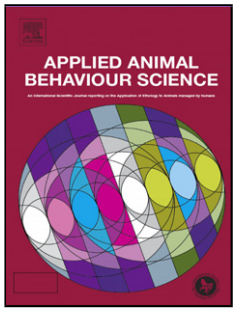

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Influence of morning maternal care on the behavioural responses of 8-week-old Beagle puppies to new environmental and social stimuli

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HIGHLIGHTS

- The amount of morning maternal care affects the behaviour of 8 weeks old puppies.
- A higher mean duration of daily maternal care produces an increase in exploration.
- A higher mean duration of daily maternal care leads to a reduced stress response.

Abstract

In mammals, maternal care represents a major constituent of the early-life environment and its influence on individual development has been documented in rodents, non-human primates, humans and recently in adult dogs. The quality and quantity of mother-offspring interactions exerts a multilevel regulation upon the physiological, cognitive, and behavioural development of the offspring. For example, in rats variations in maternal behaviour, such as mother-pup body contact and the amount of licking towards pups in the nest during the early days after parturition, influences the endocrine, emotional, and behavioural responses to stress in the offspring. This produces long-term consequences, which may remain into adulthood and can be transmitted to subsequent generations. Literature about maternal care in dogs and its effect on puppy behaviour is still scarce, although the topic is receiving a growing interest. The aim of the present study was to determine the effects of morning maternal care on behavioural responses of puppies to new environmental and social stimuli. In order to achieve this, maternal care (licking, ano-genital licking, nursing and mother-puppy contact) was assessed in eight litters of domestic dogs living in standard rearing conditions during the first three weeks post-partum. Puppies were subjected to two behavioural tests (arena and isolation tests) at 58-60 days of age, and their behavioural responses were video recorded and analysed. Data was analysed using multivariate analyses (PCA, PLS).

During the isolation test, a higher level of maternal care was associated with more exploration and a higher latency to emit the first yelp; on the contrary, a lower level of maternal care was associated with increased locomotion, distress vocalisations and destructive behaviours directed at the enclosure.

These results, comparable to those reported in laboratory rat models and to some extent to those recently reported in dog literature, highlight the importance of maternal care on the behavioural development of domestic dog puppies.

Keywords: behaviour, dog, licking, maternal care, nursing, puppy.

1. Introduction

In all mammalian species, there is an intense period of mother-infant interactions that is necessary for the survival of offspring; maternal care, especially during the early stages of postnatal life, is the main source of environmental stimuli for the progeny and a major determinant of behaviour in adulthood (Bowlby, 1988; Champagne, 2011).

Experimental evidence about the role of maternal care in mammals is commonly derived from deprivation studies. Disruption of mother-infant bonding during early lactation is known to have a great effect on the developing infant, particularly in altricial species (Mogi et al., 2011). In both primates and rodents, infants deprived of maternal care for extended periods of time exhibit dramatically increased fearfulness and anxiety, inappropriate and often excessively aggressive patterns of social behaviour, impaired cognitive development (Caldji et al. 2000b; Champagne and Curley, 2009; Levy et al., 2003; Liu et al., 2000), and enhanced neuroendocrine responses to stressors (Francis and Meaney, 1999), accompanied by epigenetic changes in the central nervous system (Weaver et al., 2004).

In *Canis familiaris* early separation from the mother at the age of 6 weeks increases disease susceptibility, weight loss and mortality in puppies (Slabbert and Rasa, 1993). Puppies prematurely separated from their mother between the ages of 30 and 40 days seem to be more likely to develop undesirable behaviours related to fear or anxiety than puppies that remain with their mothers until adoption at 60 days of age (Pierantoni et al., 2011). In addition to the presence of the mother, the amount of maternal care shown by the mother towards her puppies during the early period seems to be crucial in many species. Literature from animal models emphasizes the importance of the quantity of nursing, body and ano-genital licking, and mother-puppy body-contact, because these can shape the emotionality, reactivity to stress and social skills of the neonates (Caldji et al., 1998; Champagne et al., 2003; Starr-Phillips and Beery, 2013). Variation in the quantity of maternal care has been the subject of numerous investigations in several mammalian species and has been considered responsible for a range of effects on the brain development and on behaviour of the offspring (e.g. Caldji et al., 1998; Caldji et al., 2000a; Fairbanks, 1996; Foyer et al., 2016; Liu et al., 1997). For example, in rodents, naturally occurring variations in maternal behaviours during the first week of life, such as nursing, licking, and contact are associated in the offspring with the development of individual differences in the hypothalamic-pituitary-adrenal axis (HPA), brain morphology, neurotransmitters content in several brain regions and gene expressions (Caldji et al., 1998; Caldji et al., 2000a; Champagne, 2008;

