

## Comparative estimation of some immune parameters in three felid species

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**ABSTRACT.** Pallas' cat inhabits dry steppes in the center of Asia where winter temperatures may drop below  $-50^{\circ}\text{C}$ . We suggested that the evolution in severe climatic conditions and costly adaptations to low ambient temperatures may result in decrease of immunity of this species, because it prevents acquisition of adequate resources to develop robust immune responses. We estimated humoral immune response to a non-replicating antigen (sheep red blood cells) in ten captive Pallas' cats, twelve domestic cats and six Far Eastern wildcats. Blood-sampling was conducted prior to and 21 days after the injection. We analyzed changes in body mass, number of leukocytes, IgG concentration and hemoagglutination/hemolysis titer. Pallas' cat had lower hemoagglutination/hemolysis titer before the injection and showed lower titer than the two other felids three weeks after the injection. These results may reflect a lower level of natural antibodies in Pallas' cat than in the two other species and, possibly, lower innate immunity of this species. Domestic cats, which were serum positive to feline coronavirus, also showed lower hemoagglutination/hemolysis titer during the experiment than serum negative animals.

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## Сравнительная оценка некоторых иммунных параметров трех видов кошачьих

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**РЕЗЮМЕ.** Манул обитает в сухих степях центральной Азии, где зимние температуры могут опускаться ниже  $-50^{\circ}\text{C}$ . Мы предположили, что эволюция в жестких климатических условиях и энергозатратные адаптации к низким температурам воздуха могут приводить к снижению иммунитета у этого вида, поскольку не позволяют расходовать адекватное количество ресурсов для развития иммунного ответа. Мы оценили гуморальный иммунный ответ на нереплицирующийся антиген (эритроциты барана) у десяти манулов, двенадцати домашних кошек и шести дальневосточных лесных котов. Забор крови у животных был проведен непосредственно перед и через 21 день после инъекции. Мы проанализировали изменения массы тела, числа лейкоцитов, концентрации иммуноглобулинов G, титра гемоагглютинации/лизиса. Манул имел более низкий титр гемоагглютинации/лизиса перед инъекцией, чем два других вида, и показывал более низкий титр через две недели после инъекции. Эти результаты, по-видимому, отражают более низкие концентрации «естественных антител» у манула по сравнению с двумя другими видами и, возможно, более низкий врожденный иммунитет у этого вида. Домашние кошки, серопозитивные к коронавирусу кошек, также показывали более низкий титр гемоагглютинации/лизиса в ходе эксперимента, чем серонегативные животные.

**КЛЮЧЕВЫЕ СЛОВА:** манул, иммунный ответ, лейкоциты, коронавирус кошачьих.

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## Introduction

Immune systems in mammals play a key role in detection of and resistance to different pathogens and may determine evolutionary success of the species. The pressure of pathogens that challenges the immune system of individuals can lead to diseases and in many cases to death (Lehmann, 1993; Norris & Evans, 2000). Immune response and immune maintenance of the individuals may be affected by different factors — other pathogens (Wernicke *et al.*, 1986; Lin *et al.*, 1992), body mass and wellness (Field *et al.*, 2002; Graham *et al.*, 2015), steroid hormones level (Schleimer, 2004; Coutinho & Chapman, 2011; Bereshchenko *et al.*, 2018), heterozygosity by MHC genes (Messaoudi *et al.*, 2002; Borsetti *et al.*, 2014). Although these factors affect immunity dramatically and cause large variations in immune response, several consistent species-specific differences in innate (Zschaler *et al.*, 2014; Heinrich *et al.*, 2017) and/or adaptive (Mestas & Hughes, 2004; Mendes *et al.*, 2006) immunity have been described previously. These differences may be explained partly by the long-term species evolution in different areas that differ in environmental conditions, pathogens pressure and other factors of natural selection. Some traits of life strategies may affect immunity dramatically. The most famous example is probably the small Australian marsupial (*Anthehinus stuartii* Macleay, 1841): males mate so intensively during breeding season that they die after it due to the decrease of immunity (Bradley *et al.*, 1980). In theory, based on these life-history traits, environmental conditions and food availability, the selection pressure may strengthen some components of the immune system to provide an adequate compensatory immune response (Acevedo-Whitehouse & Cunningham, 2006; Matson, 2006).

Interspecies differences in immunity of closely related species were described quite rarely (Matson, 2006; Mestas & Hughes, 2004; Mendes *et al.*, 2006; Heinrich *et al.*, 2017). Cheetahs (*Acinonyx jubatus* Schreber, 1775), the species with the low variety in MHC loci, showed more pronounced innate immunity than leopards (*Panthera pardus* Linnaeus, 1758) (Heinrich *et al.*, 2017). In this study, the wild animals were captured in the same area (Namibia) to minimize the effect of climatic parameters on animals' immunity.

