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Original article

POWER AND COLOUR DOPPLER ULTRASONOGRAPHY FOR EVALUATION OF THE OVARIAN AND UTERINE HAEMODYNAMICS OF INFERTILE MARES

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Summary

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The present study was undertaken to investigate the haemodynamics of the ovary and uterus in infertile mares using power and colour Doppler ultrasonography. Forty-seven Arabian mares were handled in the current work through several examinations. Based on the breeding history complaints, physical, vaginal examination and Doppler examination, 12 mares were found to be suffering from abnormal ovarian structures (Experiment I). In addition, nine mares with normal cyclic activity were kept as control. Another 16 mares were found to suffer from abnormal uterine conditions and 10 mares with normal uterine findings served as control (Experiment II). Blood flows to the anovulatory follicle, ovarian inactivity, granulosa cell tumour were compared to those to the dominant follicle and luteal blood flow. Uterine blood flow of cysts, abnormal uterine fluids (endometritis) during estrus and diestrus were compared to normal uterine vascularisation during estrus, diestrus and post-mating. Results showed that granulosa cell tumour had the highest red colour blood flow and total colour blood flow area. Blue colour blood flow area of the corpus luteum was higher compared to the dominant follicle and inactive ovary. Uteri with uterine cyst and abnormal uterine fluids during estrus of infertile mares had high red colour blood flow, blue colour blood flow and power blood flow areas compared to normal uterus during estrus. It could be concluded that Doppler ultrasound could distinguish between normal ovaries with normal or abnormal structures. Moreover, the increased uterine blood flow area of mares with abnormal fluids in their uteri (endometritis) and uterine cysts could be distinguished by comparison to the normal uterine blood flow during estrus.

Key words: abnormal uterine secretion, anovulatory follicle, granulosa cell tumour, inactive ovary, uterine cyst

INTRODUCTION

At the end of the 90s, studies utilising Doppler ultrasonography to determine physiologic and pathologic alterations in the reproductive tract of the mare started their development. Uterine, follicle and corpus luteum vascular perfusion was the focus of most studies. Colour and power Doppler ultrasonography is a noninvasive pulse wave technique that has been recently used for the transrectal study of blood flow in the reproductive tract of large domestic animals (Ginther, 2007). In addition to the uterine artery (Bollwein *et al.,* 1998) and its branches, the mesometrial attachment and vessels located in the endometrium and myometrium can be evaluated for studying uterine haemodynamics (Silva *et al.*, 2005). Also, colour and power Doppler ultrasonography were used to study the uterine haemodynamic of pregnant and non-pregnant mares (Ferreira *et al.,* 2008). Doppler ultrasonography has been already performed to study uterine blood perfusion in cyclic mares (Bollwein *et al.,* 1998). It was shown that poor uterine perfusion was a reason for infertility (Rubinstein *et al.,* 1999). Doppler was also used to study uterine blood flow of sub-fertile mares (Stolla & Bollwein, 1997) during the estrous cycle in connection to ovarian hormones (Bollwein *et al.,* 2002), and in early pregnant mares (Bollwein *et al.,* 2003). Persistent anovulatory follicles are connected with the inability of a dominant follicle to ovulate (Ginther *et al.,* 2006). The incidence of a major anovulatory wave in mares preceding the ovulatory wave was 25% (Ginther *et al.,* 2004). The number of mares developing dominant anovulatory follicles during the transitional period differs among reports, from 15 of 15 horses (Ginther, 1990; Watson *et al.,* 2002), to three of eight ponies (Donadeu & Ginther, 2002). The presence of anovulatory haemorrhagic follicles during the estrous cycle of mares causes financial impacts, slowing conception and increasing the number of the services per pregnancy (Lima, 2015). The future anovulatory dominant-sized follicle is characterised by LH deficiency and reduced blood flow area (Acosta *et al.,* 2004). Haemorrhagic anovulatory follicle is the most common pathological anovulatory condition in the mare (Martínez-Boví & Cuervo-Arango, 2016). Alterations in uterine vascular perfusion have been detected during the estrous cycle, during pregnancy and in cases of infusion of inflammatory substances (Bollwein *et al.,* 1998). Also, important haemodynamic alterations in old mares, as an increase in vascular resistance, have been described. In mares, peri-glandular fibrosis of the endometrium is considered to be the major diagnosable cause of embryonic and foetal loss in older mares (Ferreira *et al.,* 2011).

