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The influence of dietary composition on food preference in Sharplanina Shepherd puppies

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ABSTRACT

Pet food manufacturers aim to balance the palatability with the nutritional value of their products. Ensuring that young canines receive the best nourishment is crucial, while also promoting long-lasting engagement and satisfaction during mealtime. The study aimed to investigate if the nutritional content of food can affect food preferences in 2-month-old Sharplanina shepherd dogs and the development of neophobia when introduced to a new diet. Three different dietary plans were tested, each with varying percentages of animal-derived proteins (97% in A, 77% in B, and 94% in C). Observations of behavior were documented on camera at the beginning and end of a 10-day feeding cycle. The observations were made during the scheduled feeding times of 7 a.m., noon, and 5 p.m. According to the study, puppies showed neophobic behavior when a new diet was introduced. The puppies displayed a notable decrease in meal rate of consumption, heightened distraction during diet consumption, and increased hesitation on the first day of each new diet, specifically on day 9 and day 10. Post-consumption interest peaked significantly on day 9 and day 10, particularly when dogs consumed diet C. Through the study, it was observed that Diet C had an impact on the puppies' feed preferences, indicating a possible link between the diet's nutritional content and their food preferences. Based on the study results, it appears that puppies need at least 9 days to reduce neophobia and adapt to new food flavors and feeding schedules.

Introduction

Pet food manufacturers strive to achieve a harmonious equilibrium between the palatability and the nutritional composition of their products. When developing new products for pet food manufacturers, it is imperative to ensure that canines receive optimal nourishment while simultaneously fostering sustained engagement and satisfaction during mealtime. Tobie et al. (2015) stated that attractiveness and acceptance are crucial attributes for pet foods and that even the best-formulated diets may not be popular among pet owners if the dog refuses to eat it. According to Vučinić et al. (2023), owners are increasingly interested in the quality and safe nutrition of their companions due to the special social status that dogs have in their owners' families. Because it is difficult for pets to evaluate the taste, smell, texture, and look of food, indirect approaches were devised to assist pet owners in ranking different items based on their dogs' feeding patterns and reactions (Tobie et al., 2015). The same authors considered pet owners' perceptions of their pets' feeding enjoyment and palatability of diets and assessed several palatability evaluation strategies to quantify this. According to pet food manufacturers, the main reasons for dog owners to switch food are higher-quality and healthier foods, coat and skin quality of dogs, the fact that their dogs did not enjoy the previous food, and price (Callon et al., 2017). Factors that can affect a dog's willingness to eat are palatability of food, or the subjective dog's preference of a food based on odor, texture, appearance, and taste (Griffin and Beidler, 1984; Bradshaw, 2006). There are several factors that can influence a

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dog's food preferences, some of which include early-life experiences and genetics (Bhadra and Bhadra, 2013). Health status, age, and environmental conditions can influence a dog's perception of a food source and dog's feeding behavior (Ventura and Worobey, 2013; Callon et al., 2017).

Establishing a balanced diet early on is crucial for the long-term health and well-being of puppies. Overcoming neophobia to novel foods and introducing them to a variety of nutritious and appealing pet foods can help ensure they grow into healthy adults with diverse dietary preferences.

Since dogs are unable to communicate their food preferences explicitly, this can be determined by comparing the dogs' relative acceptance of various diets (Callon et al., 2017). The two-pan test and one-pan test are common methods to assess food preference in dogs (Araujo and Milgram, 2004).

The one-pan test entails presenting a pan of food to the dog and observing it interacts with it. This test presents the dog with one pet food, measures its consumption, and compares it to one or more other feed types (Callon et al., 2017). This method makes it easy to compare dog food and measure its preferences and responsiveness to dietary changes (Callon et al., 2017). Unlike the two-pan test, the one-pan test eliminates food interactions that could affect palatability (Araujo and Milgram, 2004). In a two-pan test, the foods may interact and affect the dog's preference or response. For instance, a strong-smelling food may impair the dog's ability to sense and taste other foods. A one-pan test reduces the possibility of such interactions because all the items are in the same pan and do not interfere (Araujo and Milgram, 2004). This makes the one-pan test a more accurate and reliable way to evaluate a dog's food preferences and response to a new diet.

This is the first study which has used a single-pan test as a simple and effective way to examine feeding behavior in 2 months old Sharplanina shepherd puppies; however, similar approaches have been utilized in other animals, such as adult dogs (Callon et al., 2017), cats (Becques et al., 2014), rats (Grill and Norgren, 1978), and human and non-human primates (Steiner et al., 2001).

Sharplanina Shepherd dog has been registered by the Fédération Cynologique Internationale (FCI) since 1939 under the designation "Ilirski Ovcar" (Illyrian Shepherd Dog). In 1957, the FCI accepted a motion proposed by the Yugoslavian Federation of Cynology to change the name of the breed to "Yugoslavian Shepherd Dog - Sharplanina".

The aims of the study were to determine whether the nutritional composition of sustenance can exert an influence on the preference for food in canines at the age of 2 months and the potential manifestation of neophobia when presented with an unfamiliar dietary composition.

