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Authors: N.A. Sugianto, C.D. Buesching, M. Heistermann, C. Newman, D.W. Macdonald



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

Sugianto N. A.^a, Buesching C. D.^b, Heistermann, M.^c, Newman, C.^d, Macdonald D.W.^e
^{a,b,d,e} Wildlife Conservation Research Unit, Department of Zoology, University of Oxford,
 Recanati-Kaplan Centre, Abingdon Road, Tubney House, Tubney, Oxfordshire OX135QL,
 UK.

^cEndocrinology Laboratory, German Primate Center, Kellnerweg 4, 37077 Göttingen,
 Germany.

Corresponding author: Christina D. Buesching, Wildlife Conservation Research Unit -
 Department of Zoology, University of Oxford, Recanati-Kaplan Centre, Tubney House,
 Abingdon Road, Tubney, Oxfordshire, UK, OX13 5QL.

Email: christina.buesching@zoo.ox.ac.uk

Tel: +44 (0)1865 611 100

Fax: +44 (0)1865 611 101

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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 we analysed sex steroid hormones 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 testes volume; females: swollen, intermediate, normal vulva). Sex-steroid levels exhibited seasonal patterns, broadly reflecting the seasonal variation in genital condition. Nevertheless, on an individual level, male testosterone levels correlated significantly with testes condition and testes volume only during the mating season in spring, and neither oestrone nor oestradiol differed between female EGM categories. We thus conclude 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 (Hildebrandt 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 testis) 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. maniculatus*, *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 noticeably 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).

Nevertheless, 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 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, we investigate the relationship of circulating plasma sex-steroid hormones with the condition of the external genitalia of wild-living males and female European badgers (*Meles meles*; henceforth "badger"). 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 Sugianto et al., *subm.*). 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 (Tinneland et al., 2015), when average oestrone and oestradiol levels are highest (details see Sugianto et al. *subm.*), although some females can display vulval swelling also at other times throughout the summer/autumn while oestrone levels remain elevated (Sugianto et al. *subm.*). 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).

Here, we i) analyse the seasonal profiles in EGM (males: testes volume and degree of testicular descent; females: degree of vulval swelling) and sex-steroid hormones (males: testosterone; females: oestrone and oestradiol); and ii) test the hypothesis that higher sex-steroid-levels are reflected in EGM on the individual level. We discuss our results 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.

MATERIAL 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 processing 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 ($n_{\text{females}} = 3237$; $n_{\text{males}} = 2569$ with testes volume measured in $n = 1599$). 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 descended (both 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 testes 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): Testes 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 ($n_{\text{males}}= 216$; $n_{\text{females}}= 179$; 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) vacutainer 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 (see also Sugianto et al. *subm.*): 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 20 μl aliquots of diluted plasma, oestrone standard (0.195-200 pg/well) and quality controls were combined with 50 μl 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 (50 μl) and antiserum (50 μl) 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°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 aqueous 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 peroxidase (testosterone-HRP) as label (Palme and Möstl, 1994). Plates were incubated overnight at 4°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

i) Seasonal profiles of EGM and sex steroid hormones

Seasonal variation in EGM profiles was analysed with chi-square 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 testes 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 $P < 0.05$. Testes volume measures were log₁₀ transformed for best model fit. Average seasonal hormone levels (testosterone, oestrone, and oestradiol) with standard deviations were calculated across animals.

iii) 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: $\text{lmer}(\log_{10}(\text{Testosterone}) \sim \text{Testes condition} + \text{Season} + (1|\text{ID}))$. 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 analysis. Thus, we could not assess the significance of EGM (if any) for specific seasons. Instead, we visualized this relationship 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 testes volume as predictor, season as covariate, as well as including the interaction between testes volume and season using a linear mixed model (ID as random effect). Testosterone levels were log₁₀ transformed for best fit: $\text{lmer}(\log_{10}(\text{Testosterone}) \sim \text{Testes volume} + \text{Season} + \text{Testes volume} * \text{Season} + (1|\text{ID}))$. 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: $\text{lmer}(\text{Oestrone} \sim \text{Vulva condition} + \text{Season} + \text{Vulva condition} * \text{Season} + (1|\text{ID}))$. 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): $\text{lm}(\text{Oestrone} \sim \text{Vulva condition} + \text{Pregnancy status})$.

