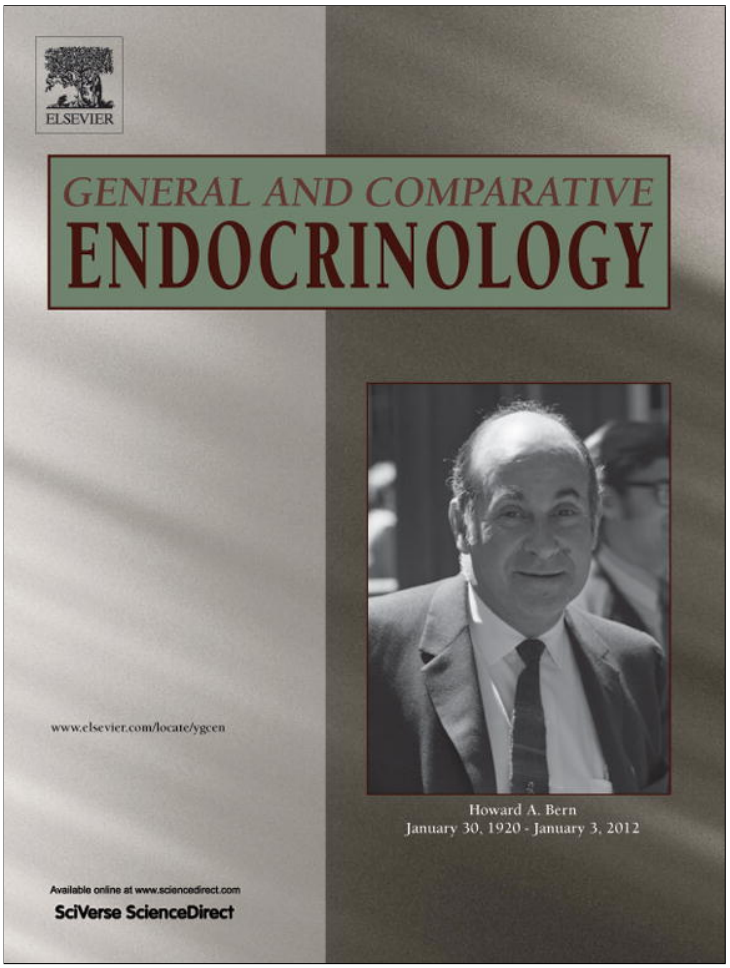

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The influence of reproductive hormones on the torpor patterns of the marsupial *Sminthopsis macroura*: Bet-hedging in an unpredictable environment

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ABSTRACT

Seasonal cycles of reproduction are common in many mammals and these are combined with the necessary energy budgeting for thermoregulatory challenges. Many mammals meet the challenge of changing environmental temperatures in winter by using torpor, a controlled reduction in body temperature and metabolic rate. We aimed to determine the effects of photoperiod and reproductive hormones on the seasonal cycles of reproduction and torpor use in a marsupial that commences reproduction in winter, the stripe-faced dunnart, *Sminthopsis macroura*. Males and females were placed under LD 14:10 and natural reproductive hormones blocked by either flutamide (males) or mifepristone (females) or tamoxifen (females). Reproductive parameters, metabolic rate and torpor variables were determined. The same animals were then placed under LD 10:14 and given testosterone (males) or progesterone (females) or oestrogen (females). Reproductive parameters, metabolic rate and torpor variables were measured. Body mass and tail widths (fattening indicator) in males were significantly affected by testosterone, and the effects were reversed by hormone blockers. Reproductive parameters were unaffected. Resting metabolic rate and ability to use torpor were not affected by treatment in males, however torpor characteristics, especially torpor bout duration, were affected by presence of testosterone in males. In females, body mass was unaffected by hormone presence, although tail widths were affected. Disruption of reproductive cycles occurred with hormone blockers in females, however, resting metabolic rate was not affected, and only presence of progesterone affected torpor characteristics in females. Our results differ from those found for rodents, where presence of testosterone abolishes the use of torpor in males, and oestrogen inhibits torpor use in females. Our study suggests that, in this mammal, metabolic responses to the presence or absence of reproductive hormones differs between males and females, and there is no absolute endocrinologically-driven reproductive season demarcated from the torpor season.

1. Introduction

Seasonal cycles of reproduction are common in many organisms. Mammals, including marsupials [75,47], will generally time the energetically costly activity of reproduction to correspond to the most advantageous time of the year, that is, for periods of food abundance. Cues such as rainfall, temperature and photoperiod have been mooted as Zeitgeber for onset of reproduction, however, photoperiod is the most reliable in indicating external environmental changes, no matter where a mammal lives. This is in contrast to rainfall and temperature, which can be variable from year to year. The obvious parameter of the day/night cycle is the duration of day, and this photoperiodic attribute is commonly thought to be the main proximate cue regulating the neuroendocrine cascade that ultimately leads to production of young [70,9].

In marsupials, photoperiodic cues are known to promote reproductive activities in species from several orders. These include the insectivorous marsupials *Antechinus* spp. [49,51], *Sminthopsis* spp. [26,68], wallabies *Macropus* spp. [61,52,45], the brush-tailed possum *Trichosurus vulpecula* [24] and the common bandicoot *Isoodon macrourus* [23]. The neuroendocrine control of reproduction in those mammals studied so far, including marsupials e.g. [61,23,45,52,50], is initiated by the hormone melatonin, via the pineal gland, in response to photoperiod [62]. The neuroendocrine cascade results in gonadal and reproductive hormonal changes in response to these changes in photoperiod, and these have been best described in some hamsters and ground squirrels [27,11,2,30,31,66]. Preparation for reproduction includes changes in body condition, increasing body mass, initiating gonadal recrudescence and changing fur colouration [27,11,2,30,31,66].

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However, in conjunction with the timing of reproduction, small mammals must also budget for thermoregulatory challenges. Both reproduction and thermoregulation at low ambient temperatures (T_a) can be energetically expensive for small mammals. Many small mammals use torpor especially during periods of low T_a and food scarcity [16]. Torpor is characterised by a controlled reduction of body temperature (T_b) and metabolic rate (MR) and is the most effective means for energy conservation available to mammals [16]. The seasonal use of torpor is common in the colder months of the year when food supply is low, and this is coincident with short photoperiods [17,18,40]. Seasonal use of torpor has also been shown to be driven by photoperiod in some species [27,11,22]. Importantly, reproduction and torpor use seem mutually exclusive for many heterothermic rodent species [29,27,60,65,57]. In contrast, a monotreme, some marsupials and bats enter torpor during the reproductive season, and females can use torpor even while pregnant or lactating [19,20,29,21,41,54,69].

Clearly mammals use contrasting methods for budgeting for energy costs while promoting the energetically costly process of reproduction. Some studies have been performed to elucidate the role that reproductive hormones have in torpor use. Testosterone administration inhibits torpor in hamsters and ground-squirrels [29,27,44], and prolactin administration inhibits torpor in non-breeding female Djungarian hamsters [65]. Paradoxically, in arctic ground squirrels, androgens do not decrease in response to onset of winter, and this is believed to promote anabolism of muscle in preparation for catabolism during the long hibernation season [5]. In female rodents the effects of steroid sex hormones on thermal physiology are mixed, with either oestradiol or progesterone administration affecting thermoregulatory activities in some species, and having no effect in others [29,11,44,42,57]. Alongside this are pronounced changes in body condition indicators promoted by the sex hormones [30,31]. Nevertheless, despite the limited understanding of the interactions between reproduction, hormones and torpor, little work has been conducted for the past 15 years specifically on the topic of reproductive hormonal influences on torpor in any species [57].