Materials and Methods

All experiments and procedures were approved by the Ethical Committee for the Protection and Welfare of Laboratory Animals at the Faculty of Veterinary Medicine, University of Belgrade (No. 03-04/2023). This behavioral study was part of a larger study focused on the effect of different meal formulations on the health status, proper development, well-being, and behavior of puppies. In this experiment, dry pet food was used, also known as kibble, a type of commercial pet food that is dehydrated and in a dry form. Dry pet food was produced utilizing an extrusion procedure, wherein the constituent elements were amalgamated, subjected to thermal treatment, and subsequently expelled through a die under conditions of elevated pressure and temperature. In this study, the subjects consisted of puppies at the age of 2 months. These puppies were administered a diet consisting of high-quality, age-appropriate complete feed. This feed was specifically formulated to contain the necessary nutrients required for optimal growth and development in this particular stage of their life cycle. The formulation of foods was deliberately engineered to cater to the unique dietary needs of large-breed puppies, with the primary objective of facilitating optimal bone and joint development. Furthermore, the size of the kibble was meticulously adjusted in accordance with the specific breed of the puppies, aiming to foster a favorable chewing behavior that promotes overall oral health. Three distinct dietary regimens were incorporated. The three diets exhibited distinct nutritional compositions, characterized by variations in the origins of starch, fibers, animal protein, the extent of mechanically separated meat inclusion, and the proportions of animal-derived proteins.

Subjects and facilities

Sharplanina shepherd puppies from the same kennel were chosen to guarantee a consistent group of puppies of the same age for participation in the experiment. At the Dog Training Center, puppies were held in kennels. Puppies (n = 18) were separated into three groups (A, B, and C), with nine males and nine females of the same age (2 months old) and body weight ranging from 9.6 to 11.0 kg (median = 9.6 kg, range = 8.7-11.0 kg). Puppies were housed in the groups of six containing three males and three females of similar average body weight. The kennel size was 220 cm × 380 cm that were opened with sliding doors to allow for group housing for the majority of the day, except during feeding. Kennels had the same environmental conditions (temperature, light).

Habituation and socialization were included during the experiment. Sharplanina shepherd puppies were safely exposed to a range of different stimuli such as noises, visual stimuli, and other sensory experiences, as well as social interactions with humans, conspecifics, and other species during this period of rapid neurological and emotional development. Very first socialization exposure commenced when puppies were around 3 weeks of age. The second phase of socialization exposure started when puppies were 45 days of age. Socialization, provided by the researchers and an employee at the Dog Training Center, involved group training of all 18 puppies. This regime was kept consistent for each puppy throughout the duration of the experiment.

Diets

Puppies were exposed to diets for 10 days. The three diets were A) ancestral grain inclusive diet with 97% of protein of animal origin, B) cereals inclusive diet with 77% of protein of animal origin, and C) grain free diet with 94% of protein of animal origin (Table 1).

Diets were designed to be similar in terms of kibble size, texture, moisture content, and density. Diet A, diet B, and diet C were formulated to meet or exceed FEDIAF nutrient standards for medium and large-breed puppies 2 months old. More animal fat and oils (chicken fat and fish oil) were added in vacuum coating system at the end of the production process of diet A kibble (11.4%) and diet C kibble (12.0%), compared with diet B kibble where 6.2% of animal fat (chicken fat, pork fat, and fish oil) was added in coating process. The percentage of proteins of animal origin was the lowest in diet B—77%, higher in diet C—94%, and the highest in diet A—97%. Diet A contained chicken, turkey, and fish as source of proteins of animal origin. Diet B contained chicken, turkey, pork, and lamb as source of proteins of animal origin. Diet C contained chicken as source of proteins of animal origin. Diets A and C contained the same chicken liquid palatant 2 compared to diet B which contained chicken liquid palatant 1.

The amount of diet provided to each puppy (g/day) was determined based on the energy density of each diet and the maintenance energy requirements of each puppy. These requirements were determined based on the initial body weight of each puppy at the beginning of the study. This individualized approach helps

Table 1
Ingredient profiles and nutrient analysis for diets 0, A, B, and C.

a) Ingredient profiles of diets

Ingredients (g/kg diet as is basis)	Diet 0	Diet A	Diet B	Diet C
Chicken MDM	240	210	/	130
Turkey MDM	/	170	/	/
Lamb MDM	/	/	40	/
Herring MDM	50	/	/	/
Dehydrated chicken protein	220	120	260	250
Dehydrated turkey protein	/	100	/	/
Dehydrated pork protein	/	/	25	/
Dehydrated herring protein	50	/	/	/
Chicken fat	130	74	50	80
Pork fat	/	/	10	/
Hydrolyzed poultry proteins	/	45	20	/
Hydrolyzed chicken proteins	/	/	/	81
Hydrolyzed fish proteins	/	42	/	/
Fish oil	30	40	2	40
Dried pumpkin	50	/	2	/
Pea starch	140	/	/	134
Dried potato	/	/	/	140
Corn	/	/	260	/
Wheat	/	/	260	/
Spelt	/	20	/	/
Brown rice	/	20	/	/
Rice	/	/	10	/
Barley	/	20	/	/
Oats	/	20	/	/
Millet	/	20	/	/
Corn gluten	/	/	2	/
Chicken liquid palatant 1	/	/	17.6	/
Chicken liquid palatant 2	/	21.7	/	25
Dried eggs	28	10	/	40
Linseed	/	5	/	20
Dried beet pulp	5	5	2	4
Alfalfa meal	5	5	2	4
Pea fiber	5	5	2	4
Dried fruits and vegetables blend	10	10.5	8.5	10.5
Chicory inulin, fructo-oligosaccharides, yeast extract	6	10	4	8
Ascophyllum nodosum	/	1	/	0.5
Calcium carbonate	1	1	2	2
Monocalcium phosphate	1	1	2	3
Potassium chloride	1	1	2	2
Sodium chloride	1	1	1	1
Dried brewer's yeast	2	1	2	4
Dried botanical blend	3	1.56	0.3	0.4
Glucosamine and chondroitin sulfate	0.8	0.8	0.8	0.8
Vitamin premix	11.2	12.25	8.7	10.23
Mineral premix	1.9	2	1.9	1.9
Amino-acids	8.1	4.19	2.2	3.67

