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Effects of Estradiol on Adult Zebra Finch Behaviour

Martin Drouin LeMay

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Abstract

I orally exposed zebra finch, *Taeniopygia guttata*, to 4.7 or 720 μg estradiol/g diet for eight days to test the hypothesis that estrogens can affect their behaviour. In a two choice preference test, low estradiol males spent ($P = 0.01$) more time near other males after six days of treatment and their courtship also diminished. Singing scores decreased ($P = 0.01$) in both treated groups, dancing score ($P = 0.01$) in the high estradiol and mounting in the low estradiol males were lower ($P = 0.02$) compared to controls on day 4 of the treatment period. Pecks and chases targeting males were lower in both treatment groups. More high estradiol females performed tail quivering on day 4 ($P < 0.01$) and their score for this behaviour was also significantly higher ($P < 0.01$). These females also accepted mounts by the stimulus male more often.

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List of abbreviations

CPM	counts per minute
CNS	central nervous system
DDE	dichlorodiphenyltrichloroethylene
DDT	dichlorodiphenyltrichloroethane
DES	diethylstilbestrol
DHT	dihydrotestosterone
DPM	disintegrations per minute
E2	17β-estradiol
EE_2	ethinylestradiol
irLH	immuno-reactive luteinizing hormone
LH	luteinizing hormone
PCB	polychlorinated biphenyl
T	testosterone
TCDD	tetrachlorodibenzodioxin

1 Abstract

Several studies have shown that some environmental contaminants are estrogenic and may interfere with the hypothalamo-pituitary-gonadal axis of animals. Sex hormones play a major role in sexual and agonistic behaviours of avian species and if adult passerine birds are exposed to these compounds, their behaviour can be affected in a way that reduces their chances of successful reproduction. This investigation was conducted to test the hypothesis that exogenous estrogens can affect behaviours linked to reproduction in adult song birds. I orally exposed adult zebra finch, *Taeniopygia guttata*, to either 4.7 or 720 μg estradiol/g diet for a period of eight days. While the results from an estradiol radioimmunoassay optimized and validated for this species showed no increase in plasma estradiol levels in a group of males treated with the high dose, I did observe changes in sexual and agonistic behaviours in both male and female finches. In a two choice preference test, males treated with the low concentration of the hormone spent significantly ($P = 0.01$) more time near other males after six days of treatment. Their courtship behaviours also diminished significantly during treatment. Singing scores decreased significantly ($P = 0.01$) in both treated groups, dancing score was significantly lower ($P = 0.01$) in the high estradiol group 4 days after the start of treatment, and mounting behaviour was lower than control on day 4 in the low estradiol males ($P = 0.02$). Agonistic behaviours also tended to be lower in estradiol fed males. Pecks and chases targeting the male stimulus were lower in the low and high estradiol groups than in the control group. This difference was statistically significant for the low estradiol group on day 6 after treatment began ($P = 0.02$). In contrast, the sexual receptivity of treated females was increased by the estradiol treatment. Significantly more females fed the high estradiol diet performed tail quivering on day 4 ($P < 0.01$) and their score for this behaviour was also significantly higher ($P < 0.01$). These females also accepted mounts by the stimulus male more often than the controls. Dietary estradiol, at the low dose, appeared to increase the female's agonistic behaviours. On day eight of treatment, the standard score for threatening behaviour targeting the male stimulus was significantly higher in the low estradiol group compared to the control group ($P = 0.02$). These data

support my hypothesis that oral exposure to estrogens can affect behaviours that play an important role in the reproduction of passerine species.

Résumé

Plusieurs études ont démontré que certains contaminants environnementaux sont estrogéniques et peuvent affecter l'axe de hypothalamo-pituitaires-gonadal des animaux. Les hormones sexuelles jouent un rôle majeur dans le contrôle des comportements sexuels et agonistiques des oiseaux adultes et des changements comportementaux provoqués par une exposition à ces xénobiotiques pourraient réduire leur succès reproducteur. Notre étude a été menée pour tester l'hypothèse stipulant qu'une exposition à un estrogène par voie orale peut affecter des comportements critiques à la reproduction des passereaux. Nous avons exposé oralement des diamants mandarins, *Taeniopygia guttata,* adultes à 4.7 ou à 720 µg estradiol/g millet pour une période de huit jours. Bien que les résultats d'un radio-immuno-essay pour l'estradiol, optimisé et validé pour cette espèce, n'ont montré aucune augmentation des niveaux de cette hormone dans le plasma de mâles exposés à la plus haute dose, nous avons observé des changements comportementaux sexuels et agonistiques chez des mâles et des femelles. Dans un test de préférence sexuelle à deux choix, après six jours de traitement, les mâles nourris avec la basse concentration d'estradiol ont passé plus de temps à proximité des mâles stimulus ($P = 0.01$). La présence d'estradiol dans la diète des mâles a causé une diminution importante des comportements sexuels. Le chant ($P = 0.01$) a été diminué par les deux concentrations d'estradiol, la danse ($P = 0.01$) par la haute concentration au jour 4 du traitement, et les tentatives de copulation ($P = 0.02$) par la basse concentration au jour 4. Les comportements agonistiques ont aussi été réduits par l'estradiol. Les mâles des deux groupes traités avaient moins tendance à poursuive agressivement le mâle stimulus. Cette diminution a été statistiquement significative ($P = 0.02$) au jour 6 chez les mâles traités avec la basse concentration d'estradiol. La réceptivité sexuelle des femelles, par contre, a été augmenté par l'estradiol. Plus de femelles nourries d'une diète traitée avec la haute concentration d'estradiol ont performé des frémissements caudales au jour 4 du traitement ($P < 0.01$) et le score de ce comportement était aussi considérablement plus haut ($P < 0.01$). Ces femelles acceptaient de copuler plus souvent avec le stimulus mâle. L'estradiol semble aussi avoir augmenté les comportements agonistiques des femelles.

Au jour 8 du traitement, le score des menaces visant le stimulus mâle était significativement supérieur (P = 0.02) chez les femelles traitées avec la haute concentration d'estradiol. Ces données supportent notre hypothèse qui stipule qu'une exposition orale à des estrogènes peut affecter des comportements importants dans la reproduction d'espèces passerines.

2 Introduction

While some studies have demonstrated the major role of sex hormones in the sexual and reproductive behaviour of numerous species of wild birds, others have documented the negative impact of endocrine disrupting chemicals on the sexual behaviour and reproductive success of birds living in environments contaminated by those chemicals.

The known and suspected endocrine disrupting chemicals belong to diverse families which are used in a variety of applications. These include various pesticides, detergents, metals, and industrial products and bi-products such as tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCBs), alkylphenolic surfactants, dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyltrichloroethylene DDE, methoxychlor, lindane (γ-hexachlorocyclohexane) and vinclozolin.

Wild birds of different species exposed to endocrine disrupting chemicals in their natural habitat developed physical anomalies and/or exhibited abnormal sexual behaviour, either or both of which may reduce their reproductive success (ex. Fox, *et al.*, 1988; Frank *et al.*, 2001; Fox, 1992; Fry, 1995).

To better circumscribe the question I will, in the first part of this introduction, give examples of reproductive and behavioural anomalies linked to exposure to endocrine disrupting chemicals and then describe some factors affecting avian sensitivity to those contaminants. Afterwards, I will consider the vulnerability of the developing embryo and then explore how contaminants can also affect breeding adults. In the second part of the introduction I will give information about the present investigation.

2.1 Background

2.1.1 Reproductive and behavioural anomalies linked to exposure to contaminants

DDT, DDE and lindane are associated with delayed breeding and reproduction in several avian species. These contaminants can up-regulate hepatic steroid hydroxylase, inhibit gonadotropin secretion and alter thyroid function. As a result, birds can behave abnormally and may fail to form pairs (Barnett *et al.*, 1984). Herring gull (*Larus argentatus*) embryos exposed to DDT and DDE develop impaired reproductive and nervous systems as adults (Fry & Toone, 1981; Fry, 1995). These alterations may lead to abnormal sexual behaviours such as those seen in the early 1970s in Santa Barbara Island, California, where multiple female Western Gulls (*Larus occidentalis*) were reported to engage in shared breeding attempts of supernormal clutches (Fox, 1992). Injections of herring gulls with DDT, DDE or methoxychlor concentrations comparable to those found in contaminated seabird eggs in the 1970s (20-100 ppm), induced abnormal ovarian development in females and the formation of oviducts and shell glands in males (Fry *et al.*, 1981; Fox, 1992).

Egg exchange experiments with Great Lakes herring gulls living in a clean and a contaminated area demonstrated that poor reproductive success may be associated with the toxic effects of organochlorine. Both the parents and the embryo were affected as low reproductive success was observed both in clean adults given contaminated eggs and in parents living in a contaminated area given clean eggs (Peakall & Fox, 1987).

Eggshell anomalies may also result from DDT exposure. This pesticide was shown to disrupt secretory cilia function and calcium transport, and cause the swelling of the shell gland epithelium (Rattner *et al.*, 1984).

Direct parental toxicity by lindane (γ-hexachlorocyclohexane) was also shown to interfere with avian reproduction. Lindane administered by stomach tube to laying female ducks (*Anas platyrhynchos*), interrupted the egg laying in these birds. Subsequently, the treated birds had fewer eggs and the laying frequency was reduced.

Further, total hepatic RNA concentration together with hepatic, plasma and ovary vitellogenin levels were reduced. These effects were reversed by a single administration of the estrogen stilbesterol suggesting that lindane inhibits estradiol synthesis and/or release (Chakravarty *et al.*, 1986).

Thyroid lesions have also been reported in Great Lakes piscivorous birds (Leatherland, 1998). Since thyroid hormones are involved in neurological development and maintenance of the embryo, changes in this hormone's levels could lead to disorganization of the cerebellar cortex and be functionally manifested in motor, metabolic or behavioural disorders. The structural similarity between PCBs, TCDD and thyroid hormones gives them comparable binding characteristics. These toxicants can therefore compete with thyroid hormones for various receptors, including Aryl hydrocarbon (Ah) and thyroid receptors and thyroid hormone binding proteins (Porterfield, 1994; Sher, Xu, Adams, Craft & Stein, 1998).

Several colonies of herring gulls around Lake Ontario exposed to DDE, PCB, mirex, and photomirex suffered from reproductive failure (Jefferies, 1975; Porterfield, 1994) in the form of abnormal incubation behaviour and absence from their nests for long periods of time. The decreased nest defence and nest behaviour resulted in increased egg breakage or decreased nest temperature and reduced the overall hatching success. Subsequent studies demonstrated a correlation between the incidence of hypothyroidism in individuals from Great Lakes colonies and the proximity to areas polluted with polyhalogenated hydrocarbons (Fitchko, 1986; Porterfield, 1994).

Laboratory experiments with other bird species including the pigeon (*Columbia livia*), Japanese quail (*Coturnix japonica*) and ring doves (*Streptopelia risoria*) confirmed the link between chlorinated hydrocarbon contamination, hormone disruption and the behavioural observations made in wild avian colonies (Barnett *et al.*, 1984; Porterfield, 1994). For example, ring doves fed mixtures of DDE, PCB, and mirex also exhibited behaviour abnormalities similar to those observed in Great Lakes gulls (McArthur *et al.*, 1983; Porterfield, 1994). Additionally, males treated with these compounds had reduced circulating total androgens and females had reduced estrogens and progesterone. Both sexes had elevated T4 levels (Barnett *et al.*, 1984; Porterfield, 1994).

2.1.2 Factors affecting avian sensitivity to endocrine disrupting chemicals

Several factors affect the vulnerability of avian species to endocrine disrupting chemicals and species at higher risk include those high in the food web, species that reproduce in zones that are treated routinely with pesticides and those that live near contaminated industrial areas. Over 450 species and representatives from every family of North American birds migrate south of the United States in colder months (Sibley, 2001). During the migration, these birds often aggregate in large numbers to feed such that large number of birds can be affected when these transient habitats are polluted. Furthermore, species that migrate to Mexico, Central and South America or Africa can be exposed to xenobiotics that are banned in Canada and the United States because of their known impact on the environment. For example, DDT, a persistent pesticide that was used in countries around the world to control agricultural pests and insect vectors of tropical diseases was banned in Canada and the United States during the 1970s because it was associated with endocrine disruption in birds (Smith, 1992), but because of its low cost (Walker, 2000), it continues to be used in tropical countries to control anopheles mosquitoes that transmit malaria.

Many of the known or suspected endocrine disrupting chemicals are lipophilic and thus they partition into cell membranes and fat stores where they are protected against physical, chemical and biochemical degradation processes. Under periods of starvation, stress, or during energetically expensive periods such as migration, lipophilic compounds stored in fatty tissues may be released in high concentration into the blood and reach concentrations which can affect the health of the organism.

2.1.3 The vulnerability of the developing embryo

The developing embryo is often more sensitive than adults to chemical insult for a number of reasons. One is that developing organisms are smaller and therefore have a higher surface: volume ratio than their adult counterparts. This may enhance absorption and distribution of certain chemicals through the integument (or eggshell). The dynamic nature of hormone receptors during ontogeny may also infer special sensitivity to an organism during those critical periods of its development. Hormone receptors have high binding affinity for their effector messenger molecules. The binding of a ligand to its receptor results in a cascade of events leading to a functional change in the cell. This ultimately leads to organizational or activational modifications at the levels of the tissue, of the organ, and finally of the whole organism.

The type as well as the number of receptors change during the development and the life of an organism. The appearance of more "primitive", less specific, or orphan hormone receptors in various tissues during specific development periods may constitute targets for some xenobiotics (Bern, 1992; McLachlan *et al.*, 1992; Porterfield, 1994). Cells may exhibit receptors even before they are capable of producing the hormones that bind to them. For example, cells of the chick gastrula have receptors for insulin-like growth factor I (IGF-I) on the day of laying even though the embryo does not synthesise this hormone until several days later (Scavo *et al.*, 1989). Testosterone, estrogen, and thyroid receptors are also found in avian embryo and before embryonic sources of these hormones are present (Grisham *et al.*, 1997; Schwabl, 1996a; 1996b). There is evidence that some embryonic receptors respond to maternal hormones which are transferred to the egg. In the canary (*Serinus canaria*), for example, testosterone levels increase in each subsequent egg laid in a clutch. In this species, yolk testosterone levels, which reflect the mother's hormonal status during egg formation, enhance post-hatching growth and cause an increase in the begging behaviour of the hatchlings (Schwabl, 1996b; Grisham *et al.*, 1997). This suggests that the maternal hormonal status can affect the organization of the neural circuits responsible for dominance in her offspring. This is a good example of the importance of timing of exposure. Because of their short half-life, new pesticides are generally believed to be safer for non-target organisms. However, if birds are exposed to an endocrine disrupting chemical during egglaying, even small, transient changes in the mother's hormone status may affect her offspring. A more important alteration of the egg endocrine environment can lead to severe physiological and behavioural organisational changes in birds. This has been demonstrated in laying Japanese quails injected with estradiol benzoate. The eggs of treated females have elevated yolk estradiol which leads to a higher incidence of persistent right oviduct in the females (Adkins *et al.*, 1995). These behavioural and physiological effects of estradiol result from a disruption of the normal development of the embryo.

In avian species, the heterogametic sex is the female (ZW) and the phenotypic sex is determined by the expression of some genes on the W chromosome which regulate the differentiation of the gonadal primordia into ovarian tissue (Elbrecht & Smith, 1992). Contrary to the testis, the ovary has a high concentration of aromatase (CYP 19) which is responsible for the synthesis of estrogens from androgens. In chicken, the expression of this enzyme is first observed on day 6 of incubation (Yoshida *et al.*, 1996). Ovarian estradiol inhibits the development of the masculine copulatory system (Balthazart *et al.*, 1992; Arnold, 1997) and induces the regression of the right gonad. Alternatively, the male phenotype develops in the absence of estrogens (Eusebe, *et al.*, 1996).

Disruption of the endocrine environment of the embryo can affect the development of its neuroendocrine and reproductive systems. During egg production, large quantities of lipid and protein are mobilized. High amounts of vitellogenin are produced by the liver in response to elevated circulating estrogens and circulate in the blood to the ovaries. During this period, lipophilic contaminants may be mobilized from the adipose tissues and are passed to the offspring via the egg causing an important body burden during critical developmental periods. Precocial species like quails, ducks, and many species of waterbirds develop considerably inside the egg before hatching and may be particularly sensitive to these chemicals (Ottinger *et al.*, 2002).

Several investigations have studied the effect of *in ovo* steroid hormones on the development of birds. Female chicken embryos treated with an aromatase inhibitor on day 5 of incubation develop male phenotypes. As adults, masculinized females have the appearance of normal cocks, they display a cock's mating behaviours, and they develop bilateral testes capable of complete spermatogenesis (Elbrecht *et al.*, 1992; Abinawanto *et al.*, 1997). Similar treatments of Japanese quail before day 12 of incubation results in females that behave like males during adulthood (Balthazart *et al.*, 1992).

On the other hand, male Japanese quail treated with estrogens prior to day 12 of incubation lose the ability to perform normal copulatory behaviour during adulthood, even when treated with androgens (Balthazart *et al.*, 1992). Demasculinisation of behaviour has been observed to occur at the same estradiol concentration that induces a significant change in testis weight asymmetry (Halldin *et al.*, 1999). An abnormal neuroendocrine system organization may reduce the chances of a bird reproducing successfully. The birds may behave abnormally and may be incapable of finding a mate or defending a territory, and their reproductive tract may not be functional. For example, laboratory investigations have shown that DDT exposure on the first day of incubation can decrease the number and the hatchability of eggs (Bryan, Gildersleeve & Wiard, 1989).

In contrast to precocial birds, the development of the neuroendocrine system of altricial song birds, hawks, and herons, continues after hatching. This may make those birds more sensitive to non-lipophilic endocrine disrupting xenobiotics to which they may be exposed orally. The effects of exogenous exposure to sex hormones after hatching was studied in various altricial species, including the zebra finch and the European starling. As observed in chicken and quails, estrogens demasculinise sexual behaviours in male zebra finches. For example, there are decreases in dance behaviours, attempted mounts, nest material carrying and undirected singing in adult males treated with the hormone as hatchlings (Gurney, 1982; Adkins-Regan & Ascenzi, 1987). Adult male zebra finches (Gurney, 1982; Wade *et al.*, 1997) and European starlings (Casto & Ball, 1996) treated with estradiol as hatchlings also develop abnormal (smaller) testis. Female zebra finches are masculinised by exogenous estrogens when exposed early after hatching. These females can dance (Adkins-Regan *et al.*, 1994) , sing (Simpson & Vicario, 1991; Gurney & Konishi, 1980) and prefer to pair with other females (Adkins-Regan, 1999).

2.1.4 Estrogens and the organization of the song system in oscine birds

In many species of songbirds, males use complex vocalisations to attract conspecific females and ward off other males. The neural circuits associated with song are composed of several nuclei found mostly in the telencephalon. These nuclei are linked to form two well characterized circuits involved in song learning and the control of the highly developed syrinx. Females of different species have different signing abilities. For example, while the zebra finch female never sings (Airey & Devoogd, 2000), both male and female bay wrens (*Thryothorus nigricapillus*) participate in intricate song duets (Brenowitz & Arnold, 1985).

Estrogens induce a masculinisation of telencephalic song control nuclei in female zebra finches (Gurney *et al.*, 1980; Simpson *et al.*, 1991) which allows them to sing. The expression of song, however, is greatly improved by androgens (Adkins-Regan & Ascenzi, 1987). Because these effects are not observed when normal females undergo the same treatment as adults, it was postulated that estrogens play an organizational role early during the development of the sexually dimorphic nuclei in the zebra finch and that, after puberty, androgens play an activational role, enabling the production of song (Gurney, 1982). Several attempts have been made to alter the morphology of the male song system using aromatase inhibitors (Wade & Arnold, 1994; Foidart & Balthazart, 1995; Wade *et al.*, 1996; Grisham *et al.*, 1997). To date, however, no laboratories have been able to fully block the development of the song control system in male zebra finches.

From these observations, it would appear that estrogens have the ability to masculinise the male song system and feminise/demasculinise the reproductive tract and the organisation of several nervous circuits involved in copulatory behaviour. This would imply that sexually dimorphic mechanisms exist to render estrogens available to specific target tissues during critical organisational periods while protecting other sensitive sites from exposure to the hormone.

2.1.5 The importance of sex hormones during adulthood

Endocrine disruption during early development results in irreversible alterations of biochemical processes which, in turn, can have functional repercussions through the life of the organism. On the other hand, after the animal has reached sexual maturity, hormones are mostly involved in activational processes that temporarily change the activity of their target tissues.

Many birds that live in temperate climates only breed in spring when the climatic conditions become favourable for reproduction and the survival of their offspring. The sex hormone levels in these species fluctuate according to season and play a key role in preparing the sex organs for reproduction and act on the brain to facilitate the behaviours associated with territory and mate protection, nest building, reproduction, egg laying, incubation and care of the chicks.

Wingfield and Farner (1978) studied the endocrine status of a natural breeding population of white-crowned sparrow (*Zonotrichia leucophrys*). In this species, males are the first to arrive at the breeding grounds and they establish territories before the females arrive. During this period, they exhibit increasing levels of immuno-reactive luteinizing hormone (irLH) and testosterone which peak after the females arrive and pairs form. Females arrive at the breeding grounds with basal levels of irLH and testosterone that increase along with estrone and 5α-dihydrotestosterone (5α-DHT) through the pairing and courtship periods to reach a maximum during incubation. All of these hormones then decline until the period during which the fledglings are being fed. Once the first brood has fledged, a second nest is built and a second brood is raised. With the exception of testosterone, which remains low, reproductive hormones rise, peak during incubation and subsequently decline to levels similar to those of the first clutch.

In female house sparrows (*Passer domesticus*), LH and estradiol (E2) concentrations are maximal during the egg-laying period, they decrease during incubation and rise again during the nestling stage. Testosterone and DHT are highest during the incubation period. In males, the concentration of corticosterone is correlated with high or increasing sex steroid levels (Hegner & Wingfield, 1986).

In the ring dove, vasoactive intestinal polypeptide (VIP) is suspected of stimulating the release of prolactin which is associated with incubation and brooding (Silver, 1990). In male doves that are paired with receptive females, LH, testosterone and DHT rapidly rise during the courtship period and subsequently decline to a minimum prior to the incubation period and remain low through the brooding and feeding offspring periods (Feder *et al.*, 1977; Ramsey *et al.*, 1985). Females also exhibit high LH levels during the courtship period. Ovulation does not occur unless this LH surge is accompanied with a drop in FSH concentrations (Cheng & Balthazart, 1982). Unlike other species, prolactin increases only 7 to 15 days after the start of the incubation period and remains high until the fourth day after the nestlings have hatched. During this period, the crop sac develops and 'milk' is produced to feed the offspring (Goldsmith *et al.*, 1981).

