



Small Mammal Research

Effect of prenatal overfeeding on the growth performance and postweaning behavioral parameters in rabbits



Panagiotis Simitzis^{a,*}, Athanasios Kiriakopoulos^a, George Symeon^b,
Michael Goliomytis^a, Antonis Kominakis^a, Iosif Bizelis^a, Stella Chadio^c,
Olga Pagonopoulou^d, Stelios Deligeorgis^a

^a Department of Animal Breeding and Husbandry, Faculty of Animal Science and Aquaculture, Agricultural University of Athens, Athens, Greece

^b Research Institute of Animal Science, HAO-DEMETER, Paralimni, Giannitsa, Greece

^c Faculty of Animal Science and Aquaculture, Department of Anatomy and Physiology of Farm Animals, Agricultural University of Athens, Athens Greece

^d Department of Physiology, Medical School, Democritus University of Thrace, Alexandroupoli, Greece

ARTICLE INFO

Article history:

Received 1 November 2018

Received in revised form

8 May 2019

Accepted 1 June 2019

Available online 8 June 2019

Keywords:

embryonic programming
prenatal overfeeding
postweaning behavior
rabbit

ABSTRACT

Does are usually fed *ad libitum* during pregnancy, a fact that can result in intrauterine overfeeding and can have detrimental effects on embryonic programming with permanent changes in the physiology and behavior of the offspring. An experiment was therefore conducted to examine the effect of does' overfeeding during two different periods of pregnancy on the behavior of kits after weaning. Fifteen does were allocated into three groups: control (M) (100% of maintenance energy requirements [MER] throughout pregnancy), O1 (129% of MER between the 7th and 19th of pregnancy), and O2 (138% of MER between the 20th and 27th day of pregnancy). At the ages of 50 and 65 days, the behavior of 24 randomly selected rabbits in home cages was recorded for three consecutive days, while an open field test that constitutes a good indicator of locomotion score and stress was conducted for another 24 rabbits. Rabbits in O1 and O2 groups had more meals in shorter duration and moved and explored their environment less than the controls ($P < 0.05$). No significant effects on the exhibition of behavior were found during the open field test with the exception of rearing that was displayed more by the O1 and O2 rabbits. Comfort behaviors and resting had increased values, although frequency and duration of eating and drinking were significantly decreased at the age of 65 days ($P < 0.05$). Males had higher values for frequency of eating than the females ($P < 0.01$). We concluded that behavior of rabbits born from overnourished does was modified, with decreased values for locomotion and exploration.

© 2019 Elsevier Inc. All rights reserved.

Introduction

Extensive epidemiological data present an association between maternal nutrition during pregnancy and offspring metabolic function. In particular, the nutritional intrauterine environment to which the fetus is exposed during prenatal and perinatal development could have long-term implications on the function of the appetite-regulating neural network and therefore the way in which an individual regulates energy balance throughout later life (Bouret, 2010; Muhlhausler, 2006). Maternal overfeeding during pregnancy

induced permanent alterations in the hypothalamic neurons that express the appetite-regulating neuropeptides and results in increased fetal plasma glucose and insulin concentrations leading to persistent hyperphagia, glucose intolerance, and associated obesity in rats (Chen et al., 2008). At the same time, higher glucose concentration and relative subcutaneous fat mass were observed in lambs born from overnourished ewes (160% of metabolizable energy requirements) during their early postnatal life (Muhlhausler et al., 2006). Glucose, insulin, and leptin act through a range of mechanisms to alter hypothalamic expression of the orexigenic neuropeptides “neuropeptide Y and agouti-related peptide” and the anorexigenic precursors “pro-opiomelanocortin and neuropeptide” and cocaine- and amphetamine-regulated transcript and thereby regulate appetite and energy balance (Karla et al., 1999; Williams et al., 2001). Moreover, high glucose levels in late prenatal life increase the expression of genes within adipose cells that are

* Address for reprint requests and correspondence: Panagiotis Simitzis, Department of Animal Breeding and Husbandry, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece. Tel: +302105294427; Fax: +302105294442.

E-mail address: pansimitzis@aua.gr (P. Simitzis).

responsible for the promotion of lipid storage and the development of new adipocytes (Muhlhauser et al., 2007).

Despite the well-known programming effects of maternal high-fat diet on metabolism, scarce data exist concerning the possible consequences of maternal overfeeding on the behavioral parameters of the offspring. According to previous studies in rats and primates, exposure to excess nutrient supply during critical windows of fetal development may also cause brain developmental abnormalities in offspring's hypothalamic and hippocampal areas and neural pathways, such as the serotonergic, dopaminergic, and opioid systems, which result in increased anxiety, impairment in spatial learning and memory, and desensitization of the reward system (Cordner and Tamashiro, 2015; Sullivan et al., 2014; 2015).

It is generally assumed that maternal high fat intake could negatively affect aspects of anxiety-related behavior, exploration, learning, and motivation behavior in offspring of rats (Bilbo and Tsang, 2010; White et al., 2009), mice (Peleg-Raibstein et al., 2012), and nonhuman primates (Sullivan et al., 2010). However, there is also a study that illustrates reduced anxiety levels in the male offspring of Wistar rats as an effect of maternal obesity (Wright et al., 2011).

Overfeeding is also related with increased risk of behavioral disorders associated with anxiety in humans, effects that are observed more frequently in females (Desai et al., 2009; Rofey et al., 2009). At the same time, male and female rodents typically show different scores in tests used to assess emotional and exploratory behaviors (e.g., open field test) that are generally attributed to differences in emotionality, latency to interact, or fear responses (Archer, 1975). Age could also influence behavioral parameters of rabbits because they appear to display increased values for resting with age (Simitzis et al., 2013, 2015).

Animal models of diet-induced obesity have been important tools for understanding the influence of overfeeding on metabolic development (Bouret, 2009). In general, rats and mice are considered as “model species” for research related to brain development, but other animals such as guinea pigs, ferrets, and rabbits could also be used (Clancy et al., 2007). Rabbits, especially, could serve as an excellent potential model for studying the critical age-related changes in the brain that are involved in the neurobiology of learning and memory (Woodruff-Pak, 1988). Despite the critical windows of development during which exposure to altered levels of nutrients can have long-term effects on the function of appetite, the regulating neural networks may differ between species (Muhlhauser et al., 2008). The rabbit can be classified as a perinatal brain developer like the human infant because the peak in brain growth and development occurs close to the time of birth (Eichenwald and Fry, 1969). The present study was conducted to determine possible effects of prenatal overfeeding (150% of the recommended energy maintenance requirements) during different developmental windows on behavioral parameters of offspring at the ages of 50 and 65 days in rabbits.

