et al., 1994b). Androgens have an inhibitory effect on the responsiveness of the HPA axis. Several studies have revealed sex differences in the stress response (Handa, 1994; Astheimer et al., 1994), which probably reflect adaptations to the different tasks fulfilled by females and males during the breeding season. For example, in the Arctic where reproduction is restricted to a narrow time window, females suppress their stress response, presumably to avoid the loss of a clutch (Wingfield et al., 1994).

1.5. The study species : The European stonechat

The species studied for my thesis is the European Stonechat (Passeriformes, Muscicapidae, Turdinae), a sexually dimorphic bird that weighs between 13 and 17 grams. It breeds in the southern Palaearctic region. The southern and western populations are mostly resident, while those of central and southeastern Europe migrate to their wintering sites in the Middle East and northeastern Africa.

The stonechat is one of the few species in which a pair holds and defends a territory not only during the breeding but also during the nonbreeding season. The wintering pairs are not necessarily breeding partners. Their territories vary in size from 0.5 to 1 ha and are usually found in bushy grassland.

During the breeding season male stonechats arrive first and immediately start to establish a territory. During this period male-male interactions and singing frequency are high. One or more days later females arrive and choose their mate. At that time males are conspicuously sitting on top of trees or bushes and singing. As soon as a breeding pair has formed and weather conditions are favourable, the female starts to build a nest and proceeds to lay ~5-6 eggs. The female alone incubates the eggs for 13 days. Both the male and the female feed their nestlings for approximately 15 days. After the young have fledged, the female can have one or two more broods, while the male takes care of the fledglings until they form flocks and float in the breeding area.

In late summer the breeding pair splits up and the pair partners migrate separately to the south. At the wintering sites males again establish a territory and form a pair with a new female.

A particularity of female stonechats is that they become alert and behave aggressively when a territory intruder is perceived, especially towards female intruders (Gwinner et al., 1994b). One function of winter pair formation may be to improve alertness against both intra- and interspecific intruders (Rödl, 1999b). The presence of female stonechats intensifies a male's aggressive territorial defence. Males that are paired with a female during the nonbreeding season are more aggressive towards a conspecific than are single males (Rödl, 1999b). As during this period stonechats are paired in a non-reproductive context, the increased aggressiveness in paired males is not related to reproductive interests.

2. AIM OF THE THESIS

The aim of my thesis was to investigate the role of steroid hormones in the control of territorial aggression in the European stonechat.

In the first study I tested the 'challenge hypothesis' on captive European stonechats during the breeding and nonbreeding seasons. According to this hypothesis, androgen levels should increase in response to a simulated territorial intrusion (STI) in both seasons, and should be low during the nonbreeding season. I compared the hormone levels before and after an STI and between the seasons. I analysed the three main androgens: androstenedione (AE), 5α–dihydrotestosterone (DHT), testosterone (T). Additionally I measured corticosterone (CORT), which could be involved in the control mechanisms of aggressive behaviour.

In the second study I tested experimentally whether T and/or its androgenic or oestrogenic metabolites are involved in the control of aggressive behaviour during the breeding and/or nonbreeding season in the European stonechat. In particular, I tested whether simultaneous pharmacological inhibition of androgen receptors and oestrogen production reduce aggressive behaviour during an STI and whether this differs seasonally.

This experiment was conducted with captive birds held in aviaries to optimise timing in blood sampling. However, in captivity many environmental cues that could influence the motivation of territorial aggression are lacking. Therefore, in the third study, I tested whether blocking androgen receptors and the conversion of T into E2 affect the aggressive behaviour of free-living male stonechats in response to an STI. I compared the behavioural findings for a stonechat population breeding in Hungary with those for a population wintering in Israel.

In stonechats not only males but also females aggressively defend the territory, although at a lower intensity than males. Territorial aggression and its control mechanisms in females have as yet been scarcely investigated. Therefore, in the fourth study I tested whether a male territorial intrusion induces a hormonal response in female stonechats. It is known that social interactions within a pair are important for hormonal and behavioural synchronisation of reproduction. As a consequence, a territorial intrusion might affect the hormonal response of

females either directly or indirectly via the behavioural response of the male. This issue was investigated in both seasons by measuring the hormonal response to a male decoy of captive female stonechats that were paired with pharmacologically castrated and control males.

3. GENERAL METHODS

3.1. Animal maintenance

3.1.1. Animals

The European stonechats originated from eastern Austria (48°13'N, 16°22'E). Birds were collected from a free-living population as nestlings and subsequently handraised in the laboratory (Gwinner et al., 1987). While still nestlings, they were divided into groups of 8 members (4 males and 4 females) that were not from the same nest. Aggressiveness within these groups was measured and the ranks of each member could be determined (Koenig et al. in prep.). Stonechats were put pairwise in individual aviaries. The male and the female of a pair usually had the same rank and were not relatives.

Experiments started 2-3 weeks after the pairs had been moved to aviaries when the birds had habituated to their new environment and partner. Each pair was observed daily to see, whether the partners accepted one another. When a pair combination did not fit, as indicated for instance by increased aggressive attacks towards the partner, I tried a new combination. Free-living stonechats migrate in the late summer and establish new pair bonds in autumn after arrival in their winter quarters. The following spring, therefore, I transferred the birds into another aviary, recombining them into new pairs according to the same criteria as above.

3.1.2. Aviary

Birds were kept in indoor aviaries under a photoperiod simulating that of 48°N, 11° 11'E. They were fed with a standard food mixture ad libitum (for composition see Gwinner et al. 1987) plus 10-15 mealworms per bird per day. Twice a week, the drinking water was enriched with vitamins (Vitin, Chevit GmbH).

The aviaries measured between about 2 x 1 x 2 m and 3 x 2 x 3 m (h/w/l m). Nine of these aviaries were in a common room. Birds were visually, but not acoustically separated from each other. Three aviaries were in isolated rooms, in which birds could hear neighbours at a

much lower intensity. A one-way mirror was installed in each aviary, so that tested birds could not see the observer. All aviaries were provided with bushes and branches in such a way that the birds could easily be observed. During the simulated territorial intrusion tests (STI) a stuffed male stonechat (decoy) was fixed on a pole in the centre of each aviary. To avoid breeding activity I did not provide any nesting materials. However, during the spring experiment some birds showed some nesting behaviour. In this case I immediately destroyed the 'nest', to keep the pre-breeding period.

3.1.3. Implants

Birds were implanted with silastic tubes (Dow Corning, USA, inner diameter 1.47 mm, outer diameter 1.96 mm), with an effective length of 8 mm. They were filled either with an androgen receptor blocker Flutamide (F; Ratiopharm GmbH & CO., Germany) or with an aromatase inhibitor 1-4-6 androstatrien-3,17 dione (ATD; Steraloids, USA). Control birds were implanted with empty silastic tubes. The end of the tubes were sealed with an adhesive glue (Dow Corning). Twelve hours before implantation the tubes were soaked in a 50% ethanol solution to accelerate the secretion of the drug.

3.1.4. Implantation

A small incision was made in the skin of the back between the wings. A cavity under the subcutis was made with a probe in order to facilitate the insertion of the implant. Following implantation the skin was closed with a tissue glue (Histoacryl,Braun surgical Gmbh,Germany).

3.2. Measurement of plasma levels of steroids

The AE, DHT, T, oestradiol (E2) and corticosterone (CORT) were measured by radioimmunoassay (RIA) after extraction and partial purification on diatomaceous earth (celite) micro-columns using a modification of the methods described by Wingfield and Farner (1975).

3.2.1. Reagents

Antisera were obtained from Endocrine Sciences (Tarzana, USA): AN6-22 (AE), DT3-351 (DHT), T3-125 (T), E17-94 (E2) and B3-163 (CORT). The cross-reactivity of the antisera with other steroids is shown in Table 3.1. Standard steroids were purchased from Sigma (USA), and tritiated steroids from New England Nuclear-Dupont (USA). All the solvents used are of analytical grade. The assay buffer for androgens and oestradiol is a 1.0 M phosphate-buffered saline with 1% gelatine and 1% sodium azide (PBSG), pH 7.0. The assay buffer for corticosterone is a 0.05 M borate buffer.

Table 3.1. Cross-reaction (%) of antibodies used for radioimmuno-assays with other steroids. Steroids with a cross-reaction above 5% are listed.

	Assay:	AE	DHT	T	E2	CORT
Steroid	Antibody:	AN6-22	DT3-351	T3-125	E17-94	B3-163
Androstenedione (AE)		100	0.2	2	<0.1	-
5α-Dihydrotestosterone (DHT)		0.5	100	44	0.2	-
Testosterone (T)		2	47	100	<0.1	-
Oestrone		-	<0.1	<0.2	130	-
Oestradiol (E2)		-	<0.1	0.5	100	-
1,4-androstadiene-3,17-dione		40	0.2	-	-	-
5α-androstan-3,17-dione		35	0.7	-	-	-
5ß-androstan-3,17-dione		35	0.1	-	-	-
Delta-1-testosterone		-	14.7	41	-	-
Delta-1-dihydrotestosterone		-	-	18	-	-

3.2.2. Extraction of steroids from plasma

Plasma contains a large amount of lipophilic compounds, which might interfere with the sensitivity of the assay. Therefore steroid extraction is essential.

Plasma samples (±50 µl) were transferred to glass extraction tubes. To determine extraction efficiency (recovery) 1500 dpm each of tritiated AE, DHT, T, E2 and 3000 dpm of tritiated CORT in 25 µl PBSG were added; the samples were incubated over night at 4°C and then extracted twice with re-distilled dichloromethane for 12 h at 4°C. The organic phase was separated from the aqueous phase by plunging the extraction tube into an ethanol - dry ice bath; the aqueous phase freezes within a few sec, after which the organic phase can be decanted into a clean glass tube. The organic phase was then dried under a nitrogen stream in a 40°C water bath prior chromatography. The dried extracts were re-dissolved in 0.5 ml of 2% ethyl acetate (EA) in isooctane.

3.2.3. Chromatography on celite micro-columns

With this step several steroids in a sample can be separated on the basis of their polarity.

Before preparation of the micro-columns, celite has to be heated up to 500°C for several hours to eliminate any organic impurity and then cooled down.

The columns were prepared by packing 5 ml serological pipettes with 0.5 ml of a celite:water mixture (2:1, w:v) and 1.5 ml of a celite:propandiol:ethylenglycol mixture (4:1:1, w:v:v). The columns were then first packed with the celite:water mixture (‘water trap’) and then with the celite:glycols mixture by means of a glass rod. The water trap prevented the exit of the glycols from the columns when high concentrations of polar solvent are used. A glass pearl was inserted at the bottom of the pipette to prevent leaking of the celite from the tip of the columns.

Columns were mounted on a holder and exposed to a nitrogen stream with a constant pressure, which washed out the solvents. After the columns had been washed twice with 4 ml isooctane, re-suspended extracts (samples) were loaded on the celite columns. The columns were washed again with 4 ml isooctane. Then steroid hormones were separated on the basis of their polarity by eluting the columns with increasing concentrations of EA in isooctane. In the first fraction AE was eluted with 2% EA, in the second DHT with 10% EA, in the third T with 20% EA, E2

in the forth with 40% EA and CORT in the fifth with 50% EA. Each fraction containing an individual steroid was collected in an extraction tube, which was fixed under the columns. The fractions were dried under nitrogen in a water bath (40°C) and then re-dissolved in 300 µl PBSG.

The CORT fraction, which was eluted with more than 40% EA, occasionally contains glycols. Therefore this fraction was further processed to remove these glycols. After the CORT fraction had been dried under nitrogen, 0.5 ml ddH2O and 2 ml dichlormethane were added; the combination was vortexed for 30 min and stored at 4°C over night. Then samples were centrifuged (200 g, 2 min, 4°C) and the organic phase was freeze-decanted into a clean tube. This procedure was repeated twice and the final organic phase was dried under a stream of nitrogen. Then samples were resuspended in 300 µl borate buffer.

All samples were kept at 4°C overnight for equilibration. Aliquots (90 µl) of each fraction were transferred to scintillation tubes, mixed with scintillation liquid (Ready Safe, Beckman, USA) and counted to an accuracy of 2-3 % to estimate the recoveries. The residuals were stored at –40°C until radioimmunoassays were conducted.

3.2.4. Radioimmunoassay

Androgens and oestradiol. With this technique, an unknown amount of plasma steroids compete with a known amount of tritiated steroids for the binding of a known amount of antibody. Concentrations of steroids in plasma samples can be calculated by comparison with a standard curve.

A standard curve was set up by serial dilution of a stock standard solution. Aliquots of the corresponding fractions were transferred in duplicate (2x100 µl) to glass assay tubes. The antiserum was added to the assay tubes, followed after 30 min by 5000 dpm of the labelled hormone (8000 dpm for T). Samples were then incubated for 20 h at 4°C (25°C for DHT).

Free steroids were separated from the bound fraction by addition of dextran-coated charcoal and centrifugation. The aqueous phase was decanted in scintillation vials, mixed with scintillation liquid and counted to an accuracy of 2%.

Corticosterone. The extracted fraction was dried under a N_2 stream and re-dissolved in 300 µl borate buffer. Single aliquots (90 µl) were transferred in scintillation vials, mixed with 4 ml of scintillation fluid (Ready Safe, Beckmann, USA) and counted to an accuracy of 2% to determine recoveries. Duplicate aliquots (100 µl) were transferred in assay tubes and incubated for 30 min with CORT antibody (final dilution 1:80; 12.000 dpm) at 37°C before adding the tritiated CORT. After 20 h incubation at 4°C, free steroids were separated from the bound fraction by adsorption on 0.5 ml dextran-coated charcoal in borate buffer and centrifugation. The decanted fraction was mixed with 4 ml of scintillation fluid in scintillation vials and counted to an accuracy of 2%. The detection limits for the assay, intra-assay variation and inter-assay variation are given in the respective chapters.

3.2.5. Data calculation and quality controls

Standard curves were determined by 4-parameter logistic interpolation. The lower detection limit of the standard curves was determined by the first point outside the 95% confidence intervals for the zero-standard. Water blanks were always below the lower detection limit. The average recoveries were between 64 and 94%.

4. HORMONAL RESPONSE TO AN INTRUSION IN CAPTIVE MALE STONECHATS

4.1. Introduction

In the last decade the 'challenge hypothesis' has been tested not only in over 20 bird species (Wingfield et al., 1990b; Beletsky et al., 1992), but also in mammals (Goymann, 2000; Creel et al., 1993; Cavigelli & Pereira, 2000), fishes (Francis et al., 1992; Oliveira et al., 2001) and reptiles (Klukowski & Nelson, 1998; Smith & John-Alder, 1999). One of the predictions is that a 'challenge', e.g. by an STI, induces an elevation in circulating T concentration (see Section 1.3.; Wingfield & Wada, 1989a; Wingfield et al., 1990b). However, this has not been confirmed in all cases (Sorenson et al., 1997; Klukowski & Nelson, 1998; Wingfield & Lewis, 1993; Thompson & Moore, 1992).

Most studies have tested the 'challenge hypothesis' during the breeding season. In fact this hypothesis applies only in a reproductive context, since during the nonbreeding season the gonads are regressed and plasma levels of T are usually low.

However several bird species aggressively defend a territory even during the nonbreeding season, despite low plasma T levels (Logan & Wingfield, 1990; Gwinner et al., 1994b; Schwabl, 1992). In some species dominance formation and aggressiveness are positively correlated with plasma levels of androgens in autumn, but not in late winter (Schlinger, 1987; Schwabl et al., 1988). Nonbreeding levels (*level a*) of T (which are mostly in an undetectable range) might be sufficient for the expression of territorial aggression during this period but it is also possible that, if the social system becomes unstable or if an individual is challenged by a conspecific, T-levels increase even during the nonbreeding season. Wingfield and Hahn (1994) tested whether an STI increases plasma levels of T during both the breeding and nonbreeding season in the resident song sparrow *(Melospiza melodia morphna*) and the migratory white-crowned sparrow (*Zonotrichia leucophrys pugetensis*) (Wingfield & Hahn, 1994). White-crowned sparrows had significantly elevated T levels following STI, whereas in the song sparrow this increase was not significant. However, neither species responded with elevated

T levels after an STI during the nonbreeding season (Wingfield & Hahn, 1994). White-crowned sparrows winter in flocks and song sparrows form ‘alliances’, with several males and/or females sharing a winter territory (Hegner & Wingfield, 1987b; Wingfield, 1994a). In view of this difference in social structure between seasons, it is difficult to compare the ‘challenge hypothesis’ directly between the breeding season and the nonbreeding season.

In contrast to the song-sparrow and the white-crowned sparrow, the European stonechat, a migratory passerine, establishes territories both on the breeding grounds in spring and in its wintering quarters in autumn and winter. It also forms heterosexual pairs not only in spring but also in autumn. Therefore, in this species, the relationship between aggressive behaviour and androgens can be tested in a reproductive and in a non-reproductive context. It is possible that the control mechanisms of winter territoriality vary among species depending on their wintering strategy. As stonechats defend their territory pairwise during both seasons, it is possible that plasma levels of T are elevated in response to an STI both in spring and in winter. T might also act in its metabolic form, as it can be reduced to DHT, which has a much stronger affinity to the androgen receptors than T (Balthazart, 1983). On the other hand T is involved in other functions, such as reproduction, which are not activated during the nonbreeding season. Therefore it is possible that during the nonbreeding season the inactive androgen AE occurs at higher concentrations in order to be quickly converted when T is needed. Alternatively, other mechanisms might regulate aggressive behaviour during the nonbreeding season. In several studies on other species T did not increase after STI (Wingfield & Lewis, 1993; Thompson & Moore, 1992), but CORT did (Knapp & Moore, 1995; Greenberg et al., 1984). Increased plasma levels of CORT are usually associated with stress (Wingfield & Ramenofsky, 1999; Siegel, 1980). However, a positive relationship between aggressive behaviour and CORT has been shown in recent studies. In male northern fence lizards (*Sceloporus undulatus hyacinthinus*) CORT levels were elevated after a ‘challenge’ only during the postbreeding season (Klukowski & Nelson, 1998). An increase in CORT levels after a short-term interaction might be beneficial in that it facilitates energetic demands via increased gluconeogenesis (Knapp & Moore, 1995). Also, in pintails (*Anas acuta*) increased male-male interactions are accompanied by increased CORT levels but not by elevated T levels (Sorenson et al., 1997).

In this study I tested endocrinological predictions of the 'challenge hypothesis' during the breeding and nonbreeding seasons in the European stonechat. Specifically I asked whether an STI causes an increase in the plasma levels of T. Moreover, I analysed two other androgens, AE and DHT, that might also be involved in controlling aggressive behaviour. Additionally I measured CORT levels, since some studies suggested that CORT might play an important role in the control of aggressive behaviour.

4.2. Methods

4.2.1. Experimental animals

For a detailed description of the aviaries and animal maintenance see Chapter 3. The experiment was conducted with 12 male and 12 female yearling stonechats.

4.2.2. Experimental design

First, a plasma sample was taken from all male stonechats. Approximately 4 days later, an STI was carried out by fixing a stuffed male stonechat (decoy) on a pole in the centre of the aviary. Behavioural responses directed towards the decoy were recorded for 20 min. Immediately following the STI a second blood sample was taken (see also Fig. 4.1.).

