Reproductive biology of the European badger (*Meles meles*): Endocrinological insights into lifetime reproductive events, strategies and cub development in response to ecological factors



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ABSTRACT

Knowledge of reproductive adaptions and physiological mechanisms are essential in wildlife conservation as they impact species survival. As markers of bodily functions, hormones mirror reproductive activity and reveal baseline information including reproductive cycles and strategies, lifetime reproductive events such as puberty and senescence, as well as responses to ecological factors, which are all profound factors of wildlife population growth. In this thesis, the reproductive biology of the European badger (*Meles meles*) is examined by utilising endocrinological measures with complementary somatic and ecological data. **Research chapter I** establishes endocinological mechanisms of the flexible delayed implantation and superfoetation mating strategy, where number of additional mating seasons varies with population density across badger geographical range. **Research chapter II** demonstrates that despite hormone levels and external genitalia morphology (EGM) showing similar seasonal patterns, EGM in males is a reliable indicator of reproductive status only during the mating season, while in females EGM is a less precise proxy. **Research chapter III** reveals that asynchronous timing in attaining minimum body size, required for sexual maturity, results in two heterochronous phenotypes (early- and late- developers) in male cubs (less evident in females), while **Research chapter IV** showcases the decline in sex-steroid levels and somatic condition with age, leading to a post-reproductive lifespan (PRLS), while also showing two reproductive phenotypes (high and low hormone levels) in older individuals of both sexes. **Research chapter V** demonstates that sexual selection is unlikely to be the driving force for sexual size dimorphism in badgers, but social and environmental factors, as well as endocrinological mechanisms, affecting juvenile diverging growth patterns and end body sizes are likely the primary physiological process of this phenomenon. **Research chapter VI** illustrates that ecological changes can be reflected in hormone levels and the regulation of these changes differs between sexes, likely linked to their respective reproductive strategies. **Research chapter VII** establishes that urinary metabolite measurement may reliably assess endocrine function in badgers as a non-invasive technique, especially in males. Collectively, these research chapters give a comprehensive understanding of the badger's reproductive processes and how it interacts with ecological factors.

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PART I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Importance of reproductive biology in animal studies

Knowledge of physiological biology is an important first step in understanding how animals regulate all aspects of their life cycle (Touma and Palme et al. 2005; Dehnhard et al. 2008). The study of reproduction, in particular, is fundamental to understanding species survival, as this is the only means by which a population can sustain and rejuvenate itself naturally (Graham 2004; Schwarzenberger 2007). In the field of conservation biology, there has been a growing interest in gaining insights into basic reproductive processes, and related factors, that impact the survival of threatened wild species (Hindle et al. 1992; Graham 2004; Schwarzenberger 2007); however, investigations can often be sample-size limited, favouring fundamental discovery from empirical investigations using more common, analogue species.

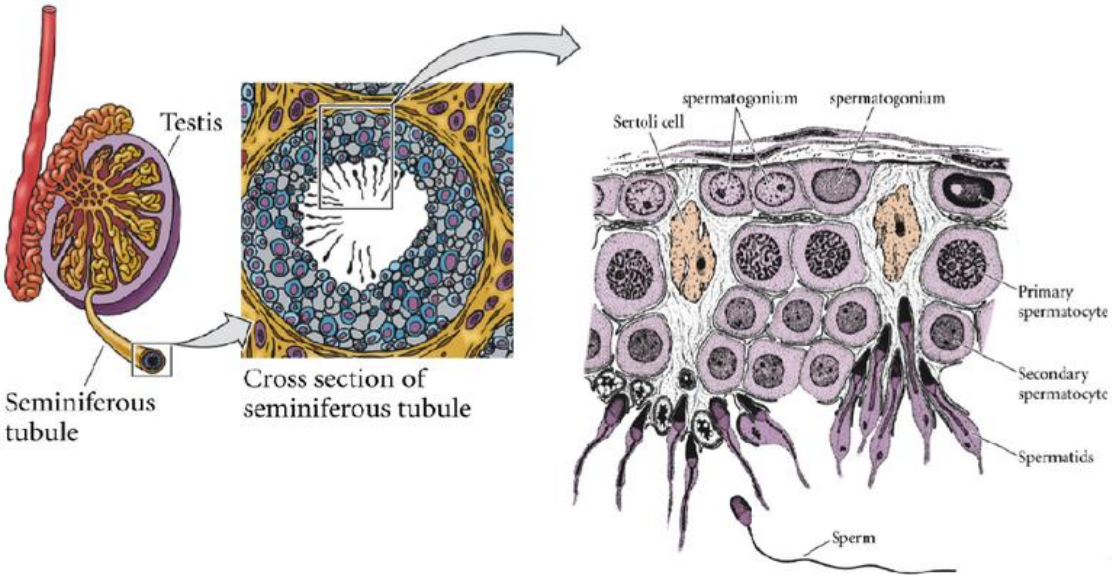
Reproductive adaptation and physiological mechanisms may be highly specialized and vary from species-to-species according to evolutionary distinctiveness, resulting in species-specific reproductive strategies, even within the same taxonomic family (e.g. the number of subtle reproductive adaptations in carnivores are almost as varied as the number of species; Wildt et al. 2003). As a consequence, species-specific studies, establishing baseline reproductive information may significantly enhance not only academic interests, but also facilitate practical applications to species management and conservation efforts both in the wild and in captivity (Graham 2004; Schwarzenberger 2007). Furthermore, animal reproductive physiology also integrates with other disciplines, such as animal health, genetics, ecology, evolution, behaviour, and immunology which could help explain complex ecological processes relating to individual life-histories and population dynamics (Adkins-Regan 2005). Despite this importance, reproductive mechanisms are properly understood for only a small proportion of the world's 40,000 vertebrate species (Wildt et al. 2003).

Endocrinology is central to reproductive physiology, although hormones also provide biomarkers of, health, stress, metabolic activity, development, and expression of behavior (Adkins-Reagan 2005; Touma and Palme 2005; Adkins-Reagan 2005). Hormones are secreted by endocrine glands and are released into the bloodstream providing a communication system between cells (Touma and Palme 2005; Dehnhard et al. 2008). Changes in reproductive hormone levels (Wade and Schneider 1992; Gesquiere et al. 2011)

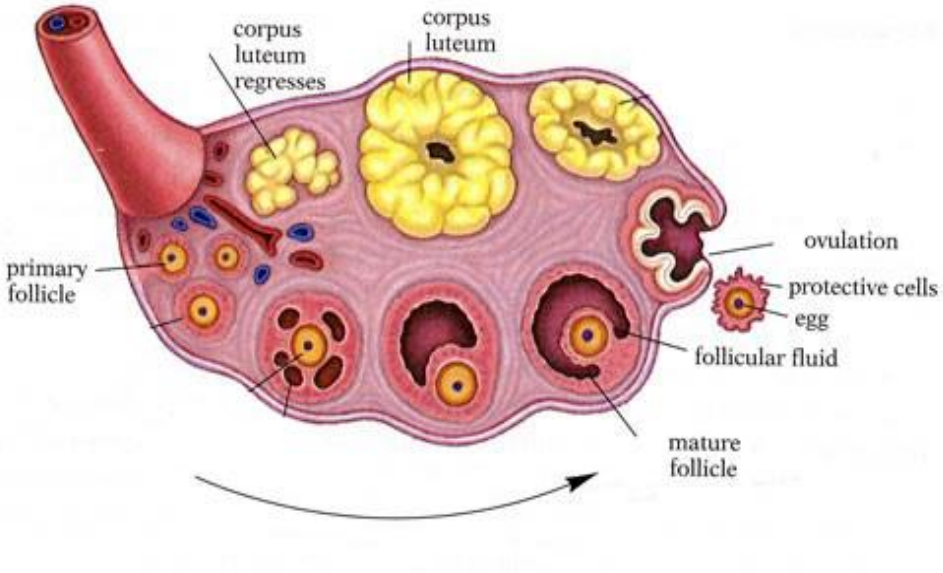
can thus signify changes in breeding condition as well as revealing lifetime reproductive processes such as puberty and senescence, which interact with extrinsic ecological factors, to profoundly influence population success (Graham 2004; Pukhazhenthi and Wildt 2004; Schwarzenberger 2007).

Lifetime reproductive events

Reproductive patterns in mammals typically follow a bell shaped curve, where they increase after puberty, peak at prime age, and decrease thereafter until reproductive cessation, or death (Jorgenson et al. 1997). Puberty in females results in the first occurence of ovulation/ oestrus, and in males with the onset of spermatogenesis (Evans and O'Doherty 2001). This arises via the full activation of the hypothalamic-pituitary-gonadal (HPG) axis in both sexes, when episodic secretion of gonadotropin releasing hormones (GnRH) by the hyphothalamus activates the anterior pituitary gland to secrete Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) which trigger the generation of gametes and release of sex steroids (Plant 2006). In males, after puberty, testosterone production by the Leydig cells (of the testes) is stimulated by the increase of LH, while testicular growth and the maturation of sperm cells in the seminiferus tubule (from spermatogonium, spermatocyte, spermatid, to sperm, Fig. 1a) are induced by an increase of FSH (Adkins-Regan 2005). Once sperm production is triggered after sexual maturity, males will continuously undergo spermatogenesis (Cohen 2004). While in sexually mature females, FSH stimulates ovarian follicle(s) growth and maturation (from primary to mature follicles, Fig. 1b). Mature follicles will then produce oestrogen that gradually causes follicles to increase in size (Jameson et al. 2013). A rise in follicular oestrogen inhibits FSH production by the pituitary gland and increases LH production instead, which triggers ovulation (Halazs and Szekenes-Bartho 2013). If fertilization occurs, any remaining ovarian tissue transforms into a corpus luteum (CL), which then produces progesterone contuinuously to support implantation and pregnancy (placenta formation), otherwise it degenerates and this initiates a new cycle (Halazs and Szekenes-Bartho 2013; Jameson et al. 2013). Hence, throughout the prepubertal period, oestrogen and testosterone levels remain low but increase immediately prior, during and after puberty, until levels reach adult concentrations (Beehner et al. 2009).



(a)



(b)

Figure 1. Spermatogenesis in males (a) and oogenesis in females (b).

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Gilbert Scott F. (2006) - https://es.wikipedia.org/wiki/Archivo:T%C3%BAbulo_seminifero.png, CC BY 3.0.

The age at which puberty occurs is not only influenced by intrinsic genetic factors, but also moderated by ecological factors such as food availability (affected by weather/ climate; Parent et al. 2003; Abeygunawardena and Dematawewa 2004; Newman et al. 2017), environmental conditions (De Jonge et al. 1996; Onyango et al. 2013), as well as presence of competing conspecifics (Macdonald and Johnson 2015). Hence, as individuals experience

different post-natal environments (Onyango et al. 2013), the timing of sexual maturity varies considerably among members of a species (Delemarre-van de Waal et al. 2002) or even within a population (Freetly et al. 2011; Onyango et al. 2013; Plaistow et al. 2004; Alberts 2012; Pusey 2012). Once an individual has reached sexual maturity (and follows the adult hormone level and pattern), as well as pursuing its own fitness and liftetime reproductive success (LRS; Gustafsson 1986) it will also start to contribute to population growth rate, as it is recruited into the reproductively active component of its population (Promislow and Harvey 1990).

After, or possibly even before, reaching prime age (where reproductive fitness peaks), an individual may experience physiological/endocrine and cellular/somatic deterioration as it ages (Nussey et al. 2008; Torress and Velando 2007). The former involves a decline in the ability to produce offspring (reproductive senescence; Finch 1990), while the latter refers to a decline in general health and condition until death (somatic senescence; Reed et al. 2008). The mechanism determining how males and females senesce (reproductively and somatically) differ according to their different allocation strategies (underpinning reproductive success) which produce sex-specific traits (e.g. investment in reproduction, somatic maintenance), which affects longevity and aging rates (Bonduriansky et al. 2008). In most mammals, males are able to sustain spermatogenesis and remain reproductively active until the end of their realized lifespan (Cohen 2004). Nonetheless, older males still tend to undergo a decrease in physical condition, sexual traits (controlled by testosterone, e.g. reproductive advertisment), mating performance, as well as sperm quality (Möller et al. 2009) which may affect their reproductive fitness (Cohen 2004). In females, aging may not only cause gradual somatic decline (where repeated breeding takes a toll on female condition), but also loss of physiological functions as well as hormone capacity due to reproductive cessation (RC), a general trait among female mammals which is triggered by oocyte depletion as age increases (Cohen 2004). The trajectory of senescence also varies among individuals, with different strategies evolving to achieve optimal trade-off between LRS and somatic maintenance under extrinsic constraints (Bonduriansky et al. 2008; Jones et al. 2008; Campbell et al. 2017).

Breeding seasons and reproductive quiescence

Due to earth rotation around the sun on a tilted axis, regions of increasing latitude (closer to the pole) are exposed to larger seasonal and daily environmental variation (i.e., radiation,

temperature, photoperiod) compared to regions closer to the equator (low latitude; Lincoln and short 1980; Dardente et al. 2016). Thus, animals in temperate regions adapt by developing endogenous biological mechanisms, typically using photoperiodic cues, to maximise fitness by timing important life events (e.g. breeding) during the optimal environmental conditions (Badness et al. 1993; Staples et al. 1992; Dardente et al. 2016). Photoperiodic stimulus is captured by the retina and stimulates melatonin release which then affects gonadal activity, where responses are species-specific (Gerlach and Aurich 2000). Melatonin which is secreted during periods of darkness, will decrease the secretion of GnRH in long-day breeders (resulting in regression of gonads e.g., the ferret *Mustela putorius furo*, Jallageas et al. 1994 and Basenji *Canis lupus familiaris*, Concannon 2012) whilst increasing secretion in short-day breeders (causing activation of the gonads e.g., the mink *Neovison vison*, Jallageas et al. 1994; red fox *Vulpes vulpes*; and badger *Meles meles*, Maurel et al. 1984). Hence, in seasonal breeders, reproduction is timed to optimise chances of mating, parturition, lactation, and cub rearing during high food availability and favourable weather to increase reproductive fitness and offsping survival (Malpaux 2006; Schwarzenberger 2007; Dehnhard et al. 2008; Schwarzenberger and Brown 2013).

During the mating season or periods close to it, seasonal breeders typically show physiological and morphological signs of mating such as an increase in testosterone levels, testes size and testes weight in males (Klonisch et al. 2006), as well as (in some mammals) testes movement from an ascended position in the abdominal cavity to descend into the scrotum (scrotal testes; Millesi et al. 2004; Crait et al. 2006) while females show an increase in oestrogen levels and vulval swelling and/or reddening as well as mucosal secretion (e.g. primates: Dixon, 1998). Behavioural changes induced by hormones also occur during the mating season e.g., males may roam extensively (Eberle and Kappeler 2004; Odden and Wegge 2007) or produce distinctive mating calls (Parijs and Clark 2006; Wong et al. 1999); whilst (especially in Carnivora) females may exhibit lordosis (Carlson and Gese 2008).

Seasonal breeding males also exhibit periods of reproductive quiescence (inactivity), coinciding with when females do not undergo oestrus cycles (Curlewis 1992; Revel et al. 2007). During this period, testicular size, testosterone secretion, and reproductive behaviour is down regulated (Gerlach and Aurich 2000), where in some cases spermatogenesis completely ceases (e.g. red deer *Cervus elaphus*; Clarke et al. 1995; brown bear *Ursus arctos*; Tsubota et al. 1997) or may still be maintained at reduced levels (stallion *Equus caballus*;

Gerlach and Aurich 2000). Examples of seasonal breeders include the black bear (*Ursus americanus*; Garshelis and Hellgren 1994), the European sousliks (*Spermophilus citellus*; Millesi et al. 2004), the European badger (*Meles meles*; Yamaguchi et al. 2006), the river otter (*Lontra canadensis*; Crait et al. 2006), the black rhino (*diceros bicornis*; Garnier et al. 2002), and baboons (*P. hamadryas ursmus*; Weingrill et al. 2004).

Reproductive cycles and strategies

As resource allocation to competing processes (e.g. reproduction, maintenance, and growth) is limited and varies according to ecological conditions, a diversity of strategies to maximise fitness have evolved among species, linked to reproductive strategies (Clutton-Brock and Harvey 1978), resulting in differences in conception to birth processes among species (Roff, 1992). The basic linear sequence of reproduction in mammals typically progresses from insemination to fertilisation (conception), implantation, placental development and gestation, until parturition (Austin and Short 1984). The ovulation cycle (as described in **lifetime reproductive events**) may be spontaneous (with ovarian steroid hormone regulation; Adkins-Regan 2005; Concannon et al. 2009; Jewgenow et al. 2014) or induced (by external factors, e.g. mate presence; coitus-induced ovulators; Wildt 1991; Bakker and Baum 2000; Lariviere and Ferguson 2003, Concannon et al. 2009); with single (monoestrus) or repeating cycles (e.g., every few days, weeks, months; polyestrus) within a specific time period (e.g., throughout the year, seasonally; Adkins-Regan 2005; see examples in Table 1).

Following fertilization and production of blastocysts, some species exhibit sequential implantation, while others undergo embryonic diapause, where pre-implanted blastocysts temporarily arrest development and remain suspended in the uterus (Mead 1989; Sandell 1990, Thom et al. 2004). This strategy allows offspring to be born during the most favourable annual environmental conditions, irrespective of gestation length, maximizing reproductive success (Sandell 1990). Species that exhibit this reproductive strategy include the American mink (*Mustela vison*; Yamaguchi et al. 2004), the European badger (*Meles meles*; Yamaguchi et al. 2006), spotted skunks (*Spilogale putorius*; Mead and Eik-Nes 1969), and black bears (*Ursus americanus*; Garshelis and Hellgren 1994), among others.

Table 1. Reproductive cycle and ovulation type in different species.

Species	Cycle type	Ovulation type	Reference
Giant panda (*Ailuropoda melanoleuca*)	Monoestrus	Seasonal spontaneous	Snyder et al. 2004; Steinman et al. 2006
Lynx (*L. canadensis, L. lynx, L. pardinus, L. rufus*)	Monoestrus	Seasonal spontaneous	Jewgenow et al. 2014
Domestic ferret (*Mustela putorius furo*)	Polyoestrus	Seasonal Induced	Lindeberg 2008
European mink (*Mustela lutreola*)	Polyoestrus	Seasonal Induced	Amstislavsky et al. 2009
Wolf (*Canis lupus*)	Monoestrus	Seasonal spontaneous	Asa and Valdespino 1998; Seal et al. 1979
Dog (*Canis lupus familiaris*)	Monoestrus	Non-seasonal spontaneous	Concannon 2011
Stoat (*Mustela erminea*)	Monoestrus	Seasonal induced	Nagl et al. 2015; King and Moody 1982
North American river otter (*Lontra canadensis*)	Monoestrus	Seasonal induced	Bateman et al. 2009; Jewgenow and Songsasen 2014
Tiger (*Panthera tigris*)	Polyestrus	Seasonal induced	Blevins et al. 2008

Physiological and somatic responses to ecological factors

When exposed to ecological vicissitude (in the form of environmental and social factors), individuals preceive signals (cues) and undergo 'allostasis', which is the the process by which the body responds to stressors in order to regain/ maintain homeostasis. (McEwen and Wingfield 2003). For daily self-maintenance, all activities and experiences manifest into 'allostatic load', where individuals can cope only up to certain thresholds (McEwen and Wingfield 2003, Korte et al. 2005). During extreme ecological events (catastrophic flooding, drought, habitat loss, fire, etc), the stimulus an individual receives may push it into 'allostatic overload'; a state requiring an organism to make physiological, morphological and behavioural adaptation and compromises in order to survive and maintain individual fitness (Whitten and Turner 2009; Gesquiere et al. 2011; Wingfield 2006). Thus changes in hormone levels and somatic condition (serving as as a proxy) may provide mechanistic understanding of how environmental changes and threatening processes impact an individual, which in turn will influence ecological function, population persistence, and species survival (Wikelski and Cooke 2006; Hansen 2009; Gesquiere et al. 2011).

During unfavourable extrinsic conditions, requiring increased energetic investment (Wade and Schneider 1992), reproduction is often affected as a trade-off response in favour of self-maintenance (Jensen 1996; Crews 1992; Gesquiere et al. 2011). This is manifested as changes in reproductive hormone levels in both males and females (Wade and Schneider 1992; Gesquiere et al. 2011) which vary based on their phenotypic quality (e.g. body size, age; Whitten and Turner 2009; Gesquiere et al. 2011; Gaillard et al. 2000). Factors such as food availability (Hansen 2009; Gesquiere et al. 2011), ambient temperature (Lynch et al. 2002; Hansen 2009) and rainfall (Kretzschmar et al. 2004; Gesquiere et al. 2011), as well as social interaction with conspecifics (may it be reproductive activity or competition; Strier et al. 1999; Kotrschal et al. 2000) and predation risk (Creel et al. 2009) are all known to have significant effects on reproductive hormone levels. Examples of such cases abound across Mammalia, such as a decrease in testosterone levels for Merino rams (*Ovies aries*; Martin et al. 1994) that undergo food deprevation; lower testosterone levels observed in yellow baboons (*Papio cynocephalus*) that experience above normal temperatures during the dry season (Gesquiere et al. 2011); lower oestradiol levels in Shiba goats during higher ambient temperatures resulting in heat stress (*Capra hircus*; Ozawa et al. 2005); elevated androgen concentrations during months of high rainfall (September–February) in the male white rhinoceros (*Ceratotherium simum simum*; Kretzschmar et al. 2004); as well as higher testosterone levels in gray-cheeked mangabeys (*Lophocebus albigena*) when higher number of receptive females were present (Arlet et al. 2011).

Factors causing allostatic overload can be particularly effective during development (Essex et al. 2011). Extrinsic factors that neonates and juveniles are exposed to during sensitive growth periods can substantially influence an individual's growth rate and end size, (Derocher and Stirling 1996) and its age of first reproduction (Kingsley et al. 1988, Garshelis and Hellgren1994; Derocher and Sterling 1998), which in turn can affect its survival probability and lifetime reproductive success. This, however, may vary not only among individuals but also between sexes, if one sex has a greater vulnerability towards the particular environmental condition in question (Leberg and Smith 1993; Post et al. 1999; Leblanc et al. 2001). Therefore, the extent to which extrinsic factors affect the end body size and reproductive capacity of each sex is often influenced by sexual dimorphism of a species (Isaac 2005). For example, male and female Bighorn lambs (*Ovis canadensis*) respond differently to maternal care deprivation during weaning, where negative effects were observed on subsequent adult body size, mass, horn size, probability of parentage, and overall

lifetime fitness in males, while in females no negative effect was observed (Festa-Bianchet et al. 1994).

Because lifetime reproductive events and strategies, as well as physiological and somatic responses to ecological factors, vary among species, sexes and individuals (Wildt and Wemmer 1999; Wildt et al. 2003), it is crucial to better understand how reproduction can impact population dynamics, population growth rate, individual LRS and support informed conservation programs and management of wildlife (Wikelski and Cooke 2006). This is especially important for species that do not follow typical linear reproductive processes, but are atypical to standard mammal models.

The European Badger (*Meles meles*) as a model species

The European badger (*Meles meles;* henceforth *'badger'*) provides a highly informative model species with which to advance conceptual understanding of reproductive biology paradigm and answering a variety of behavioural, endocrinological and evolutionary questions (Stopka and Johnson 2000), because these (facultatively) social carnivores exhibit a range of flexible reproductive strategies and adaptations (e.g. delayed implantation) across their extensive geographic variation in accord with their social organization (Macdonald et al. 2015).

Taxonomy and life traits

The European badger (*Meles meles*) belongs to the Mustelidae family which is the most diverse and largest family in the order Carnivora (Powell 1979; Sandell 1989; Macdonald and Newman 2017). It is classified in the Melinae (true badger) subfamily together with the Asian badger (*Meles leucurus*), the Japanese badger (*Meles anakuma*) and the hog badger (*Arctonyx collaris*; Wolsan and Sato 2010) – see Zhou et al. (2017). The other subfamilies of the Mustelidae (consisting of around 70 extant species; Macdonald and Newman 2017) include Taxidiinae (the American badger; *Taxidea taxus*), Guloninae (Martens; *Martes sp.* and Wolverine; *Gulo gulo*), Mellivorinae (the honey badger; *Melivora capensis*), Helictidinae (the ferret badger; *Melogale moschata*), Mustelinae (weasels and polecats; *Mustela sp.* and minks; *Neovison sp.*), Lutrinae (otters), Ictonychinae (weasels, polecats, and grisons; *Galictis*, *Lyncodon patagonicus, Vormela peregusna, Poecilogale albinucha, Ictonyx*; Wolsan and Sato 2010; Sato et al. 2012).

The word ‘badger’ comes from French *bêcheur,* meaning digger (Roper 2010). They are morphologically distinctive, presenting a highly aposematic black-and-white striped face and white margins to the ears (Newman et al. 2005). Badgers have a silver-grey back, black extremities and abdomen, as well as conspicuous long fore limb claws (Neal and Cheeseman 1996; Harris and Yalden 2008). These species are fossorial (adapted to digging and spend a large proportion of their time underground; Noonan et al. 2014; 2015a) and are primarily nocturnal with very poor eyesight, thus dependent on their sense of smell and hearing (Newman and Buesching 2018).

Geographic distribution

The European badger has a broad geographical distribution spreading throughout Europe, eastwards to the west of the River Volga (Russia), The Caucasus, Iran, Iraq, Israel, The Pamir-Alai mountains, Kopet Dagh mountains, the South and West Tien Shan mountains, and also the islands of Crete, Rhodes, Ireland, and Great Britain (Abramove 2001; 2002; 2003). Though native to most of Europe, population densities differ between countries (Palphramand et al. 2007). Very low population densities are found in Poland (0.1 badger/km^2: Kowalczyk et al. 2000), Portugal (0.36–0.48 badgers/km^2; Rosalino et al. 2004), and Spain (between 0.2-1 badger/km^2; Revilla and Palomares 2002); and low densities occur in the Republic of Ireland (1.9 badger/km^2; Corner et al. 2015; Byrne et al. 2012). Within Great Britain, moderate densities occur in Bristol, UK (4.4-7.5 badger/km^2; Service et al. 2002); while higher densities are present in south-west England (25 badger/km^2; Cresswell et al. 1992; Page et al. 1994; Carpenter et al. 2005) and very high population densities occur in Wytham Woods, Oxfordshire, UK (40 badgers/km^2; Macdonald et al. 2009).

Habitat, sett use, and diet

Badgers occupy varied habitats (Silva et al. 2017) such as woods, copses (deciduous, coniferous, and mixed), agricultural land and grassland (Feore and Montgomery 1999; Kruuk 1978; Neal and Cheeseman 1996), where they excavate large underground dens, called setts. These setts are large-scale systems of underground tunnels and nesting chambers that are occupied extensively by badger groups (Roper 1992; Noonan et al. 2015b) for breeding, providing shelter, protection, thermoregulation (Roper 1992; Kaneko et al. 2010; Tsunoda et al. 2018) as well as reducing environmental stress (Butler and Roper 1995; Kinlaw 1999; Noonan et al. 2014). These underground dens have been classified (by size and function) as main setts (large number of holes, complex system, abundant soil heaps, recognizable paths

from and between sett entrances, well used and location where breeding occurs: Kruuk 1989; Roper 1992; Woodroffe and Macdonald 1993), annex setts (close and connected to the main sett, moderate number of holes, not necessarily frequent in use) and outliers (only one or two holes, no connecting path to main sett, used sporadically; Thornton 1988; Davison et al. 2008).

Across their geographic range, badgers show a wide variation in diet and feeding strategies (Roper 1994; Johnson et al. 2002; Fischer et al. 2005; Rosalio et al. 2005). In general, badgers are opportunistic foragers (Roper 1994; Roper and Mickevicius 1995; Fischer et al. 2005; Remonti et al. 2011) that are capable of adapting to a wide range of habitat types and environmental conditions by consuming different food resources that are most abundant in the area (Roper 1994). In lowland England, badgers feed predominantly on earthworms (*Lumbricus terestris*; Kruuk 1978b; Goszczynski et al. 2000), where earthworm availability is sensitive to microclimatic conditions (Jimenez and Decaens 2000; Curry 2004). As the availability, distribution, and abundance of earthworms may vary temporally and spatially (da Silva et al. 1993; Johnson et al. 2001; Kowalczyk et al. 2003), when availability is reduced (during summer droughts and winter frosts; Kowalczyk et al. 2003; Nuutinen and Butt 2009), badgers increase the relative consumption of insects, bird eggs, carrion, small mammals, seeds, cereal, and fruits (Shepherdson et al. 1990; Roper 1994).

Group Living

Badgers have a flexible social system, largely affected by population density (Johnson et al. 2000), where they can be solitary (Woodroffe and Macdonald 1993; Goszczynski 1999; Kowalczyk et al. 2000), pair living (Revilla and Palomares 2002; Do Linh San et al. 2007) or form large social groups with up to 25 members (da Silva et al. 1993; Johnson et al. 2000; Macdonald et al. 2015). Social groups often consist of individuals that were born into the group, through high rates of natal philopatry, where most animals stay in the same social group all their life (Macdonald et al. 2008; 2009; Noonan et al. 2015b). Due to ecological constraints (Fell et al. 2006), dispersing between social groups is rare in high density populations (e.g., Wytham Woods where only 19.1% badgers permanently changed social groups; Woodroffe et al. 1993; Macdonald et al. 2008), although short-term visits are frequent (Macdonald et al. 2008; but for contrast see Byrne et al. 2014).

Interestingly, even group-living badgers show very limited cooperative behaviours, with no cooperative hunting, vigilance, or predator avoidance (Macdonald 1983; Kruuk and Macdonald 1985; Woodroffe and Macdonald 1993), resulting in them being classified as "primitively social" (Christian 1994; Woodroffe and Macdonald 2002; Stewart and Macdonald 2003) – although here the terminology 'facultatively social' is preferred. This flexible group formation can best be explained by the Resource Dispersion Hypothesis (RDH; Macdonald 1983; Macdonald and Johnson 2015), which posits that social groups will emerge under conditions where abundant resources are clumped heterogeneously, and/or vary in availability temporally (as do earthworms). When this occurs, the size of the smallest territory that could be economically defended by a pair of badgers (primary occupants), is still extensive, causing territory defence to be costly (Johnson et al. 2000). Thus, the cost of sharing a territory with secondary animals (Macdonald 1983) is low, and the formation of a group territory is thus at minimum cost (Carr and Macdonald 1986; Blackwell and Macdonald 2000; Johnson and Macdonald 2003), which then leads to a shift in the cost-benefit balance of group living (Johnson et al. 2000; see Macdonald et al. 2015).

When resources are widely dispersed, territory size increases (Johnson et al. 2000), while the quality and quantity of resource patches (Macdonald 1983), as well as spatio-temporal availability, determines group sizes (Johnson *et al*. 2000). Hence, in areas where food is abundant and availability is highly predictable, high densities occur, badgers form groups, occupy distinctive main setts, and defend small territories. While, in areas where food is scarce and availability is unpredictable due to seasonal change, population densities are lower and badgers live in small groups, or are even solitary, occupying large home ranges with several setts in them (Goszczynski 1999; Kowalczyk et al. 2000; Macdonald et al. 2004). This is relevent to questions regarding reproductive physiology as it is linked to density dependent constraints, cohort effects on puberty and development, sexual size dimorphic traits, as well as the effect of conspecific presence towards reproductive status.

The social dynamics of badger groups is strongly associated with olfactory communication, as scent is essential to communicate within and between groups (Buesching et al. 2003; Christina and Stanovitch 2017). Badgers may use urine (Service 1997; Harris 2001; Hutching et al. 2001), faeces and several glandular secretions from the inter-digital glands, the anal gland and the subcaudal gland (Harris and Yalden 2008, Buesching et al. 2003). The subcaudal gland is a skin gland unique to badgers, used for scent-marking their environment

(Buesching and Macdonald 2004) and each other (allomarking; Buesching et al. 2003). It consists of a 20-80 mm wide narrow opening, in-between the root of the tail and the anus, with a sac-like structure that secretes a lipid-rich paste (Buesching et al. 2002a; Buesching et al. 2003). Both females and males possess this gland (Buesching et al. 2002a) and scent-mark frequently, to convey individual-level information such as sex, age, reproductive status, body condition as well as group membership (Buesching et al. 2002a,b,c), at boundary latrines (shared among social groups, defining social group territories) and peripheral paths of their territory (Buesching and Macdonald 2001; Buesching and Macdonald 2004; Kilshaw et al. 2009). The frequency of scent-marking is significantly higher in males than in females, across seasons, linked to larger pouch size (Buesching et al. 2002a), which also produce and store higher amounts of subcaudal gland secretion (Buesching et al. 2003). This gland is relevent to questions regarding reproductive physiology as it is especially important during the mating season where secretion production and scent marking activity is highest in both sexes (Buesching et al. 2002a), as it encodes reproductive status and is used for reproductive advertisement (Buesching et al. 2003).

Although group living may benefit the badger byalleviating cost of territory defence (Kruuk 1989), there is little evidence of active territorial defence (Stewart et al. 2001; Macdonald et al. 2002b; Kilshaw et al. 2009; Tinnesand et al. 2015). Interactions between badgers from different social groups at boundary latrines is frequent (Christian 1995; Ellwood et al. 2017) and trespassing beyond this social group territory does not usually result in conflict with resident group members. Instead it appears that boundary latrines mark an optimal point of foraging for each social group where resource depletion has already occured (as explained in Passive Range Exclusion Hypothesis (PREH): Stewart et al. 1997; Kilshaw et al. 2009). This is especially pertinent to reproductive physiology questions as extra-group matings result from these transgressions, where males and females typically search for mates outside of their social group (causing extra-group paternity/ EGP, see below).

Badger body condition (measured by scoring body-fat along the spine and pelvis) also varies extensively with season and between individuals (Macdonald et al. 2002); nevertheless, there is little evidence of dominance hierarchy in badgers (Macdonald et al. 2002b), and body condition does not influence mating frequency (although better condition males are assigned more offspring; Dugdale et al. 2011b). There is, however, some evidence for density-dependent limitations on body size and reproduction (Macdonald et al. 2002a; Delahay et al.

2006). Individuals that are members of larger social groups (which may have twice the number of an average group size of 10) have been shown to have lower body condition (±9% difference), body weight (±12% difference), and reproductive success (±20 difference) compared to those of smaller groups (Macdonald et al. 2002a). This is relevent to questions regarding reproductive physiology because growth rate is likely affected by group size and competition, affecting timing of puberty or somatic development.

Reproductive cycle and strategies

As in the case for most species in the Mustelidae family, badgers are seasonal breeders (Amstislavsky and Ternovskaya 2000) that produce one litter annually (ranging 1-4 cubs; Macdonald et al. 2015). Cubs are typically born during mid-February in the UK (76% of births, although parturition can occur between mid January – mid March; Neal and Cheeseman 1996; Yamaguchi et al. 2006). The principal mating season is during the post-partum oestrus in January-March (which may extend until May/ June; Yamaguchi et al. 2006). Nevertheless, across badger populations, the extent to which they mate throughout the year (beyond the main mating season) has been shown to be variable (Corner et al. 2015), ranging from a single mating season in high-density areas (e.g., Wytham Woods, Oxfordshire, UK: Woodroffe 1995; Buesching et al. 2009; Dugdale et al. 2011a) to two mating peaks in medium density areas (e.g., South-west England: Cresswell et al. 1992; Page et al. 1994) to several oestrus cycles over the summer (Service et al. 2002) or matings throughout the year in low density populations (Corner et al. 2015). This is a curious and unique phenomenon, which I will investigate further in this thesis.

During autumn, badgers go through reproductive quiescence; males ascend their testes and females are anoestrous, coinciding with when they reach their heaviest weight and peak body condition (Macdonald et al. 2010; Woodroffe and Macdonald 1995a). During the main mating season in winter/spring, all male badgers have elevated testosterone levels and scrotal testes (Buesching et al. 2009; Woodroffe and Macdonald 1995a, Fig. 1). Nevertheless, while some individuals ascend their testes and reduce their testosterone levels in early/ mid September, others maintain elevated testosterone levels (comparable to the main mating season) and retract their testes later on at the end of October (Buesching et al. 2009; Woodroffe and Macdonald 1995a). Similarly, in females, vulva condition varies during the reproductive season with the majority exhibiting a swollen, pink and everted vulva (Fig. 1)

predominantly in late winter/ spring (Tinnesand et al. 2015). As examination of external genitalia morphology (EGM) is often used as a proxy for mammalian reproductive condition in the field, I will validate how these EGM-based assumptions match with the badgers physiological state in this thesis.

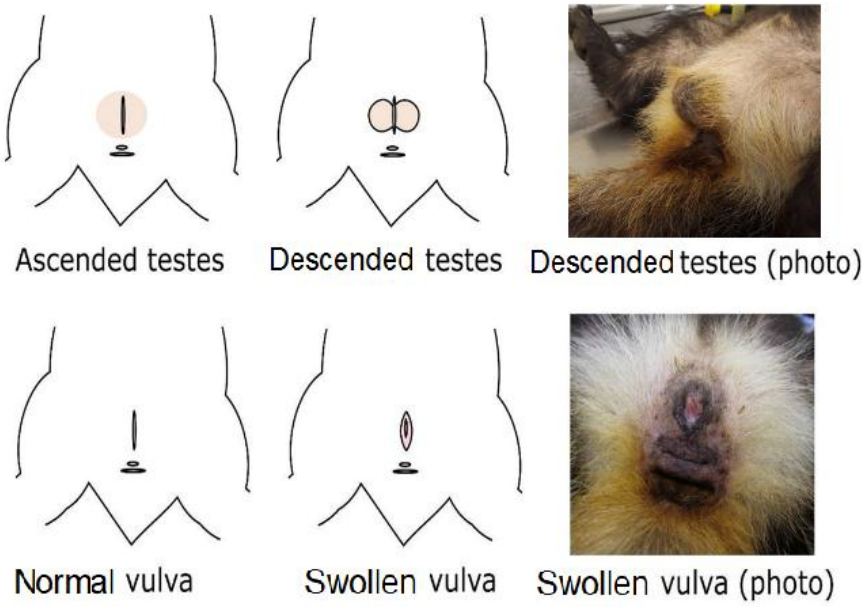


Figure 1. External genitalia morphology of male (above) and female (below) European badgers.

Badgers have a promiscuous polygynandrous mating system, where during a single ovulation each male can mate with several females and each female can mate with several males (Dugdale et al. 2007; Dugdale et al. 2011b). During this single ovulation, several eggs can be produced, which can then be fertilized by sperm from more than one male (superfecundation), resulting in multiple paternity; that is, in Wytham 45% (64/143 individuals) of litters were sired by multiple males (Annavi et al. 2014a; see also Capenter et al. 2005; Dugdale et al. 2007). This plural mating system has several advantages for the female such as potentially disguising the true paternity of cubs, thus reducing the risk of infanticide by other males (Wolff and Madonald 2004; Yamaguchi et al. 2006). Other advantages potentially include fertilization assurance, promotion of sperm competition, and the increase of genetic diversity (Chapman et al. 2003; Wolff and Macdonald 2004; Yamaguchi et al. 2006).

Although almost all mature badgers mate, only 32.37% (201/612) of males and 45.2% (228/502) of females are assigned young (based on identification of parent-cub relation from genetic pedigree data; Macdonald et al. 2015) per year; however, while there is some evidence of reproductive suppression (Wasser and Barash 1983) among females (Woodroffe and Macdonald, 1995b, see below), up to 1.6 females and 1.5 males breed on average per group (see Macdonald et al. 2015).

In Wytham Woods, 48% of paternities were the result of extra-group mating (Annavi et al. 2014a), with 84% of these involving neighbouring-group males (Annavi et al. 2014a; Macdonald et al. 2015). Although Geetha Annavi (2012) investigated at length if this extra-group mating enhanced reproductive fitness in her thesis and papers therein, there was little support that there were any quantitative or qualitative benefits, such as avoiding inbreeding depression (Annavi et al. 2014a) or achieving heterozgosity (Annavi et al. 2014b), nor did Simon Sin detect any assortative mating benefits with regard to MHC diversity (Sin et al. 2015) or pathogen resistence (Sin et al. 2014). This lead Macdonald et al. (2015) to speculate that female badgers may simply not have (yet) evolved to be discriminating of mating opportunities, with high-badger density being a relatively new phenomenon, arising through the high carrying capacity of British farmland. Alternatively, multiple mating may be needed to induce ovulation, or may serve to confuse paternity, as mentioned above (see Palombit 2015).

Although mating and fertilization can potentially occur throughout the year for badgers, implantation and gestation is restricted to mid-winter (December-January; Canivenc and Bonnin 1981; Woodroffe 1995; Dugdale et al. 2003; Yamaguchi et al. 2006) as badgers undergo delayed implantation (DI: Mead 1981; Mondain-Monval et al. 1980, 1983; Sandell 1990; Renfree and Shaw 2000; Yamaguchi et al. 2006). Thus, fertilized ova temporarily cease cellular division at the blastocyst (64 cell) stage and remain suspended in utero (instead of directly being implanted) for up to several months (Mead 1989). Implantation is triggered by short-day photoperiod in winter (Bonnin et al. 1978), at which point a true gestation commences lasting around 47-51 days (Canivenc and Bonnin 1981). Delayed implantation may decouple the time when females mate from the time parturition occurs, hence offspring can be born at the most favourable time of the year (typically at the start of spring), irrespective of the mating season and gestation length (Sandell 1990). If badgers tried to mate 50 days prior to parturition in mid February, they would face very cold and hostile weather,

whereas they have evolved the capacity to use torpor over winter to conserve energy (Newman et al. 2011; Noonan et al. 2014). Badgers, are thus evolutionary distinct in that they have one of the longest durations of DI reported in any species of up to 330 days (Canivenc and Bonnin 1981; Mead 1981; Woodroffe 1995). Delayed implantation is thought to arise due to insufficient hormonal secretion from the pituitary gland (mainly prolactin and LH; Mead 1993, Renfree and Shaw 2000, Yamaguchi et al. 2006), causing incomplete formation of the corpus luteum (small and poorly vascularised; Canivenc and Bonnin 1981) thus reducing luteal hormonal secretion, such as progesterone (Canivenc and Bonnin 1981; Mead 1993; Renfree and Shaw 2000).

This low progesterone secretion during delayed implantation is likely sufficient to maintain uterine conditions for pre-implanted blastocysts, but insufficient to inhibit further ovulation, causing superfoetation (Yamaguchi et al. 2006; Corner et al. 2015). Superfoetation allows additional oestrus cycles (multiple ova release with possible nonsynchronous fertilisation) to occur during DI, enabling simultaneous occurrence of more than one stage of developing offspring in a single female (Yamaguchi et al. 2006; Mainguy et al. 2009; Roellig et al. 2011), although implantation is synchronous, leading to synchronous parturition in individuals. In this thesis, I will investigate further the hormonal balance necessary to achieve delayed implantation and superfoetation in wild female badgers.

Not all blastocysts produced by females will be implanted as natural reabsorption may occur to adapt to litter size capacity (the mean litter size in Wytham Woods is 1.4±0.06, range of 1-4 cubs, where 93% litters comprise less than 3 cubs: Macdonald et al. 2015). Nevertheless, failure or delay to implant (as well as post-implantation reabsorbtion or abortion) may also occur if a female does not attain sufficient body condition to breed (Woodroffe 1995; Delahay et al. 2006; Dugdale et al. 2009). This may be closely linked to the scarcity of food , especially during dry summers or frosty winters (Dugdale et al. 2008; see also Bilham et al. 2018). In Wytham Woods, during the driest summers, only one cub per lactating female is produced; while during wetter summers (more food is available) up to 2-3 cubs are produced (Macdonald and Newman 2002).

Failure of breeding may also occur due to social stress. Genetic pedigree data for the Wytham badger population have shown that higher breeding success occurs when there are fewer females in the group (72% breeding success with 1-3 females, 46% with 4-5 females, 31%

with 6-10 females, and 21% with more than 11 females; Annavi 2012); noting that this implies that socio-spatial redistribution reduced competition and suppression through the 90s, allowing the population to grow; see Macdonald et al. 2004). Previous studies have shown that in higher badger density populations implantation rate is lower (44% in south-west England; Cresswell et al. 1992). Meanwhile in low density areas the implantation rate is higher (77% in Sweden; Ahnlund 1980), suggesting that higher density areas involve more social stress (note, all wild populations tend to exploit available environmental carrying capacity, and so simple density dependence is not a completely satisfying explanation), which could drive interference when foraging (limiting weight gain and blasocysts implanation; Woodroffe 1995) and reproductive suppression (Creel and Creel 1991; competition between breeding females, may cause stress that results in blastocysts re-absorption due to endocrinological imbalance; Woodroffe and Macdonald 1995b). Alternatively, lower number of cubs produced in higher densities, may be a strategic response to maximise fitness in females (investing more in quality rather than quantity, thus increasing chances of cub survival when resources are limited).

Furthermore, genetic pedigree data have also shown changes in breeding success and assigned litter size with age (Dugdale et al. 2011c). Despite the confirmed maximum lifespan in badgers ranging up to 14 years (in Wytham Woods; Macdonald et al. 2015), fewer older females were assigned cubs, starting the decline at age of 3 years whilst in males commenced later at age 5 - 6 years (Dugdale et al. 2011c), indicating an age-dependent reproductive decline. Later in this thesis, I will investigate if and when reproductive cessation (RC) and post-reproductive life span (PRLS) occurs in badgers based on endocrinological and somatic parameters.

Cub development

Neonatal badger cubs are highly altricial, their eyes and ear canals do not open until they reach 5 weeks of age (Roper 2010 c/w 2 weeks in domestic dog puppies). Cubs are typically weaned at 6-8 weeks of age, although in some cases lactation may be extended up to early May (due to drought or low alternative food availability; Woodroffe and Macdonald 2000). First emergence from underground sett occurs around the age of 8 weeks or even earlier (Fell et al. 2006; Kaneko et al. 2010) and full integration into the social group will occur at 14-16 weeks of age (Fell et al. 2006), when milk (deciduous) teeth are replaced by permanent

dentition (Woodroffe and Macdonald 2000). Cubs start producing subcaudal gland secretion when they are approximately 4 months old (Buesching et al. 2002a), but prior to this anoint themselves with secretion from other adults ('scent-theft' behaviour; Fell et al. 2006), illustrating the importance of this secretion in badger sociality (Buesching et al. 2003).

Growth rate varies among cubs depending on resource availability, which is largely affected by climatic conditions (Macdonald et al. 2010; Nouvellet et al. 2013), social pressure (Fell et al. 2006), natal sett quality (Kaneko et al 2010; Tsunoda et al. 2018; see Noonan et al. 2015a) as well as infection with endo-parasitic coccidia (Newman et al. 2001; surviving infected cubs reached a shorter adult body length by 5 cm in males and 3.5 cm in females; Newman et al. 2001). Cub development and mortality probability correlate negatively with extreme weather, such as much wetter or drier conditions than normal (Macdonald et al. 2010). Before reaching semi-independence in July, cubs are especially vurnerable to thermal stress (Kaneko et al. 2010; Tsunoda et al. 2018) arising from excessive rainfall, frost, and floods (Macdonald and Newman 2002). As extrinsic factors to which cubs are exposed during the growth period may influence end body size, in this thesis, I will further investigate how vulnerability towards a specific factor may differ between sexes, likely moderating patterns and degrees of sexual size dimorphism.

As a consequence, in high density areas (e.g., Wytham Woods: 44.55 ± 5.37 (SE) badgers/km^2; Annavi et al. 2014a, Macdonald et al. 2015), cubs take longer to reach adult size than in lower density areas (Macdonald et al. 2002a). Reports of when cubs reach sexual maturity vary considerably, ranging from 9-12 months (Ahnlund 1980; Neal and Cheeseman 1996) to 18 months (Buesching et al. 2002a). Nevertheless, female badgers would not be able to breed until reaching the age of 2 years due to delayed implantation (Macdonald et al. 2015). Further in this thesis, I will examine the endocrinological changes and concomitant ontological development of male and female genitalia during puberty, and investgate if cubs mature at the same rate or if there is evidence for different ontological strategies.

Climatic factors affecting badger populations

Aside from disease and culling regulations, badgers in the UK do not have any natural predators (Macdonald et al. 2015) and the factor most strongly mediatingtheir survival rate and reproductive rate, is prevailing weather conditions (Macdonald and Newman 2002;

Macdonald et al. 2009, 2010; Nouvellet et al. 2013). In the context of climate change higher numbers of badgers have been associated with warmer weather, both in Wytham (Macdonald and Newman 2002), the UK and Ireland (Byrne et al. 2015; Silva et al. 2017) and across Europe (Bevanger and Lindstrom 1995). As the primary driver of body condition variability in badgers is seasonality in food availability (Noonan et al. 2014), individual fitness is largely affected by climatic changes (i.e. ambient temperature and rainfall; Macdonald and Newman 2002; Macdonald et al. 2009, 2010; Nouvellet et al. 2013). Where conditions are favourable, food resources are abundant, self-maintenance will be fullfilled and access energy may be allocated to reproduction (Sapolsky 1986). It is known that extreme below average temperature during winter (causing frost) as well as above average temperature (causing drought) during summer are both detrimental for badgers as food resources (mainly earthworms) are scarce during these conditions (Kowalczyk et al. 2003; Nuutinen and Butt 2009). For both sexes, individuals are 20% heavier in warmer winters compared to colder ones (Macdonald et al. 2015). Predictability of seasonal normative conditions also plays a large role in badger survival and recruitment (Nouvellet et al. 2013), as rates varied with changing annual rainfall and temperature (Macdonald et al. 2010). The badger's physiological responses towards environmental changes based on individual phenotypic quality will be further investigated in this thesis.

Thesis structure and aims

To advance understanding against the key conceptual paradigms of reproductive endocrinology, outlined above, and utilizing the particularly apposite badger model species, this thesis aims to provide insights into lifetime reproductive events/schedules (e.g., puberty, reproductive senescence) and reproductive strategies, considering how these processes repond to ecological factors. This thesis is divided into seven parts comprising seven research chapters (see Diagram 1), where all research chapters are presented as independent papers either published or to be submitted. I conclude with a discussion, synthesising the badger's reproductive biology to advance comprehensive understanding of lifetime reproductive processes and how these are influenced by ecological factors in the wild.

Part I of this thesis provides the general introduction where the importance of reproductive biology in animal studies is described, as well as providing a literature review presenting the European badger as a model species for reproductive endocrinology.

Part II explores the badger's reproductive strategies and seasonality which consist of two reseach chapters:

R**esearch chapter I. Delayed implantation combined with superfoetation provide a flexible mechanism for assuring mating success in the European badger (*Meles meles*): Evidence from hormonal measures.**

The European badger (*Meles meles*) is evolutionarily distinct because it combines delayed implantation (DI) with superfecundation (SF). To investigate the hormonal balance necessary to achieve DI*SF, enzyme-immunoassays were used to measure cross-sectional population-level seasonal variation of circulating oestrogen and progesterone levels in wild female badgers during and after delayed implantation. Findings are discussed in the context of intra-specific endocrinological variation as an intra-sexual density-dependent adaptation in mating strategy and the evolution of female Alternative Reproductive Tactics (ART), and I consider how this flexibility allows badgers to use different mating strategies to succeed at radically different population densities across its extensive biogeographic range.

Research chapter II. Linking plasma sex steroid hormone levels to the condition of external genitalia morphology (EGM) in European badgers (*Meles meles*): A critical evaluation of traditional field methodology.

Accurate assessment of an animal's reproductive status is fundamental to many biological questions and immediate assessment is often necessary, especially in the field. Field biologists often rely on examination of external genitalia morphology (EGM) as a proxy for mammalian reproductive condition. In seasonally breeding European badgers, scrotal testes have been used as an indicator of spermatogenesis, while a pink and swollen vulva with mucosal secretion has been assumed to indicate oestrus. Nevertheless, systematic validation of these EGM-based assumptions is lacking. I analyse the relationship between circulating plasma sex-steroid hormones with the EGM condition (males: testes descendence and volume; females: vulva condition) during the winter mating season, the spring post-weaning period, the summer minor mating peak, and autumn reproductive quiescence. I test the hypothesis that higher sex-steroid-levels are reflected in EGM on the individual level and discuss results in the context of the utility of employing these frequently used field methods to infer reproductive status in European badgers and other seasonal breeders.

Part III investigates lifetime reproductive events in badgers, which consist of two research chapters:

Research chapter III: Heterochrony of puberty in the European Badgers (*Meles meles*) can be explained by growth rate and group-size: Evidence for two endocrinological phenotypes.

Puberty is a key stage in mammalian ontogeny, involving endocrinological, physiological as well as behavioural changes, and is marked by the development of secondary sexual characteristics. Although the age at puberty depends predominantly on intrinsic genetic factors, its timing can be moderated by a variety of additional extrinsic factors (food, environmental conditions, such as food availability, presence of conspecifics) as well as dynamic interactions between these factors. Consequently, not all members of a species, or even all individuals within one population, mature simultaneously or at the same rate, leading to heterochrony. I describe, male testosterone and female oestrone profiles from first capture (3 months, post-weaning) until 28 months (attaining sexual maturity and final body size), along with metrics of somatic growth, scent gland development and maturation of external reproductive organs. I examine mechanisms of how onset of puberty can be affected by body size and intra-specific competition resulting in developmental heterochrony.

Research chapter IV: Reproductive and somatic senescence of the European badger (*Meles meles*): Evidence through lifetime sex-steroid profiles.

Senescence is the deterioration of physiological and cellular functions caused by aging. Two categories of senescence have been be distinguished; reproductive senescence, which involves a decline in the ability to produce offspring after the reproductive peak, and somatic senescence, which refers to a decline in somatic condition until death. Within species, senescence schedules (reproductive and somatic) differ according to sex-specific resource allocation strategies, underpinning reproductive success. Combining long-term longitudinal data on age-dependent reproductive and somatic decline for multiple individuals from all ages groups (up to the age of 13 years), with cross-sectional endocrinological profiles for each sex, I investigate if and when individuals exhibit: 1) age-dependent reproductive declines in sex-steroid levels during spring, summer, and autumn; while also determining the prime age of reproduction; 2) age-dependent declines in somatic condition and reproductive advertisement, with reference to morphometric records and subcaudal secretion volume against age; 3) changes in their reproductive strategy as they grow older, through comparison of seasonal hormone profiles and levels between age classes. I then discuss implications for Reproductive Cessation (RC, debility to reproduce) and Post-Reproductive Lifespan (PRLS,

number of living years after RC has commenced) in the context of these findings, building on previous genetic work examining changes in breeding success and assigned litter size with age.

Part IV of this thesis decribes the badger's physiological and somatic responses to changes in ecological factors, which consist of two research chapters:

Research chapter V: Extrinsic factors affecting cub development contributing to sexual size dimorphism in the European badger (*Meles meles*). Sexual size dimorphism is common among mammals, with males usually larger than females. This phenomenon develops in most species through sex specific differences in growth rate and growth duration. Rensch's rule states that, within a lineage, sexual size dimorphism becomes greater with mean body size in taxa with larger males, while the opposite occurs when females are larger. Nevertheless, not all taxa apply to this rule as musteloidea, for example, demonstrate a hypo-allometry. Previous studies have found that the European badger exhibits relatively little sexual size dimorphism with a male and female body mass ratio of only 1.1:1, thus the extent to which extrinsic factors affect the end body size is likely significant in determining sexual size dimorphism. In this chapter I analyze how males and females differ in growth rate (during the first 2 years of life) examining morphometric measures (head-body length, zygomatic arch width, mean pastern length, as well as measurements of appendicular skeletal lengths) in relation to predictive factors (social: conspecific presence and climatic: temperature and rainfall) and how this affects final adult size (sexual size dimorphism).

Research chapter VI: Somatic, social, and climatic factors affecting reproductive hormone levels in the European badger (*Meles meles*).

Animals in the wild experience extrinsic pressures in the form of environmental and social conditions, to which they respond through physiological, morphological and behavioural alterations based on their individual phenotypic quality (i.e. body size, age) in order to maximise individual fitness. As an intensive and highly energetic investment, reproduction is often affected as a trade-off response in favour of self (somatic)-maintenance during unfavourable environmental conditions, and this should be detectable through manifest changes in reproductive hormone levels. I examine here how somatic factors (age, weight, head-body length), social factors (number of female and male adults in social groups and total number of adults in population), and also climatic factors (rainfall and temperature) affect male (testosterone) and female (oestradiol, oestrone) hormone levels across seasons as well as during the main mating season. I hypothesise that these patterns will not only differ

among individuals, but also between sexes according to their respective reproductive strategies.

Part V of this thesis further explains different methods in hormone analyses, consisting of one research chapter:

Research chapter VII: Measuring sex-steroid metabolites in urine with enzyme immunoassay (EIA) to establish a new non-invasive method for determination of reproduction status in the European badger (*Meles meles*).

Conventionally, hormone analyses is conducted using blood samples, as this gives a direct measure of the immediate circulating hormone level at the time of sampling and is considered to be most accurate in reflecting endocrine fuction. Nevertheless, routine blood sampling may not always be practical for wild animals and may induce stress which will affect accuracy of analyte levels. Therefore, development of alternative hormone measurement methods to blood sampling is of interest. Circulating hormones are ultimately metabolized through different routes, either via the kidneys to be excreted through urine or by the liver to be excreted through faeces; residual metabolites may be deposited in hair, while a small portion may be present in saliva. These trace metabolites can be utilized in non-invasive hormone measurement methods as a proxy to estimate circulating hormone levels and determine reproductive parameters. As urinary hormone metabolites have a significantly lower time lag compared to faecal and hair hormone analysis, and are more stable to pulsatile changes than plasma and saliva hormone analysis, I used urine in this chapter to establish a new non-invasive homone analysis method for badgers. Circulating hormone levels (testosterone in males; oestradiol in females) with urinary metabolites from paired samples across seasons were correlated; and urinary metabolites were tested to see whether they tracked the seasonal reproductive cycle pattern, as an additional biological validation. Results were used to validate and assess the usefulness of urinary testosterone and oestradiol metabolite measurement for reliably assessing endocrine production in the badger.

Part VI includes the general discussion synthesising the importance of reproductive hormone monitoring for wildlife management and conservation, using the European badger as a model species. This chapter will also discuss the biology of badgers to advance comprehensive understanding of reproductive processes and how these are influenced by ecological factors in the wild.

Part VII contains the appendices of this thesis, includes further original co-authored papers as well as detailing the laboratory procedures for hormone measurements.

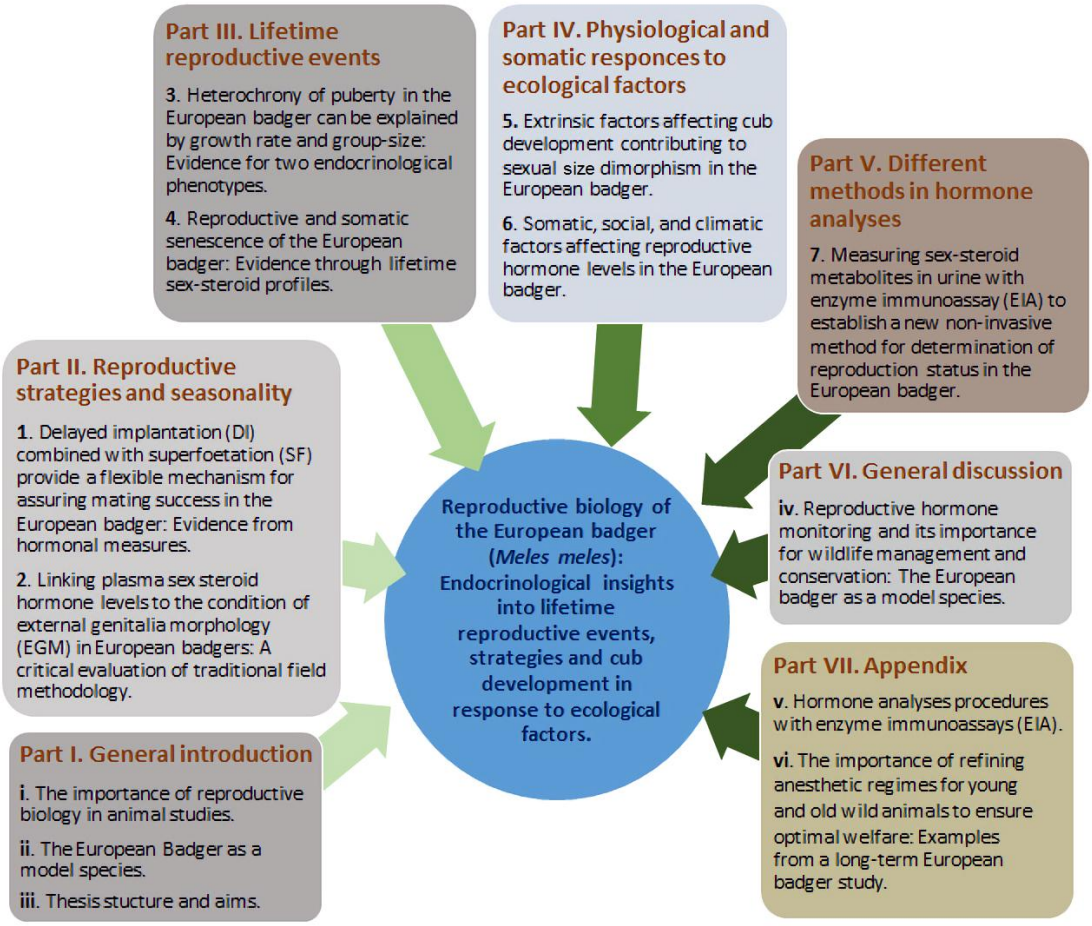


Diagram 1. Thesis outline

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PART II

REPRODUCTIVE STRATEGIES AND SEASONALITY

RESEARCH CHAPTER 1

Delayed implantation combined with superfoetation provide a flexible mechanism for assuring mating success in the European badger (*Meles meles*): Evidence from hormonal measures

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ABSTRACT

Population density can affect mating strategy evolution; however few studies have investigated intra-specific density-dependent variation. The European badger (*Meles meles*) is evolutionarily distinct because it combines delayed implantation (DI) with superfecundation (SF). To establish the tactical mating flexibility afforded by DI*SF, enzyme-immunoassays were used to measure cross-sectional population-level seasonal variation of circulating sex-steroids for 97 wild female badgers. Oestradiol was highest in spring in females without cubs, decreased in summer, and remained low during following seasons, evidencing control of spring oestrus. Oestrone was consistently higher than oestradiol; it was elevated in spring, lowest during summer, peaked in autumn, and remained elevated for pregnant females in winter, indicating that oestrone sustains pre-implanted blastocysts throughout DI. Progesterone was low throughout, and increased significantly only during winter pregnancy, associated with implantation and luteal development. Oestradiol data evidence that, at high-density, females exhibit only one oestrus, contrasting multiple oestruses reported by lower-density studies. It is postulated that, while additional oestruses during DI at low-density enhance fertility assurance, at high-density, when coitus is frequent, fertilisation is assured, precluding further cycles and associated mating risks. This endocrinologically flexible DI*SF mating strategy likely represents a form of balancing selection, allowing badgers to succeed at a range of regional densities.