In theory, interspecies differences in innate and adaptive immunity may cause the differences in susceptibility to different pathogens. In felids, there are few cases when the pathogens have a different effect on different species. Canine distemper virus is critically dangerous for tigers (*Panthera tigris* Linnaeus, 1758) (Seimon *et al.*, 2013), lions (*Panthera leo* Linnaeus, 1758) (Roelke-Parker *et al.*, 1996), leopards (Sulikhan *et al.*, 2018), lynxes (*Lynx canadensis* Kerr, 1792; *Lynx rufus* Schreber, 1777; *Lynx lynx* Linnaeus, 1758) (Daoust *et al.*, 2009) but not for domestic cats (*Felis catus* Linnaeus, 1758) (Ohishi *et al.*, 2014) and probably some other small cats (Naidenko *et al.*, 2018). Feline leukemia virus has a lethal effect on *Puma*, *Lynx* and *Felis* genera (Hoover & Mullins, 1991;

Sleeman *et al.*, 2001; Cunningham *et al.*, 2008), but no deaths caused by FLV were described for other species.

*Toxoplasma gondii* (Nicolle et Manceaux, 1908) is one of the most successful parasites, infecting all species of mammals, but the sexual reproduction of this pathogen occurs only in the intestines of felids (Dubey *et al.*, 1988). Consequently, *Toxoplasma gondii* is dependent on felid species as primary hosts for survival and reproduction. For most felids, infection by this pathogen is not fatal, but may cause problems in pregnancy and embryonic losses (Givens & Marley, 2008). However, *Toxoplasma* infection does result in high mortality of two felid species — Pallas' cat *Felis manul* Pallas, 1776 (Dubey *et al.*, 1988; Ketz-Riley *et al.*, 2003; Basso *et al.*, 2005) and sand cat *Felis margarita* Loche, 1858 (Pas & Dubey, 2008). It was hypothesized that this pathogen is absent in habitats of Pallas' cat in the wild (Brown *et al.*, 2005). However, more recently the presence of *Toxoplasma gondii* was confirmed within the range of Pallas' cat in Russia (Naidenko *et al.*, 2014), and was reported in prey species as well as sympatric populations of feral cats (Pavlova *et al.*, 2016).

One possible reason for high susceptibility of these felid species to this pathogen (and to others) may be lower activity of some components of their immune system. An adaptation of the Pallas' cat to the extreme weather conditions (winter temperature up to  $-50^{\circ}\text{C}$ , summer temperature up to  $+50^{\circ}\text{C}$ ) and high energy costs of survival and reproduction in this climate may restrict energy resources for the immune system. We hypothesized that Pallas' cat may be different in innate and/or adaptive immunity of other small cats. The aim of this study was to compare some parameters of innate and adaptive immunity in three feline's species of the same size: Pallas' cat, feral cat and Far Eastern wildcat *Prionailurus bengalensis euptilurus* Elliot, 1871. All three species have similar size and inhabit the same climatic zone of Russia.

## Materials and methods

### Study site and husbandry conditions

The study was conducted in three different study sites. Pallas' cats were held in Moscow Zoo (four animals) and Volokolamsk Breeding Center — a remote facility of Moscow Zoo (six animals). The total number of subjects was 6 males and 4 females. Domestic outbred cats (10 males and 2 females) and Far Eastern wildcats (2 males and 4 females) were held at the Biological Station "Tchernogolovka" of A.N. Severtsov Institute of Ecology and Evolution of Russian Academy of Sciences, 50 km from Moscow. All animals were kept separately from conspecifics (excluding one Pallas' cat pair (m:f) in Moscow Zoo). All animals at the Tchernogolovka Station were fed six days per week. This regime was intended to mimic conditions to the wild, where carnivores often do not feed for one to multiple days. At the Tchernogolovka Station animals were fed chicken meat (about 0.3–0.5 kg/day/individual) daily, while Pallas' cats

were fed common quail (*Coturnix coturnix*). All animals had access to water *ad libitum*.

### **Animals**

All animals were sexually mature (more than 1 year old) but not advanced in age. Age was determined by date of birth or tooth status — for the wild-caught Pallas' cat (Naidenko *et al.*, 2014). All animals were kept in captivity in Moscow Zoo, Volokolamsk Breeding Center or at Tchernogolovka Station as breeding colonies for many years. Some Pallas' cats were captured three years before in the wild, other Pallas' cats and domestic cats were 1<sup>st</sup>–2<sup>nd</sup> generation in captivity. Far Eastern wildcats were 3<sup>rd</sup>–5<sup>th</sup> generation in captivity as offspring of two zoo breeding lineages. For the experiment all animals were handled (physically restricted) without immobilization for 3–5 minutes for weighing and sampling blood and returned to their respective facilities. No animals were euthanized after the experiment and all animals stayed in Moscow Zoo, Volokolamsk Breeding Center and Tchernogolovka Station.

