

The effects of sex steroids on spatial cognition  
in the zebra finch (*Taeniopygia guttata*)

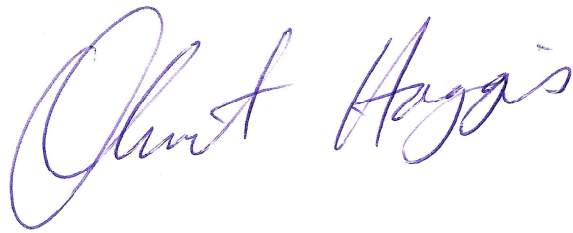


Olivia Haggis

A thesis submitted in  
application for the degree of Doctor of Philosophy  
University of Edinburgh  
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## Declaration

The work contained within this thesis is my own and has not been done in collaboration, except where otherwise stated. The text does not exceed 70,000 words, and no part of this thesis has been submitted to any other university in application for a higher degree.

A handwritten signature in blue ink, reading "Olivia Haggis". The signature is written in a cursive style with a large, looping initial 'O'.

Olivia Haggis

### Ethical note

All animal treatment, husbandry and experimental procedures were carried out in accordance with the Animals Scientific Procedures Act 1986, U.K. and the associated Guidelines under Home Office Project licence number 60/3666 and Personal licence number 60/11090. At the end of each experiment, all birds were humanely euthanased by decapitation.

## Abstract

It is well established in mammals that chronic, long-term elevations in sex steroids are associated with improvements in spatial cognition. It is less clear the extent to which short to medium term elevations in sex steroids improve spatial cognition and change hippocampal morphology, particularly in birds. The avian hippocampus expresses both androgen receptors (AR) and oestrogen receptor alpha (ER $\alpha$ ) and high levels of the enzyme aromatase that converts testosterone to oestrogen. I began by comparing spatial cognition, hippocampal sex steroid receptor and aromatase expression between males and females. There were no differences in spatial or visual cognition or in hippocampal sex steroid receptor expression between the sexes, although hippocampal aromatase mRNA expression was higher in males. I then addressed the effects of acute and medium-term sex steroid treatment on spatial cognition and hippocampal aromatase and sex steroid receptor expression. A single treatment of testosterone 30 minutes or four hours prior to cognitive testing improved spatial performance. Additionally, when testosterone and oestrogen were given daily for five days spatial cognition in both sexes was improved. The testosterone-induced improvement was blocked when testosterone was administered in conjunction with the aromatase inhibitor fadrozole but not when administered with saline. These findings suggest that spatial cognition is improved by an oestrogenic effect. Thirty minutes following acute testosterone treatment, plasma testosterone levels, hippocampal AR and ER $\alpha$  mRNA expression all increased. Five days of oestrogen treatment increased plasma oestrogen levels, hippocampal ER $\alpha$  mRNA and N-methyl-D-aspartate (NMDA) receptor levels in males and females; all were positively correlated with enhanced spatial cognition on day five of treatment. Finally, I determined which genes were differentially expressed as a result of five days of oestrogen treatment. Nineteen genes, identified as being involved in learning and memory were differentially expressed in the hippocampus, eleven of which were up-regulated and eight were down-regulated. Taken together these results demonstrate that oestrogen can improve spatial cognition in birds. It is plausible that oestrogen acts to improve spatial memory in the hippocampus through upregulation of genes that control neurotransmitter release, reuptake and receptor levels.

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# Chapter 1

## General Introduction

Sex differences in cognition can attract considerable, and enthusiastic, attention. Take, for example, the reaction sparked in response to Lawrence Summers' controversial hypotheses on the under-representation of women in the top levels of academia, which resulted in his resignation as Harvard's president. Aside from the general interest in differences in cognition between the sexes, there are implications for the treatment of disorders, including age-related illness such as Alzheimer's disease, which women are at greater risk of developing than men (Check Hayden, 2010; Gao et al., 1998). Understanding more about these differences and the underlying mechanisms will aid the development of appropriate, potentially sex-specific, treatments for disorders involving a cognitive component. The biggest, most reliably demonstrated differences between the sexes are in spatial cognition (as noted in Summers' talk), in non-human mammals as well as in humans, with males outperforming females on mental rotation, map reading and maze tasks (Astur et al., 1998; Astur et al., 2004; Collins and Kimura, 1997; Driscoll et al., 2005; Galea et al., 1995; Galea et al., 1994a; Galea and Kimura, 1993; Gaulin et al., 1990; Gron et al., 2000; Jonasson, 2005; Vandenberg and Kuse, 1978). Spatial cognition involves the acquisition, organization, storage and utilization of information about the spatial environment, which for many species facilitates successful navigation around an environment (Hart and Moore, 1973). Although when sex differences are observed, it is invariably males that perform better than do females, the considerable variation in the magnitude and consistency of that difference begs the question of the cause. There may be an evolutionary basis that explains a greater demand for spatial performance in males, for example, sexual selection may have favoured males with increased spatial abilities for either better navigational skills in hunting or to enable an increased territory size (Jones et al., 2003; Joseph, 2000). It is still not known to what degree sex differences are rooted in cultural biases and early learning experiences and the involvement of biological factors, including stress hormones

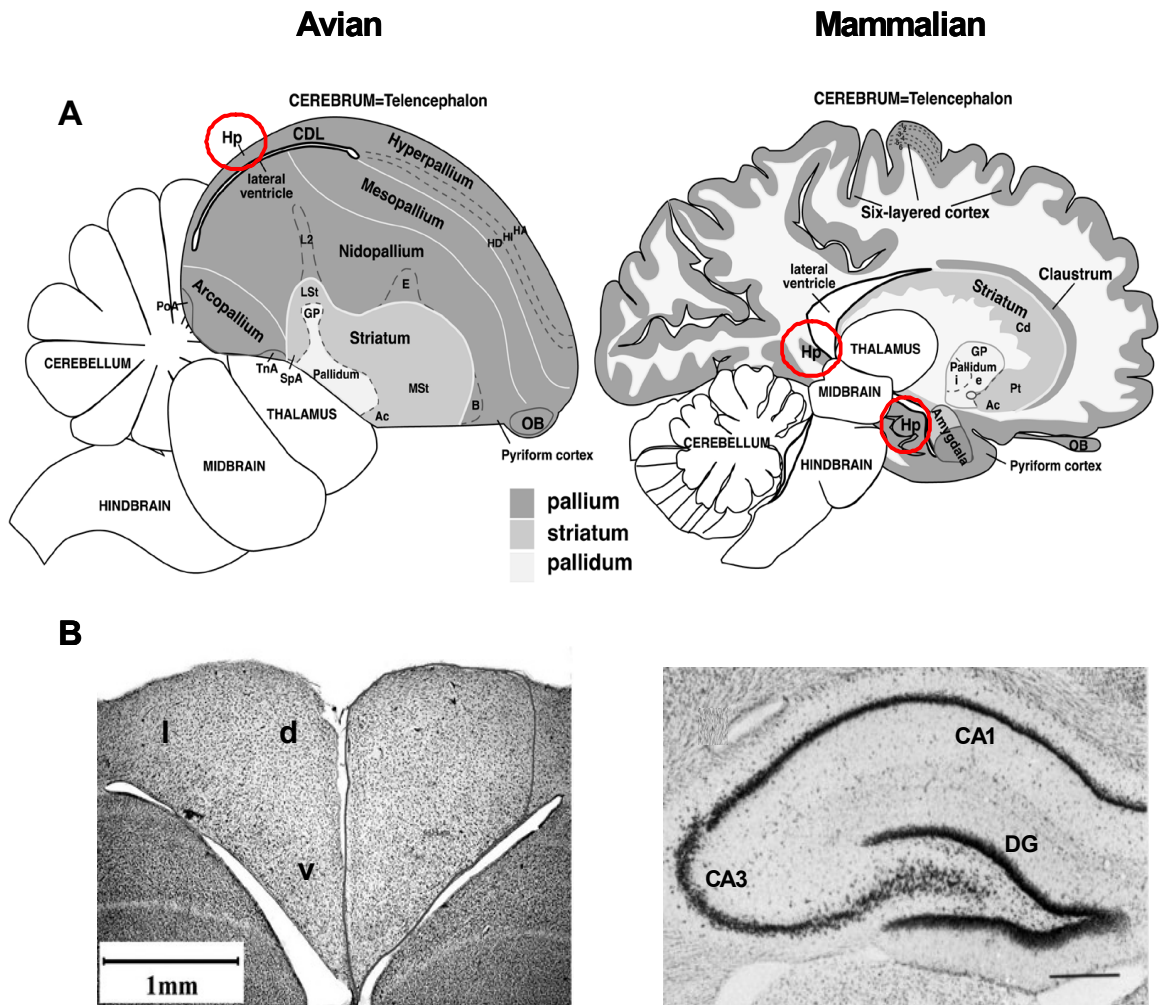
(Beiko et al., 2004; Feng et al., 2007; Lupien et al., 2007; McGillicuddy-De Lisi, 2002; Montello, 1995; Shors, 2006a; b).

Whatever the ultimate cause, it is plausible that sex steroids mediate this variation, in some way. The effects of sex steroids on sexual differentiation and maturation of the gonads and secondary sexual characteristics are well known but they also have important central actions to modulate a range of behaviours including spatial cognition. For example, male deer mice (*Peromyscus maniculatus*) only outperform conspecific females on spatial tasks in the breeding season, when testosterone levels are highest and in humans, testosterone supplementation in older men and young women improves spatial performance (Aleman et al., 2004; Cherrier et al., 2001; Galea et al., 1996; Galea et al., 1994a; Gray et al., 2005; Janowsky et al., 1994; Postma et al., 2000). In the reverse situation, lowering circulating levels by gonadectomy in rodents or chemical castration and prostate cancer in men leads to impairments (Almeida et al., 2004; Cherrier et al., 2003; Gibbs and Johnson, 2008; Kritzer et al., 2001; Salminen et al., 2004). In birds, the effects of chronic manipulations of sex steroids have been well described for the song system (Brenowitz, 2004). Following chronic testosterone treatment, males will sing more and adult gonadectomized female canaries under the influence of testosterone acquire male-like song (Bernard and Ball, 1997; Bernard et al., 1997; Dloniak and Deviche, 2001; Gulledge and Deviche, 1997; Nottebohm, 1980; Smith et al., 1997b). In addition to song, testosterone also affects parental behaviour such that male dark-eyed juncos (*Junco hyemalis*) with testosterone implants spend less time at the nest feeding the young compared to unmanipulated controls (Chandler et al., 1994; Ketterson et al., 1992; Schoech et al., 1998). Moreover, implanted birds have home ranges that are two to three times larger than those of control males, which is associated with greater success at extra-pair copulations (Chandler et al., 1997; Chandler et al., 1994; Raouf et al., 1997). Although it is generally agreed that sustained elevation of oestrogen and testosterone (typically via implants) leads to improved spatial cognition in mammals, the effects of short-term fluctuations are more controversial (Daniel et al., 1997; Heikkinen et al., 2002; Lacreuse et al., 2002;

Rissanen et al., 1999). For example, across the oestrous cycle, it has been shown that female rats perform better when in proestrous (high oestrogen levels), finding the platform in the Morris Water Maze (MWM) faster than in other phases of the cycle (Healy et al., 1999). In contrast, there are other reports that they only perform better on the day of oestrous or that their abilities do not vary at all (Berry et al., 1997; Sutcliffe et al., 2007; Warren and Juraska, 1997). The bird literature currently adds little to this debate. Currently there are only a handful of experiments in which sex steroids have been manipulated and their effects on spatial memory investigated (Hodgson et al., 2008; Oberlander et al., 2004; Spence et al., 2009).

There is an abundance of evidence to demonstrate that the vertebrate hippocampus is involved in spatial information processing. In birds, as in mammals, the hippocampus is part of a complex circuit and outputs from the hippocampal formation (hippocampus and parahippocampal area) are sent to brain regions including the septum, hypothalamus, amygdala and thalamus (Atoji and Wild, 2006; Bird and Burgess, 2008). Although there are differences between mammals and birds in the location and anatomy of the hippocampus (Figure 1), it seems that it plays a similar role in spatial processing. For example, the hippocampus is activated when humans are navigating a virtual maze and hippocampal lesions impair performance on spatial tasks in monkeys and rodents and in birds (Alvarez et al., 1995; Bailey et al., 2009; Bingman et al., 1988; Bingman and Mench, 1990; Bischofe et al., 2006; Colombo et al., 1997; Fremouw et al., 1997; Gron et al., 2000; Hampton and Shettleworth, 1996; Maguire, 1997; Maguire et al., 1998; Maguire et al., 1996; 1997; Maguire et al., 2000; Morris et al., 1982; Murray and Mishkin, 1998; Patel et al., 1997a; Sherry and Vaccarino, 1989; Sutherland et al., 1982; Sutherland et al., 1983; Watanabe and Bischof, 2004). In the course of spatial learning and recall, in mammals and birds the hippocampus shows high expression of immediate early genes (IEGs), genes which are activated transiently and rapidly in response to neuronal activity, although the distribution of expression in the hippocampus differs across the two groups (Bischofe et al., 2006; Bozon et al., 2002; Guzowski et al., 2001; Mayer et al., 2009; Poirier et al., 2008; Smulders and DeVoogd, 2000). It also

appears that the size of the hippocampus is associated with an extra demand for spatial learning and memory: meadow voles that have a larger home range size compared to pine voles, also have a larger hippocampus and solve mazes with fewer errors (Jacobs et al., 1990; Sherry et al., 1992). In humans, the posterior region of the hippocampus of London taxi drivers is significantly larger than it is in age-matched people who do not drive taxis (Maguire et al., 2000). Furthermore, hippocampal volume is larger the longer the time spent as a taxi driver (Maguire et al., 2000). Food-storing birds, which remember large number of cache locations, not only have a larger hippocampus (relative to telencephalon size) than do non-food storers, but the hippocampus is larger the more food is stored (Basil et al., 1996; Hampton et al., 1995; Healy and Krebs, 1992; Healy and Krebs, 1996; Krebs et al., 1989; Sherry et al., 1989). Migration also exerts extra spatial demands and, indeed, the migrant Garden Warbler (*Sylvia borin*) has a larger relative hippocampus than does a closely related resident species, the Sardinian Warbler (*S. melanocephala*) (Healy et al., 1996). There are also sex differences in spatial demand and in hippocampal volume within species. Male meadow voles (*Microtus pennsylvanicus*), who range widely during the breeding season to gain access to multiple mates, have a larger relative hippocampal volume than do females and outperform their conspecific females on spatial tasks (Galea et al., 1999). Conversely, male and female pine voles (*M. pinetorum*) that occupy the same territory do not differ in hippocampal volume or spatial cognition (Galea et al., 1999). Spatial demand also appears to explain the larger hippocampal volume in females relative to conspecific males in a parasitic cowbird species in which the females search for and relocate host nests alone (shiny cowbirds *Molothrus bonariensis* and brown-headed cowbirds *M. ater*). In contrast, in screaming cowbirds (*M. rufoaxillaris*), in which the males and females search together, there are no sex differences in hippocampal size (Reboreda et al., 1996; Sherry et al., 1993).

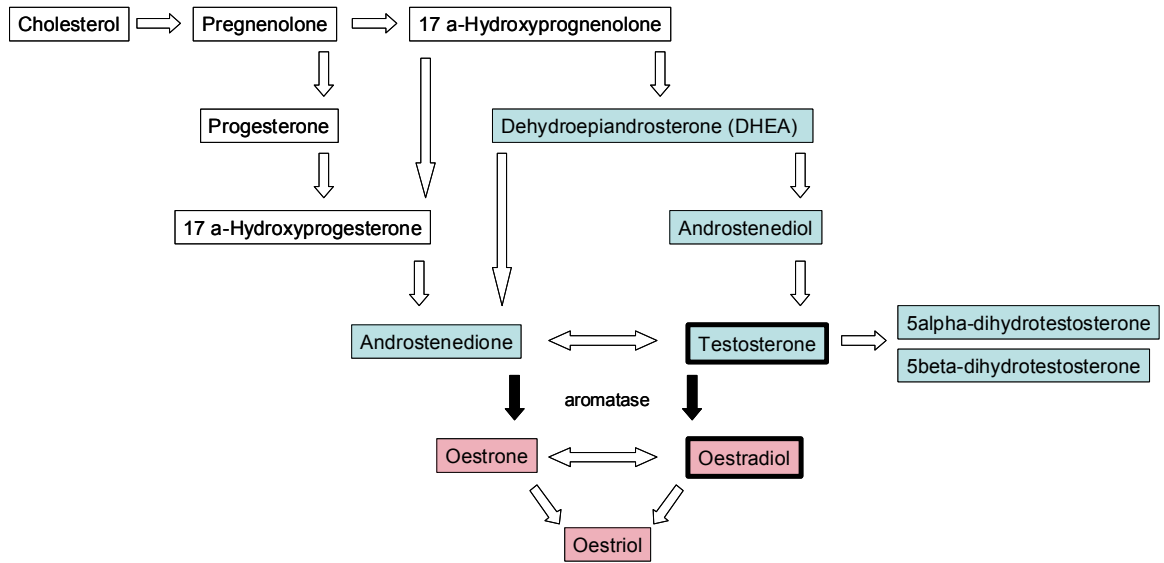


**Figure 1** A) The modern consensus view of avian and mammalian brain relationships according to the conclusions of the Avian Brain Nomenclature Forum, highlighting the location of the hippocampus (Hp) (adapted from (Reiner et al., 2004)). Abbreviations: Ac, accumbens; B, basorostralis; Cd, caudate nucleus; CDL, dorsal lateral corticoid area; E, entopallium; GP, globus pallidus (i, internal segment; e, external segment); HA, hyperpallium apicale; HD, hyperpallium densocellulare; HI, hyperpallium intercalatum; Hp, hippocampus; L2, field L2; Lst, lateral striatum; MSt, medial striatum; OB, olfactory bulb; PoA, posterior pallial amygdala; Pt, putamen; TnA, nucleus taeniae of the amygdala; SpA, subpallial amygdala. B) Coronal sections of the avian and mammalian hippocampus, showing the ventral (v), dorsal (d) and lateral (l) regions of the avian hippocampus and the dentate gyrus (DG), *Cornu Ammonis* (CA1 and CA3) in the mammalian hippocampus (black-capped chickadee (Tarr et al., 2009), rat (Puurunen, 2001)).

In mammals, variation in sex steroid levels correlates with changes in hippocampal physiology and function. For instance, in meadow voles ‘high’ testosterone males have larger hippocampal volumes (more cells) than do ‘low’ testosterone males (Galea et al., 1999; Roof, 1993; Roof and Havens, 1992). Both testosterone and oestrogen can increase hippocampal spine density, with female rats showing cyclic variations in hippocampal dendritic spine density positively correlated with circulating oestrogen levels (Leranth et al., 2003; Leranth et al., 2004b; Woolley et al., 1990). Synaptic plasticity also varies across the oestrous cycle with enhanced long-term potentiation (LTP) corresponding to high levels of oestrogen (Warren et al., 1995). Oestrogen also leads to enhanced LTP in males (Foy et al., 1999). Although in birds little is known about the effects of sex steroids on hippocampal morphology, seasonal structural changes in the song system in response to fluctuations in sex steroids illustrate the capacity for plasticity in the avian brain (De Groof et al., 2009; Tramontin and Brenowitz, 2000). Both testosterone and oestrogen contribute to seasonal growth of the song system in males, particularly the HVC, and this increase in volume is positively correlated with rates of singing (Ball et al., 2002; Bernard and Ball, 1997; Bernard et al., 1997; Brenowitz, 2004; Dloniak and Deviche, 2001; Gullledge and Deviche, 1997; Smith et al., 1997b). Testosterone treatment also increases the size of two vocal control nuclei (the HVC and RA) in adult gonadectomized female canaries (Nottebohm, 1980). Furthermore, there are increases in dendritic growth in the song system due to increases in sex steroid levels in ovariectomized adult female canaries (DeVoogd and Nottebohm, 1981). Although there are seasonal changes in hippocampal volume and neurone number in the black-capped chickadee, and oestrogen-treated male zebra finches have larger hippocampal cells compared control birds, how these changes relate to spatial abilities is still unclear (Barnea and Nottebohm, 1994; Oberlander et al., 2004; Smulders et al., 1995; Smulders et al., 2000).

A key factor in determining the level of testosterone and oestrogen in the brain is the activity of the enzyme aromatase that converts testosterone to oestradiol. In addition to aromatisation, testosterone can be converted to the active androgen 5 $\alpha$ -

dihydrotestosterone (DHT) by  $5\alpha$ -reductase or can be inactivated by conversion into  $5\beta$ -DHT by  $5\beta$ -reductase (Figure 2). In most birds,  $5\alpha$ -reductase and  $5\beta$ -reductase are expressed widely in the brain (Hutchison and Steimer, 1984; Schlinger and Callard, 1987; Schumacher and Balthazart, 1987; Vockel et al., 1990). In contrast, aromatase has a restricted distribution in the brain of non-songbirds, being concentrated in nuclei of the hypothalamus and in the nucleus taeniae of the telencephalon, which is homologous to portions of the mammalian amygdala (Hutchison and Steimer, 1986; Schumacher and Balthazart, 1987; Thompson et al., 1998). While songbirds express aromatase in these same brain regions, they also show widespread aromatase expression in the telencephalon, especially in the hippocampus and caudal neostriatum (Callard et al., 1978; Forlano et al., 2006; Saldanha and Schlinger, 1997; Saldanha et al., 2000b; Shen et al., 1994; Shen et al., 1995; Vockel et al., 1990; Wehrenberg et al., 2001; Yague et al., 2008). In both mammals and birds, testosterone is synthesized from cholesterol *de novo* in the brain, adrenals and the testes, regulated by luteinizing hormone (LH) and follicle stimulating hormone (FSH) which are produced by the anterior pituitary gland and released under control by gonadotropin-releasing hormone in the hypothalamus (the hypothalamic-pituitary-gonadal axis) (Hojo et al., 2004; Meethal and Atwood, 2005; Mukai et al., 2006). However, the sources of oestrogen in males differ in mammals compared to song birds; in the latter aromatase activity is undetected in testes, adrenals, or other tissues and the main source of oestrogen comes from aromatisation of testosterone in the brain (Schlinger and Arnold, 1991; 1992). In female zebra finches oestrogen is also synthesized in the ovaries as well as in the telencephalon, as it is in mammals (Schlinger and Arnold, 1991). Song birds may provide a particularly good system to investigate the seemingly fundamental role of aromatase in oestrogen production and its regulation of spatial cognition, as they have particularly high expression in the hippocampus (Saldanha et al., 1999; Saldanha et al., 1998; Saldanha and Schlinger, 1997; Saldanha et al., 2000b; Shen et al., 1994; Shen et al., 1995). Additionally, as the zebra finch is one of the song birds that is an opportunistic breeder, aromatase expression tends not to fluctuate throughout the year (Vleck and Priedkalns, 1985).

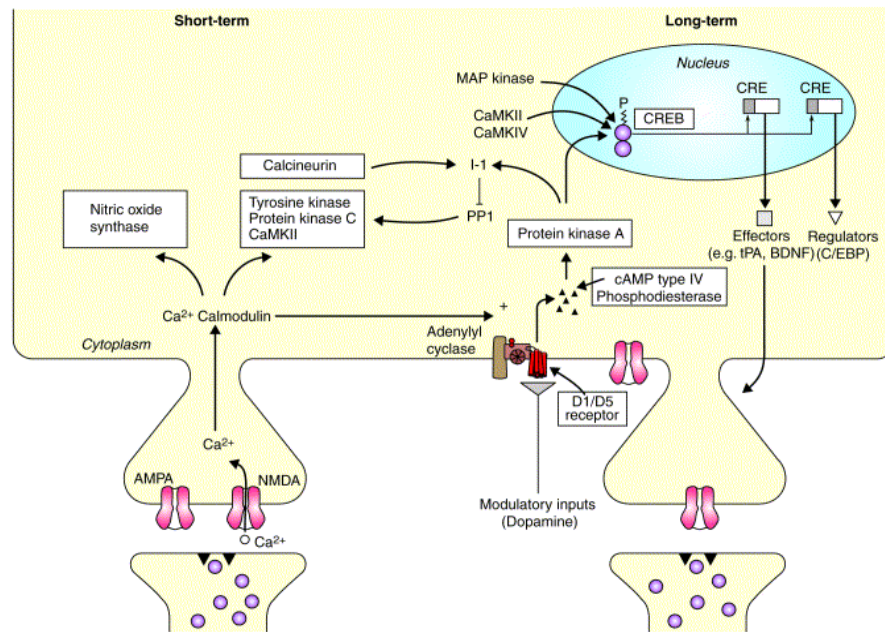
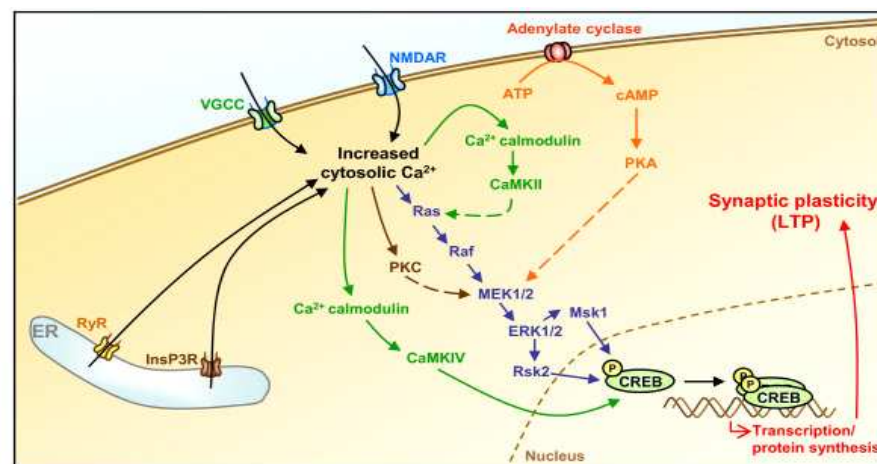


**Figure 2** The synthesis of androgens and oestrogens from cholesterol (adapted from (Tsilchorozidou et al., 2004) and (Janowsky, 2006)). The full range of components of certain types of behaviours requires both androgenic (blue boxes) and oestrogenic (pink boxes) metabolites.

To enhance spatial memory, testosterone and oestrogen modulate the pathways involved in memory formation. Memory formation requires changes in synaptic plasticity achieved by the synthesis of new proteins such as the glutamate AMPA ( $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptor and structural proteins involved in the remodelling of dendritic spines (Leuner et al., 2003). The synthesis of new proteins is triggered by the activation of the glutamate N-methyl-D-aspartate (NMDA) receptor, which occurs through simultaneous binding of glutamate to the NMDA receptor and postsynaptic membrane depolarization (e.g. through AMPA receptors), which removes the voltage-dependent magnesium ion block (Adams et al., 2001c; Bannerman et al., 1995; Bliss and Collingridge, 1993; Bolhuis and Reid, 1992; Kawabe et al., 1998; Lee and Kesner, 2002; Niewoehner et al., 2007; Tsien et al., 1996). The resulting influx of calcium across the postsynaptic membrane through the NMDA receptor is fundamental in the control of synaptic activity and memory formation (Figure 3). The calcium influx triggers the activation of specific calcium-dependent signal transduction pathways involving key protein effectors, such as  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases (CaMKs), mitogen-activated



protein kinases/extracellular signal-regulated kinases (MAPK/ERKs e.g. the MAPK kinase kinase MEK and signal-regulated kinase ERK) and adenylyl cyclase, which produces cyclic adenosine monophosphate (cAMP) that then activates Protein kinase A (PKA). The activation of these pathways results in the phosphorylation and activation of the transcription factor cyclic AMP/cAMP-responsive-element-binding protein (CREB), leading to changes in the transcription of genes important in memory (Figure 3B).

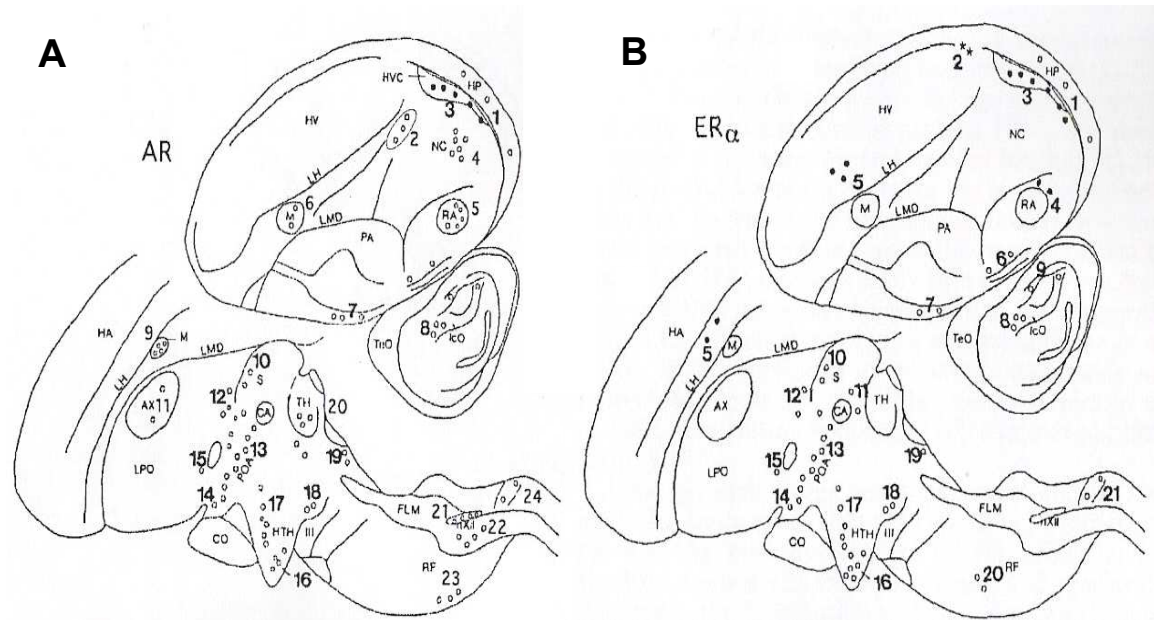
**A****B**

**Figure 3** A) The molecular events that underlie the early and late phases of long-term potentiation. Adapted from (Abel and Lattal, 2001). B) The signalling cascades activated by increased cytosolic calcium critical for protein synthesis-dependent synaptic plasticity, through cell surface calcium channels, e.g. voltage-gated calcium channels (VGCCs) or N-methyl-D-aspartate receptor (NMDAR) or via endoplasmic reticulum (ER) calcium efflux. Molecules in the cascade include  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases (CaMKs), mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERKs e.g. the MAPK kinase kinase MEK and signal-regulated kinase ERK), which then phosphorylate cyclic AMP/cAMP-responsive-element-binding protein (CREB). Adapted from (Marambaud et al., 2009).

The changes induced by sex hormones in spatial performance, the size of the hippocampus and hippocampal dendritic densities are mediated by NMDA receptors, at least in mammals. For example, in rats oestradiol-induced increases in dendritic spine density are blocked by NMDA receptors antagonists (Woolley and McEwen, 1994; Woolley et al., 1997). The effects of oestrogen on NMDA receptors may be due to an increased sensitivity of hippocampal cells to NMDA receptor mediated synaptic input, and/or increased NMDA NR1 subunit level in hippocampal dendrites (Gazzaley et al., 1996; Weiland, 1992; Woolley et al., 1997). Whether there is a generalised mechanism for sex hormone-induced changes in hippocampal morphology through NMDA receptors is unknown, but it seems feasible that they could play a role in avian spatial memory as NMDA receptors are present in the avian hippocampus and their expression is increased in response to oestrogen (Margrie et al., 1998; Meehan, 1996; Saldanha et al., 2004; Shiflett et al., 2004). However, whether this oestrogen-induced increase in NMDA receptors leads to improvement in spatial abilities is not clear.

Testosterone and oestrogen exert their modulatory actions on memory pathways through binding to androgen (AR) and oestrogen receptors (alpha or beta; ER $\alpha$  and ER $\beta$ ) (Behl, 2002; Belcher and Zsarnovszky, 2001; Janowsky, 2006). Many brain regions, in addition to the mammalian and avian hippocampus, contain these receptors and they are a target for sex steroid regulation of aggressive, copulatory, courtship and singing behaviour (Balthazart and Surlemont, 1990; Beyenburg et al., 2000; Fusani, 2008; Gahr, 2001; Gahr et al., 1993; Gahr and Metzdorf, 1997a; Hodgson et al., 2008; McGinnis et al., 1996; Metzdorf et al., 1999; Sarrieau et al., 1990; Siegel et al., 1986; Sperry et al., 2010). Comparative studies of the central distribution of ER and AR expressing cells in vertebrates have shown that the brain regions that typically contain such cells are evolutionarily conserved, which include the hypothalamic-preoptic areas, certain limbic forebrain structures and the striatum of the mesencephalon ((Gahr, 2001); Figure 4). In both male and female birds AR and ER $\alpha$  are distributed in the same brain regions, with certain exceptions such as the

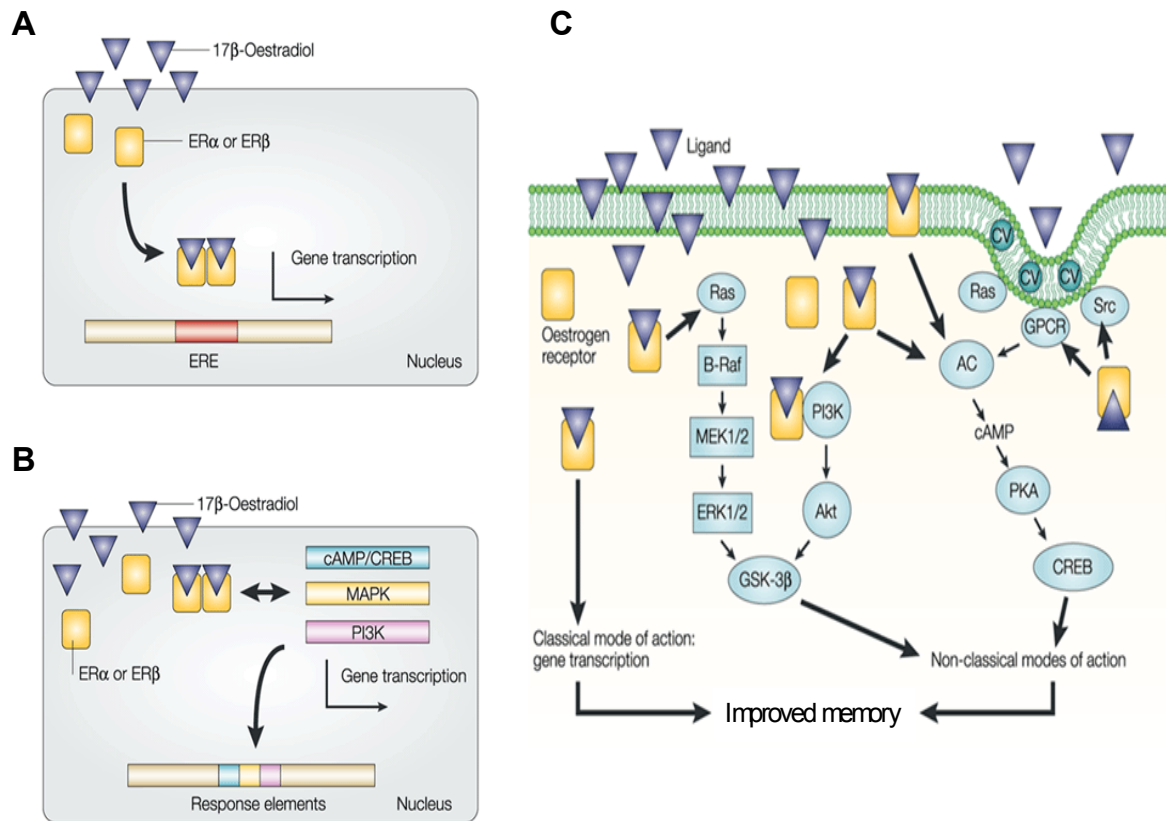
absence of ARs in the forebrain vocal areas of female songbirds e.g. the zebra finch, in which the females do not sing.



**Figure 4** The distribution of sex steroid receptors in the avian brain shown in schematic sagittal drawings of a lateral (top) and medial (bottom) view. A) The distribution of androgen receptors (AR). B) The distribution of oestrogen receptor alpha ( $ER\alpha$ ). Abbreviations: AX, Area X; CA, anterior commissure; CO, chiasma opticum; FLM, fasciculus longitudinalis medialis; HA, hyperstriatum accessorium; HP, hippocampus; HTH, hypothalamus; HVC, hyperstriatalis ventrale pars caudale; LH, lamina hyperstriatica; LMD, lamina medullaris dorsalis; LPO, lobus parolfactorius; NC, neostriatum caudale; nXII, n. hypoglossus pars tracheosyringalis; PA, paleostriatum augmentatum; POA, preoptic area; RA, n. robustus archistriatalis; RF, reticular formation; TeO, tectum opticum; TH, thalamus; III, nervus oculomotoris. Adapted from (Gahr, 2001).

In the classical mode of signalling, in which testosterone and oestrogen exert a genomic action, they diffuse across the cell membrane and activate their receptors which then undergo a structural change allowing movement into the nucleus where they bind directly to specific DNA response elements, regulating gene transcription and *de novo* protein synthesis, which can lead to changes in memory formation ((Dudai, 2004; Silva and Giese, 1994); Figure 5). The oestrogen-receptor complex mainly binds to oestrogen response elements (EREs) in the DNA, however, it can

bind to other motifs such as serum response elements (SREs) (Musgrove and Sutherland, 2009). In addition, ERs can interact directly with various intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, leading to CREB phosphorylation and thereby affecting the transcription of other target genes (Behl, 2002; Belcher and Zsarnovszky, 2001; Bi et al., 2001; Manthey et al., 2001; Singer et al., 1999); Figure 5C). CREB phosphorylation has been implicated in oestrogen-induced spine formation in the hippocampus, which may occur through actions of activated CREB on the NR1 subunit of the NMDA receptor (Lau et al., 2004; Murphy and Segal, 1996; Zhao et al., 2005). Oestrogen can alter transcription of hippocampal genes encoding proteins important in memory formation after chronic treatment or following a single dose of oestrogen, occurring within one hour (Aenlle et al., 2009; Pechenino and Frick, 2009). One group of genes that are targeted by oestrogen code for structural proteins, such as neurofilament and microtubulin-associated proteins, thereby allowing for modulation of the cellular architecture (Brueggemeier et al., 2001; Ferreira and Caceres, 1991; Scoville et al., 1997; Shughrue and Dorsa, 1993). Sex steroids also have rapid non-genomic effects on behaviour which occur too quickly to involve mechanisms requiring gene transcription (Balthazart, 2010; Cornil, 2009). These rapid actions may involve binding to transmembrane ion channels such as GABA<sub>A</sub>, NMDA and serotonin type 3 (5-HT<sub>3</sub>) and the modulation of G-protein-coupled receptors (GPCRs), in part by rapidly increasing protein kinase A and C (PKA and PKC) activities, which has the ability to modulate the coupling of GPCRs to their effector systems (Kelly and Wagner, 1999; Moss et al., 1997; Rupprecht et al., 2001; Valverde et al., 1999; Wetzel et al., 1998; Woolley, 1999).



**Figure 5** A) Oestrogen can have a classical mode of action by binding to oestrogen receptor (ER) and binding the oestrogen response element (ERE) in genes to directly alter gene transcription. B) ERs might interact directly with various intracellular signalling pathways, including the cyclic AMP/cAMP-responsive-element-binding protein (CREB), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, thereby affecting indirectly the transcription of several other target genes. C) The intracellular signaling molecules activated by oestrogen. Adapted from (Behl, 2002).

There are also a variety of other hormones, neurotransmitters and neuropeptides that, in addition to sex steroids, act on the hippocampus in mammals and birds to affect memory. The neurotransmitters glutamate, GABA, noradrenaline, serotonin and dopamine have well established roles in memory formation ((Gibbs, 2008; Gold, 2003; González-Burgos et al., 2008; Krebs et al., 1991; Matsukawa et al., 1997; McEntee, 1992; Myhrer, 2003; Riedel et al., 2003; van Stegeren, 2008); Figure 3A). In addition to neurotransmitters, the stress hormones cortisol/corticosterone affects memory formation, which involves interactions with sex hormones. In mammals,

the effect of stress on memory is in different directions in males and females, associated with opposite effects on dendritic spine density (Shors, 2002; Shors et al., 2001). Furthermore, the stage of oestrous mediates the stress-induced impairment of associative learning in the female rat (Shors et al., 1998). In mammals, stress hormones also interact with other neuromodulatory systems, such as the noradrenergic system (McGaugh et al., 2008). The avian hippocampus has both mineralocorticoid and glucocorticoid receptors and spatial abilities are impaired in zebra finches selectively bred to respond to an acute stressor with high plasma corticosterone levels (Hodgson et al., 2007). It is therefore likely that there are similar interactions between sex and stress hormones in the avian brain. Another class of neuromodulator involved in memory in mammals is the neuropeptides, some of which are also present in the avian hippocampus, such as substance P, vasoactive intestinal polypeptide, vasopressin, cholecystokinin, neuropeptide Y and somatostatin (Crawley and Corwin, 1994; Dutar et al., 2002; Erichsen et al., 1991; Goodson and Bass, 2001; Masuo et al., 1993; Stäubli and Huston, 1980; Tokita et al., 2005; Tomaz and Nogueira, 1997).

None of the work to date has followed manipulation of sex steroids from behaviour through to changes in gene transcription in one system. To do this, there are several crucial components that need to be in place: (1) it must be possible to acutely manipulate hormone levels (2) there must be a suitable sensitive spatial task to detect a response (3) appropriate receptors must have been identified and techniques must exist for measuring a response. The aim of my thesis was to follow the effects of sex steroids on spatial cognition using the zebra finch as a 'model' system. The reasons for choosing the zebra finch were several fold: (1) sex steroids can be administered orally and appear to lead to changes in spatial cognition (Hodgson et al., 2008). This non-invasive method has the advantage of enabling investigation into the effects of short-term variation in hormones on spatial cognition without the side effects of stress resulting from the more usual methods of administering hormones via injection or implant; (2) in birds, spatial performance can be assessed by the ability to relocate hidden food after a retention interval, using an experimental tray in which several

wells are covered with cardboard, one of which is rewarded (Hodgson and Healy, 2005; Hodgson et al., 2007; Oberlander et al., 2004; Patel et al., 1997a). This is a useful experimental method for testing cognitive ability as it is relatively easy to train birds to remove cardboard pieces to locate food rewards, there can be multiple different locations so that in each trial a different rewarded location could be used, the duration of the retention interval can be manipulated to examine how long birds can remember locations and the proximity of covered wells to each other can be manipulated to investigate the effects of spatial interference. As the location of the rewarded well is trial-specific and changed daily, it is a test of spatial working memory, which is important as oestrogen appears to selectively improve spatial working memory in mammals and not spatial reference memory, and NMDA receptors appear to have a specific involvement in working memory (Fader et al., 1999; Niewoehner et al., 2007). The task is hippocampal dependent as lesioning the hippocampal formation impairs spatial performance of zebra finches in a similar task (Patel et al., 1997a). Furthermore, this model allows investigation of spatial cognition in a way that minimises the possible impacts of stress on task performance. Birds can be trained and tested in their home cage which has the advantage of eliminating the stress experienced as a result of handling and no food deprivation is required. In contrast, the commonly used spatial tasks in mammals, the MWM and the radial arm maze (RAM) are stressful (Morris, 1984; Morris et al., 1982). The MWM involves swimming in opaque water to find a platform and seems to be particularly stressful for females, which may introduce bias when looking at sex differences, and the RAM depends on food reward and requires food deprivation which is a stressor (Daniel et al., 1997; Harris et al., 2008). Scrub jays have a very complex memory for caches, remembering not only what and where they cached but also when. However, these birds are not a model organism in which to address questions about the underlying neuroscience due to small sample sizes and because the birds are expensive to keep, are long lived and take a long time to train (Clayton and Dickinson, 1998; Emery and Clayton, 2001). In contrast, zebra finches are easy to breed in captivity, relatively cheap to house and are opportunistic not seasonal breeders which allows for investigation without complications of seasonal



fluctuations in reproductive hormones (Vleck and Priedkalns, 1985). (3) The zebra finch is a good model in which to investigate neural components involved in the effects of sex steroids on spatial cognition as it is already known that the hippocampus is involved in spatial memory function and contains testosterone, oestrogen and NMDA receptors and there are established techniques available to detect aromatase, sex steroid and NMDA receptor mRNA, protein and binding levels in the avian brain (Hodgson et al., 2008; Jacobs et al., 1999; Margrie et al., 1998; Meehan, 1996; Oberlander et al., 2004; Patel et al., 1997a; Perlman et al., 2003; Saldanha and Coomaringam, 2005; Saldanha et al., 2004; Shiflett et al., 2004; Stewart et al., 1992).

## **Thesis questions**

The aims of this thesis were two-fold: 1) to characterise the effects of sex steroids on short-term variation in spatial cognition; 2) to decipher the neural basis of these effects through investigation of hippocampal sex steroid and NMDA receptor changes.

The following questions were addressed in this thesis:

### **1) Does a variation in sex steroid levels underlie differences in spatial abilities?**

- Is performance linked to circulating testosterone levels?
- Is variation in hippocampal sex steroid and aromatase expression linked to circulating testosterone levels?

I predicted that there would be sex difference in circulating testosterone, with males having higher levels. I predicted that this would lead to sex differences in performance in the spatial task and hippocampal receptor expression. I predicted that such effects would be limited to the spatial domain.

### **2) Does artificially increasing sex steroid levels improve spatial ability**

#### **a) when acutely administered as a one off treatment?**

- Does testosterone treatment improve spatial performance?
- Does testosterone increase hippocampal AR, ER $\alpha$  and aromatase mRNA expression?

I predicted that a single testosterone treatment would lead to increased circulating plasma testosterone levels and that corresponding improvements in spatial performance and increases in hippocampal sex steroid mRNA and aromatase expression would be seen in both sexes.

**b) when administered medium term?**

- Does five days of sex steroid treatment improve spatial performance?
- Is oestrogen, via its conversion from testosterone, important for spatial performance?
- What effect does sex steroid treatment have on hippocampal AR, ER $\alpha$  and aromatase?

I predicted that testosterone and oestrogen would improve spatial performance over five days, and that this improvement would be blocked in the testosterone treated group by the aromatase inhibitor fadrozole. I also predicted that improvement in spatial performance would involve NMDA receptors.

**3) What genes are altered by medium term oestrogen treatment?**

I predicted that five days of oestrogen treatment would lead to the up-regulation of genes involved in learning and memory cascades, compared to control groups. I predicted that some genes might show sex specific changes as hippocampal memory pathways may differ between the sexes.

## Chapter 2

### General materials and methods

#### **Subjects**

Birds were sexually mature, captive-bred adult male and female zebra finches (*Taeniopygia guttata*), obtained from institutions within the UK (ranging from one year to four years of age). They were individually caged in wire mesh cages (77cm long x 44cm wide x 44cm high). All birds were in full visual and auditory contact with each other. They were maintained on a 15:9 hour light:dark cycle (0600:2100) at 19-22°C, humidity 25-50%, with free access to water (vitamin supplemented) and food (bird seed mixture), supplemented with dried cuttlefish bone. Birds were trained and tested in their home cage. Animal care and use protocols were approved by the University of Edinburgh. All work was carried out under UK home office licence guidelines and regulations.

#### **One trial associative memory task**

##### **Apparatus**

Birds were presented with a clear plastic tray (12.4cm x 8.2cm) with 12 wells, 22.1mm in diameter. White polyfiller was used to fill the gaps between the wells so the birds could not see the reward in the well. The reward was a small piece of cucumber, approximately 40mm<sup>3</sup>. Coloured cardboard flaps, 2.6cm x 2.6cm, were used to cover the wells. To start with the flaps were unweighted, and then they were lightly weighted with a small piece of blu-tack underneath and finally became fully weighted by attaching a small washer to the small piece of blu-tack.