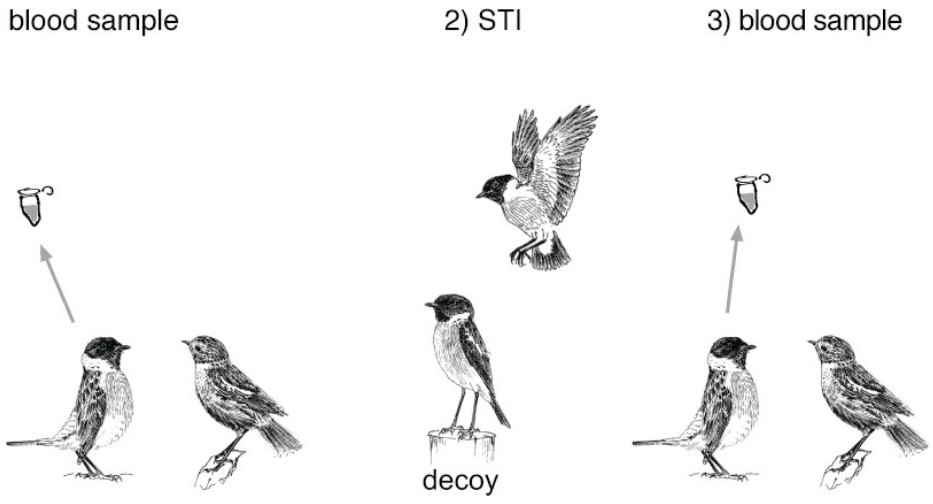

Fig. 4.1. Design of the challenge experiment. See text for details.

Aggressive behaviour in captive stonechats is less pronounced than in nature (personal observation). Free-living stonechats usually attack the decoy physically. Captive male stonechats, in contrast, approach the decoy without physical contact; the stonechat flies towards the decoy but does not touch it. These approaches are part of the threat display (Gwinner et al., 1994a; Rödl, 1999b). Several aggressive displays can also be seen in this context such as wing- and tail-flicking or presentation of white wing patches (see, e.g., Fig. 1.1. in Chapter 1). It seems likely that these approaches are a reduced form of aggressive behaviour.

During the STI the following behavioural parameters were recorded for each successive one-minute interval: *Latency of the first approach*: time interval between the beginning of the test and the first approach towards the decoy. Males that did not approach the decoy were assigned a latency of 20 min, i.e. the duration of the test. *Number of approaches* towards the decoy. *Number of songs:* I recorded how often a male sang during the test. Experiments were conducted between 9.00 and 12.00 AM in order to reduce the effects of possible circadian variations in territorial behaviour and hormones.

4.2.3. Hormonal analyses

Blood samples were taken from the wing vein within ±3.2 min (in detail see Fig. 4.2.) from the time I entered the aviary. Blood was collected in heparinized capillaries (Bayer diagnostics, Germany) and immediately centrifuged with a mini-centrifuge (Bayer diagnostics) at 11500 rpm for 8 min. Plasma samples were stored at -80°C until analysed. The androgens AE, DHT and T, as well as the stress hormone CORT, were measured by RIA after extraction. Detailed descriptions of extraction, chromatography and RIA methods are found in Section 3.2. All samples were analysed in duplicate and were run in two assays for each hormone. Intra- and inter-assay variations are presented in Table 4.1.

Table 4.1. Detection limits, intra-assay variations (%) and the inter-assay variation (%) of the two assays for each hormone assayed.

Steroids		detection limit (ng/ml)	intra-assay variation (%) First assay	intra-assay variation (%) Second assay	inter-assay variation (%)
Androstenedione	AE	0.25	12.2	20.9	8.4
Dihydrotestosterone	DHT	0.09	5.0	5.4	3.8
Testosterone	T	0.078	14.7	24.53	10.4
Estradiol	E2	0.04	3.0	4.9	10.46
Corticosterone	CORT	1.1	5.5	11.3	23.2

4.2.4. Statistics

Seasonal differences in the number of approaches and in the latency of approach to a decoy during an STI were analysed with a t-test.

To test for changes in hormone levels following an STI and between seasons I used a repeated measures ANOVA, with the factors season (breeding and nonbreeding season) and STI (before and after STI). One missing point during the breeding season was interpolated (SPSS). Correlations between hormones and behaviour were analysed with a parametric Pearson correlation for both seasons. During the nonbreeding season, plasma levels of androgens were undetectable. Therefore, no correlation between androgen levels and behaviour was calculated for this season. Singing activity was not normally distributed and a non-parametric Spearman correlation was therefore used in this case. The significance level was set at $\alpha = 0.05$. Statistical analyses were performed with SPSS for Windows, SPSS Inc.

4.3. Results

4.3.1. Hormones

In both seasons the androgens T and DHT did not increase after an STI (see Table 4.2. and Fig. 4.1.). Plasma levels of AE were undetectable in both seasons. However, it should be noted that the detection limit of AE was relatively high (<250 pg/ml). In both seasons plasma levels of CORT were increased after the STI test (see Table 4.2. and Fig. 4.2.). This increase in CORT was not an artefact of bleeding time, since the time spent for catching and bleeding the birds was similar in all bleeding procedures (see Fig. 4.3.).

Table 4.2. Changes in plasma levels of steroids between seasons and after the STI test.

	Season		*STI*		*Interaction*	
	F	*p*	*F*	*p*	*F*	*p*
Testosterone	14.3	0.004	0.13	0.73	0.03	0.87
DHT	3.2	0.1	0.08	0.8	0.4	0.5
AE undetectable		---		---		---
CORT	0.33	0.58	6.33	0.03	0.02	0.9

Plasma levels of T were higher during the breeding season than during the nonbreeding season (Table 4.2. and Fig. 4.2.). There were no seasonal differences in the plasma levels of DHT and CORT (Table 4.2. and Fig. 4.2.).

4.3.2. Behaviour

On average male stonechats approached the decoy more often during the breeding season than during the nonbreeding season, but the difference was not significant (*t-test*; p=0.062; see Fig. 4.4.). Similarly the latency of the approach to a decoy did not differ between seasons (*t-test*; p=0.3; see Fig. 4.4.). In both seasons the approach latency was not correlated with the plasma levels of T, DHT or CORT (for statistics see Table 4.3. and Fig. 4.5.) nor was the

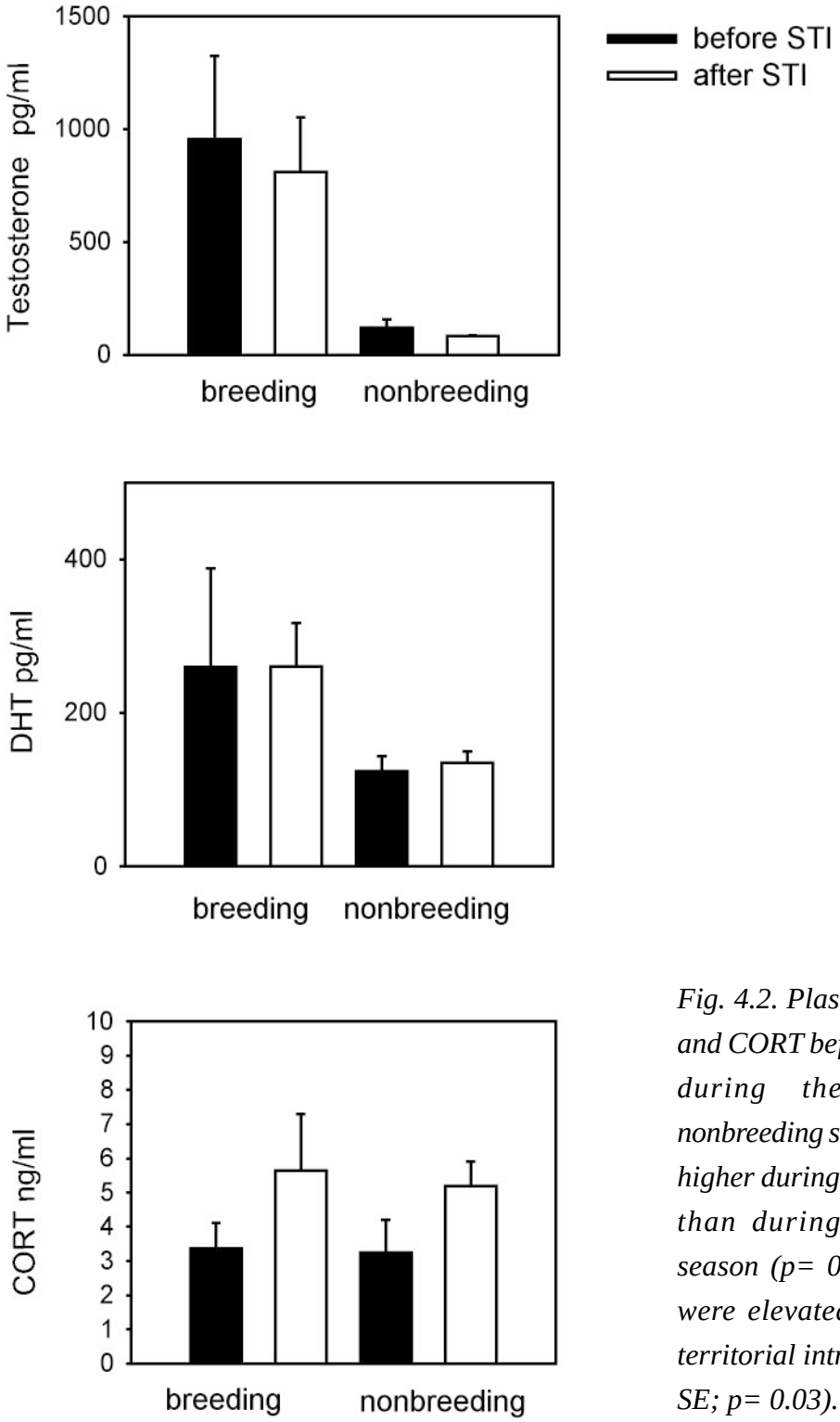

Fig. 4.2. Plasma levels of T, DHT and CORT before and after an STI during the breeding and nonbreeding seasons. T levels were higher during the breeding season than during the nonbreeding season (p= 0.004). CORT levels were elevated after a simulated territorial intrusion (STI; mean, ± SE; p= 0.03).

number of approaches to a decoy correlated with T, DHT or CORT in either season (for statistics see Table 4.3.). However, in the breeding season the number of songs during an STI was positively correlated with plasma levels of T after the STI (r_s=0.688; p=0.013) although the statistical significance of this correlation is lost if the outlying datapoint at song activity

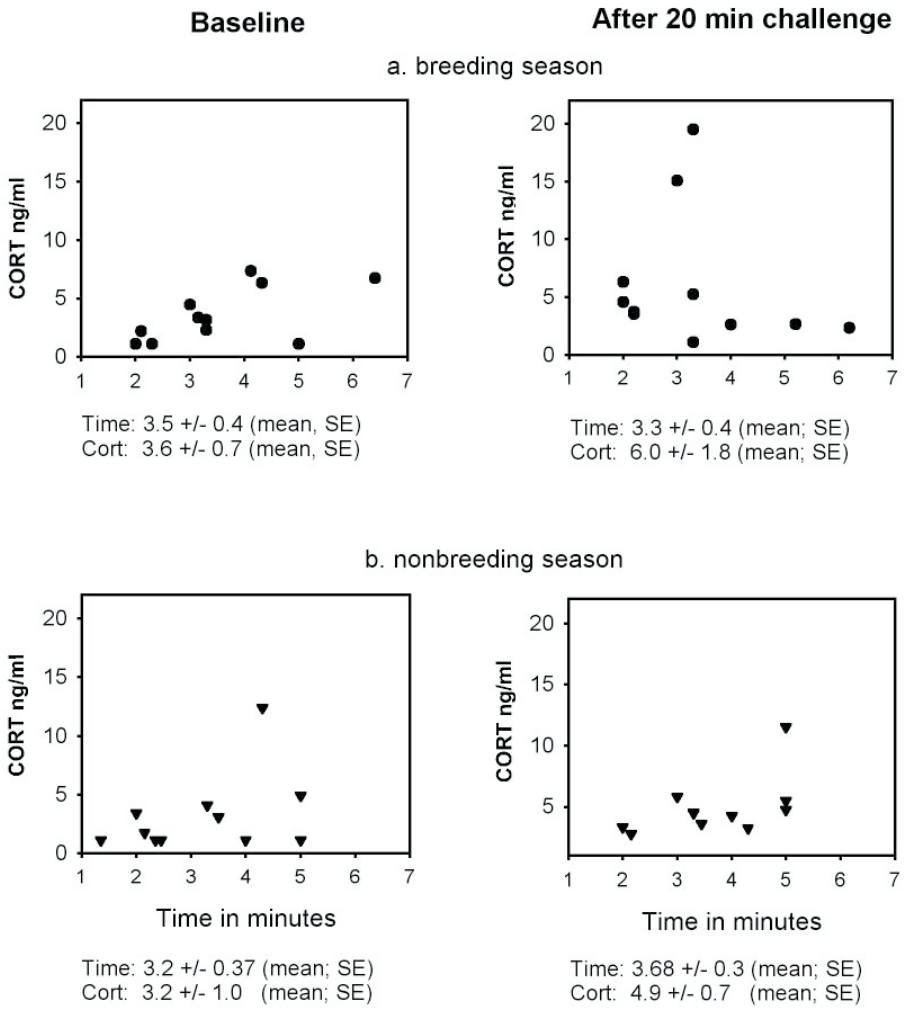

Fig. 4.3. Plasma levels of CORT plotted against the duration of blood sampling. Comparison between baseline levels (left) and after an STI of 20 min (right) during the (a.) breeding season and (b) nonbreeding season. The increase of CORT levels after an STI is not an artefact of blood sampling.

Table 4.3. Correlations between aggressive behaviour and plasma levels of T, DHT and CORT after an STI for each season separately.

	Breeding season			Nonbreeding season		
	T	DHT	CORT	T	DHT	CORT
Latency to approach	r_P= -0.213	r_P= -0.047	r_P= -0.21	r_P = 0.31	r_P = -0.12	r_P = -0.4
	p=0.5	p=0.89	p= 0.52	p= 0.354	p= 0.73	p= 0.22
Number of approaches	r_P= 0.45	r_P= 0.033	r_P= 0.1	r_P = -0.17	r_P = 0.062	r_P = -0.282
	p= 0.14	p= 0.92	p=0.74	p= 0.6	p= 0.85	p= 0.37

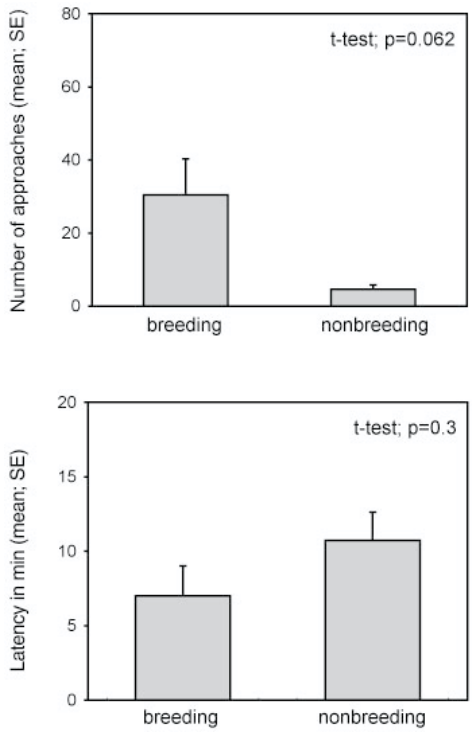

Fig. 4.4. Seasonal comparison in the aggressive behaviour of a male during an STI. Captive male stonechats tend to approach a decoy more often during the breeding season than during the nonbreeding season (p=0.062). There is no seasonal difference in the latency of the approach to a decoy.

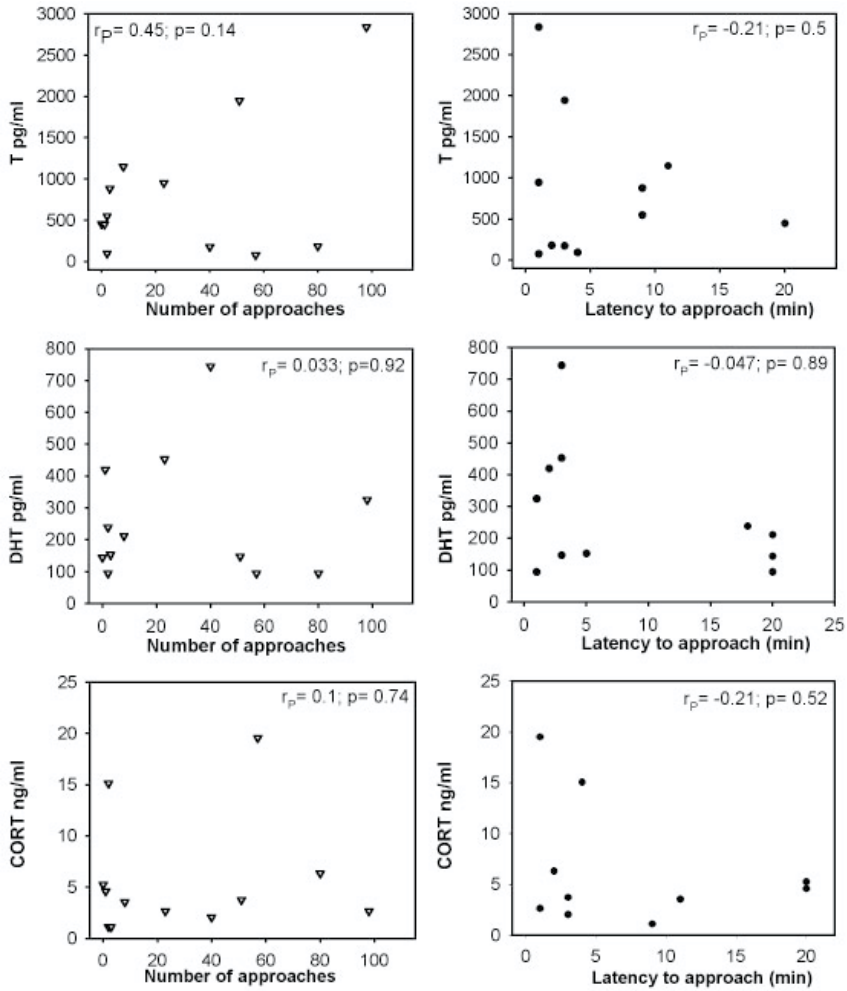

Fig. 4.5. Correlation between plasma levels of T/ DHT/ CORT and (a) number of 'approaches' and (b) the approach latency during an STI in the breeding season.

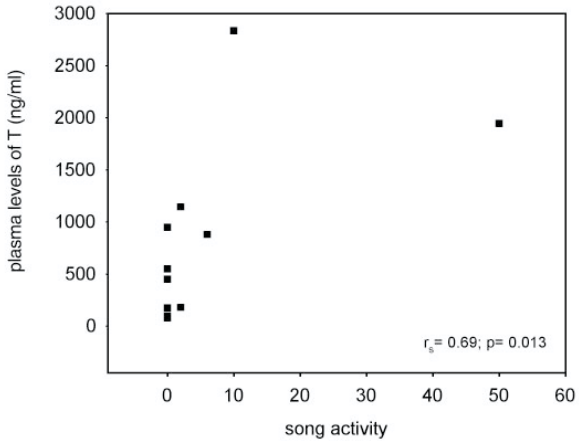

Fig. 4.6. Correlation between plasma levels of T and song activity (only during the breeding season).