Material and methods

Animals

Fifteen Hyla Nouvelle Generation commercial hybrid does (*Oryctolagus cuniculus*) of similar body weight ($4,498 \pm 154$ g) and age (14 months) were purchased from a breeding farm (Farma Trompetas, Megara, Greece). After their arrival at the experimental facilities, does were kept indoors in individual home cages ($0.40 \times 1.00 \times 0.39$ m), and 7 days later, they were artificially inseminated with the sperm of Hylamax bucks. Seven days after the artificial insemination, does were randomly allocated to 3 groups: the control group (C) ($n = 5$) that was fed 100% of the recommended energy

maintenance requirements throughout pregnancy and lactation, and the two overfed groups O1 ($n = 5$) and O2 ($n = 5$) that were offered 150% of the recommended energy maintenance requirements between the 7th and 19th day and between the 20th and 27th day of pregnancy, respectively. Gestation in the rabbit can be divided into 3 periods: period between fertilization of the ovum or ova and implantation (7th day), the period of organogenesis (between 7th and 18th–19th day), and the period of fetal growth (the last third of gestation). As a result, the time points for the dietary maternal manipulation were selected according to these three periods (implantation, organogenesis, and rapid growth) (Anderson and Henck, 1994). After the experimental period, feed was *ad libitum* offered to all treatment groups. Maintenance energy requirements were estimated according to the study by Xiccato and Trocino (2010). The diet consisted of a commercial concentrate mixture (in pellets) (Viozokat SA, Katerini, Greece) containing barley (25%), wheat bran (14%), soybean meal (9%), sunflower meal (10%), alfalfa meal (20%), sugar beet (15%), soybean oil (1%), molasses (3%), limestone (1%), monocalcium phosphate (1%), salt (0.3%), lysine (0.1%), methionine (0.1%), threonine (0.05%), choline (0.15%), and a vitamins and minerals premix (0.2%). Does had free access to water. At days 0, 7, 14, 21, and 28 prepartum, the does were individually weighed. Feed intake was also recorded individually on daily basis.

Rabbit kits were born after 31.5 ± 0.3 days of gestation. At the second day postpartum, kits were cross-fostered within treatment groups to ensure equal litter sizes per doe. After weaning (35th day of age), 48 (16 animals per treatment group and 24 per sex) of a total of 156 rabbits were randomly selected and kept indoors in individual home cages ($0.41 \times 0.33 \times 0.29$ m) with wire mesh floors, under the following environmental condition: temperature: $22.5 \pm 3.5^\circ\text{C}$, relative humidity: $50 \pm 20\%$, and lightning: 12 h/12 h light/dark cycle. Each cage was equipped with a metal feeder and an automatic nipple drinker. Feed was provided *ad libitum*, and rabbits had free access to water. The diet consisted of a commercial concentrate mixture (Viozokat SA, Katerini, Greece) (in pellets) containing barley (17%), wheat bran (23%), soybean meal (7%), sunflower meal (11%), alfalfa meal (20%), sugar beet (15%), soybean oil (1%), molasses (3%), limestone (1%), monocalcium phosphate (1%), salt (0.4%), lysine (0.1%), methionine (0.1%), threonine (0.05%), choline (0.15%), and a vitamins and minerals premix (0.2%). Individual weight of the kits was recorded at parturition and at the ages of 21, 35 (weaning), 50, and 65 days. Feed intake was recorded weekly between the 42nd and 70th day of age.

Behavioral recordings of offspring in their home cages

At the age of 50 and 65 days, behavioral parameters of 24 rabbits (four males and four females from each treatment group) were recorded on three consecutive days. The behaviors observed could be categorized as maintenance behaviors (eating and drinking), resting, comfort, and locomotory-investigatory behaviors. The definitions of these behavioral patterns are presented in Table 1, according to previous studies (Gunn and Morton, 1995; Morisse and Maurice, 1997). The behavior of the rabbits was recorded using four video cameras with infrared lighting (TX-14300A, Turbo-X). Each camera was placed in a fixed position to record behavior patterns in 6 cages of rabbits. The recorded data were stored in a digital video recorder equipped with a hard disk (TX168; Telexper Inc., USA). One person decoded all the recordings and was blind to group for all the behavioral measurements.

Open field test

At the ages of 50 and 65 days, rabbits that were not included in the behavioral recordings in home cages (four males and four

Table 1
Behavioral parameters recorded in home cages and their definition according to the existing literature (Gunn and Morton, 1995; Morisse and Maurice, 1997)

Behavioral parameter	Definition
Frequency of eating	Oral seizing of feed and chewing/munching of it or having the muzzle over the feeder for over 10 s
Duration of eating (min)	Cumulative duration of acts of eating from a feeder
Frequency of drinking	Drinking water from nipple drinkers
Duration of drinking (min)	Cumulative duration of acts of drinking from a nipple drinker
Resting	Sleeping, lying at any position (unsleeping, stretched), and sitting
Comfort behaviors	Any behavior form connected with the own body of the animal (washing, licking, starching)
Locomotory and investigatory behaviors	Any voluntary change of position (treading, running, hopping, prancing) and behavior forms connected to the cage or to its equipments (rubbing, licking, smelling, marking with the chin).

females from each treatment group) were tested in an open field once a day on 2 consecutive days between 08:00 am and noon (in March; under natural light). Open field test is generally used as a paradigm to measure emotionality (i.e., exploratory behavior) and as an indicator of stress because time spent in the center of the field is typically used as a measurement of anxiety behavior (Candland and Campbell, 1962; Kersten et al., 1989; Sasaki et al., 2013).

The open field measured 2 × 2 m was surrounded by 80-cm-high polished plywood walls and was situated in a corner of the barn in which the rabbits were housed. Each test lasted 5 min, and after its end, the box, floor, and walls were wiped with a cloth moistened with an odorless detergent to remove odor traces. For testing, the rabbits were removed from their pens, placed in a wooden box, and carried to the open field. The box was placed in the center of the test area, where the rabbits were released without human contact. To minimize further human contact, the experimenter stayed away from the field during testing and behavior was continuously video-recorded.