Keywords*: alternative reproductive tactics, delayed implantation, European badger, mating strategies, sex steroids, superfoetation*

INTRODUCTION

Population-density, and thus the number of available mates, has been shown to affect the evolution and shaping of reproductive strategies (reviewed in Kokko and Rankin 2006). Nevertheless, although selective pressures likely differ between high and low-density populations (Kokko and Rankin 2006), few studies have investigated potential density-dependent effects within the same species. Most research on Alternative Reproductive Tactics (ART: Taborsky et al. 2008) has focussed on males (reviewed in Kokko and Rankin 2006; Taborsky and Brockmann 2010); however, females likely also display a degree of flexibility in their reproductive behaviour and/ or physiology to adapt to situations of high or low mate availability (Kokko and Mappes 2005). Generally, once fertilisation has been achieved, females, as the limiting sex, are likely to suffer from too much – rather than too little – sexual attention from males, particularly at high population densities, and are thus likely selected for reproductive strategies to discourage costly male attention (Kokko and Rankin 2006; Wolff and Macdonald 2004). In mammals, this is especially important if additional oestrus cycles (e.g., Buchanan 1966) or superfluous matings (e.g. presenting risks of contracting sexually transmittable diseases: Thrall et al. 2000) have the potential to be harmful to the female herself and/ or to female reproductive output (Chapman et al. 2003).

Delayed implantation (DI) is a strategy that arrests the typical linear progression of reproduction, i.e., fertilisation, implantation, gestation, parturition. Instead of fertilised ova implanting into the uterus wall immediately and forming a placenta, their development undergoes a period of diapause, typically at the blastocyst stage, and remains suspended in the uterus lumen for up to several months (Mead 1989). In its fundamental form, DI has evolved to decouple the time when females mate from the time when they give birth. Offspring can therefore be born at the most appropriate times of the year, irrespective of mating season and gestation length (Sandell 1990). In temperate ecosystems, this optimal birth period is typically at the start of the productive spring season, where DI circumvents the need for mating to take place during the preceding winter (Ferguson et al. 2006; Thom et al. 2004), when individuals may lack the body-condition to mate (Hewison and Gaillard 2001), or may seek to avoid harsh and energetically costly conditions through relative inactivity (Noonan et al. 2014). This is particularly important in low-density populations where finding and choosing appropriate mates would entail increased – and potentially dangerous - roaming

behaviour (Macdonald et al. 2010). Obligate DI has been recorded in 53 mammal species belonging to seven different orders and 10 families (Sandell 1990, Thom et al. 2004), approximately half of which are members of the Mustelidae (see Macdonald and Newman 2017; Renfree and Shaw 2000; Thom et al. 2004).

A second phenomenon that can complicate simple linear pregnancies is superfoetation (SF), where additional oestrus cycles during pregnancy release multiple ova with possible non-synchronous fertilisation, thus allowing the simultaneous occurrence of more than one stage of developing offspring in a single mother (Mainguy et al. 2009; Roellig et al. 2011; Yamaguchi *et al*. 2006). SF may promote cryptic female choice (e.g., sperm competition) and greater genetic diversity, it can assure fertilization and may result in paternal confusion, which could inhibit infanticide (Orr 2012). In pregnancies without DI, SF is achieved via a second oestrus immediately prior to parturition of the foetuses that developed from the first ovulation event (e.g. brown hare *Lepus europaeus*: Caillol et al. 1991; North African gundi *Ctenodactyles gundi*: Gouat 1985; casiragua *Proechimys semispinosus*: Weir 1974). Therefore, although the second ovulation occurs prior to parturition, each set of ova develops separately in essentially two parallel pregnancies leading to two distinct parturitions (Yamaguchi et al. 2006).

In species without DI, further oestrus cycles are inhibited by one or several corpora lutea (CL), which produce progesterone that triggers implantation (Halazs and Szekenes-Bartho 2013) and maintains pregnancy (Adkins-Regan 2005; Austin and Short 1984). Subsequently, progesterone secreted by the foeto-placental unit inhibits the secretion of Follicle Stimulating Hormone (FSH) from the pituitary, via chorionic gonadotrophic feedback, preventing further ovulations. Concomitantly, oestradiol secretion ensures uterus inactivity during pregnancy, and plays a major role during parturition (Cohen 1985; Goodwin 1999), whereas oestrone maintains a suitable uterine environment for the survival of pre-implanted blastocysts, extending the life-span of the CL and preparing the uterus for implantation (Heap et al. 1975; Heap et al. 1979). Previous research on species with DI has found that progesterone levels follow a similar pattern as in species that implant directly (Corner et al. 2015; Moller 1973; Sinha and Mead 1975). However, this poses a problem because blastocysts must be prevented from implanting for days, or even months, despite the presence of functional CL(s) and associated increase in progesterone levels (Mead 1993; Renfree and Shaw 2000; Yamaguchi

et al. 2006) and uterine (menstrual) shedding must be prevented from aborting the foeto-placental unit.

Among eutherian mammals, only two mustelid species, the American mink (*Neovison vison*: Yamaguchi *et al*. 2004) and the European badger (*Meles meles*: Corner *et al*. 2015), combine delayed implantation with superfoetation (DI*SF). In mink, DI is of short duration (average 27.5 days; Macdonald and Newman 2017) and superfoetation occurs during a single additional oestrus. Badgers, however, are evolutionarily distinct in that they have one of the longest durations of DI reported in any species of up to 330 days (Canivenc and Bonnin 1981; Mead 1981; Woodroffe 1995), where females commence mating during a post-partum oestrus around mid-February - marking the start of DI - after which superfoetation can occur during one or several additional oestrus cycles (Canivenc and Bonnin 1981; Corner *et al*. 2015; Roellig et al. 2011; Woodroffe 1995; Yamaguchi et al. 2006), concluding once blastocyst implantation is triggered by short-day photoperiod in winter (Bonnin et al. 1978). True gestation then lasts 47-51 days (Canivenc and Bonnin 1981). This DI*SF mating system, with extended female receptivity, is also reflected in male circulating plasma testosterone titres (Buesching et al. 2009) and spermatozoa levels (Page et al. 1994), which peak around March, coinciding with the main post-partum mating season, and then decline to a minimum in October or November when testes ascend into the body cavity. Female badgers cannot be coerced into matings (Dugdale et al. 2011c) and the observed mating patterns are best explained by promiscuous convenience polygynandry (Annavi et al. 2014a, b).

Interestingly, however, the extent to which badgers have been found to mate throughout the year (i.e., outside of the February peak), varies substantially between studies, apparently linked to population density (Corner et al. 2015; Roper 2010). From post-mortem data, supported by progesterone analysis, Corner et al. (2015) reported the presence of secondary and tertiary follicles throughout the year from badgers at relatively low population density (1.9 badgers/km^2) in the Republic of Ireland, with blastocyst turnover during delayed implantation, indicating that mating was taking place throughout the period of DI. This corroborates Service et al. (2002) who, based on sex hormone measurements from urine, reported up to five oestrous cycles between late winter/spring and autumn, with an average interval of 28 days at moderate population density (4.4-7.5 badgers/km^2; Bristol, UK). In contrast, only two distinct mating periods per year have been observed at medium population density (south-west England, UK, 25.3 badgers/km^2: Cresswell et al. 1992; Page et al. 1994;

Woodchester Park, UK, 25 badgers/km^2: Carpenter et al. 2005), the main peak being post-partum in late winter/ early spring, with a secondary peak in late summer, where Cresswell et al. (1992) report (from post-mortem) that ca. 40% of females had conceived blastocysts by the end of March, increasing to 80-90% by the end of April. Ultimately, when badger populations are at very high density, as at this particular study site (Wytham Woods, Oxfordshire, UK: 40 badgers/km^2: Macdonald et al. 2009), mating is largely restricted to a single mating season (Buesching et al. 2009; Dugdale et al. 2011a; reviewed in Macdonald et al. 2015; Woodroffe 1995), although it is currently unclear if these behavioural observations are also reflected in female reproductive physiology.

Here, the hormonal balance necessary to achieve DI*SF in this badger model was investigated by measuring changes in circulating oestrogen and progesterone levels during and after delayed implantation, using cross-sectional population-level data from 97 wild females. The findings are discussed in the context of intra-specific endocrinological variation as an intra-sexual density-dependent adaptation in mating strategy (Kokko and Rankin 2006) and the evolution of female ARTs (Taborsky et al. 2008), and consider how this flexibility allows badgers to use different mating strategies to succeed at radically different population densities, linked to regional environmental carrying capacity, across its extensive biogeographic range (Johnson et al. 2002).

MATERIALS AND METHODS

Study population and sample collection

Blood samples from 97 different females of different age and reproductive status caught between February 1990 and June 2015 from a high-density badger population in Wytham Woods, Oxfordshire, UK (51:46:26 N / 1:19:19 W; for site details see Macdonald et al. 2015; Savill et al. 2010) were analysed (Table 1),. As part of an ongoing long-term research project, and following the methodology described in Sun *et al*. (2015), badgers were trapped three to four times annually in cage traps baited with peanuts: (1) in *winter* (January) during the first pregnancy trimester, (2) in *spring* (May/June) after cubs were fully weaned, (3) in *summer* (August) during lowest food abundance, and (4) in *autumn* (November) during

reproductive quiescence when badgers reach their maximum body weight. All protocols and procedures employed were approved by the Animal Welfare and Ethical Review board of Oxford University's Zoology Department and procedures were conducted under the Animals (Scientific Procedures) Act, 1986 (PPL: 30/3379).

Table 1. Number of plasma samples per associated reproductive status used for oestrone, oestradiol, and progesterone measurements.

Season	**Reproductive state**	**Sample size**		
		Oestrone (E1)	**Oestradiol (E2)**	**Progesterone**
Spring	Delayed implantation & Lactated	11	6	12
	Delayed implantation & Not-lactated	12	7	12
Summer	Delayed implantation & potential superfoetation	12	8	11
Autumn	Delayed implantation & Reproductive quiescence	9	9	10
Winter	Pregnant	10	6	11
	Not-pregnant	5	-	5
Total samples		59	36	61

*Note: Samples used for oestrone and progesterone analyses were from the same individuals (n=61) while oestradiol samples were collected from different individuals (n=36).

Traps were checked between 6.30-8.00 am, and captured animals were transferred to holding cages and transported to a central field station, before being sedated by intramuscular injection of 0.2 ml ketamine hydrochloride/ kg body weight (McLaren et al. 2005; Thorton et al. 2005). At first capture (67% as cubs), all badgers received a permanent unique tattoo in the left inguinal region. Thus, all animals could be identified individually, sexed, and aged (Macdonald et al. 2009; Macdonald and Newman 2002). Cubs and yearlings (i.e., all individuals younger than 2 years) were excluded from this study to ensure sexual maturity (Buesching et al. 2009; Dugdale et al. 2011c). Although badgers are not among the species known to undergo menopause (Ward et al. 2009), no females older than 10 years of age were

included in the dataset, because female reproductive success has been shown to senesce in older badgers (Carpenter et al. 2005; Dugdale et al. 2011c).

Following Dugdale et al. (2011b), recent lactation was inferred from teat size in spring (and, where possible, corroborated by genetic pedigree: for methodology see Annavi et al. 2014a, b) and categorised as lactated (teats swollen and elongated; as cubs were fully weaned at this point, no milk could be expressed), or not-lactated. In winter, ultrasound scans were performed to detect pregnancy, and females were categorised as pregnant (one or several vesicles/ embryos detected in uterus) or not-pregnant (no vesicles/ embryos detected).

Plasma samples

Blood samples (not exceeding 2 ml) were collected by jugular venipuncture in K2-EDTA (di-potassium ethylene diamine tetraacetic acid) vaccutainer tubes (Becton-Dickinson), and centrifuged at 10°C for 10 min at 2,500 rpm within 30 minutes. Plasma was transferred into Eppendorf tubes and frozen immediately at -20°C. Sampling times were standardised (collected between 08.30am and 10.30am) to account for circadian variation in hormonal profiles (Buesching et al. 2009).

Hormone analyses

All sex steroid levels were measured using microtitreplate enzyme immunoassays (EIA). Oestrone and progesterone analyses were performed at the Chester Zoo Endocrinology Laboratory, UK; oestradiol was analysed at the Endocrinology Laboratory at the German Primate Centre, Germany (Details of hormone measurement is shown in Appendix 1).

Oestrone was measured in microtitreplates coated with polyclonal antiserum raised against oestrone EC R522 (Munro 1991). Plasma samples were used directly, i.e. un-extracted, for measurement after 1:10 dilution with assay buffer. Duplicate 20µl aliquots of diluted plasma, oestrone standard (0.195-200 pg/well) and quality controls were combined with 50µl oestrone glucuronide coupled to horseradish peroxidase (oestrone-glucuronide-HRP) as label, and incubated at room temperature for 2 hours. After incubation, plates were washed five times and blotted dry, followed by an addition of 100 µL peroxidase substrate solution (ABTS) to each well. Plates were then covered, incubated at room temperature until the '0' wells reached about 1.0 optical density and read at 405 nm using a Spectrophotometer Opsys MR (Dynex). Assay sensitivity at 90% binding was 3.1 pg. Intra-assay coefficient of variation (CV), calculated as the average value from the individual CVs for all of the sample

duplicates, was 2.6% (expected to be <10%). Inter-assay coefficient of variation (CV), calculated from repeated measurements of high and low-value quality controls across plates (expected to be <15% for both high and low quality controls), was 9.0% (high) and 15.5% (low).

For oestradiol analysis, plasma samples (250 µl) were extracted twice with 10 volumes of diethylether by vortexing for 10 minutes. After separation of the ether phases from the aqueous phases by snap-freezing with methanol/dry ice, the two extracts were combined, evaporated until dry, and reassembled in assay buffer (250 µl; Buesching et al. 2009). Oestradiol was measured using an antiserum raised against oestradiol-17-HS-BSA (Meyer et al. 1990) together with oestradiol coupled to horseradish peroxidase (oestradiol-HRP) as label. Duplicate 50 µl aliquots of oestradiol standards (0.24-62.5 pg/well), sample extract and quality controls were combined with label (50µl) and antiserum (50µl), mixed thoroughly and incubated overnight at 4^{o}C. Following incubation, plates were washed 4 times and blotted dry. Peroxidase substrate solution (TMB; 150µl) was added to each well and plates were incubated for a further 1-1.5 hours. Absorbance was then measured at 450 nm using a spectrophotometer (BioTek Instruments EL 808). Sensitivity of the oestradiol assay at 90% binding was 0.4 pg. Inter-assay CVs of high and low-value quality controls were 4.5% (high) and 7.4% (low), while the intra-assay CV was 6.2%.

For the progesterone assay, plasma samples were diluted 1:8 with assay buffer. Duplicate 50µl aliquots of progesterone standards (0.78-200 pg/well), samples and controls were combined with 50µl progesterone coupled to horseradish peroxide (progesterone-3-CMO-HRP) as label in plate wells coated with a monoclonal CL425 progesterone antiserum (Pantex 2010). After incubation at room temperature for 2 hours, plates were washed five times and blotted dry, followed by an addition of peroxidase substrate (ABTS, 100 µL) to each well. As for oestrone measurements (see above), plates were then covered, incubated at room temperature until '0' wells reached about 1.0 optical density and read at 405 nm, using a Spectrophotometer Opsys MR (Dynex). Assay sensitivity at 90% binding was 0.78 pg. Inter-assay CVs of high and low-value quality controls were 4.9% (high) and 2.4% (low), while the intra-assay CV was 3.1%.

Statistical analyses

Prior to statistical analyses, a mixed model was constructed to account for inter-annual hormone level variation between years: hormone level ~ season+ (1|year). However, as no variation was attributed to year as a random effect, only linear models were used in the analyses. To test for seasonal variation of female hormone levels during DI, ANOVA was used (season was treated as a categorical variable with six phases: spring lactated, spring not-lactated, summer, autumn, winter pregnant, winter not-pregnant) to establish overall variation. A log-transformation was conducted on oestradiol levels to obtain best model fit; other hormones were run without transformation. A-priori contrast tests were then used to compare hormone levels between reproductive states (progesterone: lactated/not-lactated, lactated/all levels, not-lactated/all levels, pregnant/not-pregnant, pregnant/all levels; oestrone: lactated/not-lactated, lactated/all levels, not-lactated/all levels, pregnant/not-pregnant, not-pregnant/all levels, summer/all levels, autumn/all levels, not-pregnant/summer, and pregnant/autumn; oestradiol: lactated/not-lactated, lactated/all levels, not-lactated/all levels, and spring (not-lactated + lactated)/all levels).

To test if oestrone and oestradiol levels followed different seasonal patterns, a two way ANOVA was used including season/reproductive state (with the same levels as above) and hormone type (two levels: oestrone and oestradiol). As measurements of oestrone and oestradiol levels were conducted in different labs, hormone levels were standardised by dividing each sample value by the maximum value across phases of each hormone. In the presence of an interaction, ANOVAs were carried out separately for each level of season/reproductive state, with hormone type as the predictor. Patterns of residuals, normality, and mean variance relationships for each model were checked using diagnostic plots in R. All statistical analyses were performed using R Studio (0.99.896) and R (R-3.2.4).

RESULTS

Variation in progesterone levels across seasons and reproductive stages.

Progesterone levels differed significantly among the six reproductive states tested ($F_{5,55}$= 21.31, $p < 0.001$; Fig. 1). Levels remained low throughout spring, summer, autumn, and were elevated only during winter for pregnant females (Table 2).

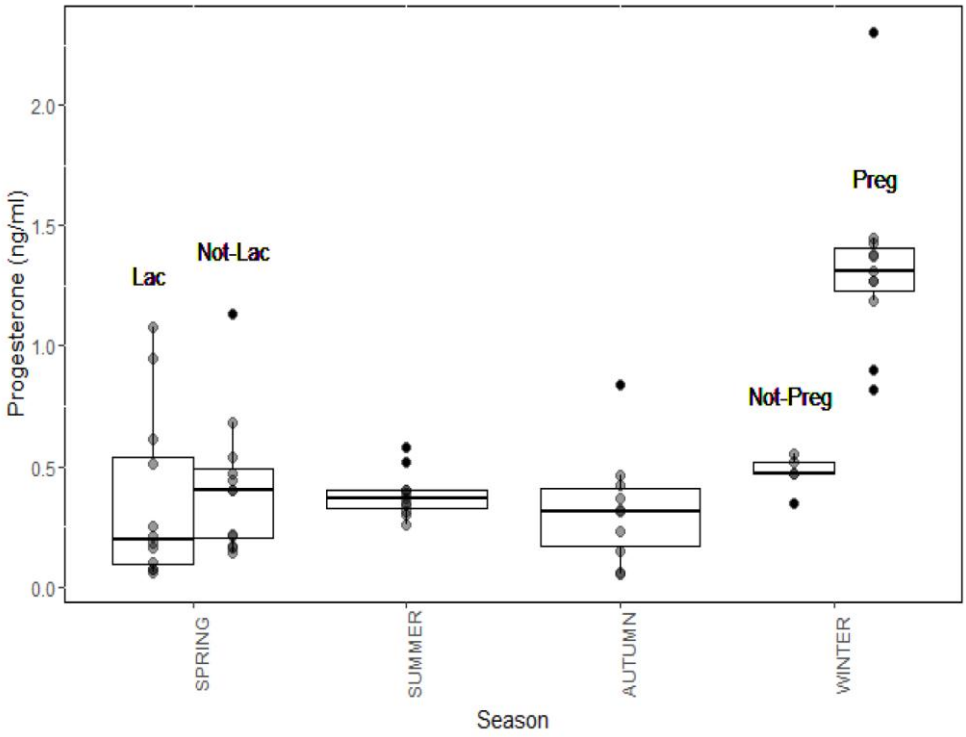


Figure 1. Variation in progesterone levels (ng/ml) with season and reproductive stage. Lac= lactated females during spring, Not-Lac= females that did not lactate during spring, Not-Preg= females that were not pregnant in winter and Preg= pregnant females in winter.

Table 2. Mean progesterone levels and standard deviation throughout reproductive stages.

Season/ Reproductive state	**Number of samples**	**Mean hormone level (ng/ml)**	**STD**
Spring lactated	12	0.35	0.35
Spring not-lactated	12	0.41	0.28
Summer	11	0.38	0.09
Autumn	10	0.32	0.23
Winter not-pregnant	5	0.47	0.08
Winter pregnant	11	1.34	0.38

Progesterone levels did not differ with lactation status in spring (lactated/not-lactated: estimate= 0.059, std. Error= 0.145, t= 0.408, p= 0.685) nor did they vary with other states (lactated/all: estimate= 0.206, std. Error= 0.121, t= 1.695, p= 0.095, not-lactated/all: estimate= 0.1730, std. Error= 0.1473, t= 1.174, p= 0.245). Pregnant females exhibited

significantly higher progesterone levels than any other category of females (pregnant/all levels: estimate= -0.159, std. Error= 0.015, t= -10.51, p <0.001; pregnant/not-pregnant: estimate= 1.170, std. Error= 0.181, t= 6.460, p <0.001).

Variation in oestrone levels across seasons and reproductive states

Oestrone levels varied significantly between the six reproductive states tested ($F_{5,53}$= 7.72, p < 0.001; Fig. 2). During spring, levels were high in all females, significantly decreased in summer (summer/all levels: estimate= 40.620, std. Error= 9.339, t= 4.349, p<0.001), peaked in autumn (autumn/all levels: estimate= -27.09, std. Error= 11.52, t= -2.352, p= 0.022), and remained elevated in winter only for pregnant females (pregnant/all levels: estimate= -21.15, std. Error= 11.22, t= -1.885, p= 0.064; autumn/pregnant: estimate= -3.448, std. Error= 14.993, t= -0.230, p= 0.8189); but matched summer lows in not-pregnant females (not-pregnant/all levels: estimate= 32.870, std. Error= 14.960, t= 2.198, p= 0.0321; summer/not-pregnant: estimate= -29.449, std. Error= 16.245, t= -1.813, p= 0.075) (Table. 3).

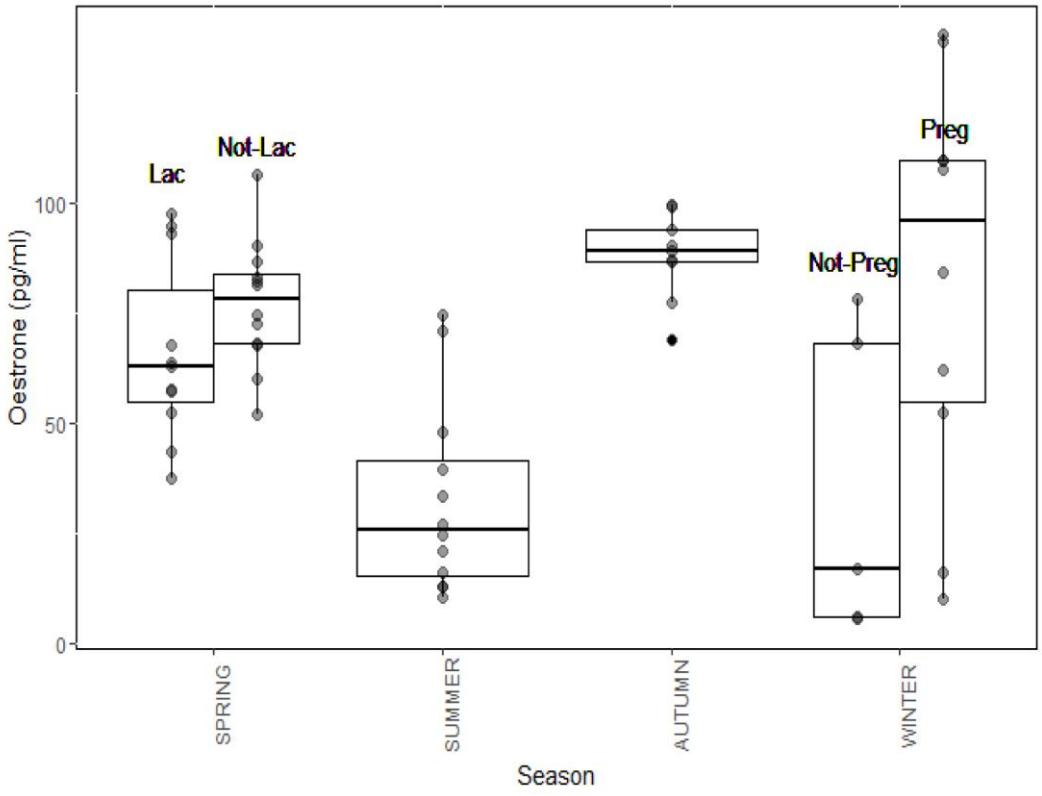


Figure 2. Variation in oestrone levels (pg/ml) with seasons and reproductive stage. Lac= lactated females during spring, Not-Lac= females that did not lactate during spring, Not-Preg= females that were not pregnant in winter and Preg= pregnant females in winter.

Table 3. Mean oestrone levels and standard deviation throughout reproductive stages.

Season/ Reproductive state	Number of samples	Mean hormone level (ng/ml)	STD
Spring lactated	11	66.09	20.51
Spring not-lactated	12	77.00	14.53
Summer	12	32.62	21.91
Autumn	9	87.94	9.80
Winter not-pregnant	5	34.90	35.26
Winter pregnant	10	82.55	46.04

Lactation had no effect on oestrone levels (lactated/not-lactated: estimate= 10.954, std. Error= 11.423, t= 0.959, p= 0.342; lactated/all: estimate= 1.362, std. Error= 11.138, t= 0.122, p= 0.903; not-lactated/all: estimate= -12.571, std. Error= 8.826, t= -1.424, p= 0.16).

Variation in oestradiol levels across seasons and reproductive stages

There was a near-significant seasonal variation in oestradiol levels among the 5 states tested ($F_{4,31}$= 2.58, p=0.057; Fig. 3). Oestradiol levels were higher during spring in not-lactated females ($spring_{not\text{-}lactated}$/all levels: estimate= -0.255, std. Error= 0.139, t= -1.824, p= 0.077), then becomes very low in summer (summer/all levels: estimate= 0.288, std. Error= 0.130, t= 2.211, p= 0.034) and remained low in autumn (summer/autumn: estimate= 0.186, std. Error= 0.166, t= 1.123, p= 0.269) and winter for pregnant females ($autumn/winter_{pregnant:}$ estimate= 0.060, std. Error= 0.181, t= 0.333, p= 0.741; as all trapped females in this sub-sample were pregnant, not-pregnant females could not be included in this analysis; Table. 4).

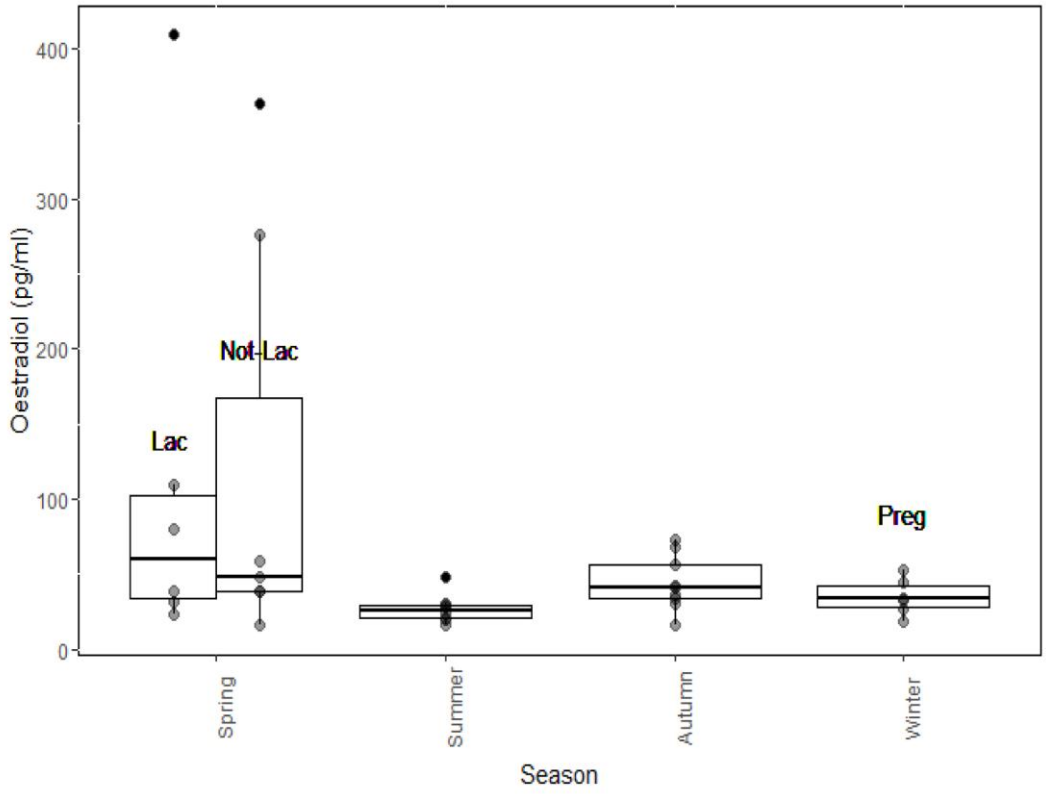


Figure 3. Variation in oestradiol (pg/ml) with season and reproductive stage. Lac= lactated females during spring, Not-Lac= females that did not lactate during spring and Preg= pregnant females in winter.

Table 4. Mean oestradiol levels and standard deviation throughout reproductive stages.

Season/ Reproductive state	**Number of samples**	**Mean hormone level (pg/ml)**	**STD**
Spring lactated	6	115.37	147.6
Spring not-lactated	7	119.97	139.34
Summer	8	26.85	10.01
Autumn	9	44.00	18.37
Winter pregnant	6	34.93	12.17

Again, there was no evidence for differences in oestradiol levels associated with lactation (lactated/not-lactated: estimate= 0.032, std. Error= 0.193, t= 0.168, p= 0.867; lactated/all levels: estimate= -0.245, std. Error= 0.149, t= -1.639, p= 0.110).

Comparison of oestrone and oestradiol levels between different reproductive states

The predominant oestrogen varied significantly between seasons and reproductive states (2-way ANOVA interaction term: $F_{4,84}$= 3.26, p= 0.015; Fig. 4). Mean standardized oestrone levels were consistently higher than oestradiol concentrations; although this was statistically significant only for spring not-lactated (p= 0.021), summer (p= 0.008), autumn (p<0.001), and winter pregnancy (p= 0.002).

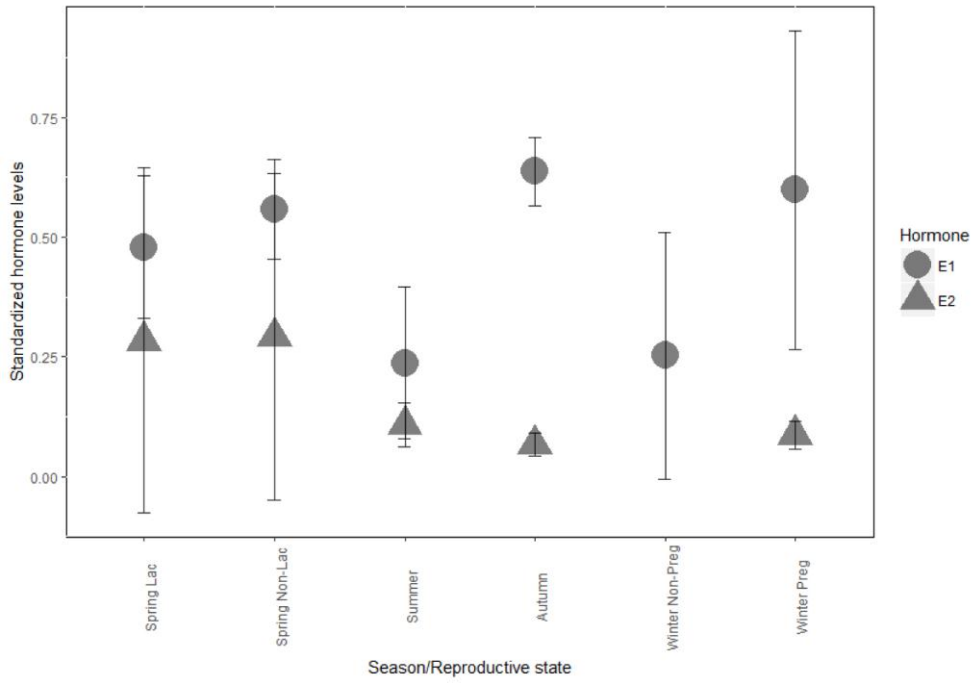


Figure 4. Comparison of mean standardized oestrone (E1) and oestradiol (E2) levels (with STD bars) during different reproductive states (Spring Lac= lactated females during spring, Spring Not-Lac= females that did not lactate during spring, Winter Not-Preg= females that were not pregnant in winter and Winter Preg= pregnant females in winter, Summer and Autumn= females captured in summer and autumn respectively).

DISCUSSION

In this study, high oestrone and progesterone levels were clearly diagnostic of post-implantation pregnancy (levels were significantly lower pre-implantation), versus non-pregnant females, while oestradiol levels were low for post-implantation females. This suggests that badgers secrete oestrone as their predominant oestrogen during DI to support the viablity and development of their blastocysts, and levels of oestrone were consistently

higher than oestradiol in all seasons. The comparatively high oestrone levels in pre-implanted pregnant females likely act to maintain the CL, supporting pre-implanted blastocysts, before signalling to the uterus to prepare for implantation (Heap et al. 1975; Heap et al. 1979), because with DI there is typically insufficient hormonal secretion from the pituitary gland (mainly prolactin and luteinising hormone) to cause complete differentiation of the CL (Canivenc and Bonnin 1981; Mead 1993; Renfree and Shaw 2000; Sundqvist et al. 1988). Consequently, it appears that the low levels of progesterone secreted by the CL that was observed during embryonic diapause (i.e. from postpartum oestrus in mid-February until implantation in mid-December) are likely insufficient to inhibit further ovulation, but sufficient to maintain uterine conditions without menstrual shedding and associated loss of pre-implanted blastocysts. The low levels of progesterone reported here are also in accord with findings on the morphology and appearance reported by Canivenc and Bonnin (1981), who describe that the CL in badgers are particularly small and poorly vascularised, leading to low levels of activity, likely due to limited prolactin and LH secretion from the pituitary (Mead 1993, Renfree and Shaw 2000, Yamaguchi et al. 2006).

We found that oestrone levels always exceeded those of oestradiol (statistically significant only during spring in not-lactated females, summer, autumn, and winter for pregnant females; see also Mondain-Monval et al. 1980). This implies that oestradiol mainly controls spring oestrus, whereas oestrone is continuously secreted throughout DI to sustain blastocysts until implantation occurs in winter. This is consistent with measures of female oestrogen levels during DI in female black bears (*Ursus americanus;* 7-8 months DI; Garshelis and Hellgren 1994; Tsubota et al. 1987), where oestradiol levels were also only high during oestrus and low during the period of DI. Similarly, in spotted skunks (*Spilogale putorius;* 6-7 months DI; Mead and Eik-Nes 1969), oestradiol levels were only detectable during oestrus, late stage of pre-implantation, and post-implantation.

In winter, it was found that badgers secreted higher levels of progesterone, associated with the timing of implantation and luteal development, triggered by a short day photoperiod (Bonnin et al. 1978; Yamaguchi et al. 2006). In terms of the late winter (March) post-partum oestrus peak (Bonnin et al. 1978; Cresswell et al. 1992; Yamaguchi et al. 2006), the hormone measurements did not detect any significant difference in sex-steroid levels between females that lactated and those that did not. Therefore, females were able to breed while lactating, shortly after parturition. This is contrary to effects typically seen in other mammal species

where lactation inhibits oestrus because high levels of prolactin cause a decrease in GnRH, thereby inhibiting ovarian folliculogenesis (causing low levels of oestrogens) and the pulsatile LH secretion required for ovulation and subsequent CL formation (Jameson et al. 2013).

In this high-density (40 badgers/km^2: Macdonald et al. 2009) badger population, a hormone profile indicative of a single distinct peak in reproductive receptivity (Hadley 2000) was detected. This contrasts with the second late summer oestrus detected in Cresswell et al.'s (1992) medium density (25.3 badgers/km^2) study, and Service et al.'s (2002) results from urinary sex-steroid analyses showing multiple cycles throughout the summer months at low density (4.4-7.5 badgers/km^2) populations of badgers in England. It is also contrary to Corner *et al.*'s (2015) report of different stages of embryonic development present simultaneously in the same female throughout the year, indicative of multiple mating periods at low density (1.9 badgers/km^2) in Ireland.

Convenience polyandry (Lee and Hays 2004; Rowe 1992) has been suggested as the most likely explanation for the observed badger mating patterns (Annavi et al. 2014a, b), where modelling predicts that, if multiple matings result in females gaining enhanced fecundity but suffering higher mortality, females re-mate more willingly when population density is low (Härdling and Kaitala 2005). Consequently, unless resisting harassment is more costly than accepting matings, (and female badgers cannot be coerced into mating by males: Dugdale et al. 2011c), selection should favour females exhibiting strategies to resist multiple matings and female mating strategies can be expected to differ with population density (Kokko and Rankin 2006): In low-density populations, female badgers are unlikely to encounter a large number of potential mates during a single oestrous period, and indeed, Corner et al. (2015) posit that the breeding rate in their low density Irish population may be limited by failure to conceive, rather than failure at any other stages of the breeding cycle. Thus, under these conditions, the DI*SF strategy likely not only facilitates that females can secure (multiple) matings – ensuring fertilization, but also enables a degree of cryptic mate choice by providing potential for selective implantation of only the 'most suitable' blastocysts (Anderson and Simmons 2006).

Conversely, at very high densities, coitus is frequent (Dugdale et al. 2011a), and fertilisation will be assured (Anderson and Simmons 2006). Under these circumstances, further oestrus

cycles may not be advantageous due to a risk of life-threatening endometriosis (reported in mustelids after failure to mate: Newman and Byrne 2017), increased risk of contracting sexually transmitted diseases (e.g. genital herpes: Kent et al. 2018) during superfluous matings, additional risks associated with increased roaming behaviour typical for the mating season (Macdonald et al. 2010), as well as increased intra-specific aggression and bite wounding (Delahay et al. 2006).

Particularly in promiscuous mating systems, population- (and thus mate-) density must be taken into account when investigating physiological and behavioural processes on a population-level that shape mating systems (Kokko and Rankin, 2006), because mate competition is unlikely to be fundamentally different from other forms of intra-specific resource competion (Emlen and Oring 1977).

It is postulated that the observed endocrinological flexibility of female badgers enables ARTs (Taborsky et al. 2008) that may represent a form of balancing selection, as densities fluctuate over time as well as spatially, thus opposing evolutionary canalisation (Siegal and Bergmann 2002). Although the DI and SF strategies have evolved for different reasons (Yamaguchi et al. 2006), the resulting 'pre-adaptedness' (or exaptation: Gould and Vrba 1982) from the DI*SF combination provide the European badger with an evolutionarily distinctive physiological mechanism to thrive in a wide variety of geographically and environmentally distinct habitats at carrying capacity. By adapting its social system (Johnson et al. 2000) as well as its reproductive physiology, badgers can achieve maximal fitness across a continuum of socio-ecological conditions without incurring sub-optimal fitness at either extreme.

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RESEARCH CHAPTER 2

Linking plasma sex steroid hormone levels to the condition of external genitalia morphology (EGM) in European badgers (*Meles meles*): A critical evaluation of traditional field methodology

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ABSTRACT

Field biologists often rely on examination of external genitalia as a proxy for mammalian reproductive condition. In seasonally breeding European badgers, scrotal testes are used as an indicator of spermatogenesis, while a pink and swollen vulva with mucosal secretion is assumed to indicate oestrus. Systematic validation of these assumptions is lacking. Here, sex steroid hormones were analysed from plasma samples collected from sexually mature adults during the winter mating season, the spring post-weaning period, the summer minor mating peak, and autumn reproductive quiescence, to compare circulating testosterone levels in males (n=216), and oestrone (n=143) and oestradiol (n=36) levels in females with concurrent external genitalia condition (males: descended, intermediate, ascended testes, as well as testis volume; females: swollen, intermediate, normal vulva). Sex-steroid levels exhibited seasonal patterns, broadly reflecting the seasonal variation in genital condition. Nevertheless, on individual level, male testosterone levels correlated significantly with testes condition and testis volume only during the mating season in spring, and neither oestrone nor oestradiol differed between female EGM categories. It is thus concluded that, in males, EGM is a reliable indicator of reproductive activity during the mating season, but can provide only a tentative indication of physiological reproductive status outside this period, while in females EGM is generally less precise for assessing female reproductive status.

Keywords: *European badger, external genitalia condition, oestrogens, reproductive status, testosterone*

INTRODUCTION

Accurate assessment of an animal's reproductive status is fundamental to many biological questions. Sex steroid hormone measures from plasma or serum are arguably the most reliable indicator of reproductive activity (Adkins-Regan 2005) and have been validated and used in a wide variety of mammals (Hildebrant et al. 2006; Brown et al. 2010; O'Brien and Robeck 2012; Pukazhenthi et al. 2013). Sex-steroids are produced mainly by the gonads (ovaries and testes) and are secreted directly into the bloodstream to bind to specific receptors that will contribute to all aspects of reproductive activity (e.g., development of reproductive organs, reproductive activity/ oestrus, as well as reproductive behaviour: Adkins-Regan 2005). Their metabolites are then excreted either in urine (e.g., testosterone and dihydrotestosterone in male African elephants *Loxodonta africana*: Lasley and Kirkpatrick 1991) or faeces (e.g., progestagens in female black rhinoceros *Diceros bicornis minor*: Garnier et al. 1998; androgens in male white rhinoceros *Ceratotherium simum simum*: Kretzschmar et al. 2004). Nevertheless, hormone assays, particularly if based on non-invasive samples such as urine and faeces (Lasley and Kirkpatrick 1991), are often species-specific and need careful validation (Möhle et al. 2002), whereas the more universal blood-based assays require the animal to be sufficiently restrained (often through sedation) for blood sampling, typically entailing a multitude of licensing as well as welfare considerations. In addition, samples need to be fresh and frozen immediately to prevent decay (Brown et al. 2010; O'Brien and Robeck 2012). Thus, the associated costs, logistics, and lab requirements of endocrinological assays make them unsuitable for many field applications (Khan et al. 2002; Haarsma 2008). Field biologists therefore often rely on examining the morphological condition of mammalian external genitalia (henceforth "external genitalia morphology"; EGM) as a proxy for reproductive condition (Johnson 2013; Pagel 1994).

In many female mammals, an increase in oestrogen, particularly follicular oestradiol levels, during the peri-ovulatory period causes vulval swelling and/or reddening as well as mucosal secretion (e.g. primates: Dixon 1998). Thus, a swollen and pink vulva is often used as an indicator of oestrus in a wide variety of species (e.g., giant panda (*Ailuropoda melanolauca*): Durrant et al. 2003; dairy cattle (*Bos taurus, Bos indicus*): Rae et al. 1999; yak (*Bos grunniens*): Yu et al. 1993; steppe pole cat (*Mustela eversmanni*): Mead et al. 1990; chimpanzee (*Pan troglodytes*): Emery and Whitten 2003; peccary (*Tayassu tajaru*): Mayor et

al. 2007; buffalo (*Bubalus bubalis*): Drost 2007; river otters (*Lontra canadensis*): Crait et al. 2006), but experimental evidence that females ovulate and/ or can conceive only during the time of vulval swelling is rare (Reichert et al. 2002; Emery and Whitten 2003).

In males, testes size and shape are generally assumed to reflect testosterone levels (Klonisch et al. 2006). The testes of most mammals are located outside the body cavity in the scrotum, where below-bodycore temperatures provide a suitable environment for spermatogenesis (Hansen 2009). Particularly in seasonal breeders, where the testes are often retracted into the abdominal cavity, males with scrotal testes are typically categorised as "in reproductive condition", whereas ascended testes are assumed to indicate reproductive quiescence (e.g. Massachusetts small rodents (*C. gapperi, M. breweri, M. pennsylvanicus, P. manculatus, S. hispidus*): McCravy and Rose 1992; black bear (*Ursus americanus*): Garshelis and Hellgren 1994; European sousliks (*Spermophilus citellus*): Millesi et al. 2004; river otter (*Lontra canadensis*): Crait et al. 2006; Asiatic mouflan (*Ovis orientalis*): Lincoln 1989). Because testes size and weight increase as the diameter of the seminiferous tubules that produce spermatogenic cells enlarges (Klonisch et al. 2006), in some mammals, spermatogenesis is not only indicated by descended testes, but also by a noticably distended cauda epididymis and enlarged testes volume (brown long-eared bat (*Chiroptera vespertilionidae*): Entwistle et al. 1998), although in many species the immediate link between testes morphology, size and reproductive activity has not been proven experimentally (Lincoln and Davidson 1977; Preston et al. 2012; Ramaswamy and Weinbauer 2014).

While EGM records are non-invasive and easy to collect during the time of capture (and thus provide the field biologist with an immediate indicator of the focal animal's apparent reproductive condition), EGM changes gradually and might thus not reflect current reproductive activity accurately (esp. sudden short-term events such as ovulation: Reichert et al. 2002; Emery and Whitten 2003 or the onset of spermatogenesis: Lincoln and Davidson 1977; Preston et al. 2012). In contrast, while hormone levels may also fluctuate due to factors other than reproduction (e.g. circadian rhythm: Irvine et al. 1994; social encounters, stress, health status: Adkins-Regan 2005), at least during the mating season reproductively active mammals exhibit sex steroid profiles that are clearly distinctive from those in other seasons (Garnier et al. 2002; Lynch et al. 2002; Lanyon et al. 2005).

Despite the wide-spread use of genital morphology to indicate male and female reproductive condition in mammalian field research, its degree of precision in reflecting specific reproductive stages generally remains unresolved, and in those studies where EGM was compared to sex steroid titres, the two methods often yielded different results (Preston et al. 2012; Reichert et al. 2002). For instance, in the female bonobo (*Pan Paniscus*), vulval swelling did not allow researchers to pinpoint the timing of ovulation, with 33% of ovulations occurring several days after the maximum swelling during vulval deflation (Reichert et al. 2002). Similarly, in male Soay sheep (*Ovis aries*), testicular diameter increased weeks or even months prior to mating and exhibited a stronger correlation with testosterone levels during this period compared to the actual mating period (Lincoln and Davidson 1977), while no correlation was found between testosterone level and testes size outside of the mating season (Preston et al. 2012) with similar asynchrony of EGM and circulating sex-steroid profiles being encountered in rodents (McCravy and Rose 1992).

Here, the relationship between circulating plasma sex-steroid hormones with the condition of the external genitalia of wild-living male and female European badgers (*Meles meles*; henceforth "badger") were investigated. Like most mustelids (Amstislavsky and Ternovskaya 2000), badgers are seasonal breeders and produce one litter of 1-5 cubs annually, born between mid-January – mid-March (76% of cubs in the UK are born in mid-February: Fell et al. 2006; Yamaguchi et al. 2006). Their main mating season is restricted to a post-partum oestrus in January-March (Yamaguchi et al. 2006) with some studies reporting a secondary mating peak in August/ September (Cresswell et al. 1992), several oestrus cycles over the summer months (Service et al. 2002), or even evidence for (successful) matings throughout the year (Corner et al. 2015; reviewed in Research Chapter 1). After fertilisation, females undergo delayed implantation with blastocysts remaining in suspended animation for up to 330 days (Yamaguchi et al. 2006) until implantation is triggered by a combination of Luteinizing Hormone (LH) and prolactin increase in mid-December. Post-implantation pregnancy is 47-51 days (Corner et al. 2015).

All male badgers have elevated testosterone levels and scrotal testes during the main mating season between January and May, but while some individuals retract their testes and decrease their testosterone levels in early/ mid September, others maintain elevated testosterone levels (i.e., comparable to those during the main mating season) and retract their testes weeks later at the end of October (Buesching et al. 2009; Woodroffe and Macdonald 1995). In females,

vulva condition varies during the reproductive season with most exhibiting a distinctly swollen, pink and everted vulva predominantly in late winter/ spring (Tinnesand et al. 2015), when average oestrone and oestradiol levels are highest (Research Chapter 1), although some females can display vulval swelling also at other times throughout the summer/autumn while oestrone levels remain elevated (Research Chapter 1). Thus, in the field, scrotal testes and a swollen and everted vulva are often used as proxies for reproductive activity (e.g. Johnson 2013; Pagel 1994).

This research was conducted: i) to analyse the seasonal profiles in EGM (males: testis volume and degree of testicular descent; females: degree of vulval swelling) and sex-steroid hormones (males: testosterone; females: oestrone and oestradiol); and ii) to test the hypothesis that higher sex-steroid-levels are reflected in EGM on the individual level. The results were discussed in the context of the utility of employing these frequently used field methods to infer female oestrus and male reproductive activity in European badgers and other seasonal breeders.

MATERIALS AND METHODS

Badger trapping and sampling

Data were collected from a high-density badger population in Wytham Woods, Oxfordshire, UK (51°46'26" N, 1°19'19" W; for details see Macdonald et al., 2015) between 1987-2016, as part of an ongoing long-term research project. Following the methodology described in Macdonald and Newman (2002; 2009), badgers were trapped three to four times annually: (1) in *winter* (January) at the start of the main mating season for males and females and first term of post-implantation pregnancy in reproducing females, (2) in *spring* (May/June) during the post-weaning period for females and at the end of the main mating period, (3) in *summer* (August/September) during lowest food abundance and second mating peak previously reported in other badger populations, and (4) in *autumn* (November) during reproductive quiescence. Traps were checked between 6.30-8.00 am, and captured animals were transported to a central field station and sedated (typically starting no later than 8 am) by intramuscular injection of 0.2 ml ketamine hydrochloride/ kg body weight (McLaren et al. 2005; Thornton et al. 2005), where induction time was between 3-4 minutes. A typical

procedure (from initial sedation to completion of data collection and handling) required 10-15 minutes per badger (Sun et al. 2015) with proccesing of all captured badgers completed before noon. All badgers received a permanent unique tattoo at first capture (typically as cubs or yearlings as identifiable by toothwear: Harris et al. 1992) allowing individual identification (ID) and accurate aging thereafter.

Classification of EGM

External genitalia were examined in adults between 2-10 years of age ($n_{females}$= 3237; n_{males}= 2569 with testis volume measured in n= 1599) (see Table 1). Cubs and yearlings were excluded to ensure sexual maturity, and very old individuals were excluded because there is evidence that reproductive success diminishes with senescence (Buesching et al. 2009; Carpenter et al. 2005; Dugdale et al. 2011). Female vulva condition was categorised as swollen (pink, swollen and everted with moist mucous membranes), intermediate (skin-coloured, slightly swollen, and dry or moist), or normal (skin-coloured, non-swollen/ flat and dry; Tinnesand et al. 2015). The degree of testes descent from the body cavity into the scrotum was categorised as fully decended (testes clearly discernable by visual inspection separated by the scrotal septum and mobile in scrotal sack), intermediate (testes slightly ascended and restricted mobility) or fully ascended (testes not visible, fully retracted into the body cavity; Buesching et al. 2009; Woodroffe and Macdonald 1995). For scrotal and intermediate testes (excluding individuals with ascended testes, as measurements were not possible), the length and width of the left testicle was measured (to the nearest mm) with a pair of callipers while the sedated badger was positioned on its back, and the thickness of the scrotal sack (to the nearest 0.5 mm) measured by gently pinching the skin between the two testicles between the calliper jaws. As testis height cannot be measured reliably in live animals, and our post-mortem examinations (Buesching et al. unpubl. data) indicated that badger testicles are cylindrical in shape, testicular width was used as a proxy for height. Testicular volume was thus calculated using the following equation (Paltiel et al. 2002): Testis volume (mm^3) = (L x W x H) x 0.71, where L= testicle length – scrotal pinch, W= testicle width – scrotal pinch, and H= testicle width – scrotal pinch.

Blood sampling and hormone measurements

For endocrinological analyses, blood samples (n_{males}= 216; $n_{females}$= 179; see Table 1, selected at random from the available data set to represent key reproductive stages) were collected by jugular venepuncture in K2-EDTA (ethylene diamine tetraacetic acid) vaccutainer tubes

(Becton-Dickinson). Blood samples were centrifuged at 10°C for 10 min under 2,500 rpm/ 1470G within 30 minutes of collection. Plasma was transferred into Eppendorf tubes and frozen at -20°C immediately. Sampling times were standardized to samples collected between 8.30am and 10.30am to account for circadian variation in hormonal profiles (Buesching et al. 2009).

All sex steroid titres were analysed using microtitreplate Enzyme-immunoassays (EIA). Oestrone (n=143) and oestradiol (n=36) were measured using the following methodology (Research Chapter 1, Appendix 1): Oestrone was quantified from un-extracted plasma samples after 1:10 dilution in assay buffer in microtitreplates coated with polyclonal antiserum raised against oestrone EC R522 (Munro 1991). Duplicate 20µl aliquots of diluted plasma, oestrone standard (0.195-200 pg/well) and quality controls were combined with 50µl oestrone glucuronide coupled to horseradish peroxidase (oestrone-glucuronide-HRP) used as label, and incubated for 2 hours at room temperature. Plates were then washed five times, blotted dry, and an addition of 100 µL peroxidase substrate solution (ABTS) was added to each well. Afterwards, plates were covered, incubated at room temperature until the '0' wells reached about 1.0 optical density and read at 405 nm using a Spectrophotometer Opsys MR (Dynex). At 90% binding, assay sensitivity was 3.1 pg. Intra-assay coefficients of variation of high and low-value quality controls were 8.2 % (high) and 6.1 % (low); inter-assay variation of high and low-value quality controls were 14.0 % (high) and 13.6 % (low), respectively.

For oestradiol measurements, plasma samples (250 µl) were extracted twice with 10 volumes of diethylether by vortexing for 10 minutes. After separation of the ether- aqueous phases by snap-freezing with methanol/dry ice, the two extracts were combined, evaporated until dry, and reassembled in assay buffer (250 µl; Buesching et al. 2009). Oestradiol measurements was conducted using an antiserum raised against oestradiol-17-HS-BSA (Meyer et al. 1990) together with oestradiol coupled to horseradish peroxidase (oestradiol-HRP) as label. Label (50µl) and antiserum (50µl) were combined with duplicate 50 µl aliquots of oestradiol standards (0.24-62.5 pg/well), sample extract and quality controls, then mixed thoroughly and incubated overnight at 4^{o}C. Plates were washed 4 times and blotted dry after incubation. Plates were incubated for a further 1-1.5 hours after peroxidase substrate solution (TMB; 150µl) was added to each well. Absorbance measurement at 450 nm was done using a spectrophotometer (BioTek Instruments EL 808). At 90% binding, sensitivity of the

oestradiol assay was 0.4 pg. Intra- and inter-assay coefficients of variation were 5.9 % (high) and 6.4 % (low) and 4.5 % (high) and 7.4 % (low) respectively.

Testosterone analyses were carried out using two different methodologies: 50 samples were analysed following the methodology described in Buesching et al. (2009) where samples (150µl) were extracted twice with diethylether (10 volumes) by 10 min of vortexing prior to analyses. The ether and aquaeous phases were then separated by snap-freezing in methanol and dry ice, followed by a combination and evaporation (to dryness) of both extracts, and reconstitution in assay buffer (150µl). Sample extracts were diluted 1:2 – 1:100 (depending on season) with EIA buffer. Duplicate 50µl aliquots of testosterone standards (0.31-40 pg/well), samples and quality controls were combined in plate wells coated with antiserum raised against testosterone 3-CMO-BSA with 50µl horseradish peroxide (testosterone-HRP) as label (Palme and Möstl 1994) . Plates were incubated overnight at 4^{o}C, washed 4 times and blotted dry, followed by an addition of substrate 150 µL/well. Plates were then further incubated by shaking in the dark at room temperature for 90 minutes, then read at 450 nm on spectrophotometer. Assay sensitivity at 90% binding was 0.3 pg. Intra-assay coefficient of variation was 6.1 % (high) and 7.6 % (low), and inter-assay variation of high and low-value quality controls were 9.1 % (high) and 11.8 % (low). The remaining 166 samples were analysed by diluting them 1:4 with assay buffer. Duplicate 50µl aliquots of testosterone standards (2.3-600 pg/well), samples and quality controls were then combined with 50µl horseradish peroxidase (testosterone-HRP) as label in plate wells coated with anti-testosterone R156/7 (OEM-Concepts, UK). After incubation at room temperature in the dark for 2 hours, the plates were washed 5 times and blotted dry, followed by an addition of HRP-substrate (100 µL) to each well. Plates were then covered, incubated at room temperature until the '0' wells reached 1.0 optical density and were then read at 405 nm using a Spectrophotometer (Opsys MR; Dynex). Assay sensitivity at 90% binding was 1.6 pg. Intra-assay coefficients of variation of high and low-value quality controls were 14.7 % (high) and 6.2 % (low), and inter-assay variations were 9.2 % (high) and 5.2 % (low). Although measurements from both testosterone assays were within the same range, data rescaling/ standardization was conducted using STD of all samples to fit all data points into analyses.

Statistical analysis

Seasonal profiles of EGM and sex steroid hormones

Seasonal variation in EGM profiles was analysed with chi-quare tests. Season (spring, summer, autumn, winter) was used as a factor against testes status (descended, intermediate, ascended) in males, and against vulva status (swollen, intermediate, normal) in females. The seasonal variation in testis volume was investigated using a linear mixed model (including ID as a random effect, assumption tests were conducted beforehand), followed by a-priori contrast tests when results were statistically significant at $p<0.05$. Testis volume measures were log10 transformed for best model fit. Average seasonal hormone levels (testosterone, oestrone, and oestradiol) with standard deviations were calculated across animals.

Within-individual correlation of EGM and sex-steroid titres

To investigate how testosterone levels correlated with testes condition, a linear mixed model was conducted using testosterone levels as response with testes condition as predictor and season as covariate, as well as including badger ID/tattoo number as random effect. Testosterone level was log10 transformed for best fit: lmer(log10(Testosterone) ~ Testes condition + Season + (1|ID). When significance was established, an a priori test was conducted. Interaction between testes condition and season was intended to be included in the model (to test whether testes condition only correlated with testosterone levels at certain times of the year), but the number of paired data points for testes condition and testosterone level per season was not large enough for this analyses. Thus, the significance of EGM (if any) for specific seasons could not be assessed. Instead, this relationship was visualized by plotting EGM as integers (Ascended=1, Intermediate=2, Descended=3) vs hormone levels and fitting a trend line for each season.

Testosterone levels were also analysed against testis volume as predictor, season as covariate, as well as including the interaction between testis volume and season using a linear mixed model (ID as random effect). Testosterone levels were log10 transformed for best fit: lmer(log10(Testosterone) ~ Testis volume + Season + Testis volume*Season + (1|ID). As an a priori test, the same analyses as described above were conducted using a restricted data set including spring only as this was the main mating season when >98% (67/68) of males had fully descended testes (linear regression as within this season individuals were independent).

For females, a linear mixed model was carried out to investigate the correlation between oestrone levels and vulva condition, using oestrone levels as response with vulva condition as predictor, season as covariate, vulva condition-season interaction, and ID as random effect: lmer(Oestrone ~ Vulva condition + Season + Vulva condition*Season + (1|ID). To account for the effect of pregnancy status, a further analysis using data from winter only (i.e., the time of pregnancy) was conducted. Oestrone levels were used as response while vulva condition and pregnancy status were used as predictors (linear regression, as there were no multiple samples per individual in this winter dataset): lm(Oestrone ~ Vulva condition + Pregnancy status).

To investigate the correlation between oestradiol and vulva condition, an analysis with oestradiol levels as response, vulva condition as predictor, and season as covariate was conducted using a linear model, where oestradiol levels were log10 transformed for best fit (as there were no multiple measures per individual): lm(log10(Oestradiol) ~ Vulva condition + Season). Interaction between vulva condition and season was not included in the model due to limited vulva condition and oestradiol data pairs in each season. Effects of pregnancy status were not investigated, as within this oestradiol dataset, all females in winter were pregnant.

Patterns of residuals, normality, and mean variance relationship for each model were checked using diagnostic plots in R. All statistical analyses were performed using RStudio (0.99.896; RStudio Team 2015) and R (R-3.2.4; R Core Team 2016).

Table 1. Number of samples per season and external genitalia condition used for testosterone, oestrone, and oestradiol measurements.

Season	Sample size for testes conditions and testosterone measurements					
	Descended		**Ascended**		**Intermediate**	
	Samp.	Pop.	Samp.	Pop.	Samp.	Pop.
Spring	67	668	-	45	1	65
Summer	80	619	1	97	8	161
Autumn	9	92	33	479	7	117
Winter	7	111	-	57	2	58
Total	163	1490	34	678	18	401
Total Hormone Measurement	163 + 34 + 18 = 215					
Total Testes Condition Observation	1490 + 678 + 401 = 2569					
Total Testis Volume Measurement	1599					
	Sample size for vulva conditions and oestrone measurements					
	Swollen		**Intermediate**		**Normal**	
	Samp.	Pop.	Samp.	Pop.	Samp.	Pop.
Spring	**19**	396	**18**	469	**17**	223
Summer	**8**	236	**10**	468	**23**	464
Autumn	**1**	52	**3**	181	**26**	606
Winter	**7**	44	**2**	47	**7**	51
Winter (preg.)	5	-	2	-	6	-
Winter (not-preg.)	2	-	-	-	1	-
Total	35	728	33	1165	73	1344
Total Hormone Measurement	35 + 33 + 73 = 141					
Total Vulva Condition Observation	728 + 1165 + 1344 = 3237					
	Sample size for vulva conditions and oestradiol measurements					
	Swollen		**Intermediate**		**Normal**	
	Samp.	Pop.	Samp.	Pop.	Samp.	Pop.
Spring	**4**	396	**8**	469	**1**	223
Summer	**2**	236	**2**	468	**4**	464
Autumn	-	52	**2**	181	**6**	606
Winter	**2**	44	**2**	47	**2**	51
Winter (preg.)	2	-	2	-	2	-
Winter (not-preg.)	-	-	-	-	-	-
Total	8	728	14	1165	13	1344
Total Hormone Measurement	8+ 14 + 13 = 35					
Total Vulva Condition Observation	728 + 1165 + 1344 = 3237					

*Note: From 216 males samples that were measured for testosterone, 215 have information on testes conditions (used for hormone agst. testes condition analyses) and 158 have information on testis volume (used for hormone agst. testis volume analyses). Some individuals contributed more than 1 data point. From 179 female samples, 143 were analysed for oestrone (hormone agst. season analyses); 141 have information on vulva condition (used in both oestrone level agst. vulva condition and integrated linier mixed model). Some individuals contributed more than 1 data point. For oestradiol, there were 36 samples and were all independent (used for oestradiol level agst. season analyses), only 35 have concurrent information on vulva condition (used in both oestradiol level agst. vulva condition analyses and integrated linier mix model).