MDM, mechanically deboned meat.

ensure that each puppy receives the necessary nutrition for their growth and development throughout the study. To ensure equal novelty of the diets, prior to the beginning of the study, puppies have been fed with one commercial puppy starter food of high quality (diet 0). In the last 20 days of gestation and during the lactation period, bitches were fed with the same commercial puppy food of high quality (diet 0). Diet 0 was complete and balanced food for puppies that meet their nutritional needs during weaning up to 2 months of age. Ingredient profiles and nutrient analysis of diet 0 are provided in Tables 1 and 2. Diet 0 contained chicken and herring as source of proteins of animal origin. The proportion of proteins derived from animal sources in diet 0 was 96%.

Puppies were fed 95% of their total maintenance energy requirements, in three meals per day, to ensure that there was total consumption of the diet. Dietary energy density was calculated using the predictive equations (NRC, 2006) for ME in processed foods for dogs and the analyzed macronutrient content of all three diets.

Table 2
Nutrient analysis for diets 0, A, B, and C.

b) Nutrient analysis for diets

Analyzed nutrient contents (as is basis)	Diet 0	Diet A	Diet B	Diet C
Metabolizable energy (kcal/kg)[a]	4189	4362	3898	4085
Dry matter %	93.00	93.00	93.00	93.00
Crude protein %	30.00	32.65	26.00	29.00
Crude fat%	20.00	19.40	13.00	15.70
Crude fiber %	1.10	1.60	3.00	2.50
Calcium %	1.10	1.20	1.25	1.25
Phosphorus %	0.91	0.90	0.90	0.90
Calcium: Phosphorus	1.2/1	1.3/1	1.4/1	1.4/1
Omega 6 %	3.20	3.10	2.00	2.40
Omega 3 %	0.70	0.90	0.30	0.70
DHA	0.40	0.50	0.15	0.40
EPA	0.20	0.30	0.10	0.30
% of the protein of animal origin[b]	96	97	77	94

[a] Calculated metabolizable energy based on predictive equations (NRC, 2006) for ME in processed pet foods.
[b] Calculated based on the inclusion of animal protein source ingredient in recipe.

Diets were extruded and packed at Agrosava DOO (Simanovci, Serbia) for KRAFTIA AG Switzerland. Nutrients were analyzed by near infrared spectroscopy and minerals by inductively coupled plasma analysis (Table 2). Diets were weighed in advance for each 10 days period. Kibble was provided using round stainless-steel non-slip bowl (Zolux, 24.3 cm × 24.3 cm × 6 cm). Puppies were fed individually at 7 a.m., noon, and 5 p.m., each day and had *ad libitum* access to fresh and clean water throughout all treatments.

Feeding behavior

Camcorders (Sony, Japan) were set up approximately 70 cm away from kennel doors and elevated using tripods to allow for a full view of the kennel where puppies were housed. Callon et al. (2017) found that 3-days prior to exposure to novel diet were enough for dogs to be acclimated to the cameras and feeding regime. Puppies were then video-recorded during 7 a.m., noon, and 5 p.m., on days 1, 9, and 10 (first and last 2 days) of observation period. Thompson et al. (2016) found that both shelter and pet-owned dogs display consistent preference for food, which was one reason that we did not video-recorded the feeding experience every day of the study. Puppies were separated and fed individually within the same group (group A, B, or C) in each pen. The puppies in the study received their meals simultaneously and in the same sequence every day. This was likely done to eliminate any potential bias or confounding variables that might result from changing the order of meals. Callon et al. (2017) have found that, for canine feeding behavior observation, it is optimal that recordings began approximately 10 seconds before puppies were given their meal, and ended approximately 10 seconds after all puppies within a group finished their meals (all kibble consumed). Each day after feeding was completed, the videos were reviewed to record specific feeding behaviors (ethogram) that may indicate puppies' preference for diet (Table 3). Methods for monitoring feeding behaviors in 2-month-old canines were the same as Callon et al. (2017) used in their study.

The observer who analyzed all video records was blinded to the diets that the puppies were receiving.

Statistical analysis

Data were analyzed using Graph Pad Prism software (Graph-Pad Software, La Jolla, CA). Results were described by descriptive

Table 3
Ethogram for the behaviors used to analyze the canine feeding experience (Callon et al., 2017).

Behavior	Definition
Rate of consumption	Starts with the first bite of food and ends when all kibble has been consumed
Distraction	Number of times the dog focused elsewhere. The puppy raises its muzzle out of the bowl, eyes averted, focuses on other stimuli (no food)
Hesitation	Amount of time (seconds) before the puppy took its first bite of food, after the dish was placed on the ground
Anticipation pre-consumption (0 or 1, absence or presence)	Signs of interest/excitement before eating[a] 1. Wagging tail 2. Licking air/lips 3. Pushing face through bars 4. Jumping at front of kennel
Interest post-consumption (1,2,3)	Level of interest post-consumption[b] 1. Little to no interest: puppies leave bowl soon after all kibble is consumed 2. Some interest: puppy may lick/sniff bowl/ground after consumption but loses interest in < 10 s 3. Lots of interest: dog licks bowl/ground excessively after kibble is consumed, remains focused on food source until cameras stopped recording (> 10 s)

Rate of consumption, distraction, hesitation pre-consumption, anticipation pre-consumption, and interest post-consumption were recorded to determine preference for different diets and the effect of neophobia. The specific behaviors were chosen as indicators of food preference or aversion. The presence of each of these behaviors was confirmed after analyzing the video recorded during the 3-day acclimation period.