### **Experiment design**

To estimate species-specific differences in immune system activity we injected sheep red blood cells (SRBC) subcutaneously to all animals and estimated their humoral immune response to the non-replicated antigen. Sheep blood was collected from the jugular vein of a live ram into a glass tube with glass balls (diameter 8–10 mm) and shaken gently for 20 minutes. Afterwards blood was filtered through gauze and washed three times (1:3 v/v) with a 0.9% NaCl solution. We prepared the SRBC solution in sterile 0.9% NaCl with the concentration of  $1 \times 10^6$  cells per 1 ml (SRBC were counted in hemacytometer chamber under Leica DM 5000 B microscope (Leica Microsystems, Wetzlar, Germany) with  $40 \times 10$  magnification). SRBC were injected at a concentration of  $1 \times 10^6$  cells per kg. SRBC were injected into all individuals on May 28th–31th 2015. All animals were weighed and blood sampled (1–3 ml) prior to SRBC injection, and sampled for blood for a second time exactly 21 days later for each individual (18th–21st of June). All individuals were captured by hands in their cages and physically restricted for blood-sampling (usually this procedure lasted less than 5 minutes). Blood was collected in K<sub>3</sub>EDTA tubes (0.25 ml) (Aquisel, Barcelona, Spain) for hematological analysis and smear preparation and in Eppendorf tubes (Scientific Specialties, Inc. (SSI), Lodi, California, USA), where the serum was later separated using a centrifuge (1000g, 20 min) (Eppendorf MiniSpin, Eppendorf AG, Hamburg, Germany). The serum was frozen as aliquots at -18°C and stored at this temperature until the serological and immunological tests were performed.

### **Estimated parameters**

We derived the following parameters for all individuals: body mass, total number of leukocytes, hemoagglu-

tinayion/hemolysis titer to the SRBC, total concentration of immunoglobulins and serum prevalence to four pathogens. All parameters (excluding serum prevalence) were estimated at days 0 and 21 after SRBC injection. Body mass was estimated using RST 08065 scales (RST, Lund, Sweden) with an accuracy of 5 g immediately after each blood sampling.

Total number of leukocytes was counted with a microscope (Leica DM 5000 B, magnification  $10 \times 40$ ) in hemacytometer counting chamber (MiniMed, Bryansk, Russia). Blood was diluted (10  $\mu$ l of blood : 190  $\mu$ l of 3.5% acetic acid) to lyse erythrocytes. Leukocytes were counted in 100 large sections of the hemacytometer chamber and the total concentration of leukocytes per ml was recalculated.

The titer of antibodies to SRBC (hemoagglutination/haemolysis titers) was estimated using the hemagglutination method. It reflects the activity of natural antibodies and the interaction between natural antibodies and their complement (Matson *et al.*, 2005; Pap *et al.*, 2010). The titrating was conducted in 96-well plates (with U-shaped bottom). We placed 50  $\mu$ l of sterile isotonic solution (NaCl 0.9%) in each well. We also placed 50  $\mu$ l of serum samples (one per well) into the first row of the plate (A1-H1), mixed gently with pipette aspiration and transferred 50  $\mu$ l of the mixture into the well in the second row, later repeated it with the next row. Using this method, we obtained serial dilution of 1:2, 1:4, 1:8, ..., 1:2048. Plates were placed into the thermostat for two hours and incubated at 37°C. We estimated the last well for each serum sample where the hemagglutination reaction was observed as a titer. One control sample (domestic cat with the titer 1:8 was used for all place to control inter-plate variation).

Immunoglobulin G (IgG) concentration was measured in plasma samples with a protein A enzyme-linked immunosorbent assay (ELISA) (Ross *et al.*, 1993). Plasma samples were diluted 1:80.000 with 50 mM NaHCO<sub>3</sub>. As a standard, we diluted purified cat IgG (Immunology Consultants Laboratory, Inc., Portland, USA) with a starting concentration of 4 mg/ml with 50 mM NaHCO<sub>3</sub> and created standard concentrations of 4000 ng/ml, 2000 ng/ml, 1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml and 0.5 ng/ml. We pipetted 100  $\mu$ l of diluted samples or standards in duplicates into each well of 96-well ELISA plates. Plates were incubated for 1 hour at 37°C and washed three times with Tris-Buffered-Saline-Tween-20 (TSB-T20). Gelatine was added to TSB-T20 solution and 200  $\mu$ l of 1% of this mixture was pipetted to each well to block non-specific reaction bindings. Plates were incubated for 30 min at 37°C and washed three times with TSB-T20. Then, 100  $\mu$ l of 1:12.000 solution of protein-A-horseradish-peroxidase (Protein A-HRP, Invitrogen, Catalog No. 10-1023) in TSB-T20 was added to each well and plates were incubated for 30 min at room temperature. Plates were washed five times with TSB-T20 and 100  $\mu$ l of TMB solution was added into each well (TMB; Xema, Moscow, Russia). The reaction was stopped after 10 min with 100  $\mu$ l of 1% H<sub>2</sub>SO<sub>4</sub>. We measured the absorbance of the wells in the plates at 450

nm in a photometric microplate reader (Multiscan EX; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). A linear standard curve was calculated for each plate using the standard concentrations. IgG concentrations were then calculated based on the standard curve.

