DEVELOPMENTAL, BUT NOT ACTIVATIONAL, ROLES FOR ESTRADIOL AND ITS RECEPTOR IN PATERNAL AND SEXUAL BEHAVIOUR OF PHODOPUS CAMPBELLI MALES

by

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ABSTRACT

The neuroendocrine basis of paternal behaviour is hypothesised to be homologous to that of maternal behaviour, with the expression of paternal behaviour resulting from the activation of estrogen-sensitive pathways in the brain. However, despite the fact that *Phodopus campbelli* fathers are highly paternal, previous studies have failed to identify an activational role for estradiol in the expression of this behaviour. The goal of this thesis was to exclude or define a role for estradiol in *P. campbelli* paternal behaviour.

In the first study, estrogen receptor alpha (ERα) expression was examined in three brain regions relevant to maternal behaviour (medial preoptic area, bed nucleus of the stria terminalis, and medial amygdala). Male and female *P. campbelli* and its close relative *P. sungorus* were sampled as sexually naïve adults, following mating to satiety, or as new parents. As predicted, new fathers of either species did not upregulate ERα expression in these brain regions. Mating also had no effect on ERα expression in males or females from either species. However, ERα expression was also not upregulated in any of the three brain regions in new mothers. Thus, it is possible that estradiol does not play a primary role in activating maternal behaviour in *Phodopus* females.

In the second study, *P. campbelli* males were administered the aromatase inhibitor letrozole at three different developmental stages (independence from the mother, testicular maturation, and sexually naïve adult) and tested for adult sexual and paternal behaviour. Aromatase inhibition during juvenile development resulted in diminished expression of adult paternal and sexual behaviour, suggesting that estradiol acts at critical periods in development to organize the paternal brain for subsequent behavioural
responses. Treatment of sexually naïve adults did not alter male behaviour, which further supports previous evidence that estradiol does not activate paternal behaviour in *P. campbelli* males, although sexual behaviour in females is completely estradiol-dependent in this species. These findings suggest that the activational roles of estradiol in maternal and male sexual behaviour identified in the rat do not generalize to *Phodopus*, and imply greater species to species variation in the neuroendocrine control of these behaviours than previously suspected.
CO-AUTHORSHIP DECLARATION

The results presented in Chapter 3 are the collaborative effort of ME Timonin, BS Cushing, and KE Wynne-Edwards, and this manuscript is in press in the Journal of Neuroendocrinology. Chapter 4, the joint effort of ME Timonin and KE Wynne-Edwards, is published in Hormones and Behavior, and is online (doi: 10.1016/jyhbeh.2008.08.003). All of the data analysis and writing of this thesis was conducted by ME Timonin in conjunction with KE Wynne-Edwards.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ArKO</td>
<td>Mice that lack a functional copy of the gene for ( \text{P}_{450} \text{aromatase} )</td>
</tr>
<tr>
<td>ATD</td>
<td>Aromatase inhibitor 1,4,6-androstatriene-3,17-dione</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>Cyp19</td>
<td>Cytochrome P45019A1 gene, which encodes the enzyme ( \text{P}_{450} \text{aromatase} )</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>ERKO</td>
<td>Mice that lack a functional copy of the gene for estrogen receptor alpha</td>
</tr>
<tr>
<td>ER( \alpha )</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>ER( \alpha )-ir</td>
<td>Estrogen receptor alpha immunoreactive staining</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>KPBS</td>
<td>Potassium-phosphate buffered saline</td>
</tr>
<tr>
<td>MeA</td>
<td>Medial amygdala</td>
</tr>
<tr>
<td>MPOA</td>
<td>Medial preoptic area of the hypothalamus</td>
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CHAPTER 1: General Introduction

The mechanisms regulating mammalian maternal behaviour are assumed to be deeply ancestral, and shared across species (Wynne-Edwards 2001). Thus, although the current model of the estradiol-sensitive neural circuits regulating maternal behaviour has been developed based primarily on research using the female laboratory rat, the maternal behaviour pathway, specifically the central role of the medial preoptic area (MPOA), can be generalized across species (Marques et al. 1979; González-Mariscal et al. 2005; Lee & Brown 2007; Perrin et al. 2007; Poindron et al. 2007). The endocrine changes associated with pregnancy, parturition, and lactation, including the peak in circulating concentrations of estradiol during late pregnancy, are also assumed to be responsible for synchronizing the expression of mammalian maternal behaviour with the birth of a first litter (Rosenblatt et al. 1988). A central role for estradiol in establishing maternal behaviour has been identified in several species (Kendrick & Keverne 1991; Fleming et al. 1997; Rosenblatt et al. 1998; González-Mariscal 2001). For example, female rats that have their pups delivered by Caesarean section during mid to late pregnancy will respond maternally when presented with their neonates for the first time, but females that have the ovaries removed at the same time as the uterus will not display maternal behaviour unless exogenous estradiol is administered (Siegel & Rosenblatt 1975a). Furthermore, virgin female rats are not maternal upon first exposure to neonates, but treatment with estradiol will stimulate rapid expression of maternal behaviour (Siegel & Rosenblatt 1975b). However, estradiol alone does not stimulate the expression of maternal behaviour in all species. Virgin ewes cannot be stimulated to show maternal behaviour in response to
estradiol priming even if they also receive artificial vaginocervical stimulation (Kendrick & Keverne 1991). Non-pregnant ewes with previous maternal experience require both physiological doses of estradiol and vaginocervical stimulation to show maternal behaviour, and pregnant females that do not receive vaginocervical stimulation during parturition show disrupted maternal behaviour (Krehbiel et al. 1987; Kendrick & Keverne 1991). Similarly, pregnant prairie voles (*Microtus ochrogaster*) that have their pups delivered by Caesarean section are infanticidal, while females that receive a sham surgery but give birth naturally are fully maternal (Hayes & De Vries 2007). Thus, estradiol appears to plays an important priming role, increasing the salience of the parturition-related stimuli necessary to establish maternal behaviour (Sheehan & Numan 2002).

Like females, naturally paternal males need to suppress infanticidal behaviour and activate direct paternal behaviour in synchrony with the birth of pups (Elwood 1977; Perrigo et al. 1991; Vella et al. 2005). The neuroendocrinology underlying this shift in behaviour is hypothesized to be homologous to that seen in females becoming mothers; activation of a pre-existing pathway would be easier, in evolutionary terms, than evolving a new neuroendocrine circuit for males (Wynne-Edwards & Reburn 2000; Wynne-Edwards 2001). A stimulatory role for estradiol, acting on the maternal behaviour pathway to alter the expression of paternal behaviour by new fathers, has been identified in some paternal species (Lee & Brown 2002a; Trainor & Marler 2002; Trainor et al. 2003). Conversely, the expression of paternal behaviour in other species, including the paternal dwarf hamster *Phodopus campbelli*, is independent of estradiol activation (Lonstein & De Vries 1999; Hume & Wynne-Edwards 2006). Because paternal
behaviour is not restricted to specific phylogenetic lineages, such variations in the neuroendocrine regulation of paternal behaviour in different species are not unexpected. However, before a role for estradiol in the expression of paternal behaviour in *P. campbelli* can be definitively excluded, two new hypotheses need to be tested. First, increases and decreases in hormone receptor expression in the brain alter sensitivity to that hormone, and might thereby influence behaviour independent of a change in peripheral hormone concentration. Thus, if estrogen receptor expression were upregulated in the ‘maternal behaviour pathway’ during the transition to behavioural fatherhood, even basal levels of peripheral estradiol could play a role in the expression of paternal behaviour by *P. campbelli* fathers. Second, critical roles for estradiol in *P. campbelli* paternal behaviour could be temporally disassociated from the activation of paternal behaviour. Such organizational effects of estradiol during development might influence the architecture of the ‘maternal behaviour pathway’ in males, yet not require estradiol in adulthood for activation of the pathway. This thesis tests both of these hypotheses in an animal model with exceptional paternal care, *Phodopus campbelli*. 
CHAPTER 2: Literature Review

2.1 *Phodopus* model of paternal behaviour

Direct biparental care is rare among mammals, with the majority of species relying on maternal care to successfully raise young (Wynne-Edwards & Reburn 2000). However, under some conditions the female alone is incapable of providing this level of care, and the male must contribute to ensure offspring survival and protect the female’s condition to maximize the potential for future breeding (McInroy et al. 2000; Wynne-Edwards & Reburn 2000). Thus, paternal care has evolved independently in different species in response to conditions that make the presence of the male essential to survival of offspring (Clutton-Brock 1991). For example, *Phodopus campbelli* and *P. sungorus* are so closely related that they were long considered subspecies (Ross 1995; Ross 1998; Neumann et al. 2006), are very similar in body weight, show the same reproductive timetable, and are able to form viable hybrid breeding pairs (Wynne-Edwards 1998; Timonin & Wynne-Edwards 2006). However, *P. campbelli* is an obligately biparental species, while *P. sungorus* has sole maternal care of offspring. Both *Phodopus* species are native to the steppes of central Asia and are well-adapted to living in this cold, arid climate (short tail, densely furred soles of the feet, and small ears; Ross 1995; Ross 1998). The species are not sympatric and *P. campbelli* occupies a habitat that is colder, drier, and more seasonal than the conditions experienced by *P. sungorus*. These harsh environmental conditions, along with the resultant low population densities, favoured the

2.1.1 *Benefits of paternal behaviour*

In the laboratory, the presence of highly responsive *P. campbelli* males at the time of birth and across pup development improves litter survival as well as increasing the probability that the female will invest in a second litter (Wynne-Edwards 1987; Wynne-Edwards & Lisk 1989). Solitary *P. campbelli* females raising a first litter under standard laboratory conditions (temperatures of 18 ± 1.0°C to reflect typical burrow temperatures; Wynne-Edwards 1998), raise fewer than 50% of pups to weaning (postnatal day 18), with approximately 25% of females suffering complete litter loss (Wynne-Edwards & Lisk 1989; Walton & Wynne-Edwards 1997). This is in contrast to 95% pup survival and 100% litter survival seen when the female raises her first litter with the assistance of her mate (Wynne-Edwards 1987; Wynne-Edwards & Lisk 1989). Low rates of pup survival and high litter losses are exacerbated by either increasing or decreasing ambient temperatures if the male is not present (Wynne-Edwards & Lisk 1989; Walton & Wynne-Edwards 1997). Males increase pup survival by reducing maternal hyperthermia, which results in an increase in the amount of time females spend with their pups, ensuring that the altricial pups receive the heat necessary for normal development (Scribner & Wynne-Edwards 1994a; Scribner & Wynne-Edwards 1994b; Walton & Wynne-Edwards 1997). Solitary females also lose more body weight across pregnancy and lactation than females that remain paired with their mate (McInroy et al. 2000). In contrast, *P. sungorus* females
do not require the assistance of a male to rear their litter successfully (Scribner & Wynne-Edwards 1994a; Newkirk et al. 1998; Stulberg & Wynne-Edwards 1998).

2.1.2 Ontogeny of paternal behaviour

Male *P. campbelli* show direct paternal care starting at the birth of their first litter. Males mechanically assist the female with the birth process, consume placenta, and care for newly born pups by licking and huddling over them (Jones & Wynne-Edwards 2000). Males also promptly retrieve pups that stray from the nest area, and typically contact and return an experimentally displaced pup to the nest in under 60 seconds (Schum & Wynne-Edwards 2005; Vella et al. 2005; Hume & Wynne-Edwards 2006). Although juveniles (20-25 days old) will show alloparental behaviour towards younger siblings, including midwifery behaviour during the birth and retrieval of a displaced pup, sexually naïve males are unlikely to show spontaneous paternal care towards a neonate (Vella et al. 2005). Only 25% of 35-90 day old males will retrieve an anesthetized pup, while 50% will show infanticidal behaviour (Figure 1; Vella et al. 2005). However, the use of an anaesthetized pup is likely to result in an underestimate of retrieval behaviour for males. New fathers will not attack an anesthetized pup, but only 25% of males will show retrieval behaviour (Vella et al. 2005). This is in contrast to the retrieval rates of 80-100% typically shown by new fathers tested with alert (unanaesthetized) pups from their first litter (Schum & Wynne-Edwards 2005; Hume & Wynne-Edwards 2006). *P. sungorus* juveniles are also alloparental, but retrieval of an experimentally displaced pup by non-parental *P. sungorus* fathers is rare, (although females exhibit prompt retrieval behaviour), males spend minimal time in contact with pups in the nest, and do not assist
Figure 1: Sexually naïve *Phodopus campbelli* males at various stages of development are unlikely to retrieve (panel A), and likely to attack (panel B), an unrelated, deeply anaesthetized pup. Males were tested at one of 5 ages from young adulthood (35 days) through peak reproduction (91 days), or as new parents (L3 = 3 days after the birth of a first litter). No new fathers attacked the pup (*p* < 0.05), although retrieval rates remained lower than those seen when males are tested with alert pups from their own first litter (see text for details). Adapted from Vella et al. (2005).

2.1.3 *Hormonal basis of paternal behaviour*

Based on the hypothesized homology between the neuroendocrine control of paternal and maternal behaviour (Wynne-Edwards & Reburn 2000; Wynne-Edwards 2001), *P. campbelli* males are expected to show paternal behaviour in response to increased estradiol concentrations leading up to the birth of their first litter. This is the natural mechanism regulating the high levels of maternal behaviour displayed by females immediately upon parturition (Kendrick & Keverne 1991; Fleming et al. 1997; Rosenblatt et al. 1998; González-Mariscal 2001). Non-paternal *P. sungorus* males are not expected to show dynamic changes in estradiol concentration over the same time period.

2.1.3.1 *Dynamic changes in peripheral estradiol concentrations*

Serum estradiol concentrations were measured in *Phodopus* males before pairing (sexually naïve), at four stages relative to their mate’s pregnancy (after implantation and the initiation of placentation; once placentation is complete; during the phase of rapid embryonic growth; and the day before parturition), and at four stages relative to postnatal pup development (postpartum days 1, 3, 5 and 12). Over this period, pups go from being hairless, deaf, and blind neonates incapable of independent locomotion or thermoregulation, to juveniles with a complete pelage and open ear canals and eyes, which have independent thermoregulation, and can eat solid food (Newkirk et al. 1995; Newkirk et al. 1998). However, in contrast to estradiol dynamics in pregnant conspecific females leading up to and following parturition (Edwards et al. 1994; Roy & Wynne-Edwards 1995), and contrary to the hypothesis, circulating estradiol concentration in
paternal *P. campbelli* males remains stable across their mate’s pregnancy and following the birth of their first litter (Schum & Wynne-Edwards 2005; Figure 2).