Francis et al., 2000; Gudsnuk and Champagne, 2011; Jensen and Champagne; 2012; Masís-Calvo et al., 2013; Meaney, 2001; Sequeira-Cordero et al., 2013; Zhang et al., 2005). Unlike rodents, scientific literature on maternal care shown by female domestic dogs during the first weeks post-partum is still scarce (Arteaga et al., 2012; Foyer et al., 2016; Guardini et al., 2015; Pal, 2005; Rheingold, 1963; Scott and Fuller, 1965) and its effects on the behaviour of adult offspring have been investigated only very recently (Foyer et al., 2016). In domestic dogs, several authors have examined how some characteristics of the mother, of the puppies or of the early environment may affect the personality and temperament of young and adult dogs. For instance, previous studies have focused on the effects of: parity of mother (Foyer et al., 2016; Foyer et al., 2013; Wilsson and Sundgren, 1998a), litter size (Foyer et al., 2016; Foyer et al., 2013; van der Waaij et al., 2008; Wilsson and Sundgren, 1998a), material used in the whelping box (Wilsson and Sundgren, 1998a), gender of the puppies (Beerda et al., 1999a; Beerda et al., 1999b; Courreau and Langlois, 2005; Foyer et al., 2016; Foyer et al., 2013; Svartberg, 2002; van der Waaij et al., 2008; Wilsson and Sundgren, 1998a, 1998b), weight of the puppies (Foyer et al., 2013; Wilsson and Sundgren, 1998a), and temperature and season of birth (Foyer et al., 2016; Foyer et al., 2013; van der Waaij et al., 2008; Welker, 1959; Wilsson and Sundgren, 1998a). In the above-mentioned studies, only that of Foyer and colleagues (2016) explored the mother-pup interactions (mother in the box, lying in contact, nursing, licking, sniff/poke) as influential factors for the dog adult behaviour. They analysed maternal care at four time points during the first three weeks after birth (1st, 7th, 14th 21st day post-partum) and they found that maternal care is correlated with the behaviour of the adult offspring, mainly with respect to behaviours classified as physical and social engagement, as well as aggression.

With regard to the effects of the quantity of maternal care received in early life on the behaviour and stress responses of young puppies, literature is still limited.

The aim of the current study was to evaluate the impact of maternal care on the behaviour of puppies. We hypothesize in our study, as already demonstrated in rodents' literature, that Beagle puppies that receive more maternal care during the first weeks after the birth, will be able to better cope with stressful situations and will shown better responses towards new environmental and social stimuli.

In order to achieve this, we analysed the behaviour of eight Beagle mothers towards their puppies every day for the first three weeks after birth, differently from the methodology used by Foyer and colleagues (2016).

144 Then, we analysed the behaviour towards new environmental and social stimuli of their puppies at eight
145 weeks of age, in two potentially stressful situations (arena and isolation tests).

146 **2. Materials and Methods**

147 2.1. Subjects

148 Eight litters of Beagle dogs belonging to a professional canine reproduction centre and living in standardised
149 rearing conditions were recruited, giving a total of 54 puppies.

150 Each mother was kept with her own litter in an individual enclosure (3.20 m x 1.80 m), which was
151 contiguous with other identical enclosures in the maternity area of the establishment.

152 Details of the eight litters (characteristics of the mothers and puppies) included in the study are reported in
153 table 1.

154 2.2. Protocol of the analysis of maternal care: mother-litter interactions

155 Every day, from day 1 to 21 after birth, a 15-minute video of each mother with her puppies was recorded.
156 Being an observational study and not an experimental one, it only needed to be approved by the Ethical
157 Committee of the facility where the study was carried out.

158 Videos were made in the morning, when the mother returned to the whelping box after having freely walked
159 into the corridor of the maternity area in the presence of a caregiver for approximately ten minutes. All
160 puppies were identified in two ways; using different coloured satin ribbons and shaving different small areas
161 of fur. This means that every puppy wore a coloured satin ribbon and had a small area shaved, the area being
162 different one from the other. This double identification allowed us to always be able to recognize each puppy
163 during the recording process, even when the neck or other body areas were not visible. Since all the puppies
164 underwent the same treatment, there was no difference in the quantity and quality of handling they received.