### **Pre-training**

Initially each bird was presented with four pieces of cucumber (each 40mm<sup>3</sup>) in their cage on top of an up-side-down tray. This was done once a day until the bird was approaching the tray and eating the cucumber, at which point it was presented with the tray the right way up with four pieces of cucumber in different wells. When the bird was eating all four pieces of cucumber, only one piece of cucumber was presented in the tray, once a day. After receiving a single piece of cucumber for two days, the rewarded well was half covered with an unweighted green card board flap once a day until the bird ate the cucumber for two consecutive days (the flap was in different places across days). Then a small piece of blutack was added to the bottom of the flap to lightly weight it. Once the bird moved the flap and ate the cucumber for two consecutive days, the weight of the flap was increased by attaching a small washer onto the bottom of the flap using blutack. After birds removed the flap and ate the cucumber for two consecutive days, an extra flap was introduced onto the tray over an empty well. This continued until there was a total of five flaps. Once birds were lifting the flaps and finding the cucumber within five minutes for two consecutive days then the tray was reintroduced after a retention interval of 10 minutes with a new piece of cucumber (the same five flap locations were used before and after retention interval and the same well was baited). The testing commenced when the bird found the cucumber following each presentation in less than five minutes. The number of birds that completed the pretraining and reached criterion and the time they took to achieve this varied between experiments and is reported in the individual chapters.

### **Spatial task**

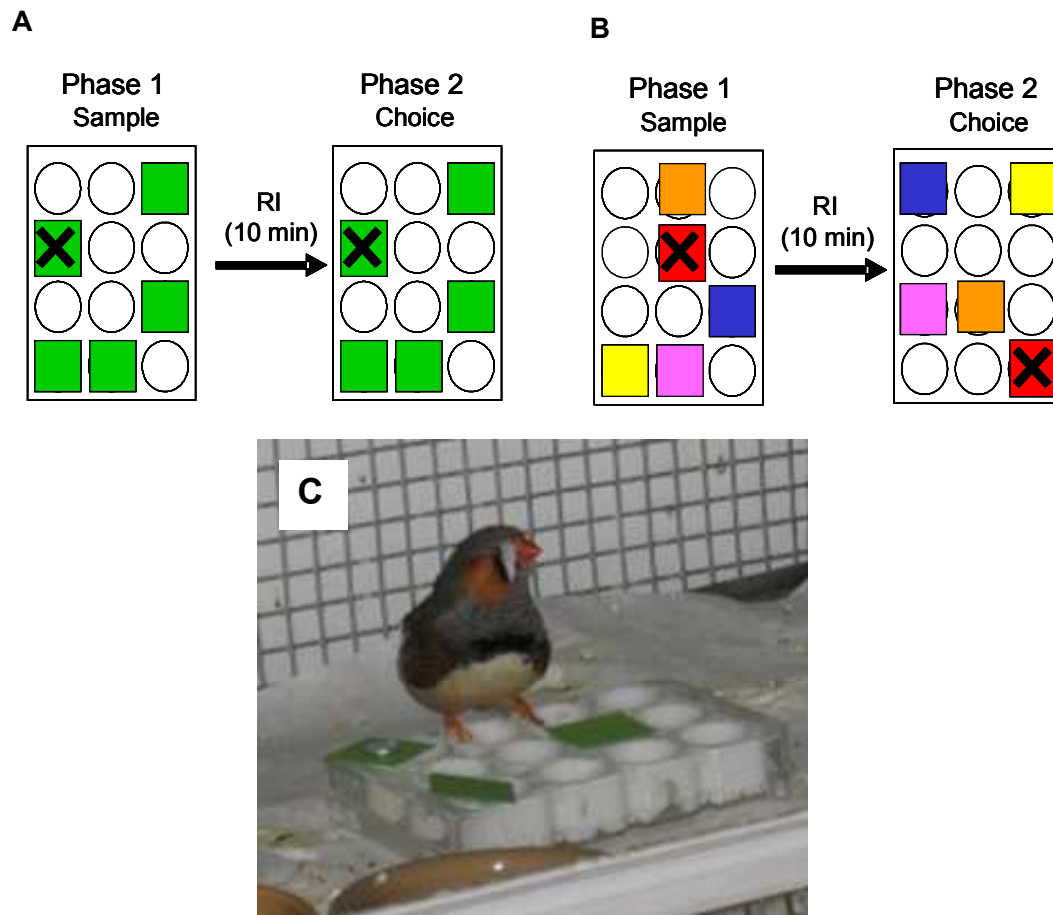
There were two versions of the memory task. The spatial task required the bird to use spatial cues to gain the reward. Each trial consisted of two phases, the sample and the choice phase, separated by a retention interval (RI). Birds received one trial per day. During the basic training the RI was always 10 minutes and birds were tested for 10 consecutive days. In the sample phase (Phase 1), birds were presented with the 12-well tray, in which five wells were covered with five green weighted

cardboard flaps, one of which was baited with a small piece of cucumber. After birds had eaten the cucumber, the tray was removed. In the choice phase (Phase 2), the tray was reintroduced having been rebaited with a new piece of cucumber in same location as in the sample phase. The five green flaps remained in the same locations as in the sample phase. The baited well is not used the day preceding or following the trial so as to avoid birds either actively avoiding it if previously unrewarded, or preferentially going there if previously rewarded (Figure 1 A). The flaps remained green throughout testing in the spatial task, but the location of both the rewarded well and the unrewarded wells changed each trial (trial specific).

Performance was measured mainly by the number of flaps lifted to locate the rewarded well in the choice phase (performance index). The time taken from when the tray was introduced in the choice phase to finding the rewarded well, latency (seconds) was also recorded.

### **Visual task**

In the visual version of the task, birds needed to use visual cues to find the rewarded well. As in the spatial version, each trial consisted of two phases separated by a retention interval (RI). However, each trial five different coloured flaps were used. The colour of all the flaps remained the same each trial including the colour of the rewarded flap, but all their locations changed between the sample and choice phases (Figure 1 B). The colour combination and locations of the flaps varied over the 10 days of testing as there were a total of 10 different coloured flaps. Each colour was rewarded only once. The colour used to cover the rewarded well was not used at all on the day proceeding or following its use, as if the colour was unrewarded previously it may have made the birds less likely to choose it and likewise if the rewarded colour was used the following day it may have increased the likelihood of selection. No colour was used for more than two consecutive days and all colours were used at least three times. The number of flaps lifted to locate the rewarded well in the choice phase was recorded as used as a measure of performance (performance index).



**Figure 1** The spatial (A) and visual (B) versions of the task and a male zebra finch solving the spatial task (C). X denotes the well rewarded with cucumber.

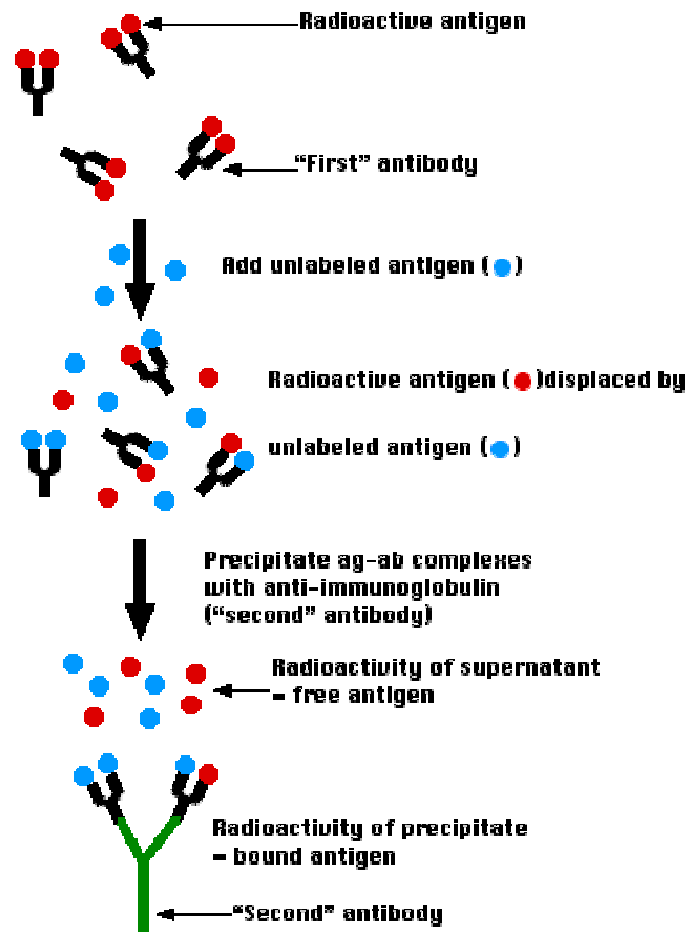
## Hormone measurement

### General principle of radioimmunoassay

Radioimmunoassay (RIA) is a very sensitive technique used to measure the concentration of antigens (such as hormone levels in the blood). To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine (the radioactive isotopes  $^{125}\text{I}$  or  $^{131}\text{I}$  are often used because of the ease with which iodine atoms can be introduced into tyrosine residues in a protein). The assay is based upon competition for a fixed

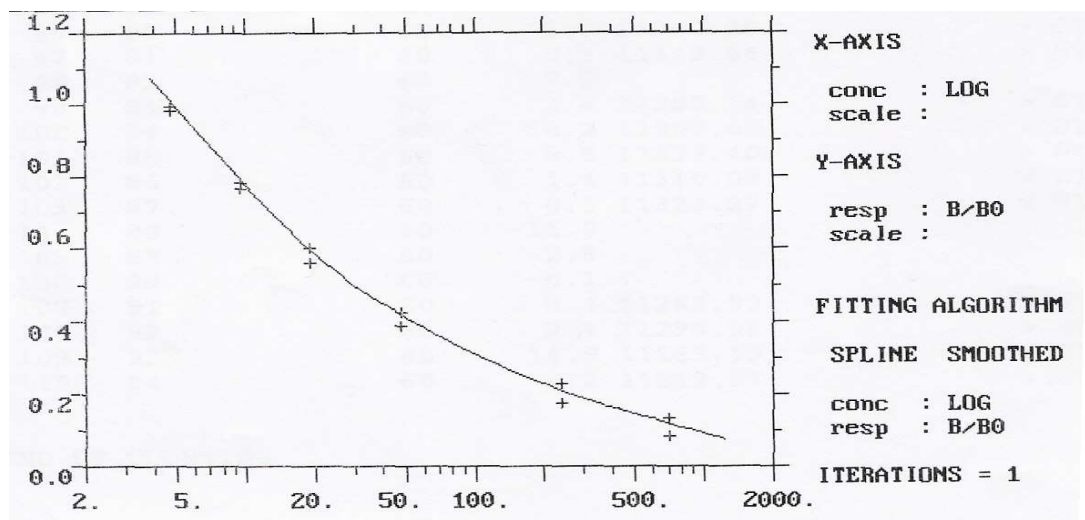
number of antibody binding sites between this radioactive antigen and the non-radioactive antigen from the sample. The amount of radioactively labelled antigen bound to the antibody is inversely proportional to the concentration of the antigen present; as the quantity of antigen in the standards or unknown samples increases, the amount of radioactive antigen able to bind the antibody is decreased. The bound antigens are then separated from the free antigens. One way of doing this is by precipitating the antigen-antibody complexes by adding a second antibody directed against the first. The radioactivity of the free antigen remaining in the supernatant or the bound can be measured (Figure 2). Using known standards, a binding curve can then be generated which allows the amount of antigen in the sample to be derived (an example is shown below Figure 3).





**Figure 2** Basic principal of radioimmunoassay

(<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/R/Radioimmunoassay.html>)



**Figure 3** Example of a standard curve (using the oestradiol RIA kit)

## **Plasma collection**

Blood samples of approximately 100-150µl were taken from the alar wing vein, by puncturing the vein with a 25G needle and collecting the blood into heparinised micro haematocrit tubes. Samples were all taken in the morning, to coincide with timing of behavioural testing. Samples were centrifuged on a micro-haematocrit centrifuge for three minutes, and plasma was recovered with a fixed needle Hamilton syringe and stored in ependorffs at -20°C until they were run in a radioimmunoassay.

## **Plasma testosterone measurement**

Plasma testosterone concentrations were measured by double antibody radioimmunoassay at the University of Glasgow using a modification of an established assay (Sheffield and O'Shaughnessy, 1989; Verboven et al., 2003). Plasma samples of 10-40µl were run in duplicate along with 40µl of testosterone standards (5, 2.5, 1.25, 0.625, 0.31, 0.16, 0.078, 0.035, 0.02, 0.01 and 0.005 ng/ml) and 40µl of testosterone controls (34 and 113 pg/ml) and to check for non-specific binding (NSB), 40µl of 0 ng/ml testosterone standard was included also run in duplicate. Samples and standards were extracted with chloroform (900µl), dried and resuspended in 50µl of testosterone antibody diluted (1:900) in assay buffer (0.05M PBS with 0.25% bovine serum albumin). NSB was resuspended in 50µl of buffer (0.05M PBS with 0.25% bovine serum albumin). Following mixing for five minutes on a multivortexer, 25µl of 3HT diluted in assay buffer (approximately 3000cpm) was added. For total counts, only 25µl of testosterone [3HT] reagent was added (estimated average cpm of 3576). After incubation for two hours at room temperature, bound material was separated by adding 400µl of charcoal in buffer (0.2g/100ml buffer) for 10 minutes. Following centrifugation at 4°C for 10 minutes, supernatant was collected and counted on a beta counter. The testosterone isotope was 9.25 MBq/250µl at the point of purchase. The limit of detection of the assay, measured as the smallest quantity of testosterone which gave rise to a response

significantly different from zero was 300pg/ml. Samples from individual experiments were also run in a single assay. The non-specific binding and intra-assay variation are reported in individual chapters. The testosterone assay used an extracted curve therefore the extraction efficiency of the curve matched the samples so there was no need to correct for it. The extraction efficiency was always >90%.

### **Plasma oestrogen measurement**

Oestradiol plasma levels were determined using an ultra-sensitive oestradiol RIA kit (DSL-4800). To validate the assay, a dilution series with avian plasma samples and standards was run and we used plasma from birds with and without oestrogen to validate the assay. Plasma samples of 100µl, 50µl and 25µl diluted to a total volume of 200µl with steroid diluent were run in duplicate to determine an optimal dilution. The binding was 24% and non-specific binding was 4.3%. The intra-assay coefficient of variation was 8.7%. For the subsequent assays, unextracted plasma samples of approximately 50µl were diluted to a total volume of 200µl with steroid diluent and run in duplicate (unless otherwise stated). 200µl of oestradiol standards (0, 5, 10, 20, 50, 250 and 750 pg/ml) and 200µl of oestradiol controls (19.4 and 237.3 pg/ml) were run in duplicate. To check for non-specific binding (NSB), 300µl of 0 pg/ml oestradiol standard was included, also run in duplicate. 100µl of oestradiol antiserum (primary antibody; polyclonal rabbit anti-oestradiol serum in a protein-based buffer with sodium azide as a preservative) was added to all tubes, except NSB. Following incubation at room temperature for one hour, 100µl of oestradiol [I-125] reagent (< 5µCi 185 kBq in a protein-based buffer) was added. After two hours at room temperature, 1ml of precipitating agent (containing goat anti-rabbit gamma globulin serum in a buffer with polyethylene glycol as a precipitating aid and sodium azide as a preservative) was added. Tubes were centrifuged for 20 minutes at 1500 xg, supernatant was aspirated and tubes were counted on a gamma counter for one minute. For total counts, only 100µl of oestradiol [I-125] reagent was added (estimated average cpm of 63782). The

theoretical sensitivity was 2.2pg/mL. The intra-assay variation is reported in individual chapters.

## **Hormone manipulations**

### **Testosterone and Oestrogen**

Testosterone and oestradiol were sonicated for five minutes to ensure mixing and were stored at room temperature. They were vortexed prior to each use. 3µl of sex steroid (in 95% ethanol) or vehicle (95% ethanol) was injected into small piece of cucumber (40mm<sup>3</sup>) and fed to the zebra finches in their home cage. A variety of different types of food, such as hard-boiled egg and pepper were tested to determine the one most preferred by the birds, and cucumber proved to be most reliably eaten by all of the zebra finches. Ethanol was used as a vehicle as testosterone does not readily dissolve in saline and oil is not easily injected into cucumber.

### **Fadrozole**

Fadrozole was sonicated for five minutes. It was made up in two separate batches, so it was freshly made for each set of five day treatments. It was aliquoted into daily epindorffs to reduce contamination and stored in foil in the fridge. It was administered by i.p. injection 24 hours prior to testing. The concentration of fadrozole of 4 µg/ml used was based on the dose used in one off injections in sparrows; weighing approximately 25g, 200µg of fadrozole was given in 50µl of saline therefore in zebra finches weighing approximately 15g, 120µg of fadrozole was given in 30µl of saline (Soma et al., 2000a).

## **In situ hybridisation**

### **General principle**

*In situ* hybridisation (ISH) is a technique which allows for the detection and quantification of mRNA. This method is very specific and sensitive, and allows for the precise localisation and identification of individual cells that contain a specific mRNA sequence and quantification of levels expressed. It can be used to compare expression levels in different anatomical regions or to look at effects on expression following treatment. However, it does not provide information on whether the mRNA is translated into protein.

In ISH, binding occurs between mRNA in the cytosol and an externally produced radiolabeled RNA or DNA probe which form, under appropriate conditions, a stable hybrid. This hybridisation is achievable through specific hydrogen bonding of complementary base pairs, which can be between two strands of RNA, two strands of DNA, or one RNA and one DNA. Detection of the radiolabeled probe is then performed by x-ray film or photographic emulsion.

There are several different types of probe that can be used. Oligonucleotide probes are short single stranded DNA oligonucleotides (generally 20+ bases), whose short length allows better tissue penetration but at the cost of a loss of sensitivity. Riboprobes are longer (approximately 200–1000 bases) single-stranded RNA molecules. They are generated by an *in vitro* transcription reaction in the presence of labelled nucleotides. The target DNA fragment to be detected is cloned into a transcription vector containing either SP6, T3 or T7 RNA polymerase promoters. This allows the transcription of either the antisense probe (complimentary so will hybridise to the mRNA of interest) or the sense probe (used as a control as it is identical so will not hybridise to the mRNA of interest) (Melton et al., 1984). The RNA-RNA hybrids are very stable and the longer length increases sensitivity. As riboprobes hybridise to non-specific sites, there is a need for pre and post-

hybridisation treatments to reduce background hybridisation. Complementary DNA (cDNA) probes are double stranded so require denaturing prior to use. Oligoprobes were used for aromatase detection and riboprobes for oestrogen receptor (ER) and androgen receptor (AR) detection as the amount of receptors are low so the more sensitive riboprobes were necessary. There are several radioactive labels available for use in ISH, such as  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{125}\text{I}$  and tritium and  $^{35}\text{S}$  was used as although it only has half the specific activity of  $^{32}\text{P}$ , it offers 30–50 times higher resolution (Polak, 1990). For RNA localisation, the type, time and concentration of the fixative are important factors in minimising RNA loss. Paraformaldehyde is a cross-linking fixative but does not cross-link proteins so extensively as to prevent penetrations of probes, unlike other fixatives such as glutaraldehyde (Polak, 1990).

### **Androgen and oestrogen receptor alpha mRNA expression**

#### *Tissue collection*

Birds were decapitated and brains were immediately removed and frozen on foil over dry ice. Brains were wrapped in foil and stored in labelled plastic bags at  $-70^{\circ}\text{C}$  until sectioned coronally at 15 microns on a cryostat (Bright 5040) and were thaw mounted onto RNase and DNase free polysine coated glass microscope slides and stored at  $-70^{\circ}\text{C}$  in sealed slide boxes containing silica gel bags to reduce condensation and tissue damage. Sections were mounted on five slides at a time with a total of five sections per slide. The slide with every fifth section was separately mounted and stained with Toluidine-Blue to determine the level in the brain and locate regions of interest and was used to select a slide with hippocampal sections at an optimal level. One slide (five sections) was used per bird.

#### *Probe generation*

The probes were generated by Dr Meddle as described previously (Hodgson et al., 2008). A 759-bp fragment encoding the nucleotides of 65-823 zebra finch AR (GenBank L25901) was subcloned into pGEM-7Zf. Antisense and sense riboprobes were generated by *in vitro* transcription, in the presence of  $^{35}\text{S}$ -UTP, with SP6- and

T7-RNA polymerase after plasmid linearization with *EcoRI* or *HindIII*, respectively. Zebra finch ER $\alpha$  sense and antisense riboprobes were generated by sub-cloning a 926 bp fragment encoding the nucleotides 751-1676 of zebra finch ER $\alpha$  (GenBank L79911) was subcloned into pGEM-7Zf. *In vitro* transcription in the presence of 35S-UTP, with T7- and SP6-RNA polymerase was performed after plasmid linearization with *BamHI* or *EcoRI*, respectively. The clones have high homology with the canary and chicken suggesting that the cloned fragments should be well conserved, at least within passerines. The clones were generously provided by Drs M. Gahr and R. Metzdorf, Max-Planck-Institute of Behavioural Physiology, Germany (Gahr and Metzdorf, 1997b).

#### *Radioactive labelling of the probe*

The riboprobe was radioactively labelled by incubating the riboprobe labelling reagents at the appropriate temperature for one to two hours (37°C for T3 and T7 or 40°C for SP6), then adding 2 $\mu$ l of DNase and returning to the hot block for a further 15 minutes during which time the DNA template was digested. The probes were then purified through Nick columns, using a separate column for each probe. TE buffer was added to the column and eluted completely into the waste pot before adding the probe directly into the gel bed in the column (approximately 10 $\mu$ l). 400 $\mu$ l of TE buffer was added to the column and dripped through. Following addition of a further 400 $\mu$ l of TE buffer, all but the first drop of the radioactively labelled probe eluted was collected into an eppendorf. 1 $\mu$ l of probe was added into a scintillation vial with 3.5ml of scintillation fluid and counted on a Beckman beta counter to determine the percentage of radioactive incorporation and probe specific activity.

#### *Preparation of slides for hybridisation*

One slide from each bird containing five sections of the hippocampus (cut at the level depicted in figure 4) were removed from -70°C and immediately incubated in 4% paraformaldehyde solution for 10 minutes to fix the tissue. After two five minute rinses in 1X PBS, slides were incubated in 300ml 0.1M triethanolamine with 0.75ml of acetic anhydride (acetylation reduces background), rinsed again in two five minute

washes of 1X PBS and dehydrated for two minutes through a series of graded ethanol (70%, 80% and 95%). Slides were then left to dry for 30 minutes at room temperature.

### *Hybridisation*

Calculating the working probe mix for the hybridisation solution:

Total hybridisation solution needed = 200µl per slide x number of slides

Deionised formamide (DF) volume = 50% total volume of hybridisation solution

DTT volume = 10ul/ml of hybridisation solution

Probe volume =  $\frac{\text{Total volume needed} \times (10 \times 10^6 \text{ cpm/ml})}{\text{Probe activity}}$

Therefore:

Vol 2X hybridisation buffer needed = Total hybridisation solution needed – DF -  
DTT - probe

Hybridisation buffer and deionised formamide (50:50) were mixed first and vortexed before addition of the labelled probe. After vortexing again, it was incubated for 10 minutes at 70°C, then cooled on ice for one minute. Once cooled the 1M DTT (Dithiothreitol) was added and the mixture vortexed. 50% formaldehyde lowers the effective melting temperature of nucleic acid chains by approximately 25°C, so that hybridisation can occur at a lower temperature.

Following incubation with 200µl/slide of pre-hybridisation solution for two hours at 50°C (in a humidifying box filled with two sheets of blotting paper and 20ml box buffer), slides were hybridised with 200µl of the hybridisation solution (5'-[<sup>35</sup>S-UTP] labelled riboprobe mixed with 50% formamide) and incubated in a humidifying box as before for 18 hours at 55°C. The probe was applied to each section at a concentration of 10x 10<sup>6</sup> cpm/ml. Post hybridization washes consisted of three five min washes in 2X saline–sodium citrate (SSC) at room temperature. Sections were then incubated with 200µl of 30µg/ml ribonuclease A (RNase-A)

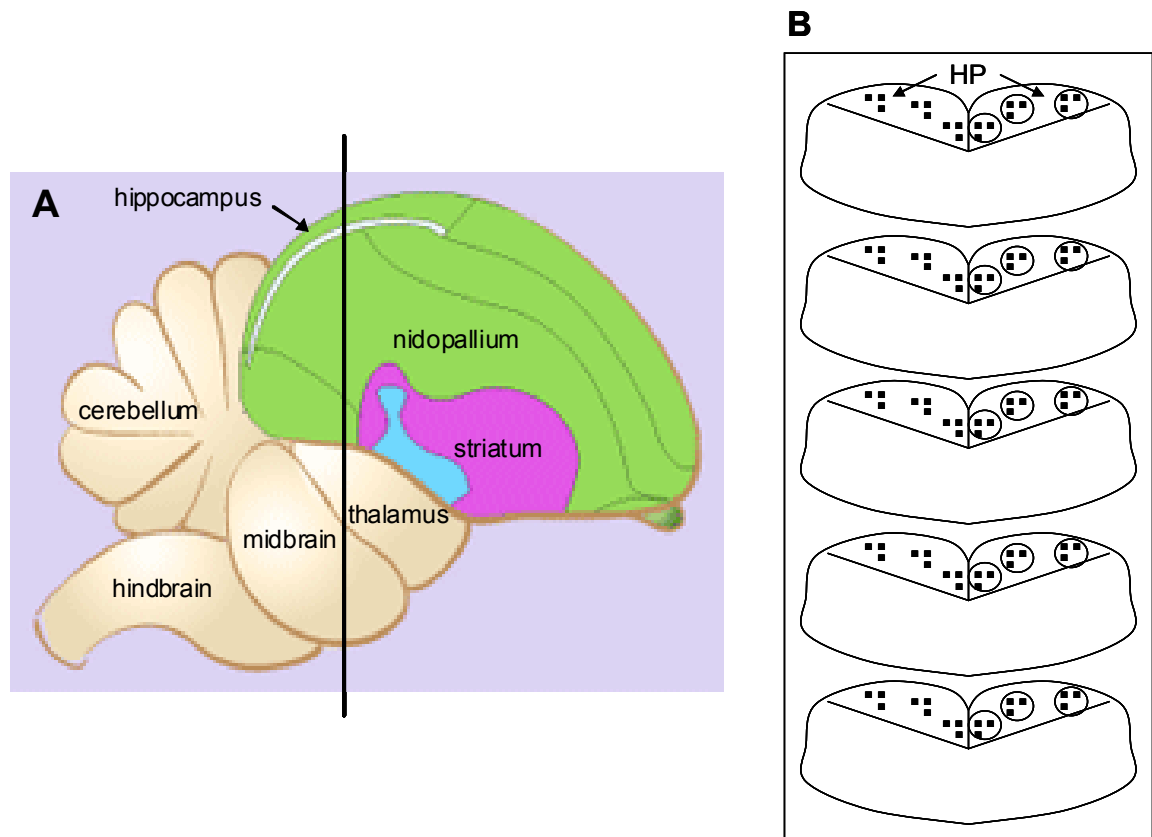


solution for 60 minutes at 37°C. RNase-A digests single stranded RNA and was included in the post-hybridisation wash to remove cytoplasmic and nuclear RNA, preventing hybridisation with the probe. Following incubation there was a 30 minute rinse in 2X SSC at room temperature and a stringent wash in 0.1X SSC at 50°C. After 60 minutes, the wash solution was changed to 0.1X SSC at 50°C and left for 60 minutes at room temperature. This was repeated, after which slides were dehydrated in a graded series of ethanol containing 300mM ammonium acetate. Slides were left overnight to dry and then dipped in autoradiographic emulsion (warmed for two hours in 40°C water bath) in the dark room with the lights out. Following dipping, slides were left to dry in the dark before being stored in the fridge in a sealed slide box, containing a silica bag, wrapped in foil and exposed for six weeks. Test slides were included to ensure optimum development time.

#### *Development and quantification*

In the dark room with the lights out, slides were immersed in developer (D-19; 80g in 500ml) for five minutes, dipped twice in distilled water then fixed by two five minute incubations in fixer (diluted 1:4; 100ml in 400ml water). This was followed by two five minute washes in distilled water. Slides were processed for background staining with haematoxylin and eosin. Slides were submerged in haematoxylin (filtered first) for five minutes then rinsed with water, prior to differentiation in acid alcohol. This was followed by another wash in water before the slides were left in STWS for three minutes, washed in water for three minutes, dipped in eosin for two minutes, rinsed in water and then dipped in potassium alum for three minutes. Finally the slides were rinsed in water then taken through a series of graded alcohols (70%, 80%, 95% and 100% for five minutes each), cleared in xylene and then mounted with DPX mountant and cover-slipped with glass microscope slides. Slides were examined at x25 on a microscope (Wang BioMedical) and quantification of mRNA expression was carried out using image analysis system (Open Lab). Anatomical identification of brain structures was based on the stereotaxic canary brain atlas (Stokes et al., 1974). Measurements of silver grain density/area were taken in the hippocampus using a 10 mm<sup>2</sup> circle as the region of interest. Three

measurements were taken in each of the central, middle and outer areas of the hippocampus on both the left and right sides of five sections per bird i.e. there was a total of 18 measurements per section giving a total of 90 per bird (Figure 4). A background measurement was also taken from each side for each of the five sections, in the lateral hyperstriatum ventrale. An average was taken of the nine measurements per side, and the background value subtracted from this. The counting was done blind to treatment group.



**Figure 4** A) A representation of the hippocampus at the level at which coronal sections were selected for analysis. B) A diagrammatic example slide from one bird depicting five hippocampal sections highlighting the three regions of interest (circled) per side of the hippocampus (HP) sampled for measurement of cell density. Adapted from (Jarvis et al., 2005) and (Oberlander et al., 2004).

## **Aromatase mRNA expression**

### *Probe generation*

A synthetic oligoprobe (cDNA) for aromatase was custom created (GeneDetect, Bradenton, FL, USA) from an existing published zebra finch (*Taeniopygia guttata*) aromatase 3188-bp sequence (48bp; Probe hybridizes to nucleotides 688-735 located within the coding sequence of ZA1; GenBank L81143.1, Shen et al 1994).

### *Radioactive labelling of the probe*

The labelling reagents (Terminal Transferase labelling kit) were combined and incubated at 37°C for one hour to radiolabel the oligoprobe. The probe was purified through a spin column. The contents of the labelling reaction were transferred to the column and washed twice by adding 500µl of PE buffer and spinning for one minute at 6000 rpm and discarding the flow through each time. This was then centrifuged for an additional one minute at 13,000rpm. To elute the radioactively labelled DNA, 50µl of elution buffer was added, and following one minute standing, it was centrifuged for one minute at 13,000rpm. A 1µl sample was added to 3.5ml of scintillation fluid and counter on a beta counter to measure activity.

### *Preparation of slides*

One slide for each bird containing five sections of the hippocampus (cut at the same level as above; Figure 4) were removed from -70°C and immediately incubated in 4% paraformaldehyde for 10 minutes to fix the tissue. After two five minute rinses in 1X PBS, slides were incubated in 300ml 0.1M triethanolamine with 0.75ml of acetic anhydride, rinsed again in two five minute washes in 1X PBS and then dehydrated through a series of graded ethanol, 100% chloroform and back through 100% then 90% ethanol. Slides were left to dry for 30 minutes at room temperature.

### *Hybridisation*

Slides were hybridised with 100µl hybridisation buffer (oligoprobe labelled with 3'[35S-ATP] and 50% formamide) for 20 hours at 37°C. Post hybridisation washes

consisted of three rinses in 1X SSC for five minutes each followed by four stringent washes of 15 minutes in 1X SSC at 55°C and two 30 minute washes at room temperature. Slides were left overnight to dry and then dipped in autoradiographic emulsion (warmed for two hours in 40°C water bath) in the dark room as previously described. Following dipping, slides were left to dry in the dark before being stored in the fridge in a sealed slide box, containing a silica bag, wrapped in foil.

#### *Development of slides*

The slides were exposed for four weeks, then developed, fixed, mounted and analysed as described above.

### **Reagents**

All reagents were autoclaved prior to use to eliminate RNase activity.

#### *Riboprobe labelling reagents*

Added in order:

4µl	5x transcription buffer
6µl	NTP (4µl 10mM ATP, 4µl 10mM CTP, 4µl 10mM GTP)
1µl	200mM DTT
2µl	Template (antisense or sense)
0.8µl	RNase, ribonuclease inhibitor
8µl	5'-[35S-UTP] (for sense use only 4µl)
2µl	RNase polymerase (T3, T7 or SP6)

RNA polymerase was included to catalyse the synthesis of the riboprobe by transcribing nucleic acid sequences downstream of the appropriate polymerase initiation site.

*TE buffer*

To 900ml ddH<sub>2</sub>O add

10ml 1M Tris (pH 7.5-8.0) = 10mM

2ml 0.5M EDTA (pH 8.0) = 1mM

The volume was adjusted to 1 litre, pH 7.4 using HCl and autoclaved

*4% Paraformaldehyde in PBS*

To 450ml DEPC water add

8.0g NaCl

0.2g KCl

1.44g Na<sub>2</sub>HPO<sub>4</sub>

450ml DEPC water was heated to 70°C and 40g paraformaldehyde was added (weighed out in fume cupboard with the fan off). To allow PFA to go into solution, 2ml of concentrated NaOH was added while mixing on a magnetic stirrer. When all solutes had dissolved completely, both solutions were combined and the volume was adjusted to 1 litre with DEPC water and the solution was cooled on ice or in the fridge overnight then the pH was adjusted to 7.2 with concentrated HCl.

*2X Prehybridisation solution (10ml)*

6.26ml DEPC water

2.4ml 5M NaCl (14.6g in 50ml)

200µl 1M Tris

400µl Denhardt's Solution (5ml of 50x Denhardt's)

40µl 500mM EDTA (pH 8.0)

50µl Yeast tRNA (ribonucleic acid, transfer)

200µl Salmon sperm DNA (5x 1ml of 11µg/ml)

Single stranded DNA was used to decrease background hybridisation signal. This DNA was already ethanol precipitated and sonicated to produce single stranded fragments for hybridisation. Denhardt's solution was used to prevent non-specific

binding to proteins, polysaccharides and nucleic acid. It was a mixture of proteins that stabilise the probe and reduces background hybridisation. EDTA (Ethylene Diamine Tetraacetic Acid) is a calcium chelator and preserves substantial amounts of RNA and decreased background signal. The working pre-hybridisation solution was prepared by combining 50% 2X pre-hybridisation solution with 50% deionised formamide. 50% formaldehyde lowered the effective melting temperature of nucleic acid chains by approximately 25°C, so that hybridisation could occur at a lower temperature.

*2X Hybridisation solution (10ml)*

7.06ml	DEPC water
2.40ml	5M NaCl (14.6g in 50ml)
200µl	1M Tris
400µl	Denhardt's solution (5ml of 50x Denhardt's)
40µl	500mM EDTA (pH 8.0)
2g	Dextran Sulphate (dissolve 2g in 5ml DEPC water at 62°C)
50µl	Yeast tRNA (ribonucleic acid, transfer)
200µl	Salmon sperm DNA (5x 1ml of 11µg/ml)

Single stranded DNA was used to decrease background hybridisation signal. This DNA was already ethanol precipitated and sonicated to produce single stranded fragments for hybridisation. Dextran sulphate is a large non-reactive polymer that increases effective probe concentration at the tissue surface.

*1M Dithiothreitol (DDT)*

0.164g	in 1ml sterile water
0.82g	in 5ml sterile water

After 30 minutes at room temperature the solution was filtered through 0.2µm acrodisc (Gelman Sciences). DTT is a reducing agent included in the hybridisation buffer to protect sulphur from oxidation. It is an antioxidant that maintains sulphur

ions in a reduced state therefore improving stability of the radioactive sulphur to uracil bonds and prevents intra-probe disulphide linkages.

*Oligoprobe labelling reagents*

Added in order:

27µl	autoclaved water
10µl	5x TdT Tailing buffer
5µl	2.5mM Cobalt chloride
5µl	3'-[35S-ATP]
2µl	Probe (working dilution 10pmol/µl)
1µl	TdT enzyme (25 units/ µl)

The reagents were incubated in 37°C for one hour. Terminal Deoxynucleotidyl transferase (TdT) catalyses template independent addition of deoxyribonucleoside triphosphates to the 3'OH end of single or double stranded DNA.

*10X Phosphate buffered Saline (PBS)*

23.12g	Na <sub>2</sub> HPO <sub>4</sub>
2.0 g	KH <sub>2</sub> PO <sub>4</sub>
2.0g	KCl

The above were dissolved in 900ml DEPC water and the volume was adjusted to 1 litre and pH to 7.4 with concentrated HCl. 1X PBS was prepared using DEPC water

*Diethylpyrocarbonate (DEPC) water (0.1%)*

1ml of diethylpyrocarbonate was added to 1 litre ddH<sub>2</sub>O and shaken well before autoclaving. DEPC inhibits RNase by acylation, as it reacts with amines and sulfhydryl groups so reagents such as tris and DTT cannot be treated directly and should be autoclaved to ensure they are RNase free.

*Triethanolamine/ Acetic Anhydride*

On the day preceding hybridisation, 0.1M triethanolamine was prepared by adding 1.49ml/100ml autoclaved water (4.47ml/300ml). Immediately before slides were immersed, 0.75ml acetic anhydride was added to 300ml 0.1M triethanolamine. The electrostatic attraction between the hybridisation probe and basic proteins in the tissue was minimised by treatment with 0.25% acetic anhydride which blocks basic groups (NH<sub>2</sub>) by acetylation (neutralises the positive charges).

*20X Saline Sodium Citrate (SSC)*

175.3g	NaCl
88.2g	Tri-sodium citrate
800ml	DEPC water

The pH was adjusted to 7.0 with NaOH and the volume was made up to 1 litre with DEPC water.

*Deionised Formamide*

Using baked glassware, spatulas etc 10g 'Amberlite'/100ml 'AnalR' Formamide was weighed out and stirred on a magnetic stirrer for at least one hour under the fume hood. The formaldehyde was then filtered from the resin using a double layer of Whatman's No. 1 filter paper and the bottle was stored at room temperature wrapped in foil (as formaldehyde is light sensitive).

*RNase box buffer*

2ml	5M NaCl
200µl	1M Tris (pH 7.5)
40µl	500mM EDTA (pH 8.0)
17.64ml	ddH <sub>2</sub> O



*Pre-hybridisation box buffer*

4ml	20X SSC
6ml	DEPC water
10ml	Deionised Formamide

*RNase solution*

1µl RNase A per 2ml RNase box buffer

*STWS (Scott's tap water substitute)*

20g	Magnesium Sulphate
3.5g	Sodium Bicarbonate (Sodium Hydrogen Carbonate)
1 litre	Distilled water

*Acid alcohol (70%)*

70ml	Absolute alcohol
300ml	Distilled water
10ml	HCl (concentrated)

*Potassium Alum (5%)*

5g	Aluminium potassium sulphate
100ml	Distilled water

*Eosin (1%)*

1g	Eosin
100ml	Distilled water

## **Immunohistochemistry for NMDA and ER $\alpha$ protein levels**

### **General principal**

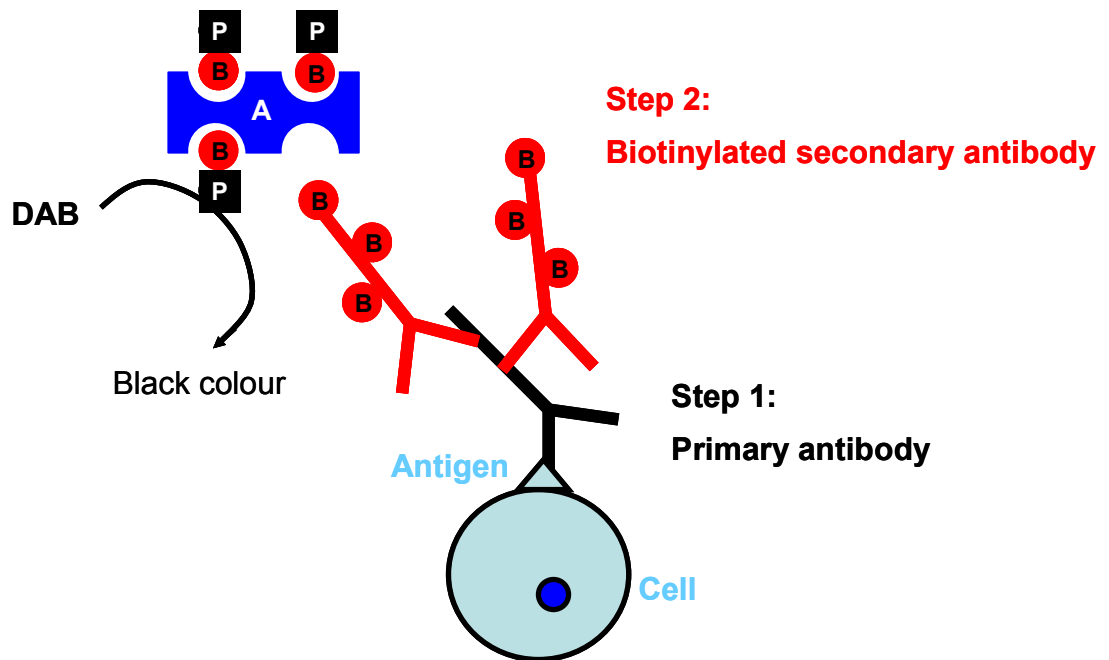
Immunocytochemistry (ICC) allows the identification of a tissue constituent *in situ* by means of a specific antigen-antibody reaction tagged by a visible label (Polak, 1983). It involves the use of target specific antibodies to localize peptides or protein antigens in the cell via specific epitopes which are then visualised using a detection system. The detection method chosen was the avidin-biotin system. ICC allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. In cases where a positive signal is found, ICC also allows researchers to determine which sub-cellular compartments are expressing the antigen. Comparisons in the levels of expression between control and treatment groups can be compared.

The first step of the procedure is to incubate the section with primary, unlabelled antibody, raised against the antigen of interest. Next, a biotin-labelled secondary antibody (raised against the IgG of the animal donating the primary antibody) is added, which introduces many biotins at the location of the primary antibody. Biotin binds the Fc portion of immunoglobulins, with several biotins able to bind to one immunoglobulin molecule. The avidin/ biotinylated enzyme complex (ABC) is then added and binds to the biotinylated secondary antibody. This complex contains avidin reacted with biotinylated peroxidase so that only three of its four biotin binding sites are taken up, leaving one free to react with the biotin on the secondary antibody. In the last step of the procedure, the tissue antigen is localized by incubation with a substrate for the enzyme, in our protocol this is DAB (diaminobenzidine tetrachloride). Nickel DAB is reduced, causing a black/brown colour which visibly labels the antigen of interest allowing for quantification (Figure 5). In this system very high amounts of label are built up which increases sensitivity. Avidin, from egg white, is a 68 000 molecular weight glycoprotein with a very high affinity ( $10^{15}\text{M}^{-1}$ ) for the small molecular weight vitamin, biotin (found in egg yolk).

As this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin is essentially irreversible. The avidin/ biotin system provides a very effective means of amplifying the signal as a secondary antibody will hold many more biotin molecules than label/enzyme molecules.

### Step 3:

#### Avidin/ Biotinylated peroxidase Complex (ABC)



**Figure 5** Avidin-biotin complex (ABC) method for antibody detection.

A: Avidin, B: Biotin, P: Peroxidase

### ICC for NMDAR1 and ER $\alpha$ protein levels in hippocampus

One slide per bird with five hippocampal sections cut at the same levels as described for the *in situ* experiments (Figure 4) were removed from the freezer and allowed to reach room temperature for 20-30 min. Following fixation in 4% paraformaldehyde (pH 7.2-7.4) in 0.1M PB in glass troughs on a shaker for 30 minutes, slides were washed for 15-20 minutes in 0.1M PB to remove fixative. Endogenous peroxidase

(which would produce a reaction product from the substrate alone) was blocked with 100µl of 0.3% hydrogen peroxide (in PB) for 15 minutes, after which slides were washed for 10 minutes in 0.1% PBT to permeabilise the membrane. Sections were drawn around using a PAP pen, and then non-specific staining was blocked with 200µl/slide of 10% normal horse serum (NMDA) or rabbit serum (ERα) in 0.1% PBT for 30 minutes in an incubation chamber. Sections were incubated in primary antibody (for NMDA antibody see (Saldanha et al., 2004) and for the ERα antibody see (Saldanha and Coomaringam, 2005)) diluted 1:1000 in 10% normal serum in 0.1% PBT overnight in the fridge in an incubation chamber (antibody dilutions of 1:100, 1:500 and 1:5000 were also tried). Slides were washed in 0.1% PBT for 10 minutes to remove excess antibody. The antibody:antigen complex was localised using the ABC vector stain kit for mouse IgG (NMDA) or rat IgG (ERα): slides were incubated in biotinylated anti-mouse/rat antibody in normal horse/rabbit serum and 0.1% PBT (200µl/slide) for one hour in an incubation chamber at room temperature. Following three 10 minute washes in PBT, sections were covered with avidin biotin complex (avidin and biotinylated horseradish peroxidase in PBT) and incubated for one hour in an incubation chamber at room temperature. After two 10 minute washes in PBT, sections were rinsed briefly in 0.1M acetate buffer. Sections were visualised using the glucose oxidase DAB method: sections were incubated in DAB solution for six minutes, then the reaction was terminated with a brief rinse in 0.1M sodium acetate (to remove the phosphate buffer) and two washes in 0.1M PB. Sections were taken through increasing concentrations of alcohols (70%, 90%, 95%, 100% x 2; five minutes in each) and cleared in xylene, coverslipped using DPX mountant and left to dry. Numbers of positively labelled cells per section were quantified using a WANG microscope under x40 magnification.

## Reagents

### *1M phosphate buffer (PB; pH 7.4)*

115g	Disodium hydrogen orthophosphate (Na <sub>2</sub> HPO <sub>4</sub> .H <sub>2</sub> O)
27.2g	Sodium dihydrogen orthophosphate (NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O)
1000ml	Distilled water (heated)

*0.1M PB (pH 7.4)*

100ml	1M PB
900ml	Distilled water

*0.1% PBT (0.1M PB with Triton X-100)*

1ml	Triton X-100
1000ml	0.1M PB

*4% Paraformaldehyde (in PB; pH 7.4)*

500ml distilled water (heated) plus:

40g	Paraformaldehyde in 500 ml distilled water (heated)
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500ml distilled water (heated) plus:

115g	Disodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ )
27.2g	Sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )

The above were then mixed to make 1 litre

*1% normal horse serum*

99ml	PBT
1ml	Normal horse serum

*0.2M acetate buffer (pH 6.0)*

16.4g	Sodium Acetate
1000ml	Double Distilled water

*0.3% Hydrogen peroxide*

1ml	30% Hydrogen Peroxide
99ml	0.1M PB

*DAB Visualisation solution*

198ml	0.1M acetate buffer
0.16g	Ammonium Chloride
2.5g	Nickel ammonium sulphate
0.8g	Glucose
2ml	Diaminobenzidine (DAB; 25mg/ml)
0.006g	Glucose oxidase (type VII)- added immediately prior to use

**Autoradiography for NMDA binding activity****General principal**

The study of a receptor protein could be carried out in a number of ways; the protein could be localized by the use of antibodies (immunocytochemistry), its mRNA could be detected (*in situ* hybridization), or it could be localized by virtue of its binding by a radiolabeled ligand (receptor autoradiography). Each of these methods provides a distinct class of information. While immunocytochemistry reflects protein localization, and *in situ* hybridization can give information about the cell bodies of origin and amount of a specific mRNA, receptor autoradiography reflects the location and amount of binding activity of the receptor protein itself. The ability to quantify receptor binding, and thereby estimate number and affinity of receptor binding sites, allows the study of regulation of receptor systems in a large number of tissues, systems and conditions.

In autoradiography, the specimen itself is the source of the radiation, which originates from radioactive material incorporated into it. This is achieved by incubating the tissue with a radiolabeled ligand, during which the receptor–ligand complex forms. Following exposure for the required period, it is then possible to quantify the binding. Such quantification is usually undertaken to compare specific

binding across anatomical regions, to evaluate receptor subtypes within a tissue region, or to study changes in receptor levels within regions after various treatments.