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): $\text{lm}(\log_{10}(\text{Oestradiol}) \sim \text{Vulva condition} + \text{Season})$. 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

RESULTS

i) 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

FIGURE 2

Testes 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

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.

ii) 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 ($X^2_{\text{season}} = 40.953$, $df=3$, $p<0.001$) 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; but no difference was found between the latter ($X^2 = 0.092$, $df=1$, $p=0.762$; 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

FIGURE 4 a, b

Although testosterone levels were significantly correlated with testes 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 testes volume over the whole year ($n=158$), only season proved a significant effect ($X^2_{\text{season}}=29.042$, $df=3$, $p<0.001$), while testes volume ($X^2_{\text{testes.volume}}=2.299$, $df=1$, $p=0.130$) and the interaction term were not ($X^2_{\text{interaction}}=3.669$, $df=3$, $p=0.300$, Table 3, Fig. 5)

TABLE 3

FIGURE 5

Females

Season was the only significant predictor of oestrone levels ($X^2_{\text{season}}=41.724$, $df=3$, $p<0.001$), while neither vulva condition ($X^2_{\text{vulva condition}}=1.094$, $df=2$, $p=0.579$, 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 ($X^2_{\text{interaction}}=3.849$, $df=6$, $p=0.697$) 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).

TABLE 4

TABLE 5

TABLE 6

FIGURE 6a, b

DISCUSSION

Our results show that badger sex-steroid levels follow distinct seasonal patterns, which broadly coincide with the seasonal variation in EGM as well as male testes 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, testes 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 to EGM 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; Sugianto et al., *subm.*). In fact, the slightly lower oestrone levels (pg/ml) in females with vulval swelling likely indicate an absence of pre-implanted blastocysts (Sugianto et al., *subm.*). 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, we 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 occurred 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 female reproductive status (as determined by necropsy) in only 58-85%.

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 of testosterone levels and testes 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 our analyses, we propose 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).

We therefore conclude that, 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), we recommend this method 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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TABLES

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 Testes 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 testes volume (used for hormone agst. testes volume analyses). Some individuals contributed more than 1 data point. From 179 females 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).

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

Complete model coefficients								
	Estimate	SE	df	t	P			
(Intercept)	-0.309	0.077	178.180	-4.008	<0.001			
TestesDescended	-0.298	0.136	208.690	-2.183	0.030			
TestesIntermediate	-0.524	0.144	207.500	-3.631	<0.001			
SeasonSpring	0.699	0.125	190.810	5.571	<0.001			
SeasonSummer	0.460	0.119	199.310	3.857	<0.001			
SeasonWinter	0.967	0.179	202.610	5.389	<0.001			
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)								

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

Complete model coefficients								
	Estimate	SE	df	t	p			
(Intercept)	-0.744	0.154	152.960	-4.824	<0.001			
Testes.Volume	0.054	0.036	126.990	1.506	0.134			
SeasonSpring	0.671	0.156	145.280	4.308	<0.001			
SeasonSummer	0.420	0.153	147.110	2.748	0.007			
SeasonWinter	0.881	0.203	138.030	4.346	<0.001			
Testes 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) ~ Testes Volume + Season + (1 Tattoo)								
model 2: log10(Testosterone) ~ Season + (1 Tattoo)								
model 3: log10(Testosterone) ~ Testes Volume + (1 Tattoo)								
model 4: log10(Testosterone) ~ Testes Volume + Season + Testes Volume* Season + (1 Tattoo)								
Spring only model coefficients								
	Estimate	SE	t	p				
(Intercept)	-0.286	0.133	-2.146	0.036				
Testes Volume	0.114	0.051	2.237	0.029				
Spring only significance of terms								
	Df	Sum Sq	Mean Sq	F	p			
Testes Volume	1	0.780	0.780	5.003	0.029			
Residuals	59	9.196	0.156					