In many species, females have higher circulating estradiol and males have higher circulating testosterone during the breeding season. Numerous studies have shown that circulating testosterone can be metabolised to estradiol, 5α-dihydrotestosterone or 5β-dihydrotestosterone by the brain. These steroids can then act on proximal receptors or can be released into the peripheral circulation (Schlinger & Arnold, 1992; 1993) (Fig. 1). For example, male aggression and song production are promoted by testosterone but are reduced by aromatase inhibitors (Adkins-Regan, 1999; Soma *et al.*, 2000). Subsequent administration of exogenous estradiol reverses this inhibition (Soma *et al.*, 2000). Estrogens have also been associated with increased libido (Balthazart, Reid, Absil, Foidart & Ball, 1995) and copulatory behaviour (Watson, Abdelnabi, Wersinger, Ottinger & Adkins-Regan, 1990) in both male and female Japanese quail. Female Japanese quails injected with estradiol benzoate also exhibit higher sexual receptivity, an effect that is reversed by the administration of the anti-estrogen tamoxifen (Delville & Balthazart, 1987). These roles of estrogens are also observed in song birds. When implanted with estradiol, female song sparrows (*Melospiza melodia*) exhibit sexual vocalisations ("chitters") as well as copulation solicitation at a higher rate than non-treated females (Wingfield & Monk, 1994). These studies suggest that estradiol plays a key role in male

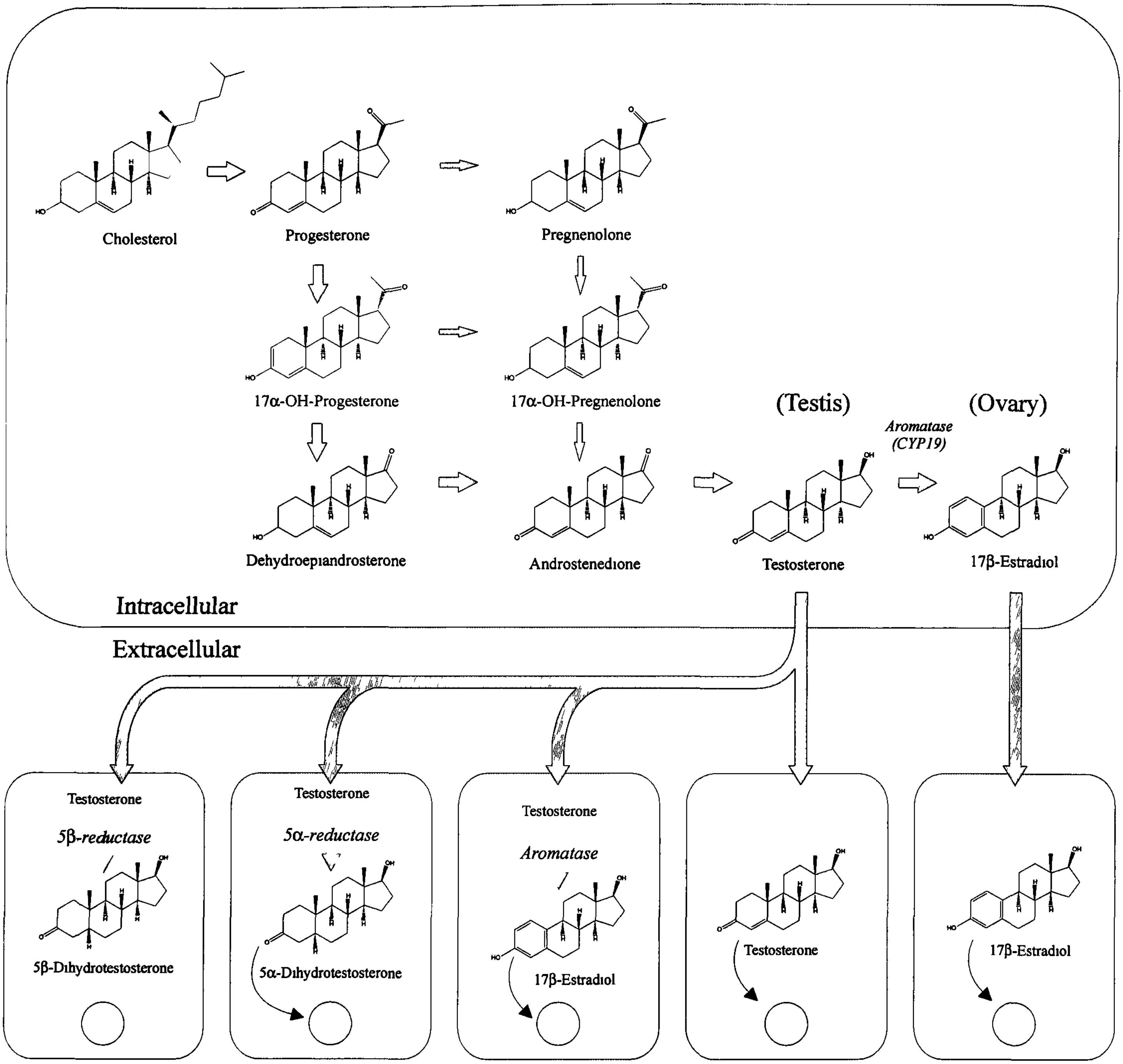


Figure 1. Sex steroid biosynthesis. Testosterone and estradiol are produced from cholesterol by the gonads and are released into the general circulation. After reaching their target tissue, the hormones can either be transformed by cytosolic enzymes or bind to their cytosolic receptor and enter the cell nuclei to initiate de novo protein synthesis. In some tissues, testosterone may be deactivated to 5β-dihydrotestosterone Modified from Hadley (1984).

and female reproductive behaviour. Modulation of sex hormones during the breeding period may lead to abnormal behaviours and altered physiological states that interfere with reproduction.

While circulating hormones play a key role in promoting reproductive behaviours, the sensitivity of target tissues and the activity of hormone metabolising enzymes around the target tissue, are also very important considerations.

The expression of steroid receptors and estrogen metabolising enzymes is modulated by photoperiod and by interactions with conspecifics. For example, in canaries, the number of estrogen receptors in the brain is up-regulated in autumn when circulating estradiol is low. This allows the brain to continue facilitating behaviours like singing and territory defence outside the breeding season (Fusani *et al.*, 2000).

In other circumstances, there is an up-regulation of enzymes or receptors when estradiol levels are highest. This results in a positive feedback that greatly increases the occurrence of important behaviours. For example, the aromatase levels in the caudomedial neostriatum are higher during the breeding season (Fusani *et al.*, 2000). Synapse density and morphology of the hypoglossal nucleus, involved in the control of the syrinx, have also been shown to be influenced by the season and testosterone in this species (Clower *et al.*, 1989).

Differences in target tissue sensitivity to hormones are also observed in sex-role reversed species. Unlike most birds, the spotted sandpiper (*Actitis macularia*) can be polyandrous. Females are the first to arrive at the breeding grounds and fight for territories. When males arrive, they compete for access to a breeding territory and a female. After pairing, nest building, and laying, the female may leave the male, find another territory, and pair with another male (Strokes & Strokes, 1990).

As in other avian species, plasma testosterone and dihydrotestosterone (DHT) in the spotted sandpiper, are higher in males than in females prior to incubation. After pairing, E2 levels are higher in females than in males. Testosterone concentration rapidly declines during egg laying and is always lower in females than in males, even during the period of territorial fights (Fivizzani & Oring, 1986; Gratto-Trevor *et al.*, 1990) .

The Wilson's phalarope (*Phalaropus tricolor*) is another sex-role reversed shore bird. As in the spotted sandpiper, the differences in reproductive behaviour and in parental care are not explained by a reversal of sex hormones in the males and females. Levels of testosterone are greater in non-incubating males and females and are associated with aggressive behaviour during territorial defence and mate courting. Males, which provide all of the incubation and brooding duties (Fivizzani *et al.*, 1990), have higher testosterone and DHT and higher progesterone levels during these periods. Females have testosterone and DHT levels similar to those of incubating males but have higher levels of E2 (Fivizzani *et al.*, 1986).

2.2 Objectives of the present investigation

As reviewed above, estradiol is involved in the activation of reproductive behaviour in female birds and is converted from testosterone to activate singing and agonistic behaviours in males.

This study is intended to identify behaviours modulated by oral exposure to exogenous estrogens that are exhibited by adult zebra finches during the early part of the breeding cycle. Hormone modulated behaviours, if sensitive enough, could eventually be used as endpoints to screen endocrine disrupting chemicals. This is not intended to replace high throughput in-vitro screening assays currently being developed. However, once chemicals with undesirable biological activity have been identified, it will be important to test their impact on the full life cycle of sensitive organisms. This study is designed to contribute to this effort by studying the effects of 17β-estradiol, a positive control for estrogenic xenobiotics, on sexual and agonistic behaviours in the zebra finch, *Taeniopygia guttata.*

The prediction being tested is that exogenous estrogens can affect behaviours linked to reproduction in adult song birds. To test this prediction, the following hypotheses will be tested:

H1. Oral estradiol elevates the plasma concentration of this hormone in male zebra finches;

H2. Oral estradiol affects the sexual preference of male and female zebra finches;

H3. Oral estradiol increases sexual receptivity of female zebra finches;

H4. Oral estradiol increases copulatory behaviour and agonistic behaviour in male zebra finches.

2.3 The zebra finch as a model species

Currently, most avian toxicological studies rely on two game species, the bobwhite quail (*Colinus virginianus*) and the mallard (*Anas platyrhynchos*). There is, however, high heterogeneity in the sensitivity of birds to xenobiotics (Mineau *et al.*, 1994). Altricial birds, which hatch only partially differentiated and rely on their parents for survival, may be at a greater risk of being affected by a wide variety of endocrine disrupting chemicals.

The zebra finches, a relatively easy to breed species of altricial birds, are colonial songbirds originating from Australian semi-arid grasslands. In Australia, they are located in over 75% of the country's area and have adapted to the highly irregular rainfalls of arid and semi-arid regions found in wide areas of the interior of the continent (Zann, 1996). Because the onset or the duration of the rainy period is unpredictable, the zebra finch must be able to respond quickly to environmental conditions favourable for reproduction. Some adaptations include the participation of both parents in nest building, the ability of the birds to reproduce continually and as long as the conditions are favourable, and the short maturation period of the offsprings. Zebra finch males have functional testes 70 days after hatching and can maintain their spermatogenic activity year round, even when the birds become mildly dehydrated (Vleck & Priedkalns, 1985). Females develop ovarian follicles to a pre-egg stage 70-90 days after hatching (Zann, 1996).

Mature zebra finches kept in captivity under favourable breeding conditions exhibit hormonal cycles similar to those observed in temperate climate species (Vleck *et al.*, 1985) (Table 1). Sex steroids are correlated with immunoreactive luteinizing hormone (irLH) and are highest early in the reproductive cycle, during successful mating and the beginning of incubation. Similar trends are observed in several species of birds living in temperate regions (Feder *et al.*, 1977; Wingfield & Farner, 1978; Ramsey *et al.*, 1985; Hegner & Wingfield, 1986; Fivizzani *et al.*, 1986; Fivizzani & Oring, 1986; Fivizzani *et al.*, 1990).

Table 1. From Vleck and Priedkalns (1985). Reproductive hormone levels in Zebra Finches breeding in aviaries.

	irLH ng/ml±SD (n)		Male plasma androgen ng/ml(n)[a]	Female plasma estrogens ng/ml(n)[a]
	Male	Female		
Unsuccessful courtship[b]	0.94±0.25 (9)	0.41±0.18 (10)	0.50 (8)	<0.13 (5)
Successful courtship	1.04±0.38 (10)	0.89±0.50 (9)	0.80 (9)	0.32 (8)
Early incubation	1.24±0.83 (7)	0.70±0.14 (4)	1.06 (4)	0.18 (5)
Late incubation	0.63±0.41 (7)	0.38±0.28 (7)	0.24 (5)	<0.16 (4)
Feeding nestlings	0.49±0.25 (6)	0.79±0.28 (4)	0.25 (4)	0.45 (3)
Feeding fledglings	1.12 (1)	0.77±0.21 (2)	-	-

[a] For the sex steroids, the values are from pooled samples of blood and the sample size indicates the number of birds whose blood was added to the pool.

[b] Unsuccessful courtship refers to birds that actively courted but did not successfully nest during the following week.

2.3.1 Reproductive behaviour of the zebra finch

To test my hypothesis related to anomalies in reproductive and courtship behaviours, a description of normal behaviour is needed. The zebra finch reproductive behaviour is well characterized and is described by Zann (1996) and by Morris (1954).

The courtship of the male comprises two main sequences preceding mounting and copulation. The first one is a waltz of which the first step, usually initiated by the male, is a greeting or courtship invitation. The male flying to and fro, lands next to the female and, his body in a rather stiff and upright posture, bows with the Head Tail Twist. This is followed by the waltz proper consisting of hopping to and fro with bows and beak wipes. At that point the female joins the male in the waltz. When the female stops moving and crouches on the branch, the male proceeds to the second sequence consisting of his song and dance. Standing upright, twisting his head and tail towards the female, he sings to her. The female does not face him but lowers her body across the branch. The male approaches her turning himself from side to side.

After some time, the female crouches lower and performs an important component of her courtship: tail-quivering in up and down vibrations and, simultaneously, lowering her head slightly. This last behaviour on the part of the female seems to constitute the invitation to the male who stops singing, mounts her and copulates. After this, the male may perform tail-quivering himself.

Some of these stereotyped courtship and copulatory behaviours can be exaggerated or omitted by individual birds. Morris (1954) stipulates that those variations are the "result of varying strengths, both relative and absolute, of the three drives to attack, flee and mate, which are present in both (male and female) birds". When the birds are stressed by a social encounter, they may also exhibit an out-of-context behaviour. These "irrelevant" reactions are called displacement behaviours. In zebra finches, these include foraging, feeding, autopreening and beak wiping (Morris, 1954).

2.4 Route of exposure

To date, studies conducted to monitor the effects of exogenous hormones have used various implants or injections as a method of administration. I have chosen to dose my birds with 17β-estradiol (E2) in their diet to minimize handling. This also ensured an experimental environment that is relevant to field exposure conditions. However, this route of exposure has some drawbacks. In my colony, the bird's normal diet was composed of millet supplemented with mealworms and a commercial vitamin and amino acid preparation dissolved in their drinking water. The millet seeds are covered with a cuticle that is cracked open and rejected by the bird. Because the whole seed is not eaten, it is difficult to precisely quantify the amount of bioavailable estradiol to the bird. Several generally unsuccessful attempts were made to dose the birds in "treats" but my zebra finches were reluctant to eat novel food.

One exception was that mealworms which were consumed by the majority of the birds. I attempted to inject estradiol into the worms but the oil carrier came out of the hole left by the needle.

I finally decided to administer estradiol in the millet. To do this, estradiol was dissolved in 99% ethanol. The solution was poured over millet into a shallow pyrex container that was subsequently sealed with a cellophane sheet. This container was gently rocked for a period of 24 hours. The ethanol was then allowed to evaporate under a fume hood.

Since the millet was the only source of food, I monitored the palatability of the treated diet to make sure the birds accepted it. There were no differences in food consumption between treated and untreated diet.

I confirmed that estradiol penetrated the seed shell by conducting the above steps with 125 000 DPM tritiated estradiol. After the evaporation of the ethanol, the shells of approximately one gram of millet was manually removed from the seeds. These seeds were weighed and homogenized. The same procedure was done with unshelled seeds and the radioactivity of the homogenates was measured. Under these conditions it was established that 32% of the estradiol mixed in the ethanol was in the whole seed, and 16% was in the seed under its shell.

2.5 Estradiol dose

I wanted to use a dose that could elevate the plasma estradiol concentrations of males to a high physiological level, one that was known to produce behavioural or physiological changes in birds.

The maximal zebra finch plasma estradiol concentrations reported in the literature range between 450 pg/ml for females feeding their nestlings (Vleck *et al.*, 1985) to 690 pg/ml in non-laying females (Hutchison *et al.*, 1984). Estradiol concentrations remain relatively constant between 100 and 200 pg/ml in adult zebra finch males (Pröve, 1983) but may reach higher concentrations. An estradiol concentration of 360 pg/ml was measured in isolated males (Hutchison *et al.*, 1984).

My high estradiol dose was 720 μg estradiol/g millet, and my low dose was 4700 ng estradiol/g millet. I estimated that approximately 16% of the estradiol was in the grain that was eaten by the birds. The ingested doses were therefore ,respectively, estimated to be 115 μg estradiol/g millet, and 750 ng estradiol/g millet. Since the finch ate an average of 3.5 g millet per day, it was estimated that the birds from the high estradiol group ingested approximately 400 μg estradiol per day and the birds from the low estradiol diet 2.6 ng estradiol per day. These exposures fall in the same range as those used in other studies. For example, Williams (1999) found that immature zebra finch females injected with four 20 μg estradiol i.m. had elevated levels of yolk precursors similar to breeding females. This treatment would result in dose of 80 μg estradiol per bird via a route of exposure that bypasses hepatic first pass metabolism.

3 Materials and Methods

3.1 Aviary

The zebra finches were originally obtained from Flikkema Aviaries but were subsequently bred in my colony in the animal care facility of the University of Ottawa. The birds were kept in an aviary maintained at a constant temperature of 22°C and a long daylight regimen (14L:10D). Adult birds were initially grouped in unisex cages equipped with numerous perches, nests, cuttlefish bones and a large water bath. Food and water were provided *ad libitum*. Water and food dishes were cleaned on a daily basis.

Birds used for the measurement of plasma estradiol during the breeding cycle were housed in Hagen canary breeding cages. These cages measured 30 cm wide x 30 cm high x 60 cm long and were equipped with perches, a cuttlefish bone, a commercial bamboo finch nest and some cotton nesting material. Pairing was done semi-randomly by picking birds from the unisex group cages while the lights were out. In these conditions, the birds remained calm and stress was kept as low as possible.

Birds used in the behavioural tests were housed under the same conditions as those used for the plasma estradiol determination, except that there was only one bird per cage.

The birds were fed a commercial finch diet composed primarily of millet (Hagen or Bulk Barn). A vitamin and amino acid supplement (Prime, supplied by Hagen) was mixed with the drinking water. Mealworms were offered to the birds once per week.

3.2 Estradiol radioimmunoassay

3.2.1 Optimization of the assay

To test the first hypothesis that oral estradiol elevates the plasma concentration of this hormone in male zebra finches, I optimised and validated an estradiol radioimmunoassay (RIA) that was originally developed by McMaster *et al.* (1992).

The first step in the development of the RIA is the quantification of the amount of antibody that will be used (Fig. 2). This was done by serially diluting the antibody and incubating it with a given volume of label. Because the zebra finches are small (13 g), it is only possible to sample small volumes of blood from the birds and, consequently, the estradiol label volume must also be small. In this study, I used 20 μl of ^{125}I-estradiol, which corresponds to approximately 9300 counts per minutes (CPM). For this experiment, the incubation was conducted for 90 min at a temperature of 37°C. I subsequently conducted a series of incubations to determine the optimal incubating period and temperature. This was done by incubating 20 μl of label with 100 μl of the antibody for various periods of time at 4°C, 25°C, and 37°C (Fig. 3).

Sex steroids are lipophilic and are transported in the blood bound to proteins. Contrary to amphibians, reptiles and mammals, birds appear to lack specific high affinity binding globulin for testosterone and estradiol (Wingfield *et al.*, 1984; Deviche *et al.*, 2001). These sex hormones are believed to travel bound to albumin and, testosterone possibly also to corticosterone binding globulin. These proteins can interfere with the radioimmunoassay by competing with the antibody for the estradiol. This would lead to an overestimation of the sample's hormone concentration. For this reason, plasma samples were mixed with organic solvents to separate the steroids from aqueous proteins (Wingfield & Farner, 1975; McMaster *et al.*, 1992). The organic fraction is then re-suspended in a matrix that is also used to build a standard curve. In this investigation, the efficiency of the extraction and resuspension procedure was confirmed by quantifying the recovery of tritiated estradiol spiked into a plasma sample. The average activity of the resuspended samples was 115 DPM and that of the control was 114 DPM, suggesting that all the steroids were extracted and resuspended.

A test for the linearity of dilution can be used to screen for the presence of interfering compounds in the prepared plasma samples. The estradiol concentrations of samples diluted with the matrix should be constant when multiplied back by their dilution factor. If there is a component of the matrix that interferes with the assay, the estimated estradiol concentration will be biased (McMaster *et al.*, 1992). Because I expected plasma estradiol concentrations around 200 pg/ml in adult male zebra finches, I spiked the plasma sample prior to dilution to prevent the estradiol levels from dropping below the detection limit of the assay (40 pg/ ml). A 1 ml zebra finch plasma sample was spiked with 400 pg/ml estradiol. The estradiol was extracted from the sample with ethyl acetate hexane (3:2) and was re-suspended in phosgel. Phosgel was used to dilute the steroid resuspension.

The accuracy of the radioimmunoassay can be tested by adding known amounts of unlabelled estradiol to a prepared sample. The expected estradiol concentrations can then be compared to the measured concentration. Finally, the plasma estradiol concentrations of untreated zebra finches should correspond to those reported in the literature.

3.2.2 Reagents

The estradiol antiserum and the ^{125}I-17β-estradiol label were purchased from ICN Biomedicals (catalog no: 07-138216 and 07-138226).

A dextran-coated charcoal suspension was used to separate the estradiol and the antibody-estradiol complex from the assay mixture at the end of incubation: 0.55g dextran-coated charcoal into 100 ml phosgel.

Phosgel:

5.75g Na_2HPO_4, 1.28g $NaH_2PO_4*H_2O$, 1 g gelatin, 0.1g Thimerosol, 800 ml distilled H_2O. Heated at 50°C until gelatin is dissolved and cooled. Adjusted to pH 7.6 and diluted to 1L with distilled H_2O.

Standards:

The standards were made fresh for each assay by serial dilution of a 5000 pg/ml stock estradiol dissolved into phosgel. The stock was kept in aliquots at –20°C.

3.2.3 Blood sampling

The plasma used for the estradiol radioimmunoassay validation was obtained from adult zebra finches. Because the birds are small and only small volumes of plasma can be obtained from single birds, the plasma from several birds was often pooled. This allowed me to use a stock of homogenous plasma for my experiments.

Birds used to build a pooled stock were sampled by cardiac puncture under isofluorane anesthesia with heparinised syringes. Good samples yielded between 700 µl and 1100 µl of blood.

The blood was slowly placed into a labelled centrifuge tube and put on ice. When all birds were sampled, the blood was centrifuged at high speed for 2 min. Plasma samples were pooled and vortexed in sterile plastic tubes and frozen at –20°C in aliquots.

3.2.4 Extraction of steroids from plasma

A plasma sample was pipetted into a new unused 8 x 75 glass test tube and the volume was adjusted to 200 µl with distilled water. 200 µl ethyl acetate:hexane (3:2) were added and the tube was vortexed vigorously for 60 sec. The samples were allowed to rest for at least 10 minutes to allow the organic and aqueous fractions to separate. Ethanol supercooled with dry ice was used to rapidly freeze the aqueous fraction and allow the transfer of the organic phase into a clean tube. The extraction procedure was repeated twice. The organic phase was evaporated at 40°C under nitrogen.

3.2.5 Resuspension of plasma extract

Once the organic phase was evaporated, 200 µl phosgel was added to the test tube that contained the dry steroid extract. The sample was vigorously vortexed for 1 min, covered with paraffin, heated at 40-50°C for 10 min, and vortexed for another min.

3.2.6 Incubation of reagents

On the first day of this two day process, the radioimmunoassay was carried out using 50 µl antibody, 20 µl standard / or the complete volume of steroid resuspended in phosgel. The volume of all tubes was adjusted to 300 µl with phosgel and 20 µl ^{125}I-estradiol was added. Care was taken to always use the same order when adding the reagents to the assay tubes. The samples were incubated overnight.

The next day, the samples were cooled on ice for 10 min and the dextran-coated charcoal suspension was placed in a water and ice filled beaker and stirred with a magnetic stirrer to keep the charcoal in suspension.

To stop the incubation, 200 µl of charcoal suspension were added to the number of sample tubes that could fit into the centrifuge. The tubes were closed and the whole rack was shaken vigorously for 20 sec. The samples were allowed to rest for 20 min at 4°C and were centrifuged at 2500 rpm for 15 min at 4°C. The samples were then placed on an ice tray, 400 µl of the supernatant were transferred to gamma counter tubes and their activity was immediately measured.

The standard curve was calculated from the raw data by plotting the percent bound estrogen as a function of the estradiol concentration on a semi-log scale. Sample estradiol concentrations were estimated from the linear regression equation.

