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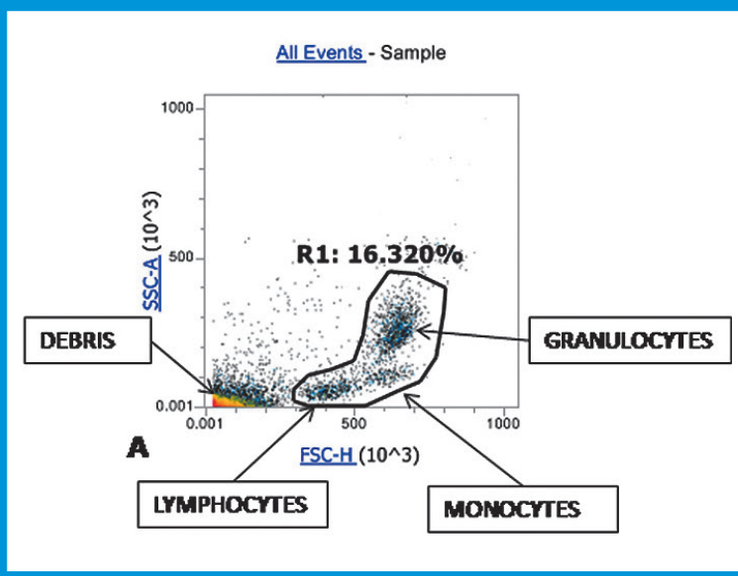

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Review Article

APPLICABILITY OF FLOW CYTOMETRY IN IDENTIFYING AND STAGING LYMPHOMA, LEUKEMIA AND MAST CELL TUMORS IN DOGS: AN OVERVIEW

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Abstract: Lymphomas, leukemias and mast cell tumors belong to the most important group among all neoplasms affecting dog species. Diagnosis, staging and determining the cell type involved in a specific tumor represent a challenge for researchers and clinicians, and plays a crucial role in treatment efficacy and prognostic purposes. Many different gold standard techniques such as cytology, histopathology, immunohistochemistry and cytochemistry are used to routinely diagnose and stage these tumors. In the recent years flow cytometry is becoming more applicable in veterinary medicine since a wide number of health conditions can be analyzed in a short period of time with a high accuracy. Multiparametric analysis performed by flow cytometry is considered as one of the main advantages of this technique since cell populations can be analyzed for different superficial markers at the same time. Immunophenotyping and staging of tumor cell populations performed by flow cytometry can help in reaching a confirmatory diagnosis and appropriate prognosis of the disease. Moreover, many flow cytometric results have been linked to a high prognostic relevance especially in neoplastic disorders. However, flow cytometry results are compatible and should be interpreted in compliance with data obtained by histopathology, immunohistochemistry and cytology.

Key words: flow cytometry; antibodies; diagnosis; lymphoma; leukemia

Introduction

Neoplastic disorders are considered as one of the most frequent pathologies not only in human medicine, but also veterinary medicine as well. Neoplastic conditions may affect dogs just as all other animals (1).

The term lymphoma often refers to a type of neoplasia where lymphocytes are strictly involved. The updated Kiel Classification is commonly used to classify lymphomas in two categories based on their grade of malignancy, as Low and High grade (2, 3). Further, histopathology is recommended to determine the neoplastic entity of the tumor. The classification proposed by Valli and colleagues (4) classifies dog lymphoma in six most common entities: diffuse large B cell lymphoma (DLBCL), marginal cell lymphoma (MZL), peripheral T cell lymphoma, T-zone lymphomas, T cell lymphoblastic lymphoma, and follicular lymphoma.

Different categories of dog lymphomas have great similarities to human and mouse lymphomas as well. Actually, a study performed by Morse and colleagues have compared similarities between mice and human lymphoid neoplasms (5). This study has also made a classification of mice lymphoid neoplasms following the World Health Organisation (WHO) classification using an appropriate terminology. Another study has classified the non-lymphoid neoplasms of mice aiming to create a consensus among clinical pathologists regarding this issue (6).

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After diagnosis has been established, staging and prognostic features of the disease in peripheral blood, bone marrow, spleen and liver using cytology, western blotting, histopathology, immunohystochemistry and immunocytochemistry should be performed in order to design an adequate therapy protocol (7). Moreover, it has been showed that immunohystochemistry can be used for the identification of lymphoid cells in normal or inflammatory conditions (8)

Among leukemias, three main types are distinguished; acute, chronic and lymphoma with a leukemic phase. Acute leukemias usually arise in bone marrow due to genetic mutations preventing the cells to maturate and proliferate, and are classified as lymphoid, myeloid or undifferentiated. Most acute leukemias in dogs have myeloid origin (9). Diagnosis of acute leukemia is based in finding more than 20-25% bone marrow infiltration from blasts (10). On the contrary, chronic leukemia's are characterized by a highly abnormal presence of mature cells in the peripheral blood (11). Chronic leukemia's can have lymphoid or myeloid origin and diagnosis for both is difficult since it is based on clinical sings and mostly by excluding other pathologies that can affect the number of mature cells in the peripheral blood circulation.

Mast cell tumors (MCTs) are among the most frequent skin tumors in dogs with an overall incidence of 7-25% (12). The cytological evaluation is recommended as a first approach since it can reach to a diagnosis on 96% of cases when a MCT is suspected (13). After cytology, the histopathologic evaluation is needed to confirm diagnosis and to define the grade of the MCT. Two main systems define the tumor grade: a) Patnaik, classifying the tumor in three grades (I, II, III); and b) Kiupel, classifying tumors in high and low grade (14, 15). Further, immunohistochemistry can be of great relevance when evaluating prognostic biomarkers such as Ki67 and CD117 (16, 17). Staging of MCTs can be performed by different techniques but the histological and cytological examination of lymph nodes (18), spleen and liver, and cytological examination of bone marrow and peripheral blood are the main procedures used to identify mast cells infiltration. However, staging of MCTs is very challenging since mast cells can be found in different tissues also in normal or reactive conditions (19).

Other than indicating the many clinical values of Flow Cytometry (FC) in diagnosing and staging lymphomas, leukemias and MCTs, one of the main aims of this review is to promote this relatively new technique for the western Balkan region.

Flow Cytometry

Flow cytometry technique discovered in the sixties, allows the measurement of physical and fluorescent characteristics of separated cells or any other particle, such as nuclei chromosome preparations, microorganisms in a suspension, passing through a light source. There are two different types of flow cytometry, sorting and non sorting. The difference between these types is that with the sorting one, the technician has the possibility to sort different population of cells or particles (20). This type of flow cytometer provides the opportunity to analyze a specific cell population which can be of major interest by identifying its composition. Briefly, a flow cytometer is composed by the sample chamber, a sample stream, a photodetector system and one or more lasers (21). Cells in saline suspension pass through a laser beam in a single row.

The light passing through a single cell is refracted and diffracted. This information is captured and converted in scatter/dot plots providing information on cell size and complexity (Figure 1).

When cells are labeled, the laser beam excites the fluorochromes conjugated to the antibodies giving a positive signal expressed in percentages. Moreover, multiparametric analysis can be performed obtaining data of interest on cell populations for different antigen expression at the same time (22).

As in other tests it is important to run negative control sample to avoid false positive signals, incorrectly considering a cell population as positive. In most cases isotype controls are used, but also a negative cell population to a specific antibody (ex: T-Lymphocytes to CD21) can be adequate for this purpose. Fluorescence minus one (FMO) tubes should be performed when a new flow cytometric experiment is taking place in order to set the correct instrument compensation (23).

Other than immunophenotyping, flow cytometry technique can be used for a wide variation of analysis such as; Minimal Residual Disease, DNA content, apoptosis, proliferation markers (ex. Ki67), immunoresponse to vaccines, external cell activities such as in allergies, etc.

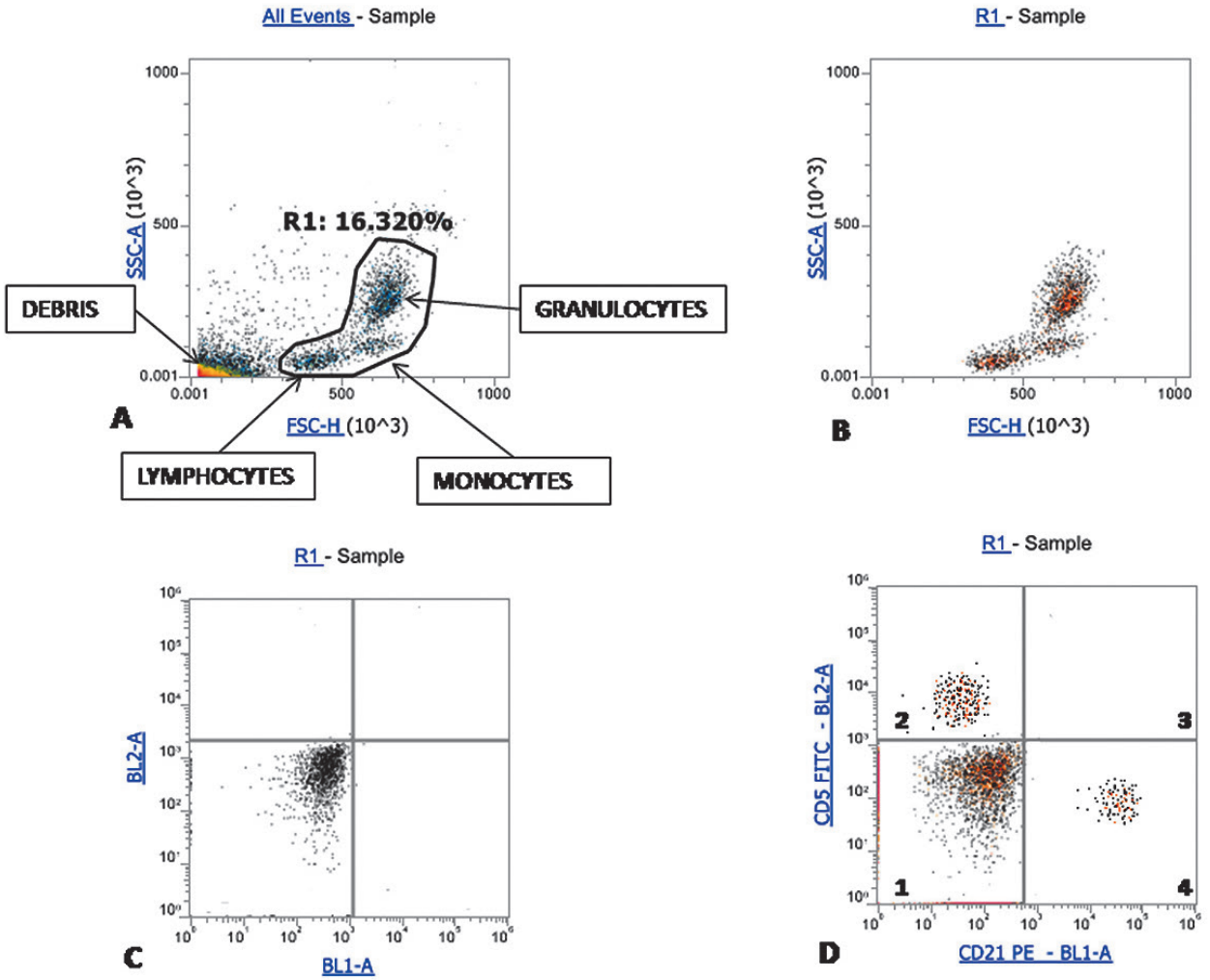

Figure 1: Normal peripheral blood sample dot plot routinely analyzed with an NxT Attune Flow Cytometer. Forward scatter (FSC) indicates cell size while side scatter (SSC) indicates cell granularity or complexity (A)

A - Gate of analysis designed to exclude debris from evaluation. Debris are presented as the less complex and smallest events (out of the analysis gate R1) followed by lymphocytes, monocytes and granulocytes.
B - The gate of analysis R1 activated in another dot plot in order to have a clear view of the cells of interest
C - FL1 and FL2 without application of antibodies
D - Two antibodies are applied CD5 (T-Cell Lymphocytes) and CD21 (Mature B-Cell Lymphocytes). Granulocytes are negative to both antibodies (quadrant 1), T-Cells positive to CD5 (quadrant 2), no events positive to CD5 and CD21 are present (quadrant 3), and B-Cells positive to CD21

Sample preparation for flow cytometric analysis

Since cells morphology can alter during conservation or transportation, sample analysis with flow cytometer should take place as soon as possible, but a period of time up to 24 hours from collection is considered suitable for the sample to maintain a good quality. Samples of Lymph nodes (LNs) and MCTs are collected using fine needle aspiration biopsy (FNAB) technique and placed in RPMI 1640 tubes (24, 25). For lymphoma usually seven to eight mass aspirations should be enough to collect an adequate number of cells for the analysis, while for MCTs more mass aspirations should be applied since mast cells are fragile compared to other white blood cells and furthermore degranulation can occur spontaneously in these cells. Peripheral blood and bone marrow samples are placed in ethylenediamine-tetra acetic acid solution (EDTA) tubes in order to prevent cell clotting.

