Impact of contraceptive hormones on the reproductive potential of male and female commensal black rats (*Rattus rattus* Linnaeus, 1758)

Running title: Fertility control of *Rattus rattus*

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Ethical approval: The study was conducted in laboratories of the Pest Management Centre, Sokoine University of Agriculture, Morogoro, Tanzania. All trials were approved by the university's ethics committee and followed the guidelines of the American Society of Mammalogists (Sikes 2016).

Abstract

The black rat is considered one of the world's top pests. With increased restrictions on rodenticides, new alternatives to manage rats are urgently needed. Research on the use of contraceptive hormones, levonorgestrel (LE) and quinestrol (QU), have been evaluated against some rodent species, and this research is the first study to assess these on black rats. Hormones were incorporated into rodent bait at 10 ppm and 50 ppm concentrations singly and in combination (EP-1). Groups of 10 animals of each sex were fed the baits over 7 days. Lower bait consumption was observed with slight body mass reductions. On dissection, it was observed that the uterus was in a state of oedema and male reproductive organs weighed less with reduced sperm counts/motility. The two most promising baits, 50 ppm QU and EP-1, were used to assess impact on pregnancy and litter size. Pregnancy was reduced from 70% success when both males and females consumed untreated bait, down to 30% when males had consumed contraceptive bait but females had not, and down to 0% when females had consumed

contraceptive bait, regardless of whether they had paired with a treated or untreated male. Litter size in the untreated pairs was 8 pups, but only 4 pups in those cases where the male only had consumed the contraceptive. Further studies should investigate how long the effect lasts and its reversibility. Field studies at the population level may also shed light on the practicality of using contraceptive baits for black rats in different habitats.

Key words: Ecologically-based rodent management; Fertility control; Contraceptive bait; Levonorgestrel; Quinestrol

Introduction

Originating in southeastern Asia, *Rattus rattus* (Linnaeus, 1758) is a cosmopolitan rodent species found on every continent except Antarctica and is one of the most globally important agricultural and urban pests with respect to disease transmission, agricultural crop damage and post-harvest losses (Singla *et al.* 2008, Aplin *et al.* 2011, Feng and Himsworth 2014, Thomson *et al.* 2014, Belmain *et al.* 2015). Commonly known as the black rat, ship rat, tree rat or roof rat, *R. rattus* is omnivorous, agile and highly adaptive to different habitats, explaining its invasiveness and establishment in most urban areas as well as in many cropping systems (Horskins *et al.* 1998, Brown *et al.* 2005, Drazo *et al.* 2008, Shiels *et al.* 2014). Its long association with human activities, often living in close proximity to humans and domestic animals, has promoted *R. rattus* involvement in the transmission of many infectious diseases, including zoonoses such as plague, hepatitis E, leptospirosis, capillariasis, hymenolepis and many others (Meerburg *et al.* 2009).

Like other pest rodents, the management of black rats almost exclusively relies on the use of mortality-based control methods, particularly the use of anticoagulant rodenticides (Buckle and Smith 2015, Jacob and Buckle 2018). Anticoagulant poisons can be highly effective when used correctly, but their mis-use and over-use has led to the development of physiological resistance within rodent populations (Berny *et al.* 2018) and environmental accumulation that negatively affects many non-target organisms (Rattner and Mastrota 2018, Elmeros *et al.* 2019). Mortality control normally has a short-term effect on a population as rodents reproduce quickly and their numbers can be rapidly replaced, often leading to population resurgence through short-term increases in survival (Stenseth *et al.* 2001, Singleton *et al.* 2007).

Due to negative environmental problems and resistance development, the use of anticoagulants is increasingly restricted with some regions considering outright bans (Quinn *et al.* 2019). As other rodent poisoning options can also be problematic through accidental poisoning (Rother 2010), low efficacy (Schmolz 2010), and humaneness (Mason and Littin 2003), there is a growing need to find alternative methods of rodent control that are environmentally sustainable, with good levels of efficacy, safety and humaneness to meet increasing demands from regulatory authorities and the general public. The use of fertility control has been argued to be more ecologically sound (Stenseth *et al.* 2001) whereby infertile animals can remain in the population, therefore sustaining density dependent feedback to recruitment and survival (Zhang 2000); although some compensation at the population level can still occur through higher survival of juveniles (Jacob *et al.* 2004, Williams *et al.* 2007). Managing populations through limiting fertility has also been argued to be more humane (Barlow 2000), and more safe and cost effective (Hone 1992) than the use of mortality control. Fertility limiting compounds can cause permanent or temporary sterility in either sex, reduce the number of offspring or impair the fertility of offspring produced (Humphrys and Lapidge 2008) through a reduction in either fertility or fecundity (Bomford and O'Brien 1992). Several anti-fertility compounds have been used in controlling reproduction in various animal species in the field, through contraception or sterilization (Kirkpatrick and Turner, 1985, Jacob *et al.* 2008, Fagerstone *et al.* 2010, Massei and Cowan 2014). Commercialized use of fertility control products for rodent control is not currently widely available, and limited to a single product available in the USA, which has been evaluated against *R. rattus* (Pyzyna *et al.* 2018, Siers *et al.* 2020).