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

Complete model coefficients								
	Estimate	SE	df	t	p			
(Intercept)	81.271	5.318	135	15.283	<0.001			
Vulva.ConditionHalfbud	2.894	6.387	135	0.453	0.651			
Vulva.ConditionRosebud	-4.322	6.434	135	-0.672	0.503			
SeasonSpring	-7.437	7.205	135	-1.032	0.304			
SeasonSummer	-41.597	7.118	135	-5.844	<0.001			
SeasonWinter	-12.305	9.317	135	-1.321	0.189			
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 model coefficients

	Estimate	SE	t	p	
(Intercept)	5.455	28.396	0.192	0.8509	
Vulva.ConditionHalfbud	-25.674	33.645	-0.763	0.4601	
Vulva.ConditionRosebud	6.067	22.621	0.268	0.7931	
Reproductive.statusPregnant	76.969	27.565	2.792	0.0163	
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 model coefficients

	Estimate	SE	t	p	
(Intercept)	1.592	0.119	13.342	<0.001	
Vulva.ConditionHalfbud	-0.055	0.143	-0.386	0.702	
Vulva.ConditionRosebud	-0.028	0.165	-0.171	0.865	
SeasonSpring	0.285	0.171	1.669	0.106	
SeasonSummer	-0.165	0.166	-0.994	0.329	
SeasonWinter	-0.044	0.185	-0.237	0.814	
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		

LIST OF FIGURE LEGENDS

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.

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

Figure 4a. Testosterone levels (ng/ml) of males exhibiting different testes conditions (descended, intermediate and ascended).

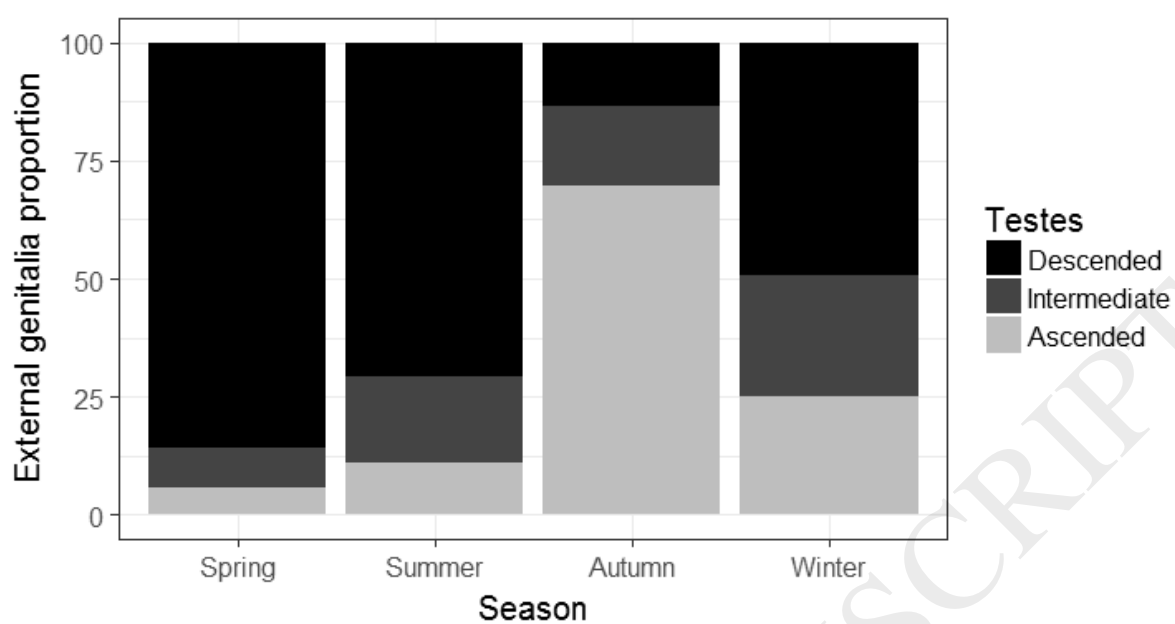
Figure 4b. Visualisation of testosterone levels (ng/ml) against testes condition (as integer: Ascended=1, Intermediate=2, Descended=3) for each season (spring, summer, autumn, Winter) in males.