Whenever possible it is strongly recommended to make cytological smears at the same time of sample collection. Before performing flow cytometric analysis it is necessary to quantify

the total number of cells/µl. For this purpose cell count analyzers (26, 27) or a flow cytometer can be used (28). A total number of 10^6 cells/ tube are considered adequate for the analysis of lymphomas and leukemia's, while for MCTs a minimum of 30 cells/µl is required to consider the sample suitable for the analysis (29).

Cells can be prepared for both staining methods, superficial and intracellular, depending on the specific analysis (30). In order to create a well-designed gate, to exclude debris from the analysis and to evaluate the samples quality, propidium iodide (PI) which binds the DNA of the fragmented cells can be used (31).

For the superficial labeling, after cell count has been performed, an aliquot of each sample is added to the FC tubes containing the specific monoclonal antibodies. Cells with the antibodies incubate at 4° C in a dark environment for 20 - 30 minutes. After incubation time, a red blood cells (RBC) lysis step is performed, using ammonium chloride lysis buffer since, RBC can interfere with the gate of analysis when white blood cells are analyzed. RBC lysis is not performed or it is modified in cases when erythroid origin leukemia is suspected. Lysis step lasts from five minutes in cases of MCTs to fifteen or more depending on the concentration of RBC in the sample. In some cases when analyzing peripheral blood or bone marrow, lysis can be done prior to the incubation, and RPMI medium can be added in order to stabilize the sample depending on the nature of the analysis. After lysis, samples are centrifuged usually at 1200 RMP for 5 minutes and then the supernatant is discarded while the pellet is resuspended with phosphate buffered saline (PBS). Lysis and PBS solutions are commercially available and ready for use, but they could also be prepared by own laboratory.

On the other hand, if intracellular staining is applied different steps including permeabilization of the membrane and fixation of the cells are necessary. Usually intracellular staining is used to detect intracellular cluster of differentiation (CD) or intracellular markers such as Ki67 and DNA content (32, 24).

Antibodies

Antibodies used in veterinary medicine can be both conjugated and non-conjugated. When antibodies are non-conjugated, a fluorochrome should be added since the laser can excite the fluorchrome conjugated to the antibody and not the antibody itself. Most common fluorochromes used in veterinary medicine include fluorescein (FITC), allophycocyanin (APC), phycoerythrin (PE), PE tandem dyes (PE-Cy5) and Alexa Fluor (AF488 and AF647) (33). Prior to its use, an antibody should always be titrated in order to exclude false positive signals.

Usually, antibodies used in FC are chosen based on the target cells under investigation. When a lymphoma is suspected the target cells are lymphocytes. Based on this criteria, specific antibodies which detect certain CD (CD3, CD5, CD4, CD8, CD34, CD21, CD45, MHC-II) are routinely used for the superficial labeling, while CyCD79b and CyCD3 are used for intracellular antigen detection (34, 35). Often these antibodies are combined together and placed in the same tube in order to make possible a multiparametric analysis. Thus, CD5, CD21, CD34, and CD45 can be placed in the same tube to detect the nature of the lymphocytes under investigation, and after that CD3, CD4, CD8 can be used if a CD5 positive lymphoma is identified. Different lymphomas can express different immunophenotypes (Table 1).

When leukemia is suspected a set of antibodies are used as a first approach and later other antibodies can be added, depending of the first analysis results. Since in cases of leukemia any of the cell lineages can be interested, more antibodies are needed for the immunophenotype characterization. For this purpose antibodies such as CD11b, CD14, CD61, CD90, CD5, CD21, CD11b, CD34, CD45 and CD44 can be applied (36). Staging of the tumors is made based on the

Table 1: Most common immunophenotypes expressed in main types of dog lymphomas.

Lymphomas	Common immunophenotype detected
B-Cell Lymphoma	CD45-Positive, CD21-Positive, CD34 Negative CD5,CD3,CD4,CD8-Negative,
T-Cell Lymphoma	CD45-Positive, CD21-Negative, CD34 Negative CD5,CD3-Positive
T-Zone Lymphoma	CD45-Negative, CD21-Positive, CD5-Positive, CD34-Negative

Table 2: Surface and intracellular antibodies used in cases of lymphoma and leukemia in dogs

Antibodies	Main Target Cells
CD5	T – Cells
CD3	T – Cells
CD4	Helper T – Cells
CD8	Cytotoxic T – Cells
CD21	Mature B – Cells
CD45	All Leukocytes
CD34	Hematopoietic stem cells
CD11b	Common myeloid marker
CD14	Monocytes
CD61	Platelets, Leukocytes, Endothelial cells
CDcy79b	B – Cells
CDcy3	T – Cells

immunophenotype found in the primary mass in case of lymphoma, and in the peripheral blood or bone marrow in leukemia cases. Most common antibodies used in veterinary medicine for lymphoma and leukemia are described in Table 2. When a MCTs is suspected specific antibodies for the identification of mast cells are needed. The first antibodies used are Immunoglobulin E (IgE) and CD117 since they have a high specificity for mast cells, especially CD117. These antibodies can be combined with primary lymphoid markers (CD5, CD21) when staging of MCTs take place in lymph-nodes in order to exclude lymphocytes from the gate of analysis. Pan leukocyte and myeloid markers can be added since mast cells have a myeloid nature. A list of antibodies used in MCTs in dogs is found in Table 3.

Table 3: List of superficial antibodies used in dog MCT

Antibodies	Marked cells
IgE	Mast cells, Basophils, Eosinophils, Macrophages
CD117	Mast cells, Basophils, Common Myeloid Progenitors, Multipotent Progenitors, Hematopoietic Stem Cells
CD18	Pan Leukocyte Marker
CD11b	Common myeloid marker
CD44	Most Hematopoietic Cells
CD5	Mature T - Cells
CD21	Mature B – Cells

Practical applicability of flow cytometry

One of the advantages of flow cytometry is the possibility to perform multiparametric analysis, which gives the possibility to analyze a group of cell populations for different marker expression at the same time. The first application of flow cytometry on lymphomas, leukemias and MCTs is the immunophenotypic characterization. Immunophenotype of the tumor provides information regarding the cell composition and nature of each tumor. In some cases, such as in chronic lymphocytic leukemia, the immunophenotype can predict the survival time (29). After exploring immunophenotype characteristics of the population and confirming diagnosis, the staging of the tumor takes place. Minimal residual disease can be performed after a specific chemotherapeutical protocol or treatment to observe the remaining percentage of the malignant cells.

Staging of lymphoma is usually performed on liver, spleen, peripheral blood and bone marrow. Flow cytometric staging is based on the immunophenotype of the specific lymphoma. When a diffuse large B cell lymphoma (DLBCL) diagnosis is confirmed, staging will be based in finding large CD21 positive cells in all tissues. Staging can be based also in aberrancies of the cells such as the presence of CD34 positive cells. In other cases, such as T-Zone lymphomas, it is based on the unique lymphoma immunophenotype (CD5+, CD21+ Low, CD45-). (22). According to the Canine Lymphoma Network, the majority of clinicians reported the definition of immunophenotype as the main reason for requiring flow cytometry, followed by the lymphoma subtype definition, checking minimal residual disease and differentiating lymphoma from reactive conditions (37).

Same as for lymphomas, the staging of MCTs is based on the immunophenotypic characteristics. Absence of normal cell antigens or presence of aberrant markers can be used to detect mast cells in tissues. Staging of MCTs in lymph nodes, peripheral blood and bone marrow is quite challenging due to the fact that mast cells can be present in normal conditions or in a reactive lymph node situation. However, flow cytometry is able to easily identify and quantify mast cells in lymph nodes (25).

Challenges and pitfalls of Flow Cytometry

Despite all the advantages mentioned in the previous sections, the Flow Cytometry technique has various challenges that have to be addressed when a new experiment take place, especially if using the technique for the first time.

Mainly, these challenges are related to technical and experimental design issues. One of the main problems when performing a new experiment is the choice of the antibodies and reagents. Antibodies should be chosen very carefully and in fully accordance with the cell populations of the researcher interest. Further, antibodies should be stored in dark in 4° C (38). Parameters of the owned FC should be always taken in consideration when purchasing conjugated antibodies, since the color and the number of lasers determine which fluorochrome can be excited by one specific FC.

Collection and manipulation of samples can be a challenge for the researches. When cells are separated such as in peripheral blood, bone marrow, and lymph node samples, FC analysis can be performed quite easily comparing with samples from solid tissues which needs a certain treatment (chemical or not) to separate the cells.

Results provided from Flow Cytometry are considered very accurate, but in many cases there are discordances with other techniques such as immunohistochemistry (39). Many antibodies that works for FC does not work for IHC and vice versa. Thus, interpretation of FC results with other techniques results may be challenging.

As a conclusion, all procedures that helps performing the analysis in an adequate way such as: use of propidium iodide to exclude debris, designation of a gate of analysis, fluorescence minus one procedure, use of isotype controls, choose of the antibodies and reagents, and designation of an experiment, needs a continuous commitment from the users to better determine and apply all of the above.

Conclusion

This is a first overview which aims to highlight the usefulness of Flow Cytometry, its importance and accessibility of using this method in the Balkan area conditions as the new generation of Flow Cytometers are getting more affordable for the research facilities and private laboratories.

Based on the authors experience and literature review, the flow cytometry technique is a coherent and useful method which can improve the diagnostic and research work. Flow Cytometry can identify and stage lymphomas, leukemia's and mast cell tumors. Multiparametric analysis, large number of events analyzed in a short period of time and the fast turn-around time to provide results, makes flow cytometry particularly appealing for the routine diagnosis of these malignancies. The technique is compatible with other methods, but results provided by FC must be interpreted along with data gained by histopathology, immunohistochemistry and cytology, in order to have a large and valuable quantity of information.

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UPORABNOST PRETOČNE CITOMETRIJE PRI PREPOZNAVANJU IN DOLOČANJU STADIJA LIMFOMA, LEVKEMIJE IN TUMORJEV MASTOCITOV PRI PSIH – PREGLED

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Izvleček: Limfomi, levkemije in tumorji mastocitov so najpomembnejše skupine neoplazem, ki prizadenejo pse. Diagnostika, določanje stopenj tumorja in tipa celic v določenem tumorju predstavljajo izziv za raziskovalce in klinike in igrajo ključno vlogo pri učinkovitosti zdravljenja in postavljanju prognoze. Za rutinsko diagnosticiranje in določanje stopenj teh tumorjev se uporablja veliko različnih temeljnih metod, kot so citologija, histopatologija, imunohistokemija in citokemija. V zadnjih letih je pretočna citometrija vse bolj uporabljana metoda v veterinarski medicini, saj je mogoče v kratkem času in z visoko natančnostjo analizirati veliko število zdravstvenih stanj. Ena izmed najpomembnejših prednosti te tehnike je multiparametrična analiza, s katero lahko v populaciji celic istočasno analiziramo različne površinske označevalce. Določanje površinskih označevalcev in stopenj populacij tumorskih celic s pretočno citometrijo lahko pripomore k potrditvi diagnoze in postavitvi ustrezne prognoze bolezni. Številni rezultati pretočne citometrije so imeli pomemben prognostični pomen zlasti pri neoplastičnih obolenjih. Vendar je rezultate pretočne citometrije potrebno združiti in razlagati v skladu s podatki, pridobljenimi s histopatologijo, imunohistokemijo in citologijo.

Ključne besede: pretočna citometrija; protitelesa; diagnoza; limfom; levkemija

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Original Research Article

EVALUATION OF COMMERCIAL TORTOISE AND TURTLE FEEDS

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Abstract: Captive chelonians should be fed a natural diet to achieve a growth rate similar to that of free-ranging animals. A wide range of commercially formulated foods dedicated to chelonians is available. Feeding commercial foods has the advantage of convenience. On the other hand, species-specific information on the nutritional requirements of chelonians is not available yet. The aim of this study was to analyse and evaluate commercial pellets and feeds for chelonians. Commercial pellets ($n_{tortoise}$ = 7, n_{turtle} = 7, from 6 companies) dedicated to carnivorous aquatic turtles and herbivorous terrestrial tortoises, and other aquatic turtle feeds (lyophilised beef heart, dried aquatic invertebrates, and whole frozen fish) were bought in pet shops. Whole frozen fish served as a reference feed for carnivorous aquatic turtles. The chemical composition as well as calcium (Ca) and phosphorus (P) contents were determined. Single-sample t-test was used with the label information as null hypothesis and the results of own parallel analyses for crude protein (CP), ether extract (EE), crude fibre (CF), Ca and P. The labelling of some of the pellets was deficient as nutritive values, Ca or P data were missing (tortoise pellets: 4 out of 7; turtle pellets: 5 out of 7). The label data differed significantly ($p<0.05$) from the results of our own analysis for 13 out of the 14 pellets. None of the tortoise pellets met the requirements of the animals completely. Because of the inadequate Ca:P ratio only one turtle pellet could be accepted. Accordingly, none of the commercial pellets can be recommended as main or only feed.

