

## IMMUNODEFICIENCY ASSOCIATED WITH MULTIPLE CONCURRENT INFECTIONS IN CAPTIVE PALLAS' CATS (*OTOCOLOBUS MANUL*)

Cornelia J. Ketz-Riley, D.V.M., Jerry W. Ritchey, D.V.M., Dipl. A.C.V.P., John P. Hoover, D.V.M., Dipl. A.C.V.I.M., Calvin M. Johnson, D.V.M, Ph.D., and Michael T. Barrie, D.V.M.

**Abstract:** Five neonatal Pallas' cats (*Otocolobus manul*) at the Oklahoma City Zoo died from toxoplasmosis with concurrent herpesvirus infection. These multiple infections suggested underlying immunodeficiency, perhaps caused by concurrent infection with feline immunodeficiency virus (FIV); so blood samples were collected for serology, serum protein electrophoresis, lymphocyte proliferation assays, and cytokine analysis by reverse transcriptase–quantitative competitive polymerase chain reaction (RT-qcPCR). Resulting data were compared with data from FIV-infected and control domestic short-haired cats. In addition, peripheral blood mononuclear cell cultures were propagated to detect FIV virus by both RT-qcPCR and detection of reverse transcriptase activity. Serum protein electrophoresis showed that four of six Pallas' cats had increased alpha<sub>2</sub> globulins. At least two Pallas' cats had decreased lymphoproliferation responses to mitogen, and all three tested animals exhibited defective interleukin-12 gene expression. Although these clinical and laboratory findings suggested an immunodeficiency syndrome, FIV infection could not be confirmed. On the basis of repeated blood test results, it can be concluded that nutritional, metabolic, or other systemic problems probably did not contribute to the disease syndrome. Further investigation of other possible causes of immunodeficiency, including a possible genetic component, in this population is needed.

**Key words:** Immunodeficiency, Pallas' cat, *Otocolobus manul*, toxoplasmosis, feline Herpesvirus, RT-qcPCR.

### INTRODUCTION

Sixteen rare Russian wild-caught Pallas' cats (*Otocolobus manul*) have been distributed to five North American zoological parks since 1994 to establish captive-breeding populations. These cats have bred successfully, but a neonatal mortality rate of over 60 % has been observed, with acute *Toxoplasma gondii* infection being the main cause of death.<sup>25</sup>

Two breeding pairs arrived at the Oklahoma City Zoo. Individuals of one pair had immunoglobulin (IgG) titer against *T. gondii* on arrival, and the other two developed titers after they were reunited during quarantine. Only IgG levels were measured. Both pairs produced litters of four kittens, and all eight kittens developed signs of acute toxoplasmosis on the day after birth. Four kittens died within a few days. They all showed macroscopic and histopath-

ologic signs of encephalitis, interstitial pneumonia, and systemic toxoplasmosis on necropsy, and immunohistochemical stains confirmed the presence of *T. gondii*.

In 1999, the remaining eight cats developed signs of upper respiratory infections that resisted treatment. A pair of black-footed cats (*Felis nigripes*) housed in the neighboring suite developed identical signs 6–7 mo later. Conjunctival cultures and serology revealed feline herpesvirus (FHV) infection. One of the offspring of the original Pallas' cats died after encephalitis and seizures developed. Postmortem findings were consistent with feline panleukopenia. This unusual occurrence of acute toxoplasmosis in the offspring of seropositive queens, persistent active FHV infection, and panleukopenia suggested that the cats were immunodeficient.

We attempted to characterize the humoral and cellular immunity of the Oklahoma City Zoo Pallas' cats and to identify any sources of immunodeficiency. We also evaluated the feasibility of using these tests to measure the immune responses of other Pallas' cats.