165 To assess maternal care given to each puppy, a list of behaviours from Guardini et al. (2015) was used.
166 Behaviours observed included: mother-puppy physical contact (later referred as contact), licking, licking the
167 ano-genital area (later referred as licking and licking-ag, respectively), and nursing. For each behaviour
168 included in the list, the interaction within a specific mother-puppy dyad was analysed.

169 2.3. Behavioural tests for puppies

170 At 58-60 days of age, each puppy was subjected to two behavioural tests on the same day; first the arena test
171 (Gazzano et al., 2008), and then, after 1-3 hours, the isolation test (Gazzano et al., 2008).

172 2.3.1. Arena test

173 The arena test aimed at evaluating the behaviour of the puppies in a novel environment in the presence of an
174 unknown human being and a variety of objects (a puppy Kong®, a puppy plaited rope of Trixie®, a plastic
175 disk of Trixie®, a small ball of Trixie®).
176 The arena was similar to that used by Gazzano et al. (2008): 3.6 m x 2.2 m, and divided into twenty-four 55
177 cm x 60 cm rectangles with a central circle of 1.60 m in diameter. The arena itself was located in a room that
178 was unfamiliar to the puppies.
179 The arena was enclosed on three sides by the walls of the test room and for the remaining short side by a 91
180 cm high metal fence covered externally with a dark cloth.
181 A stranger (a woman unfamiliar to all the puppies) sat on a stool at the centre of the circle maintaining a
182 neutral pose, without making eye contact with or interfering with the behaviour of the puppy.
183 Each puppy was individually placed by a familiar caregiver in the same starting square ("the first square"),
184 located in a corner of the arena. The test lasted 5 minutes and was recorded using two videocameras, which
185 covered the whole arena. The test order of puppies within each litter was randomised.
186 Any urine or faeces deposited during the test were removed before testing the next puppy.
187 To assess the behaviour of each puppy, five groups of behaviours were analysed: non-social behaviours,
188 vocalizations, stress behaviours, social behaviours and other behaviours (see table 2).
189 2.3.2. Isolation test
190 In the isolation test (modified from Gazzano et al., 2008) each puppy was individually placed inside an area
191 enclosed with a 91cm high metal fence, creating a square of approximately 1.5 m^2 on the floor. The fence
192 was located in a room that was unfamiliar to the puppies. Each puppy remained alone in the fenced isolation
193 area for 3 minutes whilst being videoed. To assess the behaviour of each puppy, a list of four groups of
194 behaviours was used: stress behaviours, vocalizations, non-social behaviours, and other behaviours. Stress
195 behaviours and other behaviours were the same as described for the arena test. Vocalisations were exactly
196 the same as for the arena test, with the addition of the latency to the first yelp/whine, defined as the time that
197 passed between the beginning of the test and the first yelp/whine emitted by the puppy (Gazzano et al.,
198 2008). The group of non-social behaviours was different from that of the arena test and is reported in table 3.
199 **3. Data analysis**

Every video was analysed multiple times, each time using a focal sampling on a specific mother-puppy dyad. For each video analysis, the observation was performed using a continuous sampling. The analysis consisted in recording the duration (in seconds) of each analysed behaviour.

Data was mean-centred and unit-variance scaled before principal components analysis and projection to latent structures analysis (SIMCA-P+® 12). The significance of models was calculated for PLS (projection to latent structures) models using the ANOVA (analysis of variance) of the cross-validated residuals (CV-ANOVA; Eriksson et al., 2008). Individual inter-variable correlations were performed using a Spearman rank correlation after the D'Agostino-Pearson omnibus normality test confirmed that data was not normally distributed (Graphpad Prism 6®).

4. Results

Four measures of maternal care were recorded in the videos (contact, licking, licking-ag and nursing). In order to reduce these to a single variable representing an overall measure of maternal care, principal components analysis (PCA) was used for dimension reduction. Mean daily duration of each behaviour was calculated for each mother-pup dyad. Data was then mean centred and unit variance scaled prior to multivariate analysis. PCA generated a model with a single principal component (R^2=0.582, Q^2=0.251). Scores from this model for each mother-pup interaction were used as the regression (Y) variable for subsequent models that investigated the relationship between maternal behaviour and puppy performance in the arena and isolation tests.

4.1. Amount of maternal care (mother-pup dyad behaviours) and arena test

A cross-validated projection to latent structures (PLS) model was created using the PCA score for each puppy with mother-pup interaction as the regression variable (Y) and the arena test data as the set of X variables. No model could be generated, indicating that there was no systematic relationship between maternal care and performance of puppies in the arena test.