### **Autoradiography for NMDA and AMPA receptors**

One slide per bird with five sections of hippocampus were removed from storage at -70°C and brought to room temperature overnight in the fridge. Autoradiography was based on a previous protocol (Stewart et al., 1992). After fixation in 0.1% paraformaldehyde (in tris-citrate buffer) for two minutes, they were preincubated in 50mM Tris-citrate buffer (pH 7.4) at 4°C for one hour to remove endogenous glutamate. Ligands were prepared in 50mM Tris-citrate buffer (pH 7.4). Solutions of each ligand were added as a drop to cover the sections ([<sup>3</sup>H]glutamate: 70.4 nmol/l, specific activity 49.6 Ci/mmol; [<sup>3</sup>H]AMPA: 77.8 nmol/l, specific activity 42.1 Ci/mmol), then were incubated for 20 minutes at 4°C in a humid chamber (a humidifying box filled with two sheets of blotting paper soaked in distilled water). Sections were washed three times in ice cold Tris-citrate buffer to terminate the incubation, then dipped in ice-cold distilled water to remove buffer salts, and were air dried. Slides were put on film and exposed in the dark for seven weeks. Carbon-14 standards were included. Test slides were included to ensure optimum development time, which were developed after five, six and seven weeks of exposure. In the dark, slides were developed in Agfa G150 developer (80g in 500ml double distilled water) for five minutes and rinsed in double distilled water for five minutes. Slides were then fixed in Ilford Hypam fixer (1:4 dilution) for five minutes, which was repeated before slides were washed in double distilled water for five minutes and then rinsed under running water. The densitometry of the film autoradiograms was measured on the image analysis system MCID core 7.0. Three measures from each side of the hippocampus were taken, for five sections per animal. A background measure from the film was subtracted from the values. The 30 values were averaged to give a single value per bird.

*Calculation of radioactive ligands*Glutamate

$$\begin{aligned}
 [\text{Radioligand}] &= \frac{\text{Radioactive concentration (Ci/ml)}}{\text{Specific activity (Ci/mMol)}} \\
 &= \frac{1 \text{ mCi/ml or } 0.001 \text{ Ci/ml}}{49.6 \text{ Ci/mMol}} \\
 &= 0.0000202 \text{ mMol/ml or } 20.2 \text{ nMol/ml or } 20\,200 \text{ nMol/litre}
 \end{aligned}$$

We wanted 70.4 nMol/litre so there was a dilution factor of 286.9 (20 200/ 70.4).

We had 50 $\mu$ l ligand so 1:20 dilution means we added 715 $\mu$ l of buffer.

Total volume was 765 $\mu$ l.

AMPA

$$\begin{aligned}
 [\text{Radioligand}] &= \frac{\text{Radioactive concentration (Ci/ml)}}{\text{Specific activity (Ci/mMol)}} \\
 &= \frac{1 \text{ mCi/ml or } 0.001 \text{ Ci/ml}}{42.1 \text{ Ci/mMol}} \\
 &= 0.0000238 \text{ mMol/ml or } 23.8 \text{ nMol/ml or } 23\,800 \text{ nMol/litre}
 \end{aligned}$$

We wanted 77.8 nMol/litre so there was a dilution factor is 305.9 (23 800/ 77.8).

We had 25 $\mu$ l ligand so 1:40 dilution means we added 191 $\mu$ l of buffer.

Total volume was 216 $\mu$ l.

**Reagents***Tris-citrate buffer (50mM)*

6.055g	Tris (hydroxymethyl)methylamine
1000ml	Distilled water
	Citric acid solution to get pH 7.4



### *0.1% Paraformaldehyde in 50mM Tris-citrate Buffer*

To 450ml 50mM Tris-citrate buffer we added:

0.8g	NaCl
0.2g	KCl
1.44g	Na <sub>2</sub> HPO <sub>4</sub>

We added 1g of paraformaldehyde to 450ml 50mM Tris-citrate buffer (heated). When dissolved, we mixed the two solutions and adjusted the volume to 1 litre with buffer and, once cooled, the pH was adjusted to 7.2.

## **Gene expression**

### **General principal**

A novel approach to identifying signalling mechanisms involved in memory, complementary to biochemical and pharmacological methods, has emerged with the development of DNA microarray technology (Schena et al., 1995). In the past, RNA fingerprinting has been used to identify genes up-regulated in the hippocampus of water maze trained rats, however, in these studies only a small fraction of the genes that may be differentially expressed were screened (Cavallaro et al., 1997; Zhao et al., 2000). Microarray technology provides a powerful tool for examining multifaceted transcription processes through the ability to monitor the parallel expression of thousands of genes, using a grid of DNA spots, each containing a unique DNA sequence (Cavallaro et al., 2002b).

A DNA microarray consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, each containing picomoles of a specific DNA sequence, known as probes that are used to hybridize cDNA or cRNA samples under high-stringency conditions. Following amplification, the samples are labeled, for example with a fluorescent dye, allowing probe-sample hybridization to be detected and

quantified to determine the relative abundance of nucleic acid sequences in the sample (Schulze and Downward, 2001). Fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the strength of the hybridization determined by the number of complementary paired bases (strength of bonding), the hybridization conditions (such as temperature), and washing after hybridization (only strongly paired strands remain hybridized). Microarrays use relative quantitation in which the intensity of a gene is compared to the intensity of the same gene under a different condition, and the identity of the gene is known by its position.

The microarrays used in this experiment were custom made spotted single-channel arrays. In spotted microarrays, the probes are oligonucleotides, cDNA or small fragments of PCR products that correspond to mRNAs. The probes are synthesized prior to deposition on the array surface and are then "spotted" onto glass. A common approach utilizes an array of fine pins or needles controlled by a robotic arm that are dipped into wells containing DNA probes and then deposited at designated locations on the array surface. The resulting "grid" of probes represents the nucleic acid profiles of the prepared probes and is ready to receive complementary cDNA or cRNA derived from experimental or clinical samples. In single-channel microarrays or one-color microarrays, only a single dye is used and the data collected represents absolute values of gene expression. A benefit of the single channel is that data are more easily compared to arrays from different experiments but twice as many microarrays are needed to compare samples within an experiment.

## **Hippocampal gene expression**

### *Gene arrays*

The microarrays used were custom made Agilent spotted one colour (single channel) arrays developed and kindly supplied by the Jarvis lab at Duke University. One array was used per bird and there were four arrays per slide. The Agilent songbird array used contained over 43 000 unique transcripts, many of which can be used to

distinguish between specific splice variants. The sequences used for generating probes on the array were from sequencing projects performed at the Keck Centre of the University of Illinois, at Rockefeller University and Duke University. All of these sequences are stored in the [songbirdtranscriptome.net](http://songbirdtranscriptome.net) website. In addition to these sequences, over 450 features on the array were designed from clones found in Genbank.

*RNA preparation (total RNA isolation)*

The hippocampus was dissected out while still frozen using a sterile razor blade (wiped with RNA-free water between brains) and immediately placed into in Lysing Matrix D beaded tubes with 1ml of Tri Reagent and homogenised by agitation on FastPrep FP120 at 4m/s for 20 seconds. Samples were then incubated for five minutes at room temperature before addition of 0.2 ml of 1-bromo-3-chloropropane (BCP) and subjected to vigorous shaking for 15 seconds. Samples were then incubated at room temperature for three minutes and centrifuged at 12 000xg for 15 minutes at room temperature to separate the phases. 500µl of the upper aqueous phase (containing the RNA) was then transferred to a new labelled tube. RNA precipitation was carried out by adding 1µl of linear acrylamide (5mg/ml), followed by 500µl of isopropyl alcohol after which the samples were incubated on ice for 10 minutes then centrifuged at 12 000g for 30 minutes. The RNA precipitate formed a gel like pellet at the bottom of the tube. To wash the RNA, the supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. The samples were then vortexed and spun for 30 minutes at 12 000xg (room temperature) after which the supernatant was removed and the RNA pellet was air-dried for five to 10 minutes. The pellet was then resuspended in 30µl of MilliQ water, vortexed and left for 30 minutes. The RNA concentration was quantified using a nanodrop ND-1000 spectrophotometer (Labtech Int.) to measure the absorbance. An Agilent kit, including a 12 well RNA 6000 Nano chip, was then used to measure the quality of the RNA and ensure that it was above the criteria recommended to run the samples on the arrays.

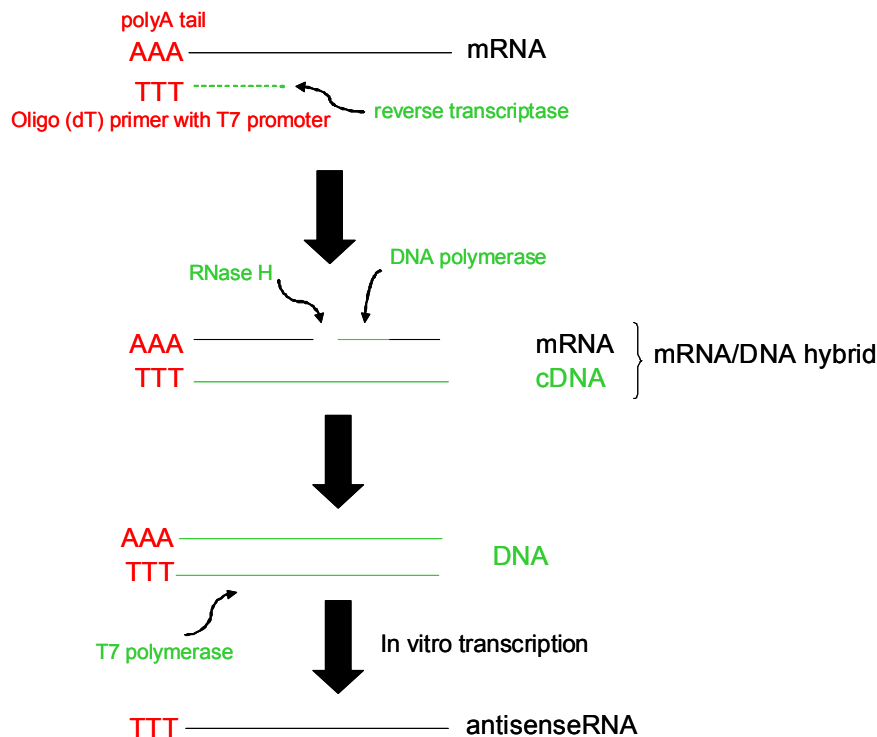
### *Single dye RNA amplification and dye coupling*

RNA Amplification was carried out using the MessageAmp kit from Ambion (Ambion 1750), based on the RNA amplification protocol developed in the laboratory of Dr Eberwine (Van Gelder et al., 1990). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter and *in vitro* transcription of the resulting cDNA with T7 polymerase to generate hundreds to thousands of antisense RNA (aRNA) copies of each mRNA in a sample (Figure 6). Amino allyl UTP was incorporated into the aRNA (aaUTP aRNA) during the *in vitro* transcription reaction. This was then used directly in a dye coupling reaction (to fluorescently label the aRNA).

Prior to using the kit, 11.2ml of 100% ethanol was added to the cDNA wash buffer, and 20ml to the aRNA wash buffer. The Agilent kit containing Spike-in controls (One-colour Spike-in Kit, Agilent Technologies 5188-5282), made from bacterial genes, was used in addition to the sample total RNA in the amplification and act as a quality control for the software and to validate the amplification process. To prepare the Spike-in dilutions, the stock solutions were defrosted, heated at 37°C for five minutes then mixed on a vortexer and briefly spun. Serial dilutions of spike in RNA were carried out (1:20, 1:25 then 1:10), using dilution buffer from the Spike-in kit. First strand cDNA was then synthesized: 1mg of total RNA was added to individual wells of a PCR plate, then to each well 5µl of Spike-in positive control and 1µl of Oligo(dT) primer was added. The total volume was made up to 12µl using nuclease free water and samples were incubated for 10 minutes at 70°C on the PCR block (Dyad PCR machine) then spun for five seconds (<5K rpm) and placed on ice. 8µl of Reverse Transcription master mix was added to each sample, after which they were incubated on the Dyad at 42°C for two hours. Samples were centrifuged for five seconds (<5K rpm) and placed on ice. Second strand cDNA synthesis reagents were made up as a master mix then added to each 20µl cDNA sample and gently pipetted up and down then incubated at 16°C for two hours in a PCR block. To purify the cDNA, the cDNA reaction was transferred to a clean RNase free tube, 250µl of cDNA Binding Buffer was added then samples were pipetted onto an equilibrated

filtered cartridge (filter cartridge was placed in a 2ml tube and 50µl of cDNA Binding Buffer was added and incubated at room temperature for five minutes). Samples were spun for one minute at 10 000xg, the flow through was discarded and the filtered cartridge replaced. 500µl of cDNA wash buffer was added to each filter cartridge and samples were spun for one minute at 10 000xg. Again, the flow through was discarded and the filter cartridge replaced and samples were spun for a further minute at 10 000xg to remove trace amounts of ethanol. The filter cartridge was transferred to a cDNA elution tube and 10µl of Nuclease-free water (preheated to 50°C) was added, after which samples were left at room temperature for two minutes then spun for one and a half minutes at 10000xg. The elution was repeated with a second 10µl of preheated Nuclease-free water. Antisense RNA (aRNA) was then synthesized using *in vitro* transcription (Figure 6). The cDNA solution (16µl) was transferred to a 0.2ml sterile, RNase free PCR tube. An aminoallyl master mix was made up containing T7 DNA polymerase, necessary for amplification, aminoallyl UTP (aaUTP) in preparation for the dye labeling stage (as the dye undergoes a chemical reaction and binds to it) and 'cold' UTP to prevent incorporation of a run of UTPs slowing down the reaction (due to the steric hindrance of the aminoallyl side group). 25µl was added to each tube, and after a brief spin, samples were incubated for 24 hours at 37°C. To remove the template DNA from the aRNA, 2µl DNaseI was added to each reaction which was then incubated for 30 minutes at 37°C. To purify the aRNA, 60µl of elution solution was added to each sample, then samples were transferred to a fresh nuclease free 1.5ml microfuge tubes. Following addition of 350µl of aRNA binding buffer then 250µl of 100% ethanol to each sample, samples were pipetted onto an aRNA filter cartridge and spun for one minute at 10 000xg. The flow through was discarded and the filter cartridge replaced (in the same 2ml tube). 650µl of aRNA wash buffer was applied to each filter cartridge, after which samples were spun for one minute at 10 000xg, the flow through was discarded and samples were spun for an additional minute to remove trace amounts of ethanol. The filter cartridge was transferred to a fresh aRNA collection tube and 50µl of Nuclease-free water (preheated to 50°C) was added and left to filter at room temperature for two minutes before being spun for

one and a half minutes at 10 000xg. The elution was repeated with a second 50µl of preheated Nuclease-free water. As a QC, the aRNA yield and quality was assessed by running 1µl of the purified aRNA per well on an Agilent Bioanalyzer RNA Nano Chip, to confirm that all the products had approximately the same electropherogram profile on the Bioanalyser.



**Figure 6** The amplification of RNA. Antisense RNA is generated, which binds to the sense RNA probes on the gene array.

The aRNA was then labelled using a dye coupling reaction. 1.2µg aminoallylUTP (aaUTP) aRNA was placed in 20µl MilliQ water in individual wells of a PCR plate, which was then dried down in the Jouan vacuum centrifuge. The RNA was then resuspended in 5µl of coupling buffer (Stratagene Fairplay kit), vortexed and spun briefly. The PCR plate was then placed on Dyad PCR block at 37°C for 30 minutes, to aid the resuspension, after which they were vortexed and spun briefly to return the contents to the bottom of the wells. 5µl of the Cy3 dye was added to the aRNA and incubated for one hour at room temperature in the dark. The dye coupled aRNA was

then purified using DyeEx 2.0 spin columns (in a 2ml collection tube). After the columns were spun using the Eppendorf microcentrifuge 5415D at 3000rpm for three minutes, each spin column was transferred to an amber tube (to minimise light exposure) and the sample was slowly applied to the gel bed in the spin column. The columns were spun at 3000rpm for three minutes, then the spin columns were removed from the tubes and discarded (the eluate contained the purified labelled aRNA). The aRNA was quantified using the nanodrop and on an agarose gel. Before proceeding to the hybridisation step, the yield should be  $> 1.65\mu\text{g}$  and the specific activity  $> 9.0\text{pmol Cy3 per } \mu\text{g aRNA}$  for one colour experiments.

The yield and specific activity of each reaction was determined as follows:

1. aRNA yield:

Concentration of aRNA( $\text{ng}/\mu\text{l}$ ) \*  $10\mu\text{l}$  (elution volume)/1000 =  $\mu\text{g}$  of aRNA

2. specific activity:

(Concentration of Cy3 or Cy5)/(concentration of aRNA)\*1000 = pmol Cy dye per  $\mu\text{g}$  aRNA.

#### *Hybridisation to arrays and scanning*

To measure levels of gene expression, test and control total RNAs are used to produce fluorescently labelled amplified RNA targets. These are hybridised onto Agilent manufactured arrays using Agilent hybridisation chambers and oven. The resulting fluorescent intensities, detected on scanning the slides with the appropriate lasers to excite the fluorescent label, provide information on the levels of expression of the particular DNA product in the tissues from which the test and control RNAs were derived.

The hybridization samples were prepared by adding the Fragmentation Mix to individual wells of a PCR plate (for each microarray) and mixing well, then incubating at  $60^{\circ}\text{C}$  in a Dyad PCR block for 30 minutes to fragment the RNA.  $55\mu\text{l}$  of hybridisation buffer (2x GE Hybridization Buffer HI-RPM) was then added to stop the fragmentation reaction. Samples were then spun for one minute at room temperature at 4000rpm in Eppendorf 5810 centrifuge, and placed on ice before

being loaded onto the array. The hybridization assembly was prepared by loading a clean gasket slide into the Agilent SureHyb chamber. 100µl of hybridization sample was slowly dispensed onto the gasket well. An array was placed “active side” down onto the SureHyb gasket slide, so that the “Agilent”-labelled barcode was facing down and the numeric barcode was facing up. The SureHyb chamber cover was placed onto the sandwiched slides and the clamp assembly was slid onto both pieces and the clamp was tightened. The assembled slide chamber was placed in a hybridization oven and rotated at 10 rpm at 65°C for 17 hours to hybridise. The hybridised array slides were then washed using Gene Expression Wash Buffer 1 at room temperature for one minute then using Gene Expression Wash Buffer 2 at 37°C for one minute. The slides were then dried by spinning in the Beckman GS-3 centrifuge at 1800rpm for six minutes and scanned according to the manufactures instructions, at 5µm on the Axon 4200AL scanner using the Gal file supplied by Agilent for autoPMT (Photo multiplier tube; altering the PMT voltage alters the sensitivity of detection). The raw image data were then extracted using Feature Extraction (Agilent software) for further analysis using Partek Genomics Suite. The data were normalised using RMA (Robust multi-array average), recommended by the manufactures of the software. Within the Partek software an ANOVA was used to compare the groups of interest and to create gene lists of significantly changing genes between groups. Downstream analysis was carried out using Ingenuity Pathway Analysis software.

## Reagents

### *Reverse Transcription master mix*

10x First Strand Buffer	2ul
Ribonuclease Inhibitor	1ul
dNTP mix	4ul
Reverse Transcriptase	1ul



*Second strand synthesis master mix*

Nuclease free water	63 $\mu$ l
10x second strand buffer	10 $\mu$ l
dNTP mix	4 $\mu$ l
DNA Polymerase	2 $\mu$ l
RNase H	1 $\mu$ l

*Aminoallyl Master mix*

double stranded cDNA	16 $\mu$ l
5-(3-aminoallyl)-UTP	3 $\mu$ l
T7 ATP Solution (75mM)	4 $\mu$ l
T7 CTP Solution (75mM)	4 $\mu$ l
T7 GTP Solution (75mM)	4 $\mu$ l
T7 UTP Solution (75mM)	2 $\mu$ l
T7 10x Reaction Buffer	4 $\mu$ l
T7 Enzyme Mix	4 $\mu$ l

*Fragmentation Mix*

Cy 3 labelled aRNA	1.65 $\mu$ g
10 x Blocking Agent	11 $\mu$ l
Nuclease free water	bring vol to 52.8 $\mu$ l
25 x Fragmentation buffer	2.2 $\mu$ l

**Analysis**

Statistics were carried out using Sigma Stat 11.0 and Graph Pad Prism 5. Analysis was done blind to treatment groups.

## **Materials**

1-bromo-3-chloropropane BCP	Sigma B9673
5-(3-aminoallyl)-UTP	Ambion 8437
ABC kit mouse IgG	Vectastain PK6102
ABC kit rat IgG	Vectastain PK6104
Acetic anhydride	Sigma A6404
[ <sup>3</sup> H]AMPA	Perkin Elmer NET833025UC
Agilent Spike in Kit	Agilent Technologies 5188-5282
Agilent Microarray Hybridization Chamber Kit	G2534A
'Amberlite' IRN-150 (monobed mixed resin)	BDH 551794K
Ammonium chloride	BDH A4514
'AnalR' Formamide	BDH 10326R
ATP, CTP, GTP	Promega
Autoradiographic emulsion	Ilford K.5
Blocking Agent (10 x)	Agilent p/n 5188-5281
Bovine serum albumin	Sigma, UK
Chloroform	Sigma, UK
Citric acid	Aldrich 24 062-1
Cobalt chloride	Sigma, UK
Coupling Buffer from the Fairplay kit	CS 003
Coverslips	BDH, UK
Cy3 monoreactive dye pack	CS 006
Developer Kodak D19	Ilford Ltd, UK
Denhardt's Solution	Sigma D-2532
Dextran sulphate	Sigma D-8906
Diaminobenzidine (DAB)	Sigma D5637
Diethylpyrocarbonate	Sigma D-57580
Disodium hydrogen orthophosphate	BDH 103835H
Dithiothreitol (DTT)	Sigma D-9779
DMSO from the Fairplay kit	CS 003

DPX mountant	Sigma 44581
DyeEx 2.0 spin kit	EQU 053
EDTA	Sigma E-7889
Eosin	BDH 341973R
Ethanol (absolute)	Fisher Scientific 64-17-5
Fadrozole	Novartis MTA
Fixer	Ilford Hypam rapid
Gene Expression Hybridization Kit	p/n 5188-5242
Gene Expression Wash Buffer 1	p/n 5188-5325
Gene Expression Wash Buffer 2	p/n 5188-5326
Glucose	Sigma G7528
Glucose oxidase VII	Sigma G7016
[ <sup>3</sup> H]Glutamate	Perkin Elmer NET1082050UC
Haematoxylin	Thermo electron corporation
Heparinised capillary tubes 75mm	Fisher Scientific, UK
Horse serum	Sigma H1270
Hydrochloric acid (HCl)	BDH, UK
Linear acrylamide	Ambion 9520
Lysing Matrix D beaded tubes	Fisher MBR-247-110Y
Magnesium Sulphate	Sigma M7506
MessageAmp aRNA Kit	Ambion 1750
Microscope slide (frosted)	BDH, UK
Multiwell culture plate (12 well flat bottom)	Sigma CLS3512
Needle 25G	BD microlance 300600
Nickel ammonium sulphate	Fisher Scientific 15699-18-0
Nickel column (Sephadex G-50)	Amersham 17-0855-01
NMDAR1 antibody (mouse monoclonal)	BD Pharmingen 556308
Oestradiol	Sigma E1024-1G
Oestradiol RIA kit	DSL-4800
Oestrogen receptor alpha antibody (H222)	Santa Cruz biotech sc-53492
PAP pen	VWR 720-0169

Paraformaldehyde	Sigma P-6148
Polysine slides (25x 75x 1.0mm)	VWR
Potassium Aluminium Sulphate	BDH 100093E
Potassium Dihydrogen Phosphate	Sigma, UK
Potassium Chloride (KCl)	BDH, UK
Rnase A	Sigma R4642
RNAlater stabilization reagent	Qiagen, UK
RNA polymerase (T3, T7, SP6)	Promega
RNase Zap	Ambion AM9780
Salmon sperm DNA	Sigma D-9156
Scintillation fluid (Utlima Gold)	Perkin Elmer
Slide boxes	VWR International 631-0100
Sodium Acetate	Sigma S9513
Sodium Bicarbonate	Sigma S8875
Sodium Chloride (NaCl)	BDH, UK
Sodium Citrate	BDH, UK
Sodium dihydrogen orthophosphate	BDH 102455S
Sodium Hydroxide (NaOH)	Sigma, UK
Sodium Phosphate (Na <sub>2</sub> HPO <sub>4</sub> )	Sigma, UK
Steroid diluent	MP Biomedicals, UK
Testosterone	Sigma T-1500, 1g
Testosterone antiserum	Guildhay Antisera (Surrey, UK)
[ <sup>3</sup> H]Testosterone	Amersham TRK 921-250
Terminal transferase labeling kit	Roche 220 582
Toluidine-Blue	Sigma, UK
Transcription buffer	Promega
Triethanolamine	Sigma T1377
Tris(hydroxymethyl)methylamine	VWR International 103156X
Tri Reagent	Ambion AM 9738
Tri-sodium citrate	Sigma S-4641
Triton X-100	Sigma T9284

Vectastain ABC kit

Xylene

Yeast tRNA

Vector PK 6102, mouse IgG

BDH, UK

Sigma R-9001

## Chapter 3

### Sex differences in spatial performance & hippocampal sex steroid receptors

#### **Abstract**

Testosterone often improves spatial learning and memory in mammals both during development and throughout life. The influence of sex steroids on avian spatial abilities has received little attention and, consequently, little is known about the actions of sex steroids on spatial cognition and the hippocampus in birds. The avian hippocampus is important for spatial learning and memory and contains both the androgen receptor (AR) and oestrogen receptor alpha (ER $\alpha$ ) and the enzyme aromatase that converts testosterone to oestrogen. The aim of this experiment was to investigate spatial abilities and hippocampal sex steroid receptor and aromatase expression in adult zebra finches and to establish if sex differences exist. Zebra finches were tested on a one-trial associative memory task (both spatial and visual versions), sampled for plasma testosterone analysis and their brains were collected for *in situ* hybridization for hippocampal AR, ER $\alpha$  and aromatase mRNA expression. Although males had significantly higher levels of testosterone on average, the males that were tested on the cognitive task from this population did not have higher testosterone levels compared to females. Circulating testosterone levels did not explain variation in task performance. Although there was a positive correlation between plasma testosterone levels and hippocampal AR mRNA expression, there was no sex difference in baseline hippocampal AR or ER $\alpha$  mRNA expression. Hippocampal aromatase mRNA expression was significantly higher in males compared to females. Taken together, although the sexes did not differ in spatial cognition, the differences in hippocampal aromatase levels may lead to effects on spatial cognition as testosterone levels increase.

## **Introduction**

In general, male mammals outperform female conspecifics on tasks involving spatial abilities. Sex steroids (e.g. testosterone, oestradiol) provide a mechanistic explanation as to why there is such a difference in spatial cognition between the sexes: in mammals, testosterone and males' superior spatial abilities both fluctuate during the breeding season. For example, in the breeding season when testosterone levels are high, male deer mice learn the platform location in a Morris Water Maze (MWM) faster than do conspecific females, whereas males are not any better than females when the animals are tested in the non-breeding season (Galea et al., 1996; Galea et al., 1994a). Furthermore, in rodents, lowering androgen levels by gonadectomy leads to impairment in performance on maze learning behaviour while testosterone replacement restores performance (Aleman et al., 2004; Cherrier et al., 2001; Edinger and Frye, 2004; Gibbs and Johnson, 2008; Gray et al., 2005; Janowsky et al., 1994; Kritzer et al., 2001; Postma et al., 2000). In humans, testosterone supplementation in older men improves spatial cognition and a single injection of testosterone in young women improves performance on mental rotation tasks and object-location memory within hours (Aleman et al., 2004; Cherrier et al., 2001; Edinger and Frye, 2004; Gibbs and Johnson, 2008; Gray et al., 2005; Janowsky et al., 1994; Kritzer et al., 2001; Postma et al., 2000). On the other hand, men who have undergone chemical castration, and who are then left with lowered testosterone levels, are impaired on spatial tasks (Almeida et al., 2004; Cherrier et al., 2003; Salminen et al., 2004). Oestrogen also enhances spatial memory performance in women and male and female non-human primates and rodents (Gibbs, 1999; Gresack and Frick, 2006; Heikkinen et al., 2002; Lacreuse et al., 2002; Rissanen et al., 1999; Sherwin, 1996). Moreover, reducing oestrogen levels in female rats by ovariectomy leads to a decrease in spatial memory performance, which is restored by oestradiol treatment (Gibbs and Johnson, 2008). Hippocampal morphology varies across the different stages of the oestrous cycle in rats, with reports of females in oestrus (low oestrogen) displaying significantly fewer dendritic spines than those in prooestrous (Woolley et al., 1990; Woolley and McEwen, 1992). However, associated behavioural changes are controversial and there are reports of

no effects of the oestrous cycle on spatial performance or opposite effects with rats in oestrous performing better on a spatial task than those in proestrous (Berry et al., 1997; Sutcliffe et al., 2007; Warren and Juraska, 1997). Assessing the effects of cycle on behaviour are complicated by the fact that the oestrous cycle of the laboratory rat fluctuates rapidly with animals cycling every four to five days, and the oestrogen peak of proestrous only lasts approximately 12 hours. Moreover, in as little time as 16 hours, oestrogen levels drop from the high concentrations seen in proestrous to the lowest point of oestrus (Huang et al., 1978). As Berry *et al* point out, many hippocampal-dependent learning tasks are inappropriate in that the amount of training to establish learning usually exceeds the duration of the cycle phase of interest (Berry et al., 1997). The interpretation of results is further complicated by the involvement of many other changing hormones, such as progesterone which may impact on performance and also the effects of increased oestrogen may not be apparent until days later, when the levels have dropped again.

Although there has been less effort addressing the effects of sex steroids on spatial cognition in birds, testosterone and oestrogen treatment for five days in great tits lead to improvements in spatial memory (Hodgson et al., 2008). Additionally, oestrogen and testosterone delivered by implant for 10 days to adult male zebra finches following castration improved performance in a spatial memory task (trained prior to surgery) (Oberlander et al., 2004). Oestrogen also appears to affect spatial memory in female zebra finches as blocking oestrogen synthesis (with fadrozole) impaired performance in a spatial task compared with spatial performance by females in which the oestrogen synthesis block was coupled with oestrogen replacement (Spence et al., 2009).

Sex steroid-induced alterations in cognitive performance are accompanied by alterations in hippocampal morphology in mammals. The size of the hippocampus is positively correlated with an extra demand for spatial learning and memory, and male meadow voles with high testosterone levels have significantly larger hippocampal volumes than do low testosterone males (Galea et al., 1996; Galea et al., 1994a; Galea et al., 1999; Gaulin et al., 1990; Jacobs et al., 1990; Sherry et al.,



1992). Testosterone also affects the female hippocampus and treatment in female rats increases the number of cells in and the thickness of the dentate gyrus, a major subregion of the hippocampus (Roof, 1993; Roof and Havens, 1992). Testosterone can also enhance memory by influencing excitatory synaptic transmission through alterations in dendritic spine density. For example, gonadectomy reduces spine density in the hippocampus in both rats and monkeys and testosterone treatment increases spine synapse density to levels comparable with those of intact males (Leranth et al., 2003; Leranth et al., 2004b). Studies on oestrogen-dependent structural plasticity of the mammalian hippocampus have shown that it can also have effects beneficial for learning and memory (Foy et al., 1999; Gould et al., 1990; Gupta et al., 2001; Murphy and Segal, 1996; Ormerod et al., 2003; Pozzo-Miller et al., 1999; Stone et al., 1998; Tanapat et al., 1999; Vouimba et al., 2000; Warren et al., 1995; Woolley et al., 1990; Woolley and McEwen, 1992; 1993; 1994; Woolley et al., 1997; Yankova et al., 2001). Oestrogen-induced increases in hippocampal spine density are associated with improved spatial performance in adult female rats and ovariectomized rats fed a high phytoestrogen diet (Luine et al., 2006; McLaughlin et al., 2008). It seems that the route of hormone administration may be important, as ovariectomized rats given implanted capsules containing oestrogen (for 15 days) showed improved spatial performance and increased spine density, whereas those given oral dosing did not (Garza-Meilandt et al., 2006). Like the mammalian hippocampus, the avian hippocampus is sex-steroid sensitive and is involved in spatial memory function (Hampton et al., 1995; Krebs et al., 1996; Patel et al., 1997b; Petersen and Sherry, 1996; Sherry and Vaccarino, 1989). Although substantially less is known about the relationship between sex steroids and the avian hippocampus, testosterone treatment of male adult castrated zebra finches leads to an increase in the size of cells in the rostral hippocampus compared to controls (Oberlander et al., 2004). Even less is known about the neural mechanisms through which sex steroid-induced changes in the hippocampus are occurring.

The effects of sex steroids on spatial performance are mediated through androgen and oestrogen receptors (AR and ERs), both of which are present in the hippocampus of birds, as in mammals (Jacobs et al., 1999; Perlman et al., 2003). Sex steroids

exert their effects by regulating gene expression in target neurones and through binding to their respective receptors they cause the up and down regulation of mRNA, including encoding for their own steroid receptor proteins (Rories and Spelsberg, 1989; Spelsberg et al., 1989). By altering the concentration of steroid receptor protein, steroids can alter the sensitivity of target cells to further steroidal stimulation (Nastiuk and Clayton, 1995). The mechanisms controlling the expression of sex steroid receptors of birds, similar to mammals, are area-specific and depend on brain intrinsic genomic mechanisms and a variety of physiological factors (Lauber et al., 1991; Lisciotto and Morrell, 1993; Lynch and Story, 2000). In general, circulating sex steroids down-regulate steady state levels of ER and AR mRNA within specific brain regions (Berthois et al., 1990; Burgess and Handa, 1993; Lauber et al., 1990; Nastiuk and Clayton, 1995; Saceda et al., 1988; Shughrue et al., 1992; Simerly and Young, 1991; Yuri and Kawata, 1991). For example, castration of male rats increases ER mRNA and AR mRNA in the brain, which decreases with dihydrotestosterone and oestradiol benzoate treatment (Burgess and Handa, 1993; Lisciotto and Morrell, 1993). Oestrogen treatment induces a decrease in oestrogen receptor ER mRNA expression and ovariectomized female rats exhibit elevated ER mRNA levels (Berthois et al., 1990; Lauber et al., 1990; Shughrue et al., 1992).

There is growing evidence that the aromatisation of testosterone to oestrogen is an important step in testosterone-mediated learning and memory in mammals and in birds, including spatial memory (Iivonen et al., 2006; Oberlander et al., 2004; Saldanha et al., 2004; Schlinger and Arnold, 1991). For example, in adult castrated male zebra finches, those given oestrogen learnt a spatial task faster than those given testosterone (Oberlander et al., 2004). Aromatase is particularly abundant and widespread in the zebra finch telencephalon compared to adult mammals and non-songbirds and in male zebra finches the brain is the major source of oestrogen (Saldanha et al., 2000; Schlinger and Arnold, 1991; Shen et al., 1994; Shen et al., 1995). Although male mice have higher hippocampal aromatase expression than do females, sex differences in aromatase mRNA expression in the avian hippocampus have not been investigated nor has the relationship between mRNA expression and

circulating sex hormone levels (Ivanova and Beyer, 2000). It is likely that locally produced oestrogen is important in spatial cognition in birds, and possible that males may have higher levels of hippocampal aromatase expression leading to higher levels of local oestrogen and better spatial performance.

The aim of this study was to investigate baseline sex differences in spatial abilities and hippocampal sex steroid receptor and aromatase expression in the adult zebra finch. It was hypothesised that testosterone would be important for superior spatial performance. I made four predictions: (1) males would have higher levels of circulating testosterone compared to females (although levels in males would be low as birds are in non-breeding conditions); (2) plasma testosterone levels would be positively correlated with performance on the spatial but not the visual task and, consequently, males would outperform females only on the spatial task; (3) circulating testosterone levels would be positively correlated with hippocampal AR mRNA expression and, therefore, males would have higher expression than females; (4) plasma testosterone levels would be positively correlated with hippocampal aromatase mRNA expression and, therefore, males would have higher expression than females.

## **Materials and Methods**

### *Subjects*

Birds were sexually mature, captive-bred adult (3-4 years of age) male (n = 19) and female (n = 16) zebra finches, obtained from St Andrews University, UK.

### *One trial associative memory task*

Birds were tested once a day between 10 and 11.30am, for 10 consecutive days, with a retention interval (RI) of 10 minutes. Performance was measured by the number of flaps a bird lifted to find the baited well. The latency to solve the task was also recorded, which was the time from the presentation of the tray in phase 2 to when the bird found the piece of cucumber. There were 35 birds (male n = 19, female n = 16) that started the pretraining, of which 14 (male n = 6, female n = 7) made it to criterion within four months of training. Birds were tested on the spatial version (males n = 6, females n = 7), followed by the visual version (males n = 5, females n = 5). Birds were then retested on the spatial version (males n = 3, females n = 4) to control for effects of improvement across the course of the experiment. The sample size decreased due to the deaths of birds from old age before completion of the testing. In the spatial version of the task, all flaps were the same colour on all 10 trials (green). Each trial, all flaps remained in the same location, however the location of the rewarded well changed daily. The baited well was not used the day preceding or following the trial so as to avoid birds either actively avoiding it if previously unrewarded, or preferentially going there if previously rewarded. In the visual version of the task, the colour of the flap covering the rewarded well was only rewarded on one trial. The other four flaps were all different colours, which again remained the same between phases, but their locations changed. There were 10 colours and the rewarded colour was not used on the preceding or following day.

### *Retention Interval manipulation*

Following completion of both spatial and visual baseline testing, birds were tested for five additional trials, with retention intervals of 20, 30, 40, 50 and 60 minutes over 5 consecutive days. The order of the retention intervals were counterbalanced

for different birds. Birds were tested with increasing retention intervals on both the spatial and visual tasks, with half the birds completing the spatial retention interval testing first and the others the visual retention interval testing first.

#### *Hormone measurement*

Blood samples of approximately 150µl were taken from the alar wing vein (both from birds which were tested in the task and those which were not; male n=19, female n=16). Samples were taken in the morning to coincide with timing of behavioural testing. Blood samples were taken following completion of testing. Plasma testosterone concentrations were measured by double antibody radioimmunoassay at the University of Glasgow using a modification of an established assay (Sheffield and O'Shaughnessy, 1989; Verboven et al., 2003). Samples were run in a single assay. The binding was 8.4% and non-specific binding was 49%. The intra-assay coefficient of variation was 8.2%. Extraction efficiency was < 90%.

#### *In situ hybridization for androgen receptor, oestrogen receptor and aromatase mRNA expression in the hippocampus*

Following completion of behavioural testing, the test birds (females n=8, males n=10) were decapitated, blood collected and their brains removed and frozen immediately on dry ice. Brains were stored at -70°C until in situ hybridisation was carried out for hippocampal AR, ER alpha and aromatase mRNA expression (one slide per bird with five hippocampal sections per slide). The sample size was increased from the behavioural testing by 4 for females and 7 males (see Table 1 in Chapter 4 for diagram of sample sizes for the Chapter 3 and 4).

#### *Analysis*

The effects on the performance index of the day of testing and sex were measured using two-way repeated measures ANOVA (independent variables: day and sex).. For the retention interval data, the effects of increasing interval and sex on the performance index were measured using two-way repeated measures ANOVA (independent variables: RI and sex). The post hoc test used was the Holm-Sidak

method. Sex differences in testosterone levels and mRNA expression levels were compared using unpaired t-tests. Correlations between latency or testosterone levels and performance, and testosterone levels and mRNA expression were measured using linear regression.

## **Results**

### *Performance*

The performance index on the spatial task (number of flaps lifted) did not change across the 10 days of testing ( $F_{9,99} = 0.77$ ,  $p = 0.65$ ) and there were no significant differences between the sexes ( $F_{1,99} = 0.73$ ,  $p = 0.41$ ). There was no interaction between sex and day ( $F_{9,99} = 0.28$ ,  $p = 0.98$ ). In order to ensure that the birds were not using olfactory cues from the cucumber to solve the task, a probe trial was carried out on day 11. There was no reward provided in the choice phase of this trial. If the birds were using olfactory cues from the cucumber to solve the task, one would predict that performance would be impaired in the probe trial. There was no difference in performance between the 10 days (averaged) and the probe trial on the 11<sup>th</sup> day for males ( $t_5 = 0.40$ ,  $p = 0.71$ ) or females ( $t_6 = 0.37$ ,  $p = 0.72$ ). Spatial performance was better than chance (three flaps lifted) for males ( $t_5 = 18.7$ ,  $p < 0.0001$ ) and females ( $t_6 = 5.55$ ,  $p < 0.001$ ).

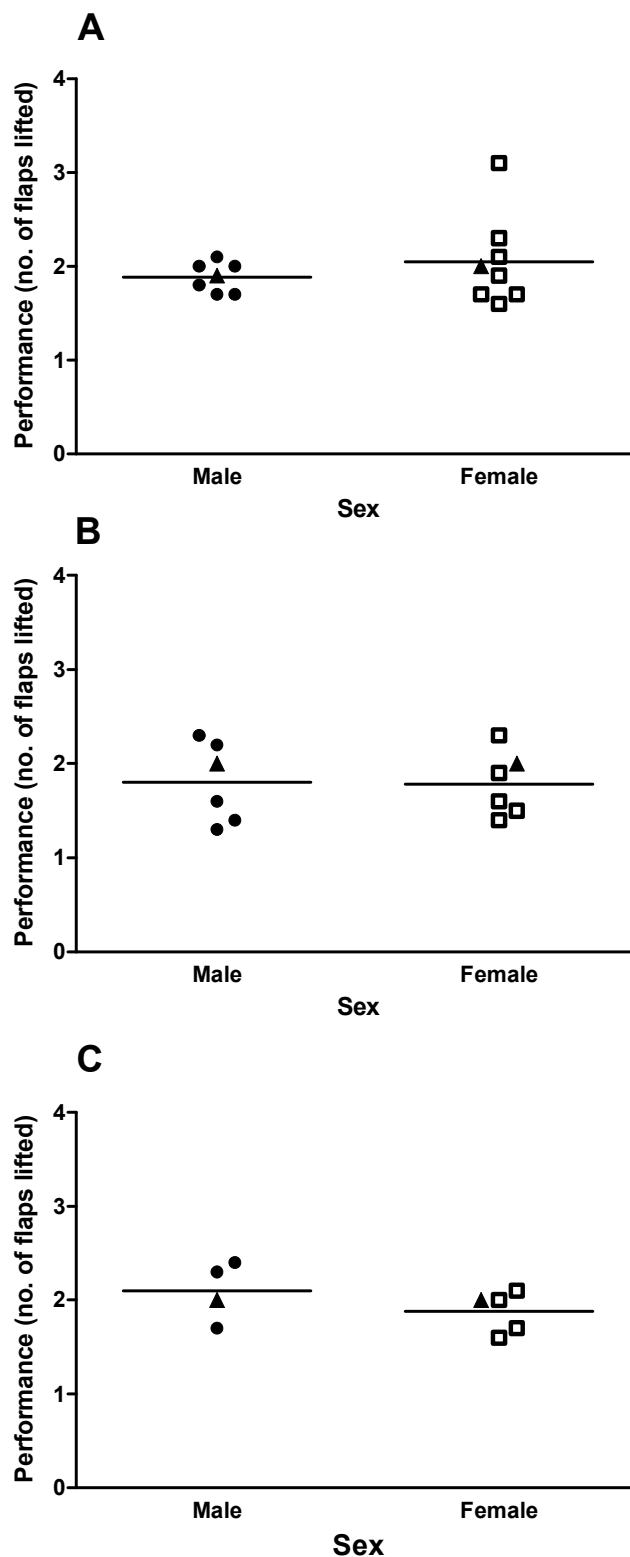
The performance index (number of flaps lifted) on the visual task also did not vary across the 10 days of testing ( $F_{9,72} = 1.30$ ,  $p = 0.25$ ) and the sexes did not differ ( $F_{(1,72)} = 0.03$ ,  $p = 0.88$ ). There was no interaction between sex and day ( $F_{9,72} = 0.98$ ,  $p = 0.47$ ). Performance for each individual was therefore averaged over the 10 trials for the spatial and visual tasks (Figure 1). Visual performance was better than chance for males ( $t_4 = 6.93$ ,  $p < 0.001$ ) and females ( $t_4 = 8.69$ ,  $p < 0.01$ ).

Prior to commencing testing, in order to check for any innate colour preferences which might affect performance on the visual task, birds were given a selection of 10 coloured flaps and tested once a day for five days to see if a particular colour was selected more often ( $n = 20$ ). None of the colours were rewarded. On average over the five days, there was no colour that was selected significantly more often than the others ( $F_{9,36} = 0.08$ ,  $p > 0.99$ ) and this was the case each day ( $F_{4,36} < 0.001$ ,  $p > 0.99$ ).

There were no differences in performance between the spatial task before or after the visual task. There was also no difference in performance between the spatial and the

visual tasks ( $F_{2,10} = 0.55, p = 0.60$ ). This was the same for males and females ( $F_{(1,10)} = 4.06, p = 0.10$ ), independent of the different task types ( $F_{2,10} = 1.27, p = 0.32$ ).

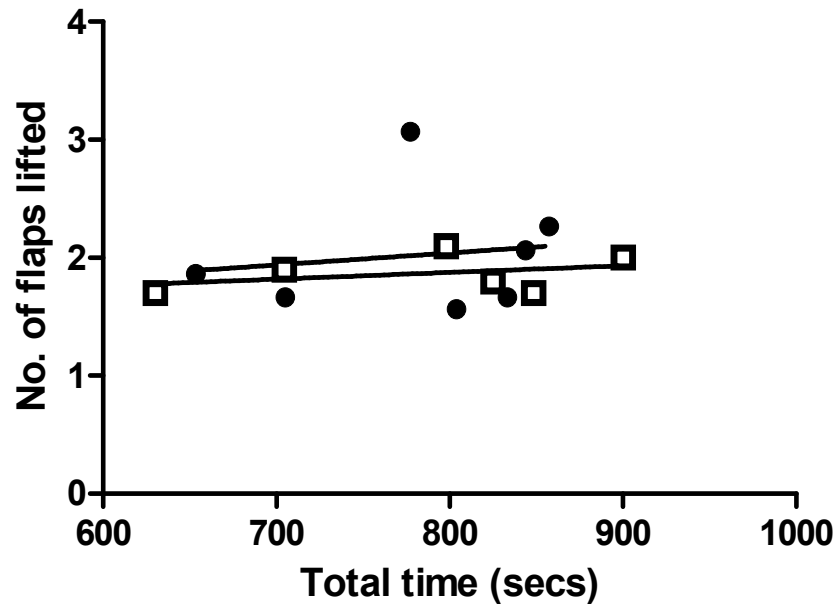




**Figure 1** Performance on the one trial associative memory task (A) spatial version (Males,  $n = 6$ ; Females,  $n = 7$ ) and (B) visual version (Males  $n = 5$ , Females  $n = 5$ ), and also (C) on the spatial task assessed after completion of the visual task (Males  $n = 3$ , Females  $n = 4$ ). Performance was measured as the number of flaps lifted to find the rewarded well and was averaged over 10 trials. There were no differences between males and females. The mean of the data is represented as a line. The average performance on probe day 11 of the birds is shown (triangle).

### *Latency and performance*

In the first spatial task, there was no correlation between the time taken to make the correct choice in Phase 2 and the performance index (number of flaps lifted) in either sex (male  $r^2 = 0.12$ ,  $p = 0.50$ ,  $n = 6$ ; female  $r^2 = 0.02$ ,  $p = 0.75$ ,  $n = 7$ ; Figure 2).



**Figure 2** The effect of latency (in seconds) to find the rewarded well in the first spatial task on performance (data points represent performance averaged over 10 trials) in males ( $n = 6$ , squares) and females ( $n = 7$ , circles). Linear regression lines are shown.

### *Retention Interval*

The birds' memory retention was tested using retention intervals (RI) of 10, 20, 30, 40, 50 and 60 minutes. As birds were only tested once at each of the RI of 20-60 minutes, performance on the 10<sup>th</sup> day only for the RI of 10 minutes was used in the analysis.