### MATERIALS AND METHODS

Six of the Oklahoma City Zoo Pallas' cats (*O. manul*), including four adults and two juveniles, were studied initially. All cats had IgG antibody titers against *T. gondii* (>1:2048; tested at Cornell Diagnostic Laboratory, Cornell University, Ithaca, New York 14852, USA) and had persistent signs of FHV infection. They were immobilized with med-

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From the Oklahoma City Zoological Park, Oklahoma City, Oklahoma 73111, USA (Ketz-Riley, Barrie); the Departments of Veterinary Pathobiology (Ritchey) and Veterinary Clinical Sciences, College of Veterinary Medicine (Hoover), Oklahoma State University, Stillwater, Oklahoma 78048, USA; and the Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611, USA (Johnson). Present addresses (Ketz-Riley): Topeka Zoological Park, 635 SW Gage Boulevard, Topeka, Kansas 66606, USA; (Barrie): Columbus Zoo, 9990 Riverside Drive, P.O. Box 400, Powell, Ohio 43065-0400, USA. Correspondence should be directed to Dr. Ketz-Riley.

etomidine (Domitor, Pfizer, Exton, Pennsylvania 19341, USA; 0.04 mg/kg, i.m.) combined with ketamine (KetaFlo, Abbott Laboratories, North Chicago, Illinois 60064, USA; 2.0–2.5 mg/kg, i.m.). Butorphanol (Torbugesic, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA; 0.15 mg/kg, i.m.) also was given when a lower dose of ketamine was used. Blood was collected from the jugular vein into tubes with ethylenediaminetetraacetic acid as anticoagulant (Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey 07417, USA) for complete blood count (CBC) and peripheral blood mononuclear cell (PBMC) isolation and in tubes without anticoagulant for serum collection to evaluate biochemistry profiles, IgGs via serum electrophoresis, and serology. The serum protein electrophoresis profiles from these Pallas' cats were compared with reference values obtained from healthy domestic cats seen at Boren Veterinary Medical Teaching Hospital, Stillwater, Oklahoma 78048, USA. The cats were given atipamezole (Antisedan, Pfizer; 0.2 mg/kg i.m) to reverse the sedative effects of medetomidine.

Measurements of IgG anti-*T. gondii* titers used a kinetics-based, enzyme-linked immunoassay (KELA, used by the Cornell Diagnostic Laboratory). All the cats had IgG titers for *T. gondii* 2 yr earlier, so IgM were not tested. Anti-feline immunodeficiency virus (FIV) antibodies were measured with the KELA and Western blot test. Feline leukemia virus (FeLV) p27 antigen and feline infectious peritonitis (FIP) corona virus antibody titers were quantified with the enzyme-linked immunosorbent assay (ELISA) and FHV antibody titers by serum virus neutralization. The whole feline viral panel was performed at the Cornell Diagnostic Laboratory. Active shedding of FHV was confirmed by virus isolation from conjunctival culture swabs. Virus isolation and serum IgG by serum protein electrophoresis were performed at the Oklahoma Animal Disease Diagnostic Laboratory (Stillwater, Oklahoma 78048, USA).

PBMCs were harvested using a Percoll gradient technique as described for domestic cats.<sup>27</sup> The isolated PBMCs were distributed to duplicate cultures. One set of cultures was used for lymphocyte proliferation assays using mitogen (Concanavalin A; 10 g/ml) stimulation.<sup>5</sup> The duplicate cultures were used for quantitation of constitutive and induced (Concanavalin A; 10 g/ml for 4 hr) cytokine gene expression using a reverse transcriptase–quantitative competitive polymerase chain reaction method optimized for domestic cats.<sup>23</sup> On the basis of a previous report that correlates immunodeficiency in FIV-infected domestic cats with altered interleukin-

10 (IL-10) and interleukin-12 (IL-12, p40 subunit) gene expression,<sup>16</sup> we limited the cytokine analysis from the Pallas' cats' PBMCs to include quantitation of IL-10 and IL-12 only. Additionally, the ribonucleic acid (RNA) collected for cytokine analysis was screened for FIV p26 gag by reverse transcriptase–polymerase chain reaction (RT-PCR).<sup>5</sup> Lastly, PBMCs were cocultured with FCD4-E cells<sup>5,11</sup> and supernatants assayed for Mg<sup>++</sup>-dependent RT activity.<sup>12</sup> Each culture was repeated in five wells. Negative controls contained  $2 \times 10^5$  FCD4-E cells only, and positive controls contained  $2 \times 10^5$  FCD4-E cells, with dilutions of a standard cell-free supernatant previously generated and characterized. A concentrated virus stock was used as a positive control for the RT assay. Results were expressed as the median RT activity from the five replicate wells. A sample was considered positive if the median RT activity exceeded the lower quartile of the positive control values.

