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Experimental elevation of wildlife testosterone using silastic tube implants

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ABSTRACT

Testosterone (T) is a key androgen that mediates vertebrate molecular, cellular, and behavioral processes. Its manipulation is therefore of interest to a vast number of researchers studying animal behavior and reproduction, among others. Here, the usage of silastic implants across wildlife species is reviewed, and a method to manipulate rock hyrax (*Procavia capensis*) testosterone levels using silastic implants is presented. Using a series of in-vitro and in-vivo experiments, the secretion patterns of silastic tubes and silastic glue were tested and were surprisingly found to be similar. In addition, we studied endogenous T levels in wild-captured rock hyraxes (*Procavia capensis*), and using T implants succeeded in elevating T to the maximal physiological concentrations recorded during the mating period. The number of implants that were inserted was the only predictor of T levels, and seven 20 mm implants were found to be the optimal dose. Implants induced sexual behaviors in the non-reproductive period. The duration of time that the implants were in the hyrax was the only significant factor that influenced the amount of T left over in the implant once it was removed. All together we affirm that T implants may offer a versatile tool for wildlife behavioral research by elevating T levels in the non-breeding period to maximal breeding levels.

1. Introduction

Testosterone (T) is a steroid hormone that regulates the cellular activity of target tissues in the central and peripheral nervous system (Ketterson and Nolan, 1992), affecting a wide range of behaviors. T is related to natural seasonal (Girard-Buttoz et al., 2015), and inter-individual (Vera et al., 2013) variations, and to spatial orientation (Ostatnikova et al., 2002), among others. According to The Challenge Hypothesis (Wingfield et al., 1990), T may be related to aggressiveness in males during social destabilization. Social destabilization can occur for example during establishment of dominant relationships, territorial disputes, or during the mating season (Wingfield et al., 1990). In contrast, aggression decreases when social status and territoriality are stable. Thus, T levels increase in high-aggression periods, when males interact, and decrease in low-aggression periods, when male-male interactions decrease. T levels are therefore expected to be at their peak during the mating season. The Challenge Hypothesis has received experimental support. For example, in males across taxa, T levels are correlated with aggression and dominance (e.g., Bouissou, 1983; Mazur and Booth, 1998; Mehta and Beer, 2010). Male rock hyraxes (*Procavia capensis*) compete aggressively among themselves before the mating season, and their social status is positively related to their T levels (Koren et al., 2006). However, unlike the conventional assumption, Koren et al. found that in female rock hyrax there is a negative relationship between T levels and social status (Koren and Geffen, 2009). In females rock hyrax, as in humans and most of mammals the relationship between T levels and dominance is more obscure (Christiansen, 2001; Girard-Buttoz et al., 2015; Mazur and Booth, 1998; Ngun et al., 2011; Staub and De Beer, 1997).

Hormone manipulation using inserted implants may be used to study the relationship between hormones and behavior. By using implants, T levels can be elevated or reception blocked, by T or receptor inhibitor implants, thus isolating T's effect from other factors and testing its role. The advantages of implants are the ability to use them for a long time frame, due to their slow and moderate releasing rate. The classic implant is made of polydimethylsiloxane (PDS) tube. Implants are widely used in veterinary medicine, and are inert, thus, do not irritate the animal (Dziuk and Cook, 1966). Inside the tube, dry crystallized steroids can be packed, and the tube is traditionally sealed at both ends with silastic glue made of PDS mixed with methyltriacetoxysilane. Hence, the glue and implant materials share similarities, yet include important differences in multiple qualities that effect materials' transmission. For example, Sahores et al., 2013 showed that mixing steroids, such as progesterone, 17β-estradiol, medroxyprogesterone acetate or mifepristone, with the glue provides a slower release of the hormone. However, the effect of the silastic glue on the passage of materials without mixing the materials with the glue was not tested.