The behavioral elements recorded during the open field test and their definition are presented in Table 2, in accordance with previous studies (Kersten et al., 1989; Meijsser et al., 1989). The behavior of the rabbits during the open field test was recorded using a Panasonic PV120 video camera fitted with a 3.6-mm lens. The camera was placed in a fixed position to continuously record behavior patterns in the open field. The recorded data were also stored in the digital video recorder, as previously described.

Statistical analysis

Body weight and feed intake data were analyzed using a mixed model with treatment as a fixed factor and doe as a random factor. Multiple comparisons were performed using Bonferroni's multiple range test, and significance was set at 0.05. Significant differences from normal distribution for the behavioral variables were assessed using the normal quartile plots along with values for skewness and kurtosis. Most studied variables reasonably followed analysis of variance (ANOVA) assumptions, i.e., normal distribution and homogeneity of variances within the class effects. For these parameters, standard multifactor ANOVA was used with treatment, sex, and age of testing as fixed effects

(time period of the day was also included in the case of the behavioral recordings of rabbits in home cages). However, there were certain variables that clearly deviated from a normal distribution, so generalized mixed models were used. "Duration of eating," "duration of drinking," and "locomotory and investigatory behaviors" were analyzed via zero-inflated negative binomial models. Frequencies of eating and drinking measured during the behavioral recordings of rabbits in home cages were analyzed by the application of logistic regression models. Here, a cumulative logit model and Fisher's scoring optimization technique for obtaining maximum likelihood parameter estimates were applied. In this case, values are presented in contrast with zero, used as a reference value. Negative and positive values correspond to lower and higher values compared to the reference, respectively.

Standard multifactor ANOVA was used with treatment, sex, and age of testing as fixed effects for most behavioral patterns during the open field test. Behavioral parameters such as "digging," "standing stretched," "rearing," and "moving forelegs" clearly deviated from a normal distribution, so zero-inflated negative binomial models were used for their analyses. The probability level for mean comparisons was set at 0.05. All the aforementioned analyses were carried out using the appropriate procedures in the statistical package of SAS/Stat (2011).

Results

Does' feed intake and weights

During the overfeeding periods, the O1 and O2 groups consumed significantly more feed than the controls ($P < 0.05$), but the average daily intake was lower than the offered one (150% of maintenance energy requirements [MER]). In fact, in the O1 group, the average daily feed intake between the 7th and 19th day of pregnancy accounted for the 129% of the MER ($P < 0.001$; 125.8 ± 4.8 vs. 164.2 ± 4.8 vs. 123.2 ± 4.8 g for C, O1, and O2 does, respectively), whereas for the O2 group, the relevant intake between the 20th and 27th day was 138% ($P < 0.001$; 124.3 ± 4.0 vs. 127.5 ± 4.0 vs. 169.9 ± 4.0 g for C, O1, and O2 does, respectively). However, no significant differences in body weight among doe groups were observed as an effect of overfeeding between the 7th

Table 2
Behavioral parameters recorded during the open field test and their definition according to the existing literature (Kersten et al., 1989; Meijsser et al., 1989)

Behavioral parameter	Definition
Latency	Time to first leaving of the central starting area of 30 × 30 cm
Sitting still	No movement of head, body, or legs
Standing stretched	The forelegs are forward, the hind legs stay in place, and the heels are visible behind the body
Moving forelegs	The forelegs move across the floor, whereas the hind legs stay in place
Walking	Forelegs and hind legs move alternately
Exploration	Head movements indicating investigation of the environment. Exploration is an independent category, but it can occur together with standing stretched, moving forelegs, or walking
Rearing	Forefeet are raised from the floor, the rabbit stands on its heels
Digging	Scratching with the forelegs on the floor or wall

Table 3
Effect of maternal overfeeding during different periods of pregnancy on body weight (g) and feed intake of offspring (16 rabbits per treatment) (least square means ± SEM)

Age (days)	Treatment ^c			SEM
	C	O1	O2	
Body weight (g)				
0 (birth)	65.9	70.3	83.1	7.8
21	394.8	390.6	412.9	17.0
35 (weaning)	1019.1	1013.1	1014.3	34.9
50	1903.8	1852.3	1904.7	31.5
65	2731.6	2623.7	2690.0	37.2
Feed intake (g/rabbit/day)				
42nd–49th day	133.2 ^a	114.3 ^b	134.0 ^a	5.1
49th–56th day	158.3 ^a	141.0 ^b	160.8 ^a	4.9
56th–63rd day	165.7	149.2	165.9	4.9
63rd–70th day	166.6	149.2	163.7	6.6

SEM, standard error of the means.

^{a,b} Mean values within a row and treatment with different superscripts are significantly different ($P < 0.05$).

^c C: control; O1 and O2: maternal overfeeding (150%) between 7th and the 19th day and between 20th and the 27th day of pregnancy, respectively.

and 19th day of pregnancy ($P > 0.05$; 4.76 ± 0.20 vs. 4.59 ± 0.20 vs. 4.48 ± 0.20 kg for C, O1, and O2 does on day 21, respectively) or between the 20th and 27th day of pregnancy ($P > 0.05$; 4.96 ± 0.20 vs. 4.75 ± 0.20 vs. 4.74 ± 0.20 for C, O1, and O2 does on day 28, respectively). As a result, at the end of the gestation period, the does could not be classified as obese.

Rabbit pups' weights and feed intake at birth, weaning, and ages of testing

As shown in Table 3, birth weight of kits was not significantly influenced by the maternal overfeeding ($P > 0.05$). As it was demonstrated, the birth weights (g) for the offspring born from the control and the overnourished mothers (LS means ± standard error of the means) were 65.9 ± 7.8 vs. 70.3 ± 7.8 vs. 83.1 ± 7.8 for the C, O1, and O2 group, respectively. Feed intake after weaning of the offspring of the O1 group was lower than that of the offspring of the C and O2 groups with significant differences observed between the 42nd and 56th day of age ($P < 0.05$). These feed intake differences were reflected in the lower body weights of offspring in the O1 group compared with C and O2 groups (Table 3). However, these body weight differences were not statistically significant ($P > 0.05$).