The stripe-faced dunnart, *Sminthopsis macroura*, is a small carnivorous marsupial that can use torpor during pregnancy [21]. It lives in the Australian arid zone and has a seasonal reproductive period as photoperiod increases from late June (winter solstice) to January, a few weeks after the summer solstice (late December), followed by reproductive quiescence for the rest of the year (while photoperiod decreases to the winter solstice in late June; [47]). The long breeding season in this species is believed to be a response to the unpredictable environment, allowing replacement of any lost litters [47]. Torpor use by dunnarts during early pregnancy is also believed to allow energy savings in preparation for the long period of lactation [21]. Because of the overlap between torpor and some reproductive activity we investigated the effect of major reproductive hormones on body condition and torpor patterns of both male and female *S. macroura*. Specifically, we aimed to determine in both sexes whether physiological levels of reproductive hormones administered to non-reproductive animals during short photoperiod (reproductively quiescent period) affect body condition and torpor patterns, and also to determine whether the absence of reproductive hormones in animals during long photoperiod (reproductively active period) affects body condition and torpor patterns.

2. Material and methods

2.1. Animals

Twenty (10 male and 10 female) adult *S. macroura* (1–2 years) were used. The animals were obtained from a captive colony maintained at the University of New England. Due to their territorial nature, the animals were housed individually. Cages consisted of translucent plastic (23 × 44 × 32 cm) with metal mesh (1 cm) lids and a floor covering of clean wood shavings. Nesting boxes with shredded paper were provided. Small cardboard rolls and boxes were given to the animals for environmental enrichment and to provide cover outside the nest box, and a variety of toys were also provided (running wheels, bird swings or climbing toys). Animal cages and nest boxes were exchanged once a week. The animals were maintained in a temperature-controlled room at 19 °C (±2 °C). Artificial light was supplied by three 75 watt broad spectrum incandescent lamps (150 Lux). The animals were fed a mixture of water soaked macerated cat biscuits (Friskies "Go Cat") and either tinned cat food (Whiskas non-fish varieties) or tinned dog food (Pedigree Pal). Vitamins (PetVite) were added to the food once a week. The food mixture consisted of 77.9% water (dried 24 h in 60° oven), 8.9% ash (determined using a muffle furnace) and 21.1 KJ/g dry (determined by bomb calorimetry, Parr Instruments, Inc). Freshly mixed food was provided once a day approximately 1 h before the onset of darkness. Food was always provided in excess to the animals' needs. Mealworms (*Tenebrio* larvae) and cooked chicken egg were provided at irregular intervals. Water was always available *ad libitum*. All animal experiments were undertaken with permission of the University of New England Animal Ethics Committee, and NSW National Parks and Wildlife Service.

2.2. General experimental protocol

Animals were exposed to short photoperiod (L:D 10:14) from mid-February to mid-June 2002 for the males and from mid-February the beginning of August 2002 for the females. Males were in long photoperiod (L:D 14:10) from mid- June to the end of August 2002. Females remained in long photoperiod from the beginning of August to mid-October 2002. The photoperiod schedule was selected because these are the shortest (L:D 10:14) and longest days (L:D 14:10) that the animals experience in their habitat range.

Animals were acclimated to both short and long photoperiods for minimum of 4 weeks before the commencement of hormone or blocker injections. At the end of the photoperiodic acclimation period, animals exhibited reproductive parameters consistent with the photoperiod exposure see [47]; that is, reproductive quiescence under short photoperiod, and reproductive activity under long photoperiod.

2.3. Hormone injection regime

Hormone injections for animals exposed to short photoperiod were calculated based on the results of blood samples taken during a period of long photoperiod and assayed by RIA. Males were injected with testosterone equal to circulating levels found in animals during long photoperiod, and half the level of testosterone circulating in animals during long photoperiod. Males were given Durateston (consisting of Testosterone propionate 6 mg/ml, Testosterone phenylpropionate 12 mg/ml, Testosterone isocaproate 12 mg/ml, Testosterone decanoate 20 mg/ml; in oil, Intervet) a long acting form of testosterone, following McAllan [46]. This long acting formula was used to test whether the hormones had to be circulating in the blood for an extended period of time to influence torpor. Females were injected with both oestrogen (β-estradiol 98%, Sigma) only and progesterone (99%, Sigma) only equal to circulating levels and half the circulating concentrations during long photoperiod for both hormones (table 1). Because the information was not available at the time of the experiment, pregnancy level progesterone values, from the literature, were used to calculate doses. The averaged pregnancy level of progesterone for the East-

ern Quoll, Kowari, brown *Antechinus* and Bandicoot was 10 ng/ml (Table 2). All females received oestrogen injections first followed by progesterone injections. The females remained in short photoperiod longer than the males because of the delay in determining progesterone values.

For animals experiencing long photoperiod, circulating levels of hormones were blocked so that they were similar to circulating levels present when animals were reproductively quiescent. Two treatments were experienced by animals, they would either be injected with enough blocker to fully block the hormones, or they would be injected with enough blocker to block half the circulating hormones. In males, testosterone as well as DHT was blocked using flutamide (Sigma), a pure androgen receptor antagonist. In females, oestrogen activities were blocked by tamoxifen (citrate salt 99%, Sigma), a protein kinase C inhibitor and a mixed oestrogen agonist/antagonist. In females, progesterone actions were blocked using RU486 (Mifepristone 98%, Sigma), which is a progesterone receptor antagonist. Doses of blockers were calculated assuming one molecule of blocker was equal to one molecule of hormone (table 1).

Injections were based upon the assumption of 8% blood volume and all doses were made up to 0.1 ml (based on 21 g BM) which were adjusted to BM of individual animals. All solvents were dissolved and diluted in ethanol (analytical grade, AnalaR). Peanut oil (Crisco) was used as a depot because it allowed the substance to be released over a period of 24 h (Bradley, personal observation). All injections were administered at 0900 h on the day the animals were to be placed in the metabolic chamber in the afternoon. Injection at this time allowed the full amount of the substance to be circulating in the blood when the animals would normally enter torpor. Injections were administered subcutaneous into the scruff of the animal's neck with a 27G needle.

2.4. Assessment of body condition

The body condition and reproductive status of all animals was assessed every one-two weeks, usually during the time of weekly cleanings, throughout the experimental period. Body mass (BM) was measured to the nearest 0.1 g on an electronic balance. Tail width (TW) has been observed to be an indicator of general fat stores in a closely related species, *Sminthopsis crassicaudata* [34], thus widths of the widest part of the tail were taken using Vernier callipers. Body mass and tail width were analysed at the beginning of the treatment regime, where they were compared to the body masses of animals at the beginning of all experiments. They were also analysed for differences between control treatment groups under the different photoperiod regimes, and they were compared after treatment with the control treatment groups for each photoperiodic regime.

2.5. External assessment of reproductive status

Scrotal widths, bulbourethral gland size and the presence of sperm were used to determine the reproductive status in males. Scrotal widths were measured weekly using Vernier callipers. In *S. macroura*, scrotal width decreases slightly, and bulbourethral glands increase with the onset of spermatorrhoea [78]. The scrotal length was determined for a single testicle and width was measured across both testes [49]. The presence of sperm in the urine was checked when a urine sample was available. Urine was collected on a clean slide and examined by a light microscope under 100× magnification [78]. The size of the bulbourethral glands were also monitored weekly and assigned to one of three categories [49]. These glands are located in all marsupials just under the crus penis and adjacent to the penis on both sides [64].

Oestrus was determined in females by monitoring the urogenital sinus for the presence of cornified epithelial cells, which are shed at the onset of oestrus [79,67]. Smears were taken by gently running a clean slide across the urogenital sinus and examined for cells under a light microscope. Changes in the depth, fur covering and colour of the pouch, have been described [79,67] and were quantified following McAllan et al. [49]. A minimum score of 2 is assigned for females that are sexually mature, but not cycling, and this state is usually seen in autumn and early winter when animals are reproductively quiescent. A maximum score of 8 is assigned when animals demonstrate full development of the pouch, urogenital sinus and maximum cytology. Assessment of reproductive parameters in both sexes was performed a few days before the metabolic rate measurements and these values are reported in the present study.