Unexpectedly, non-paternal *P. sungorus* males show the expected increase in estradiol on the day before parturition, followed by a decrease on the day after birth to concentrations equivalent to those seen in sexually naïve animals of either species (around 100pg/ml; Erb & Wynne-Edwards 1993; McMillan & Wynne-Edwards 1998; Schum & Wynne-Edwards 2005). Estradiol concentration reaches peak levels for a second time when pups are 12 days old (Schum & Wynne-Edwards 2005), which is similar to the increase seen in *P. campbelli* females that are lactating and concurrently gestating a litter conceived during postpartum estrus (Roy & Wynne-Edwards 1995). This increase in estradiol concentration the day before birth, with a return to normal levels the day after birth, might be parallel to the dynamic changes in estradiol shown by paternal black-tufted ear marmoset (*Callithrix kuhlii*) fathers (Nunes et al. 2000). Marmoset fathers have increased urinary estradiol concentrations 1-2 weeks prior to the birth of young, low levels in the first 4 weeks of infant development, and a second increase in estradiol when young are 5-6 weeks old (Nunes et al. 2000). Thus, estradiol levels are high during the female’s postpartum estrus, but low during the period when males express high levels of paternal behaviour, implying that estradiol concentration is related to sexual rather than paternal behaviour in this species (Nunes et al. 2000). Likewise, the changes in serum estradiol concentration observed in non-paternal *P. sungorus* males leading up to the birth of their first litter may also be related to postpartum mating, since estradiol plays an activational role in the expression of male
Figure 2: Despite the expectation that paternal males would show an increase in peripheral estrogen just before the birth of their offspring, paternal *Phodopus campbelli* (open bars) males do not have a significant increase in serum concentration of estradiol (pg/ml) on the day before the birth of their first litter (G17; shaded region). Non-paternal *Phodopus sungorus* (solid bars) do show a significant increase in serum concentration of estradiol both on the day before birth (G17; panel C) and when pups are 12 days old (L12; panel D). Error bars indicate SEM. Adapted from Schum & Wynne-Edwards (2005).
Before pup stimuli

With pup stimuli

Estradiol
sexual behaviour in some species (Baum & Vreeburg 1973; Sodersten et al. 1986; Balthazart et al. 2004; Arteaga-Silva et al. 2005; Arteaga-Silva et al. 2007). However, contrary to the hypothesis that paternal behaviour is regulated by activation of a homologous ‘maternal behaviour pathway’ in paternal males, there was no increase in peripheral estradiol concentration leading up to the birth of a first litter in *P. campbelli* males (Schum & Wynne-Edwards 2005).

### 2.1.3.2 Direct manipulation of estradiol

Additionally, evidence also indicates that estradiol does not play an activational role in the expression of paternal behavior by *P. campbelli* fathers (Hume & Wynne-Edwards 2005; Hume & Wynne-Edwards 2006). If estradiol priming leading up to the birth were necessary for the natural expression of this behaviour, direct manipulation of estradiol in *P. campbelli* males during their mate’s mid to late pregnancy would be expected to decrease direct (paternal responsiveness) and indirect (pup development and survival) measures of paternal behaviour. Hume and Wynne-Edwards conducted two studies to determine the role of estradiol in the regulation of paternal behaviour in *P. campbelli*.

#### 2.1.3.2.1 Castration

In the first experiment (Hume & Wynne-Edwards 2005), males were castrated on day 7 of their mate’s pregnancy, which resulted in a more than 10-fold decrease in the concentration of estradiol in peripheral circulation (from 100pg/ml to 15pg/ml; Figure 3). However, over 80% of castrated males retrieved an experimentally displaced 3-day old pup from their first litter in less than 100 seconds, which was not different from levels of
**Figure 3**: Castration revealed that the testes are the primary source of peripheral estradiol in *Phodopus campbelli* males. Males castrated (solid bars) 5 days after the birth of their first litter have significantly lower (*p < 0.0002; A*) serum estradiol concentrations (pg/ml) than males that received a sham-castration (open bars). Testosterone, the principal gonadal androgen in males, and the primary substrate for estradiol production, is also significantly reduced in serum as a result of castration (*p < 0.0002; B*). Error bars indicate SEM. Adapted from Hume & Wynne-Edwards (2005).
paternal responsiveness displayed by sham-castrated controls (Hume & Wynne-Edwards 2005). Similarly, virgin male prairie voles (*Microtus ochrogaster*) castrated during adulthood continue to show normal levels of spontaneous paternal behaviour (huddling over and licking pups) upon first exposure to a 1-4 day old neonate (Lonstein & De Vries 1999). In contrast, California mouse (*Peromyscus californicus*) fathers castrated three days after the birth of their first litter spent significantly less time huddling with and grooming their pups (Trainor & Marler 2001). In that case, paternal behaviour was fully restored as a result of treatment with estradiol (Trainor & Marler 2002). Thus, naturally paternal rodent species have at least two patterns of response to castration, and *P. campbelli* do not show diminished paternal responsiveness following adult castration.

2.1.3.2.2 AROMATASE INHIBITION

Although castration identified the testes as the primary source of estradiol in *P. campbelli*, castrated males still have detectable serum estradiol, and these concentrations may be high enough to act upon the brain to elicit paternal behaviour (Hume & Wynne-Edwards 2005). This residual estradiol may be produced through an extragonadal biosynthetic pathway starting with the adrenal androgen dehydroepiandrosterone (DHEA; Soma & Wingfield 2001), which is present in *Phodopus* (K Wynne-Edwards, personal communication). Furthermore, serum levels of estradiol do not necessarily reflect the role that this hormone plays in a particular behaviour; male California mice have undetectable levels of estradiol in peripheral circulation, but aromatase in specific brain regions is responsible for local aromatization of androgen to estradiol, stimulating paternal behaviour (Trainor & Marler 2002; Trainor et al. 2003). Thus, in their second experiment
Hume and Wynne-Edwards treated male *P. campbelli* with the non-steroidal aromatase inhibitor letrozole, to prevent peripheral and neurological estradiol synthesis (Hume & Wynne-Edwards 2006). Males received 1mg/kg/day of letrozole dissolved in propylene glycol via osmotic pumps. Osmotic pumps containing either letrozole or vehicle were surgically implanted on day 10 of the mate’s pregnancy. Serum estradiol concentrations were significantly reduced on postpartum day 5 in males administered letrozole compared to vehicle-treated males (17pg/ml versus approximately 100pg/ml), but measures of direct and indirect paternal behaviour were the same in both groups. During a standard pup retrieval test, all fathers returned an experimentally displaced pup to the nest, with most retrievals occurring in less than 100 seconds (Hume & Wynne-Edwards 2006). Furthermore, litter survival, pup survival, and pup development were not altered by letrozole treatment. However, only 38% (3/8) of letrozole-treated males sired a second litter, compared to 67% (4/6) of vehicle-treated males; this was not a significant difference, but raised the possibility that letrozole treatment during adulthood might adversely affect the sexual behaviour of *P. campbelli* males. Thus, letrozole treatment was as effective as castration in reducing serum estradiol concentrations, equally ineffective in reducing paternal responsiveness in *P. campbelli* fathers, and may alter adult sexual behaviour (Hume & Wynne-Edwards 2006).

Together, these results fail to support an activational role for estradiol in the expression of paternal behaviour by *P. campbelli* fathers. However, one aspect of estrogen control of paternal behaviour that is not considered in the above studies is the distribution of estrogen receptor in behaviourally relevant brain regions following the
transition to fatherhood. An upregulation of estrogen receptor in the maternal behaviour pathway during the transition to behavioural fatherhood would increase the sensitivity of these circuits to the actions of estradiol, and support an activational role for this hormone in the expression of paternal behaviour.

2.2 The maternal behaviour pathway

Research using the female laboratory rat to examine the neuroanatomy underlying maternal behaviour has defined an estradiol-sensitive maternal behaviour pathway (Figure 4), including the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST) and the medial amygdala (MeA; Numan & Sheehan 1997). Neurons in these three brain regions are activated in response to pup interaction and the expression of maternal behaviour: expression of Fos protein, an immediate-early gene product that is used as a marker of neuronal activation, increases in the MPOA, BNST and MeA of virgin male and female prairie voles, and in rat mothers, following pup interactions (Fleming et al. 1994; Kirkpatrick et al. 1994b; Numan & Numan 1994; Lonstein et al. 2000; Stack et al. 2002). Both the MPOA and BNST are essential for the normal expression of maternal behaviour (Numan 1974; Numan & Numan 1996; Stack et al. 2002). Females that receive electrical or axon-sparing lesions to the MPOA have severely disrupted expression of maternal behaviour including huddling over pups in a nursing posture, retrieval, and nest building, when exposed to neonates (Numan 1974; Numan et al. 1977; Numan et al. 1988). Similarly, axon-sparing lesions to the BNST result in severely disrupted maternal behaviour in postpartum rats (Numan & Numan 1996). The
Figure 4: Representative line drawings of (A) frontal and (B) sagittal sections of the rat brain at the level of the medial preoptic area (mpo), a key brain region in the regulation of mammalian maternal behaviour. The bed nucleus of the stria terminalis (st) and the medial amygdala (not visible) are also key regions in the maternal behaviour pathway. Abbreviations: a – nucleus accumbens; aa – anterior amygdala area; AC – anterior commissure; ah – anterior hypothalamic area; CC – corpus callosum; cg – central gray; cp – caudate putamen; db – nucleus of the diagonal band; dm – dorsomedial nucleus of the hypothalamus; F – fornix; h – hippocampus; lpo – lateral preoptic area; ls – lateral septal nucleus; m – mammillary bodies; mpo – medial preoptic area; ob – olfactory bulb; OC – optic chiasm; sc – superior colliculus; st – bed nucleus of stria terminalis; th – thalamus; vm – ventro-medial hypothalamic nucleus; vta – ventral tegmental area. Adapted from Numan (1988).
essential role of the MPOA and BNST in the expression of maternal behaviour can be
generalized to mothers in other species including sheep and goats (Perrin et al. 2007;
Poindron et al. 2007), rabbits (González-Mariscal et al. 2005), golden hamsters (Marques
et al. 1979), and California mice (Lee & Brown 2007). Male California mice (Lee &
Brown 2007) and rats (Rosenblatt 1967) also require the MPOA to exhibit paternal
behaviour towards pups. In contrast, the MeA plays an inhibitory role in the rat: MeA
lesions result in the rapid expression of maternal behaviour in typically non-maternal
females (Fleming et al. 1980; Numan et al. 1993; Mann & Babb 2004). However, the
exact role the MeA plays in the expression of parental behaviour differs by species;
unlike female rats, adult virgin male prairie voles given lesions to the MeA spend less
time in contact with a pup (Kirkpatrick et al. 1994a), and ewes that receive an infusion of
the anaesthetic lidocaine into the MeA show normal maternal behaviour but not maternal
selectivity (Keller et al. 2004).

2.2.1 Estrogen receptor alpha in the maternal behaviour pathway

In females, estradiol acts through estrogen receptor alpha (ERα) to promote the
expression of maternal behaviour. Stimulation of the MPOA and BNST with estradiol
leads to the rapid expression of maternal behaviour in pregnant female, virgin female,
and male rats (Rosenblatt & Ceus 1998; Rosenblatt et al. 1998), and neurons in these
brain regions are able to bind estradiol (Pfaff & Keiner 1973). Additionally, neurons
activated in MPOA, BNST and MeA by interacting with pups also express ERα
(Lonstein et al. 2000).
2.2.1.1 Role in onset of maternal behaviour

There is a direct relationship between maternal behaviour and ER\(\alpha\) in the MPOA. Virgin female mice that lack a functional copy of the gene for ER\(\alpha\) (ERKO mice) show reduced pup retrieval and increased infanticide compared to controls (Ogawa et al. 1998a). Furthermore, mothers that show high levels of pup licking and grooming have higher expression of ER\(\alpha\) in the MPOA compared to mothers that show low levels of pup licking and grooming (Champagne et al. 2003). Thus, an upregulation of ER\(\alpha\) expression in the brain should increase the sensitivity of the maternal brain network to circulating estradiol, promoting maternal behaviour. This is the case in the rat: estradiol binding capacity, gene expression, and the number of cells expressing ER\(\alpha\) in the MPOA increase in anticipation of the peak in circulating estradiol levels associated with parturition and the onset of maternal behaviour (Giordano et al. 1990; Giordano et al. 1991; Wagner & Morrell 1995; Wagner & Morrell 1996). In female mice, ER\(\alpha\) is upregulated in the MPOA, BNST and MeA during pregnancy, and receptor levels remain high into lactation (Koch & Ehret 1989). Ewes do not upregulate ER\(\alpha\) expression in either the MPOA or the MeA during late pregnancy compared to females in estrus, and the expression of maternal behaviour immediately after parturition is associated with lower ER\(\alpha\) expression levels in both regions compared to females during estrus or late pregnancy (Meurisse et al. 2005). However, ewes with previous maternal experience show higher levels of maternal responsiveness towards lambs than first-time mothers, and multiparous females express higher levels of ER\(\alpha\) in both the MPOA and the MeA than primiparous ewes (Meurisse et al. 2005). Thus, although the direct relationship between high levels of ER\(\alpha\) in the MPOA
and the expression of maternal behaviour appears to generalize across species, the upregulation of ERα expression in the brain during late pregnancy does not.

2.2.1.2 Role in paternal behaviour

There is also limited evidence that ERα plays a role in the expression of paternal behaviour. Male ERKO mice show increased infanticide compared to controls, but unlike females, they show normal levels of pup retrieval (Ogawa et al. 1998b). Additionally, male mice that exhibit pup-searching and retrieval behaviours have a pattern of ERα expression that is distinctly different from that of virgin males. As virgins, males express lower levels of ERα in the MPOA compared to females. However, ERα is upregulated in fathers so that ERα expression in these males resembles that of a virgin female (Ehret et al. 1993). Phodopus males also express ERα in regions of the maternal behaviour pathway, with lower levels of expression than conspecific females in the BNST and MeA but not the MPOA (Cushing & Wynne-Edwards 2006; Figure 5). In addition, paternal P. campbelli males have lower ERα expression in the BNST than non-paternal P. sungorus males, implying that low levels of ERα in this region are permissive for the expression of paternal behaviour (Cushing & Wynne-Edwards 2006). This association between low ERα expression in the BNST and social behaviour is also seen in voles. Monogamous, biparental prairie voles express lower levels of ERα compared to polygamous, non-paternal males from the same genus (Cushing et al. 2004; Cushing & Wynne-Edwards 2006). However, unlike Phodopus males, low levels of ERα in the MeA are also associated with monogamy and paternal behaviour in prairie voles (Cushing et al. 2004; Cushing & Wynne-Edwards 2006). Similarly, ERα expression is increased in the MeA of
Figure 5: Estrogen receptor alpha expression was examined in the brains of sexually naïve biparental male and female *Phodopus campbelli* (Djungarian, grey bars) and uniparental *Phodopus sungorus* (Siberian, black bars). The medial amygdala (MeA; panel C) and bed nucleus of the stria terminalis (BST; panel B) have sexually dimorphic ERα expression in both species (indicated by *), with females expressing higher levels of ERα than males for both species. Male *P. campbelli* have significantly lower levels of ERα expression in the BST (indicated by a vertical block arrow in panel B) than male *P. sungorus*. There was no species difference or sexual dimorphism in ERα expression in the MPOA (indicated by horizontal block arrow) despite differences in parental behaviour between the two species. The MeA, BST and MPOA (outlined with black border) are of interest due to the role of these brain regions in maternal behaviour. Adapted from Cushing & Wynne-Edwards (2006).
P. sungorus as a result of short photoperiod exposure (Kramer et al. 2008), an environmental cue associated with increased aggression in that species (Jasnow et al. 2000). Thus, although an ERα upregulation in the MPOA appears to be related to the onset of paternal behaviour, the effect of increased ERα expression in the BNST and MeA differs by species.