Therefore, the present study aimed to reveal the accuracy of Doppler ultrasound application through monitoring of ovarian and uterine haemodynamic for the diagnosis of reproductive problems in mares.

MATERIALS AND METHODS

Experimental location

This study was conducted from January 2015 to September 2017 at the Equestrian Club (El Basateen), Tora prison horse and Abassia Horse studs belonging to Police department, Ministry of the Interior in addition to some private horse studs located in Giza province, Egypt. The experiments with the animals have been conducted with the ethical approval of the

Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt.

Experimental animals, feeding and design

Forty-seven Arabian and native brood mares aged between 4 and 18 years of age, weighing 400–600 kg were handled in the current work through several examinations. Mares were kept under natural light and temperature and artificial light was used at night within the paddock. They were maintained on a commercial pelleted ration and hay with free access to water. All animals were healthy and kept under strict control measures for internal and external parasitism, undergoing a periodical deworming and prophylactic vaccination against the endemic diseases. During the course of the work (based on the breeding history complaints, physical, vaginal examination and Doppler examination), 12 mares were found to suffer from abnormal ovarian structures (Experiment I). In addition, nine mares of normal cyclic activity were kept as a control group. Another 16 mares were found to suffer from abnormal uterine condition and 10 mares with normal uterine finding served as control (Experiment II).

- *Experiment I: mares with abnormal ovarian structures*

Mares were assigned to four groups based on the ultrasound scanning of ovarian structures into normal dominant follicle (n=5), normal corpus luteum (n=4), anovulatory follicle (n=4), granulosa cell tumour (n=6), and inactive ovary (n=2).

- *Experiment II: mares with abnormal uterine findings*

Twenty-six mares were classified into seven groups: normal uterus during estrous phase (n=4) and diestrus (n=6); uterine cyst during estrus (n=4) and diestrus phase (n=3); abnormal uterine secretions during estrus (n=3), and diestrus phase (n=3), and abnormal uterine secretions after mating (post-mating endometritis; n=3). Estrus was detected using a male. Teasing was done as massive teasing to all mares to detect those in heat. All mares were scanned daily during one estrous cycle to determine the stage of estrus. A trans-rectal pulsed-wave Doppler ultrasound scanner equipped with 12 MHz linear-array transrectal transducer (Sono Ace R3; Medison, Samsung, South Korea) was used for the examination of the uterus and all structures on both ovaries. Both uterine horns and uterine body were scanned with colour and power Doppler modes. All scans were performed by the same operator during the experiment. Examinations were performed from 8 AM to 11 AM to avoid the high temperature and humidity.

Uterine blood flow measurement

To evaluate which colour or the single colour power Doppler modes would be recommended for future evaluation of uterine vascular perfusion, both colour and power Doppler modes were used during this study. Vascular perfusion of the uterus (all layers combined) was estimated subjectively by one operator during the study period from the cross sections of a horn using single colour power and colour flow mode. Only the colour signals that appeared to be within the limits of the uterine body or horns were considered. The uterine vascular perfusion was estimated subjectively using the percentage of uterine tissue with colour signals of blood flow during real-time cross sectioning of the uterus. Multiple cross sections were viewed because of animal and uterine movements and the variation resulting from angles of insonation (Bollwein, 2002). Each blood flow mode was activated, and the blood flow area within the

uterine wall was quantified from the colour images. All scans were done at a pulse-repetition frequency, and identical colour gain settings were used. The colour mode was used to determine the blood flow area within the uterine wall.