We estimated serum prevalence of all cats to four different pathogens to exclude/estimate their effect on the animals' immune system activity. We estimated the presence of antibodies to feline immunodeficiency virus (FIV), feline coronavirus (FCoV), *Toxoplasma gondii* and antigens to feline leukemia virus (FeLV). The presence of antibodies to the first three pathogens makes the presence of these pathogens per se highly likely. These methods have been described earlier (Naidenko *et al.*, 2014, 2018; Pavlova *et al.*, 2015). Briefly, the presence of FCoV antibodies was detected by a commercial enzyme-linked immunosorbent assay (Immunocomb®; Biogal, Galed Labs, Acs Ltd., Kibbutz Galed, Israel) according to the manufacturer's instructions. Animals were considered serum positive with a titer >1:20 for FCoV and serum negative with a titer <1:20. The immunochromatography tests (snap tests; BioVetTests (BVT) Groupe Virbac, La Seyne sur Mer, France) were used to detect the presence of FeLV group-specific antigen, as well as the presence of FIV antibodies. Tests for antibodies (IgG) to *Toxoplasma gondii* were conducted using EIA commercial kits (Xema, Moscow, Russia) using the "cutoff" method.

### Statistical analysis

We used Kruskal-Wallis (K-W) test to assess differences between all three species. To estimate intraspecific changes over time (before and after SRBC injections) we used Wilcoxon matched pairs test (W). To compare independent data (like body mass of males and females

of the same species) we used Mann-Whitney test. For statistical analyses we did not use the titer per se, but dilution level/well number (titer logarithm ( $\log_2$ )) from 1 to 11 where 1 corresponds to titer 1:2, 2 — 1:4, ..., 11 — 1:2058.

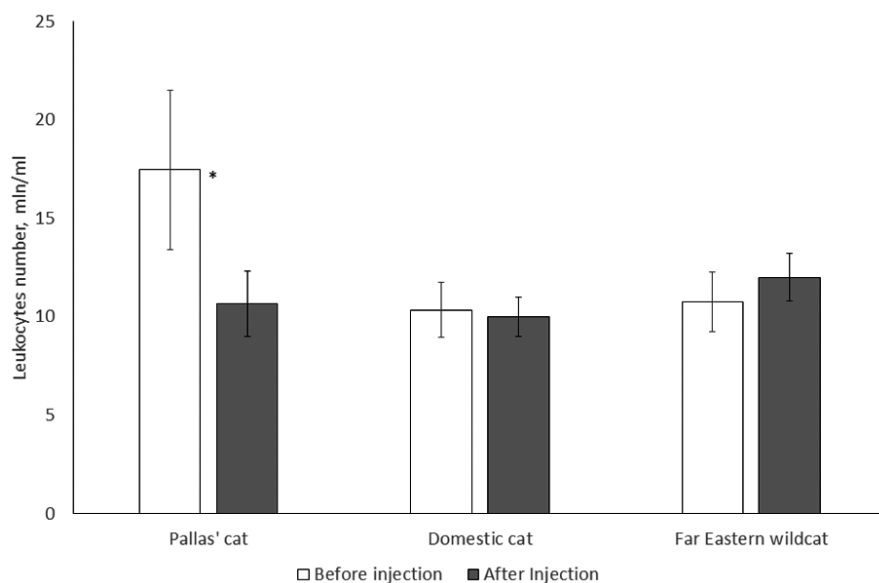
### Animal welfare

All experimental procedures did not have any visible adverse effects on the animals. No special permission for use of these captive cats in researches is required in Russian Federation. The Commission on Regulatory of Experimental Research (Bioethics Commission) in A.N. Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences was created only in 2017. The study was conducted in accordance with the ASAB/ABS (2012), Guidelines for the Treatment of Animals in Behavioural Research and Teaching and with the laws of Russian Federation, the country where the research was conducted.

