Lack of a response of the sub-tropical rodent (*Saccostomus campestris*) to a secondary plant compound, 6-methoxybenzoxazolinone – consequences for reproductive strategy

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A potential strategy for southern African small mammals to maximise reproductive success is to cue breeding activity to rainfall and subsequent vegetative growth via a secondary plant compound such as 6-methoxybenzoxazolinone (6MBOA). This study investigated whether the sub-tropical rodent *Saccostomus campestris* utilised this compound to enhance reproductive activity. 6MBOA or control vehicle were injected into adult females and into peri-pubertal females under long day (LD) and short day (SD) photoperiods (adults n = 6 to 8; peri-pubertals n = 5 to 10/treatment). There was no significant effect of 6MBOA or photoperiod on uterine or ovarian masses nor on ovarian development in sacrificed animals, nor on age at vaginal opening of peri-pubertals. It was concluded that 6MBOA is not a major stimulant of reproduction in this species. Rather, we propose that seasonal breeding occurs in response to multiple environmental cues. This reproductive strategy and omnivory permit an opportunistic reproductive response to the unpredictable environment of the southern African region.

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Small mammals in southern Africa live in environments which are often characterised by unpredictable and limiting resources (White, Kerley & Bernard 1997). Their breeding patterns may thus be seasonal to optimise utilisation of available resources. The timing of reproduction of some southern African rodents has been noted to occur post-precipitation (Neal 1986; Christian 1980) and rainfall appears to be a major environmental determinant of resource availability, particularly in arid and semi-arid areas (Neal 1984; Happold & Happold 1992). Rainfall stimulates the growth and development of vegetation (Neal 1984; Hoffman 1989; Hoffman, Barr & Cowling 1990; Happold & Happold 1992), with grasses responding particularly rapidly with the production of fresh green shoots (Hoffman et al. 1990).

The growth of fresh greens after rain has been proposed to be a reproductive stimulant for rodents in North American deserts (Beatley 1969; 1976; Bradley & Mauer 1971; Van de Graaf & Balda 1973; Reichman & Van de Graaf 1975) and it has been suggested that this effect is due to the water or vitamin content of fresh greens (Beatley 1969; 1976; Kenagy & Bartholomew 1981). However, the effects of feeding sprouting greens to *Microtus montanus* are mediated via a secondary plant compound, 6-methoxybenzoxazolinone (6MBOA) (Sanders, Gardner, Berger & Negus 1981; Berger, Negus, Sanders & Gardner 1981) which acts as a short-term cue stimulating reproduction by signalling an increased feed availability. 6MBOA is present in young green growing shoots, especially those of grasses and sedges (Negus & Berger 1987).

6MBOA has since been shown to have stimulatory effects on the reproduction of a number of other species, including two African rodents, *Gerbillus burtoni* (Alibhai 1986) and *Masomys coucha* (I.inn 1991), and other species: *Microtus townsendii* (Korn & Tait 1987). *Dipodomys ordii* (Rosemätt & O’Connor 1989), *Microtus pinctorius* (Schadler, Butterstein, Faulkner, Rice & Weissinger 1988), *Mus musculus* (Nelson & Shiber 1990) and *Rattus norvegicus* (Butterstein, Schadler, Lysegorgski, Robin & sipperly 1985; Vaughan, Little, Vaughan & Reiter 1988). However, negative results have been obtained from the African Tatera leucogaster (Neal & Alibhai 1991) and other small mammals including *Microtus ochrogaster* (Moffat, Bennett & Nelson 1991), *Peromyscus maniculatus* (Korn 1989), inbred *Mesocricetus auratus* (Anderson, Nachman & Turek 1988; Urbanski, Kim & Connolly 1990) and in some experiments with *Rattus norvegicus* (Vaughan et al. 1988). Effects of 6MBOA appear to be mediated more through the female than the male, particularly through the peri-pubertal female (Berger, Negus & Rosemätt 1987, Vaughan et al. 1988) and effects can be dose dependent (Gower & Berger 1990). The mechanism of action is not clear but 6MBOA appears to potentiate the action of FSH (Butterstein & Schadler 1988; Schadler et al. 1988) and interaction with the thyroid axis has been proposed (Vaughan et al. 1988). In some species 6MBOA alters the reproductive response to photoperiod (Gower & Berger 1990; Nelson & Shiber 1990) and effects of 6MBOA, albeit at high doses, on the melatonin receptor have been demonstrated (Yuwiler & Winters 1985; Sweat & Berger 1988; Daya, Pangerl, Pangerl, Troiani & Reiter 1990).