2.2.1.3 Role in sexual behaviour

The role of ERα in the maternal behaviour pathway, however, is not restricted to the control of maternal behaviour. Castrated ERKO males do not ejaculate, even when other aspects of sexual behaviour are restored through androgen treatment (Ogawa et al. 1998b), non-copulating male rats (apparently normal males that fail to mate) express lower levels of ERα in the MPOA (Portillo et al. 2006), and male rats that mate to satiety have increased ERα expression in the MPOA compared to controls (Phillips-Farfán et al. 2007). Similarly, ovariectomized ERKO females resist mounting behaviour by a stud male and fail to show lordosis when mounted, and these behaviors cannot be restored through estrogen, or estrogen and progesterone, treatment (Ogawa et al. 1998a).

Additionally, c-fos activity is stimulated in neurons expressing ERα as a result of mating during postpartum estrus in female prairie voles (Katz et al. 1999). ERα expression is also altered relative to the estrus cycle in females (Shughrue et al. 1992; Zhou et al. 1995). Thus, although there is support for increased ERα expression in the maternal behaviour pathway relative to the onset of maternal, and potentially paternal, behaviour, these changes may actually be related to the expression of concurrent behaviours such as mating during a postpartum estrus. Because ERα distribution in Phodopus has only been
measured in adult virgins, the effects of mating and parenting experience on ERα expression in the maternal behaviour pathway are unknown.

2.3 Developmental effects of estradiol

Although manipulations of estradiol during the period leading up to the birth of a first litter are ineffective in reducing the expression of paternal behaviour in *P. campbelli*, estradiol might act during a developmental stage temporally displaced from the expression of adult behaviour. Gonadal steroids, including estradiol, play an important role during development by organizing the hormone sensitive neural circuits activated during adulthood (Arnold & Gorski 1984). The organizational effects of gonadal steroids are typically limited to one or more ‘critical’ periods, with the majority of hormone-mediated development occurring during prenatal or early neonatal development (Arnold & Gorski 1984). However, the neural organization begun during early development is often completed as a result of the pubertal rise in steroid hormones (Romeo et al. 2003). For example, male Syrian hamsters (*Mesocricetus auratus*) castrated as pre-pubertal juveniles (postnatal day 21) have decreased mount, intromission, and ejaculation frequencies as adults (Schulz et al. 2004). Unlike males castrated as adults, normal sexual behaviour cannot be restored in pre-pubertal castrates through the administration of testosterone for the week prior to sexual behaviour testing; thus, organization of the neural pathways underlying the expression of adult male sexual behaviour must be completed at puberty (Schulz et al. 2004).
2.3.1 *Paternal behaviour*

Very little work on the developmental role of steroid hormones in establishing adult paternal behaviour has been done. However, a few studies examined the effects of neonatal inhibition of androgens and estradiol on the spontaneous paternal behaviour typically displayed by virgin male prairie voles. Castration during adulthood has no effect on the proportion of males that act paternally (huddle over and lick) towards a 1-4 day old pup (Lonstein & De Vries 1999). Similarly, suppression of estradiol synthesis through treatment with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) during late prenatal development (gestation days 15-19 of a 22 day pregnancy), or the first seven days after birth, has no effect on virgin adult paternal behaviour (Lonstein & De Vries 2000). In the same study, pups were also treated with the anti-androgen flutamide to rule out an androgenic effect on the development of paternal behaviour, but this treatment was equally ineffective in reducing paternal responsiveness in adult voles (Lonstein & De Vries 2000). Even males treated with a combination of ATD and flutamide prenatally continue to show normal levels of paternal behaviour as adults (Lonstein et al. 2002). However, only 50% of males that are castrated 2-12 hours after birth show paternal behaviour compared to the 80% of paternal controls, and treatment with testosterone at 70-75 days of age does not restore paternal behaviour in neonatally castrated males (Lonstein et al. 2002). Thus, a combination of androgenic and estrogenic activity during early development is necessary to organize the brain circuitry underlying the adult expression of paternal behaviour in the prairie vole.
2.3.2 Sexual behaviour

Estradiol also plays a role in the development of male sexual behaviour. Male rats treated with the aromatase inhibitor ATD for the first 21 days of postnatal development have reduced ejaculation frequencies as adults (Brand et al. 1991). Mice that lack a functional copy of the gene for P450 aromatase (Cyp19), the enzyme responsible for converting androgens to estradiol (Figure 6), have also proved to be a useful model for examining the role of estradiol in adult sexual behaviour (ArKO mice). ArKO males show reduced mount, intromission, and ejaculation frequencies as adults, resulting in low fertility rates (ability to sire a litter) despite normal spermatogenesis (Toda et al. 2001; Matsumoto et al. 2003). Treatment with estradiol benzoate during adulthood does not restore mount, intromission, or ejaculatory frequencies to the levels seen in controls, indicating that estradiol must act during development to organize sexual behaviour (Bakker et al. 2004). However, male ferrets treated with an aromatase inhibitor (ATD) for postnatal days 1-15 have normal sexual behaviour (mounting, pelvic thrusting and neck gripping) as adults (Baum et al. 1983). Similarly, male prairie voles treated with ATD for the first week of neonatal development show normal mounting, thrusting, and ejaculatory frequencies compared to oil-treated controls, and the proportion of males displaying these behaviours is unaltered (Northcutt & Lonstein 2008). Thus, estradiol action during development is responsible for organizing adult sexual behaviour in some, but not all, species.
Figure 6: The steroid biosynthetic pathway, showing the sequence of estradiol production from cholesterol (C\textsubscript{27} lipid). Progestins (C\textsubscript{21} steroids) are produced by cleaving the long chain of carbons from the top of cholesterol; androgens (C\textsubscript{19} steroids) are produced by cleaving the ethyl group from progestins; estrogens (estradiol; C\textsubscript{18} steroids) are produced by cleaving the carbon at C\textsubscript{19} and aromatizing the corresponding 6-carbon ring. The enzyme P\textsubscript{450}aromatase (P\textsubscript{450}arom; outlined with black border) is responsible for converting androgens to estrogen. Adapted from Speroff (2005).
2.4 Hypotheses

In Chapter 3, ERα distribution in the MPOA, BNST, and MeA of male and female *Phodopus* will be examined in sexually naïve adults, recently mated adults, and new parents to test the hypothesis that: **upregulation of ERα expression in the maternal behaviour pathway of Phodopus will reflect the activational role of estradiol in the expression of sexual and parental behaviour.** In Chapter 4, adult *Phodopus campbelli* paternal responsiveness and copulatory patterns will be examined following aromatase inhibition at three different life stages to test the hypothesis that: **developmental estradiol is necessary to establish adult paternal and sexual behaviour in Phodopus campbelli males.**
CHAPTER 3: In three brain regions central to maternal behaviour, neither male nor female Phodopus dwarf hamsters show changes in oestrogen receptor alpha distribution with mating or parenthood\(^1,2\)

3.1 Abstract

Oestrogen receptor alpha (ER\(\alpha\)) immunoreactivity in three brain regions relevant to maternal behaviour (medial preoptic area, bed nucleus of the stria terminalis, and medial amygdala) was measured in two species of dwarf hamster that both mate during a postpartum oestrus, but differ in expression of paternal behaviour. Male and female P. campbelli and P. sungorus were sampled as sexually naïve adults, following mating to satiety, and as new parents. In all brain regions females expressed higher levels of ER\(\alpha\) than males. Species did not have an effect on ER\(\alpha\) distribution except in the medial amygdala, where P. sungorus females had higher expression levels than all other groups. Behavioural status was not associated with altered ER\(\alpha\) expression. These results were not expected for females and suggest that a primary activational role for oestrogen, acting through ER\(\alpha\) in these regions, does not generalise to maternal behaviour in Phodopus. In males, these results are consistent with previous manipulations of the ER\(\alpha\) ligand,

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oestrogen, and suggest that paternal behaviour in *P. campbelli* is likely to be regulated by developmental effects of oestrogen on the brain during early life (like *Microtus ochrogaster*), rather than through activation by oestrogen at the time of fatherhood (like *Peromyscus californicus*).

### 3.2 Introduction

Paternal behaviour is hypothesised to occur as a result of pre-existing maternal behaviour pathways, homologous to those found in females, being activated in the male brain (Wynne-Edwards & Reburn 2000). However, because paternal behaviour has evolved independently in response to conditions that make the presence of the male essential to offspring survival (Clutton-Brock 1991), variations in the regulation of this behaviour are expected to exist when different paternal species are compared. Conversely, because maternal behaviour is essential in mammals, the mechanisms regulating this behaviour are assumed to be deeply ancestral, and shared across species (Wynne-Edwards 2001). As such, the weight of evidence supports the existence of a common maternal behaviour pathway, including the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST), and medial amygdala (MeA), which is activated by oestrogen priming during pregnancy (Rosenblatt et al. 1998; Numan 2006).

There is evidence supporting diversity in oestrogen roles in males. Although a role for oestrogen in the regulation of paternal behaviour is supported in some naturally biparental species, it does not consistently generalise across genera. For example, paternal behaviour in the California mouse (*Peromyscus californicus*) is regulated by the
action of oestrogen in the brain (Trainor & Marler 2002; Lee & Brown 2007), but the same behaviour occurs independent of oestrogen activation in both the prairie vole
(Micrurus ochrogaster: Lonstein & De Vries 1999), and the Djungarian hamster

Although the hypothesis predicts common neuroendocrine pathways to maternal behaviour in females, the role of sex steroids in the regulation of behavioural receptivity has evolved (McMillan & Wynne-Edwards 1999) since P. campbelli and its closest relative, Phodopus sungorus shared a common ancestor 0.8-1.0 million years ago (Neumann et al. 2006). Thus, since the activational role of oestrogen in maternal behaviour has not been examined in Phodopus, the a priori hypothesis that oestrogen is responsible for the control of maternal behaviour might also fail to generalise to Phodopus.

A receptor for oestrogen, oestrogen receptor alpha (ERα), is also involved in parental behaviour pathways. In female rats and mice, ERα expression is upregulated (compared to levels seen in virgin females) in key maternal brain regions, including the MPOA, BNST and MeA, leading up to the birth of pups (Koch & Ehret 1989; Wagner & Morrell 1995; Wagner & Morrell 1996). In mice, these levels remain high during early lactation (Koch & Ehret 1989). Male mice that exhibit pup-searching and retrieval behaviours also show dynamic expression of ERα in the brain: five days after the birth of their first litter, paternal males have a pattern of ERα expression distinctly different from that of sexually naïve males (Ehret et al. 1993). Thus, in species where oestrogen plays an
activational role in parental behaviour, changes in ERα expression render specific brain regions more sensitive to the actions of oestrogen.

However, males that do not require oestrogen activation for the expression of paternal care have a contrasting pattern of ERα expression when sexually naïve. In *Microtus*, high levels of male social behaviour (such as paternal care) are associated with low ERα expression levels in the MeA and the BNST (Cushing et al. 2004; Cushing & Wynne-Edwards 2006). Similarly, in sexually naïve *Phodopus*, paternal *P. campbelli* males express lower levels of ERα in the BNST than females, or non-paternal *P. sungorus* males, implying that low, rather than high, ERα expression in this region is permissive for the expression of paternal care (Cushing & Wynne-Edwards 2006). As predicted by the hypothesis that pathways would be common, intraspecific differences in ERα expression were not identified in sexually naïve adult females in either genus (Cushing et al. 2004; Cushing & Wynne-Edwards 2006).

The current experiment was conducted to test the hypothesis that ERα expression would increase in the established maternal behaviour pathway (Stack et al. 2002) of *Phodopus* females, but not in biparental males becoming fathers. Specifically, if oestrogen plays an activational role in maternal behaviour in *Phodopus*, ERα expression should increase in behaviourally relevant brain regions of mothers of both species, compared to ERα levels expressed in sexually naïve, and recently mated, adults. Alternatively, if *P. campbelli* females regulate maternal behaviour independent of oestrogen activation, such as is seen in *P. campbelli* males (Hume & Wynne-Edwards 2006), no increases in ERα would be anticipated as a result of motherhood.
3.3 Materials and Methods

3.3.1 Animal model

*Phodopus campbelli* and *P. sungorus* are so closely related that they were long considered subspecies (Ross 1995; Ross 1998; Neumann et al. 2006), are very similar in body weight, show the same reproductive timetable, and are able to form viable hybrid breeding pairs (Wynne-Edwards 1998; Timonin & Wynne-Edwards 2006). However, *P. campbelli* is an obligately biparental species, while *P. sungorus* has sole maternal care of offspring. In *P. campbelli*, the presence of highly responsive males at the time of birth, and across pup development, prevents maternal hyperthermia, reduces the amount of weight lost by the female (Walton & Wynne-Edwards 1997; McInroy et al. 2000), and increases pup survival rates from less than 50% to 97% (Wynne-Edwards 1987). In contrast, *P. sungorus* females do not require the assistance of a male to rear their litter successfully (Scribner & Wynne-Edwards 1994a; Newkirk et al. 1998; Stulberg & Wynne-Edwards 1998), retrieval of an experimentally displaced pup by males is rare, (although females exhibit prompt retrieval behaviour; Timonin & Wynne-Edwards 2006), and males spend minimal time in contact with pups in the nest (Timonin & Wynne-Edwards 2006), are not typically present at the time of birth in the wild (Wynne-Edwards 1995), and do not assist in the birth process (Jones & Wynne-Edwards 2000).

3.3.2 Brain regions

Three brain regions with known involvement in the expression of maternal behaviour were examined: the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA). Both the MPOA and the BNST are
essential for the normal expression of maternal care in the rat, since lesions to these regions disrupt maternal behaviour (Numan 1974; Numan & Numan 1996; Stack et al. 2002). In contrast, the MeA plays an inhibitory role; damage to this region in sexually naïve female rats leads to reduced latencies to show maternal-like behaviour towards neonates (Fleming et al. 1980; Numan et al. 1993), and early onset of maternal behaviour in pregnant rats (Mann & Babb 2004). Neurons in these three brain regions are activated by interactions with a neonate (rat: Stack et al. 2002; prairie vole: Kirkpatrick et al. 1994b), and these activated neurons also express ERα (Lonstein et al. 2000). ERα expression is upregulated in the MPOA and BNST of male mice, and all three brain regions of female mice, five days following the birth of pups (Koch & Ehret 1989; Ehret et al. 1993).

3.3.3 Details of handling

Male and female *P. campbelli* and *P. sungorus* were sampled at three discrete life stages: sexually naïve adults, recently mated/pair-bonded adults, and parents with 3-day old pups. The sample size was 5-6 animals for each sex, species, and group. Animals in all three groups ranged in age from 90 to 120 days, with animals either singly housed, or group housed with same-sex siblings, prior to experimentation.