Figure 5. Testosterone levels (ng/ml) versus testes volume (mm^3) for each season (spring, summer, autumn, winter).

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

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

Figr-1



Figr-2

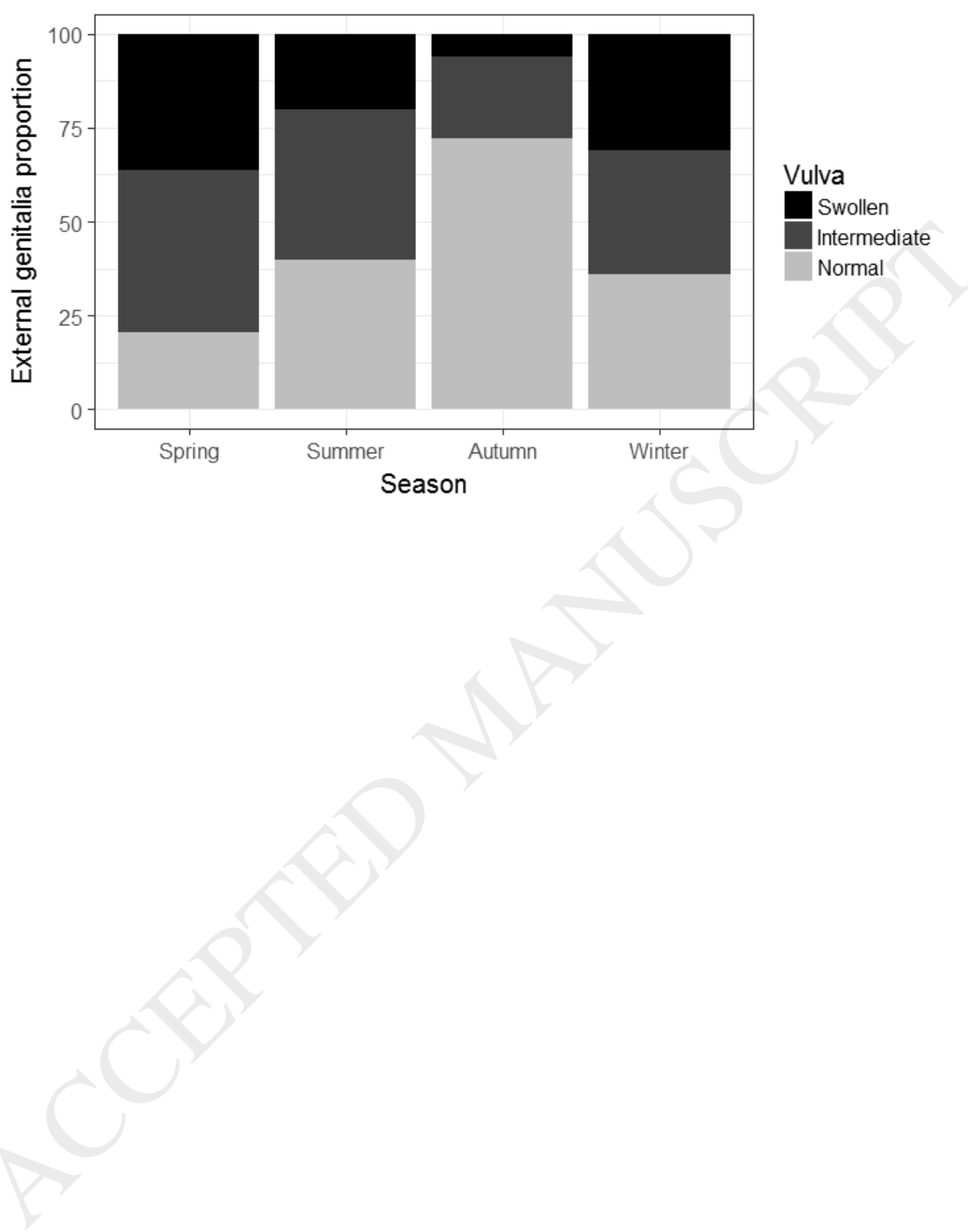
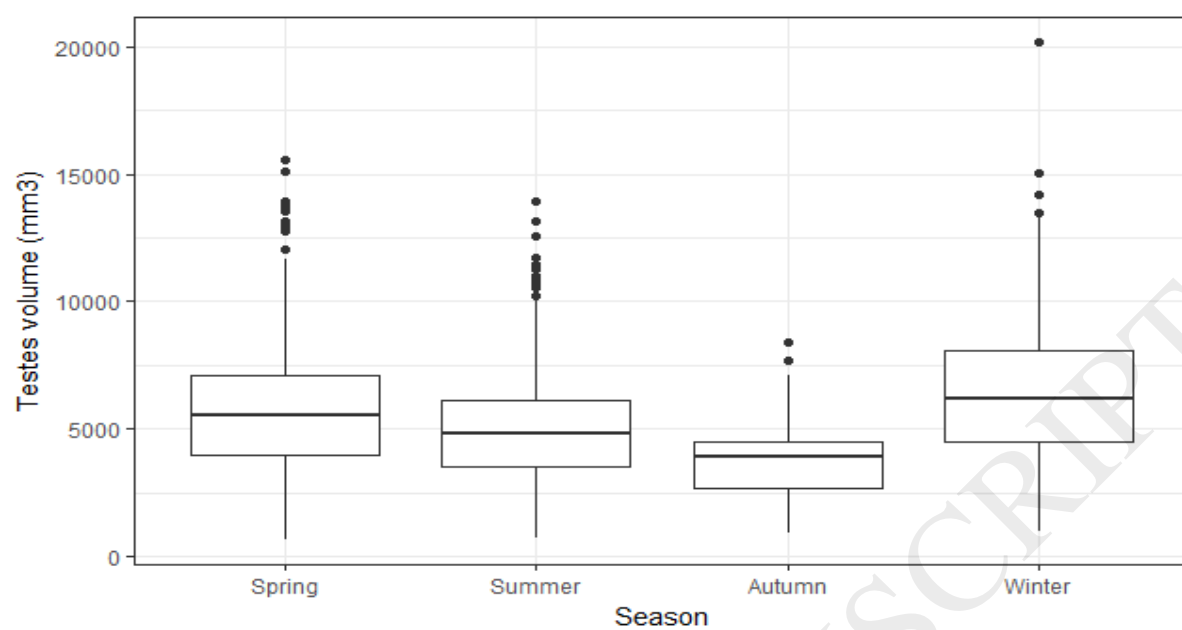
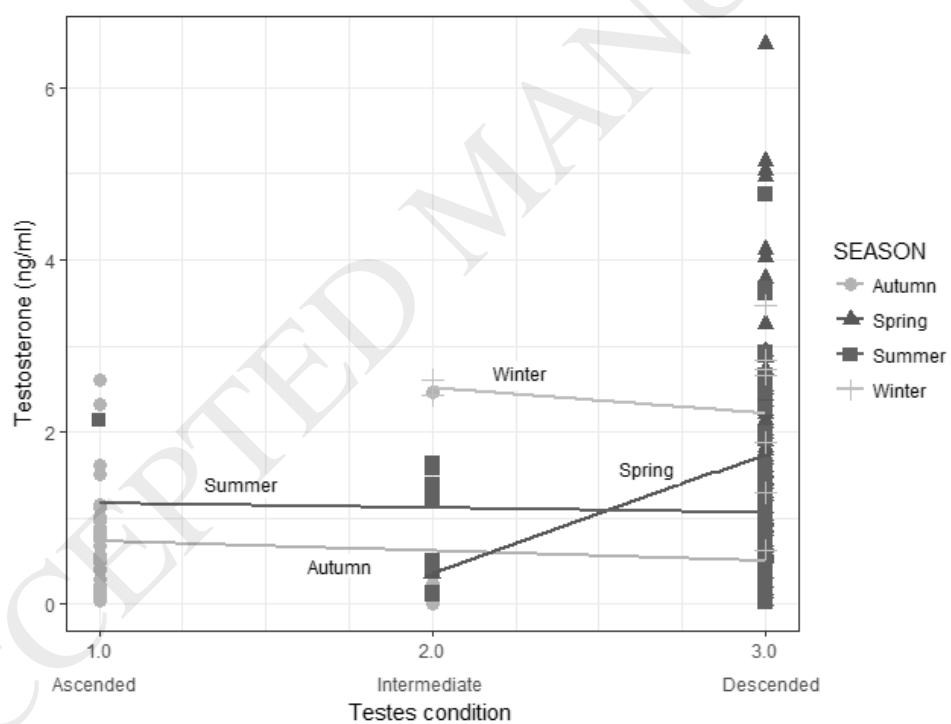
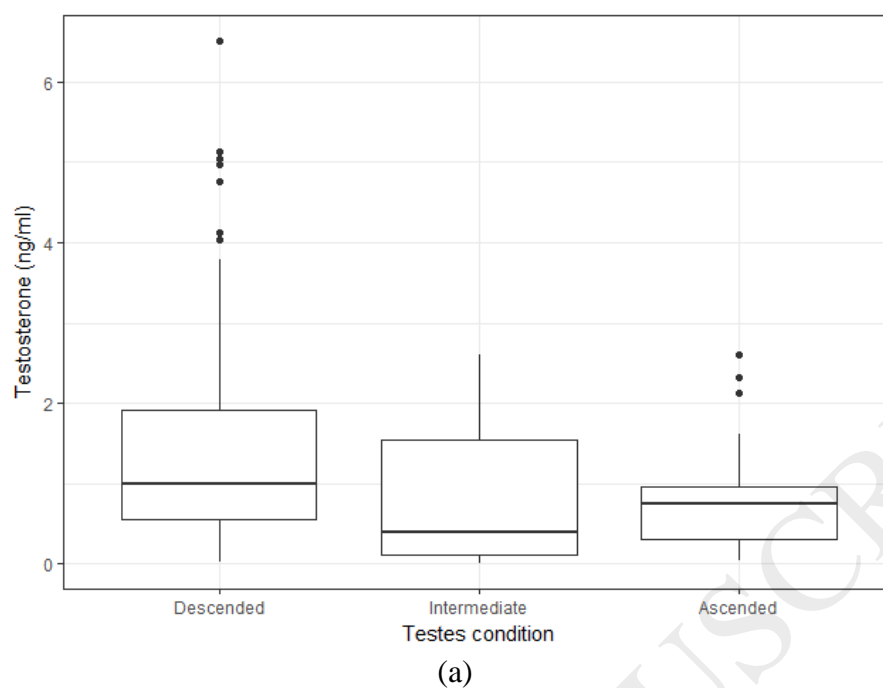


