Study of Anogenital Distance in Rabbits: Effect on Sexual Behavior and Litter Size Biological Components

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Abstract. *The aim of this work is to study the relationship between the anogenital distance (AGD) measured before mating and plasma cholesterol and hormone concentrations (testosterone and 17-βestradiol), sexual behavior, litter size and its biological components (ovulation rate and prenatal survival) and the sex ratio in rabbits. In total, 48 rabbit does were used. The females were classified according to their AGD in 2 groups (AGD long or AGDL, n = 24, and AGD short or AGDS, n = 24). Blood samples were collected before mating, receptivity of the females was tested and their behavior was observed. Endoscopy was performed at day 12 of pregnancy. The number of total born, alive, dead and the sex ratio were recorded at birth. The plasma testosterone and cholesterol concentrations were significantly higher in the AGDL group of females (14% and 24%, respectively). The AGDL females presented a higher rate of receptivity (31%; P < 0.05), they were more aggressive (78%; P < 0.05) and marked more frequently their territory using the spontaneous chin marking than the AGDS females (34%; P < 0.05). The number of implanted embryos was significantly higher in the AGDS group (9.12 vs. 8.66 embryos). The embryonic, fetal and prenatal survival were significantly higher in the AGDS females. In addition, the AGDS females presented a higher litter size at birth (8.96 vs. 7.83; P < 0.01) and sex ratio in favor of males (61.60% vs. 41.00%; P < 0.01). In conclusion, the AGD measured before mating can be used as a predictor of the testosterone level, sexual behavior, litter size at birth and the sex ratio in rabbits.*

Introduction

In mammals, litter size and reproductive performances can be influenced by the animal's previous intra-uterine position (IUP). Thus, it has been the subject of numerous studies (see review of Ryan and Vandenbergh, 2002), in order to show its influence on reproductive parameters (hormone levels, development of external genitalia and sexual behavior).

Except for this type of a purely anatomical position, there is another particularity related to hormones. Any fetus not located at one end of the uterus will be positioned between two males (2M), two females (0M), or one male and one female (1M). This IUP has important and far-reaching effects on fetal development in animals as well as in humans (MacLusky and Naftolin, 1981). These effects are essentially related to the *in utero* interaction between the different hormones to which the fetus is exposed throughout gestation (Even et al., 1992). Among these hormones, testosterone plays a primordial role in the masculinization process. Indeed, male fetuses produce testosterone earlier and in greater quantities than female fetuses (Arnold, 2002). This hormone can diffuse between fetuses through fetal membranes and amniotic fluid (Wallen and Baum, 2002). Therefore, both 2M male and female fetuses (positioned between two males) have higher blood testosterone and lower estradiol than 0M fetuses (positioned between two females) (Vom Saal et al., 1990). *In utero*, elevated testosterone concentrations in several species affect fetal body development, behavior, physiology and morphology in adulthood (Ryan and Vandenbergh, 2002). However, the most significant impact of testosterone is on anogenital distance (AGD) (Ryan and Vandenbergh, 2002).

Indeed, 2M females have a long AGD compared with that measured on 0M females. In contrast, 1M females have an intermediate AGD (Hernandez-Tristan, 1999). This phenomenon has been described in mice (Zielinski et al., 1991), rats (Meisel and Ward, 1981) and rabbits (Bánszegi et al., 2009). This morphological difference persists from birth to adulthood in rabbits and in several rodent species. On the other hand, females with a higher AGD show higher blood testosterone concentrations, tend to be more aggressive, are less attractive to males and show low prolificacy with a male sex ratio (Rohde Parfet et al., 1990; Bánszegi et al., 2009). Thus, this parameter, revealing prenatal exposure to androgens, is frequently used as a biological marker for some reproductive parameters in animals such as mice (McDermott et al., 1978), rats (Meisel and Ward, 1981), Mongolian gerbils (Clark and Galef, 1998), pigs (Drickamer et al., 1997), cows (Gobikrushanth et al., 2016) and rabbits (Bánszegi et al., 2012).

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In a first published study on rabbits, Kerkouche et al. (2014) noted higher embryonic and fetal mortality in rabbits with a long AGD and, consequently, a lower number of implanted embryos (estimated at 12 days of gestation by scarification of females) compared with females with a small AGD. Therefore, studying the relationship between IUP, AGD, litter size and sex ratio could help in the selection of the best performing animals for breeding. This experiment follows up on the previous work, and aims to study the relationship between the AGD measured before mating and the sexual behavior of female rabbits, the level of sexual hormones (testosterone and 17β-estradiol), litter size at birth and its main biological components (ovulation rate and prenatal survival) as well as the sex ratio in rabbits.