Ovarian blood flow measurement

For each ovary during estrous cycle, the number of follicles, the diameter of the largest one, the presence of the corpus luteum and the diameter of the corpus luteum was recorded using B-mode, colour and power Doppler modes. The diameter of the ovarian follicles per ovary was measured with the electronic calipers of the ultrasound. The others (>15 mm) were counted and their diameter was estimated using the electronic calipers of the ultrasound. Follicles were grouped into two categories. The first large follicle with a maximum diameter (35 mm) on either ovary during the follicular phase and accompanied by uterine oedema and estrous behaviour is considered the dominant follicle (preovulatory). The presence of a follicle of diameter ≥ 3 cm with an absence of uterine estrous oedema and estrous behaviour was considered anovulatory follicle. The corpus luteum (CL) of diameter ≥ 3 cm accompanied with no estrous oedema was considered as the luteal phase. A dominant preovulatory follicle was monitored before failure in the presence of uterine oedema and estrous behaviour, but instead to ovulate, it persisted and became anovulatory which lasted for more than 12 days without estrous behaviour. The blood flow end focuses for all ovarian structures were quantified by trans-rectal colour Doppler ultrasonography using colour mode and power flow mode.

Image analysis

Digital video recording was exported from the hard disk of the ultrasound machine to a PC. Three good pictures of CL (in B-, colour- and power-mode) were chosen. Afterward, they were processed using image analysis software (Adobe Photoshop CC software 1990–2013, Adobe Systems) for further detailed digital image analysis. The region of each CL was manually selected by the magnetic lasso tool in Photoshop and the following parameters were analysed: luteal blood flow (LBF) area (in pixels) away from the probe, which coloured blue (in pixels), blood flow area towards the probe, which coloured red (in pixels) and single colour power blood flow area (in pixels). The mean values of the three selected images were calculated and one mean value was used as referential. Percent of the coloured pixels were calculated by dividing coloured power area (in pixels) by the total CL area (in pixels) and was used as a quantitative index of the changes in blood flow within the luteal tissue. For Doppler blood flow determinations, the flow mode was activated, and the blood flow area within the follicular wall was quantified from the colour images. Areas of colour represent regions with a flow velocity higher than 10 mm/s. All scans were performed at a pulse-repetition frequency of 6 Hz. Identical colour gain settings were used for all scans. The colour mode was used to determine the blood flow area in the follicular wall. The angle of insonation was not calculated because of the small diameter of vessels, but care was taken to obtain the maximum colour intensity. Identical colour gain settings were used for all scannings.

Statistical analysis

Data were subjected to the statistical analysis using Statistical Package for Social Science (SPSS 16, 2007). Data are represented as means ± standard error of the mean (SEM). Simple one-way ANOVA was done to identify the effect of infertility problem in ovary or uterus on both colour and power Doppler blood vascularisation area. The Duncan's multiple range test was used to discriminate significant means.

RESULTS

Colour Doppler of different ovarian structures

The area of the colour signal toward probe (red/pixel) was significantly increased ($P<0.05$) in granulosa cell tumour than in the inactive ovary. However, no significant differences were noticed among anovulatory follicles, dominant follicles and corpus luteum (Table 1). On the other hand, the area of the colour signal away probe (blue/pixel) significantly ($P<0.05$) decreased in inactive ovary compared to corpus luteum, while there was no significant difference among inactive ovary, dominant follicle, granulosa cell tumour and anovulatory follicle groups (Table 1).

The mean red plus blue colour blood flow was similar among different ovarian structures but was significantly ($P<0.05$) higher for granulosa cell tumour as compared to the inactive ovary (Table 2). Data in Table 2 showed the changes in values of power blood flow vascularisation area in different ovarian structures. The data revealed that the average power Doppler blood flows were significantly ($P<0.05$) higher in the corpus luteum than those obtained in the dominant follicles.

Uterine colour and Doppler blood flow

During estrus, the blood flow area toward the probe (red) and the flow away from the transducer (blue) of the uteri with abnormal uterine secretions or with endometrial cysts were significantly higher compared to those of the normal uterus (Table 3). During diestrus, the red blood flow to the uterus with uterine endometrial cyst and when post-mating endometritis was recorded, were insignificantly lower than those of the normal or the abnormal one with uterine secretions. The normal uterine red or blue colour blood flow vascularisation area during estrus were lower than those recorded during diestrus. The reverse was true when abnormal uterine

Table 1. Effect of ovarian structure on red and blue colour blood flow vascularisation area (in pixels) in mares with different ovarian structures (mean ±SEM)

Ovarian conditions	Number	Colour Doppler mode	
		Red colour	Blue colour
Dominant follicles	5	616 ± 64^{ab}	735 ± 180^{ab}
Corpus luteum	4	1354 ± 481^{ab}	1974 ± 647^{b}
Granulosa cell tumour	6	2101 ± 565^{b}	1611 ± 172^{ab}
Anovulatory follicle	4	1288 ± 225^{ab}	1570 ± 297^{ab}
Inactive ovary	2	66 ± 66^{a}	113 ± 297^{a}

means with different superscripts in the same column (a, b) are significantly different at $P<0.05$.

