Aus dem Friedrich-Loeffler-Institut
eingereicht über den Fachbereich Veterinärmedizin
der Freien Universität Berlin

Validation of blood sampling with *Dipetalogaster maxima* for serological and blood chemical analyses and application of this method in an epidemiologic study on *Toxoplasma gondii* in small exotic felids in European zoos

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Meiner Familie

"Nature is the only book that teems with meaning on every page."

Inscription on the Kankai temple
(Quammen, 2004)

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3 LIST OF ABRREVIATIONS

°C	Degree Celsius
AIC	Akaike Information Criterion
Alb	Albumin
ALT	Alanine aminotransferase
AMY	Amylase
AP	Alkaline phosphatase
B0	Blood out of bug at point 0
B1	Blood out of bug at point 1 (after 1 hr)
B2	Blood out of bug at point 2 (after 2 hr)
B4	Blood out of bug at point 4 (after 4 hr)
BUN	Blood urea nitrogen
Ca	Calcium
CFT	Complement Fixation Test
Chol	Cholesterol
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CK	Creatine kinase
Cl	Chloride
cm	Centimeter
CO_2	Carbon dioxide
CORR	Correlation
Crea	Creatinine
DAT	Direct agglutination test
DNA	Deoxyribonucleic acid
DT	Sabin-Feldman dye test
e.g.	For example (Exempli gratia)
EAZA	European Association of Zoos and Aquaria
EEP	European Endangered species Programme
EGTA	ethylene glycol tetraacetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
EN	Endangered
ESB	European Studbook
EU	European Union
Exam.	Examined
f	Feminine
FeLV	Feline leukemia virus
FHV1	Feline Herpes virus 1
FIP	Feline infectious peritonitis
FITC	Fluorescein Isothiocyanate
FIV	Feline immunodeficiency virus
GGT	Gamma-glutamyltransferase
GLDH	Glutamate dehydrogenase
Glob	Globulin
Glu	Glucose
Hb	Hemoglobin
HCO_3	Bicarbonate
Hct	Hematocrit
hr	Hour
IB	Immunoblot
ICZN	International code of zoological nomenclature
i.e.	That is (id est)
IFAT	Indirect fluorescent antibody test
IgG	Immunoglobin G

IgM	Immunoglobin M
IHA	Indirect hemagglutination
ISIS	International Species Information System
IUCN	International Union for Conservation of Nature
K	Potassium
kg	Kilogram
L1-5	Larvae 1-5
LAT	Latex agglutination test
LC	Least concern
log_{10}	Logarithm with base 10
m	Masculine
m^2	Square meter
MAT	Modified Agglutination Test
Mg	Magnesium
min	Minutes
mo	Month
Mon	Monitored Programme
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
Neg.	Negative
No. Exam.	Number examined
NT	Near Threatened
NWM	New World monkeys
P	*P*-value
PALP	Pyridoxlphosphat
PBS	Phosphate-buffered saline
pCO_2	Partial pressure of carbon dioxide
PHOS	Phosphorus
Pos.	Positive
Pr(>|z|)	p-values computed from the z-values
PVDF	Polyvinylidene Difluoride
RBC	Red blood cell
Ref.	Reference
RHDV	Rabbit hemorrhagic disease virus
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sig.	Significance
σ	Standard deviation
Std. Error	Standard error
T	T-test
T. gondii	*Toxoplasma gondii*
TAG	Taxon advisory group
TB	Tuberculosis
TBil	Total bilirubin
TCa	Total calcium
TCO_2	Total carbon dioxide
TgSAG1	*T. gondii* tachyzoite Surface Antigen 1
TMg	Total magnesium
Toxo	Toxoplasma
TP	Total protein
Tri	Triglyceride
UAE	United Arabic Emirates
USA	United States of America
UV	Ultraviolet
V	Conventional venous blood sample

vs.	versus
VU	Vulnerable
W	Wilcoxon signed rank test
WBC	White blood cell
WHO	World Health Organisation
wk	Week
$\bar{x}$	Mean
yr	Year
z value	Standard score (regression coefficient divided by its standard error)
ZIMS	Zoological information management system

4 INTRODUCTION

4.1 Objectives of the study

Plasma samples can be used for assessing the health status of an animal. Blood sampling, however, is a challenge, especially in exotic species. To obtain the blood sample needed for analysis, handling of the animal is necessary. In some species, restraint without anesthesia is extremely stressful and difficult, if not impossible, without major risks to the animal or the person handling it. Moreover, many cat species in zoos are considered endangered and chemical immobilization always includes a risk to the patient. This risk is even higher in animals that are not totally healthy (Bille et al., 2012). When anesthetizing an exotic animal in a zoological collection, reliable data for the health status like blood analysis are usually not available beforehand. The actual risk to the animal due to anesthesia can hardly be determined. Therefore, anesthesia should be avoided whenever possible.

In felids, several blood parameters (assessed in plasma) can be highly valuable to determine the health status of an individual. Clinical blood chemistry data are of major interest for its characterization. Furthermore, serologic tests as indicators for infection, e.g. with *Toxoplasma gondii*, may provide valuable information.

Toxoplasma gondii is a parasite, whose definitive hosts are felids (Elmore et al., 2010). Serological studies showed that *T. gondii* infection is widely spread in wild animals (Bakal et al., 1980; Dubey, 2002; Dubey et al., 1999; Quinn et al., 1976; G. P. Riemann et al., 1975). In addition, *T. gondii* infections are often reported in captive mammals and birds in zoos (Alerte, 2008; Alvarado-Esquivel et al., 2013; de Camps et al., 2008; Minervino et al., 2010; Ratcliffe & Worth, 1951; Riemann, Behymer, et al., 1974; Sedlak & Bartova, 2006; Silva et al., 2001; Tidy et al., 2017).

Some species of small exotic felids seem to be more susceptible to infection with *T. gondii* than others. Captive Pallas' cats (*Otocolobus manul*) and to a certain extent Sand cats (*Felis margarita*) showed a high mortality ratio, for which *T. gondii* was suspected to be the cause (Brown et al., 2005; Dubey et al., 2010; Kenny et al., 2002; Swanson, 1999).

However, a thorough survey of the serological *T. gondii* status of the captive small cat population managed in zoos of the European Association of Zoos and Aquaria (EAZA) has not been done. This makes it difficult to define the impact *T. gondii* has on wild felids in captivity. Due to this fact and also for pre-shipment tests in zoos, testing exotic felids for *T. gondii* antibodies can be necessary.

At this point, a minimally invasive technique using reduviid bugs, which has been described by various authors, can be a valuable alternative to conventional blood sampling (Arnold et al., 2008; Becker et al., 2005; Markvardsen et al., 2012; Thomsen & Voigt, 2006; Voigt et al., 2004; Voigt et al., 2006; Vos et al., 2010).

One objective of this study was to investigate, whether reduviid bugs can be used to collect blood for *T. gondii* serology and for clinical blood chemistry to determine the patients' health status. A second objective was to use reduviid bug sampling in an epidemiological study to estimate the prevalence of *T. gondii* in small exotic felids managed in European Endangered Species Programs (EEPs), European Stud Books (ESBs) or in monitoring programs in EAZA zoos. Furthermore, a risk factor analysis for *T. gondii*-seropositivity was performed.

5 LITERATURE REVIEW

5.1 *Toxoplasma gondii*

5.1.1 General information

"Toxoplasma gondii is one of the most well studied parasites because of its medical and veterinary importance." (Dubey, 2010b). It is an obligate intracellular, coccidian parasite belonging to the phylum Apicomplexa, class of Coccidea, order Eimeriida, family Sarcocystidae, genus Toxoplasma (Eckert et al., 2008). It is the only species in the genus Toxoplasma. Only felids are able to serve as its final host, while numerous mammalian and bird species represent intermediate hosts of this parasite (Beck & Pantchev, 2006).

In the life cycle of *T. gondii* (Figure 5.1), cats get infected by ingesting one of the three infectious stages of *T. gondii*: tachyzoites, bradyzoites out of tissue cysts or sporozoites, which originate from sporulated oocysts (Beck & Pantchev, 2006). Among domestic cats, almost all individuals infected with bradyzoites shed oocysts, while less than 50% of the cats infected with tachyzoites or oocysts started oocyst shedding (Dubey & Frenkel, 1972, 1976). In experimental trials, most of the domestic cats seroconverted two to three weeks after ingesting oocysts (Dubey & Thulliez, 1989).

Tissue cysts disintegrate in the small intestine of the final host and bradyzoites invade epithelial cells where they proliferate asexually. Two days after ingestion of tissue cysts, the sexual cycle starts with the development of oocysts, which are excreted unsporulated after a prepatency period of three to ten days. It takes one to five days for the oocysts to sporulate and to become infective in the environment (Eckert et al., 2008) (Figure 5.1).

Infection usually leads to intermittent excretion of oocysts for 7-21 days. During their lifetime, cats may develop a strong immune response that may prevent excretion of oocysts if the animal is re-infected. An experiment showed that four of nine domestic cats had lost this level of protective immunity after 77 months (Davis & Dubey, 1995; Dubey, 1995). Re-shedding due to reactivation of a latent *T. gondii*-infection may for example be induced by infection with *Cystoisospora felis* (Dubey, 1976). It has also been shown that cats can excrete tachyzoites with their milk for as long as three weeks after giving birth. This might be a major risk factor of infection for kittens, which might be more susceptible than adult cats (Powell et al., 2001).

In intermediate hosts, ingested tissue cysts or oocysts lead to bradyzoites or sporozoites invading the intestinal wall and infecting cells in the lamina propria, where tachyzoites develop. This rapidly and asexually multiplying stage spreads then into various tissues where it multiplies asexually in the infected cells. In felids, oocysts may develop in parallel to tachyzoites (Figure 5.1).

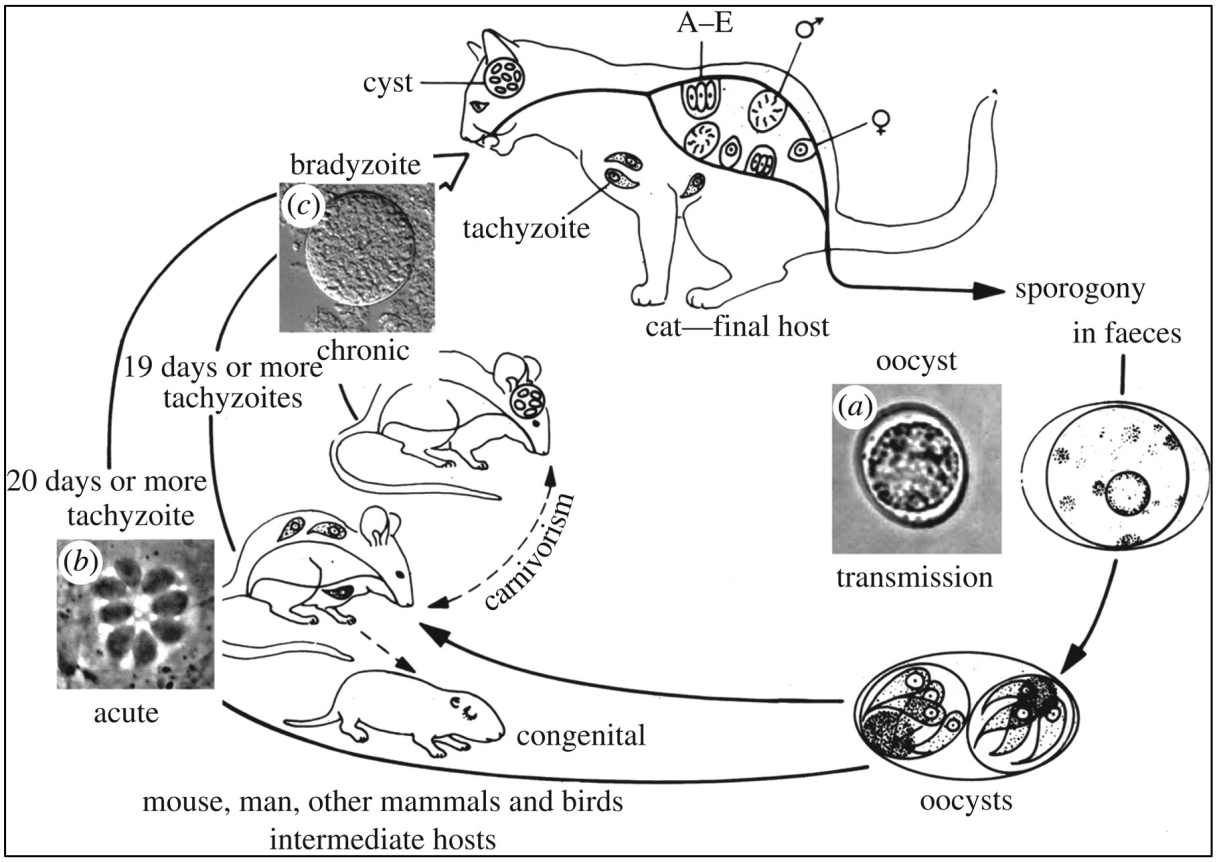

Figure 5.1: Life cycle of *Toxoplasma gondii* (Sibley et al., 2009)
a: oocyst; b: tachyzoite; c: bradyzoite

Tachyzoites spread via lymph and blood into lymph nodes and various organs like liver, lung or muscles. In cells of these organs, tachyzoites continue to multiply and infect more cells (Eckert et al., 2008). If this process occurs during pregnancy, transplacental infection can occur, causing disease in the fetus or abortion. Especially sheep and goats seem to be susceptible (Dubey & Jones, 2008), while transplacental infection does not seem to play a major role in cats (Dubey & Hoover, 1977). When infection progresses, some tachyzoites develop further into the bradyzoite stage. This is a process occurring spontaneously or induced by the immune system of the host. It is regarded to be stimulated by interferon γ. Intracellular bradyzoites divide slowly and establish tissue cysts, which may contain thousands of bradyzoites. They are often found in muscles, including the heart and the diaphragm, and in the brain. Encysted bradyzoites represent a permanent stage of the parasite and allows *T. gondii* a lifelong persistence in intermediate hosts until it is re-activated (for example after ingestion by a cat or after immunosuppression of the host) (Eckert et al., 2008) (Figure 5.1).

Cats infected with Feline Immunodeficiency Virus (FIV) were more likely to get infected with *T. gondii* and to exhibit a generalized toxoplasmosis (Davidson et al., 1993). They showed higher IgM antibody levels, an increased seroprevalence and a higher replication rate of *T. gondii* than FIV-negative cats (Hartmann, 1998; Witt et al., 1989). The susceptibility of cats for *T. gondii* increased even more, when FIV-infected cats were also infected with Feline Leucosis virus (FeLV) (Akhtardanesh et al., 2010; Davidson et al., 1993).

For domestic cats, the worldwide prevalence for *T. gondii* was estimated at 30-40%, but there is a distinct regional variation (Elmore et al., 2010). The prevalence seemed to be higher in areas with a warm climate and low altitude than in regions with a cold climate and high

altitude. Furthermore, the prevalence was higher in areas with a climate of high humidity than in arid regions (Dubey, 2010b).

Infections in domestic cats are typically asymptomatic, but congenitally infected kittens may develop clinical signs. Common symptoms include: fever, anorexia, ocular inflammation, dyspnea, lethargy, abdominal pain or discomfort and neurologic disorders (Elmore et al., 2010).

The following measures have been recommended to prevent infections of animals with *T. gondii*.

For herbivorous animals contaminated hay, straw or grain may be a source for *T. gondii* exposure. Therefore, efforts should be made to keep barns, where hay is stored, cat-proof. It is also important to prevent that stray cats enter enclosures, especially in areas where highly susceptible animals are kept (Garell, 1999).

In domestic cats, the exposure risk can be reduced by keeping cats indoors to avoid ingestion of possibly infected rodents or birds and to implement regular pest control to minimize the risk of contact to various potentially infected intermediate host species (Dubey, 2010b). Meat should not be fed raw, but cooked until it has reached an internal temperature of 61°C for at least 3.6 min (Dubey, Kotula, et al., 1990) or stored frozen at -12°C for at least seven days to destroy tissue cysts (Dubey, Kotula, et al., 1990; Kotula et al., 1991; Kuticic & Wikerhauser, 1996). Also, litter boxes should be cleaned daily as oocysts need at least 24 hours to sporulate and become infective (Elmore et al., 2010). Dogs should be kept away from litter boxes to avoid ingestion and passage of oocysts (Frenkel et al., 1995; Schares et al., 2005).

These precautions hold also true to prevent toxoplasmosis in humans, where it is in addition important to follow some hygienic rules like regular hand washing, avoiding the use of knifes or cutting boards, which have been used to cut raw meat, for preparing raw fruit or vegetables (Dubey, 2010b).

Immune-competent people who get infected after birth rarely develop any symptoms. They may, however, sometimes show fever, malaise or lymphadenopathy. An infection with *T. gondii* during pregnancy can cause severe neurologic or ocular disease in the fetus or abortion (Dunn et al., 1999; Elmore et al., 2010). The risk of prenatal infection and the severity of symptoms depends on the stage of gestation, at which the infection has occurred. It has been postulated that infection is less frequent in early pregnancy. However, fetuses, which become infected at this early stage, are usually more severely diseased than those who acquire the infection in late pregnancy (Dubey, 2010b).

5.1.2 Serological diagnosis

In the course of a *T. gondii* infection, IgM antibodies appear early (after 7 days), while the development of IgG antibodies takes longer (one to three weeks after IgM) (Jost et al., 2011). After recovery, IgM antibodies disappear faster than IgG (Hill & Dubey, 2002).

The best way to preserve antibodies for serology, is the storage of serum or plasma at low temperatures (-20°C or lower). However, antibodies were shown to be still reactive after 6 months when preserved on dried filter papers with silica gel at 25°C (Nogami et al., 1992).

Many serologic tests have been used to detect IgG or IgM antibodies against *T. gondii*, e.g. the complement fixation test (CFT), enzyme-linked immunosorbent assays (ELISA), indirect fluorescent antibody tests (IFAT), indirect hemagglutination (IHA), latex agglutination (LA), the modified agglutination test (MAT) and the Sabin-Feldman dye test (DT) (Dubey, 2010b).

5.1.2.1. Complement Fixation Test (CFT)

The CFT reveals reproducible results, but it is not routinely used to detect *T. gondii* antibodies. The procedures are complex, time-consuming, expensive and lack standardization

of the antigen and the reagents. Due to these facts the CFT is not the method of choice to determine antibodies against *T. gondii* (Ondriska et al., 2003).

5.1.2.2 Enzyme-linked immunosorbent assay (ELISA)

The ELISA works, depending on the type of assay, with soluble antigen or antibodies bound to a microtiter plate or slide as the solid phase. To detect specific antibodies, an enzyme-linked antibody or antigen system is employed. To detect antibodies that bind for example to tachyzoite antigens coupled to the plate or slide, enzyme-conjugated anti-species immunoglobins are used. A substrate for the enzyme is added and its conversion measured by spectral photometry. An advantage of the ELISA is that it can be automated, which makes rapid testing of large numbers of sera possible (Dubey, 2010b). ELISA for antibodies to *T. gondii* have been standardized in some laboratories (Meireles et al., 2004). The sensitivity and specificity of the test highly depends on the antigen used (Gamble et al., 2005; Maksimov, 2013; Tenter et al., 2000).

An antibody response, as determined by ELISA, to TgSAG1, one of the tachyzoite surface antigens, was shown to be a sensitive and specific indicator for *T. gondii* infection in different animal species such as mice, dogs, cats, sheep, goats and water fowl (ducks and geese) (Bessières et al., 1992; Kimbita et al., 2001; Maksimov et al., 2011; Sager et al., 2003; Santos de Azevedo et al., 2010; Velmurugan et al., 2008).

5.1.2.3 Indirect fluorescent antibody test (IFAT)

In the IFAT, whole tachyzoites fixed to glass slides are incubated with serum (Camargo, 1963). To detect antibodies bound to tachyzoite antigens, fluorescent-labeled anti-species immunoglobins are used. These secondary antibodies can bind to the primary antibodies present in the serum. They are visualized under a fluorescence microscope (Fritschy & Härtig, 2001). IFATs have been standardized in some laboratories. Disadvantages of the technique include the need for a fluorescence microscope and species-specific conjugates. Further drawbacks are cross- reactions of antibodies with rheumatoid factors and antinuclear antibodies (Carmichael, 1975).

5.1.2.4 Indirect hemagglutination (IHA)

In this test, red blood cells coated with soluble antigen from tachyzoites are agglutinated by sera containing antibodies directed against the tachyzoite antigens. Commercial IHA kits are available. The IHA is a simple and rapid method, but some technical variabilities make the test somewhat impractical. Other tests, like the Sabin-Feldman dye test (DT), are capable of detecting antibodies at an earlier stage of infection than the IHA. Consequently, there is the risk of missing acute infections with the IHA. It has also been reported that the IHA is frequently negative in congenital infections (Palmer et al., 1976).

5.1.2.5 Latex agglutination tests (LATs)

Latex particles coated with soluble *T. gondii* antigen are agglutinated by immune serum. Commercial kits (ToxoTest, Eiken Chemical Co., Tokyo, Japan) are available and LATs are easy to perform. For human sera, one of the LATs seemed to be a good substitute for the DT, in contrast to an IHA test (Balfour et al., 1982), but in livestock the sensitivity of the LAT tests still needed improvement (Dubey, 2010b). For feline serum, a LAT did not produce reliable results (Lappin & Powell, 1991).

5.1.2.6 Modified agglutination test (MAT)

Fulton and Turk (1960) developed a simple direct agglutination test, which does not require special equipment or conjugates. This test was modified and called the Modified Agglutination Test (MAT) by Desmonts and Remington (1980) and Dubey and Desmonts (1987). This test has been used with human and animal sera. Titers for both were equivalent to those measured by DT. The test detects only IgG antibodies, as the conventional MAT incorporates 2-mercaptoethanol to remove non-specific IgM antibodies (Dubey, 2010b). This entails the risk of false-negative results during the early stage of acute infections. A MAT is commercially available (Toxo-Screen DA, bioMerieux, Charbonnieres Beins, France). Modifications of the MAT are able to detect specific IgM antibodies (Dubey, 2010b).

5.1.2.7 Sabin-Feldman dye test (DT)

The Sabin-Feldman dye test uses a complement-mediated antigen-antibody reaction to detect antibodies against *T. gondii*. Live tachyzoites are incubated with complement and human serum. A dye (methylene blue) is then added to stain tachyzoites in the absence of *T. gondii* antibodies. In presence of *T. gondii* antibodies the parasite is not stained. The titer is determined by the dilution of serum, at which 50% of the tachyzoites are left unstained (Beverley & Beattie, 1952; Frenkel & Jacobs, 1958).

Although the DT is expensive, potentially unsafe (live, potentially infectious organisms are used) and requires a high degree of expertise, it is still regarded as the "gold standard" test for the serologic diagnosis of toxoplasmosis in humans as it has been shown to be highly specific and sensitive. To ensure comparable results, the WHO and the Statens Seruminstitut, Copenhagen, Denmark established an international standard anti-Toxoplasma serum (Dubey, 2010b).

The DT works well in many hosts, but fails to work with sera of some bird species (Frenkel, 1981).

5.1.2.8 Western Blotting (Immunoblotting)

Western blotting is a technique to transfer proteins to nylon or Polyvinylidene Difluoride (PVDF) membranes. The proteins are first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight. Antigens (e.g. from *T. gondii*), which have been separated by SDS-PAGE and transferred to a membrane are probed with sera or plasma, so that specific antibodies can bind the separated antigens. Bound antibodies are detected with a conjugate and an appropriate substrate reaction that stains the membrane at those positions, where antibodies have bound. This results in a typical pattern. The level, by which a band is stained, correlates to the amount of protein detected or to the level of specific antibodies, but it does not allow determination of an antibody titer (Mahmood & Yang, 2012). Immunoblot results can be used as an aid to confirm results in conventional serological tests (Dubey, 2010b) and has been also used to distinguish IgG antibodies from mothers and their infants (Gross et al., 2000).

5.1.3 *T. gondii* in exotic species

Serological analyses suggests, that *T. gondii* is widely spread in wild animals (Bakal et al., 1980; Dubey, 2002; Dubey et al., 1999; Elmore et al., 2010; Quinn et al., 1976; G. P. Riemann et al., 1975). Antibody reactions are also often seen in mammals and birds in zoos (Alerte, 2008; Alvarado-Esquivel et al., 2013; Bartova & Sedlak, 2012; de Camps et al., 2008; Minervino et al., 2010; Ratcliffe & Worth, 1951; Riemann, Behymer, et al., 1974; Sedlak & Bartova, 2006; Silva et al., 2001; Tidy et al., 2017).

Although evidence for *T. gondii* infections was found by serology or direct detection of parasite DNA in tissues of a large variety of species, there are only few reports of clinical disease in these animals (Table 5.1).

Buffaloes and cattle are considered resistant to disease that may be caused by *T. gondii*. However, specific antibody reactions have occasionally been shown in these species indicating that they are susceptible, but they apparently do not develop clinical disease (Dubey, 2010b). Also in camelids, infections seem to be rather rare, although there is a report of clinically apparent toxoplasmosis in a camel (Hagemoser et al., 1990) and even a toxoplasmosis-associated abortion in an Alpaca has been observed (Dubey et al., 2014).

In many birds, *T. gondii*-specific antibodies have been demonstrated in serological studies. However, the available data should be interpreted with care, as not all serological tests work reliably with avian sera (Frenkel, 1981). Cases of severe toxoplasmosis were reported in canaries (*Serinus canaries*) in several studies (Gibbens et al., 1997; Lindsay et al., 1995; Vickers et al., 1992; Williams et al., 2001).

While Old World monkeys are resistant to toxoplasmosis, New World monkeys are highly susceptible and may show severe symptoms (Dubey, 2010b). In particular squirrel monkeys (*Saimiri sciureus*) can develop clinical disease or die suddenly without symptoms (Cunningham et al., 1992; Inoue, 1997; Salant et al., 2009).

Marsupials are highly susceptible to toxoplasmosis, but their susceptibility may vary between species. There are reports of fatal disease in zoos and in the wild (Canfield et al., 1990). Sudden death without previous clinical disease is not uncommon, which makes ante-mortem diagnosis difficult as the patients may die even before developing detectable antibodies (Dubey, 2010b). The geographical isolation of Australia and the relatively recent introduction of felids to the continent are discussed as possible reasons for this phenomenon, which can be seen as a lack of adaptation. Australian marsupials may thus have evolved in the absence of *T. gondii* oocysts (Innes, 1997). Similarly, prosimians were also mentioned as highly susceptible, which may be due to a reduced evolutionary exposure to felids (Garell, 1999).

Table 5.1 presents a referenced list of reports of clinical disease in various species except for felids. Further information on toxoplasmosis in exotic felids is provided in chapter 5.1.4.

Table 5.1: Clinical toxoplasmosis in exotic species except felids

Species	Reference
Artiodactyla	(Baszler et al., 2000; Bulmer, 1971; Calero-Bernal et al., 2013; Crawford et al., 2000; Dubey et al., 2014; Dubey, Lewis, et al., 2002; Dubey, Tocidlowski, et al., 2002; Hagemoser et al., 1990; Junge et al., 1992; Oksanen et al., 1996; Oliveira et al., 2000; Sedlak et al., 2004; Stover et al., 1990)
Birds (including nine different orders)	(Casagrande et al., 2015; Cooper et al., 2015; Dubey et al., 2001; Gerhold & Yabsley, 2007; Gibbens et al., 1997; Hartley et al., 2008; Howerth et al., 1991; Howerth & Rodenroth, 1985; Jokelainen & Vikøren, 2014; Junior et al., 2012; Lindsay et al., 1995; Mason et al., 1991; Mikaelian et al., 1997; Ploeg et al., 2011; Quist et al., 1995; Ratcliffe & Worth, 1951; Szabo et al., 2004; Tackaerthenry & Kageruka, 1977; Vickers et al., 1992; Williams et al., 2001; Work et al., 2002)
Marine mammals including sea otters	(Bossart et al., 2012; Bowater et al., 2003; Di Guardo et al., 2010; Dubey et al., 2004; Dubey et al., 2009; Dubey et al., 2003; Gonzales-Viera et al., 2013; Holshuh et al., 1985; Honnold et al., 2005; Inskeep et al., 1990; Jardine & Dubey, 2002; Migaki et al., 1977; Migaki et al., 1990; Mikaelian et al., 2000; Miller et al., 2008; Resendes et al., 2002; Roe et al., 2017; Thomas et al., 2007)
Marsupials	(Basso et al., 2007; Bermúdez et al., 2009; Boorman et al., 1977; Donahoe et al., 2015; Dubey & Crutchley, 2008; Dubey et al., 1991; Dubey, Ott-Joslin, et al., 1988; Fernandez-Aguilar et al., 2013; Hartley, 2006; Hartley et al., 1990; McColl, 1983; McOrist & Smales, 1986; Miller et al., 1992; Obendorf & Munday, 1990; Patton et al., 1986)
Miscellaneous	Three-toed sloth (Tury et al., 2001), Flying-foxes (Sangster et al., 2012)
Non-human primates	(Antoniassi et al., 2011; Borst & van Knapen, 1984; Brack et al., 1998; Carme et al., 2009; Cunningham et al., 1992; Dietz et al., 1997; Epiphanio et al., 2000; Epiphanio et al., 2001; Epiphanio et al., 2003; Gyimesi et al., 2006; Inoue, 1997; Juan-Salles et al., 2011; Juan-Salles et al., 1998; Pertz et al., 1997; Salant et al., 2009; Spencer et al., 2004; Wohlsein et al., 1999)
Rodents	(Bangari et al., 2007; Carrasco et al., 2006; Christiansen & Siim, 1951; Dubey, Brown, et al., 1992; Dubey et al., 2006; Duff et al., 2001; Fayyad et al., 2016; Forzan & Frasca, 2004; Green & Morgan, 1991; Gustafsson et al., 1988; Jokelainen & Nylund, 2012; Kik et al., 2015; Leland et al., 1992; Medway et al., 1989; Morales et al., 1996; Rodhain & Hendrix, 1948)
Wild carnivores, felids excluded	(Basso et al., 2009; Burger et al., 2017; Burns et al., 2003; Dubey, Hamir, et al., 1992; Dubey, Hamir, et al., 1990; Dubey & Lin, 1994; Frank, 2001; Huffman & Roscoe, 2014; Juan-Salles et al., 1997; Kelly & Sleeman, 2003; Kottwitz et al., 2004; Margalit Levi et al., 2017; Pas & Dubey, 2008c; Sorensen et al., 2005; Thornton, 1990)

5.1.4 *T. gondii* in exotic felids

It is unknown how infectious the different stages of *T. gondii* are for exotic felids. A high susceptibility for *T. gondii* infection and subsequent toxoplasmosis is suspected for species that get rarely into contact with the parasite in the wild (for example Pallas' cats or Sand cats).

A mortality of 58% (from 24 kittens) was reported for newborn Pallas' cat kittens born in captivity in an Austrian zoo, where the suspected cause was an acute *T. gondii* infection (Basso et al., 2005). In North America, the seroprevalence for *T. gondii* was 100% in nine captive Pallas' cats in three zoos (Brown et al., 2005). A mortality of 35% (6/17) due to acute toxoplasmosis was recorded in Pallas' cat kittens in Denver Zoo. Since five kittens disappeared and were not available for necropsy, a mortality rate of up to 65% (11/17) could have been suspected if those individuals were affected as well (Kenny et al., 2002). In the Czech Republic, 12 fatal cases of suspected toxoplasmosis in Pallas' cats were recorded between 2004 and 2013. In eight cases (66.6%), toxoplasmosis was confirmed (Bartova et al., 2014). The reasons for

the increased susceptibility of Pallas' cats for toxoplasmosis are not fully understood, but a study on Pallas' cats in Oklahoma suggests an immunodeficiency (congenital or acquired), since multiple diseases occurred in the examined population. In this study, an immunodeficiency similar to that caused by FIV infection is suspected to play a role (Ketz-Riley et al., 2000; Ketz-Riley et al., 2003). It seems that the disease is usually asymptomatic for adults (Riemann, Fowler, et al., 1974). However, Dubey and colleagues reported a case of fatal toxoplasmosis in an adult Pallas' cat (Dubey, Gendron-Fitzpatrick, et al., 1988).

A serologic study confirmed a high exposure to *T. gondii* in adult Pallas' cats in North American zoos. A proportion >80% of the animals tested positive for antibodies to the parasite (Swanson, 1999). This contrasts with studies on wild Pallas' cats in Mongolia and Russia, which suggested a low incidence of *T. gondii* infection in this species. In 2000-2001, 15 Pallas' cats, 15 domestic cats and 45 prey animals were captured in Mongolia. Only two Pallas' cats (13%) showed a positive Toxoplasma-antibody titer in an EIA. No evidence for exposure to *T. gondii* was found in domestic cats or prey animals (Brown et al., 2005). In 2010 and 2011, 16 wild Pallas' cats were caught in Russia close to the Mongolian border. Like in the study of Brown and colleagues (2005), only 13% of the individuals showed positive *T. gondii* antibody reactions in an EIA (Naidenko et al., 2014).

It is suspected that animals like Pallas' cats, which live in dry habitats with very severe winters and in high altitude, get rarely into contact with *T. gondii* in nature. The climatic conditions in the natural habitat of Pallas' cats may also reduce the viability of *T. gondii* oocysts. This seems to be the reason, why these species have a minimal chance to be naturally exposed to *T. gondii* (Brown et al., 2005; Zenker et al., 2004).

A similar situation can be observed for Sand cats. Natural spread of *T. gondii* seems to occur less in hot and arid climates, the typical habitat of Sand cats (Dubey, 2010b). Lack of exposure to *T. gondii* during phylogeny might be one of the reasons for the increased susceptibility to infection or disease, possibly due to impaired immune reaction against *T. gondii* or lack of adaptation to the parasite. There are reports of deaths in Sand cats that are suspected to have been caused by toxoplasmosis (Dubey et al., 2010; Pas & Dubey, 2008a).

It is not known, to which extend the European captive population of small exotic felids is infected with *T. gondii*. Seroprevalences in captive felids were examined in Brazil, Thailand, the United States of America (USA) and the United Arabic Emirates (UAE). In Brazil, three independent studies were performed. In a first study, felids of 8 species in Brazilian zoos were analyzed and an overall prevalence of 55% (472/865) was found (Silva et al., 2001). A second study demonstrated a seroprevalence of 63.4% (102/161) in a sampled population of wild felids belonging to 14 different species (Andre et al., 2010). A third study was performed at the Itaipu Binacional Wildlife Research Center. This study included felids of five species, among which a prevalence of 66.7% (38/57) was detected (Ullmann et al., 2010). In Thailand, 12 feline species were tested and a total seroprevalence of 15.4% (21/136) found (Thiangtum et al., 2006). In the USA, samples of 17 species kept in captivity were analyzed and a seroprevalence of 59% (35/59) reported (Spencer et al., 2003). In the UAE, 29 captive Gordon's wildcats (*Felis silvestris gordoni*) were tested. All individuals were seropositive in the MAT (Pas & Dubey, 2008b).

In addition, several studies were performed to determine the prevalence of antibodies to *T. gondii* in different cat species in the wild. Among cougars (*Felis concolor vancouverensis*) from Vancouver Island, Canada, the seroprevalence was 92% (11/12) (Aramini et al., 1998). In Northern California, bobcats (*Lynx rufus*) were tested, resulting in a seroprevalence of 71.4% (15/21) (H. P. Riemann et al., 1975).

Cheadle and colleagues analyzed sera of exotic captive and free-ranging felids (lions, leopards and cheetahs) from South Africa. 74% (50/68) of the animals tested antibody- positive for *T. gondii* (Cheadle et al., 1999).

Ramos Silva and colleagues studied risk factors for seropositivity to *T. gondii* in captive neotropical felids from Brazil. They concluded that the most effective way to reduce the risk of exposure of captive neotropical felids would be to freeze meat to -12°C for more than one week before feeding it to the cats (Silva et al., 2007).

Despite reports of a high mortality due to toxoplasmosis in Sand cats and Pallas' cats, there are only few reports of clinical or fatal toxoplasmosis in other exotic felids. Congenital toxoplasmosis was reported in a one-week old bobcat (*Lynx rufus*) (Dubey et al., 1987) and there is another confirmed case of *T. gondii* in a six months old bobcat (Smith et al., 1995). Furthermore, acute disseminated toxoplasmosis was reported in a juvenile cheetah (*Acinonyx jubatus*) (Lloyd & Stidworthy, 2007), a juvenile Siberian tiger (*Panthera tigris altaica*) (Dorny & Fransen, 1989) and two juvenile captive lions (*Panthera leo*) (Ocholi et al., 1989).

5.2 Reduviid bugs

5.2.1 General information

The Reduviidae represent a large family of insects in the suborder Heteroptera. The name of the family comes from the Latin reduvia meaning "remnant" or "hangnail". Various genera contain in total about 7000 species. The subfamily Triatominae (also called "kissing bugs") with about 140 species (Galvão et al., 2003; Schofield & Dolling, 1993; Schofield & Galvao, 2009) is strictly hematophagous (or feeding on hemolymph in invertebrates). Their entire life cycle, with a development through five larval stages called nymphs or instars (Schaub, 2008), is dependent on hematophagy (Lehane, 2005). The majority of triatomine species has their habitat between Michigan (USA) and Southern Argentina. They tend to live close to their hosts and colonize burrows, caves and nests of birds and mammals (Gaunt & Miles, 2000; Meiser & Schaub, 2011).

The bugs are attracted by different stimuli such as components of sweat, an increased proportion of CO_2 in exhalated air or ammonia in the feces left by engorged bugs (Barrozo & Lazzari, 2006; Schofield, 1979). Their antennae and also mouth parts are equipped with receptors perceiving thermal differences including infra-red stimuli (Ferreira et al., 2007; Schmitz et al., 2000).

Triatomines are not pool feeders taking up blood from wounds, but vessel feeders ingesting blood directly from a blood vessel (Lavoipierre, 1965). Their mouth parts consist of a labrum and a labium, which form the proboscis. They protect the internal mandibles, which in turn protect the maxillae. When approaching a host, the animals swing these mouthparts forward from a resting position below the thorax (Wenk et al., 2010; Wirtz, 1987). The skin is punctured with a jerky movement of the head and the upper layers of the skin are pierced by the serrated proximal ends of the mandibles. The maxilla penetrates deeper layers of the skin while trying to find a blood vessel. This process causes minor hematomas. Several trials might be needed until the probing phase is successful (Ferreira et al., 2007; Lavoipierre et al., 1959). The duration of the probing phase as well as the amount of ingested blood varies depending on species and developmental stage (Meiser, 2009; Soares et al., 2000)

When retaining blood from a host, it is important for the bug that it does not attract the attention of the host to avoid mechanical defense reactions (Andrade et al., 2005). Except from *Triatoma rubrofasciata*, the bite of most species does not cause any pain (Ryckman & Bentley, 1979). However, only in *T. infestans*, a protein has been identified that acts similarly to local anesthetics (Dan et al., 1999).

There are additional features that make hematophagous ectoparasites well adapted to blood ingestion. Reduviid bugs are confronted with the complement system of the host and blood clotting (Andrade et al., 2005). To meet those challenges, components in the saliva and in the stomach, are relevant (Schwarz et al., 2009). While apyrases inhibit the primary hemostasis

(Ribeiro et al., 1998; Sarkis et al., 1986), other proteins in the saliva of *R. prolixus* inhibit the collagen-induced adhesion of platelets and yet another compound avoids thromboxane A2-mediated aggregation (Ribeiro & Garcia, 1981; Ribeiro et al., 1998). In different triatomines, the saliva inhibits the classical or alternative pathway of the complement system (Barros et al., 2009; Cavalcante et al., 2003). In the stomach of triatomines, highly potent anticoagulatory proteins are secreted. They impair the coagulation cascade and have been characterized in the stomach of various species including *D. maxima, R. prolixus, T. infestans* and others (Meiser et al., 2010; Mende et al., 1999).

The saliva of triatomines is known to cause possible allergic reactions in humans when repeatedly used. The most severe reactions have been observed for the bite of *R. prolixus* (Meiser & Schaub, 2011), followed by *T. infestans* (Hoffman, 1987; Lapierre & Lariviere, 1954), while *D. maxima* only produced mild skin reactions (Marsden, 1986). In *R. prolixus*, a protein was found in the saliva that removes histamine, which is commonly found at the site of insect bites (Ribeiro & Walker, 1994). During the intensive use of *D. maxima* to bleed zoo animals, no allergic reactions were evident in any of the animals (Stadler et al., 2011).

Triatomines have a tubular digestive tract without lateral diverticula. After the foregut, the ingested blood passes into the midgut. The anterior part of the midgut consists of a short cardia and an extendable stomach. Small portions of stomach content are passed on to the posterior midgut for digestion (Kollien & Schaub, 2000). Fully engorged bugs have a large round abdomen that makes movements almost impossible. They encounter this situation with the most effective excretion system of the animal kingdom (Maddrell, 1969; Maddrell et al., 1991). Excretion of blood components without nutritional value already starts during engorgement (Maddrell, 1963). In *R. prolixus*, about 45% of the weight of ingested blood is excreted within the first 4 hours. In *T. infestans*, 60% of the ingested blood components have been excreted 24 hours after engorgement (Eichler & Schaub, 1998; Maddrell, 1969).

After ingestion, the concentrated blood is stored primarily undigested during the first few days. During this time period, lysis of erythrocytes occurs and some changes induced by the increasing activity of glycosidases and lipases (Azambuja et al., 1983; Bauer, 1981; Canavoso et al., 2004; Garcia et al., 2010; Ribeiro & Pereira, 1984; Schaub, 2009). Hemoglobin may crystallize depending on the host species (Bauer, 1981; Oliveira et al., 2007) and the blood usually appears in a jelly-like consistency (Lehane, 2005).

Triatomines as hemimetabolic insects are vectors of *Trypanosoma cruzi*, the etiologic agent of Chagas disease (Costa & Lorenzo, 2009; Schaub, 2009).

5.2.2 *Dipetalogaster maxima* species information and rearing of bugs

The reduviid bug *Dipetalogaster maxima* (Figure 5.2) is the biggest species of all triatomines. It is found in Mexico (Baja California Sur) (Lent & Wygodzinsky, 1979; Ryckman & Ryckman, 1963). It was originally named Dipetalogaster maximus, but Schofield suggested that according to article 30 of the International Code of Zoological Nomenclature, it should be named *D. maxima* instead (ICZN; Marsden, 1986; Schofield, 1979).

Figure 5.2: *D. maxima*, L5

The development of *D. maxima* consists of five larval stages (L1-5) until the imago hatches. The amount of blood ingested by the bug depends on the larval stage (0.1 g, 0.2 g, 0.6 g, 1.2 g and 2.5 g from L1-5) (Stadler et al., 2011), each with a characteristic size (e.g. L1 = 0.4 cm,

Imago = 4 cm) (Voigt et al., 2004). Generation time in laboratories lasts at least five to six months (Schaub & Breger, 1988).

D. maxima is diurnal and naturally feeds on small mammals, reptiles and birds living on the ground (Lent & Wygodzinsky, 1979; Ryckman & Ryckman, 1963). There are strict export regulations of Mexico, causing all non-Mexican laboratory colonies to be based on collections of Ryckman and Ryckman (1963) or Mersden et al. (1979) (Stadler et al., 2011).

At the Ruhr-Universität, Bochum, Germany, Faculty of Biology and Biotechnology, working group Zoology/Parasitology, *D. maxima* are reared at a temperature of 26-28°C, a relative humidity of 70% and with a photoperiod of 12/12 hr (light/dark). During the time of rearing, they are kept in beakers (15 cm x 18 cm) covered by a nylon cloth. A commercial rubber ring is used to fix the nylon cloth to the beakers. To increase the area for the bugs to sit on, a crossed cardboard is provided. Also, filter paper is placed in the bottom of the beakers to soak excretions of the bugs. One colony of 100 first instar larvae is kept in one two-liter beaker until they reached adult stage. When raised in the laboratory, the bugs get fed on blood of cocks or hens for one hour (Schaub, 2011 personal communication; Stadler et al., 2011).

Laboratory-raised *D. maxima* are free from known diseases and possess only their own, normal bacterial flora. There are no reports that this flora can cause infection or disease in the hosts (Schaub, 2013 personal communication).

5.2.3 The use of reduviid bugs for blood collection

Reports of the use of reduviid bugs were found as early as in 1971, when they were tested as an alternative bleeding method for geckos instead of the common practice of a toe-nail clip. Blood samples were used to investigate the protein profile of geckos (Will, 1971, 1977).

Later reduviid bugs were successfully used to collect blood for the determination of antibody titers for various disease such as rabies in mice (*mus musculus*) (Vos et al., 2010), rabbit hemorrhagic disease virus (RHDV) in domestic rabbits (*Oryctolagus cuniculus*) (Voigt et al., 2006), Tuberculosis (TB) in Malayan tapir (*Tapirus indicus*), Lowland tapir (*Tapirus terrestris*), Baird's tapir (*Tapirus bairdii*), Indian rhinoceros (*Rhinoceros unicornis*), Giraffe (*Giraffa camelopardalis*), South American sea lion (*Otaria flavescens*) and Harbor seal (*Phoca vitulina*) Blue tongue virus in Black faced sheep (*Ovis ammon f. aries*), Yellow-backed duiker (*Cephalophus silvicultor*), Dromedary (*Camelus dromedaries, dom.*), Siberian ibex (*Capra ibex sibirica*), White-lipped deer (*Cervus albirostris*), Giraffe (*Giraffa camelopardalis*) and Lowland tapir (*Tapirus terrestris*) and Brucellosis in Red river hog (*Potamochoerus porcus*) and Giraffe (*Giraffa camelopardalis*) (Stadler et al., 2011).

The use of reduviid bugs has been validated in endocrinologic studies where corticosterone in Common terns (*Sterna hirundo*) and progesterone as well as testosterone in rabbits were measured (Arnold et al., 2008; Voigt et al., 2004). Hubmer et al. (2010) used blood sucking bugs for pregnancy monitoring in captive Elands (*Taurotragus oryx*). Although hormones where successfully determined in other studies, one investigation showed a substantial difference between the resulting values in conventional blood samples and samples taken with reduviid bugs (Hubmer et al., 2010). A *D. maxima*-based blood sampling was also used for pregnancy diagnostics in Iberian lynx (*Lynx pardinus*) (Braun et al., 2009).

In other studies, blood taken by bugs was used to determine levels of various blood parameters: cholesterol, triglyceride, uric acid in birds (Bauch et al., 2010) and energy budget studies using the Doubly-labelled water technique in bats (Helversen & Reyer, 1984; Helversen et al., 1986; Voigt et al., 2003).

Biochemical blood analysis was performed by a number of authors who came to different conclusions. Stadler et al. (2007, 2009) analyzed blood from 39 animal species in zoos for the following parameters: sodium (Na), chloride (Cl), potassium (K), total carbon dioxide (TCO_2), urea, glucose (Glu), hematocrit (Hct), partial pressure of carbon dioxide (pCO_2), bicarbonate

(HCO_3), hemoglobin (Hb), albumin (Alb), alkaline phosphatase (AP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBil), blood urea nitrogen (BUN), calcium (Ca), phosphorus (P), creatinine (Crea), total protein (TP) and globulines (Glob). They concluded that sampling with reduviid bugs was reliable for most parameters.

Further tests of chemical blood parameters as well as hematology were performed by Markvardsen et al. (2012). On 5 rabbits, venous blood samples and samples using 4 reduviid bugs each were collected. While many parameters (Alb, Glu, TP, ALT, Crea, AMY, Mg) did not show statistically significant differences, others such as AP, Chol, lipase, PHOS, GGT, Ca and Na declined, while K increased statistically significantly in the samples taken with bugs. For hematology, there was no evidence for statistically significant differences for Hb, Hct, reticulocytes and eosinophilic granulocytes, whereas the WBC count, the RBC count, platelets, neutrophilic and basophilic granulocytes, lymphocytes and monocytes statistically significantly dropped in the bug samples (Markvardsen et al., 2012).

Depauw (2012) tested the applicability of *D. maxima* for biochemical analyses in seven captive cheetahs (*Acinonyx jubatus*). Despite a high grade of hemolysis, they found statistically significant elevations for TP, ALT, TBil, P, K and Mg in plasma obtained by reduviid bugs, while Na and Cl were statistically significantly lower when compared to conventional blood sampling. Only Alb, urea, Crea and Ca were not affected by the sampling technique (Depauw, 2012).

In Montagu's Harriers (*Circus pygargus*) reduviid bugs were successfully used to collect blood samples for genetic analysis (Janowski, 2010). Furthermore, lymphocytes for karyological analysis were isolated from bug-derived blood to identify bat species (Helversen et al., 1986).

Different methods have been described to use reduviid bugs for blood sampling. The easiest way is probably the direct placement of the bug onto the host. In this case, recovery of the bug can be a challenge. Marking the bugs with a thread (glued or knotted to the bug) can make recovery easier.

Another possibility is the use of a small container, which is covered with gauze. When applied to the host, the bugs are able to draw blood through the netting. This method requires a direct contact of the container to the animal. In addition, it is necessary to avoid movement of the container. This method works well with trained animals or when a chute for restraint is available (Stadler et al., 2007).

For small individuals, transport boxes can be designed, which hold a wire netting at the bottom. The bugs are placed into a separate container fixed to the netting from below. When the host enters the box and ideally lies down, the bugs are able to approach the host and bite through the netting (Stadler et al., 2007; Thomsen & Voigt, 2006).

Other techniques include applying bugs in small containers. For bleeding incubating birds hollow, artificial eggs containing a bug were placed in the nest (Bauch et al., 2010; Becker et al., 2005; Janowski, 2010). Other investigators used small containers on a collar in Meerkats (*Suricata surikatta*), which were trained to accept a person to tie around and shortly after removing the collar again (Habicher, 2009).

The risk of introducing a foreign species in Northern Europe is relatively low since the bugs will not survive the winter. When using the 5th larval stage, sex determination of the bugs is easily possible. Using male bugs only for blood collection can ensure that reproduction of escaped bugs is impossible (Stadler et al., 2007). Also in Mediterranean areas, e.g. southern Europe, survival of the species is rather unlikely as high temperatures in combination with low humidity are lethal for *D. maxima*. Already at 34°C the mortality of eggs reaches 50%. A mortality of 40% is reached at a relative humidity of 20% (Johnson et al., 1984). In any case, the invasion of this foreign species caused by escaping individuals must be avoided.

In their natural habitat triatomine bugs are vectors for *Trypanosoma cruzi*, the causative agent for Chagas disease. Due to increasing migration of people from Chagas disease-endemic countries to Europe, especially to Spain, the number of people infected with *Trypanosoma cruzi* increased during the last years. Anyway, in the absence of an adequate vector in Europe, transmission is limited to blood transfusions, organ transplantation or congenital infections (Gascon et al., 2010). This situation underlines the need to avoid the introduction of triatomines as a foreign species and a potential vector even more.

5.3 Exotic felids tested for *T. gondii* in zoos

5.3.1 Species information

For the evaluation of the prevalence of *T. gondii* in EAZA zoos, only small cat species were chosen. Due to their body size and laryngeal structure, small wild cats are not able to roar in contrary to the "big cats" (Grzimek, 2003). Another requirement for the inclusion of the species was, that it is managed in a special breeding program, like the EEP (European Endangered Species Programme) and the ESB (European Studbook) or at least monitored on a regular basis (monthly intervals) (for further information, see chapter 5.3.2).