Materials and methods

This study was approved by the Scientific Council of Biotechnology Laboratory of Animal Reproduction, University of Saad Dahlab Blida, Institute of Veterinary Sciences (Algeria).

Our experiment was carried out in the rabbitry of the Experimental Station, University Blida I, Algeria. The rabbits were housed in individual flat-deck cages and fed *ad libitum* with commercial pelleted diet (17.1% crude proteins, 16.5% crude cellulose and 3.2% fat). The rabbit does were submitted to a constant photoperiod of a 16L:8D light cycle during the whole experiment period.

Animals

The rabbits used in this experiment belong to the ITELV2006 line. The characteristics of this line are described in Ezzeroug et al. (2020). Forty-eight (48) females were selected and placed in individual cages. The selection criteria were parity (multiparous at the third parity), a homogeneous weight at mating (3005 ± 47g) and a good health status. Eight males (4230 ± 284 g) were used to mate the females with a rhythm of 3 mating acts per week and a rest of one day between two consecutive mating acts.

Measurement of AGD

AGD is measured between the center of the anus and the vulva using the method described by Bánszegi et al. (2012). It is measured three times for each female, by different operators and using a digital caliper. The mean of the three observations is calculated (AGDM). The rabbits are then classified according to their AGDM into two classes: the first class concerns females with a short AGD or AGDS (AGD is equal to or lower than the average AGD of all rabbits). In contrast, the second class includes females with a long AGD (above the average of all rabbits or AGDL (Drickamer et al., 2001).

Mating and evaluation of sexual behavior

The females were mated in the morning, between 9 and 10 am. Before each presentation to the male, the female was weighed. The receptivity of the female was evaluated by examining the vulva color and its turgidity (indirect method). The female is considered receptive when the vulva is pink or red and turgid. On the other hand, it is non-receptive when it has a pale pink or white and non-turgid vulva (Theau-Clément et al., 2015). Receptivity was also assessed during mating (direct method: acceptance or refusal of mating). In addition, the behavior of the female during mating with the male was also studied. Four parameters were noted: aggression, mounting, chin and urine marking.

Blood sampling and hormones analysis

Thirty minutes before mating, a blood sample of each female was taken by puncture of the auricular marginal vein. The blood was collected in heparinized tubes and immediately centrifuged at 3000 rpm/15 minutes. The plasma was stored at –20°C for subsequent analyses of cholesterol, 17β-estradiol and testosterone. Plasma levels of the different parameters were assayed in duplicate for each plasma sample and using RIA (I^{125} Immunotech® kits) for hormones and spectrophotometry (Spinreact® kits) for cholesterol.

Endoscopy

At 12 days *post coitum*, the diagnosis of pregnancy is made by abdominal palpation. The endoscopy was performed according to the method described by Santacreu et al. (1990). The following variables were measured: ovulation rate (OR) measured by counting the number of *corpus luteum* in both ovaries, number of implanted embryos (IE) estimated as the number of implantation sites, number of alive embryos (AE) estimated as the number of normal uterine swellings, number of resorbed embryos (RE) estimated as the number of small uterine swellings with reduced vascular supply, number of total newborn at third parity (TNB), number of born alive (BA), percentage of mortality at birth (M) measured as the number of kits found dead the day of parturition divided on TNB, embryonic survival (ES) estimated as AE+RE/OR, fetal survival (FS) estimated as AE/TNB, and prenatal survival (PS) estimated as TNB/OR. Finally, the sex ratio was recorded.

Statistical analyses

The results are described by the mean and standard deviation. They were subjected to a one-factor analysis of variance (ANOVA) to determine the effect of the AGD on all measured parameters (hormone concentrations, litter size traits and its biological components). The analysis of parameters used for the evaluation of sexual behavior at mating was performed by the $\chi 2$ test. Analyses were performed using the Statview program (Abacus Concepts, 1996. Inc., Berkeley, CA94704-1014, USA).

Results

Classification of females according to their AGD

The classification of females according to their AGD is presented in Table 1. The AGDM of the females used in this experiment was 26.33 ± 1.30 mm. The females with AGDL and AGDS had distances of 28.49 mm and 24.17 mm, respectively.

