PATHOLOGY AND SEROLOGICAL ASPECTS OF BOHLE IRIDOVIRUS INFECTIONS IN SIX SELECTED WATER-ASSOCIATED REPTILES IN NORTH QUEENSLAND

Thesis submitted
by

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ABSTRACT

Bohle iridovirus is a pathogen with a broad host range and high virulence in susceptible native animals in northern Australia. The virus is inactivated at temperatures above 32°C and therefore potential hosts are limited to poikilothermic animals. The only vertebrate class of poikilothermic animals which has not yet been studied with respect to Bohle iridovirus infections is the Reptilia. This thesis reports on investigations of the pathological and serological responses to Bohle iridovirus in selected water-associated reptiles.

The reptiles were selected on the basis of their association with either animals or environments that were known to potentially harbour Bohle iridovirus. Certain species of fish and frogs are known to be highly susceptible to Bohle iridovirus and they are both found in or near freshwater habitats. In Australia, tortoises, freshwater crocodiles and certain species of colubrid snakes prey on fish and amphibians and dwell in aquatic or riparian habitats. The species chosen for this study are endemic to north Queensland where Bohle iridovirus was first isolated, and consist of the tortoises, *Emydura krefftii* and *Elseya latisternum*, the freshwater crocodile, *Crocodylus johnstoni*, the colubrid snakes, *Boiga irregularis*, *Dendrelaphis punctulatus* and *Amphiesma mairii*.

An enzyme linked immunosorbent assay was developed for the detection of serum proteins reactive to Bohle iridovirus in the selected reptiles. Reptilian test sera were used as the capture antisera in a sandwich enzyme linked immunosorbent assay which constitutes the first serological test for detection of reactivity to an iridovirus in reptiles. It enabled the monitoring of possible antibody responses to Bohle iridovirus in experimentally infected reptiles.

All six species were found to be capable of producing detectable levels of serum reactivity to Bohle iridovirus. Experimental animals were monitored for four weeks following either a single inoculation of live Bohle iridovirus, cohabiting with inoculated animals or by being fed artificially-infected prey. Within treatments, animals did not consistently produce a detectable response.

Tortoise hatchlings of both *Emydura krefftii* and *Elseya latisternum* were found to be extremely susceptible to Bohle iridovirus. Within four weeks hatchlings died with pathological changes in the liver, kidney, spleen, submucosa and pancreas. The virus was re-isolated from experimentally infected individuals.

Adult tortoises, yearling freshwater crocodiles and the three species of snakes did not appear to be adversely affected during the experimental period by exposure to Bohle iridovirus. There were no mortalities or pathological changes in these animals which could be directly attributed to a Bohle iridovirus infection. The virus was re-isolated from a single *Boiga irregularis* individual at four weeks following inoculation with live virus.

A serum survey of wild populations of the above-mentioned reptiles in selected water courses of north Queensland revealed individuals with Bohle iridovirus-hyperimmune sera at several locations. For *Emydura krefftii* and especially *Crocodylus johnstoni* a strong trend was found for larger animals to have higher levels of serum reactivity to Bohle iridovirus than the smaller animals in the sample.

The tendency for juveniles to be highly susceptible in comparison to adults which was reported for both fish and amphibians, seems also to be true for reptiles. Bohle iridovirus is extremely virulent in hatchling tortoises under the experimental conditions used, whereas adult tortoises and snakes and yearling crocodiles are not adversely affected in the short term.

Pre-experimental mortalities and intercurrent disease were thoroughly investigated as part of the monitoring of the well-being of experimental animals, but also to determine if these incidental findings were in some way influenced by the pathogen studied, or vice versa. In the freshwater crocodiles such investigations resulted in the recording of concurrent gout and hypovitaminosis A in hatchlings and also the first diagnosis of mycobacteriosis in young freshwater crocodiles.

In the wild, juveniles exposed to the virus may either succumb to infection or they may be excluded from Bohle iridovirus infection by certain behavioural features, because only serum from the larger animals were found to have high levels of Bohle iridovirus reactivity. Adult tortoises are abundant across the Australian continent. They are easily captured and can readily survive exposure to Bohle iridovirus while producing Bohle iridovirus antibodies at levels detectable by enzyme linked immunosorbent assay. Therefore they are good indicator species for a past presence of Bohle iridovirus in a particular freshwater environment. The use of such sentinel animals together with the enzyme linked immunosorbent assay described here, can survey the spread of Bohle iridovirus in northern Australia, and help to confirm the potential dangers to native fauna from this agent.

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Ellen ARIEL
June 1997

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Ellen ARIEL
June 1997

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LIST OF ABBREVIATIONS

ABTS	2'2-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid)
BDS	Bovine donor serum
BF2	Bluegill Fry 2
BIV	Bohle iridovirus
BSA	Bovine serum albumin
CBC	Carbonate bicarbonate buffer
CPE	Cytopathic effect
CSL	Commonwealth Serum Laboratories modified DMEM
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
EHNV	Epizootic haematopoietic necrosis virus
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
FBS	Foetal bovine serum
FV-3	Frog virus 3
g_{max}	Relative centrifugal field at maximum radius
HAT	Hypoxanthine Aminopterin Thymidine
HMW	High molecular weight
HT	Hypoxanthine Thymidine
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgN	Immunoglobulin N
IgY	Immunoglobulin Y
IgY(ΔFc)	Immunoglobulin Y- truncated form
JCU	James Cook University
KLH	Keyhole limpet haemocyanin
LMW	Low molecular weight
O/N	Overnight
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PVC	Polyvinyl chloride
QDOE	Queensland Department of Environment
R/T	Room temperature
RPM	Revolutions per minute
SPF	Specific pathogen free
$TCID_{50}$	Tissue culture infective dose 50
TEN-T	Tris EDTA NaCl -Tween 20 buffer
TEN-TC	Tris EDTA NaCl-Tween 20 Casein
TH-1	Box turtle cell line 1
TRIS	Tris (hydroxymethyl aminomethane) buffer
VEN	Viral Erythrocytic Necrosis
WEEV	Western Equine Encephalitis Virus