2.6. Assessment of reproductive hormones

Blood samples from animals exposed to short photoperiod (8 of each sex) and from animals under long photoperiod (8 of each sex) were taken after acclimation ended during each photoperiod. A 27G needle was used to puncture the tail vein and blood was collected in a heparinized capillary tube, transferred to a microcentrifuge tube, centrifuged cold at 1500 g until the plasma separated. Plasma was stored at −80 °C until needed. Radioimmunoassay (RIA) was used to determine the concentrations of testosterone for males and oestrogen and progesterone for females. Blood samples were not pooled. Results of the assays were used to calculate the injections for the hormone experiments.

2.7. Steroid RIA

Plasma and saliva samples (20 μl) were mixed with 0.05 M phosphate buffer (pH 7.4) to make 1 ml of solution. This was added to a Strata X solid phase separation column (Strata X polymeric sorbent column 30 mg/1 ml, 8B-S100-TAK, Phenomenex, USA) and drawn under vacuum into a waste collection tube. Protein and other substances that might interfere with the radioimmunoassay were washed through the column using 1 ml distilled water followed by 1 ml of 35% methanol in water. The steroid attached to the solid phase matrix was eluted using 1 ml absolute methanol. This elution process was monitored by internal recovery checks using small quantities (2,000dpm) of 3H-steroids giving recoveries

Table 1
Doses and control regimes for hormones and hormone blockers injections for reproductive hormone experiments. For both short and long photoperiod exposure, animals were first given no injection (control for injection) followed the next week by oil injection (control for the actions of injection). Because some of the solutions, once mixed, only remained active for a short time, all hormones and blockers were then administered in a random order once a week until all animals had received all doses. To ensure all animals received the same concentration of hormones/blockers, all animals were injected from the same batch of solution.

Short photoperiod (L:D 10:14)					Long photoperiod (L:D 14:10)				
Hormone	Control for injection	Control for vehicle	Half dose	Full dose	Blocker	Control for injection	Control for vehicle	Half dose	Full dose
Testosterone	No injection	Peanut oil	20 ng/ml	40 ng/ml	Flutamide	No injection	Peanut oil	38 ng/ml	76 ng/ml
Oestrogen	No injection	Peanut oil	1.5 ng/ml	3 ng/ml	Tamoxifen	No injection	Peanut oil	0.6 ng/ml	1.2 ng/ml
Progesterone	No injection	Peanut oil	5 ng/ml	10 ng/ml	RU486	No injection	Peanut oil	14 ng/ml	28 ng/ml

Table 2
Progesterone levels during pregnancy of four species of marsupials.

Species	Plasma progesterone concentration	Authors
Eastern Quoll	8–10 ng/ml	Hinds (1989)
Dasyurus viverrinus	pregnant	
Kowari	9.9–11.5 ng/ml	Fletcher (1989)
Dasyuroides byrnei	23–30 days oestrus/pregnancy	
Brown *Antechinus*	11–15 ng/ml	Hinds and Selwood (1990)
Antechinus stuartii	15–4 days before parturition	
Northern Brown Bandicoot	3.9–12.6 ng/ml	Gemmell et al. [25]
Isoodon macrourus	between 11–4 days before parturition	

between 92% and 97%. Prior to the RIA step, the eluted steroids were allocated to separate 12 × 75 mm polypropylene test tubes and dried at 37 °C under vacuum in a Büchler vortex evaporator. Radiolabelled steroids [1,2,6,7-^{3}H]-oestradiol, [1,2,6,7-^{3}H] – testosterone and [1,2,6,7-^{3}H]-progesterone, were purchased from the Radiochemical Centre (Amersham, U.K). Non-radioactive steroids were obtained from Sigma Chemical Co., USA. All other reagents used were of analytical grade and purchased from commercial sources.

Plasma testosterone concentrations were determined by radioimmunoassay (RIA) as described by Bradley [7] using a titre of 1/5000. Plasma oestradiol and progesterone was determined using antisera raised in New Zealand white rabbits using the procedure described by Bradley [7]. Oestradiol 3-o-carboxymethyloxime-BSA conjugate and progesterone 3-o-carboxymethyloxime-BSA conjugates were purchased (Sigma Chemical Co) for the production of antisera. The working titres of oestradiol and progesterone antisera was 1/7500 and 1/6000, respectively. Testing of both antisera revealed cross reactivities of <10% for any related steroid (Bradley, unpublished). Assay sensitivity for the three steroids were less than 10 pg. Inter-assay variation for oestradiol, progesterone and testosterone assays were 8.6%, 8.5% and 8.9%, while intra-assay variability was 8.4%, 8.9% and 7.9%, respectively.

After separation of free from bound steroid using dextran coated charcoal (DCC), 150 µl aliquots of supernatant were added to plastic picovials containing 2 ml of CytoScint (ICN Biomedicals, Coata Mesa CA, USA) and counted to an accuracy of <2% standard deviation in a Beckman LS6000TA liquid scintillation spectrometer. Standard curve construction and conversion of activity in unknown samples was performed using AssayZap software (Biosoft, Cambridge, UK). Serial dilutions of *S. macroura* plasma were made using charcoal stripped plasma and assays run against the standard curves prepared using known concentrations of steroid. The standard curves for oestradiol, progesterone and testosterone were parallel to that of a serially diluted plasma sample when measured by the appropriate RIA.

2.8. Respirometry

Metabolic rate (MR) was measured as the rate of oxygen consumption (VO_2) using open flow respirometry systems. Animals were placed in 750 ml glass chambers in a temperature controlled cabinet (±0.5 °C). Paper towelling lined the chambers to absorb waste and to act as a non-conductive surface for the animals to rest on. Oxygen consumption was measured over 23 h after each treatment the animal received. Single animals were measured in individual isolated metabolic chambers, but, as the respirometry system allowed for multiple measurements, animals were measured in groups of three of the same sex at the same time. Individuals measured on the same day were given the same injection. Animals were measured once a week to allow time for the full clearance of hormones and blockers.

Oxygen consumption was measured by a single channel system which was able to measure three animals in sequence. Automatically operating solenoid valves enabled oxygen consumption to be determined for each animal sequentially for 3 min in a 12 min cycle including one outside air reference reading for 3 min. The oxygen concentration was measured on a sub-sample of air, collected downstream of the animal chamber, using a single channel oxygen analyser (Ametek Applied Electrochemistry S-3A/1, Pittsburgh) that had been fitted with a high resolution output board (80335 SE). A flow rate of approximately 450 ml/min was maintained using rotameters (7908, Aarlborg, New York) and measured via a mass flowmeter (FMA-5609, Omega, Stanford) that had been calibrated with a bubble meter. This flow rate/chamber combination gave a 99% equilibrium in approximately 8 min using the equation from Lasiewski et al. [43]. This flow rate ensured that the oxygen concentration in the chambers did not fall below 20%. All air was dried by silica before entering the animal chambers and before concentrations were measured. Air pumps (Optima A-807) were used to provide the air flow.

The T_a in the animal metabolic chambers was measured using copper-constantan thermocouples that had been calibrated to the nearest 0.1 °C with a Dobros precision mercury thermometer (R6578) that was traceable to national standards. The thermocouple output was amplified by a digital thermometer (Omega DP116). T_a was measured at the same time as VO_2 in the corresponding animal chamber. Analogue outputs from the oxygen analyser, flowmeter, and digital thermometer were interfaced with a personal computer fitted with a 14 bit analogue to digital converter (Flytech). Oxygen consumption was calculated using Eq. 3a from Withers [77]. Processing was performed by software written by G. Körtner. Light was provided by a 12 V 15 W lamp and was programmed to the acclimation photoperiod experienced by the animals at the time of the MR measurement. The animals were isolated from the other animals throughout the measurement period.