Table 5.2: Population of small exotic felids in European zoos; Species and distribution (MacDonald & Loveridge, 2010; Nowell & Jackson, 1996; Sunquist & Sunquist, 2002), Numbers at the end of the blood sampling period (ZIMS, 2014)

Species	**Natural distribution/habitat**	**Number of institutions**	**Number of individuals**
New world felids			
Geoffroy's cat (*Leopardus geoffroyi)*	Southern South America; high variety of habitat types from desert to marshlands	19	44
Jaguarundi *(Puma yagouaroundi)*	Neotropics (Central and South America); high variety of habitat types from desert to rainforests	16	39
Margay *(Leopardus wiedii)*	Neotropics (Central and South America); tropical and subtropical forests	13	40
Oncilla *(Leopardus tigrinus)*	Neotropics (Central and South America); rainforests to savannahs	4	16
Old world felids			
Asian golden cat *(Catopuma temminckii)*	Asia-Tropical-Temperate; tropical and subtropical evergreen to mixed and dry deciduous forest	7	27
Black-footed cat *(Felis nigripes)*	Southern Africa; short grasslands	1	3
Fishing cat *(Prionailurus viverrinus)*	Asia-Tropical-Temperate; wetland	35	89
Pallas' cat *(Otocolobus manul)*	Eurasia; steppes, deserts, rocky country	36	83
Rusty-spotted cat *(Prionailurus rubiginosus phillipsi)*	Asia-Tropical-Temperate; dry forest types, scrub and grassland	9	48
Sand cat *(Felis margarita)*	South-west Asia, Africa; True desert	19 (+ 3 UAE)	57 (+ 51 UAE)

On the following pages (chapter 5.3.1.1-5.3.1.10) the different cat species are described in alphabetical order.

5.3.1.1 Asian golden cat (*Catopuma temminckii*)

The Asian golden (Figure 5.3) cat has a head and body length of 73-105 cm (Grzimek, 2003) and a weight varying from 8-16 kg (Sunquist & Sunquist, 2002). Its color varies from golden brown or red to greyish brown. The underparts are white and their face is characterized by black and white streaks (Grzimek, 2003).

Figure 5.3: Asian golden cat

Its distribution comprises Asia, in an area located between southern China, Nepal, Fukien in China, and Sumatra (Figure 5.4). In this area, the habitat of the Asian golden cat is primarily characterized by tropical and subtropical evergreen to mixed and dry deciduous forests (Nowell & Jackson, 1996). Asian golden cats were occasionally also seen in open habitats with rocky areas (Choudhury, 2007).

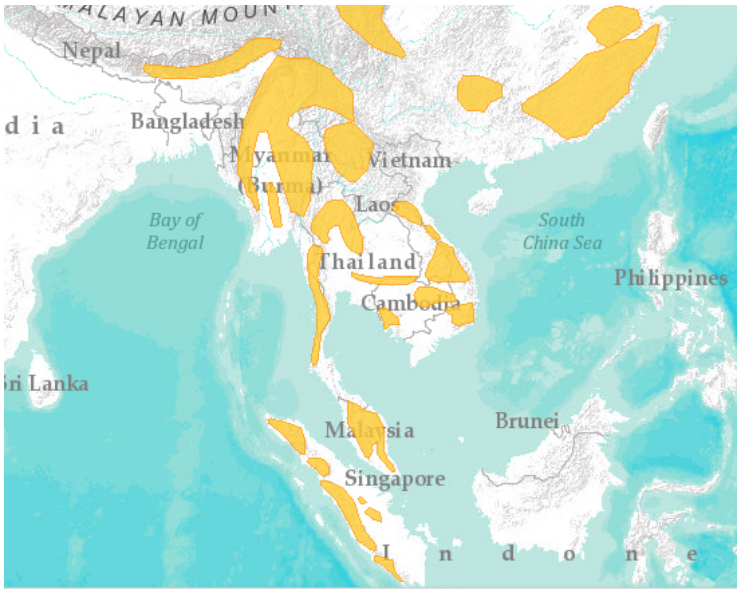

Figure 5.4: Distribution, Asian golden cat (IUCN, 2014)

The Asian golden cat is predominantly nocturnal. Although it is capable of climbing trees, it usually lives on the ground. Its diet consists of small animals like wild hares, small deer, birds and lizards. The Asian golden cat is also known to kill sheep, goats, and buffalo calves (Grzimek, 2003).

This cat is classified as 'near threatened' by the IUCN due to habitat loss, illegal hunting, and depletion of the wild ungulate prey base (IUCN, 2014; MacDonald & Loveridge, 2010).

Figure 5.5: Black-footed cat © A. Sliwa

5.3.1.2 Black-footed cat (*Felis nigripes*)

Black-footed cats (Figure 5.5) are the smallest cats in Africa with an average body length of 40-45 cm and a weight of 1.3-1.9 kg (Sliwa, 2004). It has a ground color from dark-ochre to pale-ochre. The body is covered with a bold pattern of round dark brown to black spots. Each cheek is marked with two black stripes and it has striped forelegs (Grzimek, 2003; Sliwa, 2013b).

Black-footed cats are endemic to the short grasslands of the Karoo and Kalahari in Namibia, Botswana and particularly South Africa (Figure 5.6). No other African cat species has such a restricted distribution (Nowell & Jackson, 1996).

Figure 5.6: Distribution, Black-footed cat (IUCN, 2014)

This solitary cat is nocturnal and strongly territorial, feeding on a high variety of pray items such as small mammals, birds but also, to a lesser degree, invertebrates, amphibians and reptiles (Olbricht & Sliwa, 1997; Sliwa, 2006).

This cat is classified as 'vulnerable' by the IUCN due to habitat loss, in adverted poisoning and other heedless methods of pest control (IUCN, 2014; Nowell & Jackson, 1996; Sliwa, 2008).

5.3.1.3 Fishing cat (*Prionailurus viverrinus*)

Figure 5.7: Fishing cat

Fishing cats (Figure 5.7) are medium-sized cats with a head-body length of 65-74 cm (MacDonald & Loveridge, 2010), weighing 7.7-14 kg (Mattern & McLennan, 2000). They have a deep-chested body on relatively short legs with their front feet partially webbed, which makes them well adjusted for catching fish. This cat has a rather short tail, small close-set ears and claw tips that protrude from their sheath even when retracted (Haque & Vijayan, 1993; Mukherjee, 1989; Sunquist & Sunquist, 2002).

Fishing cats have a broad but discontinuous distribution in Asia (Figure 5.8). They were found in different areas in India, in Bangladesh, Bhutan, Cambodia, Indonesia (Java), Lao People's Democratic Republic, Myanmar, Nepal, Sri Lanka, Thailand and Vietnam. (IUCN, 2014). Over the past decade, the species became increasingly hard to find. Fishing cats are strongly associated with wetlands. Their habitat is characterized by swamps and marshy areas, oxbow lakes, reed beds, tidal creeks, and mangrove areas. Although most records are from lowland areas, they were observed at an elevation of up to 1525 m (Nowell & Jackson, 1996).

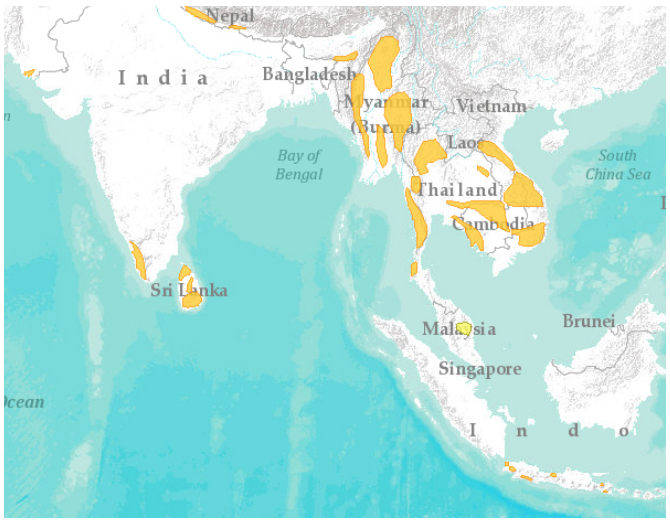

Figure 5.8: Distribution, Fishing cat (IUCN, 2014)

As the animals are strong swimmers, the main part of their diet consists of fish, but birds, insects, small rodents, mollusks, reptiles and amphibians are on their menu as well (Haque & Vijayan, 1993).

Fishing cats are classified as 'endangered' by the IUCN, among other reasons wetland destruction, depletion of fish stocks from overfishing and thoughtless snaring, trapping and poisoning seem to play a major role (IUCN, 2014).

Figure 5.9: Geoffroy's cat

5.3.1.4 Geoffroy's cat (*Leopardus geoffroyi*)

The Geoffroy's cat is a small solitary cat with a body length of 45-70 cm and a weight of 2-4.8 kg (Figure 5.9). The color of the coat varies from silver-grey to brownish-yellow with uniform small black spots (Grzimek, 2003).

Its distribution ranges from south-eastern Bolivia, Paraguay and Argentina (east of the Andes) to southern Brazil, Uruguay and all the way to the Strait of Magellan in Chile, from sea level to 3300 m (Figure 5.10). The habitat of Geoffroy's cat includes a high variety of landscape types in the subtropical and temperate zones of southern South America, including scrubby woodland, dry forests and savannahs, alpine saline desert, grasslands, marshlands, etc. in both pristine and disturbed areas. (IUCN, 2014; Nowell & Jackson, 1996; Oliveira, 1994).

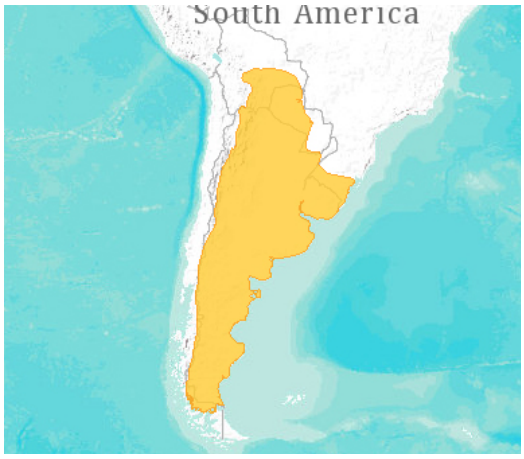

Figure 5.10: Distribution, Geoffroy's cat (IUCN, 2014)

Geoffroy's cats are strong climbers and swimmers. They live primarily nocturnal (Hunter & Barrett, 2011). Their diet consists mainly of small rodents, but also birds, fish and amphibians (Manfredi et al., 2004; Sunquist & Sunquist, 2002).

This species is classified as 'near threatened' by the IUCN due to habitat loss and fragmentation (IUCN, 2014).

5.3.1.5 Jaguarundi (*Puma yagouaroundi*)

The Jaguarundi (Figure 5.11) has an elongated low-slung body with short legs and a long tail. Its head and body length is about 60-70 cm and it has a weight of 4-9 kg (Grzimek, 2003). With its unusual shape, it has long been a taxonomic enigma (Sunquist & Sunquist, 2002), but genetic analysis groups it with the puma and the cheetah (Johnson et al., 2006; O'Brien & Johnson, 2007). The Jaguarundi comes in two color morphs. The first one is blackish-grey except for two white spots next to the nose on the upper lip and possibly some white markings on the belly. The second color phase is reddish brown, except for white marks on throat and lips (Grzimek, 2003).

Figure 5.11: Jaguarundi

The Jaguarundi occurs in an area extending from the eastern and the western lowlands of Mexico, all the way south to southern Brazil, Paraguay, Uruguay and south through Central Argentina (Figure 5.12). It predominantly ranges in heights of up to 2000 m, but in Colombia it has been reported up to an elevation of 3200 m (IUCN, 2014). The Jaguarundi is probably extinct in the USA (Southern Texas) (Sunquist & Sunquist, 2002). The habitat of this species varies from closed primary rainforest to open desert, scrub, and grassland, although in open areas it sticks to patches of dense cover (Nowell & Jackson, 1996; Oliveira, 1998a).

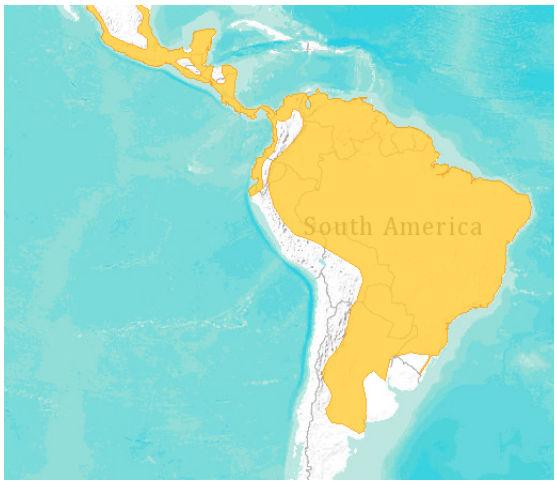

Figure 5.12: Distribution, Jaguarundi (IUCN, 2014)

This cat is predominantly diurnal (activity peaks in the late morning and late afternoon) (MacDonald & Loveridge, 2010). It lives solitarily and preys preferably on birds, but also small mammals, amphibians and fish. (Grzimek, 2003).

Although the Jaguarundi is classified as 'least concern' by the IUCN, habitat loss and fragmentation, especially for largescale agriculture and pastures, are a threat for the species (IUCN, 2014).

5.3.1.6 Margay (*Leopardus wiedii*)

The Margays' color ranges from grayish to cinnamon, with dark brown spots in longitudinal rows (Figure 5.13). The underparts of this small and slender cat are white. It has a head and body length of 46.3-79 cm and weighs about 2.6-3.9 kg (Grzimek, 2003). A long tail and flexible ankles with broad feet make them perfectly suited for an arboreal life (Nowell & Jackson, 1996). The flexible ankle joint, which extends and rotates through 180° allows them to descend a tree, head first, holding on to it with their hind legs (Taylor, 1989).

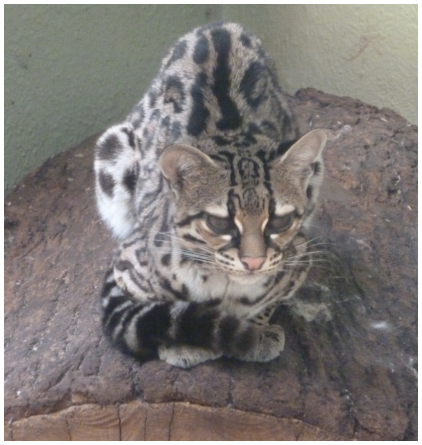

Figure 5.13: Margay

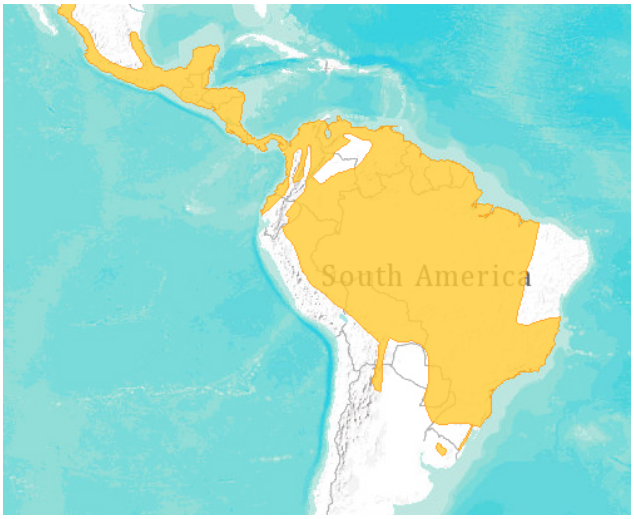

Figure 5.14: Distribution, Margay (IUCN, 2014)

The range of the Margay extends from the tropical lowlands of Mexico south through the Amazon basin, southern Brazil and Paraguay (Nowell & Jackson, 1996) to northern Argentina and Uruguay (IUCN, 2014) (Figure 5.14). It usually occurs from 0-1500 m; however, it has been recorded up to 3000 m in the Andes (Oliveira, 1994). The Margay inhabits tropical and subtropical forests with tree cover both evergreen and deciduous, reaching greatest abundance in the lowlands (MacDonald & Loveridge, 2010)

Margays show a predominantly nocturnal-crepuscular activity (Oliveira, 1998b). Prey species include small mammals, birds and reptiles (Sunquist & Sunquist, 2002).

This small cat species is classified as 'near threatened' by the IUCN. Current threats include habitat loss, fragmentation through roads and illegal trade (for pets and pelts) (IUCN, 2014).

5.3.1.7 Oncilla (*Leopardus tigrinus*)

Upperparts of the Oncilla are light to rich ochre showing rows of large dark spots (Figure 5.15). The underparts are paler and less spotted. The tail has 10-11 rings and a black tip. Their head to body length is about 40-55 cm (Grzimek, 2003). It weighs about 2.4 kg (Oliveira & Cassaro, 2005)

Figure 5.15: Oncilla © A. Sliwa

The distribution of the Oncilla extends from north Costa Rica to southern Brazil and north-east Argentina (Figure 5.16). There are only few records showing that it was found above 3000 m (IUCN, 2014). Its habitat ranges from montane cloud forest in Central America and parts of northern South America to rainforests and dry deciduous forest, savannahs, semi-arid thorny scrub, and degraded secondary vegetation in close proximity to human settlement in Brazil (MacDonald & Loveridge, 2010).

The Oncilla is nocturnal-crepuscular but shows also considerable daytime activity. It usually consumes small rodents, frogs, rabbits and birds (Grzimek, 2003).

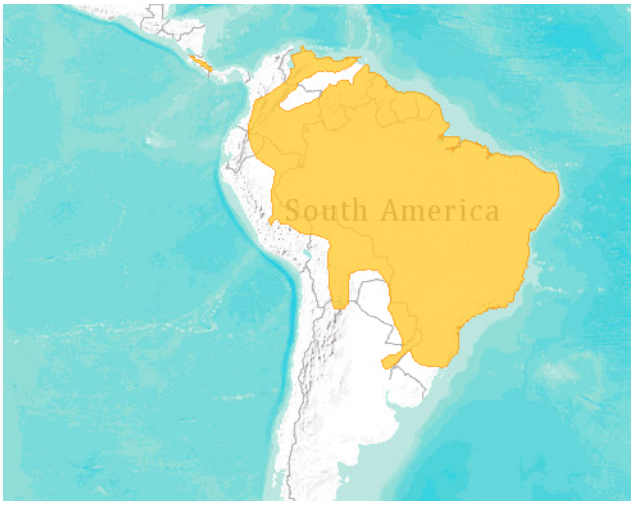

Figure 5.16: Distribution, Oncilla (IUCN, 2014)

Current threats to this species include habitat loss, fragmentation, roads and illegal trade (pets and pelts). Another potential threat could be a change in the native species dynamics (predator/competitor), notably its competition with the Ocelot (*Leopardus pardalis*). Due to these reasons, the Oncilla is classified as 'vulnerable' on the IUCN red list (IUCN, 2014).

Figure 5.17: Pallas' cat

5.3.1.8 Pallas' cat (*Otocolobus manul*)

The Pallas' cat (also known by its Russian name Manul) has a light gray to yellowish buff and russet, frosted appearance (Figure 5.17). The face is marked by two dark streaks across each side of the head. The tail shows four rings and a dark tip. They have a long dense coat, a massive body, short legs and a short broad head. The head and body length is about 50-65 cm with a weight of 2.5-3.5 kg (Grzimek, 2003).

The Pallas' cat is primarily distributed to central Asian steppe grass-land regions of Mongolia, China and the Tibetan Plateau, where Pallas' cats were recorded up to an elevation record of 5050 m (Fox & Dorji, 2007). In Russia, Pallas' cats are sporadically found in the Transcaucasus and Transbaikal regions (IUCN, 2014) (Figure 5.18). Their habitat is characterized by steppes, deserts, rocky country and an extreme continental climate with little rainfall, low humidity, and a wide range of temperatures. At a continuous snow cover of 15-20 cm the Pallas' cat reaches its ecological limit (Sunquist & Sunquist, 2002).

Its activity pattern is predominantly crepuscular, although it can be active at any time of the day (MacDonald & Loveridge, 2010). Prey species are mainly small mammals including gerbils (*Meriones* spp.) and jerboas (*Dipus sagitta* and *Allactaga spp*.). During spring, even lambs of Argali sheep (*Ovis ammon*) are taken (Murdoch et al., 2006).

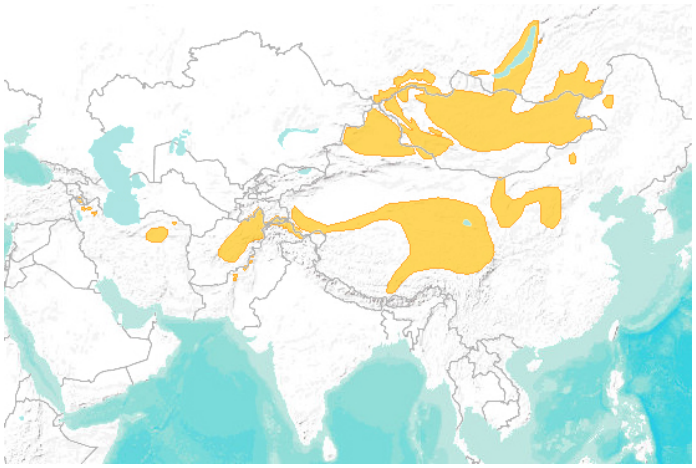

Figure 5.18: Distribution, Pallas' cat (IUCN, 2014)

The Pallas' cat is classified as 'near threatened' by the IUCN red list. The most serious threat may be depletion of their prey base (pikas, *Ochotona* sp.) because of poisoning and over-hunting (Nowell & Jackson, 1996). In addition, habitat degradation by domestic livestock and agriculture and illegal trade in skins and for traditional medicine is considered a problem (IUCN, 2014).

5.3.1.9 Rusty-spotted cat (*Prionailurus rubiginosus*)

Figure 5.19: Rusty-spotted cat

The Rusty-spotted cat is the world's smallest cat with a weight of about 0.8-1.1 kg and a head-body length of 32.9-41.6 cm (Figure 5.19). It has elongated rust-brown spots that stripe the rufous grey fur of its back and flanks (MacDonald & Loveridge, 2010).

Rusty-spotted cats occur only in India and Sri Lanka (Sunquist & Sunquist, 2002), where they live in a habitat characterized by dry forest types as well as scrub and grassland (Figure 5.20). They are likely absent from evergreen forest in India but have been found in the midst of agricultural and settled areas (Nowell & Jackson, 1996).

Figure 5.20: Distribution, Rusty-spotted cat (IUCN, 2014)

Figure 5.21: Sand cat

Rusty-spotted cats are arboreal (Sunquist & Sunquist, 2002) and mostly observed at night. Their main prey species are probably small rodents, but they have also been seen hunting frogs (IUCN, 2014).

This species is classified as 'vulnerable" by IUCN. Although there are several records of Rusty-spotted cats observed in cultivated and settled areas it is not known whether they are able to persist in that environment. In both India and Sri Lanka habitat loss and the spread of cultivation are serious problems for wildlife (Nowell & Jackson, 1996).

5.3.1.10 Sand cat (*Felis margarita*)

The Sand cat has a color from pale sandy to grey straw, the back is darker and the belly white (Figure 5.21). The face is marked with two reddish streaks and large ears. The tail shows two or three rings and a black tip. The head and body length measures about 45-57.2 cm (Grzimek, 2003) with a weight from 1.35-3.2 kg (Sunquist & Sunquist, 2002). The underside of the Sand cats' feet are thickly furred, this might be an adaptation to its life in the desert. It may help it to move across shifting sands and protect the feet from hot sand (Nowell & Jackson, 1996; Sunquist & Sunquist, 2002).

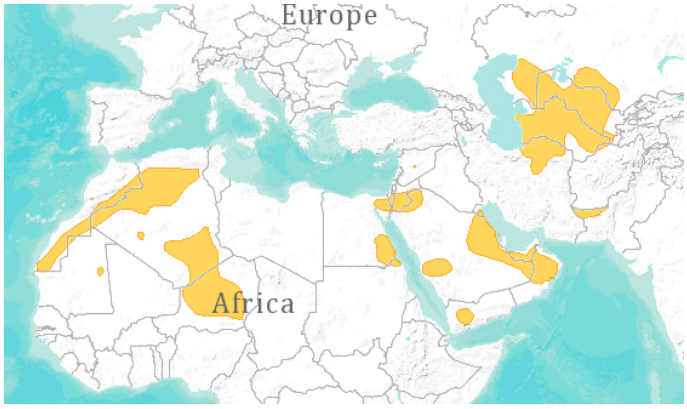

Figure 5.22: Distribution, Sand cat (IUCN, 2014)

The Sand cat is the only felid that occurs primarily in the true desert. It is widely but apparently disjunctive distributed from through the deserts of Northern Africa and southwest and Central Asia (Figure 5.22). It is unknown whether the gaps in range are due to a lack of records or actually reflects the absence of the species (Nowell & Jackson, 1996; Sliwa, 2013a).

It is a solitary and nocturnal cat (Nowell & Jackson, 1996) that preys on small mammals, birds and reptiles (Abbadi, 1993). Sand cats do not rely on drinking water; they are able to satisfy their moisture requirements with metabolic water (MacDonald & Loveridge, 2010).

This small felid species is classified as 'near threatened' by IUCN due to habitat degradation. There are other local threats such as the introduction of feral and domestic dogs and cats, creating direct competition and competition through predation as well as disease transmission (IUCN, 2014; Nowell & Jackson, 1996; Sliwa, 2013a).

5.3.2 Species protection status

The International Union for Conservation of Nature (IUCN) documents the status of biodiversity of wildlife keeping the international "Red List of Threatened Species" up to date. The IUCN is the world's oldest and largest global environmental organization and the only one providing a global list of endangered species. It is considered to be the most authoritative index of species status, particularly for mammals (Schipper et al., 2008).

"CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) is an international agreement between governments. Its aim is to ensure that international trade in specimens of wild animals and plants does not threaten their survival" (CITES, 2013). Every year, international wildlife trade is estimated to be worth billions of US dollars compromising hundreds of millions of plant and animal specimens. Specimens range from life animals and plants to large numbers of various products derived from them. This type of trade is capable of depleting populations heavily. Therefore, the existence of an agreement to ensure sustainability in trade is of enormous importance. CITES accords varying degrees of protection to more than 35000 species (animals and plants). These species are listed in three Appendices according to the degree of protection needed (CITES, 2013).

In European zoos, the most intensive population management is conducted by the European Endangered Species Programme (EEP). An EEP coordinator (a person working in an EAZA zoo or aquarium with special knowledge and interest in the species concerned) works together with the Species Committee to perform their tasks (collecting information on the status of all animals in his or her EEP, demographical and genetic analysis on the species, the establishment and maintenance of a studbook and planning the future management of the species including recommendations for breeding and the transfer of animals in between zoos).

A less intensive breeding programme is conducted by the European Studbook (ESB). The responsible studbook keeper collects data on birth, deaths, transfers, etc. from a certain ESB species in all EAZA zoos and aquariums. The information is entered in a special database, which enables the studbook keeper to carry out analyses of the population of the managed species. Studbook keepers may be asked for recommendations on breeding or transfer by EAZA institutions. They can also judge whether the species is reproducing well in EAZA

zoos/aquariums or if a more stringent management is needed to maintain a healthy population. The studbook keeper may suggest that the species should be managed as part of an EEP programme (EAZA, 2011).

The lowest level of species monitoring is realized by the Monitoring Programmes (Mon) of the EAZA. Species that are monitored under this simple follow-up program are not threatened in the wild and their management in captivity is not a priority. Monitoring can be conducted by a single person or by the Taxon advisory group (TAG).

Table 5.3: Conservation/protection status of small exotic felids managed in European breeding programs

Species	**IUCN Red List**	**Breeding program in EAZA zoos (EEP/ESB)**	**CITES Appendix**
New world			
Geoffroy's cat (*Leopardus geoffroyi*)	NT	EEP	I
Jaguarundi (*Puma yagouaroundi*)	LC	Mon	I (Central and North American populations) II (others)
Margay (*Leopardus wiedii*)	NT	EEP	I
Oncilla (*Leopardus tigrinus*)	VU	ESB	I
Old world			
Asian golden cat (*Catopuma temminckii*)	NT	EEP	I
Black-footed cat (*Felis nigripes*)	VU	EEP	I
Fishing cat (*Prionailurus viverrinus*)	EN	EEP	II
Pallas' cat (*Otocolobus manul*)	NT	EEP	II
Rusty-spotted cat (*Prionailurus rubiginosus phillipsi*)	VU	ESB	I (Population of India) II (others)
Sand cat (*Felis margarita*)	NT	EEP	II

LC: Least concern (relatively widespread and abundant); NT: Near threatened (likely to qualify for a threatened category in the near future); VU: Vulnerable (high risk of extinction in the wild); EN: Endangered (Very high risk of extinction in the wild) (IUCN, 2014)

EEP: European Endangered Species Programme; ESB: European Studbook; Mon: Monitored Programme (EAZA, 2011)

Appendix I: includes species threatened by extinction. Trade in specimens of these species is permitted only in exceptional circumstances.

Appendix II includes species not necessarily threatened with extinction, but in which trade must be controlled to avoid utilization incompatible with their survival.

Appendix III contains species that are protected in at least one country that has asked other CITES Parties for assistance in controlling the trade. (CITES, 2013)

6 ANIMALS, MATERIALS AND METHODS

6.1 Method validation on domestic cats

6.1.1 The animals (domestic cats) used for the test

To examine the relationship between *Toxoplasma gondii* antibody titers or blood chemical parameters in plasma separated from blood collected via reduviid bugs and those measured in plasma obtained by conventional blood sampling, domestic cats were used. Sample collection was approved by the Lower Saxony State Office for Consumer Protection and Food Safety according to §8 protection of animals act (33.9-42502-04-11/0436). Blood samples from 70 domestic cats were collected after the owners' consent had been obtained. Samples were taken only from cats that had been anesthetized for other reasons at a small animal clinic. Anesthesia was necessary in these animals because of diagnostic procedures or treatments (e.g. castrations and wound or dental treatment). Anesthesia was performed using 80 µg/kg Medetomidin (Domitor) and 7.5 mg/kg Ketamin (Ketamin 10%) intramuscular. All individuals were closely monitored during the procedure.

6.1.2 The bugs - *D. maxima* used for blood sampling

Larval instars of the reduviid bug *D. maxima* were used for blood collection in this study. To be able to obtain enough blood for analyses from each bug, large larval instars L4 or small instars L5 were used. Selecting bugs of the same size assured that approximately the same amount of blood was obtained from each bug (0.8-1.2 ml). Bugs were used only once to rule out cross contamination and transmission of diseases to other hosts. To assure that only completely "empty" bugs were used, individuals with a paper-thin abdomen were chosen. Waiting eight weeks after the last blood meal for completing digestion avoided contamination of samples with bug hemolymph (Lehane, 2005).

To keep the bugs at their preferred temperature of 26°C, they were housed and transported in an incubator "Kunstglucke FB 50 E-Reptilien" (Jäger und Pfrommer Brutapparate, Wächtersbach, Germany), with a 12-volt plug, suitable for use inside the car. An adequate adapter made indoor use possible as well.

Immediately after blood withdrawal, the bugs were killed by decapitation.

6.1.3 Obtaining and processing of blood samples

To obtain blood samples, reduviid bugs were placed in a modified medical urine container. The lid of the container was closed with netting that allowed the proboscis of the bugs to cross the meshes and reach the cat. The container itself was divided into four chambers using cardboards assembled to a cross.

In each chamber, a reduviid bug was placed. The readiness of the bugs to obtain a meal was assessed by blowing gently into the container. Only individuals that showed immediate interest by elevating their proboscis and searching for the source of potential prey were used to perform blood collection.

The modified urine container was placed either on the lateral chest or the abdomen of the anesthetized cat depending on the procedure and the position of the patient, to ensure that the container did not disturb the medical procedure performed on the animal.

The time when each single bug started obtaining blood was noted as well as the time when it detached fully engorged. Full engorgement took between 10 and 20 minutes. After that time, the blood was immediately obtained from the first bug (sample B0). The other bugs were placed in a room with a temperature of about 23°C. The blood sample from the second bug (B1) was

obtained after one hour, from the third bug (B2) after two hours and the fourth bug (B4) after four hours.

To collect the blood samples from the bug, a 1.2 x 40 mm needle (Braun, Melsungen, Germany) on a 3 ml syringe was used. Blood aspiration from the abdomen of the bug was performed from dorsal while holding the thorax of the bug (Figure 6.1). This approach facilitates the retrieval of the maximum amount of blood out of the bug (Schaub, 2011 personal communication). Immediately after the blood was withdrawn, it was transferred into a tube supplemented with lithium heparin (1.3 ml; Sarstedt). The tubes were centrifuged in a Spectrafuge™ Mini centrifuge (Labnet International, Edison, New Jersey, USA) at 6000 rpm for 5 min and plasma was removed and kept frozen at -20°C until further analysis.

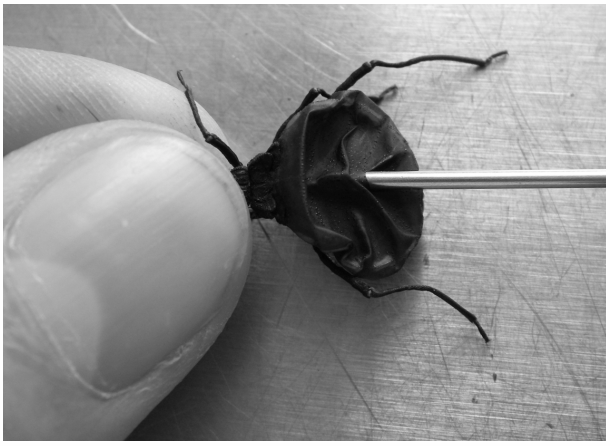

Figure 6.1: Blood aspiration from *D. maxima*

Besides the blood sampling via reduviid bugs, each domestic cat was sampled by puncture of the *Vena saphena* (V) using a 0.7 x 30 mm needle (Braun, Melsungen, Germany). The blood was collected in a tube with lithium heparin and immediately processed in the same way as the blood collected by bugs.

In 32 individuals, out of 70 domestic cats sampled, one out of the four bugs failed to obtain blood. In this case, it was decided to skip sample no. B2 and to collect only samples B0, B1, B4 and V. Data gaps in the blood chemical analysis were caused by insufficient volumes of plasma in some samples.

6.1.4 Tests used for blood analysis

Plasma was screened for the following blood chemical parameters:

Table 6.1: Chemical parameters determined using the Pentra 400 (ABX-Horiba; Justus-Liebig-Universität Giessen, 2009), Reference ranges in square brackets

Parameter [*Reference range*]	**Method used**
Organic components	
Alanine aminotransferase (ALT) [*0-70 U/l*]	Optimized Ultraviolet (UV)-Test without Pyridoxalphosphat (PALP), forward + reverse, kinetic-photometric
Albumin (Alb) [*21,0-33,0 g/l*]	Bromocresol green method, kinetic-photometric
Alkaline phosphatase (AP) [*0-39,7 U/l*]	Kinetic-photometric, p-nitrophenylphosphat,
Cholesterol (Chol) [*2,46-3,37 mmol/l*]	Accelerator Selective Detergent Method
Creatine kinase (CK) [*<205 (143) U/l*]	Optimized UV-Test, forward + reverse, kinetic-photometric
Creatinine (Crea) [*0-168 µmol/l*]	Enzymatic
Globulin (Glob) [*26,0-51,0 g/l*]	Assessed by calculation (TP – Alb)
Glucose (Glu) [*3,89-6,11 mmol/l*]	Enzymatic using hexokinase, kinetic-photometric
Glutamate dehydrogenase (GLDH) [*0-11,3 U/l*]	Nicotinamide adenine dinucleotide (NADH)-alpha-Ketoglutarat-method, kinetic-photometric
Total bilirubin (TBil) [*0-3,4 µmol/l*]	Dichloranilin-method, kinetic-photometric
Total protein (TP) [*54,7-78,0 g/l*]	Pyrogallol red-molybdate-complex-method, kinetic-photometric or Biuret-method with end-point measurement accordingly
Triglyceride (Tri) [*0,57-1,14 mmol/l*]	Enzymatic
Urea [*7,14-10,7 mmol/l*]	Enzymatic
Inorganic components	
Chloride (Cl) [*110-125 mmol/l*]	Ion sensitive electrode
Phosphorus (PHOS) [*0,8-1,9 mmol/l*]	UV-method with phosphomolybdat
Potassium (K) [*3,6-4,8 mmol/l*]	Ion sensitive electrode
Sodium (Na) [*141-150 mmol/l*]	Ion sensitive electrode
Total calcium (TCa) [*2,3-3,0 mmol/l*]	O-Cresolphtalein-Complexon-method, kinetic-photometric
Total magnesium (TMg) [*0,6-1,3 mmol/l*]	Xylidilblue-method with ethylene glycol tetraacetic acid (EGTA), kinetic-photometric

Outliers were excluded from the statistical evaluation. They were defined as values that exceeded the interquartile range by the factor 2.5 or more.

Specific antibodies against *T. gondii* were determined in an immunoblot based on the tachyzoite surface antigen TgSAG1 obtained by immunoaffinity chromatography using the mouse monoclonal antibody (mAb) IgG2a P30/3 (ISL, Paignton, UK) (Maksimov et al., 2011). For Western-blotting, purified TgSAC1 of *T. gondii* RH was used as described by Maksimov and colleagues (2011) with few modifications. A quantity of 0.05 µg TgSAC1 was incubated in non-reducing sample buffer (2%[w/v] sodium dodecyl sulfate (SDS), 10 %[v/v] glycerol, 62 mM TrisHCl, pH 6.8) for 1 min (94°C), separated in 12%[w/v] SDS polyacrylamide minigels of 60 x 70 x 1 mm size and transferred to a PVDF membrane (Immobilon-P, Millipore) (Azevedo et al., 2010). After transfer, the membrane was blocked using PBS-TG (PBS with 0.05 % (v/v) Tween 20 (Sigma) and 2% (v/v) liquid fish gelatine (Serva, Germany)), cut into 50 stripes and examined as described below. Cat plasma was diluted 1:100 in PBS-TG. The reactivity of plasma samples with a single band of 30 kDa Mr was recorded (no reaction, very

faint reaction, clear reaction). In all immunoblots, peroxidase conjugated anti-cat IgG (H + L) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used diluted 1:250 in PBS-TG.

In addition, an immunofluorescent antibody test (IFAT) using the *T. gondii* strain RH (Sabin, 1941) was performed. 10 µl of a suspension of cell culture-derived *T. gondii* RH strain tachyzoites (5 x 10^6 ml^{-1}) in phosphate-buffered saline (PBS) were used to sensitize IFAT slide wells. Slides were air-dried and stored frozen at -20°C until used. The slides were fixed with ice-cold acetone for 10 min and then incubated in PBS for 10 min. Cat plasma was titrated in PBS in 2-fold steps starting at a plasma dilution of 1:50. The test was performed as described for *N. caninum* (Schares et al., 1998) but with the following modification: Anti-cat IgG (H&L) produced in goat and coupled to FITC (Fluorescein Isothiocyanate) [Rockland Immunochemicals] diluted 1:50 in PBS, 0.2% Evans Blue was used to detect primary antibodies. The slides were examined under an Olympus AHBT3 microscope (Olympus, Hamburg, Germany). Only complete peripheral fluorescence of the tachyzoite was considered specific. The positive cut-off was a titer of 1:100 (Maksimov et al., 2013).

To determine the statistical significance of titer differences among the different sampling methods (conventional and blood sampling with reduviid bugs), only positive *T. gondii* IFAT titers were taken into account, as titer changes were not expected in Toxoplasma-seronegative samples.

6.2 Sample collection in zoos

6.2.1 Animals (exotic felids) tested at the zoos

Ten species of small exotic cats were sampled in 51 EAZA zoos: Asian golden cats (*Catopuma temminckii*), Black-footed cats (*Felis nigripes*), Fishing cats (*Prionailurus viverrinus*), Geoffroy's cats (*Leopardus geoffroyi*), Jaguarundis (*Puma yagouaroundi*), Margays (*Leopardus wiedii*), Oncillas (*Leopardus tigrinus*), Pallas' cats (*Otocolobus manul*), Rusty-spotted cats (*Prionailurus rubiginosus*) and Sand cats (*Felis margarita*).

In addition to samples collected with the use of reduviid bugs, samples provided out of serum banks were analyzed. In these cases, samples from other species were also used, later on referred to as "others". Additional samples came from the following species: one Clouded leopard (*Neofelis nebulosa*), one Eurasian lynx (*Lynx lynx*), three Gordon's wildcats (*Felis silvestris gordoni*), one Jungle cat (*Felis chaus*), two Ocelots (*Leopardus pardalis*), 3 Servals (*Leptailurus serval*) and seven Wildcats (*Felis silvestris*).

6.2.2 Keeping of bugs

To keep the bugs at their preferred temperature of 26°C and a humidity between 60% and 70%, they were housed and transported in an incubator "Kunstglucke FB 50 E-Reptilien" from Jaeger, as mentioned previously. Temperature and humidity were controlled using a digital thermo- hygrometer with two separate probes (Terra exotica). Humidity was increased placing a wet sponge inside the incubator if necessary.

6.2.3 Sample collection box for the application of *D. maxima*

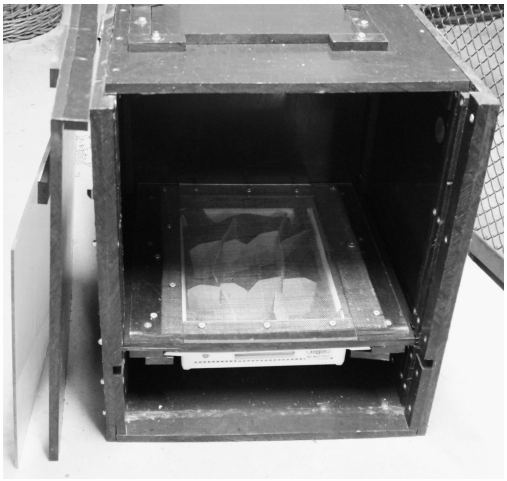

Figure 6.2: Sample collection box

Figure 6.2 shows the sample collection box, which was built at the Ree-Park, Ebeltoft Safari (Ebeltoft, Denmark). It has a window on the top, through which the animal can be monitored. A camera can also be mounted for continuous monitoring. Each side has big holes for ventilation. Either end can be opened with a trap door. One end consists of a double door; the outer black door goes all the way down to the bottom while the inner plexiglas door stops on the same level as the drawer for the bugs. This allows save removal of the bugs even while the host is inside the box. The drawer is equipped with a wire netting, stable enough to support the weight of a small felid and contains a little container (white in the picture). The container can be detached from the drawer for cleaning and for replacement, or collection of bugs. Its mesh-like structure allows fluids to run through, just in case the cat urinates during its stay in the box. The container is covered with a thin rubber net, which cushions the room inside to minimize the sound caused by bugs when walking around. First trials of the box showed, that the sound made by the bugs walking in the container below the cats disturbed the cats, so that they did not settle as fast as cats in later trials with the rubber cushion. Pieces of crossing cardboard were placed inside the bug-container allowing the bugs to climb up towards the host.

Before the box was shipped to another institution for sampling, it was completely disassembled, thoroughly washed and disinfected with Virkon S (Arovet AG). Virkon S is a highly potent disinfectant, effective at low temperatures and in hard water. Also, the solution used for disinfection does not cause skin, mucosa or eye irritations upon contact. It is virucide, bactericide and fungicide and guarantees an effect against all important infective agents from those groups.

6.2.4 Obtaining and processing of blood samples

Blood samples were obtained by placing two to four bugs inside the bug-container in the transport box. The drawer was then placed inside the box and the cat caught, or encouraged with food to move inside the box. All doors were closed and a camera for monitoring installed. The cats were left inside the box for one hour to give them time to settle, so that the bugs could proceed with their blood meal. No later than after one hour, the cats were released. Blood was obtained immediately from the bugs and processed as described in chapter 6.1.3. If more than one bug successfully finished their meals, the plasma samples were pooled.

Blood sampling was restricted to warm summer months, as the reduviid bugs are most effective at temperatures of at least 20°C.

6.2.5 Tests used for blood analysis

Blood samples from exotic felids in zoos were analyzed for *T. gondii* antibodies as described in chapter 6.1.4.

A Toxoplasma seropositive or seronegative result was recorded when both tests (TgSAG1 recognized in the immunoblot and IFAT titer $\geq$1:100) had equivocal results (both tests positive or negative). When the immunoblot showed an inconclusive result, the IFAT result was accepted as valid. In 25 cases, where the results of both tests differed, the final judgement was

considered as not reliable and the result marked as not available (NA). Individuals with an unclear result were excluded from further analysis.

6.2.6 Data from the Zoological Information Management System and questionnaire

Individual data on every felid that took part in the study was gathered from the Zoological Information Management System (ZIMS). Admission was granted by the Ree-Park Ebeltoft Safari (Ebeltoft, Denmark), allowing a read-only access to the desired data. ZIMS is a global database for the zoological community containing information on over 6,800,000 animals (living and historical), more than 21,000 species and over 167,000,000 husbandry records (ZIMS, 2016). All individuals sampled were registered at ZIMS, thus major specifications were available.

Data collected from ZIMS included the age of the animal or the date of birth, rearing specifications, relevant identifiers (studbook no., ZIMS ID, Microchip No.), sex and life history (number of institutions the animal had lived in).

A questionnaire (Appendix chapter 13.2.1) was designed to get further information about the institutions, where the sampling took place, and possible *T. gondii* infection routes for the felids. In addition to details about the animal collection (number of small exotic felids at the institution), information on animal keeping and husbandry was collected. Data on feeding and food storage, husbandry as in cleaning habits and enclosure interior, details about pest control, and a survey about known incidences of toxoplasmosis as well as other typically cat associated disease (FHV1, Calicivirus, FeLV, FIP, FIV, Feline Distemper) were also requested. Furthermore, the questionnaire contained questions about prophylactic measures such as vaccinations and parasite control.

To make the results comparable, questions were designed as closed-ended questions if possible. Multiple answers were accepted. Questionnaires were filled in by a representative of the institution or by the author during the visit of the institution.

For further analysis, data from the questionnaire and information on individual animals from ZIMS were transformed into the variables described in Table 13.69. Further modifications of the data were done, summarizing answers to more specific variables as described in Table 13.70 (Appendix chapter 13.1).

6.3 Statistical tests and computer programs used for analysis

Data was analyzed and graphs created using Microsoft Excel 2010.

Statistical tests were performed in IBM Superior Performing Software Systems (SPSS) Version 24 (SPSS Inc., Chicago, IL, USA).

The statistical tests used to analyze data for method validation in domestic cats are summarized in Table 6.2.

The statistical analysis aimed at the comparison of paired data obtained on venous plasma (V) and bug-derived plasma (B), whereby each individual time point was analyzed separately. This procedure was preferred, as especially two time intervals were regarded as most relevant in terms of practicability, when using the reduviid bugs on exotic animals i.e. B0 and B1. B0 seemed to be most relevant in animals where direct contact and application of bugs would be possible. This procedure would allow blood withdrawal right after engorgement. B1 would refer to the placement of an "unmonitored bug" within reach of an animal (for example in a sampling crate). In that case retrieving the bug after one hour would be suggested to ensure full engorgement.

In a first step, data were analyzed for normal distribution, using the Shapiro-Wilk test. The outcome of this analysis was used to decide whether parametric or non-parametric statistical tests had to be applied in the further analyses (Table 6.2).

If data followed a normal distribution, mean differences were assessed by the paired sample T-test and the level of correlation by the Pearson correlation test (Table 6.2). Level of correlation was only assessed if $P < 0.05$.

If data were not normally distributed, median differences were examined by the Wilcoxon signed rank test and the level of correlation by the Spearman correlation test (Table 6.2). Again, the level of correlation was only assessed if $P < 0.05$.

Since venous and bug-sample data determined for individual cats were not independent, tests like the analysis of variance (one-way ANOVA) or the Kruskal-Wallis test were not suitable for statistical analysis in this context.

Table 6.2: Summary of statistical tests performed in SPSS to analyze data for the method validation in domestic cats.

Test	Objective	Data analyzed	Requirement	Interpretation
Shapiro-Wilk test	Test for normal distribution	Blood chemical parameter (V, B0, B1); *T. gondii* antibody titers (log_{10}) (V, B0, B1, B2, B4)	Independent random variables	$P < 0.05$ not normally distributed $P > 0.05$ normally distributed
Paired sample T-test	Comparing mean differences between pairs	Blood chemical parameter (V *vs.* B0, V *vs.* B1)	Normal distribution	Statistically significant difference: $P < 0.05$ No statistically significant difference: $P > 0.05$
Pearson correlation test	Test for correlation	Blood chemical parameter (V *vs.* B0, V *vs.* B1)		Table 6.3 (Bühl & Zöfel, 2002) Statistical significance: $P < 0.05$
Wilcoxon signed rank test	Comparing median differences between pairs	Blood chemical parameter (V *vs.* B0, V *vs.* B1); *T. gondii* antibody titers (log_{10}) (V *vs.* B0, V *vs.* B1, V *vs.* B2, V *vs.* B4)	No normal distribution	Statistically significant difference: P < 0.05 No statistically significant difference: P > 0.05
Spearman correlation test	Test for correlation	Blood chemical parameter (V/B0, V/B1); *T. gondii* antibody titers (log_{10}) (V *vs.* B0, V *vs.* B1, V *vs.* B2, V *vs.* B4)		Table 6.3 (Bühl & Zöfel, 2002) Statistical significance: $P < 0.05$

The interpretation of correlation coefficients followed the interpretation guidelines in Bühl and Zöfel (2002) (Table 6.3). Anyway, correlations were regarded as non-correlating when P-values exceeded 0.05.

Table 6.3: Interpretation of correlation coefficients (Spearman r or Pearson's r) following Bühl and Zöfel (2002)

Correlation coefficient r	Interpretation
r = 0	No correlation
0 < r < 0.2	Very limited correlation
0.2 < r < 0.5	Limited correlation
0.5 < r < 0.7	Moderate correlation
0.7 < r < 0.9	Good correlation
0.9 < r < 1	Very good correlation
r = 1	Perfect correlation

Fisher's exact test (2 tailed) was performed to evaluate different grades of hemolysis between the different sampling methods in domestic cats. For the calculation of P-values, the following program was used: http://www.langsrud.com/stat/Fishertest.htm. Statistical significance was accepted at $P < 0.05$.

In zoo samples, the serological results for *T. gondii* were considered as the dependent variable. Because felids came from different zoos, random effects that might have been caused by different zoos were included in each of the models calculated. Because seropositivity clearly increased with age (details in the Results section), data on the age (in years) of the individual animals were included into each of the models calculated. Animals, for which no sampling date was available (n=13), were excluded from the analysis.

For the identification of potential risk factors, bivariable-multilevel-modelling (generalized linear mixed modelling fit by maximum likelihood (Laplace Approximation)) was performed, using R (http://www.R-project.org), version 3.3.1, by applying the package "Lme4". Because seropositivity clearly increased with age (see chapter 7.4.1) and age was regarded as an important effect-modifying explanatory variable, data on the age (in years) of the individual animals were included into each of the models calculated.

To find out whether the input variables were independent from each other in the data set, a factor analysis (assuming a maximum number of possible factors, respectively) was done using the command "factanal" (scores='Bartlett'). Factor loadings of > 0.5 were regarded as an indication of dependence between explanatory variables. Dependent variables were reduced to one, choosing the variable with the best AIC in the bivariable analysis, or by excluding variables that cannot have a relevant biological effect on *T. gondii*-seropositivity.

In a last step, all relevant and independent variables were included into a generalized linear mixed model to determine potential risk factors for Toxoplasma-seropositivity in captive felids. After optimization by a stepwise reduction of variables, excluding those variables, which – if removed – did not cause an increase in AIC, the final linear mixed model was generated (Figure 6.3).