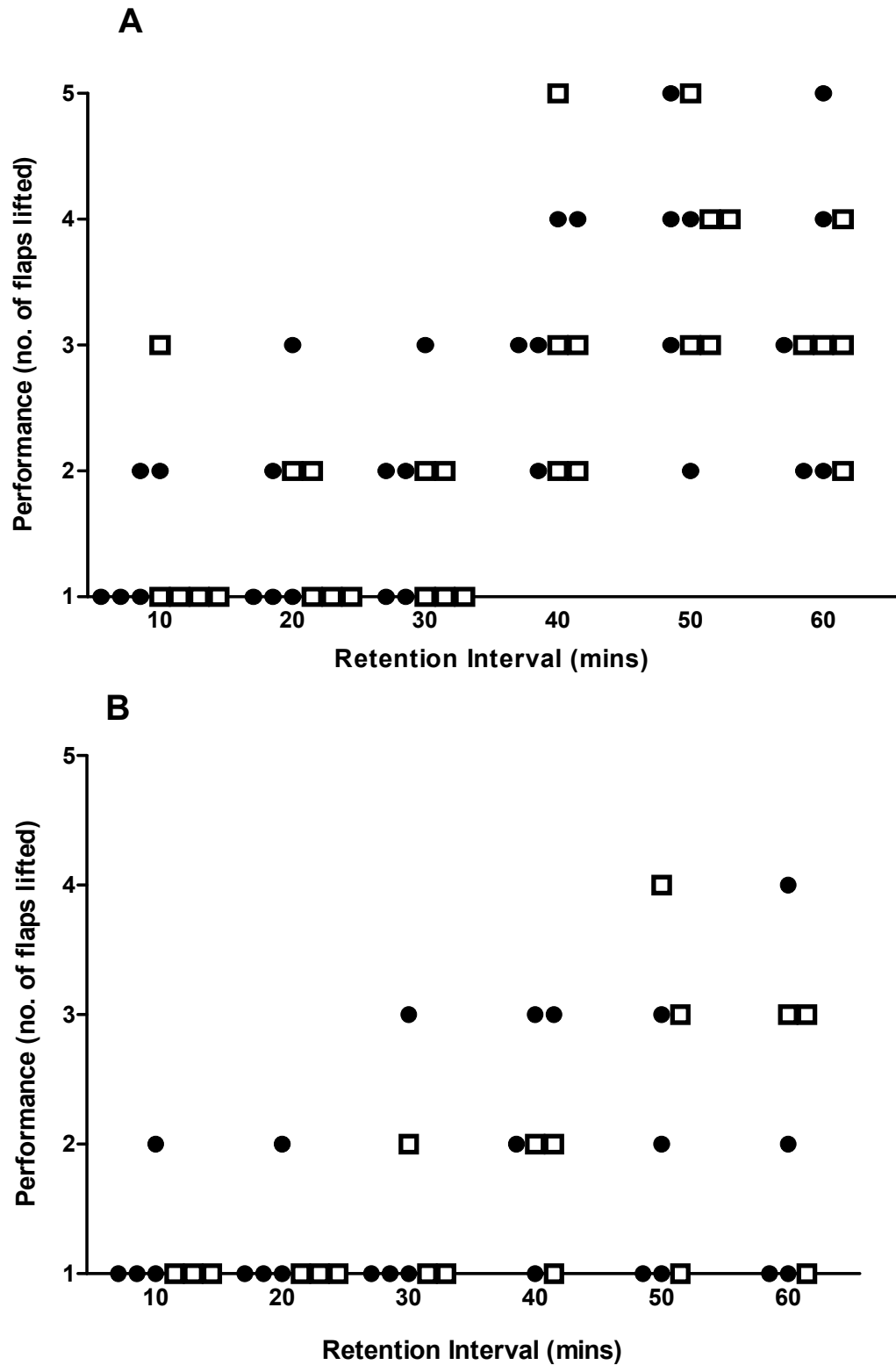
Performance on the spatial task declined with increasing RI ( $F_{5,40} = 11.51$ ,  $p < 0.001$ ; Figure 3A). Post hoc tests (Holm-Sidak) revealed that there was no difference in performance with RI between 10, 20 and 30 minutes or after an RI of 40, 50 or 60 minutes, but performance after 10, 20 and 30 was better than after an RI of 40, 50 or

60 minutes (see Table 1 below). The sexes did not differ in performance ( $F_{1,40} = 0.76$ ,  $p = 0.41$ ) and the effect of RI was not dependent on the sex of the bird ( $F_{5,59} = 0.12$ ,  $p = 0.99$ ).

Retention Interval Comparison		Significance
10 min vs 20 min	$t = 0.24$	$p = 0.81$
10 min vs 30 min	$t = 0.47$	$p = 0.64$
10 min vs 40 min	$t = 4.03$	$p < 0.001$
10 min vs 50 min	$t = 5.45$	$p < 0.001$
10 min vs 60 min	$T = 4.03$	$p < 0.001$
20 min vs 30 min	$t = 0.24$	$p = 0.81$
20 min vs 40 min	$t = 3.80$	$p < 0.001$
20 min vs 50 min	$t = 5.21$	$p < 0.001$
20 min vs 60 min	$t = 3.79$	$p < 0.001$
30 min vs 40 min	$t = 3.56$	$p < 0.001$
30 min vs 50 min	$t = 5.00$	$p < 0.001$
30 min vs 60 min	$t = 3.56$	$p < 0.001$
40 min vs 50 min	$t = 1.42$	$p = 0.16$
40 min vs 60 min	$t = 0.05$	$p = 1.00$
50 min vs 60 min	$t = 1.42$	$p = 0.16$

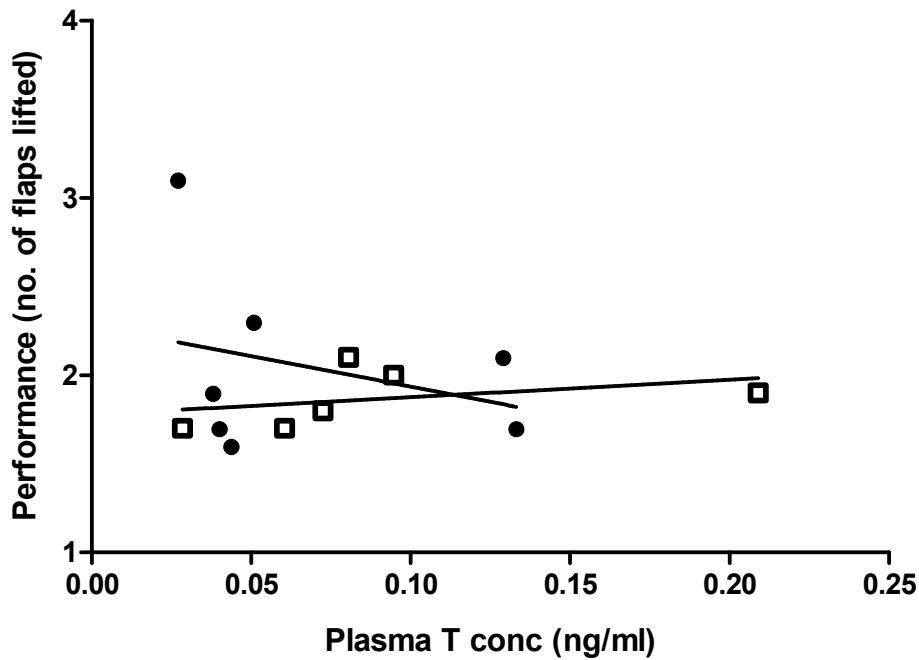
**Table 1** The results of post-hoc analysis of increasing retention interval on spatial performance

Performance (number of flaps lifted) on the visual task, however, did not decline with increasing RI ( $F_{5,25} = 2.09$ ,  $p = 0.10$ ; Figure 3B) with no effect of sex ( $F_{5,25} = 0.59$ ,  $p = 0.71$ ). The effect of RI was not dependent on the sex of the bird ( $F_{1,25} < 0.01$ ,  $p > 0.99$ ).



**Figure 3** The effect of retention interval on performance in the (A) spatial task for males ( $n = 5$ ; squares) and females ( $n = 5$ ; circles) and (B) in the visual task for males ( $n = 3$ ) and females ( $n = 4$ ). Data points represent one trial for each RI. On the spatial task, performance was significantly better with RI 10, 20 and 30 min compared to 40, 50 and 60 min.

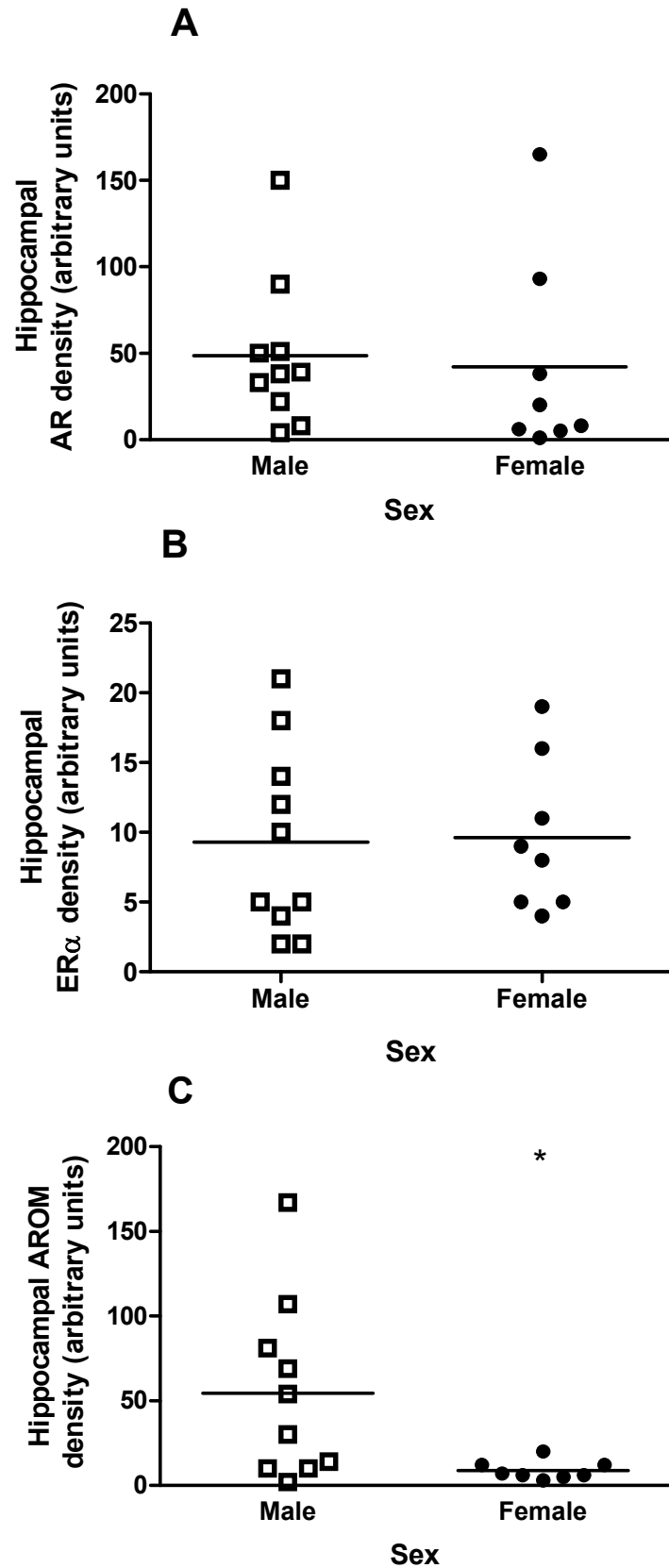




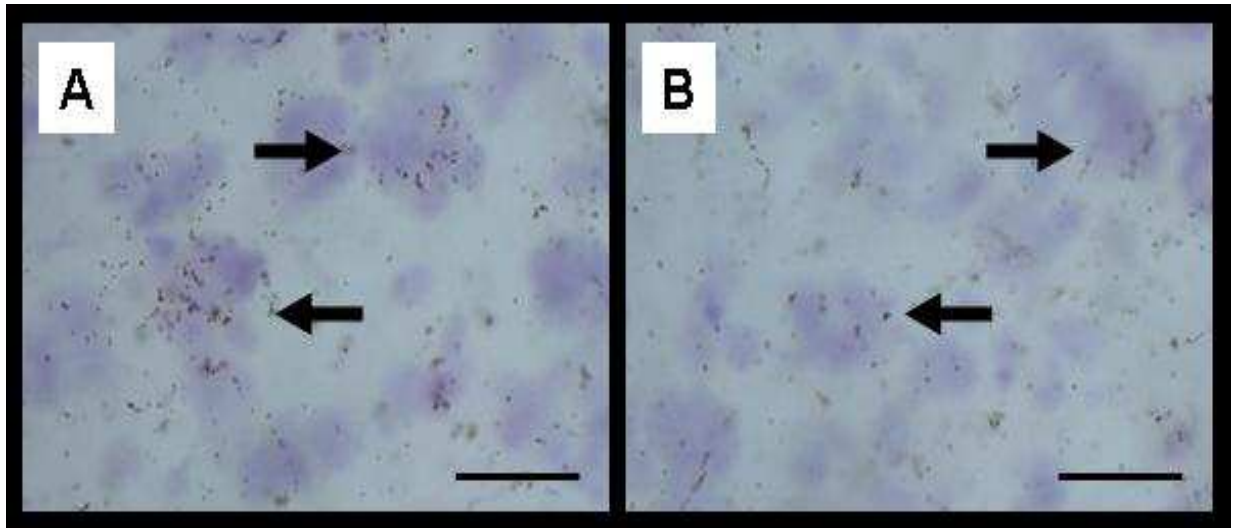
**Figure 5** Testosterone (T) levels and spatial performance for males (n = 6; squares) and females (n = 7; circles). The data points are each an average over 10 trials. Linear regression lines are shown.

#### *Testosterone and hippocampal mRNA expression levels*

Males and females did not differ in baseline AR mRNA expression levels ( $t_{16} = 0.27$ ,  $p = 0.79$ , males  $n = 10$ , female  $n = 8$ ; Figure 6A) or in ER $\alpha$  levels ( $t_{16} = 0.11$ ,  $p = 0.91$ , males  $n = 10$ , female  $n = 8$ ; Figure 6B). Males had significantly higher levels of hippocampal aromatase mRNA expression compared to females ( $t_{16} = 2.4$ ,  $p = 0.029$ , males  $n = 10$ , female  $n = 8$ ; Figure 6C and Figure 7). AR mRNA expression was positively correlated with plasma testosterone levels in males and females ( $r^2 = 0.40$ ,  $p < 0.05$ ,  $n = 10$  and  $r^2 = 0.56$ ,  $p = 0.03$ ,  $n = 8$ , respectively; Figure 8A). There was no correlation between plasma testosterone levels and ER $\alpha$  mRNA expression in either males or females ( $r^2 = 0.10$ ,  $p = 0.36$ ,  $n = 10$  and  $r^2 = 0.13$ ,  $p = 0.39$ ,  $n = 8$ , respectively; Figure 8B). This was also the case for aromatase mRNA expression in males and females ( $r^2 = 0.03$ ,  $p = 0.63$ ,  $n = 10$  and  $r^2 = 0.03$ ,  $p = 0.67$ ,  $n = 8$ , respectively; Figure 8C).

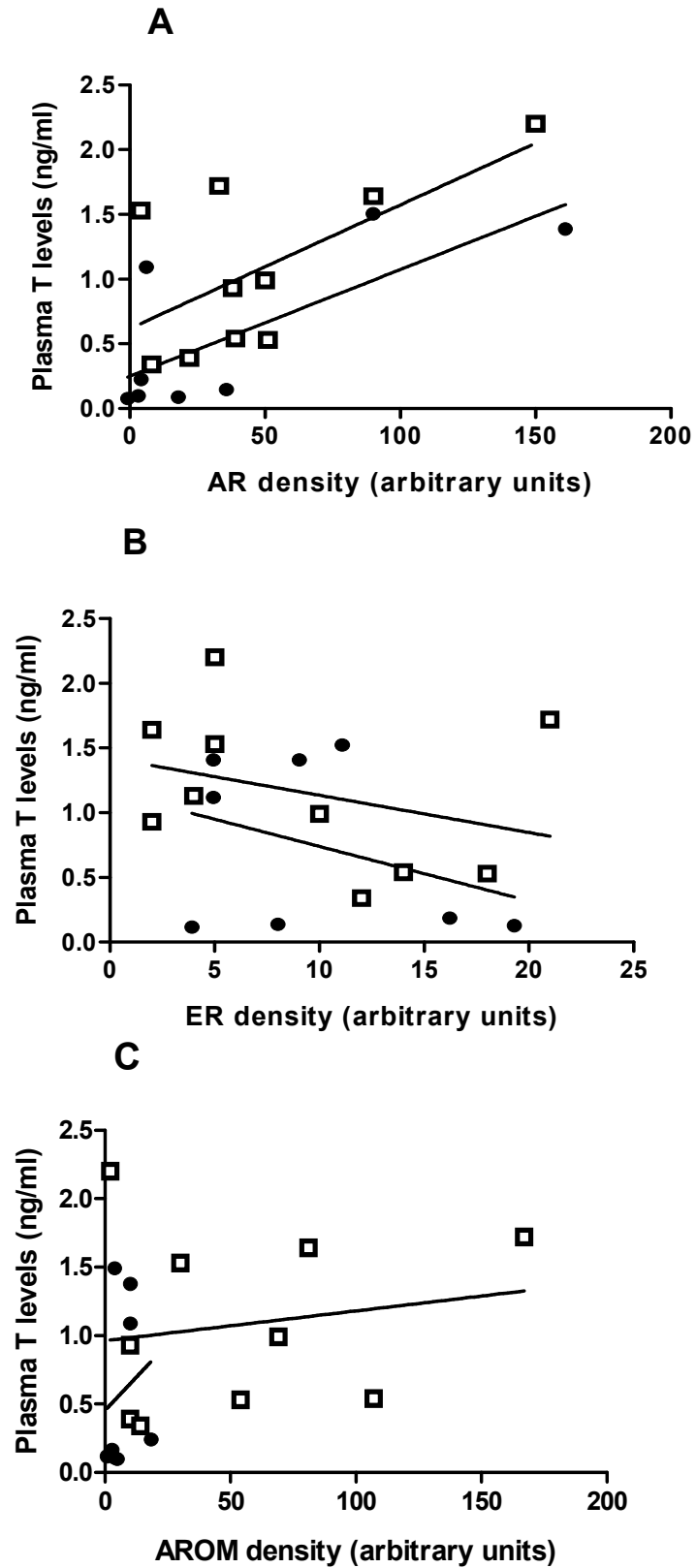


**Figure 6** The levels of hippocampal (A) androgen receptor (B) ER alpha and (C) aromatase mRNA expression in males (n = 10) and females (n = 8). Males had significantly higher aromatase levels compared to females. \* P < 0.05.



**Figure 7** Aromatase mRNA expression in the zebra finch hippocampus in males (A) and females (B). Expression was significantly higher in males compared to females ( $p < 0.05$ ). Magnification x 100, scale bar = 25  $\mu\text{m}$ .





**Figure 8** The relationship between plasma testosterone (T) levels and hippocampal (A) androgen receptor (AR) mRNA (B) oestrogen receptor alpha (ER) mRNA and (C) aromatase (AROM) mRNA expression in males ( $n = 10$ ; squares) and females ( $n = 8$ ; circles). T was significantly positively correlated with AR mRNA expression in males and females ( $p < 0.05$ ).

## **Discussion**

Male zebra finches had higher levels of circulating testosterone compared to females, although the males that reached criterion level, and were subsequently tested on the memory tasks, did not. The sexes did not differ in performance on either the spatial or visual task and performance did not differ between the task types. Male and female performances on the spatial task declined with retention intervals greater than 30 minutes, but for the visual task performance did not differ with increasing retention interval. Plasma testosterone levels and performance were not correlated in either sex. Hippocampal AR and ER $\alpha$  mRNA expression did not differ between the sexes. However, aromatase mRNA expression was significantly higher in males compared to females (mix of previously tested and not tested birds).

As male mammals generally outperform conspecific females on tasks involving spatial abilities and variation in performance is correlated with higher testosterone in males, it was predicted that male zebra finches would outperform females, specifically on the spatial task (Astur et al., 1998; Astur et al., 2004; Christiansen and Knussmann, 1987; Collins and Kimura, 1997; Driscoll et al., 2005; Galea et al., 1996; Galea and Kimura, 1993a; Galea and Kimura, 1993b; Gouchie and Kimura, 1991; Gron et al., 2000; Moffat et al., 2002; Shute et al., 1983; Silverman et al., 2000; Vandenberg and Kuse, 1978). There was no sex difference in either memory task and testosterone levels in tested birds did not differ, although testosterone levels in the group of males that included males that did not reach performance criterion were higher than testosterone levels in all females. It remains possible that testosterone may impact on spatial cognition in these birds, but when testosterone levels are low such effects are not apparent. It seems surprising that male birds with higher testosterone levels did not reach criterion and learn the task as their testosterone levels remained within physiological range and this seems to counter the hypothesis. However, birds with lower testosterone levels also did not reach criterion so it is difficult to know if this was just chance. Whether birds with higher unmanipulated circulating testosterone levels do not learn the task could be further investigated by increasing the sample size and selecting birds with naturally varying

testosterone levels and recording the relationship between testosterone levels and performance.

The birds' performance in the spatial task was impaired once the retention interval became longer than 30 minutes. In contrast, increasing the retention interval in the visual task did not significantly impair performance, which suggests the birds may find the task easier than the spatial task. Variation in cue preference has been observed within non-storing species, both in mammals and birds. In both groups, males prefer to use spatial cues while females either have no preference or prefer to use visual cues (Sovrano et al., 2003; Vallortigara, 1996; Williams et al., 1990; Williams and Meck, 1991). Variation in testosterone levels appear to underlie the sex difference in mammals as females with enhanced levels of testosterone both prefer to use location cues and perform as well as males on spatial tasks (Roof and Havens, 1992). As both male and female birds in this experiment had low testosterone levels, they may have had a preference for, and been better at remembering visual cues. Increasing testosterone levels may lead to a preference in both sexes for spatial cues, and testing birds on these tasks while experimentally manipulating testosterone levels would allow investigation of this possibility.

Males did not differ from females in baseline plasma testosterone levels, hippocampal AR or ER $\alpha$  mRNA expression. Plasma testosterone levels were positively correlated with AR mRNA expression, consistent with previous findings of positive effects of steroids on receptor expression. For example, androgenic-anabolic steroid (AAS) treatment induced upregulation of AR immunoreactivity in a range of rat brain regions, in both intact and castrated males. Additionally, a significant decrease in AR immunoreactivity was observed in all brain regions examined 14 days after castration (Lynch and Story, 2000; Menard and Harlan, 1993). Also, in young female rats, oestradiol treatment significantly increased ER $\alpha$  in the hippocampus and prefrontal cortex relative to control treatment (Bohacek and Daniel, 2009). However, contrary to this, other literature suggests that sex steroids down-regulate receptor levels, suggesting a negative feedback regulation. For example, in male adult rats castration (four days) resulted in significantly increased

AR mRNA in the anterior pituitary gland (AP) and hypothalamic-preoptic area, but not the amygdala. In the AP, administration of dihydrotestosterone or oestradiol benzoate reversed this effect. Surprisingly, five days of oestradiol treatment resulted in a significant increase in AR mRNA content so there appears to be tissue and hormone-specific regulation of AR mRNA (Burgess and Handa, 1993). Down-regulatory effects of sex hormones are also observed in the songbird HVC (Fusani et al., 2000). Additionally, in female rats, oestradiol treatment reduced levels of ER $\alpha$  mRNA in the hypothalamus (ventromedial and arcuate nucleus) relative to levels in both ovariectomized control animals and intact oestrous females (Lauber et al., 1991; Lauber et al., 1990; Simerly and Young, 1991). However, this treatment failed to down-regulate ER $\alpha$  message levels in castrated male rats. There was no significant effect of sex or oestradiol treatment on ER $\alpha$  mRNA levels in the amygdala (Lauber et al., 1991). Thus, the mechanisms controlling the expression of sex steroid receptors are likely to be sex steroid and area-specific, so it is possible that what we are seeing is a hippocampal-specific effect. Complications in interpretation of the literature may also arise due to timing effects of how soon hormone is given following gonadectomy; in middle-aged female rats, immediate oestradiol treatment significantly increased ER $\alpha$  in the hippocampus, but not the prefrontal cortex. However, delayed oestradiol treatment failed to significantly increase ER $\alpha$  protein levels in the hippocampus, but did so in the prefrontal cortex (Bohacek and Daniel, 2009).

Aromatase mRNA expression levels were significantly higher in male zebra finches compared to females, which is consistent with sexual dimorphism in other avian brain areas, such as higher male aromatase mRNA expression in the preoptic nucleus (POM) and mediobasal hypothalamus (MBH) of quail and more aromatase-positive fibres in the caudomedial neostriatum (NCM) and the preoptic area (POA) and a greater number of aromatase-containing presynaptic boutons in male zebra finches compared to females (Peterson et al., 2005; Rohmann et al., 2006; Saldanha et al., 2000; Voigt et al., 2007). There is also higher aromatase expression in the hippocampus of male mice compared to females (Ivanova and Beyer, 2000). As there is evidence that aromatase expression is regulated by both testosterone and

oestrogen in mammals and birds, we would have expected that the higher aromatase mRNA expression in the male hippocampus compared to the female hippocampus would have been associated with higher testosterone levels in males than in females but we did not see this (Fusani et al., 2001; Roselli et al., 1998; Saldanha et al., 2000b; Zhao et al., 2008). It is not clear why there was not a positive correlation between testosterone levels and aromatase expression. It may be that the regulation of aromatase is area-specific and expression in the hippocampus is not regulated by testosterone, but just by oestrogen. In male zebra finches, aromatase expression is down-regulated by circulating oestrogen in the hippocampus (but not the caudomedial neostriatum or preoptic area), so if this is also the case for females it is possible that the females had higher levels of oestrogen and therefore lower hippocampal aromatase expression (Saldanha et al., 2000b). Insight could be gained by looking at the relationship between circulating oestrogen levels and hippocampal aromatase expression. Testosterone treatment rapidly induces a significant reduction of AR mRNA levels in the nucleus HVC of female canaries (Nastiuk and Clayton, 1995). It is also possible that a threshold level of testosterone is needed above which we would have seen a positive correlation between aromatase expression and testosterone levels. In orchidectomized/ adrenalectomized rats where levels of testosterone are very low, aromatase mRNA expression is reduced (Zhao et al., 2008). It may be that in the present studies baseline levels of testosterone are not low enough to reduce aromatase expression or high enough to enhance it. To investigate this further, testosterone and oestrogen levels could be experimentally elevated and hippocampal aromatase mRNA expression analysed to look at the relationship between plasma hormone levels and hippocampal enzyme expression.

In conclusion, while we found evidence for sex differences in hippocampal aromatase mRNA expression, in the subset of birds tested we saw no sex differences in circulating testosterone levels, spatial performance, hippocampal AR or ER $\alpha$  mRNA expression. Circulating testosterone levels do not appear to be responsible for sex differences in hippocampal aromatase expression, which may actually depend on oestrogen levels. In the following studies we will investigate whether experimental elevation of sex steroids improves spatial performance in both sexes

and whether this is accompanied by changes in hippocampal receptor and aromatase expression.

## Chapter 4

# The effects of acute testosterone on spatial cognition and hippocampal androgen receptor and oestrogen receptor alpha mRNA expression

### **Abstract**

Non-breeding zebra finches have low circulating levels of sex steroids and the sexes do not differ in spatial cognition. In mammals, sex differences in spatial abilities are often seen only during the breeding season when testosterone levels are at their highest. Experimentally elevating testosterone in mammals can rapidly improve spatial memory but the mechanisms underlying these effects are still unclear. Little is known about the effects of testosterone on spatial cognition and hippocampal morphology in birds. In this study, circulating testosterone levels in adult zebra finches were elevated 30 minutes or four hours prior to behavioural testing on a one-trial associative memory task (spatial and visual versions). Brains were collected after cognitive testing for *in situ* hybridization to investigate the acute effects of testosterone on hippocampal androgen receptor and oestrogen receptor alpha (AR and ER $\alpha$ ) mRNA expression. Oral ingestion of testosterone in cucumber resulted in a peak in plasma testosterone levels after 30 minutes that returned to baseline by 60 minutes. Testosterone treatment led to a rapid improvement in spatial performance. 30 minutes after testosterone treatment hippocampal AR and ER $\alpha$  mRNA expression was significantly increased but these returned to baseline within four hours. Hippocampal aromatase mRNA expression was not significantly altered by testosterone treatment. A single oral dose of testosterone appears to be sufficient to enhance spatial cognition and increase AR and ER $\alpha$  mRNA expression in the hippocampus.

## **Introduction**

The effects of sex steroids on spatial cognition have been observed following natural changes in hormone levels in mammals, for example during the breeding season (Galea et al., 1996; Galea et al., 1994a). The short term, rapid regulation of hormones in the context of spatial behaviour have also been investigated in mammals, with improvement in spatial memory occurring as rapidly 30 minutes, or within hours, after acute administration in rats and humans (Aleman et al., 2004; Balthazart et al., 2006; Cornil et al., 2006a; Frye et al., 2007; Gresack and Frick, 2006; Luine et al., 2003; McEwen and Alves, 1999; Naghdi et al., 2005; Postma et al., 2000). This rapid improvement in spatial ability is accompanied by functional and morphological changes in the hippocampus. For example, oestradiol increases neuronal excitability and synaptic transmission and results in dendritic spine formation in the adult rat hippocampus within 30 minutes to two hours of treatment (Balthazart and Ball, 2000; Foy et al., 1999; Gu et al., 1999; Gu and Moss, 1996; Ishii et al., 2006; Ito et al., 1999; Lambert et al., 2003; MacLusky et al., 2005; Mukai et al., 2007; Remage-Healey and Bass, 2006; Shibuya et al., 2003; Teyler et al., 1980; Vouimba et al., 2000; Woolley, 2007). These rapid effects on behaviour occur too quickly to involve mechanisms requiring gene transcription and are likely mediated via non-genomic mechanisms (Balthazart, 2010; Cornil, 2009). In mammals, non-genomic effects appear to be mediated via membrane-associated receptors, which are capable of activating G-protein-coupled receptors (Boulware and Mermelstein, 2009; Kumar et al., 2007; Levin, 2009a; b; Mermelstein, 2009; Mermelstein and Micevych, 2008; Micevych and Mermelstein, 2008; Ramirez and Zheng, 1996; Ramirez et al., 1996; Razandi et al., 1999; Zheng and Ramirez, 1997). There is some evidence that steroids might have non-genomic effects in birds as corticosterone treatment increases locomotor activity and improves caching within five to 15 minutes following treatment. Furthermore, oestrogen has rapid effects on copulatory behaviour in quail but the rapid effects of sex steroids on spatial abilities and the corresponding rapid changes in hippocampal morphology have not, to our knowledge, been investigated so far (Balthazart et al., 2006; Balthazart et al., 2009b;



Breuner et al., 1998; Breuner and Wingfield, 2000; Cornil et al., 2006b; Cornil et al., 2006c; Hodgson et al., 2008; Saldanha et al., 2000a).

In this study we examined the effects of acute testosterone treatment on spatial performance and hippocampal androgen receptor (AR) and oestrogen receptor alpha (ER $\alpha$ ) mRNA expression. Although it may take days for genomic mechanisms to affect behaviour through changes in protein expression, the initial effects of increases in circulating testosterone levels on mRNA expression can be very rapid, occurring within 45 minutes (Nastiuk and Clayton, 1995). In this study we therefore looked at the effects of acute testosterone treatment on the expression of both hippocampal AR and ER $\alpha$  mRNA as any effects on spatial ability are likely to be mediated by these receptors (Ishii et al., 2006). In the avian brain testosterone is converted to oestrogen by the enzyme aromatase so we also looked for effects of acute testosterone treatment on hippocampal aromatase mRNA expression (Forlano et al., 2006; Saldanha et al., 2000b). In the auditory system of male zebra finches, the caudo-medial nidopallium (NCM), aromatase activity is increased following male song stimulation and blocking aromatase activity with the inhibitor fadrozole decreases brain oestradiol levels and increases testosterone levels (Remage-Healey et al., 2008; Saldanha et al., 2000b). As male zebra finches have higher hippocampal aromatase expression than do females (Chapter 3), this may lead to a higher rate of synthesis of locally-produced oestrogen and increasing circulating levels of testosterone (either naturally or experimentally) may lead to males with spatial abilities superior to females.

In this study we gave a single oral treatment of testosterone to male and female zebra finches and tested their spatial performances. In addition we quantified their hippocampal sex steroid receptor and aromatase mRNA expression. We made four predictions: (1) oral ingestion of testosterone would increase circulating testosterone levels in both males and females within the physiological range; (2) circulating testosterone levels would be positively correlated with spatial performance in both males and females. As sex hormones have been shown to selectively improve spatial memory, it was also predicted that improvements would be limited to the spatial task

and not the visual task (Janowsky et al., 1994; Lewin et al., 2001; Sutcliffe et al., 2007; Vandenberg and Kuse, 1978); (3) testosterone would rapidly (i.e. within four hours) increase the expression of AR and ER $\alpha$  and aromatase mRNA levels.

## Materials and Methods

### *Subjects*

Birds were sexually mature, captive-bred adult (3-4 years of age) male and female zebra finches, obtained from St Andrews University (UK). They were individually caged in wire mesh cages (77cm long x 44cm wide x 44cm high). All the birds were in full visual and auditory contact with each other. They were maintained on a 15:9 hour light:dark cycle (0600:2100) at 19-22°C, humidity 25-50%, with free access to water (vitamin supplemented) and food (bird seed mixture), supplemented by dried cuttlefish bone. The sample sizes for behavioural testing and in situ hybridisation are in Table 1. Some of the birds used were those from Chapter 3; the males (n = 3) and females (n = 4) plus 2 extra males and 3 extra females (which reached criterion) were tested in the spatial and visual task. Following behavioural testing, these birds and an additional 23 males and 20 females were included in the *in situ* hybridisation experiments.

Sample size:	Chapter 3		Chapter 4		In situ:	Chapter 3	Chapter 4
	Spatial	Task: Visual	Spatial	Spatial + Visual task		control	treatment
<b>Male</b>	6	5	3	3 + 2 extra = 5	5 + 18 extra = 23	10	13
<b>Female</b>	7	5	4	4 + 3 extra = 7	7 + 13 extra = 20	8	12

**Table 1** The sample sizes for behavioural testing and in situ hybridisation in Chapters 3 and 4

### *Plasma testosterone*

Prior to behavioural testing, blood samples of approximately 100-150µl were taken from the alar wing vein of each bird, by puncturing the vein with a 25G needle and collecting the blood into heparinised micro haematocrit tubes. Samples were all taken in the morning, to coincide with the typical time of behavioural testing. Samples were centrifuged on a micro-haematocrit centrifuge for three minutes and plasma was recovered with a fixed needle Hamilton syringe and stored in ependorffs at -20°C. Plasma testosterone levels were measured using a radioimmunoassay at Glasgow University with a 1:300 dilution of antibody, using a modification of an

established assay (Sheffield and O'Shaughnessy, 1989; Verboven et al., 2003) (as described in Chapter Two). All samples were run in a single assay. The binding was 21.6% and non-specific binding was 3.6%. The intra-assay coefficient of variation was 8.9%. The extraction efficiency was > 90%.

#### *Effect of testosterone on performance*

Birds previously trained on a memory task (female n = 7, male n = 5) were retrained until they reached the criterion of completing the sample and choice phases each within five minutes. All birds were successful in reaching criterion within one week.

Birds were tested on both the spatial and visual one-trial associative memory task in which birds, presented with a tray with one rewarded well (sample phase) had to relocate the rewarded well following a retention interval of 40 minutes (choice phase). To ensure that the vehicle did not alter performance, prior to commencing behavioural testing birds were given either just a small piece of cucumber (approx. 40mm<sup>3</sup>) or cucumber injected with vehicle (95% ethanol) and tested 30 minutes later on the spatial task. Birds were tested on two consecutive days, with half the birds receiving cucumber injected with 3µl of vehicle on day one and just cucumber on day two while the other half received just cucumber on day one, then vehicle on day two. Two days later, birds were given testosterone (3µl of 1mg/ml in 95% ethanol) or vehicle (3µl of 95% ethanol) injected into cucumber either 30 minutes or four hours prior to testing (choice phase). All birds experienced all four conditions over four consecutive days, counter-balanced for order effects. The concentration of testosterone and the time point used were determined following preliminary work described in the Appendix at the end of this chapter. Birds were bled during the experiment 30 minutes following oral testosterone ingestion once the birds had completed both phases of the task (See Figure 1).

#### *Effect of testosterone on hippocampal AR and ERα and aromatase mRNA expression*

Three weeks following completion of behavioural testing, birds from the testosterone experiment (female n = 7, male n = 5), along with untested birds (female n = 13, male n = 18; caged in single sex groups of between four and eight birds per cage)

were divided into five groups (see below), and were decapitated 30 minutes or four hours following oral testosterone ingestion. Trunk blood was collected following decapitation to determine circulating testosterone levels. ER $\alpha$ , AR and aromatase mRNA expression in the hippocampus was quantified using *in situ* hybridisation (as described in Chapter Two).

The groups were as follows:

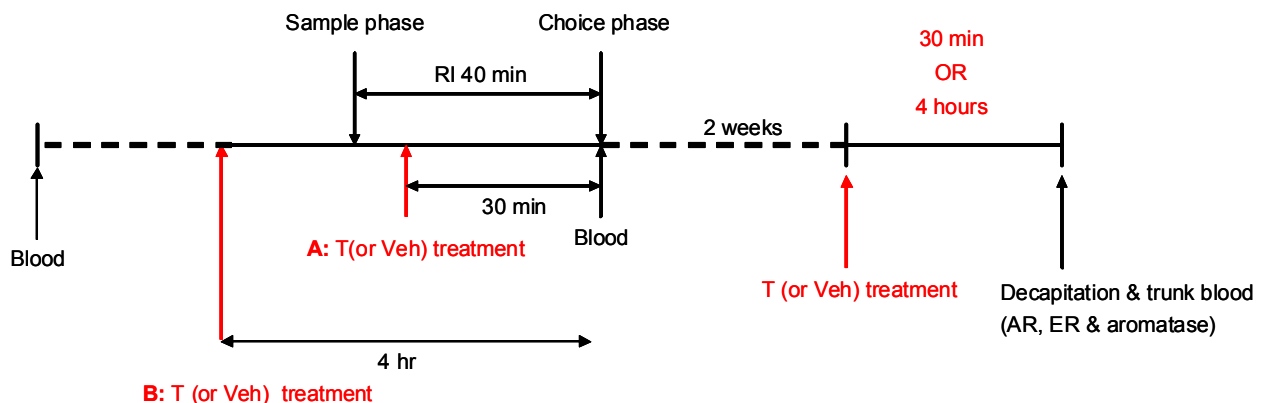
**Group 1** Controls that received cucumber only prior to decapitation (female n = 8, male n = 10);

**Group 2** Testosterone (3 $\mu$ l of 1 mg/ml) 30 minutes prior to decapitation (female n = 3, male n = 3);

**Group 3** Vehicle (3 $\mu$ l of 95% ethanol) 30 minutes prior to decapitation (female n = 3, male n = 3);

**Group 4** Testosterone (3 $\mu$ l of 1 mg/ml) four hours prior to decapitation (female n = 3, male n = 4);

**Group 5** Vehicle (3 $\mu$ l of 95% ethanol) four hours prior to decapitation (female n=3, male n=3).



**Figure 1** A time line of the experimental design, highlighting when birds were bled, treated with testosterone (T) or Vehicle (Veh; ethanol) and tested on the spatial or visual task and when brains were removed for analysis. Birds received T or Veh 30 min (A) or 4 hours (B) prior to testing (all birds received all 4 conditions). After 2 weeks, birds were decapitated either 30 min or 4 hours after T or Veh treatment.

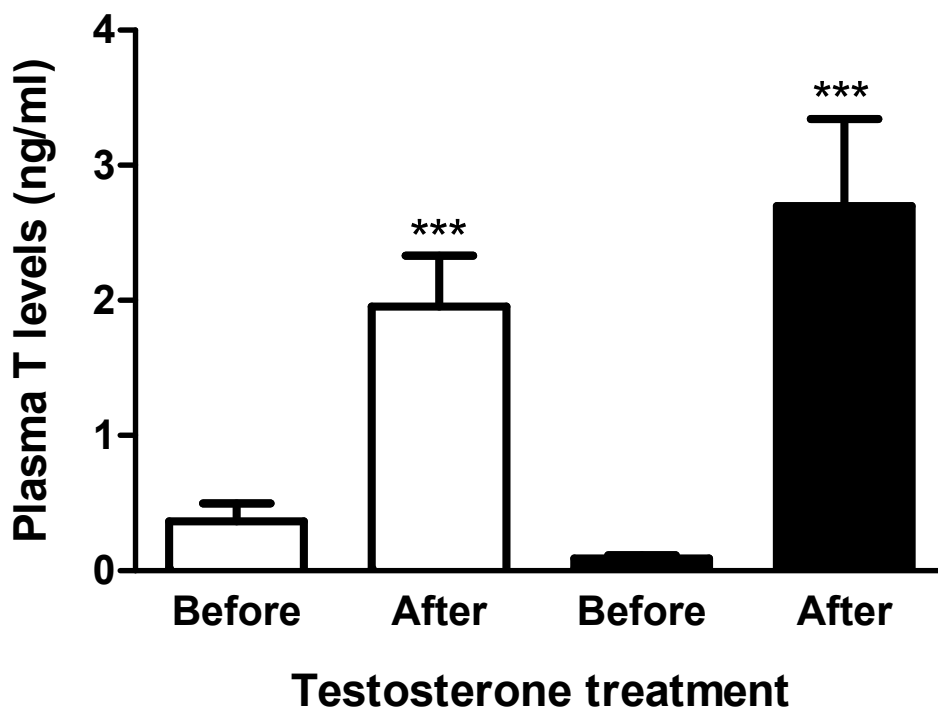
*Analysis*

The data were analysed using Sigma Stat 11.0 and Graph Pad Prism 5. Repeated measures two-way ANOVAs were used to investigate the effects of testosterone treatment on plasma levels after 30 minutes and on cognitive performance (female n = 7, male n = 5). We used two-way ANOVAs to look at the effects of testosterone treatment on hippocampal mRNA expression (n = 43; female n = 20, male n = 23). The post hoc test used was the Holm-Sidak method. Linear regression was used to investigate the relationship between spatial performance and testosterone levels 30 minutes after treatment (female n = 7, male n = 5) and the relationship between plasma testosterone levels and mRNA expression (female n = 20, male n = 23). The data were normally distributed.

## Results

### *The effects of testosterone treatment on plasma levels and performance*

Ingestion of testosterone significantly increased plasma testosterone levels compared to baseline after 30 minutes (Repeated measures two-way ANOVA;  $F_{1,11} = 26.1$ ,  $p < 0.001$ ; Figure 2). The sexes did not significantly differ in plasma testosterone levels following treatment ( $F_{1,11} = 0.38$ ,  $p = 0.55$ ) and the effect of treatment did not depend on sex ( $F_{1,11} = 1.54$ ,  $p = 0.24$ ). Although not significant, there was a trend for males to have higher testosterone levels than females prior to testosterone treatment (unpaired t-test;  $t_{11} = 2.18$ ,  $p = 0.052$ ,  $n = 13$ ).



**Figure 2** Testosterone (T) plasma levels before testosterone treatment (baseline) and 30 minutes after treatment in males (white;  $n = 6$ ) and females (black;  $n = 7$ ). The data are means + SEM. Testosterone treatment significantly increased plasma T compared to baseline levels in both sexes. \*\*\*  $P < 0.001$ .

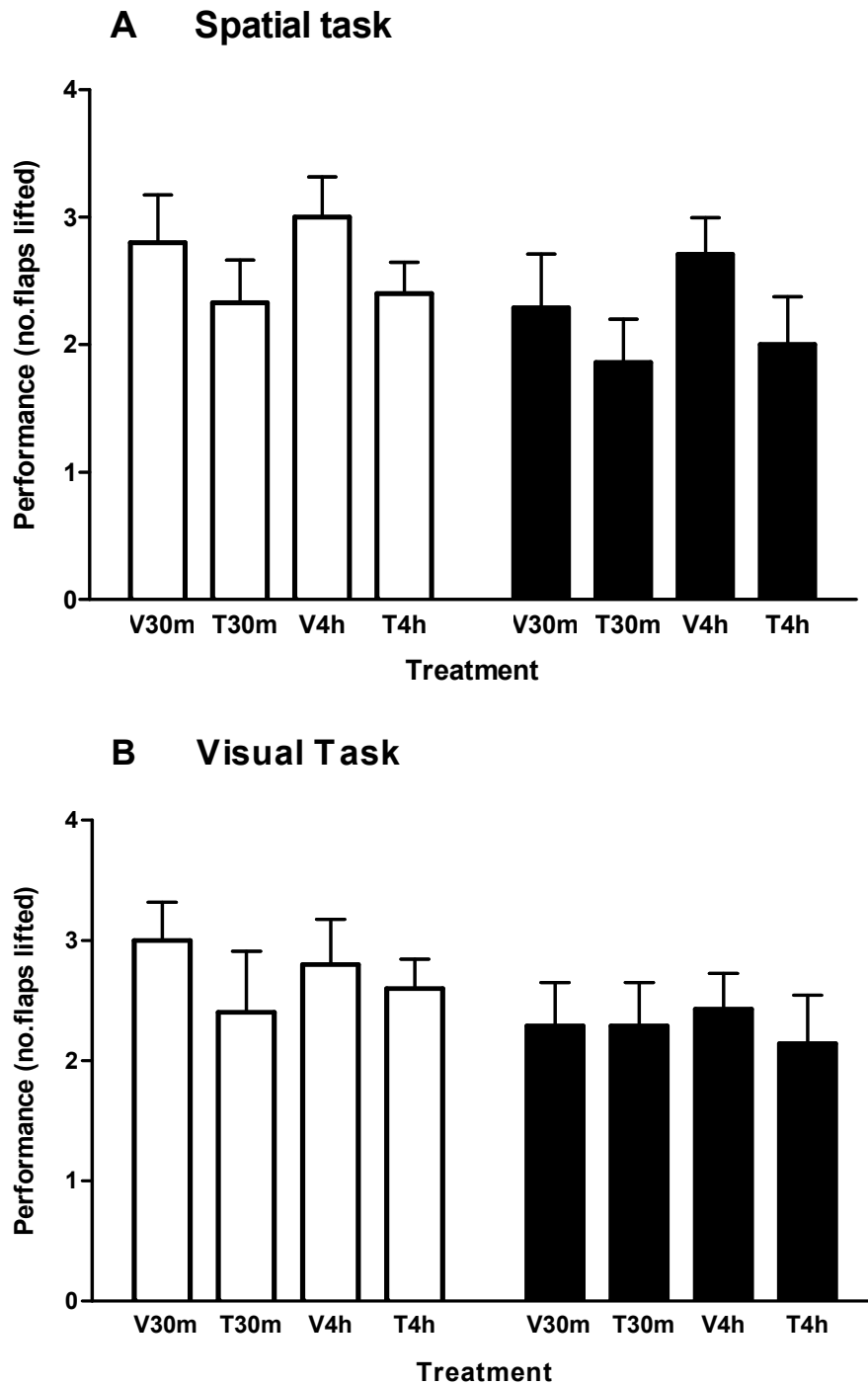
In the spatial task, testosterone tended to increase spatial performance (Repeated measures two-way ANOVA;  $F_{3,30} = 2.88$ ,  $p = 0.052$ ; Figure 3). The effect of treatment was the same in both sexes ( $F_{1,30} = 1.24$ ,  $p = 0.29$ ) and there was no

interaction between sex and treatment ( $F_{3,30} = 0.05$ ,  $p = 0.99$ ). Testosterone treatment did not change performance in the visual task (Repeated measures two-way ANOVA;  $F_{3,30} = 0.44$ ,  $p = 0.72$ ) in either sex ( $F_{1,30} = 1.55$ ,  $p = 0.24$ ). The sex-treatment interaction was not significant ( $F_{3,30} = 0.27$ ,  $p = 0.84$ ).

