Epigenetic effects in rabbits:

Impacts of intrauterine position

Outline of Ph.D. Thesis

Bánszegi Oxána
Department of Ethology, Biological Institute, Faculty of Science, Eötvös University, Budapest, Hungary

Supervisor
Dr. Altbäcker Agnes
(Department of Ethology, Eötvös University, Budapest, Hungary)

Consultatnt
Dr. Altbäcker Vilmos
(Department of Ethology, Eötvös University, Budapest, Hungary)

Ph.D. School of Biology
Head of PhD School: Prof. Dr. Erdei Anna

Ph.D. Program of Ethology
Head of Ph.D. Program: Dr. Miklósi Ádám

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I. Introduction

Sexual differentiation – physiology, morphology and behavior in adulthood – of a female offspring is known to be affected in utero by different types and amounts of steroid hormones (Phoenix, Goy et al. 1959) in several mammalian species. In litter-bearing mammals, these hormones may originate not only from the mother, but also from neighboring embryos, depending on their sexes (Clemens, Gladue et al. 1978; vom Saal 1989). Testosterone produced by adjacent male fetuses is able to diffuse through the amniotic water and fetal membranes (Even, Dhar et al. 1992; vom Saal and Dhar 1992).

The amount of testosterone - reaching a fetus - during the prenatal development is essential and has a long-term effect. Females with 2 male adjacent littermates in utero (2M female) are exposed to a larger amount of testosterone and show stronger masculinization than those with one or no adjacent males (1M and 0M females) (vom Saal and Bronson 1980). The masculinization manifests in many ways, for example in physiology (increased testosterone concentration in the blood and the amniotic fluid, testosterone sensitivity, reduction of the reproductive capacity), in morphology (weight, anogenital distance, change in the 2:4 finger ratio) and in behavior (increased aggressiveness and marking activity, changes in the reproduction behavioral, etc.).

In rodents the short and long term effects of the intrauterine position on morphology and behavior is widely discussed. The aim of the present dissertation was to test whether this phenomenon and its consequences exist in the Lagomorph group, especially in wild type rabbits (*Oryctolagus cuniculus L.*) and in its domesticated form. Rabbits are often used as experimental subjects because caring and breeding is simple. They can also be used as model animals since they are polytocous, they have uterus duplex and hemochorial placentation (as in rat, house mouse and humans) (Flexner and Pohl 1941; Faber and Hart 1967). Due to their unique reproductive behavior and parental care they had become an ideal model animal.

Rabbits are active at night; thus their most sophisticated sensory organ is their noses. Sexual dimorphism is not apparent, but there are some differences in morphology and behavior between males and females. The Department of Ethology at ELTE had a number of studies on sexual differentiation in rabbits, in which several correlations were found between certain morphological, physiological and behavioral variables (Dombay 1997; Dombay, Bilkó et al. 1997; Csatádi 2007; Csatádi, Altbäcker et al. manuscript).

These accomplishments of the department and the extensive literature available greatly helped me to design and set up my research.

II. Thesis Aims and Methods

In this doctoral dissertation, I studied the consequences of prenatal exposure to testosterone, either produced by littermates in utero or administered artificially. The laboratory experiments were done at the Biological Research Station of Eötvös University, Göd in the majority of cases and the field test took place around Paks. The experiments were planned as long-term investigations; the same animals' variables were investigated at birth and in adulthood.

- **The influence of intrauterine position on the anatomy and behavior in domestic rabbits**

My first study investigated whether the intrauterine position has any effect on the sexual development within sexes; more precisely: can the male neighbors influence a female's morphology and behavior at birth and/or adulthood? Since rabbits have two-horned uterus, before the mating of the does unilateral ovariectomy – the removal of one uterine horn with the adjacent ovary – was performed (Blasco, Argente et al. 1994). Using this method, we could determine the pups' intrauterine position (IUP) by the order of their appearance at birth. The anogenital distance (AGD) is a widely used biomarker for prenatal androgen exposure studies (Gandelman, vom Saal et al. 1977; McDermott, Gandelman et al. 1978; Zielinski, Vandenbergh et al. 1991; Vandenbergh and Huggett 1995), therefore we chose this variable to study the morphological changes. The spontaneous chin marking activity was also investigated as a behavioral test at adulthood.