[a] Presence (1) or absence (0) of behavior recorded. Anticipation calculated as sum of four behaviors.

[b] Level of interest recorded as one of the three levels (1, 2, or 3).

statistics (mean value, standard deviation). The distribution of behaviors was tested by the Kolmogorov-Smirnov distribution fitting test, which showed normal distribution. The differences between the mean and standard deviation of behaviors between different diets (A, B, and C) were calculated using one-way analysis of variance and compared by Tukey's HSD test at 5% level of significance. Differences were considered significant at $P < 0.05$.

Results

There were no food refusals throughout this study. In this research, it was determined that during the observation period puppies which consumed diet A spent significantly more ($P < 0.001$) time-consuming food on day 1 (99.06 ± 1.21) and day 10 (82.22 ± 1.17) compared to day 9 (71 ± 0.77). Distraction and hesitation were significantly higher ($P < 0.001$) on day 1 (3.17 ± 0.79, 3.78 ± 0.81) compared to day 9 (1.61 ± 0.50, 2.06 ± 0.64) and day 10 (1.67 ± 0.49, 1.94 ± 0.42) during observation (Table 4). Interest after food consumption was significantly higher ($P < 0.05$) on day 9 (2.44 ± 0.51) compared to the beginning of the observation (1.89 ± 0.47). No significant difference was found in the anticipation of food pre-consumption between different observation days ($P > 0.05$).

Puppies which consumed diet B during observation period ate very significantly slower on day 1 (108.02 ± 1.40) and day 10 (88.50 ± 0.92) compared to day 9 (83.11 ± 0.76). Distraction and hesitation were significantly higher ($P < 0.001$) on day 1 (3.06 ± 0.64, 3.78 ± 0.81) compared to day 9 (1.56 ± 0.51, 2.33 ± 0.49) and day 10 (1.67 ± 0.49, 1.479) during the period of observation (Table 5). The anticipation pre-consumption was significantly higher ($P < 0.05$) on day 9 (0.83 ± 0.86) and day 10 (0.89 ± 0.96) compared to the first day (0.22 ± 0.43) of observation (Table 5).

In this study, it was determined that during observation period puppies which consumed diet C spent significantly more ($P < 0.001$) time-consuming food on day 1 (99.44 ± 1.10) and day 10 (84.78 ± 0.73) compared to day 9 (76.56 ± 1.72). Distraction and hesitation were significantly higher ($P < 0.001$) on day 1 (3.17 ± 0.86, 3.94 ± 0.99) compared to day 9 (1.78 ± 0.43, 1.94 ± 0.54) and day 10 (1.67 ± 0.49, 2.22 ± 0.43) of observation (Table 6). Interest after food consumption was very significantly higher ($P < 0.001$) on day 9 (2.61 ± 0.50) and day 10 (2.40 ± 0.51) compared to the beginning of the observation (1.72 ± 0.46). No significant difference was found in the anticipation of pre-consumption of food between different observation times ($P > 0.05$).

It was determined that the puppies consumed diet B significantly longer ($P < 0.001$) compared to diets A and C (Table 7). Interest after consuming diet A (2.20 ± 0.59) and diet C (2.24 ± 0.61) was significantly higher ($P < 0.001$) compared to diet B (1.61 ± 0.49) (Table 7). In other indicators of the behavior of the puppies, no significant difference was found between the consumption of different feeds ($P > 0.05$) (Table 7).

Table 4
Mean ± SD level of specific behaviors for diet A.

Specific behaviors	Day 1	Day 9	Day 10
Rate of consumption	99.06 ± 1.21^{AB}	71 ± 0.77^{AC}	82.22 ± 1.17^{ABC}
Distraction	3.17 ± 0.79^{AB}	1.61 ± 0.50^{A}	1.67 ± 0.49^{B}
Hesitation	3.78 ± 0.81^{AB}	2.06 ± 0.64^{A}	1.94 ± 0.42^{B}
Anticipation pre-consumption	0.33 ± 0.49	0.89 ± 0.90	0.67 ± 0.91
Tail wagging	0.33 ± 0.49	0.56 ± 0.51	0.39 ± 0.50
Licking air/lips	0	0.22 ± 0.43	0.17 ± 0.38
Pushing face through bars	0	0.11 ± 0.32	0.11 ± 0.32
Jumping at front of kennel	0	0	0
Interest post-consumption	1.89 ± 0.47^{a}	2.44 ± 0.51^{a}	2.28 ± 0.67

A, B - Capital letters indicate significant difference (P < 0.001) between specific behaviors. a - Small letter indicate significant difference (P < 0.05) between specific behaviors.

Table 5
Mean ± SD level of specific behaviors for diet B.

Specific behaviors	Day 1	Day 9	Day 10
Rate of consumption	108.02 ± 1.40^{AB}	83.11 ± 0.76^{AC}	88.50 ± 0.92^{ABC}
Distraction	3.06 ± 0.64^{AB}	1.56 ± 0.51^{A}	1.67 ± 0.49^{B}
Hesitation	3.78 ± 0.81^{AB}	2.33 ± 0.49^{A}	1.89 ± 0.47^{B}
Anticipation pre-consumption	0.22 ± 0.43^{ab}	0.83 ± 0.86^{a}	0.89 ± 0.96^{b}
Tail wagging	0.22 ± 0.43	0.44 ± 0.51	0.50 ± 0.51
Licking air/lips	0	0.22 ± 0.43	0.17 ± 0.38
Pushing face through bars	0	0.06 ± 0.24	0.11 ± 0.32
Jumping at front of kennel	0	0.17 ± 0.38	0.11 ± 0.32
Interest post-consumption	1.50 ± 0.52	1.67 ± 0.49	1.67 ± 0.49

A, B - Capital letters indicate significant difference (P < 0.001) between specific behaviors. a, b - Small letters indicate significant difference (P < 0.05) between specific behaviors.