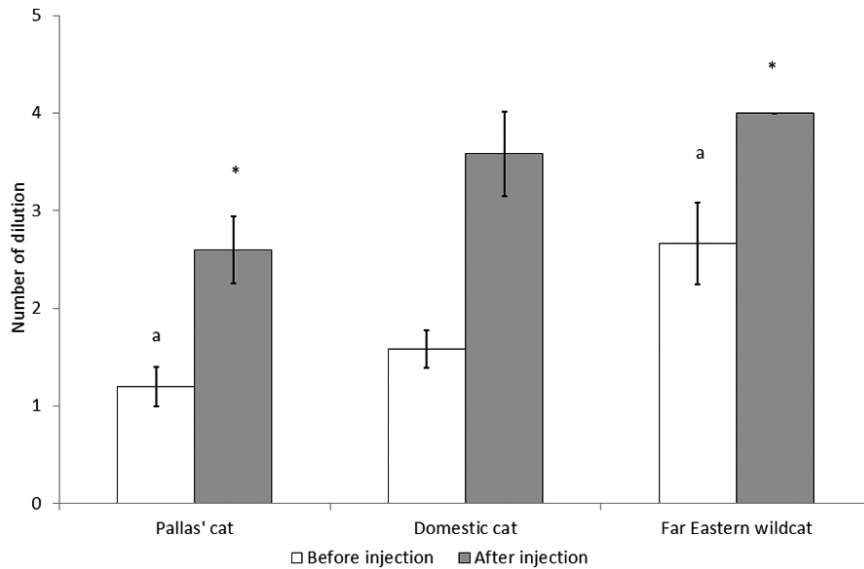
## Results

### Body mass

We found no difference in body mass between sexes of Pallas' cat both before and after injection of SRBC (M-W:  $Z=0-0.42$ ;  $p=0.76-1.00$ ). Mean body mass of Pallas' cat was  $4.17\pm 0.47$  kg before the SRBC injection and  $4.00\pm 0.42$  kg three weeks later. Although body mass of Pallas' cats declined on average  $-1.1\pm 8.4\%$ , but this change was not significant (W:  $Z=0.15$ ;  $p=0.88$ ). Body mass also did not differ significantly before and after the injection in domestic cats ( $3.59\pm 0.13$  and  $3.58\pm 0.14$  kg; average changes were  $-0.6\pm 1.0\%$ ) and in Far Eastern wildcats ( $4.02\pm 0.26$  and  $4.07\pm 0.39$  kg, with average changes of  $0.5\pm 3.3\%$ ). To sum up, the changes of body



**Fig. 1.** Total leukocytes number in three cat species before and after injection. Asterisk shows significant differences in Pallas' cats before and after injection.



**Fig. 2.** Average number of dilution (titer) of studied serum samples to sheep red blood cells. Letters and asterisks show significant differences of Pallas' cat and Far East wildcat parameters.

mass during the experiment were similar between all three species (did not over 1.1%). We may conclude that body mass stayed stable over the experiment in all species. Body mass of males and females did not differ significantly in domestic cats and Pallas' cats (respectively  $Z=1.93$ ;  $n_1=2$ ;  $n_2=10$ ;  $p=0.06$  and  $Z=1.36$ ;  $n_1=2$ ;  $n_2=10$ ;  $p=0.22$ ).

#### Total leukocytes number

Total leukocyte number before the experiment was slightly higher in Pallas' cats (K-W:  $H=1.066$ ;  $df=2$ ;  $p=0.587$ ), but was similar in the two other species (Fig. 1). Total leukocyte number decreased significantly in Pallas' cats (W:  $Z=2.43$ ;  $p=0.015$ ), but did not change significantly in two other species (W:  $Z=0.24-0.52$ ;  $p=0.60-0.81$ ). We did not find significant differences between sexes in leukocyte counts in any of the cat species (M-W:  $Z=1.07-1.93$ ;  $p=0.053-0.283$ ).

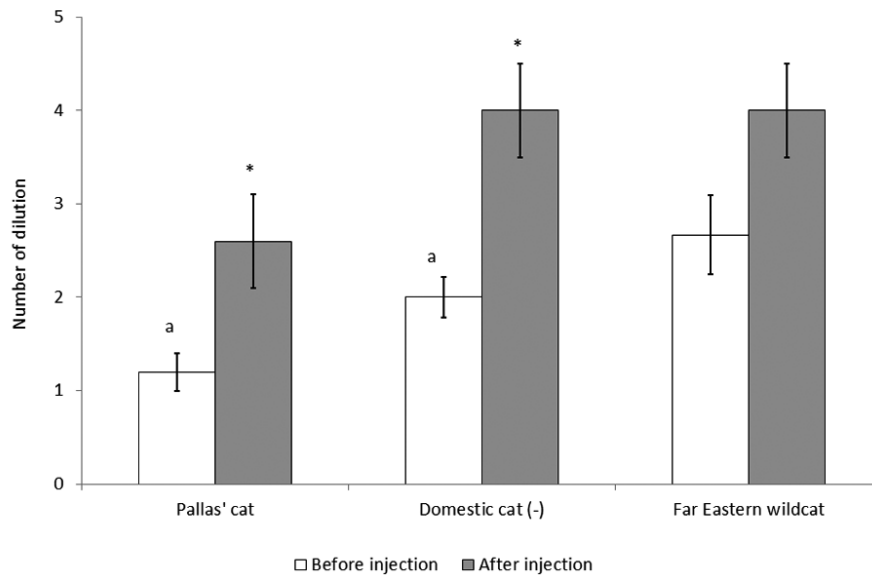
#### Humoral immune response

Titer of antibodies to SRBC differed significantly in three cat species (K-W:  $H=8.67$ ;  $df=2$ ;  $p=0.013$ ). It was significantly higher in Far Eastern wildcats than in Pallas' cat (K-W:  $Z=2.75$ ;  $p=0.018$ ) before the test (Fig. 2). Three weeks after the injection the titer of antibodies to SRBC was also different in three felid species (K-W:  $H=9.84$ ;  $df=2$ ;  $p=0.007$ ). It did not differ significantly between Far Eastern and domestic cats, but was still higher in Far Eastern wildcats than in Pallas' cats ( $Z=2.47$ ;  $p=0.040$ ). We did not find any differences in antibodies titer between Pallas' cat and domestic cats at the sampling points 0 and 1 of the experiment. In all species titer of antibodies to SRBC significantly increased after injection.