3.3 Estradiol concentration in breeding zebra finches

The blood samples were drawn from the jugular with a 26 G heparinised needle. Approximately 100 µl blood were drawn at one time. The blood samples were kept on ice and manipulated as described in the above radioimmunoassay section.

Male and female zebra finches were blood sampled and transferred from unisex group cages into individual smaller cages equipped with nests and nesting material. Blood samples were subsequently taken from each bird at various times during the breeding cycle. The first sample was collected prior to pairing the birds. The second sample was taken when the first egg was laid (end of nest construction); the third when uninterrupted incubation started (both sexes participate in incubation, approximately four eggs are found in the nest, and the eggs feel warm to the touch); the fourth during nestling care, when the offsprings hatched; and the fifth on the day the first offspring fledged from the nest.

3.4 Elevation of plasma estradiol

The blood of 16 male zebra finches was sampled on day 0, prior to treatment and the birds were housed four to a cage. From day 2 to 8, eight of the males were fed with estradiol treated diet (720 µg/g) and the other eight were fed with ethanol (vehicle) treated diet. On day 8, a second blood sample was taken from the birds and their diets were changed to untreated millet for a period of 8 days (until day16). On day 16, a third blood sample was taken from the birds. The blood samples collected during this experiment were taken to estimate the plasma estradiol levels in the treated males that would be used in the behavioural experiments.

3.5 Effects of dietary estradiol on behaviour

This second part of my investigation aimed at testing the second, third, and fourth hypothesis, each of which has to do with the effects of dietary estradiol on behaviour.

3.5.1 Experimental design

The birds were randomly assigned to one of three groups which were fed different diets during the treatment period. This was done by first transferring male and female adult zebra finches from unisex group cages to smaller cages (60 cm long x 30 cm wide x 40 cm high) and subsequently assigning them a diet treatment using random numbers generated with Excel. Birds from the control group were fed millet treated with ethanol (vehicle). The low estradiol group was fed millet treated with 4.7 μg estradiol/g millet and the high estradiol group fed millet treated with 720 μg estradiol/g millet. There were eight birds of each sex per group.

The two tests used, the "two-choice preference test" and the "daily pair test", were based on those developed by Adkins-Regan & Ascenzi (1987). These tests as they were performed, aimed at placing the birds in contexts that triggered their sexual and territorial behaviours. The behaviours that were recorded during the daily pair test are presented in Table 2.

The birds were allowed to acclimatize to the holding conditions for at least two weeks before testing. Both tests were conducted on the same day for each tested bird to minimize differences in the exposure period between tests. Birds from the alternate treatment groups were tested sequentially. The birds were tested three times before and three times after their diet was changed. To allow the birds to eat, testing started approximately 3 h after the lights were switched on.

3.5.2 Two-choices preference test

This test was modified from Adkins-Regan *et al.* (1996). The two-choice preference test was performed by placing the tested bird in the middle of three cages. This cage was equipped with two perches situated at the opposite extremities of the cage. The adjacent cages housed respectively, a group of 6 stimulus females and a group of 6 stimulus males. The time spent in proximity of each stimulus group was monitored for a period of 30 min with the help of a data acquisition system (computerboards' digital board model CIO DIO24) that detected the presence of the bird on the perches. After the test, a score was obtained by subtracting the time spent near the same sex stimulus from the time spent near the opposite sex stimulus. There were four setups like this in the room, and each test cage was visually isolated from the others. To control for the effect of the room, half of the male stimulus bird cages were placed on the far side of the room and half were placed nearer to the door. The tested birds were never tested twice in the same setup. Two birds from one treatment group and one bird of the other two groups were tested at the same time. In this way, the treatment groups alternated between setups. This also allowed to control for the effect of the time of day.

3.5.3 Daily pair test

The subjects were tested in their individual cages in an area visually isolated from the rest of the colony. Two wooden nest boxes equipped with trap doors were installed on the test cage to house stimulus birds. The test began when the experimenter, hidden behind a blind, remotely activated the trap door to allow the first stimulus bird to enter the cage. The first stimulus bird was always of the sex opposite to the tested bird. A series of behaviours were recorded with the help of a laptop computer for a duration of 6 minutes (Table 2). At the end of this period, the second stimulus bird was allowed in the cage and the behaviours exhibited by the tested bird recorded. The second stimulus bird and the tested bird were of the same sex. The behaviour of the birds was recorded with the use of a software called *The Observer*, version 2.0, by Noldus Information technology. The compilation of the data was done with Microsoft Excel. For statistical analysis, beak chatter and threats were considered the same behaviour and are presented as threats. Undirected song was not observed during the tests and was not presented in the result.

Table 2. Behavioural measures (Categories: **S**: sexual, **A**: agonistic, **D**: displacement; **Du**: duration recorded, **F**: frequency recorded; **MT**: male typical, **FT**: female typical) (Modified from (Adkins-Regan *et al.*, 1987; Balthazart *et al.*, 1994; Morris, 1954)

Behaviour pattern	Category	Du/F	MT/FT	Description
Directed song	S	Du	MT	Song observed while bird is dancing or sitting close to and facing the stimulus female.
Undirected song	S	Du	MT	Song to no obvious targets.
Dancing	S	Du	MT	Hopping along a perch toward the stimulus bird in an erect posture, reversing direction on the perch with each jump.
Mount and Mount attempts	S	F	MT	The bird attempts and eventually succeeds in landing with both feet on the back of the stimulus bird.
Cloacal contact movements	S	F	MT	The experimental subject, after a successful mount, lowers its tail and tilts it under the tail of the female to attain cloacal contact.
Beak wiping	S	F	MT	Occurs after feeding, drinking, aggressive encounters or during courtship. Recorded only in a sexual

				context (not recorded if coming immediately after eating or drinking).
Tail quivering	S	F	FT	Horizontal posture with tail vibration. Tends to indicate female receptivity (proceptive copulation solicitation posture).
Accepts mount	S	F	FT	Stays in an horizontal position and allows male to mount.
Rejects mounts	S	F	FT	Flies away or fights the attempted mount.
Auto-preening	D	F		Preening of own plumage.
Foraging	D	Du		Moves around the bottom of the cage pecking at the litter.
Feeding	D	Du		Feeding or drinking
Beak chattering	A	F		Horizontally extended position with rapid beak movements.
Beak fencing	A	Du	MT	Two finches rapidly and repeatedly thrust their beaks together.
Threatening	A	F		Horizontally extended position, often followed by chase.
Pecks and chases	A	Du		Aggressive acts often observed in sequence.

3.6 Statistical analysis

3.6.1 Estradiol concentration during the breeding cycle

The Kruskal –Wallis exact test was used to test for differences in plasma estradiol concentration in male and female zebra finches during different periods of the breeding cycle.

3.6.2 Estradiol elevation

The Wilcoxon test for two related samples was used to compare the plasma estradiol concentration before treatment and on day 8 after treatment. The Friedman test for repeated measures was used to compare the plasma estradiol concentration on day 0, 8 and 16 for the second part of this study. A one-tailed test was used since an elevation of plasma estradiol was expected. Levine's test for homogeneity of variance was used to compare the variance of the residuals.

3.6.3 Two-choices preference test

A Friedman exact test was used to confirm that there were no significant differences in the three pre-treatment time scores. To account for intra-group (between subject) variation, the pre-treatment data were averaged and subtracted from each of the post-treatment values. The Kruskal-Wallis exact test for multiple independent samples was used to test for differences among the corrected values of groups. If a statistical difference was found, the Mann-Whitney U test was used for pair-wise comparison between the control group and each of the treatment groups. A Bonferroni adjustment was used to adjust the alpha level.

3.6.4 Behaviour quantification

Sexual, agonistic, and displacement behaviours were quantified with the daily pair test. As described above, the test was first performed with the opposite sex stimulus bird alone and subsequently the test was repeated after allowing the same sex stimulus into the cage. These two scenarios represent distinct social environments under which sexual and agonistic behaviours can be exhibited. However, given the short duration of the test, some of the behaviours were rare and often not performed by all birds. For this reason the results from the two parts of the daily pair test were analysed together.

Initially the study was designed so that the result could be analysed with a two way repeated measure ANOVA. However, preliminary data analysis revealed that some of the ANOVA assumptions were being violated. Because of the nature of the data, I decided to use non-parametric exact tests for the analysis. These exact tests were performed with Cytel's *StatXact 4* statistical software which allows one "to make reliable inferences by exact and Monte Carlo methods when (the) data are sparse, heavily tied, or skewed, and the accuracy of the corresponding large sample theory is in doubt" (Cytel Software, 1998). The exact p-values are calculated by first computing the appropriate statistical test with the observed data and subsequently repeating the test with every possible combination of the variates (Manly, 1997). "The exact p-value is the sum of the exact probabilities of those outcomes in the reference set that are at least as extreme as the one actually observed" (Cytel Software, 1998).

3.6.4.1 Number of birds performing the behaviours

The Kruskal–Wallis exact test was used to analyse between group differences during each testing day (Fig. 9, 12, 15, 18, 21, and 24).

3.6.4.2 Behaviour measures

A Friedman exact test was used to confirm that there were no significant differences in the three pre-treatment measures of the behaviours quantified in the investigation. To account for intra-group (between subjects) variation, the pre-treatment data was averaged and subtracted from each of the post-treatment values. These **corrected** values were then transformed and tested (see below).

The behaviours were categorized as sexual, agonistic or displacement behaviours (Morris, 1954; Zann, 1996). The behaviours within a category occurred in different scales (frequency or duration), and the occurrence of each behaviour varied from bird to bird and were sometimes low, yielding several individual zero scores. Because of this, the raw data was transformed into standardised scores (z) that have an equal mean and variation. The separate behavioural scores can then be presented graphically side by side on the same figure (Fig. 10, 13, 16, 19, 22, and 25). This allows for direct comparison of changes in the various behaviours within and between treatment groups. This transformation also allows me to combine the behaviours from each category into a single composite score (Martin & Bateson, 1993, p. 123) that represents the overall sexual, agonistic or displacement behaviour of each subject (Fig. 11, 14, 17, 20, 23, and 26).

The standardisation of data is routinely performed during statistical analysis to compare data with standard curves (ex. with the normal curve). The z score was calculated for each **corrected** behaviour datum.

For each behaviour, the standardisation of the **corrected** data was done by subtracting the mean **corrected** measure of the three groups of birds from each datum and then dividing by the standard deviation (Equation 1). This transformation was done with the *SPSS* statistical software.

$$z = \frac{x - \mu_{(time\,a)}}{\sigma_{(time\,a)}}$$ **Equation 1**

Before calculating the composite scores, different weights were given to each of the sexual behaviours in order to emphasize biologically important behaviours that are more likely to affect reproductive success. This was done by multiplying the *z* scores with the weight specified in Tables 3 and 4. The composite scores were subsequently obtained by taking the average of the weighted standardised scores for each behaviour within the categories.

Table 3. Weights given to the male sexual behaviours for the calculation of the composite sexual scores.

Behaviour	Weight
Directed song	1
Beak wipes	2
Dancing	3
Mounts and mounts attempts	4
Cloacal contact movements	5
Tail quivering	2

Table 4. Weights given to female sexual behaviours for the calculation of the composite sexual scores.

Behaviour	Weight
Beak wipes	1
Accepted mounts/attempted mounts	3
Tail quivering	2

The Kruskal-Wallis exact test for multiple independent samples was used to test for differences between the standardized scores of groups during each of the post-treatment test sessions (days 4, 6, and 8). If a statistical difference was found, the Mann-Whitney U test was used for pair-wise comparison between the control group and each of the treatment groups. A Bonferroni adjustment was used to adjust the alpha level. These tests were done on each behaviour and on the composite scores.

The data are presented as the mean +/- S.E. of 8 birds. The probabilities reported are two-tailed.

4 Results

4.1 Estradiol radioimmunoassay

The antibody dilution curve (Fig. 2) was constructed using 4000 CPM ^{125}I-estradiol at a temperature of 37°C . The optimal antibody concentration should bind between 30% and 50% of the label. Under the present assay conditions, the optimal antibody volume was between 75μl and 150μl. This study used 100 μl of antibody.

The time needed for the assay to reach equilibrium was estimated by incubating the label with 100 μl of the antibody for various periods of time at 4°C, 25°C, and 37°C (Fig. 3). The fraction of label bound to the antibody rapidly increased during the first few hours and stabilized at approximately 40% for the assays conducted at 25° and at 37°C. The assays conducted at 4° were more variable and did not appear to reach a plateau after a 20 h incubation period. The assay in my study were conducted at room temperature with an incubation period of at least 16 h.

A sample standard curve built under the above conditions is presented in Fig. 4. The linear range of the curve lies between 40 pg/ml and 2500 pg/ml. The ED50 is approximately 300 pg/ml. Note that the curve has the expected sigmoid shape and has a low accuracy outside the linear range.

The next step in the validation of the assay was to test for interfering compounds in the matrix by serially diluting a prepared zebra finch plasma sample. The results presented in Table 5 represent the estradiol concentrations multiplied by their dilution factor.

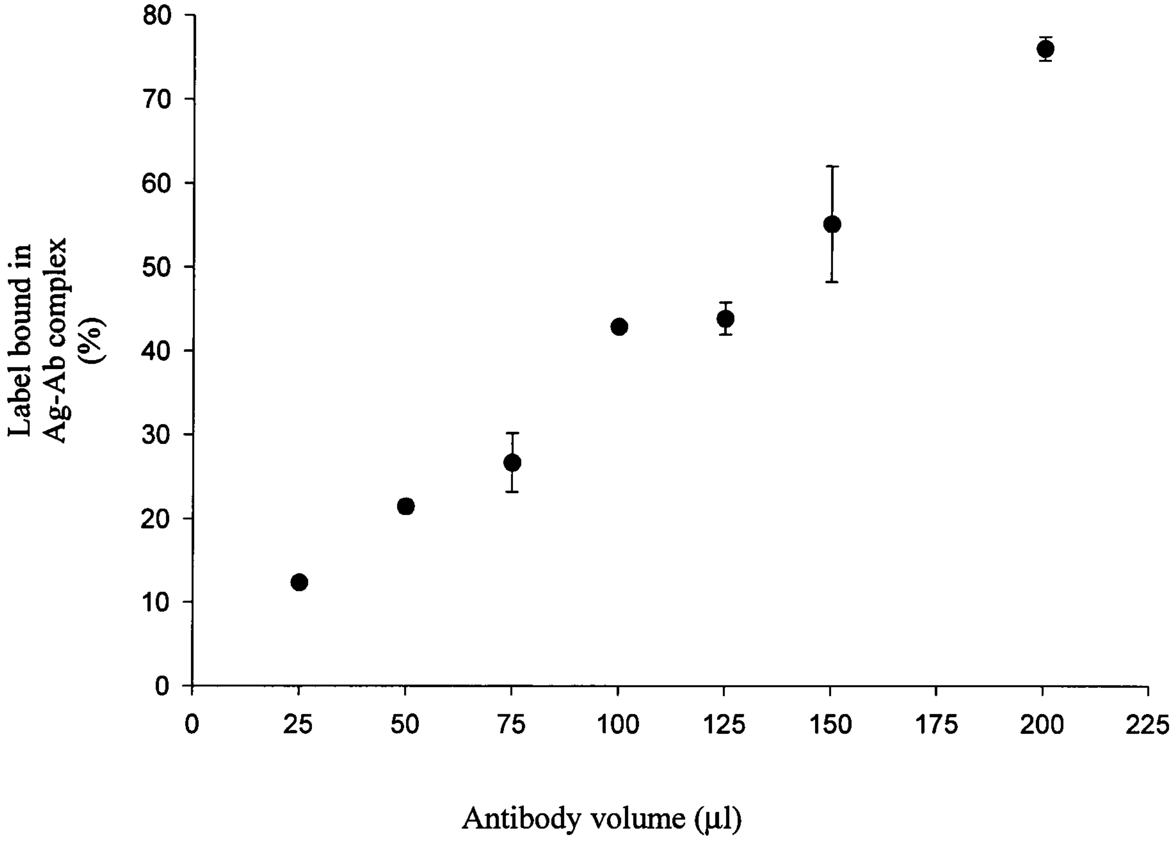


Figure 2. Antibody dilution curve. A range of antibody volumes were incubated with 20 µl ^{125}I-estradiol (4000 CPM) for 90 min at 37°C. The antibody concentration to be used in the assay should bind between 30% and 50% of the label. The experiment was repeated twice with duplicate samples. Data therefore represent the mean ± standard error, N=4.

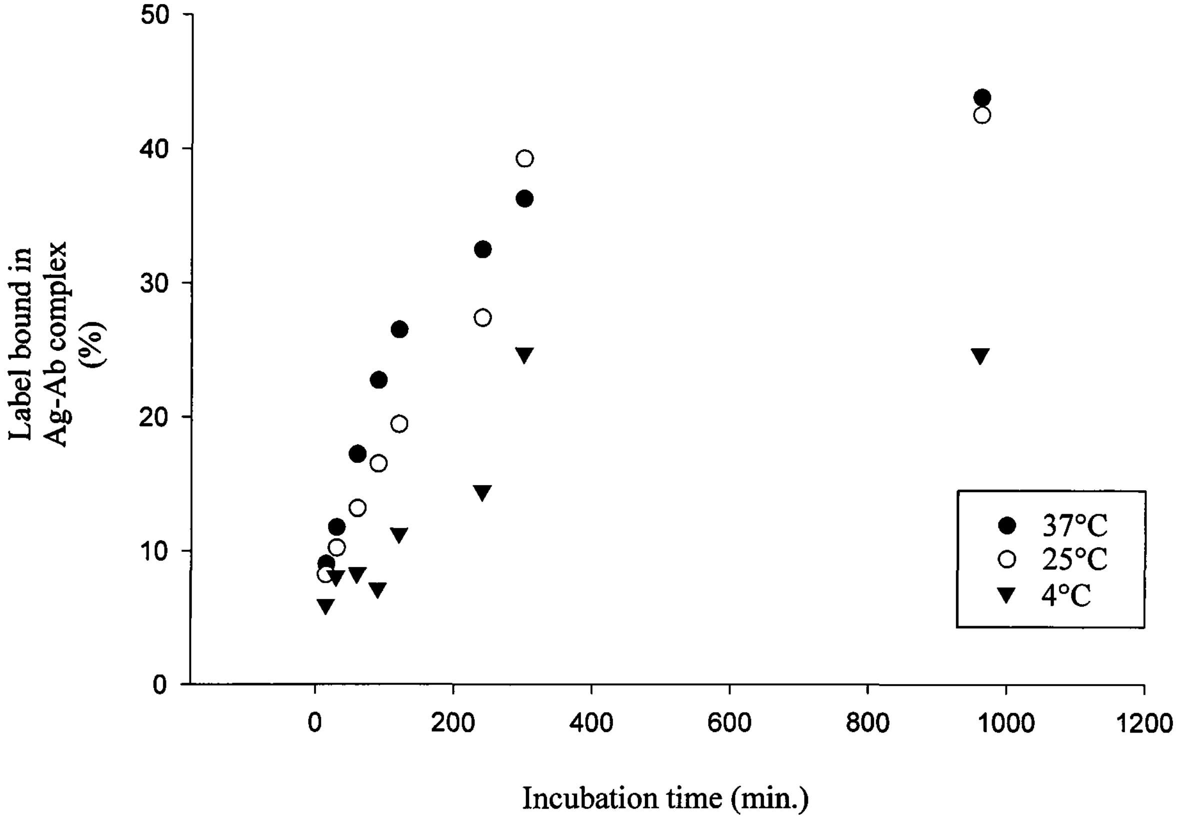


Figure 3. Saturation of the estradiol antibody incubated at different temperature. 20 µl ^{125}I-estradiol (9300 CPM) was incubated with 100 µl antibody in 500 µl standard steroid diluent for different periods of time. The equilibrium is reached after a 16 h incubation at 25° or 37°C. Each point represents the mean of 2 samples.

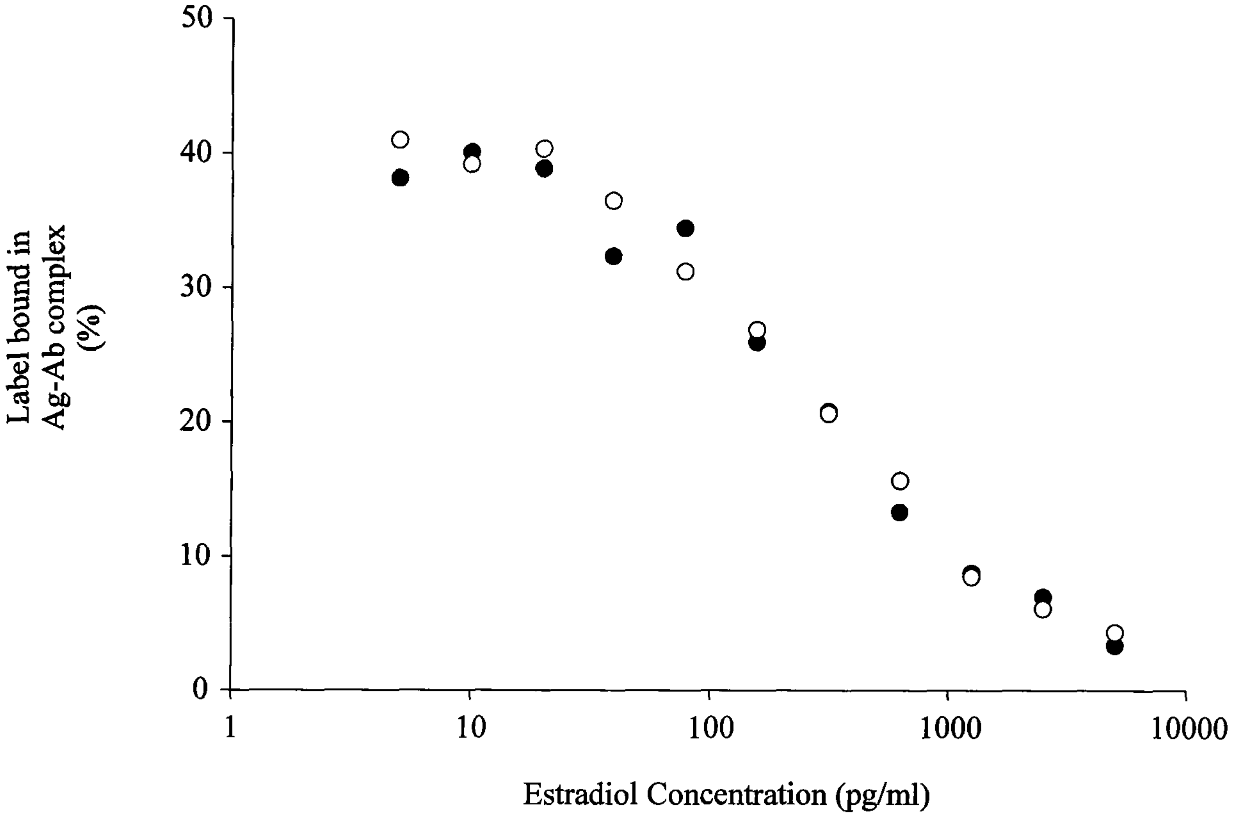


Figure 4. Standard curve for the estradiol radioimmunoassay. Incubation time was 20 h at 25°C; 100 μl estradiol antibody, 20 μl standard and 20 μl ^{125}I-estradiol (9300 CPM). The value obtained for each duplicate is presented.

Table 5. Prepared zebra finch plasma samples diluted with phosgel. The results represent the sample estradiol values multiplied back by the indicated dilution factor.