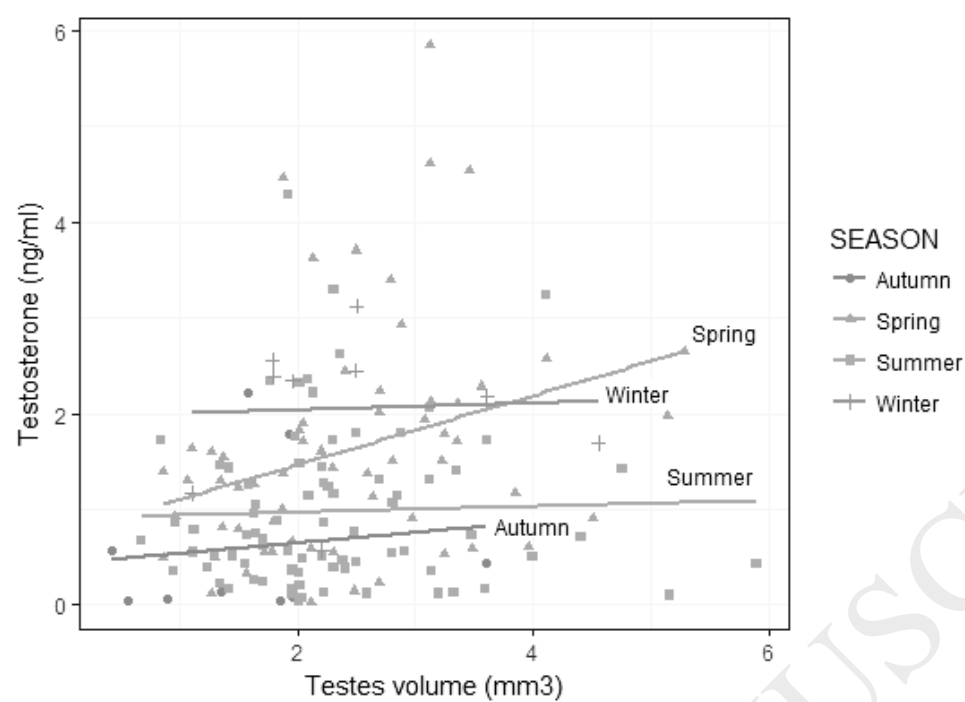
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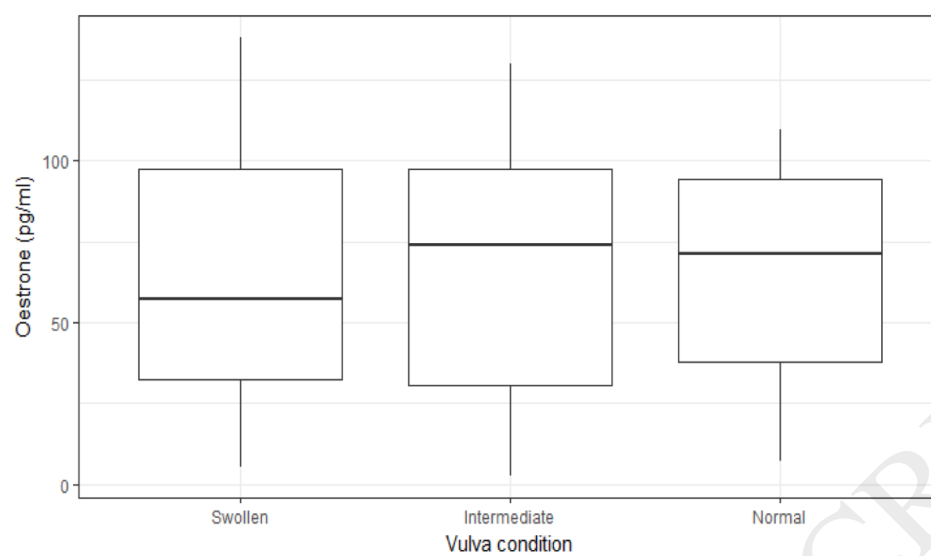
Figr-4