RESULTS

Seasonal profiles of EGM and sex steroid hormones

There was a significant seasonal effect on the degree of testes descent (X^2= 153.12, df=6, p < 0.001; Fig. 1) and vulva condition (X^2=64.364, df=6, p < 0.001; Fig. 2). In autumn, during reproductive quiescence, the majority of males had ascended testes (13.37% descended, 17.01% intermediate, 69.62% ascended; n=688), with winter being a transitional period (49.12% descended, 25.66% intermediate, 25.22% ascended; n=226), and most males having scrotal testes in spring (85.86% descended, 8.35% intermediate, 5.78% ascended; n=778), and summer (70.58% descended, 18.35% intermediate, 11.06% ascended; n=877).

This pattern was similar in females, where during autumn quiescence (n=839), the majority of females had normal vulvas (72.22%; 21.57% = intermediate, 6.19% = swollen), and the proportion of females with a swollen vulva was highest during the mating season in winter and spring (winter (n=142), 30.99% = swollen, 33.09% = intermediate, 35.92% = normal; spring (n=1088): 36.39% = swollen, 43.11% = intermediate, 20.49% = normal; summer (n=1168), (20.21% = swollen, 40.07% = intermediate, 39.73% = normal).

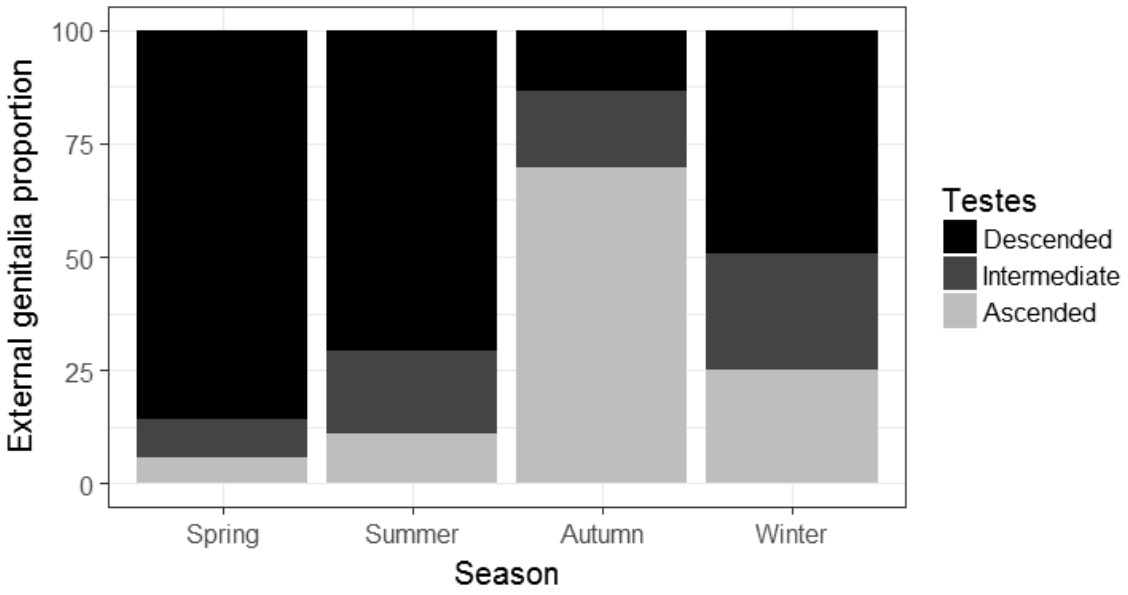


Figure 1. Proportion (%) of testes conditions (descended, intermediate, ascended) throughout seasons (spring, summer, autumn and winter) in males.

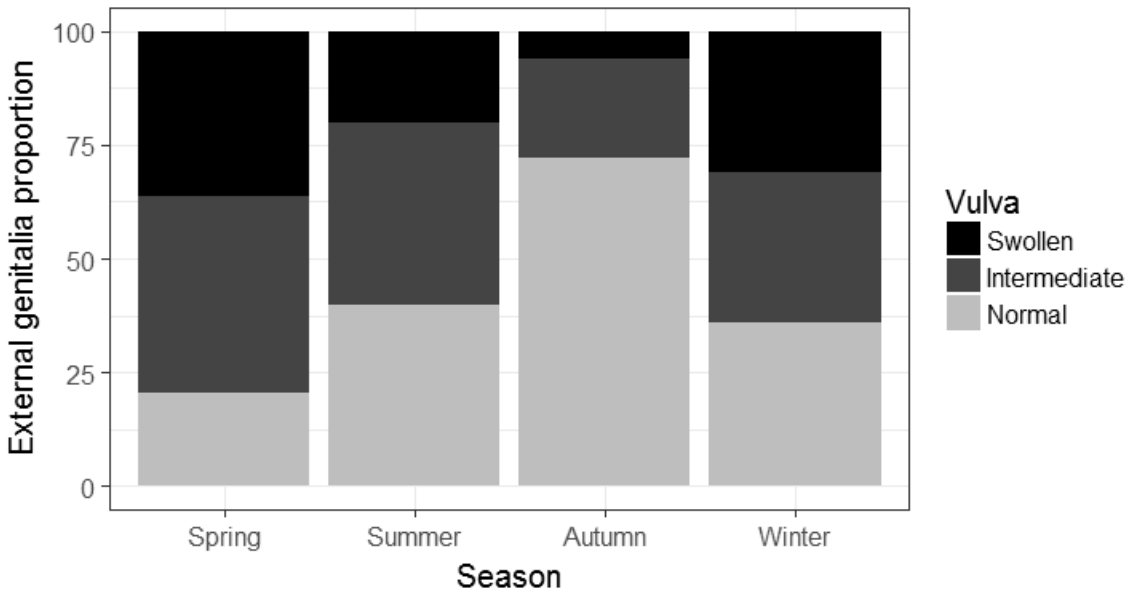


Figure 2. Proportion (%) of vulva conditions (swollen, intermediate and normal) throughout seasons (spring, summer, autumn and winter) in females.

Testis volume (in mm^3, Fig. 3) differed significantly between seasons (X^2=110.66, df=3, p<0.001). Volume was largest in winter (6650.82 ± 3039.76, n=131, X^2 = 27.74, df=1, p< 0.001), remained large in spring (5776.31 ± 2390.58, n=680, X^2=37.48, df=1, p<0.001), decreased in summer (5012.31 ± 2059.78, n=719, X^2=40.46, df=1, p<0.001), and was smallest in autumn (3816.24 ± 1500.04, n=69, X^2=41.37, df=1, p<0.001).

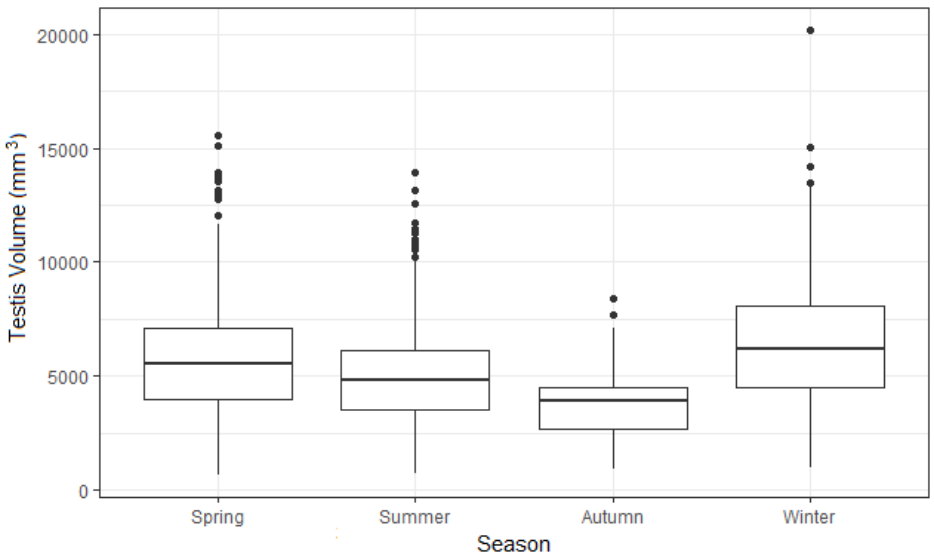


Figure 3. Testis volume (mm^3) througout seasons (spring, summer, autumn and winter) in males.

Average sex-steroid levels also showed distinct seasonal patterns: In males, average seasonal testosterone levels (ng/ml) varied significantly with season (X^2=74.17, df=3, p<0.001; spring: 1.66±1.32, n=71; summer: 1.07±0.91, n=83; autumn: 0.68±0.64, n=49; winter: 4.83±4.14, n=13). In females, average oestrone (X^2=52.22, df=5, p<0.001) and oestradiol levels ($F_{4,31}$=2.574, p=0.057) differed significantly among seasons. Oestrone reached titres (pg/ml) of 73.28±28.06 (n=54) in spring, 39.54±23.16 (n=41) in summer, 81.42±22.91 (n=30) in autumn, 80.81±43.06 (n=13) for pregnant females and 34.9±35.26 (n=5) for not-pregnant females in winter. Average oestradiol levels (pg/ml) reached 117.85±137.08 (n=13) in spring, 26.85±10.01 (n=8) in summer, 44±18.37 (n=9) in autumn, and 34.93±12.17 (n=6) during winter pregnancy.

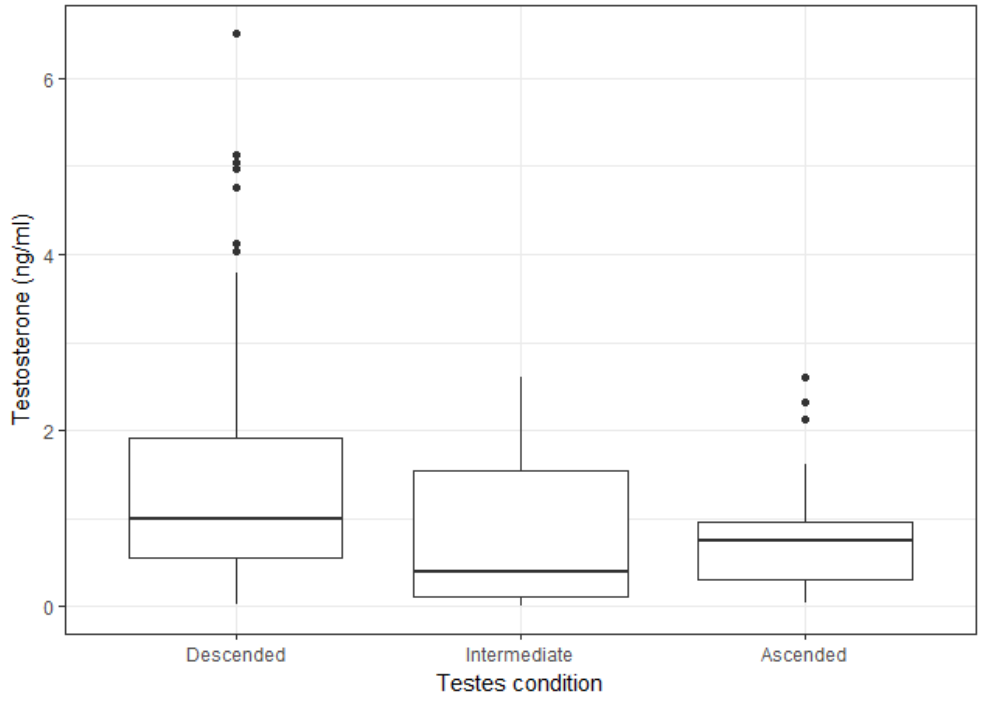
Within-individual correlation of EGM and sex-steroid titres

Males

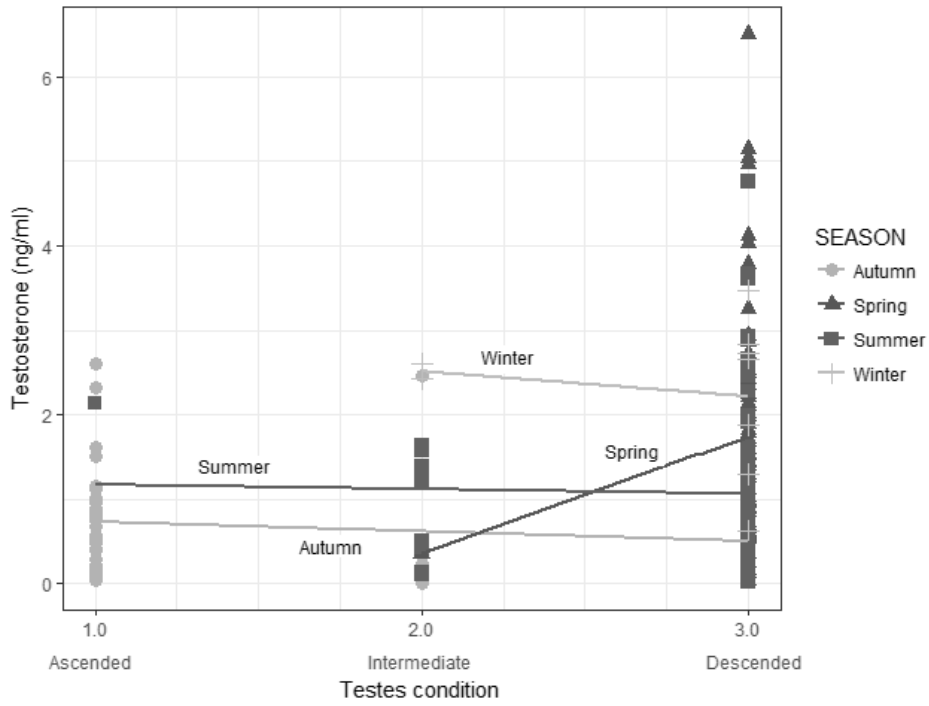
Testes condition ($X^2_{\text{testes condition}}$= 13.221, df=2, p=0.001; descended: 1.37±1.16, n=163; intermediate: 0.87±0.92, n=18 ascended: 0.79±0.63, n=34; Fig. 4a) and season (X^2_{season}= 40.953, df=3, p<0.001) proved significant predictors of testosterone levels (Table 2) where males with descended testes had significantly higher testosterone levels compared to those with ascended (X^2= 10.451, df=1, p=0.001) or intermediate (X^2= 16.999, df=1, p<0.001) testes condition. No differences in testosterone levels was found between ascended and intermediate testes (X^2= 0.092, df=1, p= 0.762; a priori contrast test). When plotting EGM as integers against testosterone levels for each season a distinct (positive) correlation was only apparent in spring (Fig.4b).

Table 2. Mixed model analysis results of testosterone levels against testes condition, with season as covariate.

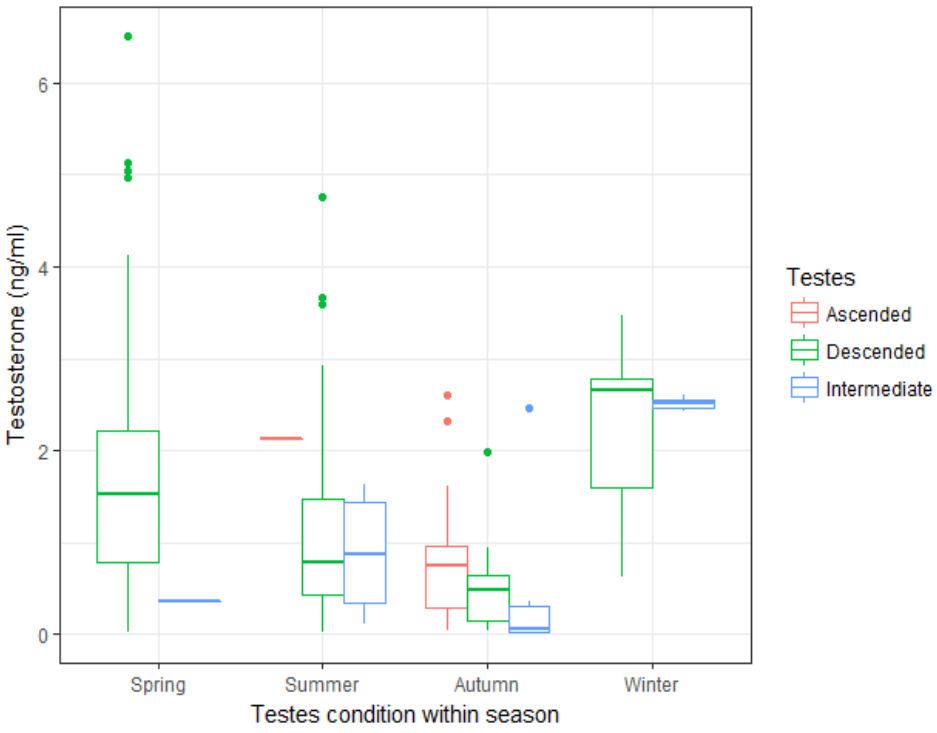
Testes condition term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	P
model 2	6	271.060	291.290	-129.530	259.060			
model 1	8	261.840	288.810	-122.920	245.840	13.221	2	0.001
Season term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	P
model 3	5	296.800	313.650	-143.400	286.800			
model 1	8	261.840	288.810	-122.920	245.840	40.953	3	<0.001
model 1: log10(Testosterone) ~ Testes condition + Season + (1 | Tattoo)								
model 2: log10(Testosterone) ~ Season + (1 | Tattoo)								
model 3: log10(Testosterone) ~ Testes condition + (1 | Tattoo)								



(a)



(b)



(c)

Figure 4. Testosterone levels (ng/ml) of males exhibiting different testes conditions (descended, intermediate and ascended) (a), visualisation of testosterone levels (ng/ml) against testes condition (as integer: Ascended=1, Intermediate=2, Descended=3) for each season (spring, summer, autumn, winter) in males (b) and breakdown of testosterone levels against testes condition for each season (c).

Although testosterone levels were significantly correlated with testis volume during the spring mating season ($F_{1,59}$=5.003, p=0.029, n=61, a priori test, Table 3; Fig. 5), when testosterone levels were tested against testis volume over the whole year (n=158), only season proved a significant effect (X^2_{season}=29.042, df=3, p<0.001), while testis volume ($X^2_{testis.volume}$=2.299, df=1, p=0.130) and the interaction term were not ($X^2_{interaction}$=3.669, df=3, p=0.300, Table 3, Fig. 5)

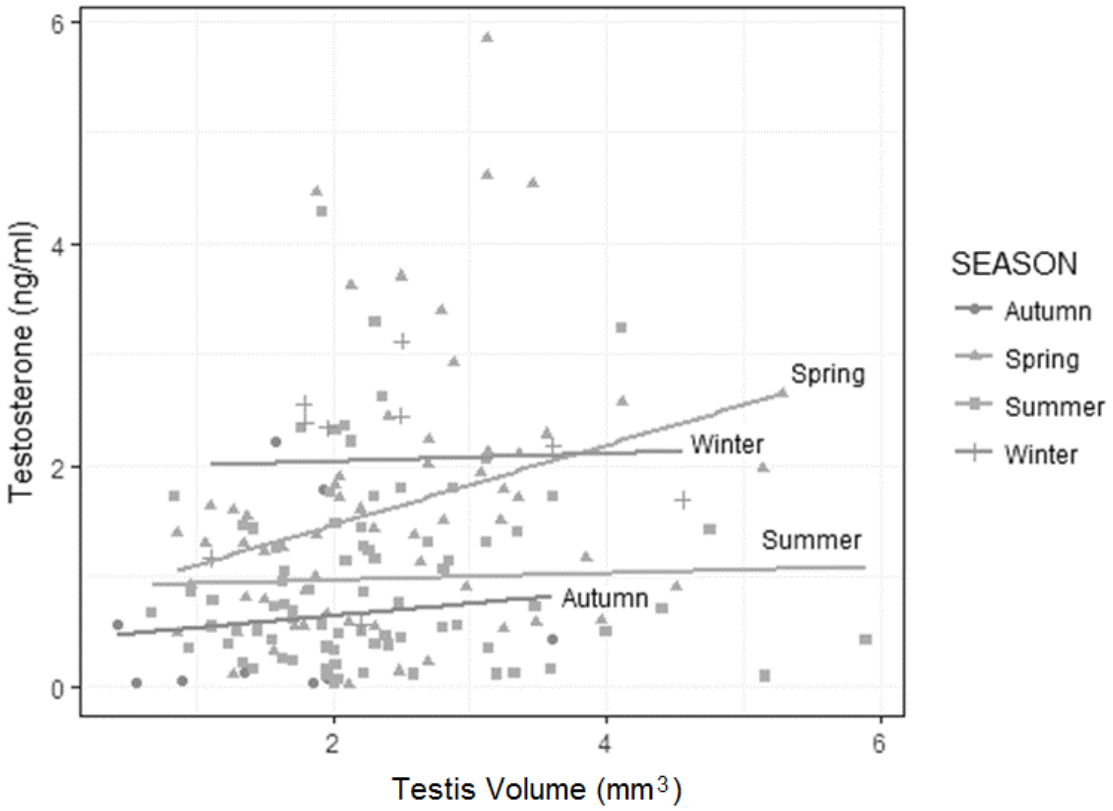


Figure 5.Testosterone levels (ng/ml) versus testis volume (mm^3) for each season

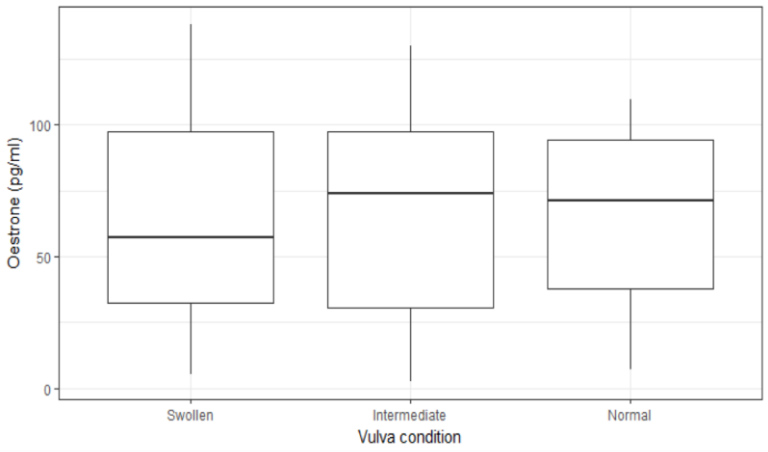
Table 3. Mixed model analyses of testosterone levels against testis volume, with season as covariate; similar model with data restricted to only spring.

Testis volume term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	p
model 2	6	191.500	209.870	-89.748	179.500			
model 1	7	191.200	212.640	-88.599	177.200	2.299	1	0.130
Season term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	p
model 3	4	214.240	226.490	-103.120	206.240			
model 1	7	191.200	212.640	-88.599	177.200	29.042	3	<0.001
Interaction term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	p
model 4	7	191.200	212.640	-88.599	177.200			
model 1	10	193.530	224.150	-86.764	173.530	3.669	3	0.300
model 1: log10(Testosterone) ~ Testis volume + Season + (1 | Tattoo)								
model 2: log10(Testosterone) ~ Season + (1 | Tattoo)								
model 3: log10(Testosterone) ~ Testis volume + (1 | Tattoo)								
model 4: log10(Testosterone) ~ Testis volume + Season + Testis volume* Season + (1 | Tattoo)								
Spring only model coefficients								
	Estimate		SE		t		p	
(Intercept)	-0.286		0.133		-2.146		0.036	
Testis volume	0.114		0.051		2.237		0.029	
Spring only significance of terms								
	Df	Sum Sq		Mean Sq		F	p	
Testis volume	1	0.780		0.780		5.003	0.029	
Residuals	59	9.196		0.156				

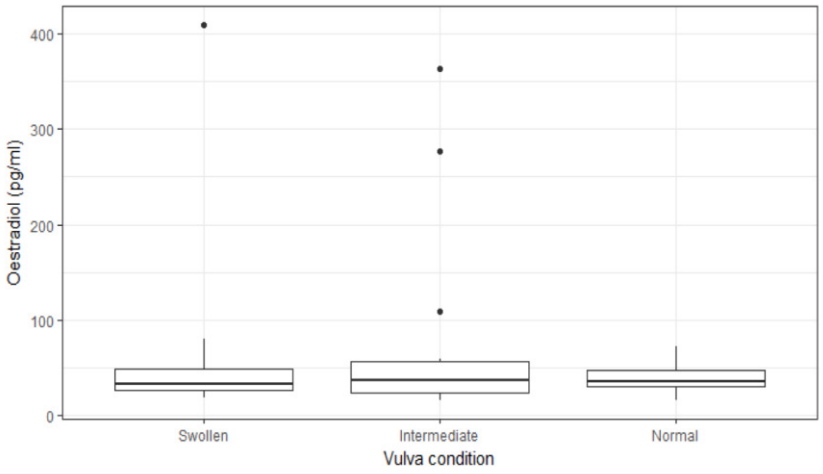
Females

Season was the only significant predictor of oestrone levels (X^2_{season}=41.724, df=3, p<0.001), while neither vulva condition ($X^2_{vulva\ condition}$=1.094, df=2, p=0.579, swollen: 60.94±37.66, n=35; intermediate: 66.76±35.26, n=33; normal: 65.25±29.56, n=73, Fig. 6a) nor the interaction term of vulva condition-season ($X^2_{interaction}$=3.849, df=6, p=0.697) were significant (Table 4). When pregnancy during winter was accounted for, only pregnancy status significantly affected oestrone levels ($F_{1,12}$=7.797, p=0.016), while vulva condition did not ($F_{2,12}$=0.100, p=0.906, Table 5). As in oestrone, season was also the only predictor found to

significantly affect oestradiol levels ($F_{3,29}$=3.079, p=0.043), while vulva condition did not ($F_{2,29}$=0.464, p=0.633, Fig.6b; Table 6).



(a)



(b)

Figure 6. Oestrone levels (pg/ml) of females exhibiting different vulva conditions (swollen, intermediate and normal) (a) and oestradiol levels (pg/ml) of females exhibiting different vulva conditions (swollen, intermediate and normal) (b).

Table 4. Mixed model analysis of oestrone levels against vulva condition, with season as covariate.

Vulva condition term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	p
model 2	6	1355.1	1372.8	-671.54	1343.1			
model 1	8	1358	1381.6	-670.99	1342	1.0944	2	0.5786
Season term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	p
model 3	5	1393.7	1408.5	-691.86	1383.7			
model 1	8	1358	1381.6	-670.99	1342	41.724	3	<0.001
Interaction term significance using maximum likelihood test								
	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	p
model 4	8	1358	1381.6	-670.99	1342			
model 1	14	1366.1	1407.4	-669.07	1338.1	3.8494	6	0.697
model 1: Oestrone ~ Vulva condition + Season + (1 | Tattoo)								
model 2: Oestrone ~ Season + (1 | Tattoo)								
model 3: Oestrone ~ Vulva condition + (1 | Tattoo)								
model 4: Oestrone ~ Vulva condition + Season + Vulva condition * Season + (1 | Tattoo)								

Table 5. Analyses results of oestrone levels against vulva condition and pregnancy status.

Pregnancy status significance of terms					
	Df	Sum Sq	Mean Sq	F	p
Vulva condition	2	346.1	173	0.0996	0.9059
Reproductive status	1	13541	13541	7.7968	0.01628
Residuals	12	20840.8	1736.7		

Table 6. Analysis results of oestradiol levels against vulva condition, with season as covariate.

Oestradiol significance of terms					
	Df	Sum Sq	Mean Sq	F	p
Vulva condition	2	0.096	0.048	0.464	0.633
Season	3	0.957	0.319	3.079	0.043
Residuals	29	3.005	0.104		

DISCUSSION

The results show that badger sex-steroid levels follow distinct seasonal patterns, which broadly coincide with the seasonal variation in EGM as well as male testis volume, and reflect the established seasonal breeding pattern of this species (reviewed in Macdonald et al. 2015). During the period of reproductive quiescence in autumn, sex steroid levels were low in both sexes, testicular volume reached an annual minimum, the vast majority of males had retracted their testes completely into the body cavity (mirroring results from Buesching et al. 2009 evidencing two different mating strategies in males), and only very few females exhibited vulval swelling. In contrast, at the end of the main mating season (i.e. spring: Buesching et al. 2009; Dugdale et al. 2011), male testosterone was high, testis volume was largest, almost all males had descended, i.e. scrotal, testes, and more than one third of females had a swollen vulva, coinciding with comparatively high levels of oestradiol. However, these apparent correlations between seasonal proportions of EGM and seasonal reproductive hormone levels could not be confirmed statistically on the individual level: In females neither oestrone nor oestradiol differed between EGM categories.

In the case of oestrone, this lack of any correlation withEGM is not surprising, because particularly in species with long periods of delayed implantation, such as badgers (Yamaguchi et al. 2006), the main function of this hormone is to protect pre-implanted blastocysts (Heap et al. 1975; Heap et al. 1979; Research Chapter 1). In fact, the slightly lower oestrone levels (pg/ml) in females with vulval swelling likely indicate an absence of pre-implanted blastocysts (Research Chapter 1). In contrast, oestradiol, produced by ovarian follicles (Emery and Whitten 2003; Thomson et al. 1992; Whitten and Russel 1996), controls female oestrus (Adkins-Regan 2005), and is commonly associated with vulval swelling, which in diurnal species often functions as a visual oestrus advertisement signal (Emery and Whitten 2003). Despite higher proportions of vulva swelling during spring coinciding with the highest seasonal oestradiol levels, in individual badgers vulval swelling was not always reflective of elevated oestradiol levels. Instead, It is found that levels showed higher variation amongst females with a swollen or intermediate EGM (see Fig. 6b), indicating not only that the endocrinological mechanisms controlling female EGM are more complex than often assumed (see also Reichert et al. 2002), but potentially also (inter-observer) variation in the visual classification of female EGM categories between “swollen” and “intermediate” in the

field. Additionally, possibly in part due to their nocturnal and largely fossorial lifestyle, badgers do not use EGM as a visual reproductive advertisement signal, but use olfactory communication instead (scent marking using subcaudal and anal gland secretions, urine, and faeces: Buesching et al. 2002). Nevertheless, even in animals that do use EGM as a form of visual reproductive advertisement (such as in primates: Emery and Whitten 2003; Deschner et al. 2004), EGM is not always a reliable indicator of sex-steroid levels (Reichert et al. 2002). For instance, in chimpanzees (*Pan troglodytes*) vulval swellings were found also during pregnancy and adolescent sterility (Emery and Whitten, 2003), and although in reproductive females, oestrogen levels typically corresponded with EGM, vulval swellings were shown to begin earlier and progress more rapidly than circulating follicular oestradiol levels (Emery and Whitten, 2003), and ovulation generally occured towards the end rather than the onset of maximum swelling (Deschner et al. 2004). Also in rodents (*C.gapperi, M.breweri, M.pennsylvanicus, P.manculatus, Shispidus*: (McCravy and Rose 1992), EGM proved to be a reliable indicator of reproductive status (as determined by necropsy) in only 58-85% females.

In males, testes condition showed a significant correlation with testosterone levels. Nonetheless when visualized for specific seasons, correlation was restricted to spring (when the majority of males had fully descended testes). Because male spermatogenesis is regulated by testosterone produced by the interstitial Leydig cells (Colenbrander et al. 1978) and requires scrotal testes for temperature regulation (Hansen 2009), an increase in testosterone production is typically restricted to males with scrotal testes and is reflected in larger testicular volume due to an increase in Leydig cells (Klonisch et al. 2006). Our data, however, confirm a correlation between testosterone levels and testis volume only during the mating season reflecting results from other seasonally breeding mammals, where testicular diameter has been shown to increase several weeks before testosterone levels begin to rise (Lincoln and Davidson 1977). Testes size several weeks or even months prior to mating can also show a stronger correlation with testosterone levels than during the actual mating period, while outside the reproductive season, testicular diameter is often not correlated with testosterone levels (e.g., soay sheep *Ovis aries*: Preston et al. 2012; rodents: McCravy and Rose 1992). This is due to the increase of testosterone levels being controlled by an increased production of Gonadotropic Releasing Hormones (GnRH) in the hypothalamus (Preston et al. 2012), resulting in an increase in Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) during the mating season (Preston et al. 2012; Ramaswamy and Weinbauer

2014). FSH, in turn, activates sperm production by increasing germ cells (spermatogenia and spermatocytes), thus causing testes enlargement, whereas LH stimulates testosterone production (Preston et al. 2012). Therefore, in seasonally breeding males, a strong correlation between EGM and testosterone levels may only be observed during the mating season, whereas outside of the mating season, the activity of FSH (responsible for testes enlargement) and LH (triggering testosterone secretion) does not show any shared response (Lincoln and Davidson 1977), mirroring our results on badgers.

Based on the analyses conducted here, it is proposed that inferring the reproductive status of wild badgers from the appearance of their external genitalia will result in accurate assessment only in males during the mating season, but does not deliver accurate results in females or in males at any other season. Because in both sexes sex-steroid levels likely change considerably earlier and/ or faster than EGM (as confirmed in a range of species: rodents: McCravy and Rose 1992; bonobos *Pan panicus*: Reichert et al. 2002; chimpanzees *Pan troglodytes*: Deschner et al. 2004; soay sheep *Ovis aries*: Preston et al. 2012), this method needs to be applied with caution, particularly as, in badgers, some males may extend their mating chances until late summer/ autumn, displaying descended testes as well as high testosterone levels, while at the same time some males with fully descended testes have low testosterone (Buesching et al. 2009).

In the field, male EGM can likely be used in most mammals as a reliable indicator of reproductive activity during the mating season, but, due to this time lag between endocrinological and morphological changes (Lincoln and Davidson 1977; Preston et al. 2012) as well as the reported high inter-individual variation (McCravy and Rose 1992), should be taken under advisement during other times of the year (Lincoln and Davidson 1977; Preston et al. 2012; Ramaswamy and Weinbauer 2014). Because female EGM is typically even less precise for assessing reproductive condition (McCravy and Rose 1992), and in most species is only indicative of the peri-ovulatory period (Drost 2007; Durrant et al. 2003; Rae et al. 1999; Reichert et al. 2002), this method is recommended to be used mostly as an in-situ field methodology, and the resulting assumptions should later be confirmed in the lab with more accurate methodologies, such as vaginal cytology and/or reproductive hormone analyses (Gordon 1997) to help determine female reproductive status more precisely in badgers as well as in other species.

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PART III

LIFETIME REPRODUCTIVE EVENTS

RESEARCH CHAPTER 3

Heterochrony of puberty in the European badger (*Meles meles)* can be explained by growth rate and group-size: Evidence for two endocrinological phenotypes

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ABSTRACT

Puberty is a key stage in mammalian ontogeny, involving endocrinological, physiological and behavioural changes, moderated by intrinsic and extrinsic factors. Thus, not all individuals within one population achieve sexual maturity simultaneously. Here, using the European badger (*Meles meles*) as a model, male testosterone and female oestrone profiles (using Enzyme-immunoassays) from first capture (3 months, post-weaning) until 28 months (attaining sexual maturity and final body size) were described along with metrics of somatic growth, scent gland development and maturation of external reproductive organs as well as intra-specific competition. In both sexes, endocrinological puberty commenced at ca. 11 months. Thereafter, cub hormone levels followed adult seasonal hormone patterns but at lower levels, with the majority of cubs reaching sexual maturity during their second mating season (22-28 months). Interestingly, there was evidence for two endocrinological phenotypes among male cubs (less evident in females), with early developers reaching sexual maturity at 11 months (first mating season) and late developers reaching sexual maturity at 22-26 months (second mating season). Early developers also attained a greater proportion of their ultimate adult size by 11 months, exhibiting faster growth rates than late developers (despite having similar adult size). Male cubs born into larger social groups tended to follow the late developer phenotype. These results support the hypothesis that a minimum body size is required to reach sexual maturity, which may be achieved at different ages, even within a single population, where early maturity can confer individual fitness advantages and enhance population growth rate.

Keywords: *developmental heterochrony, European badger, oestrone, puberty, sexual maturity, testosterone*

INTRODUCTION

Puberty represents a key stage in mammalian ontogeny during which a variety of endocrinological, physiological, and behavioural changes occur (Onyango et al. 2013). It is marked by the development of secondary sexual characteristics (Adkins-Reagan 2005), the first occurence of ovulation/oestrus in females and the onset of spermatogenesis in males (Evans and O'Doherty 2001). During puberty, typically both sexual and somatic maturation are completed (Tena-Sempere 2013), but some species may continue to grow even after reaching sexual maturity (Stamps and Krishnan 1997). Although the age at puberty depends predominantly on intrinsic genetic factors, its timing can be moderated by a variety of additional extrinsic factors, such as food availability, seasonal variation, environmental conditions (De Jonge et al. 1996; Parent et al. 2003; Abeygunawardena and Dematawewa 2004) and/ or the presence of conspecifics (Evans and O'Doherty 2001), as well as dynamic interactions between these factors (Tena-Sempere 2013). Thus, typically not all members of a species (Delemarre-van de Waal et al. 2002), or even all individuals within one population (Onyango et al. 2013; Freetly et al. 2011; Plaistow et al. 2004; Alberts 2012; Pusey 2012) mature simultaneously or at the same rate, leading to heterochrony (Klingenberg 1998; Smith 2002). In mammals, the onset of puberty typically depends on attaining a minimum body size (often a certain functional proportion of the final adult body size: (Freetly et al. 2011; Day and Rowe 2002), and conspecifics may reach this minimum required body size at different ages (e.g., dairy calves require 56-60% of adult body weight which they may reach between 49.8 and 58.2 weeks of age: Freetly et al. 2011). Individuals that experience restricted resources during development therefore tend to undergo puberty at an older age than do individuals that experienced abundant resourses and are thus in better nutritional condition (Pusey 2012), consequently there can be a trade-off between somatic growth and puberty/ reproductive activity (Green and Rothstein 1991).

A distinctive endocrinological feature of puberty is the full activation of the hypothalamic-pituitary-gonadal (HPG) axis. This involves the episodic release of gonadotropin releasing hormones (GnRH) by the hyphothalamus, which in turn activates the anterior pituitary gland to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH) that instigate the generation of gametes and release of sex steroids (Plant 2006): in males, LH stimulates

testosterone production from the interstitial cells of the testes (Leydig cells), and FSH stimulates testicular growth and enhances the production of an androgen-binding protein by the Sertoli cells, which are a component of the testicular tubules necessary for sustaining maturing sperm cells (Adkins-Reagan 2005). In females, FSH stimulates the ovarian follicle(s), causing one/ several ovum to grow, and triggers the production of follicular oestrogen. This rise in oestrogen then causes the pituitary gland to cease production of FSH and to increase LH production instead. This rise in LH levels in turn causes the ovum to be released from the ovary, resulting in ovulation (Adkins-Reagan 2005). Therefore, oestrogen and testosterone levels are low throughout the prepubertal period, but increase immediately prior, during and after puberty, until they reach adult concentrations (Fitzgerald and Butler 1982; Beehner et al. 2009).

Nevertheless, although the physiological processes of puberty have been the subject of numerous studies (De Jonge et al. 1996; Abeygunawardena and Dematawewa 2004; Sisk and Foster 2004), for many mammals, especially in wild-living populations, knowledge regarding the factors driving sexual development and potential heterochrony in puberty onset is still lacking (Parent et al. 2003; Beehner et al. 2009; Sisk and Foster 2004). Particularly in seasonal breeders, it is often difficult to determine sexual maturity unambiguously, because even adults exhibit periods of reproductive quiescence when males cease spermatogenesis and females do not undergo oestrus cycles (Curlewis 1992; Revel et al. 2007). Most carnivores (for exceptions see Goeritz et al. 2006; Jewgenow et al. 2006; Jewgenow et al. 2014), and all mustelids (Amtislavsky and Ternovskaya 2000), undergo such periods of seasonal reproductive quiescence, and in many the timing of their sexual maturity is still being evaluated (Jewgenow et al. 2014). Here, the European badger (*Meles meles*; henceforth "badger") was used as a model of a seasonally breeding carnivore to investigate the endocrinological changes and concomitant ontological development of male and female genitalia during puberty, and to examine how onset of puberty can be affected by body size and intra-specific competition resulting in developmental heterochrony.

Badger reproduction and development

The badger's mating season is restricted mainly to January-March, although further matings can occur throughout the summer months (Yamaguchi et al. 2006) with local population

density determining the number of additional oestrous cycles ranging from nil to monthly (Corner et al. 2015; Research Chapter 1). During the mating season, scent marking activity increases (Buesching and Macdonald 2001), where particularly the subcaudal gland secretion plays an important role in group-cohesion and olfactory mate guarding (Buesching et al. 2002a; Buesching et al. 2003), as well as resource defense and reproductive advertisement (Buesching et al. 2002b; Buesching and Macdonald 2004). This is reflected by significant elevation in the production of subcaudal secretion (Buesching et al. 2002a) as well as changes to the secretion's chemical composition (Buesching et al. 2002b). During the mating season, all mature males have large scrotal testes, and females exhibit a distinctly swollen, pink and everted vulva (Research Chapter 2). In contrast, during autumnal reproductive quiescence, males have smaller testes that ascend into the body cavity, while females cease to exhibit vulval swelling (Research Chapter 2). Sex steroid levels also exhibit distinct seasonal patterns in sexually mature badgers (Buesching et al. 2009; Research Chapter 1; Research Chapter 2): In males, testosterone levels are high in spring and summer, low in autumn and peak during the winter mating season. In females, oestrone levels are high in spring, low in summer, peak in autumn and remain elevated for pregnant females in winter but decline in non-pregnant females. As in all carnivores, male badgers have a bacculum (os penis; Whelton and Power 1993) that provides mechanical support during copulation (thus enabling prolonged intromission and mate-guarding through copulatory tying; Verrell 1992; Dixson 1995), facilitates sperm transport (Macdonald et al. 2015), and helps trigger ovulation in species with induced ovulation such as badgers (Yamaguchi et al. 2006).

Badgers produce one litter annually (with mean litter size at the study site = 1.4±0.06, range of 1-4 cubs, where 93% litters comprise less than 3 cubs: Macdonald et al. 2015), born between mid January – mid March (76% of cubs in the UK are born in mid-February: Macdonald et al. 2015). Newborn cubs are highly altricial, and their eyes and ear canals do not open until they reach 5 weeks of age (Roper 2010). Cubs are weaned at 6-8 weeks of age (Woodroffe and Macdonald 2000), during which time they first emerge from their underground den, termed a sett (Fell at al. 2006), and are fully integrated into the social group at 14-16 weeks of age (Fell at al. 2006). Growth rate has been shown to vary among cubs depending on resource availability, which is affected by prevailing weather conditions, linked to food (mainly earthworm) availability (Nouvellet et al. 2013) and natal sett quality (Kaneko et al. 2010; Tsunoda et al. 2018), as well as infection with a highly pathologic intestinal

coccidian (*Eimeria melis*), where surviving infected male and female cubs exhibit, respectively, 5 cm and 3.5 cm shorter mature body-length (Newman et al. 2001).

In high density areas (such as the study population in Wytham Woods: 44.55 ± 5.37 (SE) badgers/km^2; Macdonald et al. 2015; Annavi et al. 2014), cubs take longer to reach adult size than in lower density areas (Macdonald et al. 2002; Research Chapter 5) and remain smaller than those living at lower density (Macdonald et al. 2002; Delahay et al. 2006). Cubs start producing subcaudal gland secretion when they are approximately 4 months old (Buesching et al 2002a) but anoint themselves with secretion from adults (a behaviour termed 'scent-theft') at a much younger age (Fell et al. 2006), signifying the importance of this secretion in badger sociality (Buesching et al 2003). Reports of sexual maturity vary considerably, ranging from 9-12 months (Ahnlund 1980; Neal and Cheeseman 1996) to 18 months (Buesching et al 2002a); but most studies evade the issue of exact age at puberty and simply state that female badgers would not be able to breed until reaching the age of 2 years due to delayed embryonic implantation (reviewed in Roper 2010). No studies to-date have investigated potential developmental heterochrony between individuals within the same population or cub cohort.

Here, for the first time, male testosterone and female oestrone profiles for cubs were described, commencing from the time of first capture (i.e., at the end of the closed season when cubs are 3 months of age and are fully weaned) through to the age of 28 months (i.e., when all badgers have reached sexual maturity: Ahnlund 1980; as well as full adult size: Research Chapter 5), and report on the ontological development of male and female external genitalia morphology (EGM; i.e., degree of testicular descent and vulval swelling), baculum length, and testes volume. Because in other mammals, somatic growth as well as sexual maturity have been shown to vary among individuals within the same population (Onyango et al. 2013), whether all cubs in the sample mature at the same rate was investigated, as well as whether there was evidence for different ontological strategies in terms of hormone profiles, skeletal growth and the production of subcaudal gland secretion (as reported in other species: Day and Rowe 2002).

MATERIALS AND METHODS

Badger trapping and sampling

Data were collected from a high-density badger population in Wytham Woods, Oxfordshire, UK (51°46:26 N, 1°19:19 W; for details see Macdonald et al. 2015) between 1995-2016 (although, not all years in this range were represented in the datapoints used), as part of an ongoing long-term research project. Following the methodology described in Sun et al. (2015), all badgers received a permanent unique tattoo at first capture (typically as cubs: Macdonald et al 2015; Macdonald et al 2009), allowing individual identification (ID) and reliable aging.

Badgers were trapped in every month except during the closed season under the Protection of Badgers Act, 1992 (December-April), although, in some years, additional trapping was conducted under special license in December and early January before the end of the first pregnancy trimester (Macdonald et al. 2015). The development of immature badgers could therefore be followed in: (1) *spring* (May/June) at end of the main mating period when cubs are fully weaned but spring weather can impact cub growth (Macdonald et al. 2010); (2) *summer* (July/August/September) during additional mating activity previously reported in other high-density badger populations (Corner et al. 2015) and the period of lowest food abundance (Nouvellet et al. 2013; Macdonald et al. 2009); (3) *autumn* (October/November) during reproductive quiescence and highest food abundance; and (4) *winter* (December/January) during the main mating season when cold weather may affect thermal energy balance in badgers (Byrne et al. 2015).

Somatic measurements and classification of external genitalia morphology

Head-body length (to the nearest 5mm), zygomatic arch width (to the nearest 1mm), and body weight (to the nearest 100g) were measured for all captured individuals, and a Body Condition Index (BCI) was calculated as $\log_{10}$(weight)/$\log_{10}$(body length). Subcaudal gland secretion was scooped out of the subcaudal pouch using a rounded stainless-steel spatula (Buesching et al. 2002a), and the volume estimated by eye to the nearest 0.05 ml. The spatula was disinfected between individuals using absolute ethanol (Sin et al. 2012). External

Genitalia Morphology (EGM) was recorded in both sexes and categorised according to Research Chapter 2, in females as normal, intermediate or swollen vulva, and in males as ascended, intermediate or descended testes. Male baculum length, testes length, width and scrotal thickness were measured (in mm), and the testicular volume was calculated (in mm^3) as (L x W x H) x 0.71, where L= testicle length – scrotal pinch, W= testicle width – scrotal pinch, and H = testicle width – scrotal pinch (Paltiel et al. 2002).

Blood sampling and hormone measurements

Blood samples (n_{males}= 119; $n_{females}$= 63; chosen from the available data set to represent each month – except the closed season, see above) were collected for endocrinological analyses via jugular venepuncture, using vaccutainer tubes (Becton-Dickinson) with K2-EDTA (ethylene diamine tetraacetic acid) anticoagulant. Sampling times were standardized to account for circadian variation in hormonal profiles (Research Chapter 2; Buesching et al. 2009), and blood samples were centrifuged within 30 minutes of sampling at 10°C for 10 min under 2,500 rpm/ 1470G. Plasma was transferred into Eppendorf tubes and frozen at -20°C immediately.

All sex steroid titres were analysed using Enzyme immunoassays (EIA) and analysed at the Chester Zoo Endocrinology Laboratory, UK. Oestrone was measured in microtitreplates coated with polyclonal antiserum raised against oestrone EC R522 (Munro et al. 1991). Plasma samples were un-extracted and used for measurement after dilution with assay buffer at the ratio of 1:10. Duplicate 20µl aliquots of oestrone standard (0.195-200 pg/well), diluted plasma, and quality controls were combined with 50µl oestrone glucuronide coupled to horseradish peroxidase (oestrone-glucuronide-HRP) as label, and incubated at room temperature for 2 hours. Plates were washed five times and blotted dry after incubation, followed by an addition of 100 µL peroxidase substrate solution (ABTS) to each well. Plates were covered and incubated at room temperature until the '0' wells reached approximately 1.0 optical density and read at 405 nm using a Spectrophotometer Opsys MR (Dynex). Assay sensitivity at 90% binding was 3.1 pg. Intra-assay coefficients of variation (CV, calculated as the average value from the individual CVs for all of sample duplicates), were 8.21 % (high) and 6.05 % (low); inter-assay variation (repeated measurements of high and low-value quality controls across plates) was 13.96 % (high) and 13.62 % (low) respectively.

Testosterone was measured in microtitre plates coated with anti-testosterone R156/7 (OEM-Concepts, UK). Samples (un-extracted) were analysed by dilution in 1:4 assay buffer. Duplicate 50µl aliquots of testosterone standards (2.3-600 pg/well), samples and quality controls were then combined with 50µl horseradish peroxidase (testosterone-HRP) as label. After incubation in the dark at room temperature for 2 hours, plates were washed 5 times and blotted dry, followed by addition of HRP-substrate (100 µL) to each well. Plates were covered and incubated at room temperature until the '0' wells reached 1.0 optical density and were then read at 405 nm, using a Spectrophotometer (Opsys MR; Dynex). Assay sensitivity at 90% binding was 1.6 pg. The testosterone intra-assay coefficients of variation were 14.69 % (high) and 6.18 % (low), and inter-assay variation of high and low-value quality controls was 9.15 % (high) and 5.23 % (low).

Statistical analysis

All statistical analyses were performed using RStudio (0.99.896) and R (R-3.2.4). Patterns of residuals, normality, and mean variance for each model were checked using R diagnostic plots. Generalized Additive Models (GAM) were used to generate trend lines for sex steroid levels (males: testosterone; females: oestrone) against age (3-28 months) using a smoothing function. A non-linear mixed model (random effect: badger identification/ tattoo number, ID) using the *nlme* and *sslogic* function was used to form a growth curve (providing an asymptote value as output) for baculum length against age (3-28 months, n=773). To determine the age at which the baculum ceased to grow, the percentage of the predicted baculum length towards the asymptote (in the adult population) was calculated. Testes volume (n=597) and subcaudal secretion volume (n_{male}=1233; n_{female}=1284) trend lines were generated against age (3-28 months) by fitting a GAM model. Additionally, the difference between male and female subcaudal secretion pattern with age (months) was analysed by testing the sex:months interaction term in the following linear model: lm(Subcaudal volume ~ Age + Sex + Age*Sex). Interactions between proportions of EGM (males: descended, intermediate, ascended testes, n=1136; females: normal, intermediate, swollen vulva, n=1174) with age (3-28 months) were analysed using a Chi-square test.

Developmental heterochrony

Endocrinology and EGM

The GAM average trends (above) provide legitimate basis of hormonal heterochrony in both

sexes, where some cubs appeared to reach puberty earlier than others (i.e., the existence of two discrete groups that differ in their sex-steroid hormone levels at a certain age: high levels above and low levels below the GAM line benchmark, providing two developmental categories, or phenotypes). However, because endocrinological sample sizes were limited, all analyses described below were repeated on EGM-based groups at the age of 11 months during their first mating season, which enhanced sample sizes. That is, in addition to comparing cubs with high vs low sex-steroid levels, male cubs with ascended vs descended testes and female cubs with a normal vs swollen vulva were compared, excluding intermediate conditions in both sexes to avoid ambiguity.

Somatic growth

Subsequently, head-body length, zygomatic arch width, BCI, and subcaudal secretion volume between these two groups were compared using a linear mixed model (including year as a random effect to account for established inter-annual variation in growth patterns: Newman et al. 2001; Research Chapter 5) to determine potential concurrent differences in physical development at the point of hormonal divergence. A drop 1 function was used to determine signidicance of parameters in the mixed models (giving comparison of models based on their AIC criteria). If significant differences were found in any of the skeletal size measures, the differences in head-body length and zygomatic arch width between adult size (above 28 months) to the size at the age at which divergence occurred were calculated in all individuals and compared between the respective groups, to determine heterochronous residual growth between the two groups (also using a linear mixed model with year as random affect). Growth curves for repeatedly captured individuals in each group were then constructed based on the rates of increase in head-body length (which provide a reliable indicator for overall skeletal growth and development of badger cubs: Research Chapter 5), employing a non-linear mixed model (random effect: ID) using the *nlme* and *sslogic* function. Where individuals were not recaptured at this precise target-age (within the 28 months period), the closest recapture points available for these analyses were used.

Social factors affecting the timing of puberty

In addition, whether social factors/ intra-specific competition affected the timing of sexual maturity was investigated by comparing hormone levels at the age when these phenotypic

groups diverged with the total number of adults and cubs in that cub's natal group and natal sett (as some groups utilize several setts: Macdonald et al. 2004) in a linear mixed model with year included as a random effect. The total number of resident adults and cubs was determined annually (by assigning residency according to the rules in Annavi et al. 2014; see also Research Chapter 5).

RESULTS

Endocrinological changes during the first 28 months

As predicted, throughout their first summer, all cubs had significantly lower sex-steroid levels than did adults. However, at the age of 11-12 months, i.e. during their first mating season, male cubs showed a small peak in testosterone (GAM: Edf= 8.631, R-sq.(adj)= 0.566, GCV= 2.230, Deviance explained= 59.9%, $p<0.001$, Fig 1)**,** which was then followed by the seasonal pattern of testosterone levels typical for adult males (high in spring and summer, and low in autumn: Buesching et al. 2009), with a pronounced peak that reached levels typical for adults during their second mating season (22-28 months).

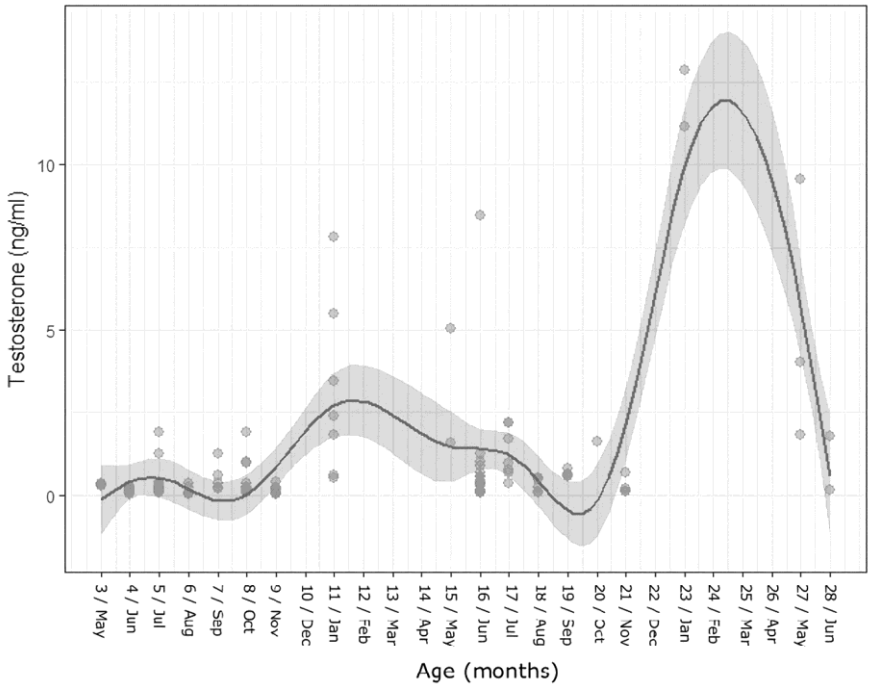


Figure 1. Testosterone levels (ng/ml) in males aged 3-28 months.

In females, oestrone levels increased gradually from the age of 3 up to 11 months, at which point they almost reached adult levels (GAM: Edf= 5.218, R-sq.(adj)= 0.216, GCV= 733.1, Deviance explained= 28.2%, p=0.009, Fig 2). Patterns then followed the seasonal oestrone pattern typical for adults, with levels being relatively high in spring (13-16 months) and decreasing in summer (17-19 months), remaining low during autumn (20-21 months, reproductive quiescence) and winter (22 months, December: implantation) after which time they increased again towards spring (27-28 months), this time reaching adult levels (73.28±28.06 pg/ml; Research Chapter 1). Nevertheless, inter-individual variation among females was considerable during the second summer (months 15-20), i.e., from the end of their first mating season until autumnal reproductive quiescence.

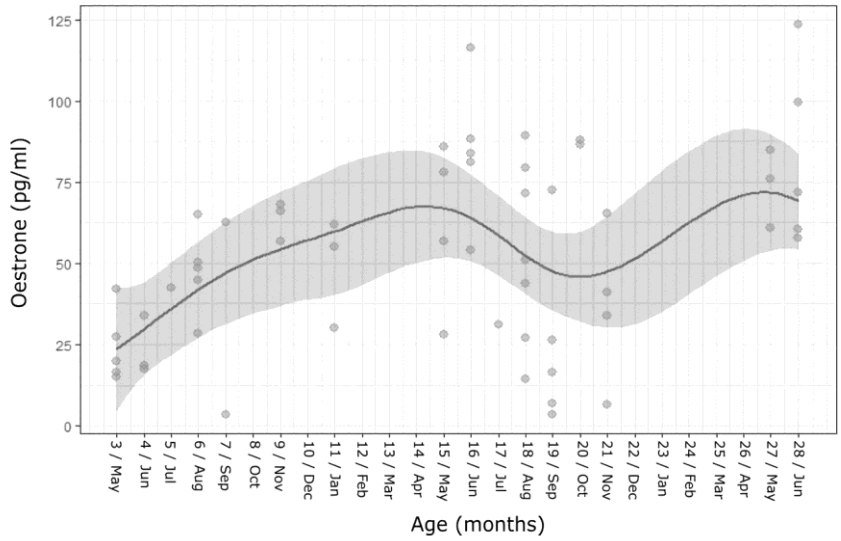


Figure 2. Oestrone levels (pg/ml) in females aged 3-28 months.

External genitalia changes during the first 28 months

In both sexes, there was a significant interaction between EGM and age (in months) ($males_{n=1136}$: $X^2 = 937.04$, df = 36, $p<0.001$; $females_{n=1174}$: $X^2 = 418.47$, df = 34, $p<0.001$; Fig 3a, b). The majority of male cubs had scrotal (i.e., fully descended) testes for the first time at the age of 5-6 months (83.3% and 70.4% respectively), while during their first autumn (8-9 months) the majority had ascended testes (63.6% and 77.1% respectively). During their first mating season in January (11 months), the largest proportion (41.5%) of male cubs had descended testes, while both ascended and intermediate proportions were each 29.3%. During

the following spring (15 months) and summer (19 months) the majority of males (94.8% and 82.9%, respectively), had descended testes and followed the adult seasonal pattern thereafter.

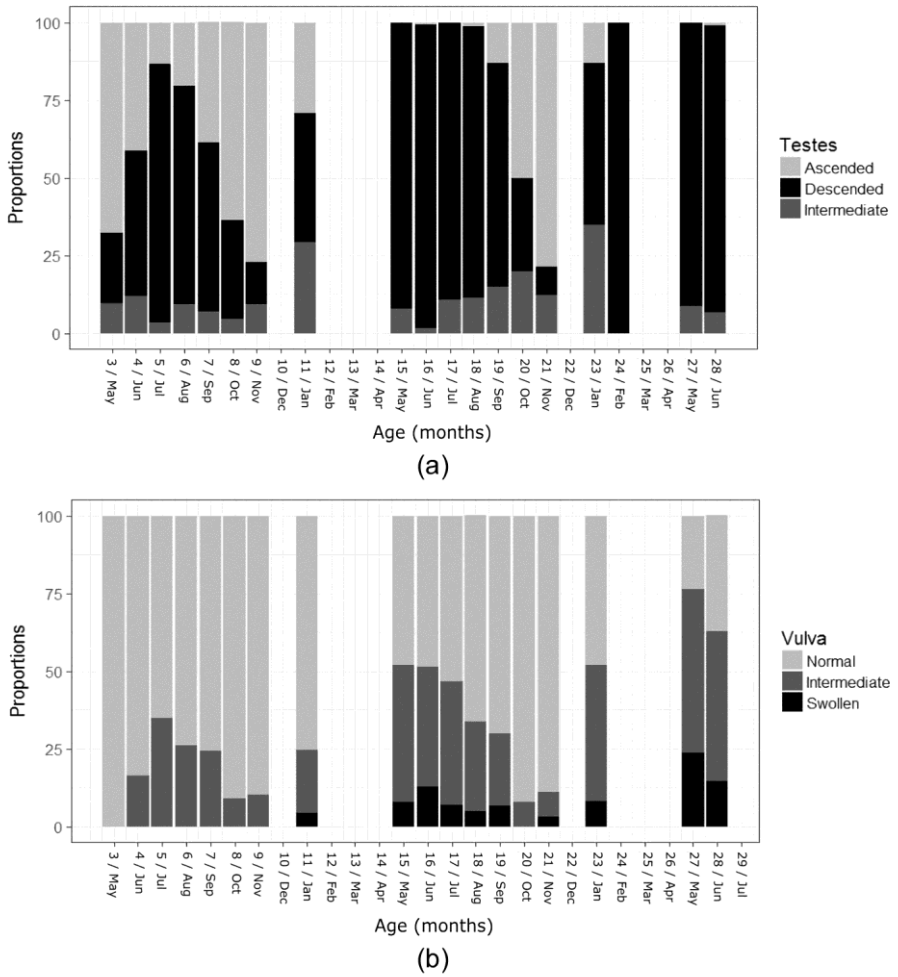


Figure 3. EGM changes in males (a) and females (b) aged 3-28 months.