Table 2. Effect of ovarian structure on red plus blue colour and power blood flow vascularisation area (in pixels) in mares with different ovarian structures (mean ±SEM)

Ovarian conditions	Number	Colour Doppler mode	
		Red+blue colour	Power blood flow
Dominant follicles	5	1351 ± 237^{ab}	2248 ± 82^{a}
Corpus luteum	4	3327 ± 870^{ab}	7383 ± 1935^{b}
Granulosa cell tumour	6	3711 ± 718^{b}	4912 ± 407^{ab}
Anovulatory follicle	4	2857 ± 483^{ab}	5045 ± 856^{ab}
Inactive ovary	2	179 ± 67^{a}	4045 ± 856^{ab}

means with different superscripts in the same column (a, b) are significantly different at $P<0.05$.

Table 3. Effect of different reproductive conditions on uterine red and blue colour blood flow vascularisation area (in pixels) in mares (means ±SEM)

Uterine conditions	Number	Colour Doppler mode	
		Red colour	Blue colour
Uterine cyst during estrus	4	1171 ± 251^{ab}	1681 ± 388^{b}
Uterine cyst during diestrus	3	565 ± 67^{ab}	1350 ± 303^{ab}
Normal uterus during estrus	4	129 ± 45^{a}	202 ± 49^{a}
Normal uterus during diestrus	6	812 ± 234^{ab}	1059 ± 232^{ab}
Abnormal uterine secretions during estrus	3	1195 ± 590^{b}	1666 ± 821^{ab}
Abnormal uterine secretions during diestrus	3	869 ± 13^{ab}	335 ± 60^{ab}
Uterus post-mating uterine secretions	3	525 ± 59^{ab}	625 ± 60^{ab}

means with different superscripts in the same column (a, b) are significantly different at $P<0.05$.

secretions or endometrial cysts are observed in the uterus. Moreover, uterine cysts and uteri with abnormal uterine secretions tended to have low red ($P=0.08$) or blue ($P<0.05$) colour blood flow vascularisation area during diestrus compared to those obtained at the time of estrus (Table 3). In contrast to the insignificant decrease of red blood flow of uteri with post-mating endometritis compared to those with abnormal uterine secretions, the blue blood flow was higher.

Maximum improvement in the total colour (red+blue) uterine blood flow was noticed in uterine cyst and abnormal secretion during estrus compared to those observed during diestrus and post-mating (Table 4). These results exhibited changes in the value of the mean power blood flow among different uterine findings. The power blood flow vascularisation area of the uterus with uterine cyst was significantly ($P<0.05$) higher during estrus compared to that during diestrus. The abnormal uterine secretions during estrus insignificantly increased the power blood flow vascularisation during estrus compared to either diestrus or post-mating. In contrast,

Table 4. Effect of different uterine conditions on red plus blue colour and power blood flow vascularisation area (in pixels) in mares (mean ±SEM)

Uterine conditions	Number	Colour Doppler mode	
		Red+blue colour	Power blood flow
Uterine cyst during estrus	4	2852 ± 633^{ab}	3830 ± 208^{b}
Uterine cyst during diestrus	3	1915 ± 238^{ab}	496 ± 44^{a}
Normal uterus during estrus	4	331 ± 93^{a}	1346 ± 549^{ab}
Normal uterus during diestrus	6	1870 ± 377^{ab}	1870 ± 689^{ab}
Abnormal uterine secretions during estrus	3	2861 ± 1399^{b}	3518 ± 725^{ab}
Abnormal uterine secretions during diestrus	3	1205 ± 74^{ab}	1877 ± 731^{ab}
Uterus post-mating uterine secretions	3	1150 ± 72^{ab}	1929 ± 284^{ab}

means with different superscripts in the same column (a, b) are significantly different at $P<0.05$.

the power blood flow vascularisation area of the normal uterus was not significantly lower during estrus compared to diestrus.