50/ plasma levels of T 2100 pg/ml is omitted (r_s=0.59; p=0.055; see Fig. 4.6.). Birds did not sing during the nonbreeding season.

4.4. Discussion

Contrary to my expectations, plasma levels of T and DHT did not increase in male stonechats after an STI during spring and winter. Thus in captive stonechats androgens seem to be unaffected by a short-term male-male interaction. In contrast, CORT levels were increased after an STI in both seasons, suggesting that CORT is involved in the physiological response to a territorial intrusion.

4.4.1. Challenge hypothesis

The 'challenge hypothesis' would have predicted an increase in T levels after the STI at least during the breeding season; therefore this study does not support the 'challenge hypothesis' (Wingfield et al., 1990b). However, it might be that the reason for the lack of an elevation in plasma T levels was that captive male stonechats expressed a reduced form of aggressive behaviour. The cause of this reduced aggressiveness in captive male stonechats is unknown. It is possible that space restriction or unlimited food availability reduced the motivation to defend a territory (discussed in detail in Chapter 5).

The ‘challenge hypothesis’ was derived from an experiment in which male song sparrows *(Melospiza melodia*) were removed from their territory during the breeding season (Wingfield, 1985). Within 2 days, new males took over the territories. These intruder males had higher T levels than control males (in an undisturbed area). In addition, neighbouring males had higher T levels than the new intruders. Thus it was hypothesised that the sudden destabilisation of the social system and the resulting increase in competition for territories caused a rise in T levels. Further studies confirmed that plasma levels of T increase following a male-male interaction during the breeding season (Wingfield & Wada, 1989a; Wikelski et al., 1999; Wingfield & Wada, 1989b; Wingfield & Hahn, 1994), as they do in lizards (Smith & John-Alder, 1999).

So far, only few studies have tested the ‘challenge hypothesis’ during the nonbreeding season, although several bird species remain territorial during this period. In most species T levels are low during the nonbreeding season; thus it has been speculated that T might not be affected by a sudden unstable situation at that time. Seasonal differences in the control of territorial aggression have been proposed by several authors (Schwabl & Kriner, 1991; Soma et al., 1999b). In lizards (*Sceloporus undulatus*) male-male interactions induce elevated T levels only during the breeding season and not during the postbreeding season (Smith & John-Alder, 1999). In song sparrows and white-crowned sparrows T levels did not increase in response to an STI in autumn (Wingfield & Hahn, 1994). As these two species form alliances or flocks during the nonbreeding season, the control of territorial aggression might vary depending on the social context in winter. In species that form social groups during the nonbreeding season aggressive behaviour is directed towards a new intruder, but not towards members of the group. In species in which males defend a territory alone or in pairs, aggressive behaviour will be expressed towards any other conspecific male, as in the breeding season. Therefore an increase of T levels following an STI might have been expected in winter. Because plasma T levels did not increase after an STI in wintering captive stonechats, it implies that the lack of elevated T levels following a challenge in winter does not depend on the social context in winter.

4.4.2. Are other androgens involved during an STI?

It is unlikely that androgens other than T play a role during a 'challenge'. High circulating levels of T during the nonbreeding season might be costly, because T activates additional systems including reproduction, which would be inappropriate for the season. It was initially conceivable that AE, an inactive androgens, is produced in higher quantity during the nonbreeding season and is quickly converted into T when the situation becomes unstable. However in both seasons AE levels were in a non-detectable range. Seasonal differences in AE levels have been reported in canaries (*serinus canaria*) (Fusani et al., 2000). In the latter study, the highest AE levels were around 250 pg/ml. This was also the detection limit in the present study, so it is possible that a rise in plasma levels of AE was not detectable. In addition, DHT was not affected by a 'challenge' and also did not correlate with any of the behavioural parameters measured during the STI (Fig. 4.2.; Fig. 4.5.).

4.4.3. Seasonal relationship between androgens and aggression/behaviour

Although in the present study T levels were not affected by a 'challenge', I found a seasonal relationship between plasma levels of T and aggressive behaviour. There was also a tendency towards seasonal differences in the number of approaches to a decoy. During the breeding season, when T levels were elevated, male stonechats approached a decoy more often than during the nonbreeding season, when plasma levels of T were undetectable. Similar seasonal differences in the intensity of aggression have been reported in a number of other studies (Schwabl, 1992; Logan & Wingfield, 1990). These findings are consistent with the hypothesis that T increases the likelihood of aggressive behaviour, namely to *facilitate* aggressive behaviour during the reproductive period, especially during 'unstable' periods (Andrew, 1975; Wingfield et al., 1990a).

If T facilitates aggressive behaviour, why don't stonechats approach faster during the breeding season than during the nonbreeding season? Possibly the motivation to defend a territory is similar in both seasons, but aggressive behaviour as such is more intense during the breeding

season. However, in contrast to the present study, other investigations did find seasonal changes in the latency to attack (approach) a conspecific decoy. During the breeding season male European robins (*Erithacus rubecula*) respond more quickly to an 'intruder' than during the nonbreeding season (Schwabl, 1992). Most studies that tested aggressive behaviour with an STI used a song playback in addition to a decoy. Song plays an important role in aggressive interactions and gives additional information to the territory owner. Thus the use of song playback might induce a quicker aggressive response to an STI. Since most bird species sing only during the breeding season, this might be one of the reasons for seasonal differences in the latency to attack a decoy in other studies. In the present study I did not use song playback, because stonechats sing only during the breeding season and I wanted to have a comparable STI test in both seasons.

4.4.4. Methodology

Some of the discrepancies between the studies reported above and my own investigation might be due to substantial differences in methodology, e.g. timing of the experiment, duration, or type of 'challenge' (e.g. Sachser & Prove, 1984; Greenberg & Crews, 1990). Further, in some studies, increased T levels were associated with rank order rather than aggression (Eberhart et al., 1980; Greenberg & Crews, 1990; Gwinner & Gwinner, 1994; Smith & John-Alder, 1999; Ramenofsky, 1984; Creel et al., 1997). Furthermore, in contrast to the present study most of the other investigations have been conducted in the field. Defending a territory in an aviary and in nature differs in several aspects, such as the size and attractiveness of a territory (which can differ between seasons), the context and environmental cues. However, the most likely explanation for the contrasting results obtained in different species are the differences in life history strategies between species.

4.4.5. Species differences

Although T is thought to be essential for aggressive behaviour, its role in the control of aggression is not fully understood (for details see Chapter 5.). Male-male aggression is not in

all species accompanied by increased T levels (in lizards: Klukowski & Nelson, 1998; Thompson & Moore, 1992; Knapp & Moore, 1995, in birds: Wingfield & Lewis, 1993; Wingfield & Hahn, 1994; Sorenson et al., 1997). Male pintails that have been selected by a female for mating subsequently have more aggressive interactions, which, however, are not accompanied by increased T levels (Sorenson et al., 1997). In the tropical white-browed sparrow weaver (*Plocepasser mahali*) aggressive attacks during an STI are not followed by increased T levels (Wingfield & Lewis, 1993). It has been proposed that in tropical birds, which are territorial throughout the year, hormones might be less important for aggression than in temperate-zone species that are territorial for only part of a year or migrate away from their breeding grounds (e.g. stonechats) (Wingfield et al., 1997). However in other tropical species such as the spotted antbird (*Hylophylax n. naevioides*), aggressive behaviour does seem to depend on the presence of T (Hau et al., 2000; Wikelski et al., 1999). The results of these studies, therefore, suggest that there are species-specific differences in the hormonal control of aggression. This hypothesis had already been proposed to explain the lack of a correlation between T and territorial aggression in some studies discussed in detail in Chapter 5. In some species aggressive behaviour seems to be dissociated from T (Eberhart et al., 1980; Logan & Wingfield, 1990; Hunt et al., 1995; Hunt et al., 1997; Creel et al., 1993). In tropical colonial weavers, secondary male helpers of a breeding pair have undetectable T levels, but are just as aggressive as the breeding males with high T levels (Wingfield & Lewis, 1993).

4.4.6. Is corticosterone involved?

In both seasons plasma levels of CORT were increased after an STI. CORT mediates survival reactions to life-threatening situations (e.g. ‘fight or flight’ reactions). CORT levels increase within 3 min after a stressful situation. Thus, it could be argued that the change in CORT levels is an artefact of handling the birds or of the experimental setup. Figure 4.3. demonstrates, however, that the time needed to catch and bleed was similar in all cases. Moreover, increased CORT levels after the STI could have been affected by the observer entering the aviary to fix

the decoy on the perch at the beginning of the STI test. This possibility is unlikely, however, because females did not show such a CORT response (see Chapter 7). Generally, increases in CORT levels after competition are associated with the winner-loser effect: in most species subordinates (loser) have higher CORT levels than dominants (winner) after a male-male interaction (Greenberg et al., 1984; Knapp & Moore, 1996). However in lizards (*Anolis carolinensis*), such an increase in CORT levels was not found in the subordinate if two castrated males were paired in a cage, although they both expressed aggressive behaviour (Greenberg et al., 1984). This indicates an interaction between the HPG and HPA axes.

Other studies found a positive relationship between aggressive behaviour and increased CORT levels, suggesting that CORT might play a role in the control of territorial aggression. Interestingly, in male pintails aggressive behaviour is positively correlated with plasma levels of CORT and not with plasma levels of T (Sorenson et al., 1997). In my experiment CORT did not correlate with the number of approaches to a decoy or the latency of the approach (Fig. 4.5.). Nevertheless in both seasons a challenge caused an increase in plasma levels of CORT (Fig. 4.2.).

In summary, this study on captive male stonechats does not confirm the 'challenge hypothesis'. In both seasons an STI induced an elevation in plasma levels of CORT, but not of androgens. Furthermore, in captive European stonechats plasma levels of androgens are not positively correlated with aggressive behaviour, although aggressive behaviour parallels seasonal changes in plasma levels of T. Additionally, androgen levels were not affected in response to an STI, so it is possible that in this bird species androgens are not involved in the control mechanism of territorial aggression. Therefore in the following Chapter I used pharmacological methods to test more directly whether androgens play a role in the control of aggressive behaviour in both seasons in captive European stonechats.

5. DO ANDROGENS CONTROL AGGRESSIVE BEHAVIOUR IN CAPTIVE MALE STONECHATS

5.1. Introduction

Several morphological and behavioural features of males such as plumage coloration, singing, courtship and territorial behaviour are androgen-dependent (Harding, 1981; Balthazart, 1983). Previous studies have shown that territorial aggression is modulated by T (Harding, 1981; Balthazart, 1983; Wingfield et al., 1987; Wingfield et al., 1990b). Administration of T during the breeding season changed the socio-sexual behaviour: Males not only had a longer period of singing activity and a prolonged courtship period and sometimes attracted a second female, but also were more aggressive and defended larger territories (Moss et al., 1994; Wingfield, 1984c; Wingfield, 1984a; Beletsky et al., 1989; Raouf et al., 1997; Silverin, 1980; Wingfield et al., 1987; Harding, 1981; Ketterson & Nolan, 1992; Ketterson & Nolan, 1999). However, other studies have indicated that the relationship between aggression and T may be more complex. For instance, in castrated male Japanese quail (*Coturnix c. japonica*) aggression was not correlated with different doses of exogenous T (Tsutsui & Ishii, 1981). In the same species, treatment with an androgen receptor (AR) antagonist did not reduce aggressive behaviour (Schlinger & Callard, 1989a). Also, aggressive behaviour persisted in male song sparrows after castration (Wingfield, 1994b). Taken together, these studies suggest that factors other than T may modulate the action of T on aggression. Some of these factors could be season-dependent.

The relationship between androgens and territorial aggression has been studied mainly during the breeding season, when androgen levels are high. However, several bird species also establish and defend a territory during the nonbreeding season, when plasma levels of T are low (Schwabl & Kriner, 1991; Schwabl, 1992; Gwinner et al., 1994b; Levin & Wingfield, 1992; Logan & Wingfield, 1990; Hau et al., 2000; Wikelski et al., 1999). The intensity of aggression in these species can reach similar levels in both seasons despite large differences in androgen levels. How can territorial aggression be expressed during the nonbreeding season, when circulating levels of T are low? It has been hypothesised that low levels of T are sufficient

to induce aggression if the brain sensitivity to T is increased, e.g. by increasing AR density (Schwabl & Kriner, 1991; Soma et al., 1999a; Wingfield & Hahn, 1994). However, in canaries AR expression in the telencephalon did not differ between late autumn and spring (Fusani et al., 2000), and in the white-crowned sparrow AR immunoreactivity is even reduced in autumn compared with spring (Soma et al., 1999a). In European robins pharmacological AR blockage reduced aggressive behaviour during the breeding season but not during the nonbreeding season (Schwabl & Kriner, 1991). Thus territorial behaviour, which appears to be androgen-dependent during the breeding season, might be androgen-independent at other times of the year. Moreover, it has been suggested that oestrogenic metabolites of T might control territorial aggression (Beletsky et al., 1990; Schlinger & Callard, 1990), since T can be converted into E2 within the brain (Schlinger et al., 1992; Steimer & Hutchison, 1981; Schlinger & Arnold, 1995). Recent results support this hypothesis. In the song sparrow territorial aggression during the nonbreeding season is reduced by inhibiting the conversion of T into E2 and these effects are reversed by administration of exogenous E2 (Soma et al., 2000b; Soma et al., 2000a).

In summary, it is still unclear whether territorial aggression outside the breeding season is androgen-dependent and whether the hormonal control of aggression changes seasonally. In particular, few studies have investigated in the same species the relationships between territorial aggression and T over different seasons (Schwabl & Kriner, 1991; Wingfield & Hahn, 1994; Soma et al., 2000a).

European stonechats establish territories and form pairs on both their breeding and wintering sites. Thus, in this species territoriality and pair formation occur both in a reproductive and in a non-reproductive context (Gwinner et al., 1994b; Rödl, 1995). In the present study, I tested whether androgens are involved in the control of territorial aggression in the European stonechat and whether the control mechanisms of this behaviour change seasonally.

I studied the aggressive response of male captive stonechats to an STI before and after blocking the action of androgens and oestrogen in both the breeding and the nonbreeding season.

5.2. Material and Methods

5.2.1. Animals

For this experiment I used the same 12 paired stonechats as in the experiment described in Chapter 4. It was conducted in November 1997 and in March 1998 one week after experiment 1 was terminated (see Chapter 4). For a detailed description of pair formation and holding conditions see Chapter 3.

5.2.2. Experimental design

I compared the response to an STI between 6 males treated simultaneously with an AR blocker and an aromatase inhibitor and 6 control males. Five days following the implantation I took an initial blood sample from the wing vein within 3 min after entering the aviary. Two days later I performed an STI test by placing a stuffed male stonechat (decoy) in the middle of the aviary. 1.4 to 6.4 min following the end of the STI test a second blood sample was taken (see Fig. 5.1.). Experiments were restricted to the morning hours between 9.00 and 12.00 AM to reduce the possible effects of variations in aggressive behaviour over the course of the day.

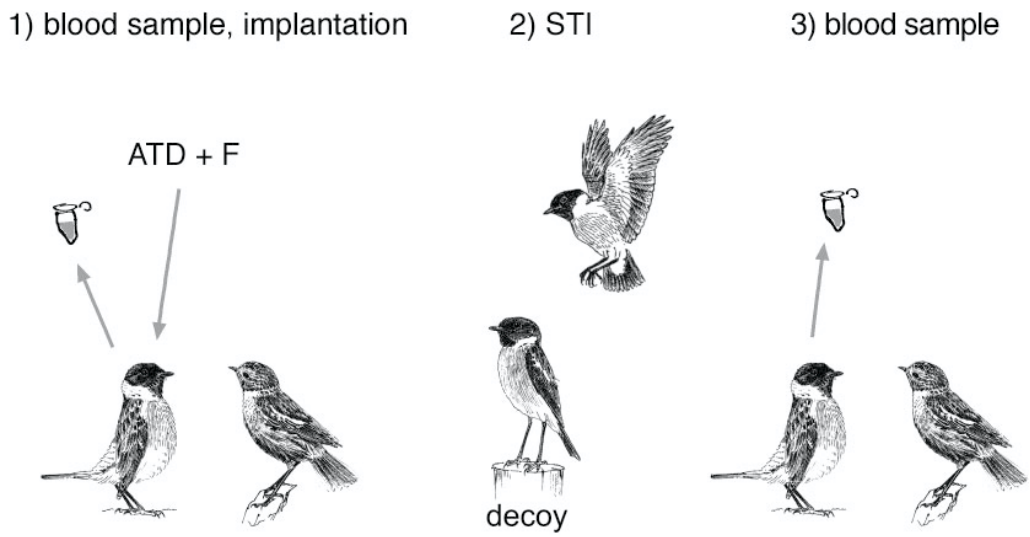

Fig. 5.1. Design of the ATD+F experiment. See text for details.

5.2.3. Simulated territorial intrusion test

To test for territorial aggressive behaviour I placed a decoy on top of the pole. During the subsequent 20 min I observed the behavioural reaction of the owner at one-minute intervals. The following parameters were quantified: *a) Latency until the first approach towards the decoy*; *b) Number of approaches towards the decoy* and c) *Number of songs* (only during the breeding season).

5.2.4. Implantation

Six males received simultaneously one implant filled with the AR-blocker Flutamide (F) and another one filled with the aromatase inhibitor 1-4-6 androstatrien-3,17 dione (ATD) (for details see Chapter 3). Males of the control group received 2 empty implants of the same size. One week after implantation I checked the implants. One bird lost the implant during the first week. It was re-implanted and the bird was tested one week later than the other birds.

5.2.5. Hormonal analyses

Blood sampling was carried out as described in Chapter 3. The following steroids were measured by RIA: AE, DHT, T, E2 and CORT. The RIA methods are described in Chapter 3. All samples were analysed in duplicate and run in two assays. The parameters of the assays of each hormone are summarized in Table 5.1.

Table 5. 1. Detection limit, the intra-assay, and the inter-assay variation of the RIAs.

	detection limit (ng/ml)	Intra-assay variation (%) First assay	Intra-assay variation (%) Second assay	Inter-assay variation (%)
Androstenedione	0.259	12.2	20.9	8.4
Dihydrotestosterone	0.095	5	5.4	3.8
Testosterone	0.078l	14.7	24.5	10.4
Oestradiol	0.040	3	4.9	10.5
Corticosterone	1.1	5.5	11.3	23.2

5.2.6. Statistical analysis

Hormonal differences between ATD+F-treated and control birds before and after the STI, and between seasons, were analysed with a repeated-measures ANOVA. Behavioural differences between ATD+F and control groups and between seasons were analysed with a repeated-measures ANOVA. Because the numbers of songs during the breeding season were not normally distributed I compared groups with a non-parametric Mann-Whitney U test. Correlations between CORT and behaviour were analysed for each season separately with a parametric Pearson correlation. Statistical significance was set at $\alpha = 0.05$. Statistical analyses were performed with SPSS for Windows NT 4.0. When not specified, values reported are means ± SE.

5.3. Results

5.3.1. Behaviour

As described in Chapter 4, captive stonechats never physically attack the decoy; their maximal response is a close approach to the decoy, accompanied by threat postures.