MR of each animal was measured for 23 h (±1 h). The animals were placed in the metabolic chambers in the afternoon 1–3 h before dark to allow time for the animals to acclimate to the chambers. No food or water was available for the duration of the measurements. MR of each animal was measured at 19 °C. Animals were weighed to the nearest 0.01 g on a laboratory balance before and after each experiment in order to calculate mass-specific metabolic rate, with a constant rate of loss in body mass assumed during the MR measurements. To determine whether duration of exposure to long-acting hormones had an effect on metabolism, animals given long-acting hormones (testosterone treatments) were measured twice, on day 1 and day 8 of administration, all other animals were measured after the series of injections were completed, that is, the last day of their hormone treatment.

2.9. Calculated variables: resting metabolic rate (RMR), average daily metabolic rate (ADMR), day metabolic rate (DMR), night metabolic rate (NMR)

Resting metabolic rate ($mlO_2/g/h$) was obtained from animals that were normothermic and post absorptive and measured at a T_a of 19 °C. The lowest values of MR of inactive resting animals

were averaged over a minimum duration of 36 min. All RMR measurements were obtained during diurnal hours since this species is nocturnal. Moreover, RMR values were taken after the animals were acclimated to the chambers, and also resting. Average daily metabolic rate (ADMR) was determined by averaging all mass-specific metabolic rate measurements over a 23 h period at a constant T_a of 19 °C. Day metabolic rate (DMR) was calculated by averaging the mass-specific metabolic rates throughout the photophase. Night Metabolic Rate (NMR) was calculated from by averaging the mass-specific metabolic rates throughout the scotophase.

2.10. Torpor frequency and Torpor bout duration (TBD)

Because T_b measurements were not available in this study, torpor was defined as the period when MR dropped below 75% RMR at the same T_a [35]. Frequency of induced torpor (no food and water provided) was defined as the percentage of animals under each treatment regime that exhibited torpor during the MR measurement period. Torpor bout duration was calculated as the time when RMR first fell below 75% of RMR (defined as torpor entry) to when the animal's oxygen consumption returned to 75% of RMR (defined as torpor arousal).

2.11. Torpor metabolic rate (TMR)

Minimum TMR was determined by the lowest consecutive MR readings over 36 min when the animal was in torpor. Mean T_a corresponding to values of TMR were also calculated.

2.12. Statistical analysis

Initial body condition measures were analysed by two-way Analysis of variance, followed by Tukey pairwise post-hoc tests if initial analysis was significant. For each of the treatment groups, data were analysed by General Linear Model Analysis of variance using the individual animals as a repeated measure. If significant, these were followed by Tukey pairwise post-hoc tests. Non-parametric data were transformed to accommodate the assumption of normality and then analysed as described. Frequency data were assessed by χ^2 analysis. Data are presented as means ± standard error of the mean.

3. Results

3.1. Body condition

3.1.1. Initial measures for each photoperiod

Body mass differed over the course of the experiment. The body mass of all individuals before exposure to changed photoperiods was significantly lower than after acclimation to the changed photoperiods (Table 3). Initial body mass, and also when body mass was measured before hormone or blocker treatment began, did not differ between sexes. There were no sex differences between body mass measurements when no injection occurred and also Peanut oil was injected (Table 3). However, body mass differed over time, with initial body mass significantly lower than when no injection occurred, but animals were acclimated to the new photoperiod regime. Tail widths differed over the course of the experiment. The tail width of all individuals before experimental photoperiod exposure began was the same as those measured under long photoperiods, and significantly smaller than those measured under short photoperiods (Table 3). Males had narrower tail widths than females initially, measurements without injection and with Peanut oil injection (Table 3). The tail widths without injection and also when Peanut oil was injected did not differ (Table 3).

3.1.2. Males LD 10:14

Body mass changed after injection under short photoperiod, with both testosterone treatments significantly increasing body mass when compared to both control groups (Table 3). Body masses after testosterone treatment remained the same (Table 3). Tail width did not differ among treatment groups (Table 3), nor did testes width (Table 3). Reproductive parameters also did not differ among treatment groups (Table 3).

3.1.3. Males LD 14:10

Body mass of males also changed significantly when injected with hormone blockers under long photoperiod (Table 3). However, here only one treatment produced a significant change: 76 ng/ml Flutamide treatment resulted in a reduction of body mass (Table 3). Tail widths differed significantly among treatments (Table 3), with flutamide treatment significantly decreasing tail width when compared with males with no injection or injected with peanut oil (Table 3). Testes width did not differ among treatment groups (Table 3). Other reproductive parameters also did not differ among treatment groups (Table 3).

3.1.4. Females LD 10:14

Body mass did not change when females were treated with hormones (Table 3), although tail width was affected (Table 3). Specifically, treatment with progesterone significantly reduced tail width compared to all other treatments. Oestrogen treatments did not affect tail widths when compared to no injection or peanut oil treatments (Table 3). Reproductive indices did not differ among treatments (Table 3).

3.1.5. Females LD 14:10

Body mass did not change significantly when animals were treated with hormone blockers, however, tail width was affected (Table 3). Treatment with both doses of tamoxifen and the higher dose of RU486 significantly reduced tail width in females when compared to no injection and other treatment groups were not significantly different from each other (Table 3). Reproductive indices differed among treatments (Table 3), with only the no injection and peanut oil treatments producing significantly different indices from one another (Table 3).

3.2. Resting metabolic rate (RMR)

When photoperiod treatment, initial injection treatments and sex was compared, RMR differed between sexes, with males having lower RMRs than females, and when animals were exposed to peanut oil (peanut oil > no injection), although photoperiodic exposure alone did not affect RMR (Table 4). When RMR was measured under LD 14:10 sex and initial treatment affected RMR with females having significantly higher RMR after injection with peanut oil (Table 4). Analysis of RMR for males treated under LD 10:14, males treated under LD 14:10 and females treated under LD 10:14 was not significant (Table 4). However, for females held under LD 14:10 RMR differed between treatments for females held under long photoperiod (Table 4). All treatments initiated higher RMRs than when animals were given no injection.

3.3. Average daily metabolic rate (ADMR)

There was no difference in ADMR between males and females under either photoperiod, nor when initial injection treatments and sex was compared (Table 4). However, when data were compared within photoperiodic treatments (i.e. between photoperiodic

Table 3
Body masses (1 in g), Tail widths (2 in mm) of both sexes, testes widths (3 in mm) of males, and reproductive score (4 no units) under different photoperiods and treatment regimes. Data are means ± 1 standard error of the mean. Two asterisks indicate that the values were significantly different from the no injection and peanuts oil control values ($p < 0.01$). One asterisk indicates that the values were significantly different to the no injection control value ($p < 0.05$). Three asterisks indicate that the values were significantly different from the no injection and peanuts oil control values, and the other hormone/blocker treatment used ($P < 0.005$).