The use of 6MBOA as a cue for initiation of reproductive activity would appear to be a good breeding strategy for southern African small mammals, permitting precise...
reproductive timing relative to availability of vegetative food sources. *Saccostomus campestris* Peters (1846), the pouched mouse, is distributed over much of the southern African subregion (Skinner & Smithers 1990). It has been classified as predominantly herbivorous (Kerley, Knight & Erasmus 1990; Kerley 1992a; b) or omnivorous (Kerley 1989; Miller 1984; Neal 1984). It is a seasonal breeder with reproduction occurring during the warm wet months of summer (Smithers 1971; Swanepoel 1976; Hoppold & Hoppold 1992). The cues for this seasonal reproduction remain obscure; photoperiod alone does not control the timing of reproduction (Bernard & Hall 1996; White & Bernard 1996), and the only significant nutritional effect on reproduction which has been identified is a reduction in the weight of accessory sex glands of males after severe feed restriction (R.T.F. Bernard, G. Tinney & R.M. White, unpublished results).

The objective of this study was to determine whether reproductive activity in this species was stimulated by 6MBOA. Because effects in other species appear to be mediated primarily through the female (Berger et al. 1987; Vaughan et al. 1988), effects were monitored only in this sex. Influences were explored in both adult and peri-pubertal females to determine whether effects were apparent both in the initiation of the breeding season in mature animals and in the timing of sexual maturity. A potential interaction with the effects of photoperiod was investigated by administration of 6MBOA under both long and short day photoperiods.

### Materials and methods

#### Animals

Animals were obtained from our laboratory stock which is derived from individuals supplied in 1992 by the Medical Research Council of South Africa from a group established in 1958 from animals originating in the Transvaal (latitude 26°). In our stock (latitude 33°), pouch mice are maintained in individual cages on wood shavings with shredded paper as bedding, a diet of rabbit pellets and sunflower seeds and water supplied ad libitum. They are kept in constant environment rooms at 22 ± 2°C. Adult females had been kept in the appropriate photoperiod for at least two months prior to the initiation of Experiment 1 and pre-pubertal animals were born and reared under the appropriate photoperiod to mothers maintained in like photoperiod for at least two months prior to parturition in Experiment 2. This study was approved by the Rhodes University Ethical Standards Committee.

#### Experiment 1

The aim of this experiment was to determine whether 6MBOA could increase uterine size and stimulate ovarian activity in adult female pouch mice. Adult nulliparous females (mean ± SEM age 252 ± 10 days) were assigned to one of four treatments by age and body weight: long day 6MBOA treated (L6dM; n = 8), long day control (LDCO; n = 7), short day 6MBOA treated (SD6M; n = 7) and short day control (SDCO; n = 6). Regular oestrous cyclicity was established by monitoring vaginal smears for two cycles and confirmation of the pattern described by Westlin-van Aarde (1988) prior to the initiation of 6MBOA treatment.

Photoperiod was long day (LD = 14L:10D, lights on 06h00) or short day (SD = 10L:14D, lights on 08h00), representing the extremes experienced at the latitude of our stock. 6MBOA was dissolved in warm propylene glycol, diluted to 5% with saline at a concentration of 500 μg/mL and administered subcutaneously at a dose of 100 μg/100 g body weight at 1600 for three consecutive days. Administration by injection was chosen because data were available on the effects of 6MBOA using similar protocols and endpoints (Sanders et al. 1981; Butterstein & Schadler 1988; Vaughan et al. 1988; Gower & Berger 1990), in small amounts of 6MBOA were required.

To control for the effects of stage of the oestrous cycle on uterine and ovarian activity, all animals were treated at the same stage of the oestrous cycle, as determined by examination of vaginal smears. 6MBOA was administered on the days of metoestrus, dioestrus and prooestrus and control animals were injected with vehicle only (the solution used to dissolve 6MBOA) to the same protocol. Animals were then sacrificed on the day of oestrus by intraperitoneal injection of Euthanase. Uteri and ovaries were dissected out and weighed. Because the corpora lutea (CL) accumulate in cycling *S. campestris* (Westlin-van Aarde 1989), ovulation rate could not be estimated from the number of CL visible in each ovary, consequently ovaries were not histologically examined.