DISCUSSION

The classic colour-flow mode uses two distinct colours, usually variations of red and blue, to represent the vascular blood perfusion of a structure. Also, coloured pixels indicate the blood red cells direction toward or away from the transducer. By convection, red coloured spots indicate blood flow moving toward the transducer, but blue coloured spots represent blood-red cells moving away from the probe (Ginther *et al.,* 2007). Power Doppler increases the sensitivity for displaying blood flow within the soft tissue from 3 to 5 times, compared to conventional colour display for Doppler sonography (Miyazaki, 1998). The greater sensitivity allows the evaluation of vessels with a small diameter or slow flow that does not appear on a conventional colour-flow image because of incompatible velocity ranges and Doppler angles. Power Doppler is angle independent and is not influenced by aliasing because velocity is not considered directly (Amso *et al.,* 2001).

Vascular perfusion of a structure can be quantitated by many coloured pixels in an image or can be estimated subjectively by the extent of the coloured spots (Ginther *et al.,* 2007). Additionally, the intensity of the coloured pixels suggests the velocity of blood flow ranging from dark to bright tonalities for slower and faster velocities, respectively (Ferreira *et al.,* 2011). In power-Doppler mode, the blood flow movement is graduated using a single colour and the colour pixels intensity vary according to the power of the Doppler signals (number of blood red cells moving at a specific velocity). Power-flow imaging has greater sensitivity to weak blood flow and is independent of the Doppler angle, its colour display is not influenced by aliasing and reduced blooming artifacts are observed when compared to the classic colour-mode Doppler (Ferreira *et al*., 2011). Regarding the presence of a substantial number of small vessels with slow blood flow that do not appear on a conventional colour-flow image, it is suggested to use power-flow mode for the vascular perfusion evaluation of follicles,

corpus luteum and uterus (Ferreira *et al*., 2008). The present study demonstrated that in mares, transrectal Doppler ultrasonography was a reliable and consistent method for the detection of velocity patterns of the ovarian blood flow during the normal and abnormal estrous cycles. The mean red colour blood flow was higher in granulosa cell tumour than those obtained in the inactive ovary. The increased ovarian vascularisation in the ovary with granulosa cell tumour in mares as expressed by significantly increased red colour blood flow area and red plus blue colour blood flow area is in agreement with the association of malignant tumour cells with an increase in microvessel density and peak systolic velocity in patients with ovarian cancer (Hata *et al.,* 1998). Also, colour Doppler showed vascularity in 97.5% of malignant ovarian tumours in contrast to only 68.1% of benign tumours and 87.5% of malignant tumours had PI less than 0.8 in contrast to only 4.54% of benign ones. Similarly, 82.5% of malignant tumours had RI less than 0.6 in contrast to only 6.81% of benign tumours (Shah *et al.,* 2013).

The ovary with granulosa cell tumour (GCT) ranged from 70 mm in diameter with a large, thick-walled cavity containing echogenic fluid to diameter 60 cm with the presence of functional contralateral ovary and no behavioural changes (Vanderwall *et al.,* 2013). GCTs had variable ultrasonographic appearance. In mares, GCT are always unilateral, slow growing, and benign. The use of transrectal ultrasonography often revealed a multicystic or honeycombed structure (Ellenberger, 2007). An affected ovary can appear ultrasonographically as a single solid mass, fluid-filled structure, heterogeneous multicystic mass, or any combination of these two. The tunica albuginea surrounding the ovary can often appear thickened (Ball *et al.,* 2008). The vascularisation of the corpora lutae monitored during the present study indicated a non-significant increase of red and blue colour blood flow area but a significant increase of power blood flow area compared to ovulatory, and anovulatory follicles. The day after ovulation significantly affected the area of CL in mares (Abdelnaby *et al.,* 2017). The anovulatory follicle recorded herein during 3rd ovulation persisted for 16 days without ovulation or regression and attained a diameter >6 cm. In this respect, the incidence of haemorrhagic anovulatory follicles (HAFs) is approximately 5% and 20% of estrous cycles during the early and late ovulatory season, respectively. Anovulatory follicles were more common in old mares (>20 years), tended to occur repeatedly in individuals, and occurred most frequently during the late follicular phase (Ginther *et al.,* 2007). The anovulatory follicle observed in the present study had the largest diameter in luteal phase compared to those observed in follicular ones. In agreement with our results, a portion of the anovulatory follicles achieved a measurement that was not recognisable from the most extreme width of an ovulatory follicle (Ginther *et al.,* 1990). Instead of ovulating and forming a typical corpus luteum (CL), the anovulatory follicle increases in diameter, with echoic particles, and eventually luteinises with active production of progesterone (Toda, 1990). The natural occurrence of the spontaneous luteinised anovulatory follicle was difficult to predict (Cuervo-Arango & Newcombe, 2012). The current study reported that mares with uterine cysts had high red+blue vascularisation area compared to ultrasonically normal mares which agree with the lower pulsatility index (PI) and greater end diastolic velocity and time-