Initial					
No. treatment	Males	Females			
	(1) 21.07 ± 0.23**	(1)21.01 ± 0.22**			
	(2) 6.80 ± 0.35	(2) 7.29 ± 0.25			
Short photoperiod (L:D 10:14)			Long photoperiod (L:D 14:10)		
Treatment	Males	Females	Treatment	Males	Females
No injection	(1) 23.60 ± 1.20	(1) 23.36 ± 0.80	No injection	(1) 25.36 ± 1.06	(1) 23.47 ± 0.70
	(2) 7.82 ± 0.38	(2) 8.70 ± 0.22		(2) 6.78 ± 0.38	(2) 7.33 ± 0.15
	(3) 10.24 ± 0.35			(3) 10.58 ± 0.19	
	(4) 2.9 ± 0.3	(4) 2.4 ± 0.2		(4) 3.6 ± 0.16	(4) 2.8 ± 0.29
Peanut oil	(1) 22.32 ± 1.10	(1) 23.11 ± 0.69	Peanut oil	(1) 24.32 ± 0.88	(1) 22.25 ± 0.64
	(2) 7.54 ± 0.28	(2) 8.69 ± 0.21		(2) 6.83 ± 0.29	(2) 6.72 ± 0.25
	(3) 10.43 ± 0.37			(3) 10.32 ± 0.18	
	(4) 2.9 ± 0.1	(4) 2.1 ± 0.1		(4) 3.44 ± 0.18	(4) 5.0 ± 0.72*
Testosterone 20 ng/ml	(1) 25.78 ± 1.17**	–	Flutamide 38 ng/ml	(1) 24.36 ± 0.68	-
	(2) 8.66 ± 0.34			(2) 6.11 ± 0.27**	
	(3) 10.81 ± 0.21			(3) 10.62 ± 0.21	
	(4) 3.3 ± 0.15			(4) 3.33 ± 0.17	
Testosterone 40 ng/ml	(1) 25.08 ± 1.01**	–	Flutamide 76 ng/ml	(1) 24.09 ± 0.84*	–
	(2) 7.90 ± 0.35			(2) 5.96 ± 0.23 **	
	(3) 10.95 ± 0.17			(3) 10.60 ± 0.14	
	(4) 3.3 ± 0.15			(4) 3.5 ± 0.22	
Oestrogen 1.5 ng/ml	–	(1) 23.74 ± 0.56	Tamoxifen 0.6 ng/ml	–	(1) 22.39 ± 0.64
		(2) 9.06 ± 0.34			(2) 6.14 ± 0.17*
		(4) 2.2 ± 0.13			(4) 3.0 ± 0.31
Oestrogen 3 ng/ml	–	(1) 23.32 ± 0.45	Tamoxifen	–	(1) 23.12 ± 0.49
		(2) 9.08 ± 0.26	1.2 ng/ml		(2)6.62 ± 0.21*
		(4) 2.0 ± 0.0			(4) 3.25 ± 0.37
Progesterone 5 ng/ml	–	(1) 23.04 ± 0.69	RU486	–	(1) 21.16 ± 0.54
		(2) 7.47 ± 0.26 ***	14 ng/ml		(2) 6.52 ± 0.23*
		(4) 2.4 ± 0.31			(4) 3.71 ± 0.45
Progesterone 10 ng/ml	–	(1) 22.99 ± 0.73	RU486	–	(1) 22.67 ± 0.55
		(2) 7.51 ± 0.22***	28 ng/ml		(2) 6.54 ± 0.18*
		(4) 2.8 ± 0.33			(4) 3.8 ± 0.53

Table 4
MR variables for males and females under different photoperiodic regimes and treatments [Resting Metabolic Rate (RMR), average daily metabolic rate (ADMR); day metabolic rate (DMR), night metabolic rate (NMR)]. Data are means ± standard error of the mean. One asterisk indicates that the values are significantly different to both female control groups for both photoperiods ($P < 0.05$). Two asterisks indicates that the groups are significantly different to the no injection control group for that photoperiodic regime and sex ($P < 0.05$). Three asterisks indicate that the groups are significantly different to both no injection and peanut oil control groups.

Sex, photoperiod regime and Treatment		Variable			
		RMR ($mLO_2\ g^{-1}h^{-1}$)	ADMR ($mLO_2\ g^{-1}h^{-1}$)	DMR ($mL\ O_2.g^{-1}h^{-1}$)	NMR ($mL\ O_2 g^{-1}h^{-1}$)
Males Short Photoperiod LD 10:14	No injection	2.83 ± 0.10*	3.78 ± 0.25	2.44 ± 0.12	4.61 ± 0.36
	Peanut oil	2.92 ± 0.07*	3.97 ± 0.30	2.68 ± 0.12	4.78 ± 0.45
	Testosterone 20 ng/ml Day 1	2.73 ± 0.07	3.69 ± 0.18	2.65 ± 0.13	4.37 ± 0.25
	Testosterone 20 ng/ml Day 8	2.74 ± 0.08	3.79 ± 0.24	2.70 ± 0.16	4.51 ± 0.31
	Testosterone 40 ng/ml Day 1	2.78 ± 0.09	3.69 ± 0.18	2.54 ± 0.14	4.57 ± 0.16
	Testosterone 40 ng/ml Day 8	2.79 ± 0.09	3.69 ± 0.20	2.53 ± 0.12	4.34 ± 0.28
Females Short Photoperiod LD 10:14	No injection	3.12 ± 0.13	3.18 ± 0.29	1.95 ± 0.14	3.98 ± 0.49
	Peanut oil	3.22 ± 0.11	3.30 ± 0.33	2.32 ± 0.17	4.34 ± 0.39
	Oestrogen 1.5 ng/ml	3.18 ± 0.09	3.23 ± 0.28	2.32 ± 0.14	3.91 ± 0.40
	Oestrogen 3 ng/ml	3.13 ± 0.10	3.45 ± 0.32	2.17 ± 0.11	4.24 ± 0.47
	Progesterone 5 ng/ml	3.26 ± 0.14	3.82 ± 0.24	2.79 ± 0.26 **	4.36 ± 0.33
	Progesterone 10 ng/ml	3.32 ± 0.08	3.97 ± 0.15	2.78 ± 0.26**	4.66 ± 0.20
Males Long Photoperiod LD 14:10	No injection	2.69 ± 0.09*	3.62 ± 0.14	2.51 ± 0.12	5.08 ± 0.20
	Peanut oil	2.99 ± 0.15*	3.59 ± 0.18	2.42 ± 0.11	5.09 ± 0.31
	Flutamide 38 ng/ml	2.90 ± 0.17	3.41 ± 0.16	2.22 ± 0.12	4.94 ± 0.21
	Flutamide 76 ng/ml	2.79 ± 0.12	3.25 ± 0.18***	2.10 ± 0.14***	4.67 ± 0.31
Females Long Photoperiod LD 14:10	No injection	3.20 ± 0.08	3.65 ± 0.19	3.01 ± 0.25	4.50 ± 0.18
	Peanut oil	3.58 ± 0.09**	4.28 ± 0.24	3.70 ± 0.34	4.95 ± 0.22**
	Tamoxifen 0.6 ng/ml	3.69 ± 0.12**	4.03 ± 0.20	3.01 ± 0.27	5.32 ± 0.16**
	Tamoxifen 1.2 ng/ml	3.70 ± 0.12**	4.14 ± 0.13	3.42 ± 0.18	5.22 ± 0.10**
	RU486 14 ng/ml	3.72 ± 0.14**	3.95 ± 0.16	3.15 ± 0.24	5.50 ± 0.27**
	RU486 28 ng/ml	3.54 ± 0.07**	4.20 ± 0.20	3.42 ± 0.32	5.23 ± 0.15**

treatments), there was a sex difference in ADMR that was not affected by injection with peanut oil under LD 10:14 with ADMR of females significantly lower than that of males (Table 4). When ADMR was measured under LD 14:10 neither sex nor initial treat-

ment affected ADMR (Table 4). ADMR did not differ between hormone or blocker treatments for males held under LD 10:14, females held under L:D 10:14 or LD 14:10 (see Table 4). However, ADMR differed between blocker treatments for males held under long photoperiod (Table 4). ADMR was significantly decreased with 76 ng/mL Flutamide treatment when compared with ADMRs measured under no injection or peanut oil treatment.

3.4. Day metabolic rate (DMR)

Before hormone or blocker treatment, DMR differed under the different photoperiod regimes in untreated animals, and also differed due to the treatment with peanut oil. DMR was significantly increased under long photoperiod. Sexes did not differ in DMR, although there was a trend (Table 4). DMR did not differ between hormone treatments for males held under LD 10:14 or for females between blocker treatments held under LD 14:10 (Table 4). However, DMR differed between treatments for males held under LD 14:10, (Table 4). DMR was significantly decreased with 76 ng/mL Flutamide when compared with DMRs measured under no injection or peanut oil treatment. Other comparisons were not significant. DMR also differed for females held under LD 10:14 (Table 4). Treatment with progesterone significantly increased DMR when compared to the no injection treatment (Table 4). All other comparisons were not significant.