Vaginal cytology is ineffective in monitoring the oestrous cycle in *Phodopus* (Wynne-Edwards et al. 1987a), and females may require exposure to a male to initiate cycling, although a large majority will mate within 4 days of first exposure to a male (Erb et al. 1993). Thus, to ensure that all female controls were cyclic (as opposed to in persistent dioestrus), and synchronous, the oestrus cycle was tracked behaviourally
(Wynne-Edwards et al. 1987a). Each female was individually exposed to the same stud male for a brief observation period (< 5 minutes) every day after the onset of the dark cycle (1900h), until the day receptivity (lordosis) was seen. The stud male was not permitted to intromit (females received no vaginocervical stimulation), and the male was removed from the female’s cage as soon as the female showed the lordosis posture, or the observer was confident that she would not. Tissue from the females that displayed lordosis was collected the following morning (approximately 8-10 hours post ovulation; Erb & Wynne-Edwards 1993), on oestrus. Male controls were not given any exposure to a female, but were individually housed for at least 24 hours prior to tissue collection.

Breeding pairs for the other two groups were created using standard procedures for this breeding colony, which preclude sibling and half-sibling mating, and provide the male with a slight (2-10 g) weight advantage. All animals were virgins at the time of pairing. The day of mating was determined through direct observation of mating including an ejaculatory lock (Wynne-Edwards et al. 1987a), and the pair was left undisturbed to mate to satiety. For the recently mated group, tissue was collected approximately 40 hours following the first observed ejaculatory lock so that the endocrinology of the mated and unmated female would have diverged (McMillan & Wynne-Edwards 1999). For the parental group, the pair cohabitated throughout pregnancy and parturition, mating ad libitum during a postpartum oestrus, and cohabitating with the litter. Parental males and females had tissue collected on the third day after the birth, at the same time of day. Postpartum mating was not directly observed; thus, the length of time between mating during postpartum oestrus and tissue collection in
the parental group was not standardised, but was at least 48 hours. All procedures were
conducted in accordance with the guidelines of the Canadian Council on Animal Care
approved by Queen’s University as protocol 2008-011.

3.3.4 Tissue processing

Tissue was collected and processed according to the methods outlined in Cushing
and Wynne-Edwards (Cushing & Wynne-Edwards 2006). Animals received a lethal dose
derine (0.1ml of 100mg/mL Rogarsetic™, Pfizer, Kirkland, QC, Canada) plus
acepromazine (0.1ml of 10mg/mL Atravet™, Ayerst Veterinary Laboratories, Guelph,
ON, Canada) by intraperitoneal injection, the descending aorta was clamped, and an
injection of heparin (Hepalean™, Organon, Toronto, ON, Canada) was given directly
into the heart to reduce blood clot formation. Animals were perfused transcardially with
20-25ml cold 0.1M potassium-phosphate buffered saline (KPBS) plus 2% sodium nitrite
as a vasodilator (pH 7.6), followed by fixation with 90-100ml cold 4% paraformaldehyde
and 2.5% acrolein in 0.1M KPBS (pH 7.6), then 20-25ml cold KPBS plus 2% sodium
nitrite. Brains were removed and stored in a 25% sucrose solution (in 0.1M KPBS) at
4°C. 30μm sections were cut using a cryostat (CM1850, Leica Microsystems, Wetzlar,
Germany), and free-floating sections were stored in cryoprotectant (Watson et al. 1986)
at -20°C until immunocytochemical staining for ERα was carried out. Sections between
the start of the anterior commissure through to the beginning of the posterior commissure
were collected. Key landmarks were identified using Sidman’s (Sidman et al. 1971) atlas
of the mouse brain.
3.3.5 Immunocytochemistry

Tissue was processed in a series of three experimental runs over a six-day period. Tissue from different sexes, species, and behavioural groups was distributed across each run. There was no effect of staining batch (all $p > 0.07$). Tissue sections were rinsed in a series of 6 washes (10 min each) in 0.05M KPBS to remove the cryoprotectant. This was followed by a 20 minute incubation at room temperature in 1% sodium borohydride in 0.05M KPBS (to reduce residual aldehydes), and repeated rinsing with KPBS. Tissue was then incubated in 0.014% phenylhydrazine in 0.05M KPBS for 15 minutes at room temperature, and rinsed in a series of 6 washes (10 min each) in 0.05M KPBS. Next, tissue was incubated for 1 hour, at room temperature, with the primary antibody, rabbit anti-ERα polyclonal antibody (C1355 lot # 33505 Upstate, Lake Placid, NY, USA), at a dilution of 1:17,500 in KPBS with 0.4% TritonX, followed by a further 48 hours incubation at 4°C. This antibody, which has been used previously in *Phodopus* (Cushing & Wynne-Edwards 2006), recognises both free receptors and those bound to ligand, which reduces staining variation as a result of differences in circulating oestrogen concentration between the experimental groups (Cushing et al. 2004). Anti-ERα is directed against the last 14 amino acids of rat ERα, a region that does not share homology with ERβ (Cushing & Wynne-Edwards 2006). Unbound primary antibody was removed in a series of ten KPBS washes (6 min each), and tissue was then incubated for one hour at room temperature with the secondary antibody, biotinylated goat anti-rabbit IgG (1:600 dilution in KPBS with 0.4% TritonX). Unbound secondary antibody was removed in a series of 5 KPBS washes (10 min each) prior to incubation for 1 hour at room
temperature in an avidin-biotin peroxidase complex (Vectastain ABC kit-elite pk-6100 standard 4.5µl A and 4.5µl B per 1ml of 0.05M KPBS with 0.4% TritonX). Prior to visualisation of ERα by incubation in nickel sulphate-diaminobenzidine chromogen solution (250mg Nickel II Sulphate, 2mg diaminobenzidine (DAB), 8.3µl 3% hydrogen peroxide in 10ml sodium acetate), tissue sections were rinsed in a series of 3 KPBS washes (5 min each) followed by three washes in 0.175M sodium acetate. Tissue was incubated in the chromogen solution for 15 minutes, and then washed three times (5 min each) in 0.175M sodium acetate, followed by three washes in KPBS. All washes and incubations, with the exception of the incubation at 4°C, were carried out on a rotational platform shaker to ensure all tissue sections were equally exposed to substrates. Sections were mounted onto coated glass slides (Micro Slides Superfrost Plus, VWR International, Mississauga, ON, Canada) and air-dried overnight at room temperature. Mounted tissue was dehydrated through ascending ethanol solutions, cleared in Histoclear, and coverslipped with Histomount. Slides were coded so that the sex, species, and behavioural status of each animal were not apparent during quantitative analysis.

3.3.6 Optimisation

Prior to staining experimental tissue, the optimal concentration of anti-ERα antibody to obtain a clear specific staining with minimal background was determined. This preliminary antibody titration indicated that a concentration of 1:17,500, and an incubation time of 15 minutes in the chromogen solution, provided optimal results. Quantitative analysis was carried out as described below.
3.3.7 Microscopic analysis

Image analysis was carried out using the program Neurolucida (MBF Biosciences Williston, VT, USA). The brain regions were identified by plate number, as indicated from Paxinos and Watson’s (Paxinos & Watson 1998) atlas of the rat brain. The MPOA corresponded to plates 19 and 20, the BNST plates 21 and 22, and the MeA plates 30 and 31. The medial habenula (plate 30), which does not express ERα (Lonstein et al. 2000), was used as a negative control region. Consecutive sections were used to quantify each brain region. The number of cells expressing ERα immunoreactivity, that is cells containing a nucleus showing dark granular staining, was counted bilaterally for each section. In each hemisphere visible landmarks (i.e. optic chiasm, lateral ventricle, anterior commissure and optic tract) were used in the placement of a polygon, which served as a counting frame. A cell density estimate was calculated by dividing the number of ERα-ir positive cells inside the counting frame by the area of the counting frame (in 100µm²). Anatomical accuracy of the scored sections was confirmed, post hoc, by a second observer.

3.3.8 Statistical analysis

All statistical analysis was carried out using JMP 7.0 (SAS Institute, Cary, NC, USA). ERα-ir expression in each section was recorded as ERα-ir density (number of cells per 100µm²) in both hemispheres. These density measures were reduced to an average density for each animal over all consecutive sections before analysis, yielding one density measure for each brain region. Results were compared using ANOVA with three class variables: sex, species, and behavioural status, and each of the three brain regions as a
dependant variable. Post hoc comparisons of sex within species and/or behavioural status were carried out as indicated by significant effects of class variables or significant interaction terms. All measures applied a critical $\alpha$ value of 0.05.

3.4 Results

In female (Figure 7A, C) and male (Figure 7B, D) *Phodopus campbelli* and *P. sungorus* oestrogen receptor $\alpha$ immunoreactive (ER$\alpha$-ir) staining was seen in all three brain regions considered, MPOA, BNST, and MeA, regardless of their behavioural status (naïve, mated or parent). Staining was never evident in the medial habenula, the negative control region.

Females expressed higher levels of ER$\alpha$-ir in the MPOA than males ($F_{1,62} = 6.0, p < 0.05$). However, no effect of species (*P. campbelli* = *P. sungorus*: $F_{1,62} = 0.02, p = 0.88$) or behavioural status (naïve = mated = parent: $F_{2,62} = 1.2, p = 0.31$) on ER$\alpha$-ir expression was found in the MPOA (Figure 8A, B). No significant interaction was found (species*sex: $F_{1,62} = 1.9, p = 0.18$; species*behavioural status: $F_{2,62} = 0.43, p = 0.65$; sex*behavioural status: $F_{2,62} = 0.02, p = 0.98$; species*sex*behavioural status: $F_{2,62} = 1.3, p = 0.06$).

Similarly, females expressed higher levels of ER$\alpha$-ir in the BNST than males ($F_{1,64} = 24.16, p < 0.0001$), but there was no effect of species (*P. campbelli* = *P. sungorus*: $F_{1,64} = 0.89, p = 0.35$), or behavioural status (naïve = mated = parent: $F_{2,64} = 1.0, p = 0.37$; Figure 8C, D). No interaction was significant.
In the MeA, *P. sungorus* had greater ERα-ir expression than *P. campbelli* \((F_{1,63} = 9.30, p < 0.005)\), females had greater ERα-ir expression than males \((F_{1,63} = 5.62, p < 0.05)\), and there was a significant interaction between species and sex \((F_{1,63} = 5.52, p < 0.05)\). In post hoc comparisons, *P. sungorus* females expressed higher levels of ERα-ir than *P. campbelli* females \((t_{1,32}, p = 0.0001)\) and males of both species. There was no effect of behavioural status on ERα-ir expression in the MeA (naïve = mated = parent: \(F_{2,63} = 0.42, p = 0.66\); Figure 8E, F).

Thus, female ERα-ir was higher than in males in all three brain regions, irrespective of species or behavioural status. The only effect of species was that *P. sungorus* females had greater ERα-ir in the MeA than *P. campbelli* females. This effect had not been reported previously by Cushing and Wynne-Edwards (2006). Behavioural status did not have an effect on ERα-ir levels in any of the brain regions of interest.
Figure 7: Oestrogen receptor-α-immunoreactive (ERα-ir) staining in *Phodopus campbelli* females (A) and males (B), and *P. sungorus* females (C) and males (D). In each panel, the left column images are from sexually naïve adults. Tissue from females was collected the morning after behavioural oestrus. Males had been separated from male siblings at least 24 hours prior to tissue collection. The central column images are from animals that had mated to satiety approximately 40 hours prior to tissue collection. The right column images are from parents that were in contact with their first litter from birth until the third day of lactation. There was a higher density of ERα-ir in the medial preoptic area (upper row), with intermediate levels in the bed nucleus of the stria terminalis (middle row), and low levels in the medial amygdala (bottom row). See Figure 8 for quantitative results. Scale bars = 50µm.
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Figure 8: Mean (± SEM) oestrogen receptor-α-immunoreactive (ERα-ir) density (cell counts per 100µm²) in the medial preoptic area (A, B), bed nucleus of the stria terminalis (C, D) and the medial amygdala (E, F). Results are presented for Phodopus campbelli (A, C, E) and P. sungorus (B, C, F), with the colour of the bar indicating behavioural status (filled = sexually naïve; open = mated; shaded = parent). Females and males are presented separately. No statistical differences are indicated on the panels because the main effects, with females expressing higher ERα-ir densities than males, were independent of species as displayed here. However, no class effects of behavioural status were observed. See text for full details.
3.5 Discussion

*Phodopus campbelli* and *P. sungorus* mothers were expected to have an upregulation of ERα in behaviourally relevant brain areas during early lactation, similar to that seen in female rats and mice. However, this was not the result. There was no increase in ERα expression in the MPOA, BNST, and MeA of lactating females relative to cyclic, but sexually naïve females, as is seen in lactating mice and rats (Koch & Ehret 1989; Wagner & Morrell 1996). In fact, the effect of motherhood on ERα expression in *Phodopus* is similar to the effect of pregnancy and parturition on ERα-ir in the ewe. ERα expression is not upregulated in the MPOA or MeA of ewes during late pregnancy, or immediately following parturition, relative to ERα expression in oestrus females (Meurisse et al. 2005). The role of oestrogen in the activation of maternal behaviour in sheep also differs from the central activational role this hormone plays in rat maternal behaviour (Siegel & Rosenblatt 1975a; Siegel & Rosenblatt 1975b). Oestrogen priming during late pregnancy is necessary for the normal expression of maternal behaviour in ewes, but oestrogen alone does not stimulate this behaviour either in experienced mothers or in virgin females (Krehbiel et al. 1987; Kendrick & Keverne 1991). Thus, if oestrogen alone is sufficient to stimulate maternal behaviour in *Phodopus*, as is seen in the rat, the lack of ERα upregulation may be a result of mating during a postpartum oestrus.

Concurrent pregnancy and lactation reduces peripheral oestrogen levels during early lactation in *P. campbelli* females (Roy & Wynne-Edwards 1995), and ERα expression is reduced as a result of a reduction in circulating oestrogen levels in female rats (McGinnis et al. 1981; Giordano et al. 1990). As such, tissue collected on the day of parturition, or
tissue from a female not permitted to mate during postpartum oestrus, might have shown an increase in ERα-ir in brain regions related to the expression of maternal behaviour. However, this does not take into consideration the effect of lactation or pup interactions on ERα distribution in the female mouse brain (Koch & Ehret 1989), or the fact that the increase in ERα expression in the female brain leading up to the onset of maternal behaviour in the rat occurs independent of an increase in circulating levels of oestrogen (Wagner & Morrell 1995). ERα upregulation may also be seen as an increase in receptor expression per cell, as is seen in female rats during late pregnancy (Wagner & Morrell 1996), rather than an increase in the number of cells expressing ERα in a given brain region. Alternatively, the fact that Phodopus females do not upregulate the expression of ERα in three key brain regions involved in maternal behaviour may reflect neuroendocrine regulation of maternal behaviour more similar to that seen in ewes, with oestrogen playing a priming, rather than a primary, role in stimulating this behaviour.