#### Serum prevalence to different pathogens and its effect on measured parameters

We found five animals with antibodies to FCoV (all domestic cats) and eight individuals serum positive to *Toxoplasma* (5 Pallas' cats, 2 domestic cats and 1 Far Eastern wildcat). We did not find any differences in analyzed parameters of the immune system between individuals serum positive and serum negative to *Toxoplasma gondii* (both in Pallas' cats M-W:  $Z=0.10-1.15$ ;  $p=0.25-0.92$  and domestic cats M-W:  $Z=0-1.93$ ;  $p=0.06-1.00$ ). However, serum prevalence to FCoV affects the antibodies titer to SRBC significantly before the SRBC injection (dilution level was  $1.0\pm 0.0$  (titer 1:2) for serum positive and  $2.0\pm 0.22$  (titer 1:4) for serum negative individuals;  $Z=2.71$ ;  $p=0.007$ ), and three weeks after the injection ( $3.0\pm 0.55$  (titer 1:8) and  $4.0\pm 0.62$  (titer 1:16) respectively; M-W:  $Z=2.71$ ;  $p=0.007$ ). Domestic cats that were serum negative to FCoV had higher titer to SRBC both before and three weeks after injection.

Since serum prevalence to FCoV significantly affected immune characteristics of domestic cats (antibodies titer to SRBC), we compared only FCoV serum negative domestic cats ( $n=7$ ) with Pallas' cats and Far Eastern wildcat. Domestic cats (serum negative to FCoV) showed higher titer of hemoagglutination/hemolysis with SRBC in comparison with the Pallas' cats (before and after the injection M-W:  $Z=2.67$ ;  $p=0.008$  and  $Z=1.81$ ;  $p=0.07$ ) (Fig. 3). "Healthy" domestic cats had significantly higher titer to SRBC than Pallas' cats and similar to Far Eastern wildcats (M-W:  $Z=0.49-1.45$ ;  $p=0.15-0.62$ ).

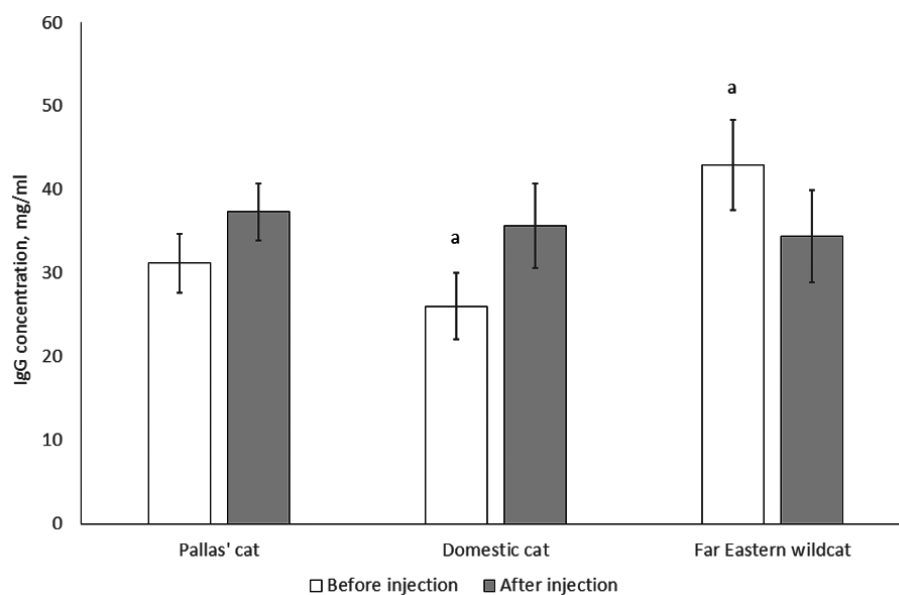


**Fig. 3.** Average number of dilution (titer) of studied serum samples to sheep red blood cells. Only domestic cats, that were serum negative to FCoV, have been included in this graph. Letters and asterisks show significant differences of Pallas' cat and domestic cat parameters.

#### *Total concentration of immunoglobulins*

IgG level did not differ between species before the injection of SRBC (K-W:  $H=1.066$ ;  $df=2$ ;  $p=0.586$ ). We expected to see an increase of total IgG level in all species after the injection. However, only two species showed some increase (Fig. 4) and only in domestic cat this increase was significant (W:  $Z=2.20$ ;  $p=0.028$ ). IgG level increased to  $53\pm 27\%$  in domestic cats,  $30\pm 18\%$

in Pallas' cats and decreased to  $18\pm 13\%$  in Far Eastern wildcats. Average concentration of IgG varied during the experiment from 17 mg/ml to 28 mg/ml. Significant interspecific differences in IgG levels were found only before injections (K-W:  $H=9.0$ ;  $df=2$ ;  $p=0.011$ ) for domestic cat and Far Eastern wildcat ( $Z=2.53$ ;  $p=0.033$ ). Domestic cats had the lowest level of IgG concentration in comparison with the other two species.



**Fig. 4.** Mean IgG concentration in three felid species before and after injection of sheep red blood cells. Letters shows significant differences.