Dilution Factor	1	1.33	2	4
Estradiol Concentration (pg/ml)	597	614	620	617
S.E.M.	10	6	12	6

The precision of the assay was tested by spiking a plasma sample with known concentrations of estradiol and comparing the resulting data with expected values (Fig. 5). The initial plasma concentration was 190 pg/ml, which falls in the expected range for this species. The estradiol concentrations measured in spiked samples corresponded to the expected values and confirmed that estradiol can be measured accurately within this range.

4.2 Estradiol concentration during the breeding cycle

Plasma estradiol concentrations of male and female zebra finches were similar at each period of the breeding cycle (Fig. 6). There were no statistical differences in the plasma estradiol concentrations among the different periods of the breeding cycle. The variability of plasma estradiol was high during the nest building and incubating period in both males and females and also during the nestling care period in males.

4.3 Elevation of plasma estradiol

The effects of oral estradiol on the plasma concentrations of this hormone were quantified (Fig. 7). For the control group (A), the pre-treatment plasma concentration of the eight males averaged around 40 pg/ml. The average plasma concentration of the

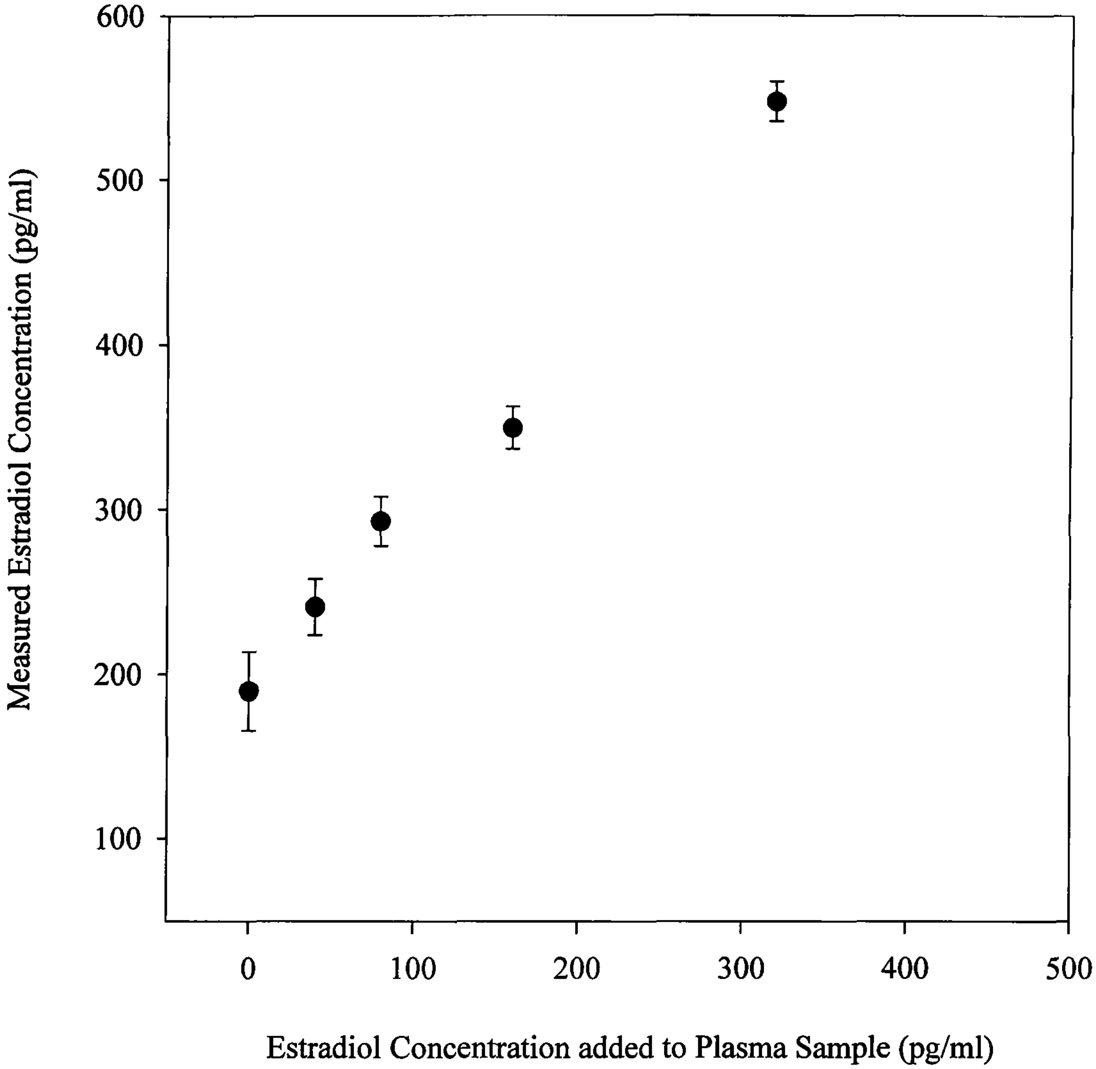


Figure 5. Accuracy test. Prepared plasma samples were spiked with 0, 40, 80, 160 or 320 pg/ml estradiol. The measured estradiol concentrations correspond to the plasma estradiol concentration plus the added estradiol concentration. Each point represents the mean ± S.E. of 4 samples prepared from the same zebra finch plasma pool.

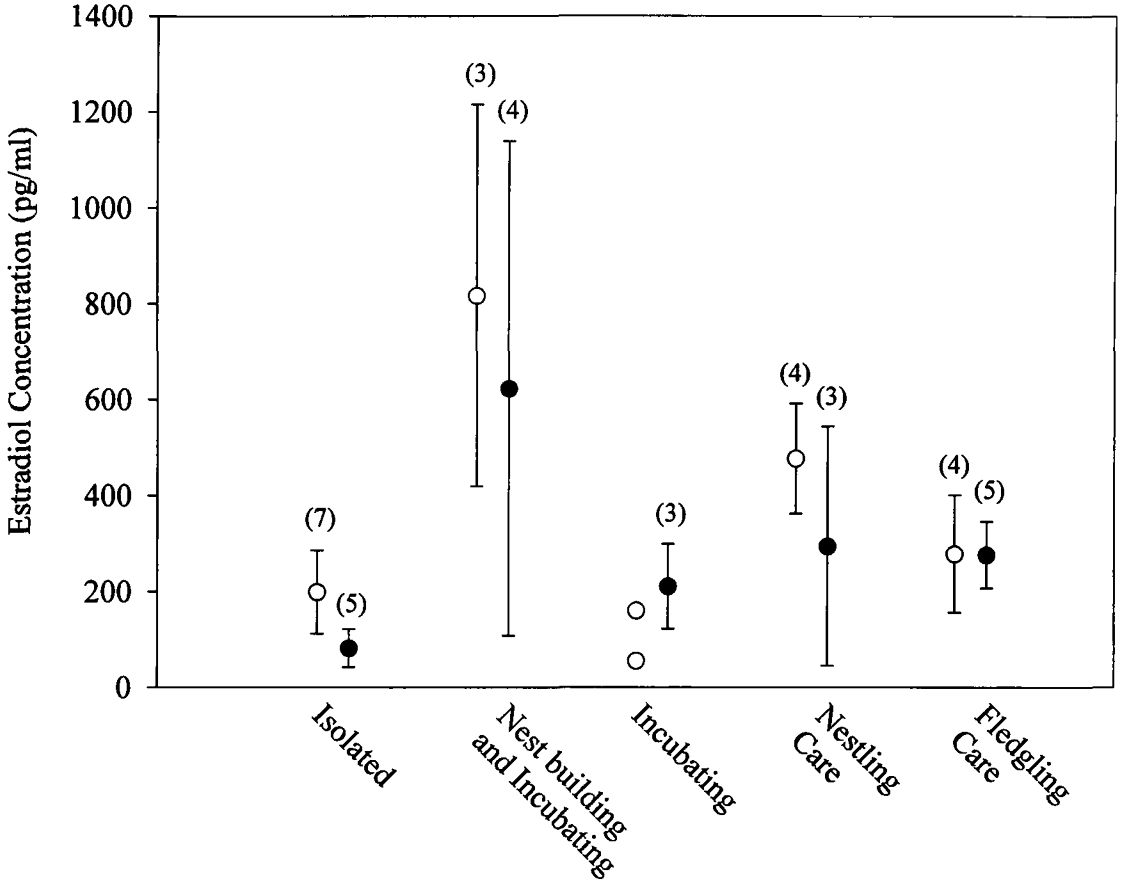
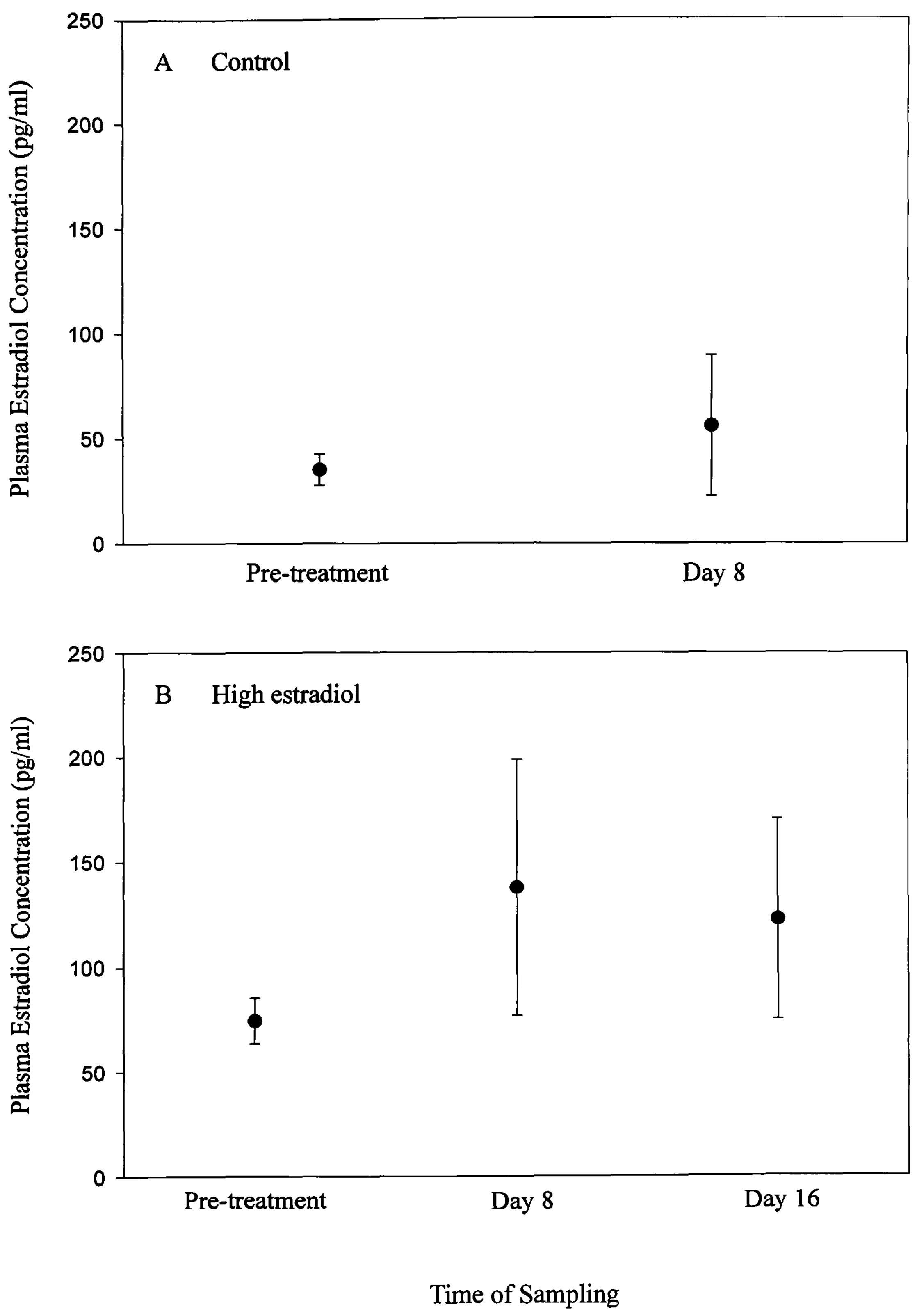


Figure 6. Estradiol concentration in male (unfilled symbols) and female (filled symbols) zebra finches during different periods of the breeding cycle. There were no statistically significant differences among the estradiol concentrations of the different life stages in either males (KW, P = 0.28) or females (KW, P = 0.31). Mean ± S.E. when N > 2 (N is given above each data point). Actual data points are given when N = 2.

Figure 7. Elevation of plasma estradiol in male zebra finches. The blood of 16 male zebra finches was sampled on day 0 prior to treatment, and the birds were housed four per cage. From day 2 to 8, eight of the males were fed with estradiol treated diet (720 μg /g) and the other eight were fed with ethanol (vehicle) treated diet. On day 8, a second blood sample was taken from the birds and their diets changed to untreated millet for a period of 9 days (until day17). On day 16, a third blood sample was taken from the birds. (mean ± S.E., n = 8)


A Control
B High estradiol
Plasma Estradiol Concentration (pg/ml)
0
50
100
150
200
250
Pre-treatment
Day 8
Day 16
Time of Sampling

same birds that were fed ethanol treated diet for eight days was 53 pg/ml. The change in plasma concentration was not statistically significant.

In the second group (B), the average estradiol concentration was 74 pg/ml before treatment. Eight days after being fed a diet treated with the high estradiol concentration, the average plasma estradiol concentration rose to 138 pg/ml. The average plasma concentration was 122 pg/ml on day 16, eight days after the group's diet had been changed back to untreated diet. Note the high variability in plasma concentration after treatment. There are no statistical differences in plasma estradiol concentrations among the three measurements. Levine's test showed that the variance on days 0, 8 and 16 were unequal.

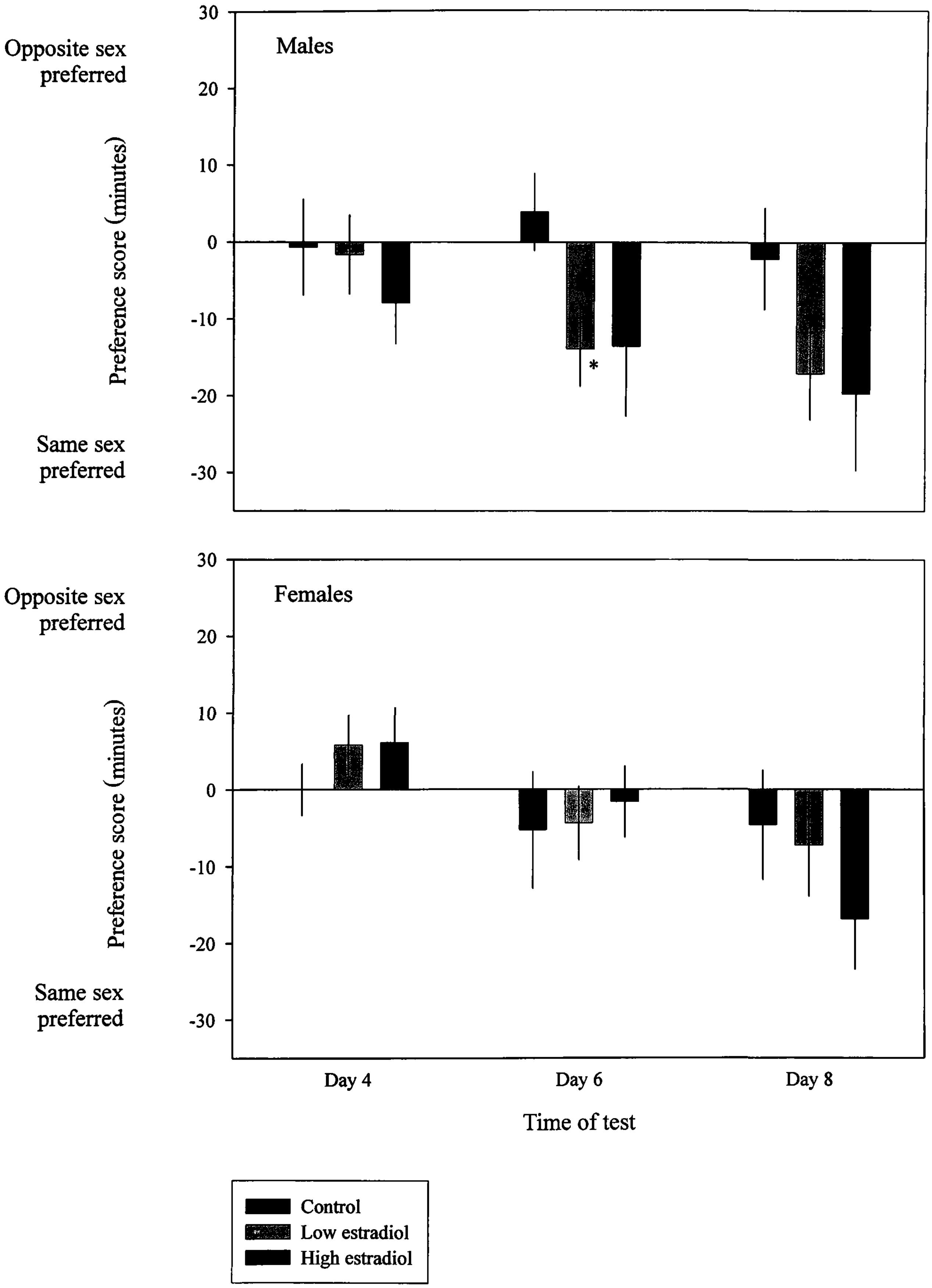
4.4 Behaviour data

4.4.1 Two choices preference test

The preference score of males appeared to decrease with time in the low and high estradiol groups (Fig. 8). There was a significant difference between the control group and the low estradiol group on day 6 (MW U: $P = 0.01$). A negative score is obtained when the birds spend more time on the male side during the post-treatment test than they did during the pre-treatment test (on average). Males treated with estradiol spent more time on the male side of the cage.

The opposite trend was observed in females. The two groups fed estradiol spent more time on the male side of the cage on day 4 after treatment. On days 6 and 8, however, the females spent more time on the female side of the cage. There were no statistical differences between the female groups.

Figure 8. The effect of estradiol on the sexual partner preference of male and female zebra finches. The two choice preference test was performed by placing a subject in the middle of a three compartment cage between a group of stimulus females and a group of stimulus males. The time spent in proximity of each sex was monitored for a period of 30 min. After the test, a score was obtained by subtracting the time spent near the same sex stimulus from the time spent near the opposite sex stimulus. The birds were tested three times before and three times after changing their diet to millet treated with ethanol (Control), low concentration (4.7 μg /g) or high concentration (720 μg /g) of estradiol. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8). * indicates a statistically significant difference from control (MW U: P = 0.01).


Opposite sex preferred
Same sex preferred
Preference score (minutes)
30
20
10
0
-10
-20
-30
Males
*
Females
Day 4
Day 6
Day 8
Time of test
Control
Low estradiol
High estradiol

4.4.2 Daily pair test

The recorded behaviours are presented by sex and category (sexual, agonistic and displacement behaviours). The data from the males are presented in Figures 9 to 17, and those from the females in Figures 18 to 26.

The behaviours within each category are presented sequentially in three figures (each on a different page). In the first, I present the number of birds from each group that have exhibited the behaviours at least once during the test. The analysis of these data gives more weight to rare behaviours. In the second figure, the standardized scores (Equation 1) for each behaviour are presented. Since the scores are calculated separately for each time (for day 4, day 6 and day 8 separately), the comparison can only be made for one day at a time. Note that the standardized scores represent distances from the average of the three groups in relation to the standard deviation and that the sum of the scores for one behaviour on any specific day is zero. The third figure presents the composite score for the behavioural category. As described in section 3.6.4, this figure represents the average score for the behaviours within the category.

4.4.2.1 Male sexual behaviour (Fig. 9, 10, and 11)

There were no statistically significant differences in the number of males performing the recorded sexual behaviours during the daily pair test (Fig. 9). There were fewer low estradiol males exhibiting sexual behaviours after treatment began compared to the pre-treatment period.

The standardized scores of song directed to the female were significantly lower in the low estradiol (MW U, $P < 0.01$) and the high estradiol (MW U, $P = 0.01$) groups (Fig. 10). The beak wipe score of this behaviour was generally lower in estradiol treated birds, but was not significantly different from the control's (Fig. 10).

Figure 9. Number of males that exhibited the recorded sexual behaviours at least once during the daily pair test. The birds were tested three times before and three times after changing their diet to millet treated with ethanol (Control), low concentration (4.7 μg /g) or high concentration (720 μg /g) of estradiol. Eight birds per group were observed.