Table 6
Mean ± SD level of specific behaviors for diet C.

Specific behaviors	Day 1	Day 9	Day 10
Rate of consumption	99.44 ± 1.10^{AB}	76.56 ± 1.72^{AC}	84.78 ± 0.73^{ABC}
Distraction	3.17 ± 0.86^{AB}	1.78 ± 0.43^{A}	1.67 ± 0.49^{B}
Hesitation	3.94 ± 0.99^{AB}	1.94 ± 0.54^{A}	2.22 ± 0.43^{B}
Anticipation pre-consumption	0.50 ± 0.71	1.06 ± 0.99	0.83 ± 0.86
Tail wagging	0.33 ± 0.49	0.56 ± 0.51	0.39 ± 0.50
Licking air/lips	0.11 ± 0.33	0.17 ± 0.38	0.17 ± 0.38
Pushing face through bars	0.06 ± 0.24	0.17 ± 0.38	0.17 ± 0.38
Jumping at front of kennel	0	0.17 ± 0.38	0.11 ± 0.33
Interest post-consumption	1.72 ± 0.46^{AB}	2.61 ± 0.50^{A}	2.40 ± 0.51^{B}

A, B - Capital letters indicate significant difference (P < 0.001) between specific behaviors.

Table 7
Mean ± SD level of specific behaviors for all dogs and diets A, B, and C.

Specific behaviors	Diet A	Diet B	Diet C
Rate of consumption	84.09 ± 11.68^{A}	93.28 ± 10.94^{AB}	86.93 ± 9.64^{B}
Distraction	2.15 ± 0.94	2.09 ± 0.87	2.20 ± 0.92
Hesitation	2.59 ± 1.06	2.67 ± 1.01	2.70 ± 1.13
Anticipation pre-consumption	0.63 ± 0.81	0.65 ± 0.83	0.80 ± 0.88
Tail wagging	0.43 ± 0.50	0.39 ± 0.49	0.43 ± 0.50
Licking air/lips	0.13 ± 0.34	0.13 ± 0.34	0.15 ± 0.36
Pushing face through bars	0.07 ± 0.26	0.06 ± 0.23	0.13 ± 0.34
Jumping at front of kennel	0	0.09 ± 0.29	0.09 ± 0.29
Interest post-consumption	2.20 ± 0.59^{A}	1.61 ± 0.49^{AB}	2.24 ± 0.61^{B}

A, B - Capital letters indicate significant difference (P < 0.001) between specific behaviors.

During observation period the puppies spent significantly more ($P < 0.001$) time in consumption of food A, B, and C on day 1 (102.2 ± 4.44) and day 10 (85.17 ± 2.77) compared to day 9 (76.89 ± 5.13) (Table 8). The number of times the puppies focused on other stimuli throughout a feeding bout (level of distraction) and amount of hesitation in consuming diets A, B, and C were significantly higher ($P < 0.001$) on day 1 (3.13 ± 0.75, 3.83 ± 0.86) compared to day 9 (1.65 ± 0.48, 2.11 ± 0.57) and day 10 (1.67 ± 0.48, 2.02 ± 0.46) (Table 8). The level of anticipation tended to be significantly higher ($P < 0.001$, $P < 0.05$) on day 9 (0.93 ± 0.91) and day 10 (0.80 ± 0.90) compared to day 1 (0.35 ± 0.55). Lip licking was significantly more pronounced on day 9 (0.23 ± 0.41) than on day 10 (0.04 ± 0.19). Interest after consuming diets A, B, and C was significantly higher ($P < 0.001$, $P < 0.05$) on day 9 (2.24 ± 0.64) and day 10 (2.11 ± 0.63) compared to the day 1 (1.70 ± 0.50) (Table 8).

Table 8
Mean ± SD level of specific behaviors for all dogs and diets A, B, and C in days 1, 9, and 10.

Specific behaviors	Day 1	Day 9	Day 10
Rate of consumption	102.2 ± 4.44^{AB}	76.89 ± 5.13^{AC}	85.17 ± 2.77^{ABC}
Distraction	3.13 ± 0.75^{AB}	1.65 ± 0.48^{A}	1.67 ± 0.48^{B}
Hesitation	3.83 ± 0.86^{AB}	2.11 ± 0.57^{A}	2.02 ± 0.46^{B}
Anticipation pre-consumption	0.35 ± 0.55^{Ab}	0.93 ± 0.91^{A}	0.80 ± 0.90^{b}
Tail wagging	0.30 ± 0.46	0.52 ± 0.50	0.43 ± 0.50
Licking air/lips	0.04 ± 0.19^{a}	0.23 ± 0.41^{a}	0.17 ± 0.38
Pushing face through bars	0.02 ± 0.14	0.11 ± 0.32	0.13 ± 0.34
Jumping at front of kennel	0	0.11 ± 0.32	0.07 ± 0.26
Interest post-consumption	1.70 ± 0.50^{Ab}	2.24 ± 0.64^{A}	2.11 ± 0.63^{b}

A, B - Capital letters indicate significant difference (P < 0.001) between specific behaviors. a, b - Small letters indicate significant difference (P < 0.05) between specific behaviors.