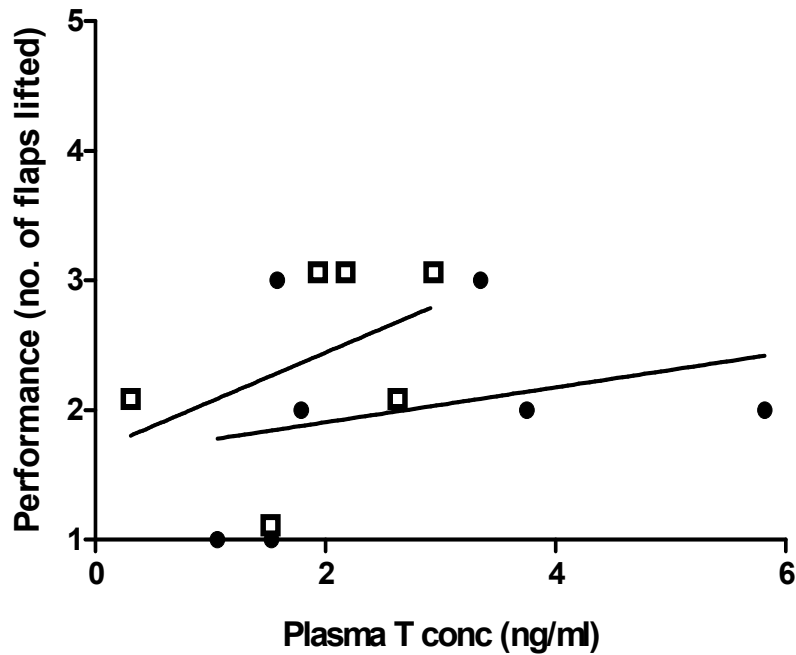
Treatment with vehicle did not effect spatial performance compared to the control group receiving only cucumber (RM 2-way ANOVA;  $F_{1,10} = 0.69$ ,  $p = 0.42$ ) in either sex ( $F_{1,10} = 0.36$ ,  $p = 0.56$ ). The effect of performance did not depend on sex ( $F_{1,10} = 0.69$ ,  $p = 0.42$ ).

Spatial performance was not correlated with plasma testosterone levels 30 minutes after testing in males or females (linear regression  $r^2 = 0.197$ ,  $p = 0.38$ ,  $n = 6$  and  $r^2 = 0.079$ ,  $p = 0.54$ ,  $n = 7$ , respectively; Figure 4).





**Figure 3** The effect of testosterone treatment on spatial and visual performance, measured as the number of flaps lifted to solve the task. A) Performance on the spatial task following treatment with testosterone (T; injected into cucumber) or vehicle (V; ethanol) 30 minutes (30m) or four hours (4h) prior to testing. B) Performance on the visual task following testosterone or vehicle treatment (male, white n = 5; female, black n = 7). Spatial performance improved after T treatment compared to vehicle treatment. The data are means + SEM.



**Figure 4** The relationship between circulating testosterone (T) levels and spatial performance, measured by the number of flaps lifted to solve the task, 30 minutes after treatment in males (squares;  $n = 6$ ;  $r^2 = 0.18$ ) and females (circles;  $n = 7$ ;  $r^2 = 0.08$ ). Linear regression lines are shown.

#### *Plasma testosterone and hippocampal mRNA expression*

Testosterone treatment significantly increased plasma testosterone levels on decapitation compared to untreated birds (Two-way ANOVA;  $F_{4,33} = 7.02$ ,  $p < 0.001$ ; Figure 5A): plasma testosterone levels were increased 30 minutes after treatment (Holm sidak post hoc test; testosterone treatment vs control/ vehicle:  $p < 0.001$ ) but not after four hours (testosterone treatment vs control birds:  $p = 0.23$ ; testosterone treatment vs vehicle:  $p = 0.83$ ). Vehicle treatment did not lead to changes in plasma testosterone compared to the un-manipulated control group (30 minutes:  $p = 0.81$ ; four hours:  $p = 0.37$ ). The effect of treatment was the same in both sexes ( $F_{1,33} = 3.57$ ,  $p = 0.07$ ). There was no interaction between sex and treatment ( $F_{4,33} = 0.18$ ,  $p = 0.95$ ).

#### Androgen receptor mRNA:

Oral ingestion of testosterone significantly increased hippocampal AR mRNA expression (Two-way ANOVA:  $F_{4,33} = 16.0$ ,  $p < 0.001$ ; Holm sidak post hoc test:

testosterone treatment 30 minutes vs control/ vehicle 30 minutes  $p < 0.001$  and testosterone treatment four hours vs control or vehicle four hours  $p = 0.59$  and  $0.98$ , respectively; Figure 5B and 6). Vehicle treatment did not lead to changes in AR compared to the control group (30 minutes:  $p = 0.65$ ; four hours:  $p = 0.59$ ). The effects of testosterone treatment were the same in both sexes ( $F_{1,33} = 1.08$ ,  $p = 0.31$ ). There was no interaction between sex and treatment ( $F_{4,33} = 1.89$ ,  $p = 0.14$ ).

Hippocampal AR mRNA expression was higher in males and females with higher plasma testosterone (linear regression;  $r^2 = 0.39$ ,  $p = 0.002$ ,  $n = 23$  and  $r^2 = 0.33$ ,  $p = 0.008$ ,  $n = 20$ , respectively; Figure 9A).

Oestrogen receptor mRNA:

Oral treatment with testosterone also significantly increased hippocampal ER $\alpha$  mRNA expression (Two-way ANOVA:  $F_{4,32} = 202.4$ ,  $p < 0.001$ ; Holm sidak post hoc test: testosterone treatment 30 minutes vs control/ vehicle 30 minutes  $p < 0.001$  and testosterone treatment four hours vs control or vehicle four hours  $p = 0.35$  and  $0.97$ , respectively; Figure 5C and 7). Vehicle treatment did not lead to changes in ER $\alpha$  compared to the control group (30 minutes:  $p = 0.11$ ; four hours:  $p = 0.35$ ). The effects of testosterone treatment were the same in both sexes ( $F_{1,32} = 1.35$ ,  $p = 0.25$ ). There was no interaction between sex and treatment ( $F_{4,32} = 2.20$ ,  $p = 0.09$ ). The sample size for the male ER $\alpha$  analysis was one less than for the AR and aromatase analyses as one of the slides was damaged during the *in situ* hybridisation and the data from that slide could not be analysed.

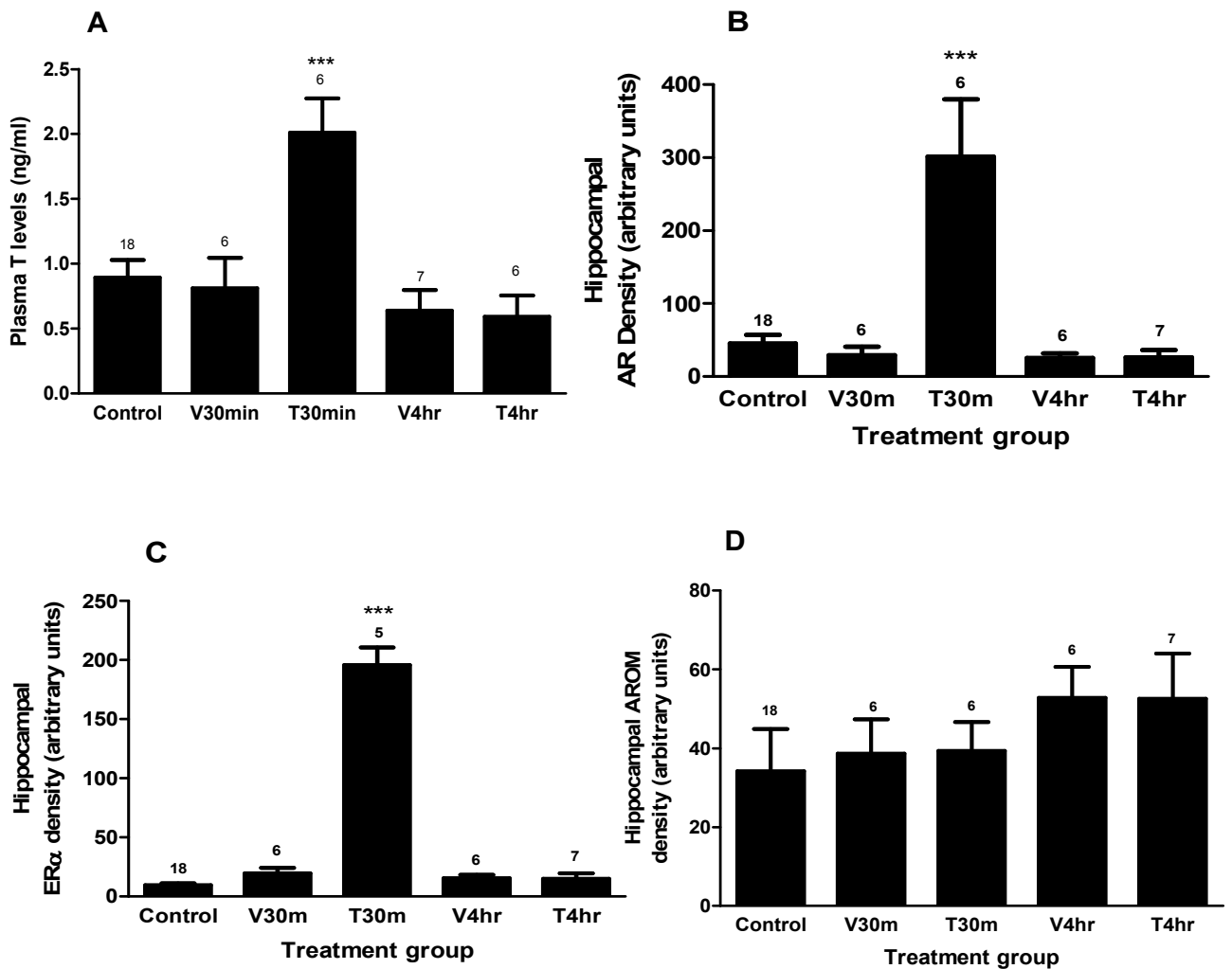
ER $\alpha$  mRNA was higher in males and females with higher circulating testosterone ( $r^2 = 0.47$ ,  $p < 0.001$ ,  $n = 22$  and  $r^2 = 0.48$ ,  $p < 0.001$ ,  $n = 20$ , respectively; Figure 9B).

Aromatase mRNA:

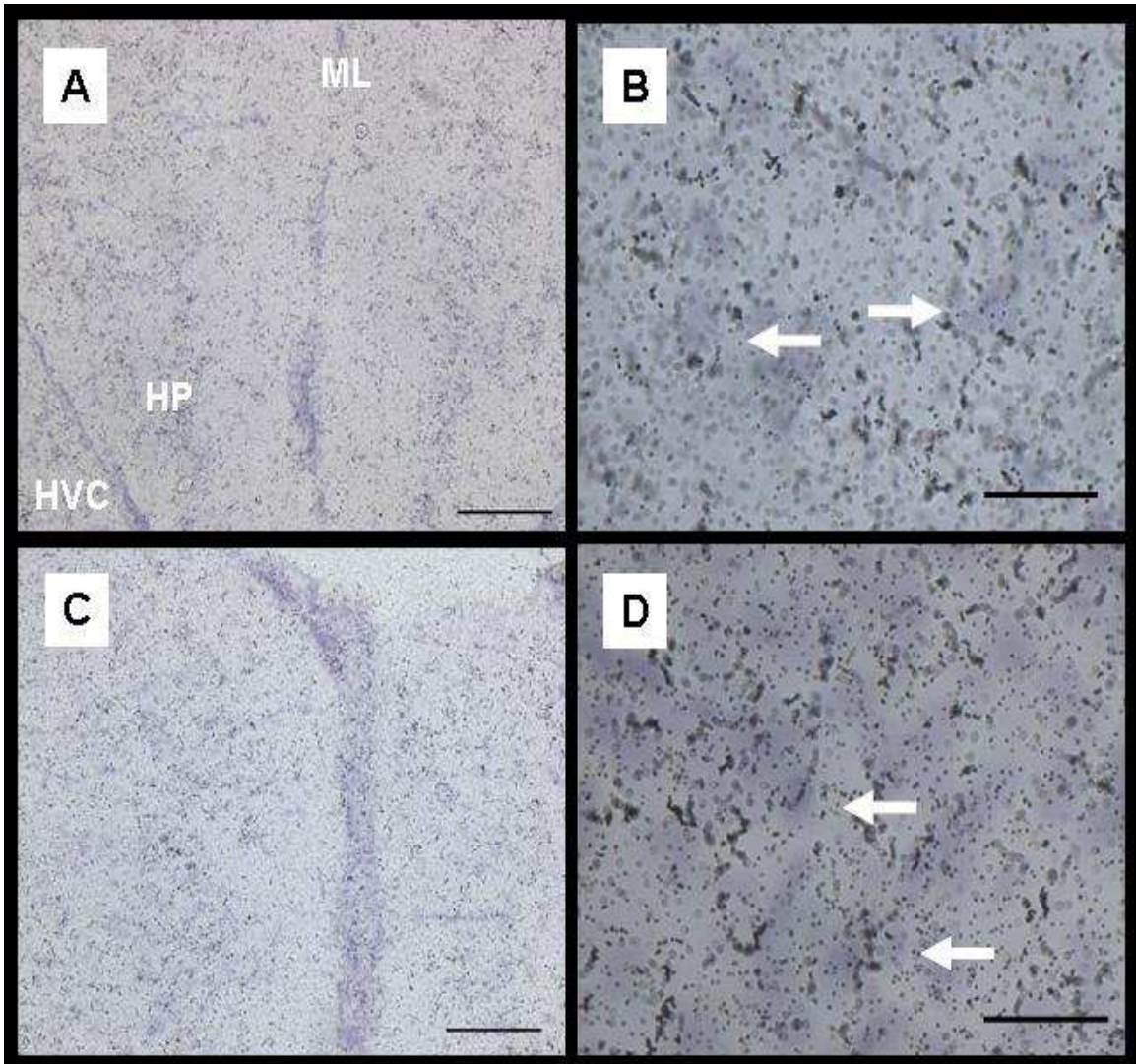
Oral testosterone ingestion 30 minutes or four hours prior to decapitation did not affect hippocampal aromatase mRNA levels (Two-way ANOVA;  $F_{4,33} = 0.50$ ,  $p = 0.74$ ; Figure 4D and 7). The effect of testosterone was the same for both sexes ( $F_{1,33}$

= 0.21,  $p = 0.65$ ). There was no interaction between sex and treatment ( $F_{4,33} = 0.02$ ,  $p = 0.99$ ).

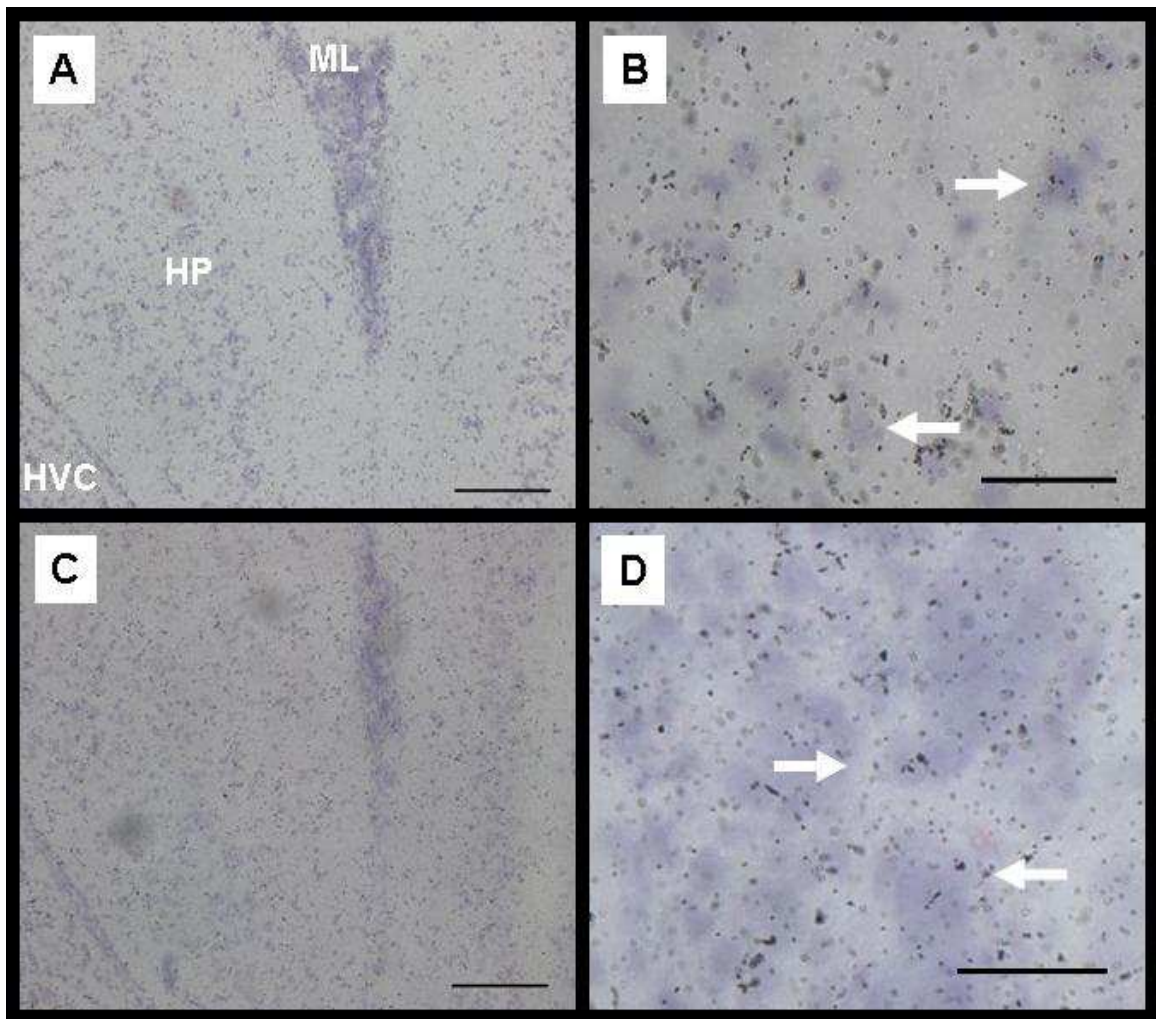
Aromatase mRNA expression was not correlated with circulating testosterone levels in either sex (males:  $r^2 = 0.016$ ,  $p = 0.56$ ,  $n = 23$  and females:  $r^2 < 0.001$ ,  $p = 0.91$ ,  $n = 20$ ; Figure 9C).



**Figure 5** The effect of testosterone (T) treatment on A) plasma T levels on decapitation and on hippocampal mRNA expression B) for androgen receptor (AR) C) oestrogen alpha receptor (ER $\alpha$ ) and D) aromatase in control birds (only cucumber), birds which received testosterone or vehicle orally 30 minutes (T 30m; V 30 m) or 4 hours (T 4hr; V 4hr) prior to decapitation. Data from males (n = 23) and females (n = 20) for each different treatment group were pooled as there was no difference in treatment between the sexes. Sample size is denoted above the bars. The data are means + SEM. T significantly increased T plasma levels, AR and ER $\alpha$  density 30 minutes after treatment compared to the other groups. \*\*\* P < 0.001.

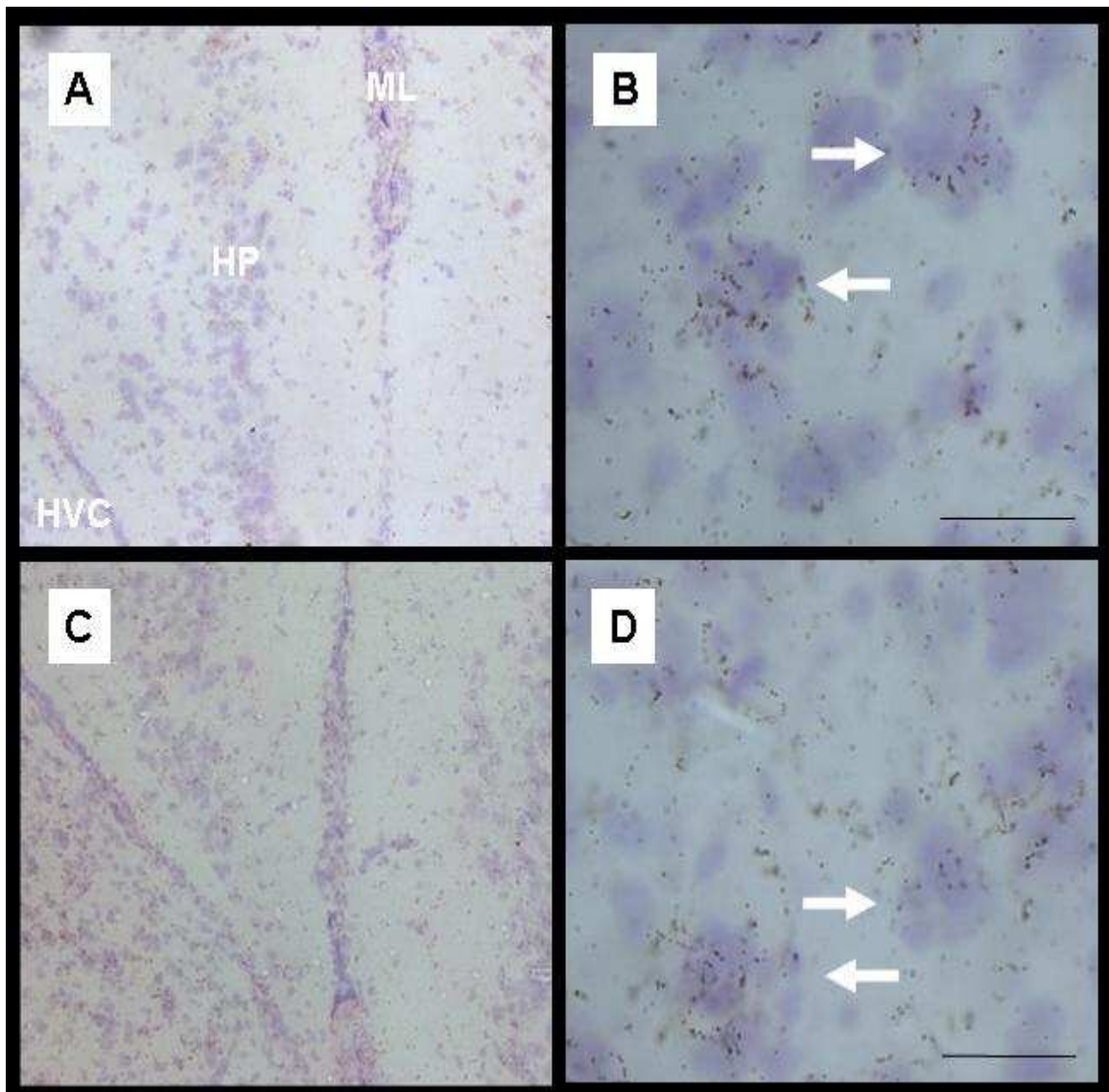


**Figure 6** The effect of testosterone treatment on hippocampal androgen receptor mRNA expression. Representative *in situ* hybridisation images 30 minutes following vehicle treatment A) x20 (scale bar = 100  $\mu$ m) and B) x100 magnification (scale bar = 25  $\mu$ m) and 30 minutes following testosterone treatment C) x20 and D) x100 magnification. Testosterone treatment significantly increased mRNA expression compared to controls. As there were no differences in the effect of treatment between the sexes, only images from males are shown. Arrows highlight examples of labelled cells. ML = midline, HP = hippocampus.



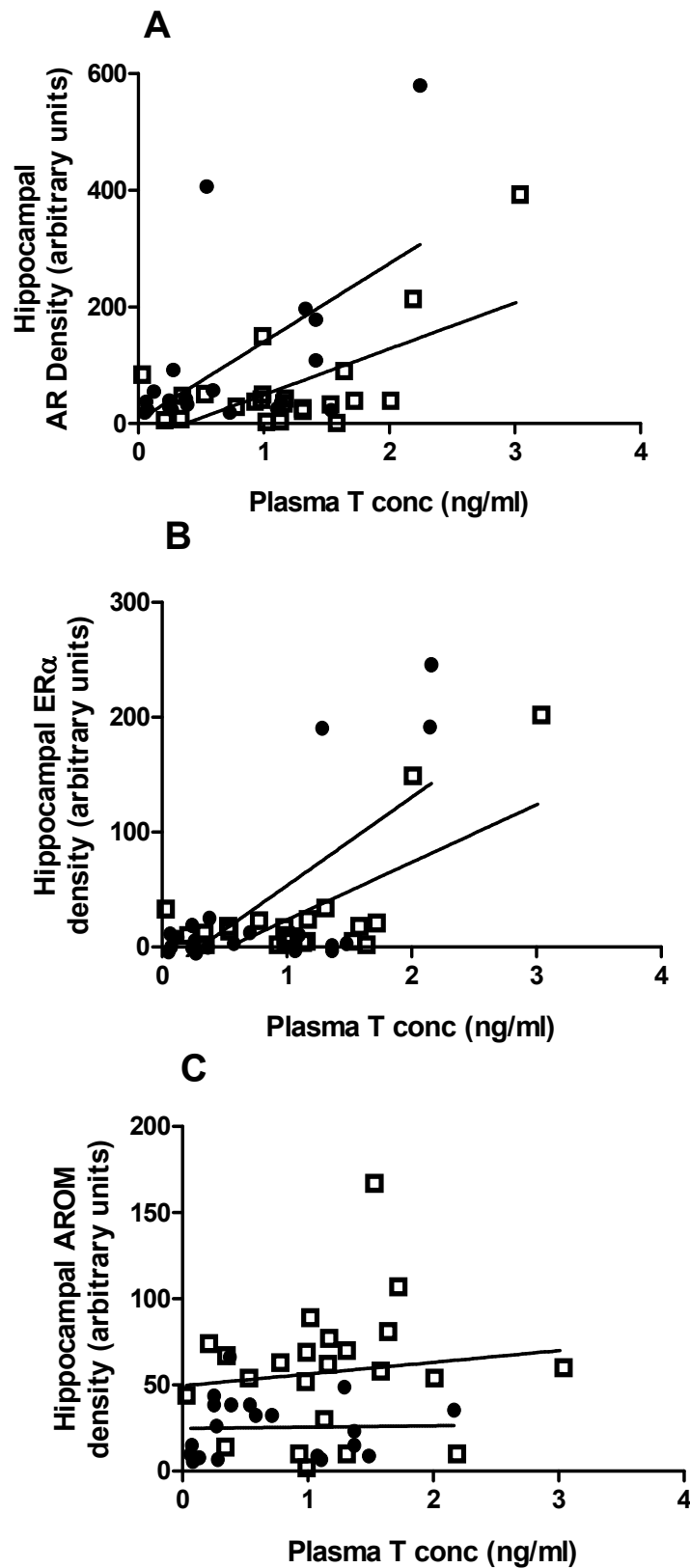
**Figure 7** The effect of testosterone treatment on hippocampal oestrogen receptor alpha mRNA expression. Representative *in situ* hybridisation images 30 minutes following vehicle treatment A) x20 (scale bar = 100  $\mu\text{m}$ ) and B) x100 magnification (scale bar = 25  $\mu\text{m}$ ) and 30 minutes following testosterone treatment C) x20 and D) x100 magnification. Testosterone treatment significantly increased mRNA expression compared to controls. As there were no differences in the effect of treatment between the sexes, only images from males are shown. Arrows highlight examples of labelled cells. ML = midline, HP = hippocampus.





**Figure 8** The effect of testosterone treatment on hippocampal aromatase mRNA expression. Representative *in situ* hybridisation images 30 minutes following vehicle treatment A) x20 (scale bar = 100  $\mu$ m) and B) x100 magnification (scale bar = 25  $\mu$ m) and 30 minutes following testosterone treatment C) x20 and D) x100 magnification. Testosterone treatment did not significantly alter mRNA expression compared to controls. As there were no differences in the effect of treatment between the sexes, only images from males are shown. Arrows highlight examples of labelled cells. ML = midline, HP = hippocampus.





**Figure 9** The relationship between plasma testosterone (T) levels and hippocampal mRNA expression 30 minutes after T treatment for A) androgen receptors (AR; males  $r^2 = 0.39$ , females  $r^2 = 0.33$ ; both sexes  $p < 0.01$ ) B) oestrogen alpha receptors (ER $\alpha$ ; males  $r^2 = 0.47$ , females  $r^2 = 0.48$ ; both sexes  $p < 0.001$ ) and C) aromatase (males  $r^2 = 0.02$ , females  $r^2 < 0.001$ ; both sexes  $p > 0.05$ ). Males (squares;  $n = 23$ ) and females (circles;  $n = 20$ ). Linear regression lines are shown.

## **Discussion**

Oral ingestion of testosterone elevated circulating testosterone levels after 30 minutes, which was enough to improve spatial but not visual performance in both male and female zebra finches. Hippocampal androgen receptor (AR) and oestrogen receptor alpha (ER $\alpha$ ) mRNA expression increased significantly 30 minutes after testosterone treatment. Aromatase mRNA levels were not significantly altered by testosterone treatment at either time point.

As predicted acute testosterone treatment improved performance, specifically on the spatial task, in both males and females. Sex steroids can, then, have rapid effects on spatial cognition in birds. Although we only looked at circulating testosterone levels, it seems likely that the oral testosterone treatment led to rapid increases in brain sex steroid levels as an injection of testosterone to male zebra finches leads to significant increases in neural testosterone and oestrogen levels in the forebrain auditory area after 30 minutes (Ramage-Healey et al., 2008). Four hours following testosterone treatment we still saw improvements in spatial behaviour even though hippocampal AR and ER $\alpha$  mRNA levels had returned to levels observed in unmanipulated birds. Improvements in spatial performance are also seen up to four hours following a single injection of testosterone in mammals (young women) (Aleman et al., 2004; Postma et al., 2000). It may be that AR and ER $\alpha$  mRNA expression were linked to circulating testosterone levels which had returned to baseline by 4 hours, but the transient increase was sufficient to triggered downstream pathways leading to changes in protein expression. It is likely that testosterone is acting through genomic and non-genomic mechanisms and while the rapid behaviour was likely mediated by none genomic, underlying genomic cascades were probably laying the foundations for more long term genomic changes. The mechanisms underlying the improvement in performance at 30 minutes and 4 hours are likely to be the same and involve non-genomic actions. The rapid effects may be occurring through a direct effect on AR or indirectly through conversion to oestrogen but are likely to involve both. The direct versus indirect effects could be elucidated by the use of a non-aromatizable 5-alpha dihydrotestosterone or an aromatase inhibitor such as fadrozole. The rapid actions on spatial behaviour may involve binding of sex steroids to transmembrane

ion channels such as GABA, NMDA and serotonin and the modulation of G-protein-coupled receptors (GPCRs) by rapidly increasing protein kinase A and C (PKA and PKC) activity and may be mediated via membrane steroid receptors as occurs in mammals (Balthazart and Ball, 2006; Cornil et al., 2006a; Kelly and Wagner, 1999; Levin, 2009a; Mermelstein, 2009; Mermelstein and Micevych, 2008; Micevych and Mermelstein, 2008; Moss et al., 1997; Qiu et al., 2008; Rupprecht et al., 2001; Valverde et al., 1999; Wetzel et al., 1998; Woolley, 1999; 2007). It seems plausible that sex steroids may be initiating rapid effects via membrane receptors in birds as the avian brain contains membrane corticosteroid receptors which likely mediate the rapid increases in locomotor activity in house and Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) and improvements in spatial performance in mountain chickadees (*Parus gambeli*) seen following corticosterone treatment (Breuner et al., 1998; Breuner and Orchinik, 2001; 2009; Breuner and Wingfield, 2000; Saldanha et al., 2000a).

Testosterone is converted to oestrogen by aromatase and although the transcription of aromatase mRNA was not altered by testosterone treatment, increases in its activity may have occurred within a rapid time frame allowing for rapid increases in oestrogen production, regulated by post-transcriptional non-genomic mechanisms such as protein phosphorylation (Balthazart et al., 2005; Balthazart et al., 2009; Balthazart and Foidart, 1993; Hutchison and Steimer, 1986; Remage-Healey et al., 2009). Thus the role of local oestrogen synthesis in modulating the rapid improvement in spatial performance is not clear and could be addressed using the aromatase inhibitor fadrozole; if the conversion of testosterone to oestrogen is involved then fadrozole treatment would block the acute improvement in spatial performance. The involvement of oestrogen synthesis in the improvement in performance might explain the lack of a significant positive relationship between circulating testosterone levels and spatial performance. This is supported by dependence on the rapid aromatisation of testosterone to oestrogen in the activation male sexual behaviour in quail which is blocked within 30 minutes by a single injection of an aromatase inhibitor (Cornil et al., 2006b).

In addition to rapid effects on behaviour, testosterone treatment increased the expression of both AR and ER $\alpha$  mRNA within 30 minutes in the hippocampus. Similar changes occur in the avian song system within 45 minutes in response to increases in circulating testosterone levels (Nastiuk and Clayton, 1995). The hippocampal AR and ER $\alpha$  mRNA levels returned to levels found in unmanipulated birds by four hours following testosterone treatment, which may represent the activation of downstream pathways leading to the synthesis of new proteins. In addition to effects mediated by the conversion of testosterone to oestrogen, testosterone itself may also be acting to increase hippocampal mRNA expression as testosterone treatment in quail increases ER levels even in the presence of an aromatase inhibitor (Schlinger and Callard, 1989). We saw no effect of testosterone on aromatase mRNA expression at 30 minutes or at four hours following treatment. Unlike its direct effects on AR and ER $\alpha$ , it appears that testosterone needs to be aromatised to oestrogen in order to increase aromatase mRNA expression in birds (Harada et al., 1993; Hutchison and Steimer, 1986). While the direct effects of testosterone on AR and ER $\alpha$  may occur within 30 minutes (when circulating testosterone levels peak), the aromatisation of testosterone to oestrogen may delay hormone-induced increases in aromatase mRNA expression such that at 30 minutes levels are not significantly increased. The regulation of aromatase appears to be different in birds compared to mammals as in rats the effects of testosterone on aromatase transcription are mediated through androgen receptors; the anti-androgen flutamide, which competes with testosterone for binding at AR, decreases aromatase activity (Roselli et al., 1987; Roselli and Resko, 1984).

In conclusion, acute manipulation of testosterone rapidly improved spatial performance in zebra finches, possibly through non-genomic mechanisms. There were also rapid effects on hippocampal gene transcription for both AR and ER $\alpha$ . Whether these testosterone-induced changes in mRNA expression lead to changes in protein levels is not clear.

## **Appendix**

### **Pilot experiment: Establishing testosterone parameters**

Preliminary work was needed to validate that ingestion of testosterone in cucumber was an efficient and reliable method of testosterone delivery, and to establish a concentration that would give rise to plasma testosterone levels within physiological range and to note the time course of testosterone elevations in the plasma.

Testosterone was administered orally and although it was not possible to confirm corresponding changes in the brain in this experiment, we know that when hormones are delivered systemically a corresponding rapid change in steroid levels in the brain is seen: an intramuscular injection of testosterone in male zebra finches causes a significant increase in local testosterone and oestrogen levels in the forebrain auditory area (caudo-medial nidopallium, NCM), peaking 30-60 minutes after injection (Ramage-Healey et al., 2008). Investigating the relationship between acute alterations in sex hormone levels and behaviour is physiologically relevant as increases in local hormone levels occur in the auditory forebrain of male zebra finches in the context of natural behaviour (social interactions), which occur on the same rapid time scale as systemic injections of sex hormones (30 minutes) (Ramage-Healey et al., 2008).

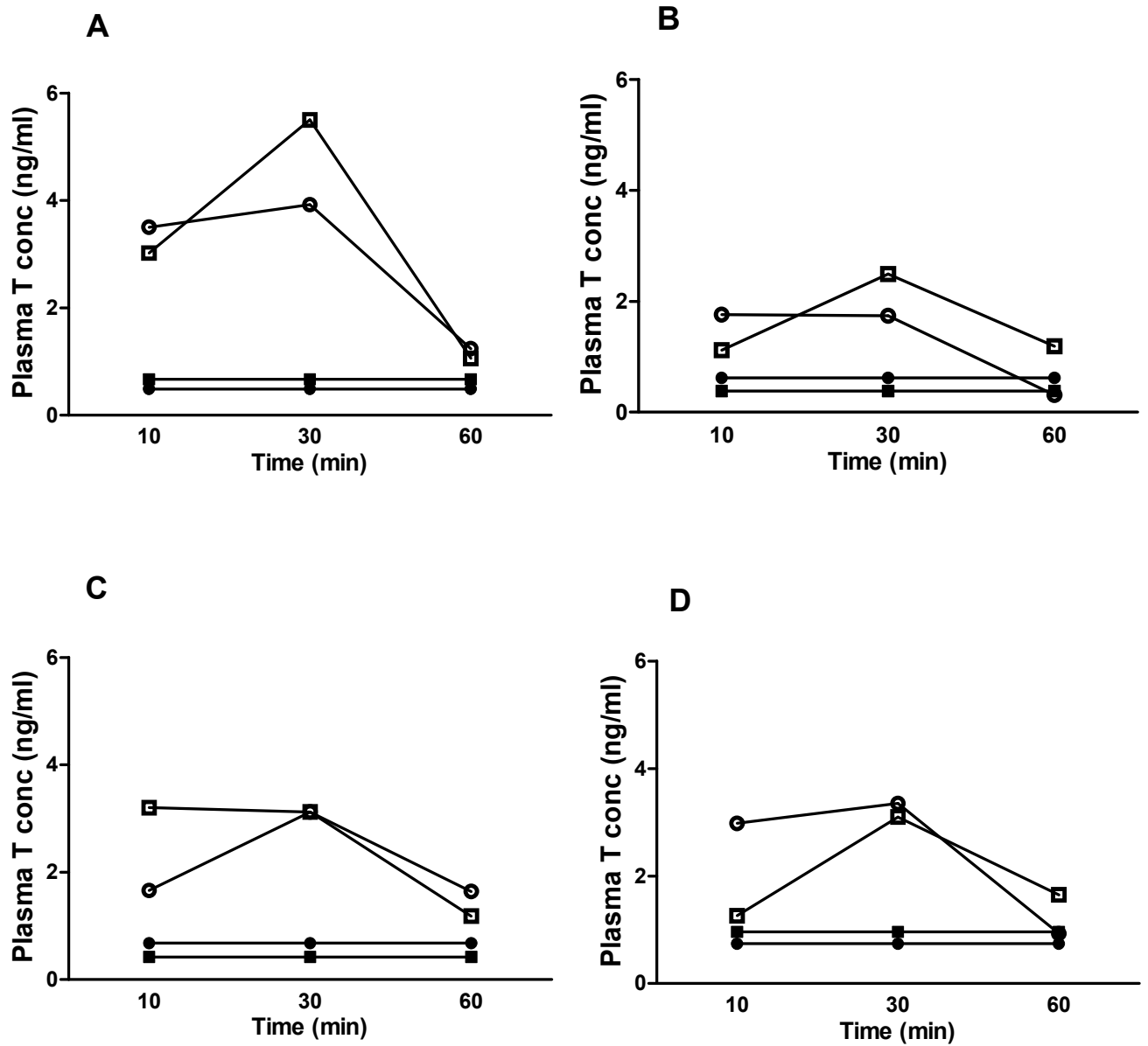
#### *Materials & Methods*

100µl blood samples were taken at 10am from the alar wing vein of male (n=12) and female (n=12) adult zebra finches using a 25G needle and collected into heparinised capillary tubes. Two weeks later, birds were given 3µl of 0.75, 1.25, 2.5 or 5 mg/ml testosterone in 95% ethanol injected in cucumber. I tested a number of foods and cucumber proved to be the food that was the most reliably eaten by all of the birds. Ethanol was used as a vehicle as testosterone does not readily dissolve in saline and oil is not easily injected into cucumber. Birds were then bled after 10, 30 or 60 minutes (one male and one female per time point and concentration combination). Samples were centrifuged, and plasma samples were recovered with a Hamilton syringe and stored at -20°C until they were processed. Plasma testosterone

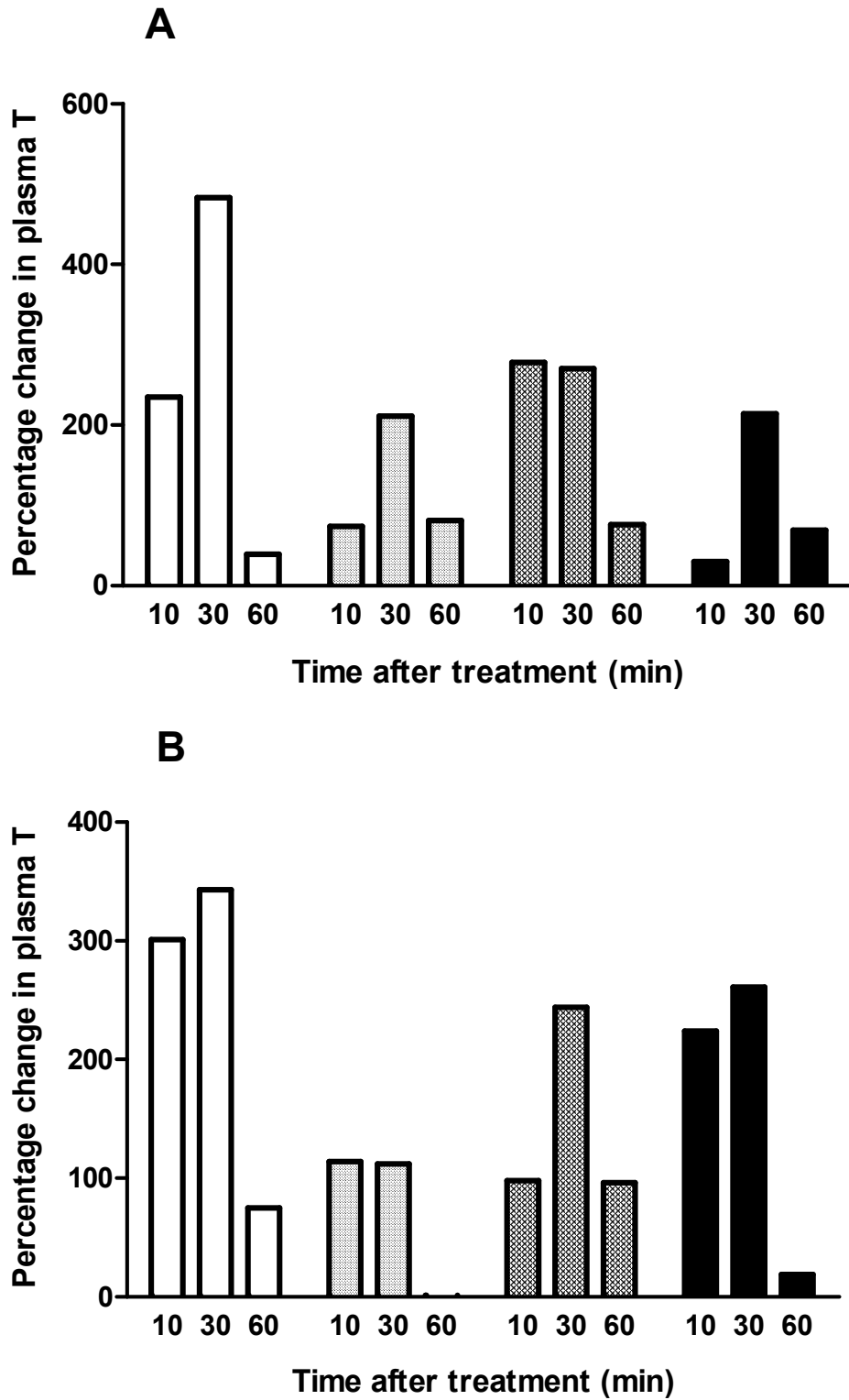
concentrations were measured by double antibody radioimmunoassay at the University of Glasgow using a modification of an established assay (Sheffield and O'Shaughnessy, 1989; Verboven et al., 2003). All samples were run in a single assay. The binding was 19.2% and non specific binding was 9.3%. The intra-assay coefficient of variation was 8.1%. The extraction efficiency was >90%.

### *Results*

As the sample size was only two for each time point/concentration combination no statistical analysis could be done. For all concentrations, testosterone ingestion appeared to increase plasma testosterone levels (visual inspection of the data: Figure 9). The smallest increases relative to baseline appeared to be in response to testosterone doses of 2.5 and 1.25 mg/ml (Figure 10). At concentrations of 5, 2.5 and 0.75 mg/ml, there appeared to be larger increases relative to baseline in females compared to males.



**Figure 9** Changes in blood plasma testosterone (T) levels at various time points following ingestion of cucumber injected with (A) 5 mg/ml (B) 2.5 mg/ml, (C) 1.25 mg/ml or (D) 0.75 mg/ml testosterone in ethanol. Males = squares; Females = circles; baseline = filled shapes and plasma T levels after 30 minutes = empty shapes. For each time point at each concentration, n=2 (1 male and 1 female)



**Figure 10** The percentage increase in plasma testosterone (T) levels following ingestion of 5 mg/ml (white), 2.5 mg/ml (spotted), 1.25 mg/ml (hatched) and 0.75 mg/ml (black) testosterone in (A) males and (B) females.  $n=2$  for each time point/concentration combination.



*Conclusion*

Ingestion of testosterone in cucumber appears to be a reliable and efficient but non-invasive method for testosterone delivery. The average peak levels at 1.25 mg/ml and 0.75 mg/ml were 3.11 ng/ml in males and 2.39 ng/ml in females, approximately two to three times higher than the physiological male maximum level of 1.06 ng/ml previously shown in zebra finches (Vleck and Priedkalns, 1985). Baseline levels of 0.67 ng/ml in males were in agreement with previous findings in male zebra finches out of breeding, which had levels averaging 0.3 - 0.5 ng/ml (Vleck and Priedkalns, 1985). In light of these results, 3 $\mu$ l of 1mg/ml of testosterone was injected into cucumber and fed to birds at 30 minutes prior to testing, when plasma testosterone levels peaked.

## Chapter 5

### The effect of five days of sex steroid treatment on spatial performance, hippocampal receptor expression & binding

#### **Abstract**

It is well established in some mammals that chronic, long-term elevations in sex steroid levels are associated with improvements in spatial cognition, for example in the breeding season or following sex steroid implants. It is less clear the extent to which short to medium term elevations in sex steroids affect spatial cognition and hippocampal morphology, particularly in birds. We manipulated circulating sex steroid levels in adult zebra finches for five consecutive days by providing food to which testosterone or oestrogen had been added. Spatial memory was assessed with a spatial one-trial associative memory task. Ingestion of oestrogen or testosterone significantly increased plasma oestrogen and testosterone levels respectively after 30 minutes in both males and females compared to control and vehicle groups, and led to improved spatial performance in both sexes. The testosterone-induced improvement was blocked with the aromatase inhibitor fadrozole, meaning that oestrogen is essential to improve spatial performance. Five days of oestrogen treatment significantly increased oestrogen receptor alpha (ER $\alpha$ ) mRNA expression in the hippocampus in both males and females and increased plasma oestrogen was associated with increased hippocampal ER $\alpha$  mRNA expression. Hippocampal ER $\alpha$  mRNA expression was positively correlated with increased spatial performance. Additionally, five days of oestrogen treatment significantly increased the number of cells expressing *N*-methyl *D*-aspartate (NMDA) receptor protein in the hippocampus in both sexes. NMDA receptor protein levels were positively correlated with both plasma oestrogen levels and with spatial performance on day five of testing. It appears that testosterone, probably through conversion to oestrogen, improves spatial cognition in birds and this effect may be mediated through an NMDA receptor mechanism.