Key words: nutrition; pellet; metabolic bone disease; chelonian

Introduction

Chelonians are commonly kept pets. Overweight, accelerated growth rate and metabolic bone disease of nutritional origin are common as a result of inadequate nutrition and housing (1, 2, 3, 4, 5). The natural diet of aquatic chelonians consists of several animal species, and seasonality is strong in the case of tortoises (6, 7, 8, 9, 10, 11, 12, 13). Captive chelonians should be fed a natural diet to achieve a growth rate similar to that of free-ranging animals (2, 3, 7, 15). The energy expenditure of reptiles is only 25–35% that of mammals (16). Feeding frequency and quantity must also be mentioned, as periodic starvation is common in the natural habitat of chelonians.

A wide range of commercially formulated foods dedicated to chelonians is available. Feeding commercial foods has the advantage of convenience. On the other hand, species-specific information on the nutritional requirements of chelonians is not available yet. Because of this, the composition of commercial pellets is not necessarily adequate for the target species. The formulation of pellets varies from manufacturer to manufacturer (14, 17). Controlled animal trials evaluating the effects of such feeds are also missing.

The aim of this study was to analyse and evaluate commercial pellets and feeds for chelonians.

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Material and methods

Commercial pellets (n=14, from 6 companies, tortoise: A = Nutrin-Aquarium Tortoise Sticks; B1 = Sera Raffy Vital Herbivor; B2 = Sera Reptil Herbivor, C1= JBL Herbil; C2 = JBL Agivert; D = Exo Terra European Tortoise Adult; E1 = Tetra Tortoise; turtle: C3 = JBL Agil; C4 = JBL Tortil; JBL = Rugil; E2 = Tetra ReptoMin Sticks; E3 = Tetra ReptoMin Energy; E4 = Tetra ReptoMin Baby, F = Panzi) dedicated to carnivorous aquatic turtles and herbivorous terrestrial tortoises, and other aquatic turtle feeds including whole frozen fish (European smelt; *Osmerus eperlanus*) and dried aquatic invertebrates (Baltic prawn [*Palaemon adspersus*] and dried freshwater crab [*Gammarus roeseli*]) were bought in pet shops. The whole frozen fish served as a reference feed for carnivorous aquatic turtles.

The chemical composition as well as calcium (Ca) and phosphorus (P) content of the pellets, lyophilised beef heart, dried aquatic invertebrates and whole frozen fish were determined according to the AOAC (16) prescriptions (10 analyses/pellet for nutrients and 2 analyses/pellet for Ca and P).

All statistical tests were conducted using R 3.5.1 software (R Development Core Team, 2009, Vienna, Austria). Single-sample *t*-test was used with the label information as null hypothesis and the results of own parallel analyses for crude protein (CP), ether extract (EE), crude fibre (CF), Ca and P. The level of significance was $p < 0.05$.

Results

The nutrient content of tortoise and turtle pellets is shown in Tables 1 and 2. In the case of tortoise pellets our own data differed significantly ($p<0.05$) from the label information on several occasions. The CP contents were significantly higher (A, B2, D and E1) or lower (B1 and C2) than those indicated on the label. This can be explained by the ingredients of animal origin (fish and fish derivatives, molluscs and shellfish) in 3 products (B1, D and E1) and the presence of alfalfa meal (A) or algae (B2). Compared to the declared value, CF was significantly higher in 4 pellets (A, C1, C2 and E1) and lower in 3 pellets (B1, B2 and D). The EE was significantly lower in 4 and higher in 3 pellets than the data on the label. The crude ash (CA) content was also significantly lower than the declared value, with the exception of two samples (A and C2). The nitrogen-free extracts (NFE) varied between 48.2–71.2% and the two cereal grain free pellets (C1 and E) had the lowest carbohydrate content).

The Ca and P contents of tortoise and turtle pellets are shown in Tables 3 and 4. Four tortoise feed labels did not declare Ca and P contents. From the remaining 3 pellets, two had significantly lower (B2 and C1) and one significantly higher (B1) Ca level than the declared value. The P concentrations were also significantly lower than those declared on the label (B1, B2 and C1). The Ca:P ration was approximately the same in all pellets.

Table 1: Nutrient content of the complete tortoise pellets on dry matter basis

Feed	CP % label[1]	CP % own[2]	CF % label	CF % own	EE % label	EE% own	CA % label	CA % own	NFE % own
A	10.0	12.9±0.17*	14.0	16.3±0.37*	2.0	1.4±0.15*	6.0	6.0±0.08	63.4
B1	18.1	16.6±0.21*	9.3	3.0±0.32*	3.4	1.9±0.23*	8.0	7.3±0.14*	71.2
B2	14.8	20.5±0.24*	13.3	4.9±0.29*	4.8	3.3±0.08*	6.3	5.5±0.16*	65.8
C1	14.0	14.2±0.08	20.0	21.5±0.52*	2.0	2.6±0.24*	14.0	13.2±0.35*	48.2
C2	12.5	10.9±0.12*	22.0	24.9±0.48*	2.5	1.9±0.18*	8.5	9.6±*0.25	52.7
D	9.0	13.8±0.22*	26.0	19.8±0.59*	2.0	2.5±0.28*	10.0	7.7±*0.12	56.2
E1	9.0	12.2±0.16*	22.0	24.5±0.65*	0.5	2.2±0.32*	10.0	7.8±*0.04	53.3

Capital letters indicate the different manufacturing companies. A = Nutrin-Aquarium Tortoise Sticks; B1 = Sera Raffy Vital Herbivor; B2 = Sera Reptil Herbivor, C1= JBL Herbil; C2 = JBL Agivert; D = Exo Terra European Tortoise Adult; E1 = Tetra Tortoise. CP = crude protein, CF = crude fibre, EE = ether extract, CA = crude ash, NFE = nitrogen-free extract, [1]label information; [2]own analysis; NA = not available; *significant difference ($p<0.05$)

Table 2: Nutrient content of the turtle pellets and feeds on dry matter basis

Feed	CP % label[1]	CP % own[2]	CF % label	CF % own	EE % label	EE% own	CA % label	CA % own	NFE % own
C3	40.0	36.8±2.51*	0.5	2.4±0.28*	7.00	6.9±0.22	8.0	7.6±0.16	46.4
C4	NA	38.5±3.04	NA	3.2±0.22	NA	5.8±0.24	NA	8.1±0.05	44.4
C5	NA	30.2±1.67	NA	1.6±0.23	NA	3.9±0.15	NA	6.9±0.08	57.4
E2	39.0	36.2±3.25*	2.0	0.5±0.18	4.5	2.7±0.07*	NA	11.6±0.17	49.0
E3	47.0	55.9±2.34*	4.0	0.05±0.04	7.0	5.8±0.18*	15.0	11.7±0.22*	26.5
E4	45.0	46.9±3.58*	2.0	0.05±0.05	8.0	5.5±0.04*	NA	11.6±0.1	35.9
F	25.0	27.3±1.28*	2.0	0.9±0.18	1.50	0.4±0.12*	7.0	3.1±0.04*	68.3
LBF	NA	64.7±3.64	NA	NA	NA	10.0±0.31	NA	10.3±0.26	NA
Shrimp	NA	70.7±0.15	NA	NA	NA	2.3±0.12	NA	17.3±0.28	NA
Gammarus	NA	49.4±0.14	NA	NA	NA	5.2±0.24	NA	19.2±0.24	NA
Fish**	NA	67.7±0.16	NA	NA	NA	12.9±0.28	NA	16.2±0.14	NA

Capital letters indicate the different manufacturing the companies. C3 = JBL Agil; C4 = JBL Tortil; JBL = Rugil; E2 = Tetra ReptoMin Sticks; E3 = Tetra ReptoMin Energy; E4 = Tetra ReptoMin Baby, F = Panzi. NA = not available; [1]label information; [2]own analysis; LBF = lyophilised beef heart, *significant difference ($p<0.05$), ** whole frozen fish, European smelt (*Osmerus eperlanus*); CP = crude protein, CF = crude fibre, EE = ether extract, CA = crude ash, DM = dry matter

Table 3: Calcium and phosphorus content of complete tortoise pellets on dry matter basis

Feed	Ca % label[1]	Ca % own[2]	P % label	P % own	Ca:P own
A	NA	1.3±0.06	NA	0.4±0.02	3.2:1
B1	1.5	2.3±0.01*	0.6	0.5±0.01*	4.6:1
B2	2.5	1.2±0.04*	0.7	0.3±0.02*	4:1
C1	2.1	1.3±0.03*	0.6	0.4±0.02*	3.2:1
C2	NA	1.1±0.02	NA	0.4±0.01	2.7:1
D	NA	1.2±0.05	NA	0.4±0.02	3:1
E1	NA	1.0±0.02	NA	0.2±0.01	2:1

Capital letters indicate the different manufacturing companies. A = Nutrin-Aquarium Tortoise Sticks; B1 = Sera Raffy Vital Herbivor; B2 = Sera Reptil Herbivor, C1= JBL Herbil; C2 = JBL Agivert; D = Exo Terra European Tortoise Adult; E1 = Tetra Tortoise. [1]label information; [2]own analysis; NA = not available; *significant difference ($p < 0.05$)

Table 4: Calcium and phosphorus content of turtle pellets and feeds on dry matter basis

Feed	Ca % label[1]	Ca % own[2]	P % label	P % own	Ca:P own
C3	NA	1.5±0.04	NA	1.1±0.01	1.4:1
C4	NA	1.6±0.04	NA	0.9±0.02	1.8:1
C5	NA	1.5±0.03	NA	1.0±0.02	1.5:1
E2	3.3	3.6±0.06	1.2	1.4±0.01	2.6:1
E3	NA	2.4±0.05	NA	1.3±0.01	1.8:1
E4	3.2	3.6±0.04	1.3	1.5±0.01	2.4:1
F	NA	0.3±0.01	NA	0.4±0.00	0.7:1
LBF	NA	2.5±0.05	NA	2.0±0.02	1.2:1
Shrimp	NA	3.9±0.03	NA	1.3±0.01	3:1
Gammarus	NA	5.0±0.02	NA	1.5±0.02	3.3:1
Fish*	NA	5.1±0.02	NA	3.3±0.01	1.5:1

Capital letters indicate the different manufacturing companies. C3 = JBL Agil; C4 = JBL Tortil; JBL = Rugil; E2 = Tetra ReptoMin Sticks; E3 = Tetra ReptoMin Energy; E4 = Tetra ReptoMin Baby, F = Panzi. NA = not available; [1]label information; [2]own analysis; LBF = lyophilised beef heart; *whole frozen fish, European smelt (*Osmerus eperlanus*)

In the case of the turtle pellets our own data differed significantly ($p<0.05$) from the label information on several occasions. The labelling of products was also very poor, lacking the declaration of nutritive value and mineral content in 2 pellets and 3 other commercial feeds. The CP was significantly lower in two pellets (C3 and E2) and higher in three pellets (E3, E4 and F) than the label information. The CP content of lyophilised beef heart, shrimp, Gammarus and fish was the highest. The CF levels were significantly lower than the declared values with one exception (C3). The EE was approximately the same as the label data for one pellet (C3) but significantly lower in the case of the others. The EE content of lyophilised beef heart and fish was much higher than that of the other feeds. The CA was significantly lower than the declared value in two pellets (E3 and F). The NFE contents varied between 26.5–68.3%. Three pellets with >45% NFE contained cereal grains as the main ingredient (C3, C5, F). In two pellets (E2 and E3) cereal grains were not mentioned but it was not specified what 'ingredient of plant origin' meant. Pellet E2 had 49% NFE content which presumably means cereal grain content. Pellets C4 and E4 also listed cereal grains but not as a main ingredient.