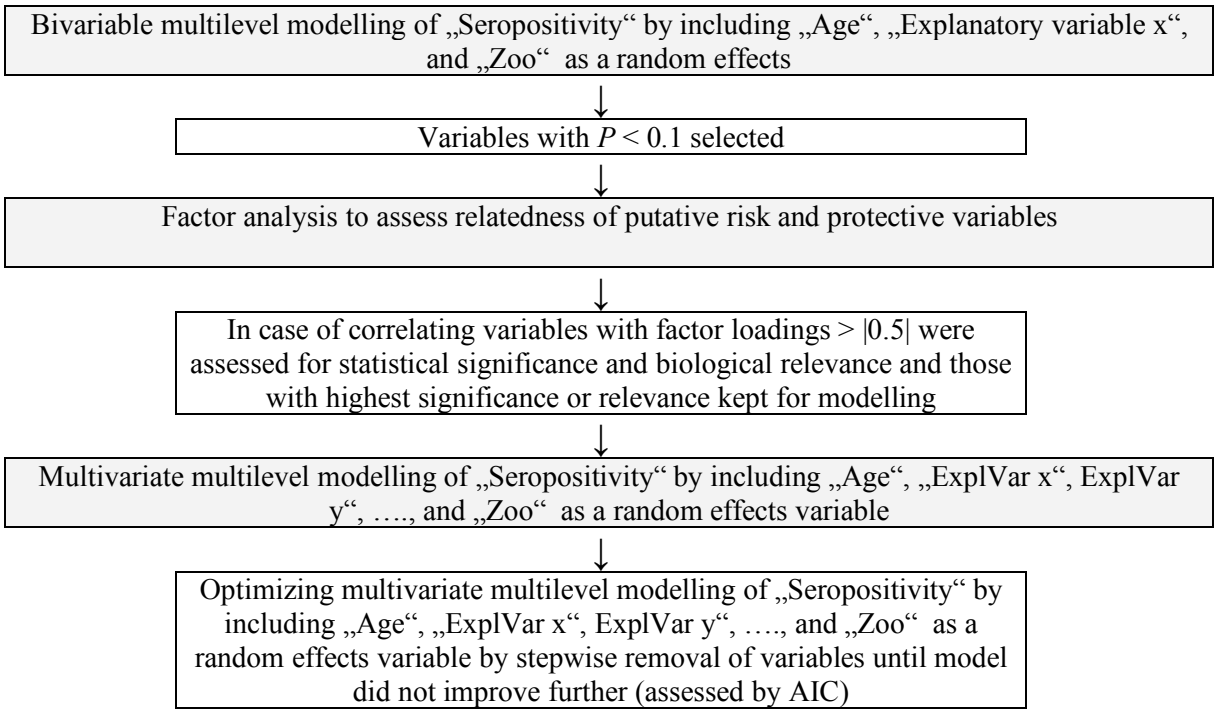

Figure 6.3: Flow chart on data analysis to assess potential risk factors for Toxoplasma-seropositivity.

7 RESULTS

7.1 Suitability of reduviid bugs for blood-sampling cats

All domestic cats exposed to reduviid bugs for blood sampling were monitored for side effects of the bites of *D. maxima*. Immediately after blood collection via bugs, the cats showed small reddish bite marks with a diameter of 1-2 mm where the bugs had bitten. A swelling could not be observed. None of the cats showed any signs of adverse reactions such as swelling, itching, pain to the bite after recovery from anesthesia. Already a short time after the procedure (within 1-2 hr), bite marks could no longer be identified macroscopically. Owners were asked to report any noticeable reactions like itching, swelling or reddening, at the site where the bugs had bitten the cats, during the following days. Individuals that received stationary treatment were investigated at the animal clinic. None of the owners reported any side effects and no side effects could be observed in stationary cats either.

In zoos, the use of reduviid bugs for collecting blood from felids was successful as well. An incubator allowed safe transport of bugs at an optimal temperature, so that they were ready to use at any time. The concept of the sampling box enabled the contact between the bugs and small cats without a risk of escaping reduviids. In addition, retrieval of fully engorged bugs from the “bug-drawer” was quick and easy.

The biggest challenge were low temperatures at some sampling sites. Especially during the first zoo visits in France, which were performed in October 2011, low temperatures reduced effectivity of the bugs. During this period, outside temperatures between 11°C and 17°C were recorded. Four bugs per cat were used to make sure that at least one fully engorged bug could be retrieved. At 11°C, however, none of the bugs was fully engorged after contact with the cats. This occurred in four individuals, so that sampling had to be repeated on another day. In 56 individuals, one or two bugs out of four finished their blood meal with full engorgement at temperatures between 13°C and 15°C. Above 16°C, the proportions of success increased

further, so that the number of bugs was reduced to three. At 17°C, almost all bugs accomplished full blood engorgement.

7.2 Validation of the methods for clinical blood chemistry in domestic cats

Blood samples were collected as described in 5.1.3. From each cat, a set of three samples was collected for blood chemical analyses: Venous plasma (V) and plasma samples collected from reduviid bugs immediately after engorgement (B0) or after one hour (B1).

Samples from 33 randomly selected individual cats were chosen, of which a sufficient volume of plasma was available for examination.

While two samples (6%) were hemolytic in visual appearance in conventional sampled plasma (V), 29 B0 samples (88%) and 28 samples (85%) in B1 were hemolytic. In both cases (B0 and B1) the number of hemolytic samples was statistically significantly higher than in V ($P < 0.001$, Fisher's exact test).

Lipemic samples (identified by the opaque appearance of the plasma) occurred in three V samples (9%), in six samples (18%) from B0 and in seven cases (21%) from B1. The number of lipemic samples was not significantly higher in bug-derived samples than in conventional plasma samples, neither in B0 nor in B1 ($P = 0.475$; $P = 0.303$), according to the Fisher's exact test.

A paired sample T-test was performed for Chol, Glob, Glu, Cl, K, and TMg to evaluate the effects of the blood sampling method on biochemical parameters. ALT, Alb, AP, CK, Crea, GLDH, TBil, TP, Tri, urea, PHOS, Na and TCa were not normally distributed; hence the non-parametric Wilcoxon signed rank test was used.

The correlation among conventional blood samples and bug-derived B0 and B1 samples was determined using the Spearman or Pearson correlation test, depending on the results for testing normal data distribution.

7.2.1 Organic blood parameters

7.2.1.1 Alanine aminotransferase (ALT)

For ALT, the data of 33 sample sets were statistically analyzed. The ALT values in venous samples showed a good correlation with the values measured in B0 with a Spearman correlation coefficient of 0.781 ($P < 0.001$). By contrast, venous samples showed only a moderate correlation with values measured in B1 (Spearman correlation coefficient of 0.611 ($P < 0.001$)) (Figure 7.1).

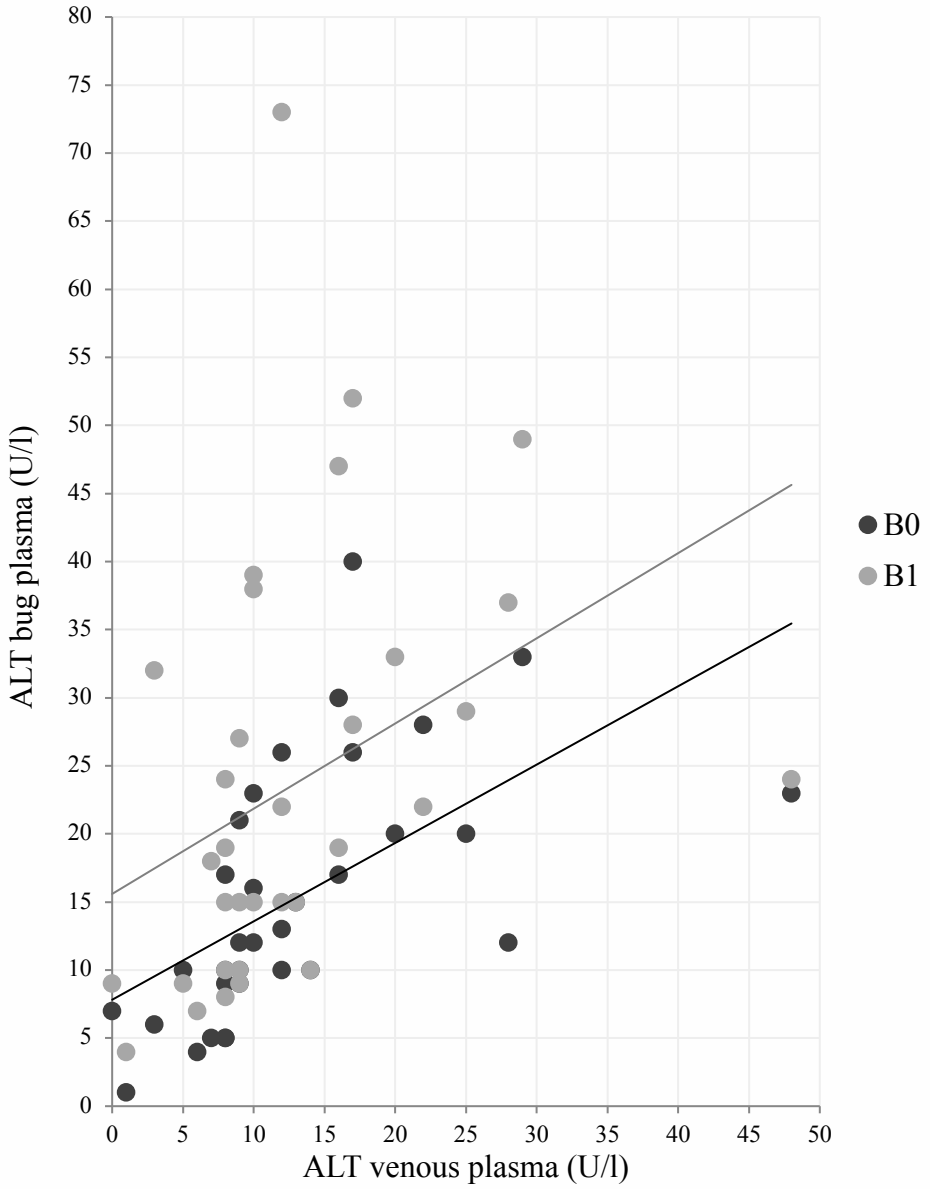

Figure 7.1: Correlation of ALT values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.781, $P < 0.001$; Spearman r for V *vs.* B1: 0.611, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for the ALT values suggest that the ALT results were spreading with increasing time between engorgement ended and sampling the bugs. In addition, the median and the 0.25 and 0.75 percentiles rose with increasing time between the end of engorgement and sampling (Figure 7.2). While the Wilcoxon signed rank test did not reveal statistically significant differences between the ALT values in V and B0 ($P = 0.053$), the difference between V and B1 was significant ($P < 0.001$).

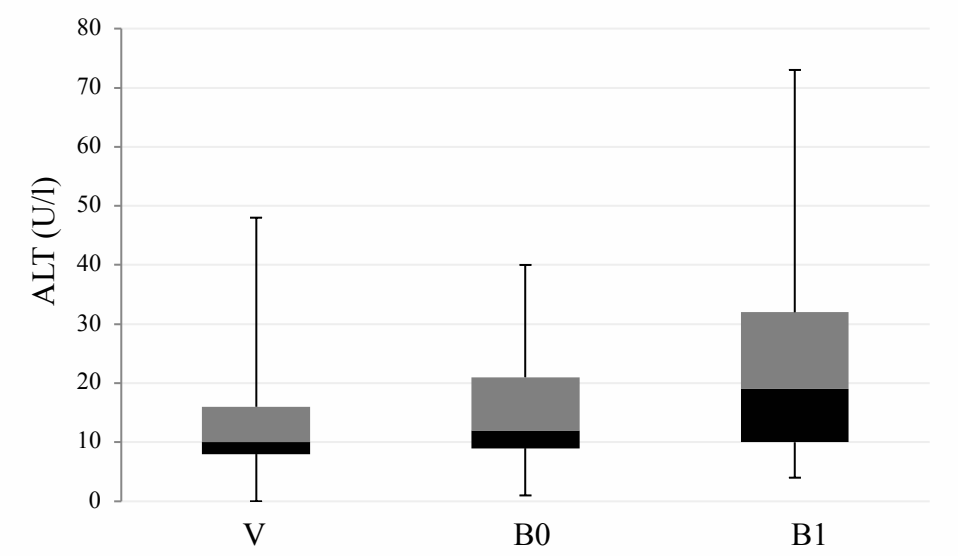

Figure 7.2: Boxplot of ALT values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

However, in one of the samples, an ALT value above the reference range (0-70 U/l) was observed in B1.

7.2.1.2 Albumin (Alb)

In 33 samples, Alb values were examined. The albumin values in venous samples showed a good correlation with those measured in B0 and B1 as indicated by Spearman correlation coefficients of 0.813 ($P < 0.001$) and 0.758 ($P < 0.001$), respectively (Figure 7.3).

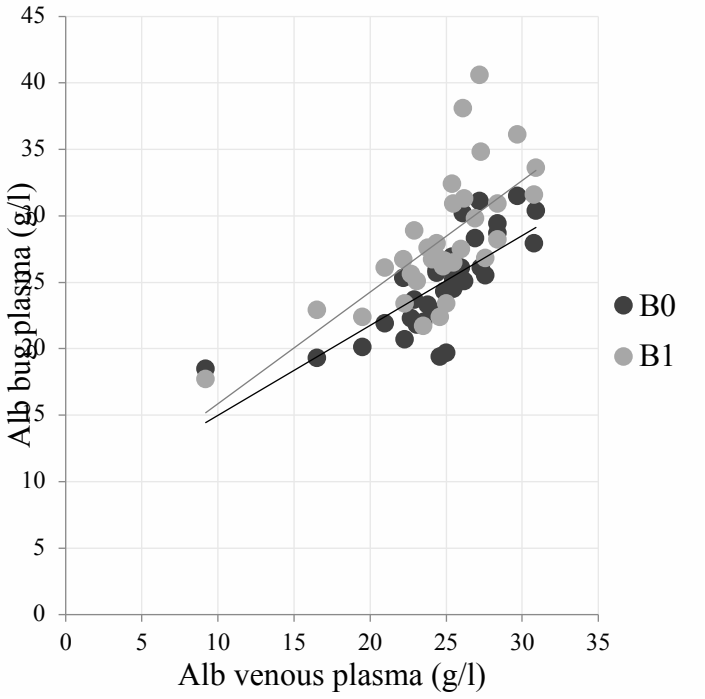

Figure 7.3: Correlation of Alb values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.0.813, $P < 0.001$; Spearman r for V *vs.* B1: 0.758, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for Alb values showed that the range of results got larger with increasing time between engorgement and sampling of the bugs. While the median values in V and B0 were almost the same, they were markedly higher in B1 than in V (Figure 7.4). The Wilcoxon signed rank test revealed no statistically significant difference between V and B0 ($P = 0.636$). In contrast, a statistically significant difference was observed in the comparison of V with B1 values ($P < 0.001$).

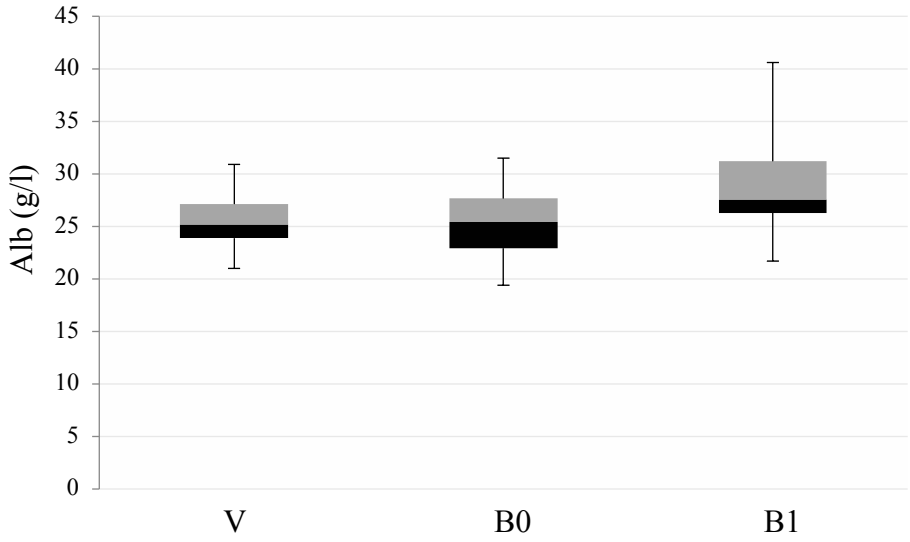

Figure 7.4: Boxplot of Alb values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

The Alb values in two V samples were below the reference range (21-33 g/l), but within the reference range in B1. Three samples that exhibited V values within the reference range tested below the reference range in B0, but had values within the reference range in B1. In four individuals, B1 samples revealed values above the reference range. One individual had Alb values below the reference range in all three samples (V, B0 and B1)

7.2.1.3 Alkaline phosphatase (AP)

For the validation of AP values, 33 samples were available. The correlation of AP values from V and those measured in B0 was characterized by a Spearman correlation coefficient of 0.640 ($P < 0.001$, moderate correlation). In contrast, the results for B1 samples showed no correlation with values measured in V, with a Spearman correlation coefficient of 0.263 ($P = 0.140$) (Figure 7.5).

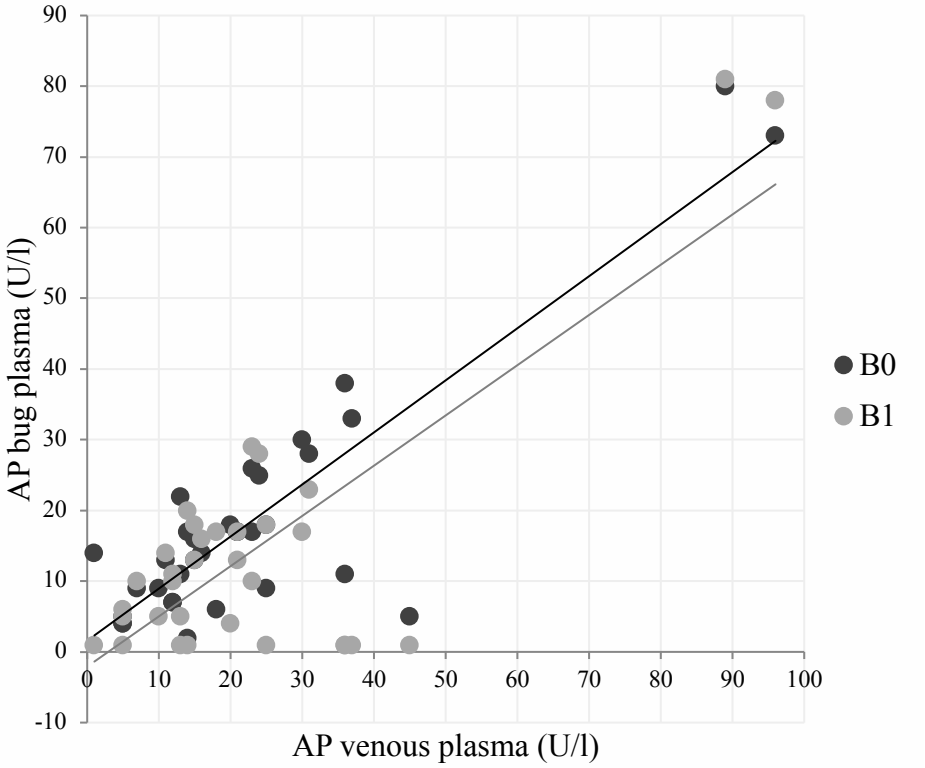

Figure 7.5: Correlation of AP values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.640, $P < 0.001$; Spearman r for V *vs.* B1: 0.263, $P = 0.140$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for AP values showed an increasing range of results from B0 to B1. The median values, the 0.25 and 0.75 percentiles for AP were lower at longer time intervals between engorgement and sampling of the bugs (Figure 7.6). AP values of V and B0 or V and B1 differed statistically significantly (Wilcoxon signed rank test; $P = 0.007$ or $P < 0.001$, respectively).

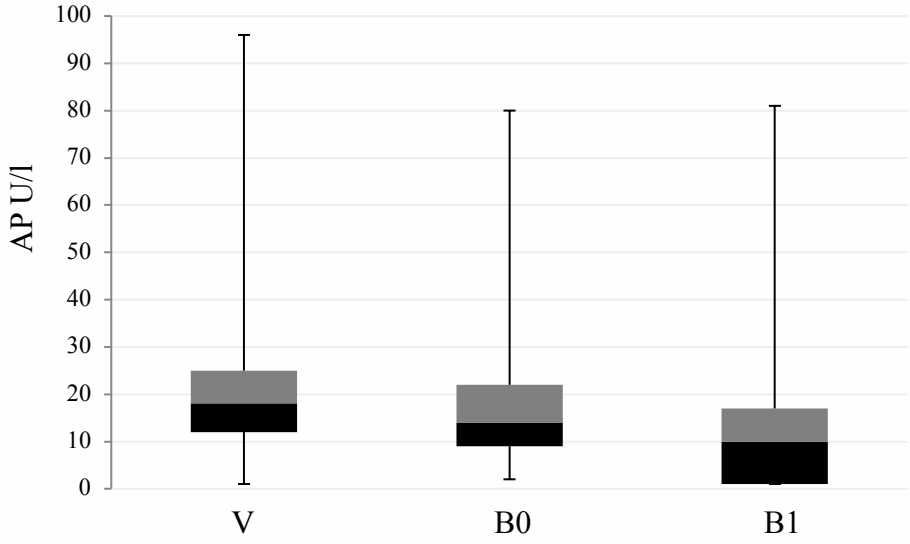

Figure 7.6: Boxplot of AP values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

In two cats, AP values in V, B0 and B1 were above the reference range (0-39.7 U/l). In one of the cats, an AP value above the reference range was measured in V. AP values in that animal were within the reference range in B0 and B1.

7.2.1.4 Cholesterol (Chol)

The Chol values of 33 venous samples showed a strong correlation with values measured in B0 and B1 with Pearson correlation coefficients of 0.842 ($P < 0.001$) and 0.838 ($P < 0.001$), respectively (Figure 7.7).

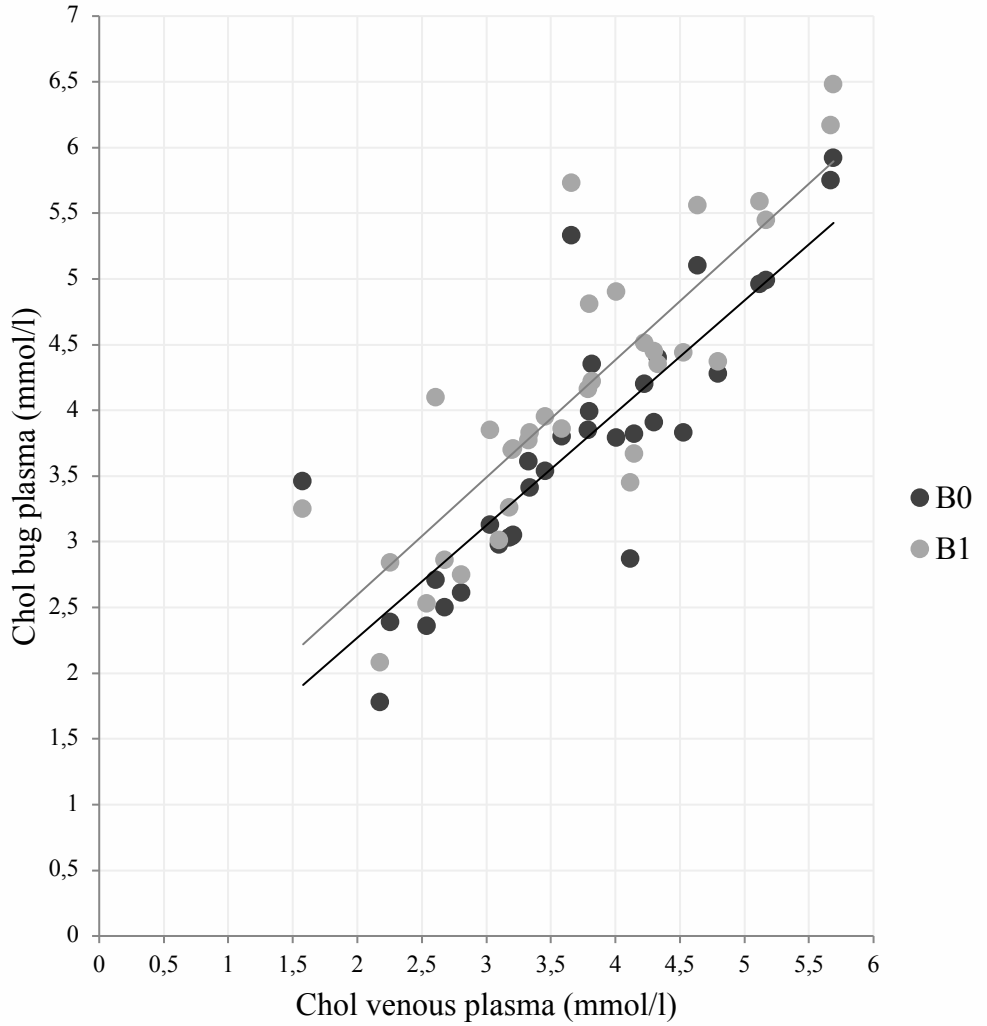

Figure 7.7: Correlation of Chol values: venous plasma (V) and bug-derived plasma (B0, B1). Pearson's r for V *vs.* B0: 0.842, $P < 0.001$; Pearson's r for V *vs.* B1: 0.838, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for Chol values displayed very similar ranges with increasing time between engorgement and sampling of the bugs. Also, the median values in V, B0 and B1 were almost the same, but rising slightly (Figure 7.8), while there was no statistically significant difference observed between V and B0 according to the paired sample T-test ($P = 0.804$). This did not account for the difference between V and B1 ($P < 0.001$).

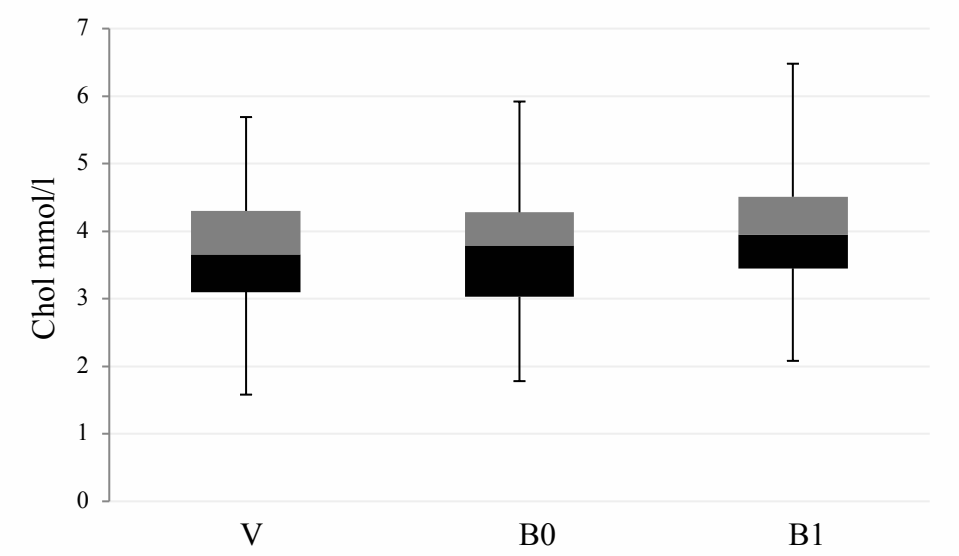

Figure 7.8: Boxplot of Chol values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

The Chol values of four B1 samples were above the reference range (2.46-3.37 mmol/l), while the samples for V and B0 were within the range. For one cat, V and B0 samples were below the reference range, while a value within the range was measured in B1. Two cats had normal Chol values in V, but values above the reference range in B0 and B1. In 23 samples, the level of the results (below/within or above the reference range) did not change with increasing time between engorgement and sampling of the bugs. From those 23 individuals 17 showed values above the range in all three samples.

7.2.1.5 Creatine kinase (CK)

For CK, only 32 samples were included in the analysis for V as one individual showed extreme CK values due to fractured bones in the patient. The resulting CK value represented an extreme outlier and the sample was excluded from the analysis to avoid bias by the outlier. In B0, one additional sample could not be analyzed due to the small sample volume. This was also the reason for the loss of further five samples in B1, resulting in a total of 26 samples in B1. The CK values in V showed good to very good correlation for the values measured in B0 and B1 with Spearman correlation coefficients of 0.801 ($P < 0.001$) and 0.925 ($P < 0.001$), respectively (Figure 7.9). Due to the distinctive spread of CK values, $\log_{10}$ transformed data was used for analysis as displayed in figures 7.9 and 7.10.

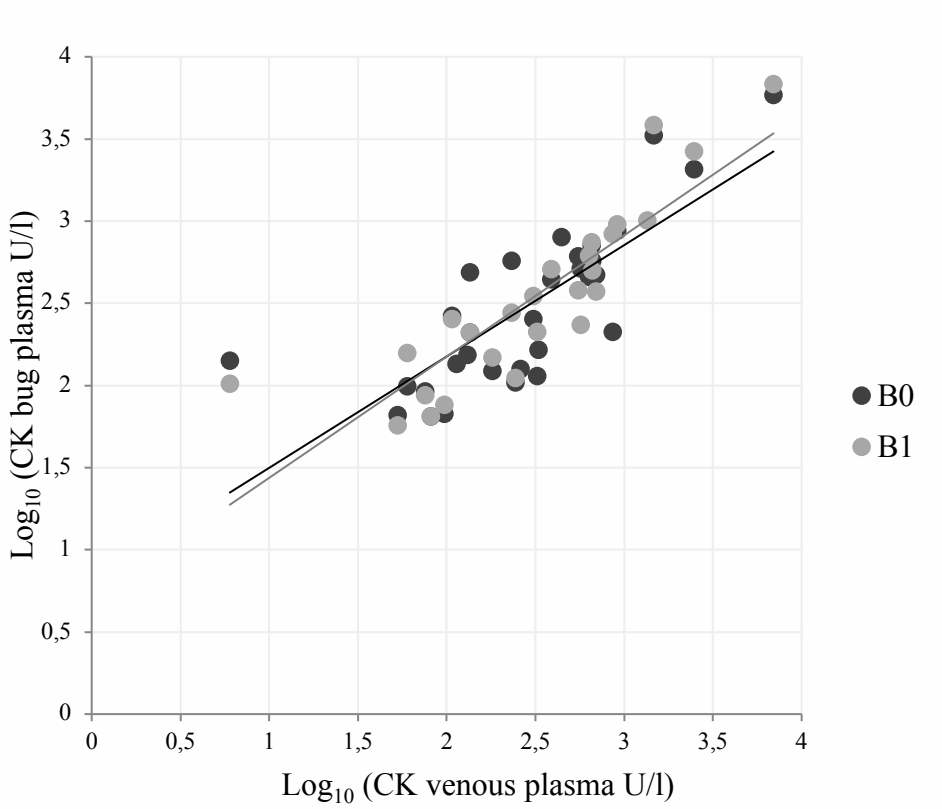

Figure 7.9: Correlation of CK (log_{10}) values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.801, $P < 0.001$; Spearman r for V *vs.* B1: 0.925, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for CK values showed very similar inter quartile ranges with increasing time between engorgement and sampling of the bugs. In addition, the median values in V, B0 and B1 were slightly different (Figure 7.10), but the differences were not statistically significant (Wilcoxon signed rank test; V and B0: $P = 0.487$; V and B1 ($P = 0.732$).

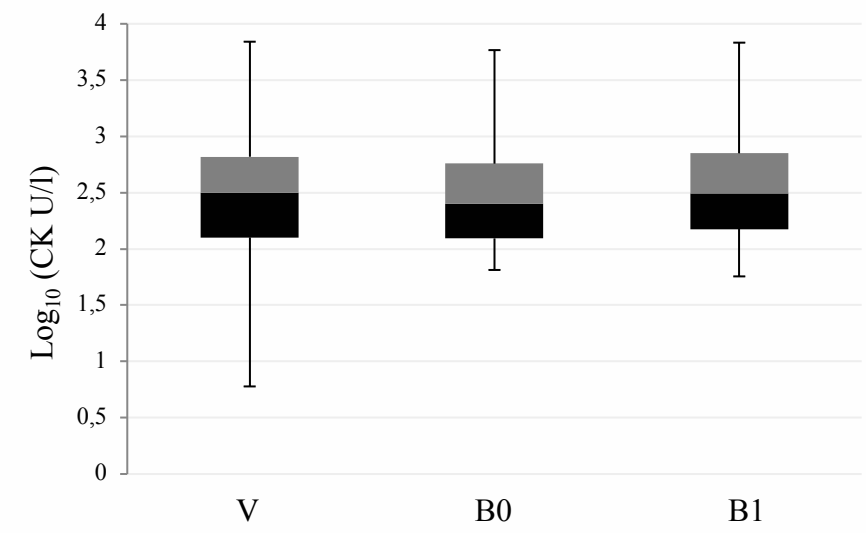

Figure 7.10: Boxplot of CK (log_{10}) values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 32; B0, n = 31; B1, n = 26.

The CK values in samples from three individuals were inside the reference range (< 205 U/l) in V and above the reference range in B0 and B1. CK values in samples from three other cats were above the reference range in V and within the reference range in B0 and B1. In 21 samples, the level of the results (within or above the reference range) did not change with increasing time between engorgement and sampling of the bugs, 14 of them showed values above the reference range. In 13 samples, CK values were higher in V samples than in bug-derived ones. In 14 samples, V values were lower than those for bug-derived plasma.

7.2.1.6 Creatinine (Crea)

In 33 sample sets, Crea values were analyzed for V and B0. For B1, 32 sample sets were included, as one extreme outlier had to be excluded. The Crea values in the V samples showed a strong correlation with those measured in B0 and B1 (Spearman correlation coefficients 0.851 ($P < 0.001$) and 0.821 ($P < 0.001$; Figure 7.11).

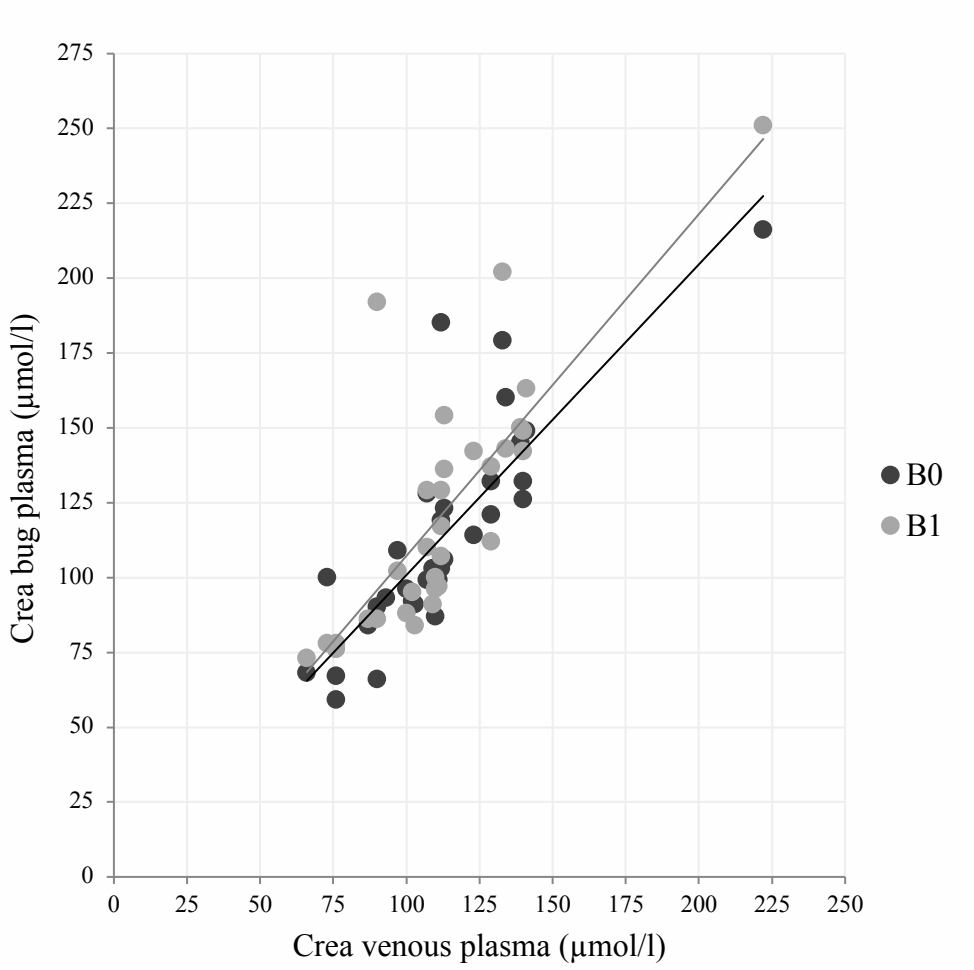

Figure 7.11: Correlation of Crea values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.851, $P < 0.001$; Spearman r for V *vs.* B1: 0.821, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for the Crea values showed that the spread of results became larger with increasing time between engorgement and sampling of the bugs, while the median values varied slightly (Figure 7.12). The differences between the values for V and B0 or V and B1 were not statistically significant (Wilcoxon signed rank test, $P = 0.474$ or $P = 0.081$, respectively).

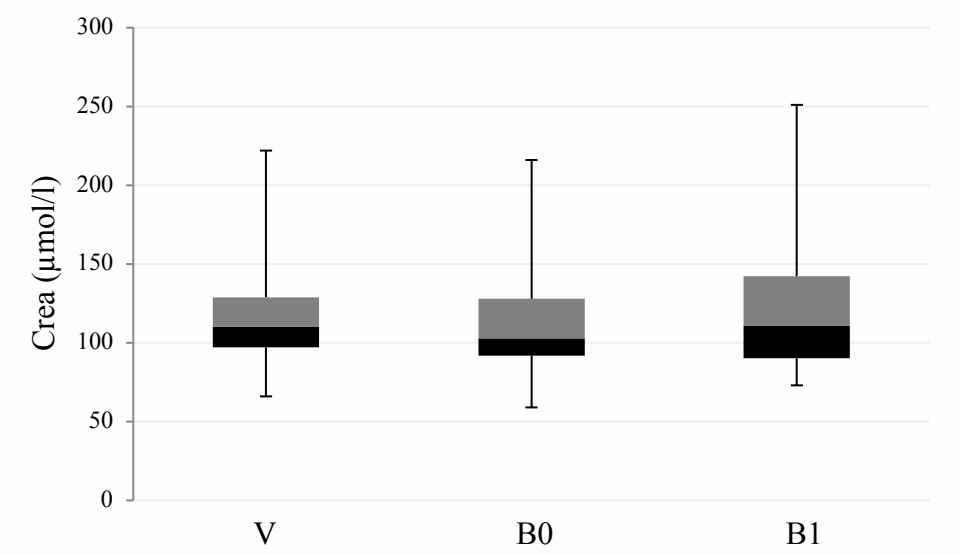

Figure 7.12: Boxplot of Crea values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 32.

Two of the samples showed a Crea change that led to values above the reference range (0-168 µmol/l) in B0 and B1. One individual had Crea values above the reference range in all three samples.

7.2.1.7 Globulin (Glob)

The Glob values in 33 V samples showed a moderate correlation with those measured in the bug-derived B0 samples (Pearson correlation coefficient 0.617, $P < 0.001$). The values for the V samples showed a limited correlation with those for the B1 samples (Pearson correlation coefficient 0.351, $P = 0.045$; Figure 7.13).

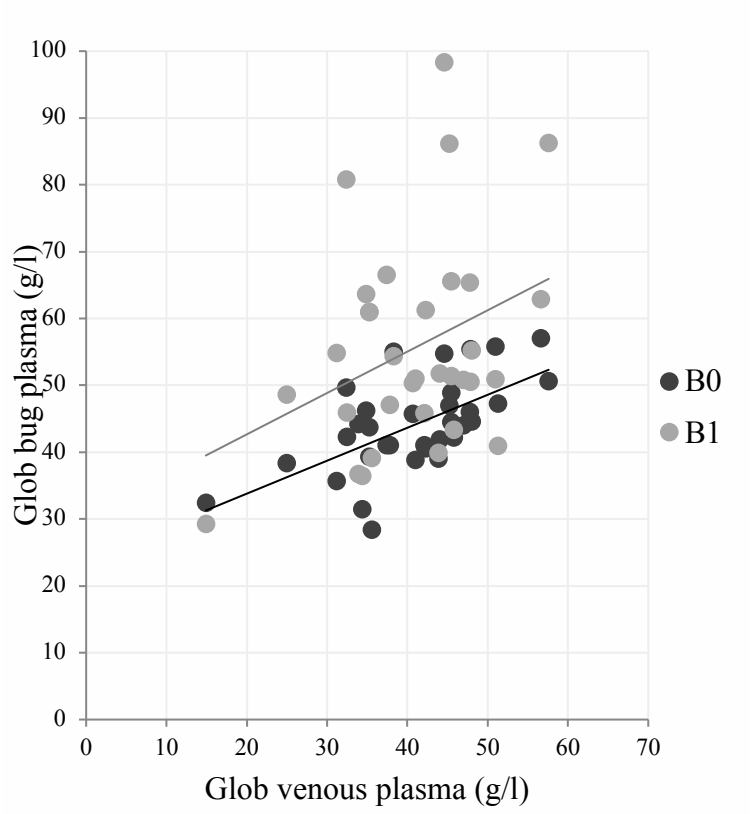

Figure 7.13: Correlation of Glob values: venous plasma (V) and bug-derived plasma (B0, B1). Pearson's r for V *vs.* B0: 0.617, $P < 0.001$; Pearson's r for V *vs.* B1: 0.351, $P = 0.045$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for Glob values showed an increasing range of results from V to B1. While the median values in V and B0 were almost the same, they were markedly higher in B1 (Figure 7.14). Glob values differed statistically significantly between V and B0 or V and B1 (paired sample T-test; $P = 0.027$ or $P < 0.001$, respectively).

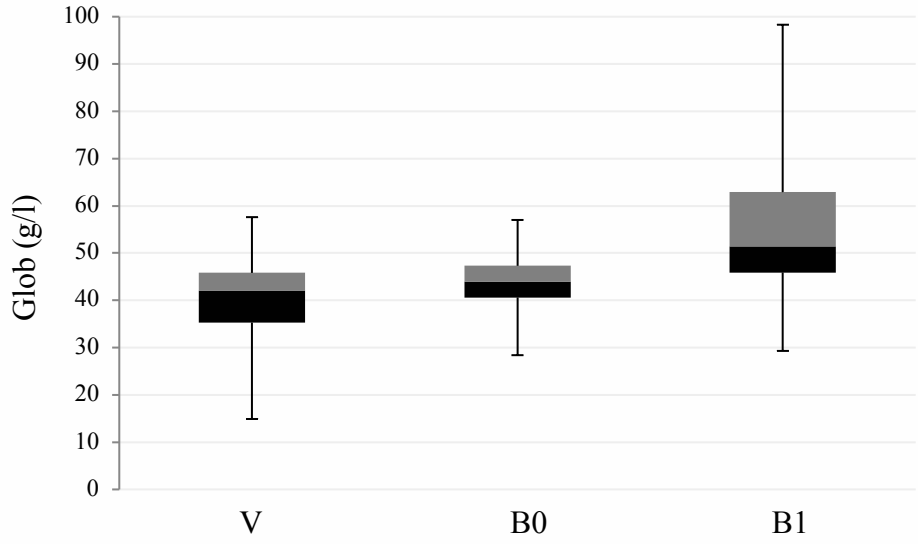

Figure 7.14: Boxplot of Glob values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

The Glob values observed in four B0 samples were above the reference range (26.0-51.0 g/l), but those for V were within the reference range. Twelve bug-derived B1 samples showed values higher than the reference range while their values in V and B0 were within the normal ranges. Four V samples showed values above the reference range, but revealed values within the reference range for B0 or B1 samples. In one of the animals, the V sample tested above the reference range, B0 within the normal range and above the range in sample B1 again. One individual showed results above the reference range in all three samples.

7.2.1.8 Glucose (Glu)

For Glu 33 sample sets were included for V and B0. For B1, only 32 samples could be analyzed due to the small sample volume obtained from one individual. The Glu values in V showed a limited correlation with values measured in B0 with a Pearson correlation coefficient of 0.497 (P = 0.003). Values obtained for V showed a moderate correlation with values measured for B1 (Pearson correlation coefficient of 0.644, $P < 0.001$; Figure 7.15).

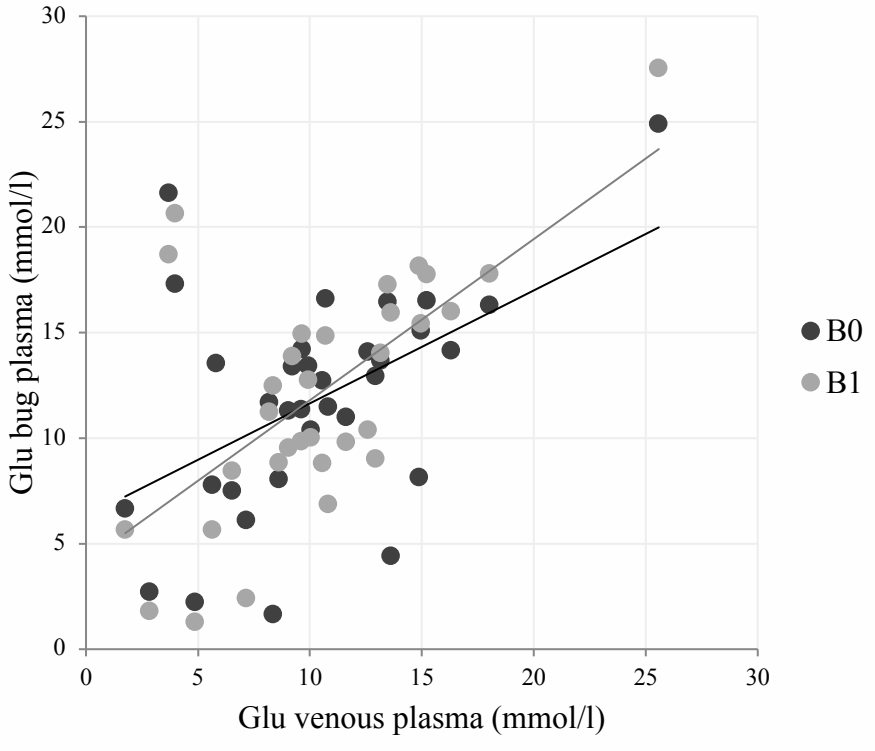

Figure 7.15: Correlation of Glu values: venous plasma (V) and bug-derived plasma (B0, B1). Pearson's r for V *vs.* B0: 0.497, P = 0.003; Pearson's r for V *vs.* B1: 0.644, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for the Glu values displayed similar ranges with increasing time between engorgement and sampling of the bugs. The median values V, B0 and B1 varied between 9.94 mmol/l and 12.72 mmol/l (Figure 7.16). While there was no statistically significant difference between V and B0 (paired sample T-test, P = 0.096), the difference between V and B1 was statistically significant (P = 0.044).

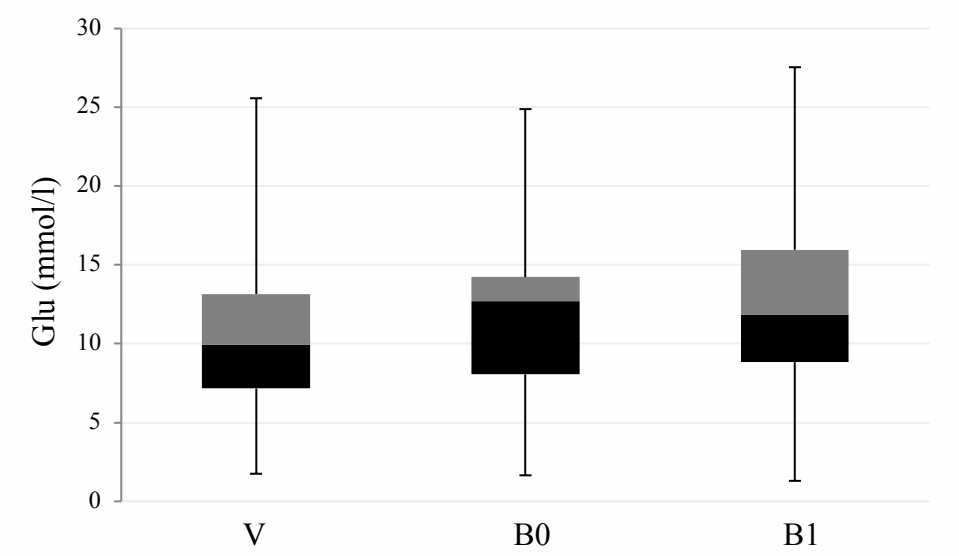

Figure 7.16: Boxplot of Glu values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 32.

23 individuals showed elevated Glu levels in all three samples. In four individuals, the Glu values were within the reference range (3.89-6.11 mmol/l) in V, but higher than the reference range in B0. One sample revealed a change from below the reference range in V to a value above the range in B0 and B1. In another individual, the results showed values below the reference range in all three samples.

7.2.1.9 Glutamate dehydrogenase (GLDH)

In 33 sample sets, GLDH values were analyzed for V and B0. For B1, only 32 sample sets could be analyzed due to the small sample volume obtained from one individual. The GLDH values in venous samples showed a limited correlation with values measured in B0 and B1 (Spearman correlation coefficients 0.451, $P = 0.008$, and 0.363, $P = 0.041$; Figure 7.17). Due to the strong spread of the GLDH values, $\log_{10}$ transformed data was used for analysis and are displayed in the Figures 7.17 and 7.18.

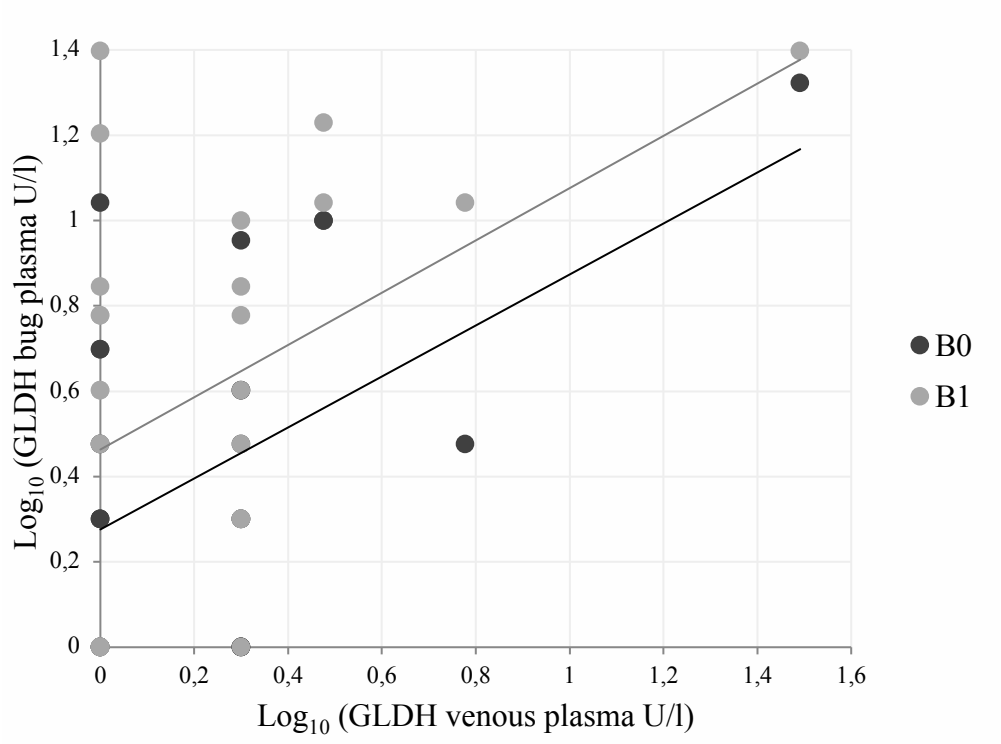

Figure 7.17: Correlation of GLDH (log_{10}) values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.451, $P = 0.008$; Spearman r for V *vs.* B1: 0.363, $P = 0.041$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for GLDH values showed that the values measured were spreading with increasing time between engorgement and sampling of the bugs. In addition, the median values were higher in B0 and B1 than in V (Figure 7.18). The Wilcoxon signed rank test did not reveal statistically significant differences between GLDH values in V and B0 ($P = 0.052$) while the differences were statistically significant between values for V and B1 ($P < 0.001$).