Discussion

The present investigation aimed to analyze various feeding behaviors exhibited by puppies at the age of 2 months, with the objective of ascertaining whether they display a preference toward specific ingredients within their sustenance. The study analyzed the rate of consumption, hesitation, and level of interest before and after consumption of different food formulations with varying ingredient combinations. These behavioral observations were used to make inferences about the puppies' preferences for certain ingredients and to identify any food compositions that they may find more appealing. The behaviors evaluated in this study may be similar to those displayed by pet puppies when introduced to new foods by their owners, because at the age of 2 months puppies can be reluctant to try new foods. Neophobia, or an aversion to anything new or unfamiliar, is common issue in dogs and can affect their willingness to try new foods. Stöwe et al. (2006) defined neophobia as "the avoidance of an object or other aspect of the environment solely because it has never been experienced and is dissimilar from what has been experienced in the individual's past." Callon et al. (2017) found that adult dogs experienced the initial neophobic effects of novel diet demonstrated by slower rate of consumption with increased distraction when first introduced to a new diet. In their study, indicators of neophobia included longer periods of hesitation, reduced interest in the food pre- and post-consumption.

In the present study, the puppies experienced neophobia at the beginning of observation period regardless of type of diet (diet A, diet B, diet C), with those effects declining by days 9 and 10. No difference was found between diet types when puppies' distraction and increased hesitation prior to eating on day 1 were observed. Puppies have consumed meals significantly faster on day 9 and day 10 comparing to the rate of consumption on the first day. It suggests that the puppies' initial response to new food, including distraction and hesitation, may be more related to the novelty of the experience rather than the specific composition of the diet. This is consistent with the idea that neophobia is a behavioral response to new stimuli, not necessarily a reaction to the taste or smell of the food itself (Callon et al., 2017; Stolzlechner et al., 2022). When presented with a new type of food, it is natural for puppies to approach it with some degree of caution and hesitation, as they need time to evaluate the safety and palatability of the food (Bourgeois et al., 2006). It might indicate that puppies 2 months old need more time to adjust to the new food. This initial hesitation may be influenced by many factors, including the sensory properties of the food (taste, smell, texture), the context in which it is presented (e.g., time of day, location), and the puppy's previous experience with similar foods (Kitchell, 1978; Bradshaw, 2006). The fact that the neophobic response decreased by days 9 and 10 suggests that the puppies may have become more comfortable with the new food over time. This is also in line with the idea that gradually introducing new experiences to a dog can help to reduce anxiety and fear (Boxall et al., 2004; Morrow et al., 2015; Serpell et al., 2016; Hakanen et al., 2020; Stolzlechner et al., 2022).

When introducing new foods to their puppies, owners should be kind and patient, allowing them the space and time to adjust and explore at their own pace. Gradual introduction and positive reinforcement can help to build their confidence, overcome these initial neophobic responses, and become more comfortable with a wider variety of foods. On the contrary, forcing or rushing puppies can lead to increased anxiety and avoidance behaviors. It is worth noting that, while faster eating rates may be seen as a positive sign of acceptance, it is important for owners to monitor their puppies' eating habits and make sure they are not eating too quickly or excessively, which can lead to digestive issues (Smith et al., 1984; Ohtani et al., 2015; De Cuyper et al., 2020). Overall, this study reinforces the importance of taking a gradual and tailored approach to puppy nutrition.

When puppies are awaiting their meals, they demonstrate excitement or anticipation. The level of excitement before eating was higher on the last two days of 10-day feeding trial, comparing to the first day. These results may indicate that the puppies were not adjusted to the feeding routine on the first day and were not anticipating a food source until last 2 days of 10-day feeding trial. Licking lips as sign of anticipation of food was more often on ninth day what can be sign that puppies get used to receive food every day in the same time, or it can be result of simple hunger caused by drop level of sugar in the blood. According to this, puppies anticipated feeding times when they got used to established routines for meals. This finding suggests that, over the course of the 10-day feeding trial, the puppies became more accustomed to their feeding schedule and learned to associate certain cues or signals with the arrival of their meals, which in turn increased their level of excitement and anticipation. This is not surprising, as dogs are known for their ability to learn and respond to cues and signals associated with meal times, such as the sound of a food bowl being filled or the smell of food being prepared (Rehn and Keeling, 2016; Lyle et al., 2017; Marshall-Pescini et al., 2017). As the puppies became more familiar with the feeding routine and cues associated with meal times, they likely began to anticipate and look forward to their meals with greater excitement.

Therefore, anticipatory behaviors on day 9 were due to feeding regimes acclimation or a result of simple hunger. In dogs, the rise and fall in blood sugar levels have been linked to several factors, including the type of diet, frequency and timing of meals, and level of physical activity (Carciofi et al., 2008). Carciofi et al. (2008) found that dogs fed a high carbohydrate diet experienced a rapid increase in blood glucose levels after eating, followed by a sharp decline within 2 hours. In contrast, dogs fed a low-carbohydrate diet had a slower and more sustained increase in blood glucose levels, with a gradual decline over several hours. Similarly, Bolli et al. (1984) reported that, in humans, blood glucose levels peak in the early morning hours and then decline gradually throughout the day, reaching their lowest levels after about 5 hours of fasting. This drop in blood glucose levels is thought to trigger hunger and increase food intake. Overall, the rise and fall in blood sugar levels play an important role in regulating appetite and energy balance in both humans and dogs. When animals are hungry, their foraging styles may be modified and they may be more likely to try new foods and show a higher preference for high-calorie, high-fat foods, even if those foods are not their usual preference. In fact, research has shown that hunger may differently affect dogs' and wolves' preference for specific food types; dogs and wolves have a higher preference for high-fat foods when they are hungry (Rao et al., 2018).