Behavioral recordings of offspring in their home cages

Rabbits born to does of the control group appeared to display a lower value for the frequency of eating than the rabbits born to

overnourished does ($P < 0.05$) (Table 4). On the other hand, duration of eating, drinking, and locomotory and investigatory behaviors were higher in offspring of controls than those of O1 and O2 groups ($P < 0.05$). As indicated, the other examined behavioral parameters were not significantly affected by maternal overfeeding ($P > 0.05$). A significant reduction in the frequency and duration of eating and drinking and an increase in duration of comfort behaviors and resting were observed with increasing age ($P < 0.05$). At the same time, male rabbits had higher values for the frequency of eating than the females ($P < 0.05$) (Table 4).

Significant differences were also observed during the time periods within the day (Figure). Values for frequency and duration of eating and drinking were increased during the last 5 hours of the dark period (8–12 h) ($P < 0.05$). Comfort behaviors had the lowest values during the dark period (2–12 h) ($P < 0.05$). Duration of locomotory and investigatory behavior was highest during the first hour of the light period ($P < 0.05$). On the other hand, values for resting behavior were decreased during the last 5 hours of the dark period and during the first 5 hours of the light period (8–17 h) ($P < 0.05$) (Figure).

Open field test

Prenatal overfeeding did not appear to influence the behavioral parameters examined during the open field test, apart from rearing that showed a higher value in prenatally overnourished pups than the control pups ($P < 0.05$). Values for latency, standing stretched, moving forelegs, walking, exploration, rearing, digging, and sitting still were not significantly different among the experimental groups (Table 5).

Rabbits spent more time sitting still at the age of 65 days than at the age of 50 days, and time spent for moving forelegs, exploration, and digging were significantly decreased by the test age ($P < 0.05$). As shown in Table 5, most of the examined behavioral elements were not significantly different between sexes with the exception of standing stretched that had greater value in males than females ($P < 0.05$).

Discussion

Rabbit pups' weights and feed intake at birth, weaning, and ages of testing

Birth weight of pups was not significantly affected by the maternal overfeeding during gestation. At the same time, the body weight of the O1 pups was slightly lighter than that of the C and O2 groups' pups after weaning. In accordance with the findings of the present study, Parigi-Bini et al. (1992) and Chen et al. (2008) demonstrated no effect of feeding level on average birth weight in rabbits and rats, respectively. Muhlhauser et al. (2006) also

Table 4
Behavioral patterns of rabbit offspring (n = 8) in home cages (min/h) after continuous 24-h recording on three consecutive days and at two different ages (least square means ± SEM^k)

Behavioral parameter	Treatment ^h			Age of testing (days)		Sex	
	C	O1	O2	50	65	Male	Female
	Frequency of eating	-0.18 ± 0.04^a	-0.12 ± 0.04^b	0 ^c	0.49 ± 0.03^d	0 ^e	0.08 ± 0.03^f
Frequency of drinking	0.01 ± 0.05	0.01 ± 0.05	0	0.18 ± 0.03^d	0 ^e	-0.04 ± 0.03	0
Duration of eating	2.36 ± 0.01^a	2.32 ± 0.01^b	2.32 ± 0.01^b	2.48 ± 0.01^d	2.20 ± 0.01^e	2.34 ± 0.01	2.33 ± 0.01
Duration of drinking	-0.28 ± 0.03^a	-0.48 ± 0.04^b	-0.44 ± 0.04^b	-0.30 ± 0.03^d	-0.43 ± 0.03^e	-0.37 ± 0.03	-0.36 ± 0.03
Comfort behaviors	10.56 ± 0.54	10.97 ± 0.52	10.76 ± 0.56	10.23 ± 0.35^d	11.30 ± 0.35^e	10.79 ± 0.41	10.74 ± 0.42
Locomotory and investigatory behaviors	1.96 ± 0.03^a	1.75 ± 0.03^b	1.83 ± 0.03^b	1.88 ± 0.02	1.82 ± 0.03	1.86 ± 0.03	1.83 ± 0.03
Resting	36.27 ± 0.51	37.23 ± 0.52	36.73 ± 0.51	35.02 ± 0.34^d	38.47 ± 0.34^e	36.73 ± 0.43	36.76 ± 0.43

SEM, standard error of the means.

^{a,b,c} Mean values within a row and treatment with different superscripts are significantly different ($P < 0.05$).

^{d,e} Mean values within a row and age of testing with different superscripts are significantly different ($P < 0.01$).

^{f,g} Mean values within a row and sex with different superscripts are significantly different ($P < 0.05$).

^h C: control; O1 and O2: maternal overfeeding (150%) between 7th and the 19th day and between 20th and the 27th day of pregnancy, respectively.

^k For frequencies of eating and drinking, a reference value (estimate–95% confidence limits) is alternatively given (logistic regression model).

found no significant difference in birth weight or body weight at postnatal day 30 between an overnourished (maternal overfeeding at the level of 160% of nutritional needs) and a control group in sheep. Several authors reached to the same conclusions because the birth weight of lambs that were born from overnourished ewes remained unaffected (Kenyon et al., 2011a; 2011b; Peel et al., 2012; Wallace et al., 2005). On the other hand, Hafez et al. (1967) suggested that the feeding level and the energy content of the diet during pregnancy influenced the prenatal environment in does because a higher birth weight was observed at medium and high than low feeding levels. Budge et al. (2000) also illustrated a significant increase in birth weight when ewes were fed at 150% of MER levels between 80 and 144 days of pregnancy. In contrast, maternal high-fat feeding in rats resulted in offspring with lower birth weights than controls (Nivoit et al., 2009).

As indicated, O1 rabbit pups consumed less feed than the pups of C and O2 groups between the 42nd and 63rd day. In contrast, an increased feed intake (hyperphagia) was observed in rat offspring

between the third and sixth week after weaning (Bayol et al., 2007; Samuelsson et al., 2008) and lambs during the first 3 weeks of postnatal life (Muhlhausler et al., 2006) as a result of maternal overfeeding. Moreover, offspring of dams fed a diet high in carbohydrates, sugar, and salt demonstrate hyperphagia, increased weight gain, and reduced skeletal muscle mass after weaning (Bayol et al., 2005; 2007). The discrepancies in body weight and feed intake that are shown in the existing literature could possibly be attributed to the species, the period of application of the respective nutritional protocols, or the level of overfeeding.