## **Introduction**

In mammals, long-term elevation of plasma sex steroid levels through implants or repeated injections is associated with improved performance on spatial memory tasks including the t-maze, the water maze and the radial-arm maze in rodents and in computer-based assessments in humans (Cherrier et al., 2001; Daniel et al., 1997; Fader et al., 1998; Fader et al., 1999; Gibbs, 2000; 2005; Gray et al., 2005; Janowsky et al., 1994; Luine et al., 1998; Sandstrom et al., 2006; Sandstrom and Williams, 2004). Considering how much is known about the effects of chronic manipulations of sex steroids on the song system, it is perhaps surprising how little we know about sex steroid effects on spatial cognition in birds (Bernard and Ball, 1997; Bernard et al., 1997; Brenowitz, 2004; Dloniak and Deviche, 2001; Gullledge and Deviche, 1997; Nottebohm, 1980; Smith et al., 1997b). One study found that castrated zebra finches with testosterone or oestrogen implants learnt a spatial task when tested over 20 days, but birds implanted with dihydrotestosterone did not (Oberlander et al., 2004). The evidence in the literature is much less clear on how acute and medium-term variation in sex hormones affects spatial cognition, for example there are mixed results over the effects of fluctuations in oestrogen levels across the oestrous cycle on spatial abilities in rodents (Berry et al., 1997; Healy et al., 1999; Sutcliffe et al., 2007; Warren and Juraska, 1997). Nonetheless, a single administration of testosterone to young women or single oestrogen injection to young female mice or ovariectomised rats improves spatial performance, which can occur as quickly as 30 minutes following administration and can improve both acquisition and retention depending on the timing of administration (Aleman et al., 2004; Frye et al., 2007; Gresack and Frick, 2006; Luine et al., 2003; Packard, 1998; Postma et al., 2000; Sandstrom and Williams, 2001). It appears that oestradiol treatment has a time-limited effect to improve retention of a recently formed hippocampus-dependent memory, for example, an intrahippocampal injection of oestradiol given immediately following training in the Morris Water Maze improves memory for the platform location in rats tested 24 hours later, but not when given two hours after training (Packard et al., 1996; Packard and Teather, 1997). In birds there is some evidence that steroids can have rapid effects on behaviour as a single administration of

corticosterone increased locomotor activity in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) and improved cache recovery in mountain chickadees (*Parus gambeli*) (Breuner et al., 1998; Breuner and Wingfield, 2000; Saldanha et al., 2000a). Over the medium term sex steroid treatment (testosterone or oestrogen once a day for five days) improved spatial performance in Great tits (*Parus major*) on a delayed-non-matching-to sample touch screen task (Hodgson et al., 2008).

These rapid improvements in spatial abilities induced by sex steroids are accompanied by changes in hippocampal synaptic physiology and morphological changes, at least in mammals (Balthazart and Ball, 2000; Foy et al., 1999; Gu et al., 1999; Gu and Moss, 1996; Ishii et al., 2006; Ito et al., 1999; Mukai et al., 2007; Shibuya et al., 2003; Teyler et al., 1980; Vouimba et al., 2000; Woolley, 2007). For example, in rats the changing oestrogen levels across the oestrous cycles have associated changes in the densities of dendritic spines, dynamic structures important for memory (Segal et al., 2002; Woolley et al., 1990; Woolley and McEwen, 1992; 1993). Spine densities can also undergo much faster morphological changes over periods of hours and even minutes as a result of oestrogen treatment. For example oestradiol can increase spine formation as measured using an electron microscope, in the adult rat hippocampus within two hours and within 30 min *in vivo* in rats (Halpain, 2000; MacLusky et al., 2005; Segal, 2001; Smart and Halpain, 2000). The underlying mechanisms may involve oestrogen acting on G protein coupled receptors, which upon activation generate inositol 1,4,5-trisphosphate (IP3) that, through its receptors on the endoplasmic reticulum, leads to the release of intracellular calcium (Ethell and Pasquale, 2005; Honkura et al., 2008; Kasai et al., ; Kelly and Wagner, 1999). The calcium triggers actin binding proteins to polymerise to rapidly extend actin filaments (Honkura et al., 2008; Pollard and Borisy, 2003). In addition, the rapid trafficking and insertion of glutamate AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors into the plasma membrane of new spines aids in synaptic plasticity (Heine et al., 2008; Kessels and Malinow, 2009; Makino and Malinow, 2009; Newpher and Ehlers, 2009). For the longer term maintenance of these changes gene transcription and protein synthesis is required so

the behavioural effects of acute and continuous oestradiol treatment may be quite different. The timing of oestrogen treatment may be important for its behavioural and neural effects: in aged (18-month-old) ovariectomized female rats short-term oestradiol treatment (two injections) increases spine density to levels normally observed in young adults, yet in those receiving continuous replacement of oestradiol (high or low dose) for many months the increase is not maintained and there is no difference in spine density compared to those who suffered long-term deprivation (Miranda et al., 1999). Furthermore, there may be sex differences in effects as older males who received short-term oestradiol treatment had decreased spine density compared to levels normally observed in young adults (Miranda et al., 1999).

The sex steroid induced changes in spatial performance and hippocampal morphology appear to involve glutamate *N*-methyl *D*-aspartate (NMDA) receptors, at least in mammals. In rats, the effect of oestradiol on dendritic spine density is blocked by NMDA receptor antagonists (Woolley and McEwen, 1994). The effects of oestrogen on NMDA receptors may be a consequence of increased sensitivity of hippocampal cells to NMDA receptor mediated synaptic input, and/or increased NMDA NR1 subunit level in hippocampal dendrites (Cyr et al., 2001a; Gazzaley et al., 1996; Weiland, 1992; Woolley et al., 1997). The levels of the NR1 subunit of the NMDA receptor are important in spatial cognition as spatial learning ability positively correlated with NR1 immunofluorescence levels in the rat hippocampus (Adams et al., 2001c). The involvement of glutamate receptors in oestrogen's effect on spine density appears to be specific to NMDA receptors as treatment with an AMPA receptor antagonist had no effect on oestrogen-induced increases in spine density (Woolley and McEwen, 1994). This may be due to the lack of an effect of oestrogen on AMPA binding in the hippocampus in rats (Cyr et al., 2000; Cyr et al., 2001a). Increases in AMPA receptors numbers may not be as important as NMDA receptors in spatial memory as, unlike the NR1 subunit, the GluR2 subunit of the AMPA receptor is not influence by spatial learning ability (Adams et al., 2001c). NMDA receptors are involved in spatial memory formation in birds, and numbers are increased in the zebra finch hippocampus following oestrogen treatment but whether an oestrogen-induced increase in NMDA receptors improves spatial abilities still

requires elucidation (Burchuladze and Rose, 1992; Margrie et al., 1998; Meehan, 1996; Saldanha et al., 2004; Shiflett et al., 2004; Steele et al., 1995; Stewart et al., 1992).

Testosterone and oestrogen alter NMDA receptor numbers through pathways activated by binding to their respective receptors the androgen (AR) or oestrogen (ER) receptor. In the classical mode of action this results in altered gene transcription and synthesis of proteins important in memory, such as receptors or structural proteins (Behl, 2002; McDevitt et al., 2008). Changes in the expression of AR and ER can alter the sensitivity of brain systems to the action of sex steroids, which appear to be influenced by changing circulating steroid levels. For example, the seasonal changes in song behaviour and the size of song nuclei in birds is accompanied by changes in the sensitivity of the song system to gonadal steroids and regulated through changes in the expression and activity of steroid receptors (Fraley et al., ; Fusani et al., 2000; Gahr and Metzdorf, 1997b; Smith, 1996; Smith et al., 1997a; Smith et al., 1997c). Additionally, increases in AR appear to regulate increases in aggressive behaviour during the breeding season in the male song sparrow (*Melospiza melodia morphna*), European robin (*Erithacus rubecula*) and red-winged blackbird (*Agelaius phoeniceus*) as blocking AR function with the nonsteroidal antiandrogen flutamide decreases aggressive behaviour (Schwabl and Kriner, 1991; Searcy and Wingfield, 1980; Sperry et al., 2010). It may be that sex steroids can also auto-regulate their activity on spatial performance by altering the hippocampal sensitivity to their actions through changes in their receptor expression.

In addition to changes in sex steroid receptors, steroid-metabolizing enzymes, such as the oestrogen-synthesizing enzyme aromatase, may play a role in the regulation of sex hormone actions. The seasonal variation in the activity of aromatase, which occurs in a range of avian species including the ring dove, the red grouse (*Lagopus lagopus scoticus*), the pied flycatcher (*Ficedula hypoleuca*), the Lapland longspur (*Calcarius lapponicus*) and canaries (*Serinus canaria*) suggests an important role at least in the regulation of sexual behaviour (Foidart et al., 1998; Fusani et al., 2000; Hutchison et al., 1986; Sharp et al., 1986; Soma et al., 1999). In songbirds, the

combination of oestrogenic and androgenic metabolites is critical in the activation of various aspects of the sexual behaviour repertoire, including song, aggression and copulatory behaviour (Fusani et al., 2003; Harding et al., 1983; Harding et al., 1988; Soma et al., 2000a; Walters and Harding, 1988). Castration of male zebra finches reduces courtship and copulatory behaviour that is only rescued by giving both oestrogenic and androgenic metabolite treatment (Harding et al., 1983). In non-breeding male song sparrows (*Melospiza melodia morphna*) treatment with the aromatase inhibitor fadrozole greatly reduces aggressive behaviour, which can be reversed by oestrogen replacement (Soma et al., 2000b). There is also some evidence that the synthesis of oestrogen is also necessary for memory, in both mammals and birds. For example, in older men testosterone supplementation showed improved verbal memory, whereas those receiving testosterone and the aromatase inhibitor anastrozole did not show any improvement (Cherrier et al., 2005). Castrated male zebra finches treated with a non-aromatisable androgen did not learn a spatial task, whereas those who received either testosterone or oestrogen did (Oberlander et al., 2004). This may be due to a role of oestradiol synthesis in modulating dendritic spine number, at least *in vitro*, as treatment with the aromatase inhibitor letrozole decreases spine density in rat hippocampal slice cultures (Kretz et al., 2004).

Although sex steroid treatment appears to improve spatial performance in the limited number of experiments performed in birds so far, the underlying mechanisms are largely unknown. As the avian hippocampus expresses a high density of NMDA receptors, which are co-localised with aromatase, local oestrogen synthesis may provide a mechanism by which hippocampal function is modulated to improve spatial memory performance via NMDA receptors (Hodgson et al., 2008; Margrie et al., 1998; Meehan, 1996; Saldanha et al., 2004; Shiflett et al., 2004; Stewart et al., 1999).

In these series of experiments we addressed three questions in the zebra finch: (1) whether medium-term sex steroid treatment improved spatial ability; (2) whether improvements in spatial cognition by testosterone depend on its conversion to

oestrogen; (3) whether improvements in spatial cognition by sex steroid occur via modifications in the expression of sex steroid and NMDA receptors. We made four predictions:

- (1) both testosterone and oestrogen treatment would enhance spatial cognition in males and females after five days. We predicted that an enhancement by testosterone would be due to its conversion into oestrogenic metabolites;
- (2) oestrogen treatment would effect the expression of hippocampal ER $\alpha$  mRNA density in males and females;
- (3) both spatial performance and plasma oestrogen levels would be positively correlated with hippocampal ER $\alpha$  mRNA expression;
- (4) oestrogen treatment would increase NMDA receptor expression and binding levels in the hippocampus but not AMPA receptors.



## **Materials and Methods**

### *Subjects*

Zebra finches were sexually mature, captive-bred adult (1-2 years of age) males ( $n = 18$ ) and females ( $n = 16$ ), obtained from Exeter University (Falmouth, UK). They were individually caged in wire mesh cages (77cm long x 44cm wide x 44cm high). All the birds were in full visual and auditory contact with each other. They were maintained on a 15:9 hour light:dark cycle (0600:2100) at 19-22°C, humidity 25-50%, with free access to water (vitamin supplemented) and food (insectivorous bird food mixture), supplemented by dried cuttlefish bone. Birds were trained and tested in their home cage.

### EXPERIMENT 1: One trial associative memory task

Birds were pretrained on the behavioural tasks as described in the general methods. Of the 45 birds that commenced pretraining, 34 birds (males  $n = 18$ , females  $n = 16$ ) reached criterion with four weeks and went on to be tested in the one-trial associative memory tasks. They were tested for 10 days on a one-trial associative memory spatial and visual task in which birds were presented with a tray with one rewarded well (sample phase) had to relocate the rewarded well following a retention interval of 10 minutes (choice phase): half of the birds (males = 9, females  $n = 8$ ) were tested on the spatial then visual task and the other half were tested in the reverse order. This was carried out to establish baseline performance.

### EXPERIMENT 2: Retention Interval

Birds were then tested on the spatial task over six consecutive days with a single trial at each retention interval (10, 20, 30, 40, 50 and 60 minutes). Trials with the different retention intervals were presented in a random order (male  $n = 18$ ; female  $n = 14$ ). The sample size for females in this experiment dropped by two, as two females ceased to lift the flaps during the experiment.

### EXPERIMENT 3: Effects of testosterone and oestrogen on spatial performance

To investigate the effects of sex steroids on spatial performance, the birds from Experiments 1 and 2 (male  $n = 18$ ; female  $n = 14$ ) were fed testosterone or oestrogen

in cucumber 30 minutes before being tested (choice phase) on the one-trial associative memory task with a retention interval of 20 minutes to ensure that their performance was at chance (based on results from Experiment 2). The birds were tested once a day in the morning for a total of 25 consecutive days, in five sequential blocks of five days. A small piece of cucumber ( $40\text{mm}^3$ ) was injected with testosterone ( $3\mu\text{l}$ ;  $0.75\text{mg/ml}$ ; Sigma), oestradiol ( $3\mu\text{l}$ ;  $1.25\text{mg/ml}$ ; Sigma) or vehicle ( $3\mu\text{l}$  95% ethanol) and fed to birds 30 min prior to testing. All birds received cucumber injected with vehicle for the first five days. On days six to ten, half the birds received cucumber injected with testosterone and the other birds received cucumber injected with oestrogen. This was followed by a further five days (11-15) of vehicle. On days 16-20, birds received cucumber injected with the hormone they had not received previously and the experiment was completed with a final five days (21-25) of birds being tested after receiving cucumber injected with vehicle.

#### EXPERIMENT 4: Effect of blocking aromatase by fadrozole on spatial performance

After a further two weeks, the birds used in Experiments 1-3 were tested again, once a day, for a further 25 consecutive days (male  $n = 17$ , female  $n = 14$ ; the sample size for males in this experiment decreased by one following the death of a male). Following five days of vehicle treatment (days 1-5;  $3\mu\text{l}$  95% ethanol in cucumber), half the birds received testosterone and saline while the other birds received testosterone and fadrozole daily for five days (days 6-10). Fadrozole ( $120\mu\text{g}$  in  $30\mu\text{l}$  sterile saline,  $4\mu\text{g/ml}$ ; MTA from Novartis) or sterile saline ( $30\mu\text{l}$ ) was injected intramuscularly 24 hours prior to testing. These five days were followed by five days (11-15) of vehicle treatment, then five days (16-20) in which the testosterone/ fadrozole treatment was given to the birds that had not received it previously (and the testosterone/ fadrozole birds received testosterone plus saline), followed by a final five days (21-25) of vehicle treatment for all birds.

#### EXPERIMENT 5: Effect of oestrogen treatment on the hippocampal expression of $\text{ER}\alpha$ and NMDA receptors

Two weeks following the completion of Experiment 4, birds were given oestrogen ( $3\mu\text{l}$  in cucumber;  $1.25\text{mg/ml}$ ; male  $n = 7$ , female  $n = 8$ ), vehicle ( $3\mu\text{l}$  in cucumber;

95% ethanol; male n = 6, female n = 5) or just cucumber (control; male n = 2, female n = 2) daily for five days, and were tested 30 min after administration (male n = 15, female n = 15; the sample size for males dropped by two as two ceased to lift the flaps and the sample size for females increased by one as a bird that had previously stopped lifting flaps reach criterion again). Immediately following testing on the fifth day, birds were decapitated and brains were removed for analysis of ER $\alpha$  mRNA expression, ER $\alpha$  and NMDA protein levels and NMDA and AMPA binding (by *in situ* hybridisation, immunocytochemistry and autoradiography respectively, as described in Chapter Two).

#### *Oestrogen plasma levels*

At the end of Experiment 5 on Day Five (between 10.30am and midday), trunk blood was collected into a heparinised micro haematocrit tubes on decapitation 30 minutes after oestrogen treatment and plasma oestrogen levels were determined. Samples were centrifuged on a micro-haematocrit centrifuge for three minutes, and plasma was recovered with a fixed needle Hamilton syringe and stored in ependorffs at -20C until samples were run in a radioimmunoassay to establish unmanipulated circulating oestrogen levels (as described in Chapter Two). All samples were run in a single assay. The binding was 18% and non-specific binding was 2.1%. The intra-assay coefficient of variation was 7.8%. The sample size for both males and females was reduced by one each as the oestradiol plasma levels were out of detection range.

#### *Analysis*

Repeated measures ANOVAs were used to analyse the effect of oestrogen treatment on plasma oestrogen levels, the effects on ER $\alpha$  mRNA expression and spatial performance after five days of treatment. Repeated measures two-way ANOVA was used to compare spatial and visual performance between males and females, the effect of increasing retention interval on performance, the effect of treatment on spatial performance and the effect of treatment on latency. The post hoc test used was the Holm-Sidak method. Correlations between spatial performance and latency for different treatments, plasma oestrogen levels and ER $\alpha$  mRNA expression, spatial

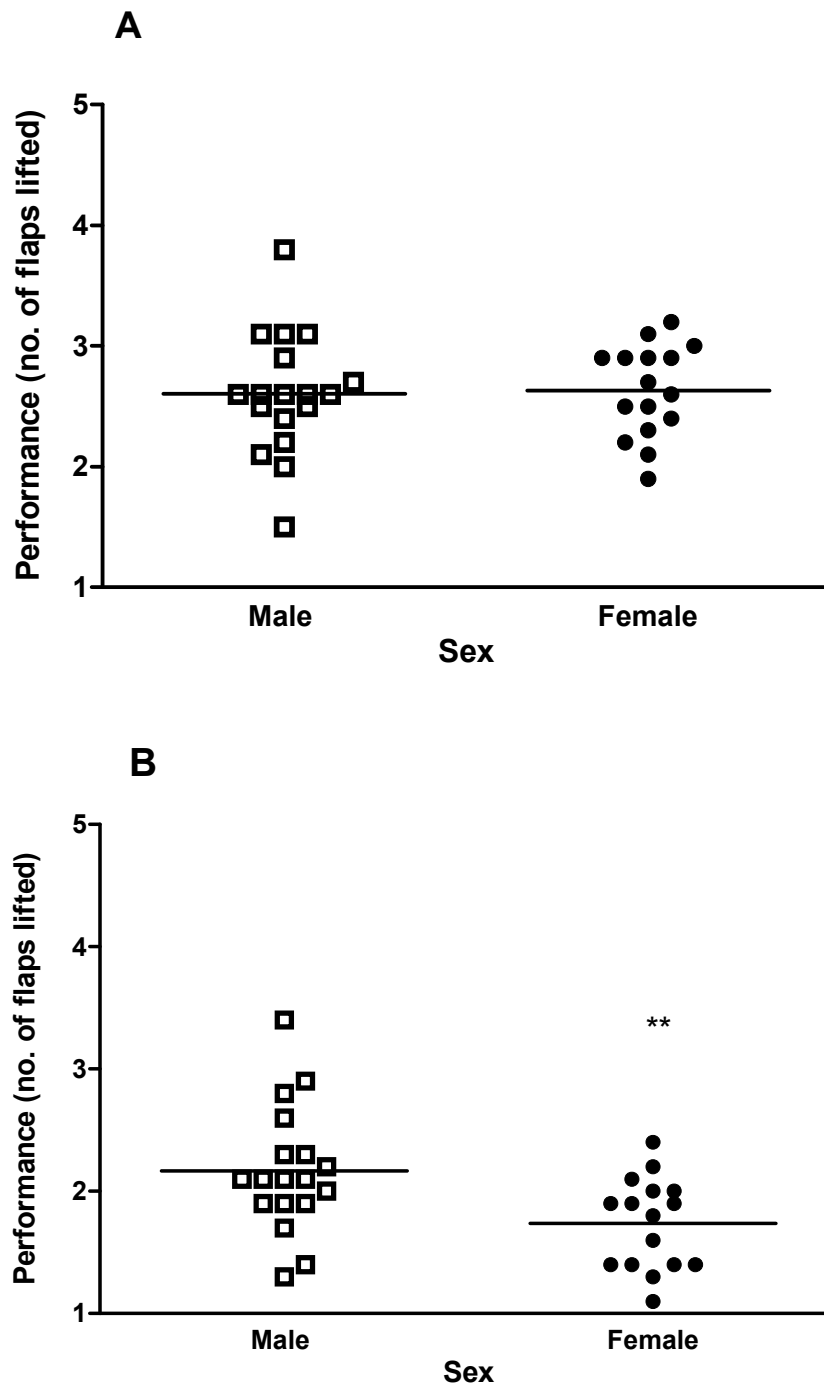
performance and both ER $\alpha$  mRNA expression and spatial performance were measured using linear regression. All data passed normality tests.

## **Results**

### *Experiment 1: Spatial and visual performance*

Spatial performance (number of flaps) did not vary significantly across the 10 days of testing ( $F_{9,288} = 1.26$ ,  $p = 0.25$ ), and there was no difference between the performance of the two sexes ( $F_{1,288} < 0.001$ ,  $p = 0.99$ ). There was no interaction between sex and day ( $F_{9,288} = 0.41$ ,  $p = 0.93$ ). Average performance over the 10 days was similar to performance on Probe Day 11 in both males (Paired  $t$ -test;  $t_{17} = 1.02$ ,  $p = 0.32$ ) and females (paired  $t$ -test;  $t_{15} = 0.29$ ,  $p = 0.77$ ). This was also the case for the visual task: there were no differences in performance across the 10 days ( $F_{9,288} = 0.75$ ,  $p = 0.66$ ), and no differences between the sexes ( $F_{1,288} = 2.97$ ,  $p = 0.09$ ). There was no interaction between sex and day ( $F_{9,288} = 0.41$ ,  $p = 0.93$ ). Average performance over the 10 days was similar to performance on Probe Day 11 in both males (paired  $t$ -test;  $t_{17} = 0.48$ ,  $p = 0.64$ ) and females (paired  $t$ -test;  $t_{15} = 1.28$ ,  $p = 0.22$ ).

As performance did not vary across the 10 days, we used the average performance to look at effects of sex and order of task presentation (spatial/visual task 1<sup>st</sup> or 2<sup>nd</sup>). It did not appear to matter in which order the tasks were presented (two-way repeated measures ANOVA  $F_{3,28} = 1.81$ ,  $p = 0.17$ ) and the mean performance did not differ between the sexes ( $F_{1,28} < 0.0001$ ,  $p > 0.99$ ). There was no interaction between sex and order of task presentation ( $F_{3,28} = 0.09$ ,  $p = 0.97$ ). We, therefore, ignored order in the following analyses. Males and females did not differ in performance on the spatial task (averaged performance over the 10 days of testing; unpaired  $t$  test  $t_{32} = 0.17$ ,  $p = 0.87$ ; Figure 1A) but they did on the visual task, with females performing better than the males ( $t_{32} = 2.76$ ,  $p = 0.0096$ ; Figure 1B).



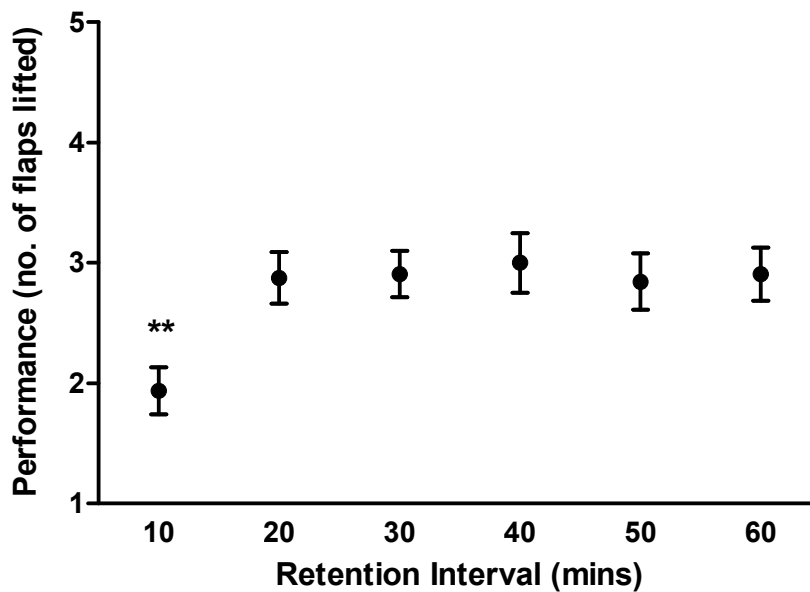
**Figure 1** Performance on the one trial associative memory task in zebra finches (A) spatial version and (B) visual version (males  $n = 18$ , females  $n = 16$ ). Performance is measured as the number of flaps lifted in up to five minutes to find the rewarded well and is averaged over 10 trials (one per day). There was no difference in performance between males and females on the spatial task but females performed better than males on the visual task (\*\*  $P < 0.01$ ). The mean for each data set is represented by a line.

### Experiment 2: Retention Interval

Increasing the retention interval significantly impaired performance on the spatial task ( $F_{5,150} = 3.76$ ,  $p = 0.003$ ; Figure 2). Performance with a 10 minute retention interval was significantly better than that after 20, 30, 40, 50 and 60 minute intervals (post hoc test results in Table 1 below). The performance of both sexes declined with increasing retention interval ( $F_{1,150} = 1.05$ ,  $p = 0.31$ ) and there was no sex-interval interaction ( $F_{5,150} = 1.94$ ,  $p = 0.09$ ).

RI	10	20	30	40	50	60
10	x	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.01$
20	$p < 0.01$	x	$p = 0.77$	$p = 0.72$	$p = 0.90$	$p = 0.88$
30	$p < 0.001$	$p = 0.77$	x	$p = 0.94$	$p = 0.89$	$p = 0.89$
40	$p < 0.001$	$p = 0.72$	$p = 0.94$	x	$p = 0.81$	$p = 0.83$
50	$p < 0.01$	$p = 0.90$	$p = 0.89$	$p = 0.81$	x	$p = 0.98$
60	$p < 0.01$	$p = 0.88$	$p = 0.89$	$p = 0.83$	$p = 0.98$	x

Table 1



**Figure 2** The effect of retention interval on performance in the spatial task. Male  $n=18$ , female  $n=14$ . The data are mean  $\pm$  SEM. Data points represent one trial for each retention interval. Males and females are graphed together as increasing retention interval had the same effect on spatial performance in both sexes. Performance following a 10 minute retention interval was significantly better than following 20-60 minutes. \*\*  $P < 0.01$ .

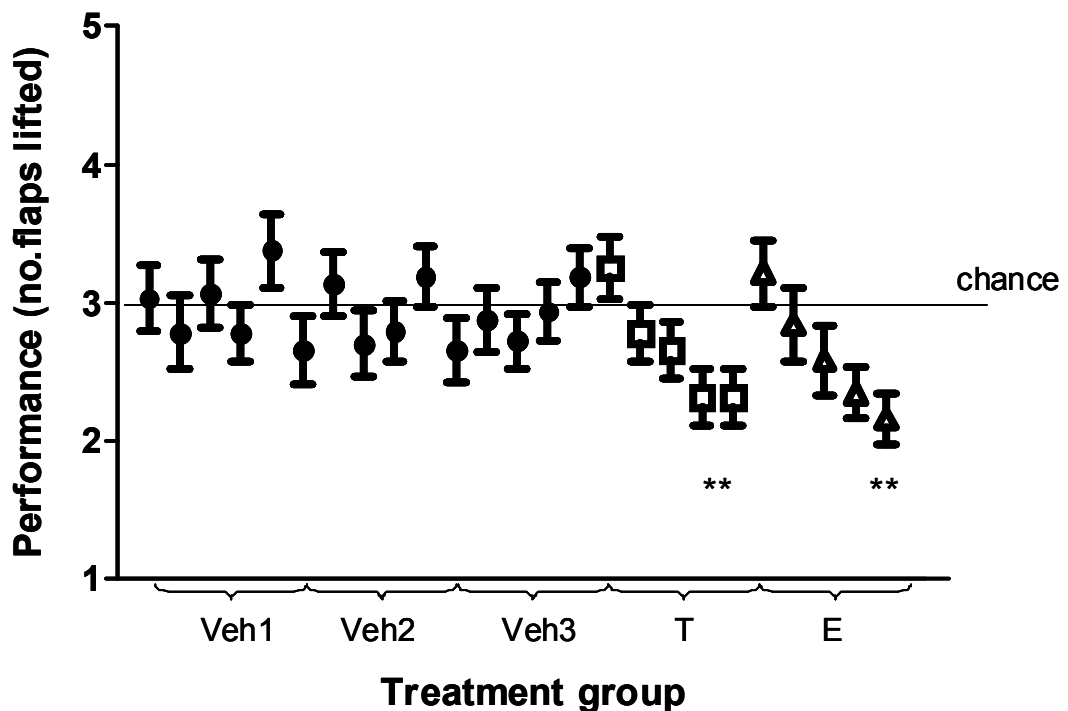
*Experiment 3: Effects of testosterone and oestrogen treatment on spatial performance*

Performance did not differ across the three blocks of vehicle treatment ( $F_{14,420} = 0.82$ ,  $p = 0.64$ ; Figure 3). Both sexes performed similarly under vehicle treatment ( $F_{1,420} = 0.23$ ,  $p = 0.14$ ) and the effect of vehicle was not dependent on sex ( $F_{14,420} = 0.81$ ,  $p = 0.66$ ). Performance improved over five days of testosterone treatment (2 way RM ANOVA;  $F_{4,120} = 4.39$ ,  $p < 0.01$ ) and there was no effect of sex ( $F_{1,120} = 1.27$ ,  $p = 0.27$ ) and no interaction between day and sex ( $F_{4,120} = 0.61$ ,  $p = 0.65$ ). Performance improved over five days of oestrogen treatment (2 way RM ANOVA;  $F_{4,120} = 3.61$ ,  $p < 0.01$ ) and there was no effect of sex ( $F_{1,120} = 2.20$ ,  $p = 0.15$ ) and no interaction between day and sex ( $F_{4,120} = 1.79$ ,  $p = 0.14$ ). Performance on the last day of each five day session was compared between treatment and vehicle groups, when treatment effects were maximal and to eliminate effects of residual hormones on the vehicle treatment: Day Five of testosterone or oestrogen treatment was compared to performance on Day Five of the vehicle treatment that preceded the hormone treatment i.e. for birds that received testosterone first, their performance on Day Five of testosterone treatment (day 10 of the experiment) was compared to their performance on Day Five of Block One of vehicle treatment (day 5), while their oestrogen performance (day 20) was compared to their performance on Day Five of the second vehicle treatment (day 15) and vice versa for those which received oestrogen first and testosterone second. Both testosterone and oestrogen treatment improved performance ( $F_{5,86} = 7.87$ ,  $p < 0.001$ ; Holm-Sidak post hoc test; testosterone first vs vehicle:  $p < 0.001$ ; testosterone second vs vehicle:  $p < 0.01$ ; oestrogen first vs vehicle:  $p < 0.01$  and oestrogen second vs vehicle:  $p < 0.01$ ). The effect of the hormones did not differ between the sexes ( $F_{1,86} = 0.29$ ,  $p = 0.60$ ) and the effect of treatment was not dependent on sex ( $F_{5,86} = 0.28$ ,  $p = 0.92$ ).

Both testosterone and oestrogen treatment improved performance regardless of which vehicle day was used in the analysis (Holm-Sidak post hoc test; Day 10 testosterone vs Day 15 vehicle:  $p < 0.001$ ; Day 20 testosterone vs Day 5 vehicle:  $p < 0.01$ ; Day 10 oestrogen vs Day 15 vehicle:  $p < 0.01$  and Day 20 oestrogen vs Day 5 vehicle:  $p < 0.01$ ).



Performance did not differ between those birds that received testosterone first and those that received testosterone second (Holm-Sidak post hoc test;  $p = 0.53$ ). There was also no difference in performance between those birds that received oestrogen first and those that received oestrogen second (Holm-Sidak post hoc test;  $p = 0.88$ ). There was no difference between oestrogen first and testosterone first (Holm-Sidak post hoc test;  $p = 0.55$ ), oestrogen second and testosterone second (Holm-Sidak post hoc test;  $p = 0.85$ ), oestrogen first and testosterone second (Holm-Sidak post hoc test;  $p = 0.97$ ) or oestrogen second and testosterone first (Holm-Sidak post hoc test;  $p = 0.66$ ).

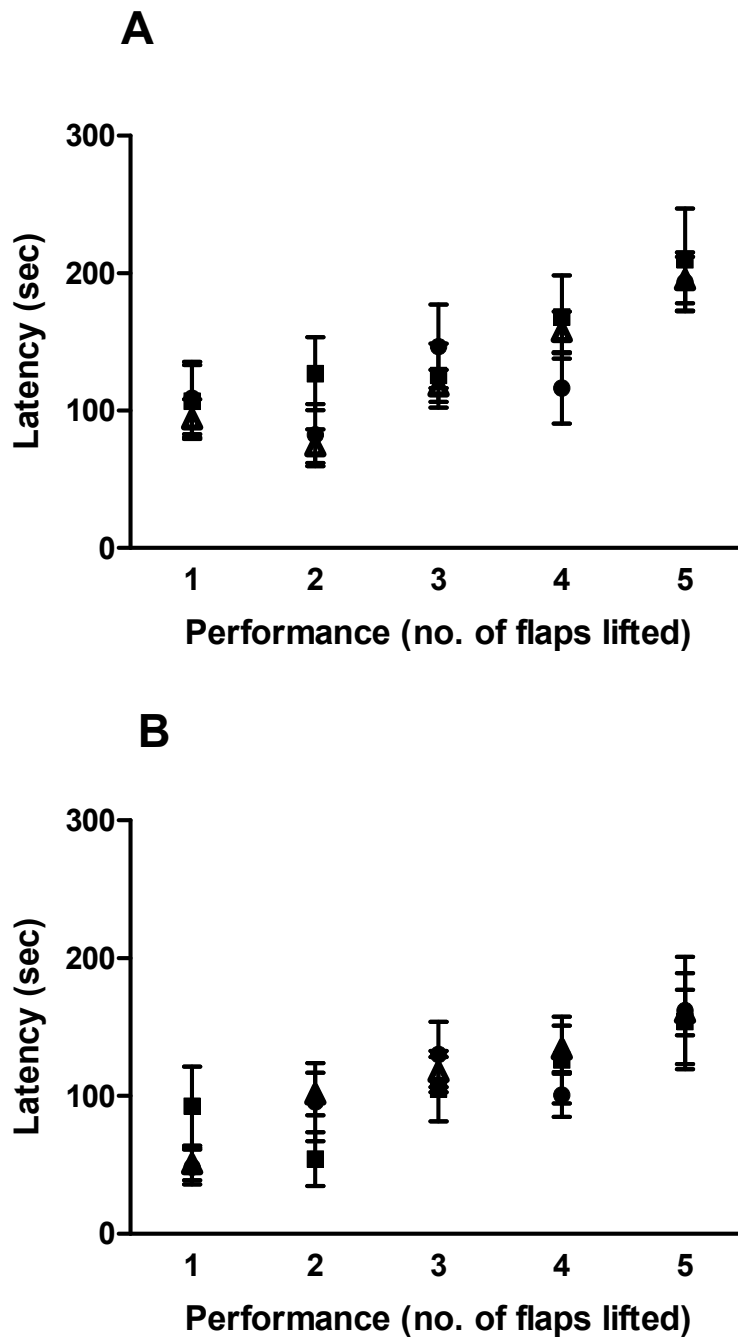


**Figure 3** The effect of testosterone (T; square), oestrogen (E; triangle) and vehicle (Veh; ethanol; circles) treatment on spatial performance in zebra finches. Male  $n=18$ , female  $n=14$ . The data are mean  $\pm$  SEM. Males and females are graphed together as hormone treatment had the same effect on performance in both sexes. Performance on Day Five of sex steroid treatment was significantly better than performance on Day Five of the preceding vehicle treatment. \*\*  $P < 0.01$ .

*Effect of sex hormone treatment on latency to solve the task*

Neither testosterone nor oestrogen treatment reduced the latency to solve the task ( $F_{4,120} = 0.67$ ,  $p = 0.62$ ), which did not differ between the sexes ( $F_{1,120} = 0.36$ ,  $p = 0.55$ ). There was no significant interaction between sex and treatment ( $F_{4,120} = 0.84$ ,  $p = 0.50$ ).

However, for each individual treatment group, there was a significant positive correlation between the latency to solve the task and spatial performance, with those birds that took longer to solve the task performing worst (linear regression; average per bird over all treatment days; vehicle treatment: males  $r^2 = 0.24$ ,  $n = 17$ ,  $p = 0.04$ , females  $r^2 = 0.34$ ,  $n = 14$ ,  $p = 0.02$ ; testosterone treatment: males  $r^2 = 0.39$ ,  $n = 17$ ,  $p = 0.006$ , females  $r^2 = 0.51$ ,  $n = 14$ ,  $p = 0.004$ ; oestrogen treatment: males  $r^2 = 0.30$ ,  $n = 17$ ,  $p = 0.02$ , females  $r^2 = 0.71$ ,  $n = 14$ ,  $p < 0.001$ ; Figure 4).



**Figure 4** The effect of latency to solve the spatial task on performance after vehicle (triangles), testosterone (circles) and oestrogen (squares) treatment in A) males ( $n = 17$ ) and B) females ( $n = 14$ ). The testosterone and oestrogen treated groups are averaged over five consecutive days of treatments and the vehicle treated group includes data from all three blocks of treatment (i.e. 15 days). The data are mean  $\pm$  SEM.

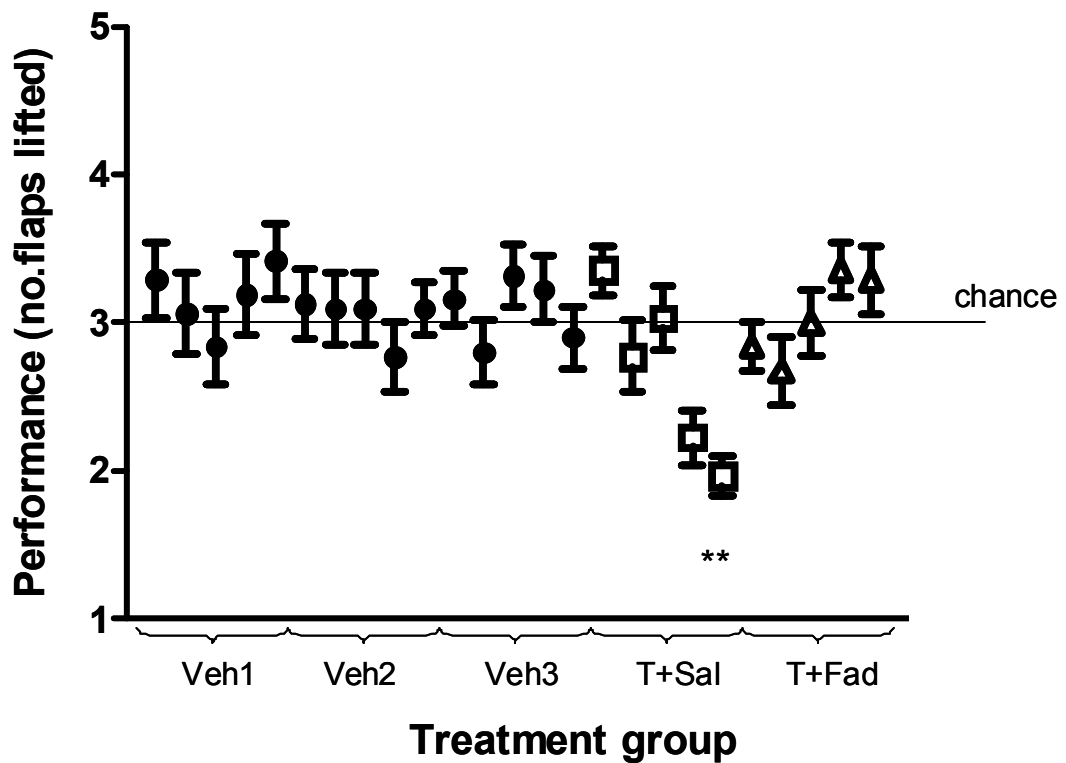
*Experiment 4: Effect of blocking aromatase by fadrozole on spatial performance*

Spatial cognition did not differ across the three periods of vehicle treatment (two-way repeated measures ANOVA,  $F_{14,406} = 0.26$ ,  $p = 0.99$ ; Figure 5), and there were no significant differences between males and females ( $F_{1,406} = 0.17$ ,  $p = 0.68$ ). The effect of vehicle treatment was not dependent on sex ( $F_{14,406} = 0.37$ ,  $p = 0.98$ ). Performance increased over the five days of testosterone and saline treatment (2 way RM ANOVA;  $F_{4,120} = 11.49$ ,  $p < 0.001$ ), there was no effect of sex ( $F_{1,120} = 0.079$ ,  $p = 0.78$ ) and there was no day/sex interaction ( $F_{4,120} = 11.49$ ,  $p < 0.001$ ). There was no change in performance in the T/ fad group over the five days of treatment (2 way RM ANOVA;  $F_{4,120} = 0.25$ ,  $p = 0.91$ ), there was no effect of sex ( $F_{1,120} = 0.02$ ,  $p = 0.89$ ) and no interaction between day and sex ( $F_{4,120} = 0.80$ ,  $p = 0.53$ ).

As above, performance on the fifth day of testosterone/ saline or testosterone/ fadrozole treatment was compared to performance on last day of the vehicle treatment that preceded the hormone treatment i.e. for birds that received testosterone/ saline first, their performance on Day 10 of treatment was compared to their performance on Day 5 of vehicle treatment while their testosterone/ fadrozole (Day 20) performance was compared to their performance on Day 15 of vehicle treatment. There was an effect of hormone manipulation on performance (two-way repeated measures ANOVA,  $F_{5,83} = 6.51$ ,  $p < 0.001$ ): testosterone/ saline treatment improved performance (Holm-Sidak post hoc test, testosterone/ saline first vs vehicle:  $p < 0.001$ ; testosterone/ saline second vs vehicle:  $p < 0.01$ ). However, testosterone/ fadrozole treatment did not improve performance (Holm-Sidak post hoc test, testosterone/ fadrozole first vs vehicle:  $p = 0.76$ ; testosterone/ fadrozole second vs vehicle:  $p = 0.78$ ). There was no significant difference between the sexes ( $F_{1,83} = 0.79$ ,  $p = 0.38$ ) and the effect of treatment was not dependent on sex ( $F_{5,83} = 0.92$ ,  $p = 0.47$ ).

Birds that received testosterone/ saline first performed as well as those that received testosterone/ saline second (Holm-Sidak post hoc test;  $p = 0.97$ ). Birds that received testosterone/ fadrozole first performed as well as those birds that received testosterone/ fadrozole second (Holm-Sidak post hoc test;  $p = 0.70$ ).

Birds that received testosterone/ saline first performed better than did those birds that received testosterone/ fadrozole treatment first (Holm-Sidak post hoc test;  $p < 0.01$ ). Likewise, birds that received testosterone/ saline treatment second performed better than those birds that received testosterone/ fadrozole second (Holm-Sidak post hoc test;  $p < 0.01$ ).

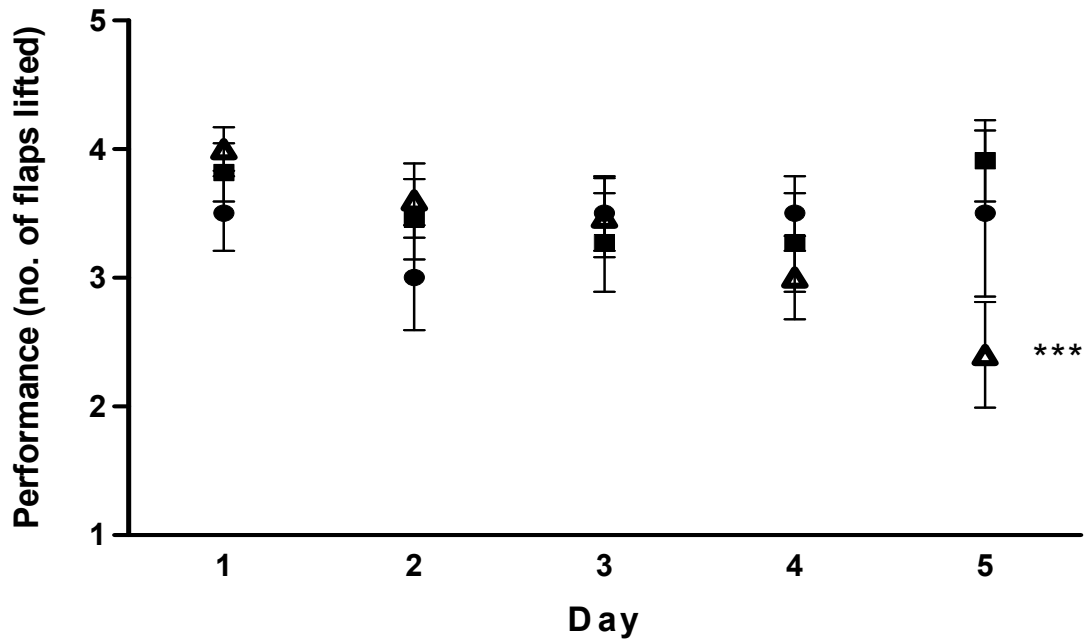


**Figure 5** The effect of fadrozole treatment on spatial performance. Zebra finches received either testosterone and saline (T+Sal; square) or testosterone and fadrozole (T+Fad; triangle). Male  $n = 17$ , female  $n = 14$ . The data are mean  $\pm$  SEM. Males and females are graphed together as hormone treatment had the same effect on performance in both sexes. Performance on Day Five following T+Sal treatment was significantly better than performance on Day Five following vehicle treatment. \*\*  $P < 0.01$ .

#### *Experiment 5: Effect of oestrogen treatment on spatial performance*

Performance on each of the five oestrogen treatment days was measured to determine that oestrogen was improving spatial performance. A performance score for each bird was calculated by subtracting the number of flaps lifted on day five of testing from the number of flaps lifted on day one of testing and these scores were compared

between birds which received oestrogen treatment, those receiving vehicle and those receiving only cucumber. Oestrogen-treated birds improved in performance more than birds receiving cucumber alone and more than birds that received vehicle ( $F_{2,24} = 8.18$ ,  $p = 0.002$ ; oestrogen vs control: Holm-Sidak post hoc test;  $p = 0.019$ ; oestrogen vs vehicle: Holm-Sidak post hoc test;  $p < 0.001$ ; Figure 6). Birds that received only cucumber did not differ in performance from those birds that received vehicle in cucumber (Holm-Sidak post hoc test;  $p = 0.88$ ). There were no significant difference between males and females ( $F_{1,24} = 0.21$ ,  $p = 0.65$ ) and effects of treatment were not dependent on sex ( $F_{2,24} = 0.77$ ,  $p = 0.47$ ).



**Figure 6** The effect of oral oestrogen treatment (in cucumber; triangles), vehicle treatment (ethanol in cucumber; squares) or ingestion of plain cucumber (control; circles) over five consecutive days on spatial performance (males  $n = 15$ , females  $n = 15$ ). Males and females are graphed together as treatment had the same effect on performance in both the sexes. The data are mean  $\pm$  SEM. Oestrogen treatment significantly improved spatial performance on Day Five compared to Day One. \*\*\*  $P < 0.001$ .

#### *Plasma oestrogen levels*

There was an effect of oestrogen treatment on circulating oestrogen levels ( $F_{2,22} = 32.22$ ,  $p < 0.001$ ; Figure 7A): the oestrogen-treated birds had significantly higher circulating oestrogen levels compared to the vehicle treated birds (Holm-Sidak post hoc test;  $p < 0.0001$ ) and the birds which received cucumber only (Holm-Sidak post hoc test;  $p < 0.0001$ ). Plasma oestrogen levels did not differ between the birds that only received only cucumber and those that received vehicle in cucumber (Holm-Sidak post hoc test;  $p = 0.918$ ). There were no significant differences between males and females ( $F_{1,22} = 1.77$ ,  $p = 0.197$ ) and the effect of oestrogen was not dependent on sex ( $F_{2,22} = 2.15$ ,  $p = 0.14$ ). There was no significant difference between oestrogen levels in males and females in the groups that did not receive oestrogen (nothing and vehicle groups combined; unpaired t-test;  $t_{11} = 0.50$ ,  $p = 0.63$ ,  $n = 13$ ).