The observation that puppies exhibited a considerably slower consumption rate for diet B compared to diets A and C presents a number of potential explanations that warrant further investigation. One possibility is that the puppies' previous experience with different types of food may have influenced their preference for specific diets, while the second possibility is that the taste and/or texture of diet B was less appealing to the puppies than the other diets.

During the last 20 days of gestation and during the lactation period, bitches were fed with diet 0. Also, prior the beginning of the study, the puppies were used to consuming a diet 0 which was high-fat diet (130 g/kg of chicken fat and 30 g/kg of fish oil). The puppies may have found diet B less palatable due to its lower fat content (50 g/kg of chicken fat, 10 g/kg of pork fat, and 2 g/kg of fish oil). On the contrary, both diets A and C were high-fat diets (100 g/kg of chicken fat and 40 g/kg of fish oil and 80 g/kg of chicken fat and 40 g/kg of fish oil, respectively).

These findings suggest that puppies could be attracted by the smell of chicken fat, which shows good attractant smell. Bauer (2006) finding highlights the important role that lipids and other macronutrients can play in the palatability of pet food. In addition to providing essential nutrients, lipids, or fats, can also function as palatants, enhancing the flavor and aroma of pet food and making it more appealing to animals. Crude fat extracts from animal sources such as chicken, beef, and lamb are particularly effective as palatants, as they have characteristic aromas that are highly desirable to pets. Callon et al. (2017) found that dogs did not show a strong preference for either the animal or vegetable ingredient-based diets. In the study mentioned, the researchers did include more fat on the outside of the vegetable ingredient-based kibble, which might have contributed to its palatability. It is important to note that palatability can be influenced by a wide range of factors, including the types of ingredients used, the processing methods, and the addition of flavorings or other additives. The current results suggest that the puppies had preference for diets A and C over diet B with the understanding that the known palatability enhancer chicken fat was applied in significantly higher quantity to the outside of the kibbles A and C than kibble B. Hepper and Wells (2005) research has shown that dogs can learn about odors prenatally through exposure to maternal diet and amniotic fluid, and that this can shape their odor preferences after birth. Both diets A and C were more similar to the diet 0 in the terms of fat type and fat content used in the diet 0. Diet A contained chicken, turkey, and fish as source of proteins of animal origin. Diet B contained chicken, turkey, pork, and lamb as source of proteins of animal origin. Diet C contained chicken as source of proteins of animal origin. Diet 0 contained chicken and herring as source of proteins of animal origin. These findings suggest that prenatal learning can play an important role in shaping odor preferences in dogs and may be a way in which animals communicate about food and other environmental stimuli across generations. Offering a variety of dietary types and flavors to pregnant and nursing bitches can help their offspring develop a broader acceptance of different foods. This is important as it can help prevent the development of fussy or discriminatory feeding habits in puppies. By exposing them to a diverse range of foods during their early development, they can learn that different flavors and textures are safe to consume. This can ultimately lead to healthier and less picky eating habits in the future. It would be interesting to research whether the food that puppies are fed during the transition period on solid food and during the last 20 days of gestation and lactation period of their mothers can affect their food preferences at 2 months of age. This research can shed more light on the critical stages of development that affect food preferences and help breeders and pet owners make informed decisions about the diets of pregnant and nursing bitches and their offspring. Such research can also help develop better dietary recommendations for puppies and improve their health, growth, and development. The level of interest in feed after eating was higher in puppies which consumed diets C and A compared with puppies which consumed diet B. The finding suggests that the puppies were more interested in continuing to eat after consuming diets C and A compared to diet B. This observation could potentially indicate two possibilities: first, that the puppies exhibited a greater preference for diets A and C due to their enhanced palatability, thereby seeking additional consumption; or second, that diets A and C were comparatively less satiating, prompting the puppies to actively seek additional food.

Interest in feed after eating was evaluated based on the puppies' tendency to lick the ground or bowl after all kibble was consumed, signifying continued interest in their meal. Diet A had 97% and diet C had 94% of proteins of animal origin. Diet B had 77% of proteins of animal origin. Keller (2011) found that plant-based proteins may be more satiating than animal-based proteins. This may be due to a variety of factors, such as higher fiber content in plant-based proteins, slower digestion and absorption rates, and fewer calories per gram of protein (since many plant-based proteins are lower in fat). These findings have important implications for weight management. Incorporating more plant-based protein sources into the diet may help to increase feelings of fullness and reduce overall energy intake, which may be beneficial for weight management. Diet B had 3% of crude fiber, diet A 1.6 %, and diet C 2.5%. A high fiber diet has been shown to increase satiety and decrease hunger. Bosch et al. (2014) have found that feeding motivation can be decreased by altering sources and levels of dietary fiber in food, since these can affect both acute and prolonged food intake control. This is believed to occur because fiber slows digestion and absorption of nutrients, leading to a gradual rise in blood sugar levels instead of a sharp spike and subsequent crash. This steady blood sugar level can help reduce cravings and feelings of hunger. Overall, these findings suggest that satiation may be an important factor in determining post-consumption interest in food, but more research is needed to fully elucidate the underlying mechanisms. In order to ascertain the satiating effects of each diet, it is imperative for future research to amalgamate the behavioral measurements pertaining to the canine feeding experience with the concentrations of satiety hormones. By conducting this experiment, it is possible to determine whether the inclination toward consuming food after a meal is a result of immediate satisfaction of hunger.