Behavioral recordings of offspring in their home cages

The frequency of eating was higher in the rabbits born from overnourished dams than those born from the controls. As it is also illustrated in Table 4, duration of eating and drinking had the lowest values in the O1 group, a fact that possibly led to the decreased feed intake of these pups between the 49th and 70th day (more meals in

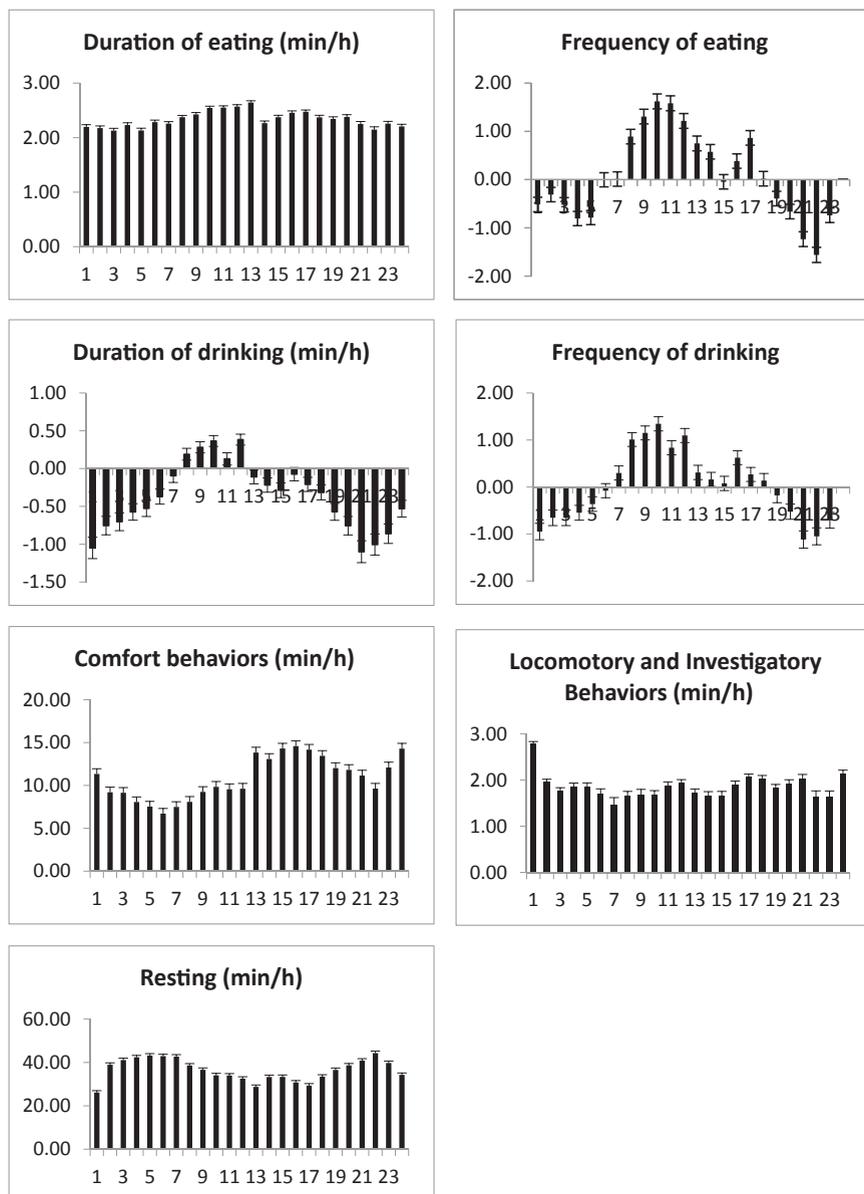


Figure. Behavioral patterns of rabbits ($n = 8$) in home cages (least squares means \pm SEM) as affected by the hour within the day (1-12: dark period, 13-24: light period). ^a For frequencies of eating and drinking, a reference value (estimate-95% confidence limits) is alternatively given (logistic regression model). Values are presented in contrast with the zero (0), used as a reference value. Negative and positive values correspond to lower and higher values compared to the reference, respectively. SEM, standard error of the means.

Table 5
Behavioral elements (s) of rabbit offspring (n = 8) during the open field test (means ± SEM)

Behavioral parameter	Treatment ^d			Age of testing (days)		Sex	
	C	O1	O2	50	65	Male	Female
Latency (log value)	1.62 ± 0.23	1.63 ± 0.20	1.76 ± 0.24	1.66 ± 0.14	1.68 ± 0.14	1.45 ± 0.19	1.89 ± 0.18
Standing stretched	2.72 ± 0.14	2.76 ± 0.11	3.06 ± 0.14	2.96 ± 0.10	2.74 ± 0.11	3.03 ± 0.10 ^c	2.66 ± 0.12 ^d
Moving forelegs	3.11 ± 0.11	3.30 ± 0.09	3.25 ± 0.13	3.44 ± 0.09 ^a	3.00 ± 0.09 ^b	3.33 ± 0.10	3.11 ± 0.09
Walking	124.92 ± 9.80	118.39 ± 8.29	109.63 ± 10.00	141.95 ± 6.12 ^a	93.34 ± 6.30 ^b	125.12 ± 7.84	110.17 ± 7.75
Exploration	135.51 ± 7.65	131.84 ± 6.58	125.25 ± 7.93	155.16 ± 5.43 ^a	106.58 ± 5.67 ^b	136.31 ± 6.22	125.43 ± 6.19
Rearing	2.50 ± 0.14 ^a	3.14 ± 0.11 ^b	3.52 ± 0.13 ^b	3.06 ± 0.09	3.04 ± 0.12	2.99 ± 0.10	3.11 ± 0.10
Digging	3.03 ± 0.08	3.14 ± 0.07	3.15 ± 0.09	3.31 ± 0.06 ^a	2.90 ± 0.07 ^b	3.09 ± 0.06	3.13 ± 0.06
Sitting still	122.85 ± 9.28	113.75 ± 8.01	122.04 ± 9.27	81.56 ± 6.80 ^a	157.54 ± 7.02 ^b	110.65 ± 7.69	128.44 ± 7.81

SEM, standard error of the means.

^{a,b,c} Mean values within a row and parameter with different superscripts are significantly different ($P < 0.05$).

^d C: control; O1 and O2: maternal overfeeding (150%) between 7th and the 19th day of pregnancy and between 20th and the 27th day of pregnancy, respectively.

shorter duration than the control pups). At the same time, display of locomotory and investigatory behaviors was lower in offspring of the O1 and O2 group than those of the C group. The other examined parameters were not significantly different among the experimental groups.