Practical use of fertility control for rodents is subject to many of the same challenges faced by rodenticide development and registration, such as overcoming poor palatability of active ingredients and potential non-target environmental impacts. Research on the contraceptive hormones quinestrol and levonorgestrel has indicated that environmental accumulation and non-target impacts are extremely low as the products break down quickly through UV light and in water or soil (Tang *et al.* 2012, 2019, Zhang *et al.* 2014). These two hormones have shown variable palatability issues depending on rodent species with clear dose-dependent relationships (Zhang 2015). Efficacy of the hormones delivered together in a food bait has been shown effective for a range of rodent species including *Lasiopodomys brandtii* (Zhao *et al.* 2007), *Rattus nitidus* (Liu *et al.* 2013), *Bandicota bengalensis* (Sidhu *et al.* 2020), *Ochotona curzoniae* (Liu *et al.* 2012) *Meriones unguiculatus* (Fu *et al.* 2013), *Tscherskia triton* (Zhang

et al. 2005) and *Mastomys natalensis* (Massawe *et al.* 2018). As *R. rattus* is a cosmopolitan species, effectively controlling its fertility could have global implications in reducing its impact on health, agriculture and the environment. This study aims to determine whether and how the fertility of male and female black rats is affected after consuming a food bait containing each hormone singly and in combination, as well as the optimum dose with respect to palatability and efficacy to limit or prevent reproduction.

Materials and methods

Experimental animals

Black rats were live trapped using modified box traps baited with a mixture of peanut butter and maize flour, tomatoes, and sardines in different housing premises in Morogoro Municipality, Tanzania (6°50'42.66"S, 37°39'29.14"E) where permission to trap was granted from the owner of each house. Equal numbers of females and males were used for the trial. Only adult animals with the weight range of >50g and < 180g were used for the experiments to ensure animals were sexually mature (Watson 1950, Tamarin and Malecha 1972, Feng and Himsworth 2014). The oestrous cycle of females was determined through visual assessment (Ajayi and Akhigbe, 2020). At the start of the trial assessing bait acceptance and physiological impact, females were at proestrus and oestrus, with the majority (>90%) at the oestrus stage. For the trial assessing pregnancy and liter size, all females were at the proestrus stage. All males were assessed as fully mature with testes fully descended. After weighing, animals were sexed and separated into large cages by sex and allowed to acclimatize for 10 days. Thereafter, animals were transferred to individual cages for a further 20 days to habituate them to standard animal rearing methods (individual cages with water and rodent pellets *ad libitum*, and wood shavings for nesting material. Thus, the total acclimatization period was 30 days, which permitted daily observation to ensure that all animals were healthy and that no females were pregnant. Any animal that appeared unhealthy (thin, poor coat condition, injury, or not feeding) was removed from the study. Animals were maintained at rodent rearing facilities located at the Pest Management Centre, Sokoine University of Agriculture. Ethical approval to collect and maintain black rats and carry out the research was granted by the ethics committee at Sokoine University of Agriculture, Tanzania, and standard protocols were used for the handling and use of wild small mammals (Sikes 2016).

Bait preparation

The bait preparation followed the protocols from Massawe *et al*. (2018). Briefly, the rodent bait was prepared by mixing maize flour, ground sardines, and crushed maize. Ten kilograms (10kg) of maize flour was mixed with 250g of ground sardines to form a maize-sardine mixture. The maize-sardine mixture was further mixed with 10 litres boiled water and cooked for 15min to obtain a stiff paste then left to cool at room temperature. The paste was mixed with crushed maize at a ratio of 1 part paste to 2 parts crushed maize, i.e. 3.3kg of the cooked paste mixed with 6.7kg of cracked maize, and the mixture was then passed through a mechanical pelletizer to create standard rodent bait, which was shade dried and stored in sacks until required.

This bait formulation was modified to create the contraceptive rodent bait as follows. Powdered quinestrol and levonorgestrel (Beijing Zizhutiangong Science and Technology Ltd, China) were weighed at 0.1g and 0.5g to make 10 ppm and 50 ppm baits, respectively. For the mixture of quinestrol and levonorgestrel (EP-1), the ratio was 1:3 (quinestrol: levonorgestrel). For each bait concentration, the weighed hormones were dissolved in 100ml of ethanol in a 60-70ºC water bath. The ethanol-contraceptive solution was then mixed with a sugar solution (200g sucrose in 1000ml of water). This solution was mixed with 6.7kg of cracked maize and then incorporated with 3.3kg of the cooked paste described above. The mixture was then passed through a mechanical pelletizer to provide 10kg of contraceptive bait.

Bait acceptance and physiological impact

Trial parameters included three contraceptive baits (levonorgestrel (LE), quinestrol (QU) and levonorgestrel + quinestrol (EP-1), evaluated at two different doses (10 and 50 ppm) as well as an untreated control bait comparison for each contraceptive. Each treatment was evaluated by providing the bait to 10 male and 10 female rodents over a 7 day period, with a total number of 180 animals (90 males and 90 females) involved. Each day 10 g of fresh bait was provided to each animal. No alternative food resources were provided. Each rodent was weighed at the start of the trial followed by daily weight measurements, recording the amount of bait consumed each day.