Similarly, male P. campbelli do not show an increase in ERα-ir in these brain regions when they are fathers; levels remain lower than those seen in females regardless of behavioural status. In comparison, male mice that have 5 days experience with pups from their first litter have a distribution of ERα very similar to that of a virgin female (Ehret et al. 1993). In particular, there is an increase in the number of ERα-ir positive cells seen in the MPOA (Ehret et al. 1993). Approximately 90% of these first-time mouse fathers exhibited pup retrieval behaviour (Ehret et al. 1993), similar levels as those exhibited by first-time P. campbelli fathers (Jones & Wynne-Edwards 2000; Hume & Wynne-Edwards 2005; Schum & Wynne-Edwards 2005; Vella et al. 2005; Hume &
The pattern of ERα-ir seen in *P. campbelli* fathers is consistent with the microtine model of paternal care, where low ERα expression in the BNST and MeA is permissive for the expression of paternal behaviour. An increase in ERα in either of these regions would be associated with increased aggression (Kramer et al. 2008), and decreased affiliative behaviour towards both pups and mates (B Cushing, unpublished data). The role of ERα in the MPOA in regulating this behaviour in voles is less clear, as ERα expression in this region is not sexually dimorphic, and is the same in both paternal and non-paternal species (Cushing & Wynne-Edwards 2006). ERα expression in non-paternal *P. sungorus* fathers is also unchanged in the MPOA, BNST, and MeA, in contrast with increased oestrogen concentrations seen in the peripheral circulation of this species on the day before the birth of a first litter (Schum & Wynne-Edwards 2005).

One obvious role for ERα across these behavioural stages is in the activation of sexual behaviour. Both species of *Phodopus* mate during a postpartum oestrus (Wynne-Edwards & Lisk 1984), and oestrogen is known to play an activational role in the expression of sexual behaviour in male rats (Baum & Vreeburg 1973), although it does not fully restore sexual behaviour in some castrated males (Arteaga-Silva et al. 2005; Arteaga-Silva et al. 2007). Mating also activates *c-fos* expression in neurons that co-express ERα in the MPOA, BNST, and MeA in female prairie voles (Katz et al. 1999; Greco et al. 2003), and sexually experienced male rats have increased ERα expression in the MPOA 24 hours after mating to satiety (Phillips-Farfán et al. 2007). However, *Phodopus* males did not show the predicted changes in ERα-ir in the brain as a result of...
mating to satiety, although this may be due to the fact that tissue was collected 40, rather than 24, hours following the onset of mating behaviour.

The current study failed to replicate patterns of ERα-ir described previously for sexually naïve Phodopus females (Cushing & Wynne-Edwards 2006). Specifically, females expressed higher levels of ERα in the MPOA compared to males, and P. sungorus females expressed higher levels of ERα in the MeA compared to P. campbelli females (Cushing & Wynne-Edwards 2006). Several methodological details might have contributed to these differences. Approximately 50% of the naïve females sampled in the previous study (Cushing & Wynne-Edwards 2006) would have been in persistent dioestrus, because approximately half of females show lordosis, and ovulation, on the third day after pairing or exposure to a male (Erb et al. 1993). In contrast, the current study used behaviour to identify the timing of spontaneous ovulation (Wynne-Edwards et al. 1987b), and ensure that all females were cycling and had experienced a rapid decline in peripheral oestrogen in the hours between lordosis (without mating) and sampling (Wynne-Edwards et al. 1987b). Thus, the most probable source of the different results is the cyclic status of the females.

However, it remains possible that, although no mating took place during behavioural testing, the cues associated with the male altered ERα expression in these females. Exposure to male odours plays a role in modulating female sexual behaviour in the rat (Bennett et al. 2002), and in stimulating sexual receptivity in female prairie voles (Cohen-Parsons & Carter 1987). However, ERα expression in the BNST and MeA of female rats exposed to male-soiled bedding for two hours was no different from
expression in controls (Bennett et al. 2002). Similarly, female prairie voles permitted to interact with a sexually experienced male without mating for 1.25 hours did not show any changes in nuclear oestrogen receptor binding in tissue pooled from the MPOA, corticomedial amygdala, and medialbasal hypothalamus (Cohen-Parsons & Carter 1987), while females given the same exposure for 18-20 hours had increased oestrogen binding in the MPOA (Cohen-Parsons & Roy 1989). This increase in oestrogen binding in the brain is associated with the onset of oestrus, as female prairie voles require male stimuli to become sexually receptive, and receptivity begins 20-70 hours following exposure to an unfamiliar male (Cohen-Parsons & Roy 1989). Thus, it is unlikely that male stimuli were responsible for the differences between Phodopus female controls from the two studies, and it is probable that the differences were related to ovarian cyclicity, and stage of the oestrous cycle.

Differences in males were also noted between the two studies. In the previous study ERα expression in the BNST was significantly reduced in P. campbelli males compared to levels seen in P. sungorus males (Cushing & Wynne-Edwards 2006). Again, sexually naïve males in the current study were more homogeneous; specifically, males were separated from their adult litter-mates for at least 24 hours prior to tissue collection. There are significant steroid and peptide hormone changes in males after isolation for 24 hours (Reburn & Wynne-Edwards 2000), and these changes might have an influence on ERα expression in the brain.

Thus, ERα expression over the transition from naïve male, to mated male, to parental male, joins the list of recent tests that reject the hypothesis that oestrogen is
important in the activation of the exceptional male paternal behaviour of *P. campbelli*. In addition, the current study challenges the assumption that oestrogen is the primary hormone responsible for the activation of maternal behaviour in all species. As a result, two new hypotheses need to be tested. First, it is possible that oestrogen activation is not sufficient to stimulate maternal behaviour in *Phodopus*. Second, it remains possible that the critical roles for oestrogen are developmental effects that are temporally disassociated from the activation of maternal behaviour.
CHAPTER 4: Aromatase inhibition during adolescence reduces adult
sexual and paternal behavior in the biparental dwarf hamster Phodopus
campbelli3,4

4.1 Abstract

Previous studies have failed to identify an activational role for estradiol in the
paternal behavior of Phodopus campbelli fathers. However, none of these studies
addressed a developmental role that estradiol might play in establishing paternal behavior
in this species. Males were orally administered the aromatase inhibitor letrozole
(1mg/kg/day) for three days at 18, 34, or 90 days of age. As adults, males were tested for
paternal and sexual behavior. Letrozole treatment at 18 days resulted in males that spent
less time huddling over pups during the birth, and had higher pup losses and male-biased
pup survival for the first litter. Letrozole treatment at 34 days resulted in males that had
altered sexual behavior; males had a longer interval between mounts and between
intromissions, and took longer to achieve ejaculations over the first three ejaculatory
series. Furthermore, these males sired smaller first litters and produced second litters with
a male-biased sex ratio. Males treated with letrozole as adults showed a modest increase
in paternal care during the birth, but pup development and survival were not altered.

3 Published in Hormones and Behavior as Timonin ME and Wynne-Edwards KE (2008). Aromatase
inhibition during adolescence reduces adult sexual and paternal behavior in the biparental dwarf hamster
4 This research was supported by research grants from the Natural Sciences and Engineering Research
Council of Canada (NSERC) and the Canadian Institutes of Health Research (CIHR) to KEWE, and an
NSERC postgraduate scholarship to MET.
There was no effect of treatment on attack or retrieval behavior either as sexually naive adults or as new fathers. Thus, the results of the present study suggest that estradiol acts during adolescence to establish the normal expression of midwifery behavior and sexual behavior during adulthood.

4.2 Introduction
In spite of male estradiol concentrations in the range of cyclic females (Schum & Wynne-Edwards 2005), and in spite of extraordinary paternal care in the form of midwifery and paternal responsiveness (Jones & Wynne-Edwards 2000; Jones & Wynne-Edwards 2001; Vella et al. 2005), an activational role for estradiol in the expression of paternal behavior by the naturally biparental dwarf hamster *Phodopus campbelli* has been repeatedly rejected (Hume & Wynne-Edwards 2005; Schum & Wynne-Edwards 2005; Hume & Wynne-Edwards 2006; Wynne-Edwards & Timonin 2007; Chapter 3). Males do not show the predicted increase in serum estradiol levels directly before the birth of their first litter (Schum & Wynne-Edwards 2005), and reduction of estradiol either in the peripheral circulation (Hume & Wynne-Edwards 2005; Hume & Wynne-Edwards 2006) or central nervous system (Hume & Wynne-Edwards 2006) in the period of time leading up to and following the birth of a first litter has no effect on paternal responsiveness or reproductive success. Additionally, estrogen receptor alpha is not upregulated in new fathers in three brain regions central to the control of maternal behavior (Chapter 3). However, none of the above studies examined the developmental role of estradiol in establishing adult paternal behavior.
An organizational role of gonadal steroid hormones during development is typically limited to one or more ‘critical’ or ‘sensitive’ periods (Arnold & Gorski 1984). For example, monogamous male prairie voles (*Microtus ochrogaster*) require the actions of gonadal steroids during early neonatal development to establish the normal expression of spontaneous paternal behavior in adulthood. Castration on the day of birth significantly reduces the number of males that act paternally as sexually naive adults, while males deprived prenatally of both estrogens and androgens, and males castrated as adults, continue to show high levels of paternal behavior (Lonstein & De Vries 1999; Lonstein et al. 2002). However, species differ in their responses to developmental gonadal steroids. For example, neonatally castrated male rats show increased ‘maternal’ behavior towards neonates as sexually naive adults (McCullough et al. 1974), while male mice and Mongolian gerbils (*Meriones unguiculatus*) require high steroid levels both pre- and postnatally to reduce the frequency of infanticidal behavior (Gandelman & Vom Saal 1975; Perrigo et al. 1989; Clark et al. 1998). Although much of the brain development that underlies sexually dimorphic adult behavior occurs during prenatal or early neonatal development (Arnold & Gorski 1984), postnatal critical periods can extend into adolescence (Primus & Kellogg 1990; Schulz et al. 2004).

In the current study, the role of estradiol in the development of adult paternal and sexual behavior in *P. campbelli* was examined at three different life stages: independence from adult care concordant with the expression of alloparental behavior (Wynne-Edwards 1995; Vella et al. 2005), the adolescent transition, and as adults. The null hypothesis was that estradiol during any of these three phases of male development would not influence
adult expression of pup-directed or sexual behavior. As the direction of predicted responses to male estradiol during development was unknown, either an increase or a decrease in behavior would provide evidence of altered behavioral outcomes as a result of altered developmental exposure to estradiol.

4.3 Methods

4.3.1 Animals

Sexually naive male and female *P. campbelli* with no alloparental experience with siblings in subsequent litters, were drawn from a breeding colony that consists of the outbred descendants of wild-caught individuals maintained at Queen’s University since 1990 (Wynne-Edwards 1995). Standard housing conditions of $18 \pm 1.0^\circ C$ and photoperiod of 14L:10D (0000h corresponding to the middle of the dark phase) were maintained throughout the experiment. To control for post-weaning social experience, which is a factor in the frequency of infanticide in some males (e.g. *Phodopus sungorus*; Gibber & Terkel 1985), males were housed in same-sexed sibling dyads from the time of separation from the natal group (15-18 days) until pairing (90 days). Females were housed in groups of up to three sisters per cage from the time of separation from the natal group until pairing. Standard caging (27x21x14 cm polycarbonate cages, Nalge Nunc International, Rochester, New York, USA) was used throughout this study. Food (Rodent Diet 5001, LabDiet®, Richmond, IN, USA) and fresh tap water were available ad
libitum. All animal care procedures complied with the guidelines set out by the Canadian Council on Animal Care under Queen’s University protocol 2008-011.

4.3.2 Treatment groups

The endocrine profile of *P. campbelli* males across development is not known. Therefore, letrozole was administered at behaviorally relevant stages. Male littermate dyads were randomly assigned to 1 of 3 groups at different developmental stages: separation from the natal group (15-18 days of age), testicular maturation (30-36 days of age), or sexually naive adult (83-90 days of age). One of the males was randomly assigned to treatment and the other to control, so that the developmental housing environment was as well matched as possible for all animals.

In the wild, pups are first seen outside of the natal burrow at around 18 days of age, and disperse soon after (Wynne-Edwards 1995). Additionally, gestation is 18 days in this species, so 18 days of age would be the first opportunity for juveniles to be exposed to younger siblings (Newkirk et al. 1997). Eighteen- to 20-day-old males will show alloparental midwifery behavior during the birth of siblings, including mechanically assisting with delivery, cleaning and huddling over the neonates, and consuming placenta (Vella et al. 2005). Approximately 70% of males present during the birth of younger siblings will also retrieve a 2- or 3-day-old sibling displaced from the nest, and none will exhibit infanticidal behavior (Vella et al. 2005). Thus, males in the youngest group (‘18-day letrozole’) were treated with letrozole upon separation from the natal group.

Thirty-five days is the approximate threshold for adult testes weight in the closely related Siberian dwarf hamster (Hoffmann 1978). In *P. campbelli*, behavior towards pups
at 35 days of age is also less ‘paternal’ than 18-day old males. Fewer than 50% of 35-day old males retrieve an anesthetized pup, while approximately 25% attack the neonate (Vella et al. 2005). Thus, a second group of males (‘34-day letrozole’) were treated at 34 days to target this adolescent transition.

Finally, males are routinely paired at 90 days in this population. Since mating is needed to inhibit infanticide in male mice (Perrigo et al. 1991), and is necessary for the establishment of a pair bond in monogamous prairie voles (Insel et al. 1995), it might contribute to the expression of paternal behavior by new fathers. Thus, a final group of adult males (‘adult letrozole’) administered letrozole prior to mating for the first time, was equivalent to naive adult males used as controls in previous studies (Hume & Wynne-Edwards 2005; Schum & Wynne-Edwards 2005; Hume & Wynne-Edwards 2006).

Based on an *a priori* expectation that controls handled at different ages would show the same behavior as adults, controls were pooled into a single reference population (‘control’) against which each of the three letrozole-treated groups was compared.

Figure 9 outlines the timing of letrozole administration and behavioral testing, as described in detail below.

4.3.3 Aromatase inhibition

Males received the aromatase inhibitor letrozole, CGS 20267 [4,4-(1H-1,2,4-triazol-1-yl-methylene)-bis-benzonitrile] orally via ingestion. Although letrozole is typically administered using oral gavage or injection, ingestion is less invasive and has been used to successfully reduce serum estradiol concentration in male boars (Berger et
Figure 9: A schematic timeline of the experimental design. “L” represents treatment with letrozole or vehicle. The upper line represents the ‘18-day letrozole’ group, the middle line the ‘34-day letrozole’ group, and the lower line the ‘adult letrozole’ group.
Pup growth

Separation (15-18 days)
Testes mature (30-36 days)
Naïve pup retrieval test (65-70 days)
Pair (90 days)

Sexual behavior test
Midwifery test

Pup growth
Pup growth

Birth

Testes mature (30-36 days)

Sexual behavior test
Midwifery test

Pup growth
Pup growth

2nd litter
al. 2007) and bonnet monkeys (*Macaca radiate*; Shetty et al. 1997). Out of each sibling dyad, one male was fed approximately 1 mg/kg (maximum dose: 1.4 mg/kg; minimum dose: 0.7 mg/kg; mean dose: 1.13 ± 0.03 mg/kg) of letrozole (Hume & Wynne-Edwards 2006) mixed into a small volume of sesame paste. The second member of each dyad acted as an age-matched handling control and was fed the sesame paste without letrozole on the same schedule as the letrozole-treated males. The day before treatment began, males were anaesthetized with remotely administered isoflurane (Reburn & Wynne-Edwards 2000), and each male was given a notch in either the left or right ear to allow for individual identification. After 15 min of recovery, individuals were placed in their own feeding station (8x8x6 cm plastic food storage box with air holes in lid) and exposed to the sesame paste (vehicle) for several minutes. If an animal did not eat the paste upon first exposure, a dish of sesame paste was left in the home cage overnight to allow for acclimatization to the novel food. For the next three days, at the same time of day, males were separated from their cage-mate, placed in their feeding station, and exposed to either letrozole or vehicle. Each male was returned to the home cage once the paste had been consumed or it was clear that the male was not going to consume it. Three days of treatment is sufficient to achieve maximal plasma estradiol suppression in humans and the half-life of letrozole in humans is 2 days (Novartis Corporation), so that estradiol suppression should continue for 48 h after the last dose of letrozole, resulting in 5 days of developmental exposure.
4.3.4 *Paternal behavior*

4.3.4.1 Sexually naive males

Animals were evaluated in a standard pup retrieval test at 65-70 days of age. All testing took place during the light phase. One male from each same-sexed sibling dyad was removed at random from the home cage, and placed in a clean cage on the floor of the testing room. Thus, this male was out of sight, but within olfactory and auditory range of the animal being tested. Unrelated litters (1-4 days old) were separated from their parents, placed in a cage containing a thin layer of bedding, and floated over a warm water-bath. Stimulus pups were euthanized at the end of the test. From the litters, a single pup was placed in the home cage, and adult behavior towards the pup was directly observed for 10 min (Jones & Wynne-Edwards 2001; Vella et al. 2004). Males were then exchanged, and the second male was tested in the same manner.