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Silastic T implants have been designed for use in sheep, rodents, and domestic species over 50 years ago. Nowadays it is widespread in experimental setups across species, since it is cheap and easy to prepare. It has been used in multiple bird species (Clotfelter et al., 2004; Hunt et al., 1997; Kellam et al., 2006; Ketterson et al., 1992, 1991; Mougeot et al., 2005; Zysling et al., 2006), cows (Godfrey et al., 1992), squirrels (Lee et al., 1990; Zucker and Boshes, 1982), hedgehogs (el Omari et al., 1989), lizards (Pollock et al., 2012; Starostová et al., 2013), quails (Busso et al., 2010), voles (Mills et al., 2009), kangaroos (Rudd et al., 1996), rabbits (Ewing et al., 1973), rats (Antonio-cabrera and Paredes, 2012; Cheung and Davidson, 1977; Nash et al., 1978; Robaire et al., 1979; Slob et al., 1981), men (Handelsman et al., 1990; Nash et al., 1978), frogs (Townsend et al., 1991) and sheep (Christensen and Kesler, 1984; Clarke et al., 2012; Dziuk and Cook, 1966). We could not find studies describing T implant usage in rock hyrax. Here, we report on our experiments with different lengths and doses of T implants in order to mimic maximal physiological T elevation in the rock hyrax. First, we reviewed the literature for studies on experimental T manipulations using silastic-tube implants in wildlife. Then, we chose a range of implant lengths and performed an in-vitro experiment with silastic implants containing T, with and without silastic glue coating, where we examined the release pattern of T to the outer medium. Thus, we aimed to determine the utility of the silastic glue in elongating release time. In the field, we established hyrax T levels year-round, and then attempted to mimic maximal levels during the non-breeding season, when steroids are at a minimum. The goal was to find the ideal implant dose, which will elevate T and maintain high levels for at least 40 days so that in the future we will be able to study the influence of testosterone on hyrax social behavior. Whereas most research on rodents tested implants for shorter time spans (e.g., Raynaud et al., 2012), several avian studies used testosterone implants that sustained elevated conditions for up to 100 days (Kellam et al., 2006; Ketterson et al., 1992, 1991; Nolan et al., 1992).

2. Materials and methods

First, we searched Web of Science (Thomson Reuters Inc.) for articles on T implants in wildlife vertebrate species that are non-domesticated. Thus, included search words were: testosterone-implant, while the excluded search words were: fish, rats, mice, chickens, quails, sheep, cows and humans. From each study we extracted the following parameters: species, sex, captive/wild, weight, metabolic rate, T levels before implant insertion, implant used (diameter and effective length), total weight of T in implant, calculated volume of T in the implant, circulating T levels with control implants, circulating T levels with implants, and time from implant insertion. Then, based on the data, we designed implants for rock hyrax (see below).

2.1. In vitro release of T implants

We prepared implants that were 10 mm long, 20 mm long, 10 mm long covered with silastic glue, and 20 mm long covered with silastic glue. Three of each type of implant were prepared by cutting 12 mm or 22 mm pieces of silastic tubing (1.47 mm, i.d.; 1.96 mm, o.d., Dow Corning, Midland, USA), sealing 1 mm of one end with silastic glue (Dow Corning), and packing them with crystallized testosterone (Sigma, UK). Then the other side (1 mm) was sealed in the same manner. The effective implant length was therefore 10 mm and 20 mm, respectively. Spreading a uniform layer, approximately 5 mm thick, made the silastic glue-covered implants. This glue layer was in addition to the glue found at the ends of all implants. Then we poured 1 mL 2% solution low melting agarose dissolved in phosphate buffered saline (PBS; Gibco, Rehovot, Israel) into a glass vial (Sigma Aldrich, 854977, 6 cm high including the orifice). Once the agarose solidified, we placed an implant on top of it, and covered it with a second agarose layer, so that the entire implant was covered with agarose. After the second layer of the agarose solidified, we added 20 mL PBS, which was replaced at predetermined times (on days 1, 7, 14, 21, 28, 35, 56, 70, 84 and 98), starting from 24 h after the experiment began, without shaking. Before replacing solution with fresh PBS, the vial was washed with 2 mL PBS, in order to wash out residual T left in the vial. Samples of the replaced PBS were kept at −20 °C until T quantification.

2.2. Field study

The rock hyrax is an African social mammal belonging to the Afrotheria lineage (Springer et al., 1997). A single social group, consisting of six hyraxes was trapped in the city of Arad (31° 15′ N, 35° 12′ E), which is located west of the Dead Sea, between the Judean and Negev Deserts, Israel. Rock hyraxes live in mixed-sex groups that comprise resident and late dispersing males and females with their pups (Koren and Geffen, 2009; Koren et al., 2006). Hyraxes are seasonal breeders, mating in July and August in the Judean Desert. Gestation period is long, 7–8 months, and litter size ranges from one to six (Mendelssohn, 1965).