After feeding on treated bait for 7 days, all animals were provided with plain untreated bait. On day 8, females (n=90) were anesthetized using halothane and then killed through cervical dislocation. On dissection the uterus and other reproductive organs were visually inspected to check for abnormalities in comparison to untreated animals, with the uterus having ovaries attached removed and weighed. Males (n=90) were killed on day 14 and the epididymis,

seminal vesicles, and testes were removed and weighed separately. Sperm motility was measured using established methods (WHO 2010). This involved dissection of the epididymis, placing it entirely in a glass petri-dish with 1ml of 0.85% normal saline and then cutting the epididymis into small pieces to release sperm. A drop of this solution was placed on a slide and examined under an ordinary light microscope at magnification 20X. The sperm motility was assessed and expressed in a percentage of mobile sperms of the total count. The remaining sample from the petri-dish was placed in a test tube with 10ml of distilled water and refrigerated for 2 hours at 4ºC to release sperm. After 2 hours of refrigeration, 9ml of 0.9% of saline was added to the solution and shaken. A drop of the solution was placed in a Fuchs Rosenthal counting chamber to count sperm following established protocols (WHO, 2010).

Assessment of pregnancy and liter size

After analyzing the data from the bait acceptance and physiological response trial, the two most effective baits, QU 50 ppm and EP-1 50 ppm, were selected for a second trial to assess their ability to suppress reproduction. A total of 140 *R. rattus* (70 males and 70 females) were involved in the trial, with 40 animals fed QU, 40 animals fed EP-1 and 60 fed untreated control bait, where all animals were assessed as being fully mature and in active sexual condition. After 7 days of feeding, animals were removed from their individual cages and placed in pairs in new cages which were provided with plain bait. Animals were paired to give 10 animals per combination, with the following paired groups: ControlFemale + ControlMale; EP-1Female + ControlMale; EP-1Female + EP-1Male; EP-1Male + ControlFemale; QUFemale + ControlMale; QUFemale + QUMale; QUMale + ControlFemale. Male-female pairs were kept in individual cages, and females were observed each morning when handling the animals during cage cleaning and replenishing food until a vaginal plug or vaginal blood (indication of successful copulation) was observed. Furthermore, a piece of plain white paper was placed on the bottom of each pairing cage for observation of any vaginal blood droplets or vaginal plug. Copulation generally took 4-7 days after pairing, with all pairs showing evidence of copulation by 7 days. After pairing, animals were returned to individual cages and left for 30 days to observe number of pregnancies and litter size. During this time females were provided with additional food supplements of fresh vegetables and sardines alongside the standard pellet. Any pups produced were counted and individually weighed.

Data analysis

Data were checked for normality by using the Shapiro-Wilk test for normality in R statistical software. Data were subjected to analysis of variance (ANOVA) with post-hoc comparison of means using the Least Significant Difference test (LSD) at the 95% confidence interval.

Results

Bait acceptance and physiological impact

Bait consumption was significantly lower when animals were fed baits containing either hormone or in combination in comparison to untreated baits. On average, both females and males consumed 7 g of bait per day when eating untreated control bait, whereas treated baits generally reduced this by about half (Fig. 1). The amount of food consumed each day by black rats was highly variable for all treatments over the 7 day period, including in untreated control groups, with linear regressions showing no obvious trends across treatments or over the 7 days (Fig. S1, Supporting Information). Generally, levonorgestrel had the least impact on food consumption compared to quinestrol and EP1. An analysis of variance (ANOVA) showed that there was no significant difference in food consumption caused by increasing the hormone concentration from 10 ppm to 50 ppm for females or males for the QU bait and only a slightly lower consumption for LE, whilst EP-1 showed reduced consumption for females only when increasing the concentration from 10 ppm to 50 ppm.

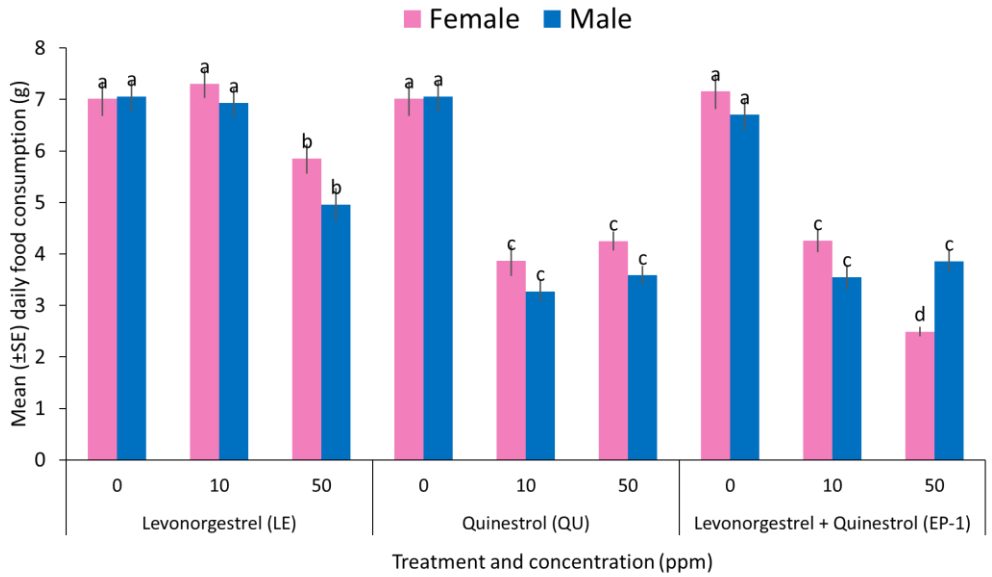

Figure 1 Mean (±SE) daily consumption over a 7 day feeding period of male and female adult *Rattus rattus* eating rodent bait containing contraceptive hormones at two different concentrations (10 and 50 ppm). Letters show significant differences between treatments by

the post-hoc Least Significant Difference test at the 95% confidence interval. (ANOVA female $F = 42.0$, $df = 8$, $p < 0.0001$; male $F = 39.9$, $df = 8$, $p < 0.0001$)