The total amount of time the male spent in contact with the pup, plus the presence/absence and latency (s) to (1) contact (sniff, lick or touch), (2) pick up, (3) retrieve (pick up and return pup to the nest), or (4) attack (bite), the displaced pup were recorded. If the male attacked the pup, the test was terminated immediately.

In addition, to avoid issues of non-independence between latencies and the presence or absence of paternal behavior, all measures were condensed into a single paternal responsiveness score, which gave penalties based on the amount of time it took the male to retrieve the displaced pup, and rewards for desired behavior. Each 100 s that elapsed resulted in a penalty of –1, such that a male that retrieved in under 100 s had no penalty, and a male that did not retrieve the pup within 10 min had a penalty of –6.
Although pup motility at this age is limited, pups occasionally locate the male’s nest unaided. If so, the male received a time penalty as if he had carried the pup to the nest. Rewards were awarded for contacting (1 point), picking up (1 point), and retrieving the displaced pup (4 points). This bonus system is employed because females that show altered maternal behavior continue to contact and pick up the displaced pup, but will not return it to the nest (Lucas et al. 1998). So, a male that contacted, picked up, and returned the displaced pup to the nest in under 100 s was given a paternal responsiveness score of +6, whereas a male that did not contact the displaced pup for the duration of the test was given a score of –6 (Jones & Wynne-Edwards 2001). Any male that attacked the pup was given a score of –6, and was not considered as either picking up or retrieving the pup, even if these behaviors were displayed prior to attack (Vella et al. 2004).

4.3.4.2 First-time fathers

4.3.4.2.1 MIDWIFERY

On gestation day 17, as determined by direct observation of ejaculation (described below), pairs were placed in a clean cage containing a thin layer of bedding, and the typical wire cage lid was replaced with a transparent Plexiglas lid. Rodent chow was made available on the floor of the cage, and slices of apple and carrot sticks were provided as a water source. Each pair was videotaped starting at 0200h on gestation day 17, and continuing as late as the onset of the dark phase (1900h) on gestation day 18. The animal holding room was illuminated with dim ambient red lighting during the dark phase.
As far as was possible based on the recording angle of the camera, all incidences of direct paternal behavior during the birth process were scored as they occurred. Male and female location relative to the nest (on or off the nest) was scored each minute, on the minute, for a 2-hour period starting with the birth of the first pup (Jones & Wynne-Edwards 2000). Ten behaviors (anogenital licking, assisting delivery, licking pups, eating placenta, carrying pup, repairing nest, huddling over pups, self-directed grooming, mate-directed grooming, and eating), as previously described by Jones and Wynne-Edwards (2000), were recorded if they occurred within the minute. Thus, all midwifery behavior results fall between 0 and 1, reflecting the proportion of the 120 scans in which the behavior occurred.

4.3.4.2.2 PUP RETRIEVAL TEST

Males that sired a litter were given a second pup retrieval test when their first litter was three days old. The female was removed from the home cage and placed in a clean cage on the floor of the testing room. A single pup was removed from the nest, weighed, and returned to the home cage in the farthest corner from the natal nest. The same behavioral measures as described above were recorded, with the addition of total time spent in contact with the natal nest during the 10-min test. The time spent in contact with the nest was combined with the time spent in contact with the displaced pup for analysis.
4.3.5 Sexual behavior

Breeding pairs were created when males were approximately 90 days old. Standard procedures for this breeding colony were followed, which preclude full and half sibling breeding pairs, and provide the male with a slight (2-10 g) weight advantage. Pairs were created in the morning, and were left undisturbed until 1h before the beginning of the dark cycle (1800h). At this time, the typical wire cage lid was replaced with a transparent Plexiglas lid and each pair was videotaped for 3 h. Videotaping was repeated for up to 5 days from the day of pairing to encompass one complete 4-day estrus cycle, including ovulation and sexual receptivity. Copulatory behaviors (Wynne-Edwards & Lisk 1987) recorded were mount frequency (the number of mounts with and without intromissions), intromission frequency (the number of mounts with intromission), the intromission to mount ratio (intromission frequency/mount frequency), the latency to ejaculate (time in seconds from first mount of the ejaculatory series to the beginning of the ejaculatory lock), the mean inter-mount interval (latency to ejaculate/mount frequency), the mean inter-intromission interval (latency to ejaculate/intromission frequency), the ejaculatory lock duration (time in seconds from the beginning of the ataxic ejaculatory lock to the separation of the male and female) and the post-ejaculatory interval (the time in seconds from the end of the ejaculatory lock until the first mount of the next series).

4.3.6 Reproductive success

For first litters, the number of pups was recorded on the day of birth, and pairs were left undisturbed for three days, until the pup retrieval test (described above) was
carried out. Females that are housed with their mate throughout pup development successfully raise 97-100% of their pups whereas solitary females wean less than half of their pups (Wynne-Edwards 1987). Thus, removing the male is an effective strategy to reveal the effect of manipulations on reproductive success (McInroy et al. 2000). Following behavioral testing the male was separated from the female and litter, and pup growth was expected to suffer in the absence of the male parent (Wynne-Edwards 1987; McInroy et al. 2000). Subsequent pup development was evaluated by measuring pup weight every three days from birth until weaning (Newkirk et al. 1995). At weaning (18 days after birth) pups were sexed by examination of the anogenital distance, and sex was confirmed by dissection. No attempt was made to track individual littermates during development, and results were reduced to an average for each litter before analysis. Pup condition was also assessed through litter survival to weaning, and through examining the proportion of ‘best pups’, that is, pups at postnatal day 18 that meet the threshold for dispersal and independent living in the wild (body weight of at least 11.5g; Wynne-Edwards 1987; Walton & Wynne-Edwards 1997; McInroy et al. 2000). Females were monitored for the birth of a second litter and pup development and condition was evaluated as described for first litters, except that pups were weighed every three days starting at postnatal day 12 until postnatal day 18.

4.3.7 Statistical analysis

All statistical analyses used JMP version 7.0 (SAS Institute, Cary, NC, USA) and applied a critical alpha value of 0.05. Controls at the 3 developmental stages were compared to ensure that there were no significant effects, and then combined to yield a
single control group against which each of the three developmental cohorts was compared. Nominal variables with two levels, such as the presence or absence of retrieval during the pup displacement test, were compared by Pearson Chi-square tests unless otherwise noted. Continuous variables, such as pup weight across litters, were tested for normality (Shapiro Wilk) and then compared by a Students t-test or a non-parametric Wilcoxon two-sample test as appropriate. In all cases where a limiting value was imposed (e.g. latency for a behavior that did not occur) comparisons were non-parametric.

4.4 Results

4.4.1 Paternal Behavior

4.4.1.1 Sexually Naive Males

All males contacted the pup, typically in under 20 s. A few males from each group neither retrieved nor attacked the pup during the test (control: 3/25; 18-day letrozole: 1/11; 34-day letrozole: 2/8; adult unmanipulated: 5/20). Of the males that picked up the pup, all retrieved it to the nest area. On average, retrieval took place in under 3 min, regardless of treatment. The proportion of males either retrieving (Figure 10A), or attacking (Figure 10B), the pup was not affected in males treated with letrozole at either 18 or 34 days of age compared to the control group. As this test preceded the adult letrozole treatment, all adults were controls (unmanipulated) and showed high retrieval and low attack rates (Figure 10A, B). The paternal responsiveness score (control: -0.6 ± 1.1; 18-day letrozole: 1.2 ± 1.7; 34-day letrozole: -3.0 ± 1.7; adult
**Figure 10**: Percent of sexually naive adult males (65-70 days of age) and fathers (approximately 110 days of age) retrieving (A, C) or attacking (B, D) a pup during a standard pup retrieval test. Sexually naive males were tested with an unrelated 1-4 day old pup placed in the home cage, and male behavior towards the pup was recorded until attack (bite) occurred, or for 10 minutes. Fathers were tested with a 3-day old pup from their first litter. Animals that attacked were not counted as retrieving even if retrieval behavior was displayed prior to attack. Letrozole treatment (open bars) occurred at 18 days of age (‘18-day’), 34 days of age (‘34-day’), or 90 days of age (‘adult’). Solid bars represent handling controls. Adult animals were untreated at the time of the naive pup test (A, B).
A: Control, 18-day, 34-day, Adult
B: Control, 18-day, 34-day, Adult
C: Naive Adults
D: New Fathers

Pup Retrieval (%)
Pup Attack (%)

Control 18-day 34-day Adult
Control 18-day 34-day Adult
letrozole: 1.0 ± 1.2), and the time (s) spent in contact with the pup (control: 453.1 ± 29.3; 18-day letrozole: 439.2 ± 39.0; 34-day letrozole: 532.2 ± 25.5; adult letrozole: 486.2 ± 20.6), did not differ between treatment groups.

4.4.1.2 First-time Fathers

4.4.1.2.1 MIDWIFERY

A total of 45 pairs were videotaped during the birth of their first litter. Males spent a large proportion of the 2-hour observation period quiet on the nest (Table 1), with no effect of letrozole treatment. As expected, the majority of males assisted in the birth process (Jones & Wynne-Edwards 2000; Jones & Wynne-Edwards 2001) through licking the females’ anogenital region and/or tugging the pup from the birth canal (control: 23/27; 18-day letrozole: 8/9; 34-day letrozole: 3/3; adult letrozole: 6/6), as well as eating placenta (Table 1; Gregg & Wynne-Edwards 2005). In addition, letrozole-treated adults significantly increased three measures of midwifery behavior: proportion of the test spent licking the females’ anogenital region ($z = 2.5, p < 0.05$), the proportion of males that manually assisted with birth (Likelihood ratio: $X^2 = 4.3, p < 0.05$), and ate placenta (Likelihood ratio: $X^2 = 4.3, p < 0.05$; Table 1). All males spent time licking and huddling over newborn pups. However, males treated with letrozole at 18 days spent a significantly lower proportion of the test huddling over pups compared to controls ($z = -2.1, p < 0.05$; Table 1). Nest repair behavior was common while carrying pups was not; neither behavior was altered by letrozole treatment.

Non-paternal behavior during birth was not altered by letrozole treatment. All males spent a large proportion of the test engaged in self-directed grooming, while mate-
Table 1: Paternal behavior displayed by males during the birth of a first litter

<table>
<thead>
<tr>
<th></th>
<th>Quiet on nest (proportion of scans ± SEM)</th>
<th>Ever assist delivery?</th>
<th>Lick anogenital region (proportion of scans ± SEM)</th>
<th>Ever eat placenta?</th>
<th>Contact with pupsb (proportion of scans ± SEM)</th>
<th>Repair nest (proportion of scans ± SEM)</th>
<th>Ever carry pup?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.81 ± 0.03</td>
<td>18/27</td>
<td>0.020 ± 0.005</td>
<td>0.09 ± 0.02</td>
<td>6/27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-day</td>
<td>0.73 ± 0.08</td>
<td>7/9</td>
<td>0.031 ± 0.016</td>
<td>0.07 ± 0.02</td>
<td>3/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34-day</td>
<td>0.75 ± 0.22</td>
<td>3/6</td>
<td>0.008 ± 0.008</td>
<td>0.04 ± 0.02</td>
<td>0/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>0.87 ± 0.03</td>
<td>6/6*</td>
<td>0.068 ± 0.021*</td>
<td>0.09 ± 0.02</td>
<td>1/6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Letrozole group significantly different from controls (p < 0.05). Significant differences are discussed in the text.

a# scans/120

bIncludes both licking and huddling over newborn pups
directed grooming (excluding anogenital licking) was rare. All males ate during the 2-hour observation period.

4.4.1.2.2 PUP RETRIEVAL TEST

On average, all fathers contacted the pup within 10 s, but unlike the sexually naive males, not all fathers that picked up the pup retrieved it to the nest area. For control and letrozole-treated males in all three groups, retrieval (Figure 10C) was typically displayed in less than 4 min, no pups were attacked (Figure 10D), and there was no effect of letrozole treatment on either the paternal responsiveness score (control: 3.2 ± 0.6; 18-day letrozole: 4.4 ± 1.1; 34-day letrozole: 1.6 ± 2.1; adult letrozole: 4.2 ± 0.8), or the time (s) fathers spent in contact with pups (control: 289.2 ± 28.3; 18-day letrozole: 275.2 ± 29.3; 34-day letrozole: 295.7 ± 59.1; adult letrozole: 339.1 ± 54.5). The mean paternal responsiveness score from the paternal pup retrieval test was significantly higher than that for the naive test because no fathers attacked the pup (paired t = 4.4, df = 42, p < 0.0001), although the scores of males were not highly correlated on the two tests (r² = 0.04).

4.4.2 Sexual Behavior

An effect of ejaculatory series on the sexual behavior of control males was expected (Wynne-Edwards & Lisk 1987). The total number of intromissions, the ratio of intromissions to mounts, the duration of the copulatory lock, and the length of time between ejaculatory series all declined across ejaculatory series (Figure 11). Of the 29 control males that ejaculated at least once, 25 (86%) ejaculated at least 3 times. There were clear differences between series 1 and 3. During ejaculatory series 1, males began
**Figure 11:** Male sexual behavior displayed by controls across ejaculatory series over the first 3 hours of mating. Each data point represents the mean (± SEM) number of mounts with intromission (a), ratio of mounts with intromission to total number of mounts (b), duration of the ejaculatory lock (c), and length of time from the end of the ejaculatory lock to the first mount of the subsequent ejaculatory series (d). *Significantly different from first ejaculatory series when compared by paired t-test (p < 0.05)
a) # Intromissions

b) I/M Ratio

c) Lock Duration (sec)

d) Post-ejaculatory Interval (min)
mating more quickly after the first ejaculatory lock (post-ejaculatory interval: $t = 2.4$, $df = 21$, $p < 0.0001$) when compared to series three (Figure 11). However, males had a longer period of time between mounts (inter-mount interval: $t = -2.7$, $df = 24$, $p < 0.05$), and took longer to ejaculate (latency to ejaculate: $t = -3.3$, $df = 24$, $p < 0.005$) compared to ejaculatory series 3. Finally, during ejaculatory series 1, males exhibited higher intromission ($t = 8.2$, $df = 23$, $p < 0.0001$; Figure 11A) and mount frequency ($t = -2.9$, $df = 23$, $p < 0.01$) compared to ejaculatory series 3. Results for each male were therefore assessed based on ejaculatory series 1, the sum of behaviors across ejaculatory series 1-3, and the sum of behaviors across all series in the 3-hour window (Table 2).