The Ca and P contents were not declared in 4 pellets and 4 other commercial feeds. Both of the remaining 2 pellets had higher Ca and P concentrations than indicated on the label. Shrimp, Gammarus and whole fish had much higher Ca content than the other feeds. The P level of dried invertebrates was similar to that of the pellets while lyophilised beef heart and fish contained more P. The Ca:P ratio showed bigger differences than that of the tortoise pellets. One pellet (F) had a very disadvantageous ratio (0.7:1) which can be explained by the extremely low (0.3±0.01%) Ca content. The Ca:P ratio of lyophilised beef heart and fish was similar to that of the pellets with one exception (F), while shrimp and Gammarus had much higher Ca:P ratios.

Discussion

The diet for captive chelonians should resemble their wild diet. Herbivorous reptiles cover their energy requirement mainly by carbohydrates (50–75% DM), of this 15–40% is the CF. Protein represents 15–35% and fat is less than 10% (14). This composition highly depends on the tortoise species. For captive tortoises it is better to reduce the protein intake and increase the fibre in order to reduce the growth rate. This diet may include garden weeds (e.g. dandelion, chickweed), dark leafy greens (e.g. mustard green, turnip top, kale, rugula, corn salad) and a small amount of vegetables (19). Tortoises fed with a diet containing less than 80% grasses and weeds in the summer tend to develop pyramidal growth syndrome (20). Fruits should be avoided or reduced to a minimum (<5%) because of their high carbohydrate content (14, 19). Some species may receive higher amount of fruits which fits their natural diet (e.g. red-footed tortoise [*Chelonoidis carbonaria*]; 21).

The natural diet of herbivorous chelonians is low in CP (approx. 15% DM; 13); however, they occasionally ingest protein of animal origin (8, 12, 22). Excess protein intake leads to accelerated growth rate, renal failure, gout and it is also associated with pyramidal growth syndrome (1, 15, 23, 24, 25). Captive chelonians may have even lower protein intake to reduce their growth rate. Five pellets met the CP requirement while one (B1) had a slightly higher level. The 20.5% CP content of pellet B2 seems to be too high for pet chelonians and thus it is not recommended.

Little is known about the fat requirement of tortoises, but it should be around 3% DM (23). The EE contents were generally lower than indicated on the label, but they probably cover the requirement. However, it is not known whether the 1.4% EE content of pellet A is sufficient if the pellet is used as the only feed.

The carbohydrate content should be around 45.5–52.3% (23). Most feeds met this requirement with three exceptions. Pellets A and B2 had approximately 10% higher NFE content while B2 had a much higher NFE concentration than optimal. Carbohydrate overload also accelerates the growth rate. It is also important to mention that some species such as the steppe tortoise (*Testudo horsfieldi*) have very low activity and spend very little time foraging (<15 min per day; 27). Thus, these chelonians can satisfy their energy requirement with a modest feeding effort.

In nature, herbivorous tortoises consume a wide range of plant species (10, 12, 13, 23, 27, 28, 29). These are typically high in CF (15–40% DM) and calcium. The high CF content of diets for captive reptiles is important for reducing the feed intake and thus the growth rate (28, 30, 31, 32). Captive

reptiles grow much faster than free-ranging ones (2, 3, 15, 25, 33, 34). In the diet of Galapagos giant tortoises (*Geochelone nigra*), CF may reach 30–40% on DM basis which might be the case in other herbivorous chelonians as well (29). In the diet of captive desert tortoise *(Gopherus agassizii)* the CF level can be 25–30% (35). Some species (e.g. the Bolson tortoise [*Gopherus flavomarginatus*]) feed on droppings of rabbits which are high (30% DM) in undigested fibre (36) and can serve as a source of trace elements as well. Herbivorous chelonians rely on gut microbes to ferment dietary fibre and produce volatile fatty acids (14, 37, 38). It seems that they can digest cellulose and hemicellulose as efficiently as herbivorous mammals (32, 39). Pellets A, B1 and B2 did not contain an adequate amount of CF. Four pellets (C1, C2, D and E1) reached the recommended minimum level but pellet D contained much less CF than the declared value (19.8 vs. 26% DM).

The natural diet is rich in Ca and tortoises feed on soil, bones, or faeces of carnivores to fulfil their Ca requirement (12, 36, 40, 41, 42). Tortoises have high Ca tolerance which can be explained by the fact that these animals grow until death (43). Higher Ca intake also leads to enhanced Ca digestibility (44, 45). In 6 pellets (A, B2, C1, C2, D and E1) the Ca concentration was around 1% which seems to be too low.

The optimal Ca:P ratio of tortoise diets is much higher (3.1–5.8:1 or even higher; 46) than the general recommendations for mammals (Ca:P = 2:1). According to experimental data this ratio may reach 6:1 without causing adverse effects (1.29% Ca on a dry matter basis; 44). In the diet of wild juvenile and adult desert tortoises the Ca:P ratio of needlegrasses (*Achantherum* spp.) is 22:1 and 13:1, respectively, while desert dandelion (*Malacothrix* spp.) forbs have a ratio of 9:1 and 14:1, respectively (9). In some plant species the Ca:P ratio may reach 32.4:1 (*Cardus australis;* 13). Five pellets reached or exceeded the minimum recommendation for Ca:P and two pellets (C2 and E1) were below that.

Carnivorous aquatic chelonians have much higher protein and fat requirements than herbivores while their fibre and carbohydrate requirements are much lower (12, 47). Whole frozen fish can be a reference feed which is available in pet shops. Freezing has the advantage that it eliminates parasites. It is advised to feed a variety of fish species to avoid the possible long-term negative effects of the exclusive feeding of one species. For example, smelt may have high thiaminase activity and can induce thiamine deficiency (48). Whole fish should be fed frequently to most freshwater turtles and should be the main feed for piscivorous species (14, 47, 48 49, 50, 51). Besides that, other whole vertebrates and invertebrates such as shrimp or Gammarus can be offered. Carnivorous reptiles mainly cover their energy requirements from protein (25–60%) and fats (30–60%), which highlights the importance of these nutrients. Carbohydrates have the lowest importance with less than 10% DM (12). Many of the freshwater turtles are opportunistic carnivores or omnivores, as they undergo an ontogenetic shift in their diet as they mature (6, 11, 14, 51, 52, 53, 54, 55, 56, 57, 58). This dietary shift can be explained by the hypothesis that larger turtle species are less able to meet their metabolic requirements on a carnivore diet, have greater capacity to store fats and can cover their energy requirements on a plant diet as well. Diet change is also linked to changes in physiological needs and specific requirements (51, 59, 60). Specialities can be mentioned as adult pond sliders (*Trachemys scripta*) become predominately herbivorous; the animal to plant matter ratio in their diet is 77:23 (54). Carbohydrates are more important for omnivorous reptiles (20–75% DM), while fats represent 5–40% and protein is between 15–40% (14).

The CP levels of pellets were much lower than those of fish or dried invertebrates. The recommended protein level for Chinese softshell turtle (*Pelodiscus sinensis*) is 39.0–47.7% DM (61, 62, 63, 64, 65, 66, 67, 68). In red-eared terrapins (*Trachemys scripta elegans)* a growth rate equal to the natural one was obtained with 25–40% CP (50). When turtles are kept as pets and not as farmed animals the protein concentrations may be lowered to prevent fast growth rate (69), but exact recommendations are missing. Sudden overfeeding with protein may lead to dysbiosis and diarrhoea while prolonged overfeeding results in obesity (49). This is why overfeeding with whole fish should be avoided by applying adequate feeding frequency. Juveniles and breeding females have much higher protein requirements (53, 66, 68), the latter may reach 61–66% (69). Too low (<30%) protein intake of growing turtles may lead to reduced growth rate (50, 64, 70). The animal:plant protein ratio of the diet should be around 3:1 (61). With one

exception (F), these commercial pellets may cover the requirements of slow-growing adults as these turtles may be fed moderate CP levels of around 26%. Pellets C5 and E4 were dedicated to young growing animals. Product E4 with its 46.9% CP content may cover the requirement but product C5 with 30.2% seems to be inadequate.

Because of packaging and storage, it is better to have pellets with lower EE content, but this macronutrient is important in the energy supply of carnivorous and omnivorous turtles (14). As Table 2 shows, the EE levels of pellets were much lower than that of the frozen fish (12.9% DM). The recommended EE level for Chinese softshell turtle in commercial farms is around 4.2–8.8% (74, 64, 65, 66, 67, 68, 70, 71, 72). High-fat diets (13.9% EE) should be avoided as they lead to the accumulation of lipids in the liver and liver injury (65). The optimal EE intake for pet turtles is not known but presumably it may be lower. Accordingly, it seems that most of the pellets can cover the EE requirements. Pellet F with 0.4% EE content is an exception. Although it is called a 'complete feed', it does not cover the requirement of the animals. Based on the EE content of whole fish, especially the European smelt, moderate and not exclusive feeding is recommended as part of a balanced diet.

The NFE requirement of turtles may vary according to their specific requirements. If we calculate with 39–46% CP, 9% EE and 4% CF for carnivorous turtles, then the NFE is approximately 41–46%. This seems to be adequate for carnivores (61, 64) and the optimal starch content for farmed juvenile soft-shelled turtle is around 30% (71). Opportunistic carnivores may have higher NFE requirements.

Little is known about the CF requirements of carnivorous or opportunistic carnivorous chelonians. Feeds of animal origin do not contain CF, thus it may only be important for opportunistic carnivores. On the other hand, fibre has a satiating effect which helps to avoid overfeeding and may have a beneficial effect on the gut microbiota of pet turtles. For juvenile soft-shelled turtles 2–8% CF seems to be adequate (71).

The Ca and P contents of the pellets and lyophilised beef heart were much lower than those of Gammarus and whole fish. Imbalanced diets having low Ca content lead to metabolic bone disease of nutritional origin in aquatic turtles as well (73). However, excess Ca intake (2.24% DM) may have a negative impact on the growth rate of aquatic turtles (74). On the other hand, the optimal Ca and P intake for Chinese softshell turtle is 5.7% and 3.0%, respectively (75). These data are very similar to the Ca and P levels of European smelt. Metabolic bone disease of nutritional origin can be prevented by providing 1.16–2.95% Ca and 0.92–2.56% P in the diet (74). Shrimp, Gammarus and whole fish are good Ca sources. As aquatic turtles feed underwater, dusting the feed with dietary minerals and vitamin supplements does little to cover the requirements. Therefore, the diet should have optimal Ca and P content.

According to studies on Chinese softshell turtle, the Ca:P ratio should be approximately 2:1. This can be reached with 5.7% Ca and 3.0% P (60). The lower Ca:P ratio may lead to shell malformations or lower growth rates. This recommended ratio was reached only in pellets E2 and E4. The Ca:P ratio of European smelt is lower than 2:1 but close to the 1.9:1 ratio recommended for Chinese softshell turtle (75). Shrimp and Gammarus have much higher Ca:P ratio than the minimum requirements; thus, they can be fed in combination with whole fish to increase the Ca:P ratio.

Conclusion

As a general recommendation, we suggest not to buy any commercial feed that does not have detailed nutritional values. For herbivorous tortoises a good-quality pellet should be low in protein (10–15%), high in crude fibre (18–20%) and its Ca:P ratio should be >3:1. Avoid feeds containing proteins of animal origin. According to the nutritional values determined by our own analysis, products C2 and E1 can be accepted but their Ca:P ratios were far from the requirements. Thus, none of the commercial feeds is recommended for use as main feed. The nutrient content of the pellets should be checked very carefully, as label information is not necessarily precise. For carnivorous turtles the nutrient content of artificial feeds should be close to the nutritive value of whole fish or the recommendations. This means 25–50% protein (for young growing animals >30%), 4–8% EE and a Ca:P ratio of >2:1. Based on their CP and EE levels, four pellets (C3, C4, E3 and E4) can be accepted, but because of the inadequate Ca:P ratio only pellet E4 can be recommended.

Based on the nutritive value of the pellets it is not advised to use them as the only or main feedstuff. Different chelonian species may have widely varying requirements, and thus a diet universally suitable for all of them cannot be formulated. Greater emphasis should be put on the proper labelling of products.