An analysis of variance in the mean starting weights of animals in the different treatment groups indicated there was no significant variation the mean weight of male black rats across the treatments (Table 1). However, there were some significant differences in the mean weight of females allocated to the different treatments, where the untreated control groups for LE and EP-1 were heavier whereas females in the EP-1 10ppm and QU 50ppm were relatively lighter (Table 1). The final weights of both male and female black rats generally showed that there were slight weight gains in the untreated control groups, compared to initial starting weights, whereas the hormone-treated baits showed slight decreases in body weight from their starting weights. Converting these data into percentage body mass weight gain or loss, i.e. (initial weight – final weight) / initial weight X 100, showed that levonorgestrel had the lowest impact on weight loss, followed by quinestrol, with most weight loss experienced by black rats consuming EP1 (Fig. 2).

Table 1 Analysis of variance on the mean weight (g) of black rats in each bait feeding treatment group (n=10) at the start of the trial (day 1) and at the end of the trial after consuming the bait for 7 days. Values in the same column followed by the same letter are not different from each other at the 95% confidence interval using the Least Significant Difference test.

Treatment and concentration	Female initial weight (g)	Female final weight (g)	Male initial weight (g)	Male final weight (g)
LE untreated	117.56 a	122.31 a	99.82 a	103.14 a
LE 10 ppm	99.44 ab	96.98 abc	90.05 a	89.12 ab
LE 50 ppm	76.82 b	76.79 c	101.27 a	101.77 ab
QU untreated	91.39 ab	87.99 abc	102.56 a	103.23 a
QU 10 ppm	83.43 ab	81.06 bc	90.23 a	84.63 ab
QU 50 ppm	65.53 b	62.13 c	77.97 a	73.49 ab
EP-1 untreated	114.04 a	113.53 ab	85.47 a	87.47 ab
EP-1 10 ppm	77.70 b	72.58 c	61.22 a	57.34 b
EP-1 50 ppm	85.42 ab	79.83 bc	87.04 a	83.59 ab
F	4.75	5.79	1.73	2.27
df	8	8	8	8

Pr > F	< 0.0001	< 0.0001	0.103	0.030
Significant	Yes	Yes	No	Yes

LE = Levonorgestrel, QU = Quinestrol, EP-1 = Levonorgestrel + Quinestrol

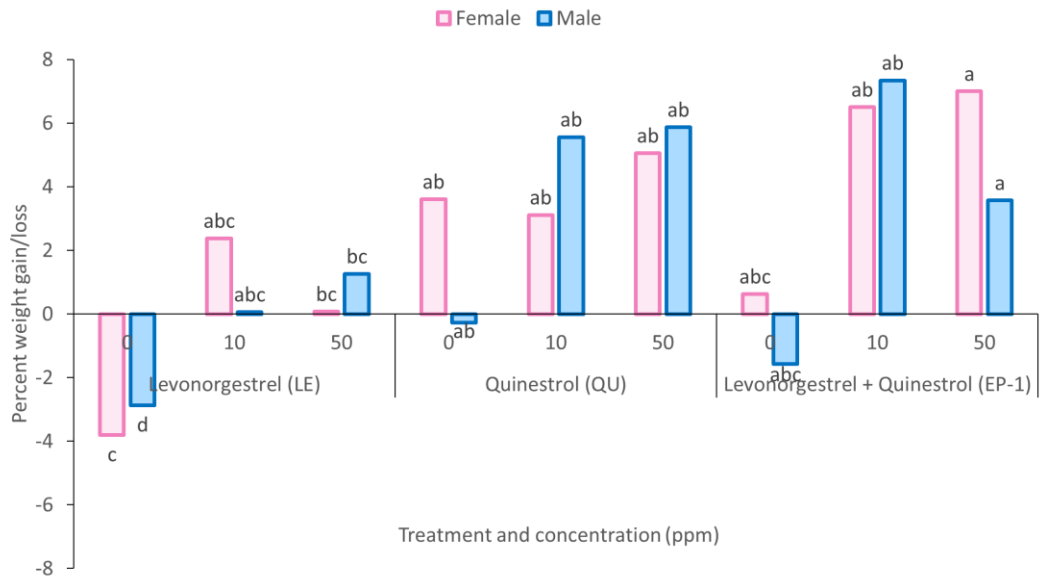

Figure 2 Mean percent change in black rat body mass after consuming hormone-treated baits for 7 days. Positive values indicated weight loss (%) whereas negative values indicated that the rats gained weight over the 7 day feeding period. Letters show significant differences between treatments by the post-hoc Least Significant Difference test at the 95% confidence interval. (ANOVA female $F = 2.02$, $df = 8$, $p = 0.054$; male $F = 4.08$, $df = 8$, $p < 0.0001$)

On physical observation of the uterus in situ, there was considerable uterine oedema in female rats consuming the hormone baits and no oedema observed in the rats consuming the untreated control baits (Fig. S2, Supporting Information). It was observed that LE resulted in the lowest degree of oedema, followed by EP-1, with QU showing highly pronounced oedema. Increasing concentration of QU and EP-1 also led to more acute oedema. The mean weight of the uterus+ovaries was generally higher with increased concentration of the hormone and following the same trend from LE to EP-1 to QU (Table 2). Similar changes were observed in male rats with reduced weights of male sex organs with increasing hormone concentration. Here, EP-1 50ppm was the most effective in reducing the weight of the testis, epididymis and

seminal vesicle. With respect to sperm count and sperm motility QU 50ppm was as effective as EP-1 50ppm (Table 2).