In previous experiments with rabbits of the same hybrid, mild (75%) or severe (50%) gestational maternal undernutrition reduced the frequency of eating and drinking behaviors in offspring (Simitzis et al., 2013; 2015), indicating that prenatal stressors may cause alterations of the hypothalamic-pituitary adrenocortical (HPA) axis and brain neurotransmitter systems of the offspring. In rats, a maternal diet rich in saturated fat derived from animal lard reduced locomotor activity of female offspring, as assessed by radio telemetric recording (Khan et al., 2003).

A significant reduction in the frequency and duration of eating and drinking and an increase in duration of comfort behaviors and resting were observed with increasing age (Table 4). A decrease in activity and an increase in resting as a result of age were also observed in previous studies that examined rabbit behavioral parameters at the ages of 50 and 65 days (Simitzis et al., 2013; 2015). On average, fattening rabbits between the ages of 6 and 10 weeks spend 60% of their time in resting, 10%–15% feeding, and 25%–30% displaying other activities (Morisse and Maurice, 1997). Display of natural locomotory activities by the rabbits is impaired by housing in individual cages, and as a result, they spend most of their time sitting and lying in “sternal recumbency” (Huls et al., 1991).

No significant effect of sex on the examined behavioral elements of rabbits was observed, with the exception of frequency of eating that was greater in males than females (Table 4). No differences in the whole pattern of behaviors between males and females were also found in previous studies with rabbits (Gunn and Morton, 1995; Simitzis et al., 2013).

Significant differences in the display of behavior by rabbits were also observed during the hours within the day (Figure). In detail, duration and frequency of eating and drinking were increased during the dark period, a finding that has been already demonstrated in previous studies (Simitzis et al., 2013; 2015) and is related with rabbit's nocturnal nature (Gunn and Morton, 1995). As it has been found, rabbits fed *ad libitum* spend more time feeding during the night than the day at a percentage of 61 vs. 39, respectively (Rommers et al., 1999). The activity of rabbits was generally increased during the dark period, a finding that is possibly explained by the fact that the ancestor of the domesticated rabbit, the European wild rabbit, is mainly active during the dusk, night, and dawn because as a prey-animal has greater chances to escape from predators during these time periods of the day (Jilge, 1991). The display of investigatory and locomotory behaviors was the greatest during the first and the last hours of the dark and light period, respectively, a finding that is in accordance with previous studies (Simitzis et al., 2015). On the other hand, Abdelfattah et al. (2013) found that

weaned rabbits had higher values for locomotion and exploration early in the morning compared with the other periods of the day.

The discrepancy between the present study and the previous literature could possibly be attributed to the different species, the type of the applied tests, the age of testing, or the level of overfeeding.

Open field test

The duration of the major behavioral elements recorded during the open field test was not significantly different between the offspring born from overnourished and control does. The only significant effect was observed for rearing; O1 and O2 rabbits displayed increased values for rearing compared with the controls (Table 5). In detail, values for latency, standing stretched, moving forelegs, walking, exploration, digging, and sitting still were not significantly different among the experimental groups, although values for walking were numerically lower in offspring of the O1 and O2 group than those of the C group (Table 5). No significant differences were also observed between sexes apart from standing stretched that was displayed more by males.

As already pointed out, the open field test is generally used as a paradigm to estimate stress- and anxiety-related behaviors (Kersten et al., 1989). According to our results, no significant effects of prenatal overfeeding on the display of stress-induced behavior were observed in rabbits. No previous data concerning the effects of prenatal overfeeding on rabbits' behavioral parameters exist. However, rats born from overnourished dams displayed decreased values for traveled distance in an open field test (Wright et al., 2011). Moreover, rat offspring born from dams fed with a high-fat diet display decreased thigmotaxic and approach behavior in an open field test (Rodriguez et al., 2012). At the same time, female rats born from dams fed a high-saturated-fat diet show increased anxiety-like behaviors in an open field task (Sivanathan et al., 2015). On the other hand, rat offspring exposed to perinatal high-fat diet did not show a difference in mobility rates during an open field test (Sasaki et al., 2013). Furthermore, although the display of anxiety-like behaviors increased in mice born to dams fed with high-fat diet, their exploratory behavior in an open field test was not affected (Peleg-Raibstein et al., 2012). In contrast, the implementation of a nutritional protocol in which mice were fed with a high-polyunsaturated-fat diet throughout pregnancy resulted in offspring with increased locomotor activity in an open field test (Raygada et al., 1998). As shown by the existing literature, results are controversial and a direct comparison between the different studies is questionable. It should be mentioned that in studies that examine the effects of maternal high-fat diet on the behavioral parameters of offspring, there is a vast diversity in the choice of animal, strain, age, diet, length of exposure, and behavioral assessment.

Rabbits spent more time sitting still at the age of 65 than at the age of 50 days, and time spent moving forelegs, walking,

exploration, and digging were significantly decreased with age (Table 5). A possible explanation of the aforementioned findings is that activity in an open field test declines with increasing age as a result of the habituation to the open field as already found in previous studies that were implemented in rabbits of the same hybrid (Simitzis et al. 2013; 2015).

Conclusion

It is concluded that maternal overfeeding between the 7th and 19th day (129% of MER) or between the 20th and 27th day of pregnancy (138% of MER) had a significant effect on the exhibition of several aspects of rabbits' postweaning behavior. Differences were observed in specific behavioral patterns recorded in the home cages, such as frequency and duration of eating, duration of drinking, and locomotory and investigatory behaviors, in the rabbits born to overnourished does compared with that born to control does ($P < 0.05$). As it was demonstrated, rabbits born to O1 and O2 does had more meals in shorter duration and moved and explored their environment less than rabbits born to control does.

No significant effects of prenatal overfeeding on the exhibition of behavior during the open field test were found with the exception of rearing that was displayed more by the rabbits born to O1 and O2 does. Duration of comfort behaviors and resting was significantly increased, although frequency and duration of eating and drinking were significantly decreased at the age of 65 days ($P < 0.05$). Male rabbits had greater values for frequency of eating than the females ($P < 0.01$). Maternal overfeeding has significant effects on the exhibition of rabbits' postweaning behavior because the offspring from the overnourished does moved and explored their environment significantly less than those from the controls. However, further experimentation is warranted to fully understand the mechanisms that connect prenatal overfeeding with the exhibition of rabbit pups' postweaning behavior.

Acknowledgments

This research project was implemented within the framework of the Action «Supporting Postdoctoral Researchers» of the Operational Program “Education and Lifelong Learning” (Action's Beneficiary: General Secretariat for Research and Technology) and is co-financed by the European Social Fund and the Greek State [LS9 (1678)].