Effect of AGD on plasma steroid hormone and cholesterol levels

Plasma concentrations of steroid hormones (testosterone and 17β-estradiol) as well as that of cholesterol are presented in Table 2. Plasma testosterone levels averaged 134 pg/mL and 115 pg/mL in the AGDL and AGDS females, respectively, a significant difference of 14% in favor of AGDL females ($P = 0.003$). The AGDL females showed elevated cholesterol levels compared with those measured in the AGDS females (24%; $P < 0.001$). In contrast, no significant difference was found between the two groups of rabbits for plasma 17β-estradiol levels.

Effect of AGD on sexual behavior of females at mating

Table 3 shows the effect of AGD on the sexual behavior of rabbit does at mating. The receptivity of females, as assessed by direct examination of the vulva or by acceptance or refusal of mating, varies significantly with the AGD. The AGDL females showed higher receptivity rates compared with the AGDS females (31; $P < 0.001$; all methods combined). A reduction in the receptivity rate between the two methods was observed (12% for the indirect method).

In addition, the sexual behavior of female rabbits at mating varied significantly between the two experimental groups. The AGDL females tended to be more aggressive (78%; $P < 0.001$) and mounted males (34%; $P < 0.001$). The AGDL females showed a significant chin marking activity compared with the AGDS females (34%; $P < 0.001$). However, only the AGDL females showed urine marking.

Effect of AGD on litter size and its main biological components

The weight of females at parturition was similar between the females of the two experimental groups (Table 4). Similarly, the ovulation rate did not vary with the AGD of the female. The number of alive embryos on day 12 of gestation was significantly higher in the AGDS females (15%, $P = 0.01$). However, the number of resorbed embryos was significantly higher in the AGDL females (91%; $P < 0.013$).

Embryonic and fetal survival were significantly higher in the AGDS females compared with those measured in the AGDL females (13% and 9%, respectively; $P < 0.01$). Similarly, prenatal survival was significantly higher in the AGDS females (25%; $P < 0.001$).

The litter size at birth estimated by the number of total newborn kits was higher in the AGDS females (13%; $P = 0.031$). The percentage of mortality at birth was significantly higher in the AGDS females (81%; $P = 0.006$). Finally, in the AGDL females, the number of males per litter was significantly higher than in the AGDS females (61.60% *vs.* 41.00%; $P < 0.001$).

Discussion

Plasma testosterone levels were significantly higher in the AGDL females compared with those measured in the AGDS females (134 pg/m *vs.* 115 pg/m). Our results corroborate those reported by several authors indicating that AGDL females have high blood testosterone concentrations (Frederick et al., 1980, in mice; Clark et al., 1992, in gerbils). In rabbits, to our knowledge, the relationship between AGD and testosterone has not been studied in the past. This hormone plays a direct role on AGD in rabbit and in several rodent species. Indeed, it has been shown in both rabbits and mice that exposure during fetal life to high concentrations of testosterone

Table 1. Classification of females according to their AGD (mean ± standard deviation).

	AGD 1 (mm)	AGD 2 (mm)	AGD 3 (mm)	AGDM (mm)
AGDT (n = 48)	26.30 ± 1.31	26.35 ± 1.32	26.32 ± 1.29	26.33 ± 1.30
AGDL (n = 24)	28.46 ± 0.65	28.47 ± 0.63	28.53 ± 0.64	28.49 ± 0.63
AGDS (n = 24)	24.17 ± 0.53	24.15 ± 0.60	24.18 ± 0.55	24.17 ± 0.53

AGDT: anogenital distance for all females; AGDL: long anogenital distance; AGDS: short anogenital distance; AGDM: medium anogenital distance

Table 2. Effect of AGD on plasma steroid hormones and cholesterol (mean ± standard deviation).

	Testosterone (pg/mL)	17β-estradiol (pg/mL)	Cholesterol (mg/dL)
AGDL (n = 24)	134 ± 22.12	254 ± 25.24	38 ± 4.61
AGDS (n = 24)	115 ± 20.45	241 ± 23.53	29 ± 5.14
P	0.003	0.071	< 0.001

AGDL: long anogenital distance; AGDS: short anogenital distance.

Table 3. Sexual behavior of females at mating according to their AGD (mean ± standard deviation).