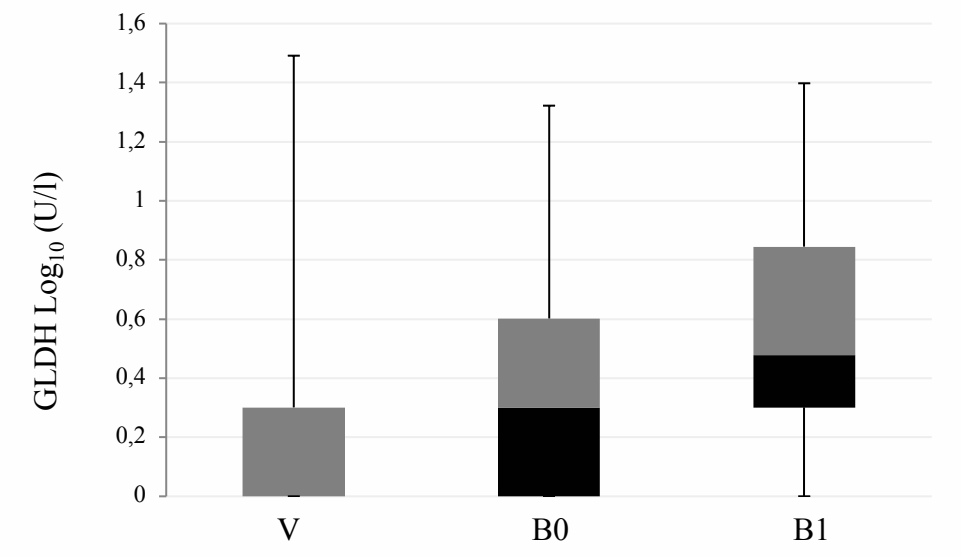

Figure 7.18: Boxplot of GLDH (log_{10}) values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 32.

In one of the animals, all GLDH values were outside of the reference range (0-11.3 U/l) regardless of the use of V or bug-derived plasma for analysis. In samples from three cats with V and B0 values within the reference range, the B1 samples tested above the range.

7.2.1.10 Total bilirubin (TBil)

33 sample sets were analyzed for TBil in V and B0. B1 samples of two individuals, which represented extreme outliers, were excluded from analysis. The TBil values in V showed a moderate correlation with those measured in bug-derived plasma B0 and B1 (Spearman correlation coefficients 0.690, $P < 0.001$ and 0.552, $P < 0.001$; Figure 7.19).

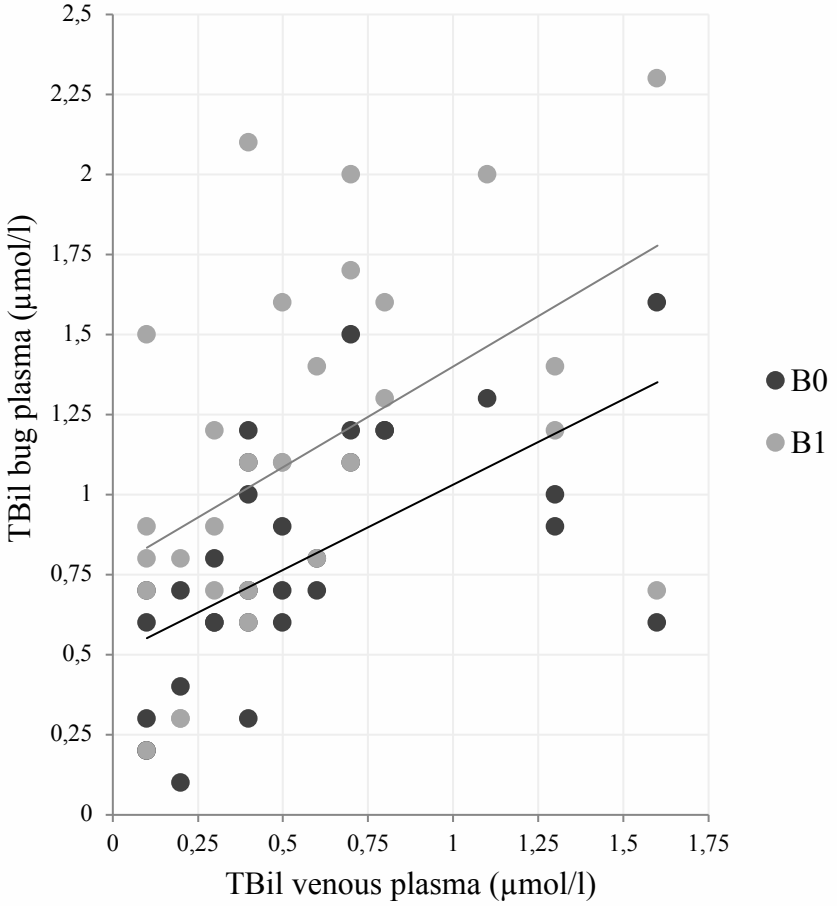

Figure 7.19: Correlation of TBil values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.690, $P < 0.001$; Spearman r for V *vs.* B1: 0.552, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for TBil values displayed a marked spread of the results in the bug-derived samples B0 and B1 compared with V. In addition, the median values obtained in V were lower than those in bug-derived plasma (Figure 7.20). B0 and B1 TBil values differed statistically significantly from the values measured in V (Wilcoxon signed rank test, $P < 0.001$).

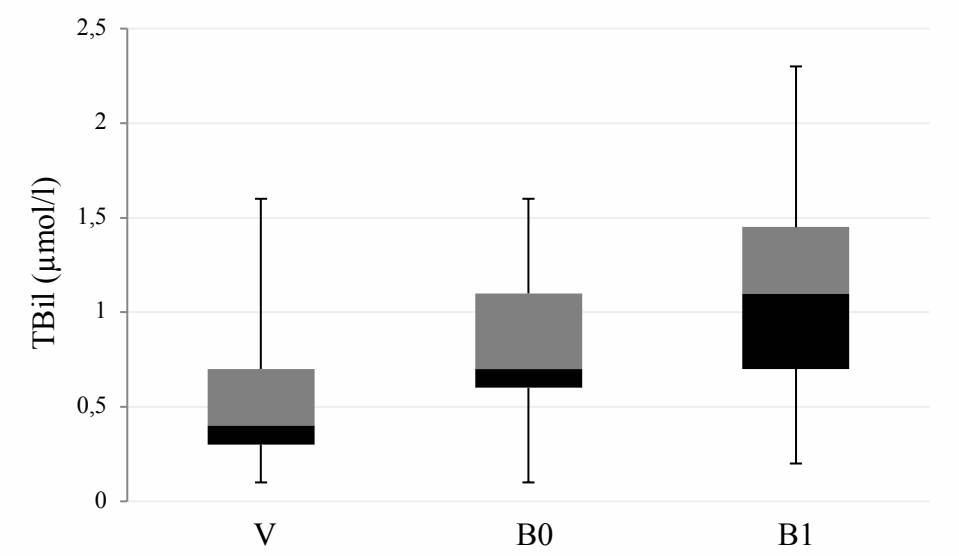

Figure 7.20: Boxplot of TBil values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 31.

All samples collected were within the reference range (0-3,4 µmol/l), independent from the sampling technique.

7.2.1.11 Total protein (TP)

The TP values in 33 V samples showed a moderate correlation with those from B0, and no correlation with values recorded for B1 (Spearman correlation coefficients 0.554, $P < 0.001$, and 0.326, $P = 0.064$; Figure 7.21).

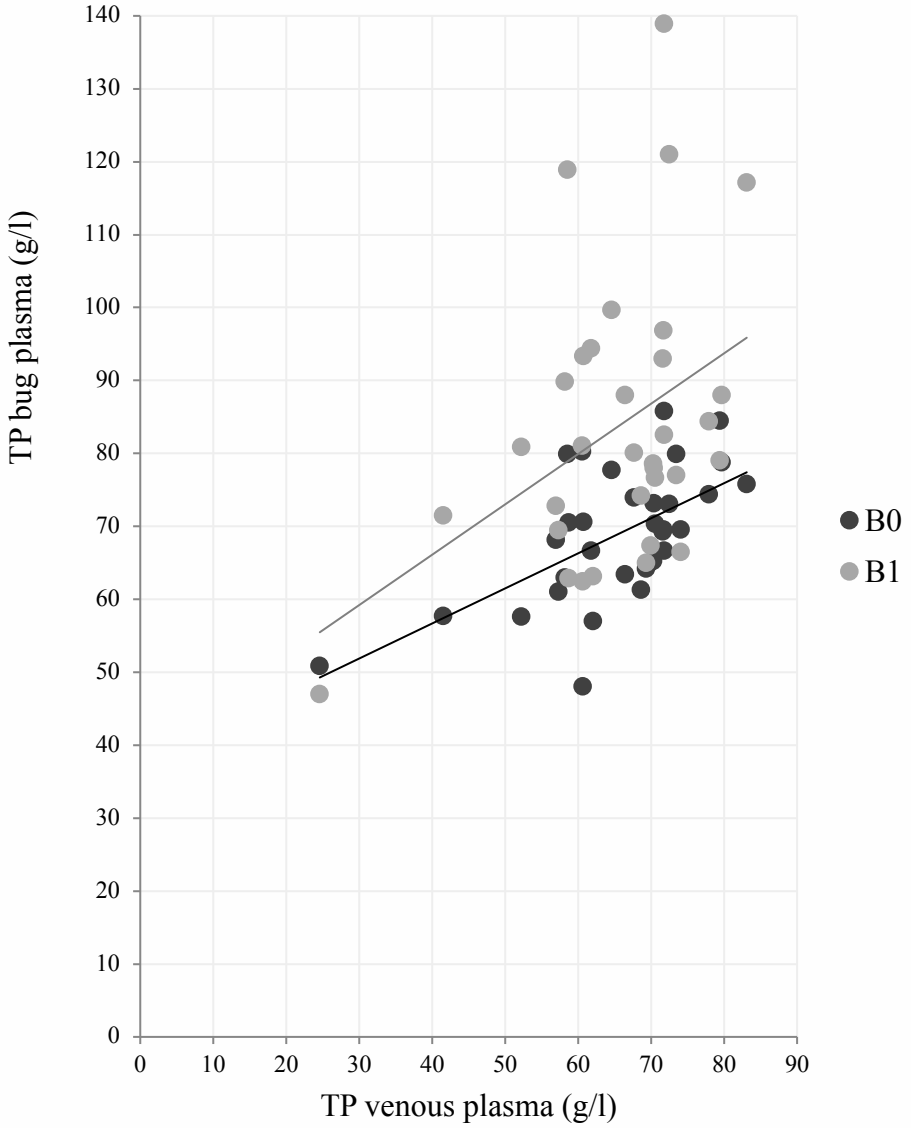

Figure 7.21: Correlation of TP values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.554, $P < 0.001$; Spearman r for V *vs.* B1: 0.326, $P = 0.064$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for the TP values illustrated an increasing range of results from V to B1. While the median values of V and B0 were almost the same, the level was higher in B1. In B1, the range revealed a distinct spread as compared to V and B0 (Figure 7.22). The Wilcoxon signed rank test showed no statistically significant difference between V and B0 ($P = 0.086$). In contrast, the difference between V and B1 was statistically significant ($P < 0.001$).

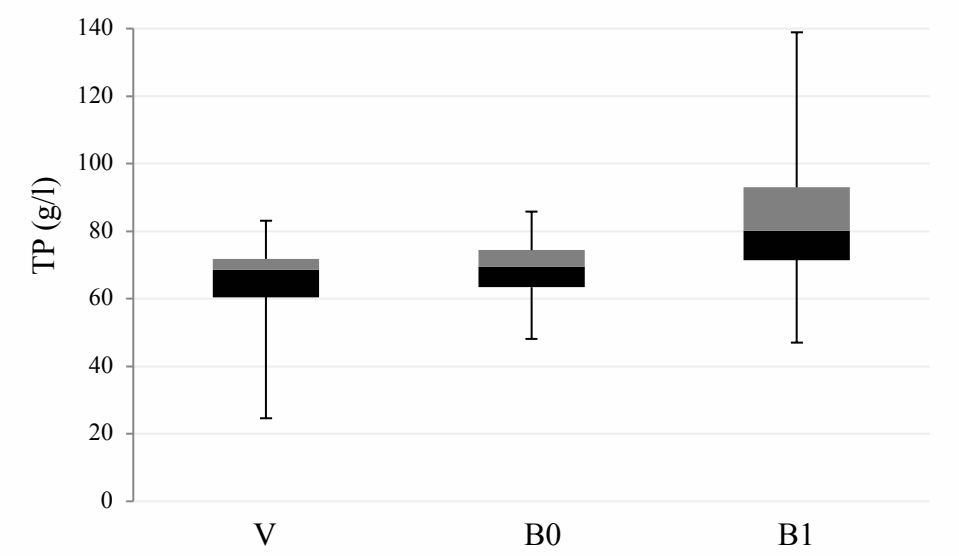

Figure 7.22: Boxplot of TP values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

The TP values of three individuals were above the reference range (54.7-78 g/l) in all three sample types. Another three samples, which had tested above the reference range in V, exhibited values within the reference range when examined in B0. Two of them showed values above the reference range for B1. The TP values for five samples in B0 and 15 in B1 were higher than the reference range, while V values for the respective animals were within the reference range.

7.2.1.12 Triglyceride (Tri)

In 33 sample sets, Tri values were analyzed. Tri values in V showed no correlation with the values measured in bug-derived plasma B0 and B1. Linear regression revealed Spearman correlation coefficients of 0.334 ($P = 0.057$) for V versus B0 and 0.102 ($P = 0.573$) for V versus B1 (Figure 7.23).

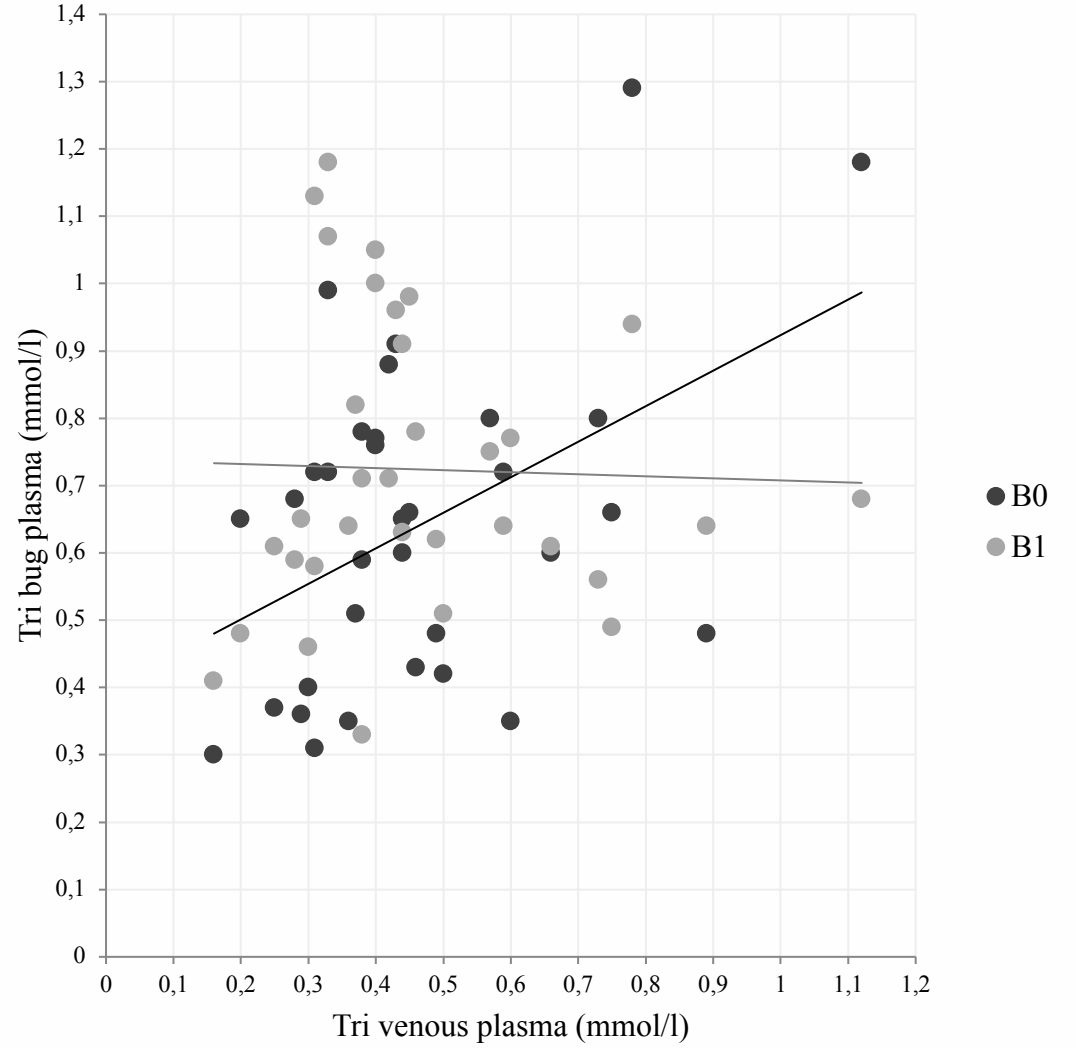

Figure 7.23: Correlation of Tri values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.334, $P = 0.057$; Spearman r for V vs. B1: 0.102, $P = 0.573$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for the Tri values showed that the interquartile range of the measured values was larger for bug-derived plasma (B0 and B1) than for V. In addition, the median values for bug-derived plasma samples were higher than in V (Figure 7.24). Tri values from V versus B0/B1 were statistically significantly different (Wilcoxon signed rank test; $P < 0.001$).

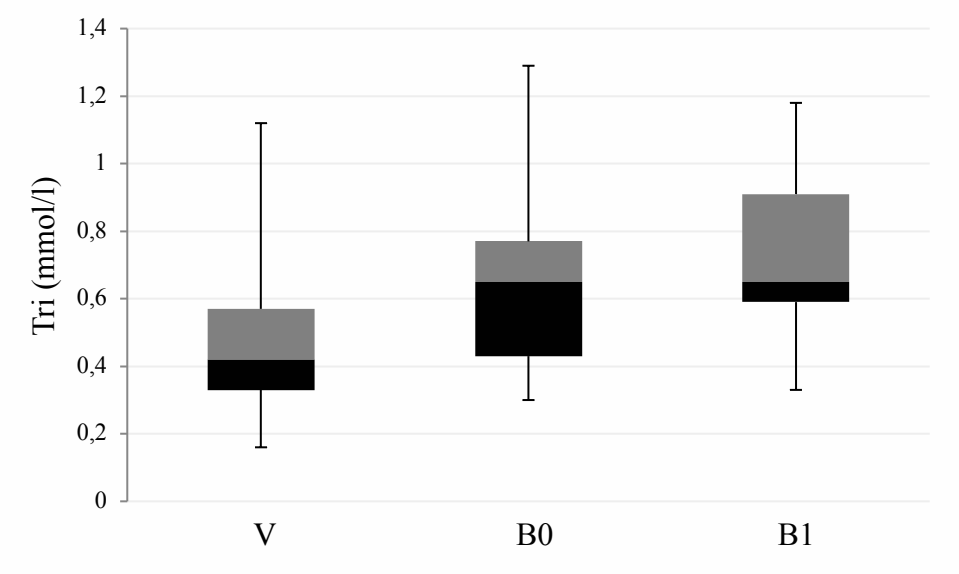

Figure 7.24: Boxplot of Tri values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

The analysis of venous samples obtained from 24 cats revealed values lower than the reference range (0.57-1.14 mmol/l). In 14 of these animals, the B0 samples showed higher values than the V samples, but were within the reference range. In contrast, the B1 samples in one individual revealed values above the reference range. In one individual, increasing values were observed from below the reference range in V, within the range in B0 to values above the range in B1. In six individuals, the categories of the results (below or within the reference range) did not change with increasing time from engorgement to sampling of the bugs.

7.2.1.13 Urea

In 33 individuals, urea was analyzed directly in plasma V samples or in plasma obtained from bugs immediately after engorgement (B0). For 32 individuals, bug-derived plasma B1 was also analyzed. One result obtained for a B1 sample represented an extreme outlier, which was excluded from analysis. The urea values in V samples showed a perfect correlation with the values measured in B0 and B1 (Spearman correlation coefficients of 0.955, $P < 0.001$; 0.963, $P < 0.001$; Figure 7.25).

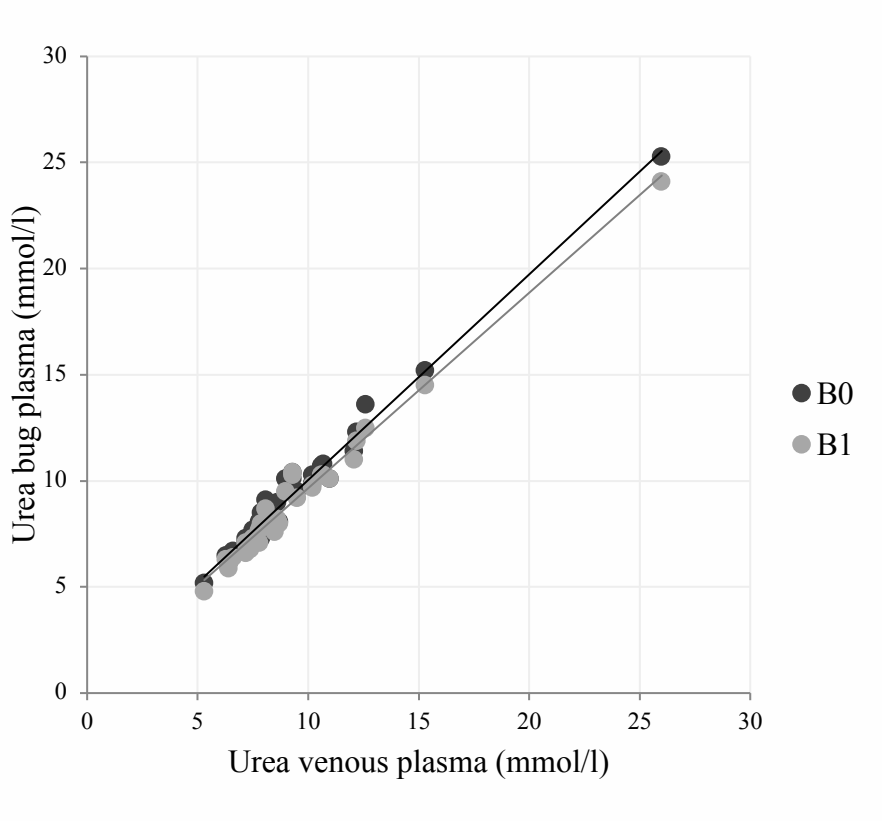

Figure 7.25: Correlation of urea values: venous (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.955, $P < 0.001$; Spearman r for V *vs.* B1: 0.963, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for the urea values showed very similar ranges, regardless whether direct V plasma or bug-derived plasma (B0, B1) had been analyzed (Figure 7.26). While no statistically significant difference could be observed between V and B0 according (Wilcoxon signed rank test, P = 0.651), the difference between V and B1 was statistically significant ($P = 0.016$).

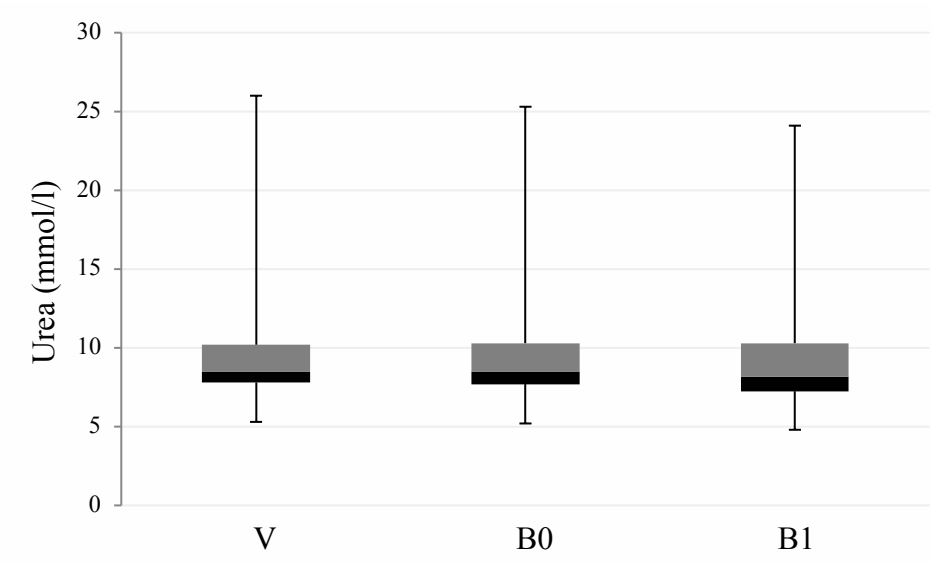

Figure 7.26: Boxplot of urea values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 32.

In 28 individuals, the levels (below/within or above the reference range) did not change regardless of the sample used for analysis (V, B0, B1). For two individuals, the urea values were lower than the reference range (7.14-10.7 mmol/l) in the bug-derived sample B1, although the results for V and B0 were within the reference range. For one cat, which exhibited a urea value higher than the reference range in V, the values obtained for the bug-derived samples B0 and B1 were within the reference range. Another individual showed a B0 value that was higher than the reference, while the results for the V and B1 samples lay within the reference range.

7.2.2 Inorganic components (electrolytes)

7.2.2.1 Chloride (Cl)

For Cl, V samples of 32 individuals were examined, while 31 B0 and 27 B1 samples could be examined. Further samples had to be excluded because the available amount of plasma was too small. The Cl values in V revealed a moderate correlation with the values measured in B0 and B1 (Pearson correlation coefficients 0.545, $P = 0.002$; 0.535, $P = 0.004$; Figure 7.27).

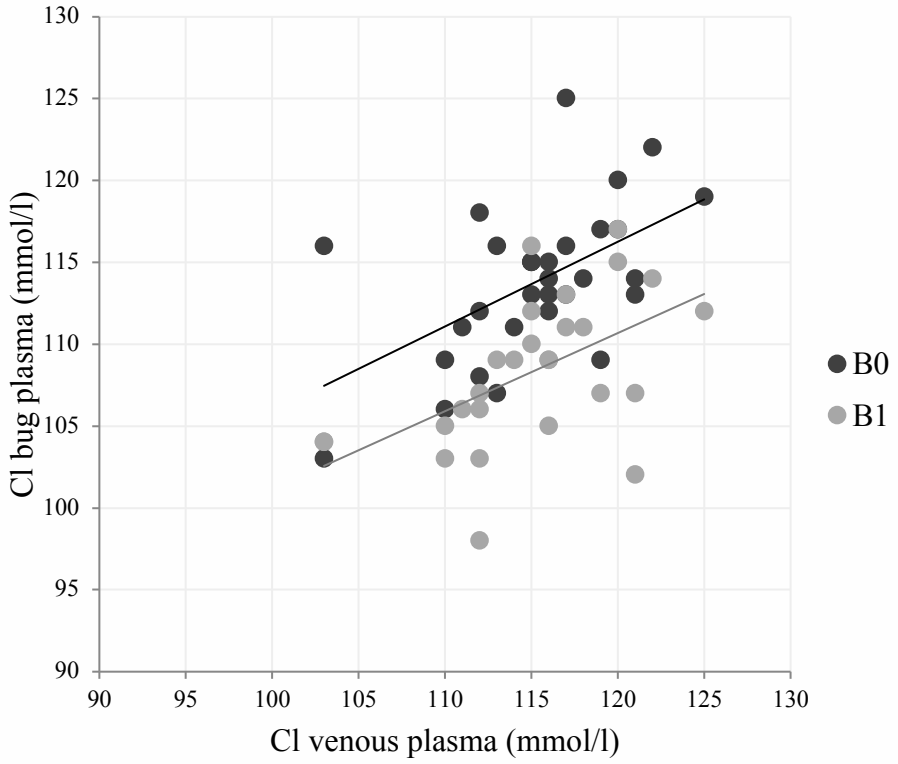

Figure 7.27: Correlation of Cl values: venous plasma (V) and bug-derived plasma (B0, B1). Pearson's r for V *vs.* B0: 0.545, $P = 0.002$; Pearson's r for V *vs.* B1: 0.535, $P = 0.004$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for the Cl values showed very similar ranges irrespective of the sample type analyzed. Also, the median values in V and in the bug-derived samples B0 and B1, were similar, with slightly lower values for B1 samples compared to B0 (Figure 7.28). While difference observed between V and B0 was not statistically significant (paired sample T-test, $P = 0.085$), the difference between V and B1 was statistically significant ($P < 0.001$).

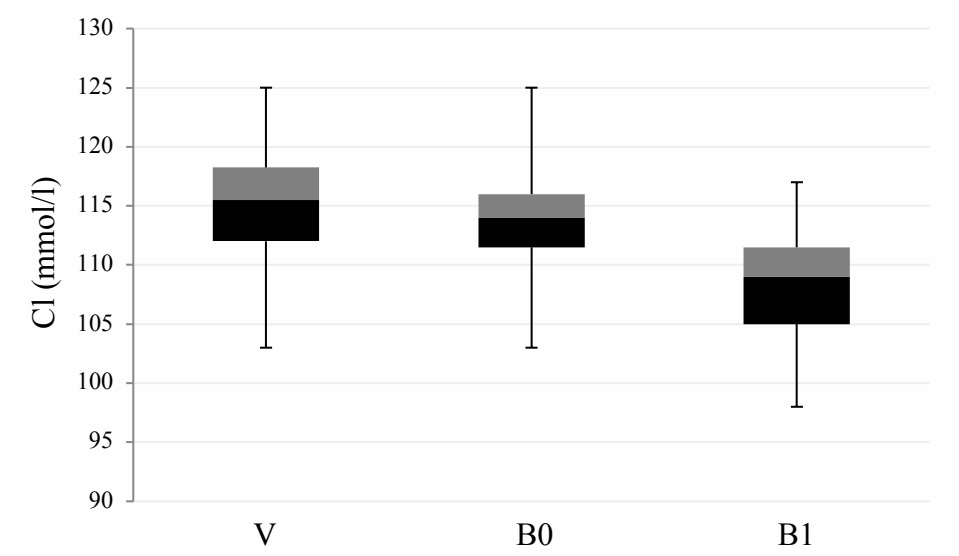

Figure 7.28: Boxplot of Cl values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 32; B0, n = 31; B1, n = 27.

In 14 individuals, all examined samples had values within the reference range. The Cl values for five individuals were within the reference range (110-125 mmol/l) in V, but the values measured for B0 were lower than the reference range. In addition, in 15 individuals the analysis of B1 samples revealed results lower than the reference range.

7.2.2.2 Phosphorus (PHOS)

The PHOS values in 33 V samples revealed a moderate correlation with those measured in the bug-derived samples B0 (Spearman correlation coefficient 0.500, $P = 0.003$) and a limited correlation when the values for V and B1 were compared (Spearman correlation coefficient 0.450, $P = 0.009$; Figure 7.29).

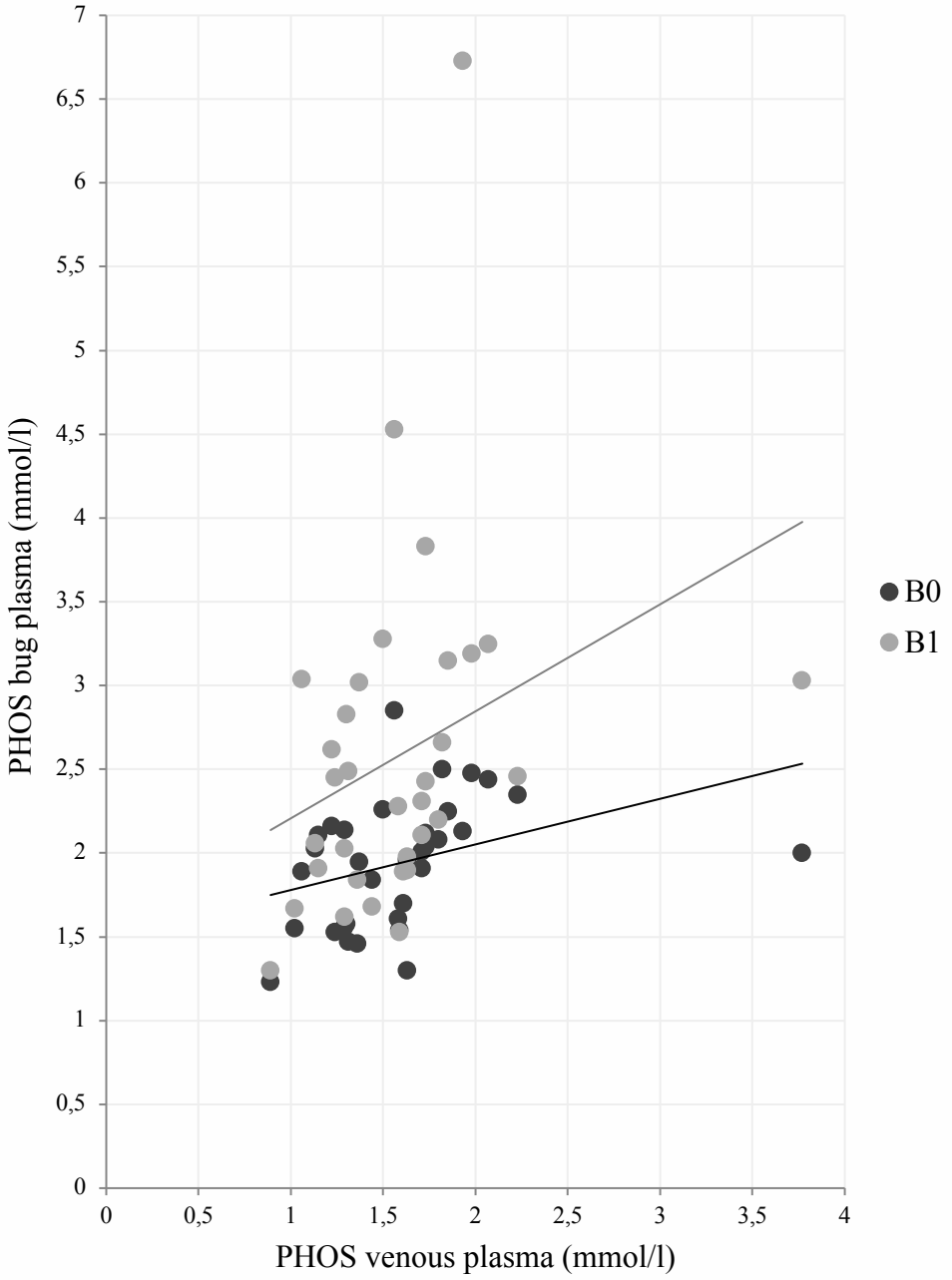

Figure 7.29: Correlation of PHOS values: venous plasma (V) and bug-derived plasma (B0/B1). Spearman r for V *vs.* B0: 0.500, $P = 0.003$; Spearman r for V *vs.* B1: 0.450, $P = 0.009$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for PHOS values showed that the range of results became larger with increasing time from engorgement to sampling of the bugs. The median values increased in B0 compared to V and even further in B1 (Figure 7.30). Statistically significant differences were found between V versus B0 and V versus B1 for the PHOS values (Wilcoxon signed rank test, $P < 0.001$).

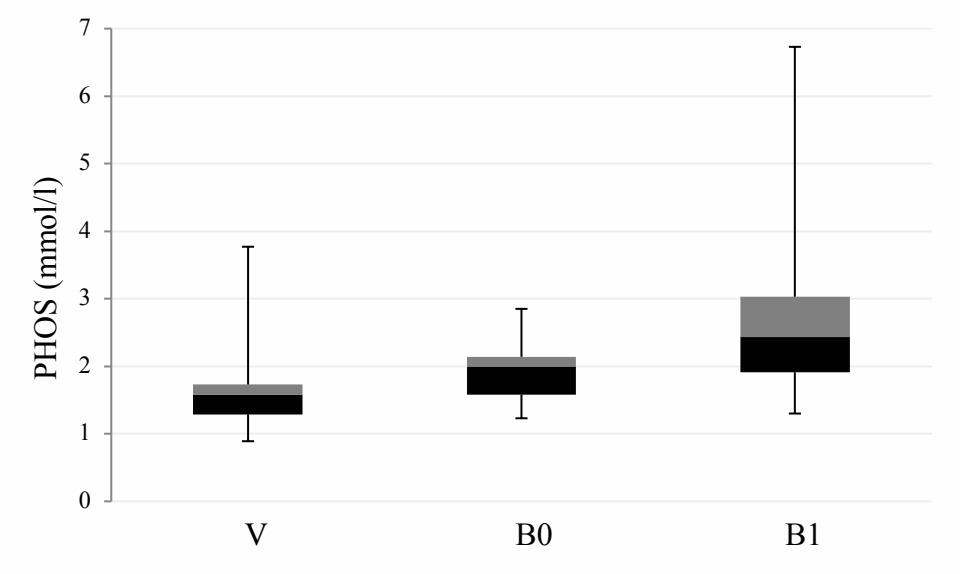

Figure 7.30: Boxplot of PHOS values for venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

In case of 15 individuals, the PHOS values determined for bug-derived samples B0 exceeded the reference range (0.8-1.9 mmol/l), while the values determined in the V sample fell into the reference range. In six further individuals, the values obtained for bug-derived samples B1 were higher than the reference range. In 12 individuals, the categories of the results (within or above the reference range) did not change with increasing time from engorgement to sampling of the bugs (from V to B0 and B1). Five of them had values exceeding the reference range.

7.2.2.3 Potassium (K)

The analysis of K values was performed in 31 and 27 individuals for a comparison of V and bug-derived samples (B0 or B1). Missing values for some cats were caused by insufficient volumes of plasma obtained from some individuals. The K values in V showed a limited correlation with the values measured in B0 (Pearson correlation coefficient of 0.366, $P = 0.043$). A moderate correlation was observed between values measured in V and the values measured in B1 (Pearson correlation coefficient 0.609, $P < 0.001$; Figure 7.31).

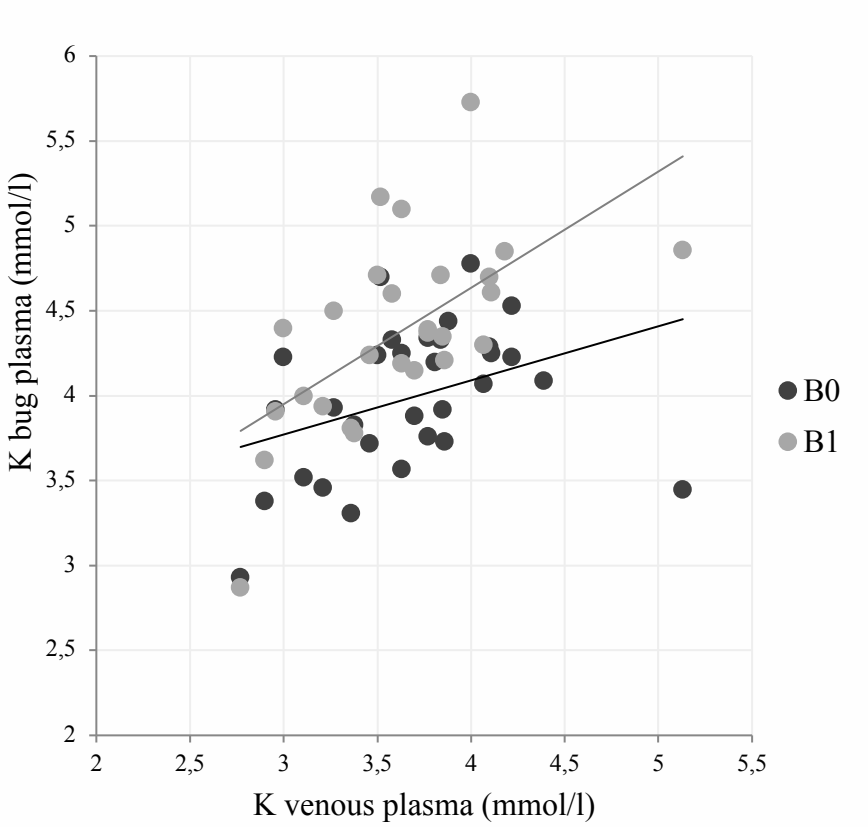

Figure 7.31: Correlation of K values: venous plasma (V) and bug-derived plasma (B0, B1). Pearson's r for V *vs.* B0: 0.366, $P = 0.043$; Pearson's r for V *vs.* B1: 0.609, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for K values exhibited very similar ranges independent of the sample type. However, the median values in the bug-derived samples B0 and B1 were higher relative to those of V and seemed to increase with the time between engorgement and sampling of the bugs (Figure 7.32). The differences of the K values in V versus B0 and V versus B1 were statistically significant (paired sample T-test, $P = 0.003$ or $P < 0.001$, respectively).

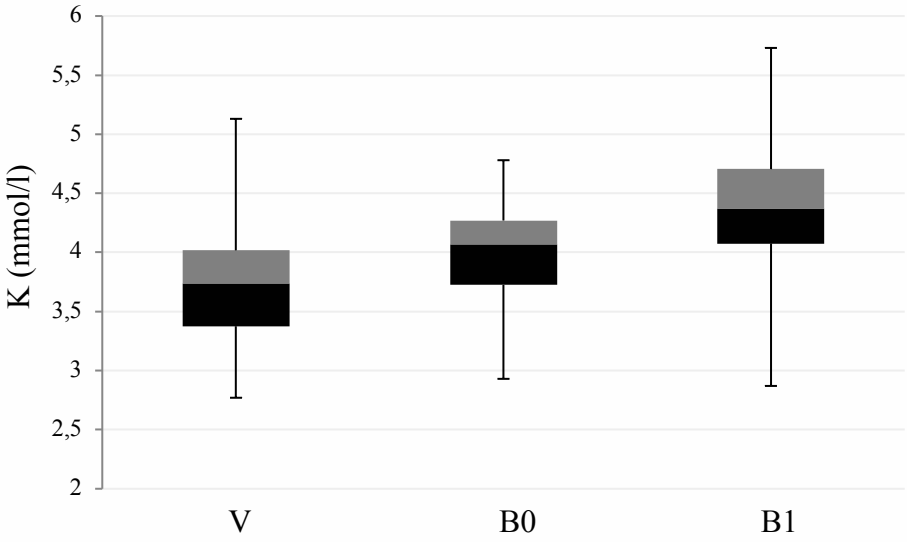

Figure 7.32: Boxplot of K values for direct venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 32; B0, n = 31; B1, n = 27.

In 15 individuals, all three samples analyzed revealed values within the reference range (3.6-4.8 mmol/l) and in one case below the range. The K values in V from eight individuals were lower than the reference range, while the corresponding bug-derived samples fell into the reference range. In four further individuals, values measured in V and B0 were lower than the reference range, while those in B1 fell into the reference range. One cat revealed results below, within and above the reference range when V, B0 or B1 samples were examined.

7.2.2.4 Sodium (Na)

For 31 and 27 individuals, comparisons between V plasma and bug-derived plasma samples B0 and B1 were performed. The Na values in V showed a limited correlation with values measured in the bug-derived samples B0 and a moderate correlation of V and B1 (Spearman correlation coefficients 0.395, $P = 0.028$, and 0.597, $P < 0.001$; Figure 7.33).

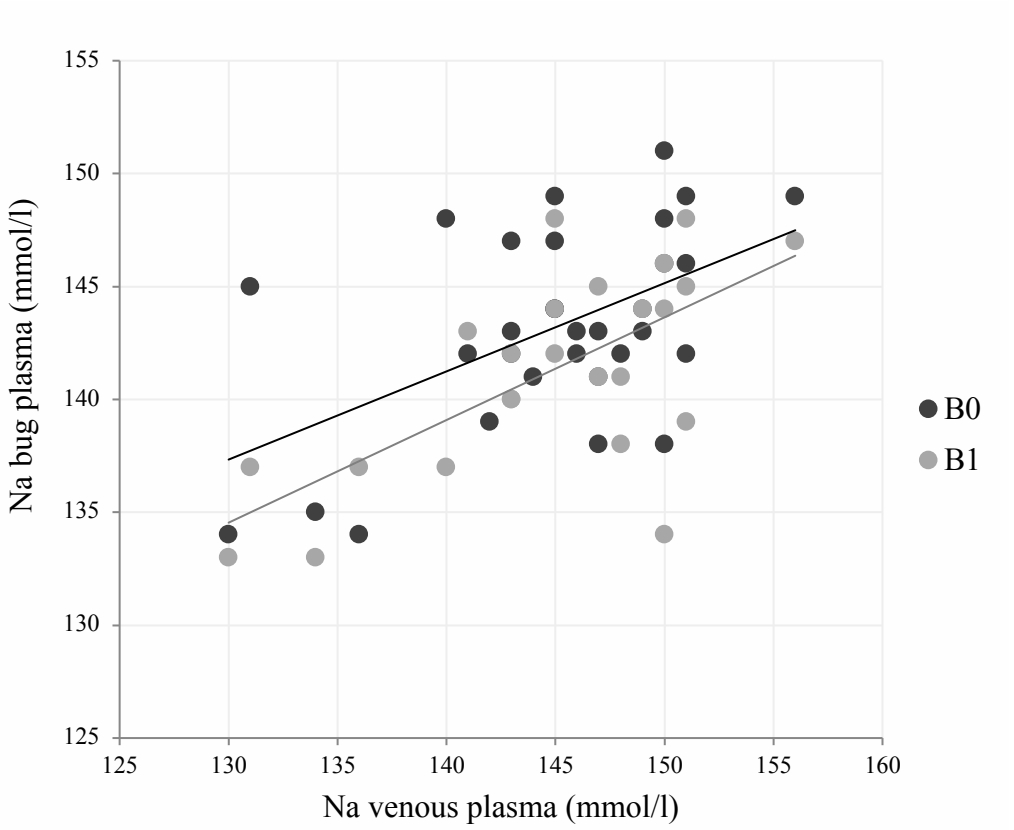

Figure 7.33: Correlation of Na values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.395, $P = 0.028$; Spearman r for V *vs.* B1: 0.597, $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for Na values showed very similar interquartile ranges independent of the sample types. However, the median value obtained for V was higher than that for the bug-derived samples (B0, B1). The median value obtained for B0 was also higher than that obtained for B1 (Figure 7.34). The differences in the Na values for V versus B0 and V versus B1 were statistically significant (Wilcoxon signed rank test, $P = 0.026$ and $P < 0.001$).

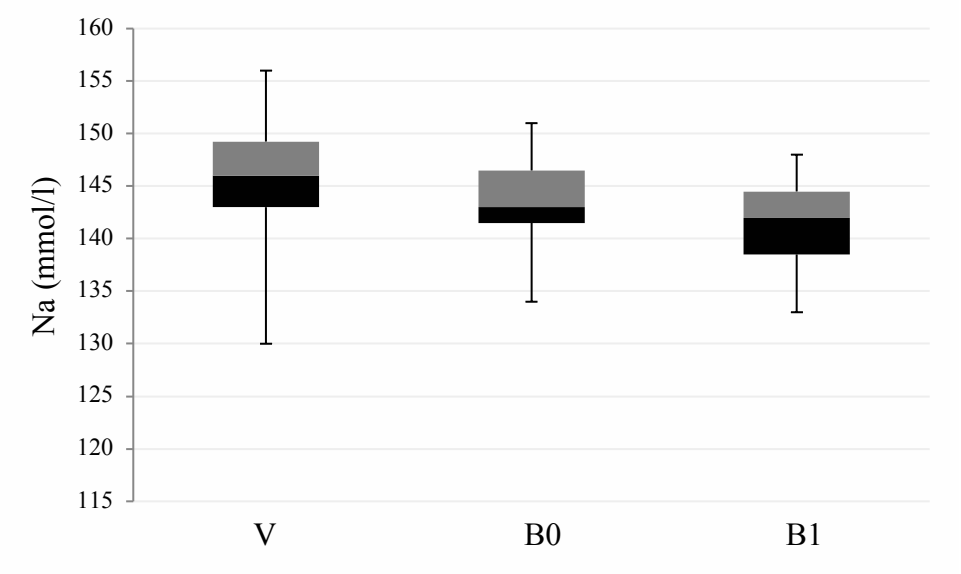

Figure 7.34: Boxplot of Na values for direct venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 32; B0, n = 31; B1, n = 27.

In 16 individuals, all three analyzed samples revealed results within the reference range (141-150 mmol/l), independent of the type of sample. In three individuals, the Na values of bug-derived samples B0 and B1 were lower than the reference range, although all results for V fell into the reference range. In three individuals, both the V and the B0 values were within the reference range, but the B1 values were lower than the reference range. Four individuals showed higher V results than the reference range while B0 and B1 values were within the reference range (three individuals) or the B0 value was within and the B1 value lower than the reference range.

7.2.2.5 Total calcium (TCa)

Samples of all 33 individuals were used to measure TCa in different types of samples. The TCa values in V showed a moderate correlation with the values measured in B0 and a limited correlation was revealed in the comparison of the V and B1 values (Spearman correlation coefficients 0.659, $P < 0.001$, and 0.443, $P = 0.01$; Figure 7.35).

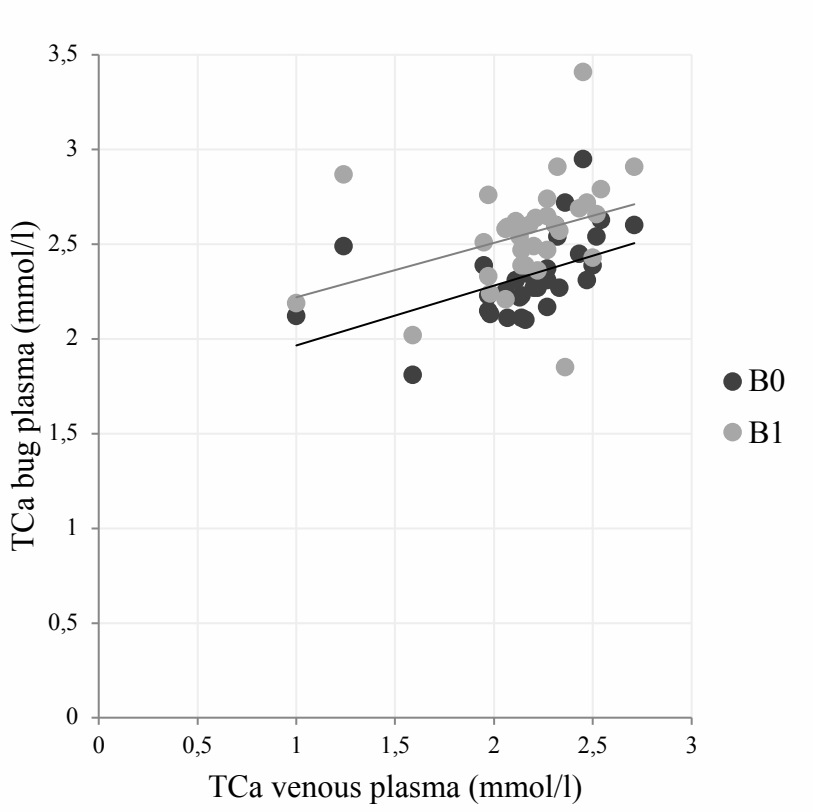

Figure 7.35: Correlation of TCa values: venous plasma (V) and bug-derived plasma (B0, B1). Spearman r for V *vs.* B0: 0.659, $P < 0.001$; Spearman r for V *vs.* B1: 0.443, $P = 0.01$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for TCa values exhibited very similar ranges with increasing time from engorgement to sampling of the bugs. The median values increased from V to B1 (Figure 7.36). B0 and B1 TCa values differed statistically significantly from those measured in V (Wilcoxon signed rank test, $P < 0.001$).