4.2. Amount of maternal care (mother-pup dyad behaviours) and isolation test

A cross validated PLS model was created using the PCA score for each puppy with mother-pup interaction as the regression variable (Y) and the isolation test data as the set of X variables. This produced a model with a single predictive component (R^2Y=0.348, Q^2=0.187, p=0.00512).

In a plot of loadings for this model (figure 1), positive loadings (upward pointing bars) indicate isolation test behaviours which were positively associated with the amount of maternal care, and negative loadings (downward pointing bars) indicate those behaviours which were negatively associated with the amount of maternal care. Height of bars is an index of the importance of that association.

PLS can be regarded as a method of feature selection, so that bivariate tests can be applied to those features which show strong loadings in the model.

In this case, exploration, latency at the first yelp, orientation to the enclosure, locomotion and whining/yelping had loadings that were of sufficient strength to warrant individual correlation tests. The D'Agostino-Pearson omnibus normality test was applied to all data, and all variables were found not to be normally distributed. A Spearman rank correlation was therefore used to analyse the correlation between each individual variable and maternal care PCA score. The results are summarised in table 4. The duration of maternal care resulted positively correlated to exploration and latency at the first yelp, and negatively correlated with orientation at the enclosure, locomotion and whining/yelping.

5. Discussion

Results from the present study confirm that in domestic dogs, the quantity of morning maternal care provided during the early development of puppies has an important influence on the future behavioural responses of young puppies, similarly to what recently found for adult dogs (Foyer et al., 2016).

In the isolation test, a higher mean duration of daily maternal care (in terms of nursing, body licking, ano-genital licking, and physical contact between mother and puppy) was found to be associated with a systematically different approach by puppies to a novel environment, i.e. a higher level of exploration and reduced signs of stress. However, this difference was not found when a stranger was present in the unfamiliar environment, as in the arena test, and no other significant correlation was found between maternal care and the behaviour of puppies in this test.

The isolation test used in the present study separates the puppy from the mother, the littermates and the familiar environment in which the puppy was born (Elliot and Scott, 1961; Fredericson, 1950, 1952; Pettijhon et al., 1977; Ross et al., 1960). By its nature, the isolation test therefore evokes a high level of emotionality and anxiety in young puppies, and consequently the appearance of the so called "protest separation behaviours" that have been previously studied and observed in the offspring of several mammal

255 species after separation from the attachment figure (Ainsworth et al., 1978; Bowlby, 1988; Coe et al., 1978;
256 Mineka and Suomi, 1978; Nagasawa et al., 2014; Nowak and Boivin, 2015; Pettijhon et al., 1977; Prato-
257 Previde et al., 2009; Ritchey and Hennessy, 1987).
258 Thus, in such conditions and because the maximum vocalisations after separation from the mother are
259 generally elicited in puppies between 6 and 8 weeks of age (Elliot and Scott, 1961), it was expected that
260 puppies would have vocalised, made attempts to escape, scratched the enclosure with their paws or jumped
261 on it. Indeed puppies did exhibit these behaviours but our results indicate that a high level of maternal care
262 during early life can mitigate the normal and adaptive distress reactions of puppies when isolated from the
263 mother and moved to an unfamiliar room. A systematically different pattern of behaviour was associated
264 with increased maternal care: increased exploration, a longer latency to first yelp, reduced orientation to the
265 enclosure (that means reduced destructiveness and attempts to escape), reduced non-exploratory locomotion
266 and reduced whining/yelping. Therefore, puppies that experienced greater maternal care showed increased
267 engagement with the environment and reduced signs of distress. On the other hand, a low level of maternal
268 care was associated with the opposite pattern: an aggravation of the emotional distress during such a stressful
269 situation. Systematic differences are potentially meaningful, because they indicate a general shift in the
270 animal's coping strategy and emotional response to dealing with the situation that it is faced with.
271 Literature from other animal models shows similar results. Rat pups that receive more maternal care during
272 the first 10 days of life show reduced fearfulness, reduced non-exploratory locomotion, and increased
273 exploration in a novel environment (Caldji et al 1998; Cladji et al., 2000a; Francis et al., 1999; Francis and
274 Meaney, 1999; Liu et al., 1997; Masís-Calvo et al., 2013; Meaney, 2001; Sequeira-Cordero et al., 2013).
275 In the present study, in order to quantify maternal care, four behaviour of each mother-puppy dyad were
276 taken into account. Two of these behaviours were body licking and ano-genital licking, which have been
277 widely demonstrated to influence the response of rat pups to stressful events (i.e. Caldji et al., 1998;
278 Champagne, 2008; Masís-Calvo et al., 2013). In fact, the reception by rat pups of high level of tactile stimuli
279 from the mother in the form of body licking and ano-genital licking has been shown to decrease DNA
280 methylation of glucocorticoid receptor genes in the hippocampus, thereby increasing the expression of those
281 receptors, leading to an enhanced glucocorticoid feedback sensitivity (Gudsnuk and Champagne, 2011; Liu
282 et al., 1997; Weaver et al., 2004). Similar physiological effects were also observed, to some extent, in boars