Hyraxes (N = 6) were caught using live box traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) placed in natural crevices. Traps were set before first light and operated until noon, inspected every 3 h. Trapped animals were anesthetized with Ketamine hydrochloride (about 0.1 mL/kg intramuscular injection), weighed, measured, and individually marked using subcutaneous transponders (DataMars SA; Bedano-Lugano, Switzerland). Then hyrax were transferred to the Safari Zoo in Ramat Gan, Israel (32° 2′ N, 34° 49′ E), where they were housed in a single group cage (≈50 m^2). Once a month, we trapped and blood sampled the hyrax. All measurements were recorded in situ, and the animals were returned to the traps for full recovery (at least 2 h), and thereafter released back to the group cage. No adverse effects of trapping and anesthesia were ever recorded. Animals resumed full normal activity following their release. Permits for capturing, handling, removing the hyrax from their natural habitat, and holding them in captivity were issued and reviewed annually by the Israeli Nature and Parks Authority. Permits for the implant experiment were issued by Bar Ilan's Animal Care and Ethics committee permits (approval number 49-12-1012, date of approval 8 January 2013).

The duration of the in-vivo experiment was approximately six months. We randomly divided the hyrax into 3 groups, each consisting of a male and a female. Every six weeks we injected implants, and removed the old implants. First, each hyrax received either: a single 10 mm, one 20 mm, two 20 mm or one 20 mm - long empty implant. However, following the first round, we found that T levels were not sufficiently elevated. Hence, we increased subsequent doses to: eight 20 mm long implants, four 20 mm long implants or a 20 mm long empty implant. The empty implant controls for the procedures that the experimental animals received, which include: capturing, anesthesia, blood sampling and the act of inserting a foreign body in the neck area. Implants were prepared as above, using the protocol we received from Prof. Ellen Ketterson's laboratory (Indiana University; Ketterson et al., 1991). For use in-vivo, implants were autoclaved before packing with T and inserted subcutaneous around the nape of the neck using trochlear gauge. This area was chosen since it has widespread subcutaneous tissue, and the animal cannot reach it. In addition, we have experience with subcutaneous chip insertion in this area in over 400 hyraxes the field since 1998 (e.g., Koren and Geffen, 2009). Insertion site was sealed with tissue sealant (Cyanoacrylate glue; Loctite, Westlake, Ohio). Blood samples were drawn every 1–2 weeks (see Fig. 4), from the cephalic vein, using a 25 G needle and a 1 mL syringe. Samples were transferred to a polypropylene tube containing anti-coagulant (Ethylenediaminetetraacetic acid; EDTA, Greiner bio-one, Austria), thoroughly mixed, and centrifuged (10 min, 2000 *g*). Separated plasma was kept at −20 °C until analysis. Testosterone was quantified in plasma using commercial enzyme-linked immunosorbent assays (ELISA) kits (DRG International Inc., item no. EIA-1559). Intra-assay

repeatability was determined using duplicates of the pool on the same ELISA plate (n = 4). The coefficient of variation was 6.76%. Inter-assay precision was determined by running duplicates of a standard on 4 different days. Inter-assay coefficient of variation was 9.14%. Serial dilutions of hyrax serum pool showed parallelism with the provided kit standards (univariate analysis of variance in SPSS; p = 0.38). Linearity was demonstrated between 0.5 and 16 ng/mL of kit standards. Recovery was calculated to be 95.2% by the addition of a known amount of testosterone to the pool.

After the implants were removed from the hyrax, we cut a small incision at one end of the implant and let it air dry for several hours until the color and texture of the material changed. Then we measured the amount of T left in the implant. Only white, powder-like substance (T) was taken into account, and not the light yellow and hard solid material left in the implant. We calculated the percent of T left in the implant, in relation to implant length.

Hyraxes were continuously observed and their social behavior monitored using two high definition video cameras that were placed in the top corners of the cage. Agonistic and sexual behaviors were then extracted into spreadsheets.

2.3. Statistical analysis

To normalize T concentrations we used the Box Cox transformation. In order to test whether implants covered with glue were different than implants that were not all covered in glue, we ran Standard Least Squares test. To test whether the number of implants or time that they were left in effects T levels we used Generalized Linear Model Fit, where hyrax ID was considered a random factor. To test whether the amount of T left in the implant after its removal was influenced by time the implant was left in the hyrax or the number of implants in the hyrax, we used Standard Least Squares with the REML method, where the hyrax ID was set as a random effect. All analysis was completed with the JMP 11.0.0 software (2013; SAS Institute Inc.), and statistically significant results ($p < 0.05$) are shown.