- **Influence of intrauterine order on birth weight in domestic rabbits**

Variation in the birth weight of pups is observed in litters of polytocous mammals. It's known from studies on New Zealand white rabbits that in the uterus the heaviest pup is the closest to the ovary, while the lightest is at the second nearest position to the cervix and those developing between them have intermediate weight (Rosahn and Greene 1936; Pálos, Szendrő et al. 1996). In these studies, the pups' order in the uterus was known from the birth order, so we could easily investigate whether the same phenomenon exists

in case of the Chinchilla breed of rabbits. This information is crucial because birth weight can cause significant biases associated with other morphological variables.

- **Impacts of testosterone treatment of pregnant rabbits on the sexual development of their daughters**

Artificial manipulation before birth may also influence the offsprings' sex differentiation. Previous rodent studies have shown that injections of testosterone given to pregnant mothers influenced the masculinization of the pups: female offspring at birth showed larger anogenital distance, delayed maturation, and elevated aggressiveness and decreased sexual behavior in adulthood (Gandelman, Simon et al. 1979; Gandelman, Rosenthal et al. 1980; Rhees, Kirk et al. 1997; Hotchkiss, Lambright et al. 2007). In case of rabbits we thus examined whether external testosterone could also mimic the adjacent male effect. Different amounts of testosterone were administered repeatedly to pregnant does (3 mg, 3 μg, sesame oil and control) around the sensitive period of sex differentiation of the offsprings. On the basis of the previous results we expected varying degrees of masculinization of the female offspring both at birth and adulthood, depending on the dose of extra testosterone. It is also expected that these will show the 'male in utero neighbor' effects as well.

- **Is anogenital distance a predictor of attractiveness, litter size and sex ratio of rabbit does?**

Previous experiments on rabbits kept in breeding pens at the Department of Ethology. Csatádi et al. (2007) showed that females with larger anogenital distance had smaller litters and the sex ratio was male biased. Since similar studies were performed mostly on laboratory species (Hirlemann, Spetz et al. 1990; Clark, Karpiuk et al. 1993) we investigated whether this phenomenon can be detected only in genetically homogeneous stocks, or it is also important in wild populations. The genetic diversity of a wild population could theoretically mask this phenomenon seen in laboratory colonies. The first goal of this study was to investigate whether the anogenital distance is a good indicator of the reproductive capacity of wild rabbits. To answer this question wild type rabbits were kept and bread in a seminatural environment and their anogenital distance was compared to their litter size and litter sex ratio.

If there is a difference among the females in fertility or/and fecundity – meaning that small-AGD females produce larger and more viable litters – raises the second question of this study: whether males are able to differentiate between females and are able to choose the high reproductive capacity females. Previous studies in mice have shown that males prefer the scent from a female with small anogenital distance over females with large anogenital distance (Drickamer, Robinson et al. 2001). In the next step, we examined in three-way choice tests whether rabbit males preferentially respond to the chin marks of females having small AGD which feature correlates with larger litter size.

III. Results and Conclusions

- **Intrauterine position influences anatomy and behavior in domestic rabbits (Banszegi et al 2009)**

Unilateral ovariectomy did not influence substantially the pups' intrauterine development. We found that neither litter size nor litter weight was significantly affected by the ectomy of one uterine horn and the adjacent ovary of the mothers. Therefore, the method developed by Blasco et al. (1994) can provide unbiased information on early sexual development in rabbits.

Our results revealed that anogenital distance was a reliable indicator of sex as male pups had larger anogenital distance than females, both at birth and later on. Adjacent male fetuses had significant effect: the more adjacent male fetuses females had, the longer anogenital distance they possessed. Anogenital distance at birth was a good predictor of the anogenital distance and behavior of adults, as females with two adjacent males in utero showed the longest anogenital distance and the highest chin marking activity among females. Anogenital distance measured at birth and at adulthood, and spontaneous chin marking activity measured in adulthood correlated positively. Thus, anogenital distance at birth can be used to predict an adult animal's anogenital distance and chin marking activity. We concluded that, similarly to rodents (Gandelman, vom Saal et al. 1977; Clemens, Gladue et al. 1978; McDermott, Gandelman et al. 1978; vom Saal and Bronson 1978), proximity to males in utero affects both anatomy and behavior in rabbits.

- **Intrauterine order influences birth weight in domestic rabbits (manuscript)**

We found that the position of the fetus in utero may influence the development of the offspring: the heaviest pups are in the last position in the uterus, closest to the ovary and the lightest are in the second nearest positions to the cervix. Our results also confirmed that the same can be said in case of Chinchilla breed of rabbits. There are several hypotheses (Rosahn and Greene 1936; Dziuk 1968; Duncan 1969) trying to explain this phenomenon, and although our study not directly tested these, our results will be used in designing further experiments.