Number of approaches. During both seasons there was no significant difference in the number of approaches between ATD+F and control males, although there was a tendency for the former to approach more frequently (Fig. 5.2.a; Table 5.2.). During the breeding season ATD+F treated males tended to approach the decoy more often than controls (Fig. 5.2.a, Table 5. 2).

Latency to approach. ATD+F-treated males responded more quickly to the STI than control males. There was a tendency towards an interaction between season and treatment (p=0.056; Fig. 5.2.a, Table 5.2.).

5.3.2. Song activity

Singing activity during the breeding season did not differ between the two groups (ATD+F: 9.3 ±5.7; control: 1.8 ±1.6; U=13.0; Z=-0.89; p=0.37).

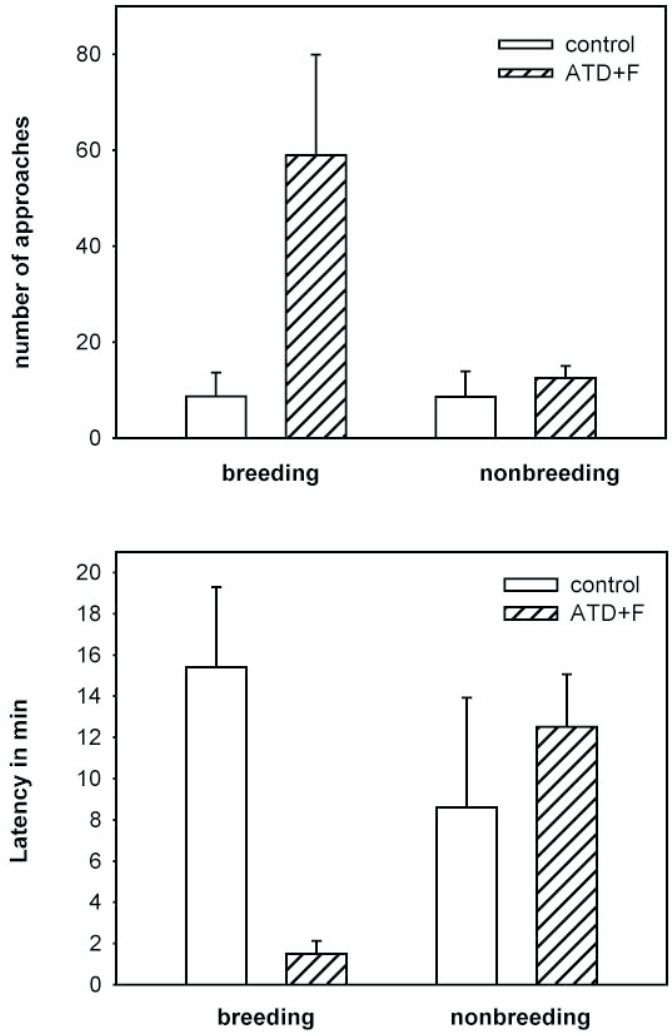

Fig. 5.2. Behavioural response of control and ATD+F-treated males to a decoy. During the breeding season the ATD+F males tended to approach more often than control males (mean, ±SE; treatment: p= 0.064; interaction season x treatment: p=0.062). ATD+F males responded more quickly to a simulated territorial intrusion during the breeding season (tretament: p=0.032; interaction season x treatment: p=0.056).

Table 5. 2. Repeated-measures ANOVA with factors: season and implant (between subjects).

	number of approaches		latency to approaches	
	F	P	F	P
season	2.94	0.12	0.96	0.35
season* implant	4.53	0.062	4.8	0.056
implant	4.4	0.064	6.4	0.032

5.3.3. Hormones

T levels were higher during the breeding season than during the nonbreeding season. Furthermore, only during the breeding season did ATD+F treatment cause a significant increase in the plasma levels of T. During the nonbreeding season both groups had low T-levels and there was no significant effect of the STI on the plasma levels of T (Fig. 5.3.). Plasma levels of DHT were also higher during the breeding season than during the nonbreeding season. Neither the treatment nor the STI affected DHT levels (Fig. 5.3; Table 5.3.). Plasma levels of AE and E2 were undetectable in all samples (det. limit: AE, 259.1 pg/ml; E2, 40 pg/ml). The baseline levels of CORT did not differ between ATD+F and control males and there was no seasonal difference in the CORT levels. However, in both seasons plasma levels of CORT were significantly increased after an STI in both groups (see Fig. 5.4.; Table 5.3.). In both seasons concentrations of circulating CORT after an STI were correlated neither with the latency of approach to the decoy (*breeding s.*: r_p=-0.5, p=0.1; *nonbreeding s.*: r_p=-0.33, p=0.33) nor with the number of approaches (*breeding s.:* r_p=0.12, p=0.7; *nonbreeding s.*: r_p=0.22, p=0.5; Fig. 5.5).

Table 5.3. Hormonal differences between ATD+F and controls before and after the STI, and between seasons, using a repeated-measures ANOVA.

	T		DHT		CORT	
	F	P	F	P	F	P
season	11.178	0.009	6.45	0.032	0.002	0.966
season x treatment	9.72	0.012	0.24	0.637	0.02	0.89
STI	2.84	0.126	0.056	0.818	11.2	0.009
STI x treatment	2.07	0.18	0.007	0.937	0.017	0.899
season*STI	2.129	0.178	0.248	0.63	0.003	0.956
season*STI*implant	1.73	0.22	0.35	0.57	0.476	0.51
implant	9.66	0.013	2.85	0.13	0.04	0.85

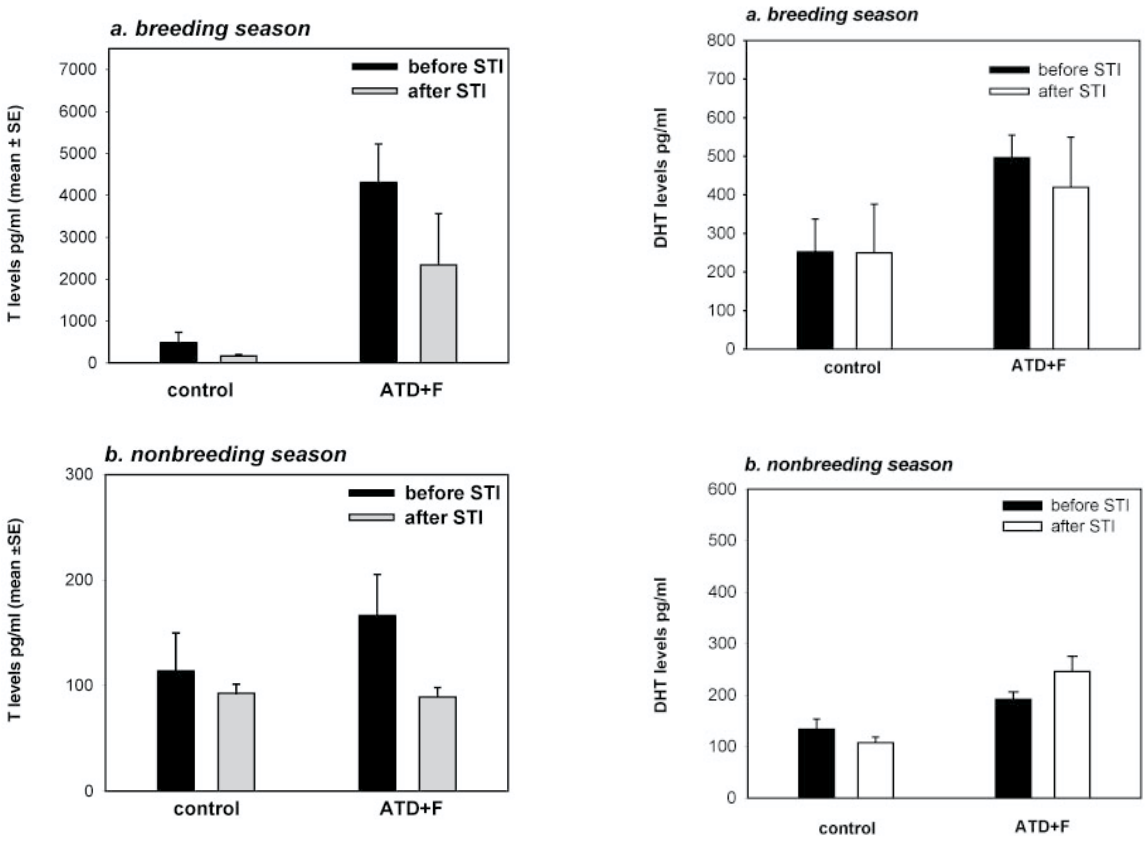

Fig. 5.3. Plasma levels of T and DHT in control and ATD+F-treated males before and after a simulated territorial intrusion (STI; mean; ±SE). In the controls, plasma levels of T were higher during the breeding season (a) than during the nonbreeding season (b) (season: $p<0.01$). ATD+F treatement affected T levels only during the breeding season (treatment: $p=0.013$; season x treatment: $p=0.012$).

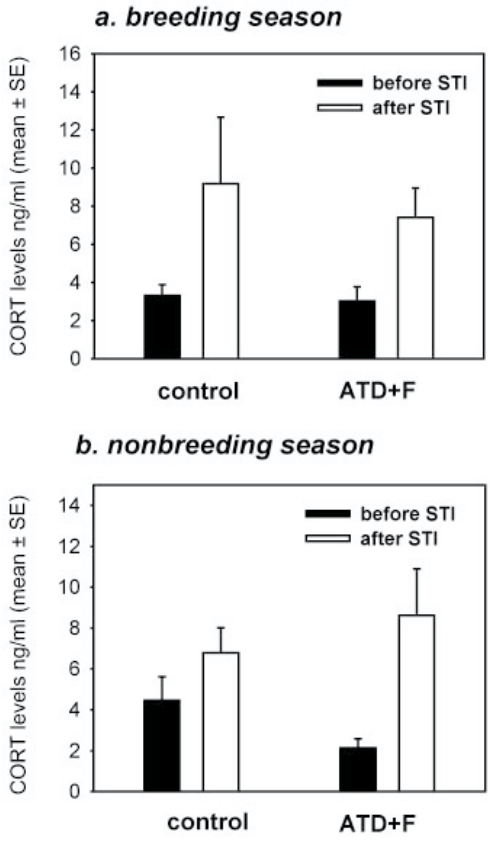

Fig. 5.4. Plasma levels of CORT in control and ATD+F-treated males before and after a simulated territorial intrusion (STI; mean; ±SE). Plasma levels of CORT were higher after an STI during both the breeding season (a) and the nonbreeding season (b; STI: $p<0.01$).

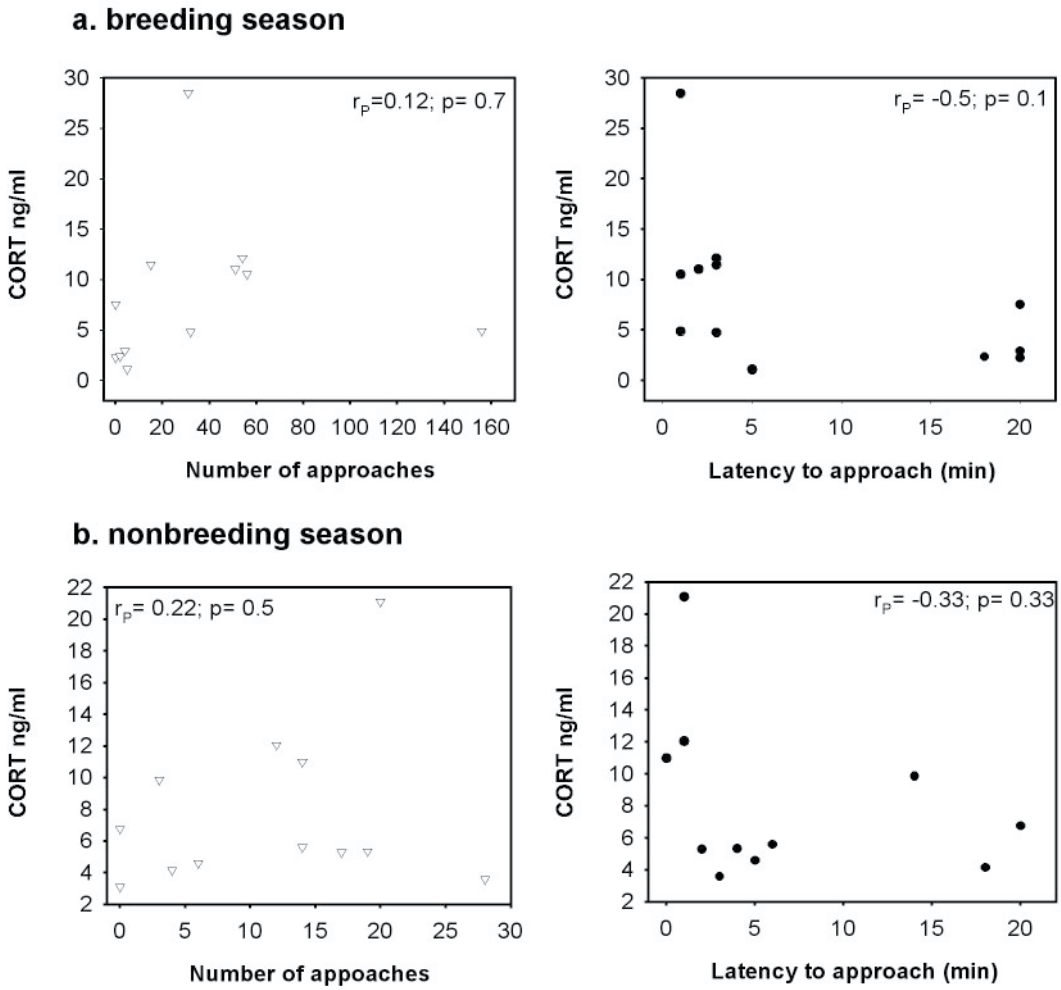

Fig. 5.5. Correlation between CORT levels and aggressive parameters during the STI between seasons.

5.4. Discussion

5.4.1. Methodology

During the breeding season T levels in ATD+F-treated males were higher than in controls. This is expected if F successfully blocks the negative feedback action of T on the HPG axis (see Section 1.2.). During the nonbreeding season, T levels did not significantly increase after ATD+F treatment, suggesting that either the regressed gonads cannot produce large amount of T and/or that the hypothalamus or pituitary does not respond to the negative feedback action of T during this period (e.g. Balthazart et al., 1981; Cho et al., 1998).

Interestingly, in European robins blocking AR with F does not induce an increase in T levels during the breeding season (Schwabl & Kriner, 1991), although the behavioural results indicate that the implant was effective. The reason for this lack of augmentation in T levels in the latter experiment might be explained by increased aromatisation of T into E2 (Schlinger & Callard, 1989a). Since T might play a role in the control of aggressive behaviour via E2, I implanted simultaneously an androgen blocker (F) and an aromatase inhibitor (ATD). In the present experiment, ATD appears to have effectively inhibited the aromatase, because in both groups plasma levels of E2 were around the detection limit and did not show any difference after treatment. In studies in which ATD implantation was less effective, an increase in E2 levels was reported (Soma et al., 1999b).

Long-term treatment with ATD+F may have physiological side effects. In the western song sparrow ATD+F implantation causes an increase in the plasma levels of E2 and CORT after 30 days, which was not observed after 7 days of treatment (Soma et al., 1999b). Since increased CORT levels can be used as an indicator of stress (Siegel, 1980), it is likely that the long-term treatment caused physiological disturbances. In the present experiment, seven days of implantation did not affect plasma levels of CORT during both seasons.

In summary, the hormonal results indicate that the inhibition of AR and aromatase was effective, at least during the breeding season.

5.4.2. Behaviour

Captive stonechats approached the decoy but did not attack it physically, as is usually the case in free-living stonechats. ‘Attack without contact’ (=approach) is a low-intensity aggressive behaviour, which seems to be characteristic of captive bird species. In the present study there was a seasonal difference in the modulation of aggressive behaviour by ATD+F treatment. During the breeding season, when T levels were elevated (Fig. 5.3.), ATD+F males tended to approach the decoy more often than during the nonbreeding season, when T levels were undetectable. Moreover, ATD+F males responded more quickly to the presentation of a decoy during the breeding season than during the nonbreeding season. In contrast singing activity did not differ between groups.

In the following two sections I shall discuss the behavioural results in the light of known effects of androgens (5.4.2.1) and corticosterone (5.4.2.2.).

5.4.2.1. Androgens

Blockage of the action of androgens and oestrogens did not reduce aggressive behaviour in both seasons. However, during the breeding season, ATD+F-treated males seemed to be even more aggressive than controls. ATD+F males responded more quickly to an STI and they also tended to approach the decoy more often than did controls. These effects of ATD+F treatment were not observed during the nonbreeding season, which is consistent with other results indicating that during the nonbreeding season aggressive behaviour may be androgen- as well as oestrogen-independent.

Unexpectedly, during the breeding season the inhibition of androgens and oestrogen action stimulated aggressive behaviour in captive European stonechats. Only one study has shown that androgens might have inhibitory effects on male aggressive behaviour during the breeding season. T-implantation in male snow buntings *(Plectrophenax nivalis)* reduced aggressive behaviour during the breeding season (Romero et al., 1998).

In general androgens are thought to be closely linked to aggressive behaviour during the breeding season (e.g. (Wingfield et al., 1990b; Balthazart, 1983; Moore, 1984). Most studies have shown that blocking AR during this period reduces aggressive behaviour (Schwabl & Kriner, 1991; Searcy & Wingfield, 1980). Flutamide treatment in male European robins increase the latency of approach to a decoy in spring, but not in winter. However such a positive relationship between T and aggressive behaviour during the breeding season is not always observed (Tsutsui & Ishii, 1981; Eberhart et al., 1980; see also Chapter 4). Several studies found no changes in aggressive behaviour following castration during the breeding season (Wingfield, 1994b).

So far, most studies have investigated the control of aggressive behaviour by either treating birds with androgens or by blocking AR. Thus the question arises whether the increase in aggressive behaviour in ATD+F-treated male stonechats was due to the additional blockage

of E2 formation. In other bird species such as the song sparrow, 30 days of ATD+F implantation reduced aggressive behaviour during the nonbreeding season (Soma et al., 1999b). Moreover, inhibition of E2 action in the song sparrow reduced aggressive behaviour during the nonbreeding season but not during the breeding season (Soma et al., 2000a).

These contrasting data in the literature on the relationship between T and aggressive behaviour could be due to species-specific actions of T (Logan & Wingfield, 1995; Moore, 1984). It might be that selection has operated on different control mechanisms of aggressive behaviour depending on the life style of each species. In some species the control of aggressive behaviour is dissociated from T (Greenberg et al., 1984; Hunt et al., 1997). For instance in an arctic bird species, the Lapland longspur (*Calcarius lapponicus*), reproduction has to take place within a few weeks. An extended period of aggressiveness may be disadvantageous for such a species because it might interfere with breeding. As a consequence, T may be involved in the control of reproduction but not of aggressive behaviour. T-implanted Lapland longspur males sing more often but are not more aggressive (Hunt et al., 1997).

This hypothesis would explain why in my experiment the blockage of androgenic and oestrogenic action did not affect song behaviour. Song is usually strongly connected with aggressive behaviour and androgens (Arnold, 1975; review Harding, 1983). However in the red-winged blackbird (*Agelaius phoeniceus*) F treatment had no effect on song activity (Searcy & Wingfield, 1980). My results are in line with results from a recent study on a population of free-living stonechats, which were singing during the first weeks on their wintering grounds, although T levels were in a undetectable range (Raess et al. 1998; pers unpubl. data). It seems that in stonechats song behaviour may under certain condition be dissociated from T.