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VREDNOTENJE KOMERCIALNIH ŽELV IN KRME ZA ŽELVE

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Izvleček: Želve v ujetništvu je potrebno hraniti z naravno krmo, da dosežejo podobno stopnjo rasti kot živali v prosti reji. Na voljo je širok izbor komercialno pripravljene hrane za želve. Prednost hranjenja želv s komercialno hrano je priročnost, vendar podatki o prehranskih potrebah za posamezne vrste želv še niso na voljo. Namen te raziskave je bil analizirati in ovrednotiti komercialne pelete in krmo za želve. V trgovinah za živali smo od 6 podjetij kupili komercialne pelete ($n_{peleti\ za\ vodne\ želve} = 7$, $n_{peleti\ za\ kopenkse\ želve} = 7$) za mesojede vodne in rastlinojede kopenske želve ter drugo krmo za vodne želve (liofilizirano goveje srce, posušene vodne nevretenčarje in zamrznjene cele ribe). Zamrznjene cele ribe smo uporabili kot referenčno krmo za mesojede vodne želve. Določili smo kemično sestavo in vsebnost kalcija (Ca) ter fosforja (P). Za ničelno hipotezo smo uporabili T-test enega vzorca s podatki na etiketi in rezultate lastne paralelne analize za surove beljakovine (an gl. *crude proteins, CP*), ekstrakt etra (angl. *ether extract, EE*), surovo vlaknino (angl. *crude fibre, CF*), Ca in P. Oznake nekaterih peletov so bile pomanjkljive, saj so manjkali podatki o hranilnih vrednostih, Ca in P ($n_{peleti\ za\ kopenske\ želve} = 4$ od 7, $n_{peleti\ za\ vodne\ želve} = 5$ od 7). Podatki na etiketi so se bistveno razlikovali ($p < 0,05$) od rezultatov naše analize pri 13 od 14 vrst peletov. Nobeni peleti za kopenske želve niso v celoti izpolnjevali potreb živali. Zaradi neustreznega razmerja Ca : P smo kot ustrezno določili le eno izmed 7 vrst peletov za vodne želve, zaradi česar nobenih od komercialnih peletov nismo določili kot priporočljivih za glavno ali edino krmo za želve.

Ključne besede: prehrana; peleti; presnovna bolezen kosti; želve

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Original Research Article

USE OF CANNABIDIOL PRODUCTS BY PET OWNERS IN SLOVENIA: A SURVEY-BASED STUDY

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Abstract: The aim of the present study was to obtain information about the experiences of Slovenian pet owners on the use of cannabidiol (CBD) products in their pets. An open online survey targeted Slovenian owners of cats and dogs who have used CBD to treat their pets. Questions pertained to demographic data, animal data, health status of the animals, CBD formulations and experiences with use. Descriptive statistics and frequency distributions were performed using the survey software. A total of 41 respondents participated in the survey, most of whom were female (87.8 %) and between 31 and 50 years old (56.1 %). Most respondents (90.2 %) were dog owners. Cannabidiol (CBD)-based products were mainly used to treat orthopedic and oncologic conditions, as adjunctive therapy to other medications. Oil formulations were used by most dog (85.2 %) and all cat owners. Participants predominantly reported positive effects, such as improved well-being, increased activity, and reduced pain. The results suggest that Slovenian pet owners who used CBD-based products as a treatment for their pets were overall satisfied with the effects of these products. However, there were still reports of some adverse effects, such as drowsiness, increased appetite, and thirst. Further research is essential to improve practices related to cannabis-based medicines for pets, especially CBD, and to put an end to the trial- and error- based therapeutic approach of pet owners and veterinarians. Long-term, large-scale research studies are needed to clarify the role of CBD as a treatment option for osteoarthritis, chronic pain, cancer, behavioral problems, and other chronic inflammatory conditions in dogs and cats.

Key words: cannabis; cannabidiol; cats; dogs; survey

Introduction

Cannabis and cannabis products have been widely used since ancient times as a remedy for many diseases (1, 2). The criminalization of cannabis occurred throughout the 20th century, beginning in the United States, where cannabis was banned for all use in 1970 with the Controlled Substances Act (1, 3), and spreading to European and other countries (4). In the 21st century, new laws were enacted to loosen the legal grip on cannabis use (1, 3), as the importance of its medicinal properties gained new attention due to renewed scientific and medical interest in the early 1990s (1, 2).

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The use of cannabis for medicinal purposes is permitted in Slovenia under the Medicinal Products Act (5) and the Pharmacy Practice Act (6). Currently, there are no cannabidiol (CBD)-based products medicinal products approved for veterinary use in Slovenia. However, CBD-based medicinal products used in human medicine could also be used for veterinary purposes according to the principle of cascade (5). In addition, products derived from *Cannabis sativa* containing mainly CBD and less than 0.2 % delta-9-tetrahydrocannabinol (THC) are sold as dietary supplements. In fact, according to the Novel Food Catalogue of European Union (7), some products derived from *Cannabis sativa* are not novel and thus legally allowed.

Cannabinoids are active chemical substances isolated from the cannabis plant that exert

psychoactive and non-psychoactive effects (8, 9). They act as exogenous ligands that interact with G-protein-coupled cannabinoid receptors (10). There are two main types of cannabinoid receptors, CB1 and CB2 (11). The CB1 receptors are widely distributed in the brain and correlate with cannabinoid effects on cognition, appetite, emotion, memory, perception, and movement control. The CB2 receptors are highly concentrated in the peripheral nervous system and immune system, where they play a role in regulating inflammation and pain (11).

One of the best-known psychoactive cannabinoids is (THC), which may cause toxicosis in dogs (12 - 14), and cats (15, 16). Cannabidiol (CBD) is the most promising non-psychoactive cannabinoid, with many beneficial effects attributed to it (8, 9).

The first studies on the effects of cannabis in pets were mostly reports of poisoning in dogs (12 - 14). In recent years, new clinical studies aimed to elucidate the role of CBD in the treatment of various diseases in dogs, such as osteoarthritis (17 - 21), seizures (22, 23), atopic dermatitis (24) and anxiety (25). In addition, recent studies investigated the safety and side effects of various cannabinoid dosages and formulations for dogs (1, 26-30) and cats (1, 26, 31). Two of the most studied canine diseases, treated with CBD-basd products, are osteoarthritis (17 - 21), and epilepsy (22, 23). While CBD appeared to improve mobility, relieve pain, or reduce the need for other analgesics in some reports of dogs with osteoarthritis (17 - 20), this has not been confirmed by other researchers (21). In addition, clinical data on the use of CBD to treat epilepsy have shown promising results (22, 23).

Pet owners also use CBD-based products as sole or adjunctive treatment for oncological diseases in their pets (32 - 34). Although there is some preclinical evidence of CBD efficacy with or without chemotherapy on canine neoplastic cells (35, 36), there are no clinical data to date to confirm these findings.

Cannabidiol-based products are purchased by pet owners to relieve pain, reduce inflammation, control anxiety (32 - 34), and improve other medical conditions such as epilepsy (22), gastrointestinal problems, and cancer (32 - 34). The socioeconomic phenomenon of cannabis-based treatments for pets has been addressed by researchers who aimed to survey pet owners (32 - 34, 37), veterinarians (38), and veterinary students (39) about their attitudes and experiences with cannabis-based products to alleviate health related problems in pets.

The aim of the present study was to obtain information about the experiences of Slovenian owners on the use of CBD products in their dogs and cats. Our main hypotheses were that CBD products are well accepted by Slovenian pet owners and that most owners observe positive effects of the products in their cats and dogs, while adverse effects are rarely observed.

Material and methods

The methods are reported according to the Checklist for Reporting Results of Internet E-Surveys (40). An open online survey targeted Slovenian owners of cats and dogs who have used CBD as a treatment for their pets. Participation in the survey was voluntary and no incentives were offered for participation. An introductory text indicated the anonymity and confidentiality of the data collected and provided information about the investigator, the purpose of the study, and the length of the survey (approximately 5 minutes). The survey was created using Google Forms, a freely available survey software, and was tested by the investigators prior to use. The survey was accessible to visitors of the website of the Small Animal Clinic, Veterinary Faculty, University of Ljubljana (www.kmz.si) from February 25 to July 7, 2019. Initial contact with potential participants was made online only, by advertising on the clinic's website and Facebook page. The nonrandomized questionnaire (Table 1) consisted of a single page with 22 questions. Responses could be reviewed and changed by scrolling the questionnaire page. The questionnaire could be submitted if all mandatory questions were answered.

Respondents were selected by convenience sampling (i.e., cat and dog owners that visited the website or Facebook page of the Small Animal Clinic, Veterinary Faculty, University of Ljubljana). View and participation rates were not recorded. Descriptive statistics and frequency distribution were performed using the survey software. Since not all questions were answered by all participants, total responses for each question vary. The percentages reported are based on the total responses for each individual question. Due to the size of the sample, results are also reported as the number of responses to each question, where appropriate.

Table 1: Contents of the questionnaire

	Demographic data
Q1	Age (18-30, 31-50, 51 or more years) *
Q2	Sex (female, male) *
	Animal data
Q3	Species (cat, dog) *
Q4	Breed
Q5	Age (0-1, 2-5, 6-10, more than 10 years) *
Q6	Sex and reproductive status (female, spayed female, male, neutered male) *
Q7	Body weight (0-5, 6-10, 11-20, 21-30, more than 30 kg; other) *
	Animal health status
Q8	The type of the condition that caused you to use CBD (orthopedic disease (e.g., joint pain, lameness), oncologic disease, skin disease (e.g., itching), neurologic disease (e.g., epilepsy), behavioral disorder (e.g., fear, anxiety), gastrointestinal disease, other) *
Q9	Please provide the name of the disease or symptoms.
Q10	Does your pet have any other known medical conditions? *
Q11	If yes, which ones?
Q12	Is the animal receiving any medications, supplements, or nutritional supplements in addition to CBD? (yes, no, other) *
	CBD formulations and experiences with use
Q13	What type of product do you use? (oil, rectal suppositories, ointment, coated tablets, capsules, other) *
Q14	What is the concentration of CBD in the product? (3 %, 7 %, 10 %, other) *
Q15	Does the product contain THC? (yes, no, other) *
Q16	What is the THC content in the product?
Q17	When did you start noticing the effects of the CBD product? (immediately after starting, within a week, within a month, more than a month after starting, other) *
Q18	What positive effects do you observe in your pet when the product is administered? (improved well-being, increased activity, decreased pain, increased appetite, decreased nausea, no effect, other) *
Q19	What negative effects do you observe in your pet when the product is administered? (fatigue, drowsiness, decreased appetite, nausea, increased thirst, excessively increased appetite, no effect, other) *
Q20	Why did you decide to use CBD? (on recommendation of a veterinarian, at my own discretion, on recommendation of others) *
Q21	Where did you get information about the use of CBD? (from a veterinarian, from a pet store, on the Internet, from other people, other)
Q22	Do you have any other opinion, comment, or advice on the use of CBD in your pet?

Q question; * mandatory question

Due to the small number (n = 4) of cat owners that participated in the study, the results are reported separately for dogs and cats, except for demographic data, and sources of information and purchase of products.

Results

A total of 41 people participated in the survey. Although view and participation rates were not recorded, a total of 281 page views were recorded for the page on the clinic's website that advertised the survey study from February 25 to July 7, 2019.

Demographic data

The respondents were mostly female (36/41, 87.8 %). Most respondents (23/41, 56.1 %) were 31 - 50 years old; 24,4 % (10/41) were 18 - 30 years old, and 19,5 % (8/41) were 51 or more years old.

Animals data

Most of the respondents (37/41, 90.2 %) were dog owners, while four (4/41, 9.8 %) had cats. Among dogs, crossbreeds were overrepresented (8/28, 28.6 %), followed by Maltese dogs (2/28, 7.1 %), Labrador Retrievers (2/28, 7.1 %), and

other (16/28, 57,1 %). Age, sex, and reproductive status of the dogs are shown in Table 2. The majority of dogs (34.1 %) weighed more than 30 kg, followed by dogs weighing 6 - 10 kg (18.9 %).

Health status of dogs

Cannabidiol based products were mostly used to treat orthopedic (e.g., joint pain, lameness), and oncologic disease (Table 3). Other conditions included gingivitis, age-related problems, thyroid disease, lack of energy, chronic sinusitis, heart disease, allergies, chronic bronchitis, cognitive dysfunction, and recovery from surgery.

The disease treated with CBD was the only health problem in 75.7 % of the dogs (28/37). Of those with multiple diseases (9/37, 24.3 %), the most common comorbidities were heart disease (3/9, 33.3 %) and chronic kidney disease (2/9, 22.2 %). In 29.7 % (11/37) of the dogs, CBD was used as the only treatment, while 70.3 % (26/37) received several different medications or supplements such as natural remedies, non-steroidal anti-inflammatory drugs (NSAID), corticosteroids or disease-specific treatments (e.g., chemotherapeutics, antihypertensives, bronchodilators, antiepileptic drugs, etc.).

Table 4: The type of CBD products used in dogs

CBD product type	n (%)
Oil	23/37 (62.2 %)
Rectal suppositories	2/37 (5.4 %)
Ointment	2/37 (5.4 %)
Coated tablets	1/37 (2.7 %)
Capsules	0/37 (0 %)
Other	9/37 (24.3 %)

Cannabidiol formulations and experiences with use in dogs

Details of the products used are given in Table 4. Other product types included resin, CBD paste, CBD oil mixed with olive oil, and tablets.