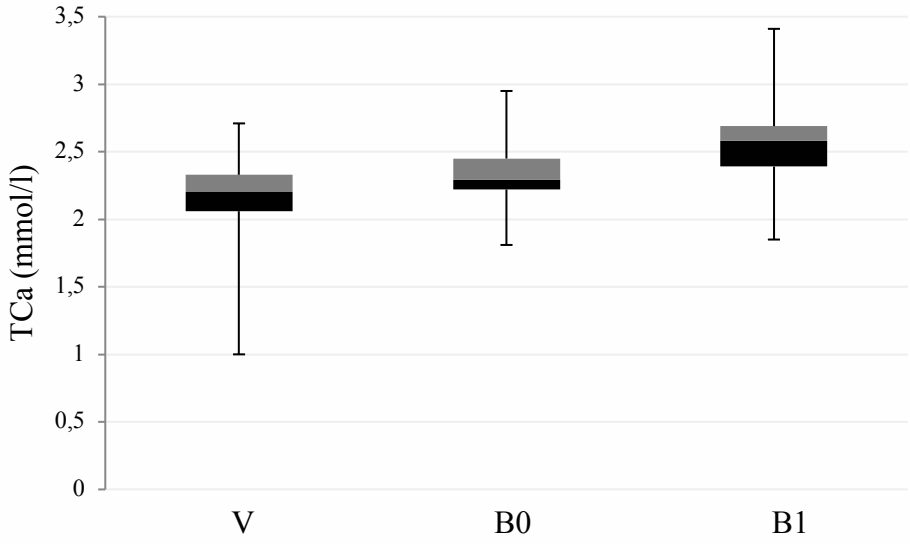

Figure 7.36: Boxplot of Na values for direct venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

Venous plasma samples (V) of 22 individuals showed values below the reference range (2.3-3.0 mmol/l). In six samples, higher values within the range could be observed in B0, while samples in 12 individuals increased into the reference range in B1. One sample had values within the range in V and B0, but above the range in B1. In 12 samples, the level of the results (below or within the reference range) did not change with increasing time from engorgement to sampling of the bugs.

7.2.2.6 Total magnesium (TMg)

The TMg values in 33 venous samples (V) showed a moderate correlation with values measured in B0 (Pearson correlation coefficient 0.570, $P < 0.001$) and no correlation with values measured in B1 (Pearson correlation coefficient 0.338, $P = 0.055$; Figure 7.37).

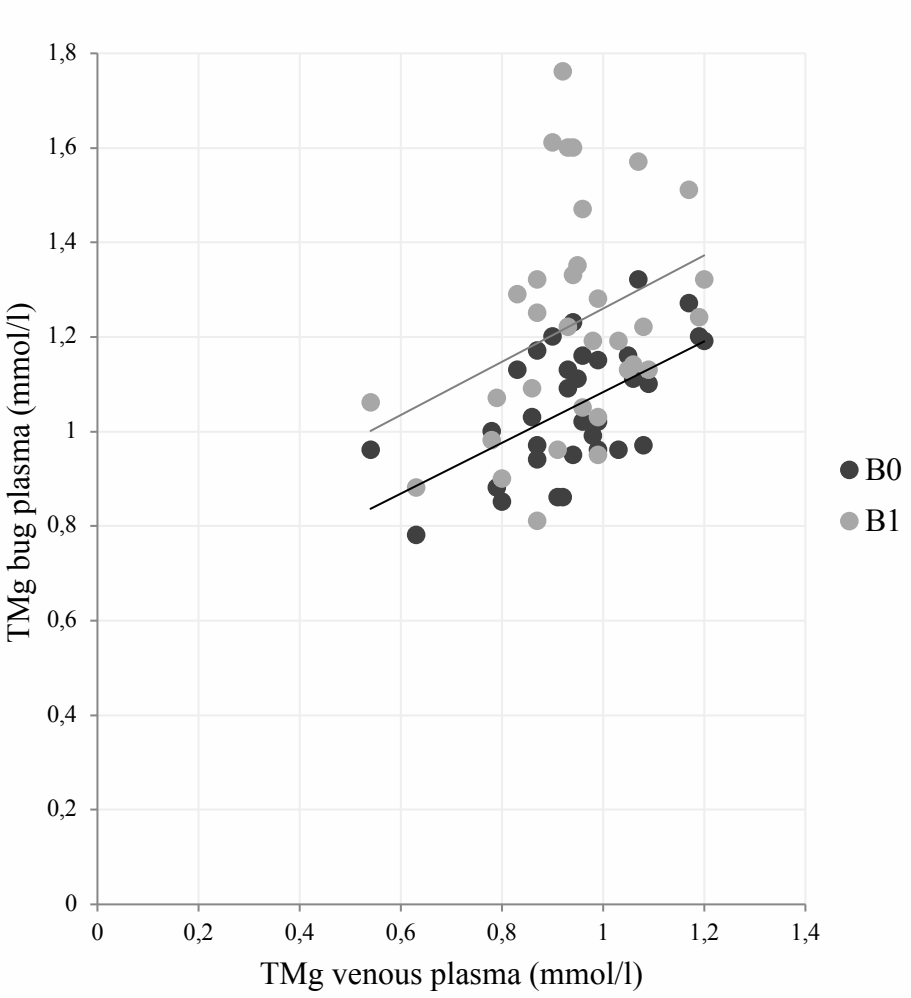

Figure 7.37: Correlation of TMg values: venous plasma (V) and bug-derived plasma (B0, B1). Pearson's r for V *vs.* B0: 0.570, $P < 0.001$; Pearson's r for V *vs.* B1: 0.338, $P = 0.055$; linear regression lines were included using Microsoft Excel 2010.

The boxplots for TMg values revealed larger ranges of results with increasing time from engorgement to sampling of the bugs. The largest range of results was observed for the B1 values. The median values in V were lower than in B0 and B1 (Figure 7.38). TMg values in V versus B0 and V versus B1 samples differed statistically significantly (paired sample T-test, $P < 0.001$).

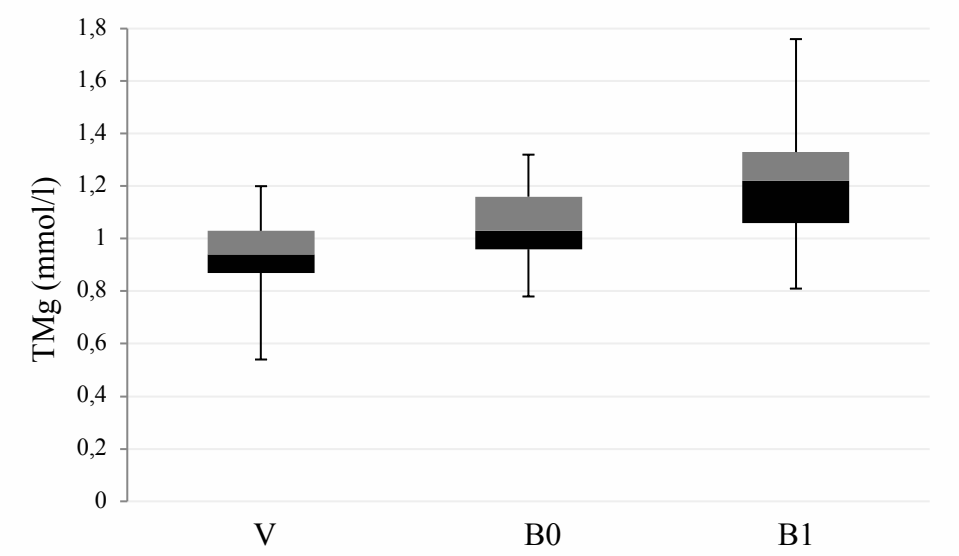

Figure 7.38: Boxplot of TMg values for direct venous (V) and bug-derived plasma (B0, B1). Sample size: V, n = 33; B0, n = 33; B1, n = 33.

In six animals, the TMg values were within the reference range (0.6-1.3 mmol/l) in V and B0, but above the range in B1. In one individual, the value of the V sample exceeded the reference range, while they were inside the range in B0 and B1.

7.2.3 Summary of the validation of blood chemical analysis

In summary, variable results were found for various analytes included in the blood chemical analysis. CK and Crea values showed good correlations between V and B0 or V and B1 values. They did not differ statistically significantly from V neither in B0 nor in B1 according to the Wilcoxon signed rank test. ALT, Alb, Chol and urea had good correlations between V and B0 or V and B1. Their V values did not differ statistically significantly from B0 values but they did in B1. The remaining blood parameters showed no correlation, a limited or a moderate correlation between V and B0 or V and B1. While no statistically significant differences were found between V and B0 in GLDH, Glu, TP and CL, this was the case for V and B1 samples. Tri, Glob, AP, TBil, K, Na, PHOS, TMg and TCa showed statistically significantly varying results between V and B0 as well as V and B1 (Table 7.1).

Table 7.1: Statistical comparison of blood chemical results of the analysis of conventional plasma samples (V) and plasma samples collected with *Dipetalogaster maxima* (B0, B1) from domestic cats.

CORR: Correlation (Spearman r or Pearson's r); Interpretation Correlation: no correlation, limited, moderate, good, very good

Test: (T), paired sample T-test; (W), Wilcoxon signed rank test (statistically significant aberration);

CH: Number of individuals for which a change from within the reference range outside the range or reversed occurred in B0 or B1 compared with V.

Parameter (Test)		**CORR** V *vs.* B0	**CORR *P*** (V *vs.* B0)	**CORR** V *vs.* B1	**CORR *P*** (V *vs.* B1)	**V** ($\bar{x} \pm \sigma$)	**B0** ($\bar{x} \pm \sigma$)	***P*** (V *vs.* B0)	**B** ($\bar{x} \pm \sigma$)	***P*** (V *vs.* B1)	**CH (B0)**	**CH (B1)**
Organic components												
Tri (mmol/l)	(W)	0.334	0.057	0.102	0.573	0.5 ± 0.2	0.6 ± 0.2	< 0.001	0.7 ± 0.2	< 0.001	18	21
GLDH (log_{10} U/l)	(W)	0.451	0.008	0.363	0.041	2.5 ± 5,2	3.5 ± 4.3	0.052	5.9 ± 6.6	< 0.001	0	3
Glu (mmol/l)	(T)	0.497	0.003	0.644	< 0.001	10.3 ± 4.9	11.8 ± 5.3	0.096	12.1 ± 5.8	0.044	8	5
TP (g/l)	(W)	0.554	< 0.001	0.326	0.064	65.4 ± 11.4	68.9 ± 9.0	0.086	83.7 ± 19.3	< 0.001	8	16
Glob (g/l)	(T)	0.617	< 0.001	0.351	0.045	40.8 ± 8.8	44.0 ± 7.0	0.027	55.6 ± 15.4	< 0.001	8	18
AP (U/l)	(W)	0.640	< 0.001	0.263	0.140	23.3 ± 20.7	18.7 ± 17.4	0.007	14.5 ±18.7.	< 0.001	1	1
TBil (µmol/l)	(W)	0.690	< 0.001	0.552	< 0.001	0.6 ± 0.4	0.8 ± 0.4	< 0.001	1.1 ± 0.5	< 0.001	0	0
ALT (U/l)	(W)	0.781	< 0.001	0.611	< 0.001	13.0 ± 9.4	15.3 ± 9.4	0.053	23.7 ± 15.6	< 0.001	0	1
CK (log_{10} U/l)	(W)	0.801	< 0.001	0.925	< 0.001	2.4 ± 0.5	2.4 ± 0.4	0.487	2.4 ± 0.4	0.732	7	3
Alb (g/l)	(W)	0.813	< 0.001	0.758	< 0.001	24.6 ± 4.1	24.9 ± 3.6	0.636	28.1 ± 4.9	< 0.001	3	6
Crea (µmol/l)	(W)	0.851	< 0.001	0.821	< 0.001	112.1 ± 28.3	113.4 ± 35,3	0.474	121.7 ± 40.9	0.081	2	2
Chol (mmol/l)	(T)	0.842	< 0.001	0.838	< 0.001	3.7 ± 1.0	3.7 ± 1.0	0.804	4.1 ± 1.1	< 0.001	5	8
Urea (mmol/l)	(W)	0.955	< 0.001	0.963	< 0.001	9.5 ± 3.6	9.5 ± 3.6	0.651	9.1 ± 3.4	0.016	2	3
Inorganic components												
K (mmol/l)	(T)	0.366	0.043	0.609	< 0.001	3.7 ± 0.5	4.0 ± 0.4	0.003	4.4 ± 0.6	< 0.001	10	15
Na (mmol/l)	(W)	0.395	0.028	0.597	< 0.001	145.1 ± 5.9	143.2 ± 4.4	0.026	141.4 ± 4.4	< 0.001	10	8
PHOS (mmol/l)	(W)	0.500	0.003	0.450	0.009	1.6 ± 0.5	1.9 ± 0.4	< 0.001	2.6 ± 1.0	< 0.001	15	20
Cl (mmol/l)	(T)	0.545	0.002	0.535	0.004	115.2 ± 4,8	113.8 ± 4,6	0.085	108.3 ± 4.6	< 0.001	6	15
TMg (mmol/l)	(T)	0.570	< 0.001	0.338	0.055	0.9 ± 0.1	1.1 ± 0.1	< 0.001	1.2 ± 0.2	< 0.001	1	7
TCa (mmol/l)	(W)	0.659	< 0.001	0.443	0.01	2.2 ± 0.4	2.3 ± 0.2	< 0.001	2.6 ± 0.3	< 0.001	7	20

7.3 Method validation for *Toxoplasma gondii* serology in domestic cats

Conventionally sampled plasma (V) or plasma sampled with reduviid bugs (B0, B1, B2, B4) from 70 domestic cats was tested for *T. gondii*-specific antibodies by both, immunoblot and immunofluorescent antibody test (Table 7.2). The study aimed to proof that antibody titers do not vary statistically significantly with varying timeframes from engorgement to blood sampling. All individuals that showed a positive titer in the venous blood also showed a positive result in all samples taken by reduviid bugs. Similarly, all animals with *T. gondii*-negative V samples tested also negative in the samples obtained through reduviid bugs.

Table 7.2: *T. gondii* specific antibodies as determined by immunblot (IB) and the immune-fluorescent antibody test (IFAT). Reciprocal IFAT titer in venous blood (Titer V) and in bug-derived plasma taken immediately (Titer B0), one hour (Titer B1), two hours (Titer B2) or four hours (Titer B4) after engorgement. Results highlighted in grey were positive (IFAT cut-off in plasma: 100). The "x" indicates that there was no sample available.

Sample No.	**IB**	**IFAT titer**				
		V	**B0**	**B1**	**B2**	**B4**
1	Pos.	1600	800	1600	x	3200
2	Pos.	3200	3200	3200	x	3200
3	Pos.	800	800	800	1600	1600
4	Neg.	<25	<25	<25	x	<25
5	Pos.	3200	6400	6400	6400	12800
6	Neg.	<25	<25	<25	<25	25
7	Pos.	800	1600	3200	x	3200
8	Neg.	50	50	50	50	50
9	Neg.	<25	<25	<25	x	25
10	Pos.	800	800	1600	x	1600
11	Neg.	<25	<25	<25	<25	<25
12	Neg.	<25	25	25	<25	<25
13	Neg.	<25	<25	<25	x	<25
14	Pos.	6400	6400	12800	12800	12800
15	Neg.	<25	<25	<25	<25	<25
16	Neg.	<25	<25	<25	<25	<25
17	Neg.	<25	<25	<25	<25	<25
18	Neg.	25	25	25	25	50
19	Neg.	25	<25	<25	x	<25
20	Neg.	<25	<25	x	x	<25
21	Pos.	800	1600	1600	x	3200
22	Neg.	25	<25	25	25	25
23	Neg.	25	x	50	x	50
24	Neg.	<25	25	25	25	<25
25	Pos.	1600	1600	1600	x	1600
26	Pos.	800	800	800	x	1600
27	Neg.	<25	<25	<25	x	25
28	Neg.	<25	<25	<25	x	<25
29	Pos.	3200	3200	3200	3200	6400
30	Neg.	<25	<25	<25	x	<25
31	Neg.	<25	<25	<25	<25	25
32	Neg.	<25	<25	<25	<25	<25
33	Neg.	<25	<25	<25	25	25
34	Neg.	<25	<25	<25	<25	<25
35	Pos.	3200	3200	3200	3200	3200
36	Neg.	<25	25	<25	x	25

Sample No.	IB	IFAT titer				
		V	B0	B1	B2	B4
37	Pos.	1600	1600	3200	x	x
38	Neg.	<25	25	<25	25	50
39	Neg.	25	25	25	x	25
40	Pos.	400	200	400	x	400
41	Pos.	1600	3200	x	x	x
42	Pos.	12800	12800	12800	12800	25600
43	Neg.	<25	<25	<25	<25	<25
44	Pos.	400	400	800	x	800
45	Pos.	800	800	1600	1600	1600
46	Neg.	<25	<25	<25	<25	25
47	Neg.	<25	<25	<25	<25	<25
48	Neg.	<25	<25	<25	x	<25
49	Pos.	1600	1600	1600	x	1600
50	Pos.	3200	3200	3200	3200	3200
51	Neg.	<25	<25	<25	<25	<25
52	Pos.	6400	6400	12800	12800	6400
53	Neg.	<25	<25	<25	x	<25
54	Neg.	<25	<25	<25	<25	<25
55	Neg.	<25	<25	<25	x	<25
56	Neg.	<25	<25	50	x	50
57	Neg.	25	50	50	x	50
58	Pos.	1600	1600	3200	3200	3200
59	Pos.	3200	3200	6400	x	6400
60	Pos.	3200	3200	3200	6400	6400
61	Neg.	<25	<25	<25	x	<25
62	Pos.	3200	1600	3200	3200	3200
63	Pos.	3200	3200	3200	x	3200
64	Pos.	3200	3200	3200	3200	6400
65	Neg.	<25	<25	<25	25	25
66	Pos.	3200	3200	3200	3200	6400
67	Neg.	25	25	50	50	25
68	Neg.	<25	<25	25	25	25
69	Neg.	50	50	25	x	50
70	Pos.	800	800	800	x	800

The data failed to show a normal distribution and were therefore statistically analyzed using the Spearman rank correlation. The titers determined in venous plasma and the titers determined in bug-derived samples were statistically significantly correlated and revealed Spearman correlation coefficients between 0.919-0.954 ($P \leq 0.001$) (Figure 7.39).

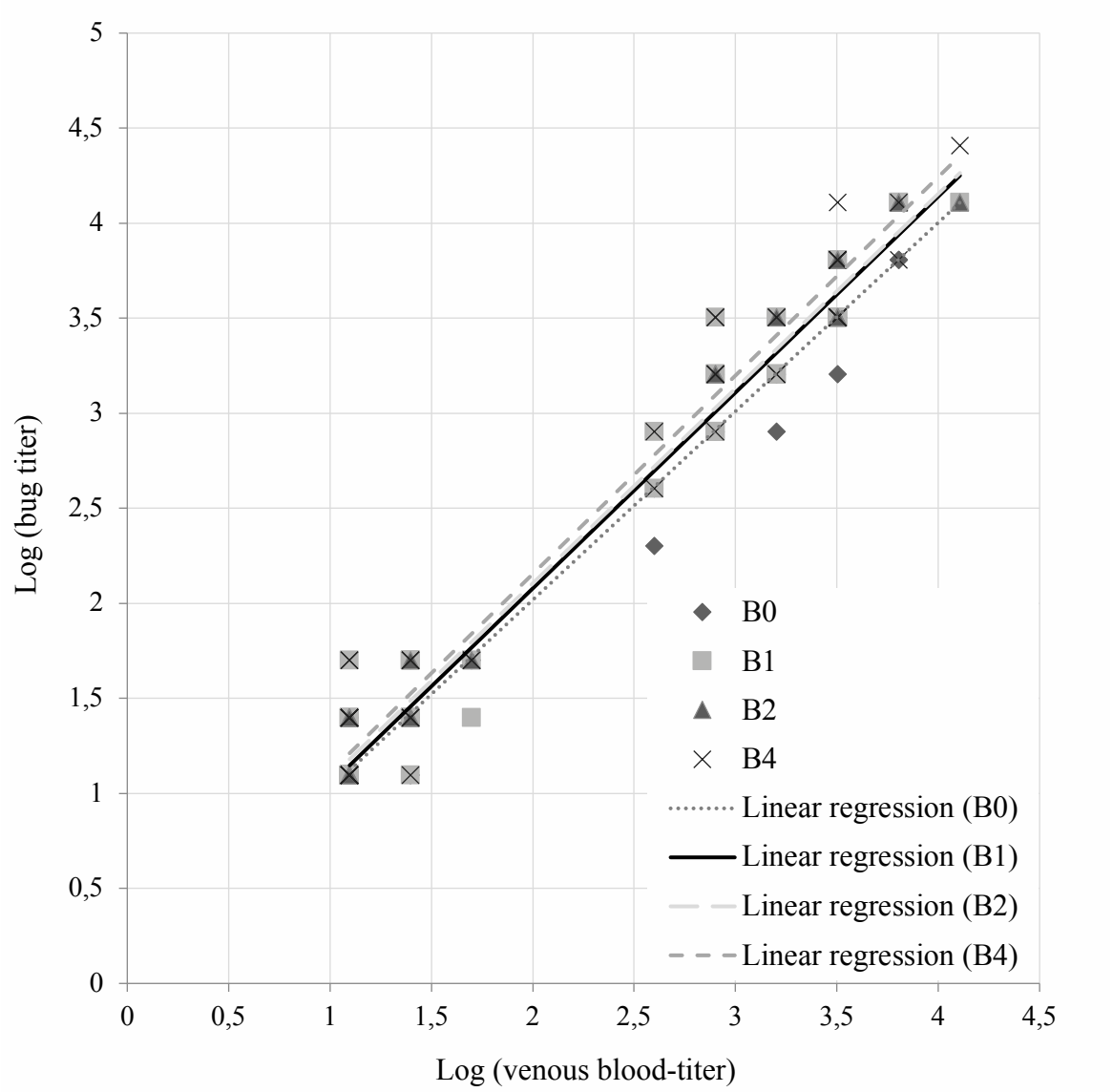

Figure 7.39: Correlation of *T. gondii* titer values in venous (V) and bug-derived plasma (B0, B1, B2, B4). Spearman r for V *vs.* B0: 0.952; $P < 0.001$; Spearman r for V vs. B1: 0.954; $P < 0.001$; Spearman r for V *vs.* B2: 0.939; $P < 0.001$; Spearman r for V *vs.* B4: 0.919; $P < 0.001$; linear regression lines were included using Microsoft Excel 2010.

Although a good correlation between the conventional venous blood sample and blood samples collected using reduviid bugs could be shown, increasing titers were observed when V titers and B0, B1 or B2 titers were compared, respectively. To characterize these differences, only samples with positive *T. gondii* IFAT titers were considered. While *T. gondii* antibody titers did not differ statistically significantly in B0, they did in B1 and B2 (Table 7.3). However, increasing titers failed to lead to a change of the overall result (from positive to negative or from negative to positive) in any of the analyzed samples. The increase of titers with time is shown in Figure 7.40. The mean titer difference (expressed as $\log_{10}$ (titer)) between V and B0, B1, B2 and B4 was 0.01 ± 0.15, 0.13 ± 0.17, 0.15 ± 0.16 and 0.2 ± 0.2, respectively. The difference was statistically significant when titers (expressed as log10 (titer)) for V were compared with those for B1, B2 or B4. When the V and B0 titers (expressed as log10 (titer)) were compared, no statistically significant difference was observed (Wilcoxon signed rank test).

Table 7.3: Statistical comparison of log_{10}-transformed titers of *T. gondii*-positive plasma samples (V) and samples collected with *Dipetalogaster maxima* (B0, B1, B2, B4) from domestic cats. CORR: Correlation coefficients (Spearman r); a non-parametric Wilcoxon signed rank test was performed for samples with positive *T. gondii* titers (log_{10}) as well as for titer differences (log_{10}); "-", not applicable.

Parameter	**CORR** (V *vs.* B0) ***P***	**CORR** (V *vs.* B1) ***P***	**CORR** (V *vs.* B2) ***P***	**CORR** (V *vs.* B4) ***P***	**V** ($\bar{x} \pm \sigma$)	**B0** ($\bar{x} \pm \sigma$)	***P*** (V/B0)	**B1** ($\bar{x} \pm \sigma$)	***P*** (V/B1)	**B2** ($\bar{x} \pm \sigma$)	***P*** (V/B2)	**B4** ($\bar{x} \pm \sigma$)	***P*** (V/B4)
T. gondii titer (log_{10})	0.919 < 0.001	0.889 < 0.001	0.890 < 0.001	0.862 < 0.001	3.3 ± 0.4	3.3 ± 0.4	0.435	3.4 ± 0.4	0.002	3.6 ± 0.3	0.011	3.5 ± 0.4	< 0.001
						B0-V ($\bar{x} \pm \sigma$)	***P*** (B0-V)	**B1-V** ($\bar{x} \pm \sigma$)	***P*** (B1-V)	**B2-V** ($\bar{x} \pm \sigma$)	***P*** (B2-V)	**B4-V** ($\bar{x} \pm \sigma$)	***P*** (B4-V)
T. gondii titer difference (log_{10})	-	-	-	-	-	0.01 ± 0.15	0.705	0.13 ± 0.17	0.001	0.15 ± 0.16	0.008	0.22 ± 0.2	< 0.001

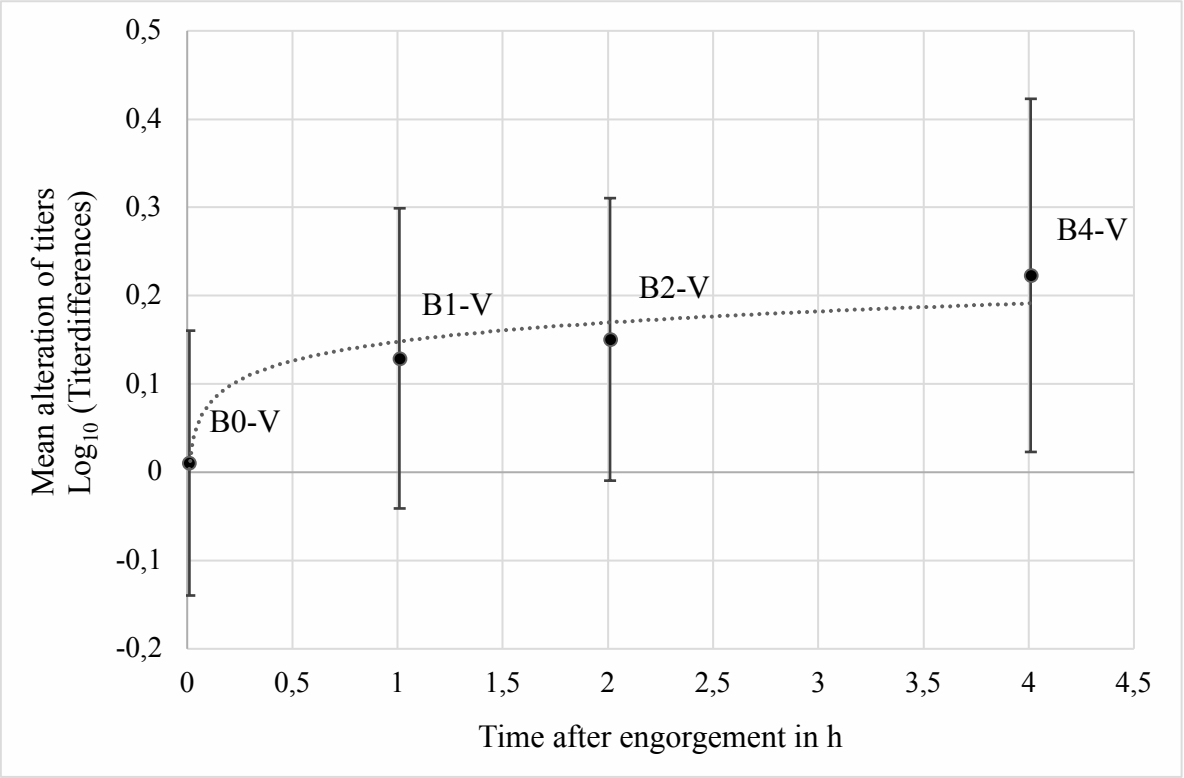

Figure 7.40: Mean differences and standard deviations between *T. gondii* IFAT titers determined in venous plasma (V) and plasma collected from bugs after varying times after engorgement (B0, B1, B2, B4).
Sampling: immediately after engorgement (B0), after 1 h (B1) after 2 h (B2) and 4 h (B4). Numbers of sets of samples: B0-V: n = 29, B1-V: n = 28, B2-V: n = 14, B4-V: n = 27. The logarithmic regression line, as determined by Microsoft Excel 2010, models the possible increase of mean titer differences with increasing time from sampling to engorgement.

7.4 Serological results of zoo-samples stratified for zoo and individual animal characteristics

336 samples from 17 felid species were collected in 51 institutions, 48 of them within Europe and three in the United Arabic Emirates. These samples were analyzed for *T. gondii* antibodies by immunoblot and IFAT. The tests revealed 196 positive and 115 negative results, 25 serological results were labeled 'not available' (NA) due to differences in the results of immunoblot and IFAT. These individuals were therefore excluded from further analysis. From 311 animals with a clear serological result, 63.0% showed a positive and 37.0% a negative antibody response. Stratification of the results according to the age of individual animals showed increasing proportions of positive cats with increasing age (Fig. 7.41). Stratification of the results for felid species showed differences between species, which can be partially explained by the age-dependency of the serological results (Fig. 7.42).

All serological results were stratified according to characteristics of the individual animals and characteristics of the zoos, i.e. according to information collected using a questionnaire.

Individual animal characteristics were represented by data collected from ZIMS including date of birth, rearing specifications (parent, foster or hand reared), sex and life history (number of institutions the animal lived in). These variables were analyzed for their potential impact on the *T. gondii*-seropositivity of individual felids.

The questionnaire aimed to analyze different variables in husbandry that might have had an impact on the *T. gondii*-seropositivity of an individual felid. Animals from three zoos, which did not submit their questionnaire, were excluded from the analysis.

Zoo-related variables were divided into food-related (food and food preparation/storage), housing-related, hygiene- and general health-related variables. Furthermore, characteristics of the individual animals were analyzed. Significance was initially examined for each variable separately with the serological status as the outcome variable using the Fisher's exact test (univariable testing). Since it became obvious that the age of an individual animal represented the major factor related to the likelihood of seropositivity, data were later-on exclusively analyzed by multilevel modelling, with age as an effect-modifying variable and the zoo, the animal lived in, as a random effects variable. The results of the univariable tests are not shown in the following, because it was expected that they were most likely biased by differences in age and potentially also by zoo-specific factors, which were not covered by the questionnaire.

7.4.1 Variables related to the individual animal

The highest percentage of *T. gondii*-seropositive animals was found in Pallas' cats with 90.4% and in Rusty-spotted cats with 96.4%. Both values were much higher than the mean proportion of *T. gondii* seropositive results in all felids tested (63%). In contrary to this, Black-footed cats showed a considerable lower percentage of seropositive animals than the remaining species (26.7%) (Table 13.65).

Animals older than five years showed a higher proportion of *T. gondii*-seropositive results (72.8%) than individuals younger than five years (44.8%) (Table 13.62).

The proportion of seropositive animals increased with age (Figure 7.41) and a generalized linear mixed model fit by maximum likelihood (Laplace Approximation) including random effects by different zoos revealed a statistically significant effect of age (Fixed effects: Estimate 0.201, Standard Error 0.003, z value 80.79, PR (> |z|) < 0.001).

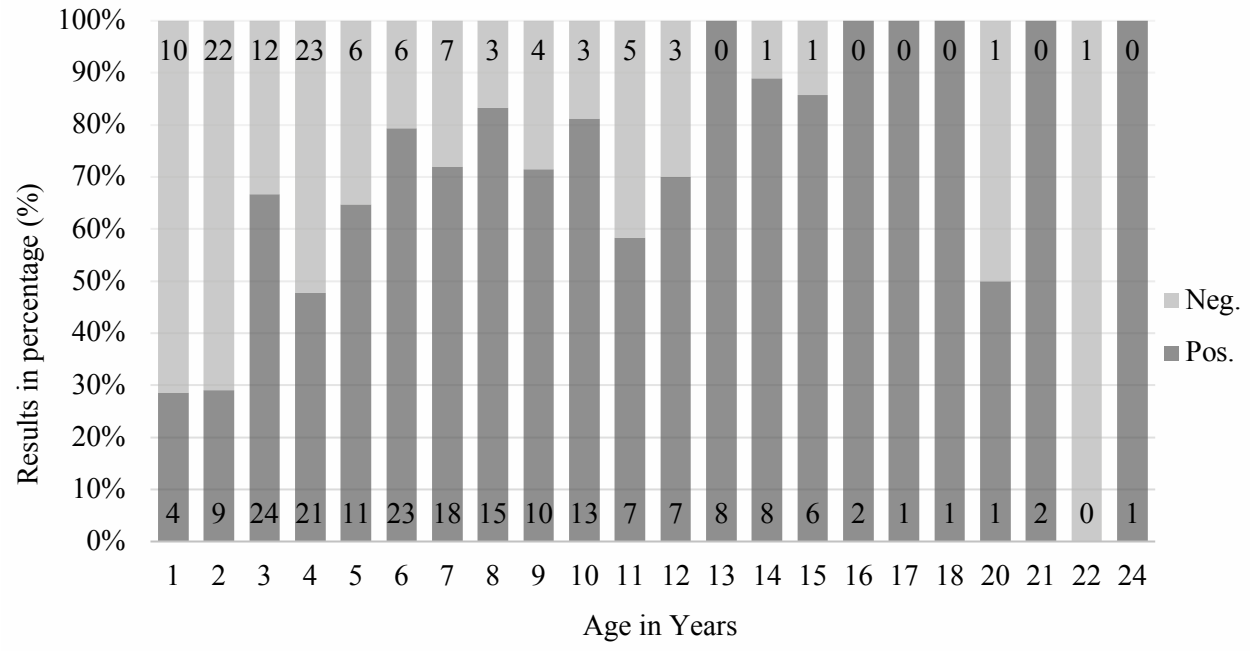

Figure 7.41: Seroprevalence regarding *T. gondii* serological results stratified by age [in years]. The numbers of seropositive and seronegative felids are displayed within the columns.

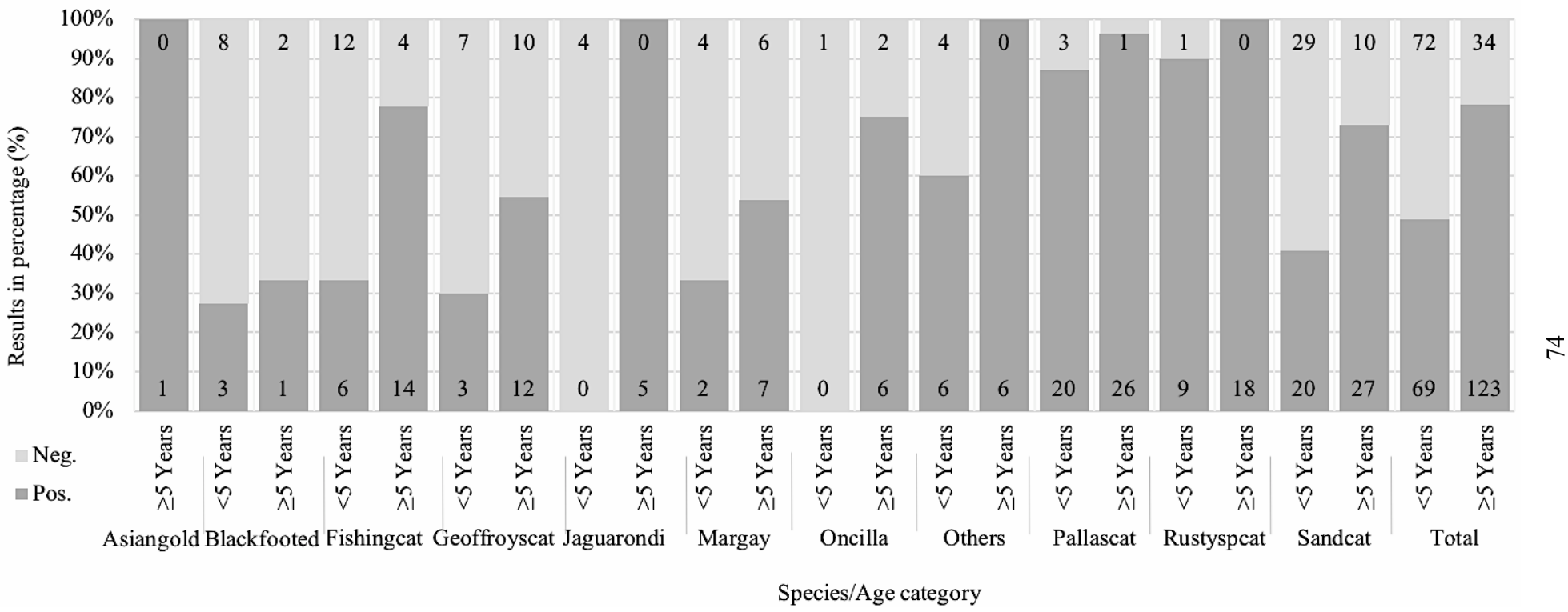

Figure 7.42: Seroprevalence regarding *T. gondii* serological by felid species.
The results are further stratified by the age of the sampled animals (< 5 years; ≥ 5 years).
The numbers of seropositive and seronegative felids are displayed within the columns.

Among all analyzed animals, 158 were males showing a prevalence of 67.8% *T. gondii*-seropositive cats. Among 151 females, 59.6% showed a positive antibody response (Table 13.64).

Most felids were parent-reared. 62.4% of these 282 individuals proved to be *T. gondii*-seropositive. Similarly, 69.2% of 13 the hand-reared animals had *T. gondii*-specific antibodies. One foster-reared individual was seropositive (Table 13.63).

Figure (7.43) shows the dependency of the serological results for antibodies to *T. gondii* relating to sex and rearing of the cats. Stratification of the serological results for hand-reared cats or individuals reared by their parents or sex showed no statistically significant difference.

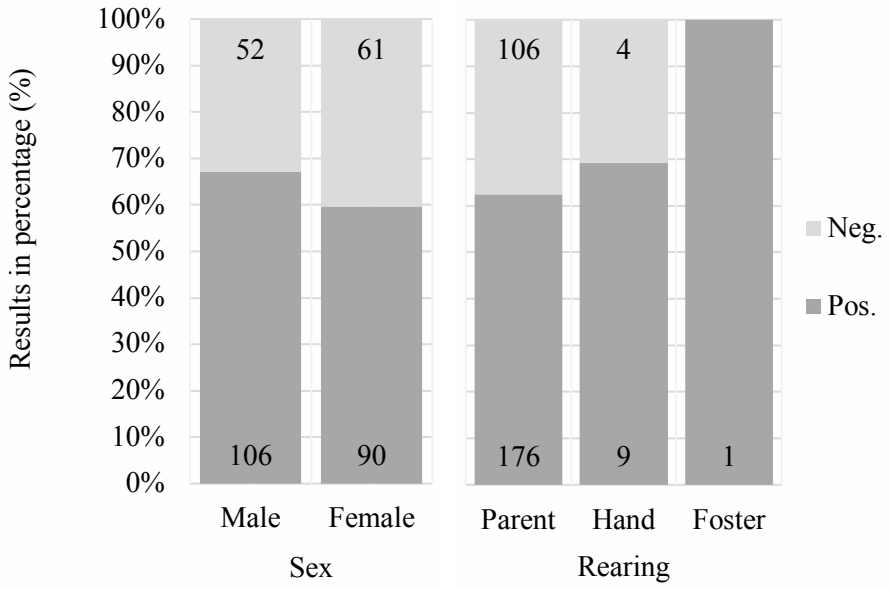

Figure 7.43: Seroprevalence regarding *T. gondii* stratified by sex and rearing of the cats. The numbers of seropositive and seronegative felids are displayed within the columns.

Among 311 individuals, 111 cats had spent their whole life in one zoo, while 198 felids had been transferred to another institution at least once in their life. The first individuals showed a proportion of 58.6%, the latter exhibited a proportion of 65.7% *T. gondii*-seropositive individuals (Table 13.31).

For Sand cats and Pallas' cats, a draft pedigree of all individuals tested could be generated, as the examined population was sufficiently large for this purpose. As the pedigrees did not reveal associations between the *T. gondii*-seropositivity of female cats and their kittens, this analysis is not documented in a figure.

7.4.2 Food-related variables

Food-related variables were recorded in 50 zoos. In addition to the treatment of food items, the different sources of meat (i.e. animal species and producer; details in Table 13.10), was recorded (feeding of previously frozen or fresh meat or carcasses) (Figure 7.44).

40 zoos fed mice to their felids, 11 of them used fresh mouse carcasses, ten institutions used mouse carcasses fresh or previously frozen and 19 zoos used only previously frozen mice. 76.1% of the cats sampled at institutions feeding fresh mice showed a positive *T. gondii* serological result. The proportion of seropositive individuals was 60.0% in zoos feeding only previously frozen mouse carcasses (Table 13.1).

While the analysis in nine of 40 institutions, which fed their felids with fresh rat carcasses revealed a proportion of 71.2% seropositive individuals, a proportion of 68.4% *T. gondii* seropositive individuals was observed in nine zoos, which fed them rat tissues fresh or previously frozen, and 61.0% in 22 zoos, which used exclusively unfrozen rats (Table 13.2).

The sources of rats and mice were variable. They were bred by laboratories, local suppliers, in own production or by commercial animal food suppliers.

Most of the ruminant tissues used to feed felids were from cattle. Six from 25 zoos used fresh bovine tissue, 76.2% of the felids in these zoos tested *T. gondii*-seropositive. 14 institutions fed bovine tissues after freezing, serological analysis revealed 45.8% individuals with antibodies to *T. gondii* (Table 13.3). All zoos, which reported feeding cattle tissues, received it from external slaughters.

Sheep tissues were fed in eight zoos. Serological analysis in institutions where sheep carcasses were used fresh, showed a proportion of 82.8% *T. gondii*-seropositive felids, while zoos that fed sheep tissues either fresh or after freezing had a proportion of 64.5% seropositive felids (Table 13.4). Sheep tissues were received from local suppliers, commercial breeders, or from slaughterhouses and butchers.

In 23 zoos, horse meat was part of the feeding scheme for felids. The highest proportion of *T. gondii*-seropositive felids was observed in four institutions feeding horse carcasses fresh or after freezing (81.1%). The proportion of seropositive felids for zoos feeding previously frozen horse tissues was 64.2%, while it was 58.6% at zoos that did not use horse meat (Table 13.5). The zoos reported that horse meat was delivered either by slaughter houses, butchers or private and local breeders.

While seven of 46 institutions fed their felids with fresh fowl with a proportion of 86.2% *T. gondii*-seropositive individuals, 12 zoos fed fresh or previously frozen fowl and 27 used fowl after freezing only. The latter showed a percentage of 57.5% *T. gondii* seropositive individuals (Table 13.6). For fowl, all kinds of sources came into account: Some zoos fed laboratory-bred animals, others fowl from local suppliers, private breeders, or commercial animal food suppliers. Some zoos used animals raised in the zoo. Most institutions fed chickens and some zoos fed pigeons shot by local hunters.

31 zoos used fish as a diet for their felids. While five zoos used fresh carcasses (these zoos had a percentage of 78.6% *T. gondii*-seropositive individuals), 22 of them used fish after freezing. These institutions had 63.6% Toxoplasma seropositive felids) (Table 13.7). Fish was bought either from local producers or from commercial animal food suppliers.

Four zoos used fruits as a source of food for their felids. In these institutions, 82.8% of the felids showed a *T. gondii*-seropositive result (Table 13.8).

12 institutions offered canned or dry commercial cat food. In four zoos that used canned food, 68.2% of the cat were serologically positive for *T. gondii*, the percentage of positive felids was 50.0% in the four institutions using dry cat food (Table 13.9). Cat food was obtained from commercial animal food suppliers.

In general, most zoos (26) received parts of the feed offered to felids from commercial animal food suppliers. 19 institutions also relied on own breeding, while 17 zoos received food from local breeders and 11 zoos from butchers or slaughterhouses. In these institutions, between 64.7% and 67.2% of the felids tested showed a *T. gondii* -seropositive result. Very few zoos received animal food from laboratories (5), hunting (3) or private owners (7). In these institutions, *T. gondii*-seropositive results were found in 75.7% to 81.3% of the individuals tested (Table 13.10).

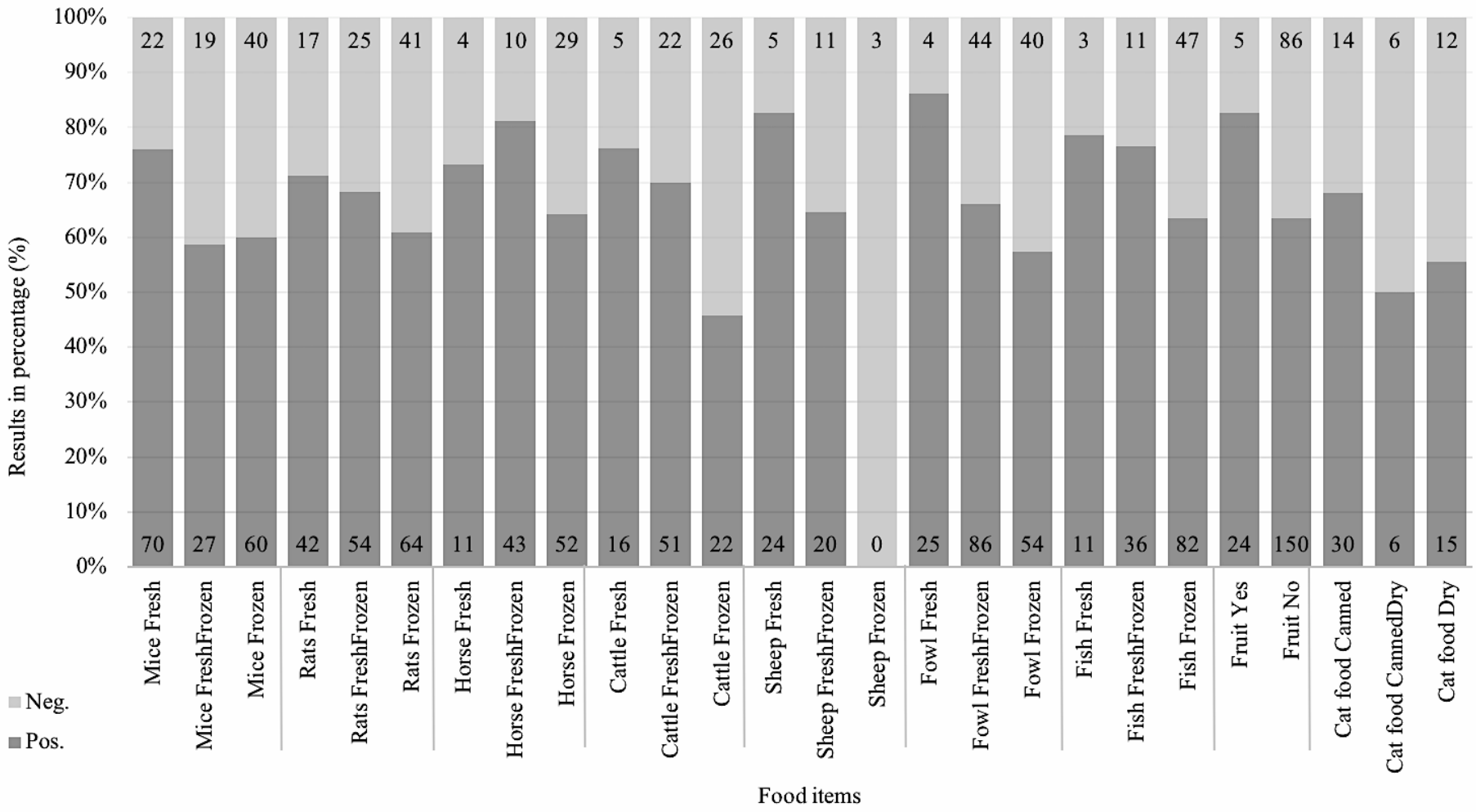

Figure 7.44: Seroprevalence regarding *T. gondii* stratified by the commonly used food items. The numbers of seropositive and seronegative felids are displayed within the columns.

7.4.3 Food storage and food preparation-related variables

Information on food storage and preparation was registered in 50 zoos. In addition to the time and temperature of food storage, it was asked if meat preparation was performed in rooms or areas, separate from rooms or areas, in which other sources of food were handled (Figure 7.45).

Four zoos used cold (-5°C to 10°C) storage for their food, 14 of them used cold or frozen storage and 27 institutions had frozen (-15°C or below) food storage. 69.2% of the felids sampled at institutions with cold storage had antibodies to *T. gondii*. The proportion of positive individuals was 61.4% in zoos that used frozen storage (Table 13.11).

Another variable was the duration of food storage under freezing condition. 14 zoos stated that the frozen storage of meat or carcasses was sometimes shorter than one week; in these zoos, 76.2% of the individuals were *T. gondii* serologically positive. Nine institutions kept carcasses frozen for at least one week (48.0% Toxoplasma seropositive felids) and most zoos (22) had a frozen storage exceeding two weeks; in these zoos, 64.3% of the felids exhibited *T. gondii*-seropositive results (Table 13.12).

While eight of 46 institutions did not separate handling of fruit/vegetables (fruits) and meat (in these zoos a proportion of 56.5% *T. gondii*-seropositive individuals was observed), ten zoos prepared fruits and meat in separate processes, either at different times of the day or on different cutting boards. In these institutions, a proportion of 79.5% *T. gondii*-seropositive individuals was observed. 29 institutions had different rooms for meat and fruits preparation. In these zoos, 61.9% of the felids had antibodies to *T. gondii* (Table 13.13).

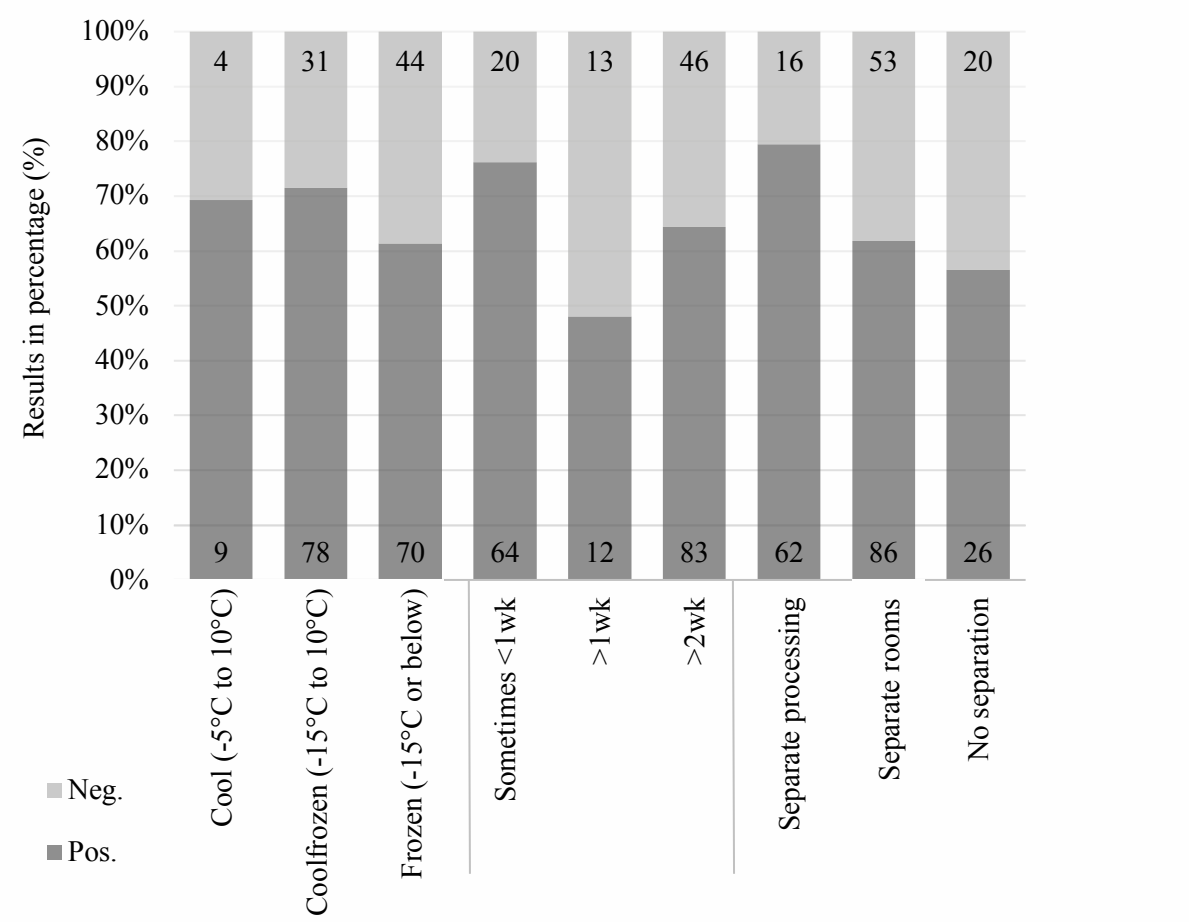

Figure 7.45: Seroprevalence regarding *T. gondii* stratified by temperature conditions and duration of food storage and the separation of processing meat/fruits.
The numbers of seropositive and seronegative felids are displayed within the columns.