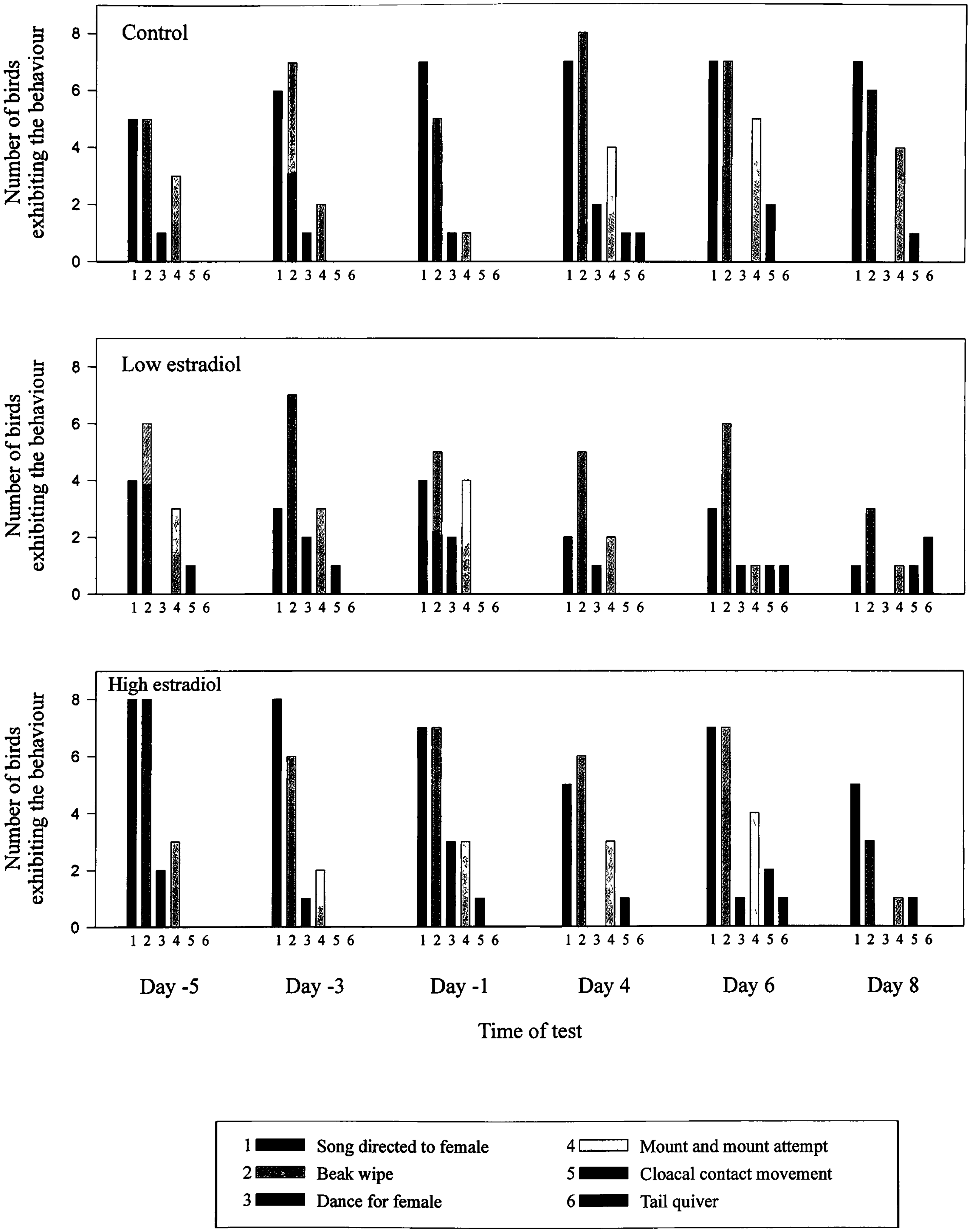
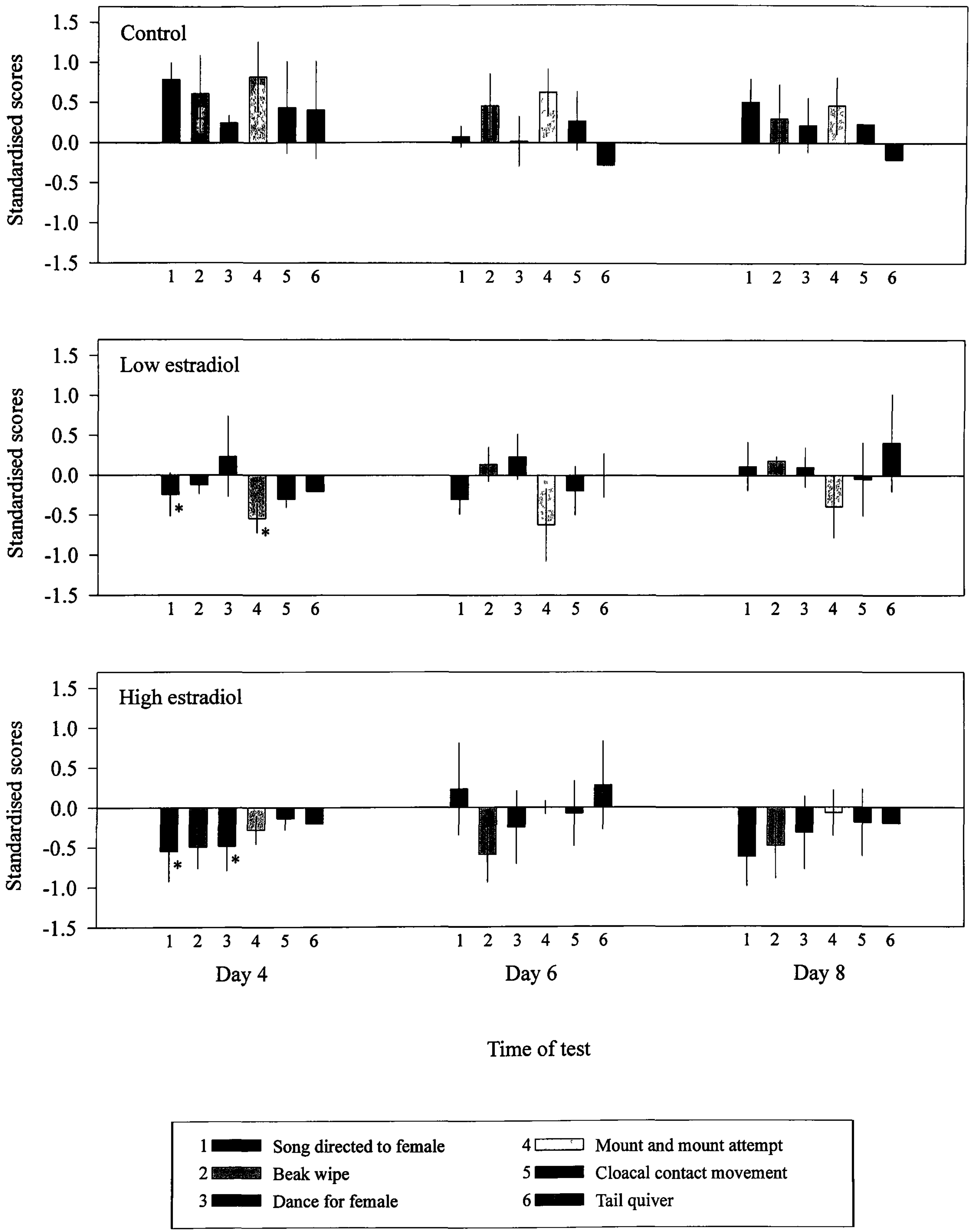

Control
Low estradiol
High estradiol
Number of birds exhibiting the behaviour
0
2
4
6
8
1 2 3 4 5 6
Day -5
Day -3
Day -1
Day 4
Day 6
Day 8
Time of test
1 Song directed to female
2 Beak wipe
3 Dance for female
4 Mount and mount attempt
5 Cloacal contact movement
6 Tail quiver

Figure 10. Standardized sexual behaviour scores of males during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8).). * indicates a statistically significant difference from control (MW U: low estradiol, $P < 0.02$; high estradiol, $P = 0.01$).


Control
Low estradiol
High estradiol
Standardised scores
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1 2 3 4 5 6
*
Day 4
Day 6
Day 8
Time of test
1 Song directed to female
2 Beak wipe
3 Dance for female
4 Mount and mount attempt
5 Cloacal contact movement
6 Tail quiver

Relatively few males danced for the stimulus females during the daily pair tests. There was a decreasing trend in the number of birds performing this behaviour in the three groups of birds. The score of the high estradiol group was significantly lower than that of the control group on day 4 (MW U, $P = 0.01$).

On day 4, low estradiol males had a lower "mount and mount attempt" score than the control group (MW U, $P < 0.02$).

Cloacal contact movement may only occur during mounting and it was observed infrequently. The score for this behaviour tended to be lower in the estradiol-treated groups than in controls, but these differences were not significant. Tail quiver, which often follows successful copulation, was also rarely observed.

The composite sexual scores for the low and the high estradiol groups were significantly lower than that of control on day 4 (MW U, $P < 0.001$) (Fig. 11). The score of the two treatment groups remained below control on days 6 and 8, but not significantly so.

In summary, oral estradiol appears to have negatively affected singing, beak wiping, dancing and mounting behaviours in males. The decrease of sexual activity in low and high estradiol males was reflected by low composite sexual scores compared to that of the control group.

4.4.2.2 Male agonistic behaviour (Fig. 12, 13, and 14)

There were no statistically significant differences in the number of males performing the recorded agonistic behaviours during the daily pair test (Fig. 12). Fewer low and high estradiol males performed the recorded agonistic behaviours (at least once) during the treatment period compared to the pre-treatment period. On day 8, all behaviours were exhibited by some control males, but only threats to the male and female stimulus and

Figure 11. Composite standardized sexual behaviour scores of males during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8). * indicates a statistically significant difference from control (MW U: $P < 0.001$).

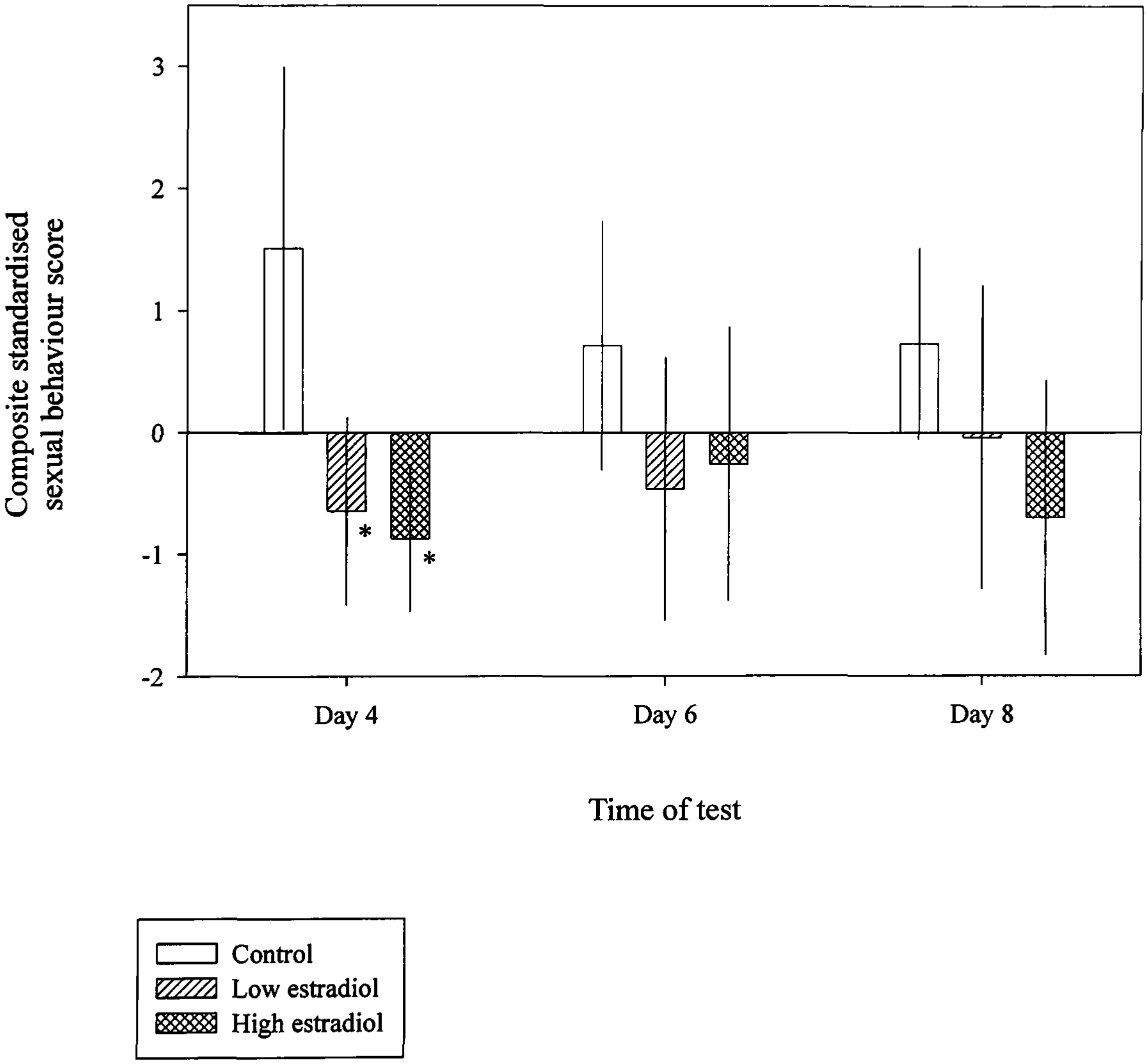
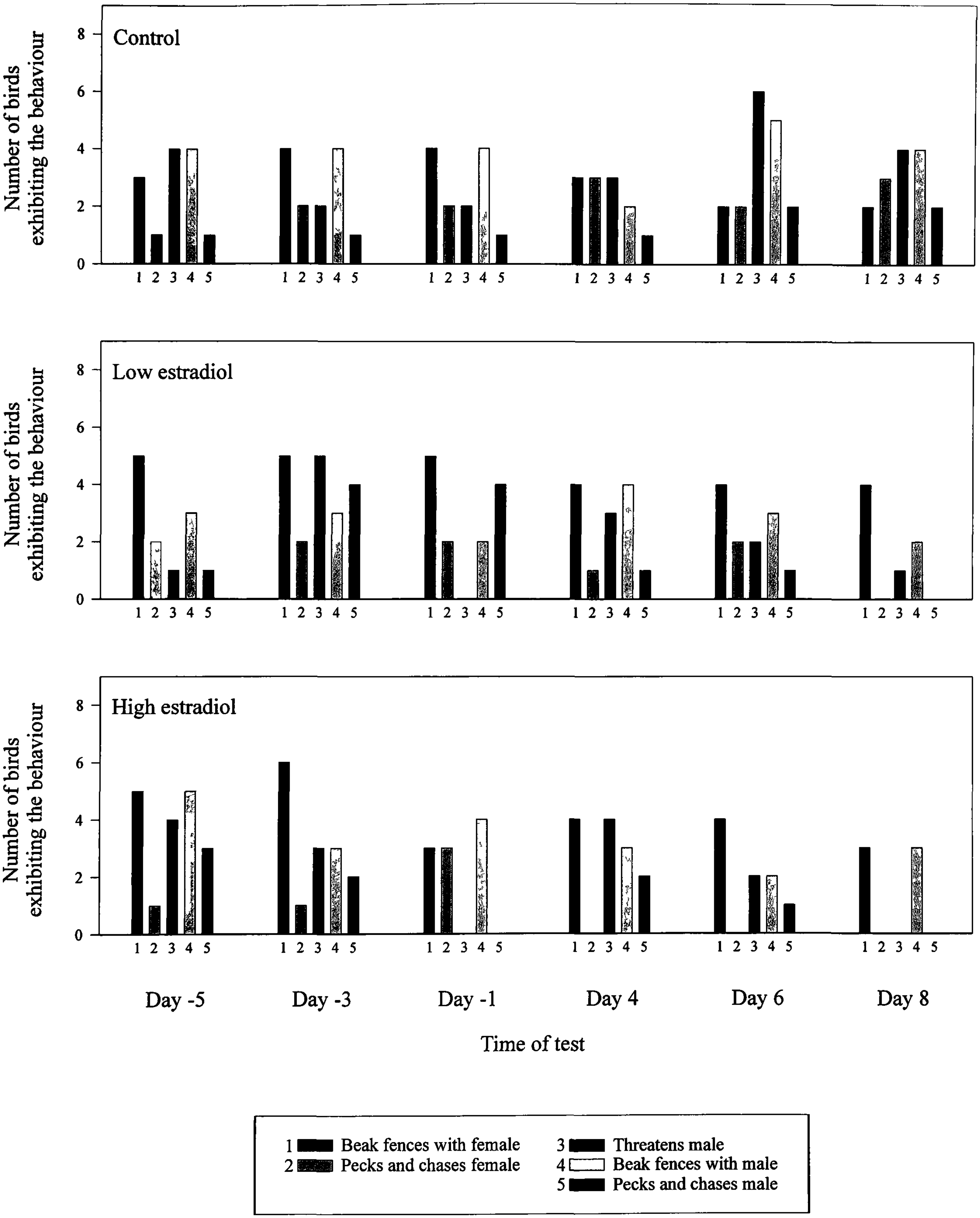

3
2
1
0
-1
-2
Composite standardised sexual behaviour score
*
*
Day 4
Day 6
Day 8
Time of test
Control
Low estradiol
High estradiol

Figure 12. Number of males that exhibited the recorded agonistic behaviours at least once during the daily pair test. Same conditions as in Fig. 9. Eight birds per group were observed.


Control
Low estradiol
High estradiol
Number of birds exhibiting the behaviour
0
2
4
6
8
1 2 3 4 5
Day -5
Day -3
Day -1
Day 4
Day 6
Day 8
Time of test
1 Beak fences with female
2 Pecks and chases female
3 Threatens male
4 Beak fences with male
5 Pecks and chases male

beak fences with the male stimulus were observed in the low estradiol males, and only beak fencing with the male or the female stimulus was observed in high estradiol males.

The scores for beak fencing with the female as well as pecking and chasing the female tended to be lower in the low estradiol group compared to the control's (Fig. 13). On days 6 and 8, the scores for threats made to the stimulus male were lower (not statistically) in the estradiol treated groups than in the control group.

The scores for beak fencing with the male stimulus varied from one test to the other without following a specific trend. There were no significant differences among the groups for this behaviour.

The pecks and chase male scores were lower in the low and high estradiol groups than in the control group. This difference was statistically significant for the low estradiol group on day 6 (MW U, $P = 0.02$).

The composite agonistic scores (Fig. 14) were significantly lower for the low estradiol (MW U, $P = 0.03$) and the high estradiol (MW U, $P = 0.01$) groups compared to the control.

In summary, the low oral estradiol concentration appears to have decreased male agonistic behaviour towards the female stimulus. Agonistic behaviour in the form of threats, pecks, and chases, targeting the male stimulus also appear to be suppressed by oral estradiol. The composite agonistic behaviour scores are highly variable but their differences are statistically significant on day 6, suggesting a decrease in agonistic behaviour in treated males.

Figure 13. Standardized agonistic behaviour scores of males during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8). * indicates a statistically significant difference from control (MW U: P = 0.02).

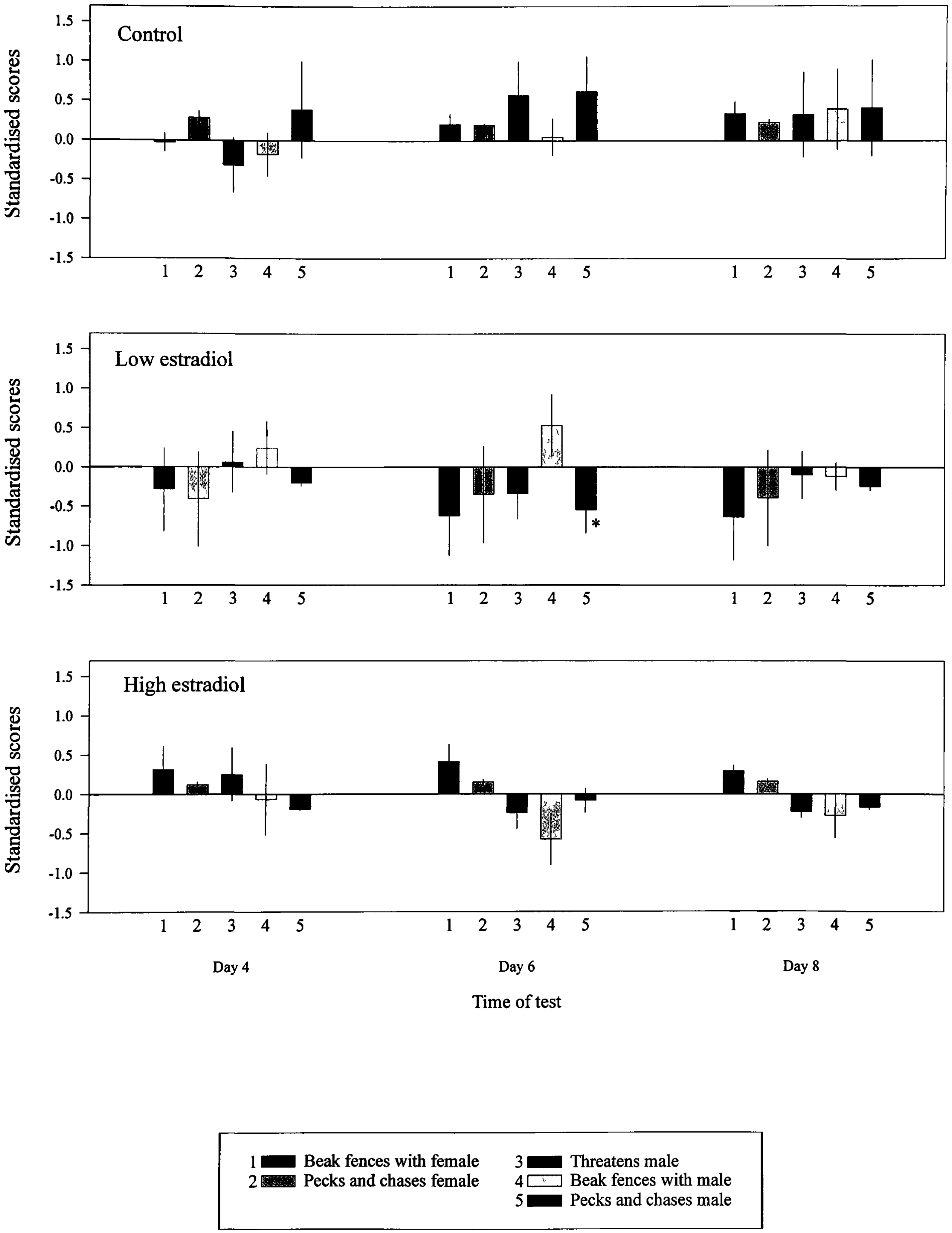
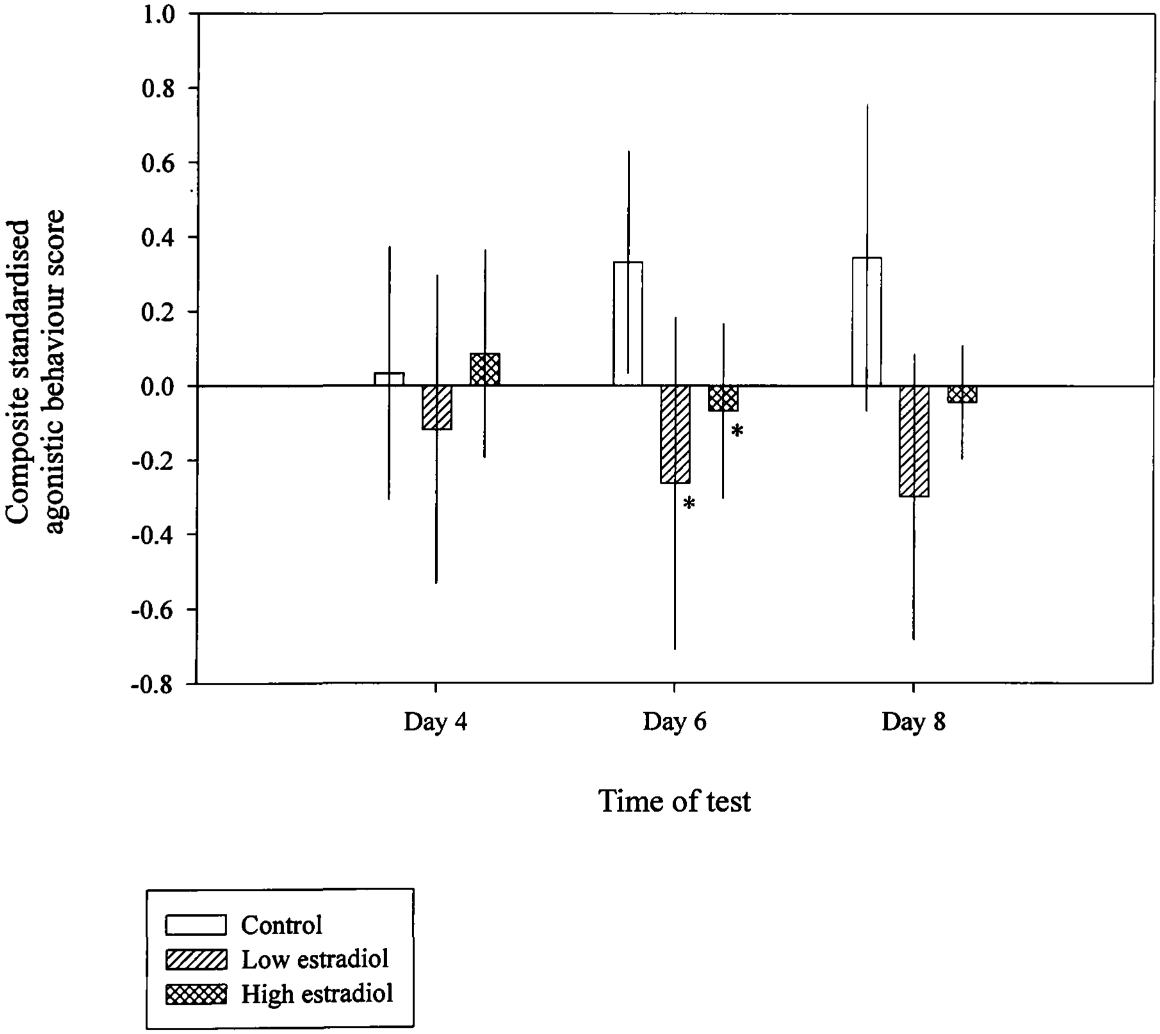

Control
Low estradiol
High estradiol
Standardised scores
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1 2 3 4 5
*
Day 4
Day 6
Day 8
Time of test
1 Beak fences with female
2 Pecks and chases female
3 Threatens male
4 Beak fences with male
5 Pecks and chases male

Figure 14. Composite standardized agonistic behaviour scores of males during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8). * indicates a statistically significant difference from control (MW U: $P < 0.05$).