In females, the earliest vulval swellings (4.4% swollen; 20% intermediate vulva) were recorded at the age of 11 months during their first mating season (Jan). The proportions of females with intermediate and swollen vulva increased during the next spring-summer (15-19 months; intermediate: spring= 41.4%, summer= 30.5%; swollen: spring= 10.3%, summer= 6.2%) and decreased in autumn (20-21 months; intermediate: 7.8%, swollen: 1.5%). During their third spring, the highest percentage of female cubs had either intermediate (50.4%) or fully developed vulval swelling (19.2 %), congruent with adult states (Research Chapter 2).

In males, testicular volume started to increase markedly at the age of 11 months (from 848.69±475.20 mm^3 at the age of 4-6 months$_{n=12}$; 1331.27±1289.97 mm^3 at the age of 7-9 month $_{n=32}$; to an average of 3449.73±1572.04 mm^3 at 11 months $_{n=18}$) and peaked during the first mating season (5326.45±2674.88 mm^3, n=60; winter-early spring; GAM: Edf= 6.921, R-sq.(adj) = 0.242, Deviance explained = 25.1%, p>0.001, Fig 4). Average testicular volume then decreased towards an autumnal minimum at 20-21 months (3209.42±2283.41 mm^3, n=20) and followed the adult seasonal pattern thereafter, reaching a slightly higher peak (5698.26±2409.85 mm^3, n=187) in the second mating season (22-28 months), with sizes comparable to adult values (winter: 6650.82 mm^3, spring: 5776.31 mm^3: Research Chapter 2).

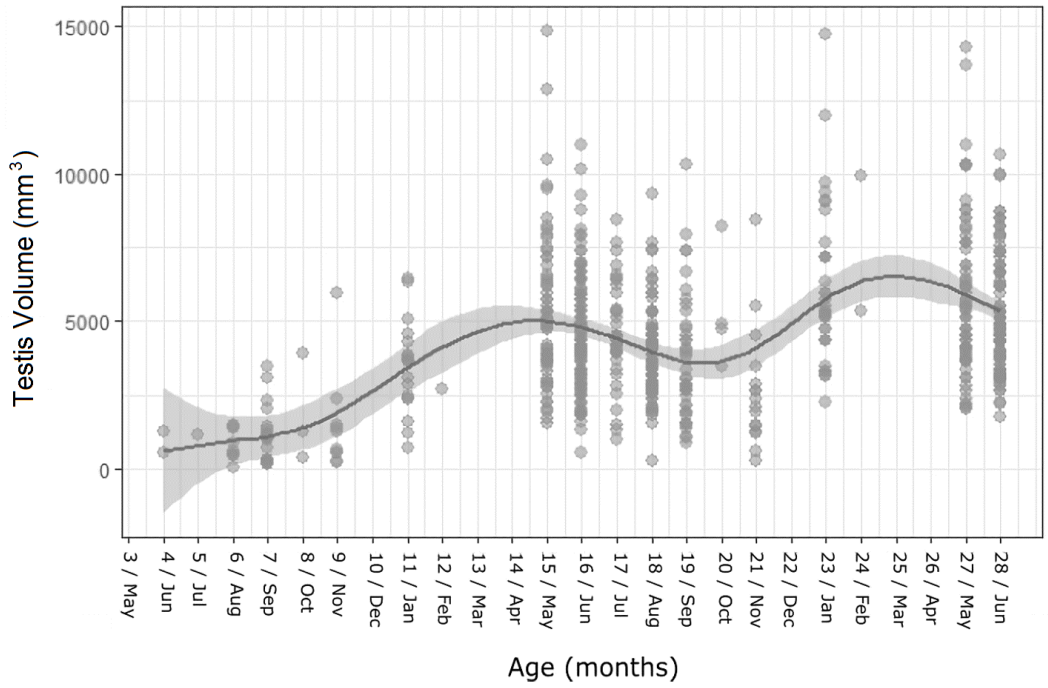


Figure 4. Testis volume (mm^3) in males aged 3-28 months.

Male baculum length increased consistently month by month for the first year, when baculum growth rates slowed and reached 99% towards the asymptote of 86.03 mm predicted by the model at the age of 23-24 months (Fig 5).

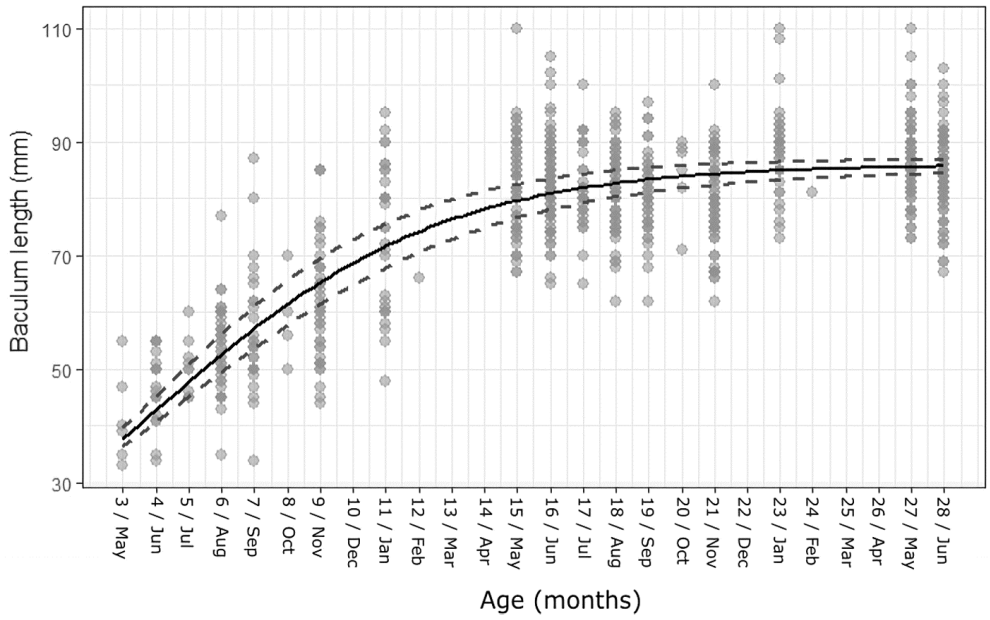


Figure 5. Baculum length growth curve at age of 3-28 months in males.

Subcaudal gland activity during the first 28 months

Both sexes started producing subcaudal gland secretion by 3 months (Fig 6). However, secretion volume was very low (unmeasurable traces in males and 0.04±0.09 ml in females $_{n=72}$) and increased only slowly towards their first mating season at age 11 months (0.38±0.36 ml in males$_{n=41}$ and 0.19±0.13 ml in females$_{n=47}$). Thereafter, secretion volume increased substantially in both sexes (GAM: male: Edf= 8.45, R-sq.(adj)= 0.56, GCV= 0.18, Deviance explained= 56.2%, $p<0.001$, Fig 6a; female: Edf= 8.83, R-sq.(adj)= 0.37, GCV= 0.04, Deviance explained= 38.2%, $p<0.001$, Fig 6b). In male yearlings, secretion volume peaked in spring-summer (13-18 months, 1.04±0.61 ml, n=292), decreased towards an autumn-minimum (20-21 months; 0.56±0.39 ml, n=97) and peaked again (with higher secretion volume) in their second winter-spring (23-28 months; 1.17±0.58 ml, n=210), following the typical seasonal pattern and secretion volume of adults (average values for spring= 1.06±0.67 ml, n=1004; summer= 0.92±0.66 ml, n=1059; autumn= 0.60±0.50 ml, n=790; winter= 0.90±0.60 ml, n=347 ml). Female yearlings showed a first slight peak in secretion in their second spring (13-16 months; 0.35±0.24 ml, n=150), after which volume decreased slightly during summer-autumn (17-19 months, 0.32±0.35 ml, n=189 and 20-21 months, 0.27±0.17 ml, n=110), but then started to increase again at the end of winter (24 months), peaking at an average of 0.52±0.41 ml in spring (27-28 months), and following the adult pattern thereafter

(average values for spring = 0.41±0.34 ml, n = 1287; summer = 0.33±0.32 ml, n = 1286; autumn = 0.25±0.24 ml, n = 933 ml; winter = 0.24±0.26 ml, n = 207). Despite having visually similar profiles, the male and female subcaudal gland secretion patterns differed when interaction of sex and months was tested (linear model sex:months term: $F_{1,2513}$=22.258, p<0.001).

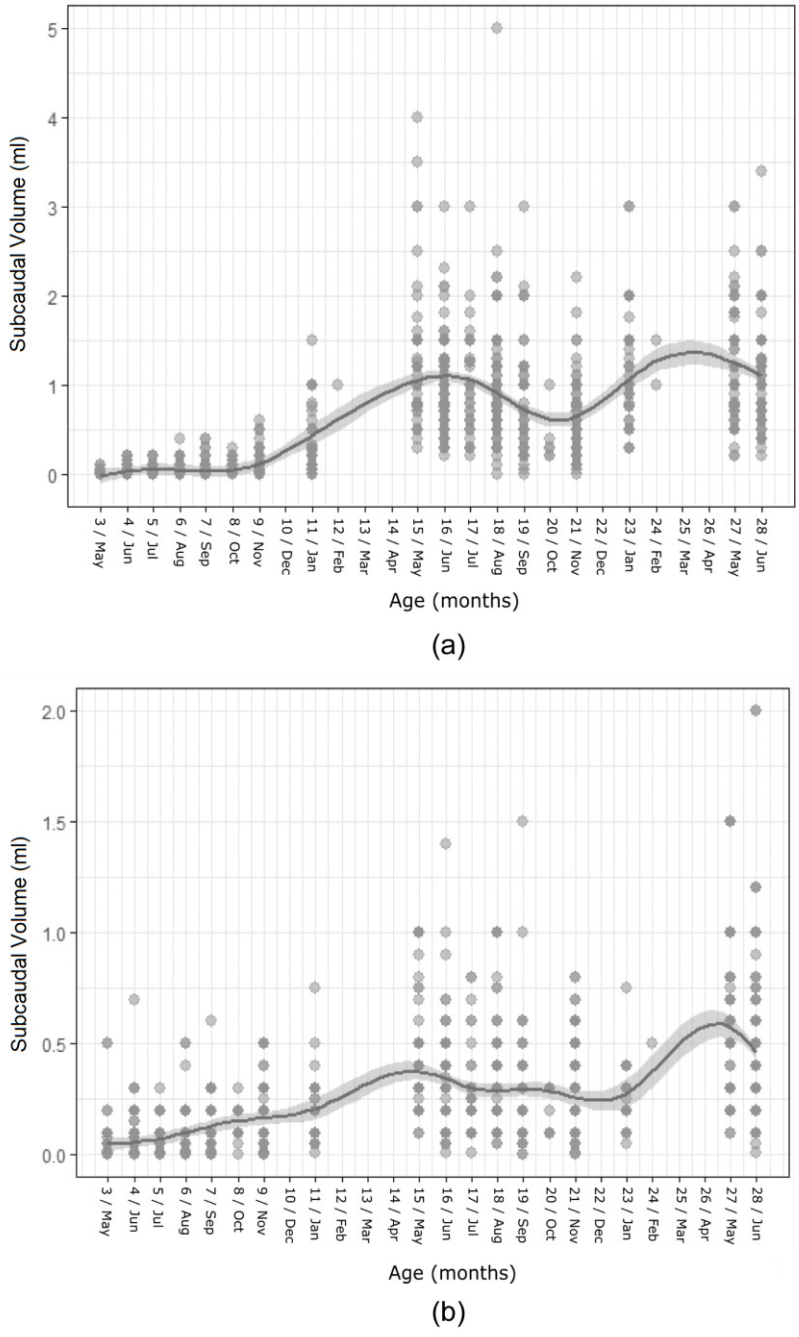
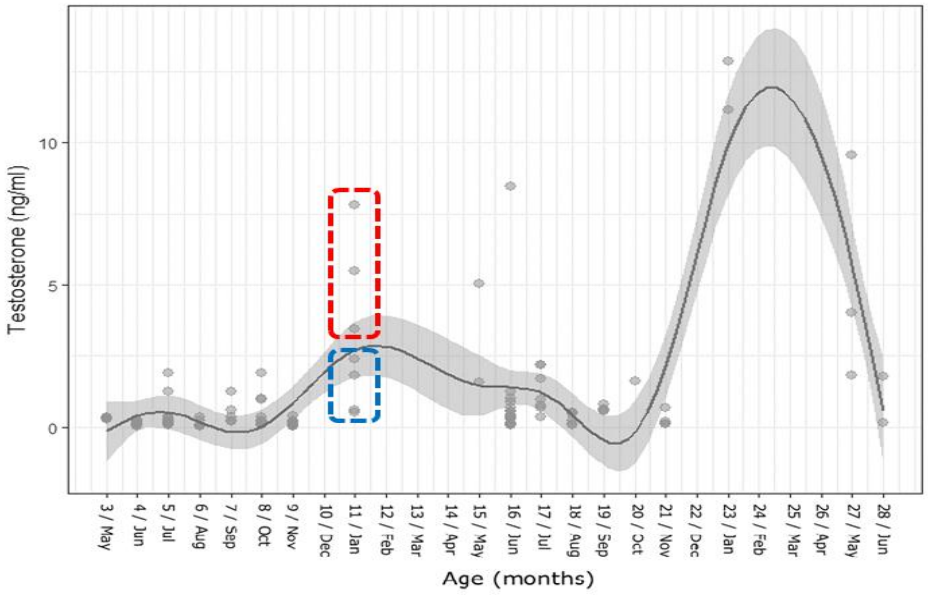


Figure 6. Subcaudal secretion volume (ml) changes in males (a) and females (b) aged 3-28 months.

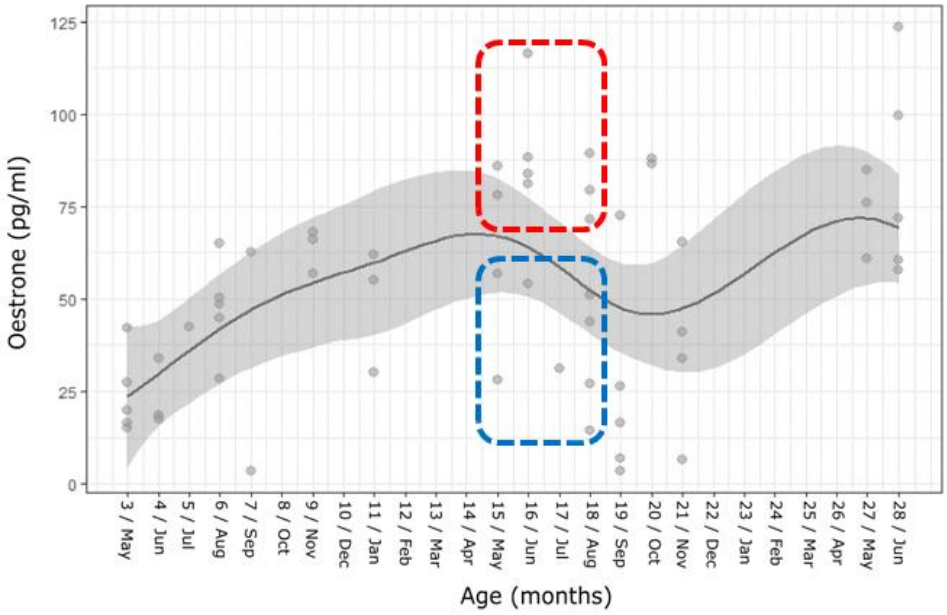
Evidence for two categories of cubs: Early- and Late-Developers

Endocrinological evidence for early and late developers

In both sexes, some individuals appeared to reach puberty earlier than others evidencing the existence of two endocrinological phenotypes: early- and late developers (Fig 7a, b), clustering into two distinct trait types according to the GAM line benchmark which exposed significantly different sex-steroid levels (for detailed results see Table 1 and 2). However, the age at which these early- and late development categories became apparent, differed between male and female cubs. For males, some (3/7= 42.9%) cubs reached puberty during their first year (HT, testosterone levels above the GAM line at 11 months of age), while the remainder reached puberty during their second year (LT, testosterone levels below the GAM line at 11 months, reaching pubescent levels at 22-28 months of age; Fig 1). It is important to note that the datapoints at the age of 11 months (when divergence occured) came from consistent years (2005-2006), although for further analyses (Table 1) year was still included as a random effect. In contrast, in females, the two possible endocrinological phenotypes diverged at age 15 -18 months (younger cubs either had more unified levels or sample sizes were too small to signify a difference; Fig 2), where some females had above-average oestrone levels (HO; oestrone above the GAM line) and some below-average levels (LO; oestrone below GAM line). In both sexes, these endocrinological phenotypes manifested independent of calendar year.



(a)



(b)

Figure 7. The two groups of male cubs at the age of 11 months based on testosterone levels (a; HT: red, LT: blue). The two groups of female cubs at the age of 11 months based on oestrone levels (b; HO: red, LO: blue).

Table 1. Differences in somatic development between early and late developers from endocrinological and EGM grouping in male cubs.

Grouping based on hormone levels in male cubs					
Endocrinological and somatic difference (at 11 months) between groups					
Endocrinological and somatic parameter	Groups based on hormone level				Linear mixed model statistics (accounting for year as a random effect)
	HT		LT		
	Avg + std	N	Avg + std	N	
Testosterone level (ng/ml)	5.58±2.19	3	1.15±1.14	4	Df=1, AIC=23.26, X^2=8.16, p= 0.004
Zygomatic arch (mm)	83.67±2.52	3	76.00±4.32	4	Df=1, AIC=48.17, X^2=6.32, p= 0.012
Head body length (mm)	656.67±23.09	3	610±23.45	4	Df=1, AIC=73.30, X^2=5.70, p= 0.017
Body Condition Index	0.31±0.01	3	0.29±0.02	4	Df=1, AIC=-31.19, X^2=5.78, p= 0.016
Subcaudal secretion volume (ml)	0.33±0.32	3	0.14±0.08	4	Df=1, AIC=10.48, X^2=1.63, p= 0.202
Residual growth (11 – 28 months) difference between groups					
Somatic parameter	Groups based on hormone level				Linear mixed model statistics (accounting for year as a random effect)
	HT		LT		
	Avg + std	N	Avg + std	N	
Zygomatic arch (mm)	5.67±0.58	3	12.25±0.96	4	Df=1, AIC=42.72, X^2=22.39, p< 0.001
Head body length (mm)	28±26.96	3	77.5±19.36	4	Df=1, AIC=70.56, X^2=12.67, p< 0.001
Grouping based on EGM in male cubs					
Somatic difference (at 11 months) between groups					
Somatic parameter	Groups based on EGM				Linear mixed model statistics (accounting for year as a random effect)
	DT		AT		
	Avg + std	N	Avg + std	N	
Zygomatic arch (mm)	83.11±3.82	9	79.40±4.33	10	Df=1, AIC=115.09, X^2=3.70, p= 0.05
Head body length (mm)	668.57±24.21	14	632.08±51.28	12	Df=1, AIC=260.00, X^2=3.19, p= 0.074
Body Condition Index	0.32±0.01	13	0.30±0.03	12	Df=1, AIC=-112.54, X^2=3.92, p= 0.048
Subcaudal secretion volume (ml)	0.54±0.44	17	0.13±0.11	11	Df=1, AIC=16.30, X^2=6.95, p= 0.008
Residual growth (11 – 28 months) difference between groups					
Somatic parameter	Groups based on EGM				Linear mixed model statistics (accounting for year as random effect)
	DT		AT		
	Avg + std	N	Avg + std	N	
Zygomatic arch (mm)	6.50±3.25	8	11.60±4.12	10	Df=1, AIC=106.88, X^2=5.38, p= 0.020
Head body length (mm)	33.21±15.14	14	70±38.79	12	Df=1, AIC=2.34, X^2=3.96, p= 0.046

Note: HT= Hight testosterone level group, LT= Low testosterone level group, DT= Descended testes group, AT= Ascended testes group.

Table 2. Differences in somatic development between assumed phenotypes from endocrinological and EGM grouping in female cubs.

Grouping based on hormone levels in female cubs					
Endocrinological and somatic difference (at 15 - 18 months) between groups					
Endocrinological and somatic parameter	Groups based on hormone level				Linear mixed model statistics (accounting for year as random effect)
	HO		LO		
	Avg + std	N	Avg + std	N	
Oestrone level (ng/ml)	86.06±12.72	9	38.31±15.32	8	Df=1, AIC=166.57, X^2 =24.74, p< 0.001
Zygomatic arch (mm)	80.44±3.81	9	81.29±3.15	7	Df=1, AIC=89.99, X^2 =0.25, p= 0.62
Head body length (mm)	672.22±23.6	9	662.5±35.15	8	Df=1, AIC=-167.54, X^2 =0.64, p= 0.423
Body Condition Index	0.3±0.02	9	0.3±0.04	8	Df=1, AIC=-68.55, X^2 =0.015, p= 0.903
Subcaudal secretion volume (ml)	0.48±0.42	8	0.9±1.46	5	Df=1, AIC=16.06, X^2 =0.44, p= 0.506
Grouping based on EGM in female cubs					
Somatic difference (at 15 - 18 months) between groups					
Somatic parameter	Groups based on EGM				Linear mixed model statistics (accounting for year as random effect)
	SV		NV		
	Avg + std	N	Avg + std	N	
Zygomatic arch (mm)	83.41±2.22	22	82.85±3.96	124	Df=1, AIC=802.84, X^2 =0.91, p= 0.340
Head body length (mm)	678.1±18.54	21	675.53±26.59	148	Df=1, AIC=1578.8, X^2 =0.59, p= 0.441
Body Condition Index	0.3±0.01	21	0.3±0.02	147	Df=1, AIC=-815.62, X^2 =0.19, p= 0.660
Subcaudal secretion volume (ml)	0.36±0.22	21	0.32±0.21	142	Df=1, AIC=-110.93, X^2 =0.96, p= 0.327
Somatic difference (at 11 months) between groups					
Somatic parameter	Groups based on EGM				Linear mixed model statistics (accounting for year as random effect)
	SV		NV		
	Avg + std	N	Avg + std	N	
Zygomatic arch (mm)	83.5±7.78	2	78.2±3.79	25	Df=1, AIC=158.97, X^2 =3.27, p= 0.071
Head body length (mm)	675±21.21	2	645.88±37.43	34	Df=1, AIC=364.26, X^2 =1.04, p= 0.309
Body Condition Index	0.32±0.02	2	0.31±0.03	34	Df=1, AIC=-157.70, X^2 =0.71, p= 0.400
Subcaudal secretion volume (ml)	0.2±0.0	2	0.18±0.14	33	Df=1, AIC=-34.14, X^2 =0.79, p= 0.373

Note: HO= High oestrone level group, LO= Low testosterone level group, SV= Swollen vulva group, NV= Normal vulva group.

Differences in somatic development between early and late developers

Males

Comparing the extent of somatic development between the two endocrinological phenotypes (at age 11 months), it was found that HT males (n=3) had significantly larger head-body length, zygomatic arch width and BCI than LT cubs (n=4); but did not differ in the volume of subcaudal gland secretion they produced. To test whether the early (endocrinological) development of individuals assigned to the HT group was simply the product of a more rapid development to adult size and were not just larger cubs but indeed early-developers (and were thus closer to being fully developed, sexually mature adults), comparison was made between the differences in head-body length and zygomatic arch width at the age above 28 months (i.e., fully developed adults) with those of cubs assigned to HT- and LT endocrinological categories at the age of 11 months. The difference in these measurements between HT-cubs and adults was significantly smaller than in LT-cubs and adults, confirming that HT-cubs were closer to being fully developed adults (see Table 1).

For males, comparing the growth curves of HT (8 repeat-measures over 33 months from 3 individuals) and LT types (32 repeat-measures over 33 months from 4 individuals) revealed a trend (albeit non-significant, likely due to limited sample sizes) for LT-cubs to grow more slowly than HT-cubs (X^2=1.873, df=7, p=0.392; non-linear mixed model), despite ultimately reaching similar adult head-body lengths, where HT had already reached 95% of the maximum head body length at the age of 11 months, whilst LT reached this percentage later at the age of 14 months. This difference in body size disappeared at the age of 19-20 months, when growth rates of HT- and LT-males equalised (Fig 8).

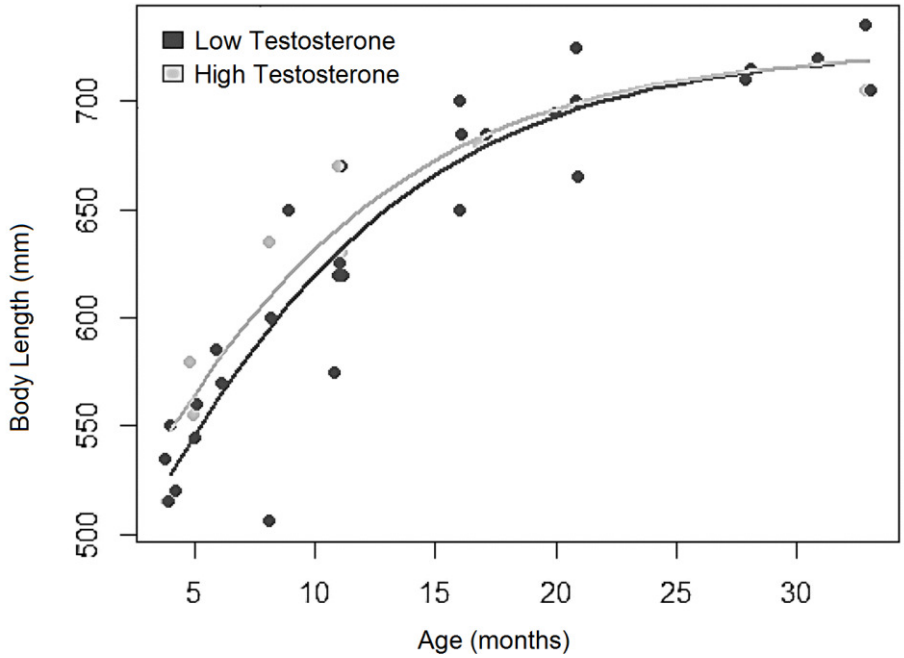


Figure 8. Body length growth curve of High Testosterone (HT, open circles) and Low Testosterone (LT, solid black circles) groups, age: 4-33 months

These analyses were repeated based on the degree of testicular descent at the age of 11 months, comparing cubs that had fully descended testes (DT; assumed to have reached puberty; n=18) with those that had ascended testes (AT; assumed to have not reached puberty; n=13). Overall, these showed similar differences in somatic development to categories arising according to endocrinological groups (for detailed results see Table 1). DT cubs had considerably larger zygomatic arch width, were in significantly better body condition/BCI, and had significantly more subcaudal secretion at 11 months of age than AT males; but no difference was found in head body length. DT cubs also had significantly smaller adult-cub differences in head-body length and zygomatic arch width than AT cubs, which corroborated that (like HT-males) they were closer to adulthood.

Mirroring the trend found in the endocrinological phenotypes, there was a significant difference in the growth curve of these two groups (Fig 9; X^2=10.087, df=8, p=0.018), with DT cubs (117 repeat-measures taken over 35 months from 18 cubs) growing faster, and reaching adult size earlier than AT cubs (99 repeat-measures taken over 35 months from 13 cubs). At the age of 19-20 months this difference disappeared and growth rates of DT and AT cubs equalised.

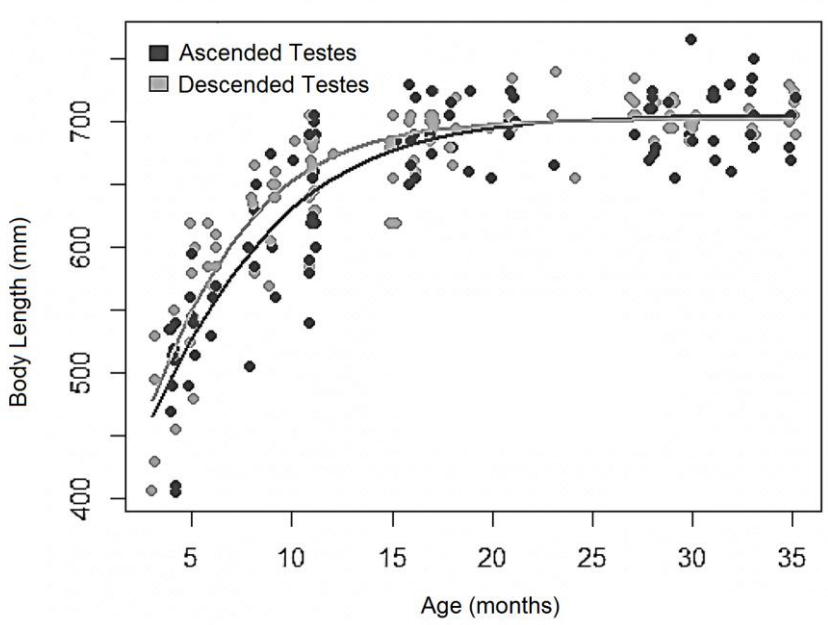


Figure 9. Body length growth curve of Descended Testes (DT) cubs and Ascended Testes (AT) cubs, age: 3-35 months

Females

In contrast, for females, no (significant) differences were found between any of the somatic parameters, nor for subcaudal gland volume, when comparing between the early (n_{HO}= 9) and late (n_{LO}= 8) developing endocrinological phenotypes (see Table 2). To ensure that the somatic similarity found between the two endocrinological phenotypes was not an artefact of the smaller sample size for the endocrinological dataset, vulva category at the ages between 15-18 months were used as the criteria defining stage of development. Again, on this basis, no significant difference was found (see Table 2) in any somatic parameters nor subcaudal gland volume between SV cubs (n= 24) and NV (n=152), evidencing that both vulva condition types exhibited similar body size by age.

However, because some female cubs first exhibit vulval swelling at the age of 11 months (n_{SV}=2, n_{NV}=34), these analyses were repeated (see Table 2) at this younger age, but again found no significant differences in BCI, head-body length, zygomatic arch width, nor subcaudal gland volume.

Social factors influencing the timing of puberty

At the age of 11 months, testosterone levels in male cubs tended to be lower in larger natal social groups (see Table 3)That is, male cubs born/ growing up in smaller social groups may be more likely to be early developers than those born/ growing up in larger groups/ setts. Using degree of testicular descent at the age of 11 months instead of the endocrinological phenotype, however, suggested no trend (see Table 3). In females, neither oestrone levels nor EGM at the age of 15-18 months showed correlation with social parameters tested (see Table 3).

Table 3. Social factors affecting the timing of puberty in male and female cubs

Social factors affecting timing of puberty in male cubs (11 months)				
Social factor	Testosterone		EGM	
	Model coeff.	Param. significance	Model coeff.	Param. significance
Number of adults in natal social group	Estimate= -0.43, SE= 0.33	Df=1, AIC=39.22, X^2 =2.05, p= 0.152	Estimate= 0.17, SE= 2.44	Df=1,AIC=194.08, X^2 =0.06, p= 0.807
Number of adults in natal sett	Estimate= -0.35, SE= 0.27	Df=1, AIC=30.47, X^2 =2.06, p= 0.152	Estimate= 1.71, SE= 1.43	Df=1,AIC=155.84, X^2 =2.13, p= 0.144
Number of cubs in natal social group	Estimate= -1.59, SE= 0.81	Df=1, AIC=35.17, X^2 =4.06, p= 0.044	Estimate= 1.05, SE= 0.90	Df=1,AIC=113.85, X^2 =1.41, p= 0.234
Number of cubs in natal sett	Estimate= -3.57, SE= 1.37	Df=1, AIC=39.22, X^2 =3.24, p= 0.072	Estimate= 0.83, SE= 0.91	Df=1, AIC=84.46, X^2 =1.29, p= 0.256
Social factors affecting timing of puberty in female cubs (15 – 18 months)				
Social factor	Oestrone		EGM	
	Model coeff.	Param. significance	Model coeff.	Param. significance
Number of adults in natal social group	Estimate= -0.29, SE= 1.43	Df=1, AIC=166.57, X^2 =0.05, p= 0.829	Estimate= -0.84, SE= 1.34	Df=1, AIC=1121.3, X^2 =0.39, p= 0.535
Number of adults in natal sett	Estimate= -0.25, SE= 1.92	Df=1, AIC=166.57, X^2 =0.02, p= 0.889	Estimate= -1.34, SE= 0.83	Df=1, AIC=898.67, X^2 =2.65, p= 0.103
Number of cubs in natal social group	Estimate= -3.18, SE= 4.79	Df=1, AIC=166.57, X^2 =0.49, p= 0.483	Estimate= 0.16, SE= 0.51	Df=1, AIC=627.17, X^2 =0.07, p= 0.788
Number of cubs in natal sett	Estimate= -2.13, SE= 9.03	Df=1, AIC=156.18, X^2 =0.06, p= 0.801	Estimate= -0.07, SE= 0.49	Df=1, AIC=391.39, X^2 =0.02, p= 0.888

Note: Coeff= Coefficient, Param= Parameter.

DISCUSSION

It was demonstrated that, in badgers, puberty begins in both sexes at ca. 11 months of age, when cubs develop similar seasonal sex-steroid patterns to adults. Furthermore, in both sexes, all parameters that support reproductive activity and mating-associated behaviours, such as

external genitalia morphology (and in males also testes volume), and subcaudal gland secretion volume, show similar developmental patterns to sex steroid levels, further corroborating the onset of puberty (Onyango et al. 2013). The increase of sex-steroid levels likely triggers changes in EGM (Research Chapter 2), as well as in the activity of species-specific subcaudal glands important in the context of reproduction and sexual advertisement (Macdonald et al. 2004). Nevertheless, cub titres typically remained lower than those reported for adults (males: Buesching et al. 2009; females: see Research Chapter 1) until their second mating season (22-26 months).

In male cubs, testosterone levels remained low and exhibited no seasonal variation until the first winter mating season, when they started to increase, reaching a smaller peak than in adults (Buesching et al. 2009). Levels then remained elevated until the end of the mating season, and followed the seasonal pattern typifying adults thereafter. By the time male cubs reached the second population breeding season, their testosterone titres had reached higher levels (compared to the first mating season) in accord with adults (Buesching et al. 2009). Baculum growth also reached the population-based asymptote by the cubs' second mating season, indicating the completion of sexual development. These findings support the hypothesis by Whelton and Power (1993), who measured baculum length post-mortem in road kill and culled badgers, positing that the observed abrupt decrease in baculum growth rate coincides with sexual maturity, although their post-mortem study was unable to verify this conclusion through endocrinological measurements.

In contrast, in females, there was a gradual increase in oestrone from the age of 3-11 months (May-January) without any noticeable seasonal variation. After age 11 months, however, cub oestrone levels started to follow the same seasonal pattern as adults (see Research Chapter 1), with high levels in spring and low levels in summer. Nevertheless, although adult female oestrone levels typically increase in autumn and remain elevated until blastocyst implantation in December (see Research Chapter 1), cub oestrone levels remained low until the next mating season (January), implying that – counter to observations from other, low-density studies (Ahnlund 1980) - no female cubs in the dataset were capable of mating successfully during their first year, corroborating genetic results from the study population reported previously (Dugdale et al. 2011).

Inter-individual variation in plasma sex-steroid levels, however, was considerable among same-age cubs of either sex, and two distinct categories: early and late developers were observed. It was inferred that these qualify as distinct phenotyptic response types, given the potential fitness advantage of early maturity (Abrams 1991), but set against the reality of resource limitation and social stress in wild populations typically precluding all individuals from engaging in the maximal developmental response (Macdonald et al. 2002). In male cubs, endocrinological profiles and EGM indicated that some had reached sexual maturity at the age of 11 months, while the remaining cubs likely achieved this only during their second winter. At this time all male cubs showed similarly large testosterone peaks comparable to adult levels and puberty had concluded. Similarly, there was substantial variation in the proportion of the final body length males had achieved by age 11 months, mirroring the differences in testosterone levels observed during this period. This increase in head body length ceased by age ca. 18 months (99 % towards asymptote; Research Chapter 5) which is also the age at which body lengths equalised when dividing males according to both testes descent categories (DT and AT as well as endocrinological categories HT and LT). Nevertheless, when cross-referenced against assigned paternity data, none of the individuals exhibiting high hormone levels at 11 months (HT=3 individuals) were assigned cubs the spring after. For female cubs, variation in oestrogen levels was high during their second summer (May-Sept/ Oct), indicating that not all females reached sexual maturity at the same age, but that puberty onset varied between 15-18 months of age.

The existence of different ontological phenotypes (i.e., early and late developers) has been described in other members of the Mustelidae, and has been linked to body size and species-specific life history traits (Amstislavsky and Ternovskaya 2000). For example, in captive sables (*Martes zibellina*), a small proportion of all individuals are reported to reproduce at 15 months of age, whereas the majority (80%) of males and females start breeding at the age of 27 months (Amstislavsky and Ternovskaya 2000). The results support observations from badgers in Sweden, where spermatozoa were first recorded in male cubs at the age of 12 months (Ahnlund 1980). Nevertheless, within a Swedish low-density population, the majority of males reached puberty in their first year, and only a minority of males did not produce spermatozoa until their second summer, or even winter (24 months: Ahnlund 1980). In this high-density population, in contrast, most males reached puberty in their second year.

Overall, these results support the hypothesis that mammals typically need to reach a threshold body size for sexual maturation (Freetly et al. 2011). Thus, the age at which puberty occurs is likely not only influenced by the gene load of the individual but also by ecological factors such as access to food (affected by weather/ climate), competition, and differences in demographic factors (Onyango et al. 2013). Resource availability tends to vary across time and space (Macdonald and Johnson 2015), and access to resources is further constrained by the number of competing conspecifics present leading to social stress (Macdonald et al. 2004). Consequently, energy budgets can differ substantially between individuals even within a single population/ year, with the potential to drive considerable variability in the timing of sexual maturity (Onyango et al. 2013). Individuals that develop under poor nutritional conditions, or subject to more social stress resulting from competition, usually reach sexual maturity at slower rates (Plaistow et al. 2004; Alberts 2012; Pusey 2012) as implied by the observation (of the 2 groups based on hormone levels alone) that male cubs born into larger social groups tended to be biased toward the late developer phenotype.

Generally, in mammals (especially those with polygynous mating systems), males tend to grow more quickly than females (Birgersson and Ekvall 1997), and ultimately attain a larger body-size (i.e., dimorphism; see Badyaev 2002). The data show that this is also the case in badgers (see also Research Chapter 5; NB, measurements were made after weaning, and thus obviating differential maternal investment effects; Clutton-Brock et al. 1985). Thus, males are likely also more vulnerable to resource limitation and social competition, with the potential to impact their degree of development by the end of their first year, explaining the observed delay in puberty in larger social groups (Leblanc et al. 2001; Fest-Bianchet et al. 1994). The findings, are congruent with those for female brown bears (*Ursus arctos*), where adult body size shows a negative relationship with population density (Zedrosser et al. 2006): female bears are larger and grow faster in areas with better environmental conditions, while with higher resource competition, females are smaller and grow more slowly. As in this badger study, bears have been shown to compensate for slower growth rates by delaying reproductive activity (Zedrosser et al. 2006) at the potential cost of lower lifetime reproductive success (Stearns 1992). Similar negative correlations between population density and individual growth rate have been reported in the northern fur seal (*Callorhinus ursinus*; Fowler 1990), polar bears (*Ursus maritimus*; in terms of smaller juvenile body length; Derocher and Stirling 1998; and adult body size; Derocher and Wiig 2002), and in

American black bears (*Ursus americanus*; with lower yearling weight; Garshelis and Hellgren 1994). Similarly, in farmed red deer (*Cervus elaphus*), Blanc and Thériez (1998) found that the growth rate of subordinate females was 2.5 times slower, and average daily weight gain of all juvenile hinds was significantly impaired, under high stocking density. Demographic effects have also been proven to affect the onset of maturation in female baboons (*Papio cynocephalus*), where first menstruation was earlier in smaller groups where individuals experience less social stress and competition (Altmann and Alberts 2003).

It is thus concluded that the asynchronous timing of puberty, leading to two heterochronous phenotypes, can occur even within a single population, and is likely caused by individuals attaining the required minimum body size according to different time scales. Ultimately, capacity to breed at a young(er) age can have profound effects on life-history trade-offs (see Campbell et al. 2017) with early-life success often being critical to an individual's fitness (Abrams 1991) and can substantially enhance population growth rate (Promislow and Harvey 1990).

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RESEARCH CHAPTER 4

Reproductive and somatic senescence of the European badger (*Meles meles*): Evidence through lifetime sex-steroid profiles

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ABSTRACT

There is sparse evidence of corroboration or substantive fitness benefits of post reproductive life-span (PRLS, number of living years after reproductive cessation; RC) among the Carnivora. In older ages, selection for age-specific detrimental genetic traits weakens as the proportion of surviving individuals decreases, resulting in eventual evolution of reproductive and somatic senescence, with trajectories differing between sexes in relation to their reproductive physiology, investment in offspring and associated resource allocation. The extent to which an individual furthur reproduces may be precluded by hormone production ('constraint') or somatic condition ('restraint'). Using cross-sectional endocrinological and morphological data, with the European badger (*Meles meles*) as a model species, it was investigated in each sex, if and when individuals exhibit: 1) age-dependent reproductive declines in sex-steroid levels in all seasons; while also determining the prime age of reproduction; 2) age-dependent declines in somatic condition and reproductive advertisement, relative to the onset of reproductive senescence. 3) changes in reproductive strategy with age; specifically what proportion of badgers attain peak reproductive age. 4) evidence for RC and PRLS with reference to numbers of cubs assigned to older individuals, from pedigree. Strong evidence was provided for gradual, not abrupt, decline in sex-steroid levels with age where both sexes followed a concave (down) quadratic trend. For both sexes, the onset of decline in somatic condition commenced earlier, starting at the age of 3 years, than any decline in reproductive hormones, starting around age 5.5 years in females and 6 years in males, with similar rates thereafter. There was also a decrease in subcaudal gland secretion volume with age, suggesting investment in reproductive advertisement also decreases. Fewer older females were assigned cubs, starting at 3 years, coinciding with the onset of somatic decline, but earlier than the hormone decline identified (5.5 years onwards). Declines in offspring assignments commenced later in males at age 5 - 6 years; concomitant with onset of testosterone decline at age 6 years. Males exhibited a similar age of onset of somatic decline in females (3 years), suggesting that declining body condition does not preclude reproductive success in males (no 'restraint') in advance of hormonal senescence ('constraint'), contrary to females. There was evidence of PRLS, with males living up to 2.02±1.50 years and females 2.59±1.29 years post RC. The implications for these findings are discussed in the context of adaptive and non-adaptive hypotheses regarding RC and PRLS.

Key words: *European badger, oestrone, post-reproductive lifespan (PRLS), reproductive strategies, reproductive cessation (RC), somatic condition, testosterone.*

INTRODUCTION

A crucial question in understanding the life-histories of wild-living mammals, and related population ecology, is whether individuals experience a post reproductive life-span (PRLS; Cohen 2004; see Medawar 1952), and what proportion of individuals live long enough to reach post-reproductive age. This leads to a second question: while older individuals may be endocrinologically capable of reproducing, what proportion actually do produce young successfully differs with what constraints influence later-life reproductive capacity, such as the somatic condition of the individual, or the age structure among competing individuals in that population (Croft et al. 2015).

The evolutionary origins of PRLS have been much debated (vom Saal et al. 1994; Austad 1997; Peccei 2001; Shanley and Kirkwood 2001), where natural selection should disfavour the maintenance of any proportion of the population not maximizing reproductive potential. A possible exception to this among social species may occur when older individuals provide genuine benefits to the reproductive success of kin. This is termed the grandmother hypothesis in primatology which generally involves a slowing of somatic senescence without favouring extension of the fertile life span (Hawkes et al. 1998; Alvarez et al. 2000). However, there is sparse evidence of corroboration or substantive fitness benefits among the Carnivora; limited to descriptions of females in the domestic dog (*Canis domesticus*; lifespan: 15 years, reproductive cessation/ RC: 9 years, 50% reach RC), domestic cats (*Felis catus*; lifespan: 20 years, RC: 14 years), wild lions (Panthera leo; lifespan: 17 years, RC: 14 years, 15% reach RC), and wild polar bears (*Ursus maritimus*; lifespan: 30 years, RC< 23 years, 1.5% reach RC), which all exhibit PRLS (Cohen 2004; but see: African wild dogs, *Lycaon pictus* – Malcolm and Marten 1982; Giant otters, *Pteronura brasiliensis*; Davenport et al. 2011).

Nevertheless, counter to this, in species/ populations where starvation, disease and predation take a systematic toll on cohorts, there are always fewer older individuals in a population than there are younger ones. Consequently, the strength of selection on alleles with age-specific fitness effects is expected to weaken with increasing age, and detrimental genetic traits (such as PRLS) can persist (or even be favoured) provided their deleterious effects are delayed until an advanced age is reached, relative to the pervading schedule of mortality. This would imply

an age beyond which selection would be too weak to prevent the onset of reproductive senescence (Croft et al. 2015), resulting in the eventual evolution of physiological mechanisms leading to reproductive and somatic senescence (Gavrilov and Gavrilova 2001). In long-lived mammals, reproductive potential typically follows a bell shaped curve, increasing after puberty to a prime age peak, then decreasing until menopause, or death (Jorgenson et al. 1997). There is, however, no evolutionary benefit of maintaining fertility if somatic senescence progresses to the stage that successful reproduction is prevented, and although onset of somatic senescence generally occurs earlier than reproductive senescence, reproductive cessation (RC; Cohen et al. 2004) typically occurs prior to somatic condition becoming inadequate to sustain reproductive activity (Berube et al. 1999; Mysterud et al. 2002; Schwartz et al. 2003; Nussey et al. 2008; Descamps et al. 2008).

This raises the important question of evaluating the extent to which further reproduction is precluded by inadequate hormone production (failure to ovulate / reduced spermatogenesis – but see below), termed 'constraint', versus the failure of reproductively capable older individuals to realize successful offspring production, due to inadequate somatic condition and/or competition from younger conspecifics, termed 'restraint' (see McNamara et al. 2009). Additionally, once individuals approach the conclusion of their reproductive lifespan, they may also make a 'terminal investment' (Clutton-Brock 1984), involving a disproportionate allocation to reproduction and sacrificing subsequent survival (see Campbell et al. 2017).

Importantly, the selective basis of senescence also differs between the sexes in relation to their reproductive physiology, investment in offspring and associated resource allocation (Bonduriansky et al. 2008), where females typically experience constraint (menopause), while males experience restraint due to reduced competitive ability (Bribiescas et al. 2012). Consequently, reproductive senescence (Finch, 1990) and somatic senescence (Reed et al. 2008) are expected to follow different sex-specific trajectories, which can occur asynchronously (Cohen 2004) leading to heterochrony across populations (Campbell et al. 2017).

Senescence in male reproductive strategies

Male reproductive strategies generally involve weaker selection for longevity, typically following a 'live fast, die young' schedule (Carranza and Pérez-Barbería 2007). In most

mammals, particularly highly polygynous species, males invest predominantly in maximizing the number of females they mate with, resulting in intra-sexual competition for mate access; an energetically costly, high-risk strategy, but one that can yield higher fitness over short time periods (Clutton-Brock and Isvaran 2007; Preston et al. 2011). This strategy can, however, incur substantial cumulative somatic damage, which is detrimental to survival (Carranza and Perez-Barberia 2007) and likely to result in accelerated physiological decline with age (Kotiaho 2001; Hunt et al. 2004; Bonduriansky et al. 2008).

Endocrinologically, aside from promoting aggressive competition (Mougeot et al. 2004; Blas et al. 2006), testosterone also causes reduced immunocompetence, increased susceptibility to oxidative stress, and increased basal metabolic rate and energy expenditure (Bribiescas 2006; Alonso-Alvarez et al. 2007; Cox and John-Alder 2007). In most mammals, males can sustain spermatogenesis until the end of their lifespan (Cohen 2004), although often of lower quality sperm (Møller et al. 2009). Testosterone is produced by leydig cells and not by the sperm itself, and thus sperm production can continue even with low circulating testosterone levels (vom Saal and Finch 1988). Nevertheless, older males tend to undergo physical decline, invest less in sexual traits (e.g. reproductive advertisment, controlled by testosterone), and have poorer mating performance, and thus reduced reproductive fitness (Cohen 2004).

Senescence in female reproductive strategies

Aside from some female social mammals investing in reproductive suppression (Creel and Creel 1991), a female's primary route to greater fitness is via allocating more available energy into offspring, leading to a more conservative lifetime reproductive strategy than in males (Bonduriansky et al. 2008). Gestation and lactation, however, incur unavoidable costs towards female fitness (see Clutton-Brock et al. 1989), and therefore physiological decline among older females can lead to less energy allocated to reproduction (Kirkwood and Austad 2000; Mysterud et al. 2001). While males are able to increase reproductive fitness by enhancing mating efforts (sacrificing longevity), females are limited by the frequency of oestrus cycles/ number of oocytes, pregnancy duration, and inter-birth interval (largely driven by hormonal homeostasis; Adkins-Regan 2005; Nussey at al. 2009). This can lead to greater variance in relative fitness between males (depending on social system) than between females (Nussey at al. 2009), due to females having less strategic flexibility and more fixed investments in attaining only a single litter per reproductive cycle (Vinogradov 1998; Clutton-Brock et al. 1981). Ultimately, oocyte depletion (affecting oestrogen levels, as they

are produced by mature follicles containing oocytes: Adkins-Regan 2005) leads to RC prior to death in menopausal mammal species (although not all undergo RC; Cohen 2004; Nozaki et al. 2015), which has the potential to be more impactful on population dynamics (depending on relative age / sex pyramids) than RC among elderly males (Rankin and Kokko 2007).

Study species: the European badger, *Meles meles*

To investigate endocrinological and somatic age-dependent decline in a long-lived, medium-sized, group-living carnivore, a 29 year - data set (1987-2016) describing the detailed life-histories of 1692 individual European badgers, *Meles meles* (henceforth 'badger') from Wytham Woods, Oxford, UK was utilized, with an extant population size averaging 183.53 (42.93) adults and 50.43 (19.20) cubs per year (Bright Ross et al. in prep), utilizing 6 km^2 range. These data, and associated inventory of blood samples, allowed us to select candidate individuals with longer realized lifespan (up to the age of 13 years), from the broader population. Genetic pedigree for this population (Annavi et al. 2011) and previous genetic analyses examining changes in breeding success and assigned litter size with age (Dugdale et al. 2011) were also used, allowing to test for reproductive restraint among older individuals that were apparently still capable of reproduction as indicated by their hormone levels.

Badgers are seasonal breeders (Amstislavsky and Ternovskaya 2000) that produce one litter annually (mean litter size = 1.4±0.06, range of 1-4 cubs, where 93% litters comprise less than 3 cubs: Macdonald et al. 2015). Cubs are typically born during mid-February in the UK (76% of births; although parturition can occur from mid January – mid March; Neal and Cheeseman 1996; Yamaguchi et al. 2006), and first emerge from underground burrow systems (termed setts) around mid-April, being weaned by mid-May (Fell et al. 2006). The main mating season occurs during the post-partum oestrus in January-March (extending until May/ June; Yamaguchi et al. 2006). Scent marking activity using subcaudal gland secretion increases markedly during the mating season, (Buesching and Macdonald 2001) to promote olfactory mate guarding (Buesching et al. 2002a, Buesching et al. 2003), resource defense and reproductive advertisement (Buesching et al. 2002b, Buesching and Macdonald 2004). During autumn, badgers go through reproductive quiescence and typically reach their maximum weight and body condition (Macdonald et al. 2010; Woodroffe and Macdonald 1995a).

Female badgers exhibit delayed implantation, where blastocysts remain suspended in the uterus lumen for up to 11 months (Corner et al. 2015), linked to an endocrinological physiology where more oestrone than oestradiol is produced to sustain pre-implanted blastocysts (see Research chapter 1). Badgers reach sexual maturity around 11 months old in both sexes (see Research chapter 3), but, in this population in Wytham Woods, typically do not start breeding until the age of 2 years (Macdonald et al. 2009; Dugdale et al. 2011). Once badgers reach sexual maturity, their circulating sex-steroid levels show a distinct seasonal pattern (Buesching et al. 2009; Research chapter 1 and 2): In males, testosterone levels are high in spring, slightly lower in summer, lowest in autumn and peak during the winter mating season. In females, oestrone levels are high in spring, low in summer, peak in autumn and remain high for pregnant females in winter but decline in non-pregnant females.

Confirmed maximum lifespan in badgers in the Wytham study population ranges up to 14 years (Macdonald et al. 2015), although only ca. 21.4% (26% females; 16.6% of males; Bright Ross et al. in prep) live up to 7 years. Breeding in badgers requires sufficient fat reserves (Woodroffe and Macdonald 1995a), and involves conspicuous reproductive advertisement (by scent marking: Buesching et al. 2003, Buesching and Macdonald 2004). Therefore, breeding has been linked to a decline in somatic condition (due to pregnancy and lactation in females; mating effort in males), greater bite wounding rates, higher parasite infestation, and anaemia (especially in males; Woodroffe and Macdonald 1995a; Macdonald and Newman 2002; Buesching et al. 2009).

Using cross-sectional endocrinological (due to gaps in individual records; see Methods) and morphological data it is investigated, for each sex, if and when individuals exhibit:

1) age-dependent reproductive declines in sex-steroid levels during spring, summer, and autumn; while also determining the prime age of reproduction;

2) age-dependent declines in somatic condition and reproductive advertisement (subcaudal volume), relative to the onset of reproductive senescence.

3) changes in reproductive strategy with age; specifically what proportion of badgers attain peak reproductive age;

4) evidence for RC and PRLS (or post-reproductive presentation, PrP, as a population measure of PRLS, see Levitis et al. 2011), with reference to numbers of cubs assigned to older individuals, from pedigree (Dugdale et al. 2011; Annavi pers comm).

Implications for these findings were then discussed in the context of adaptive and non-adaptive hypotheses regarding RC and PRLS.

MATERIALS AND METHODS

Badger trapping and sampling

Blood samples and population biometric data were collected from a high-density badger population (44.55 ± 5.37 (SE) badgers/km^2; Macdonald et al. 2015) in Wytham Woods, Oxfordshire, UK (51^{o}46:26 N, 1^{o}19:19 W; for details see Savill et al. 2010; Macdonald et al. 2015), between 1987-2016, as part of an ongoing long-term research project. Following the methodology described in Macdonald and Newman (2002; 2009; see also Sun et al. 2015), badgers were trapped three times annually, routinely over two weeks, in cage traps baited with peanuts: (1) in spring (May/June) during the post-weaning period for females where it was still within the main mating period identified for both sexes, (2) in summer (August) the second mating peak previously reported in other badger populations, and (3) in autumn (November) during reproductive quiescence when badgers reach their maximum body weight, prior to a legal closed season during late pregnancy and cub rearing (Protection of Badgers Act, 1992).

Captured animals were transferred to holding cages and transported to a central field station between 6.30-8.00 am, before being sedated by intramuscular injection of 0.2 ml ketamine hydrochloride/ kg body weight (McLaren et al. 2005; Thorton et al. 2005). All badgers received a permanent unique tattoo, allowing individual identification (ID) and subsequent aging**.** All protocols and procedures employed were approved by the Animal Welfare and Ethical Review board of Oxford University's Zoology Department and procedures were conducted under the Animals (Scientific Procedures) Act, 1986 (currently PPL: 30/3379).

Somatic measurements and subcaudal gland volume

Following sedation, somatic measurements for all individuals were carried out. Body-length was measured from the tip of the snout to the base of the sacrum (in mm) and individuals weighed to the nearest 100g; from this a Body-Condition Index (BCI) was calculated as $\log_{10}$(weight)/$\log_{10}$(body length), supplemented by a direct measure of body-condition

assessed by scoring body-fat along the spine and pelvis from 1 = emaciated to 5 = very good condition (see Buesching et al. 2009). Subcaudal gland secretion from the subcaudal pouch were also collected using a rounded stainless-steel spatula and volume was estimated to the nearest 0.05 ml. (Buesching et al. 2002a, b).

Blood sampling and hormone measurements

Blood samples were collected by jugular venepuncture in K2-EDTA (ethylene diamine tetraacetic acid) vaccutainer tubes (Becton-Dickinson) for endocrinological analyses. Following collection (within 30 minutes of sampling), blood samples were centrifuged at 10°C for 10 min under 2,500 rpm/ 1470G. Plasma was transferred into Eppendorf tubes and frozen at -20°C immediately. Sampling times were standardized to account for circadian variation in hormonal profiles (Buesching et al. 2009). To accommodate gaps in individual life-history recapture records (Cam et al. 2002; Van de Pol and Verhulst 2006) and to avoid bias that can arise if focusing only on longer lived life-histories due to selective mortality, causing the residual surviving proportion of the population to be comprised of higher quality individuals (e.g. Nussey et al. 2011), hormone samples were selected as a cross-section of the study population across years. Therefore, from the 1692 individuals (11.360 captures) on record, a single sample from 152 females and 203 males was selected, to include badgers of all ages 0-13. Had the focus was only on the longitudinal hormone profiles of longer-lived individuals, this could have failed to detect different mean hormone profile characteristics when including shorter-lived individuals.

All sex steroid titres were analysed using microtitreplate Enzyme immunoassays (EIA). Oestrone was measured following the methodology described in Research Chapter 1. The oestrone intra-assay coefficients of variation were 8.21 % (high) and 6.05 % (low); inter-assay variation of high and low-value quality controls were 13.96 % (high) and 13.62 % (low) respectively. Testosterone analyses were carried out following the methodology described in Research Chapter 2). The testosterone intra-assay coefficient of variation was 14.69 % (high) and 6.18 % (low), and inter-assay variation of high and low-value quality controls were 9.15 % (high) and 5.23 % (low).

Statistical analysis

A quadratic model (second degree polynomial curve) was fitted for testosterone and oestrone

levels against age (males: 0-13 years, females: 0-12 years) during spring (n_{males}= 67; $n_{females}$= 49), summer (n_{males}= 84; $n_{females}$= 60), and autumn (n_{males}= 52; $n_{females}$= 43) to analyse the trend of physiological senescence. To investigate whether badgers changed reproductive strategies with age at the population level, spring (i.e., peak mating season) data (for both sexes) were used to calculate the age at which mean maximum lifetime hormone curve peaked, then using the proportion of this mean, different age classes were attained. Age groups were categorized in both sexes as Cubs: < 1 year, Yearlings (Y): 1 ≤ Y < 2 years (which tended to have hormone levels < 80% of the adult peak), Young adults (YA): 2 ≤ YA < 6 years (hormone levels between 80-100% of the adult peak), Old adults (OA): 6 ≤ OA < 9 years (hormone levels declining from 100-80% of adult peak), and Very old adults (VOA) (hormone levels < 80% of adult peak): ≥ 9 years (see Figure 1).

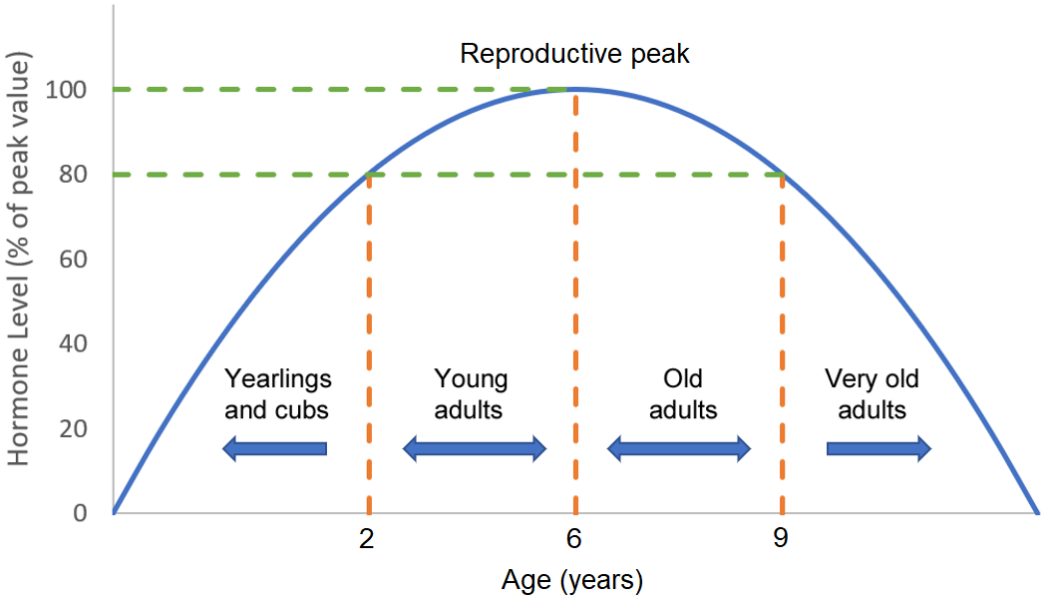


Figure 1. Classification of age groups based on the hormone quadratic curve.

The mean and standard deviation of hormone levels (for both sexes respectively) categorised by age group and season (Table 1) were plotted to inspect for any apparent changes in reproductive strategies.

Quadratic curves of sex-steroid levels during spring (n_{males}= 67; $n_{females}$= 49) were used to analyse the differences in schedules of physiological senescence between males and females, with reference against standardized (towards maximum value/concentration) hormone levels (with oestrone in females and testosterone in males on different scales) and plotted together

in Figure 6 (see RESULTS). To investigate trends in somatic senescence, metrics of BCI, body condition, and subcaudal gland secretion volume were analysed against age (years) separately in spring, summer, and autumn using a GAM (Generalized Additive Model), fitting a smoothing function (Table 2).

Table 1. Sample size of age classes (cub, yearling, young adult, old adult, and very old adult) during spring, summer, and autumn for males and females.

Season	Age group	Sample size	
		Female	Male
Autumn	Cub	5	9
	Old adult	9	11
	Very old adult	11	3
	Yearling	5	6
	Young adult	13	23
Spring	Cub	7	10
	Old adult	10	17
	Very old adult	11	11
	Yearling	5	9
	Young adult	16	20
Summer	Cub	6	18
	Old adult	18	16
	Very old adult	10	13
	Yearling	8	14
	Young adult	18	23

Table 2. Sample size of, BCI, subcaudal gland volume, and body condition in both sexes during spring summer and autumn.