The most frequent CBD concentration in the product was 10 % (10/37, 27 %) or 7 % (6/37, 16.2 %), while a 3 % CBD product was used by 5.4 % (2/37); other concentrations (19/37, 51.4 %) ranged from 5 – 50 % or were not reported. When asked about the THC content in the product, 17/31 (54.9 %) of the owners confirmed that the product contained THC. Of those who revealed the THC concentration in the product (n = 14), the majority indicated that it was less than or equal to 0.2 % (7/14, 50 %), while 4/16 (25 %) used

Table 2: Age, sex and reproductive status of the dogs

Age (years)	n (%)	Sex and reproductive status	n (%)
0-1	1/37 (2.7 %)	Female	6/37 (16.2 %)
2-5	7/37 (18.9 %)	Spayed female	16/37 (43.2 %)
6-10	14/37 (37.8 %)	Male	8/37 (21.6 %)
> 10	15/37 (40.5 %)	Neutered male	7/37 (18.9 %)

Table 3: Type of disease treated with CBD in dogs

Type of disease	n (%) *
Orthopedic disease (e.g., joint pain, lameness)	15/37 (40.5 %)
Oncologic disease	9/37 (24.3 %)
Skin disease (e.g., itching)	6/37 (16.2 %)
Neurologic disease (e.g., epilepsy)	5/37 (13.5 %)
Behavioral disorder (e.g., anxiety, restlessness)	4/37 (10.8 %)
Gastrointestinal disease	2/37 (5.4 %)
Other	7/37 (18.9 %)

*Since this was a multiple-choice question, the sum of percentages is greater than 100 %.

Table 5: The observed positive effects of CBD in dogs

Observed positive effects	n (%) *
Improved well-being	28/37 (75.7 %)
Increased activity	16/37 (43.2 %)
Reduced pain	16/37 (43.2 %)
Increased appetite	9/37 (24.3 %)
Decreased nausea	4/37 (10.8 %)
No effect	1/37 (2.7 %)
Other	12/37 (32.4 %)

*Multiple-choice question: the sum of the percentages is greater than 100 %.

products with more than 0.2 % THC (ranging from 0.3 to 70 %) and 4/16 (25 %) did not know the exact concentration. Most respondents observed an effect immediately (8/37, 21.6 %), or within a week of treatment (14/37, 37.8 %) and 24/37 (64.9 %) noted more than one positive effect (Table 5).

When asked about adverse effects, 28/37 (75.7 %) of owners reported none. The most common adverse effect was drowsiness (8/37, 21.6 %). Individual owners also reported increased appetite (3/37, 8.1 %) or thirst (2/37, 5.4 %), urinary incontinence (1/37, 2.7 %), and occasional vomiting (1/37, 2.7 %).

Results for cats

Four respondents were cat owners. One of the cats was purebred (Maine Coon), and the rest were domestic shorthair cats. Reproductive status included one female intact cat, one male neutered cat and two female neutered cats. Three cats weighed 6 to 10 kg; one cat weighed less than 5 kg. The owners used CBD for the treatment of oncological disease (n = 2), chronic respiratory disease (n = 1) and behavioral disorders (n = 1). Three of the respondents used oil products, one of respondents used only resin an one used a combination of oil, resin, and rectal suppositories. The content of CBD used for cats ranged from 3 to 16 %, and 3/4 (75 %) owners reported that the product contained THC in a concentration ranging from less than 0.2 % to 70 %. Each respondent noticed the effects of CBD at different times (i.e., immediately, within a week, within a month, never). The owners noted positive effects in 3/4 (75 %) of cats, while one cat owner reported none. Positive effects included improved wellbeing (n = 3), increased activity (n = 2), increased appetite (n = 2) and decreased pain (n = 1). Two cat owners noted adverse effects in their cats such as unspecified skin changes, and changes in cat's behavior (staring at the wall, focusing its shadow).

Sources of information and purchase of products

A 53.7 % (22/41) of respondents who answered the question, reported using commercial products while 29.3 % (12/41) used homemade products. One respondent (1/41, 2.4 %) used CBD on the recommendation of a veterinarian; the rest used the product at their own discretion (30/41, 73.2 %) or on the recommendation of others (10/41, 24.4 %). The most frequently reported sources of information were the Internet (18/41, 43.9 %) and experiences from other users (15/41, 36.6 %); 5/41 (12.2 %) of respondents obtained information from a veterinarian and 3/41 (7.3 %) from a pet store. Other responses included information from the manufacturer and other literature sources. Responses to the last question (n = 17) were overall positive (14/17, 82.4 %). Respondents described positive effects of CBD on their animals, recommended products, or expressed a reduced need for conventional medications, NSAIDs, or corticosteroids. Two respondents (2/17, 11.8 %) reported no effect. One respondent described ataxia in his dog that resolved spontaneously.

Discussion

The results of the present survey study show that most respondents observed positive effects of CBD products in their pets and that negative effects were rarely noticed. Furthermore, CBD products are well accepted among pet owners who participated in our study.

In our study, dog owners were overrepresented compared to cat owners, which was also found in other studies (32, 37). In fact, there are many more studies looking at the use of CBD products in dogs (8, 17 - 30) than in cats (26, 31). Deabold et al. (2019) emphasized the importance of further research into CBD applications in cats as CBD metabolism in cats is different than in dogs (26). In our study, most dog owners had large breed dogs, probably because these dogs are more prone to orthopedic conditions and resulting chronic pain, which is also the most common reason for using CBD products in dogs (33, 34). In addition, most of the animals in our study were adults, which may have correlated with disease incidence (41).

Orthopedic and oncologic conditions were the most common reasons for using CBD products in our study, followed by behavioral and neurologic problems, which agrees with previously reported studies (32 - 34), with the exception of skin conditions (e.g., itching), which were also strongly represented in our study. Indeed, Campora et al. (2012) suggested that the endocannabinoid system might be a target for the treatment of immune-mediated and inflammatory disorders such as allergic skin diseases in dogs (42). For the most part, pets received CBD products with other medications, implying that CBD was used as complementary therapy. These results are consistent with previous studies in which most respondents agreed that cannabis products might be appropriate as an adjunct therapy (32 - 34). However, CBD was used as the only treatment for 29.7 % of the dogs, which could mean that dog owners resorted to CBD products to help their pets before or without consulting a veterinarian, or that they were not satisfied with the conventional treatment approach (32 – 34, 37). Cannabidiol oil formulations are the most popular among CBD products and already proven to be well tolerated and effective in dogs (8, 17 - 19, 22, 26, 27, 31). Most respondents were aware of the concentrations of THC and CBD in the products used, probably because they were mostly commercial products. Nevertheless, some studies indicated that there may be discrepancies between the cannabinoid concentrations stated in the declaration and the actual cannabinoid concentrations in the product (28, 32, 43, 44).

Most respondents noticed the effect of CBD within a week or immediately. These results are difficult to discuss because the time of onset of the effect depends on the dosage. In addition, this information was not included in previous survey studies (32 - 34). The most commonly observed beneficial effects (improved well-being, increased activity, and pain relief) are generally consistent with previous studies in which pain relief was the most commonly perceived effect of CBD treatment in dogs (32 - 34). The reported adverse effects in our study such as sedation and/or drowsiness, excessive drinking, excessive appetite, skin changes and vomiting have been reported by pet owners in other studies (28, 33, 34). On the other hand, urinary incontinence, which was described by one respondent, was reported in cases of cannabis poisoning in dogs (12, 45). One dog owner reported ataxia that resolved spontaneously and as indicated by the owner, may have been the result of THC overdose (12, 45). The varying or unknown concentrations of THC in the population treated here may have influenced the occurrence of adverse effects caused by THC and not by CBD. According to research studies, the therapeutic dose of CBD for osteoarthritis and epilepsy in dogs is 2 mg/kg of a CBD-dominant product administered twice daily. However, since most of the products used also contain other cannabinoids, including THC, it is advisable to start with microdoses (i.e., 0.5 mg/kg twice daily) and titrate the dose until therapeutic effect is achieved and THC-related side effects are avoided (46, 47).

There are limited data on the safety and efficacy of CBD use in cats (1, 31). Pharmacokinetic studies show that cats have lower oral absorption kinetics and longer retention time compared with dogs, suggesting different dosing recommendations for cats and dogs (26, 31).

In our survey, most respondents reported using commercial CBD products and 35.3 % used homemade products. It is important to point out that CBD is preferred to conventional medications because it is perceived as a natural remedy (33,34, 37, 48). Many people rely on CBD formulations as a treatment for themselves (37, 48, 49) or their family members (50, 51) and pets (33, 34, 37). Cannabidiol products are often purchased from uncertified manufacturers or are homemade, and the products usually contain an unknown concentration of cannabinoids (28, 43, 44), which can lead to product inefficacy or the occurrence of adverse effects. Controlled clinical trials and pharmacokinetic studies have already demonstrated the beneficial effects of CBD in dogs and cats, and have also described the adverse effects (1, 8, 17 – 31). As highlighted in other studies, there is a need to establish standardized, independent laboratory analyses of the concentrations of cannabinoids and other elements in cannabis-based products (33, 34, 43, 44).

One of the 41 respondents started using CBD on the recommendation of a veterinarian. As noted in other studies, veterinarians are reluctant to prescribe CBD-based treatments due to lack of information, legal controversies, and limited clinical research (38). Moreover, pet owners may be discouraged to speak about the use of CBD in their pets due to the ambiguity regarding the permitted use of CBD and especially THC preparations. This

means that they must rely on other sources of information and take responsibility for treating their pets with supposedly natural remedies (33, 34, 49). The most common sources of information about CBD products in our study were the Internet and experiences of other users. This is generally consistent with similar studies in which the Internet was the most popular source of information about CBD products (33, 34, 37, 48).

The authors acknowledge certain limitations of the study. First, all survey-based studies in veterinary medicine require vicarious reporting, which may be biased by pet owner subjectivity. A caregiver placebo effect, which has been described in evaluating patient response to other treatments, may also be present in our study (21, 52). Since the survey was conducted online, selection bias may have occurred as older populations are generally not as proficient at using the Internet as middle-aged people, who were the most frequent participants in the present survey. Due to the small sample size, selection bias, and subjectivity of the responses, the results should not be generalized to all Slovenian pet owners.

The present study is the first to look into the experiences of Slovenian owners on the use of CBD products in their pets. The results suggest that Slovenian dog and cat owners who used CBD-based products as a treatment for their pets were overall satisfied with the effects of these products, although there were still reports of some adverse effects. Further research is imperative to improve practices related to CBD-based medications in pets to treat conditions such as osteoarthritis, chronic pain conditions, cancer, behavioral problems, and other chronic inflammatory conditions. Slovenian veterinarians should be informed about the indications, efficacy and current knowledge in this emerging field to enable safe use and maximize the potential of CBD-based treatment in cats and dogs, and to support their owners.

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UPORABA KANABIDIOLA PRI LASTNIKIH HIŠNIH LJUBLJENČKOV V SLOVENIJI – ANKETNA ŠTUDIJA

N. Milevoj, N. Tozon, K. Tomsič

Izvleček: Namen raziskave je bil pridobiti informacije o izkušnjah slovenskih lastnikov psov in mačk z uporabo izdelkov s kanabidiolom (CBD) pri svojih živalih. Raziskavo smo izvedli na podlagi anonimne spletne ankete, ki je bila namenjena slovenskim lastnikom psov in mačk, ki so uporabljali CBD za zdravljenje svojih živali. Vprašanja so se nanašala na demografske podatke, podatke o živalih, njihovem zdravstvenem stanju, vrste pripravkov s CBD-jem in izkušnjah z uporabo. Za opisno statistiko in frekvenčno porazdelitev smo uporabili programsko opremo za anketiranje. V raziskavi je sodelovalo 41 anketirancev, med katerimi je bilo največ žensk (87,8%) in starih med 31 in 50 let (56,1%). Večina anketirancev (90,2%) je bila lastnikov psov. Izdelki na osnovi CBD-ja so se večinoma uporabljali za zdravljenje ortopedskih in onkoloških obolenj kot dopolnilno zdravljenje. Največ lastnikov psov (85,2%) in vsi lastniki mačk so uporabljali oljne pripravke. Sodelujoči so večinoma poročali o pozitivnih učinkih, kot so boljše počutje, povečanje aktivnosti in zmanjšanje bolečine. Rezultati kažejo, da so slovenski lastniki psov in mačk, ki uporabljajo izdelke na osnovi CBD-ja za zdravljenje svojih živali, na splošno zadovoljni z učinki teh proizvodov. Poročali so tudi o nekaterih neželenih učinkih, kot so zaspanost, povečan apetit in žeja. Nadaljnje raziskave so bistvenega pomena za izboljšanje praks uporabe zdravil na osnovi konoplje za pse in mačke, zlasti CBD, in za odpravo terapevtskega pristopa lastnikov in veterinarjev, ki temelji na poskusih in napakah. Dolgoročne in obširne raziskave so potrebne za jasno opredelitev vloge CBD-ja pri zdravljenju kroničnih bolečin, raka, vedenjskih težav, osteoartritisa in drugih kroničnih vnetnih stanj pri psih in mačkah.