Because none of the Oklahoma City Zoo Pallas' cats were healthy, their data were compared with data from normal domestic short-haired cats as well as those infected with FIV. For the lymphoproliferation assays, PBMCs were collected from domestic cats in other studies. These included five specific-pathogen-free domestic short-haired cats and five specific-pathogen-free cats that had been experimentally infected with FIV for other studies. The FIV-infected cats were infected with the NCSU1 strain of FIV.<sup>16</sup> At the time of this comparison, the FIV-infected cats had been infected for approximately 6 mo and were otherwise clinically healthy. For cytokine measurements, one cat each from the SPF control or FIV-infected group was used.

## RESULTS

The CBC and serum chemistry values were within normal ranges for Pallas' cats (*O. manul*).<sup>10</sup> The total protein for all Pallas' cats was within the reference range. Although alpha<sub>1</sub>, beta<sub>1</sub>, and beta<sub>2</sub> globulin fractions were unremarkable, four of the six Pallas' cats' alpha<sub>2</sub> globulin levels were elevated ( $12.2 \pm 2.0$  g/L; normal 7.1; range 4.7–92 g/L). All the six Pallas' cats were IgG positive but IgM negative for *T. gondii*, seropositive for FHV, and seronegative for FeLV, FIP, and FIV.

Quantification of cytokine gene expression (Table 1) indicates that, as expected, the Pallas' cats and the two domestic cats (FIV infected and control) had little to no IL-10 or IL-12 gene expression in their resting or constitutive PBMC samples.<sup>23</sup> However, the Pallas' cats responded to mitogen ConA, with increased IL-10 messenger RNA ex-

**Table 1.** Cytokine analysis of wild-caught Pallas' cats (*Otocolobus manul*) and their captive-born offspring infected with *Toxoplasma gondii* and feline herpesvirus. The data represent a ratio of the cytokine (interleukin [IL]-10, IL-12) with the housekeeping gene (glyceraldehydes-3-phosphate-dehydrogenase, G3PDH), which allows comparisons between cytokines and animals.<sup>a</sup>

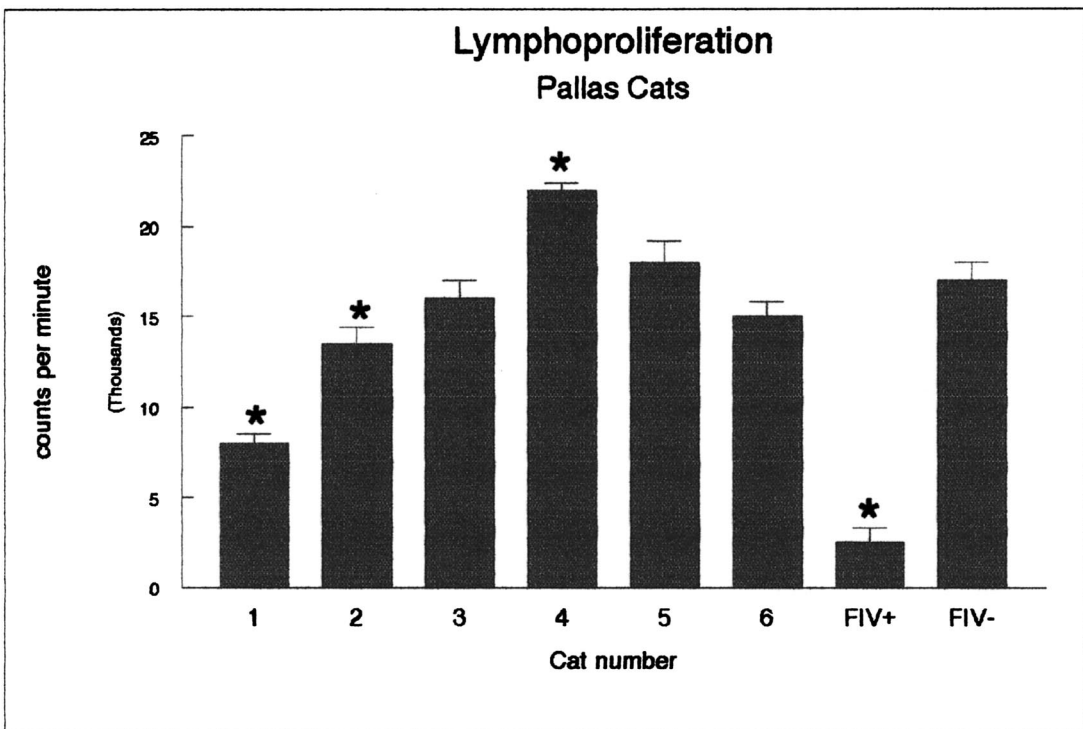
| Identity of individual(s)                   | Constitutive (resting) cytokine:G3PDH ratio |       | ConA-stimulated cytokine:G3PDH ratio |       |
|---|---|-------|--------------------------------------|-------|
|   | IL-10                                       | IL-12 | IL-10                                | IL-12 |
| Pallas' cat # 1                             | 0 <sup>a</sup>                              | 0     | ND                                   | ND    |
| Pallas' cat # 2                             | 0.025                                       | 0     | 0.646                                | 0     |
| Pallas' cat # 3                             | 0.014                                       | 0     | 0.301                                | 0     |
| Pallas' cat # 4                             | 0   | 0     | ND                                   | ND    |
| Pallas' cat # 5                             | 0.007                                       | 0     | 0.605                                | 0.003 |
| Pallas' cat # 6                             | 0   | 0     | ND                                   | ND    |
| Domestic cats FIV+ controls ( <i>n</i> = 5) | 0.050                                       | 0.1   | 0.202                                | 0     |
| Domestic cat FIV- controls ( <i>n</i> = 5)  | 0.003                                       | 0     | 0.197                                | 0.197 |