(Weaver et al., 2000), and it is reasonable to hypothesize that they may be present in the domestic dog, contributing to an explanation of our findings.

In the arena test, a wider range of interactions was measured, including interactions with toys and an unfamiliar person. The presence of different objects makes possible the evaluation of the level of puppy interaction with a complex environment, while the presence of an unknown person, as a new social stimulus, enables the evaluation of the level of the puppies' interspecific socialisation, which is presumed to be maximal at eight weeks old.

In contrast to the isolation test, no significant relationship was found between maternal care during the first 21 days after birth and individual puppy performance in the arena test. This might be explained by the fact that some of the objects of the arena and the social stimulus were not within the puppies' experience. With respect to social stimuli, it has been found that adult dogs that have been socialized with people are attracted to a person, even if that person is unfamiliar (Kaulfuss and Mills, 2008; Topál et al., 2005), and socialization has an ameliorative effect in alleviating the distress caused by isolation in a novel environment (Mariti et al., 2014; Mariti et al., 2013b; Pettijhon et al., 1977). This attraction to people increases with the level of socialization and with familiarity arising from previous social experiences (Mariti et al., 2014). Since the interspecific socialisation of the puppies in this study was restricted to a small number of caregivers, exposure to a different unfamiliar person, particularly someone who was dressed differently from the caregivers with whom the puppies were accustomed, may not have exerted any attraction for the puppies, and may instead have alarmed or disoriented them. These latest findings are different from those of Foyer et al. (2016), who found that German shepherd dogs that experienced higher levels of maternal care during the first three weeks after the birth showed, when adults, more social and physical engagement during the standardised temperament test used by the Swedish Armed Forces (SAF T-test). Such disagreement in the results may be explained in the different level of early experiences and human socialisation received by German shepherd dogs, compared to our Beagle puppies, that made the first group more prone to cooperate and engage in social interactions with humans, and less fearful and more confident with the inanimate objects used in the test. In addition, the age at which the subjects underwent to the behavioural tests was different. In the current study puppies were tested at two months of age, while Foyer et al. (2016) tested dogs between 15 and 18 months of age. During the long time from 8 weeks of age until the time of the SAF T-test,

the dog's experiences in a family and enriched environment, and all the events occurring during preadolescence and adolescence can contribute to the behaviour expression of the adult animal (Foyer et al., 2014).

In our study therefore, a lack of early experiences such as exposure to complex environments, multi-sensory stimuli and diverse human contact, could possibly have influenced the arena test result. In fact, the function of early experiences is to promote an organised and systematic response in a complex environment and in the presence of unfamiliar people.

In conclusion, to our knowledge this is the first study to show that the amount of maternal care provided during the first three weeks post-partum affects the behaviour of eight weeks old puppies reared in standardised conditions. In particular, this study provides evidence that maternal care mediates a set of responses, which allows an individual to cope with stressful situations in the absence of specific unfamiliar inanimate and social stimuli. These striking effects seem to persist in the behaviour of the adult dog, as Foyer et al. (2016) have recently demonstrated that variation of the level of maternal care significantly affected the adult behaviour of the offspring, as already shown in rodents (see e.g. Caldji et al., 1998; Zhang et al., 2005). A good level of maternal care is highly recommended as it has a big influence on the capacity of puppies to deal with stress and it leads to individuals that can better adapt to new environmental conditions.