Table 2 Analysis of variance on the mean weight (g) of reproductive organs from female and male black rats after feeding on baits containing contraceptive hormones for 7 days as well as male sperm counts (million/ml) and sperm motility (%). Values in the same column followed by the same letter are not different from each other at the 95% confidence interval using the Least Significant Difference test.

Treatment and concentration	Uterus + ovaries	Right testis	Left testis	Right epididymis	Left epididymis	Seminal vesicles	Sperm count	Sperm motility
LE untreated	0.12 d	0.98 a	0.88 ab	0.48 ab	0.48 ab	0.15 ab	119.80 a	77.50 a
LE 10 ppm	0.14 cd	0.92 a	0.92 a	0.33 abc	0.32 abc	0.16 ab	73.10 bc	74.50 ab
LE 50 ppm	0.37 b	0.89 ab	0.91 a	0.61 a	0.61 a	0.25 ab	74.20 bc	59.00 bc
QU untreated	0.12 d	0.92 a	0.92 a	0.44 abc	0.44 abc	0.28 ab	109.60 ab	76.50 ab
QU 10 ppm	0.32 bc	0.87 ab	0.86 ab	0.33 abc	0.33 abc	0.26 ab	51.70 cd	48.50 cd
QU 50 ppm	0.69 a	0.54 bc	0.52 b	0.24 bc	0.24 bc	0.09 ab	11.10 d	17.30 e
EP-1 untreated	0.12 d	0.92 a	0.92 a	0.48 ab	0.48 ab	0.23 ab	106.40 ab	76.50 ab
EP-1 10 ppm	0.30 bc	0.78 abc	0.76 ab	0.45 abc	0.45 abc	0.30 a	69.50 bc	46.50 cd
EP-1 50 ppm	0.64 a	0.52 c	0.53 b	0.17 c	0.16 c	0.07 b	27.90 d	34.00 de
F	30.70	4.80	4.10	4.30	4.20	2.90	16.70	30.10
df	8	8	8	8	8	8	8	8
Pr > F	< 0.0001	< 0.0001	0.000	0.000	0.000	0.006	< 0.0001	< 0.0001
Significant	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

LE = Levonorgestrel, QU = Quinestrol, EP-1 = Levonorgestrel + Quinestrol

Assessment of pregnancy and liter size

The contraceptive baits were effective in reducing the number of pregnancies, particularly when the female consumed the bait, regardless of whether the male had also consumed a bait containing contraceptive or the untreated bait (Fig. 3). In the untreated pairing, where both male and female did not receive contraceptives, the pregnancy success rate was 70% (n=10). The total number of offspring produced in the untreated pairing was 54 where most pregnant females had 8 pups each (one of the 7 females had only 6 pups). In cases where the pairing involved an untreated female with a treated male, the pregnancy rate was reduced to 30%. Here, the median litter size was 4 pups. Where the female had consumed either QU or EP-1 at 50

ppm, there were zero pregnancies. Results were shown to be significant through an analysis of variance (Table S1 Supporting Information).

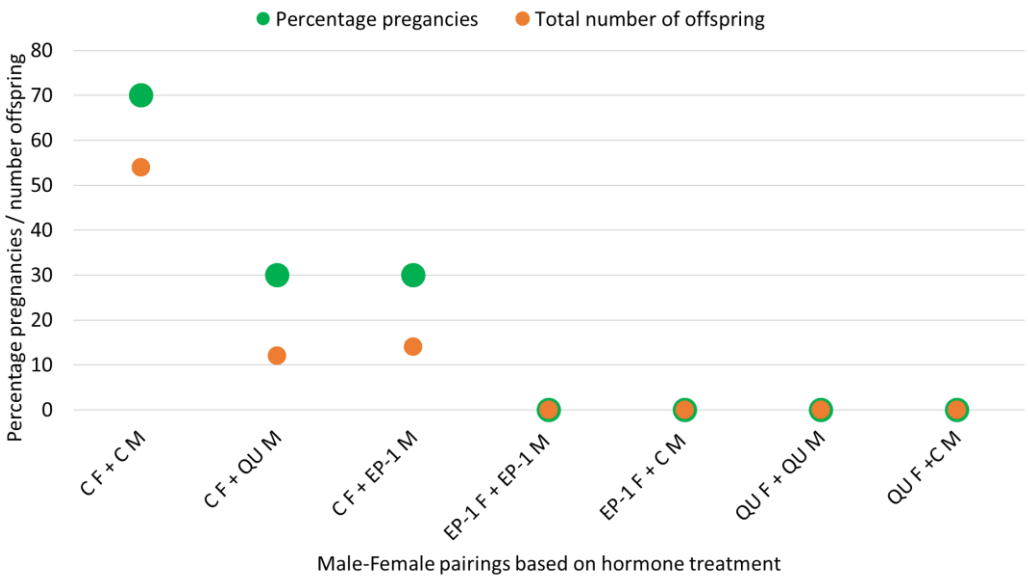

Figure 3 Effect of two contraceptive baits (QU 50 ppm and EP-1 50 ppm) fed to black rats on successful pregnancy from male-female pairings (n = 10). F = female, M = male, C = untreated control bait, QU = bait containing quinestrol at 50 ppm, EP-1 = bait containing levonorgestrel + quinestrol at 50 ppm.