#### Experiment 2

Effects of 6MBOA on peri-pubertal pouch mice were also tested because of evidence that effects may be more pronounced in advancing the age of sexual maturity in young females than in stimulating reproductive activity in adults (Berger et al. 1987; Vaughan et al. 1988). Vaginal perforation in our stock animals (when young are kept with their mother) occurred at approximately 39 days of age (R. White, unpublished information), and longer term observations indicated a mean ± SD age of 36.7 ± 2.4 days (n = 31, minimum 32 days) in LD and 39.6 ± 4.1 days (n = 22, minimum 35 days) in SD photoperiod (J. Hall, personal communication). These ages are similar to the age at which vaginal perforation occurs in young females kept with an adult female in the colony of Westlin-van Aarde (1989). Age at vaginal opening is, however, altered by social environment (Westlin-van Aarde 1989) and under different colony maintenance, mean age at vaginal opening can be reduced to 34 days (Westlin-van Aarde 1988). Age at vaginal opening was thus a potentially sensitive indicator of effects on reproduction of 6MBOA and peri-pubertal females were treated at an age at which vaginal opening could potentially be advanced.

*S. campestris* aged 30 days were therefore selected for this experiment and assigned to either LD6M (n = 10), LDCO (n = 9), SD6M (n = 6) or SDCO (n = 5) treatments as above. Within a litter, as far as possible equal numbers of animals were assigned to 6MBOA and to control treatments to allow for potential influences of litter size and genetics on sexual development. Variable litter sizes and sex ratios precluded treatment equality of numbers. Photoperiods were as described for Experiment 1 and 6MBOA treatment was similar with the exceptions that the compound was diluted to 250 μg/mL before administration at 100 μg/100 g body weight and that injection was at 18h30. Animals were treated for three days and sacrificed when aged 34 days, checked for vaginal perforation and uteri and ovaries were weighed, sectioned and
examined histologically for the presence of primary, secondary and Graafian follicles and for CL.

Statistical analysis
The effects of 6MBOA and photoperiod treatments on both absolute and relative (as a percentage of body weight) uterine and ovarian masses in each experiment were analysed using 2-way ANOVA's (SigmaStat, Jandel Scientific). Potential differences in the proportion of females with Graafian follicles in their ovaries of control and treated groups were tested by Chi-square analysis.

Results
Experiment 1
There was no significant difference between body weights at sacrifice of animals in different treatment groups (mean ± SEM 116 ± 7 g, 107 ± 5 g, 116 ± 6 g, 112 ± 11 g for SD6M, SDCO, LD6M and LDCO groups respectively). There was no significant effect of either 6MBOA or of photoperiod or an interaction between these factors on either absolute or relative uterine and ovarian masses of adult S. campestris. Because of the lack of difference in body weights, only absolute values are presented (Figure 1).

Experiment 2
Final body weights did not differ significantly between treatment groups (mean ± SEM 49 ± 3 g, 50 ± 4 g, 47 ± 2 g and 48 ± 2 g for SD6M, SDCO, LD6M and LDCO groups respectively). There was no significant effect of either 6MBOA or of photoperiod on either absolute or relative uterine and ovarian masses of peri-pubertal S. campestris. Absolute values are shown in Figure 2. Vaginal perforation had not occurred in any of these young females. Uteri appeared inactive with few endometrial glands and thin (approximately 10 μm) endometria. Primary and secondary follicles were present in all ovaries examined and Graafian follicles (mostly atretic) were observed in 8 of 15 6MBOA treated animals and 9 of 13 control animals. No CL were observed in any of the ovaries. There was no significant effect of treatment on the proportion of females with Graafian follicles ($\chi^2 = 1.99$, df = 2).