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483 Figure Captions

484

485 Figure 1 Loadings bar chart of PLS of isolation test variables versus maternal care PCA score.
486 Upward pointing bars indicate positive loadings and downward bars indicate negative loadings. Whiskers
487 indicate 95% confidence interval. **Legend**: explor (Exploration), 1st yelp latency (Latency to the first
488 yelp/whine), standing (Standing), other (Other behaviours), barking (Barking), yawn (Yawn), contact encl
489 (In contact with the enclosure), urin/defec (Urination and/or defecation), sitting (Sitting), howling (Howling),
490 growling (Growling), paw lift (Paw lifting), body shak (Body shaking), nose lick (Nose licking), lying
491 (Lying), whin/yelp (Whining/Yelping), locom (Locomotion), orientated encl (Behaviours orientated to the
492 enclosure).
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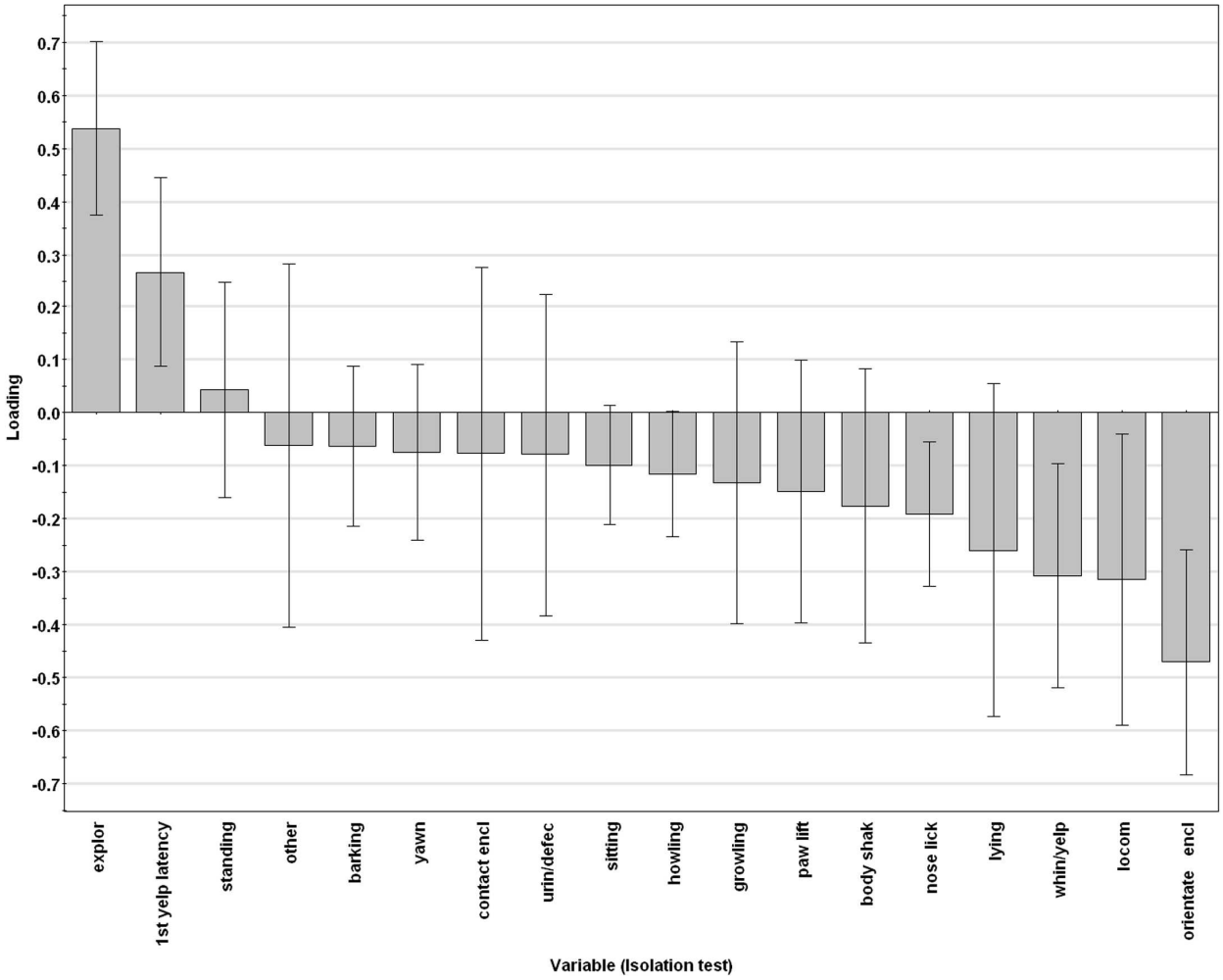

Tables

Litter	Age of the mother (in months)	Parity (n. of parturitions)	Number of male puppies	Number of female puppies	Total number of puppies
1	37	1	5	3	8
2	69	2	4	2	6
3	60	2	8	0	8
4	59	1	1	2	3
5	80	2	3	2	5
6	63	2	3	6	9
7	62	2	5	6	11
8	62	2	4	0	4
mean ± standard deviation	61.5±12.01	1.75±0.46	4.125±2.031	2.625±2.326	6.75 ± 2.71

Table 1. Characteristics of the 8 litters included in the study.