1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Composite standardised agonistic behaviour score
*
*
Day 4
Day 6
Day 8
Time of test
Control
Low estradiol
High estradiol

4.4.2.3 Male displacement behaviour (Fig. 15, 16, and 17)

The number of males that auto-preened, fed or foraged during the test did not differ significantly among groups. There were relatively more low and high estradiol males that performed the recorded behaviours at least once during the treatment period compared to the pre-treatment period (Fig. 15).

The standardized displacement behaviour scores of the treatment groups did not differ from those of the control group (Fig. 16). The composite displacement behaviour scores of the control group tended to be below those of the estradiol treated groups (Fig. 17). These trends were not statistically significant.

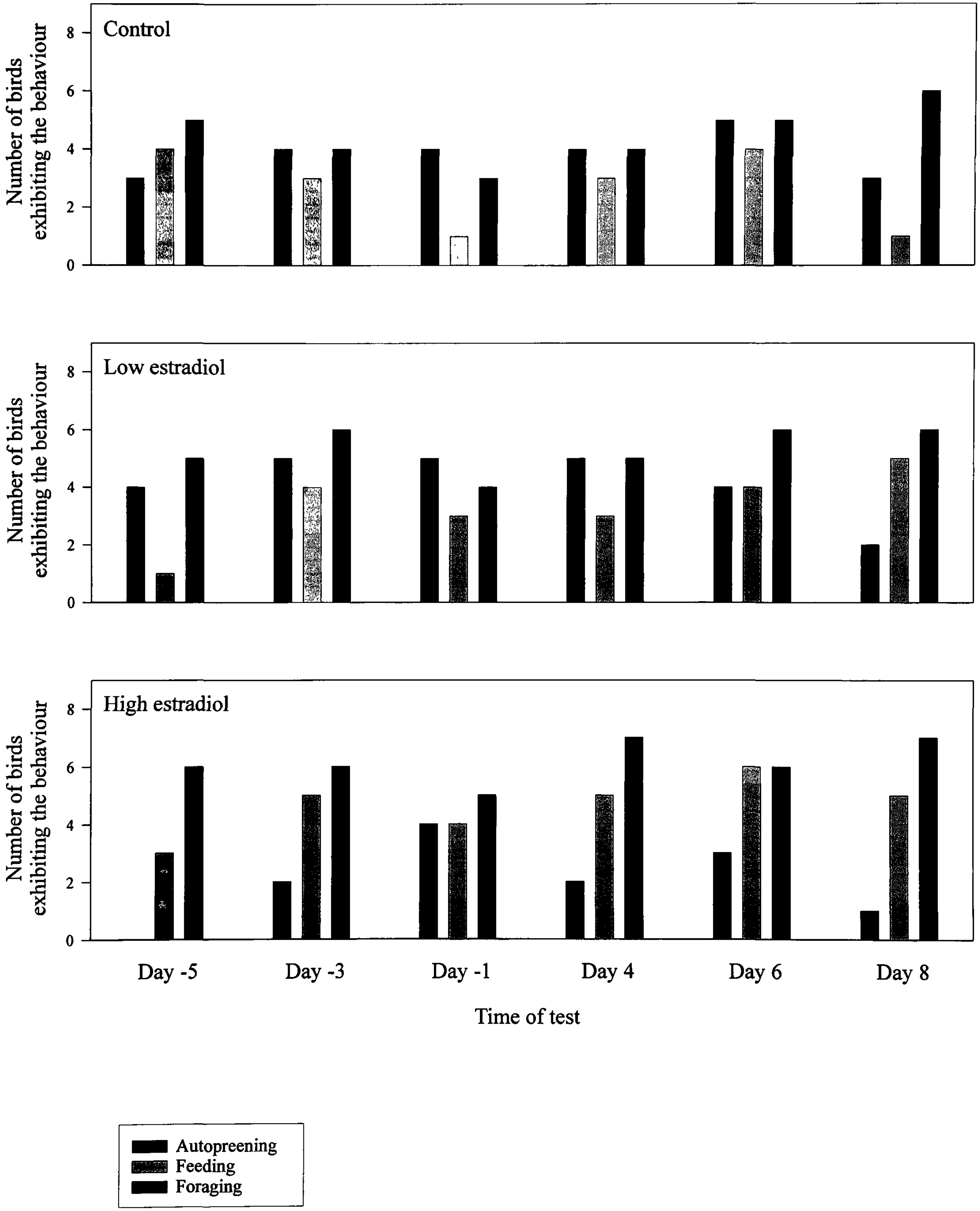
In summary, the recorded displacement behaviours were probably not affected by oral estradiol.

4.4.2.4 Female sexual behaviour (Fig. 18, 19, and 20)

The number of females exhibiting the recorded sexual behaviours was relatively constant in the control and low estradiol groups. Tail quivering was never observed in the low estradiol group (Fig. 18). In the high estradiol females, on the other hand, there was a strong increase in the number of birds that accepted mounts by the stimulus males and performed tail quivers after the start of treatment (Fig. 18). On day 4, the number of females performing tail quivering was significantly higher than in the control group (MW U, $P < 0.01$). The standardized scores for accepted mounts/attempted mounts and tail quivering (Fig. 19) were significantly higher than the control's on day 4 (MW U, accepted mounts/attempted mounts: $P = 0.05$; tail quiver: $P < 0.01$).

The composite sexual scores of the high estradiol females was significantly higher than that of the control group (MW U, $P < 0.01$). In contrast, females treated with the low

Figure 15. Number of males that exhibited the recorded displacement behaviours at least once during the daily pair test. Same conditions as in Fig. 9. Eight birds per group were observed.



Control
Low estradiol
High estradiol
Number of birds exhibiting the behaviour
0
2
4
6
8
Day -5
Day -3
Day -1
Day 4
Day 6
Day 8
Time of test
Autopreening
Feeding
Foraging

Figure 16. Standardized displacement behaviour scores of males during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8).

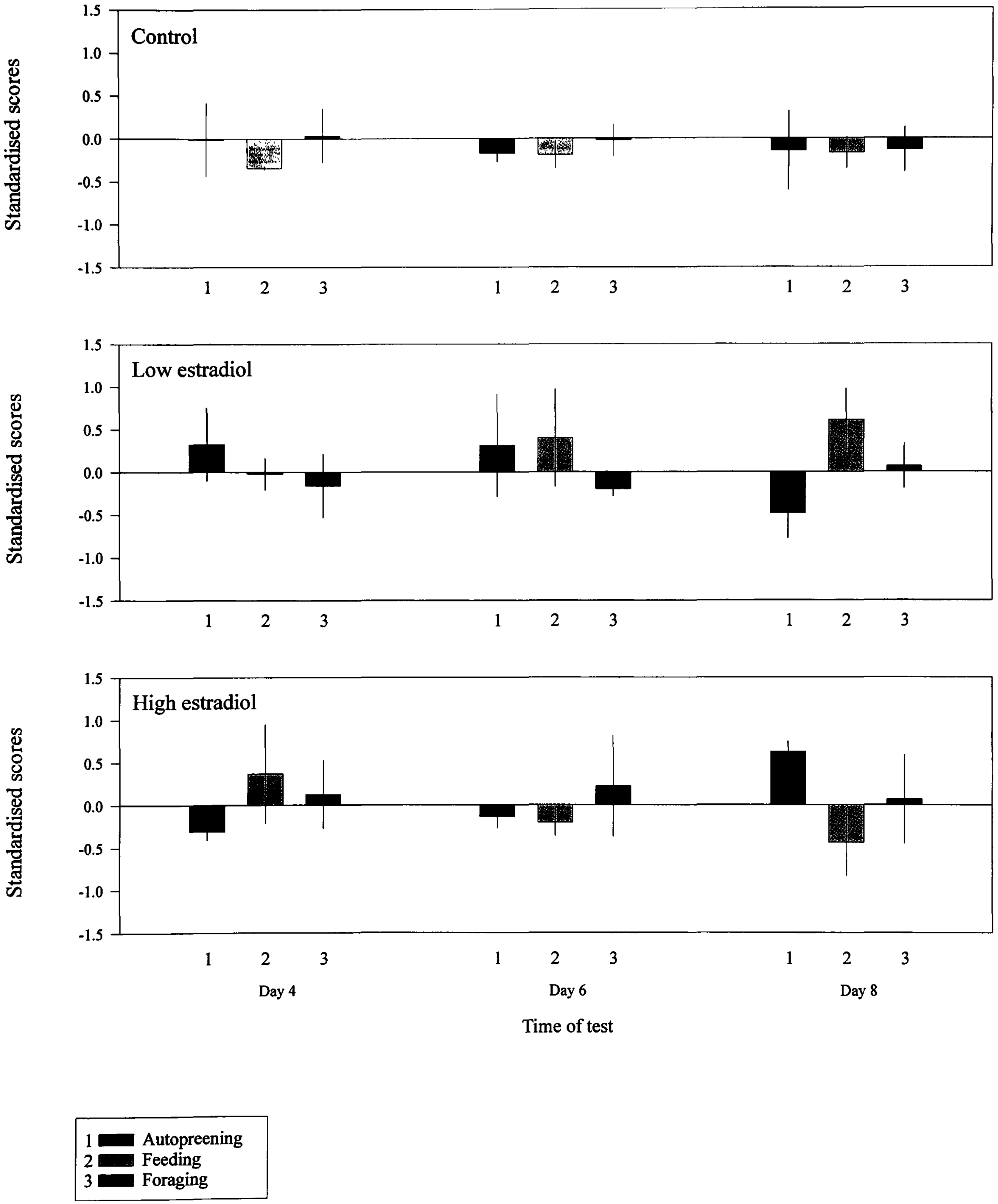
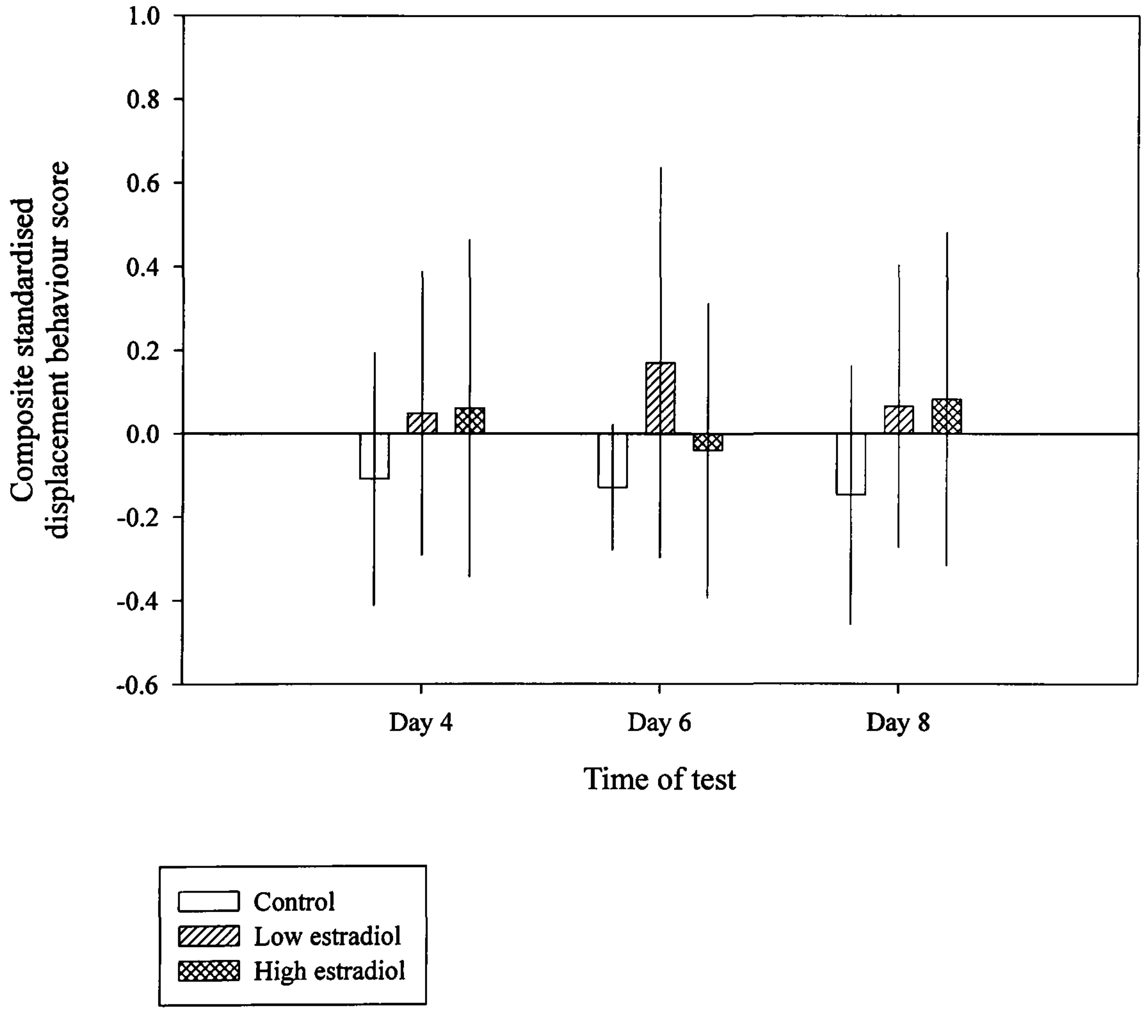

Control
Low estradiol
High estradiol
Standardised scores
Standardised scores
Standardised scores
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1
2
3
Day 4
Day 6
Day 8
Time of test
1 Autopreening
2 Feeding
3 Foraging

Figure 17. Composite standardized displacement behaviour scores of males during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8).



1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
Composite standardised displacement behaviour score
Day 4
Day 6
Day 8
Time of test
Control
Low estradiol
High estradiol

Figure 18. Number of females that exhibited the recorded sexual behaviours at least once during the daily pair test. Same conditions as in Fig. 9. Eight birds per group were observed. * indicates a statistically significant difference from control (MW U: $P < 0.01$).

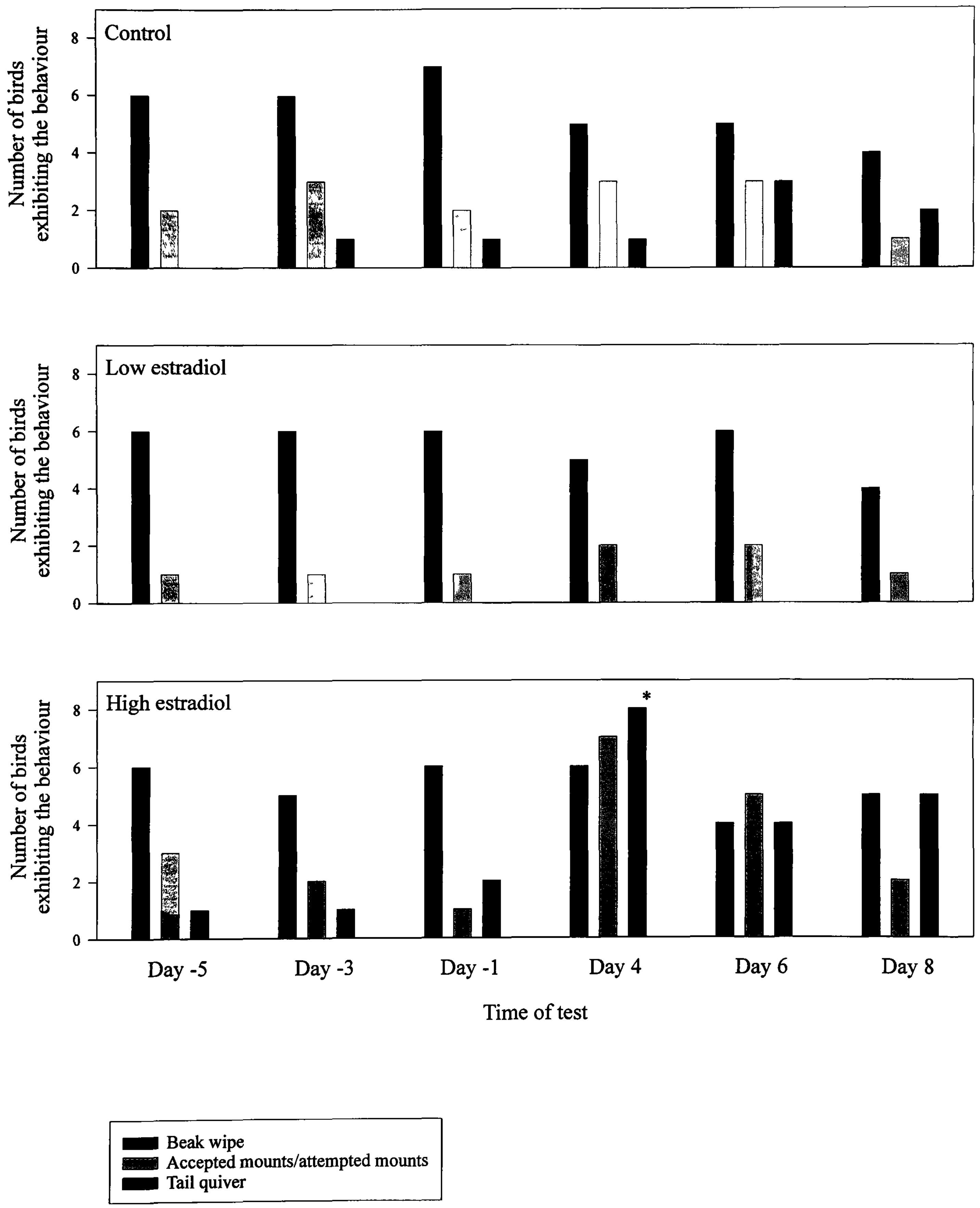

Control
Low estradiol
High estradiol
Number of birds exhibiting the behaviour
0
2
4
6
8
*
Day -5
Day -3
Day -1
Day 4
Day 6
Day 8
Time of test
Beak wipe
Accepted mounts/attempted mounts
Tail quiver

Figure 19. Standardized sexual behaviour scores of females during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., $n = 8$). * indicates a statistically significant difference from control (MW U: Accepted mounts, $P = 0.05$; Tail quiver, $P < 0.01$).

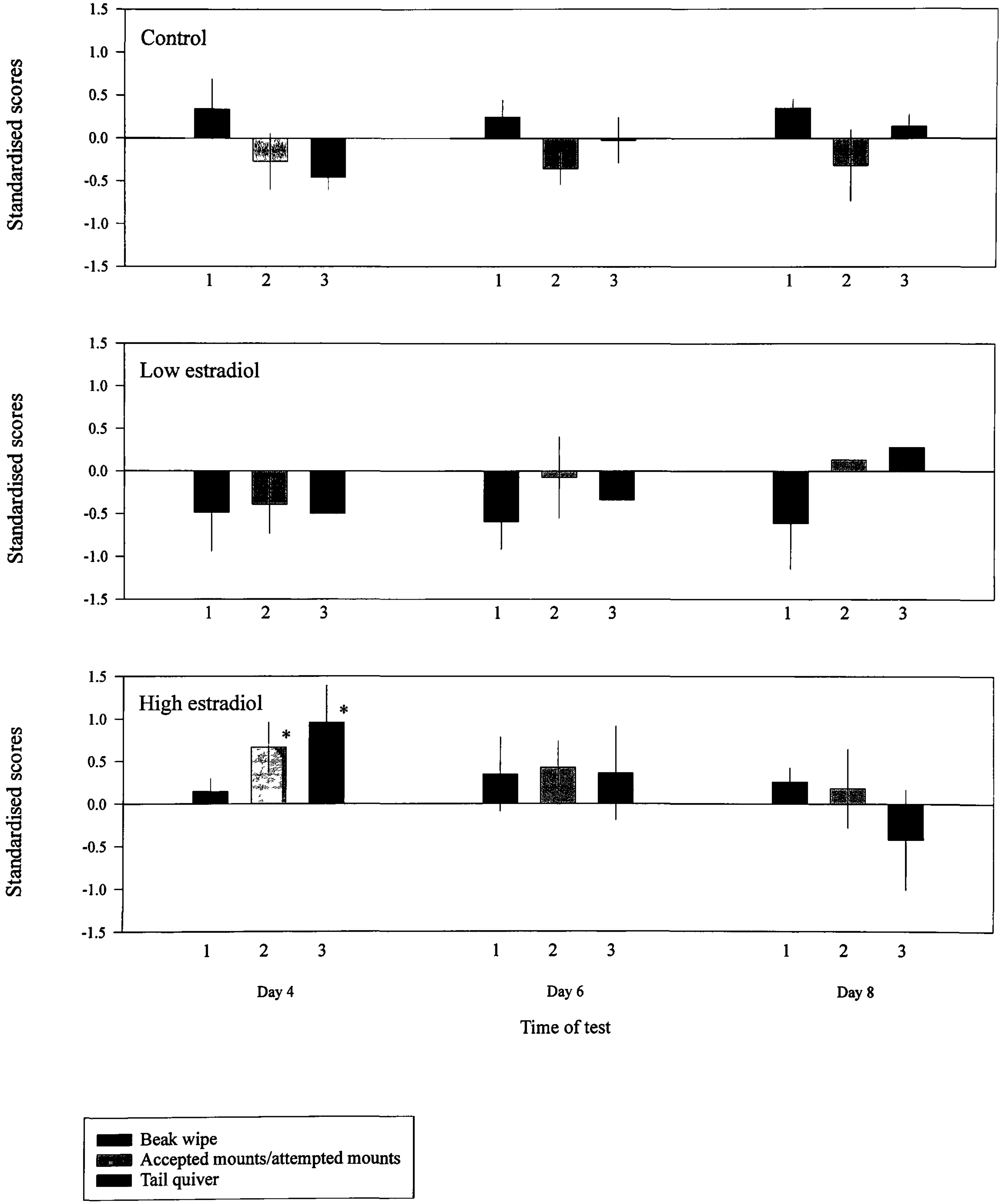
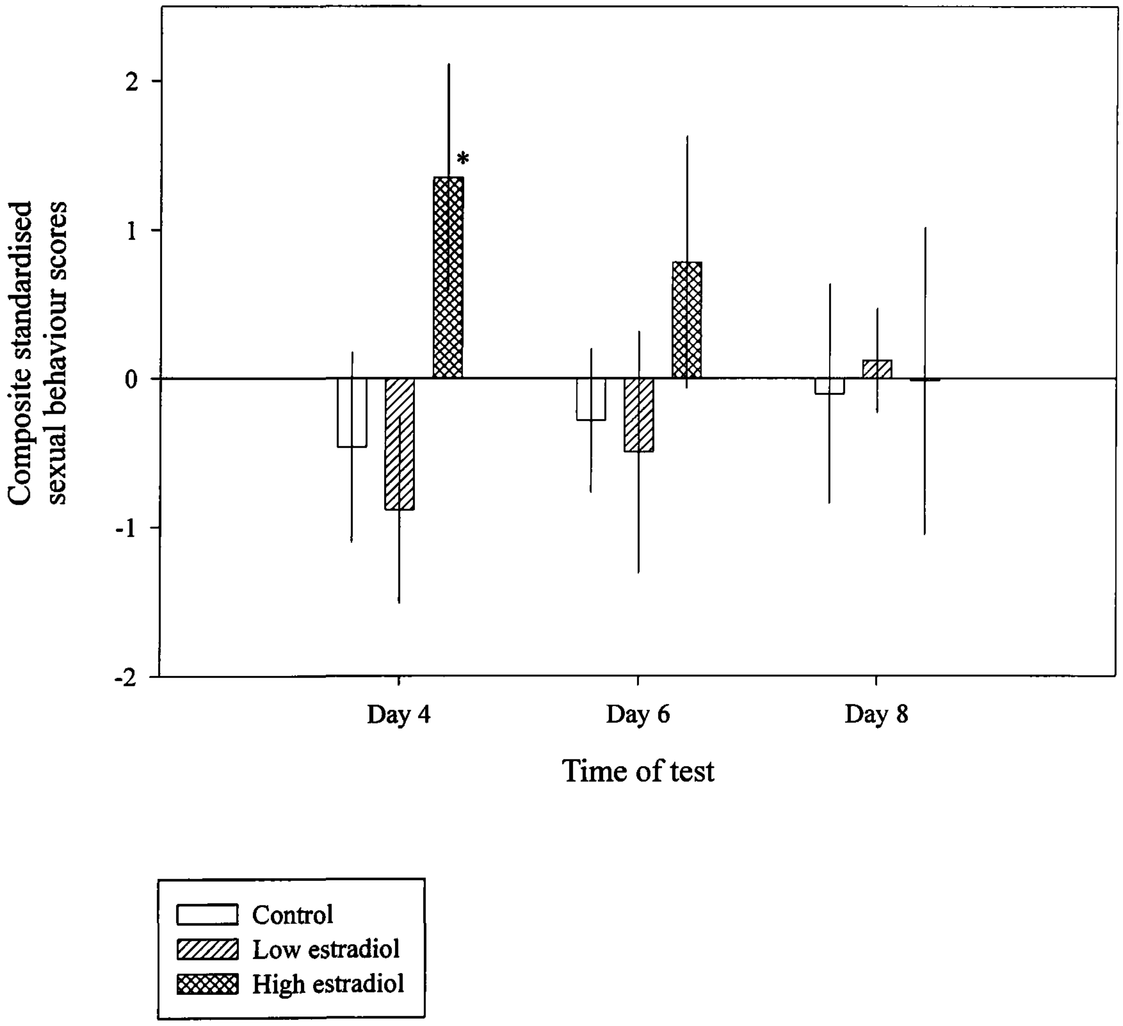

Control
Low estradiol
High estradiol
Standardised scores
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1
2
3
*
Day 4
Day 6
Day 8
Time of test
Beak wipe
Accepted mounts/attempted mounts
Tail quiver

Figure 20. Composite standardized sexual behaviour scores of females during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8). * indicates a statistically significant difference from control (MW U: $P < 0.01$).