Authors' contributions: The idea for the paper was conceived by P.S., G.S., I.B., S.C., O.P., and S.D. The experiments were performed by P.S., G.S., and A.K. The data were analyzed by A.K. and M.G. The paper was written by P.S.

Ethical considerations

The methods used in the present experiment were approved by the Research Ethics Committee of the Department of Animal Science and Aquaculture of the Agricultural University of Athens under the guidelines of “Council Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.”

Conflict of interest

Authors declare no conflict of interest.

References

Abdelfattah, E., Karousa, M., Mahmoud, E., El-Laithy, S., El-Gendi, G., Eissa, N., 2013. Effect of cage floor type on behavior and performance of growing rabbits. *J. Vet. Adv.* 3, 34–42.

- Anderson, J.A., Henck, J.W., 1994. Fetal development. In: Manning, P.J., Ringler, D.H., Newcomer, C.E. (Eds.), *The Biology of the Laboratory Rabbit*, 2nd ed. Academic Press, p. 457.
- Archer, J., 1975. Rodent sex differences in emotional and related behavior. *Behav. Biol.* 14, 451–479.
- Bayol, S.A., Simbi, B.H., Stickland, N.C., 2005. A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *J. Physiol.* 567, 951–961.
- Bayol, S.A., Farrington, S.J., Stickland, N.C., 2007. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. *Br. J. Nutr.* 98, 843–851.
- Bilbo, A.D., Tsang, V., 2010. Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. *FASEB J.* 24, 2104–2115.
- Bouret, S.G., 2009. Early life origins of obesity: role of hypothalamic programming. *J. Pediatr. Gastroenterol. Nutr.* 48 (Suppl. 1), S31–S38.
- Bouret, S.G., 2010. Role of early hormonal and nutritional experiences in shaping feeding behavior and hypothalamic development. *J. Nutr.* 140, 653–657.
- Budge, H., Bispham, J., Dandrea, J., Evans, E., Heasman, L., Ingleton, P.M., Sullivan, C., Wilson, V., Stephenson, T., Symonds, M.E., 2000. Effect of maternal nutrition on brown adipose tissue and its prolactin receptor status in fetal lamb. *Pediatr. Res.* 47, 781–786.
- Candland, D.K., Campbell, B.A., 1962. Development of fear in the rat as measured by behavior in the open field. *J. Comp. Physiol. Psychol.* 55, 593–596.
- Chen, H., Simar, D., Lambert, K., Mercier, J., Morris, M.J., 2008. Maternal and postnatal overnutrition differentially impact appetite regulators and fuel metabolism. *Endocrinology* 149, 5348–5356.
- Clancy, B., Finlay, B.L., Darlington, R.B., Anand, K.J.S., 2007. Extrapolating brain development from experimental species to humans. *Neurotoxicology* 28, 931–937.
- Cordner, Z.A., Tamashiro, K.L.K., 2015. Effects of high-fat diet exposure on learning & memory. *Physiol. Behav.* 152, 363–371.
- Desai, R.A., Manley, M., Desai, M.M., Potenza, M.N., 2009. Gender differences in the association between body mass index and psychopathology. *CNS Spectr.* 14, 372–383.
- Eichenwald, H.F., Fry, P.C., 1969. Nutrition and learning. *Science* 163, 644–648.
- Gunn, D., Morton, D.B., 1995. Inventory of the behavior of New Zealand White rabbits in laboratory cages. *Appl. Anim. Behav. Sci.* 45, 277–292.
- Hafez, E.S.E., Lindsay, D.R., Moustafa, L.A., 1967. Effect of feed intake of pregnant rabbits on nutritional reserves of neonates. *Am. J. Vet. Res.* 28, 1153–1159.
- Huls, W.L., Brooks, D.L., Bean-Knudsen, D., 1991. Response of adult New Zealand White rabbits to enrichment objects and paired housing. *Lab. Anim. Sci.* 41, 609–612.
- Jilge, B., 1991. The rabbit: a diurnal or a nocturnal animal? *J. Exp. Anim. Sci.* 34, 170–183.
- Kalra, S.P., Dube, M.G., Pu, S., Xu, B., Horvath, T.L., Kalra, P.S., 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr. Rev.* 20, 68–100.
- Kenyon, P.R., Pain, S.J., Hutton, P.G., Jenkinson, C.M.C., Morris, S.T., Peterson, S.W., Blair, H.T., 2011a. Effects of twin-bearing ewe nutritional treatments on ewe and lamb performance to weaning. *Anim. Prod. Sci.* 51, 406–415.
- Kenyon, P.R., van der Linden, D.S., Jenkinson, D.M.C., Morris, S.T., Mackenzie, D.D.S., Peterson, S.W., Firth, E.C., Blair, H.T., 2011b. The effect of ewe size and nutritional regimen beginning in early pregnancy on development of singleton foetuses in late pregnancy. *Livestock Sci.* 142, 92–98.
- Kersten, A.M.P., Meijsser, F.M., Metz, J.H.M., 1989. Effects of early handling on later openfield behavior in rabbits. *Appl. Anim. Behav. Sci.* 24, 157–167.
- Meijsser, F.M., Kersten, A.M.P., Wiepkema, P.R., Metz, J.H.M., 1989. An analysis of the open field performance of sub-adult rabbits. *Appl. Anim. Behav. Sci.* 24, 147–155.
- Khan, I.Y., Taylor, P.D., Dekou, V., Seed, P.T., Lakasing, L., Graham, D., Dominiczak, A.F., Hanson, M.A., Poston, L., 2003. Gender-linked hypertension in offspring of lard-fed pregnant rats. *Hypertension* 41, 168–175.
- Morisse, J.P., Maurice, R., 1997. Influence of stocking density or group size on behavior of fattening rabbits kept under intensive conditions. *Appl. Anim. Behav. Sci.* 54, 351–357.
- Muhlhausler, B.S., 2006. Programming of the appetite-regulating neural network: a link between maternal overnutrition and the programming of obesity? *J. Neuroendocrinol.* 19, 67–72.
- Muhlhausler, B.S., Adam, C.L., Findlay, P.A., Duffield, J.A., McMillen, I.C., 2006. Increased maternal nutrition alters development of the appetite-regulating network in the brain. *FASEB J.* 20, 1257–1259.
- Muhlhausler, B.S., Duffield, J.A., McMillen, I.C., 2007. Increased maternal nutrition stimulates peroxisome proliferator activated receptor- γ , adiponectin, and leptin messenger ribonucleic acid expression in adipose tissue before birth. *Endocrinology* 148, 878–885.
- Muhlhausler, B.S., Adam, C.L., McMillen, I.C., 2008. Maternal nutrition and the programming of obesity: the brain. *Organogenesis* 4, 144–152.
- Nivoit, P., Morens, C., Van Assche, F.A., Jansen, E., Poston, L., Remacle, C., Reusens, B., 2009. Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia* 52, 1133–1142.
- Parigi-Bini, R., Xiccato, C., Cinetto, M., Dalle Zotte, A., 1992. Energy and protein utilization and partition in rabbit does concurrently pregnant and lactating. *Anim. Prod.* 55, 153–162.
- Peel, R.K., Eckerle, G.J., Anthony, R.V., 2012. Effects of overfeeding naturally mated adolescent ewes on maternal, fetal, and postnatal lamb growth. *J. Anim. Sci.* 90, 3698–3708.