500

BEHAVIOUR	DEFINITION
NON-SOCIAL BEHAVIOURS	
Passive behaviour (Prato Previde et al., 2003; Palestrini et al., 2005)	Sitting, standing or lying down without any obvious orientation towards the physical or social environment.
Static in the 1st square (current study)	The puppy is static in the first square when left by the familiar handler.
Puppy in the 1st square (Gazzano et al., 2008)	The puppy has all four paws in the first square.
Locomotion (Prato Previde et al., 2003; Palestrini et al., 2005)	Walking, pacing or running around without exploring the environment or playing.
Exploration (Modified from: Prato Previde et al., 2003; Palestrini et al., 2005; Mariti et al., 2013a, 2013b)	Activity directed towards physical aspects of the arena or its walls/metal fence, including sniffing, close visual inspection, distant visual inspection, and gentle oral examination such as licking the arena floor, the walls/metal fence or the inanimate objects.
Individual play (current study)	Playful activity such as running, nibbling, biting, scratching the shoes/shoelace/jeans of the stranger, or the chair.
Individual play-Kong (Modified from: Prato-Previde et al., 2003; Palestrini et al., 2010; Mariti et al., 2013a, 2013b)	Any vigorous or galloping gaited behaviour directed towards the toy when clearly not interacting with the stranger, including chewing, biting, shaking from side to side, scratching or batting with the paw, chasing and tossing using the mouth. Although the pup may take the object into the mouth and carry it around the arena, destruction is not included in this category.
Individual play-rope (Modified from: Prato-Previde et al., 2003; Palestrini et al., 2010; Mariti et al., 2013a, 2013b)	Any vigorous or galloping gaited behaviour directed towards the toy when clearly not interacting with the stranger, including chewing, biting, shaking from side to side, scratching or batting with the paw, chasing and tossing using the mouth. Although the pup may take the object into the mouth and carry it around the arena, destruction is not included in this category.
Individual play-plastic disk (Modified from: Prato-Previde et al., 2003; Palestrini et al., 2010; Mariti et al., 2013a, 2013b)	Any vigorous or galloping gaited behaviour directed towards the toy when clearly not interacting with the stranger, including chewing, biting, shaking from side to side, scratching or batting with the paw, chasing and tossing using the mouth. Although the pup may take the object into the mouth and carry it around the arena, destruction is not included in this category.
Individual play-ball (Modified from: Prato-Previde et al., 2003; Palestrini et al., 2010; Mariti et al., 2013a, 2013b)	Any vigorous or galloping gaited behaviour directed towards the toy when clearly not interacting with the stranger, including chewing, biting, shaking from side to side, scratching or batting with the paw, chasing and tossing using the mouth. Although the pup may take the object into the mouth and carry it around the arena, destruction is not included in this category.
Near the enclosure (current study)	The time spent in the half of a perimeter square next to the walls or the metal fence, regardless of whether the muzzle was oriented to any other aspects of the arena.
Behaviours oriented to the enclosure (Modified from: Mariti et al., 2013a, 2013b, 2014)	All active behaviours resulting in physical contact with the walls or metal fence, including scratching the walls or metal fence with the paws, jumping on the walls or the metal fence, pulling on the walls or the metal fence with the forelegs or the mouth (including chewing, biting, shaking).
Attention oriented outdoor the enclosure (current study)	Staring fixedly outdoor at the external operator or external stimuli, either when close to the walls or the metal fence or from a distance.
Puppy in the central circle (Gazzano et al., 2008)	The puppy is in the central circle with his/her four paws.