Discussion

Bait consumption was significantly lower by black rats consuming the hormones when compared to the untreated bait. Daily food consumption was highly variable among individuals each day, and there were no clear temporal or treatment trends observed in untreated or control groups when carrying out linear regressions (see Fig. S1, Supplementary Information). The trends observed in our data with levonorgestrel having a lower impact on bait consumption than quinestrol, and both compounds generally more tolerated by females than males, is supported by some other studies (Lv and Shi 2011, 2012, Massawe *et al.* 2018). However, other researchers have indicated that quinestrol had no effect on the overall bait consumption of male Brandt's vole (Wang *et al.* 2011) or to plateau pikas (Liu *et al.* 2012). Of particular note, previous research on these species-specific differences may be related to their sensitivity to tasting bitter compounds such as quinestrol, highlighting the importance of evaluating different rodent species behavior in relation to contraceptive bait formulation and concentration of hormones used. However, reduced food intake should also be expected due to well-known

effects of gonadal hormones on food intake and weight loss/gain (Gentry and Wade 1976, Kral and Tisell 1976).

In all cases, males eating the untreated bait gained weight over the trial period. However, females eating untreated bait did not always gain weight and actually lost some weight in the untreated QU and EP-1 groups. Although the weight loss is small, it is not entirely clear why this should have happened but could have been caused by stress or poor habituation to laboratory conditions in some animals. Unsurprisingly, lower food intake by animals consuming contraceptive baits had a noticeable effect on body mass after 7 days of feeding exclusively on the bait. As observed during bait uptake, levonorgestrel had the least impact on weight loss. However, we are unable to explain why females consuming LE at 10 ppm suffered higher weight loss than females consuming LE at 50 ppm. This could be due to differences in starting weights of the two groups, where the average weight of females in the LE 10 ppm group was 99 g, compared to females in the LE 50 ppm group with an average weight of 77 g. We could argue that the lower weight animals were more in need of bait consumption and less able to rely on stored body reserves than the larger animals within the LE 10 ppm group. This argument would be supported by the lower observed food intake in the LE 50 ppm group, but where body mass remained stable. A similar phenomenon may explain the observed difference between males in the EP-1 10 and 50 ppm where males in the 50 ppm group lost less body mass than those in the 10 ppm group. Both groups had a similar consumption rate; however, the 10 ppm group was, on average, 26 g lighter than males in the 50 ppm. This could suggest that heavier animals have the reserves to compensate for lower food intake, but also are not needing to increase their weight further. We would recommend that greater effort be placed on ensuring that starting body weights are more similar across treatment groups in future research to repeat such trials. Some studies have also shown that weight loss occurs related to lower feeding rates (Lv and Shi 2011, Massawe *et al.* 2018). In contrast, other research indicated that the body weight of *R. nitidus* was not affected by consuming a quinestrol bait (Liu *et al.* 2013) nor was the weight of male *B. indica* reduced by quinestrol (Sidhu *et al.* 2020), highlighting again the importance of assessing different rodent species responses to contraceptive baits.

Changes to reproductive organs were noted in both females and males. Increased weight of the uterus was due to increased water retention and oedema, particularly in response to the higher 50 ppm dose for QU and EP-1 baits. Similar findings were reported in female multimammate rats (Massawe *et al.* 2018), Mongolian gerbils (Lv and Shi 2011), and Djungarian hamsters

(Wan *et al.* 2006) where oedema was also noted. This is in contrast with studies in *R. nitidius* (Liu *et al.* 2013) and Brandt's voles (Zhao *et al.* 2007) which showed that quinestrol did not reduce the weight of the uterus or change its morphology. Oedema in the uterus of treated animals has been suggested to be due to increases in estradiol and progesterone level and prolongation of estrogenic activity which results in swelling of the uterus (Su *et al.* 2017, Shi *et al.* 2020). Reproductive organs of males, sperm production and sperm motility were all negatively affected by both hormones. In male *R. rattus*, the consumption of quinestrol and EP-1 at concentrations of 10 ppm and 50 ppm reduced the weight of epididymis, testis, seminal vesicles, sperm count and decreased the sperm motility. On its own, levonorgestrel had relatively little impact, but when combined with quinestrol in the EP-1 bait, there appeared to be a synergy to further reduce the size of the epididymis and to a lesser extent the testis in comparison to quinestrol alone. This is similar to results reported by Liu *et al*. (2013) which showed that quinestrol reduced the wet mass of the epididymis but not the testis of *R. nitidius* after animals were fed with bait for 7days. In *M. natalensis*, quinestrol reduced the mass of seminal vesicles and testis but not epididymis and levonorgestrel alone did have effects on the mass of seminal vesicles but not on the testis and epididymis (Massawe *et al.* 2018). In Brandt's voles, levonorgestrel alone, and levonorgestrel+quinestrol did not affect sperm density but quinestrol alone had lowered the sperm count, mass of testis, and epididymis (Zhao *et al.* 2007). In Djungarian hamsters, EP-1 (levonorgestrel+quinestrol) did not affect male testis weight (Wan *et al.* 2006). The variations in the effects of contraceptive hormones on male reproductive organs from different rodent species highlights importance of species-specific evaluations to determine how best to potentially deploy contraceptive bait for population control. As the 10 ppm concentration was not very effective for any of the hormone treatments, we would suggest that 50 ppm is used in any field evaluation aiming to target *R. rattus* reproduction.