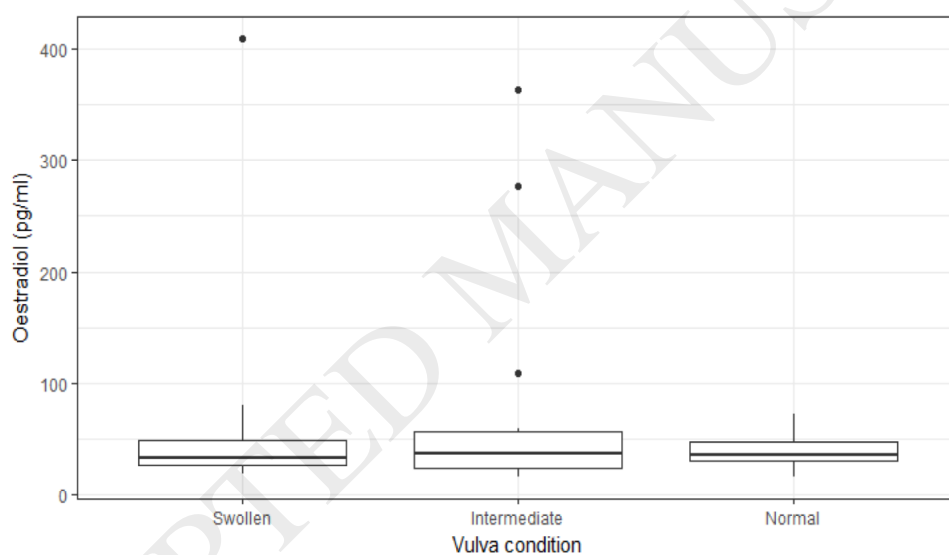
Figr-5



Figr-6



(a)



(b)