Composite standardised sexual behaviour scores
2
1
0
-1
-2
*
Day 4
Day 6
Day 8
Time of test
Control
Low estradiol
High estradiol

estradiol concentrations or the control diet had negative composite sexual behaviour scores on days 4 and 6.

In summary, the high oral estradiol treatment increased the mount receptivity and tail quivering in the female zebra finch. This was supported by an increased number of birds exhibiting the behaviour as well as high separate and composite sexual scores.

4.4.2.5 Female agonistic behaviour (Fig. 21, 22, and 23)

There was only one statistically significant difference in the number of females performing the recorded agonistic behaviours during the daily pair test (Fig. 21). On day 4, fewer low estradiol females beak fenced with the stimulus male compared to control (MW U, $P = 0.03$) but that number increased on days 6 and 8.

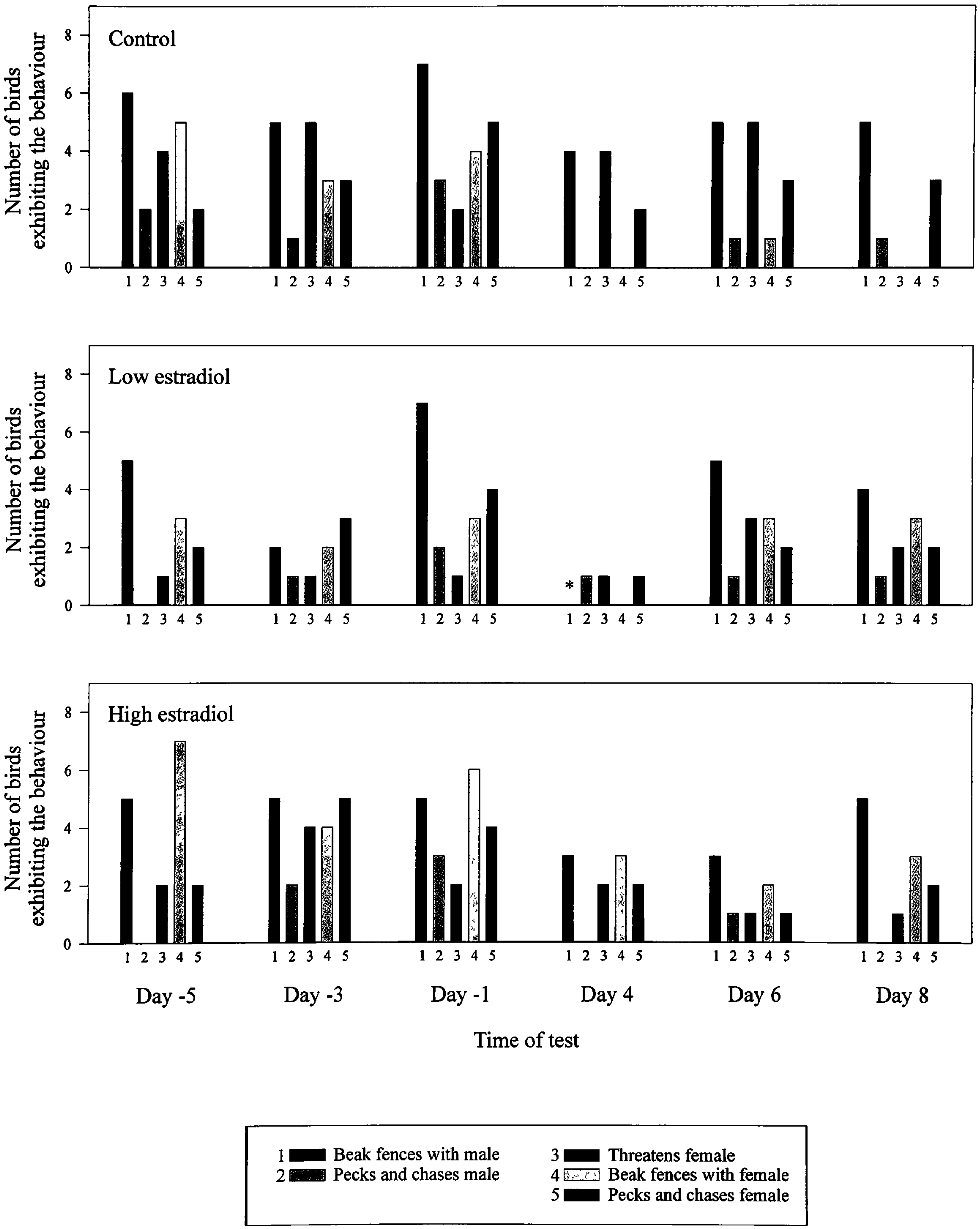
In the low estradiol group, the number of females that exhibited agonistic behaviours dropped on day 4 (Fig. 21). Similarly, in the high estradiol group, all recorded agonistic behaviours appear to follow a decreasing trend between the pre-treatment tests and day 6.

Relatively few females pecked and chased the stimulus male. The standardized score for this behaviour was highest in the control group at all times except on day 4, when the low estradiol females had the highest score (not significantly higher).

On day 8, the standard score for threatening behaviour targeting the male stimulus was significantly higher in the low estradiol group compared to the control group (MW U, $P = 0.02$).

The number of females that beak fenced with the female stimulus decreased in the control and the high estradiol females after the beginning of treatment (Fig. 21). The standardized score for this behaviour was lower for the high estradiol females than the

Figure 21. Number of females that exhibited the recorded agonistic behaviours at least once during the daily pair test. Same conditions as in Fig. 9. Eight birds per group were observed. * indicates a statistically significant difference from control (MW U: P = 0.03).


Control
Low estradiol
High estradiol
Number of birds exhibiting the behaviour
0
2
4
6
8
1 2 3 4 5
*
Day -5
Day -3
Day -1
Day 4
Day 6
Day 8
Time of test
1 Beak fences with male
2 Pecks and chases male
3 Threatens female
4 Beak fences with female
5 Pecks and chases female

low estradiol and the control groups (Fig. 21). These differences were not statistically significant.

The standardized scores for pecks and chases targeting the female stimulus did not differ significantly among the groups but in the high estradiol females, the scores for this behaviour tended to be higher than the other groups on days 4 and 8 (Fig. 22).

The composite agonistic scores of the high estradiol group was negative for days 4, 6, and 8 but did not differ from the control group (Fig. 23). The low estradiol females' composite agonistic score was significantly higher than the controls' on day 8 (MW U, $P < 0.01$).

In summary, oral estradiol, at a low dose, appears to increase agonistic behaviour and may decrease agonistic behaviour at a higher dose.

4.4.2.6 Female displacement behaviour (Fig. 24, 25, and 26)

The number of females auto-preening during the tests was relatively constant except for the high estradiol group in which there was a gradual decrease with time (Fig. 24). The number of females feeding during the test, on the other hand, was constant in the control and high estradiol groups, but appeared to increase on day 4 in the low estradiol group. In the three groups, the number of females that foraged increased on day 4.

The standardized displacement behaviour scores of the estradiol groups did not differ significantly from the control. The high estradiol group had negative auto-preening and foraging scores on days 4, 6 and 8 (Fig. 25). Except for feeding, which had a negative score on day 6, the three recorded displacement behaviour scores were positive (or very close to zero) in the low estradiol group on day 4 and 6.

Figure 22. Standardized agonistic behaviour scores of females during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8). * indicates a statistically significant difference from control (MW U: P = 0.02).

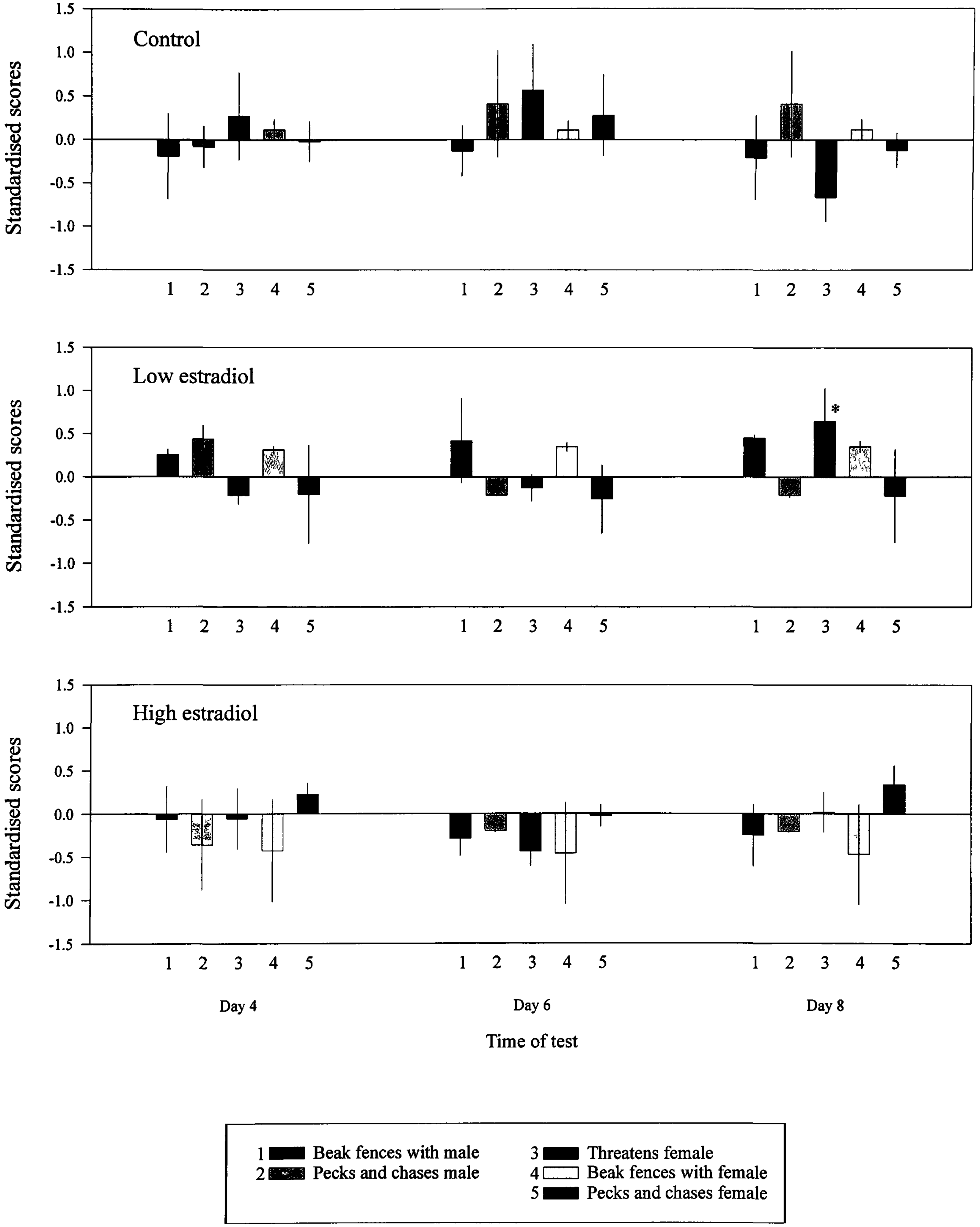

Control
Low estradiol
High estradiol
Standardised scores
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1 2 3 4 5
*
Day 4
Day 6
Day 8
Time of test
1 Beak fences with male
2 Pecks and chases male
3 Threatens female
4 Beak fences with female
5 Pecks and chases female

Figure 23. Composite standardized agonistic behaviour scores of females during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., $n = 8$). * indicates a statistically significant difference from control (MW U: $P < 0.01$).

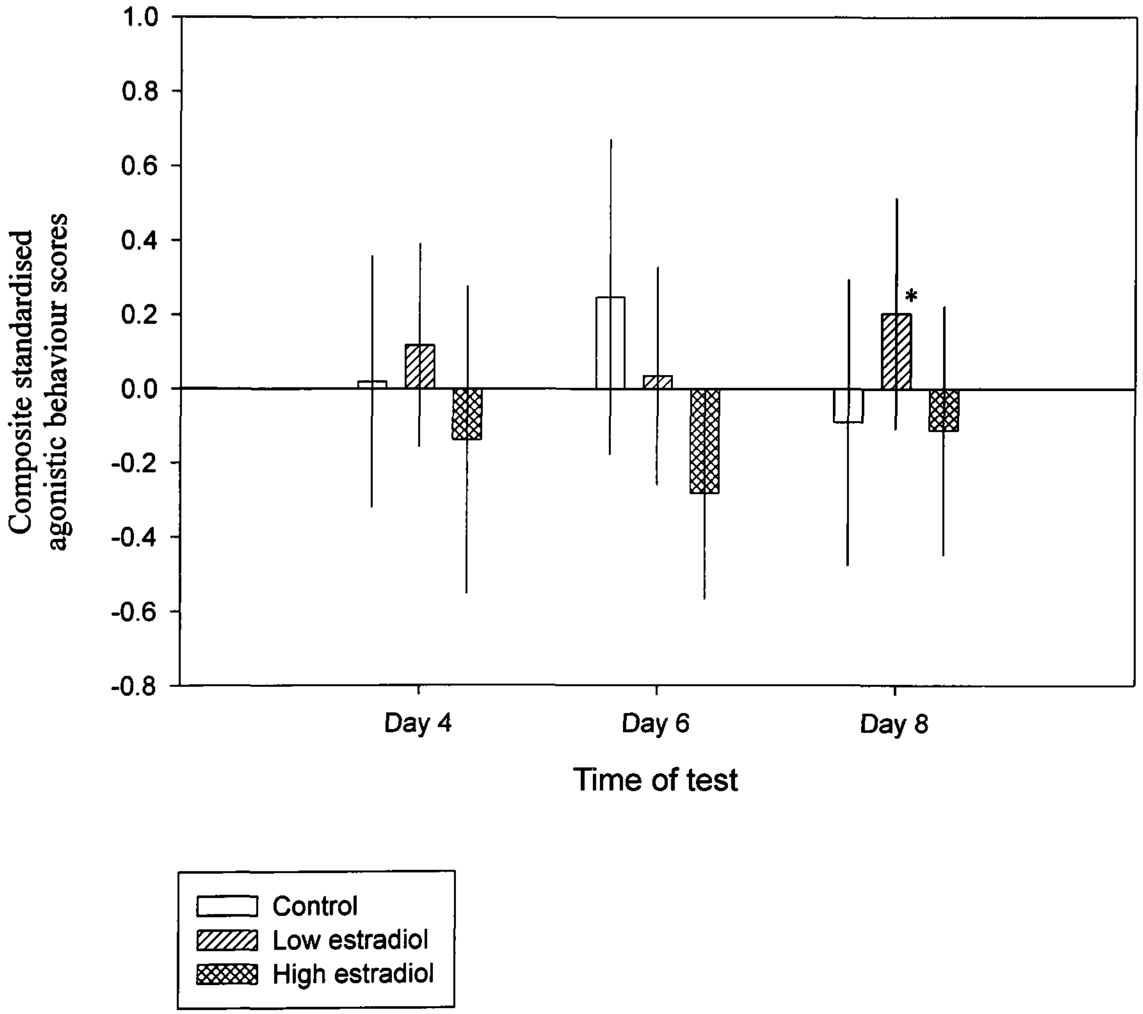

1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Composite standardised agonistic behaviour scores
*
Day 4
Day 6
Day 8
Time of test
Control
Low estradiol
High estradiol

Figure 24. Number of females that exhibited the recorded displacement behaviours at least once during the daily pair test. Same conditions as in Fig. 9. Eight birds per group were observed.

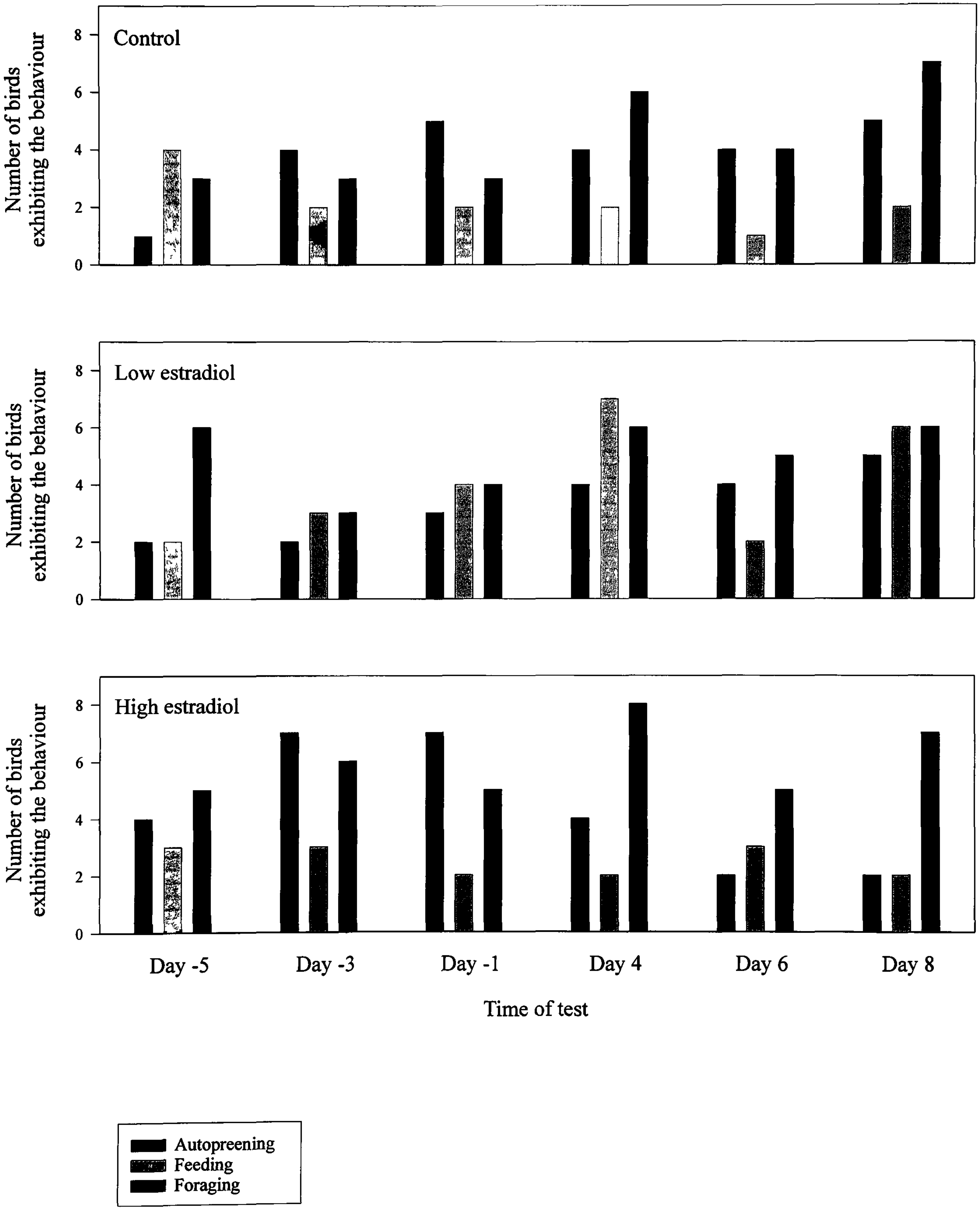
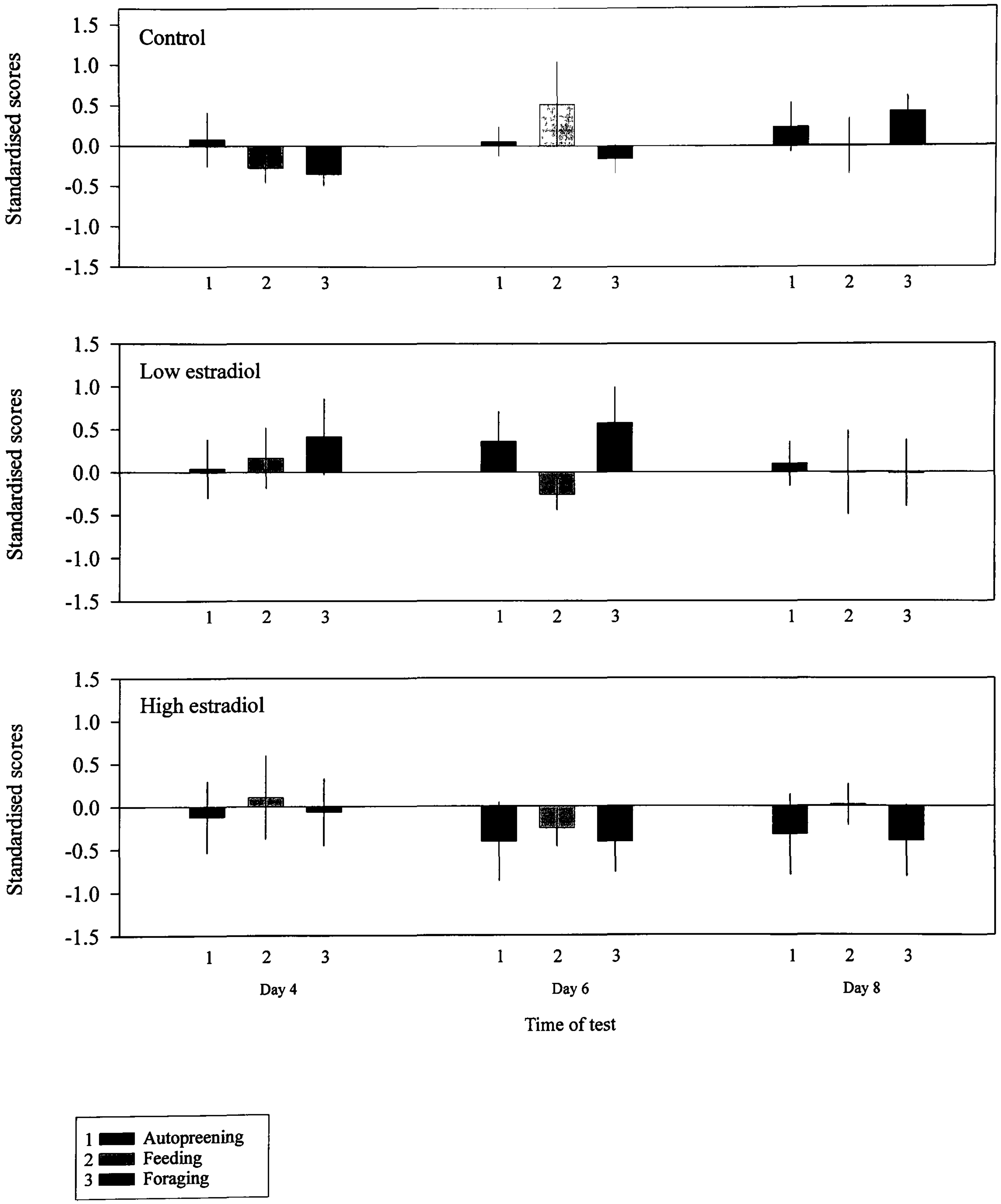