Taken together, the data suggests that androgens influence aggressive behaviour to some extent in a reproductive context. In a non-reproductive context, however, androgens have no effect on the regulation of aggressive behaviour. This seasonal difference in the regulation of aggressive behaviour makes it possible that other hormones might be involved either only during the nonbreeding season or during both seasons.

5.4.2.2. Corticosterone

In both the ATD+F and the control group and in both seasons males had increased CORT levels after presentation of a male decoy. Two points might explain the increased plasma levels of CORT following an STI:

Increased CORT levels are usually an endocrine response to a stressor (Siegel, 1980; Harvey et al., 1984) and the STI might have been perceived as a threatening situation. Aggressive interactions are stressful, particularly for the loser of a contest. Indeed individuals losing a fight have higher CORT levels than winners (Greenberg et al., 1984; Sapolsky, 1992; Moore, 1987; but Woodley et al., 2000). Similarly, subdominant animals (after a long term encounter) have increased CORT levels (Knapp & Moore, 1995). De facto I cannot exclude the possibility that a stuffed decoy appears dominant because it does not react to the threat of the resident male.

Alternatively, CORT may be involved in the regulation of aggressive behaviour. In male tree lizards (*Urosaurus ornatus)* CORT levels are elevated following a male-male encounter (Knapp & Moore, 1995). Similar results have been obtained in birds (Harding, 1983). In pintails CORT levels are positively correlated with aggressive behaviour (Sorenson et al., 1997). In my study, however, CORT levels did not correlate with the number of approaches. Thus in stonechats the intensity of aggressive behaviour does not depend on the concentration of circulating CORT. Moreover, during the breeding season ATD+F treated males approached the decoy more often than controls, although CORT levels did not differ between groups or between seasons.

Thus the most parsimonious explanation is that the increased CORT levels observed after an STI represent a stress induced by the intruder.

Why do ATD+F males react more pronounced to an STI than the control birds during the breeding season? It is known that stress has negative effects on reproduction, and conversely, that sex steroids modulate the HPA response (see Chapter 1). For instance, androgens inhibit the sensitivity of the HPA response to stressors, whereas oestrogens enhance it. Thus, castration of male rats increases the sensitivity of the HPA axis (Almeida et al., 1997) and in female rats

androgen treatment reduces it. Therefore, there is a reciprocal regulatory mechanism between the HPA and the HPG axis. The modulatory effects of sex hormones on the HPA response take place at the levels of the CNS (Handa et al., 1994a). In fact, androgen-, oestrogen- and glucocorticoid receptors are co-localised in several brain sites, including those that mediate reproductive behaviour (Handa et al., 1994a). On the basis of these results, it is likely that in the present study ATD+F treatment affected the HPA axis. Blocking AR and the conversion of T into E2 may have caused an increase in the sensitivity of the HPA axis with the consequence that the decoy was perceived as a stronger stressor than for the control males.

5.4 3. Why is aggressive behaviour reduced in captive stonechats?

A reduced territorial aggressiveness of captive birds compared with free-living ones has been observed not only in stonechats, but also in other species (e.g. European robin; Schwabl & Kriner, 1991). It is possible that captivity reduces the 'motivation' to defend a territory, because environmental cues are limited or because there is no need to be territorial as food is available ad libitum. Moreover, it is known that deficits of social experience during ontogeny cause abnormal behaviour in the black-headed gull (Groothuis & Vanmulekom, 1991). These behavioural alterations might be a consequence of morphological changes in the CNS since animals kept in aviaries experience impoverished conditions with restricted access to behavioural and spatial cues. In mammals it is known that behavioural deprivation has negative effects on some brain structures (see review Rosenzweig & Bennett, 1996). Similar results have been reported for birds (Healy et al., 1996; Barnea & Nottebohm, 1994).A recent study has shown that hippocampal formation volume is reduced in captive as compared to free-living juncos (Smulders et al., 2000). Apart from morphological changes in the brain, changes in the endocrine system might be the basis of the observed behavioural differences. Comparative studies have revealed that T levels are higher in free-living than in captive birds (see Wingfield et al., 1990a), so that captive birds might be less aggressive. Reduced T levels in captive birds are explained as a result of the suppressive action of the HPA axis on the HPG axis as captive animals are thought to be chronically stressed (see Chapter 1). However, in the present study I did not see differences in androgen levels between free-living and captive

stonechats (Fig. 5.6; for details see Chapter 6). Although captivity is supposed to be a stressful condition for animals causing increased CORT baselines, in the present study stonechats had undetectable or low CORT baselines in both groups and in both seasons.

In summary, this experiment, like the one described in the first Chapter, revealed evidence of species-differences in the control mechanisms for aggressive behaviour: In contrast to many studies (Balthazart, 1983; Schwabl & Kriner, 1991; Soma et al., 2000a; but Romero et al., 1998), blocking the action of androgens and /or oestrogens increased 'aggressive behaviour' in captive stonechats during the breeding season. However ATD+F treatment had no effect on aggressive behaviour during the nonbreeding season. Thus it seems that the relationship between androgens and aggressive behaviour is restricted to the reproductive context. As CORT was increased following an STI in both seasons, it is possible that this hormone is somehow involved in the control of aggressive behaviour. However, the results might have been strongly affected by keeping birds in captivity. Therefore I repeated this study in free-living birds of the same species.

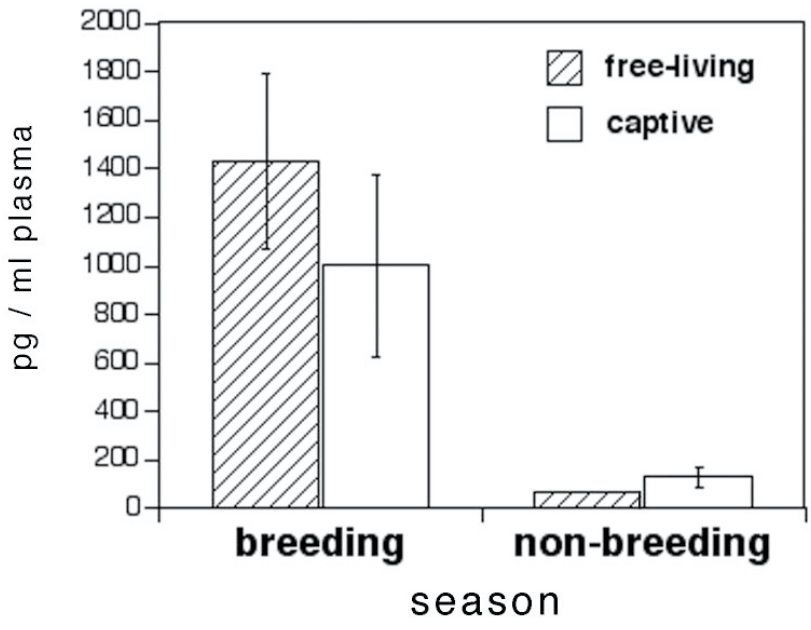

Fig. 5.6. Comparison of T levels between captive and free-living stonechats in both seasons.

6. TERRITORIAL AGGRESSION IN FREE-LIVING MALE STONECHATS

6.1. Introduction

The results obtained in the previous chapter were surprising in that blocking the action of androgens and its metabolisation to oestrogen enhanced the approaches of male stonechats towards an STI during the breeding season. Moreover, CORT levels were increased in response to each STI, suggesting that the birds might have been stressed (see discussion of Chapter 5). Since birds of this study had been kept in aviaries, the question arises whether these unexpected results are due to captivity. Captivity is often perceived as a stress situation, and may induce physiological disturbances (Carlstead & Shepherdson, 1994). Animals that have been taken out of their natural life usually lack environmental enrichment (Carlstead & Shepherdson, 1994). The difficulties of breeding animals in zoos clearly illustrate the consequences of captivity. Our own breeding attempts with stonechats are also faced with substantial problems: less than 50% of of the clutches are successful (see also Gwinner, 1991; Gwinner et al., 1995; Gwinner et al., 1987). Therefore it is likely that a complex behaviour such as territorial aggression is severely affected by captivity. Indeed, captive stonechats do not express the same intensity of aggressive display as free-living populations (e.g. Gwinner et al., 1994b; this thesis).

In this study I carried out experiments similar to those in the study presented in Chapter 5 on free-living stonechats during both the breeding and the nonbreeding season. I tested the aggressive response of free-living male stonechats to an STI before and after blocking the androgen and oestrogen action. Experiments were conducted on a stonechat population breeding in Hungary and another one wintering in Israel.

6.2. Materials and Methods

6.2.1. Study sites

The experiments were carried out at two sites. During the breeding season (May 1999 to June 1999), I investigated a population in Hungary, during the nonbreeding season (November 1997 to mid January 1998) a population in the northern Negev, near Sede Boqer (30°N, 34°E), Israel.

Hungary

The breeding population lived near Gödöllö (47°N, 19°E), northeast of Budapest, Hungary. This area, about 20 km^2, is part of the Duna Ipoly National-Park. It is a sandy and grassy area with patchily distributed bushes and trees. Some parts are cultivated fields surrounded by hedges. A railway crosses this area. Bushes growing along the railway and stones beside the rails are inhabited by a good food resource and are often a favoured habitat for stonechats.

Stonechats arrive between mid-March and early April and leave their breeding areas in late August or October.

Israel

The study site for the nonbreeding season was in the northern Negev, near Sede Boqer (30° 52'N, 34° 36'E), Israel. Most of the experiments were carried out in an area of about 12 km^2 in which a long-term study on the ecology of stonechats had previously been carried out by T. (Rödl & Gwinner, in prep.; Rödl, 1999a; Rödl, 1995; Gwinner et al., 1994b; Rödl, 1999b).

The arid part of the Negev desert is dry, rocky and sandy, but during winter the frequency of rainfall is increased (Rödl, 1999b). This provides sufficient humidity for vegetation in the wadis. A considerable number of stonechats winters in these vegetated wadis, which are covered with grassy parts and shrubbery.

6.2.2. Monitoring

I determined the approximate location of the territory of each pair by daily observations. Stonechats have clearly defined territories, where they forage and spend most of the time sitting on perches. For the present study it was not necessary to specify the exact borders of

territories, but I determined the approximate centre of a territory and the perches where the resident birds were mainly sitting and foraging.

6.2.3. Experimental design

I compared territorial aggression before and after treating males simultaneously with an AR blocker and an aromatase inhibitor or with a placebo. I simulated a territorial intrusion by placing a decoy in the centre of a male's territory. Behavioural responses towards the decoy were recorded for 20 min. Following this first STI males were caught with spring-traps or mist-nets either the same or the following day and a blood sample was taken. Males were then implanted either with a placebo or with an AR-blocker and an aromatase-inhibitor. Seven to 17 days (median: 9) after implantation I repeated the STI test as described above (see Fig. 6.1.). To control for rapid changes in the reproductive condition over time, which might affect aggressive behaviour independently of the treatment, I also tested a second control group during the breeding season. Males of this group were neither caught nor implanted, and were tested for STI twice at intervals of 3-12 days (median: 4) in the same period as for the other males.

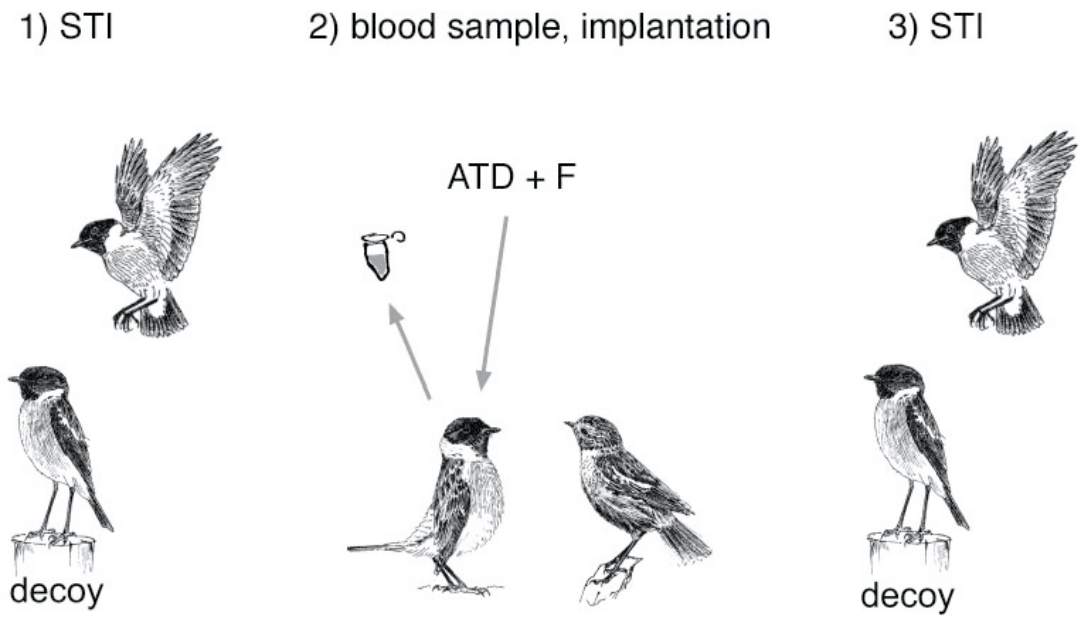

Fig. 6.1. Design of the field experiments. See text for details.

6.2.4. Simulated territorial intrusion (STI) test

Before starting an STI, I observed the pair intensively to record whether males were present and if there was any disturbance in the area, e.g. military exercises, a farmer mowing the field or presence of a predator. If any disturbing factors were present I postponed the STI until the following day.

6.2.5. Behavioural observations

I recorded at one-minute intervals the aggressive responses of each territorial male towards the decoy for 20 min, or until it attacked (with or without contact) the decoy. The following parameters were recorded. *a) Presence of aggression.* A male was scored as aggressive when it attacked the decoy, with or without contact. *b) Latency until first attack*. The time interval between the beginning of the test and the first attack of the decoy. As free-living stonechats usually attack the decoy physically and with high persistence until it is completely destroyed, it was not possible to count the number of attacks over the entire 20 min interval. Rather, the decoy was removed after the first attack. Males that did not attack the decoy were assigned a latency of 20 min, i.e. the duration of the test. Song was not recorded because stonechats do not sing during the nonbreeding season.

6.2.6. *Capture*

Animals were caught either with mist-nets or spring-traps. Mist-nets were used only at dawn (approximately between 4.00 hr and 6.00 hr). When mist-net trapping was not successful I continued to capture birds with spring-traps. I baited the trigger of the spring-traps with a mealworm to attract the birds. Catching time was between 5.00 hr and 18.30 hr. After birds were caught, a blood sample was taken. Then the birds were ringed with a unique colour combination for individual recognition, and finally two implants were inserted.

6.2.7. Blood sampling

A blood sample was taken from each bird within 5 min after capture. Blood samples were taken by puncturing the wing vein and collecting the blood with heparinised capillaries. These samples were subsequently centrifuged and plasma was transferred with a Hamilton syringe into an Eppendorf tube. Plasma samples were kept on ice until arrival at the field station and then stored at -70°C until use for lab analyses.

6.2.8. Hormonal manipulations

One group of males received one implant filled with the AR-blocker F and one filled with the aromatase inhibitor ATD. Eight males were implanted with ATD+F during the breeding season and six during the nonbreeding season. During the breeding season 5 males and during the nonbreeding season 4 males of the control group A received empty implants. During the breeding season I had an additional control group B of 10 males which were neither caught nor handled.

6.2.9. Hormonal analyses

Blood samples were taken from the alar vein using heparinised capillaries. After centrifugation plasma was collected and kept on ice for a maximum of 6 hours, then stored at -70°C. The androgens AE, DHT and T and the oestrogen E2 were measured by RIA after extraction on diatomaceous earth (celite) microcolumns using the protocol of Wingfield and Farner (1975) with modifications described in Fusani et al. (2000) (see Chapter 3). All samples were analysed in duplicate and were run in a single assay. The detection limits for the hormones were as follows: AE: 190.0 pg/ml; DHT: 123.9 pg/ml; T: 63.2 pg/ml; E2: 34.3 pg/ml. Intra-assay variation was: AE: 17.8%; DHT: 8.4%; T: 11.1%; E2: 19.8%.

6.2.10. Statistical analyses

A Fisher exact test was used to compare the presence or absence of an aggressive response after implantation between the different groups. The attack latency was compared within groups before and after implantation with a Wilcoxon signed-ranks test. Since during winter the sample size of the control group was smaller than N=5, the Wilcoxon signed-ranks test could not be used for this group. Seasonal differences in the attack latency before implantation were analysed with a Mann-Whitney U test using all tested males. The same statistical test was used for seasonal differences in the plasma levels of hormones. All tests were two-tailed and statistical tests were considered significant when $p<0.05$. When not otherwise specified, values reported are means ±SE.

6.3. Results

6.3.1. Behaviour

Presence of aggression. During the *breeding season*, 22 out of 23 males showed an aggressive response to the STI before implantation (Table 6.1.). After implantation, 6 out of 8 males treated with ATD+F did not respond to the STI, whereas all 15 control males (empty implants; control A) and unmanipulated males (control B) responded aggressively. Therefore, I compared ATD+F and control males for the presence of an aggressive response before and after implantation with a Fisher Exact test, pooling the data from the two control groups. The statistical analysis showed that during the *breeding season* the aggressive response to an STI was significantly reduced by the ATD+F treatment (Fisher Exact test: $p<0.001$). Before implantation, groups did not differ in their aggressive response to the STI (Fisher Exact test: $p=1.0$).

During the *nonbreeding season*, 9 out of 10 males showed an aggressive response to the decoy in the pre-implantation test. After implantation, all 6 ATD+F males and 3 out of 4 control males responded aggressively to the STI and there was no significant effect of the treatment (Fisher Exact test: $p>0.4$; Table 6.1.).