	Receptivity (direct method) %	Receptivity (indirect method) %	Aggression %	Mounting %	Chin marking %	Urine marking %
AGDL (n = 24)	80.14 ± 8.33	70.27 ± 7.66	25.36 ± 3.24	9.72 ± 0.18	27.17 ± 2.27	05.41 ± 1.04
AGDS (n = 24)	55.47 ± 6.25	48.51 ± 8.11	5.64 ± 1.33	2.45 ± 1.21	18.01 ± 1.98	/
P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

AGDL: long anogenital distance; AGDS: short anogenital distance.

Table 4. Effect of AGD on litter size and its biological components in rabbits (mean ± standard deviation).

Traits	AGDL (n = 24)	AGDS (n = 24)	P
WFM, g	3065.39 ± 200.55	3109.30 ± 178.78	0.427
OR, corpora lutea	10.12 ± 1.91	9.24 ± 1.75	0.103
IE, embryos	8.66 ± 2.01	9.12 ± 1.65	0.391
AE, embryos	7.69 ± 1.79	9.03 ± 1.66	0.01
RE, embryos	0.96 ± 1.61	0.09 ± 0.38	0.013
ES, %	85.62 ± 12.84	98.84 ± 3.22	< 0.001
FS, %	90.18 ± 14.33	99.01 ± 4.04	0.006
PS, %	72.83 ± 14.48	97.25 ± 5.82	< 0.001
TNB, kits	7.83 ± 1.84	8.96 ± 1.68	0.031
BA, kits	7.15 ± 1.69	7.05 ± 0.24	0.775
M, %	1.88 ± 5.94	9.73 ± 11.86	0.006
Sex ratio, %	61.60 ± 18.65	41,00 ± 11.85	< 0.001

AGDL: long anogenital distance; AGDS: short anogenital distance; WFM: weight of a female at mating; OR: ovulation rate; IE: implanted embryos; AE: alive embryos; RE: resorbed embryos; ES: embryonic survival; FS: fetal survival; PS: prenatal survival; TNB: number of total newborn; BA: born alive; M: mortality at birth.

increases significantly AGD in the newborn (Ryan and Vandenbergh, 2002; Bánszegi et al., 2010). In contrast, this effect is eliminated following treatment with antiandrogens (Clemens et al., 1978). It should be mentioned that testosterone in the rabbit is produced by the cells of the internal theca of the ovarian follicles (Erikson and Rayan, 1976), by the interstitial glands of the ovary (Hilliard et al., 1974) and by the adrenal cortex (Kolanowski et al., 1986).

Similarly, cholesterol levels were elevated in the AGDL females (24%; P < 0.001). Elevated cholesterol concentrations would be the cause of the higher plasma testosterone levels noted in AGDL females. Our results are in agreement with those noted by Okoye et al. (2016) in rabbits. Several authors have shown that testosterone synthesis depends on blood cholesterol concentration (Bender et al., 2006). This metabolite is, in fact, the main precursor in the biosynthesis of steroid hormones on the one hand and is an important component of cells, nerve fibers and sperm plasma membrane on the other hand (Wise et al., 1997; Nabi et al., 2017). In addition, testosterone is involved in cholesterol metabolism, and a deficiency in testosterone leads to increased cholesterol levels (Cai et al., 2015). In rabbits, cholesterol levels decrease steadily during gestation, rise rapidly after parturition, and then stabilize between day 8 and 14 of lactation (Quid and Ziversmit, 1986).

AGDL females are more receptive compared with AGDS females, regardless of the method used to assess receptivity (direct or indirect). A high receptivity in AGDL females would be related to the appearance of their vulvas. Indeed, receptive females generally have red or purple and very turgid vulvas. In contrast, non-receptive females have pale, non-turgid vulvas (Ilès et al., 2013). Increasing vulval volume in receptive females could, therefore, increase their AGD. Our results are in agreement with those reported by Kerkouche et al. (2014) in rabbits and those of Dusek et al. (2012) in mice. Dusek et al. (2012) has shown in mice that AGD is influenced by the estrous cycle of the female and females in estrus show the highest AGD. This morphological variation would be mainly related to the hyperhemizing action of estrogens on the genital sphere of the rabbit (Min et al., 2002). Under our experimental conditions, we did not find a significant difference in plasma 17β-estradiol levels between the two groups of females. Such results

could be related to the multitude of factors that can influence female receptivity other than blood estrogen concentrations and physiological status (see review by Theau-Clément, 2008).