## Discussion

### *Body mass*

Sexual differences in Pallas' cats were not detected for all parameters we analyzed (including body mass). In domestic cats and Far Eastern wildcats female body mass was slightly lower than male body mass, as has been described for many felids (Heptner & Sludskii, 1992; Lucherini *et al.*, 2006). The lack of significant differences in males and females body mass in our experiments was probably related to small sample size, especially for Far Eastern wildcats and domestic cats where males are usually heavier than females (Yudin, 2015). In Pallas' cats due to dramatic seasonal changes in body weight, these differences were often not observed (Naidenko *et al.*, 2014). During the experiment, the body mass of animals did not change significantly. Increased stress to the immune system does not always result in changes in body mass, although body mass may affect immune response (Sheridan *et al.*, 2012; Moreno-Ruedo, 2015).

### *Leukocytes number*

Total leukocyte count is one of the most common indicators of immune system activity although different factors may affect it (Pavlova *et al.*, 2018). In Pallas' cats the total number of leukocytes before SRBC injections was slightly higher (17.48 mln/ml) than described previously (7.33–9.75 mln/ml) (ISIS, 1999; Brown *et al.*, 2005). In the two other cat species leukocyte counts were similar to previous estimates (10.35–11.01 vs 10.28 mln/ml in domestic cats and 10.78–12.02 vs 8.25 mln/ml in Far Eastern wildcats) (ISIS, 1999). Interspecies differences in leukocytes number were not detected. Injection of SRBC to domestic cats and Far Eastern wildcats did not result in significant changes in leukocyte counts, although slight increases were noted. In Pallas' cat a significant decrease in leukocyte numbers was noted during the experimental period. Change in leukocyte count is a very important indicator of cellular immune response (Gildersleeve *et al.*, 1987; Kumar & Sharma, 2010; Hassiotou *et al.*, 2013), however, it is less related to the mounting of humoral immune response (but see also Gildersleeve *et al.*, 1987; McKinnon *et al.*, 1989). So, the absence of significant changes in leukocyte count as a response to SRBC injection was to be expected. It could be theorized that in Pallas' cat, unlike in two other species, an increase of humoral immune response may result in decrease of components of cellular immune response (and total number of leukocytes) due to the internal resources availability. However, this theory so far needs to be proven with the precise experiments.

### *Humoral immune response*

We found that antibodies titer to SRBC (and dilution number) at point 1 (immune response) was higher in two cat species (considering individuals serum negative to FCoV) than in Pallas' cat. Therefore, we may conclude that there were species-specific differences in their humoral immune response. However, these differences

were most pronounced prior to injection of SRBC (point 0), when intensity of hemoagglutination/hemolysis characterizes mainly the level of non-specific antibodies and innate immunity (complement activity) (Matson *et al.*, 2005; Pap *et al.*, 2010; Heinrich *et al.*, 2017). These non-specific antibodies (so called "natural antibodies", NABs) are unique among immunoglobulin molecules because their presence does not require previous exposure to a particular antigen. NABs react with various affinities to a wide variety of antigens including foreign red blood cells (RBCs), bacteria, viruses and toxins (Greenberg, 1985; Ochsenbein *et al.*, 1999; Belperron & Bockenstedt, 2001). The role of natural antibodies is to recognize epitopes of various antigens and initiate the complement system, a group of proteins that trigger a signal cascade which finally leads to pathogen lysis (Matson *et al.*, 2005; Gilot-Fromont *et al.*, 2012). Most natural antibodies molecules are pentameric IgM, but some IgG (monomeric) and IgA (dimeric) forms have been reported (Avrameas, 1991).

Anyway, the titer of natural antibodies at point 0 (day of injection) was significantly less in Pallas' cats than in two other species and the same differences remained when the animals formed specific immune response to SRBC three weeks later. These differences may be related to the extreme environment in which Pallas' cat has evolved. In rodents it has been shown that immune system activation from helminthes' invasion may decrease metabolic response to cold (Novikov *et al.*, 2015) and to decrease survival in severe climatic conditions. Possibly, in such climate (winter temperatures may exceed -50°C) high humoral response may be reached only by high expenditures of energy resources which may decrease survival of the individuals.

Pallas' cats formed immune response (an increase of titer from point 0 to point 1) comparable with that of other two cats. Probably, this increase is sufficient for Pallas' cats to resist infections. Harsh and severe climatic conditions may not only affect Pallas' cat but also prevent the distribution of some pathogens (Pavlova *et al.*, 2016). The data on diversity and occurrence of pathogens (based on serum prevalence estimation) supposed that pathogen pressure on Pallas' cat in the wild may be lower than on domestic cat or Far Eastern wildcat (Naidenko *et al.*, 2014, 2018; Pavlova *et al.*, 2015).