<sup>a</sup> Values of 0 as inserted are below the sensitivity of the assay used. ND, no data available.

pression, without a concomitant increase in IL-12 gene expression as previously reported, and as seen in the healthy domestic cat.<sup>23</sup>

Figure 1 compares the results of the lymphocyte

proliferation assay of the six Pallas' cats with assays of healthy FIV-negative and FIV-positive domestic cats. Compared with the control domestic cat, the adult Pallas' cats 1 and 2 had significantly



**Figure 1.** Lymphoproliferation assays from peripheral blood mononuclear cells (PBMCs) of wild-caught Pallas' cats (*Otocolobus manul*) and their captive-born offspring infected with *Toxoplasma gondii* and feline herpesvirus. Mean counts per minute with standard error of ConA (10  $\mu$ g/ml)-induced cultures from Pallas' cat PBMC samples and the FIV-infected (FIV+) domestic cat (five cats) PBMC samples were compared with the domestic cat controls (five cats) by the Kolmogorov-Smirnov comparison of two data sets. Counts per minute from nonstimulated (constitutive) cultures were  $\leq 230$ . \*Assays are significantly different at  $P < 0.05$ .

## Mg-Dependent Reverse Transcriptase Activity

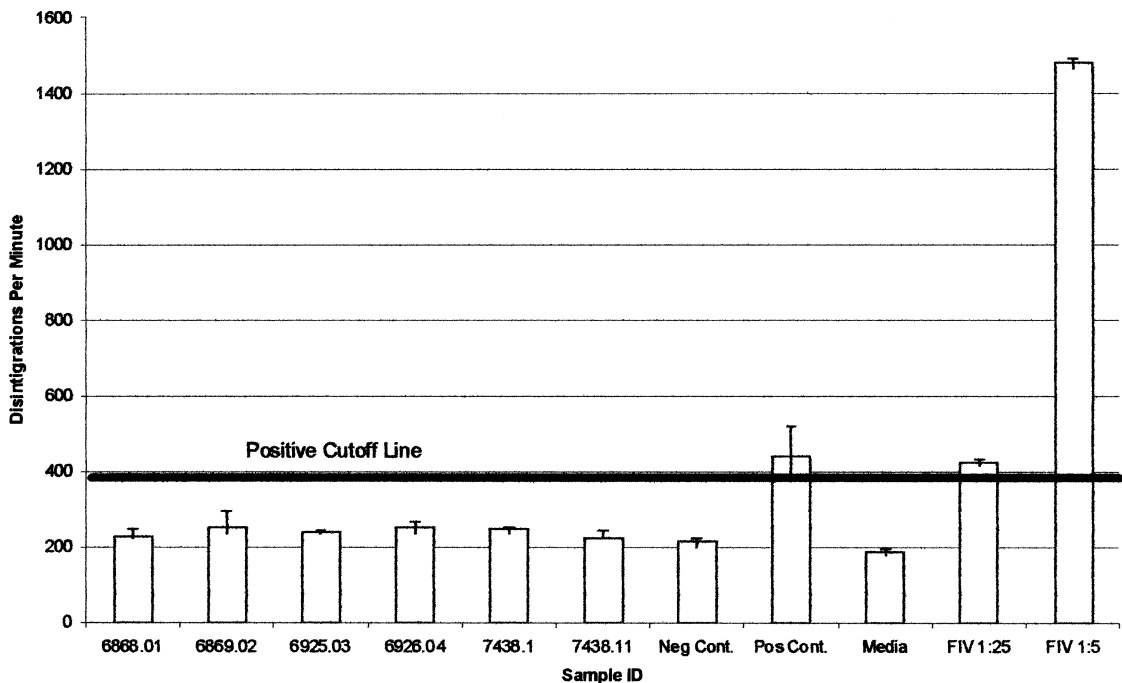


Figure 2. Mg-dependent reverse transcriptase activity of wild-caught Pallas' cats (*Otocolobus manul*) and their offspring infected with *Toxoplasma gondii* and feline herpesvirus.

lower proliferation response to ConA ( $P < 0.05$ ; Kolmogorov–Smirnov comparison of two data sets). Pallas' cat 4 had a significantly elevated ( $P < 0.05$ ) proliferation response compared with the healthy domestic short-haired control cat. But compared with Pallas' cat 4, all other Pallas' cats, with the exception of cat 5, had significantly reduced lymphoproliferation responses.

The coculture supernatants from Pallas' cat PBMC did not show any RT activity, and the cats were negative for FIV p26 gag by RT-PCR (Fig. 2). Positive and negative control supernatants and RNA samples behaved as expected.

## DISCUSSION

In domestic cats, the presence of IgG antibodies for *T. gondii* without measurable IgM antibodies indicates that *T. gondii* exposure occurred approximately 20 wk earlier.<sup>14,15</sup> On the basis of this information and the fact that only IgG antibody levels were measured during quarantine examination of the Pallas' cats, we know that only the positive individuals had been exposed to the parasite sometime before their arrival at the Oklahoma City Zoo. Two of the animals became IgG positive during ex-