Table 6.1. Presence (yes) or absence (no) of aggressive response to a simulated territorial intrusion before and after implantation of ATD+F, empty implants (control A) or no implantation (control B).

		before implantation		after implantation	
	attack	yes	no	yes	no
breeding season (N=23)	ATD+F (N=8)	8	0	2	6
	control A+B (N=15)	14	1	15	0
nonbreeding season (N=10)	ATD+F (N=6)	5	1	6	0
	control A (N=4)	4	0	3	1

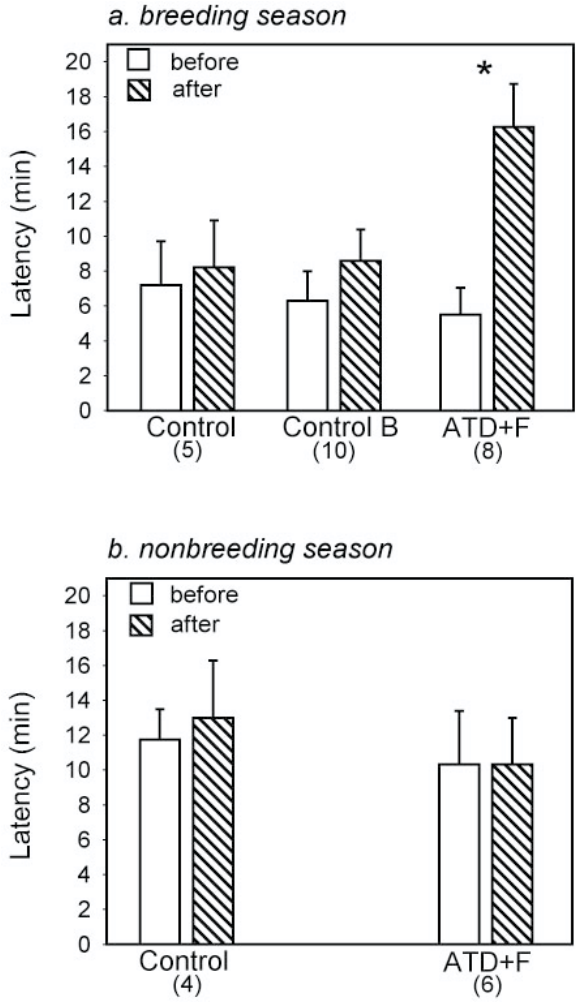

*Fig. 6.2. Latency until attack of a decoy during an STI in control or ATD+F-treated male stonechats before and after the implantation (means; ±SE; *= $p < 0.02$). Numbers refer to sample sizes.*

Latency until first attack. During the *breeding season* ATD+F administration in males affected the latency of the response to an STI (Fig. 6.2.a). After the treatment this response latency was significantly increased compared to the pre-implantation test (Wilcoxon signed ranks test: Z=-2.37; N=8; p<0.02). Males that received empty implants (control A) did not show any changes in the latency of response to an STI (Z=-0.27; N=5; p=0.78). The unmanipulated males (control B) also showed no difference in the latency of aggression between STI tests (Z=-1.3; N=10; p>0.18).

During the *nonbreeding season*, in both ATD+ F and control males the latency of aggression did not differ between the two STI tests (ATD+F: Z=-0.21; N=6; p>0.8; Fig. 6.2.b).

Seasonal difference in responsiveness. Seasonal comparison of the initial STI test shows that during the *breeding season* males attacked the decoy after an average of 6.2 ±1.0 min, whereas in winter males attacked after a mean of 10.9 ±1.9 min. This difference is significant (Mann Whitney U test: Z=-2.56; p<0.01; Fig. 6.3.).

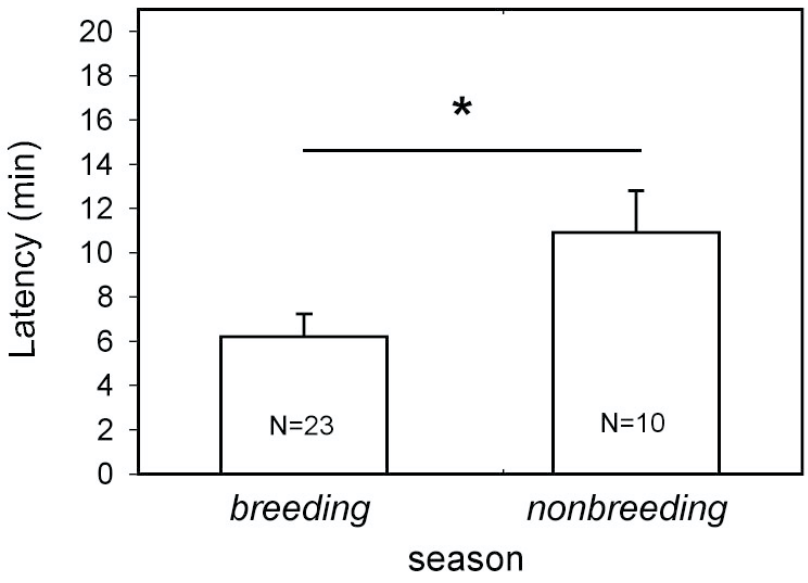

*Fig. 6.3. Latency until attack of a decoy during an STI in both seasons (means; ±SE; * = p< 0.01).*

6.3.2. Hormones

There were pronounced seasonal differences in the plasma levels of T and DHT (Fig 6.4.). During the *breeding season* the average plasma levels of T were 1384.0 ± 387.7 pg/ml. During the *nonbreeding season* all males had undetectable plasma levels of T (Mann-Whitney U-test: U=0; N=23; p<0.001). Similarly, plasma levels of DHT were detectable only during the *breeding season* (185.4 ± 26.9 pg/ml) (U=30; N=22; p<0.031). Plasma levels of AE were undetectable in both seasons. In both seasons, plasma levels of E2 were detectable only in few males and no seasonal difference was observed (U=60.5; N=23; p>0.7).

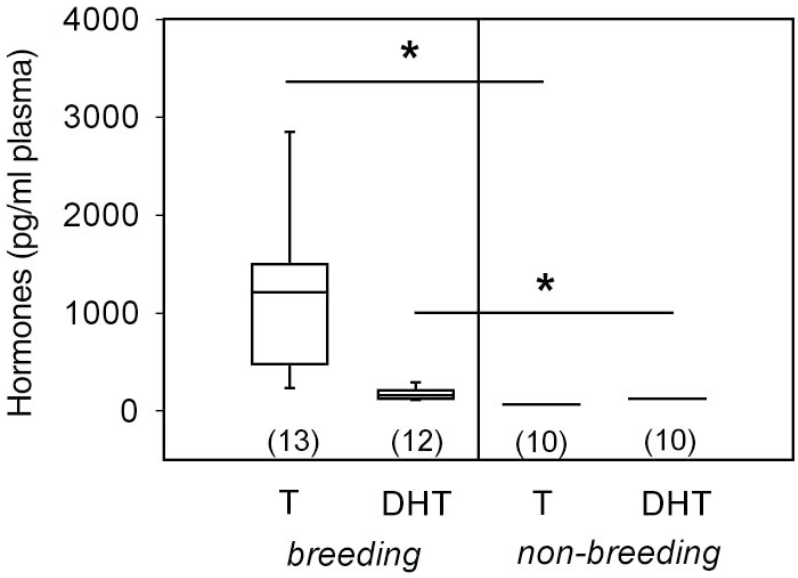

*Fig. 6.4.. Plasma levels of T and DHT during the breeding and nonbreeding seasons (medians, quartiles, ranges; * p< 0.05). Numbers refer to sample sizes.*

6.4. Discussion

This study demonstrates that there are seasonal differences in the hormonal control mechanisms of territorial aggression in free-living European stonechats. In territorial males, simultaneous treatment with the AR antagonist F and the aromatase inhibitor ATD reduced the aggressive response to an STI during the breeding season, but not during the nonbreeding season. Thus, during the breeding season territorial aggression appears to be modulated by androgens or

their oestrogenic metabolites, whereas during the nonbreeding season territorial aggression does not seem to be dependent on these hormones.

This is the first study that compares territorial aggression between seasons in one species by using an AR blocker and an aromatase inhibitor, thus blocking both androgenic and oestrogenic action. Schwabl and Kriner (1991) had observed seasonal differences in the androgen-dependence of territorial aggression in male European robins. Males implanted with the AR blocker Flutamide showed a reduced aggressive response to an STI during the breeding season but not during the nonbreeding season (Schwabl & Kriner, 1991). However, in the study of Schwabl and Kriner (1991) the aggressive response was reduced only after 3 weeks following implantation, compared to 9 days in the present study. This difference might be due to a number of factors. First, there could be species differences in the androgen modulation of aggression. There are species-specific differences in the temporal pattern of plasma levels of T (Wingfield et al., 1990b; Hunt et al., 1995). Secondly, the dose of F used in our study was lower than that used by Schwabl and Kriner (1991). However, one would expect a slower response to the treatment with a lower dose and not the opposite. Finally, we implanted the males with both F and ATD. If there was a synergism between androgens and oestrogens in controlling territorial aggression, the simultaneous blockage of androgen and oestrogen action would cause a more rapid decrease in the aggressive response. Oestrogenic effects on territorial aggression have been shown by a few authors (Schlinger & Callard, 1989b; Soma et al., 2000b; Soma et al., 2000a).

In contrast to the present results, Soma et al. (Soma et al., 1999b) showed that in male western song sparrows the aggressive response to an STI is reduced after ATD+F implantation during the nonbreeding season. In the latter study, an increase in the latency of aggression of about 60 sec could be observed after 30 days of ATD+F implantation, but no effect was seen 7 days after implantation. There are several possible explanations for the different results obtained by Soma et al. (Soma et al., 1999b) and by us. First, I might have overlooked differences in the order of seconds, because we recorded behaviour at one-minute intervals. Secondly, in the present study males attacked only after approximately 10 min during the nonbreeding season, compared with 25 sec in the study of Soma et al. (1999b). The more rapid response

observed in the latter study could be due to the use of song playback during the STI. However, effects of song are unlikely in stonechats because wintering stonechats do not respond to playback of conspecific song (Gwinner & Schwabl, unpublished). Third, there might be age or species differences in the regulation of nonbreeding aggression. In male European starlings *(Sturnus vulgaris)* castration during the nonbreeding season has age-dependent effects on aggression (Pinxten et al., 2000). In some species increased plasma levels of T correlate with autumnal sexual behaviour and increased male-male interactions (e.g. Lincoln et al., 1980; reviewed by Wingfield, 1994a). On the other hand, in several species territorial aggression during the nonbreeding season is not accompanied by increases in T (Burger & Millar, 1980; Logan & Wingfield, 1990; Schwabl & Kriner, 1991; Wingfield, 1994a; Gwinner et al., 1994b). Recent studies suggest that in the western song sparrow non-gonadal oestrogens (originating from brain or peripheral tissues) might play a role, since in song sparrows the aromatase inhibitor fadrozole reduced territorial aggression during the nonbreeding season (Soma et al., 2000b; Soma et al., 1999b), but castration did not (Wingfield, 1994b). Interestingly, the fadrozole treatment did not reduce territorial aggression during the breeding season (Soma et al., 2000a).

Why could there be species differences in the control mechanism of territorial aggression during the nonbreeding season? Western song sparrows are territorial year-round and winter in complex (hetero and/or unisexual) groups within 100 m of their breeding grounds (Wingfield & Monk, 1992). Therefore, a ‘reproductive context’ might begin during or be maintained throughout the nonbreeding season, and females and territories might be selected during this period. This view is supported by the study of Wingfield and Monk (1994) in which males associated with E2-treated females responded with an increase in T in late winter, at the very beginning of gonadal recrudescence (Wingfield & Monk, 1994). In contrast to the western song sparrows, migratory stonechats have distinct breeding and nonbreeding territories hundreds or thousands of km apart. Moreover, all evidence suggests that wintering stonechat pairs are not identical with breeding pairs (Gwinner et al., 1994b; Rödl and Gwinner, in prep). Another difference between the two species that might account for different control mechanisms is that western song sparrows sing even during the nonbreeding season (Wingfield

& Hahn, 1994; Wingfield, 1994b), while the stonechats of the wintering population we studied do not. It is well known that androgens are related to song behaviour and it is thus possible that T or its metabolite E2 are involved in winter territoriality in song sparrows, but not in stonechats.

The present study revealed clear seasonal differences in the latency of responding to a decoy. Although stonechats were aggressive in both seasons, the STI response latency was shorter during the breeding season, when androgen levels were elevated, than in the nonbreeding season, when plasma levels of T were low. It is known that T can increase vigilance, exploratory tendencies and the persistency with which certain behaviours are pursued (Wingfield, 1994b; Andrew & Rogers, 1972; Fusani et al., 1997; Andrew, 1972). It also increases overall locomotor activity (Aschoff, 1962; Gwinner & Gwinner, 1994). Hence it is possible that the quicker response of stonechats to an STI during the breeding season is due to an unspecific stimulatory action of T rather than to a specific increase of aggression.

Although during the breeding season ATD+F-treated males reduced their aggressive response to an STI (or did not respond at all), we observed that they were still able to express aggressive behaviour towards their conspecific neighbours or other species (pers. obs.). This suggests that ATD+F treatment did not 'abolish' aggressive behaviour in general, but rather reduced aggressive responsiveness specifically towards an unknown intruder. This observation supports the 'challenge hypothesis', which states that during the breeding season T is positively correlated with aggressive behaviour, when social relationships are 'unstable' (Wingfield et al., 1987; Wingfield et al., 1990b). In male quails plasma levels of androgens correlate with dominance and aggressiveness only during the first few fights. Once hierarchies are established, plasma androgen levels decline and no longer correlate with dominance and aggression (Ramenofsky, 1984; see also Schlinger, 1987). Further experiments are needed to verify this hypothesis.

In conclusion, the present study shows that the inhibition of androgenic and oestrogenic action in free-living European stonechats reduces territorial aggression during the breeding season (reproductive context), but not during the nonbreeding season (non-reproductive context). Moreover, it shows that the latency of the response to an STI differs seasonally, probably in relation to seasonal differences in circulating levels of T.

7. HORMONAL RESPONSE TO AN INTRUSION IN CAPTIVE FEMALE STONECHATS

7.1. Introduction

In many species not only males but also females express territorial aggressive behaviour. Little is known about aggressive behaviour and its endocrine control in females. Because many investigations suggested that androgens control aggressive behaviour in males, it was first thought that female aggression is controlled in much the same way as male aggression (Eens & Pinxten, 2000; Staub & De Beer, 1997). Female vertebrates produce a small amount of androgens and androgenic precursors (DHEA) in both the ovaries and adrenals. However, the few studies that have investigated this issue indicate that the endocrine regulation of aggressive behaviour in females may not depend on androgens and that its control may be more complex.

Female robins establish and aggressively defend individual territories during the nonbreeding season. During this period the blockage of AR (by Flutamide implantation) does not reduce aggressive behaviour (Kriner & Schwabl, 1991). Furthermore T-treatment does not facilitate aggressive behaviour in females during the breeding season (Kriner & Schwabl, 1991). Thus in female robins aggressive behaviour appears to be androgen-independent. In the song sparrow, females challenged by a female intruder with an additional female song playback have lower plasma levels of T than 'non-challenged' females (Elekonich & Wingfield, 2000). The authors proposed that T inhibits aggressive behaviour in females. However, a follow-up experiment showed that T- and E2 treatment neither decreases nor increases aggressive behaviour in captive female song sparrows (Elekonich & Wingfield, 2000). Thus it seems that although a simulated female intrusion may modulate androgen levels, the aggressive behaviour in females is not directly controlled by androgens.

In sex-reversed bird species like the Wilson's phalaropes (*Phalaropus tricolor*) and the spotted sandpiper (*Actitis macularia*) the behaviour of females resembles that of males. Again, it was first thought that androgens play a major role in controlling the 'male-like' behaviour of

females of these species (Fivizzani et al., 1990; Höhn & Cheng, 1967). In the 70´s it was shown that increased aggressiveness in sex role-reversed females is not based on a reversal of the androgen/ -oestrogen ratio as had previously been assumed. Females have lower androgen levels than males, just like those of non-sex-reversed species (Rissman & Wingfield, 1984; Fivizzani & Oring, 1986; Fivizzani et al., 1986). Following this demonstration, it was speculated that females have increased AR densities or an increased efficiency of enzymatic activation of androgens. But this hypothesis was also not supported in subsequent studies, which showed that the pattern of these factors is similar to that found in females of non-sex-reversed species (Fivizzani et al., 1990). Thus the regulation of aggressive behaviour in females is still unknown.

Stonechats are particular in that they defend their territory pairwise in a reproductive context and also in a non-reproductive context (Greig- Smith, 1980; Gwinner et al., 1994b; Rödl, 1999b). In general, females are more likely to attack female intruders (Gwinner et al., 1994b), but they become alert once an intruder of either sex appears. Thus, in the first experiment I asked if paired female stonechats show a hormonal response to a male STI. According to the 'challenge hypothesis' (see Chapter 4) one might expect increased plasma levels of androgens in response to an STI. However, a recent study on male song sparrows suggested that E2 controls territorial aggression during the nonbreeding season (Soma et al., 2000b; Soma et al., 2000a; see Chapter 5). E2 would indeed be a more likely candidate for the control of aggressive behaviour in females as it is the main gonadal steroid of females. Furthermore, I also analysed the 'stress-hormone' CORT, which might also be affected by an STI. As pair formation occurs in stonechats during both seasons, I compared the hormonal response to an STI between a reproductive and a non-reproductive context.

A territorial intrusion could affect the hormonal response in females directly, or indirectly through the behavioural response of their male partners. It is known that within a pair, the male and the female influence the endocrine state and consequently the behaviour of their respective partner. For instance it has been shown in wintering free-living stonechats that males paired with a female are more aggressive towards a conspecific intruder than unpaired males (Rödl, 1999b). Thus the presence of a female promotes the intensity of a male's

aggressive territorial defence. Since during this period stonechats are paired in a non-reproductive context, the increased aggressiveness in paired males cannot be related to mate or nest guarding. The mechanism is unknown, but it is likely that the behaviour of the female modulates the hormones of her mate, which in turn influence the mate's behaviour. This social influence on the endocrine system has been investigated in the 60´s (Lehrman & Friedman, 1969; Lehrman, 1964; Erickson & Lehrman, 1964); these studies revealed that the partners within a breeding pair synchronise each other's reproductive state, to optimise behaviour, energy and physiology according to the breeding conditions (Feder et al., 1977; O'Connell et al., 1981; Delville et al., 1984). The mechanism of endocrine synchronisation is driven by physiological inputs such as acoustical or visual stimuli (Lehrman & Friedman, 1969; Lehrman, 1964). Isolated female canaries exposed to the song of a male begin to build a nest earlier and ovarian development is accelerated in comparison with acoustically isolated females (Bentley et al., 2000). The strength of the response seems to depend on the males' quality. Females hearing a male sing a relatively large repertoire started their nest-building earlier than females exposed to a small repertoire (Kroodsma, 1971).

In the second experiment I tested the hormonal response to an STI of females paired with pharmacologically castrated (ATD+F) or intact males. This experiment was conducted during both seasons to test whether the hormonal response is different in spring and in winter.

7.2. Methods

The females used in these experiments were those to which the males of the experiments described in Chapter 4 and 5 were paired. A detailed description of the setup and the handling of the animals is found in Chapter 3.

7.2.1. Experiment 1

First, a plasma sample was taken from all female stonechats. Approximately 4 days later, a STI was carried out by fixing a male decoy on a perch in the centre of the aviary (see Chapter 4). Twenty minutes following the STI a second blood sample was taken.

7.2.2. Experiment 2

Two weeks after experiment 1, I compared the hormonal response towards an STI measured in 6 females paired with males treated simultaneously with an AR blocker and an aromatase inhibitor with the response of 6 females paired with control males. Five days following the implantation I took an initial blood sample from the wing vein within 3 minutes after entering the aviary. Two days later I performed an STI test by positioning a decoy in the centre of a male's territory (aviary). Immediately after the end of the STI test a second blood sample was taken.

Both experiments were conducted during the breeding and the nonbreeding season. Testing was restricted to the morning hours between 9.00 and 12.00 AM to reduce possible effects of variations of plasma hormone levels in the course of the day.

7.2.3. Hormonal analysis

In both experiments I measured the following hormone; AE, T, E2 and CORT. For a detailed description of the hormonal analysis see Chapter 3. Due to a failure I do not have the recoveries of the first assay for T. In this case I used the average recovery of the second assay (* in Table 7.1.).