3. Results

In order to choose the optimal implant type and dose, we reviewed the literature. Table 1 (Supplementary Information) contains studies published on multiple wildlife species, and includes information on implant length, dose, and duration of the experiment. It also indicates T levels that were achieved following implant insertion. We tried to match hyrax metabolic rate (0.4 mL O_2/g/h; Taylor and Sale, 1969), sex, body weight, T concentration and time effect, to produce implants for the described in-vitro and in-vivo experiments.

In-vitro, we quantified T in the PBS collected from vials that contained an implant covered with or without glue. The rational of this study was to test whether T actually leaches out from the silastic tube, or leaks out from the glue at the ends of the implant. We also wanted to test whether covering the implant with silastic glue would elongate release time. Extending steroid release time would provide a significant advantage while working with wildlife, since capturing is especially challenging, disturbing individuals and potentially influencing behavioral research. Across implant types, we found no significant differences between implants' secretion patterns or lengths (Fig. 1). T levels were similar between implants on the first experimental day, peaked on day 7, and then decreased gradually, until leaching out stopped, after approximately two months.

In order to define hyrax maximal physiological T concentrations, the target of our implant experiment, we monitored endogenous circulating T levels throughout the year. High inter-individual variability was seen, with the highest T levels (5–10 ng/mL; hence our target concentrations; Fig. 2) immediately before and during the mating period (June–July). Maximal T level measured was 10.51 ng/mL (Fig. 2). Hyrax weight (kg; mean ± SD = 2.55 ± 0.68) was a significant predictor of natural T levels ($F_1 = 8.45$; $p = 0.0265$).

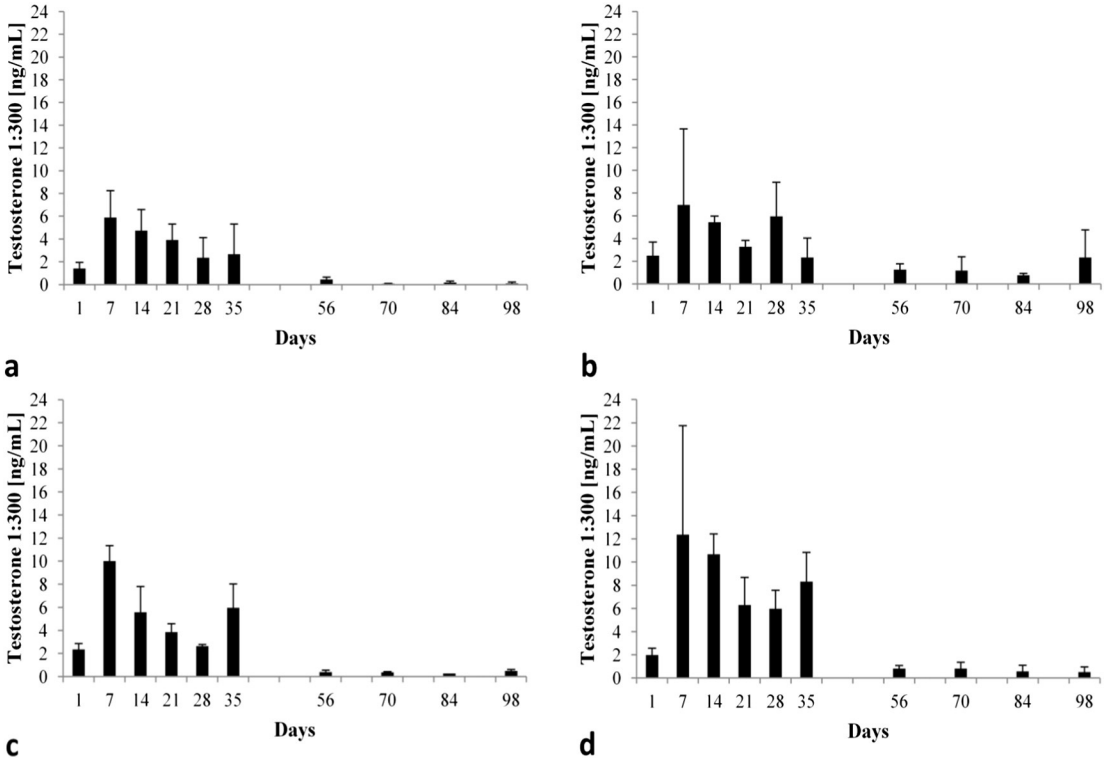

Fig. 1. Mean testosterone levels (±SD) of in-vitro experiment. a = 10 mm implant covered with glue, b = 10 mm implant without glue, c = 20 mm implant covered with glue, d = 20 mm implant without glue. N = 3 for each implant. The first collection of PBS started 24 h after the beginning of the experiment. No significant differences were seen between the different treatments.