Three of 48 zoos stated that their main water sources were rain or tap water; in these zoos, a proportion of 75.0% *T. gondii*-seropositive individuals was observed. Most institutions used tap water as a main water source, in these zoos a percentage of 66.4% of the felids had antibodies to *T. gondii*. Three zoos used both, well and tap water, while four zoos selected well water as the main water source. In these zoos, of 59.1% or 60.0% of the felids had antibodies to *T. gondii*, respectively (Table 13.14).

Most institutions performed a water change daily. A percentage of 63.3% serologically *T. gondii*-positive individuals was observed in these zoos. Four zoos changed drinking water every second week; 79.1% of the individuals tested positive for antibodies to *T. gondii* under these conditions (Table 13.15) (Figure 7.46).

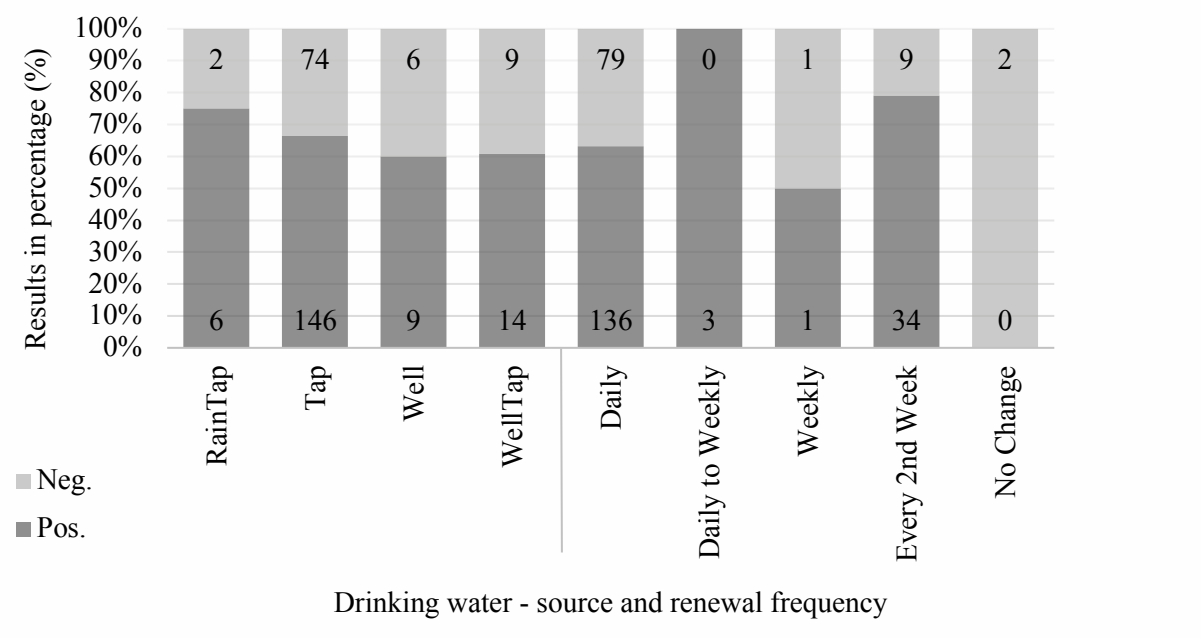

Figure 7.46: Seroprevalence regarding *T. gondii* stratified by the source of drinking water and its renewal frequency. The numbers of seropositive and seronegative individual felids are displayed within the columns.

7.4.4 General housing related variables

With regard to housing-related variables, it was asked, which species were kept close to the felids and if the animal attendants cared for other *T. gondii*-susceptible species as well. Also, the number of other felids and feline species kept at the zoos was determined (including small cat species that are not managed in EEPs or ESBs and big cats). In addition, the character of enclosures of the sampled animals was described. Information on in- and outdoor housing was obtained as well as data on size, mesh size and the occurrence of stray cats in the institution (Figure 7.47).

In total, 24 zoos kept one to 16 feline individuals (big and small cats); 63.4% of the individuals of feline species from these zoos tested *T. gondii*-seropositive. In 26 zoos, 17 or more felids were housed. The individuals tested in these institutions exhibited a proportion of 61.1% *T. gondii*-seropositive results (Table 13.66).

In 17 of the zoos, where felids had been sampled, none of the individual felids (among all cat species) had offspring during the year prior to sampling. In these zoos, 50% of the tested individuals were seropositive for *T. gondii*. In 17 zoos, one or two litters were born among all felids; 69.7% of the individuals tested positive for antibodies to *T. gondii*. In 16 zoos, three or more litters were born with 63.8% *T. gondii*-seropositive results among the individuals tested (Table 13.68).

During the last five years prior to sampling, 12 zoos had no offspring among all felids (52.8% *T. gondii*-seropositive animals among the species tested). In 20 zoos, one to nine litters and in 18 zoos more than ten litters were born during the last five years (71.6% and 60.6% *T. gondii*-seropositive individuals, respectively). Two institutions had more than 30 litters born during this period (Table 13.67).

32 zoos kept other felids close to the tested individuals (within a radius of 50 m). Of the felids examined in these zoos, 67.9% had antibodies against *T. gondii*. In 16 zoos that did not keep other felids within the 50 m-radius, 55.3% of the felids were *T. gondii*-seropositive (Table 13.16).

In 17 zoos, marsupials were kept within a 50 m-radius to the felids examined. Felids in these institutions exhibited a percentage of 67.1% *T. gondii*-seropositive individuals. The percentage of *T. gondii*-seropositive felids was 65.0% in 31 institutions that did not keep marsupials close to the felids examined in this study (Table 13.17).

12 zoos kept New World monkeys close to the examined felids. Among the felids from these zoos, 56.5% showed a *T. gondii*-seropositive result. In 36 zoos that did not keep New World monkeys within a 50 m-radius of felids, 67.6% of the cats were *T. gondii*-seropositive (Table 13.18).

In 39 zoos, animal attendants cared for more than one cat species. The tested felids in these institutions exhibited a percentage of 67.6% individuals serologically positive for *T. gondii*. The percentage of *T. gondii*-seropositive felids was 45.8% in nine institutions where animal attendants took care for one cat species only (Table 13.19).

In 18 zoos, the animal attendants who looked after the felids under study were in addition also responsible for marsupials or New World monkeys. Among those felids, 63.9% showed a positive antibody response for *T. gondii*. In 30 zoos where animal attendants did not look after marsupials or New World monkeys in addition to felids, 66.2% of the cats were serologically positive for *T. gondii* (Table 13.20).

33 of 50 zoos stated that they had no indoor housing available for their felids. These zoos had a percentage of 65.7% individuals serologically positive for *T. gondii* among the examined felids. In 15 zoos with indoor housing available in addition to outdoor enclosures, the proportion of serologically positive felids was 65.7% (Table 13.21).

40 zoos had outdoor housing, which was fenced in on all sides (including a closed top); a proportion of 63.6% of the examined felids were serologically positive for *T. gondii*. In contrast, 77.5% of the felids from eight zoos with no outdoor housing fenced in on all sides were *T. gondii*-seropositive (Table 13.22).

In nine zoos, outdoor open top housing was used to keep felids; in these zoos, a prevalence of 70.8% *T. gondii*-seropositive results was observed among felids. In 39 zoos that did not use outdoor open top enclosures, the testing revealed 62.7% of *T. gondii*-seropositive felids (Table 13.23).

26 zoos had enclosures with a size smaller than 20 m^2; these zoos had a proportion of 66.1% *T. gondii*-seropositive individuals. 35 zoos had enclosures with a size between 20 m^2 and 50 m^2; 65.0% of the felids tested serologically positive for *T. gondii* in these zoos. In 16 zoos with enclosures larger than 50 m^2, 63.7% of the individuals showed a positive antibody response (Table 13.24-13.26).

The mesh sizes of the enclosing fences were recorded. In 15 zoos, the enclosure mesh size was smaller than 2 cm in diameter (a prevalence of 74.6% *T. gondii*-seropositive individuals was observed in these zoos). In 30 institutions using a mesh size with a diameter of 2-5 cm, the *T. gondii* seroprevalence in felids was 58.7%. Six zoos reported the use of fences with mesh sizes bigger than 5 cm; the prevalence was 73.3% *T. gondii* seropositive individuals in these institutions (Tables 13.27-13.29).

In addition, the institutions were asked to estimate the number of stray cats entering the premises. 11 zoos stated that there were no stray cats present (68.9% *T. gondii*-seropositives among captive felids in these zoos), nine zoos had one individual roaming the premises (61.3% *T. gondii*-seropositives). The two zoos with the highest number of stray cats stated that they had observed up to 15 individuals on the premises; analysis in these zoos revealed 50.0% *T. gondii*-seropositive felids (Table 13.30).

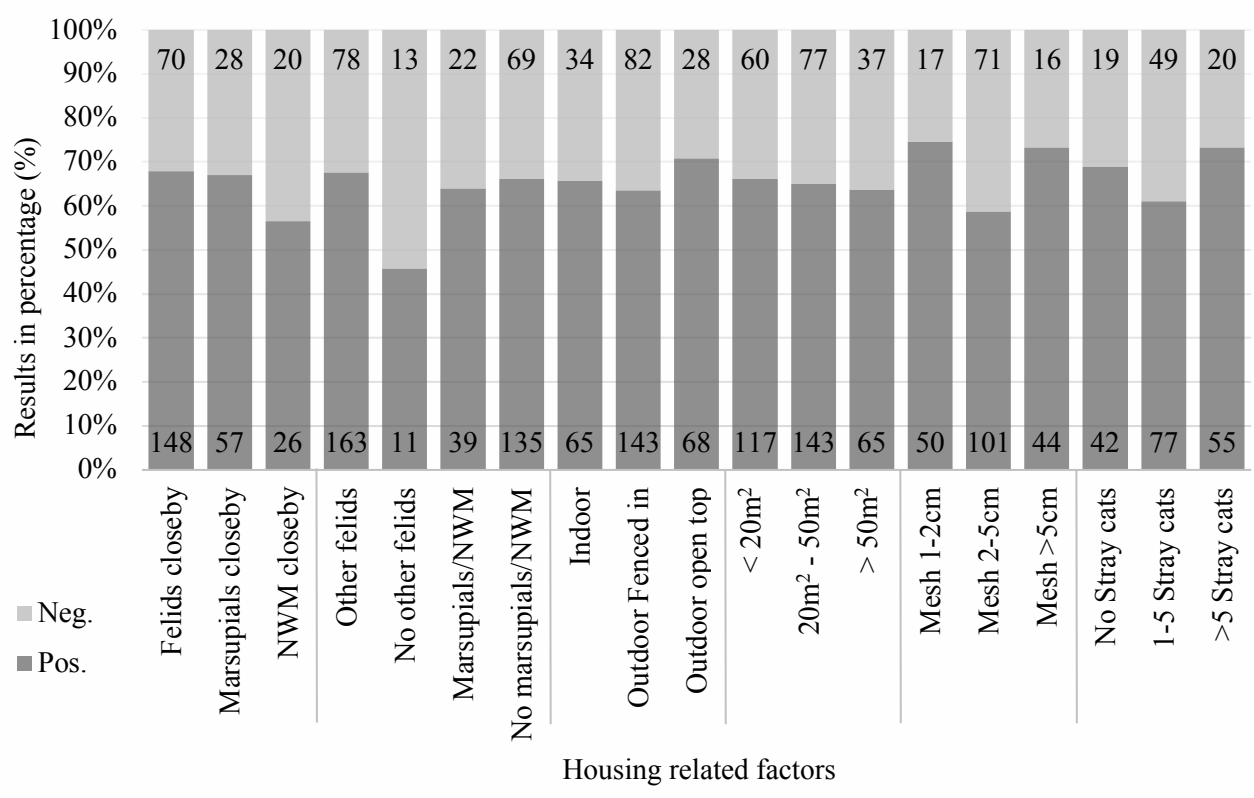

Figure 7.47: Seroprevalence regarding *T. gondii* stratified by housing-related variables: Results were stratified for animals kept close by, i.e. marsupials or New World monkeys (NWM), stratified for data on animal attendants taking care of other felids, marsupials or NWM, stratified for details about the enclosure including size and mesh size and the number of stray cats observed at the compound. The numbers of seropositive and seronegative individual felids are displayed within the columns.

7.4.5 Specific housing related variables

Further specific housing related variables of interest were the bedding material offered (shavings, hay, straw, sand bark and other materials) and the renewal frequency of the latter. In addition, the use of enrichment items (industrial toys or other items like paper bags) was inquired (Figure 7.48).

24 zoos used shavings as bedding material; in these zoos, a proportion of 64.1% *T. gondii*-seropositive individuals was observed. 15 zoos used hay and a proportion of 70.0% of the felids from these zoos tested serologically positive for *T. gondii*. Among 25 zoos using straw, 65.6% of the individuals showed a positive *T. gondii* antibody response. In 21 institutions, sand was used as bedding material; in these zoos, a proportion of 59.7% of the sampled felids tested seropositive for *T. gondii*. Five zoos stated the use of other bedding materials (not further specified). They kept a proportion of 56.7% serologically positive individuals (Table 13.32-13.36).

The change of bedding material was performed in different time intervals. Seven of 50 zoos stated that they changed bedding material on a daily basis; in these zoos, a proportion of 52.2% *T. gondii*-seropositive individuals was observed. In 17 institutions bedding material was changed weekly; a proportion of 67.4% *T. gondii*-seropositive individuals was observed. Seven zoos changed bedding materials monthly and 16 zoos changed it infrequently (i.e. as deemed necessary), proportions of 61.1% and 67.8% *T. gondii*-seropositive felids were observed in these zoos, respectively (Table 13.37).

35 of 50 zoos offered enrichment items to their captive felids; a prevalence of 64.7% *T. gondii*-seropositive individuals was found in theses zoos (Table 13.38). In 25 institutions, industrial toys were used, 28 zoos used other items for enrichment; in these zoos, percentages of 64.0% and 54.1% of the felids tested *T. gondii*-seropositive. The use of items for enrichment of natural origin like branches was recorded as well. Since all zoos used branches for decorating the interior of the enclosures, this variable was not examined further.

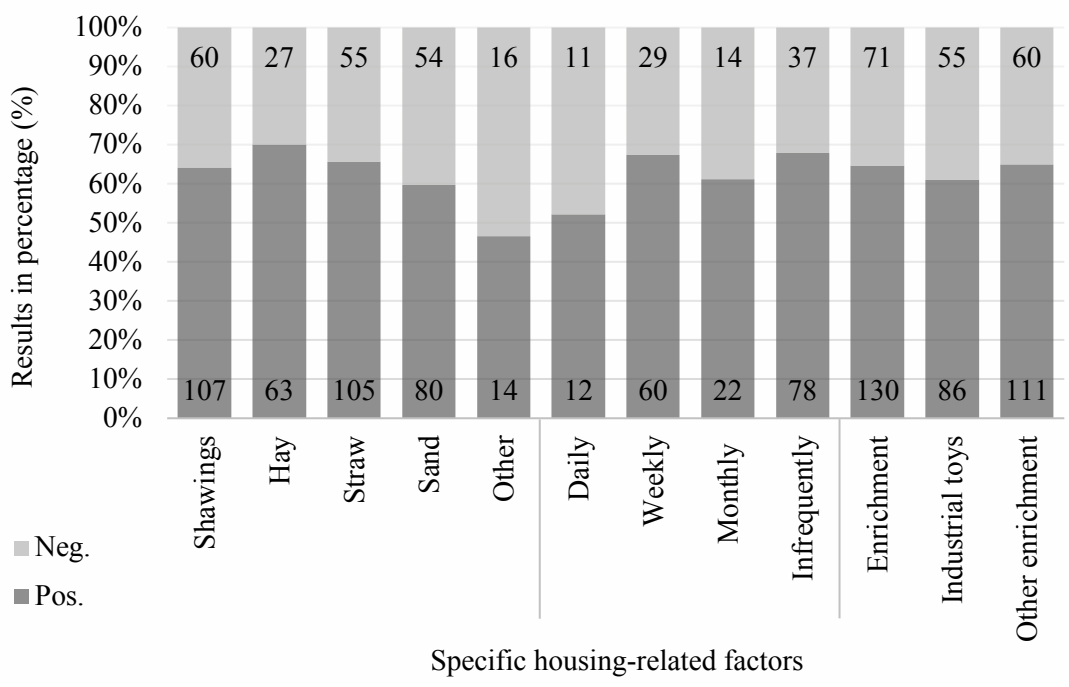

Figure 7.48: Seroprevalence regarding *T. gondii* stratified by housing-related variables. Variables were: bedding material, renewal intervals of the latter and enrichment used. The numbers of seropositive and seronegative individual felids are displayed within the columns.

7.4.6 Hygiene-related variables

To evaluate hygiene-related variables, the institutions were asked how often they removed feces from the enclosures of felids. Another subject was the availability of certain hygienic devices such as hand-wash facilities, hand disinfection or a disinfectant footbath at the enclosures and the use of disposable gloves. Furthermore, the regime of pest control was of interest for this study (Figure 7.49).

In 36 zoos, feces were removed regularly on a daily basis; a proportion of 67.0% *T. gondii*-seropositive individuals was observed in these institutions. In institutions with other time intervals for removing feces, the percentage of seropositive felids was 61.3% (Table 13.39).

23 zoos offered litter boxes to the felids (62.8% *T. gondii*-seropositive individuals), while 25 institutions did not (69.2% seropositive individuals) (Table 13.40).

37 zoos stated to take general hygienic measures (disinfection of hands or shoes, wearing gloves or shoe covers); serological analysis in these zoos revealed a percentage of 67.0% *T. gondii*-seropositive individuals. 11 zoos stated that they did not apply these general hygienic measures; they had 62.8% *T. gondii*-seropositive individuals (Table 13.41).

Institutions that had hand-wash facilities available at the enclosures had lower percentages of *T. gondii*-seropositive individuals, i.e., 13 zoos with hand-wash facilities had 64.6% *T. gondii*-seropositive individuals. In 35 zoos without hand-wash facilities, a percentage of 66.1% *T. gondii*-seropositive felids was found (Table 13.42).

Hand disinfection was applied in 12 zoos (61.8% *T. gondii*-seropositive felids). In 36 institutions without hand disinfection at the enclosures, the percentage of seropositive felids reached 67.0% (Table 13.43).

In 20 zoos where disposable gloves were used, the proportion of *T. gondii*-seropositive individuals was 51.2%, while in 28 zoos that did not use gloves, 72.4% of the felids had antibodies against *T. gondii* (Table 13.44).

Marginal differences were observed among zoos that had a disinfectant footbath available and those that had not with proportions of 66.7% (18 zoos) and 65.0% (30 zoos) *T. gondii*-seropositive individuals, respectively (Table 13.45).

One zoo used disposable shoe covers regularly. In this zoo, 20.0% of the felids tested *T. gondii*-seropositive. In 47 zoos that did not use shoe covers, 66.5% of the felids had antibodies against *T. gondii* (Table 13.46).

Different measures of pest control were applied at the zoos. 43 institutions stated that they practiced pest control in general; in these zoos, a proportion of 64.9% individuals tested serologically positive for *T. gondii*. In four zoos that did not perform pest control, 72.2% of the felids had antibodies to the parasite. The frequency of pest control varied widely. Most institutions (21) performed pest control in monthly intervals (with a proportion of 58.7% *T. gondii*-seropositive felids). Five zoos performed pest control in quarterly intervals; 68.8% of the felids were *T. gondii*-seropositive in these zoos. The proportion of *T. gondii* seropositive felids was 80.0% in three zoos that performed pest control biannually. Institutions with infrequent pest control intervals harbored a proportion of 78.4% felids with antibodies to *T. gondii* (Table 13.47-13.48).

28 zoos used traps for pest control; 63.3% of the felids in these institutions were *T. gondii*-seropositive. In contrast, zoos that did not use traps, had 69% *T. gondii*-seropositive felids. In 10 zoos, poison was used for pest control; a proportion of 66.1% *T. gondii*-seropositive felids were detected in these institutions. 14 zoos had cats on the property for pest control. The proportion of *T. gondii*-seropositive individuals in these zoos was 66.7%. Analysis in institutions that did not tolerate cats on the property revealed 66.3% *T. gondii*-seropositive felids (Table 13.49-13.51).

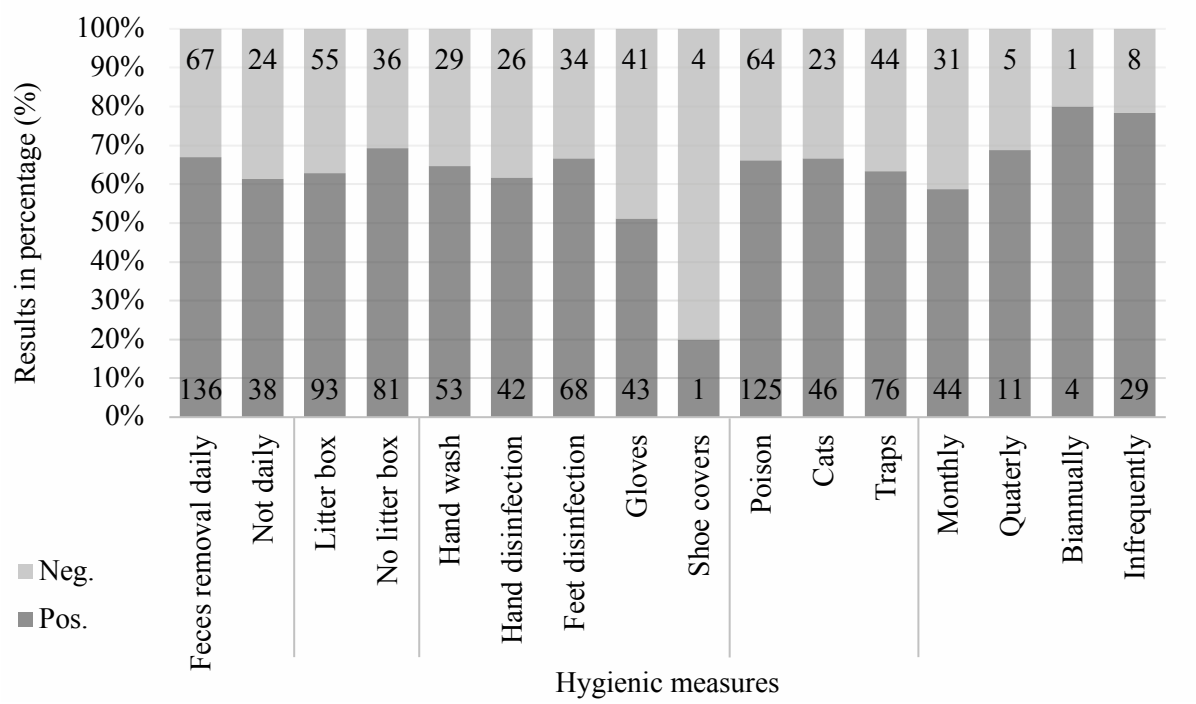

Figure 7.49: Seroprevalence regarding *T. gondii* stratified by specific hygiene related variables. Feces removal, availability of a litterbox, hygienic devices, pest control measures and frequency. The numbers of seropositive and seronegative individual felids are displayed within the columns.

7.4.7 General health-related variables

Variables of major interest were the vaccination policy and endoparasite control and treatment. While most zoos immunized their felids against infections with parvovirus and cat flu only few institutions chose to vaccinate against rabies and Feline Leukemia Virus as well. The vaccination against Feline Infectious Peritonitis (FIP) was not regularly performed (Figure 7.50).

In 37 zoos, vaccinations were performed (64.6% *T. gondii*-seropositive felids) and seven institutions did not vaccinate their felids (46.7% of the felids seropositive for *T. gondii*). 32 zoos stated to vaccinate against cat flu; these institutions had a percentage of 64.8% *T. gondii*-seropositive individuals. In contrast, the proportion of *T. gondii* seropositive felids was 52.2% in 12 institutions that did not use the vaccination against cat flu. Parvovirus vaccinations were performed in 31 zoos (63.3% *T. gondii*-seropositive results), while in 13 zoos with no parvovirus vaccination programs the proportion of *T. gondii*-seropositives was 58.9%. 17 zoos vaccinated against rabies (77.8% *T. gondii*-seropositive felids) and 27 institutions did not use rabies vaccines (53.2% *T. gondii*-seropositive felids). Leucosis vaccination was performed at 15 zoos; analysis revealed a percentage of 64.3% *T. gondii* seropositive felids. 29 zoos did not use leucosis vaccinations with a percentage of 60.9% of felids that tested positive for *T. gondii* antibodies. FIP vaccinations were used in 13 zoos (63.5% *T. gondii*-seropositive felids), while it was not performed in 31 institutions (61.8% *T. gondii* seropositive felids) (Table 13.52-13.57).

Most zoos (39) checked their felids regularly for intestinal endoparasites. In these zoos, a proportion of 62.1% of the felids tested was *T. gondii*-seropositive. Six institutions stated that they did not perform parasite checks on a regular basis (proportion of 63.6% *T. gondii*-seropositive felids). The difference in the proportions of *T. gondii*-seropositive individuals was marginal when zoos were stratified for different time intervals between the checks. In zoos that

performed quarterly checks for endoparasites, the proportion of *T. gondii*-seropositive individuals was 58.5%; in those with biannually performed checks 60.0% and in zoos with yearly performed checks 61.1%. Regular deworming protocols were carried out in most institutions. Quarterly deworming was performed in seven zoos (proportion of *T. gondii*-seropositives 63.2%), biannually in 21 zoos (proportion of 60.0% *T. gondii*-seropositives) and in yearly intervals in three zoos (proportion of *T. gondii*-seropositives 100.0%) (Table 13.58-13.60).

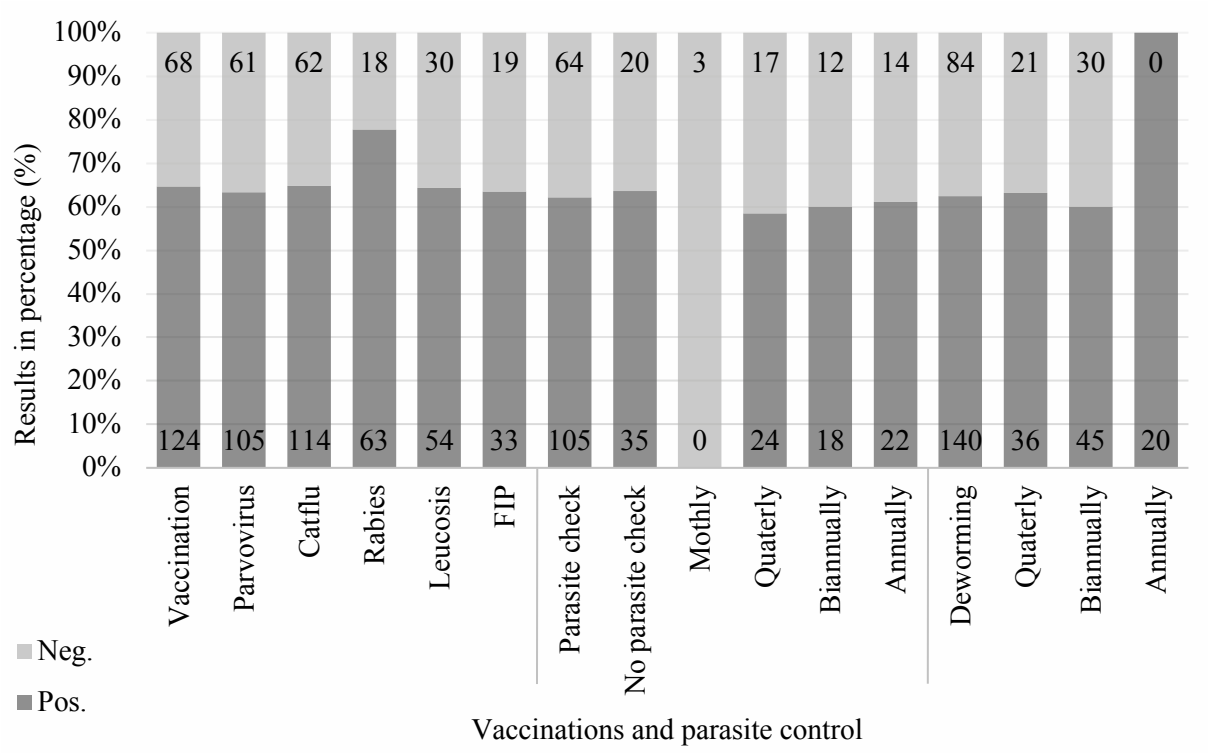

Figure 7.50: Seroprevalence regarding *T. gondii* stratified by variables concerning vaccinations and parasite control in the tested felids. The numbers of seropositive and seronegative individual felids are displayed within the columns.

Institutions were asked if they had performed any tests for *T. gondii* over the last five years. In total, 291 animals of various species had been tested, 69.8% with a *T. gondii*-seropositive and 30.0% with a negative result. 105 of the seropositive animals were felids, 15 monkeys, five marsupials and 78 belonged to various other species. Further classification of the total numbers of animals tested was not possible, as not every zoo provided the details necessary (Table 7.4).

Table 7.4: Numbers and proportions of animals tested for *T. gondii* over the last five years in zoos.

	Zoos that performed *T. gondii* tests	**Animals tested**	**Numbers of positive test results**				
			Total	**Felids**	**Marsupials**	**Monkeys**	**Other**
Total numbers	26	291	203	105	5	15	78
Proportion [%]	51.0	100.0	69.8	51.7	2.5	7.4	38.4

It was furthermore asked, how many deaths had occurred among the felid species sampled within the last five years. In 21 institutions that reported no losses in this period of time, a proportion of 65.1% *T. gondii*-seropositive individuals has been determined in this study. In 11 zoos one animal was lost, these institutions showed a proportion of 54.2% *T. gondii*-seropositive individuals. 73.7% of the felids tested were seropositive in five zoos that lost five individuals within the past five years. One institution that reported the loss of 16 felids showed a proportion of 50.0% *T. gondii*-seropositive felids (Table 13.61).

7.4.8 Identification of potential risk factors

Various routes may contribute to the infection of captive felids with *T. gondii*. Different entry routes like food, predation, hygiene and animal transport are displayed in Figure 7.51.

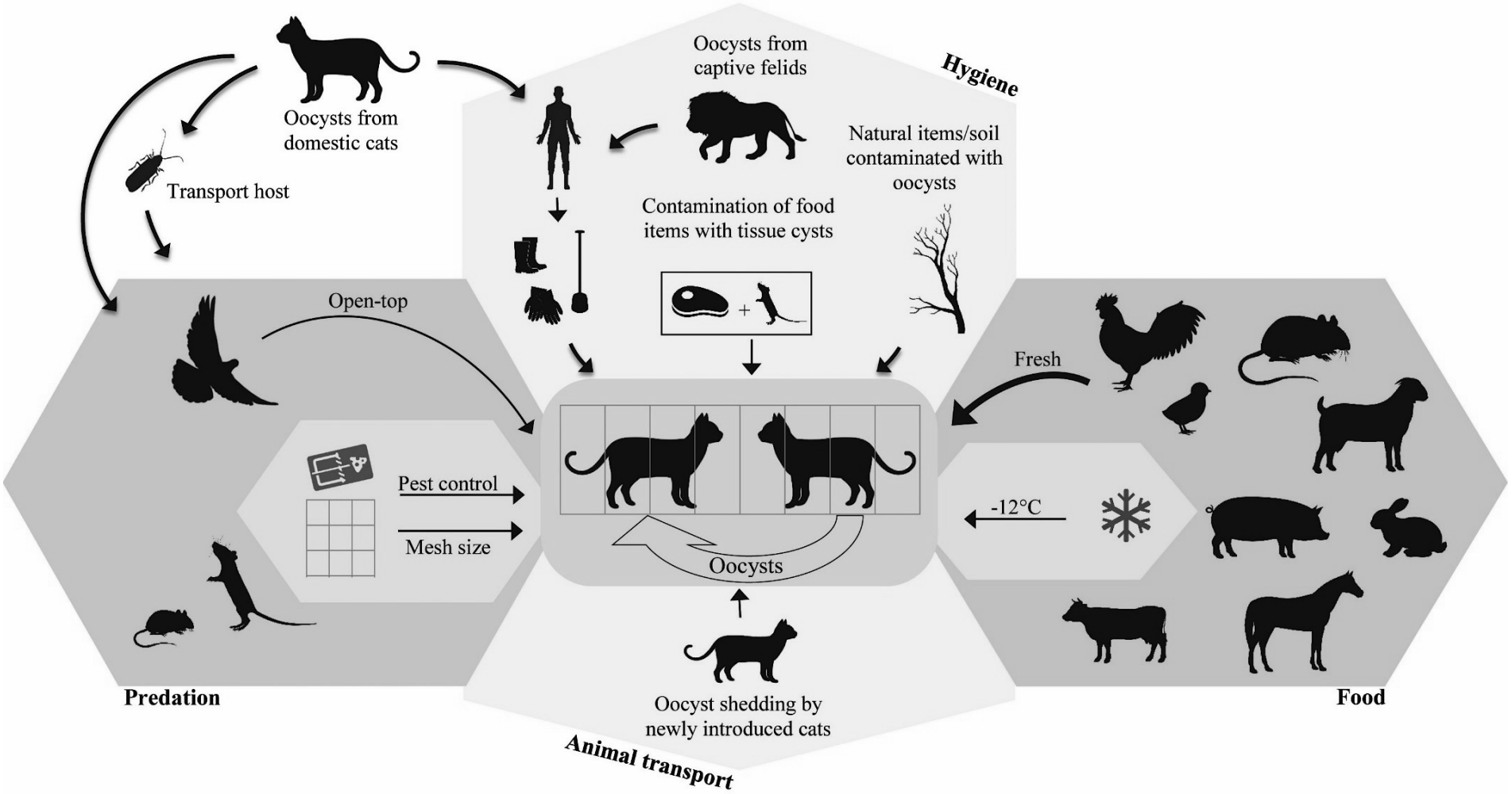

Figure 7.51: Potential entry routes for *T. gondii* in zoos and institutions keeping felids in captivity

For the identification of potential risk factors, multi-level modelling (generalized linear mixed model fit by maximum likelihood (Laplace Approximation)) was performed. The felids considered in this model came from 51 different zoos. Thus, random effects by different zoos were included in each of the models calculated. Because seropositivity clearly increased with age (chapter 7.4.1), which was regarded as an important effect-modifying explanatory variable, data on the age (in years) of the individual animals were included into each of the models. Since the age could only be calculated for animals, for which the date of birth and the sampling date was available, animals (n=13) with missing information on the age had to be excluded from the analysis.

In a first step, a generalized linear mixed model was calculated that included seropositivity as the dependent variable and, in addition to age (in years), all factors described in chapters 7.4.2-7.4.7). In all models, age remained as a statistically significant explanatory variable.

In addition to age, 15 further variables including gender, variables characterizing feeding or treatment of food (e.g. feeding mouse tissues, cattle tissues or tissues from fowl), keeping other zoo animals (e.g. NWM) close to felids, hygiene (e.g. wearing gloves), housing (e.g. outdoor housing, mesh-size of enclosures), health measures (e.g. rabies vaccination, deworming intervals (in months)) and numbers of litters produced by felids in the zoo (during the last year or during the past five years) had a statistically significant effect ($P < 0.1$) on the seropositivity of captive felids (Table 7.5-7.9).

Feeding of mice, rodents, tissues from cattle, ruminants or fowl in fresh condition was always the reference to analyze the risk of individual felids to test seropositive for *T. gondii*. Relative to these references, feeding these tissues after freezing had a statistically significant protective effect ($P < 0.05$ for meat from cattle and ruminants and $P < 0.1$ for carcasses of mice, rodents and fowl). Relative to the reference, mouse carcasses fed either fresh or previously frozen had a statistically significant protective model effect as well ($P < 0.05$).

Table 7.5: Fixed effects in generalized linear mixed models to determine potential individual risk factors for *T. gondii*-seropositivity in captive felids. The Akaike Information Criterion (AIC) was used to characterize model fit. Ref. = reference; Std. Error = standard error; z value = standard score; Pr(>|z|) = P-values computed from the z-values; Sig. = Statistical significance; Codes of significance: ***, < 0.001; **, 0.001 ≤ 0.01; *, 0.01 ≤ 0.05; ., 0.05 ≤ 0.1

Model (AIC, model fit)	Variable	Estimate	Std. Error	z value	Pr(>|z|)	Sig.
1 (354.8)	(Intercept)	-0.91455	0.38704	-2.363	0.0181	*
	Age	0.19731	0.04252	4.64	3.48E-06	***
	Sex- female (Ref.)					
	Sex- male	0.57605	0.29158	1.976	0.0482	*

Table 7.6: Fixed effects in generalized linear mixed models to determine potential food-related risk factors for *T. gondii*-seropositivity in captive felids. The Akaike Information Criterion (AIC) was used to characterize model fit. Ref. = reference; Std. Error = standard error; z value = standard score; Pr(>|z|) = *P*-values computed from the z-values; Sig. = statistical significance; Codes of significance: ***, < 0.001; **, 0.001 ≤ 0.01; *, 0.01 ≤ 0.05; ., 0.05 ≤ 0.1

Model (AIC, model fit)	Variable	Estimate	Std. Error	z value	Pr(>|z|)	Sig.
2 (300.6)	(Intercept)	0.26891	0.53793	0.5	0.6171	
	Age	0.20251	0.04734	4.278	1.89E-05	***
	Mice- Fresh (Ref.)					
	Mice- Fresh/Frozen	-1.42524	0.72147	-1.975	0.0482	*
	Mice- Frozen	-1.17735	0.66281	-1.776	0.0757	.
	Mice- No	-0.89937	0.81114	-1.109	0.2675	
3 (301.4)	(Intercept)	0.38772	0.66673	0.582	0.5609	
	Age	0.20797	0.04809	4.325	1.53E-05	***
	Rodents- Fresh (Ref.)					
	Rodents- Fresh/Frozen	-1.28399	0.7956	-1.614	0.1066	
	Rodents- Frozen	-1.38776	0.79496	-1.746	0.0809	.
	Rodents- No	-0.80939	1.14788	-0.705	0.4807	
4 (296.6)	(Intercept)	-0.01381	0.7099	-0.019	0.9845	
	Age	0.20855	0.04819	4.327	1.51E-05	***
	Ruminants- Fresh (Ref.)					
	Ruminants- Fresh/Frozen	-0.23356	0.89752	-0.26	0.7947	
	Ruminants- Frozen	-1.84804	0.86109	-2.146	0.0319	*
	Ruminants- No	-0.27029	0.76614	-0.353	0.7242	
5 (296.4)	(Intercept)	0.14541	0.7574	0.192	0.8478	
	Age	0.20861	0.04817	4.331	1.49E-05	***
	Cattle- Fresh (Ref.)					
	Cattle- Fresh/Frozen	-0.39333	0.9363	-0.42	0.6744	
	Cattle- Frozen	-2.00582	0.90769	-2.21	0.0271	*
	Cattle- No	-0.46307	0.80823	-0.573	0.5667	
6 (301.9)	(Intercept)	0.54495	0.79391	0.686	0.4925	
	Age	0.20059	0.04781	4.195	2.72E-05	***
	Fowl- Fresh (Ref.)					
	Fowl- Fresh/Frozen	-1.03852	0.86231	-1.204	0.2285	
	Fowl- Frozen	-1.50805	0.83011	-1.817	0.0693	.
	Fowl- No	-0.99211	1.3874	-0.715	0.4746	

Among the housing-related variables (Table 7.7), no litters born within one or five years prior to sampling was the reference to analyze the risk of individual felids to test seropositive for *T. gondii*. Relative to these references "Few litters born" (1-2 within one year, or 1-9 within five years) proved to be a statistically significant risk factor ($P < 0.05$). The same effect was found, when three or more litters were born during the last year ($P < 0.05$).

Keeping "NWM close by" or keeping animals in "Outdoor enclosures fenced in on all sides" showed statistically significant protective effects ($P < 0.1$) compared to the references ("No NWM close by", "No outdoor housing fenced in on all sides"). A mesh size of 5 cm and more-characterizing fencing-was the reference to analyze the risk of individual felids to test seropositive for *T. gondii*; mesh sizes "Below 5 cm" showed a statistically significant protective effect ($P < 0.05$).

Table 7.7: Fixed effects in generalized linear mixed models to determine potential housing-related risk factors for *T. gondii*-seropositivity in captive felids. The Akaike Information Criterion (AIC) was used to characterize model fit. Ref. = reference; Std. Error = standard error; z value = standard score; Pr(>|z|) = *P*-values computed from the z-values; Sig. = statistical significance; Codes of significance: ***, < 0.001; **, 0.001 ≤ 0.01; *, 0.01 ≤ 0.05; ., 0.05 ≤ 0.1

Model (AIC, model fit)	Variable	Estimate	Std. Error	z value	Pr(>|z|)	Sig.
7 (321.4)	(Intercept)	-1.67063	0.58434	-2.859	0.00425	**
	Age	0.20601	0.04552	4.526	6.01E-06	***
	Litters within 1 year- NULL (Ref.)					
	Litters within 1 year- 1-2	1.62867	0.68859	2.365	0.01802	*
	Litters within 1 year- ≥3	1.32641	0.62031	2.138	0.03249	*
8 (324.4)	(Intercept)	-1.49508	0.65609	-2.279	0.0227	*
	Age	0.20022	0.04559	4.392	1.12E-05	***
	Litters within 5 years- NULL (Ref.)					
	Litters within 5 years- 1-9	1.43293	0.6776	2.115	0.0345	*
	Litters within 5 years- ≥10	0.8376	0.6745	1.242	0.2143	
9 (297.9)	(Intercept)	-0.41259	0.4002	-1.031	0.3026	
	Age	0.21177	0.04852	4.364	1.28E-05	***
	NWM close by (Ref.)					
	NWM close by	-1.17519	0.67622	-1.738	0.0822	.
10 (298.4)	(Intercept)	0.44984	0.68933	0.653	0.514	
	Age	0.19578	0.04666	4.196	2.72E-05	***
	Outdoor housing fenced in on all sides-No (Ref.)					
	Outdoor housing fenced in on all sides-Yes	-1.21426	0.69023	-1.759	0.0785	.
11(290.4)	(Intercept)	0.77143	0.65554	1.177	0.2393	
	Age	0.19189	0.04518	4.247	2.16E-05	***
	Mesh size- more than 5 cm (Ref.)					
	Mesh size- below 2 cm	-0.73987	0.76366	-0.969	0.3326	
	Mesh size- 2-5 cm	-1.68593	0.69772	-2.416	0.0157	*

Applying general health and hygienic measures such as wearing gloves or rabies vaccination had statistically significant protective effects on the risk of individual felids to test seropositive for *T. gondii*; ($P < 0.05$) compared to the references ("No gloves", "No rabies vaccination"). (Table 7.8-7.9).

Table 7.8: Fixed effects in generalized linear mixed models to determine potential hygiene related risk factors for *T. gondii*-seropositivity in captive felids. The Akaike Information Criterion (AIC) was used to characterize model fit. Ref. = reference; Std. Error = standard error; z value = standard score; Pr(>|z|) = *P*-values computed from the z-values; Sig. = statistical significance; Codes of significance: ***, < 0.001; **, 0.001 ≤ 0.01; *, 0.01 ≤ 0.05; ., 0.05 ≤ 0.1

Model (AIC, model fit)	Variable	Estimate	Std. Error	z value	Pr(>|z|)	Sig.
12 (295.1)	(Intercept)	-0.14802	0.39021	-0.379	0.7044	
	Age	0.20289	0.04667	4.347	1.38E-05	***
	Gloves- No (Ref)					
	Gloves- Yes	-1.25221	0.51555	-2.429	0.0151	*

Table 7.9: Fixed effects in generalized linear mixed models to determine potential general health related risk factors for *T. gondii*-seropositivity in captive felids. The Akaike Information Criterion (AIC) was used to characterize model fit. Ref. = reference; Std. Error = standard error; z value = standard score; Pr(>|z|) = *P*-values computed from the z-values; Sig. = statistical significance; Codes of significance: ***, < 0.001; **, 0.001 ≤ 0.01; *, 0.01 ≤ 0.05; ., 0.05 ≤ 0.1

Model (AIC, model fit)	Variable	Estimate	Std. Error	z value	Pr(>|z|)	Sig.
13 (248.5)	(Intercept)	-1.59116	0.56244	-2.829	0.00467	**
	Age	0.22821	0.05439	4.196	2.72E-05	***
	Rabies vaccine- No (Ref.)					
	Rabies vaccine- Yes	1.59122	0.65822	2.417	0.01563	*
14 (175.8)	(Intercept)	-3.04025	1.3555	-2.243	0.024903	*
	Age	0.26781	0.07837	3.417	0.000633	***
	Deworming interval (months)	0.37112	0.19787	1.876	0.060723	

To find out, if the input variables were independent from each other in the data set, a factor analysis was done.

When the input variables “Age”, “Sex”, feeding of “Mice”, “Rodents”, “Cattle”, “Ruminants” or “Fowl”, “Litters within 1 year”, “Litters within 5 years”, keeping “NWM close by”, “Outdoor housing fenced in on all sides”, “Mesh size”, wearing of “Gloves”, “Rabies” vaccination and “Deworming interval (month)” were included, factor analysis revealed dependences (Factor loadings of absolute values >0.5) between feeding “Mice”, “Rodents” and “Fowl”, and between feeding “Cattle” and “Ruminants” (Table 7.10). Because the model including “Mice” represented the better model with an AIC of 300.6 in the initial analysis than models with “Fowl” and “Rodents” (AIC of 301.9 and 301.4, respectively), the variable “Mice” was maintained and “Fowl” and “Rodents” were excluded from further analysis. “Cattle” was preferred and “Ruminants” removed, as “Cattle” showed the better AIC than “Ruminants” (296.4 and 296.6 respectively) in the initial analysis. “Litters within 1 year” was kept and “Litters within 5 years”, was removed for the same reasons (AIC 321.4 and 324.4, respectively). Finally, factor analysis revealed a dependency between “Mesh size” and “Rabies”. “Mesh size” was maintained and “Rabies” vaccination removed because the biological relevance of this variable for seropositivity regarding *T. gondii* could not be explained.

Table 7.10: Results of a factor analysis on input variables characterizing zoo animals and putative risk and protective factors for *T. gondii* infection. Factor loadings of absolute values <0.1 are not shown. Factor loadings of absolute values >0.5 are displayed bold and was regarded as an indication of dependency. Following variables were analyzed: Age, Sex, Mice, Rodents, Cattle, Ruminants, Fowl, Litters within 1 year, Litters within 5 years, NWM close by, Gloves, Outdoor housing fenced in on all sides, Mesh size, Rabies vaccination, Deworming interval (month).

Variables	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8
Age		0.191						
Sex							-0.161	
Mice	**0.923**			-0.136		0.282	-0.165	0.106
Rodents	**0.971**			-0.146	-0.152			
Cattle		**0.808**	0.223				**0.523**	
Ruminants		**0.961**		0.106			0.198	
Fowl	**0.609**	0.181	-0.207				0.251	0.258
Litters within 1 year	-0.193	0.244	**0.768**			0.162		-0.326
Litters within 5 years		-0.105	**0.950**			-0.202	-0.111	0.129
NWM Close by	0.205					**0.666**	0.173	0.209
Outdoor housing fenced in on all sides	0.265		-0.286		-0.13	0.156	0.282	
Mesh size		-0.15	-0.264	**-0.524**	-0.159	-0.132		0.244
Gloves						0.107		0.414
Rabies vaccination	-0.337	-0.281		**0.888**		-0.1		
Deworming interval (month)	-0.175				**0.957**		0.154	-0.106

A second factor analysis (Table 7.11) included all variables, which had remained after the first factor analysis: “Age”, “Sex”, “Mice”, “Cattle”, “Litters within 1 year”, “NWM Close by”, “Outdoor housing fenced in on all sides”, “Mesh size”, “Gloves” and “Deworming interval (month)”. In this set, absolute factor loadings > 0.5 suggested dependency between “Cattle” and “Litters within 1 year”. Due to the assumption that a feeding related variable has a higher biological plausibility than the breeding-related variable “Litters within 1 year”, “Cattle” was kept for further analysis and a third analysis started.

Table 7.11: Results of a second factor analysis on input variables characterizing zoo animals and putative risk and protective factors for *T. gondii* infection. Factor loadings of absolute values <0.1 are not shown. Factor loadings of absolute values >0.5 are displayed bold and was regarded as an indication of dependency. Following variables were analyzed: Age, Sex, Mice, Cattle, NWM close by, Litters within 1 year, Outdoor housing fenced in on all sides Mesh size, Gloves, Deworming interval (month).

Variables	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
Age			**0.996**			
Sex						-0.287
Mice	0.329	-0.152			-0.233	
Cattle		**0.501**	0.103			0.204
Litters within 1 year	-0.114	**0.927**		-0.189		-0.254
NWM close by	**0.992**					
Outdoor housing fenced in on all sides	0.203	-0.102	-0.11		-0.113	**0.582**
Mesh size		-0.183		**0.974**	-0.106	
Gloves	0.21			0.12		0.19
Deworming interval (month)	-0.143				**0.930**	

A third factor analysis revealed no dependency between the remaining nine input variables “Age”, “Sex”, “Mice”, “Cattle”, “NWM Close by”, “Outdoor housing fenced in on all sides”, “Mesh size”, “Gloves” and “Deworming interval (month)”, as none of the factors showed more than one absolute factor loading >0.5 (bold numbers) (Table 7.12).

Table 7.12: Results of a third factor analysis on input variables characterizing zoo animals and putative risk and protective factors for *T. gondii* infection. Factor loadings of absolute values <0.1 are not shown. Factor loadings of absolute values >0.5 are displayed bold. Following variables were analyzed: Age, Sex, Mice, Cattle, NWM close by, Outdoor housing fenced in on all sides, Mesh size, Gloves, Deworming interval (month).