AGDL rabbits tend to be more aggressive and mount males more at mating compared with AGDS rabbits. Similar observations have been found in rabbits and several rodent species (Kerkouche et al., 2014). Indeed, several authors report that AGDL females are more aggressive, less attractive to males and mount more with them during mating compared with AGDS females (Rohde Parfet et al., 1990). According to Bánszegi et al. (2010), this behavior is related to the higher testosterone concentrations in AGDL females. Furthermore, exposure of fetuses (male or female) to higher concentrations of testosterone during fetal life increases not only their AGD, but also aggression behavior in adulthood (Bánszegi et al., 2010).

In our experimental conditions, the AGDL females showed significant chin marking activity compared with the AGDS females (27.17% *vs.* 18.01%; $P < 0.001$). The same effect was reported by Hudson and Vodermayer (1992) and could be related to the high concentration of testosterone (Arteaga et al., 2008). It could be also related to the physiological status of AGDL rabbits (females in estrous or more receptive). Spontaneous chin marking increases in estrous females and decreases significantly in pregnant and lactating females (Beyer et al., 2007). Therefore, chin marking could be used by the female as a means to communicate her reproductive status. We also noted that only AGDL females showed urine marking behavior. Our results are in agreement with those reported by Vom Saal and Bronson (1978) in mice. This behavior would also be related to the high plasma levels of testosterone in AGDL females. Urinary marking is a phenomenon that has been described previously in several rodent species, is under the control of androgens and is dependent on AGD (Palanza et al., 1995).

At 12 days of gestation, the total number of implanted embryos was 10.12 and 9.24 embryos in the AGDS and AGDL females, respectively. The total number of implanted embryos approaches that noted by Belabbas et al. (2021) in the same line and those recorded in some French and Spanish lines selected on different reproductive criteria (Blasco et al., 2005; Brun et al., 2006). However, it remains low compared with that measured in other Spanish rabbit lines selected on various reproductive parameters (Laborda et al., 2012; Agea et al., 2020). A significant difference in favor of AGDS females is noted for the number of alive embryos (15%; $P = 0.01$). Such results could be related to the low embryonic and fetal survival rates recorded in AGDL females (85% and 90%, respectively), themselves related to their hormonal status (high plasma testosterone concentrations). Indeed, several studies have shown that AGDL females have higher levels of testosterone in their blood (Van der Hoeven et al., 1992), which is in agreement with our study. This hormone is known to have a direct effect on prenatal mortality. Indeed, the increase of testosterone in pregnant females is at the origin of an increase in the incidence of abortions and embryonic resorptions (Grant, 2007).

Litter size at birth estimated by the number of total newborn was 8.4. It is comparable to that reported by Belabbas et al. (2016). However, it is lower than that obtained in French and Spanish lines with an average of 10 rabbits per litter (Ragab et al., 2012; Theau-Clément et al., 2012). The AGDS females showed a higher litter size (7.83 *vs.* 8.96; $P = 0.031$). These results corroborate those found in the literature, not only in rabbits, but also in several mammalian species, showing that AGDL females produce small litters (Bánszegi et al., 2012; Szencez et al., 2013). This may be related to higher rates of mortality during gestation observed on AGDL females.

The percentage of mortality at birth was significantly higher in the AGDS females (81%; $P = 0.006$). Data from the literature report that mortality does not seem to be influenced by the AGD of the female (Lamberson et al., 1988). A high mortality rate could be related, in part, to the maternal behavior of some females that do not prepare their nests properly, resulting in the complete loss of some litters. Also, Ezzeroug et al. (2020) report that increased litter size at birth is often associated with increased mortality.

In AGDL rabbit does, the number of males per litter was significantly higher than in AGDS females (61% *vs.* 41%). Several authors have shown that the AGD of the female can influence the sex ratio of her litter in different species (rabbit, mouse, rat, birds and pig), and females with a long AGD tend to give birth to more males per litter (Grant and Irwin, 2005; Goerlich et al., 2009; Bánszegi et al., 2012). The hormonal status of the female at conception of her fetuses could be the cause of this variation in the sex ratio (Szencez et al., 2013).

Conclusion

In conclusion, this work is the first to study the effect of the AGD measured before mating on hormones and sexual behavior in rabbits, litter size and its biological components. From the results of this study, we can conclude that the AGD could be used as a predictor of some reproductive parameters in rabbits. Indeed, it turns out that AGDL rabbits are more receptive to males, aggressive and mark their territory more. However, they have a low litter size at birth related to higher embryonic and fetal mortality.

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Conflict of interests

Authors declare no conflict of interest.

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