Ključne besede: konoplja; kanabidiol; mačke; psi; anketa

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Original Research Article

PERFORMANCE AND *Nosema* spp. SPORE LEVEL IN YOUNG HONEYBEE (*Apis mellifera carnica,* Pollmann 1879) COLONIES SUPPLEMENTED WITH CANDIES

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Abstract: We evaluated the efficacy of supplementation with protein, yeast, or sugar candies of young honeybee colonies originated from artificial swarms, by measuring strength and determining *Nosema* spp. spore level in adult bees in summer period. At the same time, we aimed to assess longevity of adult worker bees after feeding the same type of candies in controlled laboratory condition. The highest survival was found in Yeast and Protein candy group. On the contrary, the field study showed that artificial swarms produced significantly more pupae (2510.4 cm^2, p=0.0001) in the 1st period of measurement, and more larvae (964.8 cm^2, p=0.003) and frames with bees (5.6, p=0.008) in the 2nd period by feeding non-protein candy. In the 3rd period of evaluation of young colonies, the Sugar candy group had the highest number of frames covered by adult bees and honey stores, respectively (5.6, p=0.0009; 3432.0 cm^2, p=0.015). Sugar candy group produced the largest area of wax cells, however, the differences were not statistically significant. *Nosema* spp. spore level was checked quantitatively in adult bees. The lowest infection was statistically significant in Yeast candy group in June (4.35 million spores per bee, p=0.02), but insignificant in September. Supplementing artificial swarms Sugar candy offers the most promising potential for development of productive young colony. The findings of our study could help beekeepers to choose the effective candy supplement for optimal development of artificial swarms.

Key words: *Apis mellifera*; artificial swarms; young colonies; supplements; candies; development; *Nosema* spp.; longevity

Introduction

Honeybees are one of the most important pollinators for the agriculture and food production. The density of honeybee colonies can be very versatile in different parts of the world, depending mostly upon availability of nectar flow. Facing the overpopulated area with honeybee colonies can lead to decrease of health status, frequent infections with pathogens and exploitative competition with wild bees, like bumblebees (1).

A success and production in beekeeping sector always depends on the weather conditions, climate change, health condition of the colonies and changes in the environment due to human interventions. The essential honey bee colony needs must cover a proper quantity of quality food for productive development, reproduction, and honey yield (2), to overcome the stress caused by pesticides and pathogens. A honey bee colony needs pollen to meet the needs for protein, lipids, and vitamins (2, 3, 4). The lack of pollen diversity and diminished quantity affects the colony in a healthy brood production, increases infections due to deterioration of immune system defence mechanisms and shortens the life span of bees (4-11). Carbohydrates are the source of energy and structural storage polysaccharides (in plants there are as starch and inulin; in animals they are stored as glycogen and chitin). In pollen there are up to 41 % of sugars, but 50 % of them are starch and cell wall constituents and very hard to digest for the honey bees (4). Therefore, floral

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nectar and pollination drops are the richest source of carbohydrates for adult bees containing glucose, fructose, and sucrose, depending on the flowering periods and types of plants available. Honeydew is another source of carbohydrates for honey bees containing glucose, fructose, and sucrose like nectar, but also more complex sugars (maltose, melezitose etc.). It is usually available during nectar dearth and occurs on leaves of fir, larch, pine, oak, spruce etc. Nectar and honeydew are collected by bee foragers, taken to the bee hive, processed, and stored in wax cells as ripe honey. Bees need round 4 kg of honey to produce 500 g of wax or young bees at age 12 to 18 days need on average 20 kg of sugars (fructose, glucose, sucrose) and a significant amount of proteins to produce 1 kg of wax (4, 12).

Most insects can regulate protein and carbohydrates from natural food sources for optimal growth and survival (13). Different ratios of carbohydrate to protein affect social insect physiology and ability to survive. The optimal balance of nutrients can be determined by the geometric framework (13, 14). Adult honey bees lived longer on a pure carbohydrate or low protein diet (15). Paoli et al. (16) showed that honey bees of different age and behavioural have different nutritional requirements. The mortality of adult bees fed high amino acid diet was quite high. Also confirmed in ants, where Dussutour and Simpson (2012) (17) showed a reduction in ant worker lifespan when feeding a high protein diet. On contrary, Archer *et al.* (2013) (18) found that honey bees exposed to environmental stressors (e.g. low temperatures, nicotine) and fed with high protein diet (1:3=P:C) had lower mortality. However, they found that workers in this experiment did not adjust their intake to improve their survival after being exposed to the stressed condition. Feeding protein also extended the survival ability of adult bees after infection with *Nosema* sp. (19).

In beekeeping management, the establishment of young colonies is a frequently used practice in early summer to use the natural reproduction of the colony and to prevent unwanted swarming. New colonies established in May and June need round 1.5 kg of mostly young honeybees and a young, mated queen (20). As such they represent a swarm as a new young colony that must be placed by the beekeeper on another location than its primary colony. When placing the adult bees in a hive with frames and a wax comb foundation only, a newly established young colony requires constant food supplementation (20, 21) to support comb building and foragers start to bring nectar and pollen in the hive and the queen starts egg-laying. The choice of a food supplement is therefore a key factor to bring young colonies in the best shape and increase their potential of productiveness.

In our study we fed the bees with different candies in laboratory conditions to compare longevity and consumption rates. Another important aim of our study was to evaluate the performance and level of *Nosema* spp. spores in young honey bee colonies established from artificial swarms, supplemented with different candies in summer, in the apiary conditions.

Material and methods

The cage trial was established according to the standard methods for maintaining adult bees in controlled conditions (22). Combs with emerging worker bees were obtained from two colonies, placed in an incubator (34.5° C) and left overnight. On the next day, round 1.000 newly-emerged adult bees were collected and randomly put into plastic cages (8 x 12 cm) of air hoarding cages with around 80 openings of ~ 2 mm to provide circulation, and two larger holes for plastic feeders to deliver water and food. 50 bees were introduced in each cage, having 5 replicates per group. Bees were fed Yeast, Protein or Sugar candy, or sugar syrup (1:1, w:v) as a control. The cages with adult bees were kept in a darkened incubator at 28 (±1) °C. Mortality and food consumption were recorded on daily basis. The feeders were weighted on daily basis and the food replaced every 2 or 3 days.

The artificial swarms were established in spring (May) from honeybee colonies (*Apis mellifera carnica*, Pollmann 1879), weighing 1.5 kg of young, mixed-aged bees (weight of worker bees) each and transferred into boxes for swarms (Multibox®, Croatia) (Fig. 1) and left in a dark place overnight. The next day they were transferred into new LR (Langstroth hive) hives (30.5 x 50.5 x 24.3 cm) with 7 frames of AŽ (Alberti-Žnideršič, 410 x 260 mm) with a new wax foundations inserted. In total 18 hives were installed to Jable location (Middle-Slovenian region, 46°08›26.0»N 14°33›22.5»E). The young, mated queen bees were introduced, originating from the same queen breeding operation.

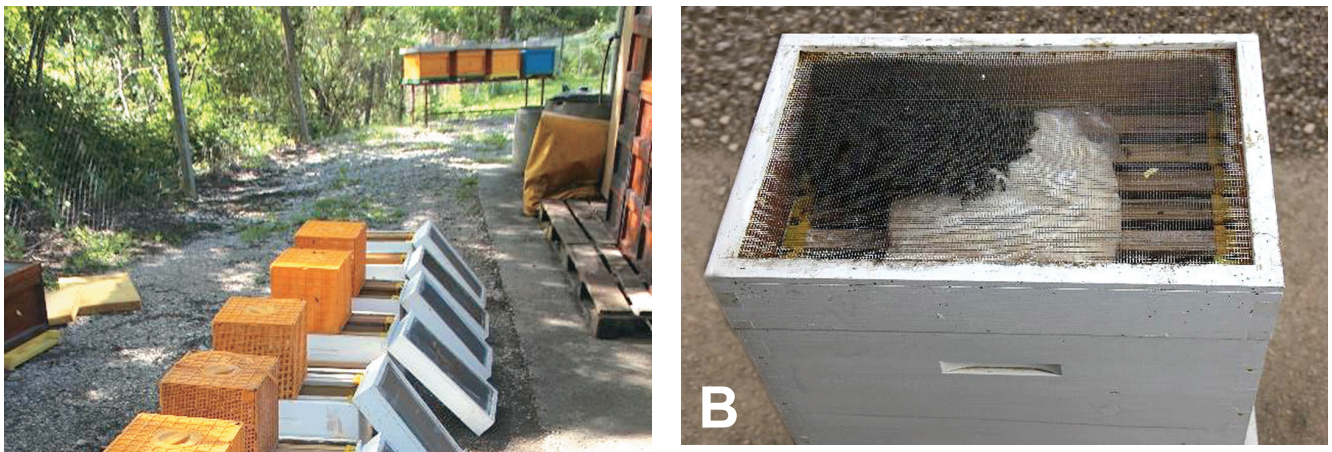

Figure 1: A – Artificial swarms in multiboxes and LR hives with 7-framed wax-foundations. B – Swarms in the hives were established and fed with candy

Artificial swarms were divided into three groups: (1) Yeast candy, (2) Protein candy and (3) Sugar candy. Colonies were continuously fed with one home-made and two commercial candies: (1) Yeast – grounded cane sugar, 5% baker's yeast and water, (2) Protein candy – Medopip plus®, Pip d.o.o., Pisarovina, Croatia, (sugar and vitamins) and (3) Sugar candy – Apifonda®, Südzucker, Germany, (sugar), Figure 1. Young colonies were evaluated three times in summer according to Liebefeld method (23).

Adult bees from the side frames were collected for *Nosema* spp. spores quantification twice, at the beginning of feeding in June and later in September. The abdomens of bees were used to estimate the *Nosema* spp. spores' prevalence and intensity as determined by light microscopy techniques, described by Cantwell (24). Briefly, bees' abdomens (20 bees, 3 replicates per colony) were macerated using a mortar and pestle in 1 ml of distilled water/bee. Further, a drop of the solution was placed on a hemocytometer and *Nosema* spp. spores were counted under a microscope, at 400 x magnification.

The experimental colonies were checked for overwintering ability by being inspected in March of the following year.

All data were analyzed using the RStudio (2021.09.0, PBC, Boston, USA). The data were expressed as mean ± standard error (SE). The lifespan was calculated using Kaplan-Meier curves of honey bee survival, and a log-rank test was performed for significant differences between curves. Measurements of colonies and *Nosema* spp. were analyzed using one-way ANOVA with Bonferroni corrected t-test.

Results

In the controlled laboratory conditions, worker bees from Yeast and Protein candy group lived longer compared to Sugar candy group (Fig. 2) having a significant difference between groups. The highest consumption was determined in group fed sugar syrup followed by Yeast candy group (Fig. 3).