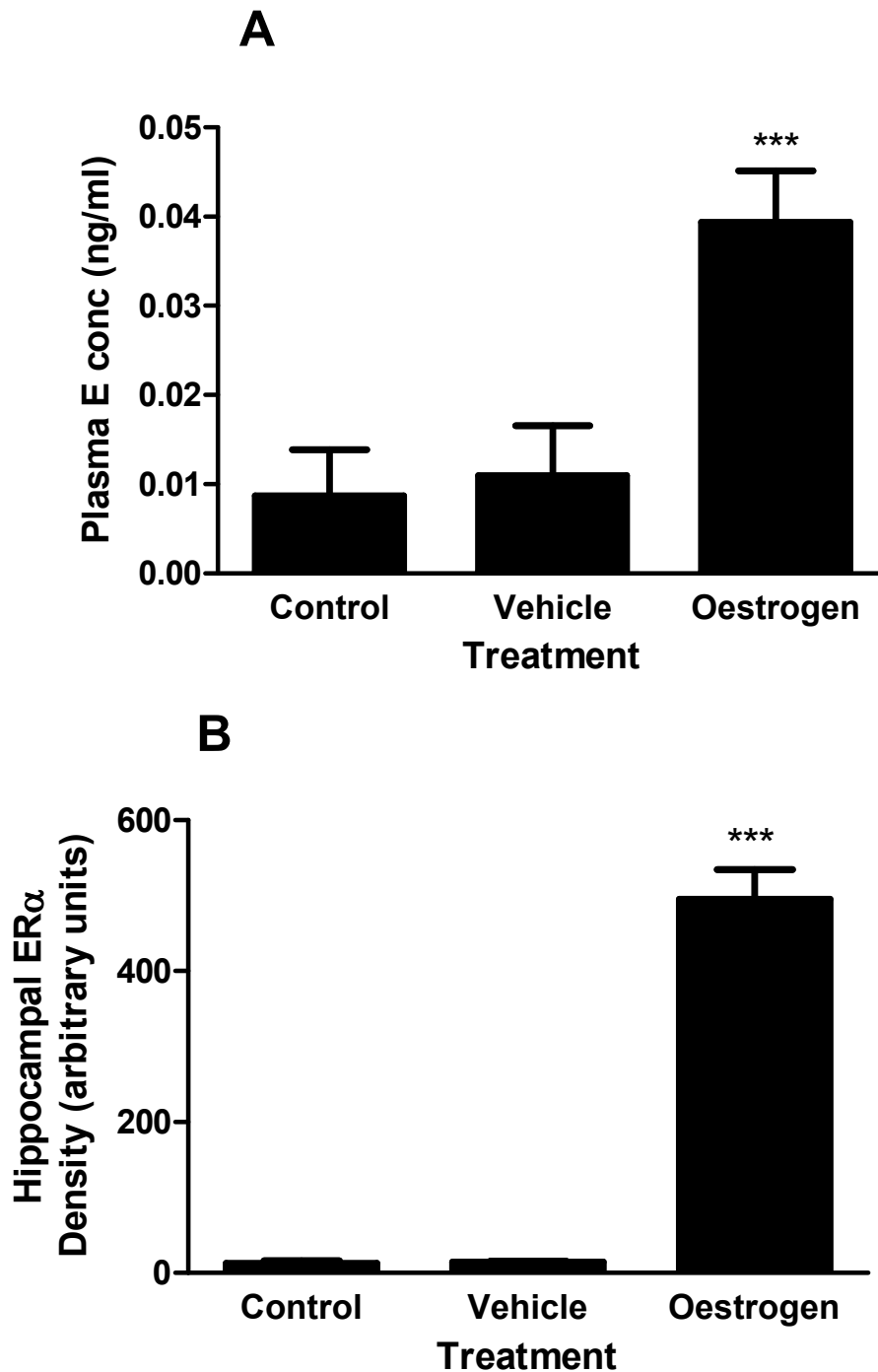
*Effect of five days of oestrogen treatment on hippocampal ER $\alpha$*

Hippocampal ER $\alpha$  mRNA expression in birds that received oestrogen was higher than expression in both vehicle-treated birds (two-way ANOVA,  $F_{2,24} = 63.59$ ,  $p < 0.001$ ; Holm-Sidak post hoc test;  $p < 0.001$ ; Figure 7B and 8A-D) or in birds that received only cucumber (Holm-Sidak post hoc test;  $p < 0.001$ ). There was no significant difference in ER $\alpha$  mRNA expression between birds that received cucumber alone and those that were vehicle treated (Holm-Sidak post hoc test;  $p = 0.98$ ). There were no significant differences between males and females ( $F_{1,24} = 0.078$ ,  $p = 0.78$ ) and there was no interaction between sex and treatment ( $F_{2,24} = 0.032$ ,  $p = 0.97$ ). Plasma oestrogen levels were positively correlated with hippocampal ER $\alpha$  mRNA expression levels in males and females ( $r^2 = 0.87$ ,  $n = 14$ ,  $p < 0.0001$  and  $r^2 = 0.59$ ,  $n = 14$ ,  $p = 0.0011$ , respectively; Figure 9A). ER $\alpha$  numbers as measured by immunocytochemistry were at the limit of detectability in both males and females in all groups in the hippocampus, although detectable in other brain regions such as the HVC and the bed nucleus of the stria terminalis (BnST), which influences reproduction and sexual behaviour and is involved in enhancing associative learning in rats after a stressful experience (Bangasser et al., 2005) (Figure 8E and F). As a consequence, no analysis was carried out on the slides.

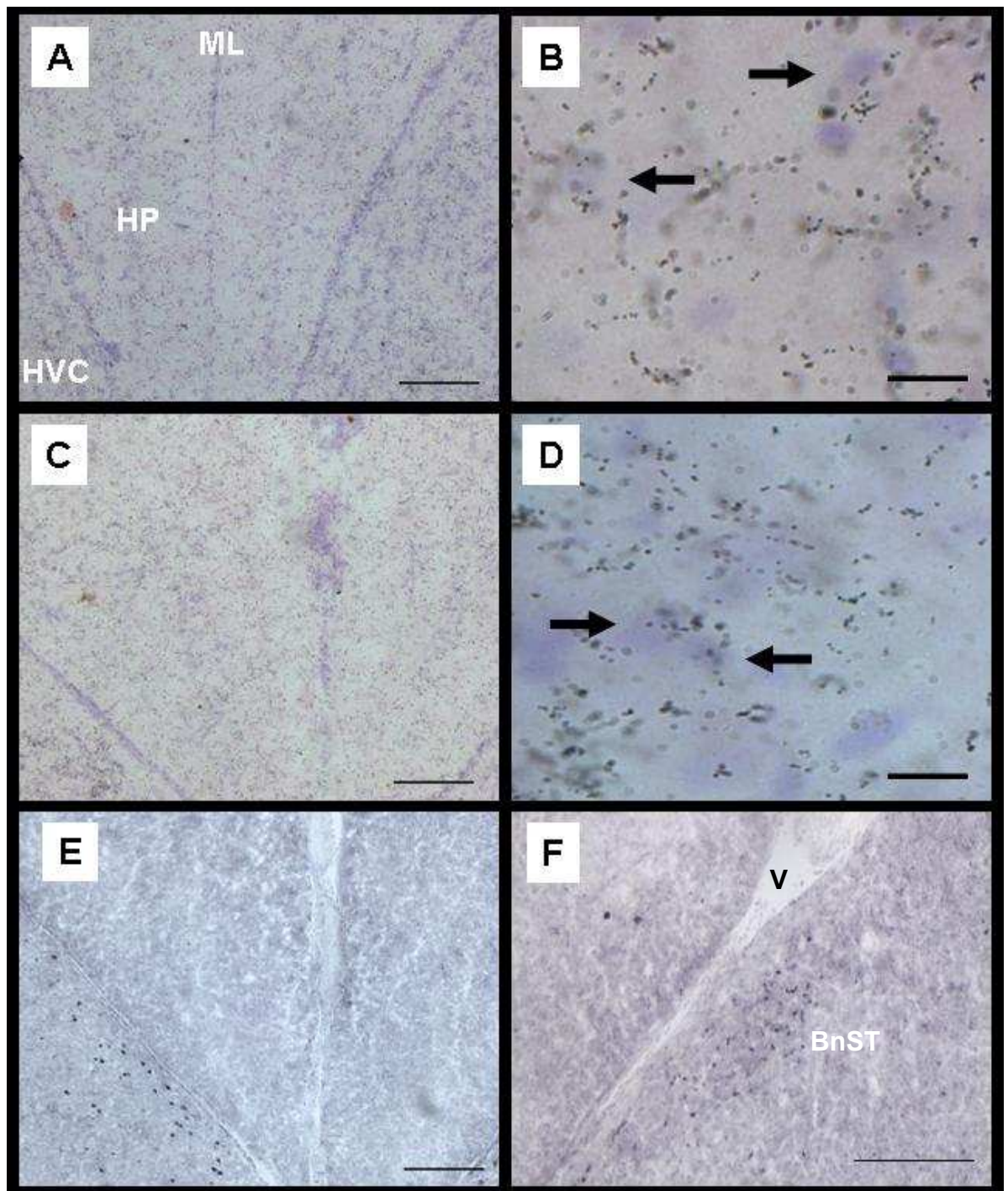
*Spatial performance, plasma oestrogen and ER $\alpha$  hippocampal expression*

Plasma oestrogen levels were positively correlated with spatial performance on day five of testing in males ( $r^2 = 0.40$ ,  $n = 14$ ,  $p = 0.015$ ) and in females ( $r^2 = 0.55$ ,  $n = 14$ ,  $p = 0.003$ ; Figure 9B). Hippocampal ER mRNA expression was positively correlated with spatial performance on day five of testing in males ( $r^2 = 0.56$ ,  $n = 15$ ,  $p = 0.0013$ ) and in females ( $r^2 = 0.36$ ,  $n = 15$ ,  $p = 0.017$ ; Figure 9C).

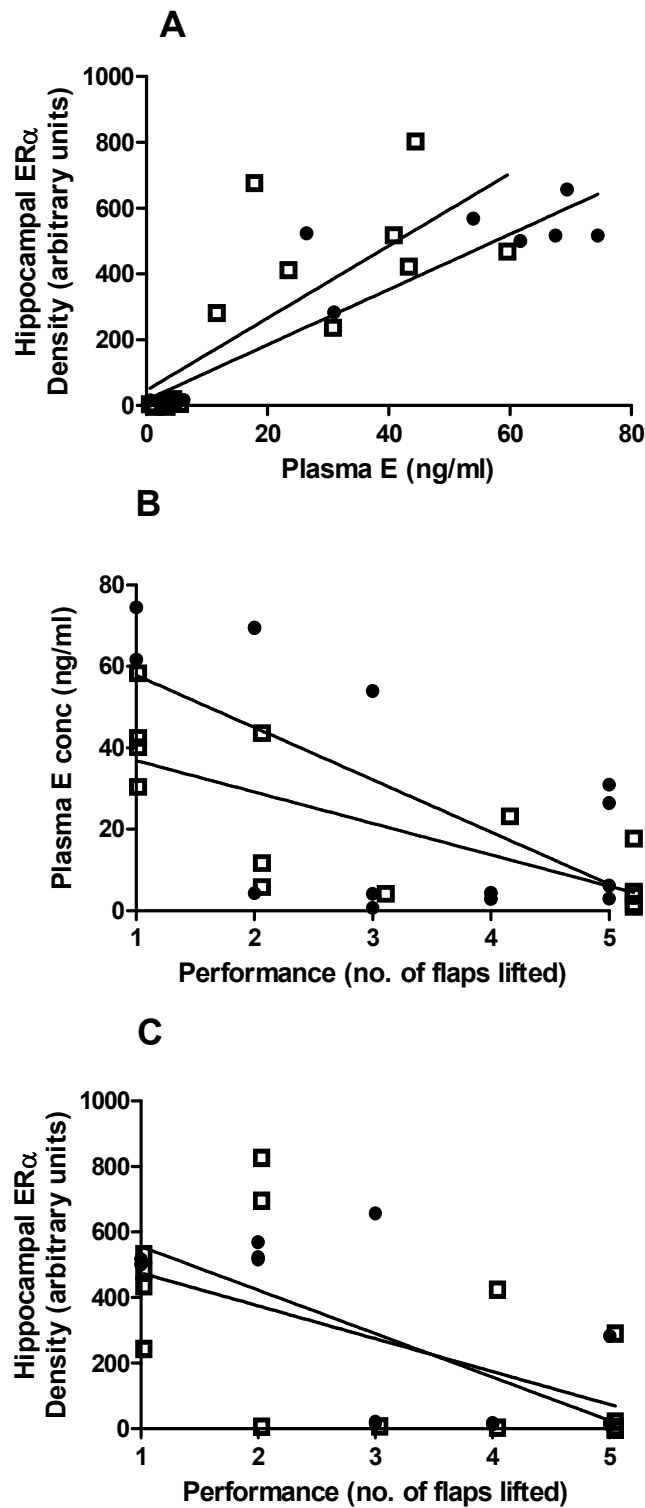




**Figure 7** The effect of five days of oral oestrogen treatment (in cucumber;  $n = 15$ ), vehicle treatment (ethanol in cucumber;  $n = 9$ ) or ingestion of plain cucumber (control;  $n = 4$ ) on A) plasma levels oestrogen (E) levels 30 min after ingestion on Day Five of five consecutive days of treatment and B) on hippocampal oestrogen receptor alpha ( $ER\alpha$ ) density. Males ( $n = 15$ ) and females ( $n = 15$ ) were combined as there were no differences between the sexes. Oestrogen treatment significantly increased plasma E and hippocampal  $ER\alpha$  mRNA compare to controls. The data are mean + SEM. \*\*\*  $P < 0.001$ .



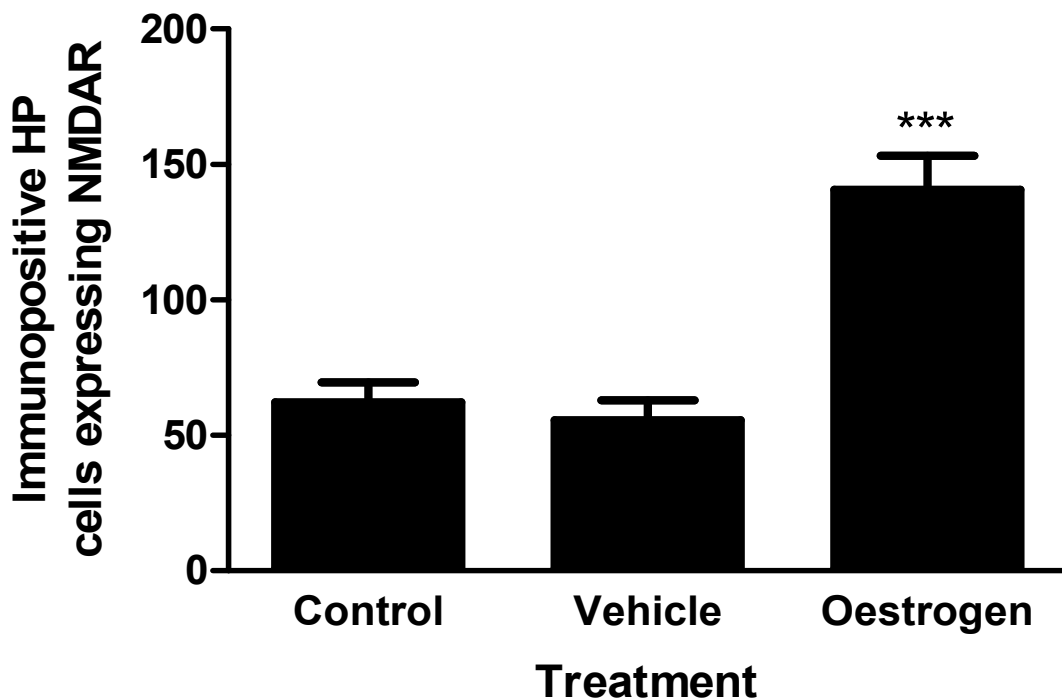
**Figure 8** The effect of oestrogen treatment for five days on ER $\alpha$  in the hippocampus (HP). ER $\alpha$  mRNA expression following vehicle treatment A) x20 (scale bar 100 $\mu$ m) and B) x100 magnification (scale bar 25 $\mu$ m) and following oestrogen treatment C) x20 and D) x100 magnification and ER $\alpha$  protein levels following oestrogen treatment in E) the hippocampus and HVC and F) the bed nucleus of the stria terminalis (BnST). ML = midline, V = ventricle.



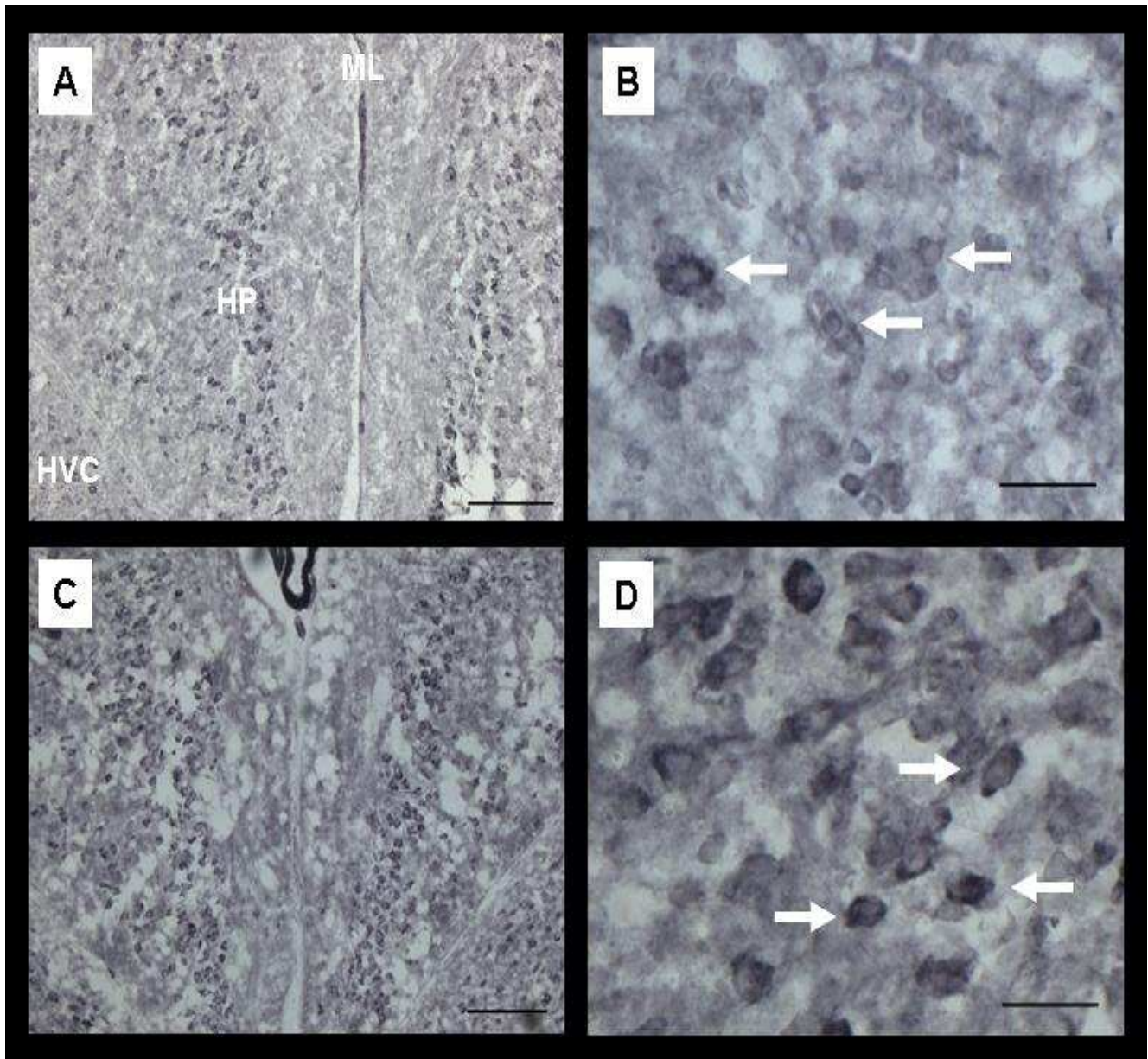
**Figure 9** The significant relationship between plasma oestrogen (E) levels and A) hippocampal oestrogen receptor (ER) mRNA density and B) spatial performance 30 min after oestrogen treatment on Day Five of five consecutive days of treatment in males ( $n = 14$ ; circles) and females ( $n = 14$ ; squares) and C) the significant relationship between ER mRNA density and spatial performance 30 min after oestrogen treatment on Day Five of five consecutive days of treatment (males  $n = 15$  and females  $n = 15$ ).

*The effect of five days of oestrogen treatment on the number of hippocampal cells expressing NMDA receptors*

The number of hippocampal cells expressing NMDA receptors in birds that received oestrogen was higher than in both vehicle-treated birds ( $F_{2,24} = 20.6$ ,  $p < 0.001$ ; Holm-Sidak post hoc test;  $p < 0.001$ ; Figure 10 and 11) and in birds that received only cucumber (Holm-Sidak post hoc test;  $p < 0.001$ ). There was no difference in expression between birds which received cucumber alone and those which were vehicle treated (Holm-Sidak post hoc test;  $p = 0.73$ ). There were no differences between males and females ( $F_{1,24} = 1.04$ ,  $p = 0.32$ ) and there was no interaction between sex and treatment ( $F_{2,24} = 2.55$ ,  $p = 0.099$ ).



**Figure 10** The effect of five days of oral oestrogen treatment (in cucumber;  $n = 15$ ), vehicle treatment (ethanol in cucumber;  $n = 11$ ) or ingestion of plain cucumber (control;  $n = 4$ ) on the number of hippocampal (HP) cells expressing NMDA receptors (NMDAR). Males ( $n = 15$ ) and females ( $n = 15$ ) were combined as treatment had the same affect in both sexes. The data are mean  $\pm$  SEM. Oestrogen treatment significantly increased hippocampal NMDA receptor numbers compared to controls. \*\*\*  $P < 0.01$ .



**Figure 11** The effect on the number of cells expressing NMDA receptors of five days of vehicle (ethanol in cucumber) treatment at x20 (A) and x100 magnification (B) and following oestrogen treatment (in cucumber) at x20 (C) and x 100 magnification (D). Scale bar for x20 = 100 $\mu$ m and for x100 = 25 $\mu$ m.

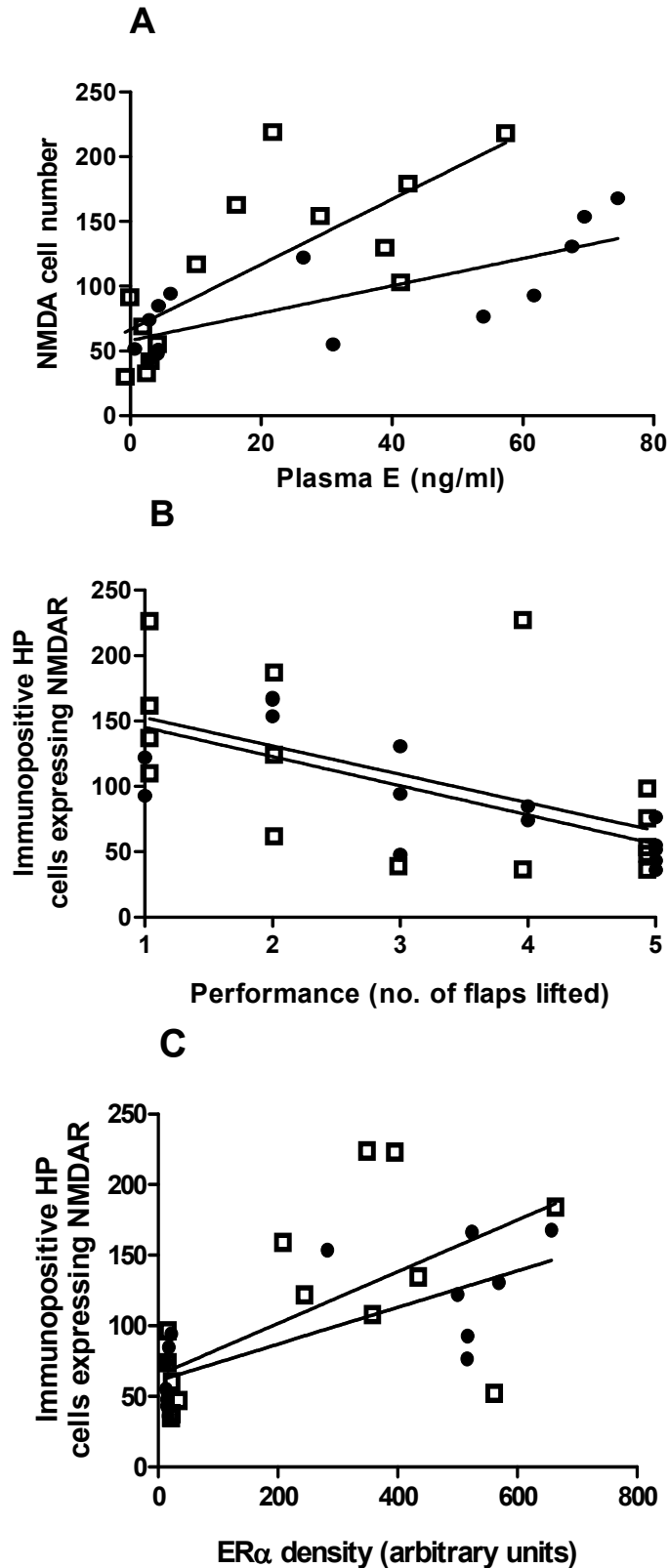


*Spatial performance and plasma oestrogen and the number of hippocampal cells expressing NMDA receptors*

The number of hippocampal cells expressing NMDA receptors were positively correlated with plasma oestrogen levels in males ( $r^2 = 0.60$ ,  $n = 14$ ,  $p = 0.0013$ ) and in females ( $r^2 = 0.56$ ,  $n = 14$ ,  $p = 0.002$ ; Figure 12A). The number of hippocampal cells expressing NMDA receptors were positively correlated with spatial performance on Day Five of testing in males ( $r^2 = 0.55$ ,  $n = 15$ ,  $p = 0.0016$ ) and females ( $r^2 = 0.29$ ,  $n = 15$ ,  $p = 0.039$ ; Figure 12B).

*Hippocampal ER $\alpha$  mRNA expression and NMDA receptor cell number*

The number of hippocampal cells expressing NMDA receptors were positively correlated with ER $\alpha$  expression in males ( $r^2 = 0.58$ ,  $n = 15$ ,  $p < 0.001$ ) and in females ( $r^2 = 0.38$ ,  $n = 15$ ,  $p = 0.015$ ; Figure 12C).



**Figure 12** The significant relationship between hippocampal (HP) NMDA receptor (NMDAR) protein levels and (A) plasma oestrogen (B) spatial performance and (C) oestrogen receptor (ER $\alpha$ ) levels 30 min following treatment on Day Five (n = 28 for A; n = 30 B & C; males = circles, females = squares).

*Effect of five days of oestrogen treatment on hippocampal NMDA and AMPA receptor binding*

Oestrogen treatment had no significant effect on NMDAR binding in the hippocampus ( $F_{2,24} = 0.35$ ,  $p = 0.71$ ). There was no significant difference between males and females ( $F_{1,24} = 0.73$ ,  $p = 0.40$ ) and there was no interaction between sex and treatment ( $F_{2,24} = 0.24$ ,  $p = 0.79$ ). Oestrogen treatment did not have a significant effect on AMPAR binding in the hippocampus (two-way ANOVA,  $F_{2,24} = 0.79$ ,  $p = 0.46$ ). There was no significant difference between males and females ( $F_{1,24} = 0.05$ ,  $p = 0.82$ ) and there was no interaction between sex and treatment ( $F_{2,24} = 0.16$ ,  $p = 0.85$ ).

Hippocampal NMDAR binding was not significantly correlated with plasma oestrogen levels in males (linear regression:  $r^2 = 0.002$ ,  $n = 14$ ,  $p = 0.89$ ) or females ( $r^2 = 0.13$ ,  $n = 14$ ,  $p = 0.20$ ). The sample size for both males and females was reduced by one as the oestrogen plasma levels were out of detection range.

Hippocampal NMDAR binding was not significantly correlated with spatial performance on day five of testing in males (NMDA:  $r^2 = 0.06$ ,  $n = 15$ ,  $p = 0.38$ ) or females (NMDA:  $r^2 = 0.06$ ,  $n = 15$ ,  $p = 0.36$ ).

Hippocampal AMPAR binding was also not significantly correlated with plasma oestrogen levels in males ( $r^2 = 0.09$ ,  $n = 14$ ,  $p = 0.29$ ) or females ( $r^2 = 0.21$ ,  $n = 14$ ,  $p = 0.10$ ). The sample size for both males and females was reduced by one as the oestrogen plasma levels were out of detection range. Hippocampal AMPAR binding was also not significantly correlated with spatial performance on day five of testing in males ( $r^2 = 0.09$ ,  $n = 15$ ,  $p = 0.27$ ) or females ( $r^2 = 0.09$ ,  $n = 15$ ,  $p = 0.28$ ).



## **Discussion**

Five days of daily ingestion of testosterone or oestrogen led to improved spatial performance in both sexes. The testosterone-induced improvement was blocked when testosterone was administered in conjunction with the aromatase inhibitor fadrozole, suggesting that testosterone exerts its effect through an oestrogenic action. Five days of oestrogen treatment increased ER $\alpha$  mRNA expression in the hippocampus in both males and females which was positively correlated with increased spatial performance. Plasma oestrogen levels were also positively correlated with ER $\alpha$  mRNA expression and with spatial performance. Additionally, five days of oestrogen treatment significantly increased the number of hippocampal cells expressing NMDA receptors in males and females, and numbers of NMDA receptor immunopositive cells were positively correlated with both plasma oestrogen levels and with spatial performance on day five of testing in both sexes. Furthermore, the number of hippocampal cells expressing NMDA receptors were positively correlated with hippocampal ER $\alpha$  mRNA expression.

It appears that oestrogen is the major contributor to the cognitive improvement we observed, as the improvements in spatial performance induced by testosterone treatment were mimicked by treatment with oestradiol and blocked by injection of the aromatase inhibitor fadrozole. Although the importance of both androgenic and oestrogenic metabolites in birds has been illustrated in the activation of male aggression (Schlinger and Callard, 1989) and copulatory behaviour (Watson and Adkins-Regan, 1989a; b), to our knowledge, it has not previously been shown that oestrogen is essential to elicit an improvement in spatial memory in both sexes. However, seasonal changes in aromatase activity occur in the hippocampus in the Lapland longspur (*Calcarius lapponicus*), with highest activity during the incubation phase, with concurrent decreases in hippocampal 5 $\alpha$ -reductase activity, which suggests that the hippocampus favours the production of oestrogens over androgens during incubation, when males range over larger areas (Hunt et al., 1995; Soma et al., 1999). Additionally, one previous study suggests the importance of the aromatisation of oestrogen in the acquisition of a spatial task in males using castrated

zebra finches which were implanted with either testosterone, dihydrotestosterone or oestradiol: birds were tested once a day in task in which the rewarded well was in the same location each day and performance over five day periods was binned and then the change in performance (improvement) between these blocks was compared to assess the rate of acquisition between the different treatment groups over a 20 day period. Birds implanted with the non-aromatisable dihydrotestosterone did not learn the task (the number of flaps lifted did not differ from chance across the 20 days) and the oestrogen group learnt the task most rapidly than the other groups (Oberlander et al., 2004).

The level to which circulating oestrogen is increased appears to be important as impairments in performance are seen when levels become too high, and there may be an inverse U-shaped relationship between plasma levels and spatial performance, as is the case with testosterone and possibly also corticosterone (Breuner et al., 1998; Gouchie and Kimura, 1991; Moffat and Hampson, 1996). In this experiment, the plasma levels of oestrogen appear to remain within the optimal range following treatment, as the increased plasma oestrogen levels were associated with better spatial performance in both males and females. It seems unlikely that the improvement in cognitive performance across the five days was due to practice at the task as we saw no improvement in cognitive performance across the five days of testing in any of the three vehicle-treated blocks. It also seems unlikely that the hormone treatment changed activity or boldness levels of the birds, as hormone treatment did not change the latency to solve the task. However, it is difficult to quantify the contribution of sex steroid actions on the reward system as both testosterone and oestrogen activate dopaminergic neurones in the nucleus accumbens, at least in mammals (Frye, 2007; Packard et al., 1998; Schroeder and Packard, 2000; Walf et al., 2007).

In addition to improving spatial performance, oestrogen appears to be positively auto-regulating its own actions and the sensitivity of the hippocampus through increases in its receptor expression as there was a positive correlation between plasma oestrogen levels and hippocampal ER $\alpha$  mRNA expression in both males and

females on day five on treatment. Oestrogen-induced increases in ER $\alpha$  mRNA expression also occur in mammals, and may be involved in oestrogen regulation of spine density: in rat hippocampal slice cultures oestradiol treatment leads to an increase in the density of spines and blocking of ER $\alpha$  (by ICI 182,780) suppresses the enhancing effect of oestradiol whereas treatment with an ER $\alpha$  agonist (propyl-pyrazole-trinyl-tris-phenol; PPT) induces the same enhancing effect of spinogenesis as that induced by oestradiol (Bohacek and Daniel, 2009; Iivonen et al., 2006; Murakami et al., 2006). Rather disappointingly, the immunocytochemical detection of ER $\alpha$  protein in the birds in the present study was at the limits of detection and not quantifiable.

It seems that in birds, as in mammals, oestrogen improves spatial performance in males and females via actions on the NMDA receptor as five days of oestrogen treatment lead to an increased number of hippocampal cells expressing NMDA receptors and there was a positive correlation between the number of NMDA receptor immunopositive cells and spatial performance. This is comparable to the findings in castrated male zebra finches following an oestrogen implant (Saldanha et al., 2004). It may be that it is an up-regulation of receptor number as opposed to increased NMDA receptor binding that underlies the improvement in spatial performance as we saw no effect of oestrogen treatment on binding in the hippocampus. In rats, in addition to oestrogen-induced increases in hippocampal NMDA receptors at the transcriptional and protein level, binding is altered by oestrogen levels, both in the short and longer-term: two days of oestrogen treatment increases hippocampal NMDA-receptor binding and ovariectomy (for 2 weeks) in rats decreases binding, which is prevented by oestrogen replacement (Adams et al., 2001a; Cyr et al., 2001b; Gazzaley et al., 1996; Romeo et al., 2005a; Weiland, 1992). However, the effects of increased binding on spatial memory were not investigated. Although the effects of oestrogen on NMDA binding in birds have not been addressed, increases in NMDA binding do seem to be involved in memory and occur following a one-trial passive avoidance task with domestic chicks (*Gallus domesticus*): training increases binding to NMDA-sensitive [3H]glutamate receptors in two regions of the forebrain (the intermediate and medial hyperstriatum ventrale

and the lobus parolfactorius), important for memory following passive avoidance training and when electric shock follows training, increases in binding only occur in electro-shocked chicks which showed recall of the aversive experience but were absent in avoidance-trained chicks rendered amnesic by electro-shock (Steele et al., 1995; Stewart et al., 1992). Our lack of change in binding is unlikely to be timing dependent as changes occurred in the chick after 30 min, and may not reflect different mechanisms involved in different types of memory or brain regions involved therein as the changes in binding in mammals occurs in the hippocampus. It is however possible that the changes in binding occurring in our study were too low to be detected. There also appears to be a complicated mechanism by which levels of NMDA binding are related to spatial memory in birds as although there are difference in [3H]glutamate binding between food storing marsh tits (*Parus palustris*) compared to non-food storing blue tits (*P. caeruleus*), only occurring in the hippocampus and parahippocampus, the binding is lower in food storers compared to non-storers which is rather surprising given the greater spatial demand in food storers (Stewart et al., 1999).

Although the number of NMDA receptor expressing cells was increased by oestrogen in males and females, the magnitude of the increase was greater in females. Sex differences in NMDA receptors in response to oestrogen are also seen in mammals, for example in rats oestrogen treatment only increases NMDA binding in gonadectomized females but not males (Romeo et al., 2005a). This may underlie sexually dimorphic effects of oestrogen on spine density changes as in females oestrogen induces increases in spines via NMDA receptors, however, whether this happens in males is unclear. It seems that testosterone may be acting directly on hippocampal androgen receptors to increase spine density in males rather than indirectly via local oestrogen biosynthesis as following gonadectomy, treatment with testosterone or dihydrotestosterone increases spine density to levels comparable with intact males, but oestradiol treatment does not (Lee et al., 2004b; Leranath et al., 2003; Murakami et al., 2006). Androgens can increase NMDA receptor binding in males, and NMDA receptors can increase spine density, but whether testosterone-induced increases in spine density are NMDA dependent has not been addressed

(Kovacs et al., 2003b; Leranth et al., 2004a; Romeo et al., 2005b). As oestrogen is important in improving spatial performance in males there must be another mechanism underlying oestrogen's actions, which may not depend as heavily on NMDA receptors as it does in females. The mechanisms underlying the difference in oestrogen's actions between the sexes is unclear but it does not appear to be mediated in the zebra finch by ER $\alpha$  receptor expression, as oestrogen treatment increased levels equally in both males and females.

In conclusion, we propose that oestrogen, produced by aromatisation of testosterone in the hippocampus, improves spatial cognition in birds through mechanisms involving increases in sex hormone and NMDA receptors. It is not yet clear what signalling cascades lead to increased NMDA receptor levels in birds and what, if any, sex differences exist (Balthazart and Ball, 2006; Bjornstrom and Sjoberg, 2005; Colbran, 2004; Cornil et al., 2006a; Lau et al., 2004; Lee et al., 2004a; Mannella and Brinton, 2006; Murphy and Segal, 1996; Pozzo-Miller et al., 1999; Qiu et al., 2008; Sawai et al., 2002; Szego et al., 2006; Tang et al., 2008; Wade and Dorsa, 2003; Woolley, 2007; Zhao et al., 2005; Zhou et al., 2005). These gaps in our current knowledge could be addressed by experiments that compare gene expression data following oestrogen treatment.

## **Appendix**

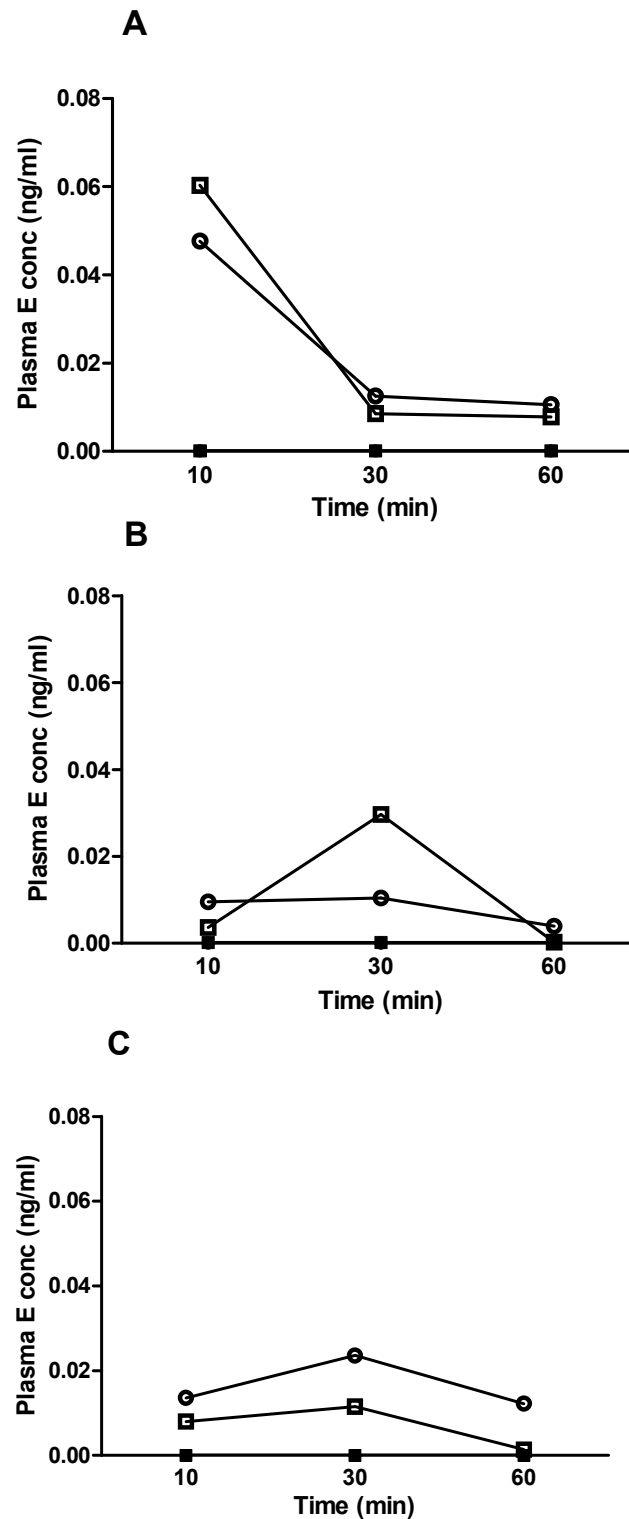
### **Pilot experiment: Establishing oestrogen parameters**

#### *Materials and methods*

Prior to behavioural testing, (n=18; male n=9, female n=9) 100µl blood samples were taken at 10am from the alar wing vein of male (n = 9) and female (n = 9) adult zebra finches using a 25G needle and collected into heparinised capillary tubes to establish baseline circulating levels of oestrogen. Samples were centrifuged, and plasma samples were recovered with a Hamilton syringe and stored at -20C until they were processed. Two weeks later birds were given either 2.5 mg/ml, 1.25mg/ml or 0.25 mg/ml (3µl in 95% ethanol) of oestradiol injected in cucumber and bled between 10 and 11am either 10, 30 and 60 minutes later. Two birds (one male, one female) were tested for each time point/ concentration combination. Oestradiol plasma levels were determined using an ultra-sensitive oestradiol RIA kit (DSL-4800) as described in Chapter Two. All samples were run in a single assay. The binding was 29.5% and non-specific binding was 2.6%. The intra-assay coefficient of variation was 8.1%.

#### *Results*

As the sample size was only two for each time point for each concentration, no statistical analysis could be done. At all three concentrations, oestrogen treatment increased circulating levels of oestrogen in both males and females. The higher dose of 2.5 mg/ml lead to a rapid increase in circulating levels (within 10 minutes), which returned close to baseline levels by 30 minutes in both males and females. The 1.25mg/ml dose of oestradiol increased plasma oestrogen levels after 30 minutes in both males and female, which returned to baseline by 60 minutes (Figure 13). The greatest increase following treatment was after 10 minutes following treatment with of 2.5 mg/ml of oestrogen, and after 30 minutes following 1.25 and 0.25 mg/ml.



**Figure 13** Changes in blood plasma oestrogen (E) levels at various time points (10, 30 and 60 min) following ingestion of cucumber injected with (A) 2.5 mg/ml (B) 1.25 mg/ml or (C) 0.25 mg/ml oestrogen in ethanol (males: squares; females: circles). Circulating oestrogen levels prior to treatment are depicted by filled shapes and plasma oestrogen levels after 30 min are depicted by empty shapes. For each time point at each concentration n = 2 (1 male and 1 female).

These results confirm that ingestion of oestrogen in cucumber is a reliable and efficient non invasive method for oestrogen delivery. As a result of these findings, a concentration of 1.25mg/ml was used in the hormone manipulation experiment and was administered in cucumber to birds 30 minutes prior to testing on the spatial task. In the experiments described in this chapter, the testosterone concentration was lowered slightly from the previous experiment described in Chapter Four (1mg/ml) as 1mg/ml lead to plasma levels slightly higher than physiological levels and in some birds with higher baseline levels, this increase had negative effects on performance.



## Chapter 6

### The effect of five days of oestrogen treatment on hippocampal gene expression

#### **Abstract**

Medium-term oestrogen treatment leads to improvements in spatial cognition in the zebra finch, with associated increases in hippocampal sex steroid and NMDA receptor expression. To elucidate the pathways involved in this improvement, we investigated the genes regulated by oestrogen. We used microarray technology to compare hippocampal gene expression in zebra finches following five days of oestrogen treatment with the expression in the hippocampus of three groups of control birds. These birds received nothing, vehicle or fadrozole plus oestrogen over five days. Five days of oestrogen treatment led to the differential expression of 823 genes in the hippocampus compared to control groups. Of these 823 genes, 19 were identified as being involved in learning and memory, the expression of 11 of which were up-regulated as a result of oestrogen treatment and eight were down-regulated. Five days of oestrogen treatment appears to improve spatial memory through effects on genes involved in neurotransmitter release and reuptake, as well as by altering genes linked to transmitter receptors levels and intracellular signalling molecules. Although there were commonalities between the sexes in the memory-related genes regulated by oestrogen, eight genes showed differential regulation between males and females.

Note: This work was carried out in collaboration with Dr Saldanha and his student Brad Walters at Lehigh University, USA, where the hormone manipulation work was carried out. The gene expression arrays were run at the Roslin Institute, Edinburgh, with the assistance of ARK Genomics.

## **Introduction**

The long term changes involved in the formation of memory, including spatial memory, depend on hippocampal gene transcription and the synthesis of new proteins such as the glutamate AMPA ( $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptor and structural proteins involved in the remodelling of dendritic spines (Dudai, 2004; Leuner et al., 2003; Silva and Giese, 1994). In mammals, the synthesis of new proteins occurs when the glutamate N-methyl-D-aspartate (NMDA) receptor becomes activated. This results in an influx of calcium that triggers the activation of specific calcium-dependent signal transduction pathways culminating in the phosphorylation and activation of the transcription factor cyclic AMP/cAMP-responsive-element-binding protein (CREB). CREB binds to response elements in the DNA leading to changes in gene transcription (Adams et al., 2001c; Bannerman et al., 1995; Bliss and Collingridge, 1993; Bolhuis and Reid, 1992; Kawabe et al., 1998; Lee and Kesner, 2002; Niewoehner et al., 2007; Tsien et al., 1996). Rodents tested in mazes (the Morris water maze and t-maze) show distinct temporal hippocampal gene expression profiles relative to naïve untrained animals, with increased expression of genes involved in calcium signalling, such as Ras activation, kinase cascades and extracellular matrix function, which may regulate neural transmission, synaptic plasticity, and neurogenesis (Cavallaro et al., 2002a; Leil et al., 2003; Luo et al., 2001).

The identification of 'memory' genes has been enormously enhanced by the advances in microarray technology as it enables the monitoring of the parallel expression of thousands of genes. This allows the examination of varied transcription processes, as opposed to the screening of only a small fraction of genes achieved using older methods such as RNA fingerprinting (Cavallaro et al., 2002b; Cavallaro et al., 1997; Zhao et al., 2000). The identification of important genes in the regulation of memory may provide targets for pharmacological modulation of pathways, allowing the development of new therapeutic approaches to improve learning and memory. For example, exogenous administration of fibroblast growth factor (FGF)-18, implicated to be important in memory retention by microarray

studies, improved spatial learning behaviour in rats (Cavallaro et al., 2002a). More recently the investigation into genes involved in behaviour in birds has been possible thanks to the creation of cDNA libraries from zebra finches and the development of high-throughput molecular resources and the use of these techniques has revealed a set of 33 genes regulated in forebrain vocal nuclei by singing behaviour (Wada et al., 2006). However, the genes involved in the regulation of spatial behaviour in birds still require elucidation.

Oestrogen-induced improvements in spatial behaviour and hippocampal morphology, such as spine density, involve changes in gene transcription (Gould et al., 1990; Gresack and Frick, 2006; Lacreuse et al., 2002; Murphy and Segal, 1996). This involves oestrogen binding with its receptor, either directly altering gene transcription through binding of the oestrogen-receptor complex to specific regions of DNA or indirectly by the stimulation of pathways leading to phosphorylation and activation of transcription factors such as CREB (Behl, 2002). CREB phosphorylation has been implicated in oestrogen-induced spine formation in the rat hippocampus, which may occur through actions of activated CREB on the NR1 subunit of the NMDA receptor (Lau et al., 2004; Murphy and Segal, 1996; Zhao et al., 2005). In mammals, oestrogen can alter transcription of hippocampal genes encoding proteins important in memory formation, following chronic treatment or even within one hour after a single dose (Aenlle et al., 2009; Pechenino and Frick, 2009). For example, oestrogen can modulate cellular architecture through targeting a group of genes coding for structural proteins in rodent neurones, such as neurofilament and microtubulin-associated proteins (Ferreira and Caceres, 1991; Scoville et al., 1997; Shughrue and Dorsa, 1993). Whether there is a generalised mechanism for sex hormone-induced changes in hippocampal morphology in vertebrates through NMDA receptors is unknown, but it seems feasible in birds as the androgen receptor (AR), oestrogen receptor alpha (ER $\alpha$ ) and NMDA receptor are all present in the avian hippocampus and seem to play a role in spatial memory and their expression is increased in response to oestrogen (Margrie et al., 1998; Meehan, 1996; Saldanha et al., 2004; Shiflett et al., 2004).