averaged maximum velocity of the mesometrial vessels in mares with uterine cysts compared to controls. The effect of uterine cyst on uterine vascularisation depended on their size (Ferreira *et al.,* 2008). However, the difference between the current findings and the results of Ferreira *et al.* (2008) may refer to the difference in the breed studied, the difference of the Doppler mode used and the effect of the phase of the estrous cycle where a small uterine cystic area did not affect uterine haemodynamics. In contrast, the normal uterus showed a non-significant increased vascularisation during diestrus compared to estrus. The only significant increase of the uterine red colour blood flow vascularisation area during estrus was observed for uteri with abnormal secretions compared to normal uterus. Using colour- and power Doppler modes, this study reported a significant difference between the ipsilateral uterine horn (dominant) and the contralateral horn (non dominant) with a reverse correlation between them (Abdelnaby *et al.,* 2016). In contrast to colour and power Doppler, spectral Doppler mode showed no differences ($P>0.05$) and a high correlation ($r=0.81$; $P<0.0001$) between PI values of the dominant and non-dominant uterine arteries (Bollwein *et al.,* 2004). In agreement with the change of the uterine vascular perfusion during diestrus compared to estrus whatever ultrasonically normal uterus, with uterine cyst or with endometrial secretions observed during this study, time averaged maximum velocity (TAMV) and pulsatility index (PI) of the ipsilateral uterine horn had mild cycle-dependent fluctuations in cyclic cows and there were no significant differences in these values between consecutive examinations (Honnens *et al*., 2008). Moreover, previous results showed day and horn dependence on uterine blood flow in non-pregnant mares (Abdelnaby *et al.,* 2017). Uterine vascular perfusion and mesometrial pulsatility index (PI) evaluated every hour from zero hour H0 (moment immediately before AI) to twelve hours (H12) revealed a pronounced and transitory increase on uterine vascular perfusion and did not vary between old and young mares or different grades of endometrial degenerations. Moreover, reduced blood flow of the uterus during the post-breeding period was strongly associated with endometrial degenerative changes in mares, regardless of age (Ferreira *et al.,* 2015).

In conclusion, colour and power Doppler ultrasound could distinguish between normal follicle, anovulatory follicle, functional corpus luteum, granulosa cell tumour and inactive ovaries. This study found out that the estrus phase affected blood flow vascularisation of uterine cyst and endometritis. Power Doppler was more suitable for evaluating uterine vascularisation during the estrous cycle. Moreover, the increased uterine blood flow area of mares with abnormal fluids in their uteri (endometritis) and uterine cyst could be distinguished compared to the normal uterine blood flow during estrus. This study confirmed that Doppler ultrasonography was a useful tool in equine reproduction and increasing the experience for its application among veterinary practice will reflect on the improved reproductive performance of farm animals. Further studies including endocrine profiling or endometrial cytology and bacteriology in subfertile mares are needed.

CONCLUSIONS

Though the ovarian neoplasm in mares had no growing follicles, it had the highest area and vascularisation with either colour

or power Doppler. The inactive ovary had neither follicles nor vascularisation and had the lowest area. The remarkably increased blood flow to the normal uterus during estrus was responsible for the secretion of estrous mucus, but the presence of abnormal secretions (endometria) increased the uterine blood flow to the systemic circulation to influence the response to uterine inflammation. Uterine secretions post-mating or abnormal/uterine endometrial cysts disturb the uterine vascularisation during the estrous cycle phases and may interrupt the implantation of the embryos after breeding.

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