Letrozole-treated males did not have altered behavior in ejaculatory series 1 (Table 2a). However, over the first three ejaculatory series, males treated with letrozole at 34 days displayed a greater inter-mount interval compared to controls ($z = 2.0$, $p < 0.05$; Figure 12B), as well as a greater inter-intromission interval ($z = 2.2$, $p < 0.05$; Figure 12C), and a longer latency to ejaculate ($z = 2.3$, $p < 0.05$; Figure 12D). No alterations were detected in 18-day letrozole treated males. The duration of the ejaculatory lock, however, was longer in adult letrozole-treated males ($z = 2.6$, $p < 0.01$).

Behavior results for the entire 3-hour test included all males irrespective of mating performance. No aspect of male sexual behavior was altered by letrozole treatment (Table 2c). Although 25/29 control males ejaculated at least 3 times, only 8/29 (23%) ejaculated at least 7 times, and the maximum number of ejaculations was 11 (1/29 males; Figure 13).
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>18-day</th>
<th>34-day</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mount freq</td>
<td>68.8 ± 22.6</td>
<td>53.6 ± 15.1</td>
<td>46.2 ± 5.1</td>
<td>43.0 ± 9.4</td>
</tr>
<tr>
<td>Intromission freq</td>
<td>24.0 ± 4.4</td>
<td>17.6 ± 3.5</td>
<td>12.7 ± 2.3</td>
<td>11.3 ± 2.6</td>
</tr>
<tr>
<td>Intromission to mount ratio</td>
<td>0.49 ± 0.06</td>
<td>0.40 ± 0.11</td>
<td>0.55 ± 0.03</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>Intermount interval (sec ± SEM)</td>
<td>23.4 ± 5.4</td>
<td>38.2 ± 17.6</td>
<td>51.6 ± 10.8</td>
<td>60.0 ± 18.3</td>
</tr>
<tr>
<td>Inter-intromission interval (sec ± SEM)</td>
<td>51.2 ± 4.1</td>
<td>85.9 ± 7.7</td>
<td>60.0 ± 11.3</td>
<td>41.0 ± 3.4</td>
</tr>
<tr>
<td>Latency to ejaculate (min ± SEM)</td>
<td>83.8 ± 13.2</td>
<td>85.0 ± 19.1</td>
<td>51.6 ± 12.7</td>
<td>38.2 ± 13.2</td>
</tr>
<tr>
<td>Ejaculatory lock duration (sec ± SEM)</td>
<td>34.9 ± 8.7</td>
<td>54.8 ± 11.9</td>
<td>26.2 ± 7.1</td>
<td>13.4 ± 3.7</td>
</tr>
<tr>
<td>Post-ejaculatory quiescent period (min ± SEM)</td>
<td>9.0 ± 5.2</td>
<td>10.4 ± 1.8</td>
<td>9.0 ± 0.9</td>
<td>9.0 ± 0.9</td>
</tr>
</tbody>
</table>

Letrozole group significantly different from controls (p < 0.05). Significant differences are discussed in the text.

Includes only males with 3 or more ejaculations.

*Includes males that did not ejaculate or sire a litter born on time with values of 0 for the number of intromissions and a maximal latency to ejaculate (10800 sec) included for these males.

Includes males with 3 or more ejaculation.

Table 2: Sexual behavior results (mean ± SEM).
**Figure 12:** Male sexual behavior across the sum of the first three ejaculatory series, including the intromission frequency (A), time between mounts (B), time between intromissions (C), time from first mount to ejaculation (D), duration of the ejaculatory lock (E), and the time between ejaculations (F). Letrozole treatment (open bars) occurred at 18 days of age (‘18-day’), 34 days of age (‘34-day’), or 90 days of age (‘adult’). Solid bars represent handling controls. *Significantly different from control (p < 0.05)*
Figure 13: Survival lines representing the number of males reaching each threshold for ejaculatory series.
4.4.3 Reproductive success

4.4.3.1 Breeding success
Litters were born to over 90% (58/64) of pairs within the expected window if conception had occurred during the first 4 estrus cycles from the time of pairing. Pups from those litters were included in all measures of reproductive success. Forty-eight of the 64 pairs were observed mating (ejaculatory lock) during the sexual behavior test, and 44 of these pairs produced a first litter 18 days following mating. Four of the pairs had a confirmed pregnancy block (pairs observed mating that failed to produce a litter on time), but 3/4 successfully mated during a subsequent cycle. Sixteen out of 64 pairs were not videotaped mating. Of these pairs, 10/16 produced a first litter whose timing indicated that the problem was the timing of the videotaping, rather than with a failure to mate within 5 days of pairing. Six of the remaining 10 pairs were not observed mating and failed to produce a litter on time, although 1 pair did mate in a subsequent cycle. Overall, the probability that a pair would conceive a first litter within 4 estrus cycles was not affected by aromatase inhibition (Table 3a), with breeding success ranging from 75% (34-day letrozole group) to 100% (adult letrozole group).

Second litters were born to 79% (46/58) of pairs, with no evidence that the probability that a pair would produce a second litter was affected by aromatase inhibition (Table 3b).

4.4.3.2 Litter quality
There was a significant effect of aromatase suppression on litter size at birth, with males treated with letrozole at 34 days producing significantly smaller first litters.
<table>
<thead>
<tr>
<th>n</th>
<th>Pairs producing a litter</th>
<th>Mean litter size at birth (± SEM)</th>
<th>Litter size at weaning (± SEM)</th>
<th>Mean proportion of pups lost (± SEM)</th>
<th>Proportion of litters with 1 or more best pup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) first litter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>36</td>
<td>33/36</td>
<td>6.5 ± 0.3</td>
<td>5.2 ± 0.3</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>18-day</td>
<td>11</td>
<td>10/11</td>
<td>6.2 ± 0.5</td>
<td>4.0 ± 0.6</td>
<td>0.32 ± 0.10*</td>
</tr>
<tr>
<td>34-day</td>
<td>8</td>
<td>6/8</td>
<td>4.3 ± 0.8*</td>
<td>3.5 ± 0.8</td>
<td>0.17 ± 0.12</td>
</tr>
<tr>
<td>Adult</td>
<td>9</td>
<td>9/9</td>
<td>5.7 ± 0.3</td>
<td>4.1 ± 0.4</td>
<td>0.26 ± 0.07</td>
</tr>
<tr>
<td>b) second litter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>33</td>
<td>25/33</td>
<td>5.8 ± 0.4</td>
<td>5.0 ± 0.4</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>18-day</td>
<td>10</td>
<td>9/10</td>
<td>6.1 ± 0.3</td>
<td>5.8 ± 0.5**</td>
<td>0.07 ± 0.06**</td>
</tr>
<tr>
<td>34-day</td>
<td>6</td>
<td>5/6</td>
<td>6.0 ± 1.3</td>
<td>4.8 ± 0.6</td>
<td>0.17 ± 0.13</td>
</tr>
<tr>
<td>Adult</td>
<td>9</td>
<td>7/9</td>
<td>6.3 ± 0.3</td>
<td>5.7 ± 0.3</td>
<td>0.08 ± 0.04</td>
</tr>
</tbody>
</table>

*Mean proportion of pups lost = litter size at birth – litter size at weaning/ litter size at birth
*Best pup = pup that weighs at least 11.5 g at weaning (dispersal threshold in wild)
*Letrozole group significantly different from controls (p < 0.05)
**Significantly different from first litter when compared by paired t-test (p < 0.05). Significant differences are discussed in the text.
compared to controls ($t = -2.1, df = 37, p < 0.05$; table 3a). There was no effect of letrozole treatment on second litter size at birth or weaning (Table 3b). The only other effect on litter size was that second litters, compared to first litters, were larger at weaning for 18-day letrozole-treated males ($t = 3.7, df = 7, p < 0.005$).

Postnatal day 12 represents the last day that maternal contributions to pup development can be measured before pups supplement their own growth with laboratory chow (Newkirk et al. 1995). On day 12 there were no differences in average pup weight for first litters (Table 4a). Pups on day 12 for second litters were heavier in all groups except adult letrozole-treated males (Table 4b). Patterns were similar on day 15 after birth (control: $t = 3.8, df = 24, p < 0.001$; 18-day letrozole: $t = 4.9, df = 7, p < 0.005$; 34-day letrozole: $t = 3.7, df = 3, p < 0.05$; adult letrozole: $t = 2.0, df = 6, p = 0.1$).

As expected for solitary *P. campbelli* mothers raising a first litter (Wynne-Edwards 1987), only 40% of control first litters contained at least one ‘best pup’ that reached a dispersal weight by postnatal day 18 (Table 3a). Also as expected, for experienced mothers (McInroy et al. 2000) this increased to 80% for second litters (Table 3b). For controls, 70% of pups were at least 11.5g at weaning for second litters, compared to less than 30% for first litters ($t = 3.9, df = 24, p < 0.001$). None of these measures of pup quality was altered by letrozole treatment (Table 3).

As expected (Wynne-Edwards 1987), there was no sexual dimorphism for body weight at postnatal day 18 for either the first or second litter. However, there were two interesting letrozole effects on female pups. First, letrozole treatment at 34 days decreased the number of females at weaning in the second litter (Table 4), resulting in male-biased investment in the second litter. Second, none of the 18 surviving females in
## Table 4: Measures of litter quality (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Pup weight on day 12 (g ± SEM)</th>
<th>Pup weight at weaning (g ± SEM)</th>
<th>Mean proportion males on day 18 (± SEM)(^a)</th>
<th>Mean proportion of male best pups day 18 (± SEM)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) First litter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>36</td>
<td>6.5 ± 0.2</td>
<td>9.0 ± 0.5</td>
<td>0.54 ± 0.04</td>
<td>0.59 ± 0.08</td>
</tr>
<tr>
<td>18-day</td>
<td>11</td>
<td>6.0 ± 0.4</td>
<td>7.9 ± 0.8</td>
<td>0.62 ± 0.10</td>
<td>1.00 ± 0</td>
</tr>
<tr>
<td>34-day</td>
<td>8</td>
<td>6.6 ± 0.9</td>
<td>9.8 ± 1.4</td>
<td>0.48 ± 0.18</td>
<td>1.00 ± 0</td>
</tr>
<tr>
<td>Adult</td>
<td>9</td>
<td>7.4 ± 0.6</td>
<td>11.8 ± 1.1*</td>
<td>0.43 ± 0.07</td>
<td>0.43 ± 0.11</td>
</tr>
<tr>
<td><strong>b) Second litter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>33</td>
<td>8.2 ± 0.4**</td>
<td>12.8 ± 0.6**</td>
<td>0.43 ± 0.05</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td>18-day</td>
<td>10</td>
<td>7.7 ± 0.3**</td>
<td>11.8 ± 0.7**</td>
<td>0.42 ± 0.07</td>
<td>0.34 ± 0.10</td>
</tr>
<tr>
<td>34-day</td>
<td>6</td>
<td>8.0 ± 0.7**</td>
<td>13.0 ± 0.8**</td>
<td>0.69 ± 0.06*</td>
<td>0.75 ± 0.03*</td>
</tr>
<tr>
<td>Adult</td>
<td>9</td>
<td>8.6 ± 0.8</td>
<td>13.7 ± 1.0</td>
<td>0.54 ± 0.08</td>
<td>0.51 ± 0.08</td>
</tr>
</tbody>
</table>

\(^a\)Mean proportion males on day 18 = \(\frac{\text{# males alive on day 18}}{\text{total # pups alive day 18}}\); values over 0.5 = male-biased sex ratio

\(^b\)Mean proportion of male best pups = \(\frac{\text{# males at least 11.5g on day 18}}{\text{total # pups at least 11.5g on day 18}}\)

*Letrozole group significantly different from controls \((p < 0.05)\)

**Significantly different from first litter when compared by paired t-test \((p < 0.05)\).

Significant differences are discussed in the text.
the first litters born to fathers treated with letrozole when they were 18 days old reached the dispersal threshold weight of 11.5g, whereas 23% of the 78 control females surpassed that threshold (Likelihood ratio $X^2 = 5.3, p < 0.05$). Wynne-Edwards (1987) did not identify an effect of sex on pup survivorship in litters raised by solitary mothers.

### 4.5 Discussion

Overall, aromatase inhibition at 18 days resulted in males that were less attentive to the birth and had decreased first litter pup survival, while the same treatment at 34 days resulted in males that had altered mating behavior and decreased reproductive success. Males treated as adults showed modestly enhanced midwifery behavior during the birth, but this was not associated with improved pup development or survival. There was no effect of treatment on attack or retrieval behavior either as sexually naive adults, or as new fathers. Thus, the results of the present study suggest that estradiol might act during the adolescent period to establish the expression of paternal and sexual behavior during adulthood.

Based on the ontogeny of paternal behavior for *P. campbelli*, the neural networks necessary for the expression of the full repertoire of paternal behavior (including midwifery and retrieval behavior) would need to be complete prior to adolescence in *P. campbelli* males to ensure the expression of alloparental behavior upon exposure to a litter of younger siblings. A critical period occurring during early development is also in line with studies conducted using the paternal prairie vole (*M. ochrogaster*). Like *P. campbelli*, male prairie voles exhibit alloparental care as weanlings (Wang & Novak
1994), and castration on the day of birth reduces the number of sexually naive males that act paternally towards a neonate as adults (Lonstein et al. 2002). However, treatment with an aromatase inhibitor during neonatal development has no effect (Lonstein & De Vries 2000), and the effect of aromatase inhibition at later stages of juvenile development has not been examined.

There was no effect of treatment on the proportion of sexually naive adult males exhibiting spontaneous paternal behavior, as was observed in male voles castrated at birth (Lonstein et al. 2002). However, unlike male prairie voles, sexually naive male *P. campbelli* show low levels of paternal care towards an unrelated neonate (53% as opposed to the 80% seen in voles: Roberts et al. 1996; Lonstein & De Vries 1999) and high rates of pup-directed aggression (31% attack as opposed to the 8% seen in voles: Roberts et al. 1996; Lonstein & De Vries 1999). Unlike the microtine model, 75% of male rats castrated on the day of birth will display ‘maternal’ behavior (retrieval, licking, crouching over a pup, and nest building) if continuously exposed to neonates for 5 days, while fewer than 10% of intact males will show the same behavior (McCullough et al. 1974). In contrast, male mice and Mongolian gerbils (*M. unguiculatus*) exposed prenatally to high levels of testosterone (which could be converted to estradiol) are less infanticidal as adults (Perrigo et al. 1989; Clark et al. 1998), and neonatal castration increases infanticide in adult mice treated with testosterone (Gandelman & Vom Saal 1975). Male mice, like male *P. campbelli* (Vella et al. 2005), become infanticidal at about 32 days (Gandelman 1973), a change in behavior that is not linked to a natural increase in circulating androgen levels (Svare et al. 1978). Thus, the developmental role of gonadal
steroids in establishing the amount of spontaneous care shown by adult virgins differs between species.