- Peleg-Raibstein, D., Luca, E., Wolfrum, C., 2012. Maternal high-fat diet in mice programs emotional behavior in adulthood. *Behav. Brain Res.* 233, 398–404.
- Raygada, M., Cho, E., Hilakivi-Clarke, L., 1998. High maternal intake of polyunsaturated fatty acids during pregnancy in mice alters offsprings' aggressive behavior, immobility in the swim test, locomotor activity and brain protein kinase C activity. *J. Nutr.* 128, 2505–2511.
- Rodríguez, J.S., Rodríguez-Gonzalez, G.L., Reyes-Castro, L.A., Ibañez, C., Ramirez, A., Chavira, R., Larrea, F., Nathanielsz, P.W., Zambrano, E., 2012. Maternal obesity in the rat programs male offspring exploratory, learning and motivation behavior: prevention by dietary intervention pre-gestation or in gestation. *Int. J. Dev. Neurosci.* 30, 75–81.
- Rommers, J.M., Kemp, B., Meijerhof, R., Noordhuizen, J.P.T.M., 1999. Rearing management of rabbit does: a review. *World Rabbit Sci.* 7, 125–138.
- Rofey, D.L., Kolkó, R.P., Iosif, A.M., Silk, J.S., Bost, J.E., Feng, W., Szigethy, E.M., Noll, R.B., Ryan, N.D., Dahl, R.E., 2009. A longitudinal study of childhood depression and anxiety in relation to weight gain. *Child Psychiatry Hum. Dev.* 40, 517–526.
- Samuelsson, A.M., Matthews, P.A., Argenton, M., Christie, M.R., McConnell, J.M., Jansen, E.H., Piersma, A.H., Ozanne, S.E., Twinn, D.F., Remacle, C., Rowlerson, A., Poston, L., Taylor, P.D., 2008. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. *Hypertension* 51, 383–392.
- Sasaki, A., De Vega, W.C., St-Cyr, S., Pan, P., McGowan, P.O., 2013. Perinatal high fat diet alters glucocorticoid signaling and anxiety behavior in adulthood. *Neuroscience* 240, 1–12.
- SAS/Stat, 2011. Statistical Analysis Systems Version 9.3. SAS Institute Inc., Cary, NC.
- Simitzis, P., Skoupa, E.-P., Goliomytis, M., Kominakis, A., Deligeorgis, S.G., 2013. The effects of mild maternal undernutrition on the post-weaning behavior of rabbits. *Appl. Anim. Behav. Sci.* 148, 157–163.
- Simitzis, P.E., Symeon, G.K., Kominakis, A.P., Bizelis, I.A., Chadío, S.E., Abas, Z., Deligeorgis, S.G., 2015. Severe maternal undernutrition and post-weaning behavior of rabbits. *Physiol. Behav.* 141, 172–179.
- Sivanathan, S., Thavartnam, K., Arif, S., Elegino, T., McGowan, P.O., 2015. Chronic high fat feeding increases anxiety-like behavior and reduces transcript abundance of glucocorticoid signalling genes in the hippocampus of female rats. *Behav. Brain Res.* 286, 265–270.
- Sullivan, E.L., Grayson, B., Takahashi, D., Robertson, N., Maier, A., Bethea, C.L., Smith, M.S., Coleman, K., Grove, K.L., 2010. Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. *J. Neurosci.* 30, 3826–3830.
- Sullivan, E.L., Nousen, E.K., Chamlou, K.A., 2014. Maternal high fat diet consumption during the perinatal period programs offspring behavior. *Physiol. Behav.* 123, 236–242.
- Sullivan, E.L., Riper, K.M., Lockard, R., Valteau, J.C., 2015. Maternal high-fat diet programming of the neuroendocrine system and behavior. *Horm. Behav.* 76, 153–161.
- Wallace, J.M., Milne, J.S., Aitken, R.P., 2005. The effect of overnourishing singleton-bearing adult ewes on nutrient partitioning to the gravid uterus. *Br. J. Nutr.* 94, 533–539.
- White, C.L., Pistell, P.J., Purpera, M.N., Gupta, S., Fernandez-Kim, S.-O., Hise, T.L., Keller, J.N., Ingram, D.K., Morrison, C.D., Bruce-Keller, A.J., 2009. Effects of high fat diet on Morris maze performance, oxidative stress, and inflammation in rats: contributions of maternal diet. *Neurobiol. Dis.* 35, 3–13.
- Williams, G., Bing, C., Cai, X.J., Harrold, J.A., King, P.J., Liu, X.H., 2001. The hypothalamus and the control of energy homeostasis: different circuits, different purposes. *Physiol. Behav.* 74, 683–701.
- Woodruff-Pak, D.S., 1988. Aging and Classical Conditioning: Parallel Studies in Rabbits and Humans. *Neurobiol. Aging* 9, 511–522.
- Wright, T., Langley-Evans, S.C., Voigt, J.P., 2011. The impact of maternal cafeteria diet on anxiety-related behaviour and exploration in the offspring. *Physiol. Behav.* 103, 164–172.
- Xiccato, G., Trocino, A., 2010. Energy and Protein Metabolism and Requirements. In: De Blas, C., Wiseman, J. (Eds.), *Nutrition of the Rabbit*, 2nd ed. CAB International, UK, pp. 83–118.