Although oestrogen improves spatial performance in both male and female zebra finches, it is possible that different signalling cascades are involved. For example, as in mammals, there may be sex differences in the kinases required (Mizuno et al., 2007; Mizuno et al., 2006). Calmodulin-dependent protein kinase kinase alpha (CaMKKalpha) is a kinase important for contextual fear memory formation in male but not female mice. In males, contextual fear conditioning induces CaMKKalpha-dependent up-regulation of hippocampal mRNA expression of brain-derived neurotrophic factor (BDNF), while in females contextual fear conditioning induces down-regulation of BDNF expression that does not require CaMKKalpha (Mizuno et al., 2006). Additionally, in male mice CaMKKbeta is required for spatial memory formation and for activation of the transcription factor CREB in the hippocampus by spatial training but it is not required for spatial memory formation in females (Mizuno et al., 2006; Peters et al., 2003). Female mice thus must activate a different kinase pathway to phosphorylate CREB and trigger memory formation as female *Camkk2* (the gene for CaMKKbeta) null mutants are not impaired in spatial memory formation and they have the same level of hippocampal CREB phosphorylation after spatial training as do female wild-type mice (Mizuno et al., 2007). Altered transcription due to alternative splicing may contribute to these sex differences in memory formation. Hippocampal mRNA expression of two splicing factors (*Srp20* and *Psf*) is altered in CaMKKbeta-deficient male mice. In wild-type mice, the basal expression levels in the hippocampus of these splicing factors are higher in males than in females. Training in hippocampus-dependent learning tasks leads to up-regulation of these factors in a sex-dependent manner; the hippocampal mRNA expression of both splicing factors is increased in males, however, the increase in *Srp20* mRNA expression does not occur in females (Antunes-Martins et al., 2007). Sex differences occur also in transcription factors important in the modulation of synaptic plasticity and memory, such as the nuclear factor-kappa B (NF-kappaB) family. Female, but not male, transgenic mice over-expressing an inhibitor of NF-kappaB have robust deficits in hippocampal-dependent learning and memory (Bracchi-Ricard et al., 2008). The effects of oestrogen on hippocampal gene transcription have previously been investigated in mice, however sex differences in expression levels were not determined as only females were used (Pechenino and

Frick, 2009). The effect of oestrogen on gene expression in birds has not been addressed and the next step is to use gene arrays to address the specific mechanisms of oestrogen on hippocampal genes and potential sex differences in these expression profiles.

The aim of this study was to determine the genes implicated in oestrogen-induced improvements in spatial memory in the adult zebra finch by examining gene expression in the hippocampus using a zebra finch microarray. In addition, potential sex differences in gene expression profiles were addressed by comparing treatment effects in both males and females.

## **Material and methods**

### *Subjects*

Thirty-two adult zebra finches (16 males, 16 females, all >90 days post hatching) were obtained from Magnolia Bird Farm (Anaheim, CA, U.S.A.) and housed in individual cages (18" x 18" x 16") in the Biological Sciences Animal Facility at Lehigh University, PA, USA. Birds were maintained at a temperature of  $74 \pm 2^\circ\text{F}$ , and on a 14:10 light:dark cycle (on at 6am) with food (bird seed mixture) and water *ad libitum*. All housing and experimental conditions were in accordance with guidelines set forth by Lehigh University's Institutional Animal Care and Use Committee.

### *Hormone manipulation*

Zebra finches were each fed one piece of cucumber (skinned and cut into square pieces approximately  $40\text{mm}^3$ ) per day for three days in order to acclimatise the animals to the novel food. Each piece of cucumber was placed in the cage on a raised white plastic platform (approx. one cubic inch in size) and 30 minutes after presentation the cages were inspected to confirm that the food had been eaten. On the fourth day, the finches were sorted into four experimental groups, each consisting of eight birds (four males and four females):

Group 1: for five consecutive days, birds were fed pieces of cucumber as before but not injected with anything

Group 2: received cucumber injected with  $3\mu\text{l}$  of vehicle (95% ethanol)

Group 3: received the aromatase inhibitor fadrozole (Wade et al., 1994) (70mg/ml in  $3\mu\text{l}$  of 95% ethanol), a gift from Novartis (East Hanover, NJ, U.S.A.)

Group 4: received fadrozole (70 mg/ml) and  $17\beta$ -oestradiol (1.25 mg/ml, Steraloids Inc. Newport, RI, U.S.A.) in  $3\mu\text{l}$  of 95% ethanol. Fadrozole was included to block in vivo synthesis of oestrogen.

The concentrations for fadrozole and oestrogen were chosen based on previous studies, where daily, oral administration of  $200\mu\text{g}$  of fadrozole will significantly diminish aromatase activity in the zebra finch hippocampus (Saldanha et al., 2004),

and daily administration of 3.75ng of oestrogen can dramatically increase circulating levels of oestrogen leading to significant improvements on a spatial memory task (Chapter 5). Throughout the experiment, the cucumber pieces were presented between 1300 and 1600 hours each day, and the cages were inspected every ten minutes following feeding to make sure that the cucumber was eaten. All of the experimental animals ingested the cucumber pieces presented to them within 30 minutes.

#### *Tissue and plasma collection*

On the fifth day of treatment, the birds were given cucumber between 1300 and 1600 hours, but in a staggered fashion to allow for the sequential collection of blood and tissue samples. Animals were checked every ten minutes after initial delivery of cucumber, and 50 minutes after confirmed ingestion (no more than 60 minutes after ingestion) the birds were decapitated with heparinized dissecting scissors and trunk blood was collected in heparinized tubes (heparin sulfate salt 0.51mg/mL of dH<sub>2</sub>O, Sigma-Aldrich, St. Louis, MO, U.S.A.). Blood samples were then stored on ice until they were centrifuged. Immediately following blood collection, the brain of each animal was rapidly dissected out and snap frozen in isopentane on dry ice. Brains were then wrapped in aluminium foil and placed on dry ice before being stored at -80°C. Subsequently, blood samples were centrifuged at 2400xg for 10 minutes at 4°C and the resulting plasma was transferred to sterile microcentrifuge tubes and stored at -20°C until being processed using ultra sensitive oestradiol kit (DSL4800) as described in Chapter 2. All samples were run in a single assay. The binding was 27% and non-specific binding was 1.63%. The intra-assay coefficient of variation was 7.3%. At the time of collection, plasma and brain tissue samples were coded with 4 digit numbers obtained using a random number generator (<http://www.random.org>).

#### *RNA preparation, amplification and dye coupling*

The hippocampus was dissected out while still frozen using a sterile blade and the hippocampal RNA for each sample was isolated then amplified using reverse transcription with an oligo(dT) primer bearing a T7 promoter and *in vitro*

transcription of the resulting DNA with T7 polymerase to generate antisense RNA (aRNA) copies of each mRNA. Aminoallyl UTP was incorporated into the aRNA (aaUTP aRNA) during the *in vitro* transcription reaction, which was then used directly in the dye coupling reaction to fluorescently label the RNA.

#### *Hybridisation to arrays and scanning*

The fluorescently labelled amplified RNA targets were hybridised onto Agilent manufactured arrays using Agilent hybridisation chambers and oven. There was one array per bird and four arrays per slide. The resulting fluorescent intensities, detected on scanning the slides with the appropriate lasers to excite the fluorescent label, provided the levels of expression of the particular DNA product in the tissues from which the RNAs were derived. The slides were then scanned according to the manufactures instructions, at 5µm on the Axon 4200AL scanner using the Gal file supplied by Agilent for autoPMT. The raw image data were then extracted using Feature Extraction (Agilent software) for further analysis.

#### *Analysis*

Three different lists of genes that significantly changed in response to oestrogen compared to the control groups (nothing, vehicle and fadrozole) were generated using Partek Genomics Suite (one way ANOVA with Bonferroni correction  $p < 0.05$ ; <http://www.partek.com/partekgs>): list 1 = nothing vs oestrogen plus fadrozole; list 2 = vehicle vs oestrogen plus fadrozole; list 3 = fadrozole vs oestrogen plus fadrozole. In order to reduce the chance of a type I error and lower the identification of false positives, a final list of differentially expressed genes was restricted to those genes whose altered expression was common in at least two out of the three lists mentioned above. Lists for genes altered by oestrogen treatment in males only and females only were generated in the same way.

The final list of genes differentially expressed by oestrogen treatment was then analysed to identify genes within it associated with learning and memory using Core Analysis in Ingenuity Pathway Analysis Software (<http://www.ingenuity.com>). Ingenuity Software provides information on the functions of different genes based on findings from peer-reviewed literature and additionally it links the genes of interest



into pathways. It therefore was used to identify the main biological categories and functions associated with the genes that were changed. I then selected genes whose function was associated with learning and memory functions.

Note:

An original five day hormone manipulation experiment and tissue collection was carried out at the University of Edinburgh, in which brains were stored in RNAlater. However, unfortunately, the hippocampal material could not be run in the arrays due to high levels of background contamination from RNA degradation problems. Due to a lack of zebra finch facilities remaining at Edinburgh University, the experiment was repeated in collaboration with Dr Saldanha. In an attempt to overcome the contamination problem, in the repeated experiment the brains were immediately frozen and remained so until the RNA was extracted.

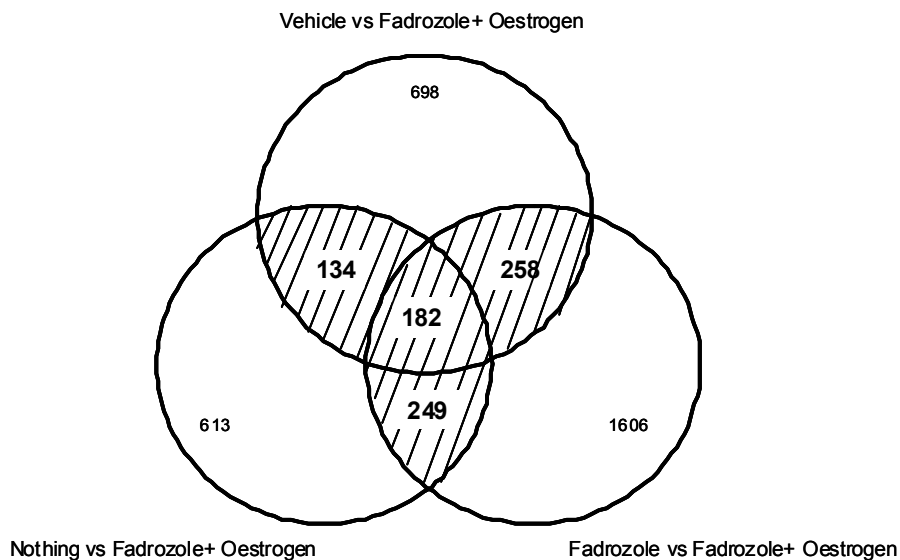
## **Results**

### *Plasma oestrogen levels*

In the control groups (nothing, vehicle and fadrozole) the oestrogen levels in 87% (20/23) of the samples were below detection levels, whereas in the fadrozole plus oestrogen group 100% of the samples fell within the detection range and the average level was  $54.4 \pm 36.3$  pg/ml (mean  $\pm$  SEM). As many of the plasma oestrogen levels were too low for detection, no statistical analysis could be carried out. However, these results confirm that oestrogen treatment lead to increases in plasma oestrogen levels compared to control birds.

### *Gene expression levels*

Using Partek analysis, list 1 comparing genes differentially regulated between the nothing control group and the fadrozole plus oestrogen group contained a total 1178 genes, list 2 comparing the vehicle control group and the fadrozole plus oestrogen group contained 1272 differentially regulated genes and list 3 comparing the fadrozole control group to the fadrozole plus oestrogen group contained 2295 genes (Figure 1). Of the genes in these three lists that were significantly altered by oestrogen treatment, 823 were significantly differentially expressed in at least two of the three groups and this list was taken as the genes regulated by oestrogen and used in the analysis to identify genes involved in memory (Figure 1). Of the 823 genes, the expression of 367 genes was up-regulated as a result of oestrogen treatment and the expression of 456 genes was down-regulated by oestrogen.



**Figure 1** The number of genes identified as being altered by oestrogen treatment (oestrogen plus fazole) compared to the three control groups (nothing, vehicle and fazole) using Partek ( $p < 0.05$ ). The 823 genes that were differentially regulated in response to oestrogen in at least two out of the three comparisons were taken as the final list of regulated genes (in the shaded area).

Based on details provided about gene function in Ingenuity, 19 genes linked to learning and memory were identified as being significantly differently expressed in oestrogen treated birds compared to controls. Details of the functions of the selected genes, as determined by Ingenuity, are shown in Table 1. Of those genes, 11 were up-regulated following treatment and eight were down-regulated (Table 2). When the gene lists for females only and males only were compared five genes were commonly regulated between the sexes, five genes were found to be altered only in females and four genes only in males.

	Symbol	Name	Function Annotation
Enzyme	ADCY1	adenylate cyclase 1 (brain)	synthesis of cyclic AMP long-term memory memory of mice memory long-term potentiation of cerebellum
	GUSB	glucuronidase, beta	spatial learning by rodents learning by rodents
	NCF1	neutrophil cytosolic factor 1	memory of mice memory
Transmembrane receptor	CHRNA1	cholinergic receptor, nicotinic, alpha 1 (muscle)	neurotransmission synaptic transmission
G-protein coupled receptor	DRD2	dopamine receptor D2	release of L-glutamic acid neurotransmission synaptic transmission long-term memory memory of mice memory learning by rodents
	NPY5R	neuropeptide Y receptor Y5	synaptic transmission
Ion channel	GABRA3	gamma-aminobutyric acid (GABA) A receptor, alpha 3	neurotransmission synaptic transmission
	KCNJ5	potassium inwardly-rectifying channel, subfamily J, member 5	memory of mice memory spatial learning by rodents learning by rodents
	KCNMB2	potassium large conductance calcium-activated channel, subfamily M, beta member 2	neurotransmission
	SCN1A	sodium channel, voltage-gated, type I, alpha subunit	neurotransmission
Transporter	NRXN3	neurexin 3	secretion of neurotransmitter neurotransmission synaptic transmission
	SLC6A1	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	neurotransmission synaptic transmission
Kinase	SGK1	serum/glucocorticoid regulated kinase 1	long-term memory spatial learning by rodents learning by rodents memory
Unidentified	SYN2	synapsin II	secretion of neurotransmitter release of L-glutamic acid neurotransmission synaptic transmission
	ADM	adrenomedullin	secretion of catecholamine suppression of cyclic GMP synthesis of cyclic AMP
	DTNBP1	dystrobrevin binding protein 1	release of L-glutamic acid
	ERC2	ELKS/RAB6-interacting/CAST family member 2	secretion of neurotransmitter
	GJC1	gap junction protein, gamma 1, 45kDa	neurotransmission synaptic transmission
	CORT	cortistatin	neurotransmission synaptic transmission

**Table 1** Details of the function of oestrogen-regulated genes indentified by Ingenuity and based on this information selected by myself as being involved in learning and memory.

Gene Name	Symbol	p-value			Fold change		
		All	Female	Male	All	Female	Male
<b>Up-regulated</b>							
0203P0034K15	ADM	0.02	0.01	0.02	1.51	1.97	1.51
0203P0021A20	ERC2	0.03			1.59		
0058P0001C09	CHRNA1	0.03		0.03	1.35		1.35
Zebra_Finch_D2_long	DRD2	0.03		0.03	1.69		1.69
0058P0008C02	DTNBP1	0.01			1.13		
0203P0036H12	GJC1	<0.05	0.04	<0.05	1.67	2.43	1.67
0205P0013K10	KCNJ5	0.01	0.01		1.14	0.49	
0203P0066C13	NRXN3	0.02		0.01	1.14		1.18
0057P0001G12	SGK1	0.01	<0.01		1.20	1.37	
0106P0007G05	SLC6A1	0.01	0.01	0.01	1.88	2.37	1.88
0105P0029D12	ADCY1	0.02	<0.01	0.02	1.69	2.34	1.69
<b>Down-regulated</b>							
0061P0017B09	CORT	<0.01	<0.01		2.14	2.57	
0205P0018P21	GABRA3	<0.01	<0.01	<0.01	1.42	1.48	1.58
0203P0033H15	GUSB	0.01	0.01		1.21	1.30	
0106P0002B09	KCNMB2	<0.05	0.02		1.16	1.32	
0205P0023K08	NCF1	0.02		0.02	1.69		1.69
0203P0017J13	NPY5R	0.03			1.35		
0202P0012J18	SCN1A	<0.05			1.65		
0202P0007A24	SYN2	0.02			1.17		

**Table 2** The learning and memory genes significantly differentially expressed by oestrogen treatment compared to controls in zebra finches; all birds, males only and females only ( $P < 0.05$ ).

## **Discussion**

Medium-term treatment with oestrogen led to differential expression of 823 genes in the hippocampus compared to control groups. Of these 823 genes, 19 were identified as being involved in learning and memory, the expression of 11 of which were up-regulated as a result of oestrogen treatment and eight were down-regulated. The differentially expressed genes fell into a diversity of different categories, including enzymes (NCF1, ADCY1, GUSB), receptors and ion channels (DRD2, NPY5R, CHRNA1, KCMNB2, GABRA3, SCN1A, KCNJ5), kinases (SGK1) and transporters (GJC1, NRXN3, SLC6A1/GAT1).

Oestrogen treatment altered the expression of genes associated with neurotransmitter release, receptor expression and reuptake. Firstly, oestrogen altered the expression of genes for ERC2 (ELKS/RAB6-interacting/CAST family member 2) and Synapsin II (SYN2) which are involved in the presynaptic release of neurotransmitters. ERC protein 2 is present in the nerve terminals active zone and regulates neurotransmitter release by binding other proteins in this area (Higa et al., 2007; Ko et al., 2006). The up-regulation of the gene for ERC2 by oestrogen in the zebra finch might act to facilitate the release of neurotransmitters involved in memory processes, such as acetylcholine (Ach), dopamine and glutamate. SYN2 is a member of the synapsin gene family that encode neuronal phosphoproteins which associate with the cytoplasmic surface of synaptic vesicles to facilitate neurotransmitter release (Rosahl et al., 1995; Sudhof et al., 1989). It is not surprising, perhaps, that we saw changes in SYN2 expression in response to oestrogen as expression of this gene also changes across the oestrous cycle in rats but, given its enhancing role in transmitter release, we would have expected an up-regulation rather than a down-regulation of its expression (Diao et al., 2008). However, synapsins are not an essential component of long-term synaptic plasticity as the induction and expression of long-term plasticity (LTP) in the hippocampus, the enhancement in transmitter release and the recovery of the vesicle pool after depletion occurs in synapsin knock-out mice (Spillane et al., 1995; Sun et al., 2006).

Five days of oestrogen treatment up-regulated the expression of the nicotinic Ach receptor gene (*CHRNA1*), which may increase cholinergic signalling and aid in improving memory as extensive evidence supports the view that cholinergic mechanisms enhance learning and memory formation (Gold, 2003). The co-existence of cholinergic receptors and oestrogen receptors on neurones and astrocytes in the rat brain is consistent with the possibility that oestrogen directly modulates cholinergic transmission, as is the block of memory enhancing effects of injection of oestradiol in ovariectomized rats by the acetylcholine receptor antagonist scopolamine (Hösli and Hösli, 1999; Hösli et al., 2001; Hösli et al., 2000; Packard, 1998; Shughrue et al., 2000). Ach regulates the potassium inwardly-rectifying channel subfamily J member 5, which may explain the increased in expression of its gene (*KCNJ5*) following oestrogen treatment. Cortistatin (CORT) is a neuropeptide which impairs memory processing in mice, possibly by antagonism of the excitatory effects of Ach so the down-regulation of its gene expression by oestrogen may enhance the positive effects of Ach on memory (de Lecea et al., 1996; Flood et al., 1997).

Dopamine is another neurotransmitter known to be important in the modulation of memory, acting on five receptor subtypes including the D2 receptor, the activation of which may enhance spatial working memory (Liggins, 2009). Oestrogen treatment up-regulated expression of the *DRD2* gene in the zebra finch hippocampus, similar to effects seen in rats, which may increase the memory enhancing actions of dopamine (Bazzett and Becker, 1994; Hruska et al., 1982; Lévesque et al., 1992). The oestrogen-increased expression of the gene for dystrobrevin binding protein 1 (*DTNBP1*) we found may lead to aid in improvements in memory through the action of dopamine. The sandy (*sd*) mouse, which has a deletion in the *Dtnbp1* gene and expresses no dysbindin-1 protein, shows impaired long-term memory retention and working memory, which appears to be linked to dopamine levels (Hattori et al., 2008; Takao et al., 2008).

Gamma-aminobutyric acid (GABA), although not solely inhibitory, is the most widespread inhibitory neurotransmitter in the vertebrate central nervous system and,

in rats, increases in dendritic spine density are achieved by reducing GABA neurotransmission (Murphy et al., 1998; Sivilotti and Nistri, 1991). Thus the down-regulation of the GABA receptor gene (GABRA3) by oestrogen treatment may reduce the inhibitory actions of GABA, thereby facilitating memory formation. This may be further advanced by the increased removal of GABA from the synaptic cleft and the more rapid termination of its synaptic signalling by the oestrogen-induced up-regulation of the gene coding for the GABA plasma membrane neurotransmitter transporter (SLC6A1 or GAT-1). It appears that the effects of oestrogen on GAT1 may be similar in birds and mammals as reducing oestrogen levels by ovariectomy decreases GAT1 mRNA in female rats and oestrogen treatment returns expression to the levels observed in intact rats and increases GABA transporter activity and GABA uptake (Herbison et al., 1995).

The release of neurotransmitters and the activation of their receptors triggers downstream cascades leading to increases in 3',5'-adenosine monophosphate (cAMP), a positive regulator of synaptic plasticity required for several forms of hippocampus-dependent memory through activation of cAMP-dependent kinases (Eckel-Mahan et al., 2008). Adenylyl cyclases (AC), a group of enzymes important in memory formation, convert adenosine-5' triphosphate (ATP) into cAMP (Poser and Storm, 2001). Several of the genes found to be differentially expressed as a result of oestrogen treatment in the zebra finch are linked to the activity of AC. Firstly, the gene for type I adenylyl cyclase (ADCY1) was up-regulated following oestrogen treatment, which, if translated into the protein, would have direct effects on cAMP production. These increases in ADCY1 may have implications for spatial memory as in transgenic mice that over-express this gene in the forebrain show elevated long-term potentiation (LTP) and increased memory for object recognition, which may be due to enhanced extracellular signal related kinase (ERK)/mitogen-activated protein kinase (MAPK) signalling (Wang et al., 2004). Additionally, the oestrogen-induced increase in the expression of the gene for adrenomedullin (ADM) might increase cAMP production through AC activation, as adrenomedullin activates AC in human cell cultures (Moreno et al., 1999). Conversely, the down-regulation of gene for the neuropeptide Y receptor (NPY5R) by oestrogen may also increase AC



activity, as one of the typical signalling responses of the neuropeptide Y receptor in mammals is the inhibition of AC (Michel, 1991; Olasmaa et al., 1986). A reduction in NPY levels and gene expression in response to oestrogen is also seen in rats, in which the oestrogen receptor alpha and neuropeptide Y are co-expressed on neurones in the hippocampus (Hilke et al., 2009; Nakamura and McEwen, 2005; Shimizu et al., 1996). The oestrogen-induced reductions in NPY expression in the zebra finch hippocampus are likely to have positive effects on spatial performance as transgenic rats with hippocampal NPY over-expression have impaired performance in the Morris water maze (Thorsell et al., 2000). One of the downstream effects of the activation of intracellular signalling cascades and protein synthesis is the growth of dendritic spines, known to improve memory (Leuner et al., 2003; Leuner and Shors, 2004). The up-regulation of the gene for serum/ glucocorticoid regulated kinase (SGK1) by oestrogen treatment in the zebra finch hippocampus may enhance memory through increases dendritic growth, as SGK1 increased the neurite formation of cultured rat hippocampal neurons through microtubule depolymerisation both via directly and indirectly, through the phosphorylation of tau (Yang et al., 2006).

In both males and females the gene encoding type I adenylyl cyclase (ADCY1) and adrenomedullin (ADM), which activates adenylyl cyclase, were up-regulated by oestrogen which suggests there may be a common mechanisms of oestrogen action between the sexes (Moreno et al., 1999). However, the mechanisms leading to AC activation or the down-stream effects may differ between the sexes. Although the GABAergic system seems to be involved in both sexes, as both the GABA receptor (GABRA3) and GABA transporter (SLC6A1) genes are down-regulated in males and females following oestrogen treatment, it is possible that the dopamine and cholinergic systems are affected to a greater degree or in different ways in males compared to females as the acetylcholine and dopamine receptors (CHRNA1, DRD2) were only up-regulated in males. It is possible that the genes for these receptors, or other subtypes, are also regulated in females but their change in expression levels were below statistical detection. In contrast, the regulation of expression by oestrogen of other factors was only detected in females, such as the kinase SGK1, peptide CORT and the potassium channels KCNMB2 and KCNJ5.

Five days of oestrogen treatment improved spatial performance in both male and female zebra finches (Chapter 4) but these differences in gene expression profiles between the sexes suggest that although there is overlap in the mechanisms involved, there is also a sex-specific component.

It appears from our hippocampal gene expression data that the effects of oestrogen involve surprisingly few 'memory' genes in common with those involved in response to a single dose of oestrogen in mice. In female mice, when a single dose of oestrogen was administered, one hour after treatment 23 hippocampal genes previously associated with learning and memory were differentially expressed (identified by the authors based on a PubMed search) (Pechenino and Frick, 2009). Although the majority (83%) of these 'memory' genes were present on our zebra finch gene array, only one of these genes was differentially expressed in response to oestrogen in the zebra finch which was the phospholipase C (PLC) gene. In our study, PLC gamma 2 (PCLG2) and delta 4 (PCLD4) were up-regulated, however in the mouse study PLC expression was down-regulated following treatment. There are multiple possible explanations for the difference in outcome between the mouse data and ours: (1) there may be differences in the definition of 'memory' genes. Our 'memory' genes may not have been identified as being involved in memory based on the authors PubMed search for memory terms. Conversely, their 'memory' genes may not have been identified by our Ingenuity Software analysis; (2) the change in expression of our 'memory' genes may have been below the authors' significance for detection; (3) the difference in results may reflect a temporal expression of genes involved in oestrogen-induced memory improvements. It might be that the genes identified in their study are especially relevant to the acute effects of oestrogen on memory whereas the genes identified in our study become significantly differentially expressed only following five days of treatment; (4) their findings only reflect changes in the female hippocampus.

Here we have shown that the avian hippocampus contains a wide range of genes important for memory, the expression of which is differentially regulated by short-

term oestrogen treatment, sometimes in a sex-specific manner. However, it remains to be determined whether the genes of interest are translated into proteins.

## Chapter 7

### General discussion

In mammals, a large and reliable difference between the sexes is observed in spatial cognition, with males outperforming females on mental rotation, map reading and maze tasks, dependent on levels of circulating sex steroids. Although it is not clear whether sex steroids also improve spatial cognition in birds, birds provide a good model in which to address questions for at least two reasons: (1) there are spatial tasks that do not require handling or food deprivation and (2) methods for the acute non-invasive oral administration of steroids are available, and the combination of these two overcome some of the shortcomings associated with mammalian models.

Coupling a one-trial associative memory task with oral sex steroid manipulations, we addressed several questions pertaining to the acute and medium-term effects of sex steroids on spatial cognition in the adult zebra finch. First we asked whether there were sex differences in spatial performance and in hippocampal sex steroid receptor and aromatase mRNA expression. We found that when circulating steroid levels were low spatial performance did not differ between the sexes, a result that is consistent with the mammalian literature (Galea et al., 1996; Galea et al., 1995). There were also no differences in hippocampal androgen receptor (AR) or oestrogen receptor alpha (ER $\alpha$ ) mRNA expression although males did have higher levels of aromatase mRNA. The next question to ask was what, if any, were the acute effects of testosterone on spatial cognition. A rapid improvement in spatial performance was observed in both sexes as a result of oral administration of a small amount of testosterone which, although not dramatic, raises the question of whether this rapid effect of testosterone is mediated via genomic or non-genomic mechanisms. Given the time frame of hours to days in which genomic effects occur, it is likely to be non-genomic. To experimentally test this hypothesis protein synthesis could be blocked with anisomycin and this would be predicted to have no effect on the testosterone-

induced improvement in performance. Whereas blocking protein kinase A or C (PKA or PKC; for example with Rp-cAMPS or Go 7874, respectively) thought to be important in mediating the rapid behavioural effects of sex steroids in mammals, would be predicted to block the rapid improvement by testosterone (Frey et al., 1988; Kelly and Wagner, 1999; Moss et al., 1997; Rupprecht et al., 2001; Valverde et al., 1999; Vianna et al., 2000a; Vianna et al., 2000b). Testosterone treatment also had rapid effects on hippocampal AR and ER $\alpha$  mRNA expression, although it was not clear whether a single physiologically relevant dose was sufficient to increase receptor protein levels. The increase in AR mRNA may mean that testosterone was acting directly to improve spatial performance, which in mammals occurs via a pathway involving NMDA receptors and PKC (Kovacs et al., 2003a; Leranth et al., 2004a; Leranth et al., 2003; Nguyen et al., 2009; Romeo et al., 2005b) (Figure 1). As ER $\alpha$  mRNA was increased it may be that testosterone also exerted effects via its conversion to oestrogen and although hippocampal aromatase mRNA expression was not rapidly altered, its activity can be increased within this time frame by phosphorylation in mammals (Balthazart et al., 2005; Cornil et al., 2006b). It might, therefore, be worth looking at the acute effects of sex steroid treatment on hippocampal aromatase activity in addition to mRNA expression and the effects of treatment with the aromatase inhibitor fadrozole on testosterone-induced rapid effects.

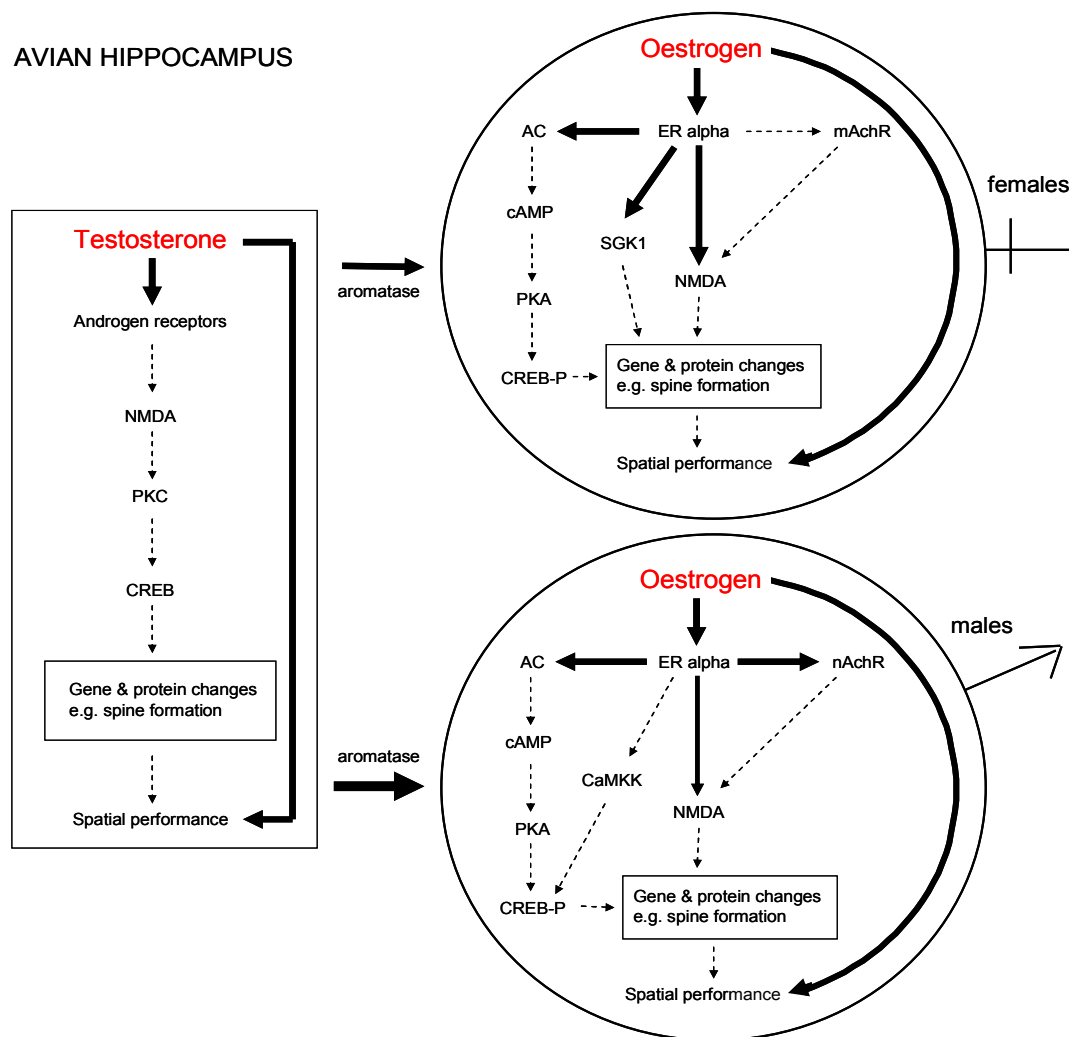
The single dose effects, although promising, were not compelling. We therefore decided to administer hormone orally for five consecutive days, following the positive effects on spatial cognition shown by Hodgson *et al.* (Hodgson et al., 2008). In the experiments described in Chapter 5, both testosterone and oestrogen treatment improved spatial performance significantly. A substantial component of the effects of testosterone appeared to be dependent on its conversion to oestrogen as fadrozole treatment in conjunction with testosterone then blocked the improvement. It may be concluded that aromatase is involved in the improvement in spatial performance, however, it is not clear whether the five days of treatment altered aromatase activity and/or mRNA expression. The underlying mechanisms of the medium-term effects of oestrogen on spatial cognition were partly addressed by looking at changes in

hippocampal N-methyl-D-aspartate (NMDA) receptors, which were increased following treatment. We then showed for the first time that five days of oestrogen treatment lead to the differential expression of nineteen hippocampal genes identified as playing a role in memory. One of the future steps is to determine whether the differential expression of these genes drives changes in hippocampal protein levels. This would allow determination of the relative importance of the different proteins in oestrogen effects on spatial cognition by targeted and selective blocking of different proteins and observing the resulting effects on spatial performance. For example, as oestrogen treatment altered adenylyl cyclase (AC) gene expression in both sexes, blocking AC should lead to impaired spatial performance in both sexes (Figure 1). On the other hand, serine/glucocorticoid regulated kinase (SKG1) gene expression was only increased in females following oestrogen treatment so blocking this kinase may impair spatial performance only in females. Other kinases may be mediating spatial memory in males, for example it appears that calcium/calmodulin kinase kinase beta (CaMKK $\beta$ ) is required for spatial memory formation in male mice but not in females so blocking this in zebra finches may only impair males (Mizuno et al., 2007) (Figure 1).

In addition to the specific points addressed in each chapter, there are several more general issues to remark on arising from the results described in this thesis. Firstly, as the local conversion of testosterone to oestrogen in the hippocampus is necessary to improve spatial cognition significantly, circulating sex steroid levels may not accurately reflect differences between the sexes because there may still be differences in central levels. This highlights the need to look at the bird as a whole when addressing differences between the sexes and not just plasma levels. While it still remains important to look at both circulating levels of testosterone and oestrogen, it may be that, of the two, oestrogen is having the greater impact in spatial cognition, at least in song birds. It is also important to consider that the relative levels of circulating oestrogen when looking at the effects on spatial cognition as, in mammals, increases in oestrogen appear to improve performance only to a certain point after which performance is impaired (Galea et al., 1995; Holmes et al., 2002; Warren and Juraska, 1997). The fertility and parental care hypothesis proposes that

the detrimental effects of high oestrogen levels may have a function in reproduction in mammals to impair spatial abilities and reduce mobility during proestrus so as to lower the risks of predation or encounters with males from other groups to mothers with newly born offspring (Sherry and Hampson, 1997). However, the underlying mechanism leading to impairments are not clear. It is possible that the impairments in spatial ability induced by high oestrogen may be linked to calcium levels and dendritic spine formation in the hippocampus, as calcium concentration effects on spine morphology depends on magnitude; moderate and transient elevations in calcium induce spine elongation and the formation of new spines while large increases cause spine shortening and collapse (Halpain et al., 1998; Korkotian and Segal, 1998; 1999a; b; Miranda et al., 1999; Segal et al., 2000; Segal, 1995). The mechanisms may also involve effects on the cholinergic system as treatment of high doses of oestradiol eliminates the dose-related increase in acetylcholine synthesis seen at lower doses in female rats (Gibbs, 1997). Finally, the reasons behind the enhanced ability of song birds to locally synthesise oestrogen due to exaggerated levels of aromatase present in the hippocampus compared to non song birds and mammals are still not clear. It is likely that the mechanisms involved in the effects of sex steroids on spatial information processing between different taxa are the same, but what the difference in the scale of aromatase levels means remains unknown, as does the functional impact of the differing distribution of aromatase which in male zebra finches is solely in the brain.

## AVIAN HIPPOCAMPUS



**Figure 1** A schematic diagram highlighting the results presented in this thesis (thick arrows) with gaps in our current knowledge based on the mammalian literature represented by dotted lines. Both testosterone (T) and oestrogen (E) treatment improved spatial cognition in zebra finches, which was associated with increases in hippocampal androgen receptors (AR) and oestrogen receptor alpha (ER alpha) and hippocampal N-methyl-D-aspartate (NMDA) receptors. The oestrogen-induced increase in NMDA receptors may be linked to acetylcholine (Ach), working through muscarinic (mAChR) receptors in females (top) and nicotinic (nAChR) receptors in males (bottom). In both sexes various signalling cascades are likely to lead to the phosphorylation of cyclic AMP/cAMP-responsive-element-binding protein (CREB), and potentially increase spine density but these cascades may differ between the sexes, involving for example serine/glucocorticoid regulated kinase (SKG1) only in females and calcium/calmodulin kinase kinase beta (CaMKK $\beta$ ) only in males. In both sexes, oestrogen treatment upregulated the gene for adenylyl cyclase (AC) which can also lead to CREB phosphorylation through increases in cyclic adenosine monophosphate (cAMP) and phosphorylation of protein kinase A (PKA). Although we found that the conversion of testosterone to oestrogen by aromatase was responsible for the significant improvement in spatial performance, testosterone may also be directly activating proteins such as protein kinase C (PKC) leading to CREB phosphorylation, increases in spine density and spatial memory improvements.



A key feature to all of the work in this thesis was the suitability of the zebra finch and the memory task for addressing the effects of sex steroid on spatial cognition. Having established that sex steroids lead to both acute and medium-term improvements in spatial cognition, perhaps the logical next question to ask would be whether chronic treatment for weeks with sex steroids also improves spatial performance, which is plausible given the enhancing effects of such treatment in mammals. Then, with a clearer picture of the activational effects of sex steroid on spatial cognition over varying time courses, one could go on to address the question of what the organisational effects of sex steroids are on spatial cognition and whether testosterone and oestrogen have similar effects if administered during development. In mammals, there is natural variation in sex steroid exposure due to the sex ratio of the litter and intrauterine position, and exposure to high sex steroid levels improves spatial abilities and alters hippocampal morphology as adults (Galea et al., 1996; Galea et al., 1994b; Grimshaw et al., 1995; Roof, 1993; Sherry et al., 1996; Williams and Meck, 1991). The zebra finch is a good model in which to investigate the effects of sex steroids during development due to the ease of sex steroid manipulation as steroid injections can be made directly into eggs, unlike in mammals in which it is difficult to manipulate the hormonal environment within the uterus (Niall Daisley et al., 2005). Additionally, in zebra finches the resulting offspring reach adulthood in a time frame short enough to allow for investigation into the interaction between organisation and activation effects of sex steroids on spatial cognition. Using this method one could address whether testosterone, oestrogen and fadrozole manipulations have same effects in development as in adulthood. It is likely that increasing or reducing sex steroid exposure during development may have very different effects to manipulations in adulthood. For example, in adult zebra finches singing behaviour is never observed in females even if they are exposed to high levels of testosterone but early treatment with exogenous oestrogens produces a masculinisation of the song system in females and testosterone-induced singing in adulthood (Foidart and Balthazart, 1995; Gurney, 1981; Gurney and Konishi, 1980; Simpson and Vicario, 1991a; b). Following on from the effects of sex steroids during development and in early adulthood, the question of whether sex steroid effects are age-dependent could be investigated by comparing the effects of sex

steroids on spatial cognition in younger and older birds to get at some of the questions raised by human work. In this thesis, when older birds were used they took longer to train and a higher proportion of the older birds did not pick up the task. Would giving oestrogen during training improve this? It is not clear. During aging and reproductive senescence there is a reduction in circulating hormone levels. In humans, the most dramatic and rapidly occurring change in women is menopause at which point cycling oestradiol production during the reproductive years is replaced by very low, constant oestradiol (Abe et al., 1983). In contrast, changes in the activity of the hypothalamo-pituitary-gonadal axis in males are slower and more subtle and during aging there is a gradual decline in serum testosterone levels (Vermeulen, 1991). The second hormonal system demonstrating age-related changes is the circulating levels of dehydroepiandrosterone (DHEA) and its sulphate (DHEAS), which gradually decline with age, resulting in “adrenopause” (Herbert, 1995; Ravaglia et al., 1996). In older men, lower levels of testosterone are associated with impaired spatial performance, which is improved by testosterone treatment (Cherrier et al., 2001; Janowsky et al., 1994; Moffat et al., 2002). There are also age-dependent changes in the hippocampus and in the effects of sex steroids in mammals. For example, oestrogen replacement in aged female rats fails to increase spine density which may be linked to an inability of oestrogen to activate its receptors as 50% fewer spines contain ER $\alpha$  in the aged compared with young hippocampus (Adams et al., 2001b; Grimshaw et al., 1995). There are also age related changes in NMDA receptors, with aged rats and mice exhibiting an decline in the number of NMDA receptors, with a selective decrease in the expression of the NR2B subunits of the NMDA receptor (Clayton and Browning, 2001; Clayton et al., 2002b; Wenk and Barnes, 2000). Additionally there is a reduction of NMDA binding, due to decreases in the densities of binding sites (Peterson and Cotman, 1989; Tamaru et al., 1991). Furthermore, aged rats fail to show activity-dependent (LTP-stimulated) changes in the surface distribution of NMDA receptors from an intracellular pool to the surface (Clayton et al., 2002a).

Whether there are such age-dependent effects on spatial cognition and the hippocampus in birds is not yet clear. However, sexual behaviour during aging has been well studied in the male Japanese quail (*Coturnix japonica*) in which

reproductive failure occurs during aging and is accompanied by a decline in sexual behaviour and decreasing plasma testosterone (Ottinger and Balthazart, 1986; Ottinger et al., 1983). In male quails there is also a dramatic decrease in the aromatase system of old senescent males, who have less cells containing aromatase enzyme (AROM-IR cells) in the medial preoptic area (POM) and the lateral septum (LS) compared to young males (Dellovade et al., 1995). This is likely to prevent production of sufficient oestrogen to stimulate neuronal circuitries controlling sexual behaviour as well as endocrine aspects of reproduction. The loss of AROM-IR containing cells during aging bears similarity to the effects of castration, which is reversed by testosterone treatment (Balthazart et al., 1992). It is possible that there may also be a reduction in aromatase in the hippocampus of zebra finches during aging which may impact on spatial cognition.

In mammals when males fully cease courtship and mating behaviour, an external supply of testosterone is not capable of restoring sexual behaviour. In contrast to this, in quail the sexual behaviour can be restored in senescent individuals by administration of exogenous testosterone (Ottinger and Balthazart, 1986). However, the dose of testosterone used to stimulate sexual behaviour in senescent males was double the effective dose for young or in adult birds (Ottinger and Balthazart, 1986). This suggests that part of the machinery does deteriorate during aging and the recovery of function may be based on a certain degree of plasticity still remaining in the old quail brain or to an overstimulation of the persisting system. In the older zebra finches used in Experiment 1, there was a range of ages and it may be that although in some birds their testosterone levels were high, the levels may not have been sufficient for spatial learning as the machinery in the hippocampus, such as ER $\alpha$  and NMDA receptors, may have deteriorated. Perhaps older zebra finches need even higher testosterone levels to learn? As we did not look at the brains of the birds which did not reach criterion we can not say. There may also be a reduction in aromatase in the hippocampus of zebra finches, reducing the local production of oestrogen and so a larger amount of testosterone might be necessary to produce adequate oestrogen for spatial cognition.

To further understand the mechanisms involved in the effects of oestrogen on spatial cognition, the involvement of acetylcholine (ACh) implicated from mammalian work could be addressed by oral treatment with oestrogen and an ACh antagonist (Bora et al., 2005; Daniel and Dohanich, 2001; Gibbs, 1996a; Gibbs, 1996b; 1997; 2002; Gibbs et al., 1994; Lam and Leranth, 2003; Luine, 1985; Luine and McEwen, 1983; Luine et al., 1986; Mufson et al., 1999; Shughrue et al., 2000; Towart et al., 2003). Furthermore, selective ACh antagonists could be used as the ACh muscarinic (mAChR) and nicotinic (nAChR) receptors, which are both important in memory in mammals, may have sex specific role (Figure 1) (Chan et al., 2007; Curzon et al., 2006; Felix and Levin, 1997; Kim and Levin, 1996; Li et al., 2007; Socci et al., 1995; Terry Jr et al., 2000; Van der Zee and Luiten, 1999). In female rats, the action of oestrogen on NMDA receptors and spatial performance involve mAChR, whereas we found that oestrogen treatment increased gene expression of nAChR only in males (Daniel and Dohanich, 2001). As the effects may be sex specific one would predict that treatment with oestrogen and a mAChR antagonist would block the improvements in performance and increases in NMDA receptors only in females and the nAChR antagonist only in males. Furthermore, the age-related changes in NMDA receptors discussed above may be linked to the decrease in cholinergic innervation of the hippocampal formation associated with AD (Kasa et al., 1997) as the activity of NMDA-evoked release of ACh is markedly decreased afterward 2 months in senescence-accelerated mice (Zhao et al., 1992). Senescence-accelerated mice show reductions in choline acetyltransferase (ChAT) activity, associated with impairments in Morris Water Maze performance and the lower ACh synthesis in AD may impact on the effects of oestrogen on NMDA receptors, which are mediated by ACh (Daniel and Dohanich, 2001; Wang et al., 2009).

Having established more clearly the mechanisms underlying the effects of sex steroids on spatial cognition, an important next step would be to look at the processes involved in their interplay with the stress system as in mammals there are sex-specific effects of stress on spatial memory and hippocampal spine density, which appear to involve oestrogen and NMDA receptors (Conrad et al., 2003; Conrad et al., 2004; Luine, 2002; Luine et al., 1996; Luine, 1994; 1997; Shors, 2002; Shors et al.,

2001; Shors et al., 2004; Shors et al., 1998). The methods used in this thesis would be useful firstly for addressing the effects of acute and medium-term effects of corticosterone alone and then for looking at what happens to spatial performance and hippocampal sex steroid, corticosterone and NMDA receptors when corticosterone is administered with oestrogen. The advantages are several fold: 1) corticosterone could be orally (i.e. non-invasively) administered, rapidly and transiently increasing circulating levels thus enabling investigation into acute and medium term effects; 2) the spatial task would be particularly advantageous for this work as the elimination of handling or food deprivation minimises the complications of uncontrollable natural fluctuations of corticosteroid levels; 3) there are established *in situ* hybridisation methods allowing the detection of mineralocorticoid and glucocorticoid receptor mRNA expression (Hodgson et al., 2007); 4) there is some indication that stress affects spatial performance and hippocampal stress steroid receptors in the zebra finch (Hodgson et al., 2007); 5) zebra finch gene expression arrays are available to look at hippocampal changes in gene expression induced by treatment with corticosterone.

In conclusion, the zebra finch has provided a useful model in which to address the acute and medium-term effects of sex steroid treatment on spatial performance and hippocampal receptor, aromatase and gene expression changes and the system and results presented here provide a strong basis on which to investigate remaining questions raised.

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