Variables	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Age		**0.994**			
Sex					-0.361
Mice	0.216		0.485		
Cattle		0.115	-0.142	-0.181	0.267
NWM close by	**0.966**		0.241		
Outdoor housing fenced in on all sides	0.161	-0.141	0.214	0.209	0.444
Mesh size	-0.129		0.204	0.46	-0.172
Gloves	0.179			0.363	0.132
Deworming interval (month)			**-0.515**	-0.196	

In a last step, all nine variables “Age”, “Sex”, “Mice”, “Cattle”, “NWM Close by”, “Outdoor housing fenced in on all sides”, “Mesh size”, “Gloves” and “Deworming interval (month)” were included into a generalized linear mixed model to determine potential risk factors for *T. gondii*-seropositivity in captive felids. This model did not converge. Removing either “Deworming interval (month)” or “Mesh size” led to a converging model. Since for 19 zoos, no data on “Deworming interval (month)” was available and in contrast, only four zoos had not answered questions on “Mesh size”, the latter variable was kept and “Deworming interval (month)” removed from full modelling. The resulting model with the remaining input variables “Age”,

"Sex", "Mice", "Cattle", "NWM close by", "Outdoor housing fenced in on all sides", "Mesh size" and "Gloves" revealed an AIC of 283.2. After optimization by a stepwise reduction of variables, excluding those variables first, which – if removed – did not cause an increase in AIC, the final linear mixed model had an AIC of 276.8 and comprised of the five variables "Age", feeding "Cattle" tissues, "Outdoor housing fenced in on all sides", "Mesh size" of enclosures and wearing "Gloves" (Table 7.13). In this model, likelihood of seropositivity increased statistically significantly by "Age". Feeding "Cattle-Frozen" relative to "Cattle-Fresh", "Outdoor housing fenced in on all sides", "Mesh size below 5 cm" relative to "Mesh size more than 5 cm" and wearing "Gloves" had a protective effect.

Table 7.13: Fixed effects in optimized generalized linear mixed models to determine potential risk factors for *T. gondii*-seropositivity in captive felids. Modelling was performed assuming random effects by "Zoo". Optimization of modelling was started with a full model (including all variables with a statistically significant effect ($P < 0.1$) in an initial bivariable generalized linear mixed modelling including always "Age (year)" in addition to the variable in question and proven independent by factor analysis) (Table 7.5-7.9). Optimization of the full model was done by a stepwise reduction of variables, excluding those variables first, which – if removed – did not cause an increased Akaike Information Criterion (AIC).

Model (AIC, model fit)	Variable	Estimate	Std. Error	z value	Pr(>|z|)	Sig.
15 (276,8)	(Intercept)	2.82244	1.00145	2.818	0.00483	**
	Age	0.20896	0.04422	4.725	2.30E-06	***
	Cattle-Fresh (Ref.)					
	Cattle-Fresh/Frozen	-0.67945	0.66093	-1.028	0.30394	
	Cattle-Frozen	-1.94808	0.7068	-2.756	0.00585	**
	Cattle-No	-0.92345	0.63174	-1.462	0.14381	
	Outdoor housing fenced in on all sides-No (Ref.)					
	Outdoor housing fenced in on all sides-Yes	-1.34488	0.62083	-2.166	0.03029	*
	Mesh size more than 5 cm (Ref.)					
	Mesh size below 2 cm	-0.60252	0.70989	-0.849	0.39603	
	Mesh size below 5 cm	-1.14835	0.5793	-1.982	0.04744	*
	Gloves-No (Ref.)					
	Gloves-Yes	-0.86919	0.38825	-2.239	0.02517	*

Fixed effects: Estimate Std. Error z value Pr(>|z|)

8 DISCUSSION

As blood sampling in exotic species in general can be very challenging, the use of reduviid bugs allows to reduce the risk for the animal sampled and the person handling the animal. Especially in felids (also in smaller species), physical restraint of the animal goes along with the risk of major injuries due to scratching or biting. Despite from that, it causes substantial stress to the sampled animal. Anesthesia, as an alternative to physical restraint, always includes a risk to the patient. This risk is even higher in animals that are not totally healthy (Bille et al., 2012). Therefore, anesthesia should be avoided whenever possible.

8.1 General applicability of blood sampling using reduviid bugs

In the present study, severe reactions to the biting of *D. maxima* were not observed in any of the sampled domestic cats. Immediately after blood collection via bugs the cats showed small reddish bite marks, but swelling or signs of other adverse reactions like itching or pain to the bite after recovery from anesthesia could not be observed. Already shortly after the bite (1-2 hours) bite-marks could no longer be recognized. The saliva of triatomines has been observed to cause possible allergic reactions in humans when repeatedly used (Stadler et al., 2011). The most severe reactions were seen after the bite of *R. prolixus* (Meiser & Schaub, 2011), followed by *T. infestans* (Hoffman, 1987; Lapierre & Lariviere, 1954), while *D. maxima* only produced mild skin reactions (Marsden, 1986). These reactions were probably hypersensitive reactions and a sensitization to the bug bites could not be excluded (Costa et al., 1981). Other authors confirmed papular, urticarial and bullous reactions due to triatomine bites in humans (Stibich et al., 2001; Wood, 1942). In animals, IgG-antibody responses to the salivary antigens of *T. infestans* has been determined. Skin reactions have not been reported so far (Schwarz et al., 2009). When reduviid bugs were used to blood-sample zoo or wild animals, no allergic reactions were evident in any of the animals (Arnold et al., 2008; Becker et al., 2005; Braun et al., 2009; Helversen & Reyer, 1984; Thomsen & Voigt, 2006; Voigt et al., 2004; Voigt et al., 2003; Voigt et al., 2005; Voigt et al., 2006; Vos et al., 2010).

While the sampling box was a valuable tool to apply the reduviid bugs safely, temperature was a critical point in the applicability of the sampling method. Own experiences during the blood collection showed, that the temperature during the sampling procedure had a major influence on the success rate. At low temperatures (below 17°C), some bugs did not start or complete engorgement, while at higher temperatures almost 100% of the bugs fulfilled complete engorgement. This experience is in accord with experiences of other authors who found that triatomines do not start sucking blood at temperatures below 20°C (Stadler et al., 2011). As a consequence, the sampling periods were restricted to warm summer months. When working at temperatures of at least 20°C, the number of bugs could be reduced to two or three per cat with a success rate of almost 100%.

8.2 Applicability of blood sampling using reduviid bugs for clinical blood chemistry

The use of reduviid bugs to obtain blood samples from wild animals for health checks could be a valuable, minimal invasive tool for veterinarians and researchers. For blood chemical analysis, a sample set of three samples was collected: Conventional venous plasma (V) and plasma samples collected from reduviid bugs immediately after engorgement (B0) and one hour after engorgement (B1). These time intervals were regarded as most relevant in terms of practicability, when using the reduviid bugs on exotic animals. However, some major aberrations in test results were detected, when bug-derived plasma was used for blood chemical analyses instead of conventionally collected plasma. Blood sampling using *D. maxima* might therefore become a valuable tool to examine some parameters, but care must be taken, when parameters are chosen for examination. Parameters, for which there was a good or very good

correlation between test results obtained for V and B0 or for V and B1, and parameters, for which at the same time no statistically significant differences were observed between the results of obtained V and B0 or for results obtained for V and B1, were considered as reliable. All other parameters were considered not reliable. Table 8.1 summarizes the classifications of blood values as identified in this study.

Table 8.1: Classification of results on blood chemical parameters obtained by bug-derived plasma samples as determined in this study.

Classification of results	Results for B0 samples	Results for B1 samples
Not reliable	Tri, GLDH, Glu, TP, Glob, AP, TBil, K, Na, PHOS, Cl, TMg, TCa	Tri, GLDH, Glu, TP, Glob, AP, TBil, ALT, Alb, Chol, urea, K, Na, PHOS, Cl, TMg, TCa
Reliable	ALT, CK, Alb, Crea, Chol, urea	CK, Crea

8.2.1 Parameters that were not reliable in bug-derived samples

Insufficient correlations for Tri, GLDH, Glu, TP, Glob, AP, TBil, K, Na, PHOS, Cl, TMg or TCa indicated that measuring these values in blood samples from reduviid bugs did not produce reliable data. In addition, there was a statistically significant difference between V and B0 values in Tri, Glob, AP, TBil, K, Na, PHOS, TMg and TCa. The same effect occurred in all blood chemical parameters except for CK and Crea comparing V and B1 samples (Table 7.1).

The rise of interquartile ranges in GLDH and median values with increasing time from engorgement to sampling of the bugs illustrated that the reliability of data decreased the longer it took to collect blood from the reduviid bug. The same was also true for increasing Tri ranges. Another study suggested that Tri values did not differ statistically significantly between different sampling methods (Bauch et al., 2010). This parameter had been evaluated with an unknown number of samples from captive herring gulls (Bauch et al., 2010). The difference between the result in this thesis and the previous findings might be based on differences in host species or in sample size.

The analysis of Glu values showed that about 87.9% of all V samples had Glu levels above the reference range (3.89-6.11 mmol/l). This can be explained by the fact that many animals sampled were not fasted prior to sampling as they were presented to the clinic with an acute problem. Also, stress (for example caused by the transport to the clinic) could be a reason for the observation of elevated Glu results (Kraft & Dürr, 2005). The median Glu values were higher in B1 as compared to V. While the difference in the results was not statistically significant in the comparison V vs. B0 it was in case of V vs. B1. In contrast to this study, Markvardsen et al. (2012) stated that Glu did not differ statistically significantly between conventional and the bug-derived samples and would thus provide reliable results. They analyzed blood from only four New Zealand white rabbits using 13 bugs for clinical blood chemistry. Also, they collected blood samples only at B0, which might have led to the difference of the outcome in addition to a lower sample size.

TP values were observed to rise from within the reference range in V above the range in B0 in 15.2% of the samples. Statistically significantly higher TP results in bug-derived samples were also observed by Depauw (2012). In this study, already 42.4% of the samples that had values within the reference range in V showed values above the range in B1. The range of results became larger with increasing time from engorgement to sampling of the bugs. While the median values in conventional plasma and the bug-derived sample B0 were almost the same, the values measured in B1 were considerably higher, which underlines the effect of elongated periods until sampling. Other authors found reliable results for TP (Markvardsen et al., 2012;

Stadler et al., 2007, 2009). This may be explained by very short time intervals between engorgement and sampling, lower sample sizes or the use of different species.

For Glob, 24.2% of the tests results changed from between inside to outside the reference or vice versa when B0 was used for the analysis instead of V. The same occurred in 54.6% of the samples when B1 was used instead of V. Thus, the diagnostic value of results on bug-derived samples for this parameter is questionable. Stadler et al. (2007) found Glob values in bug-derived samples reliable. They compared the results from samples of a single host obtained through several bugs and observed similar results in conventionally collected plasma and bug-derived plasma. They also compared conventionally collected and bug-derived samples from various species at the time B0, which might have led to the different outcome in the present and the previous study.

Already in samples collected one hour after engorgement (B1), 55.6% of the test results for Cl were below the reference range. In samples collected immediately after engorgement (B0), only 16.1% had test results below the reference range. Since the Cl values in bug-derived samples had a strong tendency to decrease with increasing time between sampling and end of engorgement, as also observed in another study (Depauw, 2012), test results for Cl above the reference range can always be interpreted as a hyperchloremia.

Another blood-chemical parameter in the present study that tended to be lower in bug-derived samples than in conventionally collected plasma was AP. This observation is in accord with results of Markvardsen et al. (2012). However, the differences in Cl and AP values between bug-derived and conventional plasma samples were not explicitly confirmed by another study, in which bugs had been sampled immediately, and where blood-chemical results were compared with those of venous samples from a variety of species (Stadler et al., 2007, 2009).

PHOS, TCa, TMg and TBil values were higher in B0 and B1 than in V and had obviously increased with increasing time from engorgement to sampling. Values measured for B1 were also higher than those for B0. In PHOS, 45.5% of the results, which were within the reference range for V, tested above the range, when B0 samples was analyzed. Due to the observation that PHOS, TCa TMg and TBil tended to be higher in samples collected via bugs, results from bug-derived samples below the reference range can be interpreted as a hypophosphatemia, hypocalcemia or hypomagnesemia, respectively. As only elevated results for TBil are an indication for illnesses, values for bug-derived samples within the reference range can be interpreted as unaltered. Higher PHOS, TMg and TBil results in samples collected with reduviid bugs than those determined in conventional plasma were also found by Depauw et al. (2012), while they did not find statistically significant differences in TCa results. By contrast, Markvardsen et al. (2012) found statistically significantly lower PHOS and TCa values in bug-derived samples than in conventional plasma, while TMg values showed no statistically significant differences. According to Stadler et al. (2007), liver-associated parameters (including TBil) did not seem to be reliable. In contrast, the authors stated that bug-based sampling would deliver reliable data for PHOS and TCa values. Different results regarding the reliability of hematological parameters measured in bug-derived plasma in different studies might be caused by differences in host species, sample size or tests used.

The ranges of K and Na values in bug-derived plasma remained very similar relative to those from conventional plasma with increasing time from engorgement to sampling the bugs. However, median K values increased with increasing time from engorgement and sampling while Na median values decreased. Other authors found the same effect with K values that were higher in bug-derived plasma, while Na values were lower than in conventionally collected plasma. In accord with this study, they found statistically significant differences between results of the two sampling methods (Depauw, 2012; Markvardsen et al., 2012). While Na values determined in bug-derived plasma were reliable according to Stadler et al. (2007), these authors

found also major variations between K values determined in conventionally and bug-derived plasma.

The results regarding ALT, Alb, Chol and urea values were reliable in B0 as compared to those in V, but not in B1. Statistically significant differences occurred in the comparison of values for V and B1. These findings on effects of time between end of engorgement and sampling could not be compared with those of other studies, because in none of the studies the bias caused by the time between engorgement and sampling from the reduviid bugs had been analyzed.

Processes that may influence the results for different blood parameters are described in chapter 8.2.3.

8.2.2 Parameters showing reliable results in bug-derived samples

The ALT, CK, Alb, Crea, Chol and urea values in conventionally collected plasma samples showed a good or very good correlation with values measured in bug-derived samples B0 and in B1, except for ALT, in which a moderate correlation between values for V and B1 was evident. All correlations between the values of conventional plasma (V) and bug-derived plasma (B0, B1) were statistically significant. No statistically significant differences were observed between V and B0 in these parameters, thus they could be considered reliable and are potentially useful for assessment of patients. In B1, most parameters showed statistically significant differences compared to V results, except for CK and Crea, which could still be considered reliable. In general, the validity of data improved, when the time between engorgement and blood sampling was shorter.

Although median ALT values were higher in B0 or B1 relative to those in V, none of the B0 samples showed values outside the reference range. Only in one B1 sample, the ALT value was outside the range although the V value was inside the reference range. Higher ALT results in bug-derived samples have also been observed in previous studies. The authors suggested that values of liver enzymes such as ALT were not reliable, when bug-obtained plasma was used for analysis (Depauw, 2012; Stadler et al., 2007). Another study supports the finding that ALT values did not differ statistically significantly between the different sampling methods (Markvardsen et al., 2012). In conclusion, it seems to be of paramount importance for these analytes to keep time from engorgement to sampling as short as possible.

Results for CK values showed no statistically significant differences between V and B0 or V and B1 in addition to good correlations between values for samples collected by the different blood sampling techniques. This led to the conclusion that CK values can be reliably determined in bug-derived samples. No other author reported results for CK; the present results could therefore not be compared with results from other studies.

A good correlation and no statistically significant differences between the test results for conventional and bug-derived blood samples (B0) suggested that Alb and Crea can be reliably determined, using the less invasive bug technique. Other authors' observations agree with this finding (Depauw, 2012; Markvardsen et al., 2012; Stadler et al., 2007). In B1, Alb values could not be considered reliable anymore due to statistically significant differences between B1 and V values. In the other above-mentioned studies, samples were only taken at B0. They could therefore not confirm the changes in results obtained at prolonged times between engorgement and sampling.

Chol values seemed to be reliable in B0 samples. This was confirmed by good correlations between test results on samples collected by different techniques and no statistically significant differences were observed. While the results of Bauch et al. (2010) corroborate these findings, Markvardsen et al. (Markvardsen et al., 2012) found statistically significantly higher Chol values in conventional blood samples as compared to bug-derived samples. The difference in

results might have been due to the sampling of different host species or to differences in sample size.

Urea values in conventionally collected plasma samples showed a very good correlation with values measured in bug-derived samples B0 and in B1. This correlation proofed to be statistically significant for both the comparisons V vs. B0 and V vs. B1. In any case, the differences between V and B0 were not statistically significant and the results in B0 samples seem therefore reliable for patient assessment. Other studies corroborate this finding (Depauw, 2012; Stadler et al., 2007).

To receive results as accurate as possible from bug-derived plasma, the time between end of engorgement and sampling should always be kept as short as possible. Even the CK and Crea results, for which reliable values had still been measured in B1, may be influenced by digestion in the bugs, which may lead to minor aberrations.

Processes that influence the results for different blood parameters are described in chapter 8.2.3.

8.2.3 Influencing factors on clinical blood chemistry

Several factors might cause aberrations in blood-chemical values; one of them is hemolysis.

The conventional collection of plasma and the collection method via bugs showed differences in the extent of hemolysis, which was determined by visual inspection. While two V samples (6%) showed hemolysis, the number of hemolytic samples was statistically significantly higher in B0 (88%) and B1 (85%) samples. In about 55% of the samples, the grade of hemolysis even increased from B0 to B1. In human clinical setups, the occurrence of hemolytic samples ranges between 3% and 3.5% (Carraro et al., 2000). Assuming that the percentage in veterinary medicine may be similar, the grade of hemolysis in bug-retrieved samples is remarkable. Typically, hemolysis occurs due to turbulences (Grant, 2003) for example caused by rough handling, but also during prolonged storage (Harvey, 2001). Although a big gauge needle was chosen in the present study to gather the blood samples from reduviid bugs, hemolysis might be caused during the process of blood collection. Most likely, bug associated factors may have played a role in causing hemolysis. Hemoglobin is an important protein source for reduviid bugs. It is made accessible by lysis of erythrocytes, a process that already occurs when the bugs begin to digest blood (Azambuja et al., 1983; Bauer, 1981; Canavoso et al., 2004; Garcia et al., 2010; Markvardsen et al., 2012; Ribeiro & Pereira, 1984; Schaub, 2009). Since hemolysis can potentially influence blood parameters (Sonntag, 1986) it might be the cause for some of the differences observed in blood chemical results between conventionally collected plasma and plasma samples collected with reduviid bugs.

This effect may be important to explain the observations made for ALT values. Since the ALT concentration in erythrocytes is approximately 7-fold higher than in blood plasma, the lysis of red blood cells most likely causes increasing levels of free ALT in bug-derived plasma samples (Sonntag, 1986). This is also valid for organic phosphates (Alleman, 1990), TMg (Heins et al., 1995; Sonntag, 1986) and K (Ramer et al., 1995; Sonntag, 1986; Stadler et al., 2007), which are more concentrated in erythrocytes than in plasma.

In addition, the concentration of Cl, Na and TCa is lower in erythrocytes than in plasma. Hemolysis can therefore cause a decrease of these parameters in blood plasma (Depauw, 2012; Heins et al., 1995; Sonntag, 1986). This process seems to have a minor effect on TCa as the predominant effect is an increase of TCa values. Furthermore, other studies described a decrease of AP values (Sonntag, 1986) and slightly lower CK values (Morgan et al., 1998) in hemolytic samples.

Hemolysis causes a rise of hemoglobin in plasma, which absorbs light at wavelengths of 417-575 mm. Similar wave lengths are often used to determine Alb, TBil and TP. Therefore, hemoglobin can have an additive effect, which might explain the increases observed in

hemolyzed sera (Ramer et al., 1995). Interferences due to increased hemoglobin concentrations in plasma were observed at concentrations above 232 µmol/l for Alb, above 195 µmol/l for TBil and above 1100 µmol/l for TP using the Pentra 400 (ABX-Horiba). Since hemoglobin values were not determined in this study and visual grading of hemolysis is not as reliable (Glick et al., 1989; Hawkins, 2002), we can only hypothesize that this effect may have played a role in the above-mentioned alterations.

The Glob values were determined by calculating the difference between TP and Alb. Aberrations in the Glob results may thus originate in aberrations caused during the measurement of these parameters (Jacobs et al., 1992).

Among conventional samples from 33 individuals, three (9%) samples were slightly lipemic (identified by the opaque appearance of the plasma). In B0, 6 samples (18%) and in B1 seven samples (21%) were lipemic. Lipemia may falsely increase TBil, TP and PHOS (Alleman, 1990), which might have influenced the results as well.

Serum phosphatases can cause hydrolysis of phosphatase esters, which implicates an increase in inorganic phosphates (Heins et al., 1995).

In the case of CK, the sampling technique can influence results as well. Not every individual allowed easy access to its veins so that muscle trauma at the injection site might cause elevated CK results (Fayolle et al., 1992). One can hypothesize that the bugs cause less trauma at the sampling site and therefor do not cause an explicit rise in CK values. In this study, samples were taken opportunistic during different procedures. As conventional samples could be taken within a very short time, the bugs were still taking up blood, while the procedure (e.g. castration) had already started. Although these factors might have influenced the results, they did not seem to play a major role as CK values did not differ statistically significantly when the values for V were compared to those observed for B0 or B1.

Other processes that can influence differences between conventional and bug-derived plasma in all parameters can be the dilution of blood from the host with hemolymph or intestinal fluid derived from the bug (Voigt et al., 2003). It was suggested that maximal engorgement of the bug could increase blood values because the proportion of fluids secreted by the bug relative to the host-derived blood will decrease (Stadler et al., 2007). Another study suggested that the dilution with hemolymph never exceeded 4% (Helversen et al., 1986). The authors determined the maximum amount of hemolymph in a bug by squeezing it. No conclusions could be drawn about this factor in the present study, as all bugs that were taken into account were fully engorged at the time of sampling.

In addition, the fact that blood samples collected from reduviid bugs can be of both, venous or arterial origin could have caused differences in blood-chemical values in any parameter (Markvardsen et al., 2012).

Furthermore, the bugs start to concentrate blood very quickly. Wigglesworth (1931) analyzed the process of fluid excretion in the reduviid bug *Rhodnius prolixus*. Almost immediately after feeding, the insect started to void fluid. This process was especially noticeable in the first three to four hours, although the frequency of excretion varied a lot between individuals. The osmotic concentration of the urine produced by engorged *R. prolixus* depended on the temperature and rose with increasing temperature (Maddrell, 1964). As *R. prolixus* belongs to the same subfamily (Triatominae) as *D. maxima* (de Paula et al., 2005), similar processes can be assumed that may possibly influence all blood parameters.

Wigglesworth (1931) found that the urine of *Rhodnius prolixus* contained appreciable amounts of Na and urea, especially in the first few hours after engorgement. In addition, K was excreted with the urine of the bugs in small quantities shortly after engorgement. Its amounts gradually increased with time. These processes are likely to happen in *D. maxima* as well. The excretion of Na and urea would explain lower levels in bug-derived samples, but this may have

a minor effect on K as the predominant effect observed in this study was an increase of K values, possibly caused by hemolysis.

Markvardsen et al. (2012) discussed the effect of K^+/H^+ antiports that exchange extracellular H^+ for intracellular K^+. This process could cause an increase in plasma K values during engorgement. In addition, the authors concluded that the passive transport of Na^+ across the basal membrane of the anterior midgut into epithelial cells driven by Na^+/K^+ ATPase might have caused a decrease in plasma Na values.

Despite the increase of PHOS, TMg, TCa and TP in the engorged blood, there is no excretion of these analytes with the urine of *Rhodnius prolixus* (Wigglesworth, 1931). It is reasonable to hypothesize that the same process occurs in *D. maxima* as well. The loss of fluids and the hold back of PHOS, Mg, TCa and TP might therefore cause a further increase of these parameters. However, the excretions of *R. prolixus* did not contain Crea either (Wigglesworth, 1931). If the same process occurs in *D. maxima*, it hardly seems to influence Crea values.

The Malpighian tubules of *Rhodnius prolixus* were shown to use glucose as an energy source for secretion (Maddrell, 1969). Although this process is likely to happen also in *D. maxima*, it seems improbable that it has a major impact on the Glu values in bug-derived plasma samples, as an increase of plasma Glu occurred.

It is important to note that the results discussed above account for domestic cats and most likely for the family of the felids, if the same laboratory techniques are used. Generalizing the results is not unproblematic as differences caused by pre-analytical factors like hemolysis, lipemia or bilirubinemia can vary depending on the method of analysis and the sampled species (Braun et al., 2015; Jacobs et al., 1992).

8.3 Applicability of bug-derived samples for *T. gondii* serology

As a result, the tests in domestic cats showed that the antibody titer from venous blood correlated statistically significantly ($P < 0.001$) with titers determined for blood taken with the help of reduviid bugs. This correlation could be confirmed even after waiting four hours to obtain the blood sample from the bug. However, only between V and B0 samples no statistically significant difference was found. This suggests that blood collection using *D. maxima* can be a valuable tool to show an antibody reaction for *T. gondii* in felids, if the period between engorgement and blood collection is reduced to a minimum. Anyway, positive or negative serological *T. gondii*-results of the individual tested were consistent in V, B0, B1, B2 and B4 in all samples. If the antibody reaction to *T. gondii* had to be determined as positive or negative, the time of sampling did not influence the diagnostic outcome. For the determination of reliable antibody titers, the use of V or B0 samples may be recommended.

This study further confirmed that reduviid bugs can be a valuable tool in antibody detection, especially in exotic animals, as previously shown for other infective agents (Table 8.2).

Table 8.2: Implementation of blood sampling for serology using reduviid bugs.

Host Species	Reduviid used	Infectious agent	Author
Domestic cat *(Felis catus)* Geoffroy's cat (*Leopardus geoffroyi*) Jaguarundi (*Puma yagouaroundi*) Margay (*Leopardus wiedii*) Oncilla (*Leopardus tigrinus*) Asian golden cat (*Catopuma temminckii*) Black-footed cat (*Felis nigripes*) Fishing cat (*Prionailurus viverrinus*) Pallas' cat (*Otocolobus manul*) Rusty-spotted cat (*Prionailurus rubiginosus phillipsi*) Sand cat (*Felis margarita*)	*D. maxima*	*T. gondii*	Present Study
Baird's tapir *(Tapirus bairdii)* Giraffe *(Giraffa camelopardalis)* Harbor seal (Phoca vitulina) Indian rhinoceros *(Rhinoceros unicornis)* Lowland tapir *(Tapirus terrestris)* Malayan tapir *(Tapirus indicus)* South american sea lion *(Otaria flavescens)*	*D. maxima*	Tuberculosis	(Stadler et al., 2011)
Black faced sheep *(Ovis ammon f. aries)* Dromedary *(Camelus dromedarius, dom.)* Giraffe *(Giraffa camelopardalis)* Lowland tapir *(Tapirus terrestris)* Siberian ibex *(Capra ibex sibirica)* White-lipped deer *(Cervus albirostris)* Yellow-backed duiker *(Cephalophus silvicultor)*	*D. maxima*	BTV	(Stadler et al., 2011)
Giraffe *(Giraffa camelopardalis)* Red river hog *(Potamochoerus porcus)*	*D. maxima*	Brucellosis	(Stadler et al., 2011)
Mouse *(Mus musculus)*	*D. maxima* *R. prolixus*	Rabies	(Vos et al., 2010)
Domestic rabbit *(Oryctolagus cuniculus)*	*D. maxima*	RHDV	(Voigt et al., 2006)

As a gentle method that causes only minimal stress, this technique might improve diagnostic possibilities, especially in exotic species including felids.

8.4 Seroprevalence of antibodies to *T. gondii* in small exotic felids in European zoos

With 63.0% *T. gondii*-seropositive results in zoo felids, the seroprevalence for *T. gondii* was remarkably high in the tested felids. Worldwide, the seroprevalence for *T. gondii* in domestic cats (*Felis catus*) was estimated as 30-40% (Elmore et al., 2010). In other studies, seroprevalences of 34.7% among zoo animals in Czech and Slovak, 54.6% among cats in Brazilian zoos, 59% among captive felids in the United States and 15.4% in captive felids in Thailand were found (Sedlak & Bartova, 2006; Silva et al., 2001; Spencer et al., 2003; Thiangtum et al., 2006). The varying proportions between different species were interesting. While Pallas' cats (90.4%) and Rusty-spotted cats (96.4%) showed high proportions of seropositivity, the proportions of *T. gondii*-seropositive animals were considerably lower in other species, e.g. the Black-footed cat (26.7%).

Differences in the susceptibility of various feline species for *T. gondii* were discussed in previous studies. Especially in Pallas' cats, but also in Sand cats, a high susceptibility to *T. gondii* has been assumed (Brown et al., 2005; Dubey et al., 2010). Vertical transmission from

an infected mother to kittens is likely to occur in these species, which is not known for domestic cats (Basso et al., 2005; Dubey et al., 2010; Pas & Dubey, 2008a; Riemann, Fowler, et al., 1974). Based on the studbook data for Pallas' cats and Sand cats, a draft pedigree of all individuals tested in this study was generated. There was no evidence for vertical transmission in the pedigree in any of the two species. However, vertical transmission cannot be ruled out completely, since the time of seroconversion is not known. Pedigree analysis furthermore revealed that the individuals, which had been tested serologically, originated from 22 founder animals born in the wild in Pallas' cats and 24 founder animals in Sand cats. It is quite likely that some of these founder animals were closely related and that genetic diversity in their progeny may thus be lower than expected. In other species, e.g. mice, there is a remarkable difference in susceptibility to oral infection with *T. gondii* among different inbred strains (Liesenfeld et al., 1996). It might be possible that genetic factors predispose some feline species for *T. gondii* infections as well.

In Pallas' cats, an immunodeficiency (congenital or acquired) similar to FIV was suspected to play a role for a higher susceptibility (Ketz-Riley et al., 2003). FIV was found in captive and wild Pallas' cats showing a unique monophyletic lineage of the virus in the population (Brown et al., 2010; Troyer et al., 2005). Brown et al. (2005) compared the general health status and indicators for chronic stress (corticoid metabolite measurement in fecal samples) of captive and wild Pallas' cats and found similar results in both populations. In contrast to captive populations, low percentages (13%) of *T. gondii* seropositive animals were found in the wild (Brown et al., 2005; Naidenko et al., 2014). Brown et al. (2005) concluded that Pallas' cats might not have co-evolved with *T. gondii* leading to a certain susceptibility for the parasite.

Variations in numbers of examined animals, tests and the age of tested felids did not allow comparisons of *T. gondii* prevalences in different studies (Table 8.3). In any case, it seems likely that the prevalences reported for Pallas' cats and Sand cats are generally higher than those in other small feline species.

Table 8.3: Prevalences of *T. gondii* among small cat species sampled in the present study (in bold) compared with other studies.

Species	Test	No. Exam.	% Positive	Location	Author
Geoffroy's cat *(Leopardus geoffroyi)*	**IB + IFAT**	**33**	**48.48**	**Europe**	**Present Study**
	Molecular/DNA	22	27.27	Brazil	(Cañón-Franco et al., 2013)
	ELISA	8	25.00	Bolivian Chaco	(Fiorello et al., 2006)
	MAT	12	83.33	Brazil	(Silva et al., 2007)
	IFAT	1	0.00	California	(Spencer et al., 2003)
	MAT	1	100.00	Brazil	(Ullmann et al., 2010)
Jaguarundi *(Puma yagouaroundi)*	**IB + IFAT**	**9**	**55.56**	**Europe**	**Present Study**
	MAT	2	50.00	Mexico	(Alvarado-Esquivel et al., 2013)
	IFAT	25	40.00	Brazil	(Andre et al., 2010)
	Molecular/DNA	22	40.91	Brazil	(Cañón-Franco et al., 2013)
	ELISA	1	100.00	United States	(Lappin et al., 1991)
	IFAT	1	100.00	Brazil	(Rivetti Júnior et al., 2008)
	IFAT	1	100.00	Czech/Slovak zoos	(Sedlak & Bartova, 2006)
	MAT	99	46.46	Brazil	(Silva et al., 2007)
	IHA + MAT	2	50.00	Brazil	(Silva et al., 2016)
	IFAT	2	0.00	California	(Spencer et al., 2003)
	MAT	3	66.67	Brazil	(Ullmann et al., 2010)
Margay *(Leopardus wiedii)*	**IB + IFAT**	**19**	**47.37**	**Europe**	**Present Study**
	IFAT	4	100.00	Brazil	(Andre et al., 2010)
	Molecular/DNA	10	60.00	Brazil	(Cañón-Franco et al., 2013)
	MAT	2	50.00	Guatemala	(Lickey et al., 2005)
	IHA	2	0.00	California	(Riemann, Behymer, et al., 1974)
	MAT	63	53.97	Brazil	(Silva et al., 2007)
	IHA + MAT	1	100.00	Brazil	(Silva et al., 2016)
	MAT	17	58.82	Brazil	(Ullmann et al., 2010)
Oncilla *(Leopardus tigrinus)*	**IB + IFAT**	**9**	**66.67**	**Europe**	**Present Study**
	MAT	2	0.00	Mexico	(Alvarado-Esquivel et al., 2013)
	IFAT	35	62.86	Brazil	(Andre et al., 2010)
	Molecular/DNA	28	28.57	Brazil	(Cañón-Franco et al., 2013)
	IFAT	1	100.00	Bolivia	(Deem et al., 2004)
	MAT	131	50.38	Brazil	(Silva et al., 2007)
	DT	9	66.67	Brazil	(Sogorb et al., 1977)
	MAT	22	68.18	Brazil	(Ullmann et al., 2010)

Species	Test	No. Exam.	% Positive	Location	Author
Asian golden cat *(Catopuma temminckii)*	**IB + IFAT**	**2**	**50.00**	**Europe**	**Present Study**
	MAT	2	50.00	Australia	(Hill et al., 2008)
	IHA	3	33.33	California	(Riemann, Behymer, et al., 1974)
	LA	8	12.50	Thailand	(Thiangtum et al., 2006)
	ELISA + MAT	6	83.33	Shanghai Zoo	(Zhang et al., 2000)
Fishing cat *(Prionailurus viverrinus)*	**IB + IFAT**	**40**	**55.00**	**Europe**	**Present Study**
	IFAT	1	0.00	Brazil	(Andre et al., 2010)
	DT	1	100.00	Thailand	(Buddhirongawatr et al., 2006)
	MAT	4	25.00	Midwestern zoos	(de Camps et al., 2008)
	MAT	4	50.00	Australia	(Hill et al., 2008)
	IFAT	1	0.00	California	(Spencer et al., 2003)
	LA	27	22.22	Thailand	(Thiangtum et al., 2006)
Pallas' cat *(Otocolobus manul)*	**IB + IFAT**	**52**	**90.38**	**Europe**	**Present Study**
	DAT + IFAT	8	100.00	Austria	(Basso et al., 2005)
	EIA + LA	9	100.00	United States	(Brown et al., 2005)
	MAT	5	20.00	Midwestern zoos	(de Camps et al., 2008)
	MAT	3	66.67	Wisconsin	(Dubey, Gendron-Fitzpatrick, et al., 1988)
	LA	4	100.00	Denver	(Kenny et al., 2002)
	ELISA	6	100.00	Oklahoma	(Ketz-Riley et al., 2003)
	IHA	3	100.00	California	(Riemann, Fowler, et al., 1974)
	IFAT	2	100.00	Czech/Slovak zoos	(Sedlak & Bartova, 2006)
	ELISA	14	78.57	Ohio	(Swanson, 1999)
Sand cat *(Felis margarita)*	**IB + IFAT**	**87**	**54.02**	**Europe**	**Present Study**
	MAT	1	100.00	France	(Alerte, 2008)
	MAT	20	70.00	UAE	(Dubey et al., 2010)
	MAT	6	100.00	UAE	(Pas & Dubey, 2008a)

No. Exam. – Number examined

8.5 Evaluation of potential risk factors for *T. gondii*-seropositivity in zoos

Different potential risk factors for *T. gondii*-seropositivity were evaluated using data collected with a questionnaire and ZIMS. Data was consolidated or stratified to improve the statistical power if necessary (Table 13.71).

Among 311 individuals included in the analysis, 111 (35.7%) had been kept only in a single institution until the time of sampling. Individuals that were transferred to other zoos (e.g. to enable breeding with a partner chosen by the stud book coordinator) were usually transported at young age. Although husbandry variables from other zoos might have influenced the serological *T. gondii* result of these individuals, 82.7% of all felids had spent most of their lifetime in the institution where they were sampled.

A first multilevel analysis in a generalized linear mixed model, in which "Zoo" was included as a random effects variable, showed that the age of the felids was strongly associated with the

likelihood to test seropositive for *T. gondii*. There are many reports showing that older felids have a higher risk to test positive than younger animals (Afonso et al., 2006; Dubey, 2010b; Silva et al., 2001; Spada et al., 2012). This is explained by the cumulative effect of the periods of potential exposure to the parasite during lifetime. Differences in the mean age might have been a reason for the variability of *T. gondii* seroprevalences between different feline species included in this study. For instance, only few of the sampled Black-footed cats were older than five years (seroprevalence 26.7%). By contrast, most Pallas' cats and Rusty-spotted cats sampled were older than five years and these two species showed relatively high seroprevalences of 90.4% and 96.4%, respectively. Thus, it was assumed that the age of an animal is an important modifier for the effects of other risk factors. Due to differences in the time of possible exposure over the lifetime of the animal, other risk factors could have had an impact on the risk of exposure to *T. gondii*. The variable "Age" was therefore included as an effect-modifying variable in all models developed to determine potential risk factors for *T. gondii*-seropositivity in captive felids. Because animals clustered in particular zoos, the records for individuals were not completely independent and "Zoo" was added as a random effect variable in the multilevel generalized models. After bivariable analysis (chapter 7.4.8), a factor analysis was performed to determine if variables, which had shown statistically significant effects, were independent. If they were not, the variables were removed from the model based on the criteria of statistical significance and biological relevance (chapter 7.4.8). In the next step, all remaining variables were included in a generalized linear mixed model. After optimization by stepwise elimination of variables, excluding those variables first, which – if removed – did not cause a decrease in model quality (i.e. did not cause an increase in AIC), a final linear mixed model was generated.

The final linear mixed model had an AIC of 276.8 and consisted of five variables including "Age", feeding "Cattle" tissues, "Outdoor housing fenced in on all sides", "Mesh size" of enclosures and wearing "Gloves" (Table 7.13). In this model, the probability of seropositivity increased statistically significantly with "Age". Feeding "Cattle-Frozen" relative to "Cattle-Fresh", "Outdoor housing fenced in on all sides", "Mesh size below 5 cm" relative to "Mesh size more than 5 cm" and wearing "Gloves" had statistically significant protective effects.

In accordance with the bivariable analysis, the final model suggested that feeding cattle tissues that were frozen previously instead of fresh beef can protect felids from *T. gondii* infection, and thus from becoming *T. gondii*-seropositive. It can be suspected, that feeding tissues from ruminants in fresh condition in general would inhere a greater risk for *T. gondii*-seropositivity. The prevalence of *T. gondii* infection in sheep is considerably high in most European countries and worldwide (Dubey, 2010b; Kijlstra & Jongert, 2009). However, in the present analysis, only very few zoos reported to feed meat from small ruminants. It is possible that this is the reason why feeding tissues from sheep did not emerge as a relevant factor in the present study. The effect of freezing on the infectivity of *T. gondii* tissue cysts in pork has been studied and recommendations have been given to keep meat stored frozen to reach an internal temperature of -12°C for at least seven days prior to use to destroy tissue cysts (Alerte, 2008; Dubey, 2010b; Dubey, Kotula, et al., 1990; Kotula et al., 1991; Kuticic & Wikerhauser, 1996). Thus, the protective effects of freezing in the present study are in accord with these findings. Other studies also recommended freezing meat prior to feeding in zoos to reduce the risk of exposure to *T. gondii* in carnivores (Alerte, 2008; de Camps et al., 2008; Garell, 1999; Tidy et al., 2017). Silva et al. (2007) hypothesized that feeding unfrozen meat in general inhered a higher risk for exposure to *T. gondii* in felids.

In addition to feeding previously frozen cattle tissues, which appeared in the final model as a protective variable, the bivariable analysis revealed that feeding carcasses or tissues from mice, rodents, ruminants or fowl after freezing had a statistically significant protective effect as well. Feeding any of these items fresh represented the reference in these analyses and seemed

to be risk factors for seropositivity. A protective effect could also be seen when carcasses of mice were fed to felids either fresh or previously frozen (relative to the reference feeding mice fresh). The prevalence of *T. gondii* in rodents and fowl produced for animal food is unknown, but they are likely to inhere a low risk to transmit *T. gondii*. Fowl or domestic small rodents are less likely to contain tissue cysts than pigs or small ruminants (Brandt, 2006; Cenci-Goga et al., 2011; Kijlstra & Jongert, 2009). Despite the fact that commercial animal food suppliers mainly offer rats, mice or fowl frozen, especially small rodents like mice and rats used for animal food are produced under laboratory-like conditions. Commercial chickens are often raised indoors. In both, laboratory or indoor-raised rodents or fowl, the risk for an exposure to *T. gondii* oocysts or tissue cysts can be considered as limited. Indoor chickens proved to have low prevalences of *T. gondii* infection compared to backyard chickens (Dubey, 2010a). Overall, it can be assumed that commercial fowl and rodents from animal food suppliers inhere a high degree of biosafety.

The final linear mixed model demonstrated that keeping felids in outdoor enclosures fenced in on all sides had a statistically significant protective effect against mounting an antibody response to *T. gondii*. This may be explained by a lower risk of wild animals entering the enclosure compared to enclosures with an open top, to which especially avian wildlife might have access readily. Other authors reported that birds and small mammals might serve as a source of infection with *T. gondii* in feline enclosures. They may either serve as transport hosts (which might include insects as well) (Chinchilla et al., 1994; Wallace, 1971, 1972, 1973) introducing oocysts to the exhibited felids or as intermediate hosts, i.e. infected pray species, thus exposing captive felids to *T. gondii* tissue cysts (Alerte, 2008; de Camps et al., 2008; Garell, 1999; Ippen et al., 1980; Riemann, Behymer, et al., 1974; Tidy et al., 2017). In general, it has been shown that cats showing hunting behavior are more likely to be infected with *T. gondii* than cats that do not hunt (Simon, 1995). The availability of enclosures fenced in on all sides goes along with the mesh size of the enclosure. Mesh size was another factor, included in both, the bivariable analysis and the final multivariable linear mixed model.

Statistical analysis suggested that keeping felids in enclosures with a mesh size below 5 cm had a statistically significant protective effect on development of a seropositive *T. gondii* results as compared with mesh sizes above 5 cm as the reference. This result may be explained by the reduction of the number of rodents or other wild animals (i.e. possible intermediate hosts of *T. gondii*) that are able to enter the enclosures, although a mesh size of less than 5 cm is not considered as rodent-proof. However, a small mesh size may reduce the number of pray animals that can enter the enclosure. To exclude rats or mice from enclosures, a netting with a minimum mesh size of 1.8 cm or 5 mm has been recommended, respectively (Rentokill, 2016a, 2016b). As not only rodents, but also other intermediate hosts such as avian wildlife, might play an important role in the transmission of *T. gondii* (Schares et al., 2016), it appears plausible that mesh sizes below 5 cm may still have a protective effect and protect captive felids from *T. gondii* infection at least to a certain level.

Finally, the use of gloves for hygienic reasons had a statistically significant protective effect against *T. gondii*-seropositivity in the final linear mixed model. The use of gloves might coincide with effective hand hygiene. This may reduce the probability of an accidental transmission of diseases including *T. gondii*. Moving contaminated items into the enclosure may lead to a contamination of non-protected hands with oocysts, which could in turn cause a secondary contamination e.g. of food preparations if these are handled without washing or protecting hands with gloves. In general, the route of transmission via contaminated hands is possible, but does not seem to be very likely. In addition, it may be assumed that institutions that make sure that staff uses gloves in their daily routines are generally more considerate about hygiene issues.

While variables that appeared to have statistically significant effects in the final model, bivariable analysis indicated that also some other variables might have had an effect on *T.*

gondii-seropositivity. Although, the impact or the biological plausibility of the effect seem to be questionable for some of them, the variables that had tested statistically significant ($P < 0.1$) in the bivariable analysis are discussed in the following.

Bivariable analysis suggested that felids from zoos, in which few litters (1-2 within one year, or 1-9 within five years) or more than three litters had been born during the last year, had a statistically significantly higher risk to test serologically positive for antibodies to *T. gondii* as compared to felids from zoos with no litters born during the past year or the past five years. There are at least two possible explanations. First, pregnancy may have an immunosuppressive effect on felids (Tizard, 2013) and it can be hypothesized that this may have increased the risk of re-shedding of *T. gondii* oocysts in felids affected by immunosuppression. However, re-shedding has so far only been reported under immunosuppressive corticoid treatment (Dubey & Frenkel, 1974), but not yet during pregnancy. Another possible explanation is based on the observation that young felids seem to be more susceptible for intestinal infection and subsequent oocyst shedding than older felids. In older felids, immunity that has developed after a prior infection may prevent subsequent infections and thus renewed oocyst shedding (Davis & Dubey, 1995; Dubey, 1995). In addition, the intensity of oocyst shedding after a secondary infection is reduced compared to the shedding after the primary infection (Dubey & Frenkel, 1974). Therefore, oocyst contaminations may occur more often and might be more intense in zoos with litters of young and thus more susceptible felids than in zoos without young felids.

Little is known on differences in the ability of various felid species to shed *T. gondii* oocysts. Some studies demonstrated that oocyst shedding occurred in exotic felids, although oocyst production was not as efficient as in domestic cats (Jewell et al., 1972; Miller et al., 1972). Shedding of oocysts in several episodes without signs of clinical disease were confirmed in Wild cats and Amour leopard cats (Lukesova & Literák, 1998). In Pallas' cats, oocyst shedding coincided with clinical disease in juvenile individuals in addition to recurrent shedding of the infected queen (Basso et al., 2005). It is not known if Sand cats are able to excrete oocysts, but in contrast to domestic cats, chronically infected, but asymptomatic Sand cat and Pallas' cat queens were suspected to transmit *T. gondii* via the transplacental route or congenitally to their offspring (Dubey et al., 2010).

Keeping NWM close to felids protected felids statistically significantly against the development of a *T. gondii*-seropositive result. It is difficult to explain this observation by the biology of the parasite. Keeping felids close to NWM or other intermediate hosts might inhere a certain risk for these animals to get infected, as a number of case reports suggest (Carme et al., 2009; Cunningham et al., 1992; Epiphanio et al., 2003; Ferreira et al., 2015; Pardini et al., 2015). Animal attendants might carry infective oocysts from one enclosure to the other as hypothesized by some authors (Cunningham et al., 1992; de Camps et al., 2008; Pardini et al., 2015). More probably, the protective effect observed for this variable is a confounder: Zoo staff members most likely know about the risk of NWM to get infected with *T. gondii*. This might increase the awareness for hygienic measures around the NWM and the feline enclosures, because felids are the final hosts for *T. gondii*, and thus explain the protective effect. Moreover, ten institutions, which had stated to keep NWM close to felids, fed their cats with carcasses from mice, rodents, or meat from cattle, ruminants or fowl fresh, on a regular basis.

Performing rabies vaccinations appeared to have had a statistically significant risk effect for captive felids to test serologically *T. gondii*- positive. It is difficult to think of any biological plausible explanation for this effect. It is therefore possible that this association is spurious, which may be explained by the limitations of the statistical model (e.g. maximum of 5% error probability at the 95% confidence level). Rabies vaccinations were mainly performed in Eastern European zoos (92.3%), while none of the institutions in Great Britain and 22.7% in Central Europe immunized their animals against this disease.

Finally, it can be concluded that feeding tissues unfrozen, keeping animals housed fenced in on all sides and in enclosures with mesh sizes below 5 cm as well as wearing gloves when working inside enclosures seem to be the most relevant protective measures to prevent *T. gondii*-seropositivity in captive felids.

9 OUTLOOK

The first part of this work contributed further data on the feasibility of blood sampling with reduviid-bugs to determine clinical blood chemical parameters in cats. Varying results, from highly reliable results to largely divergent results between bug-derived plasma and conventionally collected plasma, depending on the blood chemical parameter were shown (chapter 8.2). It will be a future challenge to provide and confirm the results of this work for blood sampling in other species or for other analytes and laboratory tests.

The use of *Dipetalogaster maxima* to collect blood for *T. gondii* serology in captive felids proofed to be a highly reliable method. The method was applied very successfully in various zoos. It may therefore be applicable for minimal invasive blood sampling, if serological analyses need to be performed in captive felids. It is likely that the technique can also be adapted to obtain blood samples from other species.

Sampling boxes as used in this study illustrate, that further creative solutions can be invented to utilize reduviid bugs for obtaining blood samples from zoo animals. "Bug-application systems" can be adapted to local needs and facilities as well as to the sampled species. Collars with small containers for the bugs, artificial eggs for birds or small application boxes integrated into the environment have been successfully used in other studies as well as free-roaming bugs or containers fixed by hand.

Seroprevalence differences among many different small cat species in EAZA zoos were observed. However, further studies are needed to investigate if species-specific risk factors or differences in susceptibility between species might play a role and if specific measures are necessary for particular species to reduce the risk of infection with *T. gondii*.

The negative effect of *T. gondii* infections on the breeding success of Pallas' cats and Sand cats has been described before. However, the breeding success of other exotic felids might also be influenced by infection with *T. gondii*. This fact should be further investigated, in particular if losses have been recorded, especially in young felids. Dead animals and aborted fetuses should be regularly necropsied and tested for *T. gondii* and felids tested for antibodies to this parasite in regular intervals.

Long term epidemiological studies are necessary to determine whether interventions by protective measures, e.g. feeding tissues only after freezing, keeping animals housed fenced in on all sides and in enclosures with mesh sizes below 5 cm as well as wearing gloves can reduce the incidence of *T. gondii*-seroconversion and the occurrence of fatal toxoplasmosis among felids in zoos. Further investigations are needed to verify if such protective measures (except the treatment of meat of herbivores) have an effect on the *T. gondii*-seropositivity of other captive species, especially those with a high susceptibility for the parasite, e.g. New World monkeys or marsupials.

10 SUMMARY

Blood sampling can be difficult in several exotic species. To obtain the blood sample needed for analysis, handling of the animal is necessary. In some species, restraint without anesthesia is extremely stressful and risky for the animal or the person handling it.

The first objective of this study was the evaluation of a non-invasive blood sampling technique for *Toxoplasma gondii*-serology and blood-chemical analysis using the reduviid bug *Dipetalogaster maxima* in domestic cats. The technique was then applied to small exotic felids managed in European Endangered species Programmes (EEPs), European Stud Books (ESBs) or in monitoring programs (Mon) within the EAZA (European Association of Zoos and Aquaria). A seroepidemiological risk factor analysis for *T. gondii* infection in small exotic felids was conducted using *D. maxima* for sample collection.

A total of 70 domestic cats (*Felis silvestris catus*) were blood-sampled by venous puncture (V) and by using *Dipetalogaster maxima*. Plasma samples collected from bugs shortly after engorgement (B0) were found reliable for the determination of alanine aminotransferase, albumin, creatinine, creatine kinase, cholesterol and urea concentrations. Values determined in B0 for alkaline phosphatase, total bilirubin, total calcium, total magnesium, phosphorus, globulin, total protein and chloride showed major aberrations from the concentrations measured in V. The comparison of V and B0 samples for sodium, potassium, triglyceride, glucose, glutamate dehydrogenase revealed only a limited correlation. As differences increased, when plasma was collected 1 h after engorgement (B1), it seemed important to collect samples soon after the engorgement to reduce the effects of digestion of the samples in the bugs.

T. gondii antibody titers determined in V and the titers determined in plasma samples collected from bugs immediately, 1 hour, 2 hours or 4 hours after engorgement (B0/B1/B2/B4) were statistically significantly correlated. No statistically significant difference was observed between V and B0. In B1, B2 and B4 titers had increased slightly and the differences were statistically significant relative to V titers. However, the distribution of positive or negative serological *T. gondii*-results of the individuals tested remained the same regardless of the sample (V, B0, B1, B2 or B4).

Felids analyzed in EAZA zoos showed an overall seroprevalence for *T. gondii* of 63%. Potential risk factors for seropositivity regarding *T. gondii* in EAZA zoos were evaluated using a questionnaire and individual data from the Zoological information management system (ZIMS). The risk factor study resulted in a final generalized linear mixed model comprised of five variables: The likelihood of seropositivity increased statistically significantly with “Age”, while feeding “Cattle-Frozen” relative to “Cattle-Fresh”, “Outdoor housing fenced in on all sides”, “Mesh size below 5 cm” relative to “Mesh size more than 5 cm” and wearing “Gloves” had statistically significant protective effects.