### *Serum prevalence*

We did not find any significant differences in studied parameters between individuals serum positive and serum negative to *Toxoplasma*, although sometimes the presence of *Toxoplasma* negatively affects the activity of immune system (Fatoohi *et al.*, 2004), mainly cellular immunity (Beran *et al.*, 2015). However, in domestic cats a decrease in immune activity (antibodies titer) was detected in FCoV-positive animals. Serum positive individuals showed lower antibodies titer both at the point 0 and point 1 (before and three weeks after RBSC injection), although positive animals did not show any clinical signs of disease. FCoV is a common infection

of cats with a tropism for the epithelial cells of the gut and for monocytes/macrophages (Addie & Jarrett, 1990, 1992). It is found worldwide (up to 40–80% of cats in a population may carry the virus) (Pedersen *et al.*, 2008). Most FCoV-infected cats remain asymptomatic. Closely related to FCoV FIP virus leads to immune suppression (Poland *et al.*, 1996), but for FCoV it is not so obvious. When FCoV starts to develop in the cat organism it starts so called antibodies-related increase of lymphocytes number (Pedersen & Boyle, 1980; Weiss & Scott, 1981). In presence of FCoV the affinity of antibodies to proteins increases and it makes easier the contact of virus with the macrophages (Olsen *et al.*, 1992). In this case macrophages of infected cats increase the differentiation rate of B-lymphocytes (Takano *et al.*, 2009). We have theorized that this increase will positively affect the production of different antibodies by B-lymphocytes, however, it seemingly does not. Antibodies titer to a non-replicated antigen (SRBC) in FCoV-positive animals was lower, probably because the animals spent a high amount of resources on FCoV-antibodies production.

#### **Total immunoglobulin G concentration**

Total concentration of immunoglobulins in all three species was inside of the range that was described for the felids and these three species in particular ([www.zims.species360.org](http://www.zims.species360.org)). Interspecies comparison of IgG concentration in three cat species in this study showed the differences between domestic and Far Eastern wildcats before the injection. Animals were kept in similar conditions and did not show any signs of diseases so we may suggest species-specific differences in concentration of IgG in these two cats. An injection of SRBC did not change the total IgG level in two species significantly but increased IgG concentration in domestic cats. It is probable that outbred domestic cats reacted more effectively to a non-specific antigen than other two cats and they produce more IgG than wild cat species. The additional research on changes of IgG and NAbs concentrations under immune pressure (like an effect of different non-replicated/replicated pathogens) should be conducted. It is necessary to note that domestic cats showed the most pronounced immune response to SRBC of all three species, and possibly, an increase of IgG specific to SRBC during the experiment increased the total IgG concentration.

We analyzed three parameters of the immune system that are related to innate immunity: total WBC number, IgG concentration and hemoagglutination/hemolysis titer in three cat species. We also monitored these parameters for three weeks after the SRBC injection. We hypothesized that Pallas' cat (as a species inhabiting the areas with extremely severe climate) will demonstrate the lowest immunity among the three cat species. However, we were able to confirm this hypothesis just for one parameter: Pallas' cat demonstrated lower hemoagglutination/hemolysis titer in comparison with two other cat species. It shows that Pallas' cat may have lower innate immunity (NAbs concentration) than domestic cat and Far Eastern

wildcat. This parameter was also lower in Pallas' cat three weeks after injection. It shows approximately the same increase (adaptive immune response) as two other cats, but zero-point was significantly lower. Thus, not all parameters (WBC number, total IgG concentration), but some parameters of innate immunity were weaker in Pallas' cats.

We assume that this trait is related to the evolution of the species in an extremely severe climate where it is more difficult to find energetic resources to sustain high immunity. The costliness of immunity is supported by several studies (Sheldon & Verhulst, 1996; Demas *et al.*, 1997; Nordling *et al.*, 1998; Moret & Schmid-Hempel, 2000), however, there were no comparison of different components (for example, production of leukocytes and antibodies). The antibodies production is very costly for the mammals (Demas *et al.*, 1997) and may be it explain why Pallas' cats were different on hemoagglutination/hemolysis titer (it means — NAbs concentration) of two other cats.

From other point of view, these severe climate conditions may decrease the number of pathogens and pathogen pressure in Pallas' cat habitats (Pavlova *et al.*, 2016). This low pathogen pressure does not require high immunity of hosts to override it. To verify this hypothesis, it will be necessary to conduct additional studies on immunity (both innate and adaptive) of these feline species.

Another important issue is that genetic diversity may affect the immunity of the species/population significantly (Bernatchez & Landry, 2003; Froeschke & Sommer, 2005; Schad *et al.*, 2005), although in some cases species with low MHC diversity show higher innate immunity (Heinrich *et al.*, 2017). We did not check the genetic diversity of the felids in this study (it is a point for future work), but only Far Eastern wildcats were bred in captivity for several generations (following their pedigree preventing the mating of closely related individuals) and did not show any signs of inbred suppression (including immune characteristics in this study). Both wildcats are very poorly studied in the wild, including their genetic parameters. So far the preliminary data did not show a decrease of genetic diversity of Pallas' cat (Sorokin *et al.*, 2015). The genetic diversity of Far Eastern wildcat so far has never been studied. The articles analyzed data on genetic diversity of the Bengal cat with unique sampling in Russia Far East (Patel *et al.*, 2017) or on insular populations of this subspecies (Tamada *et al.*, 2008). Anyway, the genetic aspect should be considered when the study on wild populations will be conducted.

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