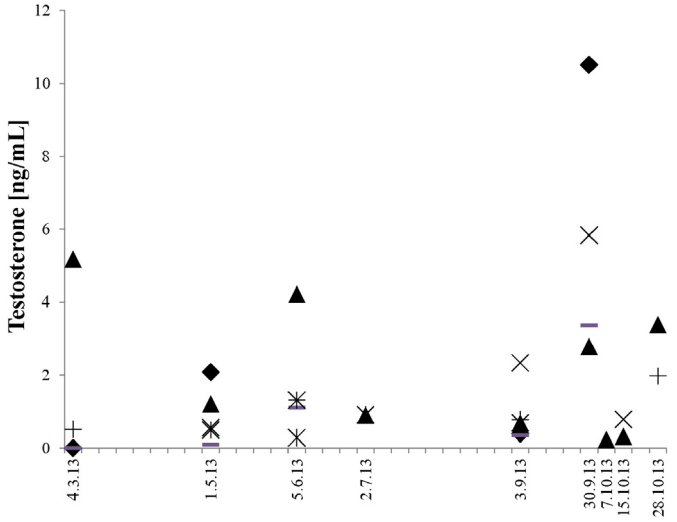

Fig. 2. Hyrax endogenous testosterone levels. A different sign designates each individual (N = 6).

In our in-vivo implant experiment, the number of implants that were inserted in each animal was the only predictor of T levels ($\chi^2_{19} = 107.7$, $p < 0.0001$; Fig. 3). Hyrax weight (kg; mean ± SD = 2.5 ± 0.4) did not influence T levels during the experiment. The time period that implants were left in the hyrax, and the interaction between time and the number of implants was also not significant. When less than two implants were used, T levels were baseline. Only when three or more implants were used, T levels were elevated. The response to three and four implants was very wide and some of the hyrax did not reach our target concentration. Although only a few hyraxes received 6–8 implants, it appears that it is the required number for providing the targeted dose. In addition to T elevation, we recorded six cases of sexual (i.e., mounting) behavior during the implant experiment.

The time that the implant was left in the animal was the only significant factor that influenced the amount of T remaining in the implant once it was removed ($F_1 = 23.3$; $p < 0.0001$), explaining 64% of the variance. The longer the implant was in the animal, the more T diffused out, and less T remained in the silastic tube (Fig. 4). The number of implants and the interaction between the number of implants and the duration the implants remained in the animal was not significant. Most of the implants that were removed were more than half empty, and some were empty following 6 weeks in the hyrax. On the other hand, we found implants that still had significant amounts of T in them following 18 weeks in the animal. In two of the six hyraxes we were not certain how long implants stayed, since we found old implants at later stages of removal. In addition, removed implants had some fluids in them, showing that diffusion was bi-directional.

4. Discussion

Through a series of in-vitro and in-vivo experiments we describe a method to construct and use T implants in wildlife. Our in-vitro experiment showed that there was no difference between the implants with or without glue (Fig. 1), and that implant length did not influence T concentrations. If T were released exclusively from the silastic tube, than we would expect that covering the implant with glue would prevent the release of T, or at least prolong the release time. Although an in-vitro system cannot fully mimic a physiological system, it provides important insight on the steroid-implant interaction, release mechanism, and dynamics. T leakage stopped after approximately two months. We assume that in-vivo releasing period is shorter than in-vitro since it involves more secreting factors and is also regulated by internal physiology and conditions, as well as cellular uptake and excretion dynamics. In the in-vivo experiment we found that after six weeks most implants were more than half empty. Since glue-covered implants showed similar dynamics as the non-covered implants, we assume that there is leakage from the silastic glue, in addition to leaching from the silastic tube. It seems that over time both the tube and glue undergo expansion, as fluids pass in and out of the implant. Consequently, we decided to proceed with the experiment using the classic implants, without glue covering.