Hewson-Hughes et al. (2012) found that dogs showed a preference for diets with specific macronutrient compositions. Dogs were given a choice between four diets that varied in their protein, fat, and carbohydrate content. The study found that dogs preferred a diet with higher protein and lower carbohydrate content. This suggests that dogs may have an innate ability to regulate their macronutrient intake to some extent, and may prefer foods with specific macronutrient profiles based on their individual nutritional needs. Prior to the beginning of the study, during a weaning period and up to 2 months of age the puppies have been fed with diet 0. In the last 20 days of gestation and during the lactation period, bitches were fed with diet 0, as well. Nutritional analyses showed that diet 0 was a high-protein (30% protein) and a high-fat diet (20% fat) compared to diet B which had a protein content of 26% and a fat content of 13%. The protein content of diet A (32.65% protein) and diet C (29% protein) is closer to the protein content of diet 0, which may be one of the reasons may have influenced puppies' preference for diets A and C. This is consistent with observations of wild canids, which also show a preference for high-protein foods (Hewson-Hughes et al., 2012). Alternatively, if they were used to consuming a diet with a specific type of protein, they may have found diet B less appealing due to the use of a different type of protein. Diet A contained chicken, turkey, and fish as source of proteins of animal origin. Diet B contained chicken, turkey, pork, and lamb as source of proteins of animal origin. Diet C contained chicken as source of proteins of animal origin. Diet A and diet C contained the same chicken liquid palatant 2 compared to diet B which contained chicken liquid palatant 1. Diet 0 contained chicken and herring as source of proteins of animal origin. These natural flavors may also provide an additional sensory cue to animals that the food is high in protein, which is an important macronutrient for growth and maintenance. Bergström et al. (2020) highlighted the importance of considering a dog's ancestral history and natural dietary preferences when formulating and selecting pet food. While dogs have evolved to eat a variety of foods, including grains and vegetables, their preference for meat-based diets is rooted in their history as carnivorous predators (Hall et al., 2017; Bergström et al., 2020). The findings from Lohse (1974) and Houpt and Hintz (1978) provide further evidence of dogs' preferences for meat-based diets. Lohse (1974) suggested that beef is the most preferred protein source for dogs, followed by lamb and chicken. This aligns with the idea that dogs have a strong preference for red meats, which are more similar to the prey they would have consumed in the wild. Similarly, the study by Houpt et al. (1978) found that dogs exhibit similar preferences for beef and pork, with higher preferences for these meats compared to chicken and lamb. This suggests that the specific type of meat may influence dogs' palatability preferences.

Diets A and C can be attractive to the puppies due to the higher level of mechanically deboned meat (MDM) included in diets where the extrusion process helps to improve the taste and aroma of the pet food by exposing the ingredients to high temperatures. This enhances the flavor and makes it more appealing to dogs. Di Donfrancesco et al. (2014) and Koppel (2020) found that, while the aroma or smell of a pet food may initially attract animals, the overall flavor is what ultimately drives their consumption of the product. It is important for pet foods to offer a balanced and appealing flavor profile to encourage regular consumption and ensure that animals are receiving the necessary nutrients from their diet. Retronasal odors refer to the aromas released when food is chewed or swallowed and travels up through the back of the throat to the nose (Koppel, 2020). This sensory experience is a critical aspect of flavor perception and plays a significant role in driving overall food preferences and consumption. Houpt et al. (1978) found that dogs prefer meat protein over high protein diets composed of non-meat products. Brown (2009) suggests that dogs may find diets lacking any animal-based ingredients less palatable due to their evolutionary history as carnivores. The taste, smell, and texture of animal proteins are more appealing to dogs because they are more familiar and natural to them. Additionally, animal-based diets are more energy-dense, meaning that they contain more calories per unit of volume, which can make them more satisfying and fulfilling for dogs. Bhadra and Bhadra (2013) conducted a study on adult Indian free-ranging dogs to investigate their scavenging behaviors and food preferences. The researchers observed that the dogs showed a significant preference for meat when scavenging for food, often targeting leftover meat scraps from butcher shops and discarded carcasses of livestock. The dogs also demonstrated a preference for food with a strong odor, suggesting that the smell of meat may play a role in their food preferences. Since dogs are often considered as primarily meat-eaters, it was expected that they would demonstrate a preference for the diets A and C due to the higher level of MDM.

Conclusions

This study could serve as a step toward the formulation of alternative approaches for evaluating canine food preference, aiming to more faithfully simulate the conditions encountered by consumers within their domestic settings. The findings of this research, although limited by the low number of diet types and only a single breed observed, indicate that it could be advisable for dog owners to allocate at least 9 days for their large dog breeds to acclimatize to a novel dietary regimen, prior to making a conclusive assessment regarding its palatability for the dog. It is plausible that any observed disparity in dogs' level of interest could be attributed to alternative factors, such as immediate satiety, specific constituents, or the utilization of distinct processing techniques aimed at stimulating food consumption. Additional research is necessary to explicate the intricate variables that affect and forecast food preference in canines, as well as the manner in which the owner perceives the act of feeding.

Authorship Statement

All authors equally were participated in the design of the study, conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Author contributions

M. Velebit and **R. Marković**: Conceptualization, Methodology. **M. Velebit:** Investigation and Writing-original draft preparation. **M. Mirilović** and **K. Nenadović:** Data curation and Formal analysis. **D. Šefer** and **B. Velebit:** Writing-reviewing and editing.

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Ethical Considerations

All experiments and procedures were approved by the Ethical Committee for the Protection and Welfare of Laboratory Animals at the Faculty of Veterinary Medicine, University of Belgrade (No. 03-04/2023).

Conflict of Interest

The authors declare they have no conflict of interest.

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