Somatic parameter	Season	Sample Size	
		Males	Females
BCI	Spring	703	869
	summer	687	744
	autumn	422	556
Subcaudal gland secretion volume	Spring	535	723
	summer	475	559
	autumn	294	451
Body condition	Spring	652	813
	summer	559	513
	autumn	360	513

Patterns of residuals, normality, and mean variance relationship for each model were checked using diagnostic plots in R. All statistical analyses were performed using RStudio (0.99.896) and R (R-3.2.4).

RESULTS

Physiological senescence

Males

For males, age explained a substantial amount of variation in testosterone levels (quadratic curve – concave down), and levels were lower among older individuals. This was especially evident in spring and summer (reproductive season; see Fig. 2a,b); but less apparent in autumn (Fig. 2c). The quadratic curves of testosterone against age for all seasons combined (i.e., annual trends) are presented in Fig. 2d.

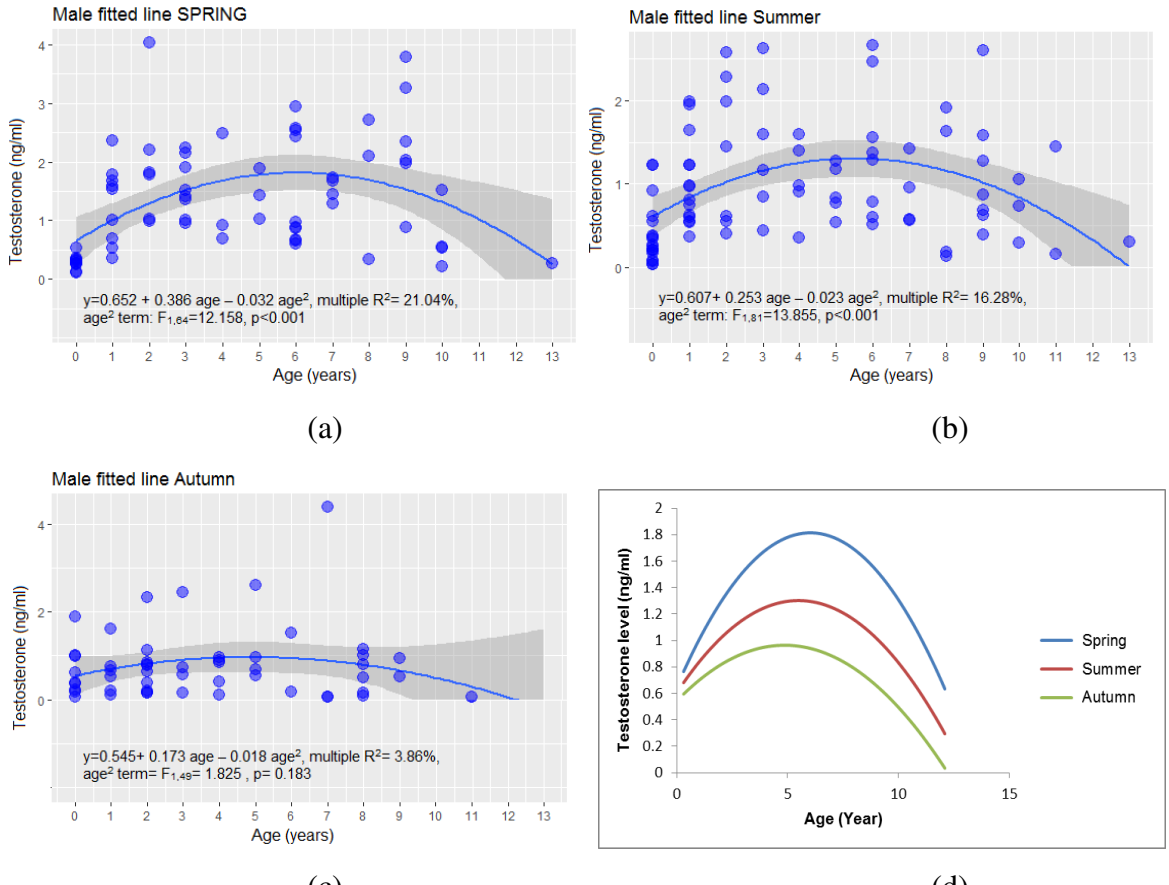


Figure 2. Quadratic curves of testosterone (ng/ml) against age in males during spring (a), summer (b), and autumn (c). Quadratic curves of all seasons combined (i.e. yearly means) (d).

With regard to male age class (Fig. 3), Young adults (YA) and old adults (OA) both had testosterone levels within 80-100% of the hormone level peak (at 6 years) and showed similar seasonal mean testosterone profiles (patterns) and levels (concentrations). Levels were highest in spring (YA = 1.64±0.77 ng/ml; OA = 1.55±0.86 ng/ml), slightly lower in summer (YA = 1.24±0.70 ng/ml; OA = 1.16±0.76 ng/ml), and lowest in autumn (YA = 0.84±0.71 ng/ml; OA = 0.90±1.26 ng/ml). Yearling (i.e., post-pubescent) males also exhibited similar seasonal profiles to young and old adults, only at lower absolute levels of concentration (spring = 1.28±0.67 ng/ml; summer = 1.01±0.53 ng/ml; autumn = 0.65±0.54 ng/ml). For very old adults (VOA), mean testosterone levels were slightly lower than for yearlings (summer = 0.92±0.68 ng/ml; autumn = 0.51±0.44 ng/ml), except for in spring (1.58±1.22 ng/ml), and thus substantially lower than in YA and OA. Intuitively, pre-pubescent cubs had different seasonal hormone profiles and levels from sexually mature males, which increased from spring (0.29±0.12 ng/ml) and summer (0.40±0.38 ng/ml) to peak in autumn (0.64±0.58 ng/ml) as they developed toward levels seen in yearlings (i.e., they attained puberty; Fig. 3).

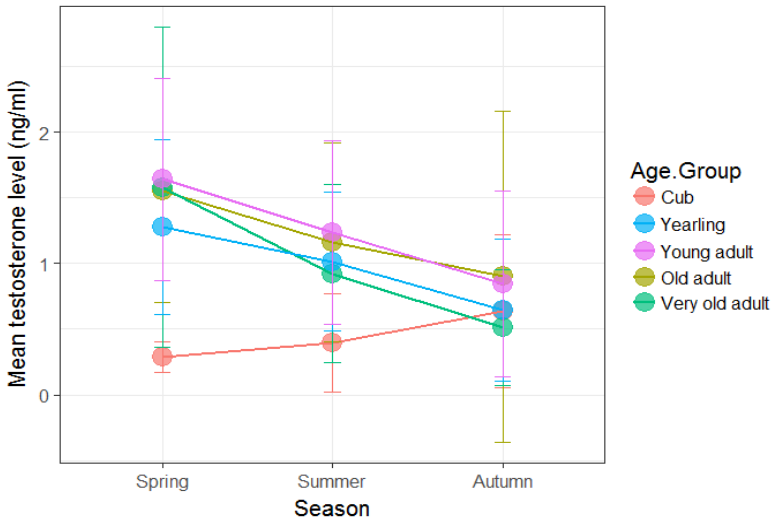


Figure 3. Seasonal mean testosterone levels (ng/ml) by age group.

Females

In females, age explained a substantial amount of variation in oestrone levels (quadratic curve – concave down), and levels were also lower among older individuals. This was especially apparent in spring and autumn (see Fig. 4 a, c). Age was not, however a good predictor of oestrone levels in summer (Fig. 4b). Quadratic curves of all seasons (i.e., annual trends) are combined in Fig. 4d.

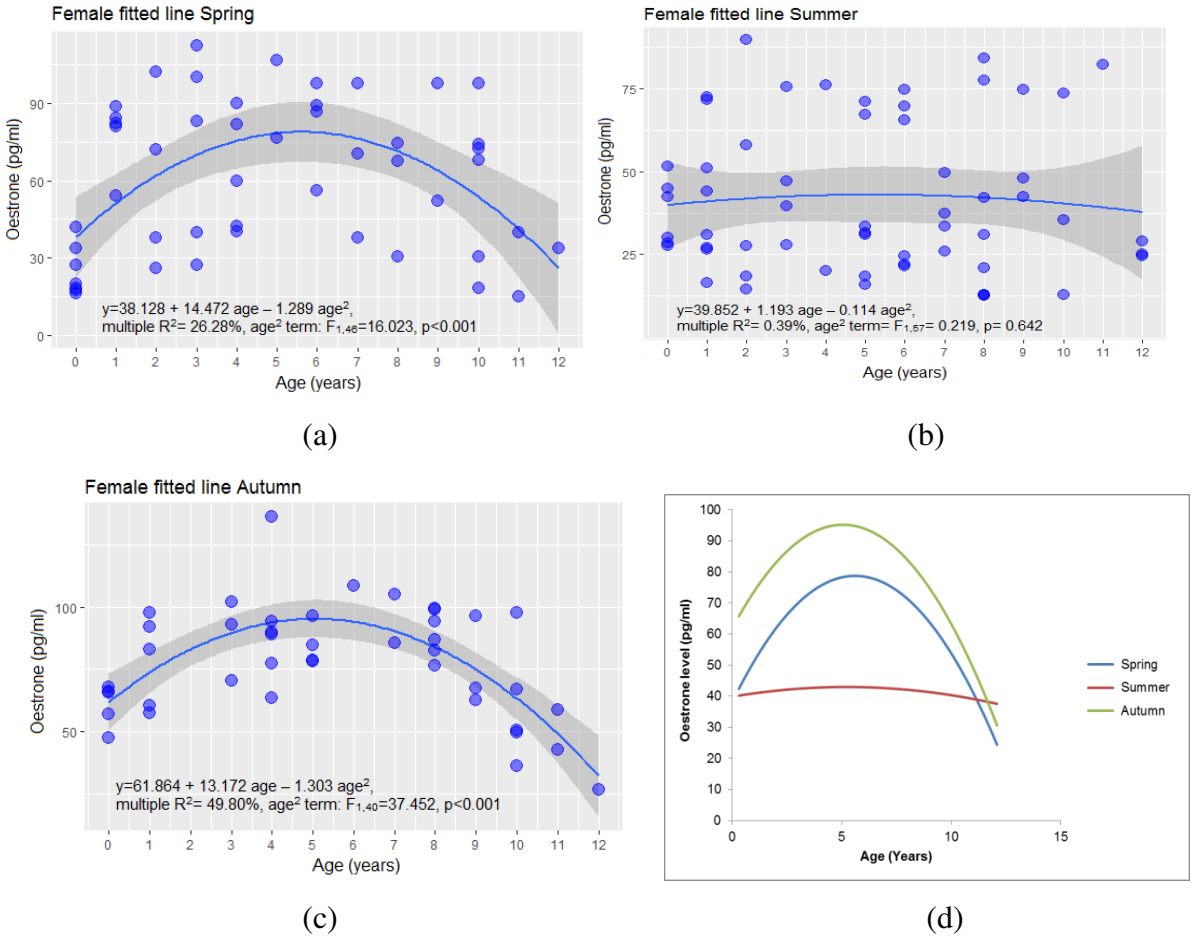


Figure 4. Quadratic curves of oestrone (pg/ml) against age in females during spring (a), summer (b), and autumn (c). Quadratic curves of all seasons combined (d).

With regard to female age class (Fig. 5), oestrone profiles (and levels) were similar among all age classes, except for (pre-pubescent) cubs, with mean oestrone levels in young and old adults being highest in spring (YA = 68.63±29.59 pg/ml; OA = 70.75±23.45 pg/ml), lowest in summer (YA = 42.36±24.27 pg/ml; OA = 39.89±24.20 pg/ml), and higher again in autumn (YA = 93.06±10.83 pg/ml; OA = 88.69± 17.92 pg/ml). Yearlings showed comparable seasonal trend patterns as YA and OA with similar hormone levels in summer (42.50±21.11 pg/ml), higher oestrone levels in spring (77.90±13.67 pg/ml) and lower in autumn (78.10±18.22 pg/ml). VOA also exhibited similar seasonal patterns as YA and OA with similar hormone levels in summer (44.75± 24.17 pg/ml), but lower concentration levels than YA, OA, and yearlings during spring (54.50±29.42 pg/ml) and autumn (59.51±22.48 pg/ml; comparable to pre-pubescent cub levels). In pre-pubescent cubs, osterone levels increased through spring (25.14±9.76 pg/ml) summer (37.50±10.14 pg/ml) and autumn (59.51±22.48

pg/ml), as they matured toward puberty. Yearling (post-pubescent) females then exhibited similar oestrone profiles to adults, but at lower levels (Fig. 5).

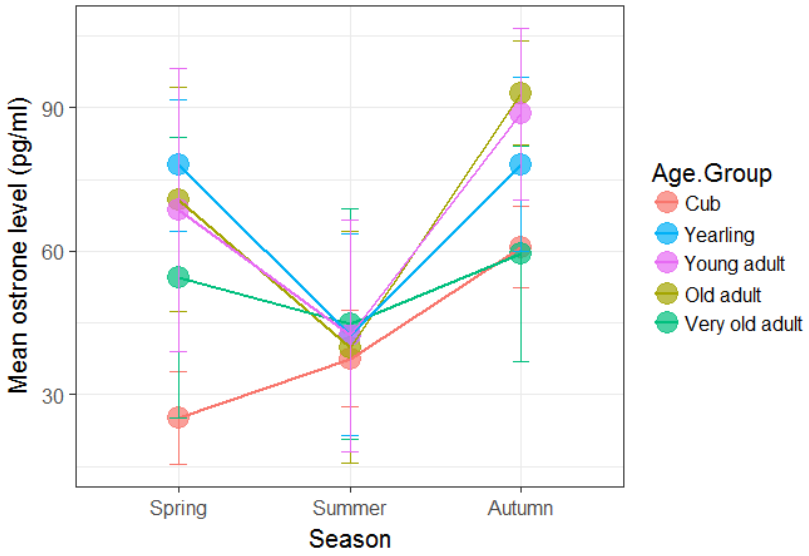


Figure 5. Seasonal mean oestrone levels (pg/ml) in 5 age groups.

Age-dependent male and female hormonal decline after reproductive prime age

Males and females exhibited similar standardized hormone profiles (quadratic concave down) in spring, when sex-steroid levels were at their seasonal peak. Females reached their reproductive peak/ prime-age at 5.5 years, slightly earlier than males, which peaked at 6 years (Fig. 6). Hereafter, the sexes showed similar schedules of hormonal decline indicating reproductive senescence.

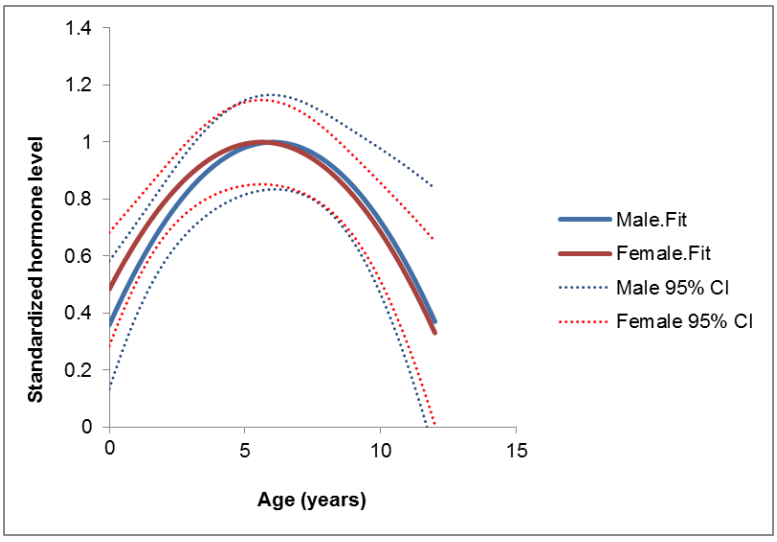


Figure 6. Comparison of male and female quadratic hormone curves during spring.

Age-dependent somatic decline

A gradual post-maturity reduction in BCI and body condition as badgers become older, was found in both sexes in all seasons. The GAM model, testing BCI values as a smoothed fuction of age, revealed that BCI (Fig. 7) started to decline after age 3 years, but more markedly after age 5 years (see Table 3). Decline for both sexes was most most evident in autumn, less so in summer and weakest in spring (Fig. 7).

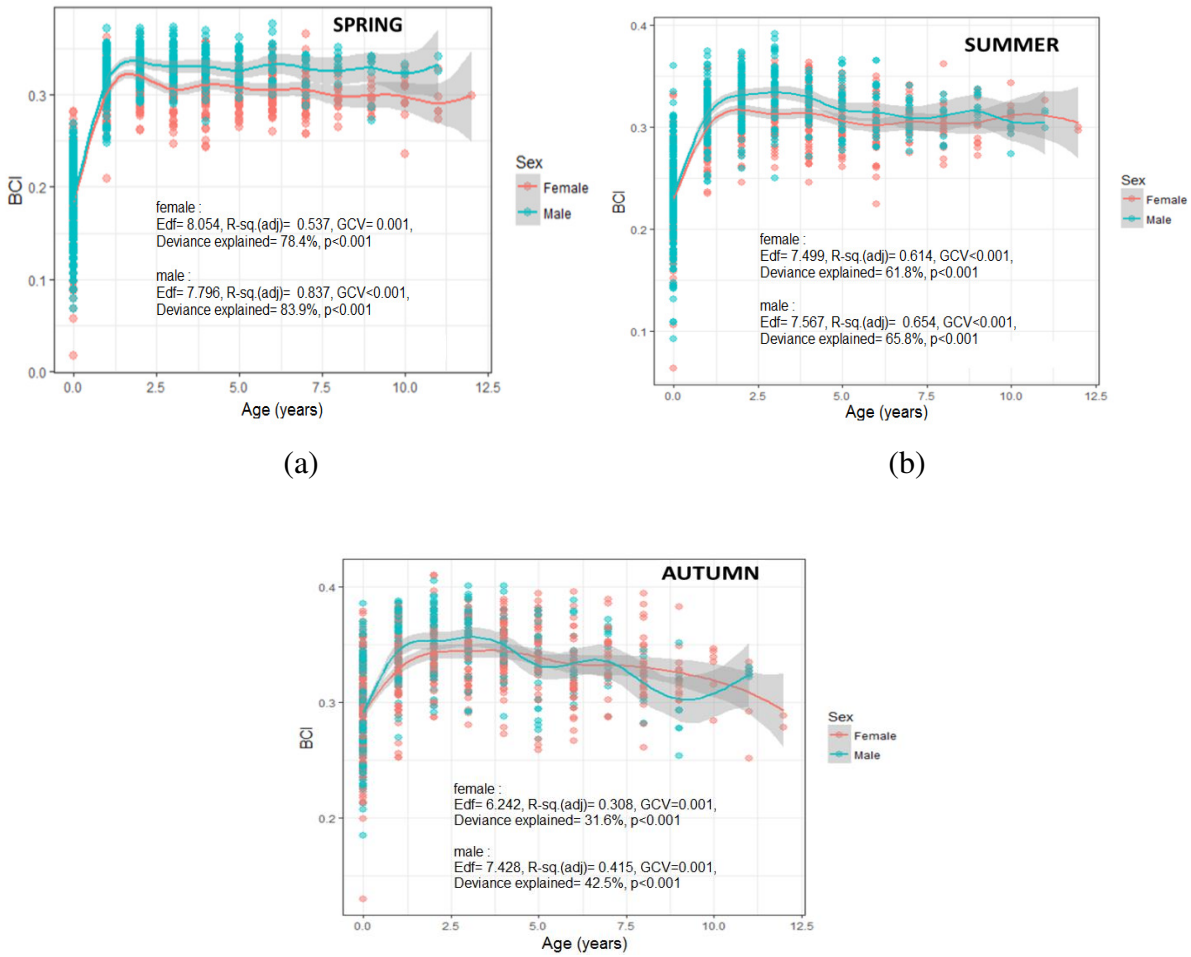


Figure 7. Body condition index (BCI) against age in males and females during spring (a), summer (b), and autumn (c).

This gradual reduction in somatic condition for both sexes with age in all seasons was further corroborated by a simple metric of body condition, with onset starting at age 3 years, but with a steeper rate of decline beyond age 5 years (Fig. 8, Table 3).

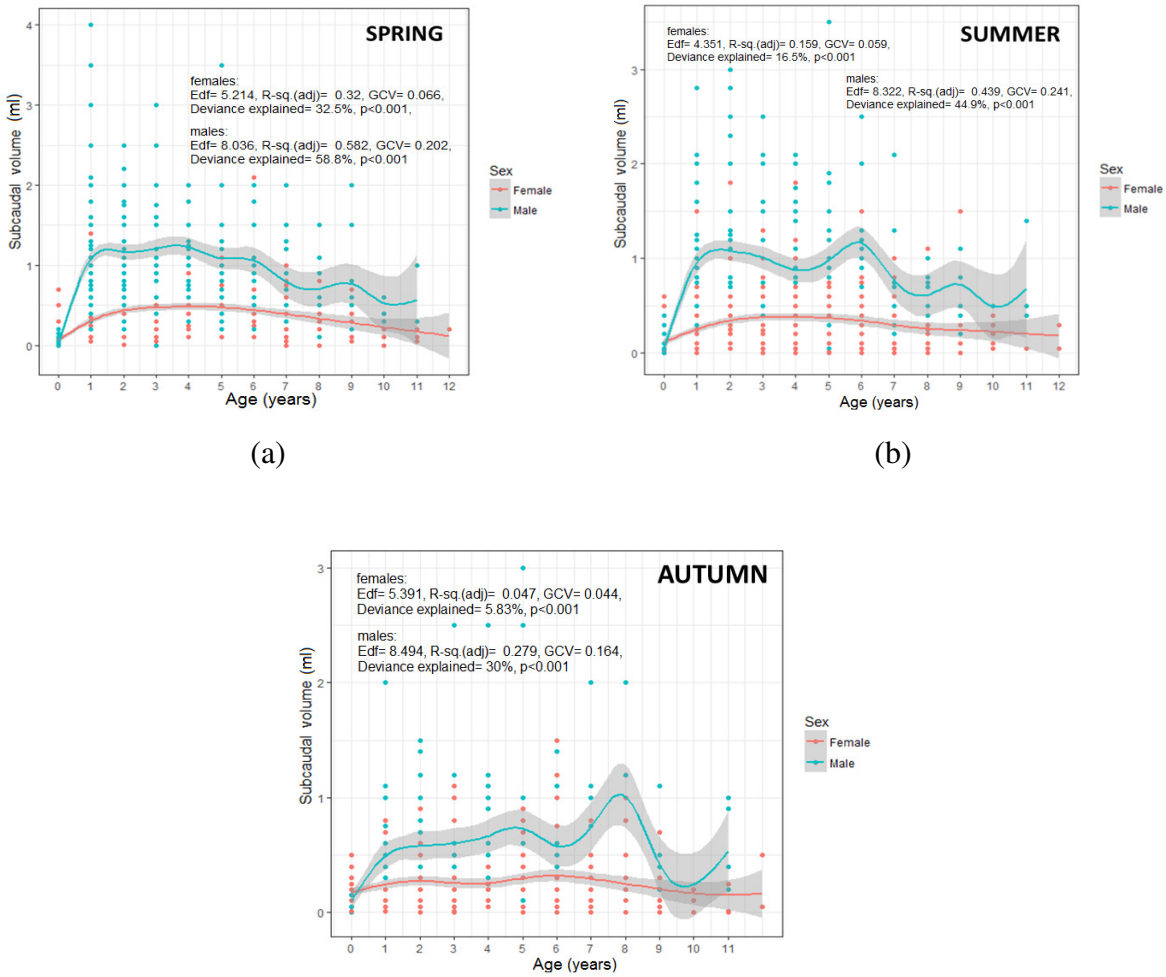
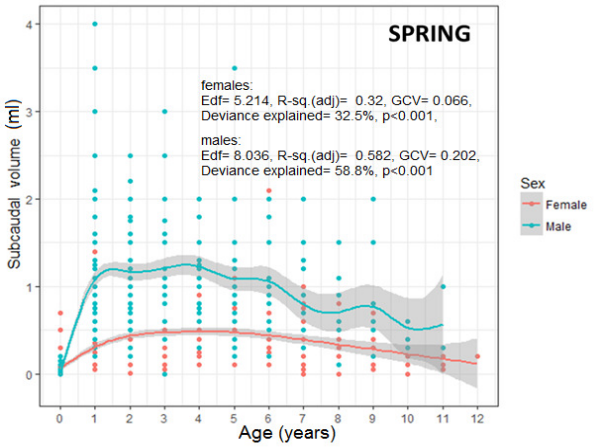


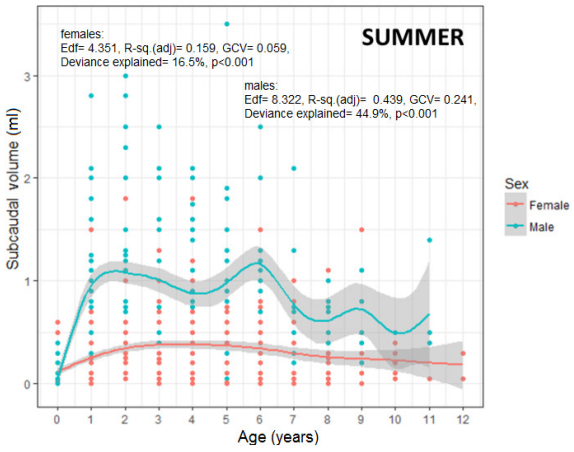
Figure 8. Body condition score against age in males and females during spring (a), summer (b), and autumn (c).

Age-dependent reduction in subcaudal gland volume as proxy of reproductive advertisement

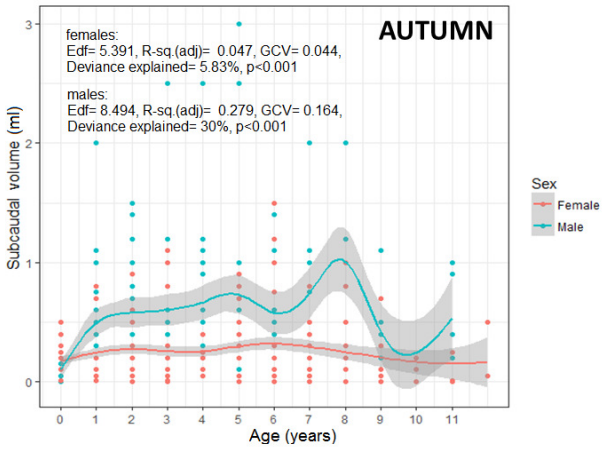
Subcaudal gland volume underwent a gradual decline with age, especially after age 5 years (Table 3), for both sexes in all seasons, but following broad typical changes in seasonal volume patterns (Fig. 9). The negative trend was more abrupt in males, while females underwent a more gradual transition, as shown in the GAM analyses (Fig. 9).



(a)



(b)



(c)

Figure 9. Subcaudal gland volume (ml) against age in males and females during spring (a), summer (b), and autumn (c).

Table 3. Decline in average BCI, body condition score and subcaudal gland volume in both sexes at older ages during spring, summer, and autumn.

Spring				
Somatic parameter	Male		Female	
	Average at age 2 – 5 years	Average at age >5 years	Average at age 2 – 5 years	Average at age >5 years
Body condition index	0.320±0.02	0.319±0.02	0.299±0.02	0.297±0.02
Body condition score	2.50±0.79	2.13±1.22	2.11±0.76	1.92±0.93
Subcaudal gland volume	1.18±0.81	0.75±0.48	0.71±1.01	0.31±0.25
Summer				
Somatic parameter	Male		Female	
	Average at age 2 – 5 years	Average at age >5 years	Average at age 2 – 5 years	Average at age >5 years
Body condition index	0.312±0.02	0.309±0.02	0.32±0.01	0.31±0.02
Body condition score	2.68±1.06	1.84±0.73	2.72±0.94	2.30±0.92
Subcaudal gland volume	1.05±0.59	0.81±0.65	0.31±0.23	0.28±0.26
Autumn				
Somatic parameter	Male		Female	
	Average at age 2 – 5 years	Average at age >5 years	Average at age 2 – 5 years	Average at age >5 years
Body condition index	0.35±0.02	0.34±0.03	0.35±0.03	0.33±0.02
Body condition score	3.95±0.77	3.00±0.94	3.92±0.86	3.35±0.93
Subcaudal gland volume	0.52±0.28	0.44±0.41	0.39±0.25	0.28±0.23

DISCUSSION

Age-dependent reproductive declines in sex-steroid levels

From the endocrinological analyses, strong evidence for a gradual, but not abrupt, decline in sex-steroid levels with age was provided. Both sexes followed a concave (down) quadratic trend, with lower testosterone levels among older males most apparent during spring and summer (i.e., the periods within and close to the main mating season: Research Chapter 2), and lower oestrone levels among older females more apparent during spring and autumn, but not summer. Oestone functions to sustain pre-implanted blastocysts during delayed implantation in badgers (Thom et al. 2004; Yamaguchi et al. 2006; Research Chapter 1), which explains why oestrone levels are higher during spring (after the mating season) and

autumn (closer to the implantation period in mid-December, Research Chapter 1), but lower during the summer during blastocyst diapause.

This is the first report for the natural occurrence of reproductive senescence in a wild-living mustelid, where the results concur with a growing body of evidence supporting reproductive senescence in wild mammals (Nussey 2008), contradicting earlier beliefs that senescence rarely occurred in natural populations (Comfort 1979), due to the various threats wild mammals face reducing the proportion of individuals in the population that survive to old age. For instance, age-dependent declines in physiological markers and fitness have been demonstrated in birds (McCleery et al. 2008; Bouwhuis et al. 2009), ungulates (Ericsson and Wallin 2001; Jones et al. 2008; Nussey et al. 2009) and primates (Nozaki et al. 1995; Sapolsky et al. 1993; Setchell et al. 2005). Male red deer (*Cervus elaphus*) exhibit reduced breeding success with age (Nussey et al. 2008), and among bighorn sheep (*Ovis canadensis*), a smaller proportion of older ewes breed (Berube et al. 1999). Similarly, lighter body weight (Mystreud et al. 2002) and smaller litter sizes occur among older ewes in domestic sheep (*Ovis aries*; Mystreud et al. 2002), whilst older female South African Kalahari meerkats (*Suricata suricatta*) have lower rates of pup survival (Sharp and Clutton-Brock 2010). Nevertheless, the occurrence of RC prior to death (resulting in PRLS) remains little known for many mammal orders, aside the Cetacea (Ellis et al. 2018), Primates, Artiodactyla, and Rodentia (Cohen 2004).

Age-dependent declines in somatic condition and reproductive advertisement relative to reproductive senescence.

For both sexes, the onset of decline in somatic condition commenced earlier, starting at the age of 3 years, than any decline in reproductive hormones, starting at 5-6 years of age; congruent with general trends in other mammal species (Berube et al. 1999; Mysterud et al. 2002). Thus it is shown that both sexes exemplify heterochrony between rates of somatic and hormonal senescence (Berube et al. 1999; Mysterud et al. 2002; Schwartz et al. 2003; Cohen 2004; Nussey et al. 2008).

Decrease in subcaudal volume with age were also observed, suggesting that investment in reproductive advertisement (Buesching et al. 2003; Allan et al. in prep.) also decreases. This was most evident among badgers older than 5 years, for both sexes in all seasons, aligned with the decline in hormone levels. In general, this decrease was more abrupt in males, and

more gradual in females, especially during the spring and summer when badgers are more reproductively active (Buesching et al. 2002a). Similar declines in sexual advertisement with age can be seen in the curvilinear decline of antler growth (linked to dominance status and reproductive success in male deer; Kruuk et al. 2002) with age in the red deer (*Cervus elaphus*; Kruuk et al. 2002) and white-tailed deer (*Odocoileus virginianus*; Scribner et al. 1989). Similarly, older male house mice (*Mus musculus domesticus*), produce lower concentrations of involatile signaling proteins in their urine (MUPs), which serve in mate attraction (Garratt et al. 2011).

These age-related declines in hormonal levels (5-6 years) and body-condition (3 years) were highly synchronous between males and females (although with different offspring assignment implications, below). This is contrary to most studies in polygynous mammals, where males typically senesce earlier than females, and thus realize shorter lifespans (Metcalfe and Monaghan 2003), leading to more rapid male mortality rates (Nussey et al. 2009; Clutton-brock and Isvaran 2007); as seen in black tailed pairie dogs *Cynomys ludovicianus*, red deer *Cervus elaphu*s, and African lions *Panthera leo* (see Clutton-Brock and Isvaran 2007)

In the study population here, however, no significant sex-bias in badger mortality rate is evident (Macdonald and Newman 2002; Macdonald et al. 2009, but see Bright Ross et al. in prep), according with the similar senescence rates between sexes reported here. Due to the badger's polygynandrous mating system, combined with the fact that females undergo delayed implantation and superfoetation (causing longer periods and more chances of mating; see Yamaguchi et al. 2006), and low rates of inter-group dispersal (19.1%; see Macdonald et al. 2008). It is postulated that sexual selection may not be as severe as in polygynous mating systems, leading to similar (somatic and hormonal) senescence schedules in the sexes.

Changes in reproductive strategy with age

Dugdale et al.'s (2011) analysis of maternal assignments, through genetic pedigree for this same population established that fewer older females were assigned cubs, starting at age 3 years; this is earlier than the hormone decline identified, evident from age 5.5 years onwards in females (attained by 31.8% of individual females born, or 55.8% of those attaining sexual maturity), implying some decoupling between potential and actual reproduction. Reduced offspring assignment with age was, however, more coincident with the onset of somatic decline in females, also occurring around age 3 years.

A similar decline in the number of offspring assignments commenced later in males than in females, at age 5 - 6 years (Dugdale et al. 2011), concomitant with the onset of testosterone decline at age 6 years (attained by 20.9% of individual males born, or 37.8% of those attaining sexual maturity; Bright Ross et al. in prep). Males exhibited a similar age of onset of somatic decline to females (3 years), indicating that declining body condition does not preclude reproductive success in males (no 'restraint') in advance of hormonal senescence ('constraint'; see Dugdale et al. 2011).

Evidence of RC and PRLS

For males, initial increase in testosterone levels among cubs (see Figure 5) initiates sperm production during puberty (Adkins-Regan 2005). Thereafter, it appears that some sperm production can persist throughout the lifetime of individuals (Vom Saal and Finch 1988; Cohen 2004) even though testosterone levels decrease with age – evidenced by the fact that some males were still assigned parentage up to the age of 13 years (Dugdale et al. 2011). Moreover, a much greater inter-individual variation in testosterone levels were observed as male badgers advanced into very old age (≥ 9 years; Figure 2a), where a proportion (5/11, 45.5%) maintained levels within 80% of peak production, while levels dropped below 80% to pre-pubescent levels in the remaining 54.5% (i.e., males are not sexually mature until they transition through 80% of the peak value, i.e., 1.76 ng/ml). This is consistent with Buesching et al. (2009), who found that two endocrinological phenotypes were apparent among especially older males in this same population, and evidences RC (that is not in terms of sperm production cessation but potential diminishing ability of reproductive performance during mating, controlled by testosterone levels, affecting fitness) in male badgers, with on average 54.5% of individuals ≥ 9 years old living up to 2.02±1.50 years (range 0.42-4.67 years) of PRLS.

Cross-referencing testosterone and pedigree data, out of the 11 individuals ≥ 9 years, only 7 had cub assignment data which revealed that 2 males sired cubs (where both were 9 years old). Of these 2 only 1 had high testosterone (2.02 ng/ml; above 80% of the age profile peak of 2.2 ng/ml) during the spring mating season, while the other had hormone levels well below 80% of the peak (0.88 ng/ml), implying that – contrary to previous suggestions (Woodroffe and Macdonald 1997; Buesching et al., 2009), a decline in testosterone levels with age does not necessarily exclude males from siring cubs (as sperm is still produced; Vom Saal and Finch 1988; Adkins-Regan 2005). This flexibility may permit a small proportion of males to

make a terminal investment (Clutton-Brock 1984) in late life reproduction. Simultaneously, 2 of these 7 very old males that maintained the high testosterone phenotype were not assigned offspring, indicating that high testosterone does not necessarily lead to breeding success in older males (indicating constraint), not least because reproductive failure in conceiving females is high (Roper et al. 1993). In combination, however, these observations suggest that more complex reproductive performance factors are involved in achieving breeding success for males, such as how testosterone influences mating effort, reproductive advertisement, roaming, and sperm quantity and quality (Cohen 2004; Møller et al. 2009; Clutton-Brock and Isvaran 2007).

While low testosterone may impair spermatogenesis, it is not extinguished (Adkins-Regan 2005); whereas, in contrast, below a certain threshold low oestrone will cause an absolute failure to ovulate in females, and thus total loss of potential fitness (Adkins-Regan 2005; Packer et al. 1998; Cohen 2004). Figures 4a and 4c illustrate that in female cubs oestrone levels develop through ca. 40 pg/ml in spring and 60 pg/ml in autumn, until puberty at ca. 11 months (Research Chapter 3), which implies that oestrus and ovulation are not possible until levels reach ca. 70 pg/ml; this being the minimum age at which conception is inferred from pedigree in a very few individuals (subject to delayed implantation). Applying this 70 pg/ml cut off on the older side of the quadratic curve, suggests that the majority (7 of 11 individuals aged 9-12) of female badgers undergo an oestrone decline consistent with functional menopause at around age 9 years. However, older females also exhibited more heterogeneity in their hormone levels, and it was identified that 4 of 11 individuals aged 9-12 maintained oestrone levels above the 70 pg/ml threshold during spring. This implies that some very old females may not experience breeding constraint and, as per males, suggests two reproductive phenotypes among older females. This agrees with previous findings that very old females may still be assigned parentage although at much lower numbers compared to younger females (with 1 individual still breeding up to 11 years; Dugdale et al. 2011). Here, offspring assignments were only available for 3 of the 11 older females in this study, where all had low hormone levels (during spring) and did not breed thereafter. Meanwhile the 4 individuals that still had high hormone levels did not have assigned parentage data, and thus it was not possible to ascertain whether they continued to reproduce.

Clearly, however, even among the high-oestrone-phenotype in older females, successful breeding is far rarer than in younger badgers, where senescence in body-condition can exert a

major restraint on embyronic implantation, delayed implantation and pregnancy (Woodroffe and Macdonald 1995a; see also Kirkwood and Austad 2000; Mysterud et al. 2001). Similar somatic, rather than hormonal, limitations on reproduction affect older bighorn ewes, where low body-mass individuals are less able to ovulate and conceive (Berube et al. 1999); and reproductive fecundity also correlates significantly with body fat in older female Eurasian beavers (*Castor fiber*; Parker et al. 2017). Thus, it was found that 7 of the 11 females in the dataset aged 9 or more underwent RC, and then on average lived a PRLS of ca. 2.59±1.29 years (range 1.25-4.75 years).

Support for hypotheses explaining reproductive senescence

The difficulties involved in collecting data from wild populations, combined with the ever-diminishing proportion of cohorts surviving to older age (Nussey 2008), resulted in reproductive senescence once being considered a rare phenomenon in nature (Comfort 1979). Gradually, however, studies are revealing that a (small) proportion of various mammal species experience RC and PRLS (see Cohen 2004), although data are lacking particularly for the Carnivora.

Nevertheless, there are difficulties in arguing for PRLS under natural selection. Non-adaptive hypotheses dismiss PRLS as simply a proportion of a population merely 'being lucky' enough to live beyond RC (Peccei 2001), arising as an epi-phenomenon of antagonistic pleiotropy favouring early life fertility (Wood et al. 2000), where PRLS provides an insurance against the risk of dying by chance prior to RC (Tully and Lambert 2011). This would fit badger biology, because early life mortality rates can be high and vary substantially inter-annually (Macdonald and Newman 2002; Macdonald et al. 2009), and thus the contribution to population fertility made by older individuals can be important.

Alternatively, under selection where older males secure more matings and offspring (the Patriarch hypothesis: Marlow 2000), the inseparability of polygenic factors would also result in the extended lifespan of females, subsequently experiencing PRLS (Tuljapukar et al. 2007). This is less plausible for badgers, given that male mating success (but not mounting success) is linked positively with body condition (Dugdale et al. 2011b), where older males show a lower BCI beyond age 3, and older males are assigned fewer, rather than more offspring (Dugdale et al. 2011a).

Cant and Johnston's (2008) adaptive kinship dynamics hypothesis, in contrast, proposes that older females should stop breeding when females of the next generation start to breed. This, however, also does not fit badger senescence patterns well, because prime-age female badgers are able to suppress the reproductive capacity of younger females (Woodroffe and Macdonald 1995b), all females mate but only ca. 45.2% are assigned offspring per year (Macdonald et al. 2015), and younger females allo-mark prime-age (but not old) females with subcaudal secretion (Buesching et al. 2003). Furthermore, high rates of inter-group visits and extra-group paternity obscure the linearity of intra-sex breeding competition.

Unlike in other group-living carnivores (Macdonald and Moehlman 1982), allo-maternal care is also not a prominent feature of badger society (Fell et al. 2006), no food is provisioned to weaned cubs, and thus helpers provide no detectable benefits (Woodroffe and Macdonald 2000; but see Dugdale et al. 2010). This offers no support for either the 'mother' hypothesis' (Williams 1957), which proposes that females that terminate reproduction in mid-life gain fitness advantages by investing in previous offspring, or the 'grandmother hypothesis' (Hawkes et al. 1998), which proposes that post-reproductive females can increase their inclusive fitness by supporting weaned grand-offspring. Furthermore, despite inter-generational overlap (generation length for this population = 3.09 years; Macdonald and Newman 2002), natal philopatry and low levels of dispersal lead to high inbreeding coefficients in badgers ($f = 0.010$; Annavi et al. 2014) and high inter-generational relatedness, offering little support for Cant and Johnstone's (2008) inter-generational Reproductive Conflict hypothesis as a driver favouring RC through the segregation of reproductive generations. In classical terms, neither does Peccei's (1995) altriciality-lifespan hypothesis fit badger biology; but noting that due to induced ovulation and delayed implantation (Macdonald et al. 2017), reproduction represents a substantial component of a female's time and energy budgets, where early life investments may be linked to later-life RC. Therefore, perhaps the most parsimonious explanation for RC in badgers – congruent with a general thesis that sociality in badgers is facultative and linked to resource dispersion, not altruism (Macdonald et al. 2015) – is that by ceasing to reproduce, females escape the increased risk of mortality associated with late-life pregnancy, and both sexes escape the demands that maintaining reproductive condition places on somatic condition (Penn and Smith 2007).

It was demonstrated for the first time an age-related decline in reproductive hormone profiles in a wild-living population of European badgers, and a heterochronic decline in somatic

condition, leading to RC and PRLS, especially in females. With regard to differential rates of reproductive senescence between the sexes, it is important to consider that germ cell production is less tightly coupled to hormonal variations in males than it is in females (Adkins-Regan 2005), causing differences in the potential influence of an age-related decline in sex-steroid levels on reproductive fitness (see Penn and Smith 2007). The ability to reproduce into old age can enhance individual lifetime reproductive success significantly (Merilä and Sheldon 2000), and in capital breeders (Festa-Bianchet et al. 1998), this can relate to the ability to accrue, or continue to be able to accrue, resources necessary for late-life reproductive effort (e.g., Campbell et al. 2017). The extent to which late-life breeding contributes to population demographics depends, however on age-specific survival. In this badger population c. 90.2% badgers die before reaching the age of 9 years (Bright Ross et al. in prep), thus only 9.8% of badgers born will ever be in a position to experience RC (or continue to breed). Consequently, while evidence that PRLS can occur in badgers, it is still subject to substantial negative selection pressure (Cohen 2004; Baudisch 2005), where the low post-reproductive presentation (PrP; Croft et al. 2015) of senescent individuals frees resources for more productive younger badgers. Conversely, juvenile mortality is often very high in badgers (Macdonald and Newman 2002; Macdonald et al. 2009, peaking at 48.5% in 2014; n = 66; Bright Ross et al. in prep), linked to parasitoses and immune development (see Macdonald et al. 2015), and thus there may be heterochronic advantages for a proportion of older individuals to retain breeding capacity to ensure population continuity if substantial failures occur in younger cohorts under adverse inter-annual conditions (Promislow and Harvey 1990); that is, to provide insurance against indeterminacy (Tully and Lambert 2011).

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PART IV

PHYSIOLOGICAL AND SOMATIC RESPONSES TO CHANGES IN ECOLOGICAL FACTORS

RESEARCH CHAPTER 5

Extrinsic factors affecting cub development contributing to sexual size dimorphism in the European badger (*Meles meles*)

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ABSTRACT

Sexual size dimorphism (SSD) is common among mammals, with males typically being larger than females, as a product of sex-specific differences in growth rate and growth duration. The Mustelids, however, are known to exhibit a hypo-allometric decrease of SSD with increasing body size (contrary to Rench's rule). A variety of extrinsic factors likely affect juvenile growth rates and end body size, where one sex may demonstrate greater vulnerability than the other towards a specific factor, moderating patterns and degrees of SSD. Here, analyses were conducted to look at how male and female European badgers (*Meles meles*) differ in their somatic growth patterns comparing the sex-specific growth curves in a range of somatic parameters and investigate what extrinsic (social and environmental) factors affect cub growth rates during the first 2 years of life and their sexual-dimorphic adult end sizes. It was found that average male end size of all skeletal measurements was significantly larger than those of females. Although male and female weanling cubs had similar body sizes, skeletal growth curves diverged significantly from ca. 11 months onwards due to continuous rapid growth of males and slowing female growth. Consequently, females always reached end skeletal growth earlier than males. In both sexes, extremities ceased to grow at an earlier age than body length and zygomatic arch width. Although all badger cubs were impacted by their social environment as well as by climatic factors, based on severity of these effects, it was found that male cubs are more sensitive to one particular social factor, remaining smaller in social groups with more adult males whereas female end size was predominantly affected by climatic factors and associated food availability. How tested extrinsic parameters can moderate the patterns of SSD described in this species is then discussed.

Keywords: *development, end size, extrinsic factors, growth patterns, sexual size dimorphism,weather*

INTRODUCTION

Sexual size dimorphism (SSD) is defined as differences in skeletal size between males and females of the same species (Lammers, Dziech and German, 2001: Badyaev 2002) and is common among mammals, where male size typically exceeds that of females (Badyaev 2002). Sexual selection is often suggested as the ultimate cause of SSD (Hoogland 2003; but see Isaac 2005), where bigger males have an advantage to compete for mates, territories, and resources (e.g., Trivers 1972). Rensch's rule states that, within an evolutionary lineage, SSD increases with mean body size in those taxa with larger males (NB, when females are larger, SSD declines with increasing body size: Rensch 1950; Abouheif and Fairbairn 1997). Nevertheless, not all taxa conform to this rule. For example, the Musteloidea demonstrate a hypo-allometry contrary to Rensch's rule, with lower SSD associated with larger body size although males are larger than females in this group (Noonan et al. 2016). Thus, Noonan et al. (2016) conclude that low energy abundant food (i.e., an omnivorous or insectivorous diet) promotes group-living (with promiscuous mating systems), and thus reduces the need of males to defend access to multiple females, and consequently decreases the competitive advantages of larger males.

Diverging growth patterns (i.e., growth rate and/ or duration: Badyaev 2002) between males and females appear to be the primary physiological mechanism leading to SSD (Isaac 2005), and a variety of extrinsic factors likely affect juvenile growth rates and end body size in both sexes (Badyaev 2002). Typically, one sex, however, demonstrates a greater vulnerability towards a specific environmental factor than does the other, moderating patterns and degrees of SSD (Leberg and Smith 1993; Post et al. 1999; Leblanc et al. 2001). For example, in some mammals such as white-tailed deer (*Odocoileus virginianus*; Leberg and Smith 1993) and bighorn sheep (*Ovis canadensis*; Leblanc et al. 2001), male adult size tends to be more sensitive to adverse social conditions (such as high population density: Leberg and Smith 1993; Leblanc et al. 2001) during juvenile development, whereas females appear better able to compensate for early growth loss (Festa-Bianchet et al. 1994) and are typically less affected by social factors (Leberg and Smith 1993; Leblanc et al. 2001). In contrast, females appear more sensitive to environmental factors such as weather and food availability (e.g., red deer, *Cervus elaphus*: Post et al. 1999).

In accord with the hypo-allometric decrease of SSD with increasing body size within the musteloids (Noonan et al. 2016), the European badger (*Meles meles*, henceforth 'badger') is an example of an exception to Rensch's rule, where SSD is lower in this species compared to smaller, more carnivorous Musteloidea (typical sex ratio, male/ female body mass ratio of 1.1:1; Noonan et al. 2016).

Environmental factors are well-known to affect juvenile growth patterns in badgers, and cub development in general is negatively correlated with aberrant climatic conditions (i.e., wetter or drier than average: Macdonald et al. 2010), where predictability of seasonal normative conditions supports optimal survival (Nouvellet et al. 2013). Variability in weather conditions, in contrast, negatively affects food availability, explaining inter-annual differences in cub numbers, growth rates, and survival (Woodroffe and Macdonald 2000). Before reaching semi-independence in July, cubs are especially vulnerable to thermal stress (Kaneko et al. 2010; Tsunoda et al. 2018) due to excessive rainfall, frost, and floods (Macdonald and Newman 2002), particularly when suffering from parasitic infections (such as coccidiosis: *Isospora melis* and *Eimeria melis*: Anwar et al. 2000) that may result in stunted growth (Newman et al. 2001) and mortality rates peaking above 90% (Newman et al. 2001; Nouvellet et al. 2013). In addition, cub development (Fell et al. 2006) and attainment of sexual maturity (Research Chapter 3) may be affected negatively also by social pressures perhaps associated with the vulnerability of cubs to infanticide (Woodroffe and Macdonald 1993) and resource competition for food and sett space with other cubs as well as adults in their natal sett/ social group or even the general population (Woodroffe and Macdonald 2000).

In this research, i) how males and females differ in their somatic growth patterns comparing the sex-specific growth curves in a range of somatic parameters during the first 2 years of life; ii) what extrinsic (social and environmental) factors affect cub growth rates during the first 2 years of life and their sexual-dimorphic adult end sizes; and iii) how these parameters can moderate the patterns of sexual size dimorphism described in this species, will be investigated

MATERIALS AND METHODS

Badger trapping and sampling

As part of an ongoing long-term research project, data were collected from a high-density badger population in Wytham Woods, Oxfordshire, UK (51°46'26" N, 1°19'19" W; for details see Macdonald et al., 2015), between 1995 – 2016. Following the methodology described in Sun *et al*. (2015), badgers were trapped three to four times annually: (1) in *spring* (May/June) at the end of the closed season, when cubs were fully weaned, (2) in *summer* (July/August/September) during lowest food abundance, period of independent foraging for cubs, (3) in *autumn* (October/November) when badgers reach maximum body weight, and (4) in *winter* (January) during the main mating season, when some cubs already achieve sexual maturity ('early-developers': Research Chapter 3). Captured animals were transported to a central field station, before being sedated by intramuscular injection of 0.2 ml ketamine hydrochloride/ kg body weight (McLaren et al. 2005; Thornton et al. 2005). All badgers received a permanent unique tattoo, allowing individual identification (ID) and aging.

Body measurements

Following sedation, head-body length (to the nearest 5mm); zygomatic arch width (mm), pastern length (mm, averaged between left and right foot), radius-ulna (mm, averaged between left and right), humerus (mm, averaged between left and right), tibia-patella (mm, averaged between left and right), patella-femur (mm, averaged between left and right), and tail length (mm) were measured using calipers.

Population density

Yearly population density was calculated from minimum–number-alive estimates (capture-mark-recapture data, from 1994 to 2016; following Macdonald et al. 2009). The total number of badgers as well as the numbers of adult males, adult females, male cubs, and female cubs at each sett, social group, and in the Wytham Woods study site overall were determined annually by assigning residency according to Annavi et al. (2014; see also Macdonald et al., 2008). Cases that were not addressed by either set of rules were accounted for in new residency rules as the following:

1. When there was a 1-year gap in an individual's capture history and the sett/social group before and after that gap were the same, the individual was assigned to that same sett/ social group for that year.
2. When there was a 1-year gap in an individual's capture history but the sett/ social group before and after that gap were different, the individual was assigned to the sett/ social group that it was captured at immediately after that gap if a clear dispersal event had occurred (see Macdonald et al., 2008 for definition). Otherwise, the individual was assigned to the sett/ social group that it was captured at prior to that gap.
3. When there was an even-numbered gap in an individual's capture history (e.g. 2 years, 4 years, etc.), a proportional assignment was made between the sett/ social group that the individual was captured at immediately prior to and immediately after the gap.
4. When there was an odd-numbered gap in an individual's capture history (e.g. 3 years, 5 years, etc.), a proportional assignment was made between the sett/social group that the individual was captured at prior to and after that gap (e.g. for a 3-year gap, an individual would be assigned to the first sett/ social group for 1 year and assigned to the second sett/ social group for 2 years).
5. When the individual was captured in the same social group but in different setts within that social group during a particular year, it was assigned to the sett that it was captured at most often throughout its entire capture history. Nevertheless, if it showed an equal preference for multiple setts, it was assigned to the sett that it was captured at initially.

Weather data

Weather data (from 1994 to 2016) were obtained from the Environmental Change Network (ECN) data centre. A long-term seasonal trend from 1994 to 2016 (spring: March - May, summer: June - August, autumn: September - November, winter: December - February) for temperature (T) and rainfall was established. Seasonal mean T and seasonal mean rainfall were calculated for the six seasons which a badger experiences between birth until reaching adult end size at 19 months: 1^{st} spring, 1^{st} summer, 1^{st} autumn, 1^{st} winter, 2^{nd} spring, and 2^{nd} summer. The deviation from the long-term mean (1995- 2016) for a given season was then calculated and used in the global model as a modification of the methodology used in Nouvellet et al. (2013).

Statistical analyses

Comparison of cub sizes at first capture (3-4 months of age)

To investigate the difference in male and female cub sizes during first capture in spring (3-4 months of age), analyses with 2 linear models (NB, all datapoints were independent) were conducted with sex as predictor and somatic parameter groups (head body length, zygomatic arch width, mean pastern length for the 1st model and radius-ulna length, humerus length, tibia-patella length, patella-femur length, tail length for the 2nd model) as response, including year as covariate to account for interannual differences. The somatic parameters were divided into 2 groups as they were from independent data sets (1st somatic parameter group obtained from 1995 – 2016 while 2nd group was obtained from 2016 only). The somatic parameters for each group were combined using principal component analysis (PCA), where only the first PCA component was used for the linear models.

Somatic growth curves

A non-linear mixed model using the nlme and sslogic function (forming a growth curve and giving an asymptote value as output), including ID as random effect, was used to analyse head body length, zygomatic arch width, mean pastern length, radius-ulna length, humerus length, tibia-patella length, patella-femur length, and tail length against age (in months). To determine at what age different skeletal measures cease to increase (i.e., end of growth), the percentage of the predicted value towards the asymptote was calculated. To investigate whether growth curves in a given somatic parameter differed between the sexes, a Maximum Likelihood (ML) test was conducted comparing the non-linear mixed model with and without sex as an independent variable. It is important to note, that any averages presented (across the remaining sample of survivors) may become more skewed towards bigger high quality individuals due to 'selective removals' (e.g. Nussey et al. 2011).

Sexual size dimorphism in adults

Simimlar to the analyses conducted in weanlings, to investigate the difference in male and female adult end sizes (18 months above), 2 linear models (NB, all datapoints were independent) were conducted with sex as predictor and somatic parameter groups (same groups as in weanlings, where somatic parameters were also combined using a PCA with only the 1st component being used) as response, including year as covariate to account for interannual differences.

Environmental effects on cub growth (global and sex-specific models)

The age when growth reached 99% of the asymptote value, was assigned as the 'end point' of head body length and zygomatic arch width growth (the two measures used as responses in the global model because they provide reliable indicators of overall skeletal growth and development in badger cubs). Two global linear mixed models with head body length and zygomatic arch width (in badgers aged 19 months when all parameters have reached end growth, males and females combined) as respective response variables were run with sex, the numbers of male and female adults in the cub's natal social group, male and female cubs in the natal group, male and female cubs in the natal sett, total adults in the population in the natal year, total number of cubs in the respective cohort, deviation of mean T from long term mean in the cubs' first spring, deviation of mean T in first summer, deviation of mean T in autumn, deviation of mean T in winter, deviation of mean T in second spring, deviation of mean T in second summer, deviation of mean rainfall from longterm mean in first spring, deviation of mean rainfall in first summer, deviation of mean rainfall in autumn, deviation of mean rainfall in winter, deviation of mean rainfall in second spring, and deviation of mean rainfall in second summer as predictors. Birth Year was assigned as a random predictor to account for inter-annual effects. Similar models were designed for males and females separately thereafter, but including only the significant parameters found in the respective global models above (where male and female data were combined). Severity of the effects of each factor was judged on the basis of whether it affected both independent growth parameters (i.e., end head body length and zygomatic arch width) or only one parameter. Before any models were run, inter-correlation between continuous parameters was analysed using Pearson correlation tests. All parameters included in the global model were confirmed to be independent.

A drop1 function giving a comparison of models based on their AIC criteria (i.e., testing whether or not the model excluding a given term is different from the model with that term), was used to determine which factors influenced end body length and end zygomatic arch width significantly in cubs at age 19 months in all models. Catagorization of results (i.e., significant parameters) from all global models elucidated which factors affected adult size in both sexes simultaneously and which factors were sex-specific.

For each model, patterns of residuals, normality, and mean variance were checked using diagnostic plots in R. All statistical analyses were carried out using RStudio (0.99.896;

RStudio Team 2015) and R (R-3.2.4; R Core Team 2016).

RESULTS

Sexual size dimorphism at weaning

There was no evidence that weanlings (aged 3-4 months) differed in size between sexes for all the measured parameters combined in model 1 and 2 (Table 1), indicating no sexual size dimorphism during early ages (first capture).

Table 1. Comparison of somatic parameters in weanling cubs at first capture aged of 3-4 months

Model 1				
Combined parameters	PCA1 variance at 3-4 months of age due to sex			
	% Variance explained by PCA1	n	F value	p
Head body length Zygomatic arch width Mean pastern length	82.54	501	$F_{1,498} = 0.785$	0.376
Model 2				
Combined parameters	PCA1 variance at 3-4 months of age due to sex			
	% Variance explained by PCA1	n	F value	p
Radius-ulna length Humerus length Tibia-patella length Patella-femur length Tail length	90.66	48	$F_{1,45} = 2.07$	0.157

Somatic growth curves

The growth curves for male and female head body length differed significantly (Table 2) with females reaching 99% of their end body length at 16 months of age, faster than males which achieved 99% at 18 months. Although at age 3-4 months both males and females show similar body length (Table 1) and rapid growth rate (parallel curves, 45.2 and 44.1 mm/months for males and females respectively; difference in growth rate was non-significant at 1.12 mm/month), growth of female cubs slowed earlier than that of males (at ca. 6 months;

divergence of average line due to larger differences in growth rates of 2.57 mm/month), whilst males continued to grow rapidly until age ca. 11 months (after which the differences in growth rate decreased to 1.48 mm/months, and continued to decrease as both slowly reached their end size). At 11 months of age, male and female body lengths diverged sufficiently for the confidence intervals between the two curves to cease to overlap (Fig. 1).

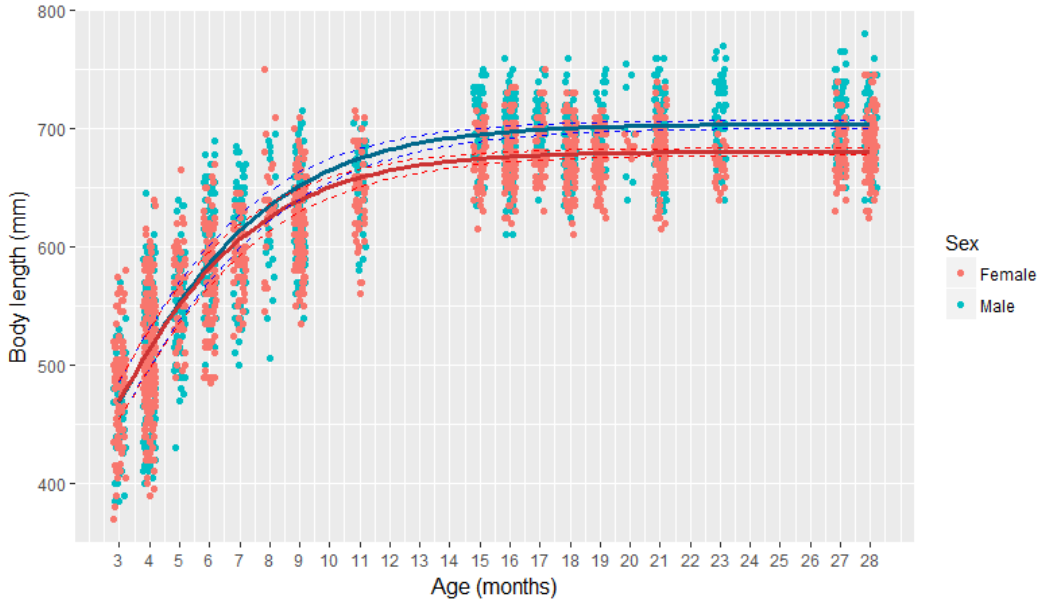


Figure 1. Head body length growth curves in males and females aged 3-28 months.

The growth curves for zygomatic arch width also differed significantly between the sexes (Table 2; Fig. 2) with both reaching 99% towards the asymptote at age 19-20 months. Although the sexes had similar widths at 3 months (Table 1), growth rates of males were always higher compared to females (4.14 and 2.25 mm/months respectively at age 3-5 months), resulting in diverging average growth curves at the age of 5 months. In females the growth of the zygomatic arch slowed at the age of 7 months, whereas male skulls continued to grow until age 9 months. The zygomatic arch width diverged between sexes (with confidence intervals no longer overlapping) at the age of 12 month (Fig. 2).