Pregnancy and litter size of *R. rattus* were affected by contraceptive consumption. Female black rats consuming either QU or EP-1 at 50 ppm resulted in zero pregnancies, regardless of whether the male had also consumed a contraceptive bait. This is in contrast to the untreated pairings where 70% of females became pregnant. The absence of pregnancy in treated females could be attributed to the formation of oedema in the uterus making it difficult for egg implantation. Previous studies have suggested the uterus of Mongolian gerbils were similarly disrupted, preventing conception (Huo *et al.* 2006). Where males had consumed the contraceptive but females had not, 30 % of the cohort became pregnant and with roughly 50% fewer offspring per litter. This should still be considered a good outcome as it shows that some

control could still be expected in field trials where not all individuals in a population are consuming the contraceptive bait.

The results from the current study have indicated that quinestrol alone and EP-1 at 50 ppm have good short-term contraceptive effects on male and female *R. rattus*. It is not yet clear how long the anti-fertility effect lasts after the conceptive is no longer consumed, and further research is necessary to understand the limitations of baiting rodent populations under field conditions. The reversibility of hormonal effects could be an important trade-off with respect to accidental non-target exposure when compared to other potential contraceptives that aim induce permanent irreversible sterility. However, there is some evidence that the impact of these hormonal contraceptives can be quite long-lasting, even after a single bait delivery in the field. For example, the single baiting administration of quinestrol in plateau pikas (*Ochotona curzoniae*) led to male infertility for the entire breeding season (approximately 2 months), with some impact lasting into the next breeding season through a cross-year effect (Liu *et al.* 2012). Research on mice (*Mus musculus*) suggests the impact of quinestrol on male fertility could last at least 20 days, with some effect even up to 50 days on sperm production (Liu *et al.* 2017).

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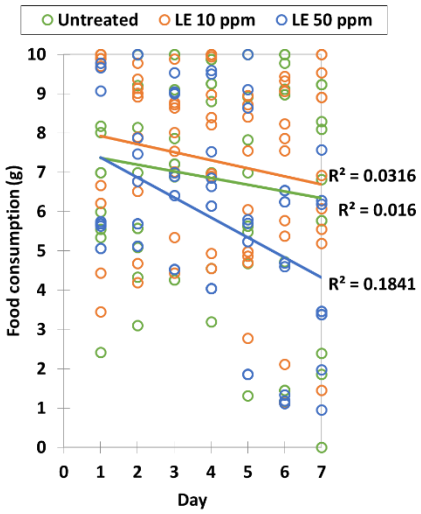
Supplementary Information

A



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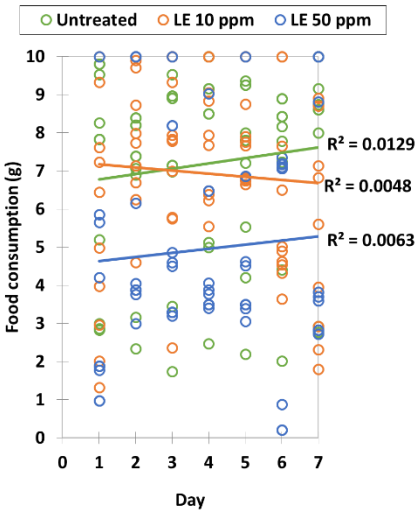

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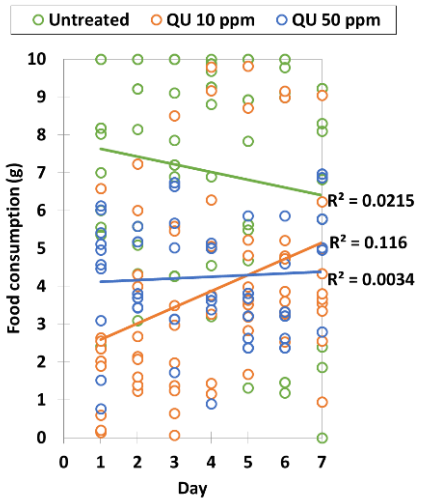

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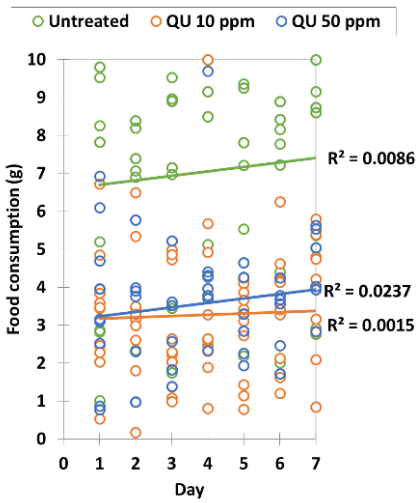