Discussion
Administration of the secondary plant compound, 6MBOA, failed to stimulate reproductive activity in the southern African sub-tropical rodent, Saccostomus campestris. Negative results from the administration of a putative reproductive stimulant are rarely totally conclusive. Nevertheless, the dose of 6MBOA administered in these experiments was carefully calculated to correspond with maximally stimulatory doses used in similar protocols (Sanders et al. 1981: Microtus montanus, 5 μg; Butterstein & Schadler 1988: 25 day laboratory rats, 100 μg; Gower & Berger 1990: M. montanus, 10 μg) after accounting for species differences in body weight. Thus although dose dependent responses to 6MBOA have been observed (Butterstein & Schadler 1988; Gower & Berger 1990), it is unlikely that a different dose would have induced a positive response in S. campestris. Although breeding
success is the ultimate test of reproductive ability, it is unlikely that an aspect of the reproductive process not monitored in this study was affected since 6MBOA has consistently induced increases in uterine and/or ovarian masses (Berger et al. 1977; Linn 1991; Rowsemitt & O’Connor 1989; Alibhai 1986). Moreover, lack of responsiveness to environmental cues resulting from multigenerational breeding in captivity is unlikely to have induced a failure to respond to 6MBOA in this study because physiological responses to 6MBOA are maintained in both laboratory bred Rattus norvegicus (Butterstein et al. 1985; Vaughan et al. 1988) and Mus musculus (Nelson & Shiber 1990). In addition, in our laboratory we have confirmed responses to photoperiod of stock animals with those of wild caught animals (J. Hall, R.T.F. Bernard & R.M. White, unpublished information). The age of treatment of peri-pubertal females was carefully selected in this study to realistically permit advancement of vaginal opening as observed in previous studies (Westlin-van Aarde 1988; 1989). We thus suggest that 6MBOA does not play a major role in the stimulation of reproduction in S. campesi·tris.

Species differences in response to 6MBOA may derive from their different ecological strategies. The microtines which respond most strongly to the environmental cue provided by 6MBOA are those which are not only herbivorous but also reliant primarily on the consumption of monocotyledons rather than dicotyledons (Negus & Berger 1987; Korn 1989). For these species, 6MBOA provides a useful signal regarding availability of their seasonal food supplies. For animals in arid or semi-arid regions of southern Africa, rainfall is believed to be the major determinant of resource abundance because it induces vegetative growth and phenological development (Hoffman 1989) and increases in insect abundance (Cumming & Bernard 1997). Since grass responds particularly vigorously to rain (Hoffman et al. 1990), 6MBOA is potentially a reasonably good indicator of food availability in southern Africa. However, the failure of S. campesi·tris to respond to 6MBOA may be because this species can be omnivorous and may consume a variety of foods including seeds and insects (Kerley 1989; Miller 1984; Neal 1984). When availability of herbage declines, such species may switch to the consumption of seed stocks (Neal 1984; Kerley 1989; Kerley 1992a; b). Behaviours such as hoarding seed in caches (Pettifer & Nel 1977; Ellison 1993) may further reduce the dependence of this species on herbage and reduce the efficacy of 6MBOA as a cue for breeding. It is also possible that plants in the southern African veld produce different secondary plant compounds from those identified in the northern hemisphere. Finally, the putative effects of rainfall on reproduction of southern African rodents may be mediated directly through the alleviation of water stress rather than through increased food abundance, because water supplementation can improve the reproductive performance of some rodent species in the Namib desert (Christian 1979). In this case too, the value of secondary plant compounds as an environmental cue would be reduced. The negative results of this study confirm the failure of the putative inhibitory secondary plant compound pCA to suppress reproduction in S. campesi·tris (White & Bernard 1996).

No effect of photoperiod on reproductive activity nor interaction between photoperiod and the effects of 6MBOA were observed in this study. These observations confirm previous findings that a short day photoperiod does not induce reproductive regression in S. campesi·tris (Bernard & Hall 1996; White & Bernard 1996). However, some metabolic photoresponsiveness appears to have been retained since thermoregulatory processes are altered by photoperiod (Ellison, Skinner & Haim 1992; Ellison, Skinner & Ferguson 1994). Social environment has the capacity to modify sexual response in S. campesi·tris (Westlin-van Aarde 1989) and experiments are underway in our laboratory to test the effects of nutrition on reproduction. The less predictable environment of the southern hemisphere may have contributed to the evolution of reproductive strategies in which there is a flexible response to the environment resulting from the ability of multiple cues to affect reproduction. In other species, response to 6MBOA may form a part of this strategy.

Responses may differ between males and females since it has been proposed that males require a reproductive strategy distinct from females as a result of the longer time for gamete production (Bronson 1985; 1989). Males may thus be cued by different factors such as more predictive cues (White et al. 1997) or may read the same cues differently.

In conclusion, although the secondary plant compound 6MBOA appeared to have potential to signal a rainfall-induced period of increase in resource availability for southern African small mammals, a positive response to this compound was not observed in S. campesi·tris. Rather, we propose that a combination of cues times reproductive activity in this species. This reproductive strategy and omnivory permit an opportunistic reproductive response to the unpredictable environment of the southern African region.

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