While monitoring endogenous circulating T levels, the maximal T level detected was 10.51 ng/mL. Based on that value and on concentrations reported in the literature (e.g., Neaves, 1973) we aimed to elevate T levels to 5–10 ng/mL. The large variation in T may be due to the differences in the time of day in which individual hyrax were captured (Resko and Eik-nes, 1966), and the time that it took to draw blood (i.e., sedate and sample the animal). Implant experiments began late September, when T concentrations are at their lowest. Rock hyraxes are highly seasonal, mating in Israel in July and August. Parturition is in March, and T is expected to rise throughout pregnancy (Leary et al., 1991). Sexual behavior during the non-reproductive period is rare in hyrax. Yet, in this

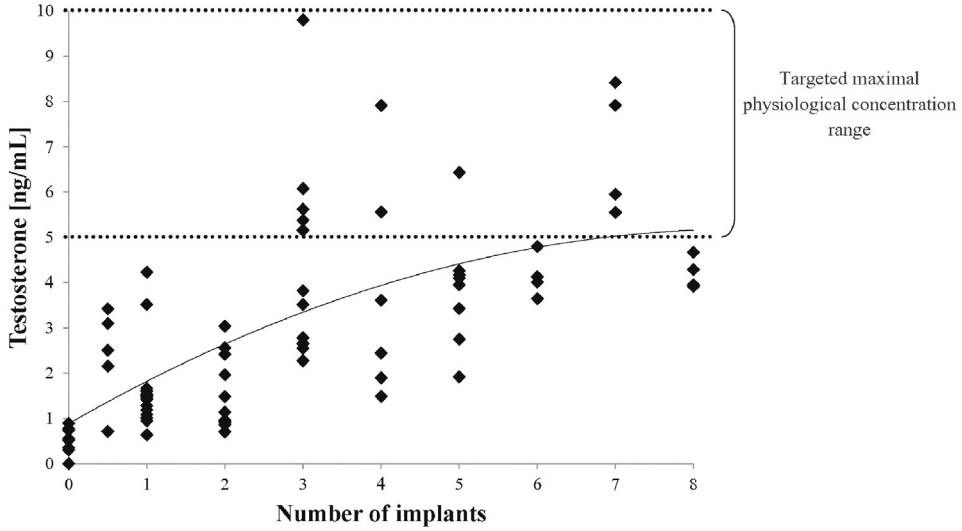

Fig. 3. Number of implants affected testosterone levels. Mean testosterone levels for the entire duration of the experiment (i.e., approximately 6 weeks), showing a polynomial response curve. Dotted lines indicate our target concentration (5–10 ng\mL). 0 are controls, which are empty implants, 0.5 indicates a single 10 mm long implant, and the rest of the implants are 20 mm long.