Control
Low estradiol
High estradiol
Number of birds exhibiting the behaviour
0
2
4
6
8
Day -5
Day -3
Day -1
Day 4
Day 6
Day 8
Time of test
Autopreening
Feeding
Foraging

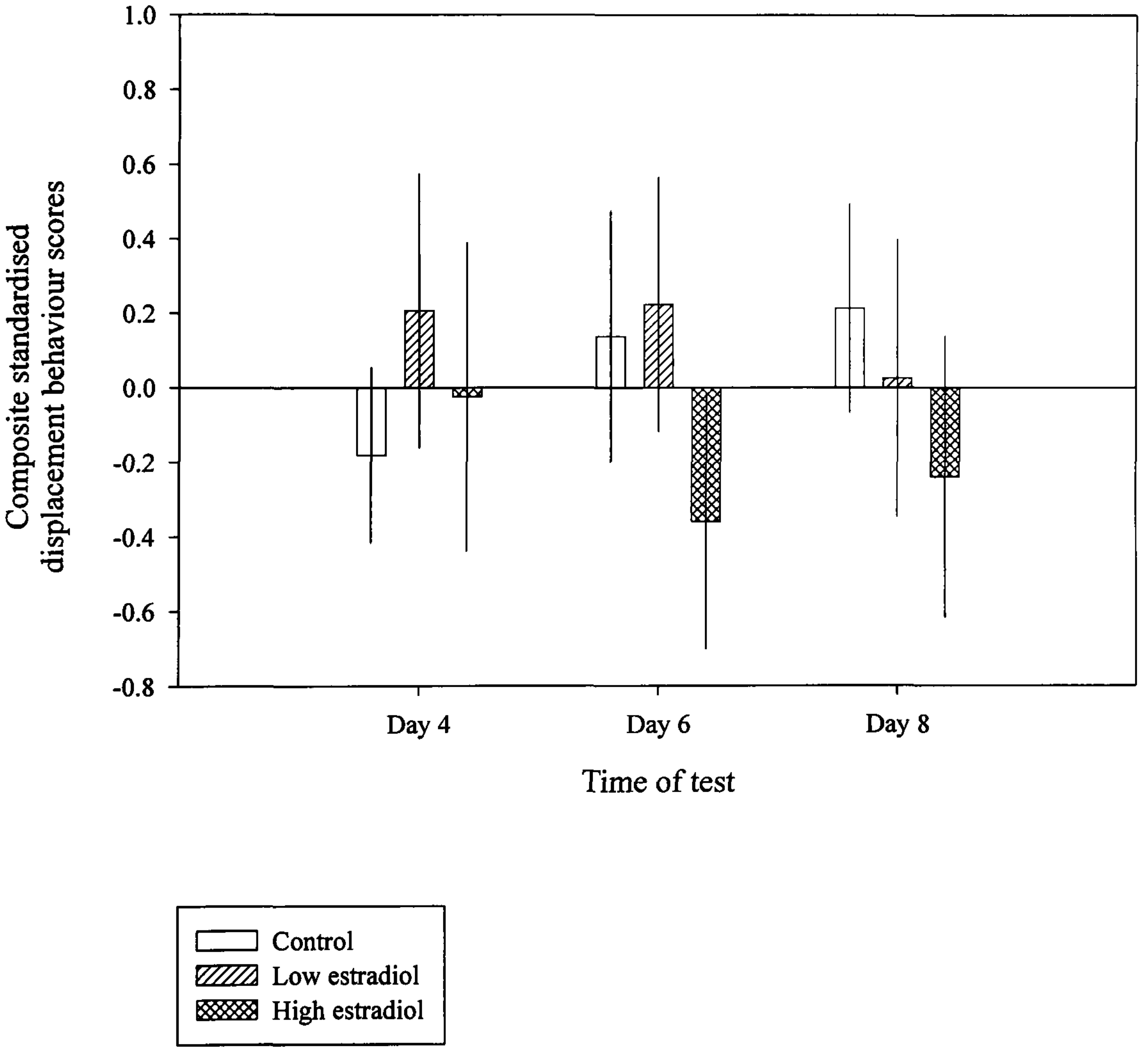
Figure 25. Standardized displacement behaviour scores of females during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8).


Control
Low estradiol
High estradiol
Standardised scores
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1
2
3
Day 4
Day 6
Day 8
Time of test
1 ■ Autopreening
2 ▨ Feeding
3 ■ Foraging

The composite displacement behaviour score did not differ significantly among groups (Fig. 26). The low estradiol group had positive score while the high estradiol group had negative scores on days 4, 6 and 8.

In summary, the data is very variable, but the weak trends indicate that oral estradiol may increase displacement behaviour at a lower concentration and decrease it at a higher concentration.

Figure 26. Composite standardized displacement behaviour scores of females during the daily pair test. Same conditions as in Fig. 9. The average pre-treatment score was subtracted from each post-treatment score. (mean ± S.E., n = 8).


1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Composite standardised displacement behaviour scores
Day 4
Day 6
Day 8
Time of test
Control
Low estradiol
High estradiol

5 Discussion

As described in the Introduction, several studies have shown that some environmental contaminants are estrogenic and may interfere with the hypothalamo-pituitary-gonadal axis. These disruptions may occur during the development of the organism or during adulthood.

Exposure to endocrine disrupting chemicals during critical developmental windows may lead to an abnormal organization of sensitive CNS and/or endocrine tissues. In one study by Halldin *et al.* (1999) Japanese quail behaviours were found to be highly sensitive to *in-ovo* exposure to estrogens. Male sexual behaviours were depressed by exposure to 6 ng/g eg ethinyl estradiol (EE_2) or by 19 ng/g egg diethylstilbestrol (DES). This non-invasive and ecologically relevant endpoint was as sensitive to EE_2 as testis weight asymmetry, and more sensitive to DES compared to any of the recorded physiological and biochemical endpoints.

Estrogens also play a key role in orchestrating sexual behaviours during adulthood.
In the present study, I investigated the effects of exogenous estradiol on some passerine bird behaviours that are important for reproduction. I exposed adult zebra finches to estradiol through their diet and predicted that this treatment would increase the plasma concentration of this hormone and change some of the courtship, copulatory and agonistic behaviours of the birds.

5.1 Estradiol concentration during the breeding cycle

To test my hypothesis, I began by optimizing and validating an estradiol radioimmunoassay that would serve to measure this hormone in plasma. The assay was first used to monitor the estradiol concentration during the breeding cycle of zebra finch pairs. This allowed me to confirm the accuracy of the assay for a range of estradiol concentrations.

Although there were no statistical differences among the plasma estradiol concentrations during different periods of the breeding cycle, the average estradiol concentrations in this study are similar to those reported by other investigators (Fig. 6). Vleck and Priedkalns (1985), for example, measured estrogen concentrations in female zebra finches during their reproductive cycle (Table 1). The concentrations from this study ranged from below 130 pg/ml to 450 pg/ml with the lowest concentrations coinciding with the "unsuccessful courtship" and the "late incubation" periods, whereas the highest concentrations were measured during "successful courtship" and at a time when the parents were actively "feeding (the) nestlings".

In the present investigation, the average plasma estradiol concentrations were lowest in isolated birds and during the incubating period. Plasma estradiol concentrations were relatively high and were more variable during the nest building and nestling care periods. Because Vleck and Priedkalns (1985) pooled the blood samples, they could not conduct statistical analysis on their estrogen concentration data nor could they measure the variability of plasma estrogen level for their zebra finch females. The peak in estradiol concentrations obtained in their study corresponds roughly with the periods when high estradiol was observed in some birds in my investigation.

Similar results were obtained by Wingfield and Farner (1978) who monitored luteinizing hormone (LH) and testosterone levels of wild breeding white-crowned sparrow. Estradiol was not monitored in males but was directly related to LH levels in other species (see Table 1, for example) (Vleck *et al.*, 1985). The LH concentration peaks during the courtship period, decreases during the incubating period, and rises again during the post-hatching period. Estradiol levels in females are lowest (approximately 50 pg/ml) in winter and peak during the courtship period (approximately 120 ±15 pg/ml). They drop to near basal level just before ovulation and slowly increase to approximately

110 ±50 pg/ml during fledgling care. In this species, estradiol concentrations appear lower than in the zebra finch but remain in the same order of magnitude.

In male mockingbirds (*Mimus polyglottos*) (Logan & Wingfield, 1995), estradiol concentrations slowly increase from a basal level of 250 pg/ml to approximately 400 pg/ml during the nestling care period. In females, basal estradiol concentrations are approximately 100 pg/ml in the fall. They rise to approximately 200 pg/ml during the courtship period, drop back to basal levels during the incubation period and rise again to approximately 800 pg/ml during the nestling care period.

In these studies, the peaks in circulating estradiol concentrations were observed during the mating season and after incubation. In the present investigation, the variability of plasma estradiol concentrations during the nest building and nestling care periods was substantial (Fig. 6). This may reflect genuine differences in hormones or could be caused by interfering chemicals in the plasma of some birds. While extraction with an organic phase helps eliminate aqueous compounds which could interfere with the assay, the samples may still contain organic molecules that are soluble in the ethyl-acetate/hexane fraction. Ideally, in a radioimmunoassay, the ligand matrix of the standards and the samples should be the same. In this way, any influence of the matrix on the antibody-antigen reaction would be accounted for. However, since estradiol influences blood chemistry, its levels will also change the matrix of a sample. For example, elevated estradiol levels in avian plasma will cause a mobilization of calcium (Qin & Klandorf, 1995), vitellogenin and cholesterol (Kern *et al.*, 1972), as well as triglycerides and phospholipids (Leszczynski, Toda & Kummerow, 1982). Some of the lipids produced in these birds could have been present in the plasma ether acetate/hexane extracts and might have interfered with the assay (Wingfield *et al.*, 1975). The birds used in the test for interfering compounds (Table 5) probably had relatively low circulating estradiol since they were housed in unisex cages and their pooled plasma did not have high levels of interfering compounds. The pooled blood samples should have been collected from successfully paired females during the nest building/early incubation period. However, I had to sacrifice the birds in order to collect enough plasma for the validation of the radioimmunoassay and could not afford to lose too many established pairs since I needed some for other experiments. Alternatively, I could have injected or implanted the birds with estradiol before collecting their blood. This would have changed their plasma chemistry and allowed me to screen for possible interfering compounds released in the circulation in response to this hormone. However, it would have been impossible to confirm if the estradiol dose was in a physiological range. The radioimmunoassay, can therefore only be considered valid for plasma concentrations under which it was tested and the steroid fraction should probably be purified further before analysis.

5.2 Elevation of plasma estradiol

Oral estradiol treatment of male zebra finches was associated with a non-significant increase in level of the hormone in their plasma (Fig. 7). The relatively low standard error in the pre-treatment samples suggests a reasonable precision of the assay and a relatively low variability between the birds. Furthermore, the estradiol concentrations I measured fall in the same range as those obtained in other laboratories. Adkins-Regan and collaborators (1990a) measured estradiol concentrations of 45 pg/ml in intact males, Pröve (1983) 100 pg/ml and Hutchison and collaborators (1984) 360 pg/ml.

It is possible that I observed only a modest increase in plasma estradiol concentration in treated males because I overestimated the bioavailability of the hormone. Alternatively, I would expect that birds exposed to an exogenous source of estradiol would reduce their endogenous production of this hormone or increase its catabolism. If the latter explanation was true, then I would predict that estrogen levels would rise and fall rapidly after feeding. I did not find any information on the pharmacokinetics of estrogens in song birds. In chicken, however, the clearance rate of exogenous tritiated estradiol was estimated to be 77 ml plasma/min/kg (Johnson & van Tienhoven, 1981). This suggests that oral estrogens are metabolized rapidly by birds.

The variance of the data increased significantly on days 8 and 16. I can infer that a change in the plasma composition in some of the birds interfered with the assay because the variance was high on day 16, eight days after the males had been switched back to an untreated diet. I did observe marked differences in the colour and in the viscosity of different plasma extracts. Some birds treated with estradiol had yellow and more opaque and viscous plasma than the control birds and extraction and re-suspension did not eliminate the colour differences.

I might have gained interesting information by withdrawing blood samples sooner and at shorter intervals after the beginning of treatment. These observations would have allowed me to see if there was a gradual change in plasma estradiol. Doing more blood samples, however, would have proven difficult given the small size of the birds.

It would have been possible to use a synthetic estrogen such as ethinylestradiol (EE_2) or diethylstilbestrol (DES) as an alternative to 17β-estradiol. These are more effective when administered orally since they are more slowly metabolized by the liver (Goldfien, 1992). However, it would have been more difficult to confirm the plasma estrogen levels in the birds since the antibody used in the radioimmunoassay is specific to 17β-estradiol. Feedback mechanisms are quite complex in zebra finches and I initially felt it would be a good idea to validate the increase in plasma estradiol after orally exposing the birds to the hormone. Some studies have shown that unexpected results can be obtained when trying to manipulate hormone levels in zebra finches. Until 1990, several teams studying steroid hormones in zebra finches tried to equate hormonal levels of the birds by gonadectomizing them (Harding *et al.*, 1983; Adkins-Regan & Ascenzi, 1990b; Vockel, Prove & Balthazart, 1990). A study by Adkins-Regan (1990a), however, proved that this procedure was not only ineffective, but unexpectedly resulted in a dramatic increase in circulating estrogens in both males and females. When implanted with estradiol benzoate, the estradiol plasma levels of gonadectomized birds dropped back to near normal. Studies have subsequently shown that circulating estradiol can be synthesized by the brain in zebra finches (Schlinger & Arnold, 1991; Schlinger *et al.*, 1992). Another argument against using synthetic estrogens is that these hormones do not all have the same physiological effects. For instance, EE_2 but not DES significantly affect testis weight asymmetry in adult Japanese quails exposed *in ovo* (Halldin *et al.*, 1999). Since my study was designed as a positive control for estradiol, I decided to use the natural hormone. In retrospect, however, it would appear that I might have gained more information by giving less importance to plasma hormone levels and spending more time investigating the effects of various estrogenic compounds on behaviour.

5.3 Behavioural data

Several studies have shown that courtship and agonistic behaviours are facilitated by the conversion of testosterone to estradiol within the avian brain (Soma *et al.*, 2000; Wingfield, Hegner & Lewis, 1992; Schlinger & Callard, 1989). I therefore expected the estradiol treatments to promote courtship and sexual behaviours in males exposed to stimulus females, and to increase the agonistic response of a simulated territorial intrusion during the daily pair test with two stimulus birds. Since estrogens are known to modulate the sexual receptivity of females in avian species (Wingfield *et al.*, 1994; Watson *et al.*, 1990; Delville *et al.*, 1987), I also predicted an increase of tail quivering and accepted mounts in females exposed to the hormone.

5.3.1 Sexual behaviours

In my study, oral estradiol affected the males in the two choice sex preference (Fig. 8) and decreased several sexual behaviours in male zebra finches (Fig. 9, 10, and 11). The interpretation of the two choice preference test must be made with caution. When the stimuli for the two choice tests are presented simultaneously, it is not possible to assess if the bird is choosing one side of the cage or avoiding the other. One way to overcome this problem is to present a third choice, with no stimulus, to the bird (Martin *et al.*, 1993). However, even then, the motivations of the subject may not be assumed without further testing in an environment that allows direct interactions of the birds. For example, the tested bird may be choosing one side because it is sexually attracted by the adjacent stimulus, or it may be trying to defend its territory against "intruders". For this reason, a test in which the subject can come in contact with the stimulus bird can provide a better insight in the motivation behind the response obtained in the preference test.

In my investigation, males tended to spend more time close to the male stimulus cage during the treatment period (Fig. 8). Data obtained with the daily pair test suggests that there was a decrease in sexual activity in males treated with estradiol. This was unexpected since numerous studies have shown that sexual behaviours are partially controlled by this hormone in numerous avian species.

Sexual partner preferences can also be affected by the social environment of the bird during its development. Adkins-Regan and Krakauer (2000) found that adult males and females that have been raised in the absence of males, do not prefer the opposite sex stimuli in the two choice sex preference test. The birds in my colony were raised by both parents and were transferred to unisex cages only at puberty. The time spent in the unisex cages varied from bird to bird, but lasted at least two months. It would be interesting to see if long term housing in unisex cages can affect sexual partner preferences.

I found no reports on the activational effects of estradiol on sexual partner preference. In other species, such as the Japanese quail, song sparrow, and the red winged blackbird (*Agelaiua phoeniceus*), estradiol implants increase libido or sexual behaviours and restore these behaviours in castrated birds (Harding *et al.*, 1988; Wingfield *et al.*, 1994; Balthazart *et al.*, 1995). For example, in castrated Japanese quail both testosterone and estradiol restore head grabbing, mounting and cloacal contact movements (Watson & Adkins-Regan, 1989a). If, however, an aromatase inhibitor is co-administered with each of these hormones, only males implanted with estradiol exhibit normal sexual behaviour (Watson & Adkins-Regan, 1989b).

Zebra finch males castrated during adulthood have elevated circulating estradiol and decreased levels of androgens (Adkins-Regan *et al.*, 1990b). Those males exhibit reduced courtship and copulation, behaviours which may be related to sexual partner preference (Arnold, 1975; Harding *et al.*, 1983; Mansukhani *et al.*, 1996). While these behavioural changes may be linked to the low androgen levels, they could also be caused by the increased circulating estradiol. It is possible that, in my study, oral estradiol treatment resulted in the up-regulation of cytochrome P450s responsible for the metabolism of endogenous steroid hormones. If this was the case, testosterone catabolism might have increased. It is also possible that estradiol reduced the production and release of testosterone by inhibiting hypothalamic and pituitary trophic hormones. This would lead to an endocrine environment similar to those observed in gonadectomized zebra finches, and the decreased sexual activity would be consistent with observations made with castrated finches.

In females (Fig. 18, 19, and 20), as observed in other studies (Wingfield *et al.*, 1994), estradiol increased sexual receptivity. The number of females displaying tail quivers and accepting mounts from the stimulus males sharply increased on day 4 after the beginning of treatment (Fig. 18). This was reflected in the frequency of these behaviours in the high estradiol females (Fig. 19). On day 4, the females treated with estradiol spent more time in proximity of males than they did, on average, during the pre-treatment period (Fig. 8). Interestingly, this tendency was reversed on days 6 and 8.

Because of my experimental set-up, the daily pair test demanded much less handling than the preference test. In the latter, the females had to be removed from their home cage and transferred to the preference test cage. Females may have been more influenced by handling than the males and may have remained inactive during the test. I observed that some of the subjects constantly moved on their perch as they tried to join the birds from the stimulus cage while others were more calm and remained quiet on one of the perches without paying much attention to the adjacent stimulus birds. Information on the hopping behaviour of the birds might have supplied interesting data on their level of activity during the test.

5.3.2 Agonistic behaviours

Oral estradiol decreased agonistic behaviours in male and female zebra finches (Fig. 12, 13, 14; and 21, 22, 23). These results were unexpected as estradiol is typically involved in territorial behaviour in other species. In males, agonistic behaviours are exhibited during territory or mate defence and these behaviours are associated with increased circulating testosterone (Wingfield *et al.*, 1992; Silverin, 1993; Romero *et al.*, 1998) which is believed to act as a pro-hormone. In male song sparrows, for example, testosterone activates and sustains aggression during the breeding season (Wingfield, 1994).

Circulating steroid hormones alone, however, cannot explain behavioural changes during the breeding season. The sensitivity of the target tissues to these hormones must also be considered. In song sparrows, aggressive behaviours are sustained in the males even after castration and, in intact males, they are observed in autumn when circulating testosterone levels are low. It is possible that, like in zebra finch, gonadectomy elevates circulating estradiol in this species since aromatase inhibitor treatment greatly reduces aggressive behaviour during the non-breeding season and estradiol reverses these effects (Soma *et al.*, 2000).

In the artic breeding Laplang longspur (*Calcarius lapponicus*), the brain activity and distribution of aromatase, 5α-reductase, and 5β-reductase change through the breeding season. The presence of these metabolizing enzymes in the target tissues plays an important role in determining the sensitivity to circulating hormones and this sensitivity changes through the breeding season. In several species, this sensitivity to circulating steroids is modulated by the photoperiod (Brown, Johnson & Bottjer, 1993; Hutchison, Steimer & Jaggard, 1986; Thompson & Adkins-Regan, 1994; Wingfield, Hahn, Wada & Schoech, 1997). While zebra finches are well studied and exhibit numerous well characterized behaviours, their ability to breed during any natural cycle of photoperiod (Zann, 1996) makes it difficult to synchronize laboratory experiments. Because of this adaptation, their neuroendocrine systems may also possess unique mechanisms for the regulation of steroid sensitivity.

5.4 Conclusion

A radioimmunoassay was optimized to quantify plasma estradiol in zebra finches during their breeding cycle and after administration of dietary estradiol. Plasma estradiol concentrations during the breeding cycle did not change significantly but there was higher variability in the hormone levels during the nest building and the nestling care periods.

While dietary estradiol did not significantly elevate plasma estradiol concentrations in male zebra finches, there was a significant increase in the variance of the assay results which, in some birds, could be attributed to an increase in plasma estradiol or to a change in plasma composition induced by the treatment. Dietary estradiol caused significant changes in some of the behaviours that are important for reproduction. Estradiol did cause the expected increase in libido in female zebra finches: females treated with the high estradiol concentration exhibited more tail quivers and accepted mounts from the stimulus males more often. In males, however, there was an unexpected decrease in sexual and agonistic behaviours. Low estradiol males sang, danced and mounted less than other males. Treated males also had lower composite agonistic scores than controls. In the two choice preference test, treated males spent significantly less time near females.

Several behaviours suggested an effect of estradiol witch was not statistically significant. Increasing the sample size might have led to a statistically cleaner study. Also, because the behaviour of individual birds varied with time, a repeated measure experimental design may not have been warranted and my resources might have been better spent if I had tested more birds, fewer times.

The behavioural tests used in this investigation attempted to recreate a small fraction of the life cycle of avian species. The preference test represents encounters with conspecifics. The daily pair tests allow the birds to directly interact with the opposite sex as well as a bird of the same sex. During these tests, however, the birds did not have the time to socialize and evaluate each other and the encounters were probably not representative of those that would occur in a natural settings. Furthermore, these short tests did not take into account the full reproductive cycle and, thus, in further studies, some behaviours that are important for the survival of the offspring should be evaluated more extensively.

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