BEHAVIOUR	DEFINITION
Number of entrances in the central circle (Gazzano et al., 2008)	Number of times the puppy enters in the central circle with his/her four paws.
Number of squares crossed (Gazzano et al., 2008)	Number of squares crossed with at least two paws simultaneously.
VOCALISATIONS	
Barking (Mariti et al., 2014)	Bark: sharp explosive vocalisation.
Growling (Modified from: Tod et al., 2005; Parthasarathy and Crowell-Davis, 2006)	Growl: deep threatening rumble, with or without exposed teeth.
Howling (Parthasarathy and Crowell-Davis, 2006)	Howl: Low-pitched, long-duration vocalization.
Whining/Yelping (Modified from: Parthasarathy and Crowell-Davis, 2006; Mariti et al., 2013a; 2013b; 2014; Beerda et al., 1998)	Whine: High-pitched vocalization. Yelp: loud (relative to whining), high-pitched vocalizations.
BEHAVIOURS POSSIBLE INDICATORS OF STRESS	
Urination and/or defecation (Tod et al., 2005)	Self-explanatory.
Body shaking (Beerda et al., 1998)	The puppy shakes his/her body.
Paw lifting (Beerda et al., 1998)	A forepaw is lifted into a position of approximately 45°.
Yawn (Tod et al., 2005)	Mouth widely opened for a period of a few seconds, then closes.
Nose licking (Tod et al., 2005)	Tongue extends upwards to cover nose, before retracting into mouth.
SOCIAL BEHAVIOURS	
Physical contact with the stranger (Modified from: Prato-Previde et al., 2003; Mariti et al., 2013a, 2013b)	Being in physical contact with the stranger.
Approach (Modified from: Prato-Previde et al., 2003; Mariti et al., 2013a, 2013b, 2014)	Approaching while clearly visually oriented to the stranger and entering in the central circle.
Attention oriented to the stranger (Modified from: Prato-Previde et al., 2003; Palestrini et al., 2005; Mariti et al., 2013a, 2013b, 2014)	Staring at the stranger, either inside or outside the central circle.
Proximity (Modified from Mariti et al., 2013a, 2013b)	Close to (not in physical contact) the stranger within the central circle or just outside the central circle.

BEHAVIOUR	DEFINITION
Attention seeking (Modified from: Mariti et al., 2013a)	Seeking attention from the stranger to play, be patted, including intentional pawing the stranger, inside or outside the central circle.
Social exploration (Modified from: Mariti et al., 2014)	Sniffing, close visual inspection, or gentle oral examination (such as licking) towards the stranger.
OTHER BEHAVIOURS Any activity not included in the behavioural catalogue, such as:	
Autogrooming Behaviours directed towards the subject's own body, like scratching, licking and biting-self. (Mariti et al., 2012; Beerda et al., 1998; Palestrini et al., 2005; Parthasarathy and Crowell-Davis, 2006)	
Digging Scratching the floor with the forepaws in a way that is similar to when dogs are digging holes (Beerda et al., 1998) **Circling** Continuous walking in short circles (Beerda et al., 1998)	

501 **Table 2.** Behaviours analysed for each puppy in the arena test

502

BEHAVIOUR	DEFINITION
NON-SOCIAL BEHAVIOURS	
Lying (Modified from: Tod et al., 2005).	Ventral/lateral lying on ground with all four legs resting and in contact with ground.
Sitting (Tod et al., 2005).	Hindquarters on ground with front two legs being used for support.
Standing (Tod et al., 2005).	All four paws on ground and legs upright and extended supporting body.
Locomotion (Modified from: Prato Previde et al., 2003; Palestrini et al., 2005)	Walking, running, pacing around without exploring the environment.
Exploration (Modified from: Topàl et al., 1998; Prato Previde et al., 2003; Palestrini et al., 2005, 2010; Mariti et al., 2013a, 2013b).	Activity directed towards physical aspects of the environment, including sniffing, close visual inspection and gentle oral examination such as licking/sniffing the floor or the puppy metal fence.
In contact with the enclosure (current study).	In contact with the metal fence.
Behaviours oriented to the enclosure (Modified from: Mariti et al., 2013a, 2013b, 2014).	All active behaviours resulting in physical contact with the puppy metal fence, including scratching the metal enclosure with the paws, jumping on the puppy metal fence, pulling on the puppy metal fence with the forelegs or the mouth (including chewing, biting, shaking).

503 **Table 3.** Non-social behaviours analysed in the isolation test

504

	explor	1st yelp latency	orientated encl	locom	whin/yelp

Spearman r					
r	0.5505	0.2878	-0.5145	-0.3224	-0.3058
95% confidence interval	0.3244 to 0.7171	0.007895 to 0.5259	-0.6918 to -0.2787	-0.5489 to -0.05166	-0.5360 to -0.03332
P value					
P (two-tailed)	< 0.0001	0.0386	< 0.0001	0.0174	0.0245

Table 4. Table of individual correlations between individual isolation test variables and maternal caregiving PCA score. **Legend**: explor (Exploration), 1st yelp latency (Latency to the first yelp/whine), orientated encl (Behaviours orientated to the enclosure), locom (Locomotion), whin/yelp (Whining/Yelping).