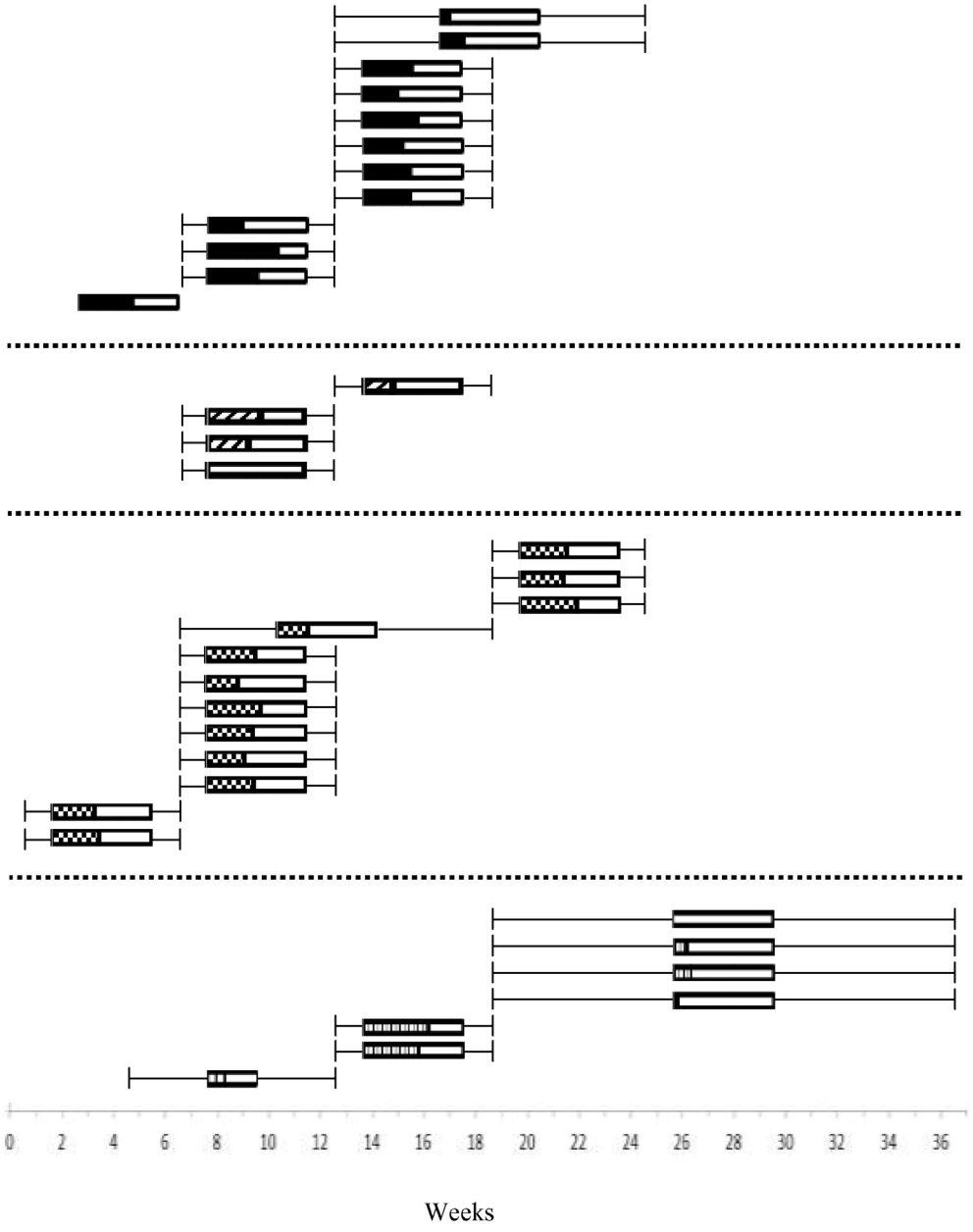

Fig. 4. Residual testosterone in removed implants. Each pattern indicates a different hyrax, separated by dotted lines. Rectangle lengths designate implant lengths. Error bars indicate the duration of time that the implant was in the hyrax.

experiment, we observed T-implanted males sexually mounting T-implanted females during the non-reproductive period. Thus, it seems that T implants may promote sexual behavior in both sexes.

Despite sealing the implant insertion site, we could not locate all implants in order to remove them. It is possible that they either fell out at some point, or became lodged in scar tissue. Thus, we considered the number of implants that were inserted in hyrax as the effective units. Our study shows that 6–8 20 mm implants are the most appropriate vehicles to elevate T to the maximal physiological concentrations, with minimal variability. This dosage could not be deduced by reviewing the literature on published T implant research in other species, and trying to match hyrax body weight or metabolic rate. Furthermore, while hyrax body weight predicted endogenous T concentrations, following the experimental manipulation, the association disappeared.

The time that the implant remained in the hyrax was the only significant factor that influenced the amount of T left over in the implant once it was removed. Implant length or the number of implants did not influence T release rate. Hence, to achieve higher T release, our data suggests that a greater surface area is needed. Several short implants may therefore be more beneficial, by providing greater opportunities for interchange, than fewer longer implants. Thus, when planning an implant study, factors including implant length, insertion technique, site, and device must be taken into account, in addition to subject size and species' metabolic rate.

5. Conclusion

In this study we found that silastic glue allows T to leak out of silastic implants, in addition to the tubing. In addition, we experimentally elevated rock hyrax T levels to maximal physiological levels using silastic T implants. Our results suggest that using multiple shorter implants may facilitate slower T release, while longer implants may be used

when a faster effect is needed. Although the total initial amount of crystalline T in the implants may be identical, leakage depends only on time, allowing different concentrations to be targeted over time. We suggest that for rock hyrax, approximately seven 20 mm long implants are the optimal dose, achieving target concentration. In the future, we will increase sample sizes in the optimal dose in order to determine implant kinetics and steroid clearance that will allow us to use this methodology in the wild to explore the effects of T on social and sexual behavior.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.rvsc.2016.07.005.

Conflicts of interest

None.

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