11 ZUSAMMENFASSUNG

Validierung der Blutentnahme mit Hilfe von *Dipetalogaster maxima* zur Bestimmung serologischer und blutchemischer Parameter und Anwendung der Methode in einer epidemiologischen Studie zu *Toxoplasma gondii* in exotischen Kleinkatzen in europäischen Zoos

Eine Blutentnahme ist bei vielen exotischen Tieren problematisch, weil direkter Tierkontakt dafür unumgänglich ist. Bei vielen Arten ist dies ohne eine Narkose nicht möglich, da das „Handling" extrem stressig und riskant für das Tier, oder auch die Personen, die an der Blutentnahme beteiligt sind, sein kann.

In einem ersten Schritt wurde die nicht invasive Blutprobenentnahme für die *Toxoplasma gondii*-Serologie und klinische Blutchemie mit der Hilfe von Raubwanzen (*Dipetalogaster maxima*) bei Hauskatzen (*Felis silvestris catus*) überprüft.

Darauf aufbauend wurde die Methode angewandt, um die Prävalenz von *T. gondii* bei exotischen Kleinkatzen in Zoos, innerhalb der „European Association of Zoos and Aquaria" (EAZA), zu untersuchen. Von Interesse waren Katzen-Spezies, welche in einem „European Endangered species Programme" (EEP) oder „European Stud Book" (ESB) geführt wurden, oder aber wenigstens den Status „Monitored" aufwiesen. Des Weiteren wurde eine sero-epidemiologische Risikofaktorstudie für *T. gondii* Infektionen bei exotischen Kleinkatzen, durchgeführt.

Von 70 Hauskatzen (*Felis silvestris catus*) wurden Blutproben konventionell aus der Vene entnommen (V) und mit Hilfe von *Dipetalogaster maxima* gewonnen. Plasmaproben, welche gleich nach dem Saugvorgang aus den Wanzen entnommen wurden (B0), lieferten verlässliche Ergebnisse für die Bestimmung von Alanin-Aminotransferase-, Albumin-, Kreatinin-, Creatinkinase-, Cholesterol- und Harnstoff-Konzentrationen im Vergleich zu den Werten, wie sie für konventionell gewonnenes Plasma (V) ermittelt worden waren. Die Parameter Alkalische Phosphatase, Bilirubin, Calcium, Magnesium, Phosphor, Globulin, Gesamtprotein und Chlorid wiesen deutliche Abweichungen in den Konzentrationen zwischen V und B0 auf. Der Vergleich von V- und B0-Werten bei Natrium, Kalium, Triglyceriden, Glukose und Glutamat-Dehydrogenase zeigte keine deutliche Korrelation der Messwerte. Da sich die Konzentrationen der verschiedenen Parameter mit zunehmender Zeit zwischen Saugvorgang und Blutgewinnung aus der Wanze in steigendem Maße veränderten, sollte dieser Zeitraum so kurz wie möglich gehalten werden, um Effekte durch die Verdauung der Wanze zu reduzieren.

T. gondii-Antikörpertiter in konventionellen Plasmaproben (V) und Proben aus Raubwanzen, die gleich nach dem Saugakt und eine, zwei und vier Stunden nach dem Saugakt (B0, B1, B2, B4) gewonnen wurden, zeigten eine statistisch signifikante Korrelation. Die Werte für die Proben V und B0 wiesen zudem keine statistisch signifikanten Unterschiede auf. Die Titer für B1, B2 und B4 stiegen nur leicht, aber dennoch statistisch signifikant an. Trotzdem war die Verteilung negativer und positiver Werte identisch, unabhängig davon, ob V, B0, B1, B2 oder B4 untersucht worden waren.

Die serologische Prävalenz von *T. gondii* bei Feliden in EAZA-Zoos lag bei 63%. Verschiedene Risikofaktoren wurden mit der Hilfe eines Fragebogens und individuellen Daten aus dem „Zoological Information Management System" (ZIMS) evaluiert. Die Risikofaktorstudie lieferte ein finales Model, welches fünf Variablen beinhaltete: die Wahrscheinlichkeit für Seropositivität stieg mit zunehmendem "Alter", während das Füttern von "Rind-Gefroren" im Vergleich zu "Rind-Frisch", "Außenhaltung - an allen Seiten eingezäunt", "Maschenweite unter 5 cm" im Verhältnis zu "Maschenweite über 5 cm" und das Tragen von "Handschuhen" statistisch signifikant schützende Effekte zeigten.

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13 APPENDIX

13.1 Data

Table 13.1: Serological results in captive felids stratified by the treatment of mice carcasses prior to feeding

Parameter	**Serological result**		
Feeding mice (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Fresh (11)	76.09	70	92
Fresh or frozen (10)	58.70	27	46
Frozen (19)	60.00	60	100
No mice (8)	62.96	17	27
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.2: Serological results in captive felids stratified by the treatment of rat carcasses prior to feeding

Parameter	**Serological result**		
Feeding rats (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Fresh (9)	71.19	42	59
Fresh or frozen (9)	68.35	54	79
Frozen (22)	60.95	64	105
No rats (8)	63.64	14	22
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.3: Serological results in captive felids stratified by the treatment of cattle carcasses prior to feeding

Parameter	**Serological result**		
Feeding cattle (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Fresh (6)	76.19	16	21
Fresh or frozen (5)	69.86	51	73
Frozen (14)	45.83	22	48
No cattle (23)	69.11	85	123
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.4: Serological results in captive felids stratified by the treatment of sheep carcasses prior to feeding

Parameter	**Serological result**		
Feeding sheep (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Fresh (5)	82.76	24	29
Fresh or frozen (1)	64.52	20	31
Frozen (2)	00.00	0	3
No sheep (40)	64.36	130	202
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.5: Serological results in captive felids stratified by the treatment of horse carcasses prior to feeding

Parameter	**Serological result**		
Feeding horse (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Fresh (4)	73.33	11	15
Fresh or frozen (4)	81.13	43	53
Frozen (15)	64.20	52	81
No horse (25)	58.62	68	116
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.6: Serological results in captive felids stratified by the treatment of fowl carcasses prior to feeding

Parameter	**Serological result**		
Feeding fowl (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Fresh (7)	86.21	25	29
Fresh or frozen (12)	66.15	86	130
Frozen (27)	57.45	54	94
No fowl (2)	75.00	9	12
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.7: Serological results in captive felids stratified by the treatment of fish carcasses prior to feeding

Parameter	**Serological result**		
Feeding fish (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Fresh (5)	78.57	11	14
Fresh or frozen (4)	76.60	36	47
Frozen (22)	63.57	82	129
No fish (17)	60.00	45	75
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.8: Serological results in captive felids stratified by the feeding of fruits

Parameter	**Serological result**		
Feeding fruit (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No fruits (43)	63.56	150	236
Yes (4)	82.76	24	29
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.9: Serological results in captive felids stratified by different kinds of cat food

Parameter	**Serological result**		
Feeding cat food (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Canned (4)	68.18	30	44
Canned or dry (4)	50.00	6	12
Dry (4)	55.56	15	27
No cat food (36)	67.58	123	182
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.10: Serological results in captive felids stratified by food sources

Parameter	**Serological result**		
Food sources (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Labs (5)	81.25	39	48
Hunting (3)	75.68	28	37
Own breeding (19)	66.67	84	126
Slaughter (11)	66.67	46	69
Local Breeder (17)	64.67	97	150
Private owner (7)	79.45	58	73
Commercial animal food supplier (26)	67.22	121	180
NA (9)	46.77	29	62
Total (50)	**63.02**	**196**	**311**

Table 13.11: Serological results in captive felids stratified by the temperature condition of carcass storage prior to feeding

Parameter	**Serological result**		
Food storage (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Cool (4)	69.23	9	13
Cool or Frozen (13)	71.56	78	109
Frozen (27)	61.40	70	114
NA (5)	52.00	39	75
Total (50)	**63.02**	**196**	**311**

Table 13.12: Serological results in captive felids stratified by the length of time of freezing carcasses prior to feeding

Parameter	**Serological result**		
Length of freezing carcasses (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Sometimes < 1 week (14)	76.19	64	84
One week at least (9)	48.00	12	25
> 2 weeks (22)	64.34	83	129
NA (5)	50.69	37	73
Total (50)	**63.02**	**196**	**311**

Table 13.13: Serological results in captive felids stratified by the separate preparation of fruits and meat

Parameter	**Serological result**		
Separation of fruit and meat (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No separation (8)	56.52	26	46
Separation by processing (10)	79.49	62	78
Separation by different rooms (29)	61.87	86	139
NA (3)	45.83	22	48
Total (50)	**63.02**	**196**	**311**

Table 13.14: Serological results in captive felids stratified by the source of drinking water

Parameter	**Serological result**		
Water source (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Rain or Tap (3)	75.00	6	8
Tap (38)	66.36	146	220
Well or Tap (3)	59.09	13	22
Well (4)	60.00	9	15
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.15: Serological results in captive felids stratified by the frequency of drinking water renewal

Parameter	**Serological result**		
Clean water (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Daily (41)	63.26	136	215
Daily or Weekly (1)	100.00	3	3
Weekly (1)	50.00	1	2
Every 2nd week (4)	79.07	34	43
No change (1)	00.00	0	2
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.16: Serological results in captive felids stratified by other felids kept close by

Parameter	**Serological result**		
Felids close by (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No Felids close by (16)	55.32	26	47
Yes (32)	67.89	148	218
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.17: Serological results in captive felids stratified by marsupials kept close by

Parameter	**Serological result**		
Marsupials close by (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No marsupials close by (31)	65.00	117	180
Yes (17)	67.06	57	85
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.18: Serological results in captive felids stratified by New World monkeys kept close by the felids

Parameter	**Serological result**		
NWM close by (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No NWM close by (36)	67.58	148	219
Yes (12)	56.52	26	46
NA (3)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.19: Serological results in captive felids stratified by animal attendants looking after other felid species

Parameter	**Serological result**		
Animal attendants care for more than one cat species (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No care for other felids (9)	45.83	11	24
Yes (39)	67.64	163	241
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.20: Serological results in captive felids stratified by animal attendants looking after New World monkeys or marsupials besides felids

Parameter	**Serological result**		
Animal attendants care for cats plus NWM /Marsupials (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No care for cats + NWM/Marsupials (30)	66.18	135	204
Yes (18)	63.93	39	61
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.21: Serological results in captive felids stratified by indoor housing

Parameter	**Serological result**		
Indoor housing (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No indoor housing (33)	65.66	109	166
Yes (15)	65.66	65	99
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.22: Serological results in captive felids stratified by outdoor keeping (fenced in on all sides)

Parameter	**Serological result**		
Outdoor housing fenced in (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No fenced in outdoor housing (8)	77.50	31	40
Yes (40)	63.56	143	225
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.23: Serological results in captive felids stratified by outdoor keeping (open top)

Parameter	**Serological result**		
Outdoor housing open top (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No open top outdoor housing (39)	62.72	106	169
Yes (9)	70.83	68	96
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.24: Serological results in captive felids stratified by enclosure size < 20 m^2

Parameter	**Serological result**		
< 20 m^2 (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Size not < 20 m^2 (22)	64.77	57	88
Yes (26)	66.10	117	177
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.25: Serological results in captive felids stratified by enclosure size 20-50 m^2

Parameter	**Serological result**		
20-50 m^2 (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Size not between 20-50 m^2 (14)	68.89	31	45
Yes (34)	65.00	143	220
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.26: Serological results in captive felids stratified by enclosure size > 50 m^2

Parameter	**Serological result**		
> 50 m^2 (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Size not > 50 m^2 (32)	66.87	109	163
Yes (16)	63.73	65	102
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.27: Serological results in captive felids stratified by enclosures with a mesh size of < 2 cm

Parameter	**Serological result**		
Mesh size < 2 cm (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Mesh size not < 2 cm (33)	62.63	124	198
Yes (15)	74.63	50	67
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.28: Serological results in captive felids stratified by enclosures with a mesh size of 2-5 cm

Parameter	**Serological result**		
Mesh size 2-5 cm (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Mesh size not between 2-5 cm (18)	78.50	73	93
Yes (30)	58.72	101	172
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.29: Serological results in captive felids stratified by enclosures with a mesh size of > 5 cm

Parameter	**Serological result**		
Mesh size > 5 cm (No. of zoos)	**Proportion of positives (%)v**	**No. positive**	**Total**
Mesh size not > 5cm (42)	63.42	130	205
Yes (6)	73.33	44	60
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.30: Serological results in captive felids stratified by the number of stray cats roaming the zoo at the time of interview

Parameter	**Serological result**		
No. of stray cats (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
0 (11)	68.85	42	61
1 (9)	61.29	19	31
2 (7)	50.00	10	20
3 (4)	66.67	6	9
4 (3)	57.14	16	28
5 (5)	68.42	26	38
6 (2)	83.33	5	6
7 (1)	81.82	27	33
8 (2)	65.39	17	26
10 (1)	100.00	2	2
15 (2)	50.00	4	8
NA (3)	44.90	22	49
Total (50)	**63.02**	**196**	**311**

Table 13.31: Serological results in captive felids stratified by individuals that had lived in one institution only

Parameter	**Serological result**		
Number of institutions lived in (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
> 1 institution (48)	65.66	130	198
Only 1 institution (22)	58.56	65	111
NA (2)	50.00	1	2
Total (50)	**63.02**	**196**	**311**

Table 13.32: Serological results in captive felids stratified by the use of shavings as bedding material

Parameter	**Serological result**		
Shavings (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No shavings (24)	68.37	67	98
Yes (24)	64.07	107	167
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.33: Serological results in captive felids stratified by the use of hay as bedding material

Parameter	**Serological result**		
Hay (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No Hay (33)	63.43	111	175
Yes (15)	70.00	63	90
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.34: Serological results in captive felids stratified by the use of straw as bedding material

Parameter	**Serological result**		
Straw (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No straw (23)	65.71	69	105
Yes (25)	65.63	105	160
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.35: Serological results in captive felids stratified by the use of sand as bedding material

Parameter	**Serological result**		
Sand (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No sand (27)	71.76	94	131
Yes (21)	59.70	80	134
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.36: Serological results in captive felids stratified by the use of other materials as bedding material

Parameter	**Serological result**		
Other bedding materials (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No other materials (43)	68.09	160	235
Yes (5)	56.67	14	30
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.37: Serological results in captive felids stratified by the frequency of changing bedding material

Parameter	**Serological result**		
Change of bedding material (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Daily (7)	52.17	12	23
Weekly (17)	67.42	60	89
Monthly (7)	61.11	22	36
Infrequently (16)	67.83	78	115
No change (1)	100.00	2	2
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.38: Serological results in captive felids stratified by the use of enrichment items

Parameter	**Serological result**		
Enrichment (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No enrichment (13)	68.75	44	64
Yes (35)	64.68	130	201
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.39: Serological results in captive felids stratified by the frequency of feces removal in the enclosures

Parameter	**Serological result**		
Frequency of feces removal (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Daily (36)	67.00	136	203
Not Daily (12)	61.29	38	62
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.40: Serological results in captive felids stratified by the availability of a litterbox

Parameter	**Serological result**		
Litterbox (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No litterbox (25)	69.23	81	117
Yes (23)	62.84	93	148
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.41: Serological results in captive felids stratified by the application of hygiene measures

Parameter	**Serological result**		
Hygiene measures (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No hygiene measures (11)	62.79	54	86
Yes (37)	67.04	120	179
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.42: Serological results in captive felids stratified by the availability of hand wash facilities

Parameter	**Serological result**		
Hand wash facilities (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No hand wash facilities (35)	66.12	121	183
Yes (13)	64.63	53	82
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.43: Serological results in captive felids stratified by the availability of hand disinfection

Parameter	**Serological result**		
Hand disinfection (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No hand disinfection (36)	67.01	132	197
Yes (12)	61.77	42	68
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.44: Serological results in captive felids stratified by the use of disposable gloves working in felid enclosures

Parameter	**Serological result**		
Gloves (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No gloves (28)	72.38	131	181
Yes (20)	51.19	43	84
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.45: Serological results in captive felids stratified by the availability of a disinfectant footbath

Parameter	**Serological result**		
Disinfectant footbath (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No footbath (30)	65.03	106	163
Yes (18)	66.67	68	102
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.46: Serological results in captive felids stratified by the use of disposable shoe covers

Parameter	**Serological result**		
Shoe cover (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No shoe covers (47)	66.54	173	260
Yes (1)	20.00	1	5
NA (2)	47.83	22	46
Total (50)	**63.02**	**196**	**311**

Table 13.47: Serological results in captive felids stratified by the implementation of pest control

Parameter	**Serological result**		
Pest control (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No pest control (4)	72.22	39	54
Yes (43)	64.90	135	208
NA (3)	44.90	22	49
Total (50)	**63.02**	**196**	**311**

Table 13.48: Serological results in captive felids stratified by the frequency of pest control

Parameter	**Serological result**		
Pest control frequency in month (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
0 (3)	53.85	7	13
0.25 (1)	100.00	4	4
1 (21)	58.67	44	75
2 (3)	71.43	15	21
3 (5)	68.75	11	16
6 (3)	80.00	4	5
12 (1)	66.67	2	3
Infreq (3)	78.38	29	37
NA (10)	58.39	80	137
Total (50)	**63.02**	**196**	**311**

Table 13.49: Serological results in captive felids stratified by the use of traps for pest control

Parameter	**Serological result**		
Traps for Pest control (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No traps used (19)	69.01	98	142
Yes (28)	63.33	76	120
NA (3)	44.90	22	49
Total (50)	**63.02**	**196**	**311**

Table 13.50: Serological results in captive felids stratified by the use of poison for pest control

Parameter	**Serological result**		
Poison for Pest control (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No poison used (37)	67.12	49	73
Yes (10)	66.14	125	189
NA (3)	44.90	22	49
Total (50)	**63.02**	**196**	**311**

Table 13.51: Serological results in captive felids stratified by the use of domestic cats for rodent control

Parameter	**Serological result**		
Cats for Pest control (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No cats used (33)	66.32	128	193
Yes (14)	66.67	46	69
NA (3)	44.90	22	49
Total (50)	**63.02**	**196**	**311**

Table 13.52: Serological results in captive felids stratified by the performance of vaccinations

Parameter	**Serological result**		
Vaccination (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No vaccination (7)	46.67	14	30
Yes (37)	64.58	124	192
NA (6)	65.17	58	89
Total (50)	**63.02**	**196**	**311**

Table 13.53: Serological results in captive felids stratified by the vaccination against feline influenza

Parameter	**Serological result**		
Cat flu Vaccination (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No cat flu vaccination (12)	52.17	24	46
Yes (32)	64.77	114	176
NA (6)	65.17	58	89
Total (50)	**63.02**	**196**	**311**

Table 13.54: Serological results in captive felids stratified by the implementation of parvovirus vaccinations

Parameter	**Serological result**		
Parvovirus Vaccination (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No parvovirus vaccination (13)	58.93	33	56
Yes (31)	63.25	105	166
NA (6)	65.17	58	89
Total (50)	**63.02**	**196**	**311**

Table 13.55: Serological results in captive felids stratified by the implementation of rabies vaccinations

Parameter	**Serological result**		
Rabies Vaccination (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No rabies vaccination (27)	53.19	75	141
Yes (17)	77.78	63	81
NA (6)	65.17	58	89
Total (50)	**63.02**	**196**	**311**

Table 13.56: Serological results in captive felids stratified by the implementation of feline leucosis vaccinations

Parameter	**Serological result**		
Leucosis Vaccination (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No leucosis vaccination (29)	60.87	84	138
Yes (15)	64.29	54	84
NA (6)	65.17	58	89
Total (50)	**63.02**	**196**	**311**

Table 13.57: Serological results in captive felids stratified by the implementation of FIP vaccinations

Parameter	**Serological result**		
FIP Vaccination (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No FIP vaccination (31)	61.77	105	170
Yes (13)	63.46	33	52
NA (6)	65.17	58	89
Total (50)	**63.02**	**196**	**311**

Table 13.58: Serological results in captive felids stratified by the implementation of endoparasite checks

Parameter	**Serological result**		
Check for endoparasites (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
No check for endoparasites (6)	63.64	35	55
Yes (39)	62.13	105	169
NA (5)	64.37	56	87
Total (50)	**63.02**	**196**	**311**

Table 13.59: Serological results in captive felids stratified by the frequency of endoparasite checks

Parameter	**Serological result**		
Frequency of endoparasite checks in month (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
0 (1)	00.00	0	1
1 (1)	00.00	0	3
2 (3)	71.43	10	14
3 (12)	58.54	24	41
6 (13)	60.00	18	30
12 (7)	61.11	22	36
NA (13)	65.59	122	186
Total (50)	**63.02**	**196**	**311**

Table 13.60: Serological results in captive felids stratified by the frequency of deworming procedures

Parameter	**Serological result**		
Deworming frequency in month (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
3 (7)	63.16	36	57
4 (1)	62.50	5	8
6 (21)	60.00	45	75
12 (3)	100.00	20	20
NA (18)	59.60	90	151
Total (50)	**63.02**	**196**	**311**

Table 13.61: Serological results in captive felids stratified by the number of deaths among the felid species tested within the last 5 years

Parameter	**Serological result**		
No. of death (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
0 (21)	65.12	56	86
1 (11)	54.17	13	24
2 (1)	100.00	2	2
3 (2)	66.67	22	33
4 (2)	80.95	34	42
5 (5)	73.68	14	19
7 (1)	40.00	4	10
9 (1)	50.00	2	4
10 (1)	80.00	4	5
12 (1)	62.50	10	16
16 (1)	50.00	11	22
NA (3)	50.00	24	48
Total (50)	**63.02**	**196**	**311**

Table 13.62: Serological results in captive felids stratified by age categories

Parameter	**Serological result**		
Age Categories (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
<5 Years (33)	48.94	69	141
>5 Years (44)	78.34	123	157
NA (6)	30.77	4	13
Total (50)	**63.02**	**196**	**311**

Table 13.63: Serological results in captive felids stratified by the way of rearing

Parameter	**Serological result**		
Rearing (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Parent (48)	62.41	176	282
Hand (11)	69.23	9	13
Foster (1)	100.00	1	1
NA (11)	66.67	10	15
Total (50)	**63.02**	**196**	**311**

Table 13.64: Serological results in captive felids stratified by sex

Parameter	**Serological result**		
Sex (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Male (45)	67.09	106	158
Female (46)	59.60	90	151
NA (2)	00.00	0	2
Total (50)	**63.02**	**196**	**311**

Table 13.65: Serological results in captive felids stratified by species

Parameter	**Serological result**		
Species (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
Asian golden cat (2)	50.00	1	2
Black-footed cat (3)	26.67	4	15
Fishing cat (15)	55.00	22	40
Geoffroy's cat (14)	48.49	16	33
Jaguarondi (5)	55.56	5	9
Margay (7)	47.37	9	19
Oncilla (3)	66.67	6	9
Pallas' cat (22)	90.39	47	52
Rusty-spotted cat (3)	96.43	27	28
Sand cat (15)	54.02	47	87
Others (5)	70.59	12	17
Total (50)	**63.02**	**196**	**311**

Table 13.66: Serological results in captive felids stratified by the total number of felids kept at the time of sampling

Parameter	**Serological result**		
No. of felids (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
1-16 (24)	63.42	163	257
≥17 (26)	61.11	33	54
Total (50)	**63.02**	**196**	**311**

Table 13.67: Serological results in captive felids stratified by the number of litters born among felids within 5 years prior to sampling

Parameter	**Serological result**		
No. of litters within 5 Years (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
0 (12)	52.78	19	36
1-9 (20)	71.58	68	95
≥10 (18)	60.56	109	180
Total (50)	**63.02**	**196**	**311**

Table 13.68: Serological results in captive felids stratified by the number of litters born among felids within 1 year prior to sampling

Parameter	**Serological result**		
No. of litters within 1 Year (No. of zoos)	**Proportion of positives (%)**	**No. positive**	**Total**
0 (17)	50.00	25	50
1-2 (17)	69.74	53	76
≥3 (16)	63.78	118	185
Total (50)	**63.02**	**196**	**311**

13.2 Questionnaire

13.2.1 Original Questionnaire

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Questionnaire on toxoplasmosis in small exotic felids

Address of your Institution:

_____

_____

_____

Name, Email and telephone number of the person answering the questionnaire:

_____

_____

Date: _____

How many small felids do you have currently in your zoo?

Species	Number of Individuals Juvenile (m,f,x)	Adult (m,f,x)
Sand cat *(Felis margarita)*		
Black footed cat *(Felis nigripes)*		
Pallas cat *(Felis manul)*		
Fishing cat *(Prionailurus viverrinus)*		
Rusty-spotted cat *(Prionailurus rubiginosus phillipsi)*		
Geoffroy's cat *(Leopardus geoffroyi)*		
Oncilla *(Leopardus tigrinus)*		
Margay *(Leopardus wiedii)*		

m: male, f: female, x: sex unknown (i.e.: Sand cat 0,0,2 3,1,0)

Feeding:			
1. What do you feed your cats with? (Multiple answers possible)	Mice	Frozen regularly □ rarely □	Fresh regularly □ rarely □
	Rats	Frozen regularly □ rarely □	Fresh regularly □ rarely □
	Sheep/ Goat	Frozen regularly □ rarely □	Fresh regularly □ rarely □
	Cattle	Frozen regularly □ rarely □	Fresh regularly □ rarely □
	Horse	Frozen regularly □ rarely □	Fresh regularly □ rarely □
	Fowl/ chicks	Frozen regularly □ rarely □	Fresh regularly □ rarely □
	Fish	Frozen regularly □ rarely □	Fresh regularly □ rarely □
	Fruit/ Vegetables		regularly □ rarely □
	Catfood	Canned regularly □ rarely □	Dry regularly □ rarely □

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Questionnaire on toxoplasmosis in small exotic felids

2. Where do you get the food (meat) from?		
3. What is the temperature of your food storage?	10°C to 5°C	□
	5°C to 0°C	□
	0°C to -5°C	□
	-5°C to -10°C	□
	-10°C to -15°C	□
	-15°C to -20°C	□
	Under -20°C	□
4. Is the preparation of meat and fruits/vegetables separated?	No	□
	Preparation in different rooms	□
	Separate areas in the kitchen	□
	Separate cutting boards in use	□
5. How long is the food frozen before feeding?	Less than 1 week	□
	1-2 weeks	□
	More than 2 weeks	□
6. Where does the drinking water come from?	Rain water	□
	Well water	□
	Tap water	□
	Bottled water	□
7. How often do you clean the water bowl?	Daily	□
	Every other day	□
	Once a week	□
	We do not change the water	□

Husbandry of small felids:			
1. How often do you remove feces in the enclosures?	Daily		□
	Every second day		□
	Once a week		□
	More infrequently		□
2. Is there a litter box?		Yes □	No □
3. Are any of these animals kept close by? (within 50 meters)	Other felids	Yes □	No □
	New world monkeys	Yes □	No □
	Marsupials	Yes □	No □
4. Do the same zookeepers care for more than one cat species?		Yes □	No □
5. Do the same zookeepers care for cats and Marsupials/ New world monkeys?		Yes □	No □
6. Do you use hygiene devices to avoid spreading of pathogenic agents?		Yes □	No □

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Questionnaire on toxoplasmosis in small exotic felids

7. If you do, what kind of hygiene devices? (Multiple answers possible)	Hand wash facilities at all enclosures	□
	Hand disinfection at all enclosures	□
	Disinfective footbath between enclosures	□
	Protective clothing: *Disposable gloves*	□
	Disposable shoe covers	□
	Disposable suits	□
8. What kind of bedding material do you use in your enclosures? (Multiple answers possible)	Shavings	□
	Hay	□
	Straw	□
	Sand	□
	Other	□
9. How often do you change the bedding material?	Daily	□
	Weekly	□
	Monthly	□
	More infrequently	□
10. Do the cats get any items for enrichment?	Yes □	No □
11. If yes, what kind of items for enrichment? (Multiple answers possible)	Branches etc. out of nature	□
	Industrial cat toys (Balls…)	□
	Other (Paperbags…)	□
12. What kind of enclosures do you have?	In a house, no contact to wildlife possible	□
	Outdoors fenced in on all sides (also from above), indirect contact to wildlife possible	□
	Enclosure open from above, direct contact to wildlife possible	□
13. Total size of the enclosure	< $20m^2$	□
	20-$50m^2$	□
	>$50m^2$	□
14. What mesh size does the fence of your Enclosures have?	≤ 1cm	□
	1-2 cm	□
	2-5 cm	□
	> 5 cm	□
15. Do damaged fences allow access for mice?	Yes □	No □

Pest control:		
1. Do you do any pest control?	Yes □	No □
2. If yes, how do you do pest control?	Traps	□
	Poison	□
	Domestic cats	□
3. How often?	Once a month	□
	Quarterly	□
	Biannual	□
	Once a Year	□
4. Do you have stray cats at the zoo?	Yes □	No □

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Questionnaire on toxoplasmosis in small exotic felids

5. If yes, how many?			
Toxoplasmosis:			
1. How many deaths did you have the last 5 years?	Species	No. of death Juvenile (m,f,x)	Adult (m,f,x)
	Sand cat *(Felis margarita)*		
	Black footed cat *(Felis nigripes)*		
	Pallas cat *(Felis manul)*		
	Fishing cat *(Prionailurus viverrinus)*		
	Rusty-spotted cat *(Prionailurus rubiginosus phillipsi)*		
	Geoffroy's cat *(Leopardus geoffroyi)*		
	Oncilla *(Leopardus tigrinus)*		
	Margay *(Leopardus wiedii)*		

2. Did you in the last 5 years look for *T. gondii* or antibodies against *Toxoplasma* in any animals (felids and non felids)?				Yes □	No □
3. If yes, please give details:					
Species	No. of individuals	Test used and results	In which context did you look for it?	*T. gondii* suspected cause of death?	
				Yes □	No □
				Yes □	No □
				Yes □	No □
				Yes □	No □
				Yes □	No □
				Yes □	No □
				Yes □	No □
				Yes □	No □
				Yes □	No □
				Yes □	No □
4. Did you treat any of your animals against toxoplasmosis?				Yes □	No □
5. If yes, what treatment did you use?					
6. Was the treatment successful?				Yes □	No □

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Questionnaire on toxoplasmosis in small exotic felids

General veterinary treatment of small felids:		
1. Are the animals vaccinated?	Yes □	No □
2. If yes, what do you vaccinate for?	Cat flu	□
	Parvovirus	□
	Rabies	□
	Leucosis	□
	FIP	□
3. Do the cats get regularly dewormed?	Yes □	No □
4. If yes, how often?	Every 3 months	□
	Half a year	□
	Once a year	□
	More infrequently	□
5. Do you regularly check for parasites in the feces?	Yes □	No □
6. If yes, how often?	Every 3 months	□
	Half a year	□
	Once a year	□
	More infrequently	□

7. Was any of the following disease diagnosed in any of your small felids during the last 5 years?		
Disease	Species infected (number of animals)	What kind of test did you use?
FHV1 (Feline Herpes virus 1)		
Calicivirus		
FeLV (Feline leukemia virus)		
FIP (Corona virus)		
FIV (Feline immunedeficiency virus)		
Feline Distemper (Feline panleuko-penia virus)		

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13.2.2 Data transformation for the evaluation of the questionnaire

Table 13.69: Description of variables: determined from the Questionnaire and from individuals using ZIMS.

Variable as determined for individual animals and as determined by questionnaire	Explanation	Categories	Modification of variables
Zoo	Zoo named by the region		
Species	Name of the species sampled		
SampleNo			
StudbookNo			
ArksID	LocalID		
ChipID	No. of Mikrochip Transponder		
Housename			
IFAT	Titer determined by IFAT		IFAT_Categories
FLIImmunoblot	FLI Immunoblot result	“positive”, “negative”, “inconclusive”	
BirthYear			Birth
BirthMonth			
BirthDay			
Sampling	Date of sample collection		
Rearing	Way of rearing the tested individual	“Parent”-, “Foster”-, “Hand”- reared	
Sex		Male: “m”, female: “f”	
BirthLocation	Zoo named by the region in which the individual was born		
Lived.in.1.Institution.only	No transport to other Zoos until sample collection.	“0”: no, “1”: yes	
Mice-fresh	Fresh mice used as food items	“0”: no, “1”: yes	Mice
Mice-frozen	Frozen mice used as food items	“0”: no, “1”: yes	
Rat-fresh	Fresh rats used as food items	“0”: no, “1”: yes	Rat
Rat-frozen	Frozen rats used as food items	“0”: no, “1”: yes	
Sheep-fresh	Fresh sheep used for feeding	“0”: no, “1”: yes	Sheep
Sheep-frozen	Frozen sheep used for feeding	“0”: no, “1”: yes	
Cattle-fresh	Fresh cattle used for feeding	“0”: no, “1”: yes	Cattle
Cattle-frozen	Frozen cattle used for feeding	“0”: no, “1”: yes	
Horse-fresh	Fresh horse used for feeding	“0”: no, “1”: yes	Horse
Horse-frozen	Frozen horse used for feeding	“0”: no, “1”: yes	

Table 13.69: Description of variables: determined from the Questionnaire and from individuals using ZIMS, continued.

Variable as determined for individual animals and as determined by questionnaire	Explanation	Categories	Modification of variables
Fowl-fresh	Fresh fowl used for feeding	"0": no, "1": yes	Fowl
Fowl-frozen	Frozen fowl used for feeding	"0": no, "1": yes	
Fish-fresh	Fresh fish used for feeding	"0": no, "1": yes	Fish
Fish-frozen	Frozen fish used for feeding	"0": no, "1": yes	
Fruit-vegetables	Fruits and vegetables used for feeding	"0": no, "1": yes	Fruit
Catfood-cannded	Feeding canned cat food	"0": no, "1": yes	Catfood
Catfood-dry	Feeding dry cat food	"0": no, "1": yes	
Foodstorage<-20°C	Temperature of food storage <-20°C	"0": no, "1": yes	FoodStorage
Foodstorage-20°C- -15°C	Temperature of food storage -20°C- -15°C	"0": no, "1": yes	
Foodstorage-15°C- -10°C	Temperature of food storage -15°C- -10°C	"0": no, "1": yes	
Foodstorage-10°C- -5°C	Temperature of food storage -10°C- -5°C	"0": no, "1": yes	
Foodstorage-5°C-0°C	Temperature of food storage -5°C-0°C	"0": no, "1": yes	
Foodstorage0°C-5°C	Temperature of food storage 0°C-5°C	"0": no, "1": yes	
Foodstorage5°C-10°C	Temperature of food storage 5°C-10°C	"0": no, "1": yes	
Preparation-rooms	Preparation of meat and fruits/vegetables in different rooms	"0": no, "1": yes	MeatFruitSeperation
Preparation-areas	Preparation of meat and fruits/vegetables in different areas	"0": no, "1": yes	
Preparation-board	Preparation of meat and fruits/ vegetables on separate cutting boards	"0": no, "1": yes	
Preparation-noseparation	No separate preparation of meat and fruits/vegetables	"0": no, "1": yes	
FoodFrozen- <1w	Freezing meat for less than 1 week	"0": no, "1": yes	FoodFrozen
FoodFrozen- 1-2w	Freezing meat for 1-2 weeks	"0": no, "1": yes	
FoodFrozen- >2w	Freezing meat for more than two weeks	"0": no, "1": yes	
Rainwater	Rain water as main water source	"0": no, "1": yes	Water
Tapwater	Tap water as main water source	"0": no, "1": yes	
Wellwater	Well water as main water source	"0": no, "1": yes	
Bottledwater	Bottled water as main water source	"0": no, "1": yes	
Cleanwater-Daily	Daily water renewal	"0": no, "1": yes	Cleanwater
Cleanwater-2ndday	Every other day water renewal	"0": no, "1": yes	
Cleanwater-Weekly	Once a week water renewal	"0": no, "1": yes	
Cleanwater-no	No water change	"0": no, "1": yes	

Table 13.69: Description of variables: determined from the Questionnaire and from individuals using ZIMS, continued.

Variable as determined for individual animals and as determined by questionnaire	Explanation	Categories	Modification of variables
FecesRemoval-Daily	Daily feces removal	"0": no, "1": yes	FecesRemoval
FecesRemoval-2ndday	Feces removal every second day	"0": no, "1": yes	
FecesRemoval-Weekly	Weekly feces removal	"0": no, "1": yes	
FecesRemoval-Infreq	Infrequent feces removal	"0": no, "1": yes	
Litterbox	Availability of a litterbox	"0": no, "1": yes	
FelidsCloseby	Other felids kept close by (within 50m)	"0": no, "1": yes	
MarsupialsCloseby	Marsupials kept close by (within 50m)	"0": no, "1": yes	
NWMCloseby	New World monkeys kept close by (within 50m)	"0": no, "1": yes	
CareOtherCats	Animal attendants look also after other cat species	"0": no, "1": yes	
CareCatsPlusNWM/Marsupials	Animal attendants look also after Marsupials and/or New World Monkeys	"0": no, "1": yes	
Hygiene	Availability of hygienic devices	"0": no, "1": yes	
HandWashing	Availability of hand wash facilities at all enclosures	"0": no, "1": yes	
HandDisinfection	Availability of hand disinfection at all enclosures	"0": no, "1": yes	
FeetDisinfection	Availability of disinfective footbath between enclosures	"0": no, "1": yes	
ShoeCovers	Availability of disposable shoe covers	"0": no, "1": yes	
Gloves	Availability of disposable gloves	"0": no, "1": yes	
HayBeddingMaterial	Hay as bedding material	"0": no, "1": yes	
StrawBeddingMaterial	Straw as bedding material	"0": no, "1": yes	
SandBeddingMaterial	Sand as bedding material	"0": no, "1": yes	
OtherBeddingMaterial	Other bedding material	"0": no, "1": yes	
BeddingChange-Daily	Daily change of bedding material	"0": no, "1": yes	BeddingChange
BeddingChange-Weekly	Weekly change of bedding material	"0": no, "1": yes	
BeddingChange-Monthly	Monthly change of bedding material	"0": no, "1": yes	
BeddingChange-Infreq	Infrequent change of bedding material	"0": no, "1": yes	
Enrichment	General use of items for enrichment	"0": no, "1": yes	
BranchesEnrichment	Use of natural materials like branches for enrichment	"0": no, "1": yes	
IndustrialToysEnrichment	Use of industrial toys for enrichment	"0": no, "1": yes	
OtherEnrichment	Use of other items for enrichment (paper bags…)	"0": no, "1": yes	

Table 13.69: Description of variables: determined from the Questionnaire and from individuals using ZIMS, continued.

Variable as determined for individual animals and as determined by questionnaire	Explanation	Categories	Modification of variables
Indoor	Housing indoors, no contact to wildlife possible	"0": no, "1": yes	
Outdoor_Fencedin-allsides	Outdoors fenced in on all sides (also from above), indirect contact to wildlife possible	"0": no, "1": yes	
Outdoor_Opentop	Enclosure open from above, direct contact to wildlife possible	"0": no, "1": yes	
SizeSmaller20	Enclosure size < 20 m^2	"0": no, "1": yes	
Size20Until50	Enclosure size 20-50 m^2	"0": no, "1": yes	
SizeLarger50	Enclosure size >50 m^2	"0": no, "1": yes	
MeshSize1-2	Mesh size 1cm-2 cm	"0": no, "1": yes	MeshSize
MeshSize2-5	Mesh size 2 cm-5 cm	"0": no, "1": yes	
MeshSize>5	Mesh size > 5 cm	"0": no, "1": yes	
PestControl	Performance of pest control in general	"0": no, "1": yes	
PestControl –Cats	Cats used for pest control	"0": no, "1": yes	
PestControl -Poison	Poison used for pest control	"0": no, "1": yes	
PestControl -Traps	Traps used for pest control	"0": no, "1": yes	
PestControl -Monthly	Frequency of pest control: "Monthly"	"0": no, "1": yes	PestControl Frequency-Month
PestControl -Quarterly	Frequency of pest control: "Quarterly"	"0": no, "1": yes	
PestControl -Biannual	Frequency of pest control: "Biannual"	"0": no, "1": yes	
PestControl -Yearly	Frequency of pest control: "Annually"	"0": no, "1": yes	
StrayCats	Number of stray cats at the compound		
ToxoTest	Implementation of *T. gondii* tests in any animal species within 5 years prior to blood sampling.	"0": no, "1": yes	
ToxoFound	Number of positive *T. gondii* tests in any animal species within 5 years prior to blood sampling.		
Death	Occurrence of deaths among the felid species tested within 5 years prior to blood sampling	"0": no, "1": yes	
NoDeath	Numbers of deaths among the felid species tested within 5 years prior to blood sampling		

Table 13.69: Description of variables: determined from the Questionnaire and from individuals using ZIMS, continued.

Variable as determined for individual animals and as determined by questionnaire	Explanation	Categories	Modification of variables
Vaccine	Implementation of vaccinations in general in small felids	"0": no, "1": yes	
Cat flu	Implementation of vaccinations against cat flu in small felids	"0": no, "1": yes	
Parvovirus	Implementation of parvovirus vaccinations in small felids	"0": no, "1": yes	
Rabies	Implementation of rabies vaccinations in small felids	"0": no, "1": yes	
Leucosis	Implementation of feline leucosis vaccinations in small felids	"0": no, "1": yes	
FIP	Implementation of FIP vaccinations in small felids	"0": no, "1": yes	
Deworming	General implementation of deworming	"0": no, "1": yes	
DewormingFrequency-Quarterly	Deworming interval: Quarterly	"0": no, "1": yes	DewormingFrequency-Month
DewormingFrequency-Biannually	Deworming interval: Biannually	"0": no, "1": yes	
DewormingFrequency-Annually	Deworming interval: Annually	"0": no, "1": yes	
DewormingFrequency-Infreq	Infrequent deworming intervals	"0": no, "1": yes	
Check	General implementation of parasite checks	"0": no, "1": yes	
CheckFrequency-Quarterly	Interval for parasite checks: Quarterly	"0": no, "1": yes	CheckFrequency-Month
CheckFrequency-Biannually	Interval for parasite checks: Biannually	"0": no, "1": yes	
CheckFrequency-Annually	Interval for parasite checks: Annually	"0": no, "1": yes	
CheckFrequency-Infreq	Infrequent parasite check intervals	"0": no, "1": yes	
SourceFood	Open question on the origin of food items used for the felids tested. Categorized after data collection.	"Laboratories", "hunting", "own production", "local suppliers", "slaughters", "private breeder", "commercial animal food supplier"	

Table 13.69: Description of variables: determined from the Questionnaire and from individuals using ZIMS, continued.

Variable as determined for individual animals and as determined by questionnaire	Explanation	Categories	Modification of variables
LabsSpecies	Food items/species produced by laboratories	“Rats”, “RatsMice”, “RatsMiceFowl”	
HuntingSpecies	Food items/species produced by hunting	“Rabbits”, “RabbitsSquirrelPigeon”	
OwnProductionSpecies	Food items/species out of own production	“Fowl”, “GoatDeer”, “Mice”, “Rats”, “RatsMice”	
LocalSupplierSpecies	Food items/species produced by local suppliers	“Cattle”, “HorseCattle”	
SlaughtersSpecies	Food items/species produced by slaughters	“Fowl”, “Fish”, “FowlFish”, “MiceFish”, “MiceRats”, “RabbitsHorse”	
PrivateBreederSpecies	Food items/species produced by private breeders	“Fowl”, “Horse”, “RabbitFowl”	
CommercialProducerSpecies	Food items/species produced by commercial producers	“Fowl”, “FowlCatfood”, “FowlFish”, “Mice”, “Rabbits”, “Rats”, “RatsFowl”, “RatsFowlMice”	
NoFelidsTotal	Number of felids (all species including small and big cats) as determined by ZIMS		
Litters within 1 year	Litters born per zoo among all feline species within 1 year prior to sampling	“1-2”, “≥3”	
Litters within 5 years	Litters born per zoo among all feline species within 5 years prior to sampling	“1-9”, “≥10”	

Table 13.70: Description of new variables based on more than one basic variable (Table 13.69).

New variables based on more than one basic variable	Explanation, based on questionnaire variable	Further modification of variables	Categories
Birth	Date of birth based on "BirthYear", "BirthMonth", and "BirthDay"		
Age	Age at sampling calculated based on "Birth" (birth date) and "Sampling" ("sampling date")	AgeCategories	"1": 0-3 years, "2": 3-7 years "3": > 7 years
IFAT_Categories	Titer determined by IFAT put into 3 categories		"1": Titer 25-50, "2": Titer 100-800, "3": Titer >800
SerologicalStatus	Based on "IFAT" and "FLIImmunoblot". Considered positive/negative when both values correspond.		"1": positive, "0": negative
Mice	Based on "mice" – "frozen" or "fresh"		"No", "Fresh", "Frozen", "FreshFrozen"
Rat	Based on "rats" – "frozen" or "fresh"		"No", "Fresh", "Frozen", "FreshFrozen"
Sheep	Based on "sheep" – "frozen" or "fresh"		"No", "Fresh", "Frozen", "FreshFrozen"
Cattle	Based on "cattle" – "frozen" or "fresh"		"No", "Fresh", "Frozen", "FreshFrozen"
Horse	Based on "horse" – "frozen" or "fresh"		"No", "Fresh", "Frozen", "FreshFrozen"
Fowl	Based on "fowl" – "frozen" or "fresh"		"No", "Fresh", "Frozen", "FreshFrozen"
Fish	Based on "fish" – "frozen" or "fresh"		"No", "Fresh", "Frozen", "FreshFrozen"
Fruit	Based on "yes" or "no"		"yes", "no"
Catfood	Based on "Catfood" – "Canned" or "Dry"		"No", "Canned", "Dry", "CannedDry"
FoodStorage	Based on the "Temperature of food storage "10°C to 5°C", "5°C to 0°C", "0°C to -5°C"		"Cool"
	Based on the "Temperature of food storage "-15°C to -20°C", "Under -20°C"		"Frozen" Any combination of both: "CoolFrozen"

Table 13.70: Description of new variables based on more than one basic variable (Table 13.69), continued.

New variables based on more than one basic variable	Explanation, based on questionnaire variable	Further modification of variables	Categories
MeatFruitSeperation	Based on the preparation of meat and fruit “in different rooms”, “in different areas”, “on separate cutting boards”, “no separation”		“No”, “Process”, “Rooms”
FoodFrozen	Based on the length of frozen storage “Less than 1 week”, “1-2 weeks”, “More than two weeks”		“Sometimes<1W”, “OneWeekatLeast”, “More>2W”
Water	Water sources based on “Rain water”, “Well water”, “Tap water” or “Bottled water”		“Tap”, “RainTap”, “Well”, “WellTap”
Cleanwater	Based on the frequency of water renewal “Daily”, “Every other day”, “Once a week”, “No water change”		“Nochange”, “Daily”, “Every2nd”, “DailyWeekly”, “Weekly”
FecesRemoval	Based on the frequency of feces removal “Daily”, “Every second day”, “Once a week”, “More infrequently”		“Daily”, “NotDaily”
BeddingChange	Based on the frequency of changing bedding material “Daily”, “Once a week”, “Monthly”, “infrequently”		
MeshSize	Minimal Mesh size based on the records on mesh size categories “< 1cm”, “1-2 cm”, “2-5 cm” or “> 5 cm”		“Mesh size more than 5 cm”, “Mesh size below 5 cm”, “Mesh size below 2 cm”
PestControlFrequency_Month	Frequency of pest control in month based on “once a month”, “quarterly”, “biannual”, “once a year”		“0”, “0,25”, “1”, “2”, “3”, “6”, “12”, “Infrequently”
DewormingFrequency_Month	Deworming frequency in month based on “Quarterly”, “Biannually”, “Annually” or “Infrequently”		
CheckFrequency_Month	Frequency of parasite checks in month based on “Quarterly”, “Biannually”, “Annually” or “Infrequently”		

14 PUBLICATIONS

2011	Stagegaard, J., Kunze, M.; *Toxoplasma gondii* – the parasite, hosts, open questions. *Presentation at the Felid TAG midyear meeting 2011, Nordens Ark, Sweden.* Stagegaard, J., Kunze, M.; DAZA – Zoolog 2011, Opdatering 2011/12. *Brief presentation informing about the status of the T. gondii research projekt at the DAZA meeting 2011, Esbjerg, Denmark.* Sliwa, A., Stagegaard, J., Kunze, M.; Toxoplasmosis in small exotic felids - research. P*resentation at the EAZA Annual Conference 2011 – Felid TAG meeting, Montpellier, France.*
2012	Stagegaard, J., Kunze, M.; Toxoplasmosis in small exotic felids - research. *Presentation at the Felid TAG midyear meeting 2012, Ammersfort, Netherlands.* Kunze, M., Stagegaard, J., Conraths, F. J., Schares, G.; Toxoplasmosis in small exotic felids, an introduction of the research project and the technique of non-invasive blood sampling with reduviid bugs and Transmission of *Toxoplasma gondii* in an urban population of domestic cats (*Felis catus*). *Presentation at the Scandinavian zoo veterinarian meeting 2012, Odense, Denmark.* Kunze, M. Stagegaard, J., Conraths, F. J., Schares, G.; Toxoplasmosis in small exotic felids, Preliminary results. *Presentation at the EAZA Annual Conference 2012 – Felid TAG meeting, Innsbruck, Austria.*
2013	Kunze, M., Conraths, F. J., Schares, G.; *Toxoplasma gondii* antibodies in small exotic felids - Preliminary report. *Article in Mourek – Proceedings of the UCSZ small cat's group.*
2017	Kunze, M., Stagegaard, J., Conraths, F. J., Maksimov, P., Schares, G.; Putative risk and protective factors associated with *Toxoplasma gondii* infection in captive felids. *Presentation at the EAZWV Zoo and Wildlife Health Conference 2017, Berlin, Germany.* Kunze, M., Stagegaard, J., Conraths, F. J., Maksimov, P., Schares, G.; Putative risk and protective factors associated with *Toxoplasma gondii* infection in captive felids. *Abstract in the proceedings of the EAZWV Zoo and Wildlife Health Conference 2017, Berlin, Germany.* Kunze, M., Stagegaard, J., Conraths, F. J., Maksimov, P., Schares, G.; Putative risk and protective factors associated with *Toxoplasma gondii* infection in captive felids. *Presentation at the EAZA Annual Conference 2017 – Felid TAG meeting, Emmen, Netherlands.* Kunze, M., Stagegaard, J., Conraths, F. J., Maksimov, P., Schares, G.; Mögliche Risikofaktoren und prophylaktische Maßnahmen im Zusammenhng mit *Toxoplasma gondii* bei exotischen Kleinkatzen. *Presentation at the 37. Arbeitstagung der Zootierärzte im deutschsprachigen Raum, Innsbruck, Austria.* Kunze, M., Stagegaard, J., Conraths, F. J., Maksimov, P., Schares, G.; Mögliche Risikofaktoren und prophylaktische Maßnahmen im Zusammenhng mit *Toxoplasma gondii* bei exotischen Kleinkatzen. *Abstract in the Tagungsband der 37. Arbeitstagung der Zootierärzte im deutschsprachigen Raum, Innsbruck, Austria.* Schares, G., Kunze, M., Stagegaard, J., Conraths, F. J., Maksimov, P.; Putative risk and protective factors associated with *Toxoplasma gondii* infection in captive felids. *Presentation at the Tagung der DVG-Fachgruppe "Parasitologie und parasitäre Krankheiten" 2017, Hannover, Germany.* Schares, G., Kunze, M., Stagegaard, J., Conraths, F. J., Maksimov, P.; Putative risk and protective factors associated with *Toxoplasma gondii* infection in captive felids. *Abstract in the conference proceedings of the DVG, Hannover, Germany.*

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16 SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 26.06.2018;

Maike Kunze