3.5. Night metabolic rate (NMR)

NMR differed under the different photoperiod regimes when photoperiod treatment, initial injection treatments and sex were compared. NMR was significantly increased under long photoperiod ($P < 0.05$ Table 4). There was no difference between sexes, although there was a trend (Table 4). When data were compared within photoperiodic regimes for untreated animals, there were no differences in NMR under LD 10:14 or LD 14:10 (Table 4). However, while NMR did not differ for hormone or blocker treatments in males held under LD 10:14, males held under LD 14:10 or females held under LD 10:14 (see Table 4). However, NMR was significantly different between blocker treatments for females held under LD 14:10 (Table 4). NMRs in females when no injection occurred were significantly lower than when treated with both tamoxifen and RU486.

3.6. Torpor frequency

Torpor use differed among photoperiod treatments and between sexes (Fig. 1a, $\chi^2 = 20.22$, $P < 0.0001$). Under short photoperiods torpor was used by all animals when measured when they had no injection, and by all but one male when measured after the injection with peanut oil. In contrast, under long photoperiod when either no injection was given, or when peanut oil was given, all males used torpor but only 40% of females used torpor. Torpor frequency did not differ between treatments for males held under LD 10:14 or LD 14:10, or for females held under LD 14:10 (Figs. 2a, 3a and d). However, progesterone administration significantly decreased torpor frequency in females held under LD 10:14 (Fig. 2d, $\chi^2 = 18.462$, $P < 0.0001$).

3.7. Torpor bout duration (TBD)

TBD was significantly decreased under long photoperiod. There was no difference between sexes, nor with injection treatment (Fig. 1b). However, when data were compared within photoperiodic treatments, TBD was significantly longer for females under

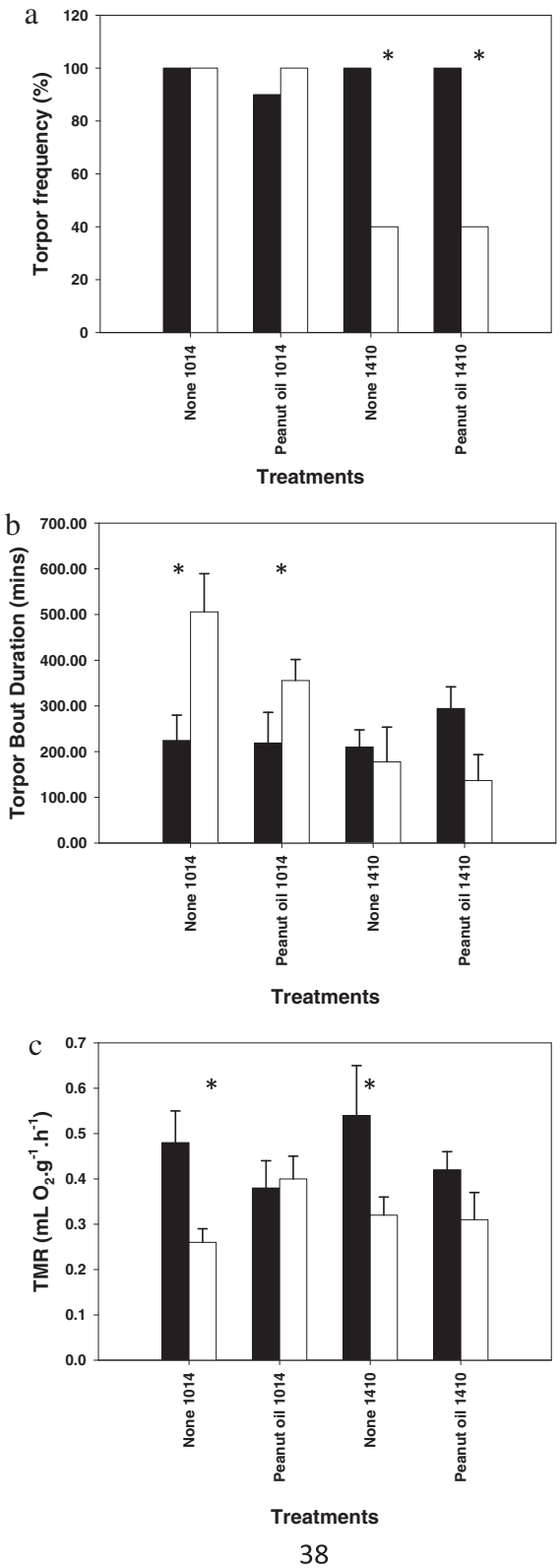

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Fig. 1. Torpor frequency (%, Fig. 1a), torpor bout duration (minutes, Fig. 1b) and Torpor metabolic rate ($mL\ O_2\ g^{-1}\ h^{-1}$, Fig. 1c) of males and females after exposure to the short photoperiods without treatment ("none 1014") or treated with peanut oil ("peanut oil 1014") and also to long photoperiods without treatment ("none 1410") or treated with peanut oil ("peanut oil 1410"). Male values are indicated by solid bars and female values are indicated by open bars. With the exception of percentages, data are means ± 1 standard error of the mean. An asterisk indicates that males and females in the group were significantly different at $P < 0.05$.