In young colonies, adult bees were building wax cells most intensively in Sugar candy group in all measured periods, but the differences were not significant (Table 1). Brood area was statistically significant in Sugar and Yeast candy group (1st Period) and later on in Yeast and Protein candy group. Comparing the amount of pollen stores there were no differences, and the highest honey storage was in Sugar group (Table 1). However, we noticed that in the same group the content in the wax cells was white assuming that workers stored candy (personal observations). Number of frames with adult bees was the highest in Sugar candy group showing statistically significant differences from

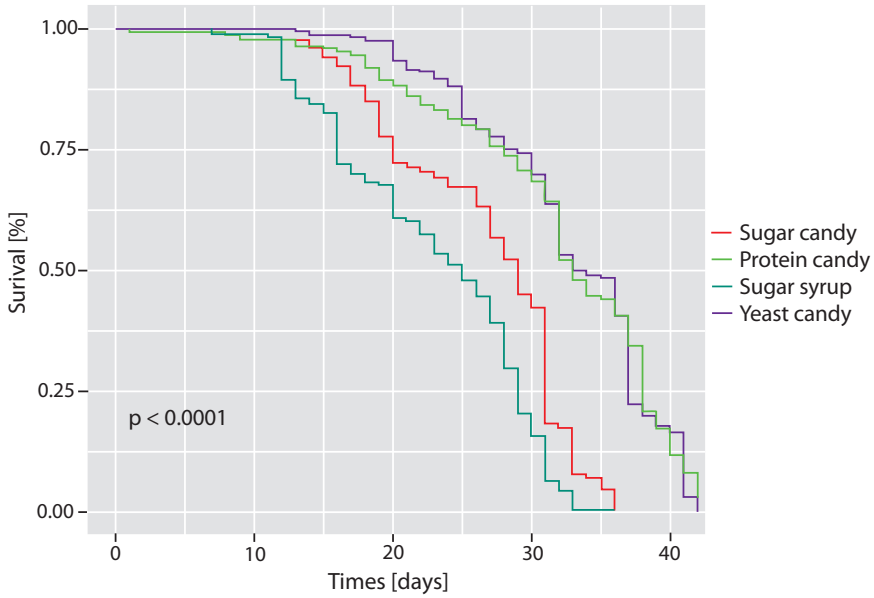

Figure 2: Survival of worker bees in controlled conditions. Kaplan-Mayer survival analysis

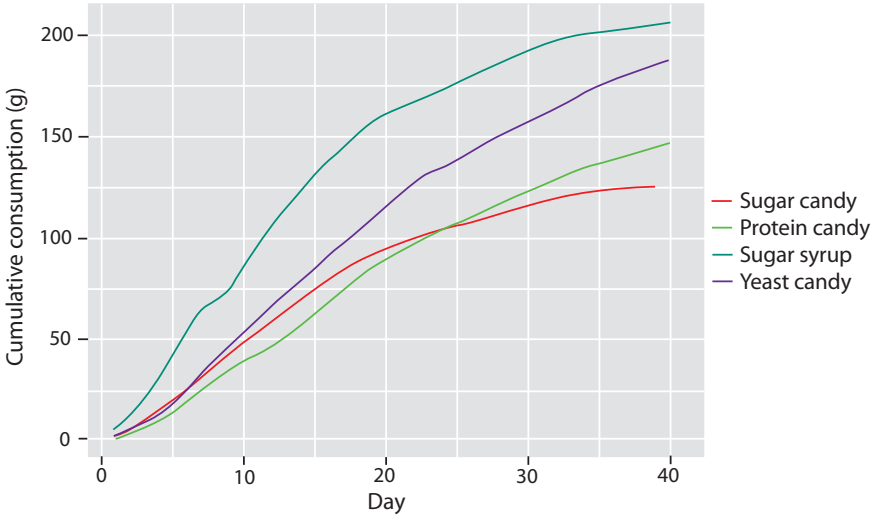

Figure 3: Cumulative consumption of candies and sugar syrup by worker bees under controlled conditions

Yeast candy group (2nd period), and later the differences were significant in all groups in the last observation period (Table 1).

In young colonies, adult bees were building wax cells most intensively in Sugar candy group in all measured periods, but the differences were not significant (Table 1). Brood area was statistically significant in Sugar and Yeast candy group (1st Period) and later on in Yeast and Protein candy group. Comparing the amount of pollen stores there were no differences, and the highest honey storage was in Sugar group (Table 1). However, we noticed that in the same group the content in the wax cells was white assuming that workers stored candy (personal observations). Number of frames with adult bees was the highest in Sugar candy group showing statistically significant differences from Yeast candy group (2nd period), and later the differences were significant in all groups in the last observation period (Table 1).

Table 1: Performance of young colonies established from artificial swarms in summer (1st period – 27.6., 2nd period – 2.7., 3rd period – 27.7.). N = 4-7, Y – Yeast candy, S – Sugar candy, P – Protein candy. SE – standard error, * significant differences (ANOVA, Bonferroni corrected t-test)

Artificial swarms	Group	1st period			2nd period			3rd period		
		Mean	±SE	p-values	Mean	±SE	p-values	Mean	±SE	p-values
Wax production (cm^2)	Y	6162	735.79	0.18	6666	909.96	0.26	8947.2	376.53	0.08
	S	7780.8	440.8		8308.8	850.61		10896	864	
	P	7073.14	453.22		8081.14	378.27		10628.57	473.42	
Eggs (cm^2)	Y	384	143.67	0.019	240	150.52	0.08	518.4	120.14	0.18
	S	840	71.6		648	109.46		624	112.83	
	P	648	54.17		480	82.48		785.14	77.45	
Larvae (cm^2)	Y	198	128.64	0.04	276*	173.48	0.003	676.8	142.47	0.85
	S	652.8	138.79		964.8	161.78		782.4	140.27	
	P	336	72.76		843.43*	50.62		754.29	105.9	
Pupae (cm^2)	Y	492*	202.35	0.0001	522*	318.45	0.001	2078.4*	531.46	0.015
	S	2510.4*	311.87		1987.2	369.19		3115.2	406.33	
	P	1690.29	138.65		2057.14*	59.88		3960*	296.72	
Bees (no. of frames)	Y	3.5	0.29	0.02	4*	0	0.008	3.8*	0.2	0.0009
	S	4.6	0.24		5.6*	0.24		5.6*	0.24	
	P	4.29	0.18		4.86	0.26		4.86*	0.26	
Pollen stores (cm^2)	Y	/			348	79.3	0.5	177.6	70.63	0.8
	S	/			235.2	98.78		249.6	84.72	
	P	/			219.43	55.78		240	76.08	
Honey stores (cm^2)	Y	/			1698	420.33	0.51	1272*	197.47	0.015
	S	/			2188.8	507.16		3432*	664.8	
	P	/			1614.86	230.42		1827.43	229.45	

In June, there were significant differences in *Nosema* spp. spore level, with the highest level in Sugar candy and the lowest in Yeast candy group ($p<0,05$). Comparing the level of *Nosema* spp. spores in September of the same year, the infection was low and non-significant (Table 2).

Table 2: Nosema spp. spores in worker bees in young colonies expressed in million per bee. N = 4-7, Y – yeast candy, S – Sugar candy, P – Protein candy. SE – standard error, *** p>0.05.** (ANOVA, Bonferroni corrected t-test).

Group	June			September		
	Mean	±SE	p-value	Mean	±SE	p-value
Yeast	4.35*	1.97	0.02	1.68	0.59	0.48
Sugar	14.96*	2.27		4.29	1.85	
Protein	12.55	2.74		4.03	2.14	

The colonies from Yeast candy group survived the winter successfully, however the other two groups lost one colony each by the time of the first inspection in early spring.

Discussion

An artificial swarm of honey bees is a very vulnerable young colony, as it possesses a small number of adult workers in comparison to the colony with brood and food stores. Young colony needs continuous food intake to allow worker bees to remain in the hive, building wax cells and later nursing young brood. Our study provides insights on how long worker bees live when fed protein or non-protein candies having no access to honey and pollen at the same time, and how

small, nucleus colonies perform during and after being fed the same type of candies.

The results of the field study show that the young colonies produced more brood feeding non-protein candy having at the same time the highest number of *Nosema* spp. spores in the same experimental group. On the contrary, adult bees in cages lived longer being fed protein or yeast candy, indicating that cage trials by itself do not provide adequate information on performance of bees. The bees in artificial swarms were of mixed age, physiologically in the stage of building wax, and therefore the nutritional needs differed from the bees in cages. However, the overwintering was less successful in groups fed commercial sugar and protein candy. This result can also be connected to some other factors that affect survival ability of honey bee colonies (Varroa, viruses etc.) (24), indicating that trials with bees in controlled conditions, among others, provide the insight in longevity and nutritional requirements (26).

For the last few decades, the apicultural sector is expected to reach a high production of honey and a strong resilience to honey bee diseases and at the same time to overwinter colonies successfully. Unfortunately, there are several factors that hinder the development of colonies and challenge the beekeepers. Changing climate affects colony development and redistribution of honey plants (27, 28, 29, 30), and adaptation cycle of plants and bees to these sudden changes is very slow. The abundance and quality of pollen and nectar is changing and is therefore very unstable natural food source for bees (27, 31). Malnourished colonies are very sensitive and susceptible to infections of pathogens and stress due to pesticide exposure (32), and even in some cases being capable to adapt, eventually those colonies will die (33). Nevertheless, beekeepers need to supply all types of colonies with food supplements and substitutes for bees to fill the gap in food shortage and according to season, health status and needs (artificial swarms, queen production etc.). There are many food supplements available on the market and beekeepers mostly prepare syrups containing white sugar (saccharose) and water (34), and some mix sugar patties with or without additives (i.e., pollen, yeast, vitamins, and minerals etc.) (34, 35, 36) to feed their colonies. In our study, we used a home-made and two commercial candies, that are commonly used by the beekeepers. We found that sugar candy (Apifonda) showed the best results in a short term to establish a young colony. Concerning *Nosema* spp. infection, several recent papers report that different additives are potentially effective to prevent or eliminate *Nosema* spp. spores and/or support development of honey bee colonies: pre/probiotics (37), EM probiotic (38), anti-nosema products (39), medicinal mushroom (40), plant extract (41, 42, 43), microalga (44), Chlorella (45), Cyanobacteria (46), pentadecapeptide BPC 157 (47), and other artificial diet (36). Sammataro and Weiss (2013) (48) compared productivity of colonies supplemented with sucrose or high fructose corn syrup (HFCS) and reported that group fed sugar syrup produced more wax, brood, and adult bee population that HFCS group. However, studies on effects of food supplements and additives on productive colonies are abundant, but there is a lack of research for artificial swarms or young colonies. Health status of worker bees in swarms and quality queen is therefore essential for optimal development and productivity of young colonies and the disease in colonies requires control (49).

Regarding the health status, young colonies originated from artificial swarms were analysed for Nosema spp. in our study. The spores were detected in adult bees due to possible previous infection of honey bee colonies that were used for the experiment, and the spore load in June was the lowest in Yeast candy group and decreased in all groups in September. Moreover, all the colonies fed Yeast candy were able to survive the winter. Microsporidia Nosema apis and N. ceranae (cause of Nosemosis type C) infect the midgut of bees and reproduce in epithelium cells of the gut (50, 51). Research of Nosema infection (52, 53, 54) shows that Nosemosis influences the strength of the colony and the honey yield (55). It should be pointed out, that through the stages of swarm manipulation (or queen production etc.) beekeepers must consider the negative impact of Nosema infection and prevent transfer of pathogen by adopting good beekeeping practice (56).

There are some differences in feeding various type of candies that might affect development and performance of young colonies. In our case we noticed differences in brood production, number of adult bees and honey stores in the hive, and longevity of workers in cages. At this point more studies of feeding supplements and effects on physiology and productivity should be done at the individual bee and colony (swarm) level to prevent colony failure.

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RAZVOJ DRUŽIN IN ŠTEVILO SPOR *Nosema* spp. PRI MLADIH DRUŽINAH MEDONOSNE ČEBELE (*Apis mellifera carnica,* Pollmann 1879), KRMLJENIH S POGAČAMI

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Izvleček: Mlade čebelje družine iz umetnih rojev smo krmili z različnimi pogačami z dodatkom beljakovin, kvasa ali sladkorja. Ocenjevali smo razvoj družin in določali število spor *Nosema* spp. pri odraslih čebelah v poletnem obdobju. V laboratorijskih pogojih smo krmili čebele delavke z isto vrsto pogač. Najboljše preživetje smo ugotovili v skupinah, ki so prejele pogačo s kvasom oziroma beljakovinami. Nasprotno pa je poskus v družinah pokazal, da je bilo v 1. obdobju merjenja bistveno več bub (2510,4 cm^2, p = 0,0001), v 2. obdobju pa več ličink (964,8 cm^2, p = 0,003) in okvirjev s čebelami (5,6, p = 0,008) pri krmljenju s sladkorno pogačo. V 3. obdobju ocenjevanja mladih družin je imela skupina s sladkorno pogačo največ pokritih okvirjev z odraslimi čebelami in zalog medu (5,6, p = 0,0009; 3432,0 cm^2, p = 0,015). Skupina s sladkorno pogačo je zgradila največjo površino satja, vendar razlike niso bile statistično značilne. Število spor *Nosema* spp. je bilo kvantitativno preverjeno pri odraslih čebelah. Najnižja okužba je bila statistično značilna v skupini s pogačo s kvasom v juniju (4,35 milijona spor na čebelo, p = 0,02), septembra pa spremembe niso bile signifikantne. Dodajanje sladkorne pogače umetnim rojem se je pokazalo kot najbolj obetavno za razvoj produktivnih mladih čebeljih družin. Ugotovitve naše študije bi lahko pomagale čebelarjem pri izbiri učinkovitega dodatka pogač za optimalen razvoj umetnih rojev.

Ključne besede: *Apis mellifera*; umetni roji; mlade družine; dodatki; pogače; razvoj; *Nosema* spp.; dolgoživost

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