Table 7.1. Detection limit, the intra-assay, and the inter-assay variation of the RIAs.

	detection limit (ng/ml)	Intra-assay variation (%) First assay	Intra-assay variation (%) Second assay	Inter-assay variation (%)
Androstenedione	0.17	12.3	18.6	20.4
Testosterone	0.06	33.5*	<1	24.7
Oestradiol	0.36	24.8	4.2	< 4
Corticosterone	0.89	12.5	4.8	< 1

7.2.4. Statistics

In the first experiment I analysed changes in hormone levels following an STI and between seasons using a repeated-measures ANOVA, with the factors season (breeding and nonbreeding season) and STI (before and after STI).

In the second experiment I compared the hormonal response to an STI between the breeding and nonbreeding season and between females paired with ATD+F-or control- implanted males using a repeated-measures ANOVA.

7.3. Results

7.3.1. Experiment 1

In both seasons, before and after an STI androgen levels (AE and T) were undetectable. Plasma levels of E2 were detectable, but did not differ between seasons and were not affected by the STI (see Table 7.1. and Fig. 7.4.). There were seasonal differences in the plasma levels of CORT (Fig. 7.1. and Table 7.2.) During the breeding season CORT was higher than during the nonbreeding season. However, plasma levels of CORT were not affected by an STI (see Table 7.1. and Fig. 7.1.).

Table 7.2. Hormonal response of females following a male STI. Hormones are compared between seasons, before and after an STI.

	Estradiol		*Corticosterone*	
	F	p	F	p
Season	0.59	0.46	16.37	0.002
STI	0.19	0.67	0.95	0.35
*Season*STI*	0.66	0.43	0.15	0.7

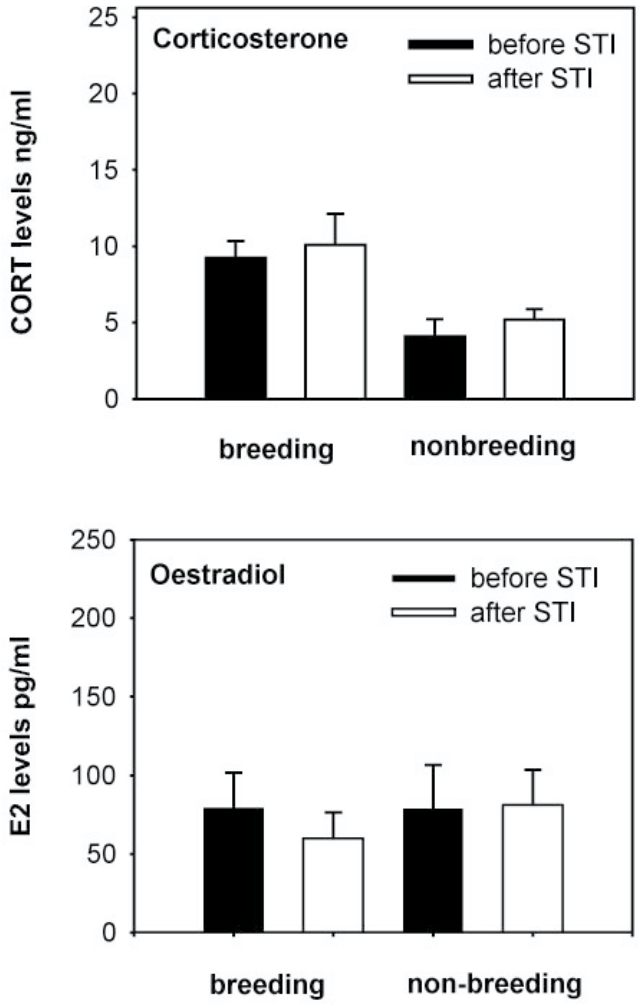

Fig. 7.1. Plasma levels of steroids in female stonechats before and after an STI during the breeding and nonbreeding seasons. CORT levels differ seasonally (p=0.002).

7.3.2. Experiment 2

As in experiment 1, androgen levels of the females of experiment 2 were undetectable (AE, T). Furthermore, E2 levels were low and did not differ between seasons (Fig. 7.2.), following an STI, or as a function of ATD+F treatment of their male partners. As in experiment 1 plasma levels of CORT were higher during the breeding season than during the nonbreeding season (Fig. 7.3.). However, in contrast to the previous experiment, in both groups and in both seasons plasma levels of CORT increased following an STI. Moreover, females paired with an ATD+F-treated male had lower CORT levels before and after an STI and in both seasons than females paired with a control male (Fig. 7.3. and Fig. 7.4.). Blood sampling took about the same time in both experiments (see Fig. 7.5.), suggesting that the increase of CORT in experiment 2 is not a methodological artefact.

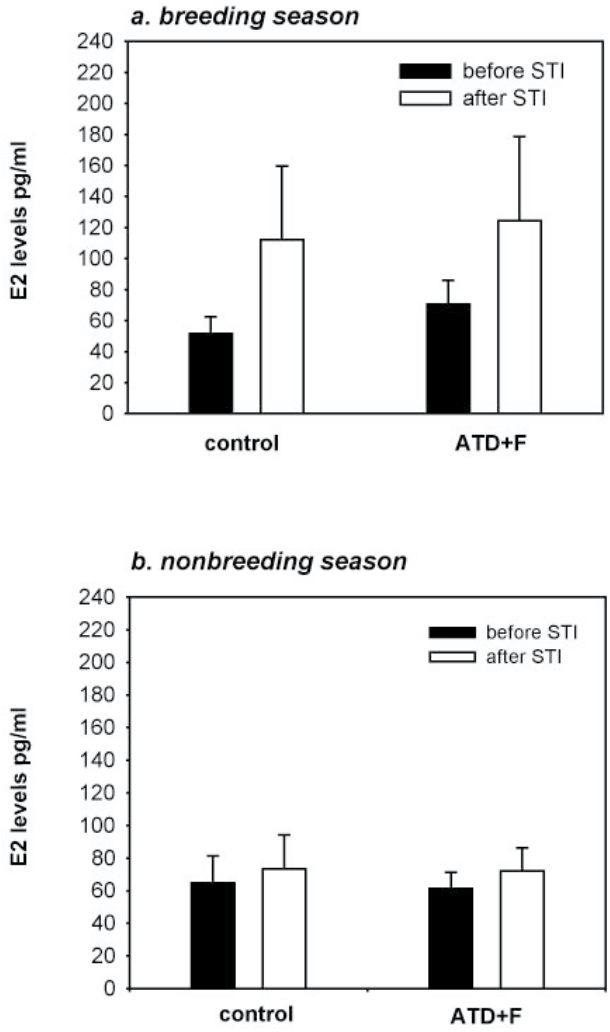

Fig. 7.2. Plasma levels of E2 in females paired with control and ATD+F-treated males, before and after an STI (experiment 2). During both the breeding season (a) and the nonbreeding season (b) plasma levels of E2 did not differ significantly.

Table 7.3. Hormonal differences between females paired with ATD+F or control implanted males, using a repeated-measure ANOVA.

	Estradiol		*Corticosterone*	
	F	p	F	p
	F	p	F	p
season	0.38	0.55	16.84	0.003
season* implant	0.53	0.49	0.003	0.96
STI	1.98	0.2	6.74	0.03
STI*implant	0.38	0.56	0.116	0.74
season* STI	0.17	0.7	0.92	0.36
season* STI*implant	0.64	0.45	0.055	0.82
implant	0.26	0.63	5.8	0.04

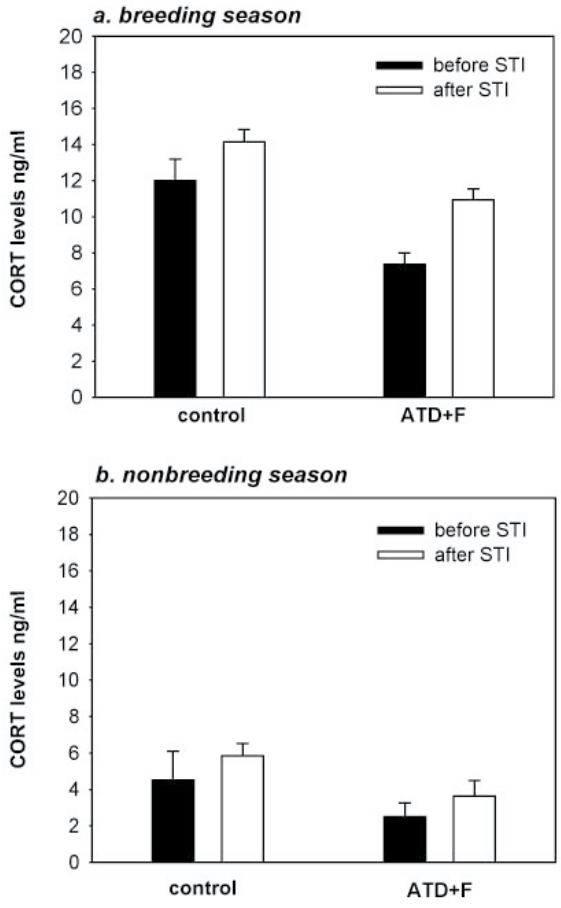

Fig. 7.3. Plasma levels of CORT in females paired with control and ATD+F-treated males, before and after an STI. During the breeding season (a) plasma levels of CORT were higher than during the nonbreeding season (b) (mean; ±SE; p=0.003). Moreover, plasma levels of CORT were lower in females paired with ATD+F males than in females paired with control males (p<0.05). Plasma levels of CORT were elevated following an STI (p=0.03).

7.4. Discussion

In the present study I tested whether an STI affects plasma levels of androgens (T, AE), E2 or CORT in paired female stonechats kept in aviaries. I also tested whether a territorial intrusion affects the hormonal changes in females directly, or indirectly via the behavioural response of the male. In the present study females neither attacked or approached the decoy. In both experiments the androgens were undetectable in both seasons. Furthermore, androgen levels were not elevated following an STI. CORT levels varied seasonally. In the second experiment CORT levels increased after an STI. Moreover, females paired with ATD+F males had generally lower CORT levels than females paired with control males.

In the following sections I shall discuss the possible involvement of androgens, oestrogens and corticosterone in the hormonal response of females to an STI.

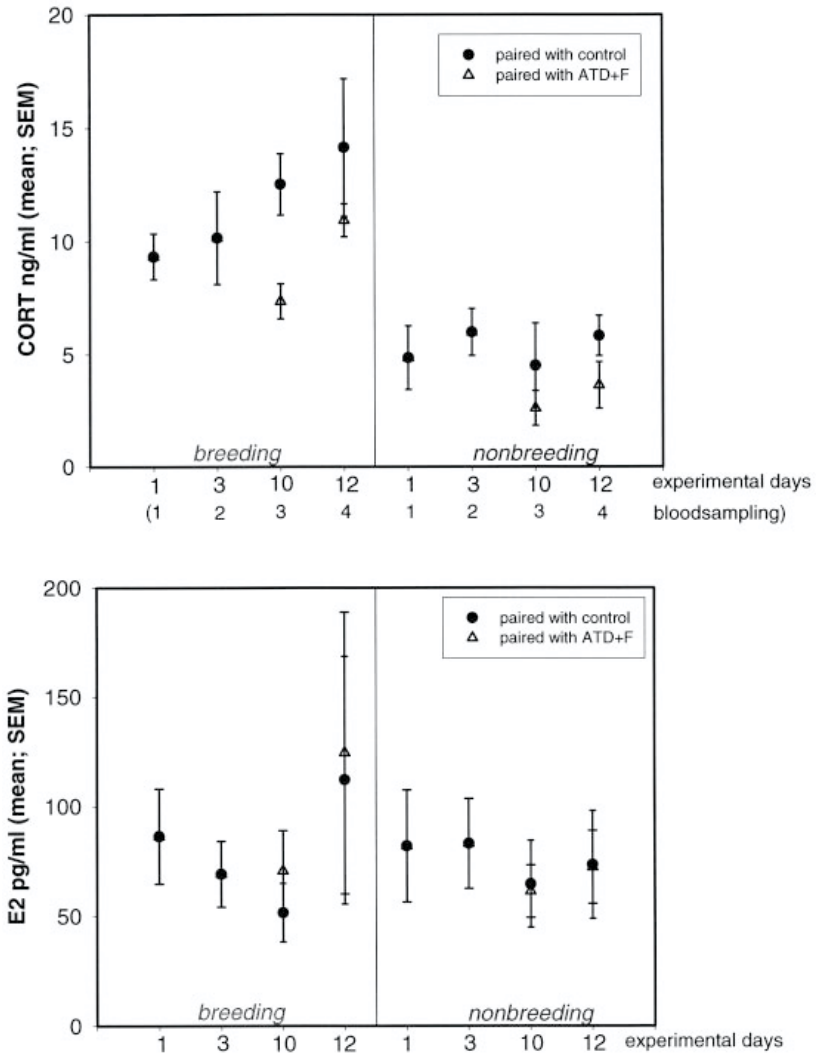

Fig. 7.4. Seasonal changes in plasma levels of steroids in females. Numbers represent the day when blood was sampled after onset of the experiment.

7.4.1. Androgens

Previous studies have suggested that androgens control aggressive behaviour in males and females (Eens & Pinxten, 2000; Staub & De Beer, 1997). In both experiments androgen levels were in the undetectable range before and after an STI. The lack of changes in plasma levels of androgens might indicate that females do not react aggressively towards a male decoy; alternatively females may not respond to an STI with an increase in androgen levels. In line with this latter possibility, Kriner and Schwabl (1991) found that aggressive behaviour

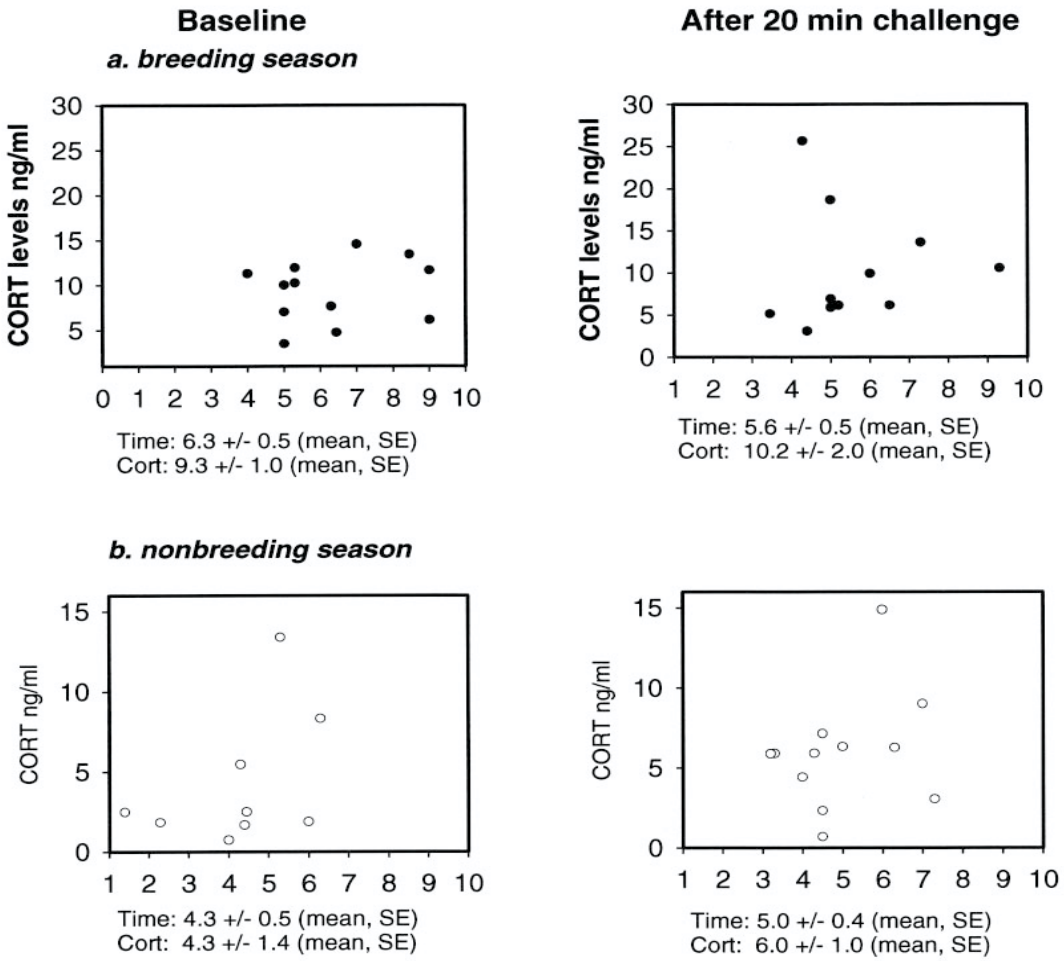

Fig. 7.5. Plasma levels of CORT plotted against the duration of blood sampling (in min). Comparison between baseline levels (left) and after an STI lasting 20 min (right) during the breeding season (a) and nonbreeding season (b). The increase of CORT levels after an STI is not an artefact of blood sampling.

in female European robins is androgen-independent. Like males, female European robins sing and establish individual territories during the nonbreeding season. Treatment with an AR blocker does not affect aggressive behaviour during the nonbreeding season. Moreover, T treatment during the breeding season increased singing but did not affect aggressive behaviour in female robins.

A recent study has shown that female song sparrows have lower T levels after a simulated female territorial intrusion than 'non-challenged' females (Elekonich & Wingfield, 2000). In fact, the authors suggested that T inhibits aggressive behaviour in females and therefore the reduction of T in females could result in disinhibition of aggressive behaviour. However, the

reduction in plasma levels of T may not be a cause, but rather a consequence of the aggressive encounter. In an additional experiment with female song sparrows neither T- nor E2-treatment altered the frequency of female-female interactions (Elekonich & Wingfield, 2000). Thus it seems that in song sparrows T is not causally involved in the control of female territorial aggression, but rather that, conversely, T levels are reduced as a result of 'challenge'. As plasma levels of androgens were undetectable throughout the experiments in female stonechats, a reduction in androgen levels following an STI cannot be excluded. It is also possible that androgens would be reduced only after an intrusion of a female. Female stonechats respond mostly to female STI but not to male STI (Gwinner et al., 1994b). Similar results were obtained in a tropical songbird, the spotted antbird. In this study, all females responded to a female STI while only few females were aggressive towards a simulated male intruder (Hau et al. 2001 in prep).

Taken together, these results highlight the complexity of the control mechanism of aggressive behaviour in females and the existence of differences among species. Furthermore, ATD+F treatment of male stonechats did not change androgen levels in their female mate. Although Ketterson et al. (1991) hypothesised that T levels will be elevated in females paired with T-treated males, this conjecture was not supported by their data.

7.4.2. Oestrogens

E2 is a possible candidate for controlling aggressive behaviour, since both sexes produce this steroid. In my study, however, plasma levels of E2 were not affected by an STI and did not differ between seasons, although plasma levels of E2 were detectable. These results are compatible with those of a study on song sparrows in which E2 levels neither were affected by a female STI nor showed a seasonal difference (Elekonich & Wingfield, 2000). Seasonal fluctuations of E2 levels are usually difficult to observe, since E2 has only a short peak preceding ovulation (Wingfield, 1984b). Furthermore, female stonechats paired with ATD+F males had plasma levels of E2 similar to those of females paired with control males.