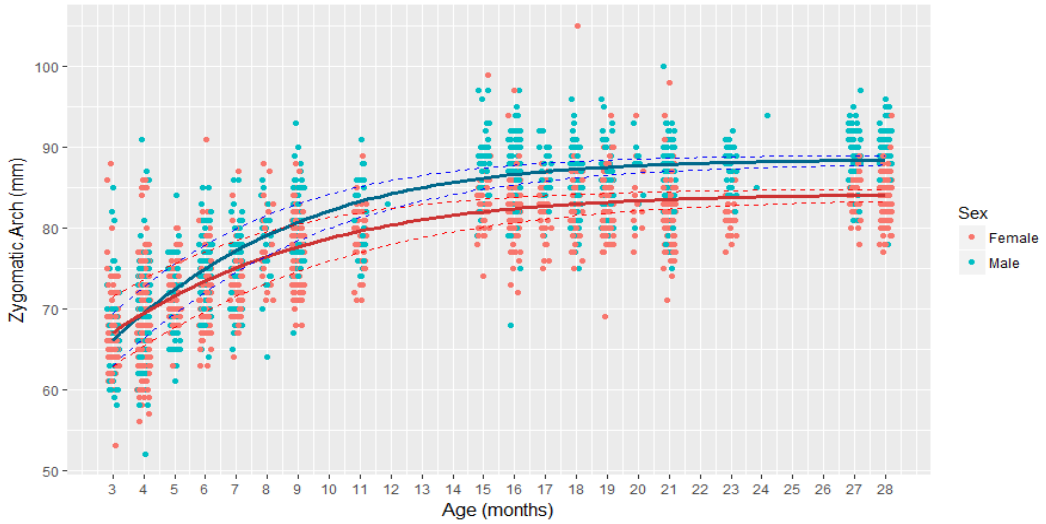


Figure 2. Zygomatic arch growth curve in males and females aged 3-28 months.

Mean pastern length growth curves also differed significantly between sexes (Table 2; Fig. 3) with males reaching 99% towards the asymptote at the age of 9 months, whereas females reached 99% towards the asymptote at age 7 months. Initial growth rates of the sexes (3-5 months) were similar, around 4 mm/months, although initial size of males at 3 months were slightly longer than that of females; Table 1). Growth in females slowed at 5 months while males continued to grow rapidly with higher growth rate until 6 months, leading to a significant divergence in growth curves at the age of 8 months (Fig. 3).

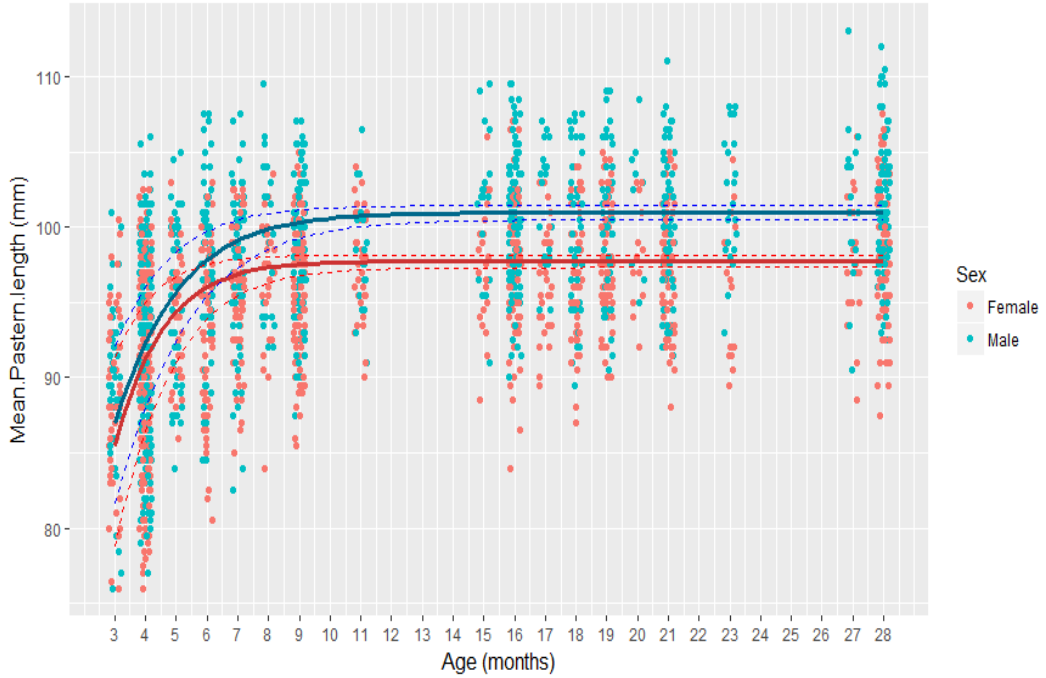


Figure 3. Mean pastern length growth curve in males and females aged 3-28 months.

In males, radius-ulna length (Fig. 4a.), humerus length (Fig. 4b.), tibia patella length (Fig. 4c.) and patella-femur length (Fig. 4d.) reached 99% of end length at 13, 15, 18 and 16 months respectively whilst in females, growth reached the 99% asymptote significantly faster at 11,14,15 and 15 months respectively. In contrast to other parameters, however, tail length (Fig. 4e.) reached 99% of the end length faster in males (at 8 months) than in females (at 10 months) although end tail length did not differ between the sexes (Table 2).

Differences in somatic growth curves between sexes and sexual size dimorphism in adult badgers

The maximum likelihood tests confirmed that growth curves of all somatic parameters (Fig. 1-4) are significantly different between sexes, except for tail length (see Table 2). The asymptotes of the growth curves show that males achieve a significantly larger mean size than do females in all skeletal measures except in the length of their tails, indicating that females have longer tails relative to their body size than do males (see Table 2 and Fig. 1-4). Combining somatic parameters as a whole (using PCA), model 1 and 2 show that male badgers have significantly larger end body size than females (see Table 2). This supports the observation of differences in growth curves in the previous section as seen from the diverging growth rates.

Table 2. Comparison of somatic growth curves and asymptot value between adult males and females.

Differences in somatic growth curves due to sex							
Parameters	Asymptote value (mm)		Male and female growth curve difference statistics				
	Male	Female	n_{male}	n_{female}	X^2	Df	p
Head body length	**704.06**	**680.81**	**1368**	**1385**	**1076.759**	**10**	**<0.001**
Zygomatic arch width	**88.57**	**84.32**	**1223**	**1298**	**725.597**	**8**	**<0.001**
Mean pastern length	**100.95**	**97.73**	**1006**	**1120**	**95.620**	**8**	**<0.001**
Radius ulna	**113.89**	**109.64**	**101**	**75**	**42.688**	**8**	**<0.001**
Humerus	**119.00**	**115.01**	**101**	**75**	**37.551**	**8**	**<0.001**
Tibia-patella length	**107.92**	**102.55**	**101**	**74**	**12.751**	**8**	**0.005**
Patella-femur length	**117.19**	**114.44**	**101**	**74**	**7.481**	**8**	**0.006**
Tail length	145.97	146.01	95	62	1.854	8	0.603

Differences in adult end size due to sex				
Model 1				
Combined parameters	PCA1 variance at adulthood due to sex			
	% Variance explained by PCA1	n	F value	p
Head body length	75.40	364	$F_{1,361} = 114.36$	<0.001
Zygomatic arch width				
Mean pastern length				
Model 2				
Combined parameters	PCA1 variance at adulthood due to sex			
	% Variance explained by PCA1	n	F value	p
Radius-ulna length	61.31	40	$F_{1,37} = 6.29$	0.017
Humerus length				
Tibia-patella length				
Patella-femur length				
Tail length				

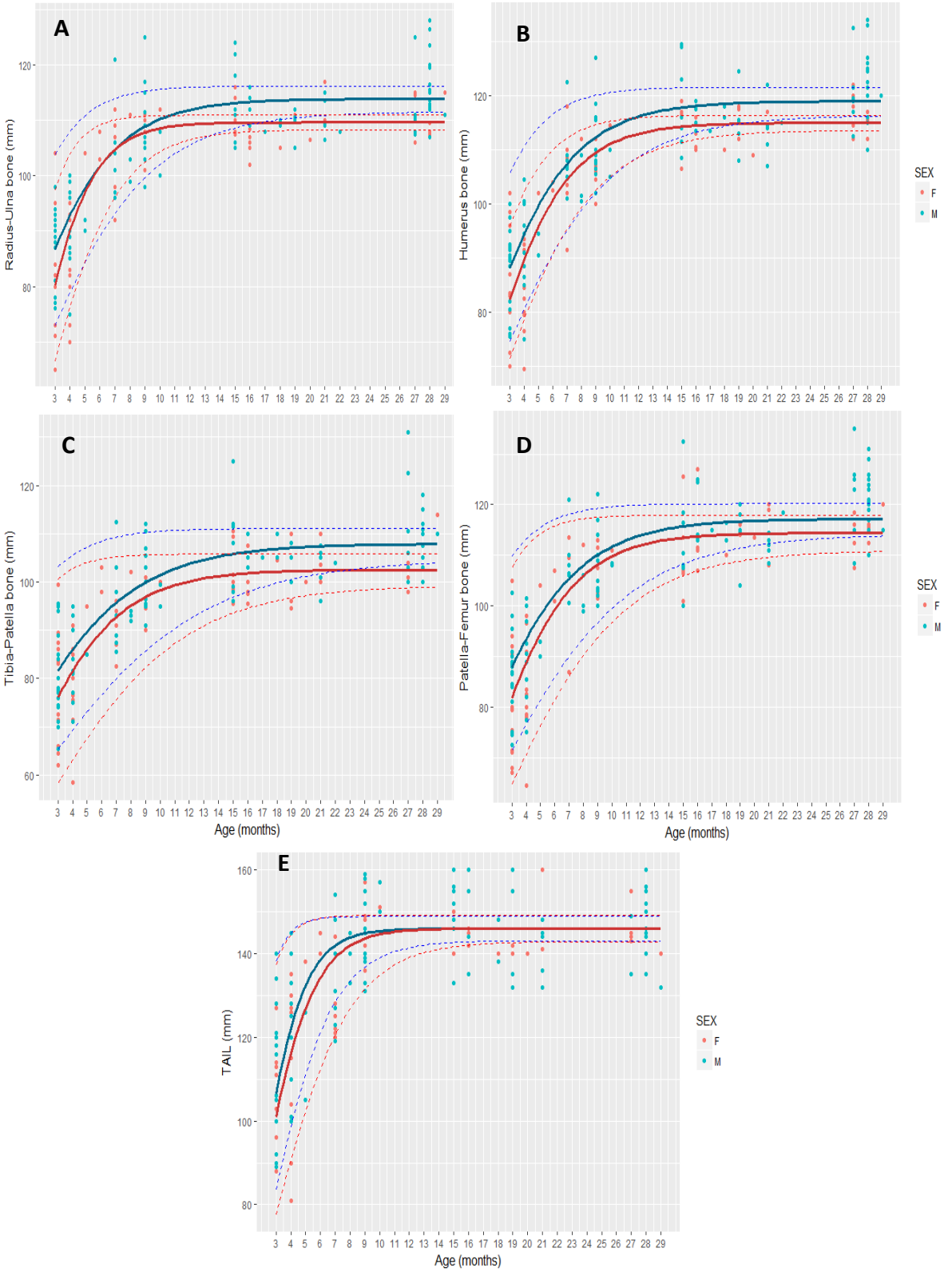


Figure 4. Growth curves of male and female radius-ulna (A), humerus (B), tibia-patella length (C), patella-femur length (D), and tail length (E) in badgers aged 3-28 months.

Environmental factors affecting adult sexual size dimorphism

End head body length (n=226; see Table 3) was affected significantly by sex, number of cubs in cohort, deviation of mean T in first spring, deviation of mean rainfall in first spring, deviation of mean rainfall in first summer, deviation of mean rainfall in autumn, deviation of mean rainfall in winter and deviation of mean rainfall in second summer.

Table 3. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the global model with body length as response

Parameters	Global model coefficients			Global model parameter significance			
	Estimate	SE	t	DF	AIC	X^2	p
Global model					2162.2		
(Intercept)	685.97	20.16	34.03				
Sex	**14.16**	**4.06**	**3.49**	**1**	**2173.0**	**12.844**	**<0.001**
Male adults in birth social group	0.58	0.76	0.76	1	2161.1	0.893	0.345
Female adults in birth social group	-0.79	0.81	-0.98	1	2161.4	1.211	0.271
Male cubs in birth social group	1.84	2.36	0.78	1	2160.9	0.744	0.388
Female cubs in birth social group	-1.76	1.72	-1.03	1	2161.6	1.425	0.233
Male cubs in birth sett	-0.87	3.03	-0.29	1	2160.3	0.106	0.745
Female cubs in birth sett	0.02	2.35	0.01	1	2160.2	0.005	0.944
Total adults in birth population	0.19	0.11	1.22	1	2162.5	2.321	0.128
Total cubs in birth cohort	**-0.58**	**0.33**	**-1.78**	**1**	**2164.5**	**4.359**	**0.037**
Dev. of meanT in 1st spring	**8.76**	**4.98**	**1.76**	**1**	**2165.0**	**4.792**	**0.029**
Dev. of mean T in 1st summer	2.97	6.53	0.45	1	2160.5	0.282	0.596
Dev. of mean T in autumn	6.31	5.83	1.08	1	2161.7	1.525	0.217
Dev. of mean T in winter	2.08	6.35	0.33	1	2160.4	0.197	0.657
Dev. of mean T 2nd spring	1.92	4.12	0.47	1	2160.5	0.287	0.592
Dev. of mean T 2nd summer	-7.21	6.16	-1.17	1	2162.2	2.048	0.152
Dev. of mean rainfall in 1st spring	**21.05**	**11.26**	**1.87**	**1**	**2165.2**	**4.976**	**0.026**
Dev. of mean rainfall in 1st summer	**-30.24**	**9.40**	**-3.22**	**1**	**2175.3**	**15.149**	**<0.001**
Dev. of mean rainfall in autumn	**-22.50**	**10.56**	**-2.13**	**1**	**2166.8**	**6.622**	**0.010**
Dev. of mean rainfall in winter	**-30.11**	**10.94**	**-2.75**	**1**	**2171.5**	**11.293**	**<0.001**
Dev. of mean rainfall in 2nd spring	-2.97	8.24	-0.36	1	2160.4	0.239	0.625
Dev. of mean rainfall in 2nd summer	**-16.95**	**8.69**	**-1.95**	**1**	**2166.0**	**5.860**	**0.015**

Note: Dev. = Deviation, T= Temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

End zygomatic arch width (n= 218; Table 4) was highly sexually-dimorphic, and was strongly affected by number of adult males in birth social group, total number of adults in birth population, number of cubs in cohort, deviation of mean T in first spring , deviation of mean T in autumn deviation of mean rainfall in first spring, deviation of mean rainfall in first summer, deviation of mean rainfall in autumn, deviation of mean rainfall in winter, as well as deviation of mean rainfall in second summer.

Table 4. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the global model with zygomatic arch width as response

Parameters	Global model coefficients			Global model parameter significance			
	Estimate	SE	t	DF	AIC	X^2	p
Global model					1177.4		
(Intercept)	84.08	3.40	24.73				
Sex	**2.96**	**0.52**	**5.65**	**1**	**1206.6**	**31.202**	**<0.001**
Male adults in birth social group	**-0.21**	**0.10**	**-2.18**	**1**	**1179.7**	**4.354**	**0.037**
Female adults in birth social group	0.07	0.10	0.65	1	1175.6	0.189	0.664
Male cubs in birth social group	0.43	0.29	1.46	1	1178.0	2.615	0.106
Female cubs in birth social group	-0.14	0.22	-0.67	1	1176.4	1.007	0.316
Male cubs in birth sett	-0.52	0.38	-1.36	1	1177.4	2.055	0.152
Female cubs in birth sett	-0.19	0.30	-0.62	1	1175.6	0.251	0.616
Total adults in birth population	**0.03**	**0.02**	**1.43**	**1**	**1182.0**	**6.627**	**0.010**
Total cubs in birth cohort	**-0.10**	**0.06**	**-1.81**	**1**	**1184.5**	**9.074**	**0.003**
Dev. of mean T. in 1st spring	**1.24**	**0.87**	**1.42**	**1**	**1183.1**	**7.727**	**0.005**
Dev. of mean T in 1st summer	0.97	1.06	0.92	1	1177.1	1.726	0.189
Dev. of mean T in autumn	**1.60**	**0.99**	**1.62**	**1**	**1181.1**	**5.726**	**0.017**
Dev. of mean T in winter	-0.14	1.10	-0.13	1	1175.4	0.035	0.852
Dev. of mean T 2nd spring	0.07	0.72	0.10	1	1175.4	0.007	0.934
Dev. of mean T 2nd summer	0.06	1.05	0.06	1	1175.4	0.025	0.874
Dev. of mean rainfall in 1st spring	**2.88**	**1.90**	**1.52**	**1**	**1182.5**	**7.084**	**0.008**
Dev. of mean rainfall in 1st summer	**-4.60**	**1.59**	**-2.90**	**1**	**1195.9**	**20.552**	**<0.001**
Dev. of mean rainfall in autumn	**-2.10**	**1.78**	**-1.18**	**1**	**1179.5**	**4.167**	**0.041**
Dev. of mean rainfall in winter	**-3.64**	**1.86**	**-1.96**	**1**	**1187.6**	**12.193**	**<0.001**
Dev. of mean rainfall in 2nd spring	-0.09	1.47	-0.06	1	1175.4	0.017	0.896
Dev. of mean rainfall in 2nd summer	**-2.06**	**1.55**	**-1.33**	**1**	**1182.4**	**7.053**	**0.008**

Note: Dev. = Deviation, T= Temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

In the following sex-specific models (where all parameters proven to be significant in the global models above were included: Table 3 and 4), male body length (n = 108; Table 5) was significantly affected only by the numbers of male adults in the cub's natal social group, deviation of mean rainfall in first summer, and deviation of mean rainfall in winter; all of which had a negative effect on end body length. Male zygomatic arch width (n= 103; Table 5) was negatively affected by numbers of male adults in the cub's natal social group, total number of cubs in the respective cohort, deviation of mean rainfall in first summer, deviation of mean rainfall in winter, and deviation of mean rainfall in second summer, whilst deviation of mean temp in autumn had a significant positive effect. Total number of adults in the population in the natal year only approached significance but was positively correlated with end zygomatic arch width.

Table 5. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the male models with head body length and zygomatic arch width as response

Parameters	**Male body length model coefficients**			**Male body length model parameter significance**			
	Estimate	SE	t	DF	AIC	X^2	p
Male global model					1034.1		
(Intercept)	701.87	25.19	27.87				
Male adults in birth social group	**-2.17**	**0.97**	**-2.24**	**1**	**1037.0**	**4.960**	**0.026**
Total adults in birth population	0.10	0.15	0.72	1	1032.9	0.857	0.355
Total cubs in birth cohort	-0.41	0.37	-1.1	1	1034.1	2.046	0.153
Dev. of mean T. in 1st spring	-1.73	6.41	-0.27	1	1032.2	0.111	0.740
Dev. of mean T. in autumn	5.44	7.13	0.76	1	1033.1	0.988	0.320
Dev. of mean rainfall in 1st spring	2.54	11.24	0.23	1	1032.1	0.066	0.797
Dev. of mean rainfall in 1st summer	**-26.34**	**11.20**	**-2.35**	**1**	**1041.0**	**8.896**	**0.003**
Dev. of mean rainfall in autumn	-4.89	13.01	-0.38	1	1032.3	0.269	0.604
Dev. of mean rainfall in winter	**-25.26**	**12.53**	**-2.02**	**1**	**1038.5**	**6.452**	**0.011**
Dev. of mean rainfall in 2nd summer	-13.14	10.61	-1.24	1	1034.8	2.706	0.100
Parameters	**Male zygomatic arch model coefficients**			**Male zygomatic arch model parameter significance**			
	Estimate	SE	t	DF	AIC	X^2	p
Male global model					577.03		
(Intercept)	87.43	3.79	23.10				
Male adults in birth social group	**-0.31**	**0.13**	**-2.37**	**1**	**580.96**	**5.929**	**0.015**
Total adults in birth population	**0.02**	**0.02**	**1.06**	**1**	**578.06**	**3.032**	**0.082**
Total cubs in birth cohort	**-0.09**	**0.06**	**-1.67**	**1**	**580.14**	**5.112**	**0.024**
Dev. of mean T. in 1st spring	0.20	0.99	0.21	1	575.22	0.194	0.659
Dev. of mean T. in autumn	**1.94**	**1.08**	**1.80**	**1**	**580.41**	**5.384**	**0.020**
Dev. of mean rainfall in 1st spring	1.61	1.76	0.91	1	576.93	1.904	0.168
Dev. of mean rainfall in 1st summer	**-5.27**	**1.76**	**-2.99**	**1**	**588.81**	**13.786**	**<0.001**
Dev. of mean rainfall in autumn	-1.76	2.01	-0.88	1	576.70	1.672	0.196
Dev. of mean rainfall in winter	**-3.95**	**1.95**	**-2.02**	**1**	**582.73**	**7.699**	**0.006**
Dev. of mean rainfall in 2nd summer	**-3.24**	**1.70**	**-1.91**	**1**	**582.34**	**7.314**	**0.007**

Note: Dev. = Deviation, T= Temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

Female body length (n = 118; Table 6), in contrast, was positively affected by numbers of male adults in the cub's natal social group, deviation of mean T in first spring and deviation of mean rainfall in first spring, whilst deviation of mean rainfall in first summer, deviation of mean rainfall in autumn and deviation of mean rainfall in winter had significant negative effects. Female zygomatic arch width model (n = 115; Table 6) was positively affected by the total number of adults in the population in the natal year, deviation of mean T in first spring and deviation of mean rainfall in first spring, whereas the total number of cubs in the respective cohort, deviation of mean rainfall in first summer, deviation of mean rainfall in winter and deviation of mean rainfall in second summer had significant negative effects.

Table 6. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the female models with head body length and zygomatic arch width as response

Parameters	Female body length model Coefficients			Female body length model parameter significance			
	Estimate	SE	t	DF	AIC	X^2	p
Female global model					1115.6		
(Intercept)	665.56	20.28	32.81				
Male adults in birth social group	**2.18**	**0.78**	**2.80**	**1**	**1122.0**	**8.355**	**0.004**
Total adults in birth population	0.09	0.11	0.83	1	1114.4	0.754	0.385
Total cubs in birth cohort	-0.25	0.31	-0.79	1	1114.3	0.686	0.407
Dev. of mean T. in 1st spring	**12.09**	**4.43**	**2.73**	**1**	**1121.6**	**7.950**	**0.005**
Dev. of mean T. in autumn	1.074	5.39	0.20	1	1113.7	0.044	0.834
Dev. of mean rainfall in 1st spring	**26.72**	**8.94**	**2.99**	**1**	**1123.1**	**9.474**	**0.002**
Dev. of mean rainfall in 1st summer	**-25.42**	**8.04**	**-3.16**	**1**	**1124.2**	**10.551**	**0.001**
Dev. of mean rainfall in autumn	**-31.13**	**8.91**	**-3.49**	**1**	**1126.0**	**12.371**	**<0.001**
Dev. of mean rainfall in winter	**-24.23**	**9.64**	**-2.51**	**1**	**1120.4**	**6.773**	**0.009**
Dev. of mean rainfall in 2nd summer	-8.300	7.77	-1.07	1	1114.9	1.253	0.263
Parameters	**Female zygomatic arch model coefficients**			**Female zygomatic arch model parameter significance**			
	Estimate	SE	t	DF	AIC	X^2	p
Female global model					584.24		
(Intercept)	83.03	2.48	33.51				
Male adults in birth social group	-0.001	0.09	-0.01	1	582.24	0.003	0.953
Total adults in birth population	**0.03**	**0.01**	**1.95**	**1**	**587.01**	**4.778**	**0.029**
Total cubs in birth cohort	**-0.09**	**0.04**	**-2.30**	**1**	**589.69**	**7.453**	**0.006**
Dev. of mean T. in 1st spring	**1.54**	**0.57**	**2.69**	**1**	**592.01**	**9.775**	**0.002**
Dev. of mean T. in autumn	0.72	0.66	1.08	1	583.88	1.642	0.200
Dev. of mean rainfall in 1st spring	**2.61**	**1.13**	**2.30**	**1**	**589.25**	**7.016**	**0.008**
Dev. of mean rainfall in 1st summer	**-3.78**	**1.05**	**-3.61**	**1**	**597.38**	**15.143**	**<0.001**
Dev. of mean rainfall in autumn	-1.49	1.14	-1.31	1	584.56	2.330	0.127
Dev. of mean rainfall in winter	**-3.63**	**1.22**	**-2.98**	**1**	**593.66**	**11.423**	**0.001**
Dev. of mean rainfall in 2nd summer	**-1.53**	**1.00**	**-1.53**	**1**	**585.84**	**3.609**	**0.057**

Note: Dev. = Deviation, T= Temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

Thus, in both sexes, end body length and zygomatic arch width were negatively affected by deviation of mean rainfall in first summer and winter, and additionally, zygomatic width arch width was negatively affected also by the total number of cubs in the respective birth cohort as well as the deviation of mean rainfall in their 2nd summer, whilst surprisingly, the total number of adults in the population had a positive effect (Table 7).

Some effects, however, are sex-specific: male body length and zygomatic arch width was negatively affected by the number of male adults in the social group, whereas in females the number of adult males in social group correlated positively with body length. Similarly, deviation of mean T in autumn correlated positively with zygomatic arch width in males, whereas in females, deviation in rainfall and T in first spring had a positive effect on both body length and zygomatic arch width.

Table 7. Combined results of significant parameters in body length and zygomatic arch global and sex specific models.

Factors	BL (Global)	BL (Male)	BL (Female)	ZA (Global)	ZA (Male)	ZA (Female)
Sex	✓			✓		
Male adults in birth social group		-	+	-	-	
Total adults in birth population				+	+	+
Total cubs in birth cohort	-			-	-	-
Dev. of mean T in 1st spring	+		+	+		+
Dev. of mean T in autumn				+	+	
Dev. of mean rainfall in 1st spring	+		+	+		+
Dev. of mean rainfall in 1st summer	-	-	-	-	-	-
Dev. of mean rainfall in autumn	-		-	-		
Dev. of mean rainfall in winter	-	-	-	-	-	-
Dev. of mean rainfall in 2nd summer	-			-	-	-

General factors affecting body size for both male and female (both global and sex specific models)		
Both BL and ZA	**BL only**	**ZA only**
Dev. of mean rainfall in 1st summer (-)	None	Total adults in birth population (+)
Dev. of mean rainfall in winter (-)		Total cubs in birth cohort (-)
		Dev. of mean rainfall in 2nd summer (-)

Sex specific factors affecting body size (for both BL and ZA)	
Male only	**Female only**
Male adults in birth social group (-)	Dev. of mean T. in 1st spring (+)
	Dev. of mean rainfall in 1st spring (+)

Other sex specific factors affecting body size (BL only or ZA only)	
Male only	**Female only**
Dev. of mean T. in autumn (ZA, +)	Male adults in birth social group (BL, +)
	Dev. of mean rainfall in autumn (BL, -)

Note: BL = Head body length, ZA = zygomatic arch

DISCUSSION

Although mustelids exhibit a hypo-allometric SSD contrary to Rensch's rule (Rensch 1950), and sexual size dimorphism is barely noticeable in adult badgers (Noonan et al. 2016), it was found that average male size of all skeletal measurements included in this study (except tail length, which shows high inter-individual variation suitable for individual recognition: Dixon 2003) was significantly larger than those of females, with inter-sexual differences greatest in head-body length (3.4%) and zygomatic arch width (5%). This mirrors results from previous studies on canine teeth measurements (male canines are approx. 10% bigger than those of

females: Johnson and Macdonald 2001), and cranial characteristics, where male badgers were confirmed to have larger skulls than do females (Abramov and Puzachenko 2005; Harmsen and Van Bree 1998). Furthermore, it was confirmed that also in badgers diverging growth patterns between males and females appear to be the primary physiological mechanism leading to sexual size dimorphism as reported for the majority of species (Badyaev 2002; Isaac 2005). Although weanling cub (3-4 months of age) size did not differ between the sexes, skeletal growth curves diverged significantly from age 11 months (i.e., the onset of puberty: Research Chapter 3) onwards due to continuous rapid growth of males and slowing female growth. Diverging growth patterns in mammals are triggered by rapidly increasing sex-steroid levels during puberty that induce the release of sex-specific growth hormones (GH: Badyaev 2002). Oestrogens trigger elevated basal GH levels in females, while testosterone stimulates high level GH pulses and the related rapid growth rates typical for males (e.g., rats: Painson et al 2000); and therefore, the average age when mammals exhibit sexually dimorphic divergence in somatic growth is a valuable indicator for the onset of puberty and can be used to estimate sexual maturity (Gatford et al. 1998; Potau et al. 1999; Painson et al. 2000).

For most measurements, female cubs reached the end of their skeletal growth (i.e., the asymptote of the respective growth curve) at age 7 months (mean pastern length) to 16 months (head body length), whereas males continued to grow 1-2 months longer until age 8 (mean pastern length) – 18 months (head body length). Similar prolonged growth patterns in males have also been documented in sea otters (*Enhydra lutris*: Bodkin et al. 1993) and in polar bears (Derocher et al 2002) where males reach 97% of their asymptotic body length at 6.2 years, whereas females cease to grow significantly earlier at 4.4 years-of-age. Even in the most dimorphic mammals, however, SSD at birth is low (although often measurable: Derocher et al. 2002), and in many species dimorphism only becomes apparent during juvenile development (e.g., giant panda, *Ailuropoda melanoleuca*, at 4 months: Peng et al. 2001; polar bear, *Ursus maritimus*, at the end of their first year of life: Derocher et al. 2005), despite maternal investment in male offspring being typically higher than that in female offspring (Clutton-Brock et al. 1985; badgers: Dugdale et al. 2003).

In both sexes, however, extremities ceased to grow at an earlier age than body length and zygomatic arch width. As cubs reach independence at the age of 15 weeks (usually in July: Fell et al. 2006), they are required to forage for their own food where longer limbs may

afford more energetically efficient movement (Pontzer 2012). In addition, (perceived) body size is important in intra-specific (e.g., badgers: Stewart et al. 1999, but see Macdonald et al. 2002b) as well as inter-specific aggressive encounters (Marti et al. 1993; Memmott et al. 2000; Carbone et al. 1999), where badger cubs stand on tip-toe and raise their hackles when encountering potential predators (Roper 2010). As badger cubs are vulnerable to infanticide by conspecifics (Woodroffe and Macdonald 1993) as well as being killed by foxes in the UK (Macdonald et al. 2004; Roper 2010) and other large carnivores in continental Europe (Shaparev 1977; Olsson et al. 1997; Jedrzejewska and Jedrzejewski 1998), longer extremities likely help to project a larger, more fearsome opponent (Blanckenhorn et al. 2000).

Nevertheless, a larger body size, and the necessary prolonged and/ or rapid growth to achieve it, are associated with high energy requirements (Blanckenhorn et al. 2000). The availability of nutrients that fluctuate with climatic conditions as well as population density therefore significantly affects mammalian growth patterns (Mahoney et al. 2001, Eifler et al. 2003). Young badger cubs experience exposure to a wide variety of stressors, ranging from social to climatic factors, that may lower their chances of survival and/or impair their growth (Echols et al. 2004; Newman et al. 2001; Macdonald and Newman 2002; Macdonald et al. 2015). Because the metabolic rate of cubs is higher than that of adults due to rapid growth (in European badgers: McClune et al. 2015), any exposure to adverse environmental conditions during this developmental period may be reflected in smaller adult body size (Ferguson and McLoughlin 2000; Derocher and Wiig 2002; Mowart and Heard 2006).

Here, analysis of environmental factors affecting end head body length and zygomatic arch width showed that, in badgers, both climatic and social factors affected somatic growth. In both sexes, growth rates were weather-dependent: Rainfall during their first summer and winter had the most severe effects, while effects of rainfall during their second summer were less severe due to cubs being closer to reaching the end of their somatic growth. Persistently wet conditions expose cubs to increased hypothermic stress, often resulting in higher parasitic infections (Newman et al. 2001; Nouvellet et al. 2010), where survival and recruitment have been reported to be lower in years that are wetter than normal (Newman et al. 2001; Nouvellet et al. 2010; Macdonald et al. 2015), particularly when summer flooding occurs (Macdonald and Newman 2002; Macdonald et al 2010). Weather conditions, especially during the first spring, however, do not only affect cubs directly, but also lactating mothers. Badger milk is very high in fat (Roper et al. 2010), lactation is very energetically demanding

(Woodroffe and Macdonald 1995; McClune et al. 2015), and suckling continues until weaning in early to mid-May (approx. 3 months of age; i.e. time of first capture in this study; Fell et al. 2006). Accelerated growth in brown bear (*Ursus arctos*) and polar bear (*Ursus maritimus*) cubs is associated with higher quality and quantity of milk produced by mothers in better body condition (Robbins et al. 2012).

In addition, social factors also affected male and female growth patterns: Head body length and zygomatic arch width had a negative relationship with the number of cubs in the cohort, mirroring results by Macdonald et al. (2002) and Delahay et al. (2006) that show that an increase in group size may have negative effects on badger growth. Also in the Ursidae, morphometric data are commonly linked to population density, where a higher population density typically results in smaller adult size (Hilderbrand et al. 1999; Ferguson and McLoughlin 2000; Derocher et al. 2002); and, in brown bears, cub-cub resource competition, rather than cub-adult competition, has been suggested as the causal mechanism to explain why average cub size is smaller in bigger cohorts (Dahle et al. 2006).

Nevertheless, the impacts of environmental factors on growth rates often differ between the sexes (Leberg and Smith 1993; Post et al. 1999; Leblanc et al. 2001). Although all badger cubs were affected by their social environment as well as by climatic factors, based on the severity of these effects, it was found that male cubs are more sensitive towards one particular social factor, remaining smaller in social groups with more adult males. In contrast, for females, end size is predominantly affected by climatic factors and associated food availability. This mirrors results from other species, where male adult size has been shown to be more sensitive to social factors, such as population density (e.g., Leberg and Smith 1993; Leblanc et al. 2001), while females are more sensitive to environmental factors, such as weather and food availability (e.g., Post et al. 1999; Atkinson et al. 1996), rather than social factors (Leberg and Smith 1993; Leblanc et al. 2001). Similarly, in big horn sheep *Ovis canadensis* (Leblanc et al. 2001) and white tail deer *Odocoileus virginianus* (Leberg and Smith 1993), population density correlates negatively with body mass in both sexes, and the effect is significantly larger in males than in females resulting in decreased sexual size dimorphism under higher population density.

This sensitivity of male offspring towards social factors is likely due to their prolonged growth period, that facilitates compensation for shorter-term factors such as adverse weather,

but not for long-term adverse social conditions (Leblanc et al. 2001; Festa-bianchet et al. 1994), whereas females lack the ability to compensate for short-term adverse climatic effects, due to their shorter growth periods, but are less affected by long-term effects. This prolonged growth in males often results in considerable inter-individual differences in attaining key developmental life stages (Metcalfe and Monaghan 2001), such as puberty/ sexual maturity (Morgan and Metcalfe 2001; Plaistow et al. 2004; Freetly et al. 2011; Alberts 2012; Pusey 2012; Research Chapter 3) and growth cessation (Morgan and Metcalfe 2001) as shown here.

In the past, sexual selection received virtually universal acceptance as an explanation for male-biased sexual size dimorphism (Mueller, 1990; Karubian and Swaddle 2001; but see Lammers et al. 2001), mostly because larger males are assumed to have a competitive advantage in mate competition (Howard et al. 1998; Hirotani 1994; Wong and Candolin 2005) and mate attraction (Hirotani 1994). Badgers, however, are highly promiscuous (Dugdale et al. 2007) without a clear dominance hierarchy governing mating patterns (Macdonald et al. 2002b; Yamaguchi et al. 2006), and reproductive suppression amongst females is likely as only a small percentage of adult females breed successfully each year (in this study population 45.2%; Annavi et al. 2014), and infanticide occurs (Woodroffe and Macdonald 1993).

In addition, female mate selection is likely to be cryptic, e.g. through sperm competition (Chapman et al. 2003; Wolff and Macdonald 2004; Yamaguchi et al. 2006) and/ or induced ovulation (Yamaguchi et al. 2006). Under these circumstances of decreased male-male competition and heightened female-female competition, however, female body size might be expected to increase and sexual size dimorphism might be expected to disappear or even become female-biased (Isaac 2005). Advantages of larger body sizes in males are indeed not immediately obvious although one potential reason could be that males spend more time on sett excavation than do females (Stewart et al. 1999) - providing a valuable breeding resource (Kaneko et al. 2010; Tsunoda et al. 2018), where longer and stronger limbs would be advantageous (Stewart et al. 1999).

We thus conclude that male-biased sexual size dimorphism in badgers is, most likely, a result of phylogenetic inertia, where smaller mustelids benefit from exaggerated size differences between males and females (Noonan et al. 2016). In badgers, sexual selection is unlikely to be the driving force for sexual size dimorphism (Isaac 2005), but social and environmental

factors affecting juvenile growth patterns and end body sizes are likely important, while diverging growth patterns governed by sex-steroid moderated growth hormones (Badyaev 2002) are the primary physiological mechanism for SSD in badgers. The sudden increase in testosterone at the onset of puberty (Research Chapter 3), and its role as a physiological trigger of somatic growth (Painson et al. 2000; Badyaev 2002), however, is subject to high evolutionary inertia likely explaining the continued existence of sexual size dimorphism in badgers.

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RESEARCH CHAPTER 6

Somatic, social, and climatic factors affecting hormone levels in the European badger (*Meles meles*)

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ABSTRACT

Animals in the wild experience extrinsic changes in the form of environmental and social factors to which they respond through physiological, morphological and behavioural alterations based on their individual phenotypic quality. Reproduction is often affected as a trade-off response in favour of self-maintenance during unfavourable environmental conditions, which is then manifested as changes in reproductive hormone levels. Here, using the European badger (*Meles meles*) as a model species, endocrinological data of male testosterone and female oestrogens across seasons as well as during the main mating season were used as response and analysed against somatic (age, weight, head-body length), social (number of female and male adults in social groups and total adults in population), and climatic factors (rainfall and temperature), to determine which factors affect the physiological state the most when accounted all together. It was found that across seasons (spring, summer, autumn, winter), testosterone in males postively correlated with rainfall and body weight; whilst oestrone in females positively correlated with total number of adults in the population and negatively correlated with temperature. When analysis was restricted to only during the mating season (spring), higher testosterone levels were linked to heavier males, lower female oestrone levels occured in above average temperatures, and higher oestradiol levels were found in longer (head-body length) females as well as conditions where more adults were present in the population. How reproductive physiological regulation differs between sexes in accordance with their respective reproductive strategies is discussed.

Keywords: *badgers, climatic factors, conspecifics, phenotypic quality, reproductive strategies, sex steroid hormone*

INTRODUCTION

In the wild, animals experience various extrinsic environmental and social changes to which they respond through physiological, morphological and behavioural alterations based on their phenotypic quality (i.e. body size, age) in order to maximise individual fitness (Whitten and Turner 2009; Gesquiere et al. 2011). In this context, reproduction as an intensive highly energetic investment (Wade and Schneider 1992) is often affected as a trade-off response in favour of more primary concerns such as self-maintenance during unfavourable extrinsic conditions (Crews 1992; Gesquiere et al. 2011). The endocrine system, as the regulator of most bodily functions, mirrors these effects on reproduction which are manifested as changes in reproductive hormone levels (Wade and Schneider 1992; Gesquiere et al. 2011). Thus, changes in reproductive hormone levels are not only caused by reproductive processes but also affected by extrinsic changes either directly or as a trade-off response (Gesquiere et al. 2011).

Environmental factors such as climate variability (i.e. temperature and rainfall) and associated resource availability are known to significantly affect the somatic condition of an individual animal which in turn may affect its reproductive fitness (Hansen 2009; Gesquiere et al. 2011). Decline in food availability causes deterioration of body condition which is then compensated for by larger efforts in foraging/feeding and hence reduced activity related to reproduction as observed in testosterone level reduction in male Merino rams (*Ovies* aries; Martin et al. 1994) and oestradiol level reduction in female Syrian hamsters (*Mesocricetus auratus*; Wade and Schneider 1992) deprived of food. These decline in testosterone levels were also observed in male tufted capuchin monkeys (*Cebus apella nigritus*; Lynch et al. 2002), yellow baboons (*Papio cynocephalus*; Gesquiere et al. 2011) and olive baboons (*Papio Anubis*; Sapolsky 1986) as caused by reduced food availability during dry seasons/ droughts where temperature was high and rainfall low. In highly seasonal habitats where changes in climatic factors is drastic but predictable, most animals take advantage of periods with favourable environmental conditions and high resource availability to rear young by adopting seasonal mating patterns (Crews 1992) which is reflected in seasonal patterns of reproductive hormone levels as seen in Suffolk rams (*Ovis aries*; Dufor et al. 1984), white deer (*Odocoileus virginianus borealis*; Plotka et al. 1977), as well as the European badger (*Meles meles*; Buesching et al. 2009; Research Chapter 1, 2). Exposure to high ambient

temperatures may also directly influence reproductive processes through heat stress (Hansen 2009) which has been observed to induce lower testosterone levels in boars (*Sus domesticus*; Wetteman and Desjardin 1979) and lower oestradiol levels in Shiba goats (*Capra hircus*; Ozawa et al. 2005).

Social interaction (*i.e.* reproductive activity or competition for resources) is found to have a significant effect on reproductive hormone levels (Strier et al. 1999). In rhesus monkeys (*Macaca mulatta*), the lack of female presence has been found to suppress seasonal patterns in testosterone levels indicating that female stimulus is required for initiation of reproductive processes (Gordon et al. 1978). Similarly, male testosterone levels in gray-cheeked mangabeys (*Lophocebus albigena*) increased with higher numbers of receptive females reflecting a larger investment in mating efforts when exposed to better opportunities of mating success (Arlet et al. 2011). Competition for resources induces testosterone elevation in males which is linked to increased aggression in antagonistic interactions (Cristobal – Azkarte et al. 2006; Arlet et al. 2011). This was observed in mantled howler monkeys (*Alouatta palliate*; Cristobal–Azkarte et al. 2006) where testosterone elevation was positively correlated with the number of outsider individuals introduced to a social group (exposing the group to potential extrinsic threats). Similarly, in gray-cheeked mangabeys (Arlet et al. 2011), resident individuals exhibited higher testosterone levels than immigrant individuals as response to increased competition from a new competitor.

Reproductive responses to extrinsic conditions will vary for each individual depending on their phenotypic quality such as age, body size and sex. In tufted capuchin monkeys, it was observed that subadult males had significantly lower testosterone levels throughout the year than did adult males (Lynch et al. 2002). Similarly, in yellow baboons, chacma baboons (*Papio ursinus*), and geladas (*Theropithecus gelada*) it was found that testosterone initially increased towards reproductive peak age and declined approaching older ages, signifying a shift in reproductive strategies as somatic and physiological conditions change with age (Beehner et al. 2009). In bighorn ewes (*Ovis canadensis*), increase in population density elevates reproductive costs (as resources availability per individual depletes) and only individuals with better body condition as well as higher body mass had good reproductive performance (Festa-Bianchet et al. 1998). Body size has been linked to fasting endurance (Lindstedt and Boyce 1985) which in turn prevents fasting induced anestrous during periods of low resource availability or high energetic costs of mating (Wade and Schneider 1992).

Sex may also have an effect on reproductive response to extrinsic changes as it has been found that effects of heat stress on reproductive hormone levels differs between sexes, as male hormones tend to return to normal levels after a certain amount of time to elevated temperature exposure (Hansen 2009), while females had more permanent detrimental effects such as slower follicle maturation rates, decline in ovulation frequency, and increased embryo mortality (Roth et al. 2000; Ozawa et al. 2005).

As there are multiple environmental, social and individual factors that can affect reproductive hormone levels, possible links between factors may exist. Thus, it is of interest to conduct analyses of the various effects of extrinsic factors to have a comprehensive understanding of how these factors interact and affect reproductive hormones. Here, the correlation between circulating plasma sex-steroid hormones with changes in extrinsic factors and individual variation/phenotypic quality were investigated, using the European badgers (*Meles meles*; henceforth "badger") as a model species.

Badgers reproductive, climatic and social aspects

Badgers are seasonal breeders (Amstislavsky and Ternovskaya 2000) in which the main mating season is during the post-partum oestrus in January-March (extends until May/ June; Yamaguchi et al. 2006). However, the number of oestrus cycles females undergo during summer has been shown to be density-dependent (Corner et al. 2015; Research Chapter 1). In the UK, cubs are typically born (1 annual litter consisting of 1-4 cubs; Macdonald et al. 2015) during mid-February (76% of births, though parturition could occur between mid January – mid March; Neal and Cheeseman 1996; Yamaguchi et al. 2006), with first emergence around mid-April (in the Wytham population; Fell et al. 2006), and are weaned by mid-May (Macdonald et al. 2015). Sexual maturity is achieved around the age of ca. 11 months in both sexes (Research Chapter 3) although it has been observed that in this population breeding starts at the age of 2 years (Macdonald et al. 2009; Dugdale et al. 2011). Once sexual maturity is achieved, circulating sex-steroid levels start to show a distinct seasonal pattern (Buesching et al. 2009; Research Chapter 1, 2). Testosterone levels in males are high in spring, slightly lower in summer, lowest in autumn and peaked during the winter mating season. While in females, oestrone levels are high in spring, low in summer, peak in autumn and stay high in winter for pregnant females and decline in non-pregnant females; and oestradiol levels are only high during spring, and low in the following seasons. Female badgers are known to undergo delayed implantation, where blastocysts can remain suspended

in the uterus lumen for up to 11 months (Corner et al. 2015). It is for this reason that they show endocrinological physiology where more oestrone (rather than oestradiol) is produced in order to sustain pre-implanted blastocysts (see Research Chapter 1).

Badgers are (facultative) social animals in which group sizes depend on population density and range from pair living in low density populations (Kruuk 1989) to large social groups in higher densities (Macdonald et al. 2015). The badger's diet predominantly consists of earthworms (*Lumbricus terrestris*) which were found to have a significant effect on social group dynamics (the dispersion of patches determines the group range whilst the richness of patches determines the number of individuals; Macdonald 1983). Nevertheless, earthworm availability is subject to climate variability where moist conditions are most favorable (Macdonald et al. 2015), while summer droughts can generate significant food shortages which may negatively affect the body condition and survival of badgers. Milder frost-free winters are beneficial for badger body weight and also correlates with elevated badger populations (Macdonald et al 2010), thus showing the significant role of climatic factors in badger wellbeing and survival (Macdonald et al 2015).

In this chapter, endocrinological data of male (testosterone levels) and female (oestradiol and oestrone levels) badgers across seasons (spring, summer, autumn, winter) and during the main mating season in spring were used as a response in ananalysis of the effect of somatic factors (age, weight, head-body length), social factors (number of female and male adults in social groups and total number of adults in population), and climatic factors (rainfall and temperature) to determine which factor affect the physiological state the most.

MATERIALS AND METHODS

Badger trapping and data collection

Data were collected from a high-density badger population in Wytham Woods, Oxfordshire, UK (51^{o}46:26 N, 1^{o}19:19 W; for details see Macdonald et al. 2015), between 1995-2016, as part of an ongoing long-term research project. Following the methodology described in Macdonald and Newman (2002; 2009), badgers were trapped 3-4 times a year: (1) in *spring*

(May/June) at the end of the main mating period, (2) in *summer* (August/September) during the second mating peak previously reported in other high-density badger populations (Cresswell et al. 1992; Page et al. 1994; Carpenter et al. 2005), (3) in *autumn* (October/November) during reproductive quiescence, and (4) in winter (December/January) during the main mating season. All badgers at first capture, typically as cubs, received a permanent unique tattoo allowing reliable aging and individual identification (ID). All animals used in this study were adults (above 2 years of age).

Somatic measurements and population count

Head-body length (mm) was measured to the nearest 5 mm and body weight (kg) was measured to the nearest 100 g. The total number of adult males and females resident to social group were determined annually by assigning residency according to Annavi et al. (2014; see also Macdonald et al. 2008). For cases not addressed by Annavi et al. or Macdonald et al. (i.e. individuals with a gap in capture history), new residency rules were applied (see Research Chapter 5).

Climatic parameters

Climatic parameter data used in this paper were produced using the methods described in Nouvellet et al. (2013). Climatic data (mean temperature and rainfall) were available daily from the start of 1987 until the end of 2016. For every calendar day in a year, a daily long-term average was calculated for climatic data within this time period. For each badger, daily climate data 70 days prior to its capture date was compared against the respective daily long-term average to produce a deviation score for each day. The deviation score from the 70 days was then averaged for analysis. The selection of the 70 day period was based on the study by Noonan et al. (2015) which found that temperature and rainfall up to 70 days (10 weeks) prior to trapping proved more influencial on the badgers than weather conditions near day of capture or seasonal variation.

Blood sampling and hormone measurements

Blood samples of adult badgers were collected by jugular venepuncture in K2-EDTA (ethylene diamine tetraacetic acid) vaccutainer tubes (Becton-Dickinson) for endocrinological analyses. Blood samples were centrifuged within 30 minutes of sampling at 10°C for 10 min under 2,500 rpm. Plasma was transferred into Eppendorf tubes and frozen at -20°C immediately. Sampling times were standardized to account for circadian variation in

hormonal profiles (Buesching et al. 2009). Sex steroid titres were analysed using Enzyme-immunoassays (EIA), where testosterone level was analysed in males (n=114); oestrone (n=117) and oestradiol (n=18) levels were analysed in females. Oestrone and testosterone levels were analysed at the Chester Zoo Endocrinology Laboratory, UK (see details in Research Chapter 1, 2); while oestradiol was analysed at the Endocrinology Laboratory at the German Primate Centre, Germany (see details in Research Chapter 1). Intra-assay coefficient of variation for oestrone (CV, calculated as the average value from the individual CVs for all of sample duplicates), were 8.21 % (high) and 6.05 % (low); inter-assay variation (repeated measurements of high and low-value quality controls across plates) were 13.96 % (high) and 13.62 % (low) respectively. For oestradiol, inter-assay CVs of high and low-value quality controls were 4.5% (high) and 7.4% (low), while the intra-assay CV was 6.2%. The testosterone intra-assay coefficient of variation were 14.69 % (high) and 6.18 % (low), and inter-assay variation of high and low-value quality controls were 9.15 % (high) and 5.23 % (low).

Statistical analysis

A linear mixed model using sex-steroid levels (testosterone, oestrone, and oestradiol respectively) as response with head body length, age, body weight, number of adult males in social group, number of adult females in social group, average temperature deviation, average rainfall deviation as predictors, and season as a covariate was analysed. Year (when badger was captured) and ID (Tattoo number) were assigned as random predictors. A drop1 function, which gives a comparison of model based on AIC criterion (explains whether or not the model without that term is different from the model with that term), was used to determine which factors significantly influenced sex-steroid levels in the mixed models. Oestrone data from pregnant females were excluded from this across-season analysis for ease of analyses, as it is known that pregnant females have high oestrone levels (and are not likely to be influenced externally).

As an a priori test, the same analyses as described above was conducted using a restricted data set including spring only, for both testosterone and oestrone, as this was the main mating season when the majority of males (>98%) showed fully descended testes (Research Chapter 2); while for females, it was the season when the highest proportion of swollen vulva occured (36.39%). Winter data (January) was not included within these restricted mating season models, as it was during the very start of the mating season where most males have not yet

shown morphological and physiological signs of mating (Research Chapter 2), and it was the season where pregnancy occured in females (Research Chapter 1).

Oestradiol levels only show variation during the mating season in spring (and levels were low in the following seasons: Research Chapter 1), hence only oestradiol levels exclusive to this season were used in the oestradiol model. As all oestradiol levels were independent data points, a linear model was conducted using the same parameters used in the testosterone and oestrone models. Before the models were run, correlation between continuous parameters were analysed with a Pearson correlation test. All parameters included in these models were independent. Patterns of residuals, normality, and mean variance relationship for each model were checked using diagnostic plots in R. All statistical analyses were performed using RStudio (0.99.896; RStudio Team 2015) and R (R-3.2.4; R Core Team 2013).

RESULTS

Male physiological responses

Based on the global model using testosterone levels across seasons, among all somatic, social, and climatic factors analysed, weight and average rainfall deviation affected testosterone levels the most in males (for details see Table 1), with both positively correlated with the response. While when testosterone levels exclusive to the mating season in spring were analysed, the models showed that the weight of males significantly affected the variation of testosterone levels, where males with a larger mass exhibited higher hormone levels (Table 2).

Table 1. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the male model with testosterone levels across seasons as response.

Parameters	Across season testosterone model coefficients			Across season testosterone model parameter significance			
	Estimate	SE	t	DF	AIC	X^2	p
Across season testosterone model					63.227		
(Intercept)	-1.74	0.99	-1.77				
Head Body Length	0.04	0.04	0.88	1	61.969	0.742	0.389
Age	-0.002	0.03	-0.05	1	61.227	0.000	0.998
Weight	**0.05**	**0.03**	**1.92**	**1**	**65.271**	**4.044**	**0.044**
Total adult males in SG	0.005	0.02	0.33	1	61.245	0.018	0.893
Total adult females in SG	-0.008	0.01	-0.63	1	61.457	0.230	0.631
Total adults in population	-0.0002	0.004	-0.07	1	61.243	0.016	0.900
Avg. T deviation	0.02	0.04	0.66	1	61.660	0.433	0.511
Avg. Rainfall deviation	**0.17**	**0.08**	**2.11**	**1**	**64.465**	**3.238**	**0.072**
Season				**3**	**87.526**	**30.299**	**<0.001**
Season Spring	0.40	0.08	4.92				
Season Summer	0.13	0.09	1.50				
Season Winter	0.92	0.32	2.91				

Note: Avg.= Average, SG= Social group, T = temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

Table 2. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the male model with testosterone levels during the mating season in spring as response.

Parameters	Spring testosterone model coefficients			Spring testosterone model parameter significance			
	Estimate	SE	t	DF	AIC	X^2	p
Spring testosterone model					48.109		
(Intercept)	-2.71	2.09	-1.30	1			
Head Body Length	0.04	0.09	0.49	1	46.275	0.166	0.684
Age	0.06	0.07	0.83	1	46.814	0.704	0.401
Weight	**0.12**	**0.08**	**1.57**	**1**	**50.128**	**4.019**	**0.045**
Total adult males in SG	0.009	0.03	0.32	1	46.139	0.030	0.863
Total adult females in SG	0.007	0.03	0.26	1	46.279	0.170	0.680
Total adults in population	0.001	0.006	0.16	1	46.204	0.094	0.759
Avg. T deviation	-0.003	0.06	-0.06	1	46.111	0.001	0.973
Avg. Rainfall deviation	0.04	0.23	0.16	1	46.253	0.1432	0.705

Note: Avg.= Average, SG= Social group, T = temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

Female physiological responses

Oestrone

For females, when oestrone levels across seasons were analysed, the total number of adults in the population and the average temperature deviation affected oestrone levels the most, with total number of adults correlated positively and average temperature deviation correlated negatively with oestrone levels (Table 3). When oestrone levels exclusive to the mating season in spring were analysed, the models showed that the only parameter significantly

affecting oestrone levels was average temperature deviation. With higher temperatures causing lower secretion of oestrone levels (Table 4).

Table 3. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the female model with oestrone levels across seasons as response

Parameters	**Across season oestrone model coefficients**			**Across season oestrone model parameter significance**			
	Estimate	SE	t	DF	AIC	X^2	p
Across season oestrone model					18.665		
(Intercept)	0.09	0.86	0.10				
Head Body Length	-0.01	0.03	-0.45	1	16.889	0.224	0.636
Age	0.03	0.02	1.40	1	18.825	2.160	0.142
Weight	0.02	0.02	0.64	1	17.122	0.458	0.499
Total adult males in SG	0.0003	0.01	0.02	1	16.666	0.001	0.981
Total adult females in SG	0.01	0.01	1.14	1	18.093	1.428	0.232
Total adults in population	**0.003**	**0.002**	**1.77**	**1**	**20.089**	**3.424**	**0.064**
Avg. T deviation	**-0.04**	**0.02**	**-1.74**	**1**	**19.974**	**3.309**	**0.069**
Avg. Rainfall deviation	-0.05	0.05	-0.93	1	17.622	0.957	0.328
Season				**3**	**61.674**	**49.009**	**<0.001**
Season Spring	-0.09	0.08	-1.08				
Season Summer	-0.37	0.08	-4.92				
Season Winter	-1.29	0.27	-4.75				

Note: Avg.= Average, SG= Social group, T = temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

Table 4. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the female model with oestrone levels during the mating season in spring as response

Parameters	**Spring oestrone model coefficients**			**Spring oestrone model parameter significance**			
	Estimate	SE	t	DF	AIC	X^2	p
Spring oestrone model					7.702		
(Intercept)	-0.66	1.24	-0.53				
Head Body Length	0.02	0.04	0.56	1	6.079	0.377	0.539
Age	0.02	0.04	0.60	1	6.139	0.437	0.509
Weight	-0.04	0.05	-0.87	1	6.606	0.904	0.342
Total adult males in SG	0.02	0.02	1.17	1	7.341	1.639	0.200
Total adult females in SG	0.01	0.02	0.30	1	5.808	0.106	0.745
Total adults in population	0.003	0.003	1.09	1	7.128	1.425	0.233
Avg. T deviation	**-0.07**	**0.03**	**-2.28**	**1**	**11.650**	**5.948**	**0.015**
Avg. Rainfall deviation	-0.06	0.08	-0.65	1	6.213	0.511	0.475

Note: Avg.= Average, SG= Social group, T = temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

Oestradiol

As oestradiol levels only showed variation during the mating season in spring (Research Chapter 1), only spring oestradiol levels were used in the oestradiol model. The only parameters that significantly affected oestradiol levels in females was head body length and total adult in population, where both of them positively correlated with the response (Table 5).

Table 5. Coefficients (Estimate, SE, and t-value) and significance of parameters (Df, AIC, X^2, and p-value) of the female model with oestradiol levels during the mating season in spring as response.

Parameters	**Spring oestradiol model coefficients**			**Spring oestradiol model parameter significance**				
	Estimate	SE	t	DF	Sum Sq	Mean Sq	F	p
Spring oestradiol model								
(Intercept)	-8.95	11.68	-0.77					
Head Body Length	**0.13**	**0.17**	**0.73**	**1**	**0.953**	**0.953**	**5.435**	**0.045**
Age	-0.03	0.12	-0.28	1	0.037	0.037	0.208	0.659
Weight	0.01	0.21	0.05	1	0.016	0.016	0.094	0.766
Total adult males in SG	0.03	0.06	0.58	1	0.234	0.234	1.334	0.278
Total adult females in SG	-0.03	0.06	-0.43	1	0.012	0.012	0.071	0.796
Total adults in population	**0.04**	**0.10**	**0.45**	**1**	**0.613**	**0.613**	**3.499**	**0.094**
Avg. T deviation	0.12	1.49	0.08	1	0.0006	0.001	0.003	0.957
Avg. Rainfall deviation	0.78	1.02	0.77	1	0.104	0.104	0.591	0.462
Residuals				9	1.578	0.175		

Note: Avg.= Average, SG= Social group, T = temperature. Threshold of significance is $p<0.05$. Significance based on drop 1 function

DISCUSSION

Circulating sex-steroid levels in both male and female badgers were significantly affected by individual variation in body size and condition, as well as extrinsic factors which include weather variables and conspesifics presence. Testosterone levels across seasons in males had a significant positive correlation with weight and average rainfall deviation, which shows that heavier males produce more testosterone as well as wetter conditions are more favourable for testosterone secretion (important for mating activity). During the mating season in spring, weight influenced testosterone levels significantly.

Across seasons, oestrone levels in females elevated with higher numbers of adult badgers in the population and decreased with higher average temperature deviation, while in the mating

season in spring, oestrone levels were significantly affected by average temperature deviation alone. Oestradiol levels during the mating season in spring had a significant positive correlation with both head body length and a close to significance positive relationship with total numbers of adults in the population.

Body weight, which may also be a proxy of body condition and health, plays a major role in testosterone level secretion in male badgers. This mirrors cases in other animals where body condition and nutritional status are ultimate factors affecting variation of testosterone levels in males (Duckworth et al. 2001). The explanation for this relates to energetics, since energy from food consumption must fulfill the requirement associated with self maintenance first, while other proccesses such as reproduction and growth are of lower priority (Sapolsky 1986). Thus, once self maintenance is satisfied, excess energy may be used for reproduction, which is exhibited by higher testosterone levels. Sexual traits and behaviour expressions related to high testosterone levels are energetically costly to produce and may cause negative effects on the immune system (Marler and Ryan 1996; Pérez-Rodríguez et al. 2006). For example, during the mating season when testosterone levels are high, male badgers typically exploit their energy resources by increasing activity (roaming for receptive females) which in turn decreases their body condition, haematological parameters (Woodroffe and Macdonald 1995), and increases bite wounds (Macdonald et al. 2004). By the time the mating season is done, males are usually observed with significant loss in body weight and body condition (Woodroffe and Macdonald 1995; Buesching et al. 2009). Hence only individuals in good body condition may express these costly reproductive traits (to a larger proportion) as they could cope with such energetic costs (Folstad and Karter 1992; Brown 1996; Duckworth et al. 2001). Observations of higher testosterone levels in animals with better body condition were also found in the hanuman langur (*Semnopithecus entellus*; Koenig et al. 1997), Long-tailed macaques (*Macaca fascicularis*; van Schaik and van Noordwijk 1985), European rabbits (*Oryctolagus cuniculus*; Monclus et al. 2006; Cabezas et al. 2007) and Orangutans (*Pongo sp.*; Knott 2005).

An alternative perspective in understanding the mechanism of how body condition affects reproductive hormone levels (and mating activity) is through the mediation of stress hormones. Stress hormones, i.e. glucocorticoids, typically increase as a consequence of a decrease in body weight (as this causes 'energetic stress': Perez-Rodriques et al. 2006; Cabezas et al. 2007; Gesquiere et al. 2012). Elevated glucocorticoid levels will in turn reach

a threshold level that inhibits reproductive hormone production (Wingfield and Kitaysky 2002). In European rabbits (*Oryctolagus cuniculus*), body mass explained a large proportion of cortisol level variation amongst individuals, where elevated cortisol levels were found in animals with lower scores of body condition (Cabezas et al. 2007). In the male red-legged partridge (*Alectoris rufa*), cortisol levels were subsequently elevated by decrease of body weight (15% from initial weight) after food shortage, causing lower secretions of androgen levels (Perez-Rodriques et al. 2006).