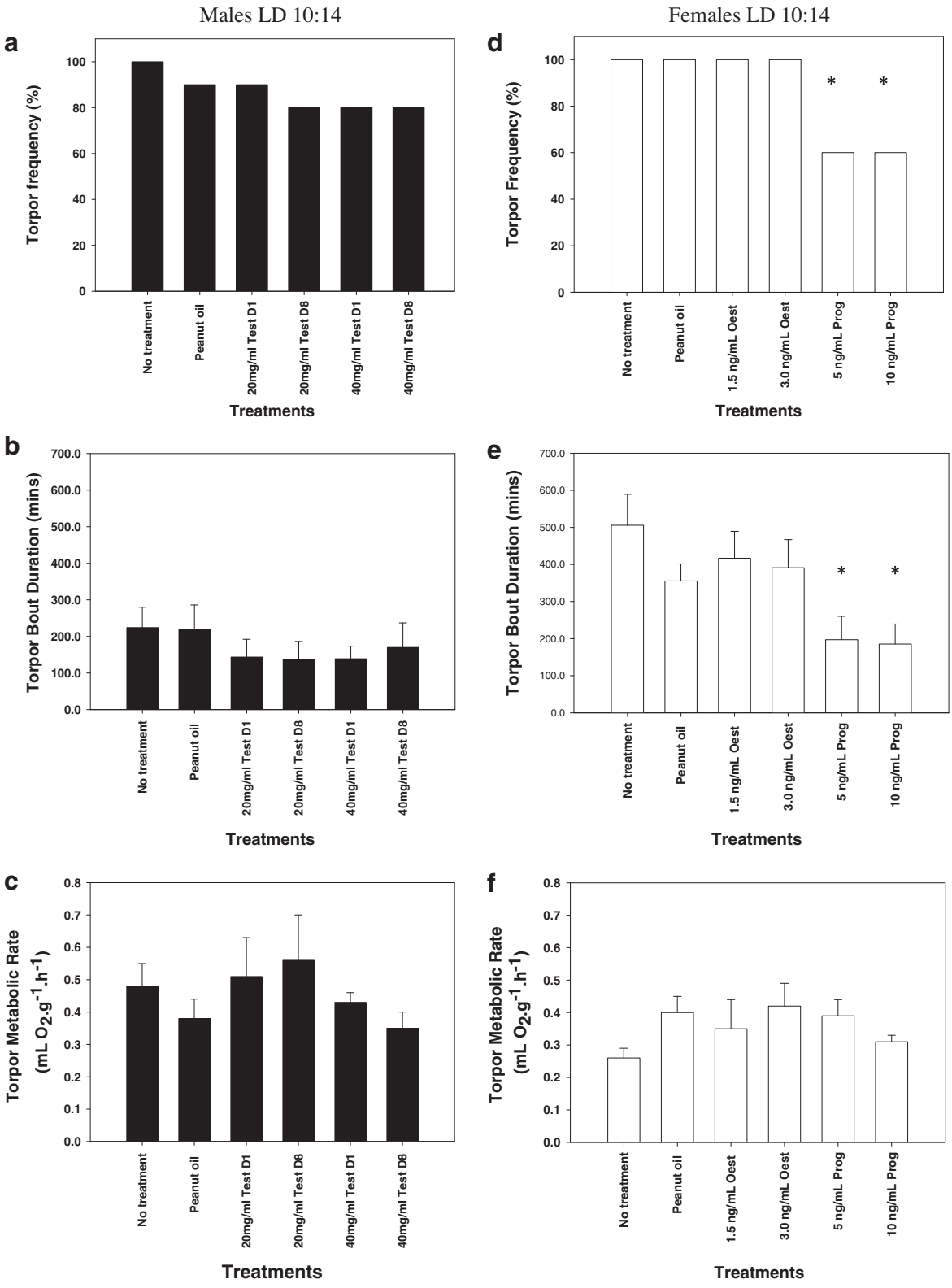

Fig. 2. Torpor frequency (%, Fig. 2a and d), torpor bout duration (minutes, Fig. 2b and e) and Torpor metabolic rate (mL $O_2\,g^{-1}\,h^{-1}$, Fig. 2c and f) of males (solid bars, Fig. 2a–c) and females (open bars, Fig. 2d–f) after exposure to the short photoperiods and with hormone treatments. With the exception of percentages, data are means ± 1 standard error of the mean. An asterisk indicates that the group were significantly different to the no injection control at $P < 0.05$.

LD 10:14 (Fig. 1b, $P = 0.003$). There were no differences in TBD when measured under LD 14:10 (Fig. 1b). TBD differed significantly between treatments for males held under LD 10:14 (Fig. 2b, $P = 0.02$). Pairwise post-hoc comparisons were equivocal, with none being significantly different. TBD differed between treatments for males held under LD 14:10, (Fig. 3b, $P = 0.004$). TBD was significantly greater when males were treated with flutamide when compared with TBDs under no injection treatment ($P = 0.025$). Other comparisons were not significant. TBD differed between treatments for females held under LD 10:14, (Fig. 2e,

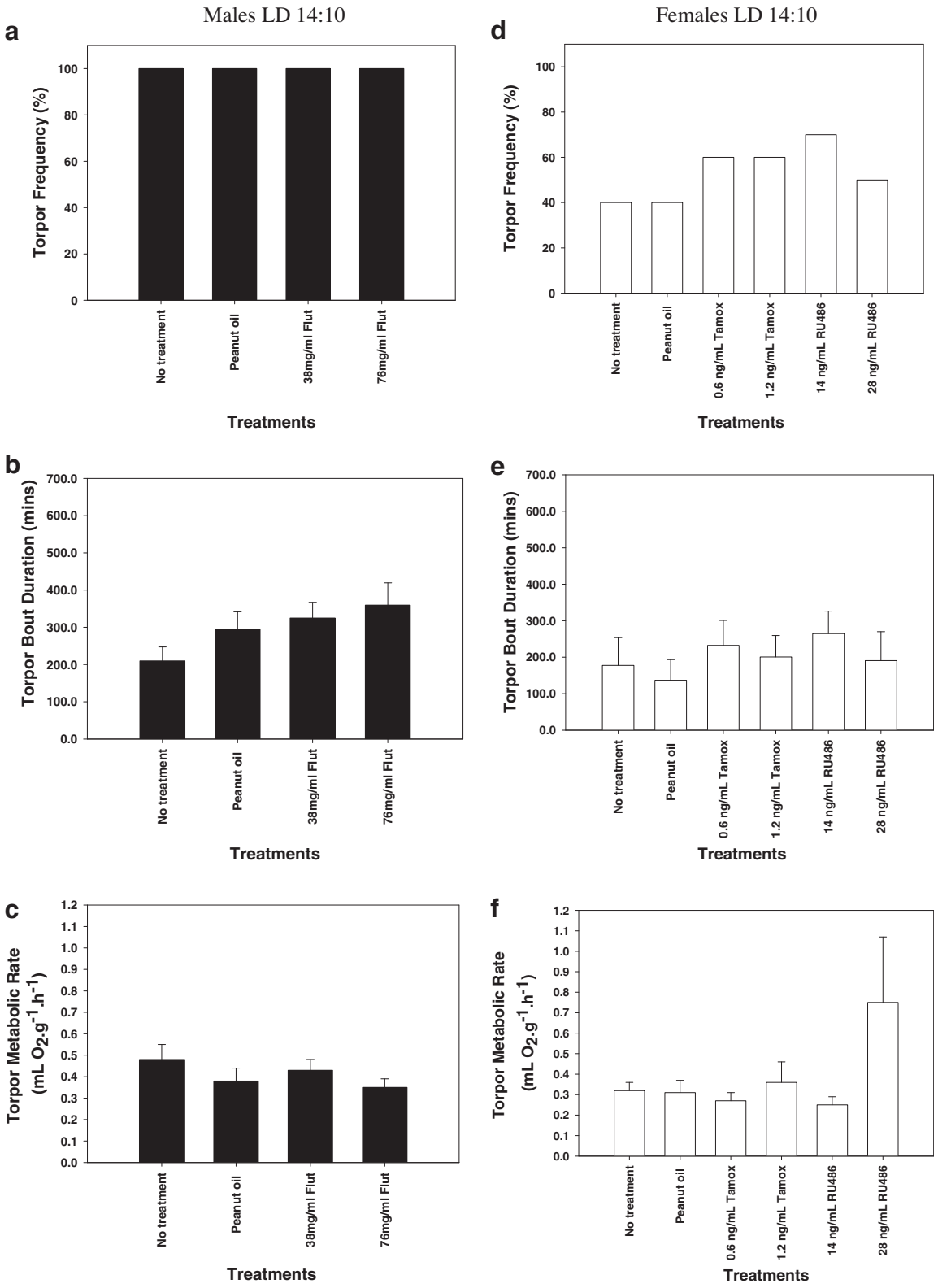

Fig. 3. Torpor frequency (%, Fig. 3a and d), torpor bout duration (minutes, Fig 3b and e) and Torpor metabolic rate (mL O_2 g^{-1} h^{-1}, Fig. 3c and f) of males (solid bars, Fig. 3a–c) and females (open bars, Fig 3d–f) after exposure to the long photoperiods and with hormone-blocker treatments. With the exception of percentages, data are means ± 1 standard error of the mean.

$P = 0.002$). Progesterone treatment significantly decreased TBD when compared to TBD measured under no injection ($P = 0.007$). Other comparisons were not significant. However for females held under LD 14:10 TBD did not differ between treatments (Fig. 3e).

3.8. Torpor metabolic rate (TMR)

TMR of males was higher than that of females when photoperiod and injection use were analysed (Fig. 1c, $P = 0.023$). When data

for no injection or peanut oil were compared within photoperiodic treatments, there were no differences in TMR under LD 10:14 or under LD 14:10 (Fig. 1c). TMR did not differ among treatments for either males or females held under either LD 10:14 or LD 14:10 (Figs. 2c and f, and 3c and 3f).

4. Discussion

Exposure to different photoperiods and hormone regimes significantly altered body condition, reproductive status and thermal energetics in *S. macroura*.

4.1. Body condition and reproductive indices

Body mass of untreated individuals was similar under both photoperiods, tail widths decreased under long photoperiods, and reproductive indices increased under long photoperiods. Testes widths of males did not change throughout the experiment. Treatment with testosterone under short photoperiods increased body mass in males, but not other parameters, and treatment with flutamide under long photoperiods decreased tail widths, but only 76 ng/mL flutamide treatment affected body mass. The response of males to testosterone treatment is similar to other studies where the anabolic effects of testosterone are immediately apparent on body mass [46,8]. However, in rats, an increase in body mass can be driven by the duration of the photoperiod on growth hormone secretion, independent of testosterone [58,36]. In contrast, in collared lemmings the peripheral conversion of testosterone to oestradiol can inhibit body mass increase [28]. In hamster species changes in other body indices, such as winter coat development, are modulated by circulating prolactin rather than circulating testosterone alone [12–14]. It may be that the lack of change in body condition indicators in animals exposed to all but the largest dose of flutamide is because other hormonal actions were not measured.