7.4.3. Corticosterone

In both seasons, females had elevated CORT levels after an STI in the second experiment and not following the first experiment. Note that in both experiments one STI was performed and the interval between the two tests was 2 weeks. The results differ from those obtained with males, in that the latter had elevated plasma levels of CORT following each STI in both experiments. Elevated plasma levels of CORT are an indicator of stress (Siegel, 1980; see also Chapter 4) which suggests that in the second, but not in the first experiment the STI elicited stress in female stonechats. Therefore it is possible that females experienced an aversive situation after the first STI, and, as a consequence, the STI in the second experiment was perceived as stressful. Moreover, it is evident that a male intruder did not affect the hormonal response of female stonechats directly, because CORT levels in females were not elevated after the first STI. The increase in plasma levels of CORT only in the second experiment is difficult to interpret, but the efficacy of the second STI indicates that males contributed to the endocrine changes in females.

In the second experiment females paired with ATD+F males had lower plasma levels of CORT before and after the STI than females paired with control males.

There are several possible ways in which males might have modulated the hormonal levels in females. First, the hormonal changes in females might have been due to the behaviour of the male towards the decoy. This possibility can be excluded because the CORT levels were already different between groups before the STI (baseline levels). Secondly, it is possible that the male changes its behaviour towards its mate, for instance by increased intra-pair competition following the first STI. Unfortunately, I did not observe the behaviour of the pair between the two experiments. Intra-pair competition has been widely neglected. It has been reported that males become highly aggressive towards their mate once a male intruder has been perceived (Birkhead & Moeller, 1992; Mougeot et al., 2001). The reason for this increased aggression could be the risk of extra-pair copulation. The risk of extra-pair fecundation is high, as females can store sperm for several days after copulation. A male makes an extra-pair copulation less likely to succed by immediately copulating with the female and behaving aggressively towards

her, which can delay the ovarian cycle. Female doves that are paired with hyperaggressive males have a delayed ovarian development compared to females paired with less aggressive males (Hutchison & Lovari, 1976). Similarly, in another study female doves that have already interacted with a male received more aggressive behaviour when confronted with a new male (reviewed in Birkhead & Moeller, 1992).

In summary, it is possible that male stonechats became aggressive towards their females after an intruder was discovered in the aviary. (Note that during the entire duration of the experiments stonechat pairs were visually isolated from neighbouring pairs.) Thus, female stonechats might have been exposed to high aggressiveness from their males after the first STI, which in turn caused those females to have increased CORT baseline levels before the second STI. Moreover, the intensity of the 'intra-pair' aggressiveness of males seems to depend on circulating levels of androgens. Females paired with control males had higher CORT levels before and after the STI than females paired with ATD+F males. As stonechats also live as pairs during the nonbreeding season, it is plausible that this kind of intra-pair aggressiveness occurs during this period too (Rödl, 1999b).

Alternatively, it is possible that ATD+F (pharmacologically castrated) males are less dominant and/or less stressful to their 'mates' than intact males. In general males are dominant over females and are more aggressive towards their females (see Harding, 1983). Since dominance establishment is androgen-dependent (Ramenofsky, 1984), it is possible that ATD+F males were less dominant and therefore less aggressive towards their females.

Seasonal differences in plasma levels of CORT in females were also found in this study. CORT levels were higher during the breeding season than during the nonbreeding season. Males, in contrast, did not show seasonal differences in plasma levels of CORT (see Chapter 4). Several possibilities could explain sex difference in CORT plasma levels.

First, females might respond more quickly to captivity-stress. Since the experiments started in the nonbreeding season, it could be that captivity stress affected the CORT levels half a year later (breeding season). In rats it has been shown that females are more sensitive to stressors than males (Handa et al., 1994a). Second, sex differences in CORT levels may

reflect an adaptation to different tasks required for reproductive success. Third, there may be a consequence of long-term intra-pair competition, given that the experiments started during the nonbreeding season. CORT levels were higher during the breeding season, after females had been together with a male for almost half a year in a cage.

In the present study I have shown that an STI does not affect the plasma levels of androgens and E2 in female stonechats, as the ‘challenge hypothesis’ would have predicted. However CORT levels were elevated following an STI in the second experiment. Moreover, females paired with control males had higher CORT levels before and after an STI than those paired with ATD+F-treated males. I suggest that these hormonal changes are a result of increased intra-pair aggression in control pairs due to the STI in the first experiment. The intra-pair competition might be androgen-dependent even in the nonbreeding season, when androgen levels are low.

8. GENERAL DISCUSSION

8.1. The role of steroids in the control of aggressive behaviour.

My study showed that in stonechats androgens play a role in the control of aggressive behaviour only in a reproductive context. Male stonechats responded more strongly to an intruder during the breeding season, when androgen levels are high, than during the nonbreeding season, when androgen levels are low. This has been demonstrated in the experiments described in Chapters 5 and 6, in which the blocking of AR and oestrogen formation (ATD+F treatment) modulated aggressive behaviour only during the breeding season. During the nonbreeding season ATD+F treatment did not affect aggressive behaviour in either captive or free-living stonechats. Aggressive behaviour was more intense in free-living stonechats than in captive stonechats. It is likely that the lack of environmental cues, such as natural light intensity, territory size or habitat, is responsible for the reduction in aggressive behaviour in captivity.

There was a seasonal difference in aggressive behaviour in both captive and free-living stonechats. In spring captive male stonechats approached a decoy more often than in winter. Free-living stonechats had a shorter latency until attacking an intruder during the breeding season than during the nonbreeding season. Thus, it appears that in a reproductive context an intruder represents a stronger stimulus to attack than outside this period. This seasonal difference paralleled the seasonal fluctuations of plasma levels of androgens.

It could be speculated that this seasonal change in intensity or persistence of aggression depends solely on the increase of circulating androgens from the nonbreeding (*a*) to the breeding (*b*) baseline (see Fig.1.3.). However, this is unlikely because ATD+F treatment affected the behavioural response only during the breeding season. Thus, there appear to be true differences between seasons in the regulatory mechanisms of aggressive behaviour.

During the breeding season, ATD+F treatment increased the number of approaches to a simulated territorial intruder in captive male stonechats whereas the same treatment reduced aggressive behaviour in free-living stonechats. In addition my results suggest that in captive

male stonechats an STI is perceived as a stressful situation: following each STI plasma levels of CORT were elevated.

On the basis of these results, I propose the following hypothesis for the hormonal control of territorial behaviour in stonechats.

8.2. Hypothesis

I hypothesise that an intruder is always perceived as a social threat and consequently the HPA axis is activated to optimise the behavioural and physiological reaction to this stressful situation. When threatened, an individual can choose between two alternative strategies: to *escape* or to *fight* ('flight or fight syndrome'). The decision as to which of the two strategies will be chosen depends on the bird's physiological condition, its experience, and/or the environmental situation. Because reproduction is costly and time-restricted, during the breeding season an *escape* response to a social threat could dramatically reduce reproductive success. Therefore, it would be beneficial to modulate seasonally the activation of the HPA axis (stress-response) following a social threat, so as to reduce the likelihood of choosing escape behaviour during reproduction. Indeed it is known that increased androgen levels suppress the sensitivity and responsiveness of the HPA axis to stressors (see Handa et al., 1994a). Thus, it is possible that in the breeding season androgens act on the HPA axis to increase the threshold for an *escape* response to a social threat. The ATD+F treatment, then, would counteract the effects of androgens and increase the sensitivity of the HPA axis to social stress. However, in captivity stonechats cannot escape from the intruder because they are confined in the aviary; hence they immediately approach the decoy (Chapter 5). Free-living stonechats, in contrast, can choose between *fight* or *flight* and therefore avoid an aggressive interaction with the intruder (Chapter 6). A study on free-living song sparrows support this hypothesis, as CORT administration during the breeding season reduces aggressive behaviour. This model would also explain the seasonal differences in the involvement of androgens in the control of aggressiveness. Furthermore, it would explain why androgen levels are often not correlated

with aggressiveness, but have a strong influence on aggressive behaviour in a reproductive context.

8.3. Sex differences

There were clear sex differences in the behavioural response to an STI. Males usually approached the decoy, whereas female did not. There were also sex differences in the levels of sex steroids. Male stonechats had higher androgen levels than females and, unlike males, females had detectable oestrogen levels. Interestingly, females had an elevated CORT baseline in spring compared to winter, whereas males did not show any seasonal fluctuation in plasma levels of CORT. Female rats usually have higher CORT baselines than males, which is supposed to be the result of the stimulatory effects of oestrogens on the HPA axis (Handa et al., 1994a). However, this kind of interaction cannot explain the striking result that when paired individuals experienced two STIs, the males had increased CORT levels after each STI but females only after the second one. This could be a result of increased intra-pair competition. Males are more aggressive towards their mates after an intruder has been perceived. This intra-pair aggressiveness appears to be androgen-dependent, even during the nonbreeding season, as females paired with pharmacologically castrated males had lower CORT levels than control females.

8.4. Future studies

First, it would be necessary to investigate seasonal changes in the expression of androgen-, oestrogen- and glucocorticoid-receptors in the brain areas controlling aggressive behaviour. It might be that seasonal differences in the regulation of aggression are controlled by seasonal changes in the sensitivity and the distribution density of receptors. In addition, it would be interesting to test whether androgens are produced in the brain itself. Recent studies suggest that sex steroids are produced not only in the gonads or adrenal gland, but also in the brain. Thus it is possible that hormones that control aggressive behaviour originate in the brain. It

would also be necessary to test whether an STI causes a rise in plasma levels of CORT in free-living stonechats as well. The hypothesis proposed in the present dissertation is a plausible explanation and one worth testing: For instance the implantation of CORT in free-living male stonechats should cause a reduction of aggressive behaviour.

Another interesting point resulting from this thesis is the evidence that intra-pair competition occurs and that males might control the endocrine and behavioural states of females by increased aggressiveness. Studies on intra-pair competition have been widely neglected.

This work has contributed to the understanding of the control of aggressive behaviour. Many studies had investigated the regulation of aggressiveness in a reproductive context, but this is one of the first studies of the mechanism controlling territorial aggression outside the breeding season. With this work I confirmed that the endocrine control of aggressive behaviour differs between a reproductive and non-reproductive context.

9. SUMMARY

In this thesis I have examined the role of androgens, oestrogen and corticosterone in the endocrine control mechanisms of territorial aggressive behaviour in European stonechats and whether this differ seasonally.

Because European stonechats form pairs and defend aggressively a territory during the breeding and nonbreeding season, the endocrine control of aggressive behaviour can be compared in a reproductive and non-reproductive context. I tested whether pharmacological inhibition of the action of androgen and/or oestrogen affects aggressive behaviour in captive and free-living male stonechats. Furthermore I asked whether hormonal levels change following a simulated territorial intrusion (STI) in both males and females. In females I was particularly interested in studying whether the hormonal response due to a male STI depends directly on the stimulus (STI) or indirectly on the effects of the STI on the male.

My study produced the following results:

In both free-living and captive male European stonechats the plasma levels of the androgens testosterone (T) and 5α–dihydrotestosterone (DHT) are elevated during the breeding season and more or less undetectable during the nonbreeding season. Male stonechats sing more and are more aggressive during the breeding season than during the nonbreeding season. However, aggressive behaviour is also expressed when androgen levels are low. Aggressive behaviour during an STI is more intense in free-living than in captive males, although there are no differences in the plasma levels of androgens. In male captive stonechats plasma levels of T, DHT and AE are not affected by an STI in either season. However CORT levels are elevated following an STI in both seasons. In captive male stonechats singing is positively correlated with plasma levels of T only at the beginning of the breeding season. In contrast, two aggressive parameters (number of approaches and approach latency) measured during the STI are not correlated with plasma levels of T, DHT or CORT. The blocking of androgen receptors AR and the conversion of androgens into oestrogen (ATD+F treatment) affects aggressive behaviour in captive and free-living male stonechats during the breeding season , but not during the nonbreeding season. The behavioural response to an STI appears to be influenced

by environmental factors, because captivity affects the quality of aggressive behaviour. ATD+F treatment enhances ‘approaches’ to a decoy in captive males, but reduces it in free-living male stonechats. In captive male stonechats singing is not reduced by ATD+F treatment.

In view of these results I propose a hypothesis, which states that an intrusion is perceived as a social threat for which reason the HPA axis is activated. During a social threat a male can chose between two coping strategies, *escaping* or *fighting*. However during breeding, *escaping* behaviour might cause a decrease of reproductive success, thus this behaviour is suppressed by the inhibitory action of androgens (or HPG axis) on the HPA axis.

Captive female stonechats have undetectable plasma levels of androgens. Plasma levels of E2 are low and do not differ between seasons. CORT levels, however, are higher during the breeding season than during the nonbreeding season. One STI does not alter plasma levels of any steroid in captive female stonechats. However, plasma levels of CORT are elevated in both seasons following a second STI. This suggests that a territorial intrusion per se does not evoke any hormonal changes in females, instead, the second STI may be perceived as a stressor. Females paired with pharmacologically castrated (ATD+F) males have lower CORT levels before and after an STI than control females. One possible explanation is that ATD+F-treated males are less stressful for their mates.

9. ZUSAMMENFASSUNG

In dieser Arbeit untersuchte ich die Rolle von Androgenen, bzw. von Östradiol und Corticosteron bei der Steuerung territorialer Aggression europäischer Schwarzkehlchen. Im Mittelpunkt stand dabei die Frage, ob sich die zugrundeliegenden Mechanismen saisonal ändern.

Europäische Schwarzkehlchen verpaaren sich und verteidigen aggressiv ihre Territorien sowohl im Brut- wie auch im Überwinterungsgebiet, weshalb die endokrine Kontrolle territorialer Aggression im reproduktiven und im nicht-reproduktiven Kontext miteinander verglichen werden kann. Ich untersuchte an Schwarzkehlchen-Männchen sowohl in Volieren als auch im Freiland, ob die pharmakologische Blockade von Androgen- und Östrogenwirkung das aggressive Verhalten hemmt. Zusätzlich stellte ich die Frage, ob die Simulation eines territorialen Eindringens (STI) in Form eines ausgestopften Schwarzkehlchen-Männchens, die Hormonwerte des Männchens und des Weibchens beeinflußt. Bei den Weibchen war ich insbesondere auch an der Frage interessiert, ob die Veränderungen in den Bluthormon-Konzentrationen direkt durch die Präsentation des Präparats oder indirekt durch das Verhalten des männlichen Partners hervorgerufen werden. Die Volieren- und Freilanduntersuchungen ergaben, daß Schwarzkehlchen-Männchen in der Brutzeit höhere Testosteron (T)- und 5a-Dihydrotestosteron (DHT)- Blutplasmawerte haben als in der Überwinterungsphase. Männliche Schwarzkehlchen waren im Frühjahr aggressiver als im Winter. Gesang war nur im Frühjahr zu hören. Obwohl die Androgenwerte im Winter niedrig waren, waren die Vögel auch zu dieser Jahreszeit aggressiv. Aggressives Verhalten während eines STI-Tests war bei freilebenden Schwarzkehlchen ausgeprägter als bei Vögeln, die in Volieren gehalten wurden, obwohl die Plasma-Androgenwerte ähnlich waren. Im Volierenexperiment rief die STI weder im Frühjahr noch im Winter Veränderungen in den Blutplasmawerte von Androstendion (AE), T, und DHT hervor. Im Gegensatz zu den Androgenwerten waren die Blutplasmawerte des Corticosterons (CORT) sowohl im Frühjahr als auch im Winter nach Präsentation eines ausgestopften Schwarzkehlchen-Männchens erhöht. Bei in Volieren gehaltenen Schwarzkehlchen war die Gesangsaktivität zumindest zu Beginn der Brutphase positiv mit T korreliert. Im Gegensatz dazu bestand keine Korrelation zwischen den zwei gemessenen

Aggressionsparametern (Anzahl und Latenz der Annäherung zum STI), die während des STI-Tests gemessen wurden, und den Blutplasmawerten von T, DHT, AE und CORT. Das Blockieren von Androgenrezeptoren und die Umwandlung von T in E2, beeinflußte das aggressive Verhalten freilebender und in Volieren gehaltener Schwarzkehlchen-Männchen nur im Frühjahr, also im reproduktiven Kontext.

Die unterschiedlichen Umweltbedingungen, denen freilebende und in der Voliere gehaltene Schwarzkehlchen ausgesetzt waren, schienen das aggressive Verhaltensmuster zu beeinflußen. Nach der ATD+F Behandlung nahm die Anzahl der Annäherungen bei den Volieren-Männchen zu, während sie bei den freilebenden Männchen abnahm .

Auf Grund der Ergebnisse schlage ich die folgende Hypothese vor: Ein Eindringling wird von einem territorialen Männchen grundsätzlich als eine soziale Bedrohung empfunden, weshalb die Hypothalamo-Hypophysen- Adrenale (HPA)-Achse aktiviert wird. Um sich dieser Bedrohung zu entziehen, kann das Männchen zwischen 2 Strategien wählen: *Flüchten* oder *Angreifen*. Während der Brutphase würde das Fluchtverhalten jedoch den reproduktiven Erfolg beeinträchtigen, weshalb diese Reaktion durch die inhibierende Wirkung von Androgenen auf die HPA-Achse gehemmt wird.

Die Androgenwerte von Schwarzkehlchen-Weibchen befanden sich im nicht meßbaren Bereich. Die Blutplasmawerte von E2 waren niedrig und zeigten keine jahreszeitlichen Unterschiede. Die Konzentration von CORT war im Frühjahr höher als im Winter. Ein erstmals präsentiertes Stopfpräparat hatte keine Veränderungen der gemessenen Hormone zur Folge. Nach der zweiten Präsentation waren die CORT-Werte dagegen erhöht. Dies läßt vermuten, daß eine STI an sich beim Weibchen keine Hormonveränderungen verursacht. Die Simulation eines Eindringlings könnte sich aber möglicherweise auf die Beziehung zwischen den Paarpartnern ausgewirkt und dadurch zur Folge gehabt haben, daß eine weitere STI beim Weibchen eine Stressreaktion auslöste. Außerdem hatten die Weibchen, die mit ATD+F behandelten Männchen verpaart waren, sowohl vor wie auch nach dem STI-Test niedrigere CORT Werte als Kontroll-Weibchen. Eine Erklärung hierfür könnte sein, daß ATD+F Männchen grundsätzlich weniger aggressiv gegen ihre Weibchen waren.

Zusammenfassend hat diese Dissertation gezeigt, daß Androgene nur im reproduktiven Kontext einen Einfluß auf die endokrine Kontrolle von territorialer Aggression ausüben. Trotzdem weisen einige Befunde darauf hin, daß Corticosteron, das normalerweise ein Stresshormon ist, auch im Kontrollmechanismus der Aggression eine Rolle spielt. Zusätzlich weisen Ergebnisse meiner Arbeit darauf hin, daß die CORT-Werte der Weibchen nicht von einem Eindringling beeinflusst werden, sondern vielmehr durch das Verhalten, welches ein verpaartes Männchen seinem Weibchen gegenüber zeigt.

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ABBREVIATIONS

AE	Androstenedione
ACTH	Adrenocorticotrophic hormone
AR	Androgen receptor
ATD	1-4-6 androstatrien-3,17 dione
CNS	Central Nervous System
CORT	Corticosterone
CRF	Corticotrophin Releasing Factor
DHT	5α-dihydrotestosterone
EA	Ethylacetate
E2	Oestradiol
F	Flutamide
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
HPA-axis	Hypothalamo-Pituitary-Adrenal axis
HPG-axis	Hypothalamo-Pituitary-Gonadal axis
LH	Luteinizing Hormone
RIA	Radioimmunoassay
STI	Simulated Territorial Intrusion
T	Testosterone

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