amination, before release from quarantine. Without IgM measurements, we can only assume that either the positive Pallas' cats were infected shortly before arrival at the zoo and infected the two initially negative cats or that the two negative cats were infected at the zoo via wild rodents through outdoor contact.

With the exception of the alpha<sub>2</sub> globulin elevations, serum protein analysis was unremarkable. Although alpha<sub>2</sub> globulins include several of the acute phase inflammatory proteins (such as haptoglobin, serum amyloid A, and ceruloplasmin) that are synthesized by the liver in response to inflammation, elevated levels are not cause specific. The elevations corroborate the clinical findings of inflammatory or infectious disease in these cats.

Immunodeficiency states may be inherited or acquired. Heritable anomalies of the immune system, undefined heritable resistance factors, and deficient maternal immunity are possible genetic causes. Concurrent illness, malnutrition, environmental factors such as high population density, poor sanitation, and the interchange of animals between populations, as well as FeLV and FIV infection, also can cause immune deficiency in cats.<sup>1,18–22,28</sup> Envi-

ronmental factors and malnutrition most likely did not affect the Oklahoma City Zoo Pallas' cats.

Because both viral and *Toxoplasma* infections were present, tests of cell-mediated immune function were selected. Although lymphoproliferation assays are not the best indicators of effector T-cell function, they are standard and classical and are routinely used to evaluate overall T-cell reactivity in many systems.

Pallas' cats 1 and 2 had significantly reduced mitogen-induced lymphocyte proliferation compared with the domestic control cats as do domestic cats infected with either FeLV or FIV.<sup>4–6,14,20,22,24,26</sup> Cat 1 had the most severe and chronic clinical signs of FHV infection, whereas cat 4 appeared to be the healthiest adult. If cat 4 represents the expected lymphoproliferation of normal immunocompetent Pallas' cats, then all but Pallas' cat 5 had significantly decreased responses ( $P < 0.05$ ; Fig. 1). Alternatively, the highest resting (nonstimulated) lymphocyte proliferation rate in cat 4, 200–250 cpm compared with levels that were  $<150$  cpm for the others, may have been due to a current illness, with mild upper respiratory infection in that cat. The clinical signs of rhinitis and conjunctivitis in this cat were attributed to persistent FHV infection. However, Pallas' cat 4 never showed any other clinical signs.