In the present study, the testes themselves appear relatively impervious to changes in circulating hormone concentrations which is consistent with another closely related marsupial, *Antechinus stuartii* [46] and to some kangaroos (see [48]). This contrasts with other mammals, where testes maintenance is dependent on circulating testosterone [12,48]. However, the insignificant effects of either testosterone or flutamide on the accessory reproductive tract contrasts to other studies on marsupials [46,10], rodents [37,1,76] and fish [3] although in other studies higher doses of flutamide were used to initiate a significant response [1,76]. Independence of the accessory tract to testosterone may suit the life history pattern of this marsupial genus, where males can move large distances, and females are more likely to hold territories when reproducing [55,21].

In female dunnarts, body mass was not significantly affected by any treatment although tail widths were significantly decreased by the administration of progesterone under short photoperiod, and by the administration of tamoxifen or the higher dose of RU486 under long photoperiod. In rats administration of either tamoxifen or RU486 slows weight gain and reduces reproductive organ weight in a dose-dependent manner [39,72,74]. Reproductive cycling appears to have been affected in some female dunnarts. Although statistical analyses of the indices were not significant, cycling in some non-cycling females under short photoperiod was prompted by addition of hormones. The equivocal response of the females may be because both oestrogen and progesterone are needed for reproductive cycles to be completed [53] and in the present study only one hormone was administered at a time. Under long photoperiod females in both the control groups are cycling as indicated by the higher indices, and this is dampened when treated with hormone blockers. *S. macroura* have an approximately 24 day oestrous cycles [67] and because they were housed singly there is no socially-induced oestrous synchrony.

In other mammals administration of tamoxifen disrupts but does not completely abolish oestrous cycles [59,74]. Tamoxifen is believed to be a partial oestrogen agonist as well as an antagonist, with studies demonstrating a range of results from complete oestrogen antagonism to oestrogen agonism, depending on the dose, sex of the animal, and the target organ examined, thus promoting a mix of body condition effects [38,39]. Administration of RU486 promotes persistent oestrus in rats [72] and more frequent LH surges in goats [71]. The effects of RU486 are believed to be on the pituitary, affecting FSH and LH production [4,71]. Commonly RU486 administration stimulates the development of follicles, without ovulatory rupture [4,71,72]. The effects of tamoxifen and RU486 on females are dose-dependent, with higher doses inflicting more disruption to oestrous cycles and body condition [38,39,71,73,59,74]. Our lower doses of tamoxifen and RU486 produced the disruption of oestrous cycles without complete abolishment of cycling, moderation of fat stores as seen by tail width modifications, and some body mass changes, similar to the effects of these doses on other species [39,38,72,73,59,74].

4.2. Metabolism

Changes in torpor metabolic variables were observed, although metabolism of normothermic animals was largely unaffected by the treatments. There were also some effects of the use of peanut oil, the vehicle control, which increased the RMR, DMR and NMR of animals under long photoperiod. The injection of peanut oil under long photoperiod was the first experience of the animals to the injection regime, and thus our results for this parameter may have been affected by the novelty of the experience, as peanut oil injection did not affect RMR under short photoperiod. Mass-specific RMR differed between sexes, with the RMR of females consistently higher than that of males. Higher RMRs have been reported for human males than for females, although when adjusted for body fat content, this sex difference is removed [6]. Sex differences in RMRs have often been associated with pregnancy and lactation [63,71]. However, the sex difference in RMR seen in our study may be more related to this species' sensitivity to the presence of female sex hormones, as they were neither pregnant nor lactating. Clearly further work is needed to explain the sex differences in RMR seen in our study.

ADMR was also higher in females than in males, and although it did not differ with treatment for females, it did for males, where the highest dose of flutamide decreased metabolism. This was also true for DMR and NMR. There are surprisingly few data on the effects of flutamide on metabolism, with a focus on body condition and reproductive disruption rather than cellular metabolism [1,76]. Interestingly, compartmentalisation of MR during the daily cycle occurred, with ADMR unaffected in females, but DMR significantly increased under LD 10:14 with progesterone treatment and NMR significantly increased under LD 14:10 with either tamoxifen or RU486. These values are averaged in ADMR so any daily cycle differences are negated.

We found the greatest effects of treatment were on the torpor variables, including significant sex differences. Untreated males did not differ in their frequency of torpor use, irrespective of photoperiod, although treatment affected TBD, with testosterone appearing to shorten TBD under LD 10:14, and flutamide administration increasing TBD under LD 14:10. In contrast, females used torpor less frequently under LD 14:10 than for LD 10:14, and progesterone treatment decreased TBD under LD 10:14, but TBD did not change under LD 14:10. TMR was higher in males than for females, irrespective of photoperiod and treatment. If animals used torpor, then the TMR was independent of other variables. This met-

abolic characteristic appears to be determined intrinsically rather than determined by photoperiod and hormonal status. It is as though the animal "decides" to use torpor, it switches on metabolic process to this species' preset torpor regime, which differs between males and females.

However, the photoperiod and treatment component of the study indicate that reproductive hormones can affect the torpor frequency and TBD. Presence of sex hormones shortens torpor duration and reduces the incidence of torpor use whereas exposure to long photoperiod shortens TBD. We have reported torpor use by *S. macroura* during early pregnancy [21] but not in the later stages of pregnancy when circulating progesterone levels are known to be high [53]. Our current data suggest that oestrogen has little effect on torpor variables in female *S. macroura*, in contrast to the few studies on oestrogenic influences on torpor use [29,11]. In other species oestradiol can partially inhibit torpor use and progesterone has no effect on other torpor variables [29,11]. The influence of testosterone on torpor variables is better described, with high circulating testosterone completely abolishing torpor use in several species of hamster [29,27,11,60] and in pouched mice [57]. The effect of testosterone on these species is absolute. In contrast, our study on *S. macroura* demonstrated that testosterone administration did not abolish torpor use, but rather affected components of torpor such as TBD. Ours is the first study to show that testosterone administration does not completely abolish torpor use in a small mammal.

To the best of our knowledge there are no studies examining at the effects of flutamide on metabolic rate and torpor. In our study, the outcomes for flutamide treated males reiterate the influences for testosterone treatment, that is, blocking testosterone had the reverse effect on torpor variables than did testosterone administration. Intriguingly, blocking either oestrogen or progesterone did not affect torpor variables, and, coupled with the incomplete effects of progesterone administration under LD 10:14, this suggests that both hormones may be interacting to affect torpor use and subsequent characteristics.

Photoperiodic effects on torpor use in other species are often absolute, with the photoperiodic effects on torpor use closely allied to gonadal involution under short photoperiod and completely absent during gonadal recrudescence under long photoperiods [27,60]. The complete inverse relationship seen between gonadal activity and torpor expression seen in the mostly hamster species studied suggests that the regulation of this is driven by the obvious strict seasonality of their natural environment. For *S. macroura* the lack of differentiation between a "torpor season" and a "reproductive season" may be related to their unpredictable environment, where spring flushes of insects may not occur and then be followed by hot summers or periods of floods. Their adaptation to unpredictability can be seen in their response to food unpredictability and food restriction [56] where torpor characteristics are modified to accommodate unpredictable food availability. Similarly, reproduction cannot be sacrificed in an unpredictable environment, thus allowing reproduction to occur while enabling energy conservation for survival is maintained by this more undifferentiated "reproduction versus torpor" strategy.

5. Conclusion

The marsupial *S. macroura* does not have a "torpor season" distinct from the "reproductive season", as has been found in some other species. Moreover the endocrinological control of these physiological seasons by reproductive hormones is also blurred. The reasons for this may have more to do with the unpredictable environment in which they live, allowing them to respond to immediate food shortages by opportunistic use of torpor, while maintaining the ability to reproduce in the longer term.

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