In the current study, the effect of letrozole treatment was seen as a decrease in direct paternal care during the birth (time spent huddling over newborn pups), and a decrease in pup survival from birth to postnatal day 18. The decrease in the amount of time males spent with pups during the birth process was not the result of the female aggressively excluding the male from the nest area, as is seen in meadow voles (Microtus pennsylvanicus; Storey et al. 1994). P. campbelli males also spent the majority of the test quiet in the nest area, but not in contact with pups, unlike Mongolian gerbil (M. unguiculatus) males, which avoid the nest area for the first 7 h after the birth of a litter (Clark & Galef 2000). Organizational effects of estradiol on midwifery behavior have not previously been identified. Prairie vole fathers do not assist with the birth process (McGuire et al. 2003), and the organizational role of estradiol in the paternal behavior of California mouse (Peromyscus californicus) fathers, who do assist with the birth (Lee & Brown 2002b), has not been examined.

Although letrozole treatment at 18 days of age had only a modest effect on measures of direct paternal behavior displayed by males as new fathers, paternal contribution to litter survival was also negatively affected. As expected, pup survival was low for all first litters, and high for second litters (Wynne-Edwards 1987; McInroy et al. 2000). P. campbelli females separated from their mate soon after mating lose over 50% of their pups, and 20% of these pups achieve a body weight of 11.5g by postnatal day 18 (dispersal threshold in the wild), in comparison 98% pup survival, with 51% of these
pups surpassing the dispersal threshold for litters raised by both parents (Wynne-Edwards 1987). Similarly, if the male is separated from his mate and first litter on postnatal day 8, 17% of pups are lost, and only 60% of pups weigh 11.5g at weaning, compared to 8% pup loss and 98% of pups reaching the dispersal weight in litters raised by both parents (McInroy et al. 2000). In the current experiment, pup losses in litters sired by 18-day letrozole males were twice as high as those seen for control litters (32% versus 15%) and only 20% of these pups surpassed the dispersal threshold (body weight of 11.5g by postnatal day 18; Wynne-Edwards 1987), compared to the 40% seen in control litters. Thus, measures of both paternal behavior and paternal contribution were reduced in these males, suggesting a developmental role for estradiol in establishing adult paternal behavior.

Overall, 80% of new fathers consistently retrieved the experimentally displaced pup to the nest in less than 100 s, regardless of letrozole treatment, which is consistent with previous manipulation of estradiol in adult males of this species (Hume & Wynne-Edwards, 2006). Retrieval behavior by fathers in this species is relatively invariant and robust (Jones & Wynne-Edwards, 2001; Brooks et al., 2005; Hume & Wynne-Edwards, 2005, 2006). It is likely that this measure of paternal responsiveness was not altered by aromatase inhibition during development, while midwifery behavior was decreased, because fathers had 3 days of continuous exposure to their pups plus prior experience with the testing protocol, before the paternal pup test.

Letrozole treatment during the adolescent transition to sexual maturity resulted in males that had an altered copulatory pattern as adults. Specifically, these males had fewer
mounts with intromission in the first ejaculatory series and across the 3-hour sexual behavior test, as well as increased ejaculation latencies and inter-mount and inter-intromission intervals across the first three ejaculatory series. In addition, these males sired significantly smaller first litters. Male Syrian hamsters (*Mesocricetus auratus*) castrated as adults and given exogenous aromatizable testosterone show normal mating behavior (Schulz et al. 2004). However, if males are castrated prior to puberty, but following neonatal development (21 days of age), mount, intromission, and ejaculation frequencies are decreased, and normal behavior cannot be restored with testosterone treatment during adulthood (Schulz et al. 2004). Mice that lack the gene for aromatase (ArKO) have low fertility rates, and typically show reduced mount, intromission, and ejaculation frequencies, compared to controls, following treatment with a combination of estradiol and androgen in adulthood (Bakker et al. 2004). These males would lack the organizational effects of estradiol during neonatal development and during puberty. Male prairie voles treated with an aromatase inhibitor as neonates exhibit normal sexual behavior as adults (Northcutt & Lonstein 2008), as do *P. campbelli* males treated with letrozole at 18 days of age. Thus, this effect of letrozole treatment on adult sexual behavior was restricted to late adolescent development.

Aromatase inhibition during adolescence resulted in a male-biased sex ratio at weaning, which might be the result of differential investment favoring male pups since *P. campbelli* sex ratios on day one after birth are female biased (Stulberg & Wynne-Edwards 1998). Solitary *P. campbelli* females raising a first litter (but not a second litter) also lose body condition (Walton & Wynne-Edwards 1997; McInroy et al. 2000), which
should theoretically result in a female-biased sex ratio (Trivers & Willard 1973; Koskela et al. 2004). However, no mechanism involving changes in sex ratio in sperm has been proposed, and these alterations are most likely to reflect a maternal response to some aspect of the male’s phenotype (McInroy et al. 2000).

An activational effect for estrogen on adult male sexual behavior was not expected. Male P. sungorus continue to mate even when serum testosterone levels are low as a result of photoperiod exposure or castration (Hegstrom & Breedlove 1999; Park et al. 2004; Timonin & Wynne-Edwards 2006). However, aromatase inhibition during adulthood did increase the duration of the ejaculatory lock. Since the length of the ejaculatory lock is related to efficient sperm transfer to the female, a longer lock should increase the probability of pregnancy and, by increasing vaginal stimulation should reduce the probability that the female will subsequently mate with another male (Wynne-Edwards & Lisk 1987).

Finally, the sample sizes were relatively small in these treatment groups, and some of the critical behaviors were rare. As a result, additional, smaller, effects of treatment would not have been detected by this experimental design, and are possible. Thus, the current study suggests that estradiol effects on sexual behavior are likely, replicates the findings of previous studies that failed to support an activational role for estradiol in paternal behavior (Schum & Wynne-Edwards, 2005; Hume & Wynne-Edwards 2006), and identifies critical periods in earlier life when estradiol may organize the paternal brain for subsequent behavioral responses.
CHAPTER 5: General Discussion

Much of our understanding of the endocrinology underlying reproductive physiology and behaviour has been gained through studies conducted on the laboratory rat, including our current model of the neuroendocrine pathways regulating the expression of maternal behaviour. Although research using the rat frequently results in a framework of understanding that generalizes to various species, there are exceptions to the accepted rule. For example, a common estradiol-sensitive maternal behaviour pathway, specifically the central role of the medial preoptic area (MPOA) and the bed nucleus of the stria terminalis (BNST), has been identified in females from diverse species (Rosenblatt 1967; Marques et al. 1979; Kirkpatrick et al. 1994b; González-Mariscal et al. 2005; Lee & Brown 2007; Perrin et al. 2007; Poindron et al. 2007). The evidence available for the neural pathways underlying paternal behaviour also supports the presence of a homologous pathway in males (Rosenblatt 1967; Kirkpatrick et al. 1994b; Lee & Brown 2007). However, the exact role that estradiol plays in activating either maternal or paternal behaviour differs between species (Kendrick & Keverne 1991; Lonstein & De Vries 1999; Trainor & Marler 2002; Hume & Wynne-Edwards 2006; Hayes & De Vries 2007).

ERα was not upregulated in the MPOA, BNST or MeA of Phodopus females three days following the birth of a first litter, which is in contrast to the pattern of ERα expression seen in females that rely on estradiol to stimulate maternal behaviour (rat: Wagner & Morrell 1996; mouse: Koch & Ehret 1989), but is similar to the effect of
pregnancy on ERα expression in females where estradiol alone is not responsible for stimulating this behaviour (ewe: Meurisse et al. 2005). Thus, a lack of upregulation in new mothers might indicate that estradiol plays a secondary, priming role in the activation of maternal behaviour in Phodopus females.

Although estradiol may act in Phodopus females to increase the salience of parturition and pup-related cues, this seems unlikely in P. campbelli males. P. campbelli fathers do not require previous experience with neonates, exposure to cues from the pregnant female, or the stimuli associated with birth (including consumption of either the amniotic fluid or placenta) to act paternally towards pups from their first litter (Jones & Wynne-Edwards 2001; Gregg & Wynne-Edwards 2005; Hume & Wynne-Edwards 2005). Furthermore, estradiol is not necessary during mating for the first time (Chapter 4), or from day 10 of the mate’s pregnancy through early pup development (Hume & Wynne-Edwards 2006), for males to act paternally and ensure high levels of pup survival. ERα expression was also not upregulated in the MPOA, BNST or MeA of P. campbelli first-time fathers with 3-day old pups (Chapter 3).

Estradiol is, however, necessary during P. campbelli development to establish adult paternal behaviour (Chapter 4): males treated with the aromatase inhibitor letrozole at 18 days of age spent less time huddling over pups during the birth process, and a greater number of pups were lost from the first litter sired by these males. Similarly, paternal behaviour in male prairie voles (Microtus ochrogaster), does not require estradiol activation (Lonstein & De Vries 1999), but gonadal hormones are necessary during neonatal development to establish adult paternal behaviour, since castration on the
day of birth disrupts this behaviour (Lonstein et al. 2002). Low levels of ERα expression in the BNST and MeA of virgin males, presumably organized during early development, are thought to be permissive for the expression of this behaviour (Cushing et al. 2004; Cushing & Wynne-Edwards 2006). However, the microtine model does not fully explain the regulation of paternal behaviour in *P. campbelli* males, as the ontogeny of this behaviour differs considerably between the two species. Male prairie voles maintain high levels of paternal care (80%) and low attack rates (8%) throughout development (Roberts et al. 1996; Lonstein & De Vries 1999). This continuation of paternal behaviour as sexually naïve adults would be beneficial in the prairie vole cooperative breeding system, which includes the formation of communal groups and philopatry (Solomon 1991; Getz et al. 1994; Wang & Novak 1994). Sexually naïve *P. campbelli*, as reported in Chapter 4, showed relatively low levels of paternal care (53% of males retrieved) and high levels of pup-directed aggression (31% of males attacked) compared to sexually naïve male voles, but showed high retrieval rates as first-time fathers. Additionally, no differences in the expression of ERα in either the BNST or MeA were identified when paternal *P. campbelli* and non-paternal *P. sungorus* males were compared as virgins or as new fathers (Chapter 3).

Sexual behaviour is also a potentially important non-hormonal cue in the regulation of paternal behaviour. Mating inhibits infanticidal behaviour in male house mice in the interval shortly before the expected date of parturition (Perrigo et al. 1991), and is necessary for the formation of a pairbond in monogamous prairie voles (Insel et al. 1995). Sexual behaviour also activates brain regions within the maternal behaviour
pathway, and mating occurs during the same timeframe as the onset of paternal behaviour in species that mate during postpartum oestrus (Katz et al. 1999; Phillips-Farfán et al. 2007). As for parental behaviour, the hormonal regulation of mating behaviour also differs between species. Estradiol can restore sexual behaviour in male rats castrated as adults, and ERα expression is upregulated in the MPOA of males 24 hours following mating to satiety (Baum & Vreeburg 1973; Phillips-Farfán et al. 2007). However, estradiol does not activate the same behaviour in males from all species (Arteaga-Silva et al. 2005; Arteaga-Silva et al. 2007). Estradiol is not necessary for the normal expression of sexual behaviour in adult male *P. campbelli* (Chapter 4), and ERα is not upregulated in the MPOA, BNST or MeA 40-48 hours following mating in these males (Chapter 3). Estradiol does, however, play a developmental role in establishing adult sexual behaviour in *P. campbelli*. Males treated with an aromatase inhibitor at around the time of testicular maturation showed abnormal mating behaviour as adults (reduced intromissions, and a longer interval between mounts, intromissions, and ejaculations compared to controls) and sired smaller litters.

In conclusion, the results of this thesis failed to identify an activational role for estradiol, acting through estrogen receptor α in the maternal behaviour pathway, in the regulation of *P. campbelli* paternal and sexual behaviour. This supports the findings of previous studies (Hume & Wynne-Edwards 2005; Schum & Wynne-Edwards 2005; Hume & Wynne-Edwards 2006), but is in contrast to the central role of estradiol in the control of reproductive behaviour in the laboratory rat (Baum & Vreeburg 1973; Rosenblatt et al. 1998). Estradiol does, however, act during development to establish
adult reproductive behaviour in *P. campbelli* males. Furthermore, the central role for
estradiol in the control of maternal behaviour, as identified in the female rat (Rosenblatt
et al. 1998), may not generalize to *Phodopus* females. If estradiol alone does not
stimulate maternal behaviour in *Phodopus* females, like paternal behaviour in *P.
campbelli* males, the neuroendocrine pathways underlying parental behaviour in this
species may still be homologous between mothers and fathers. Thus, the hormonal basis
of maternal behaviour in this species needs to be examined more closely in order to fully
test the hypothesis of homology between the neuroendocrinology of maternal and
paternal behaviour in *P. campbelli*.
LITERATURE CITED


Baum MJ, Canick JA, Erskine MS, Gallagher CA & Shim JH 1983 Normal differentiation of masculine sexual behavior in male ferrets despite neonatal


Cushing BS & Wynne-Edwards KE 2006 Estrogen receptor-alpha distribution in male rodents is associated with social organization. *Journal of Comparative Neurology* **494** 595-605.


Jasnow AM, Huhman KL, Bartness TJ & Demas GE 2000 Short-day increases in aggression are inversely related to circulating testosterone concentrations in male siberian hamsters (Phodopus sungorus). *Hormones and Behavior* **38** 102-110.


Lonstein JS, Rood BD & De Vries GJ 2002 Parental responsiveness is feminized after neonatal castration in virgin male prairie voles, but is not masculinized by perinatal testosterone in virgin females. *Hormones and Behavior* **41** 80-87.


Northcutt KV & Lonstein JS 2008 Sex differences and effects of neonatal aromatase inhibition on masculine and feminine copulatory potentials in prairie voles. \textit{Hormones and Behavior} \textbf{54} 160-169.


Perrin G, Meurisse M & Lévy F 2007 Inactivation of the medial preoptic area or the bed nucleus of the stria terminalis differentially disrupts maternal behavior in sheep. *Hormones and Behavior* **52** 461-473.


Neither reduced photoperiod, nor female-related social cues, nor increased maternal thermal stress result in a paternally responsive *Phodopus sungorus* male. *Physiology and Behavior* **88** 309-316.


Trainor BC, Bird IM, Alday NA, Schlinger BA & Marler CA 2003 Variation in aromatase activity in the medial preoptic area and plasma progesterone is associated with the onset of paternal behavior. *Neuroendocrinology* **78** 36-44.


Trainor BC & Marler CA 2001 Testosterone, paternal behavior, and aggression in the monogamous California mouse (*Peromyscus californicus*). *Hormones and Behavior* **40** 32-42.