Reproductive physiology of an individual may be largely influenced by the environmental conditions it is exposed to (Jacobs and Wingfield 2000; Graham 2004; Walker et al. 2005). This is definitely expressed in the results here which showed that rainfall played a major role in testosterone secretion across seasons, where elevated testosterone levels were found in wetter conditions. This may be due to food resources (in general, but earth worms *Lumbricus terrestri*s in particular, the predominant diet of badgers; Noonan et al. 2014; Macdonald et al. 2015) being more plentifulwhen there is more rainfall compared to dryer conditions (Macdonald and Newman 2002; Macdonald et al. 2009; Nouvellet et al. 2013). During favorable weather conditions such as this, food resource is abundant, nutritional intake increases, and body condition is improved, allowing this extra energy to be used for physiologically non-primary processes such as reproduction. In contrast, during less favourable conditions (e.g., occurance of drought), reproductive function may shut down and testosterone levels may decrease as available energy within an individual may not allow for both self-maintenance and reproduction at the same time, thus trade-offs are necessary.

During the main mating season in spring, only body weight affected testosterone levels significantly, while average rainfall deviation was no longer a significant factor. This is likely due to the fact that spring is a season when food resources are already abundant (compared to other seasons; Macdonald and Newman 2002; Macdonald et al. 2009; Macdonald et al. 2015), thus rainfall does not have much of an effect on testosterone levels, but rather the ability of the badger to gain these resources (shown by heavier body weight) does. Other examples of testosterone production favoring wetter conditions/high rainfall in other species include male baboons in the Amboseli basin (*Papio cynocephalus*) which exhibited lower testosterone and higher glucocorticoid levels during the dry season (2.6 times fold for both) compared to the wet season (Gesquiere et al. 2012); the male white rhinoceros (*Ceratotherium simum simum*) that had elevated androgen concentrations during months of

high rainfall (September–February) compared to months of little or no rainfall (March–August; Kretzschmar et al. 2004); and the tufted capuchin monkey (*Cebus apella nigritus*) that showed mean adult testosterone levels were positively correlated with rainfall across the year (Lynch et al. 2002).

Testosterone secretion intensity is a plastic response which is dependent on the current physiological and physical state of an individual (health, nutritional status, body condition; Folstad and Karter 1992; Brown 1996). Individuals are expected to self regulate their hormonal levels according to their concurrent somatic state, thus trade-offs between testosterone levels (in relation to reproductive processes) and self-maintenance may occur when faced with unfavorable conditions (Gesquiere et al. 2012). Factors affecting testosterone level secretion are important to understand as it affects male fertility such as spermatogenesis, sperm quality and quantity, as well as reproductive behaviour (Miner and DeLiberto 2008).

In female badgers, a decline in oestrone levels was linked to higher temperatures. Oestrone is known to maintain a suitable uterine environment for the survival of pre-implanted blastocysts, extend the life-span of the corpus luteum (CL), and prepare the uterus for implantation (Heap et al. 1975; Heap et al. 1979), which are all important for badger blastocysts that go through long periods of delayed implantation (see Research Chapter 2).

High temperatures may cause heat stress and energetic constraints due to the cost of thermoregulation (Hansen 2000; Gesquiere et al. 2008, 2012); by redistribution of blood flow to the periphery areas (and less to other organs, including the gonads) to regulate heat loss and also by reduced food intake during heat stress causing energy and nutrient imbalance (Roth et al. 2000; Hansen 2000). This will affect female reproduction processes through slowing down of follicle maturation, lowering ovulation frequency, and increasing embryo mortality (Hansen 2009), thus altering steroid secretion such as oestrogens (Roth et al. 2000; Ozawa et al. 2005). Examples of this phenomenon include the reduced concentrations of plasma oestradiol and Luteinizing Hormone (LH) causing delayed ovulations in goats (*Capra aegagrus hircus*; Ozawa et al. 2005); and reduced levels of oestradiol in folicullar fluids, as well as a decrease in gonadotropin receptors in granulosa cells of rats (*Rattus rattus*; Shimizu et al. 2005). Moreover, preimplanted blastocysts are found to be susceptible to maternal heat stress, as a proportion of embryos in female cows (*Bos taurus)* exposed to heat stress reduced

when examined 8 days after mating (Ealy et al. 1993); while mouse (*Mus musculus*) embryo development slowed down during heat stress due to reactive oxygen species occuring in the oviduct and embryo body (Matsuzuka et al. 2005). It is likely that for this reason, oestrone levels (as proxy of blastocyst availability) decreased during unfavourable higher temperatures.

Not only higher temperatures may negatively affect female reproduction, but below average temperatures are not favourable as well, e.g. the chacma baboons (*Papio ursinus*) which live at high latitutes cope with lower ambient temperatures by increasing inter-birth intervals (Hill et al. 2000) and cortisol levels (Weingrill et al. 2004); while gelada monkeys (*Theropithecus gelada*), also living at higher altitudes, reduce birth rates (Ohsawa and Dunbar 1984).

Another mechanism in which higher temperatures may affect oestrone levels negatively is through drought which in turn affects food availability, thus limiting nutritional resource causing low body conditions which will impair reproductive function such as decrease in frequency of ovulation, lower oestrogen and progesterone levels, and longer interbirth intervals (Gesquiere et al. 2008, 2012).

The total number of adults affected both oestrone levels across seasons and oestradiol levels during the mating season, with the number of conspecifics positively correlated with secretion in both of these hormones. It is known that social stimuli caused by conspecific presence may promote endocrine changes in individuals (Gonzales et al. 1988; Lott 1991; Kotrschal et al. 2000). When there are more individuals in the population, presumably males, female badgers are likely to encounter a large number of potential mates during the mating period (hence increasing oestradiol levels) – ensuring fertilization (producing blastocysts reflected in oestrone levels). Changes in sex-steroid levels caused by conspecific presence are also demonstrated in other species such as the American mink (*Neovison vison*) which showed an increase in oestrogen levels and also occurance of ovulation when placed in contact with males (Amstislavsky and Ternovskaya 2000), and the male white rhinoceros (*Ceratotherium simum simum*) which had elevated androgen levels when placed near receptive females, compared with males in seclusion (Kretzschmar et al. 2004).

In contrast to males, the only somatic parameter that significantly affected female sex-steroid levels was head body length (and not body weight) where larger/ longer females produce

more oestradiol during the mating season. This finding makes sense as larger animals can store more energy and so can invest more in reproduction. However, it is of interest why female reproductive hormones were not correlated with body weight as in males, but rather with something more permanent such as head body length. This is likely related to the fact that female reproductive investment is long term (pregnancy, lactation, cub rearing) compared to short-term investment in males (only during mating; Woodroffe and Macdonald 1995; Yamaguchi et al. 2006; Gesquiere et al. 2012). Male reproductive investment during the mating season is short term but energetically costly, therefore testosterone levels reflect the capacity and quantity of investment made by the male during this particular period. The capacity to invest is largely influenced by the body condition (exhibited in weight) acquired during that specific period which dictates the quantity of the investment. While in females, oestradiol levels reflect not only the capacity during the particular mating period (body condition) but also the individual specific capacity (somatic) shown in head body length to undergo the whole female reproductive process.

I conclude that sex-steroid levels reveal mechanisms by which individuals reproduce while responding to social and climatic changes in its environment, based on their respective phenotypic quality. As animals are exposed to a variety of environmental changes that may be unfavorable (McEwen and Wingfield 2003; Wingfield 2004), individuals are expected to self regulate their hormonal levels according to their concurrent physical state to maximize fitness (which may involve trade-offs between reproduction and self-maintenance). This reproductive physiological regulation may not only differ among individuals, but also between sexes according to their respective reproductive strategies.

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PART V

DIFFERENT METHODS IN HORMONE ANALYSES

RESEARCH CHAPTER 7

Measuring sex-steroid metabolites in urine with enzyme immunoassay (EIA) to establish a new non-invasive method for determination of reproduction status in the European badger (*Meles meles*)

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ABSTRACT

Conventionally, hormone analysis is conducted using blood samples as it gives a direct measure of circulating hormone levels at the time of sampling and is considered to be most accurate in reflecting endocrine function. Nevertheless, routine blood sampling may not always be practical for wild animals and may induce stress which affects accuracy of analyte measurements. Thus, development of alternative hormone measurement methods to blood sampling is of interest. Circulating hormones are metabolized through different routes, either by the kidneys to be excreted through urine or by the liver to be excreted through faeces; the rest may be deposited in hair whilst a small portion enters saliva. These traces of hormones can be utilized in non-invasive hormone measurement methods as a proxy to estimate circulating hormone levels and to determine reproductive parameters. Here, the relationship between circulating plasma sex-steroid hormones with urinary metabolites were investigated (analysed with enzymeimmunoassays) for non-invasive assessment of the reproductive status using the European badger (*Meles meles*) as a model species, by correlating circulating testosterone in males and total oestrogen in females with urinary metabolites from paired samples across seasons and test whether urinary metabolites alone, in both sexes, mirrored the seasonal reproductive cycle pattern, as an additional biological validation. On an individual level, it was found that in males, plasma and urine testosterone levels correlated significantly across seasons while in females no correlation was found. When additional biological validation was conducted, both testosterone and total oestrogen metabolite excretion patterns corresponded with seasonally mediated reproductive events known for badgers. This implies that urinary metabolite measurement may reliably assess endocrine function in badgers, especially in males; while for females, application on a wider time scale is likely more suitable due to potential longer time lag between circulating hormones and urinary metabolites (explaining the non-correlation). This information was used to validate and assess the usefulness of urinary metabolite measurement for reliably assessing endocrine function in badgers.

Keywords: *biological validation, European badgers, non-invasive monitoring, reproductive status, sex-steroids, urine metabolites*

INTRODUCTION

In conservation, knowledge of a species' reproductive physiology (i.e. reproductive status, reproductive cycle, etc.) is important (Monfort et al. 1997). Because hormones regulate almost all aspects in the life of animals, including reproduction (Adkins-Reagan 2005; Touma and Palme 2005), a detailed understanding of the interactions between reproductive physiology, endocrinology and environmental factors can help predict the response of a population to outside stimuli which in turn helps effective design and implementation of conservation management strategies (Hindle et al. 1992; Monfort et al. 1997; Graham 2004).

The main endocrinological regulators of mammalian reproduction are male testosterone (regulating male reproductive physiology and behaviour), and female progesterone (regulating pregnancy) and oestrogens (regulating ovulation and oestrus). These sex-steroid hormones are produced mainly by the gonads (testes and ovaries; Touma and Palme 2005; Dehnhard et al. 2008) and released into the blood stream, from where they are metabolized either by the kidneys (to be excreted through urine) or by the liver (through faeces: Graham 2004; Touma and Palme 2005; Heistermann 2010) either as conjugated or as unconjugated metabolites (Schwarzenberger et al. 1996; Heistermann 2010). In addition, a small proportion of these sex-steroids may be deposited in hair or enter the saliva (Heistermann 2010).

Conventionally, hormone levels are determined by analysing circulating levels in blood/ plasma samples (Heistermann et al. 1993; Monfort et al. 1997; Dehnhard et al. 2008; Heistermann 2010). This method measures the immediate "active" hormone level directly at the time of sampling and is considered to be the most accurate method in reflecting endocrine function when collected in a longitudinal data set (Heistermann et al. 1993; Graham 2005; Dehnhard et al. 2008; Heistermann 2010). However, routine blood sampling may not be practical in wild animals (Monfort et al. 1997; Touma and Palme 2005) as it may induce stress, which in turn can induce hormonal changes and/ or affect assay accuracy due to cross-reactivity (e.g., with adrenaline metabolites: Dehnhard et al. 2008; Heistermann 2010). Furthermore, blood steroid levels reflect hormone levels at the exact time of sampling and may therefore be biased by transitionary effects such as circadian variations, pulsatile secretion, and stress (Touma and Palme 2005; Heistermann 2010). Lastly, based on the principles of the "3Rs" (i.e., Replacement, Reduction, and Refinement: Animals (Scientific

Procedures) Act 1986), refinement of research methods to increase animal welfare and reduce pain should be used whenever possible (Department of Health 2014), and thus the development of alternative, non-invasive endocrinological methods to blood sampling is important (Heistermann 2010).

Non-invasive methods based on hormone traces in saliva, hair (Koren et al. 2002; Heistermann 2010), nails/ claws (Mack and Fokidis 2004; Matas et al. 2016), or based on hormone metabolites in faeces or urine (Graham 2004; Touma and Palme 2005; Heistermann 2010) have been established as a proxy to estimate the levels of circulating hormones, and to determine reproductive parameters of a wide range of wild and captive species (Hindle et al. 1992; Monfort et al. 1997; Graham 2004; Schwarzenberger 2007; Dehnhard et al. 2008); often easing data collection (especially in wild animals) as they remove the need to handle and consequently stress the animal (Graham 2004; Heistermann 2010). However, these non-invasive methods have to be validated carefully against circulating plasma hormone titres, and choosing the most appropriate one amongst them is dependent on species (Schwarzenberger 2007) and research question (Graham 2004; Touma and Palme 2005).

Hormones in saliva typically occur in their original, non-metabolized form, and exhibit a very low time-lag, but saliva is difficult to obtain without handling the animal (Heistermann 2010). Hormones deposited in hair and nails, in contrast, exhibit a very long time–lag, and are thus suitable only for long-term monitoring in the order of weeks or even months (Heistermann 2010), and moulting patterns as well as keratine deposit rates have to be taken into account (Koren et al. 2002; Mack and Fokidis 2004). Therefore, the most suitable non-invasive method is often the analysis of urinary and/ or faecal sex-steroid levels (Heistermann 2010). When analysing hormone levels in urine/ faeces, however, several considerations have to be taken into account: i) The predominant metabolic pathway (urine vs. faeces) can differ significantly between species (Schawrzenberger et al. 1996; Schwarzenberger 2007); ii) Because hormones are typically metabolised before being eliminated in excreta, the inherent time lag between plasma hormone levels and faecal/ urinary hormone levels can vary considerably dependent on species, individual, metabolism, water-/ food intake, and activity-level (Palme et al. 1996; Graham 2004; Touma and Palme 2005; Schwarzenberger 2007); and iii) Because excretions of metabolized hormones are mostly discrete events (i.e., bowel movements/ emptying of bladder), hormones are pooled in these excretions over a (more or less variable) period of time (Graham 2004; Touma and Palme 2005), and can vary according

to circadian rhythm, activity levels, and fluid/ dietary fibre intake, necessitating careful assay calibration and sample quantification/ standardisation (Miller et al. 2004). The resulting hormone profiles are, however, more stable than to profiles obtained from measuring the pulsative/ episodically released plasma hormones, while still providing a fairly accurate measure of an animal's current endocrinological status.

Faecal hormone measurements are widely considered to be the most practical in terms of sample collection in wild animals (Touma and Palme 2005; Heistermann 2010), especially if combined with faecal DNA-analysis to identify the donor individual (Kalz et al. 2006). In many species, faecal hormones are present predominantly in their unconjugated form, improving assay specificity (Schwarzenberger et al. 1996; Graham 2004; Heistermann 2010). Nevertheless, the time lag between circulating plasma hormones and faecal excretion is typically more than 12 hours (Palme et al. 1996; Graham 2004; Heistermann 2010), making this method less suitable for correlating an animal's behaviour with its immediate endocrinological profile (Heistermann 2010).

The lag time in urinary hormone profiles, in contrast, is significantly lower (2 to 8 hours; Palme et al. 1996; Heistermann 2010) making the analyses more sensitive to short-term endocrinological events than faecal hormones (Heistermann 2010; Touma and Palme 2005). However, urine can be difficult to collect from wild animals (Graham 2004), and levels vary considerably with time of day, necessitating a standardised sample collection regime (Heistermann 2010). In addition, urinary hormones typically occur in conjugated form (Graham 2004; Heistermann 2010), and assays are therefore susceptible to cross-reactivity and/ or lower sensitivity (Hindle et al. 1992).

Here, for the first time, a non-invasive, urine-based sex-steroid analysis for the European badger (*Meles meles*; henceforth "badger") was designed and validated. The badger is one of the most intensively studied mammals in the world (around 1640 scientific publications based on google scholar search of *Meles meles* in titles) and has been recognised as a suitable model species for many biological paradigms (Stopka and Johnson 2000). Badgers are facultative social mesocarnivores that exhibit an atypical reproductive strategy incorporating delayed implantation and superfoetation (Macdonald et al. 2015). Previous measurements have investigated plasma sex-steroid profiles (testosterone, progesterone and oestrogens) in the context of mammalian reproductive strategies (Buesching et al. 2009; Research Chapter 1, 2).

Nevertheless, although trapping and handling of badgers necessary for blood sample collection is regulated by strict national and international laws (Bern convention; Animal act), thus far, no non-invasive method to measure sex-steroid hormone levels has been established for this species, curtailing the possibility of a physiological component to many research topics.

The chemical composition of badger urine has been established to vary with sex and season (Service et al. 2002). Badgers use urine for scent-marking, urinating directly on the ground, on vertical objects, on "crossing" points in their paths or on top of their setts (Buesching and Macdonald 2004; Roper 2010) in addition to specific urination and defecation sites ("latrines"), which are shared between individuals and act as boundary markers for social group territories (Buesching and Macdonald 2004; Buesching et al. 2016; Hutchings et al. 2001). Understanding of badger urination behaviour allows for the establishment of non-invasive urine sample collection methods such as urine bait marking (Hutchings et al. 2001) accompanied by possible soil urine extraction (Kirkpatrick et al. 1988) or the use of artificial urination pits (L. Rosalino pers. comm.), which in turn allows for feasible non-invasive reproductive assessment.

In this study, whether urinary hormone metabolites correlate with circulating plasma sex-steroid hormones sufficiently to establish badger reproductive status was investigated by i) correlating circulating hormone levels (testosterone in males; total oestrogen in females) with urinary metabolites from paired samples across seasons, and ii) testing whether urinary metabolites alone, in males and females, mirrored the seasonal reproductive cycle pattern, as an additional biological validation. This information was used to validate and assess the usefulness of urinary testosterone and total oestrogen metabolite measurement for reliably assessing endocrine function in badgers.

Badger reproductive biology

Badgers are seasonally breeding carnivores that are members of the mustelidae family (Amstislavsky and Ternovskaya 2000). They typically produce one litter annually (with mean litter size of 1.4±0.06, range of 1-4 cubs, where 93% litters were less than 3 cubs: Macdonald et al. 2015), born between mid January – mid March (76% of cubs in the UK are born in mid-February: Neal and Cheeseman 1996; Yamaguchi et al. 2006). Their mating season is mainly

restricted to January-March (post-partum oestrus), although further matings can extend until May/ June (Yamaguchi et al. 2006) with number of additional oestrus cycles ranging from zero to monthly determined by local population density (Corner et al. 2015; Research Chapter 1).

During spring, which is still within the main mating season (Yamaguchi et al. 2006), males have large scrotal testes, and females exhibit a distinctly swollen, pink and everted vulva (Research Chapter 2). Summer coincides with the lowest food availability (Macdonald and Newman 2002) and the supposedly additional matings in other populations (Cresswell et al. 1992; Page et al. 1994; Carpenter et al. 2005). In autumn reproductive quiescence occurs (Macdonald et al. 2010; Woodroffe and Macdonald 1995), where males typically have smaller testes that ascend into the body cavity and females tend to exhibit a flat/ normal vulva condition (Research Chapter 2). Female badgers undergo delayed implantation with blastocysts remaining in suspended animation for up to 330 days (Yamaguchi et al., 2006) until implantation occurs in mid-December (reviewed in Research Chapter 1). This causes females to develop an endocrinological system to produce more oestrone than oestradiol to sustain these pre-implanted blastocysts (Research Chapter 1).

Previous measurements on plasma oestrogen, progesterone, and testosterone profiles associated with mating seasons, delayed implantation, and pregnancy have shown that circulating sex-steroid levels showed a distinct seasonal pattern in sexually mature badgers (Buesching et al. 2009; Research Chapter 1, 2): In males, testosterone levels are high in spring, slightly lower in summer, lowest in autumn and peaked during the winter mating season. In females, oestrone levels are high in spring, low in summer, peaked in autumn and remain high for pregnant females in winter, but decline in non-pregnant females. While oestradiol levels only showed a distinct peak during spring and remained low in the following seasons, showing that this study population only has a single mating season (Research Chapter 1)

MATERIALS AND METHODS

Badger trapping and sampling

Data were collected as part of an ongoing long-term research project on European badgers *(Meles meles)* from a high-density badger population in Wytham Woods, Oxfordshire, UK (51°46'26" N, 1°19'19" W; see Macdonald et al., 2015) between 2012-2017. Badgers were trapped three times annually in: (1) *spring* (May/June) towards the end of the main mating season, and a period of high food/ water abundance; (2) *summer* (August/September) during the second mating peak previously reported in other badger populations and period of lowest food/ water abundance; and (3) *autumn* (November) during reproductive quiescence and abundant food and water, following the methodology described in Sun et al. 2015. Traps were checked (6.30-8.00 am) and captured badgers were transported to a central field station to then be sedated (usually starting no later than 8 am) by intramuscular injection of 0.2 ml ketamine hydrochloride/ kg body weight (McLaren et al. 2005; Thornton et al. 2005) with an induction time between 3-4 minutes. Processing of all captured badgers was completed before noon with a typical procedure (data collection and handling) of 10-15 minutes per badger (Sun et al. 2015), from sedation to completion. At first capture, typically as cubs, all badgers received a permanent unique tattoo allowing individual identification (ID) and accurate aging thereafter. If new individuals were first caught as adults, age was estimated using toothwear (Harris et al. 1992).

Plasma and urine samples

Paired blood and urine samples were collected from captured adult individuals (66 males and 61 females) under sedation. Blood samples (not exceeding 2 ml) were obtained by jugular venipuncture in K2-EDTA (di-potassium ethylene diamine tetraacetic acid) vaccutainer tubes (Becton-Dickinson), and centrifuged at 10°C for 10 min at 2,500 rpm (1470g) within 30 minutes. Plasma was then transferred into Eppendorf tubes (1.5 ml) and frozen immediately at -20°C. Urine samples were collected in glass bottles with stainless steel layered caps (10 ml) by manual palpation (wearing a fresh pair of latex gloves after each animal) and then transferred with a plastic pipette into Eppendorf tubes (1.5 ml) and frozen immediately at -20°C. All sampling times were standardised to account for circadian variation in hormonal profiles (Buesching et al. 2009).

Sample extraction

Plasma (0.1 mL) was extracted with 2.5 mL petroleum ether. After freezing at –80°C, the organic phase was decanted, dried at 50°C under a stream of nitrogen, and dissolved in 1 mL 40% methanol. For total oestrogen as well as testosterone analyses, 20 µl aliquots were used. To 0.1 ml urine, 0.5 ml 0.05 M acetate buffer pH 4.8 was added including ß-glucuronidase / arylsulfatase from Helix pomatia and incubated for 2 h at 37°C. For extraction, 2.5 ml of a mixture of tert butylmethylether/ petroleum ether 30/70 % v/v were added. Following shaking for 20 min samples were frozen at -80°C. After freezing at –80°C, the organic phase was decanted, dried at 50°C under a stream of nitrogen, and dissolved in 1 mL 40% methanol.

Hormone analyses

Total oestrogen analyses were carried out using a polyclonal antibody raised in rabbits to 1,3,5(10)-estratrien-3-17ß-diol-17-HS-BSA and 1,3,5(10)-estratrien-3-17ß-diol-17-HS-peroxidase label. The cross-reactivities to oestrogens were as follows: 1,3,5(10)-estratrien-3,17b-diol (17ß-E2), 100%; 1,3,5(10)-estratrien-3,17-one (oestrone), 100%; 1,3,5(10)-estratrien-3,17a-diol (17a-E2), 66%; 1,3,5(10)-estratrien-3,16a,17b-triol (oestriol), 1.5%; and 0.1% for 19-nortestosterone, P4 and testosterone. Intra- and inter-assay CVs for two biological samples with low and high concentrations were 2.4 and 6.9% (n=12) and 1.3 and 4.7% (n=18) respectively. Testosterone was analysed using antibodies (provided by Prof. Meyer, Weihenstephan, Germany) raised in rabbits immunized against 17a-OH-testosterone-HS-BSA. Testosterone-3-CMO-peroxidase was used as enzyme conjugate. The cross-reactivity of the antibody with other steroids was as follows: 4-androsten-17ß-ol-3-one (testosterone: 100%), 5a-androstan-17ß-ol-3-one [dihydrotestosterone (DHT): 13.6%], 5a-androst-2-en-17ß-ol (1.5%), and < 0.1% for 5a-androstan-3ß-ol-17-one (epi-androsterone), 5a-androstan-3a-ol-17-one (androsterone), 5a-androst-2-en-17-one.

Standardisation of urinary hormone levels with specific density

All urinary hormone levels were standardised with specific density (Miller et al. 2004) to account for each badger's fluid intake. Aliquots 50 µL of urine were vortexed for approx. 2 minutes (until supernatant was clear) and measured using a KERN OPTICS digital refractometer ORD 1 PM. Hormone levels were corrected according to the following equation:

$$HC_{SD\ corrected} = HC_{Measured} \times (SD_{target} - 1.0)/ (SD_{sample} - 1.0)$$

where $HC_{SD\ corrected}$ is the specific density corrected hormone concentration, $HC_{SD\ corrected}$ is the hormone concentration measured directly from urine samples, SD_{target} is the population mean specific density (of all samples) and SD_{sample} is the specific density of each sample (Miller et al. 2004).

Statistical analyses

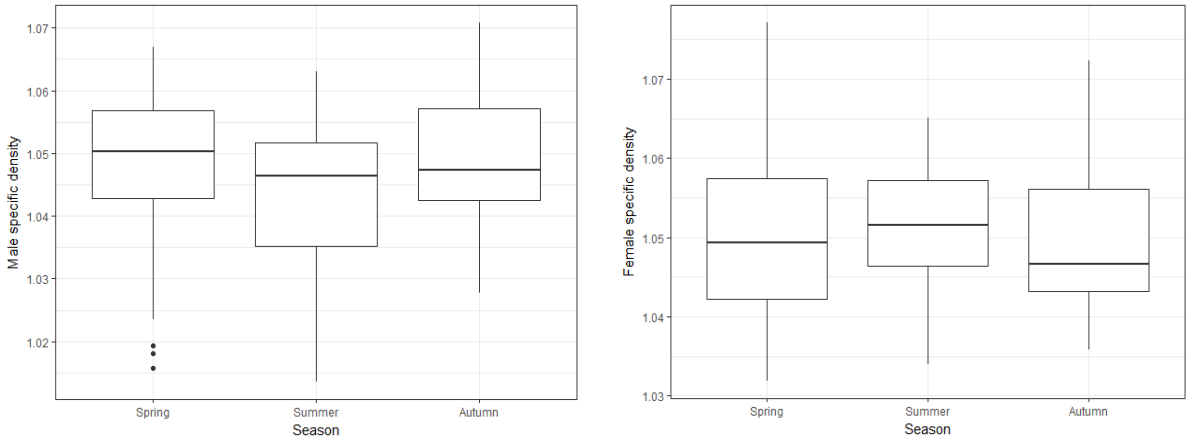
To analyse whether there were seasonal differences in specific urine density values (i.e., urine concentration) of male and female samples, a linear model using specific density as response and season as predictor was conducted for both sexes separately. In cases where significance was found, a-priori tests were conducted to further investigate the differences between seasons. To investigate correlation between plasma and urinary sex-steroid levels, a general linear model was conducted using urine hormone levels (testosterone ng/ml for males; total oestrogen pg/mg for females) as response, plasma hormone levels (testosterone ng/ml for males; total oestrogen pg/mg for females) as predictor, season (spring, summer, autumn) as covariate, and including the interaction-term between plasma hormone levels with season to investigate whether potential correlations were season-specific. For the correlation analyses in males, plasma testosterone was log10 transformed for best model fit. The following models were analysed: Males= lm(Urine testosterone (ng/ml) ~ log10(Plasma testosterone, ng/ml) + Season + log10(Plasma testosterone)*Season; Females= lm(Urine total oestrogen (pg/ml) ~ Plasma total oestrogen (ng/ml) + Season + Plasma total oestrogen*Season). All individuals used in the analyses were independent.

To analyse whether urinary hormone levels exhibited similar seasonal variation as plasma levels, a general linear model was conducted using urinary hormone levels (testosterone ng/ml for males; total oestrogen pg/mg for females) as the response and season (spring, summer, autumn) as the predictor. In case of significance, a-priori tests were conducted to further investigate the differences between seasons by comparing spring vs all seasons, as well as summer vs autumn. All individuals used in the analyses were independent. Patterns of residuals, normality, and mean variance relationship for each model were checked using diagnostic plots in R. All statistical analyses were performed using RStudio (0.99.896; RStudio Team 2015) and R (R-3.2.4; R Core Team 2016).

RESULTS

Urine specific density

Urine specific density did not vary significantly between seasons in males ($F_{2,70}$= 1.409, p= 0.251) nor females ($F_{2,79}$= 0.165, p= 0.848), implying urine concentration was relatively constant all year round (Fig. 1).



(a) (b)

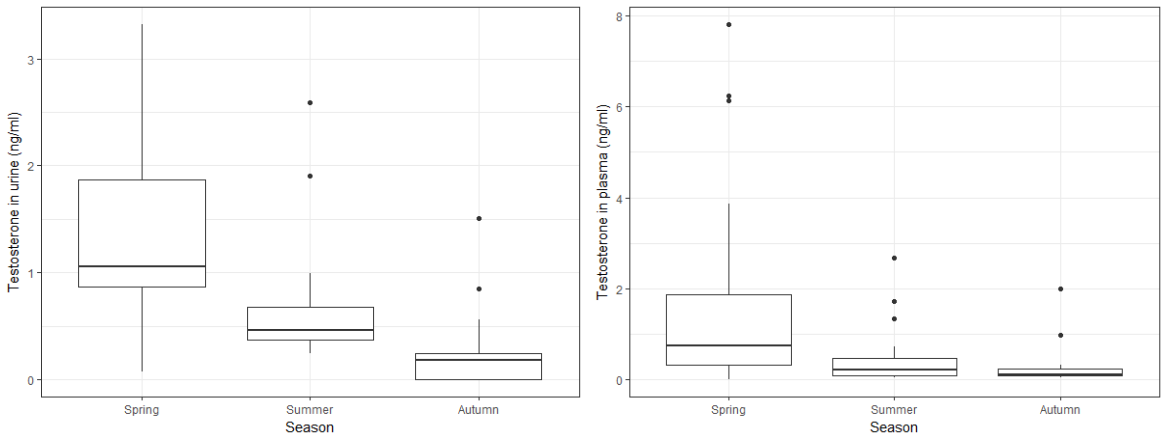
Figure 1. Specific density of seasonal urine samples for males (a) and females (b)

Seasonal hormone profiles

Testosterone levels in urine showed a significant variation with season ($F_{2,63}$= 15.43, p<0.001), where testosterone levels where highest in spring (a-priori test, spring vs summer+autumn: Estimate= 0.861, SE= 0.169, t= 5.108, p<0.001) and levels between summer and autumn did not differ significantly (Estimate= 0.384, SE= 0.266, t= 1.444, p= 0.154; a-priori test). Adult male seasonal pattern of testosterone levels (ng/ml) observed in plasma was also exhibited in urine, where levels were highest in spring (urine= 1.34±0.81; plasma= 1.63±2.03; n= 30), slightly decreased in summer (urine= 0.68±0.60; plasma= 0.49±0.69; n= 19), and was lowest in autumn (urine= 0.26±0.4; plasma= 0.28±0.49; n= 17; Fig. 2).

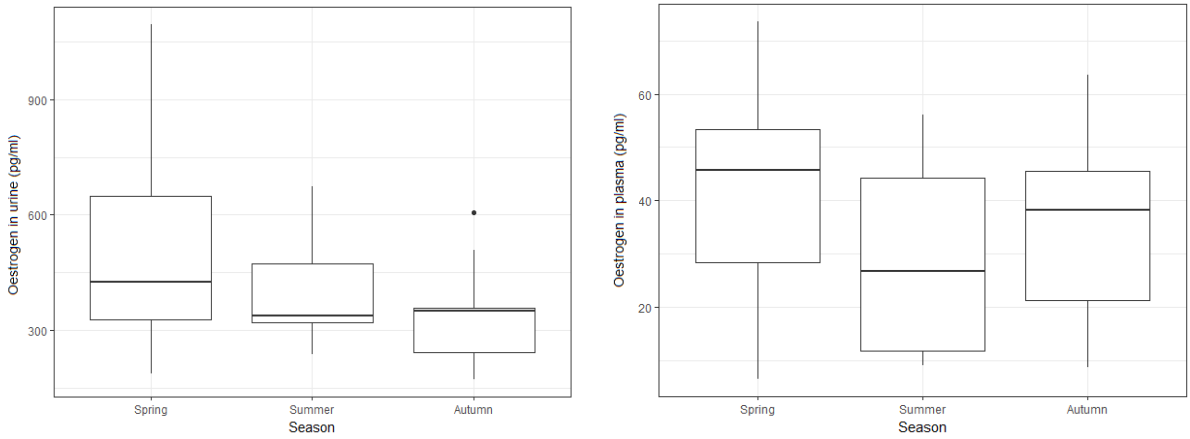
Season was a significant predictor for urine total oestrogen levels ($F_{2,58}$= 3.97, p= 0.024, Fig. 3). The highest urine total oestrogen levels were found during spring (spring vs

summer+autumn: Estimate= 131.44, SE= 49.56, t= 2.652, p= 0.010), while levels during summer and autumn did not differ (Estimate= 65.41, SE= 77.84, t= 0.84, p= 0.404; a-priori test). Seasonal total oestrogen patterns in urine showed similar patterns as plasma during spring where total oestrogen levels (pg/ml) were highest (urine= 498.88±230.36; plasma= 41.53±18.15; n= 34), while during summer (urine= 401.58±128.47; plasma= 28.29±17.17; n= 14) and autumn (urine= 330.68±122.82; plasma= 34.13±17.22; n= 12) levels remained lower.



(a) (b)

Figure 2. Testosterone levels (ng/ml) in urine (a) and plasma (b) during spring, summer, and autumn.



(a) (b)

Figure 3. Total oestrogen levels (pg/ml) in urine (a) and plasma (b) during spring, summer, and autumn.

Plasma and urine hormone level correlation

Urinary testosterone levels correlated positively with plasma levels ($F_{1,59}$= 27.71, p<0.001, Fig. 4) in all seasons (plasma testosterone-season interaction term was non-significant: $F_{2,59}$= 0.4893, p= 0.616), when season was accounted for ($F_{2,59}$= 6.07, p= 0.004).

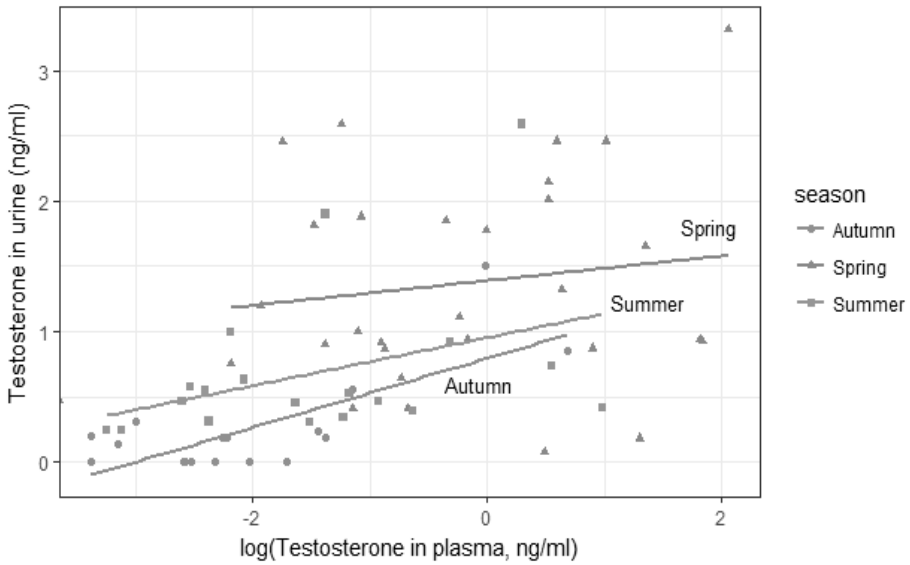


Figure 4. Correlation between testosterone levels (ng/ml) in urine and plasma during spring, summer, autumn.

Total oestrogen levels in urine did not show any correlation with plasma levels ($F_{1,55}$= 0.335, p= 0.565, Fig.5) when season was accounted for ($F_{2,55}$= 3.615, p= 0.034) and the plasma total oestrogen-season interaction term was not significant ($F_{2,55}$= 0.026, p= 0.975).

When plasma and urinary testosterone values of all individuals in every season were compared (Fig 6), it was shown that both had overall similar levels and patterns (confirming the correlation established previously). However, plasma levels tend to have pulsatile peaks while urine profiles were more stable causing slight asynchrony between the two hormone measures in some individuals (e.g. as seen in badger ID 1488 and 1659 in spring; Fig. 6a) although not as much as to break the population level correlation.

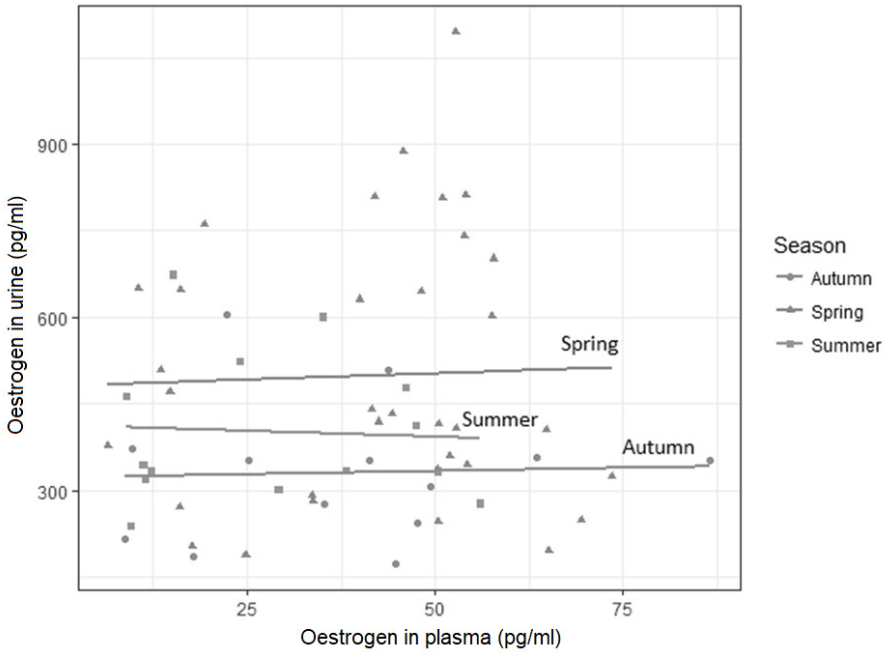
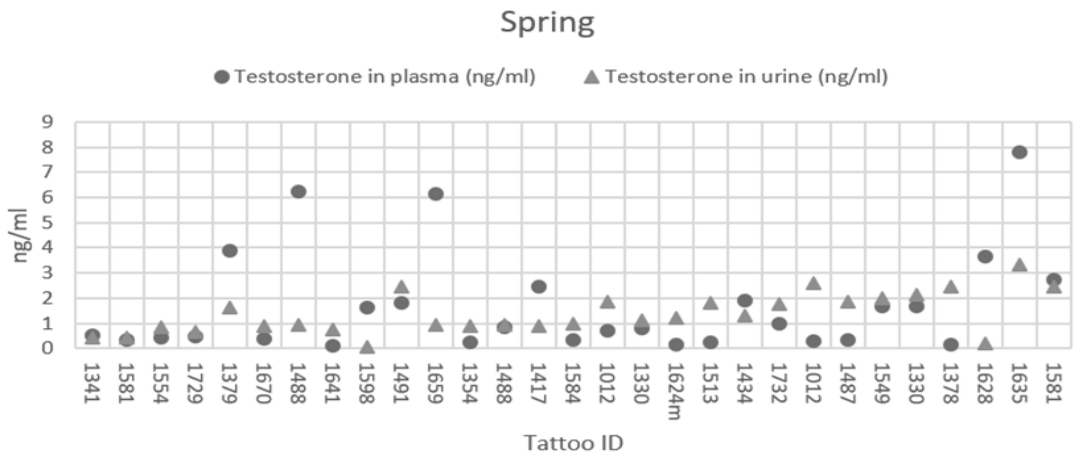
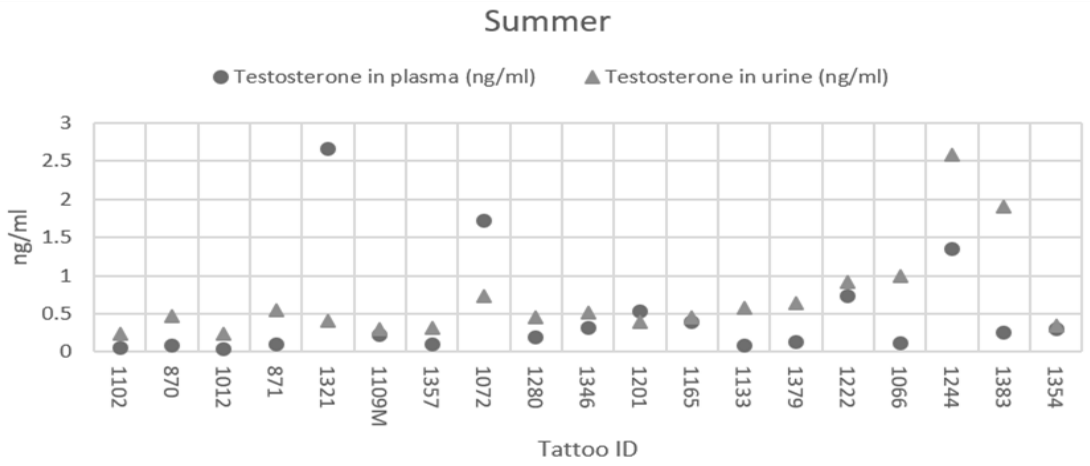


Figure 5. Correlation between total oestrogen levels (pg/ml) in urine and plasma during spring, summer, autumn.

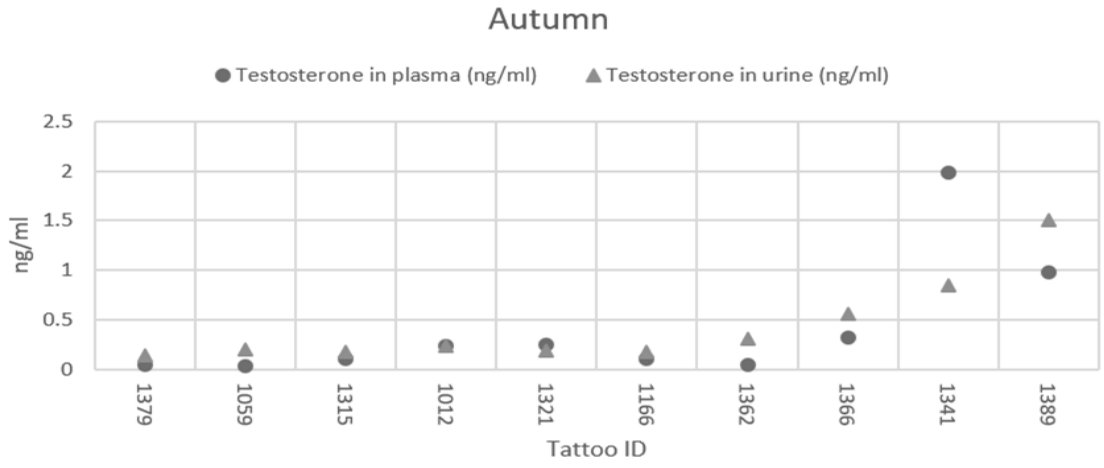
When individual urine and plasma total oestrogen data points in every season were compared, it was observed that urine levels were remarkably higher than of plasma with higher inter-individual variation (Fig. 7) compared to urinary testosterone (Fig. 2,3,7) resulting in asynchronous patterns and thus no correlation (as found previously; Fig. 5) between the two hormone measures.



(a)

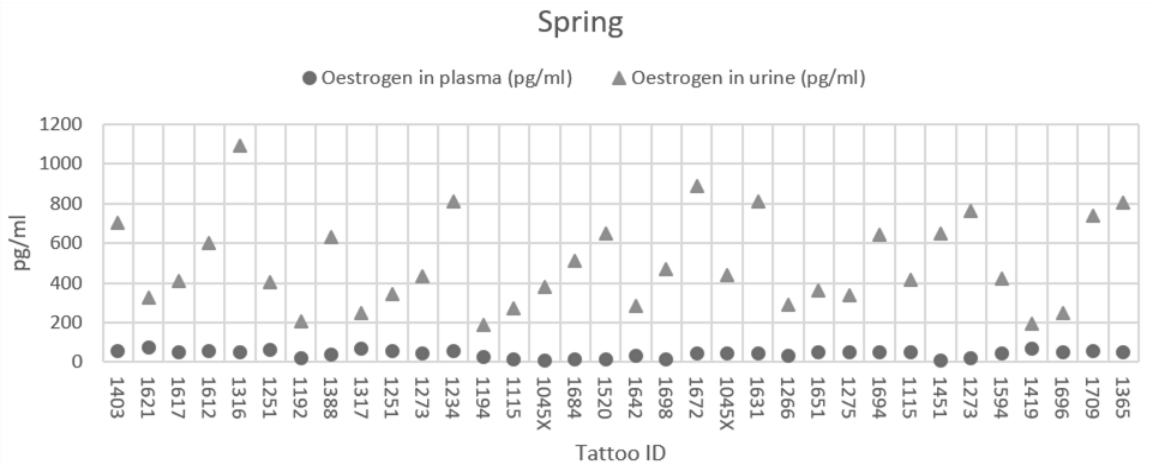


(b)

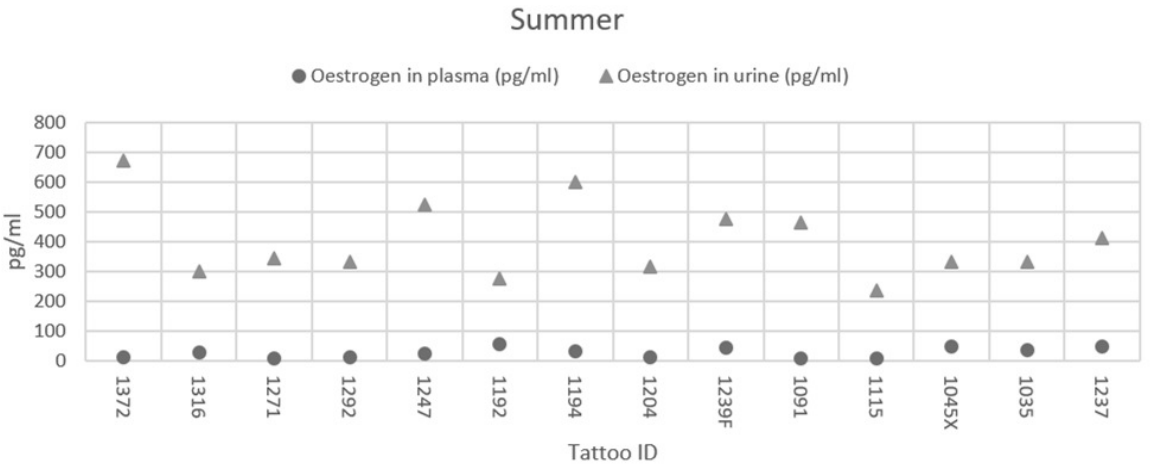


(c)

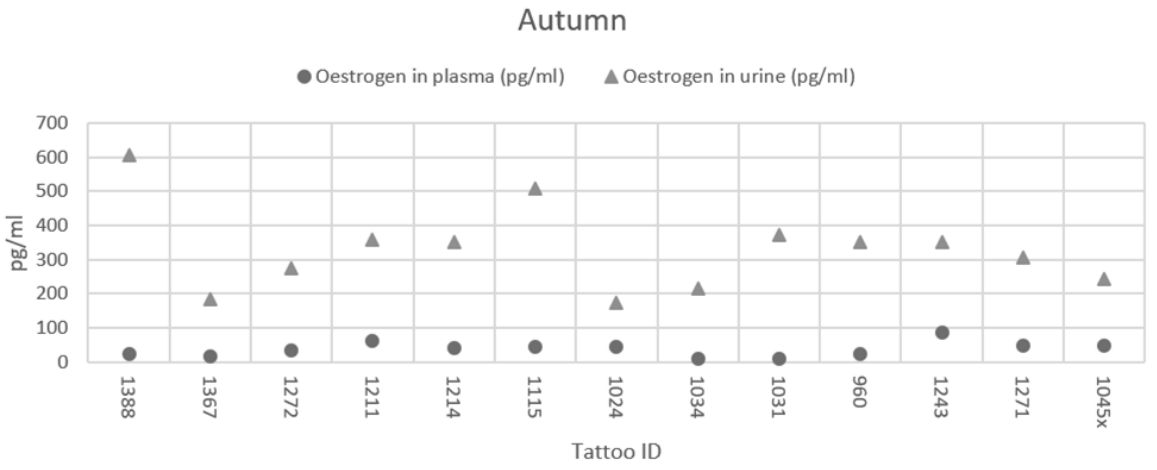
Figure 6. Testosterone levels (ng/ml) in plasma and urine of each individual from spring (a), summer (b), and autumn (c).



(a)



(b)



(c)

Figure 7. Total oestrogen levels (pg/ml) in plasma and urine of each individual from spring (a), summer (b), and autumn (c).

DISCUSSION

A wide range of studies have been conducted regarding badger urinary behaviour (location, frequency; White et al. 1993; Hutchings and Harris 1999; Roper 2010; Buesching and Macdonald 2004) and have found that badgers display consistent urinating patterns allowing for potential development of non-invasive urinary sample collection. Detailed knowledge of a species' sex-steroid hormone metabolism and their excretion is important for designing accurate non-invasive endocrinological methods to determine reproductive status that can be used remotely without the need to capture and sedate the animal (Graham 2004). Nevertheless, as hormone metabolites and excretion routes may differ between species (Heistermann et al. 2006; Palme et al. 1996), sex (Touma et al. 2003), and type of hormone (Monfort et al. 1997), analytical methods need to be validated separately in each context, and the resulting data must be interpreted cautiously. Comparison of excreted hormones to circulating plasma levels is regarded as one of the most robust validation methods (Heistermann et al. 1993; Heistermann et al. 1995; Barrett et al. 2002; Capezzuto et al. 2008), where the measured hormone metabolite patterns are expected to reflect key reproductive events/ physiological states (e.g. mating season, pregnancy, puberty or seasonality: Amstislavsky and Ternovskaya 2000; Schwarzenberger 2007).

Here, badger urinary sex-steroid excretion patterns corresponded well with established seasonally-mediated reproductive events. Urinary testosterone levels were highest at the end of the spring mating season, when male badgers are known to have elevated plasma testosterone levels (Buesching et al. 2009), and large descended testes (Research Chapter 2); the low urinary testosterone levels in autumn correspond to the period of reproductive quiescence in badgers, when males have low blood testosterone levels (Buesching et al. 2009) and small ascended testes (Research Chapter 2). Also, on an individual level, plasma and urinary testosterone levels correlated significantly across all seasons, evidencing that testosterone metabolites in urine accurately reflect changes in circulating testosterone titres and may therefore provide a reliable index in badgers of male reproductive condition.

In females urinary sex-steroid metabolites also exhibited significant seasonal variation reflecting badger reproductive stages: urinary oestrogen levels were highest during the spring mating season coinciding with elevated plasma levels, when females typically exhibit a

distinctly swollen, pink and everted vulva (Research Chapter 2), and lower during periods outside of the mating season in summer and autumn, when vulva condition is usually normal (not-swollen) or intermediate (for details see Research Chapter 2). Thus, these urinary profiles corroborate the previous findings that at high population density (such as at this study site) plasma oestradiol levels show a single peak in spring (Research Chapter 1). This biological validation of urinary oestrogen levels reflecting seasonal patterns of plasma oestradiol levels reported previously (Research Chapter 1) as well as seasonality in badger reproductive biology therefore indicate that urinary oestrogen metabolites can be used to infer female reproductive status.

Nevertheless, on a individual level, circulating total oestrogen levels did not correlate with urinary oestrogen metabolites. A potential reason could be that the assay antibody was not sensitive enough to detect/ cross-react with circulating oestrogen in badgers causing detected levels to be very low and thus not capturing accurate individual values although still maintaining seasonal relative proportions (see Fig. 6). A polyclonal antibody raised in rabbits was used against oestradiol, which cross-reacts with oestradiol (17β-E2: 100%; 17a-E2: 66%), oestrone (100%), and oestriol (1.5%). In some species, however, oestradiol is immediately metabolized after secretion by the ovaries, and thus present in blood/ plasma only in conjugated form which would not be detectable by the assay (e.g., the active circulating oestradiol is conjugated oestradiol glucuronide in African wild dogs, *Lycaon pictus*: Monfort et al. 1997; African elephants, *Loxodonta africana*: Hodges et al. 1983; and Asian elephants, *Elephas maximus*: Czekala et al. 1992). It is postulated that this might also be the case in badgers, and future use of assays with high cross-reactivity against heavily metabolised oestrogen (Graham et al. 2001; Mostl et al. 2005) or the employment of a biochemical approach, where the chemical structure of the plasma oestrogen metabolite is identified and a specific antibody is raised against it (Hindle et al. 1992), might result in more conclusive results.

The lack of individual correlation between female plasma and urinary oestrogen profiles may also be affected by the pulsative nature of circulating hormone secretion (Veldhuis 2008), which can fluctuate due to multiple factors other than reproduction (e.g. circadian rhythm: Irvine et al. 1994; stress, social encounters, health status: Adkins-Regan 2005). Sex-steroid secretion into the blood stream tends to be rapid and episodic (Veldhuis 2008), whereas urinary metabolites are filtered from the blood stream by the kidneys continuously, and

therefore reflect accumulated levels over time (rather than 'snap shot' values; Knox et al. 1992), often resulting in a considerable secretion-excretion time-lag (testosterone: sheep *Ovis aries*= 3-4 hours; ponies *Equus ferus caballus*= 3-5 hours; cortisol: chimpanzee (*P. troglodytes*), long-tailed macaque (*M. fascicularis*), and common marmoset (*C. jacchus*): 2-6 hours; Bahr et al. 2000). In males, this pulsative fluctuation is seen to cause asynchrony between urinary and plasma testosterone levels in only a few individuals and thus does not remove the significance of the correlation. In females, the negative effect of plasma oestrogen fluctuation on individual correlation may be worsened by the low detectable values.

An alternative explanation to the non-correlation is the lag time between circulating and urinary oestrogen levels which may differ from testosterone depending on the differences in metabolism of each hormone (Palme et al. 1996). Similarity between circulating and urinary oestrogen levels at the seasonal level (Fig. 2) indicates correlation on a wider time scale, whilst when compared on a 'snapshot' timescale (since both plasma and urine were collected at the same time) the correlation becomes non-existent. Radiometabolism studies, where a radioactive component is administered into the blood stream and excreta are collected over the following time period (Palme et al. 1996; Schwarzenberger et al. 1996), could help to determine the extent of this species- and hormone-specific time-lag between plasma and urinary sex-steroid profiles (Keckeis et al. 2012; Bahr et al. 2000). However, the inherent health-risks and logistical problems typically prevent this methodological approach being applied to wildlife studies in free-ranging populations (Graham 2004).

Once the relationship between steroid hormone metabolite concentrations and reproductive events has been characterized for a species, non-invasive endocrine monitoring can be used as a tool to enhance the understanding and management of the species in captivity and in the wild (Graham 2004). In badgers, this will then remove the necessity of trapping and collecting plasma samples which increases practicality and also animal welfare. From these results, it was concluded that urinary testosterone and oestrogen levels are a good indicator of seasonal endocrinological events, with urinary testosterone also being accurate for individual plasma testosterone levels.

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PART VI

GENERAL DISCUSSION

GENERAL DISCUSSION

Reproductive hormone monitoring and its importance for wildlife management and conservation: the European badger (*Meles meles*) as a model species

Hormones function as an internal communication system between cells and are specific in that each hormone generates a response in a specific target organ or group of cells (Adkins-Reagan 2005). They are secreted from glands of the endocrine system (e.g. reproductive homones are produced by the ovaries and testes) and are released into the bloodstream to affect other parts of the body (Touma and Palme 2005; Dehnhard et al. 2008). As hormones regulate all aspects of an animal's life cycle, understanding of basic endocrine patterns may reveal physiological mechanisms by which individuals reproduce, respond to environmental challenges, grow, maintain homeostasis and interact socially with conspecifics (Touma and Palme et al. 2005; Dehnhard et al. 2008; Ganswindt et al. 2012). This information is essential in both *in situ* and *ex situ* wildlife management and conservation, and may inform regulatory policies for protecting endangered species (Wasser et al. 1997; Graham 2004; Schwarzenberger and Brown 2013). Nevertheless, overall, insufficient information is available on the endocrinology of most wild-living mammal species (Monfort 2003).

In wildlife management, endocrinological data are typically used to assess reproductive status (e.g. fertility, oestrus, pregnancy, sexual maturity; using steroid hormones; Paris et al. 2002; Pereira et al. 2006; De Matteo et al. 2006), stress levels (using glucocorticoids; Touma and Palme 2005; Keay et al. 2006; Lane 2006), and health/welfare status (with glucocorticoids, growth hormones, insulin etc; Mckenzie and Deane 2005). Nevertheless, hormones can potentially be used to interpret much wider aspects of an animal's biology, especially when combined with other concurrent biometric data (somatic, environmental, and social), elucidating complex problems related to species ecology and individual life-histories, in turn informing population dynamics (Adkins-Regan 2005). As reproduction enables a population to rejuvenate itself naturally, understanding basic reproductive processes, mechanisms and related factors through reproductive hormone monitoring becomes an important research tool, especially for endangered wildlife species (Graham 2004; Schwarzenberger 2007). This will allow assessment of physiological aspects such as reproductive activity, seasonality, puberty,

development, and senescence which are all profound factors of wildlife population growth (Graham 2004; Pukhazhenthi and Wildt 2004; Schwarzenberger 2007).

Using the European badger (*Meles meles*; hereafter 'badger') as a model species, in this thesis I demonstrated how reproductive hormones can reveal various aspects of badger reproductive processes, in terms of reproductive success, developmental biology and life-history schedules. Badgers are facultative social mesocarnivores where females follow an atypical reproductive strategy involving delayed implantation and superfoetation that varies with population density, in turn a function of habitat productivity and resource distribution across their extensive biogeographical range (Macdonald et al. 2015). Because of this, badgers provide an ideal model species for studying the endocrinological mechanisms regulating reproductive physiology in mammals with regards to social and climatic factors (Stopka and Johnson 2000).

Monitoring the reproductive cycle may provide information on the mechanism/ strategies and time scale over which a population replenishes itself (Graham 2004; Schwarzenberger 2007; Dehnhard et al. 2008). It is particularly important to understand the reproductive cycle of a species which does not follow the typical line of reproductive processes. Female badgers undergo delayed implantation (DI) where blastocyst(s) produced during the main mating season (post-partum oestrus in January-March; Yamaguchi et al. 2006) temporarily arrest development and remain suspended in the uterus for up to 330 days (amongst the longest in any species) until implantation occurs around mid-December (Mead 1981; Woodroffe 1995; Yamaguchi et al. 2006; Corner et al. 2015). This phenomenon is simultaneously accompanied by another strategy called superfoetation (SF) which allows badgers to mate and conceive even if viable blastocysts are already present inside the uterus (Mainguy et al. 2009; Roellig et al. 2011; Yamaguchi et al. 2006). This reproductive strategy (DI*SF) is unique only to the badger and the American mink (*Neovison vison*; Yamaguchi et al. 2004; Roellig et al. 2011). In Research Chapter 1, I identified hormone mechanisms supporting the DI*SF reproductive strategy in female badgers by measuring circulating plasma oestrogen and progesterone levels using cross-sectional population-level data. I found that oestrone was consistently higher than oestradiol throughout DI, indicating its function to sustain pre-implanted blastocysts (with levels elevated in spring, lowest during summer, peaked in autumn, and remained elevated for pregnant females in winter); oestradiol levels were only elevated during the mating season (in spring); while progesterone was low throughout seasons and

increased only during winter pregnancy, associated with implantation and luteal development.

This reproductive cycle is influenced by ecological factors, where my investigation revealed that the extent to which badgers mate outside of the post partum oestrus (winter-late spring) varies substantially between studies linked to population density (in relation to mating opportunities/ fertilisation assurance). In the Republic of Ireland where the badger population density is low (1.9 badgers/km^2), presence of secondary and tertiary follicles with blastocyst turnover (during delayed implantation) were found all year round, indicating continuous mating (Corner et al. 2015). In Bristol (UK), where the population density is moderate (4.4-7.5 badgers/km^2), five oestrus cycles between late winter/spring and autumn were found (Service et al. 2002). Studies in higher population densities in South-west England and Woodchester Park (UK; both approx. 25 badgers/km^2) observed two distinct mating periods per year during winter/spring and summer (Cresswell et al. 1992; Page et al. 1994; Carpenter et al. 2005). While in my very high density (40 badgers/km^2) focal population in Wytham Woods, Oxfordshire (UK), I recorded only a single distinctive mating season (see also Buesching et al. 2009; Macdonald et al. 2015), as reflected in the oestradiol data I present in Research Chapter 1. This endocrinologically flexible DI*SF mating strategy likely represents a form of balancing selection, allowing badgers to succeed at a range of regional densities.

Some animals are seasonal breeders, that only breed at particular times of the year and undergo certain reproductive processess during specific seasons, thus seasonality would be a factor to consider when monitoring reproductive activity (Schwarzenberger 2007; Dehnhard et al. 2008; Schwarzenberger and Brown 2013). Plasma hormone monitoring may not always be practical in the field, when immediate results are required to interpret reproductive status, thus external genitalia morphology (EGM; vulva and testes condition) is often used to provide an immediate, if cruder, estimate of the individual's or the population's reproductive state; but EGM must be used carefully especially if validation with hormone levels has not been established (Preston et al. 2012; Reichert et al. 2002; Emery and Whitten, 2003). I investigated this in Research Chapter 2, comparing measurements of circulating hormone levels and observed EGM condition. I discovered, that for seasonally breeding badgers, EGM in males does provide a reliable indicator of reproductive activity during the mating season, but can provide only a tentative indication of physiological reproductive status outside this period. In females, EGM is generally less precise and reliable as a means for assessing

reproductive status. This illustrates the importance of endocrinological data for validation of field techniques used to assess the reproductive state.