Toxoplasmosis and FHV infections are normally controlled by cellular immune responses. In accordance with the Th1 (cell-mediated immunity) and Th2 (humoral immunity) paradigm in immune responses, a successful cellular immune response depends on timely IL-12 expression.<sup>9</sup> In contrast, IL-10 antagonized IL-12, reducing IL-12 synthesis and inhibiting such IL-12-mediated functions as the establishment or maintenance of a cell-mediated immune response.<sup>7,29,30</sup> These IL-12 and IL-10 functions have been reported in humans (*Homo sapiens*) and nearly all laboratory and domesticated animals, and Pallas' cats are probably not an exception. In this study, IL-12 and IL-10 functions from Pallas' cat PBMC mimic those seen in the accompanying FIV-infected domestic cat (Table 1). When stimulated with Con A, the Pallas' cats and the FIV-infected cat responded with inducible IL-10 expression, with no detectable IL-12 expression. In contrast, the healthy control cat responded, as expected, with elevations of IL-12 and IL-10 gene expression. Although these data are intriguing, they are limited by the small number of animals tested, and definitive conclusions cannot be drawn as to whether the Pallas' cats in this study have "FIV-like" cytokine gene expression patterns. Furthermore, there is no analogous *in vitro* data on mito-

gen-induced IL-10 and IL-12 gene expression from PBMC- of FIV-infected cats. However, this does not preclude the fact that *in vitro* IL-12 gene expression could not be induced by mitogen activation of the Pallas' cat PBMC. This is in contrast to readily inducible IL-12 expression exhibited by the control cat in this study and that reported in other healthy cats.<sup>23</sup> *In vivo* studies with FIV-infected cats indicate that susceptibility to opportunistic infections is related to disparity between IL-10 and IL-12 gene expression.<sup>3,9</sup> Although FIV infection was not confirmed in these Pallas' cats (see Discussion below), these preliminary studies suggest that the apparent clinical susceptibility to infections exhibited by these Pallas' cats could be related to alterations in cytokine expression, particularly a disparity between the expression of IL-10 and IL-12.

The responses of Pallas' cat PBMC to either lymphocyte proliferation or cytokine analysis were expected to be similar to those of the domestic short-haired cat. Unfortunately, PBMC from clinically and routinely healthy Pallas' cats were not available.

Toxoplasmosis, FHV, and other infectious agents often coinfect FIV-positive domestic cats,<sup>1,8,12,13,15,16,17</sup> and FIV can infect nondomestic felids,<sup>1,19,20</sup> so the possible role of FIV in these Pallas' cats' immunodeficiency was studied. Infection with FIV was not detected by serology, RT-PCR for FIV p26 gag, and RT activity from supernatants collected from Pallas' cat PBMC along with feline FCD4-E cells. Some of the FIV-infected domestic cats never produce detectable blood antibody levels,<sup>1,8</sup> and RT activity or virus cannot always be demonstrated in cocultures because of efficient, anti-FIV CD8+ effector function from the FIV-infected cats.<sup>2</sup> Therefore, although it is unlikely, these Pallas' cats may have had occult FIV infection.

## CONCLUSIONS

These Pallas' cats appeared to be immunodeficient, probably with a defect in the generation or maintenance of a cell-mediated immune response. Although infection with FIV could not be demonstrated or definitively ruled out, these Pallas' cats had a FIV-like clinical and immune function profile.

A control group of immunologically healthy Pallas' cats could be studied using many of the techniques that are used to characterize the immune system in the domestic cat to establish reference values for further immunologic studies.

All the Pallas' cats in the United States may have been caught in the same region in Russia, so they may all be closely related. The genetic background

and lineage of immunodeficient and normal Pallas' cats in North American and European zoos needs to be carefully examined. If Pallas' cats do not have an inherited immunodeficiency, then they are probably very stress sensitive and prone to immunosuppression associated with capture, transportation, translocation, captivity, breeding, parturition, and lactation.

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