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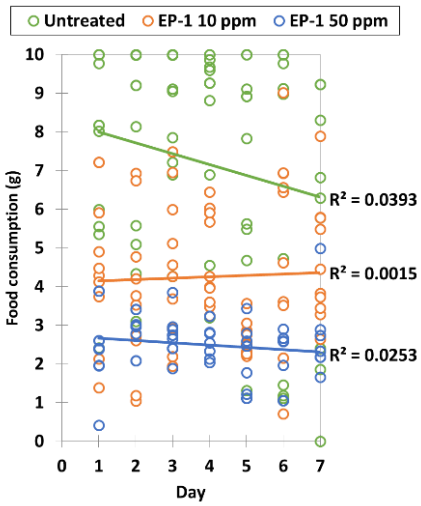

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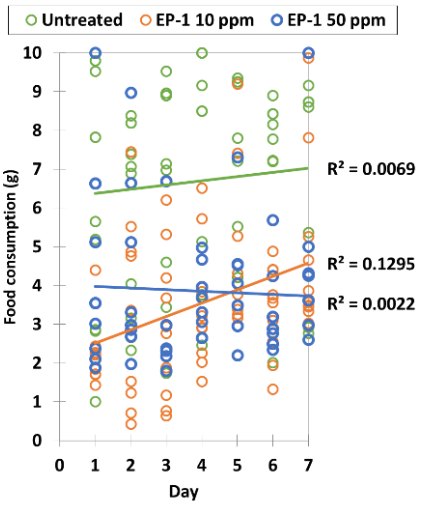

Figure S1 Linear regressions on the daily amount (g) of food consumed by black rats fed bait containing different contraceptive hormones at different concentrations. (A) Females fed on bait containing levonorgestrel (LE); (B) Males fed on bait containing levonorgestrel (LE); (C) Females fed on bait containing quinestrol (QU)); (D) Males fed on bait containing quinestrol (QU); (E) Females fed on bait containing levonorgestrel + quinestrol (EP-1); (F) Males fed on bait containing levonorgestrel + quinestrol (EP-1). R^2 values from linear regression analyses are all very low indicating rodent feeding was highly variable, with no clear trends over time or by treatment.

A **B**

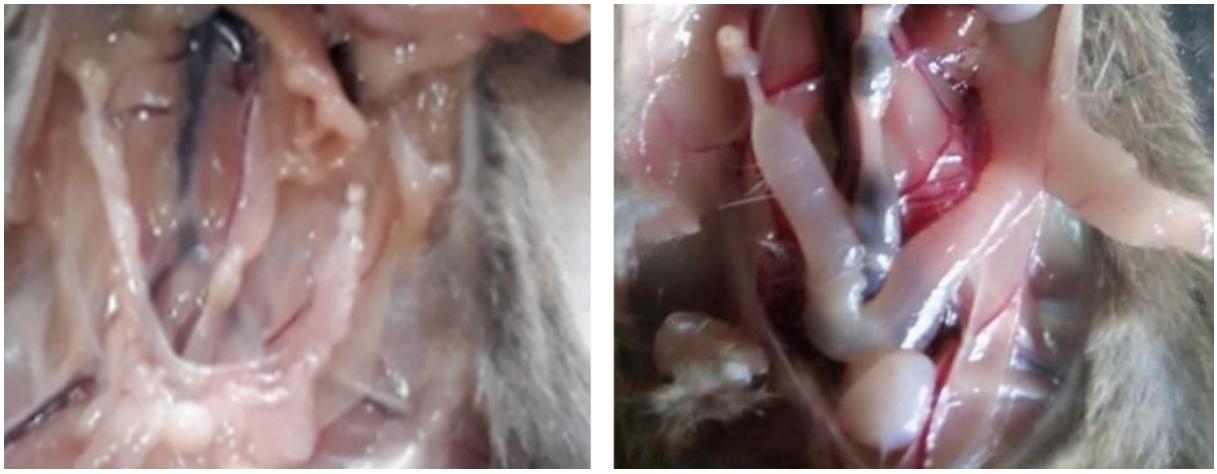

Figure S2 Female black rats dissected to show the uterus. (A) a typical uterus in a sexually mature virgin female black rat when fed untreated bait, with the uterus thin. (B) a typical oedemic uterus when female black rats were fed either QU or EP-1 at 50 ppm, showing the uterus to be full of fluid. Further research is required to understand how long this state of oedema lasts when the rats no longer consume contraceptive bait, after which it would theoretically be possible for the female to conceive again.

Table S1 Analysis of variance (ANOVA) on the percentage of male-female pairs in each treatment (n=10) that resulted in pregnancy and the mean number of offspring produced for each treatment. Values in the same column followed by the same letter are not different from each other at the 95% confidence interval using the Least Significant Difference test.

Treatment pairings (n=10)	Percentage pregnancy success	Mean number of offspring produced
ControlFemale+ControlMale	70% a	5.4 a
EP-1Male+ControlFemale	30% b	1.4 b
QUMale+ControlFemale	30% b	1.2 b
EP-1Female+ControlMale	0 c	0 c
EP-1Female+EP-1Male	0 c	0 c
QUFemale+ControlMale	0 c	0 c
QUFemale+QUMale	0 c	0 c
R^2	0.405	0.517
F	7.143	11.258
df	6	6
Pr > F	< 0.0001	< 0.0001
Significant	Yes	Yes