Knowledge regarding the onset of sexual maturity/ puberty is important to understand the age at which an individual is recruited into the reproductively active component of a wild-living population (Pukhazhenthi and Wildt 2004; Graham 2004; Schwarzenberger 2007). In Research Chapter 3, using testosterone and oestrone levels in badgers, I demonstrate that puberty in both sexes begins in a minority of individuals at ca. 11 months of age, whereafter cubs develop similar seasonal sex-steroid patterns to adults, while the majority attained sexual maturity in their second mating season (22-28 months).

I discovered that the timing of puberty was linked to ecological factors, as heterochrony of puberty was observed even within this single population. Two typologies of male cubs were found: early (reached sexual maturity at 11 months, first mating season) and late developers (reached sexual maturity in their second mating season). Early developers were larger in size (at the age of 11 months, despite having similar adult body sizes), had less remaining growth to complete, and faster growth rates compared to late developers. Male cubs born into larger social groups and bigger setts typically belonged to the late developer phenotype and vice versa. These findings support the hypothesis that a minimum body size is required to reach sexual maturity. Population density may increase intraspecific competition (as access to food quality and quantity decline; Clutton and Brock et al. 1982; 2010), resulting in a slower growth rate, as well as lower (mean) body mass and smaller size (LeBlanc et al. 2001; Zedrosser et al. 2006), which in turn will cause later sexual maturity (Onyango et al. 2013) and/ or reduced lifetime reproductive success (Stearns 1992).

Understanding when an individual experiences a decline in the ability to produce offspring (or 'reproductive senescence') is also essential to a complete understanding of population demographics and reproductive capacity (Graham 2004; Walker et al. 2005; Schwarzenberger 2007). Among a variety of reproductive parameters (seasonal number of ovulations: Nozaki et al. 1995; litter size: Mysterud et al. 2002, Descamps et al. 2008; offspring weight: Mysterud et al. 2002; offspring survival: Nussey et al. 2009; and annual breeding success: Reed et al. 2008), hormone levels can reveal age-dependent declines in reproductive capacity as demonstrated in Research Chapter 4, where I used lifetime endocrinological and morphological data to investigate age-dependent reproductive and

somatic decline at older ages. Both sexes underwent a somatic decline, starting at the age of 3 years, while endocrinological decline started later; females experienced a decline in oestrone levels at the age of 5.5 years, while male testosterone secretion declined after the age of 6 years. Cross-referencing endocrinological data with previous cub assignment data involving the same study population (Dugdale et al. 2011), I deduced that, in females, the decline in number of maternal cub assignment coincided with the decline of somatic condition; while in males, the decrease coincided with the onset of diminishing testosterone levels. This signifies different senescence patterns between the sexes, indicating different reproductive investment strategies ('restraint' for females versus 'constraint' for males). Using survival data (Bright Ross et al. in prep.) for the study population, I cross-referenced that the majority of badgers in Wytham Woods do not survive past 6 years of age where hormonal decline starts to occur. While for the remaining individuals that do survive, there is evidence that reproductive cessation (RC) occurs leading to a post reproductive lifespan (PRLS), although reproductive phenotypes with high hormone levels at older ages were also found in a small proportion of elderly badgers of both sexes. Nevertheless, this reveals that some badgers do live several years of PRLS; a phenomenon generally considered to be rare in wild carnivore populations (Cohen 2004).

Wild animals are exposed to extrinsic factors (involving changing social and environmental factors) that can be either predictable (i.e. seasons) or unpredictable (i.e. sudden disturbance, unfavorable climatic conditions, predators; McEwen and Wingfield 2003; Wingfield 2004). Responses to these changes, although intended to maximise fitness, may possibly prevent the annual reproduction and/ or disrupt the life-cycle of the individual (Jacobs and Wingfield 2000; Wingfield 2004; Walker et al. 2005). In order to understand the effects of these extrinsic changes on a population, it is necessary to know how the individuals can cope with these changes through physiological accommodation and adaptation. When perceiving signals from extrinsic changes, individuals may undergo morphological, physiological and behaviour modifications that result from impacts on the endocrine system, which can be detected through hormone levels (Jacobs and Wingfield 2000). Thus, field endocrinology and related morphological alterations can be used to understand and predict the responce of a population to different conditions such that management actions can be taken accordingly.

With regard to extrinsic effects on badger development to adulthood, I report in Research Chapter 5, when extrinsic factors (social and environmental) throughout the developmental

stage were analysed, deviation from typical mean rainfall in the first summer, winter, and second summer of their development; total adults present in a cub's natal social group; as well as total number of cubs in birth cohort; all affected the adult end size for badgers in general. These factors affected sexes differently, with females more sensitive to climatic factors (i.e. deviation of mean temperature and rainfall in first spring, associated with food availability), while males were affected more by social factors (i.e. male adults in birth social group). These differences in vulnerability towards extrinsic factors during the growth period influence divergent juvenile growth patterns and end body sizes, linked to sexual size dimorphism in badgers.

Reproductive hormone levels (used as a proxy for reproductive activity) are not only influenced by extrinsic factors (e.g. environmental and social), but also by intrinsic factors (e.g. somatic condition, age), hence it is important to understand how dynamic interactions between these influence endocrine function. In Reseach Chapter 6, I found that for males, testosterone levels varied significantly across seasons with average rainfall deviation and body weight, where both were positively correlated with testosterone levels; while during the mating season in spring, body weight was the factor that affected (positively correlated) testosterone levels the most. In females, oestrone levels varied significantly across seasons with total number of adults in the population (positively correlated) and average temperature deviation (negatively correlated); however, during the spring mating season, only average temperature deviance affected (negatively correlated) oestrone levels. While for oestradiol, head body length and total number of adults in the population affected (both positively correlated) hormone levels during the mating season. Based on their respective phenotypic quality, individuals are expected to self regulate their hormonal levels according to their concurrent physical state to maximize fitness, likely involving trade-offs between reproduction and self-maintenance, especially when exposed to unfavorable environmental conditions (McEwen and Wingfield 2003; Wingfield 2004). This reproductive physiological regulation may not only differ among individuals, but also systematically between sexes according to their respective reproductive strategies, as I found in Research Chapter 6. During the mating season, body weight affected male testosterone levels (which is fluctuative, illustrating the 'short-term' reproductive investment depending more on the capacity during that particular period), more so than bodyweight affected oestradiol in females, which were influenced predominantly by head body length (which is permanent,

illustrating the 'long-term' reproductive investment and also the individual-specific capacity to undergo the whole female reproductive process).

Conventionally, blood samples are used in hormone immunoassays (enzyme immunoassay, EIA or radioimmunoassay, RIA) to measure circulating hormone levels (Monfort et al. 1997; Dehnhard et al. 2008; Heistermann 2010), and to provide accurate and immediate information on the endocrine function (Graham 2004; Schwarzenberger and Brown 2013). However, most wildlife species are not easily managed for repeated routine blood sampling (Monfort et al. 1997; Touma and Palme 2005) and restraint may cause problematic stress (Dehnhard et al. 2008; Heistermann 2010). Moreover, as circulating hormone levels only represent a single time point, these may be biased by transitionary effects such as circadian variations, episodic changes, and stress (especially for steroid and glucocorticoid hormones; Touma and Palme 2005; Heistermann 2010).

Circulating hormones are metabolized and deposited in different tissues and excreta (such as faeces, urine, hair, nails, and saliva; von Engelhardt et al. 2000). These traces of hormones can be used as non-invasive proxies to estimate endocrine activity in a wide range of both wild and captive species (Hindle et al. 1992; Monfort et al. 1997; Graham 2004; Scwarzenberger 2007; Dehnhard et al. 2008). However, the excretion route and metabolism of hormones varies considerably among and within species (Hindle et al. 1992; Heistermann et al. 2006; Dehnhard et al. 2008), sex (Touma et al. 2003), and hormone type (Monfort et al. 1997; Graham et al. 2001; Palme 2005; Schwarzenberger 2007). Thus, each analytical method needs to be validated each time for a particular species and a particular hormone, and for the metabolite product involved (Hindle et al. 1992; Heistermann et al. 1993; Heistermann 2010) to ensure the analyte measured is suitable for assessing the target physiological parameter (Palme et al. 1996; Touma and Palme 2005).

In Research Chapter 7, I provide a biological validation for non-invasive urinary hormone (oestrogen and testosterone) monitoring in badgers, where urinary hormones revealed the same seasonal patterns as circulating plasma hormones with good correlation between measures on a seasonal time scale. Moreover, for testosterone, individual correlation on a shorter time frame (with urine and blood collected simultaneously) was significant; while for oestrogen it was not, possibly due to a longer time lag before circulating hormones metabolites enter the urine (Palme et al. 1996). This implies, that application on a wider time

scale would be more suitable (explaining the non-correlation) for females. Once non-invasive techniques are validated, they may be used to reliably assess endocrine function and alleviate the necessity to capture and restrain the study animal for blood sampling, aiding practicality and benefiting animal welfare.

Optimizing conservation activities for wildlife species requires a multi-disciplinary approach. Nonetheless, one of the most important approaches is to develop a detailed knowledge of the animal's endocrine physiological and associated reproductive traits. Furthermore, as demonstrated in my thesis, it is vital to consider how aspects of reproductive physiology may also be affected by ecological conditions experienced on an individual level, subsequently informing on population level effects, and revealing complex ecological and evolutionary processes (Adkins-Regan 2005). In the broader context of conservation, it is critical that we learn more about the endocrine function of species to allow *ex situ* sites (zoos, rehabilitation centers, etc) to maintain healthy and productive "reservoir" populations, which may be reintroduced to the wild, but also provide experimental subjects to predict how animals function in the wild; as well as understand basic physiological factors that profoundly affect the growth of wild populations.

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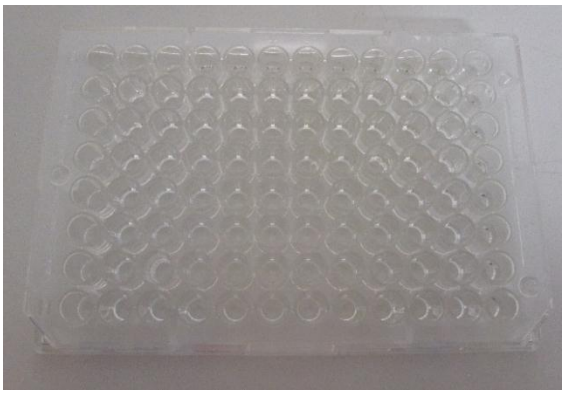
APPENDICES

APPENDIX 1

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA, EIA) FOR HORMONE MEASUREMENT

Enzyme immunoassay (EIA) is a plate-based assay technique used to detect the presence of antigens (e.g. hormones, antibodies, peptides, proteins) using the immunological concept of competition between enzyme-labeled antigen ('tracer'; which is able to change color when bounded with substrate) and unlabeled/ target antigen (e.g. hormone in samples) for limited antibody binding sites (Lequin 2005; Gan and Patel 2013). When competing, the amount of unlabeled antigen may vary in each well/ sample, while the concentration of tracer is constant, thus if more unlabeled antigen is present, lower amounts of tracer would be able to bind to the specific antibody and vice versa (Brown et al. 2004). Hormone concentrations in samples are inversely reflected by the strength of signal (colour/ optical density, OD; read photometrically) presented by the amount of tracer bound with antibody (Lequin 2005).

Components of an EIA consists of the following: (1) **Solid phase** (Fig. 1): Typically a 96-well polystyrene micro-titre plate which acts as an absorbing surface of the antibody (or other proteins) and used to immobilize and detect unknown amount of unlabeled antigen in the samples (Lenhoff and Ngo 2012).



S= Standard C= Control T= Test Sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Zero	S5	S9	T1	T5	T9	T13	T17	T21	T25	S5
B	Blank	Zero	S5	S9	T1	T5	T9	T13	T17	T21	T25	S6
C		S2	S6	S10	T2	T6	T10	T14	T18	T22	T26	S7
D		S2	S6	S10	T2	T6	T10	T14	T18	T22	T26	S8
E		S3	S7	C1	T3	T7	T11	T15	T19	T23	Zero	S9
F		S3	S7	C1	T3	T7	T11	T15	T19	T23	S2	S10
G		S4	S8	C2	T4	T8	T12	T16	T20	T24	S3	Zero
H		S4	S8	C2	T4	T8	T12	T16	T20	T24	S4	Zero

Figure 1. Polystyrene micro-titre plate (above) and plate map (below)

In the plate map above, A1 and B1 are 'blanks' or non-specific bindings (NSBs) which are used to detect non-antibody bindings in the plate and should value less than 10% of the maximum binding wells ('zeroes'; Brown et al. 2004). The maximum binding wells or 'zeroes' (well A2, B2, E11, G12 and H12) have the highest optical densities (0.8 - 1.0) as there is no presence of unlabelled antigen (standard and sample) and maximum binding (100%) of tracer and antibody occurs (Brown et al. 2004). Well C2 to D4 are standards with different dilutions given in duplicates, well E4 - H4 are quality control 1 and 2 with their duplicates, while well A5 - D11 are samples with duplicates and F11- F12 are another batch of standards, but without duplicates. (2) **Antibody:** A large, Y-shaped immunoglobulin (Ig), produced against a specific antigen which is bound to the surface of plate wells and used to capture the antigen of interest (Porstmann and Kiessig 1992). (3) **Tracer (Enzyme conjugate/labelled antigen):** An antigen that has been labelled with enzyme (which will change color when bounded with substrate) that competes and thus detect the presence of unlabeled antigen concentration in the sample. Hormone conjugated to horseradish peroxidase (HRP) is typically used for a single antibody EIA while biotin labeled hormone binding to peroxidase-labeled strepavidin is used for sandwich EIA (Brown et al. 2004). (4) **Unlabelled antigens (samples and standards):** Hormones in samples with unknown concentrations are unlabeled antigents which are of interest to detect and measure. Standards are also unlabelled antigens, but of known concentrations that are used as a reference (in the form of a standard curve) to determine the concentration of the hormones. The standard curve is a plot that shows the relationship between the percentage of bound tracer and the concentration of standard in assay wells (Maggio 2018). (5) **Quality controls:** Internal control samples that are run in every assay to monitor assay precision. Usually 2-3 controls (with low and high concentration) are used with duplicate or tripricate of each control. (6) **Substrate:** A chemical component (consisting of buffer, catalyst and chromagen) that binds with the enzyme conjugate and changes the colour of the plate, inversely reflecting hormone levels (Lequin 2005). (7) **Wash solution:** Used to wash micro-titre plates after each incubation, allowing excess unbounded components to be removed from wells. (8) **Assay buffer:** Buffer used to dilute the samples, standards, and tracer. (9) **Stop solution:** A solution primarily used in double antibody (sandwich) EIA to stop the substrate reaction (e.g. H_2SO_4) . (10) **Blocking buffer:** A buffer containing proteins such as bovine serum albumin (BSA) and used to block/ reduce non-specific binding sites, typically used in double antibody (sandwich) EIA (Brown et al. 2004).

An EIA may use a single antibody (hormone specific, ‘single antibody EIA’) or an additonal second antibody (both hormone specific and unspecific IgG/ coating matrix, ‘sandwich EIA’), largely depending on the size of the substance of interest (Lequin 2005; Gan and Patel 2013). In a sandwich EIA (see Fig. 2), the first (non-specific) antibody is coated to the plate, incubated and followed by an addition of blocking buffer (to reduce non-specific bindings) and plate washing. The second antibody (specific antibody that identifies the hormone), unlabeled antigens (samples and standards) and tracer are then added to the well and followed by plate incubation. During incubation, the second and first antibody will bind and the unlabeled antigen and tracer will compete for limited binding sites of the second antibody. After incubation, plates are washed to remove excess components and enzyme followed by substrate are added to the wells (causing colour change, which can be ceased by stop-solution when plate is ready for reading). The more colour is present in wells signifies higher amounts of tracer-antibody bindings and lower concentration of hormones, and vice versa. Interpolation of hormone concentrations is conducted by refering to the standard curve values (Lequin 2005; Lenhoff and Ngo 2012). A single antibody EIA has similar procedures as a sandwich EIA, except instead of adding a non-specific antibody and blocking buffer in the beginning, plates are directly coated with specific antibodies (Porstmann and Kiessig 1992; Gan and Patel 2013; see Fig. 3).

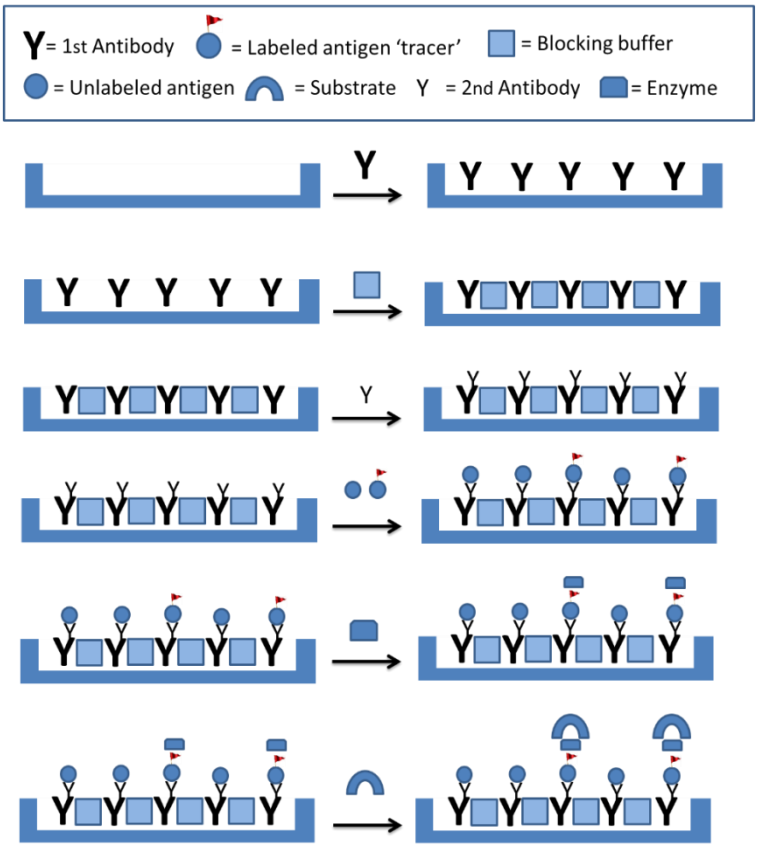


Figure 2. Sandwich EIA.

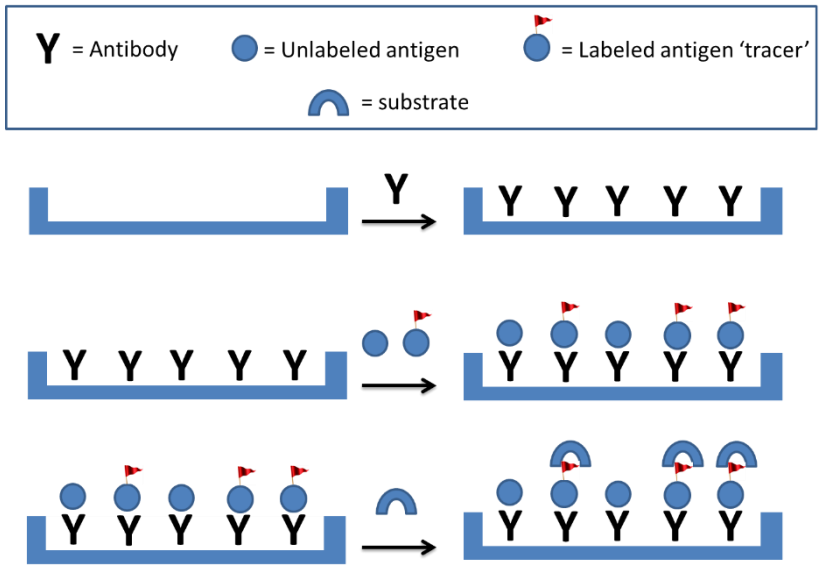


Figure 3. Single antibody EIA

To avoid misleading results, EIA validation is required before introduction into routine use or if there is change in conditions and procedures. This may include biological validation and

analytical method validation (Hindle et al. 1992; Heistermann et al. 1993; Palme et al. 1996). Biological validation demonstrates whether hormonal measures accurately reflect the physiological event(s) in question (Wasser et al. 2000), while analytical method validation tests whether analytical method is reliable and data is accurate by ensuring that target hormone(s) are measured precisely; this includes analyses such as assay sensitivity, precision/ repeatability, accuracy, and specificity (Brown et al. 2004; Lequin 2005; Gan and Patel 2013). Assay sensitivity is calculated to determine the lowest amount of analyte which can be reliably discriminated from zero values with a 95% probability, which is the steroid concentration at 90% binding (Lequin 2005; Gan and Patel 2013). To ensure an assay provides optimal precision, only values within the 'linear range' of the curve are suggested to be used as they are measured accurately, while error increases in saturation areas of the curve (Fig. 4; Heistermann 2017).

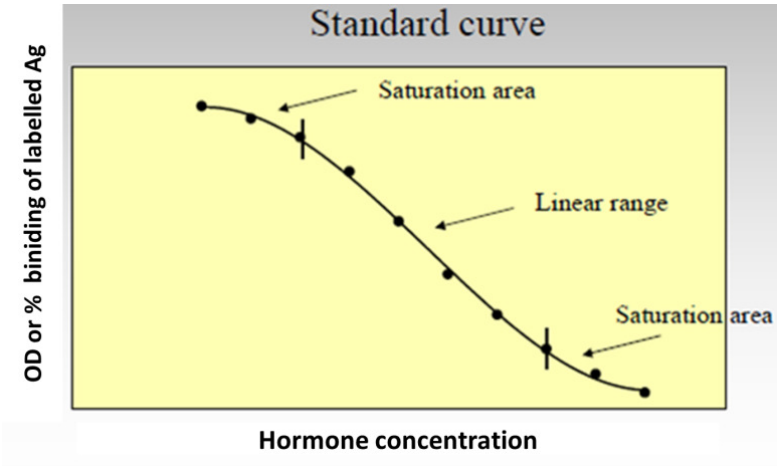


Figure 4. The linear range and saturation areas of the standard curve (Heistermann 2017).

In addition to the standard curve, assay precision is also conducted by testing the degree of scatter between series of measurements obtained from multiple sampling of the same sample under the prescribed conditions. This is done by calcualtion of intra- and inter- assay variation (Graham et al. 2001; Brown et al. 2004). The former is the within assay error (variation in running the same samples in an assay) which can be calculated as the average value from the individual CVs (standard deviation/ mean * 100) for all of the sample duplicates or CV of the internal quality controls. While the later is the between assay error (variation in running the same samples in different assays) that is determined by calculation of averages for high and low quality controls in each plate, which is then used to calculate

overall mean, standard deviation, and eventually % CV (standard deviation of plate means/ mean of plate means) across plates (Brown et al. 2004; Lequin 2005; Maggio 2018). Hormone concentrations are considered accurate when the value of intra- and inter- assay variation is below 10% and 15 % respectively (Brown et al. 2004; Gan and Patel 2013).

Assay accuracy is the degree a measured concentration corresponds to the true concentartion of the substance, which is tested by analysing analyte of known amounts and running it in assays. The amount observed (assays results) will then be tested against the amount expected (true concentration). An assay is considered accurate when mean percent recoveries of analyte are within 20% of the expected value (Lequin 2005; Gan and Patel 2013).

The specificity of an assay depends mainly on the specificity of the hormone specific antibody used in the system as well as interference of the sample matrix with a specific measurement of the substance of interest (Brown et al. 2004; Lenhoff and Ngo 2012). To understand the specificity of an assay, a test for linearity/parellelism of analyte dilutions and evaluation of antibody crossreactivity needs to be conducted. Parallelism tests whether an assay is measuring the hormone of interest and helps determine an optimum dilution for the samples used in the assays (Lequin 2005; Gan and Patel 2013). When sample dilutions run parallel to the standard curve (similar shape with no differences in slopes), this infers that matrix does not interfere the hormone measurement and the assay is measuring the hormone of interest correctly (Lenhoff and Ngo 2012; Maggio 2018). Paralellism should be conducted especially when sample of interest is newly introduced and typically dilution at 50% binding is the best option to choose as it lies in the middle of the linear range (provides enough ‘space’ to measure higher and lower concentrations without going beyond this range; Brown et al. 2004), although not all cases allow this and other bindings within linear areas of the standard curve are acceptable.

An assay is only specific, if the antibody used for detection reacts exclusively with the substance of interest, nevertheless, nearly all antibodies react also with structurally closely related compounds, thus assessment of antibody crossreactivity is essential (Porstmann and Kiessig 1992; Lequin 2005). Crossreactivity (%) of an antibody towards other hormones is determined using inhibition curves and is expressed as the standard concentration at 50% binding divided by concentration of competitive antigen at 50% binding (Brown et al. 2004; Lequin 2005; Gan and Patel 2013).

Protocols for hormone level measurement with EIA

Hormone measurements for progesterone, oestrogen, and testosterone levels in this thesis were conducted using enzyme immunoassays at the Endocrinology Lab of Chester Zoo, Chester, UK. All additional non-specific assay components (i.e. coating buffer, assay buffer, wash solution concentrate, substrate buffer, ABTS, and hydrogen peroxide) used across assays are decribed in Table 1., while specific assay components and procedures are described below. Both oestrone and progesterone level measurement were conducted using a single antibody assay while testosterone level measurements utilised a second antibody (sandwich) assay, all of which are detailed in the following sections.

Table 1. Recipes of EIA components

Substance/volume	Components	Amount	Resources
Coating buffer/ 1 Litre	Na_2CO_3 (Anhydrous)	1.59 g	Sigma, S-2127
	$NaHCO_3$	2.93 g	Sigma, S-8875
	dH_2O	1000 ml	Sigma, S-8875
	pH	9.6	Sigma, S-8875
EIA buffer/ 1 Litre	Stock A 0.2M NaH_2PO_4	27.8 g/L dH_2O	Sigma, S-9638
	Stock B 0.2M Na_2HPO_4	28.4 g/l dH_2O	Sigma, S-0876
	Stock A	195 ml	Sigma, S-0876
	Stock B	305 ml	Sigma, S-0876
	NaCl	8.7 g	Sigma, S-0876
	BSA	1.0 g	Sigma, A-7906
	dH_2O	500 ml	Sigma, A-7906
	pH	7.0	Sigma, A-7906
Wash solution concentrate/ 1 Litre	NaCl	87.66 g	Sigma, A-7906
	Tween 20	5.0 ml	Sigma, P-1379
	dH_2O	1000 ml	Sigma, P-1379
Substrate buffer/1 Litre	Citric acid (anhydrous)	9.61 g	Sigma, C-0759
	dH_2O	1000 ml	
	pH	4.0	
ABTS (40 mM) /25 mLitre	ABTS	0.55 g	Sigma, A-1888
	dH_2O	25 mL	
	pH	6.0	
Hydrogen peroxide/ 0.5 M	H_2O_2	500 µL	Sigma, H-1009
	dH_2O	8 mL	

Oestrone and progesterone assays

Stock preparation

Antibody, tracer (horseradish peroxidase/ HRP, conjugate), and standard stocks were prepared prior to running the assays. Antibody stock were produced by adding 20 µL of polyclonal anti-EC R522 to 2.0 mL coating buffer (1:100 dilution) for oestrone and 100 µL of monoclonal CL425 to 4.9 mL coating buffer (1:50 dilution) for progesterone. A 100 µL aliquot of each antibody was then placed into O-ring vials and freezed immediately. The tracer for oestrone was produced by addition of 12.5 µL oestrone-glucuronide-horseradish peroxidase to 1.238 mL assay buffer (1:100 dilution), while for progesterone 6.25 µL progesterone-3CMO-horseradish peroxidase was added to 1.244 mL assay buffer (1:200 dilution); both were then stored at 4^{o}C. Oestrone and progesterone standards were produced by adding 5 mg of oestrone-B-glucuronide (Sigma Diagnostics Cat. #E1752) and 1 mg of progesterone (Sigma Diagnostics Cat. #P0130) to 1 mL EtOH respectively, which was subsequently transfered to 99 mL EtOH to produce 5 mg/100 mL of primary stock solution for oesterone and 1 mg/100 mL for progesterone. A 100 µL aliquot of the oestrone primary stock solution was then diluted with 100 mL assay buffer while a 20 µL aliquot of the progesterone primary stock solution was diluted with 49.9 mL assay buffer, to prepare 10 ng/mL and 4 pg/µL working standard stock respectively (200 pg/well). All standard stocks were aliquot and stored at -20^{o}C.

Plate coating (Day 1)

The microtiter plates (NUNC Maxisorb) were coated the day before running the assays. The antibody solutions used were 12.5 µL antibody stock (1:100, - 20^{o}C) for oestrone and 25 µL antibody stock (1:50, -20^{o}C) for progesterone, where each were mixed with 5mL coating buffer, producing a working dilution of 1:40.000 for the former and 1:10,000 for the later. Coated plates were then left overnight and runned the next day.

Standard, samples, controls, and ‘tracer’ preparation (Day 2)

The folowing day, before the plates were runned, standards, samples, controls, and tracers were prepared. The standard values used for oestrone were 200, 100, 50, 25, 12.5, 6.5, 3.12, 1.56, 0.78, 0.39, 0.195 pg/well, and 200, 100, 50, 25, 12.5, 6.5, 3.12, 1.56, 0.78 pg/well for progesterone. Working standard stocks (10 ng/mL and 4 pg/µL for oestrone and progesterone; 200 pg/well) were diluted serially 2-fold using 200 µL assay buffer, while

plasma samples were diluted in assay buffer with appropriate dilution (determined after parallelism; described below). Pre-made quality control neat (1 and 2, of oestrone and progesterone) were prepared. The tracer used for both oestrone and progesterone assays was horseradish peroxidase conjugate (Oestrone-HRP and Pg-HRP respectively) with a working dilution of 1:35.000 for both. The HRP for oestrone was a mixture of 14.3 µL stock (1:100, 4^{o}C) with 5mL assay buffer and 28.6 µL stock (1:200, 4^{o}C) with 5mL assay buffer for progesterone, both prepared in a glass beaker.

Running the micro-titre plates

After left overnight, plates were washed with wash solution 5 times, blotted dry on paper towels to remove excess wash solution and runned immediately. Standard, samples, and control were then added to the wells in duplicate with the amount of 20 µL and 50 µL for oestrone and progesterone respectively. 50 µL of tracer (diluted oestrone-HRP and Pg-HRP respectively) was then added into every well and plates were covered and incubated in room temperature for 2 hours. After incubation, plates were washed again 5 times and blot dried. Substrate was prepared immediately after all plates were washed by combining 40 µL H_2O_2, 125 µL ABTS and 12.5 ml substrate buffer (used for both assays), 100 µL of this solution was then added in each well. Following substrate addition, plate were covered and incubated at room temperature. The plate was ready to read, at 405 nm, when the '0' wells reached 1.0 optical density. Plates were read using a spectrophotometer at 405 nm, when the '0' wells reached 1.0 optical density.

Testosterone assay

Stock preparation

Primary antibody, tracer (horseradish peroxidase/ HRP conjugate), standards, controls, secondary antibody, and blocking buffer stocks were prepared before running the assay. The primary antibody stock was produced by adding 20 µL of anti-testosterone R156/7 to 2.0 mL coating buffer (1:100 dilution) and an aliquot of 100 µL was placed into O-ring vials and freezed immediately. The tracer was produced by adding 12.5 µL testosterone-horseradish peroxidase to 1.238 mL assay buffer (1:100 dilution), which was then stored at 4^{o}C. Standards were made by adding 1.0 mg testosterone (Sigma Diagnostics Cat. #T1500) to 100 mL EtOH, producing 1 mg/100 mL (10.000 pg/µl) of primary stock solution. A 60 µL aliquot of the primary stock solution was then further diluted (1:833) with 50 mL assay buffer which prepared a 12.000 pg/ml (600 pg/well) working standard stock. All standard stocks

were aliquot and stored at -20°C. Controls were prepared by using standard stocks that binded at 30 and 70%. The secondary antibody was produced by dissolving 1 mg of anti-rabbit IgG (Sigma R2004) in 5 ml of coating buffer which was then frozen. The blocking buffer used was a mixture of 2.42 g Tris (SLS-Tris(hydroxymethyl) methylamine A.R.$NH_2C(CH_2OH)_3$), 17.92 g NaCl (Sigma S-9625), 10.0 g BSA (Sigma A-7906), 1000 ml dH_2O, and a pH of 7.5.

Plate coating (Day 1)

The microtiter plates (NUNC Maxisorb) were coated the day before running assays with the secondary non-specific antibody solution of 500 µL anti-rabbit IgG (1.0 µg/well) mixed with 25mL coating buffer. In each well (starting from A2 and down each column, including the non-specific binding wells; NSBs: A1 and B1, but not the rest of column 1) 250 µL of secondary antibody solution was added. Plates were tapped gently (to ensure antibody solution covers well bottom) and covered with acetate plate sealers, followed by overnight incubation at room temperature.

Blocking (Day 2)

The day after, before running the plates, blocking was conducted. The excess secondary antibody was removed by shaking the plate on a paper towel. 300 µL of blocking buffer was then added to each well (including NSBs), followed by a (minimum) 2 hour incubation in room temperature.

Standard, samples, controls, and 'tracer' preparation (Day 2)

Standards, samples, controls, and tracer were prepared before running the plates. The standard values used were 600, 300, 150, 75, 37.5, 18.8, 9.4, 4.7, and 2.3 pg/well and standard stock (12.000 pg/mL or 600 pg/well, -20°C) was diluted serially 2-fold using 200 µL assay buffer. Plasma samples were diluted in assay buffer with the appropriate dilution which was determined after conducting parallelism. Pre-made quality controls (1 and 2) neat were prepared before assay was runned. The primary antibody was produced by adding 20 µL of primary antibody stock (1:100, -20°C) to 5 mL assay buffer (working dilution 1:25.000). The tracer used in the assay was horseradish peroxidase (Testosterone-HRP) with a working dilution of 1:45.000, which was produced from a mixture of 11.1 µL stock (1:100, 4°C) with 5mL EIA buffer.

Running the micro-titre plates

After left overnight, plates were washed 5 times with wash solution and blotted dry on paper towels to remove excess wash solution. Plates were loaded and run immediately after being dried. 50 µL of assay buffer was added to the wells followed by the addition of 50 µL standard, sample, and control to the wells in duplicate. Diluted testosterone-HRP in the amount of 50 µL was then added in every well followed by addition of 50 µL primary testosterone-antibody (except for the NSB wells). Plates were then covered and incubated at room temperature in the dark for 2 hours. After incubation, plates were washed again 5 times with wash solution and blot dried. Substrate (in room temperature before usage) was prepared immediately after all the plates are washed by combining 40 µL H_2O_2, 125 µL ABTS as well as 12.5 ml substrate buffer, then an aliquot of 100 µL was added in each well followed by incubation at room temperature in the dark. Plates were read at 405 nm when the '0' wells reached 1.0 optical density.

Assay validation

Results from all assays were validated biologically and analytically. For biological validation, hormone levels were correlated with periods of known biological importance such as reproductive status (oestrus vs non-oestrus; pregnant vs non-pregnant) and seasonality to assure that they reflect the physiological event in question and present reliable results. For analytical validation, assay sensitivity, precision, accuracy and specificity were tested. Assay sensitivity (at 90% binding) for the oestrone, progesterone, and testosterone assay was 3.1 pg, 0.78 pg, 1.6 pg respectively. Assay precision was tested by calculation of intra-assay coefficient of variation (CV), which was 8.2% (high) and 6.1% (low) for oestrone, 14.7 (high) and 6.2 (low) for testosterone and 3.1% for progesterone; while inter-assay coefficient of variation (CV) was 14% (high) and 13.6% (low) for oestrone, 9.2% and 5.2% for testosterone and 4.9% (high) and 2.4% low for progesterone.

An accuracy check was conducted for all assays used in this thesis to measure the degree to which the measured sample concentration correlates to the real concentration of the sample. This was done by making aliquots of pooled sample with an equal amount of standard and analysing it as unknowns in the assay. The sample pool was also analysed without added standard to determine the amout of endogenous hormone present. The correlation between the amount expected (concentration of standard aliquots/2) and amount observed (concentration observed from assay results minus endogenous hormone present) was then

analysed with a linear regresion model (with an expected slope to be close to 1). Assay accuracy tests are presented in Figure 5-7 for oestrone, progesterone, and testosterone assays respectively.

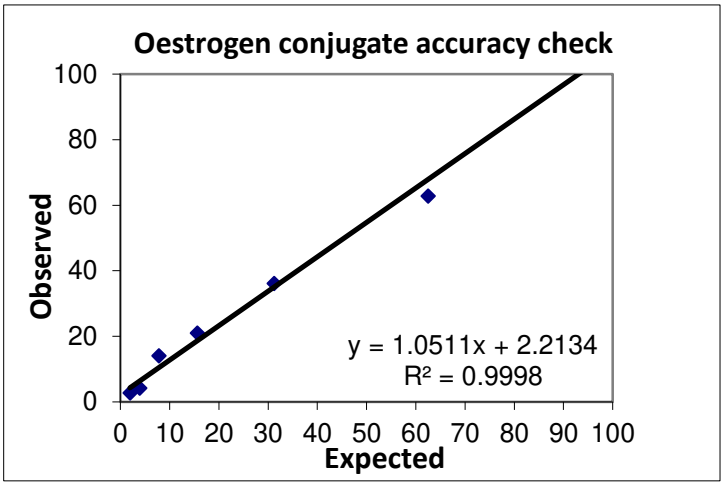


Figure 5. Accuracy check of oestrone assay.

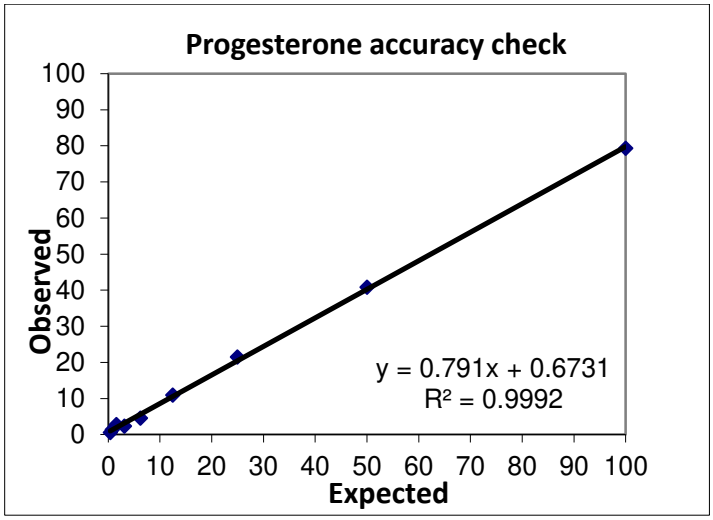


Figure 6. Accuracy check of progesterone assay.

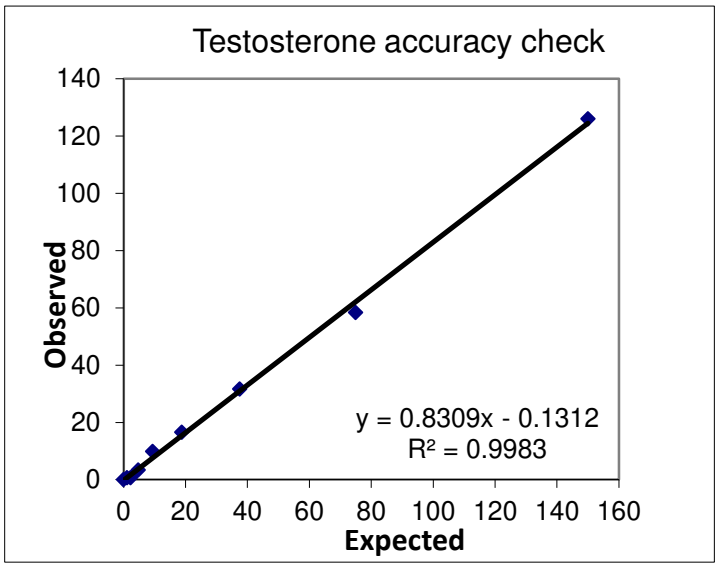


Figure 7. Accuracy check of testosterone assay.

Assay specificity was determined by analysing assay paralellism and cross-reactivity. As it was unknown whether badger plasma samples/hormone would be detected by the assay antibodies, a parallelism test was conducted for each hormone assay. This was done by pooling plasma samples to produce a neat solution which was diluted serially two-fold in assay buffer (neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, 1:8192), followed by an addition of 200 µL buffer in each tube (1:2 to 1:8192). Thereafter, 200 µL was taken out of the neat sample, placed in the 1:2 tube and vortexed to mix, followed by taking the next 200 µL from the 1:2 tube, placing it into the 1:4 tube and vortex to mix, this process was repeated until reaching the 1:8192 tube. At the end of this process, there was 200 µL in each tube and 400 µL in the last tube (1:8192), the neat pool by then have been halved 13 times. The standards and reagents were then prepared according to protocols of each hormone assay and run with the diluted neat pool. The percentage of bindings in the diluted neat pool samples were plotted by choosing an arbitrary concentration and halving the concentration for each dilution. This plot (curve) was then analysed, to see whether it was parallel to the standard curve. Parallelism between the standard curve and pool curve signifies that hormones levels in samples are similar or act the same way as the standards, therefore it can be measured in the assays correctly. After parallelism was achieved, the best dilution to run the samples was determined. For oestrone assays the chosen dilution was 1:10 (78% binding; Fig. 8), for progesterone it was 1:8 (68.96% binding; Fig. 9),

and for testosterone it was 1: 4 (42.5% binding; Fig. 10). Cross-reactivity of each hormone assay is presented in Table 2-4.

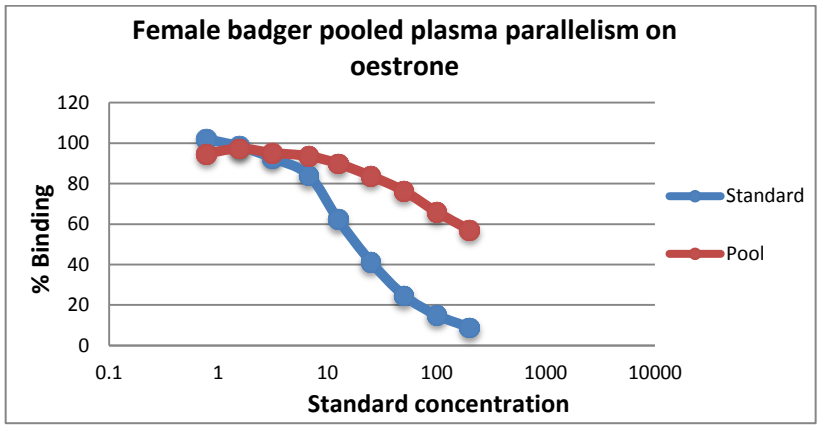


Figure 8. Parallelism of oestrone assay with badger neat pool.

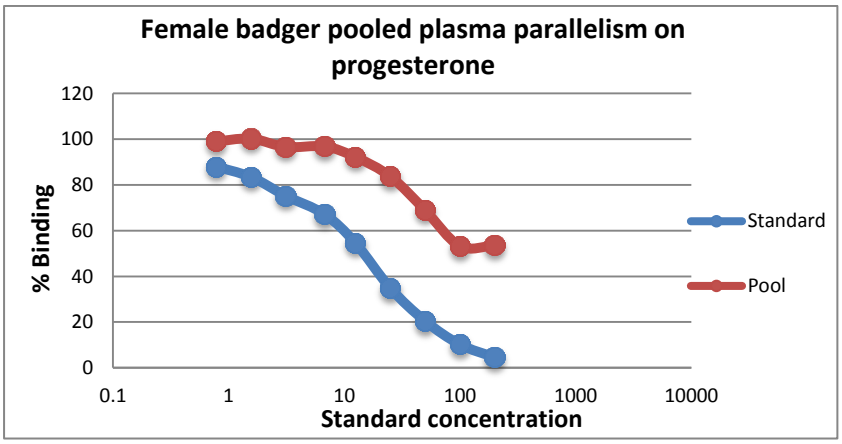


Figure 9. Parallelism of progesterone assay with badger neat pool.

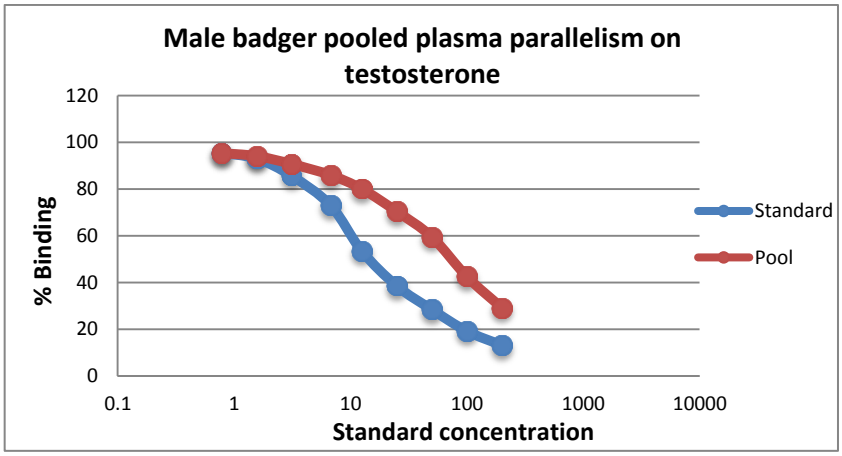


Figure 10. Parallelism of progesterone assay with badger neat pool.

Tabel 2. Oestrone antibody cross reactivity (Munro, 1991)

Oestrone cross reactivity	% Cross-reaction
Oestrone-3-glucuronide	100%
Oestrone-3-sulfate	66.6%
Oestrone	238%
Oestradiol-17β	7.8%
Ostradiol-17-glucuronide	3.8%
Oestradiol-3-sulfate	3.3%
Oestradiol-17-sulfate	0.1%
Oestradiol-17-disulfate	0.1%
Ethynyl-oestradiol-17β	<0.1%
Oestriol	<0.1%
Progesterone	<0.1%
Pregnanediol	<0.1%
Cortisol	<0.1%
Testosterone	<0.1%
Androsterone	<0.1%

Tabel 3. Progesterone antibody cross reactivity (Pantex, 2010)

Progesterone cross reactivity	% Cross-reaction
Progesterone	100%
17-αHydroxyprogesterone	0.45%
Pregnenolone	0.03%
17-OH-Pregnenolone	<0.001%
Desoxycorticosterone	1.75%
11-Desoxycortisol	0.028%
Corticosterone	0.70%
Aldosterone	0.006%
Cortisol	0.061%
Androstenedione	0.061%
Testosterone	0.017%
5α-Dihydrotestosterone	0.004%
Dehydroepiandrosterone SO4	0.008%
Androstanedione	0.04%
Oestradiol-17β	<0.001%
oestradiol-17α	<0.001%
oestriol	<0.001%
oestrone	<0.001%

Tabel 4. Oestrogen conjugate antibody cross reactivity (OEM-Concepts, 2010)

Testosterone cross reactivity	% Cross-reaction
Testosterone	100%
Dihydro testosterone	1.00%
Nortestosterone	<1.00%
Sex hormone binding globulin	0.50%
Progesterone	0.00%
Other steroids	<0.010%

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APPENDIX 2

The importance of refining anesthetic regimes for young and old wild animals to ensure optimal welfare: examples from a long-term European badger (*Meles meles*) study

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ABSTRACT

Safe and effective anesthesia is vital to the welfare of animals used in laboratory and field research, yet very young and very old animals may benefit from refinements to standard protocols and the therapeutic mitigation of adverse effects. Here instances arising during 11,645 anaesthesia procedures were reported, involving 1806 individual European badgers (1987-2017). Small cubs (<2.3Kg) require 50% of adult anaesthetic dose rates and their recovery can benefit from treatment with a glucocorticoid (0.1 mg/kg dexadreson / dexamethasone) to alleviate protracted sedation. Females > 8 yrs are susceptible to seizures, treatable with benozodiazepenes (0.2 mg/ kg midazolam). Practitioners are advised to be alert to these welfare risk factors and have appropriate treatment on hand to alleviate adverse effects.

Keywords: *adverse effects, anaesthesia, animal welfare, cubs, geriatric, seizures*

INTRODUCTION

Safe and effective anesthesia is fundamental to the welfare of a substantial proportion of animals used in various laboratory or wildlife research projects. Indeed, advising on the design and refinement of appropriate anesthetic protocols is central to the remit of the Animal Welfare and Ethical Review boards of academic institutions and biomedical laboratory operations, where, in the UK, such careful consideration is mandated under the Animals (Scientific Procedures) Act, and under similar legislation in other jurisdictions (Richardson and Flecknell 2005).

A major challenge when developing appropriate anaesthetic regimes is, however, that a protocol that might best suit the majority of subject animals may bring about adverse effects at the tail ends of the normal distribution of typical population responses, such as in very young or very old animals. In some circumstances these less-suitable research subjects may be excluded from experiments, but in ecological field research and conservation programs it is often necessary to try to capture, sedate and sample the complete population. Even if these individuals are rare in a natural population, as is usually the case with only a few elderly individuals surviving in the wild, if large numbers of animals are caught and processed by assiduous long-term studies, then ultimately a substantial number of procedures may benefit from specific anesthetic refinements. In order to ensure high standards of animal welfare for the entire population being studied it is thus crucial to report such adverse effects, as mandated under the ASPA, 1986, and to develop appropriate treatment regimes with the advice of veterinarians.

Here, particular refinements were described that have emerged as good practice as part of a long-term study of European badger (*Meles meles*) ecology in Wytham Woods, Oxfordshire, UK (Macdonald et al. 2015) based on 11,645 sedations of 1806 individual badgers between 1987 and 2018. Attention to the specific needs of both very young and very old badgers is particularly important, given the large numbers of badgers caught and sedated as part of studies and management interventions investigating their role in the epizootiology of bovine tuberculosis in the UK (Riordan et al. 2011) and Ireland (Aznar et al. 2011). Furthermore, badgers are among the wild animal species anaesthetized most often by veterinarians in general practice, treating wounds, or injuries arising from road traffic accidents (Grogan and Kelly 2013).

MATERIALS AND METHODS

Anaeasthetic options suiting badgers has been reported previously (McLaren et al. 2005; Thornton et al. 2005; see also De Leeuw et al. 2004; Davidson et al. 2007) on following the expert advice of Oxford University's Veterinary Services Department (Named Veterinary Surgeons, NVS, under the Animals (Scientific Procedures) Act, 1986; currently PPL: 30/3379). This has led to favour the use of ketamine hydrochloride (intra-muscular injection of 20 mg/kg, Vetalar® ketamine HCl injection, USP into the quadriceps; McLaren et al. 2005); finding that combinations including Medetomidine can cause peripheral vasoconstriction in badgers (evident as bluing of gum membranes; Lester et al. 2012). Also, the benefits of Butorphanol (opiod) mostly reduce pre-anesthetic anxiety (Corletto et al. 2005; Vettorato and Bacco 2011), thus potentiating sedation, where it is reported previously in Animal Welfare (Sun et al. 2015) that can be largely alleviated agitation in badgers awaiting anesthesia through good practice. Also, given that 83% of annual recapture rate (Macdonald et al. 2009) in this site, badgers in this study become somewhat accustomed to restraint. Nevertheless, in instances where animals are agitated, or would benefit from analgesia (i.e., those with naturally occurring bite wounds), Ketamine / Butorphanol (Torbugesic®) combinations (20 mg/kg ketamine + 0.4 mg/kg butorphanol) were indeed used.

Cubs

A priority of our research is to capture badger cubs in the spring in order to understand initial cohort size, which enables us to model factors affecting juvenile mortality rate (Macdonald et al. 2009). Nevertheless, care is taken not to trap until cubs are weaned, and this restricts our earliest trapping window to late May / early June (badgers are typically born in mid-Feb; Yamaguchi et al. 2006). An emergent difficulty, however, is that as the Wytham badger population density has increased, the weight and level of maturity of cubs in May/June has decreased (Fig.1; see also Macdonald and Newman 2002). Small cubs, with a typical cut-off point of under 2.3kg, need special attention during anaesthesia, where a full ketamine dose of 20mg/kg can cause unduly extended periods of anesthesia (up to 60 mins; vs average of 20-25 min), although without any atypical alteration of procedural heart rate (180 bpm), breathing rate (regular, c. 6 breaths / min), rectal temperature (38.6C) or concerning pathophysiology (zero mortality). Combinations involving Butorphanol worsen this effect

through respiratory depression (and Medetomidine would be counter-productive, reducing peripheral circulation).

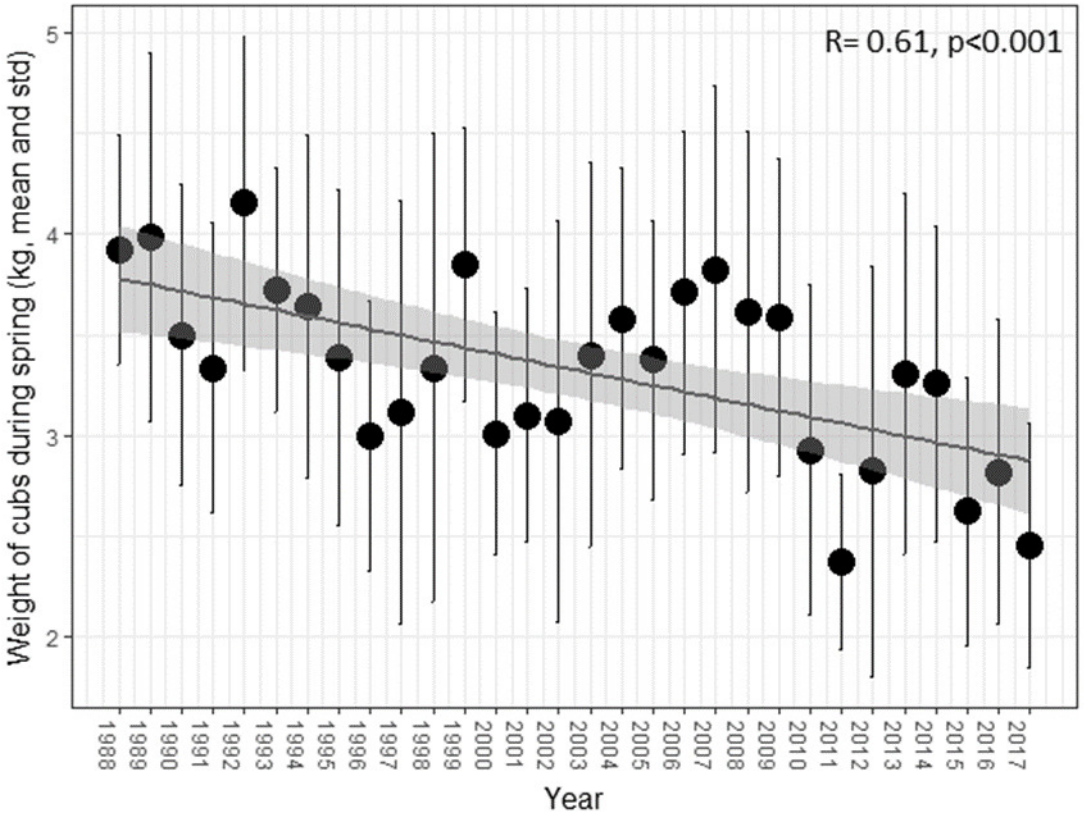


Figure 1. Weight (kg, mean and std) of cubs during spring from 1988 to 2017 with regression line and confidence interval.

Geriatric badgers

At the other end of studies into life histories, and particularly important for investigations into patterns of senescence (Dugdale et al. 2011; Research Chapter 4), is our ability to safely handle elderly badgers. Although only c. 5% of badgers live to be > 8 years old in this population, with just 1% reaching 12 (Macdonald and Newman 2002; with a maximum verified lifespan of 14 years), this still represents on average 30 procedures per year on badgers older than 8 years.

Among these elderly badgers it was noted that a phenomenon where four individuals, all female, have presented tonic-clonic seizures (1.74% of females > 8 years of age; 12 procedures) (Table 1). Notably, these elderly females also took longer for anaesthetic induction, requiring, on average 8.4 ± 4.2 mins (n=12), compared to 3-4 mins in typical

procedures (Sun et al. 2015); providing some pre-warning that adverse effects might be anticipated, to consider treatment.

Table 1. Cases of tonic-clonic seizures in four individual badgers sedated with ketamine.

Badger ID	Year of birth	Number of captures since birth	Occurence of seizures/ convulsions and pathophysiological response noted during anaesthesia	Age at seizure
911F	2003	32	15/11/10 ; padding, irregular deep respiration, epileptic seizure 08/09/ 11 ; epileptic seizure, body hunched 11/11/11 ; seizure 05/06/12 ; continuous twiching 24/08/13 ; seizure	7 years 9 months 8 years 7 months 8 years 9 months 10 years 4 months 11 years 6 months
859F	2001	27	11/09/10 ; irregular pulse, seizure 26/05/11 ; irregular deep respiration, seizure 09/09/11; epilepsy	9 years 7 months 10 years 3 months 10 years 7 months
1115F	2006	24	31/ 05/ 14 ; epileptic seizure 13/11/15 ; twicthing, high HR, seizures 08/09/17 ; repeated fits, growling, epilepsy	8 years 3 months 9 years 9 months 10 years 7 months
912F	2003	27	21/08/13 ; hard to sedate, seizures stoped after lights turned off.	10 years 6 months

RESULTS

Cubs

To mitigate protracted sedation, it was found that only a 50% adult dose rate (i.e., 10 mg/kg) is necessary to anaesthetize cubs up to a body-weight of 4kg, or until August trapping sessions, where stage of development appears ultimately more influential than simple weight. Even under this 50% regime, a proportion of cubs (1/30 = 3.3%, per cohort), still persist in a sedated state longer than required (although with good vital signs). This is particularly prevalent in cubs < 2.3kg (n=19 = 3.2%: 2000-2018). To treat this, it was found that an intra-muscular dose of 0.1mg / kg Dexamethasone (Dexadreson or equivalent glucocorticoid (synthetic predisolone analogue; Johnson – Delaney 2005; Hicks et al. 2012), in combination with warmed subcutaneous fluid (saline or lactated ringer) therapy (not more than 35-40 ml per site and not more than 50 mL/kg/day in total; NB intra-venous (iv) fluid administration was impractical in a recovering wild animal). This stimulated onset of recovery within 2-5

minutes, and all cubs have been fit for release with no latent malaise after the normal 3 hrs post-anaesthetic recovery in a dark, quiet room, as per our established protocol (Sun et al. 2015).

Geriatric badgers

Seizures present with initial muscle rigidity, followed by rapid, rhythmic convulsions. Mucous membranes become pale, but not cyanotic. Other symptoms include incontinence, hypersalivation and prolific nasal mucous section, causing sternuation (sneezing). Our best treatment results involved the intramuscular administration of 0.2 mg/ kg midazolam (a benzodiazepine, Versed®), supplemented with 0.4 mg/ kg butorphenol (administered only to badgers sedated with ketamine alone), which caused the rapid cessation of seizures (< 1 min; Voss et al. 2008; Chen et al. 2015). Fluorescent laboratory lights were also to be switched off during these compromised procedures to alleviate any contributory risks of visually-induced occipital seizures (Guerrini 1995). This midazolam treatment can repeated, if necessary, during anaesthetic recovery, after a 30 min interval. These elderly badgers were also administered subcutaneous fluids by syringe (as per cubs, above), to remediate potential dehydration arising. Affected badgers treated according to this regime proved fit for release after 3 hrs, as per our normal protocol; all except one instance (badger 1115F, case 08/09/17) where convalescence was extended through 24hrs prior to release, during which time this badger was provided (and consumed) peanut butter mixed with honey and water, ad libitum. Notably, none of these individuals exhibited any convulsive symptoms while conscious during the trapping, pre-sedation or release phases of our protocol. Furthermore, this Wytham badger population has been subject to extensive video surveillance over many years (Stewart 1997), and no seizures have been noted among subjects observed.

DISCUSSION

Ketamine hydrocloride is used widely as a dissociative anaesthetic agent in many domestic and wild mammals, particularly for carnivore species (Bush 1996; Forsyth et al. 1999; Fernandez–Moran et al. 2001), and provides good muscle relaxation, complete unconsciousness and a wide safety range in badgers; however, it is recommended that dose rates in young animals should be reduced significantly.

In geriatric badgers > 8 yrs, ketamine appears to induce tonic-clonic seizures in some individuals (Smith et al. 2008), as noted in susceptible humans (Ferrer – Allado et al. 1973) and dogs (Lervik et al. 2010; Adami et al. 2013), often associated with brain tumours (e.g. glioblastoma, astrocytoma, adenoma), Cushing's disease, or disease of the liver or kidneys (Smith et al. 2008). Furthermore, all four affected individuals were females, where Monteiro et al. (2012) report that female dogs are prone to more frequent cluster seizures than males, possibly due to pro-convulsan effects of oestrogen which enhances neuronal excitability (Van Meervenne et al. 2015). Through genetic pedigree (Annavi et al. 2014), It is also ascertained that two of these four elderly individuals were siblings, suggesting a degree of heritability of this geriatric condition, suggestive of epilepsy.

Animal welfare implications

Likely risks, similar to those shown here, affect other mammals, but have hitherto remained unreported. Veterinarians and research practitioners are advised to be alert to the risks of sedating very small/young wild-animal subjects requiring much smaller dose rates of anaesthetic, and be prepared to use steroid / fluid therapy. Similarly, special attention should be given to very elderly (evident in badgers from toothwear under examination; Harris et al. 1992) and especially older female individuals, with benzodiazepine treatment on hand, along with facilities for post-procedural care. Because these effects are comparatively rare, and most wild-animal studies comprise far smaller sample sizes of captured and sedated individuals, these effects can easily be over-looked and/ or attributed to inter-individual effects rather than age-dependency. Thus, it is postulated that similar sedation-associated risks might also be prevalent in other mammals, but have hitherto remained unreported. Therefore, it is recommended Ethical Review processes for encouraging the reporting and treatment of adverse effects.

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