- **Testosterone treatment of pregnant rabbits affects sexual development of their daughters (Banszegi et al 2010)**

The aim of this study was to test if the intrauterine position effect in rabbits can be induced by exogenous testosterone treatment. By administering different doses of testosterone to pregnant rabbits, and following the anatomical and behavioral development of their female offsprings, we induced dose-dependent changes in morphology and behavior of prenatal testosterone exposed females. Females exposed prenatally to 3 mg testosterone had longer anogenital distance and were heavier than females from the other experimental groups at birth. In adulthood, females from the 3 mg testosterone group had the longest anogenital distance, had enlarged chin glands and – additionally – these females showed the highest spontaneous marking activity.

The effect of the testosterone treatment corresponded to the variation due to intrauterine position, namely, exposure to testosterone of known external origin resulted in similar tendencies of masculinization in rabbit females as the in utero proximity to male siblings. Exposure to elevated testosterone levels generally results in masculinized anatomy and behavior in females of several rodent species (Gandelman, Simon et al. 1979; Mann and Svare 1983; Rhees, Kirk et al. 1997; Wolf, Hotchkiss et al. 2002). Our results suggest that the intrauterine position effects may have similar physiological bases across different mammalian taxa including Lagomorphs.

- **Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does (Banszegi et al 2012)**

We documented that anogenital distance variation exists in wild type rabbit females and it corresponds to breeding performance. Our results demonstrated that does with large anogenital distance have significantly smaller and lighter litters than small-AGD females, and the difference in the litter's weight was due to the smaller litter size, as these two variables positively correlated. Females with large anogenital distance produced male biased litters and the thorough examination of the sex composition of litters showed that there were significantly fewer female pups compared to the litters of does with small anogenital distance, while the number of male pups was equal. Hirlemann & Spetz (1990) presented the same results in mice. In case of rabbits, the sex specific embryo mortality and resorption are the most feasible explanation (Fuller, Zarrow et al. 1970; Pratt and Lisk 1989), since litter size decreased in large anogenital distance females, but the number of male pups did not change. The underlying mechanism behind the atypical sex ratio is not yet clear but maternal stress might mediate it (Christiansen 2004; Grant 2007). The prenatal hormonal impact on the female fetus can be such a mechanism behind the observed variation. Since this phenotypic variation also appears in genetically heterogeneous populations, and it depends on prenatal hormonal effect, it must be considered as a non-negligible effect in future developmental studies.

On the other hand, we documented that bucks showed a more definite response to chin marks of females with small anogenital distance than to marks of females with large anogenital distance. That implies that males are able to distinguish between chin marks of females with different sexual status, which correlates with anogenital distance. The present study shows that rabbit males are sensitive to certain cues in the chin marks of females reflective of their breeding capacity. Preferring small anogenital distance females seems plausible as these females would provide large litters and increase the males' breeding success. Our results suggest that variation in the prenatal hormonal environment, reflected through variation in anogenital distance, could have long-term consequences on mate choice and population dynamics.

Publications related to the theses:

Bánszegi O., Szenczi P., Dombay K., Bilkó Á., Altbäcker V. 2012. Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. Physiology & Behavior 105 (5), 1226-1230.

Bánszegi O., Altbäcker V., Dúcs A., Bilkó Á.. 2010. Testosterone treatment of pregnant rabbits affects sexual development of their daughters. Physiology & Behavior 101 (4), 422-427.

Bánszegi O., Altbäcker V., Bilkó Á.. 2009. Intrauterine position influences anatomy and behavior in domestic rabbits. Physiology & Behavior 98 (3), 258-262.

Abstract from international conferences:

Bánszegi, O., Szenczi, M. P., Altbäcker, A. 2009. Anogenital distance as a predictor of fecundity in the European rabbit (*Oryctolagus cuniculus*). 5th Ecology and Behaviour meeting, Lyon France

Bánszegi, O., Bilkó, Á., Altbäcker, A. 2007. The effect of testosterone on sexual development of rabbits. 30th International Ethological Conference. Halifax, Canada.

Bánszegi, O., Bilkó, Á., Altbäcker, A. 2005. Intrauterine position has long lasting effect on morphology and behaviour of female rabbits. 29th International Ethological Conference. Budapest, Hungary.

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