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CHAPTER 1

GENERAL INTRODUCTION

Bohle iridovirus (BIV) is one belonging to the genus Ranavirus (Speare 1995). It has been isolated only once from wild populations of newly metamorphosed ornate burrowing frogs, *Limnodynastes ornatus*, in north Queensland, Australia (Speare and Smith 1992). Experimental infections of toads, frogs and fish revealed that BIV is a pathogen of extreme virulence to certain species with a wide host range spanning both amphibians and fish (Moody and Owens 1994; Cullen, Owens and Whittington 1995). The virus is inactivated at temperatures above 32°C (Moody 1992), which limits its potential hosts to poikilothermic animals. Of potential poikilothermic vertebrate hosts for BIV, this is the first study of the only class not yet investigated: the Reptilia.

Ranaviruses are generally associated with aquatic or semi-aquatic hosts where they may cause systemic infection. Similarly BIV was initially isolated from an amphibian host and later found infective to fish. The reptiles in this study were chosen based on their association with water, either through a semi-aquatic habitat or via aquatic or semi-aquatic prey, because they were considered of greatest risk of encountering BIV under natural conditions.

Tortoises (*Elseya latisternum* and *Emydura krefftii*), freshwater crocodiles (*Crocodylus johnstoni*) and snakes (*Boiga irregularis*, *Dendrelaphis punctulatus* and *Amphiesma mairii*) with either frogs and/or fish in their diet, were studied. Because these reptiles occasionally prey on frogs and fish, which are potential hosts of BIV, they could be exposed to the virus by eating infected prey or through exposure to waterborne virions in an infected environment. These species are part of the common aquatic and riparian reptilian fauna in north Queensland.

This study aimed to clarify the potential pathogenicity of BIV in selected reptiles, with respect to host susceptibility, antibody production, and pathological lesions arising from experimental infection, and to re-isolation of the virus from infected animals. The study also aimed to develop a serological tool for the detection of previous exposure to BIV in wild populations of the selected reptiles.

To achieve these aims, I (1) reviewed the literature on the subjects of the reptilian immune system, viruses of reptiles and the iridoviruses, in particular BIV; (2) developed an ELISA for detection of BIV antibodies in reptilian sera; (3) performed experimental infections on the six reptilian species identified above, both to determine the effects of the virus, but also to

determine a discrimination level between BIV-reactive and non-reactive sera. The ELISA and the determination of a cut-off value for each species enabled the final part of the project, which was (4) a survey of wild populations of the selected species of reptiles, for the presence of anti-BIV antibodies in their sera as evidence of past exposure to BIV.

CHAPTER 2

REVIEW OF THE REPTILIAN IMMUNE SYSTEM, VIRUSES OF REPTILES AND IRIDOVIRUSES

2.1 Reptilian Immune System – General Principles

Of primary concern to the survival of a species is the ability to resist or eliminate pathogens (Hildemann 1962), thus recognition of self versus non-self is a characteristic of all animals (Marchalonis and Cone 1973). Invertebrates cope with microbial intruders by means of phagocytosis and enzymatic reactions (Gewurtz, Finstad, Muschel and Good 1966), and although vertebrates have an innate immune defence, they also possess adaptive immunity consisting of a more complex and specific array of immune mechanisms involving both the cellular and humoral systems (Hildemann 1962).

Natural haemolysin, a complement related compound, is present in invertebrates and some vertebrates (Gewurtz et al. 1966). It is able to recognise cell surfaces of bacteria and xenogenic cells and lyse these intruders. This first line of defence may rid the host of an infection without further involvement of other immune factors, thus reducing the need for a prompt cell mediated immune response and a diverse antibody repertoire (Du Pasquier 1982). In primitive multicellular animals, recognition of non-self appears to depend on cell surface macromolecules (Marchalonis and Cone 1973).

The immunoglobulin-like receptors for antigen on the surface of invertebrate phagocytic cells suggest that vertebrate immunity may well have evolved from such surface recognition systems, as increasingly diverse membrane patterns emerged (Marchalonis and Cone 1973). Phylogenetically, immunoglobulins precede plasma cells and organised lymphoid tissue (Good and Papermaster 1966), and all vertebrates tested possess the capacity to respond to specific foreign antigens by producing circulating antibody, even when lacking the sophisticated lymphoid architecture characteristic of mammals. Additionally, the vertebrate species studied so far are able to reject allografts by means of cell mediated immunity (Borysenko 1978). Although all vertebrates possess both humoral and cell mediated immunity, for the ectothermic vertebrates, the kinetics of such responses, as with all their physiological functions, depend on the ambient temperature (Evans 1963a).

The immunology of reptiles has been studied less than that of fish, amphibians, mammals and birds for several reasons. First, reptiles are of little commercial value compared to animals raised

in aquaculture, poultry-keeping and mammal farming. Second, certain reptiles are often difficult to maintain in captivity due to their low stress tolerance, specific habitat and food requirements, protected status and venom or other dangerous attributes (Jurd 1994). Third, there are only few inbred reptile strains available for large scale immunological studies. Following the above, the information available on reptile immunology is still disjunct and does not cover all members of the class. Therefore an investigated species often stands as a representative of its order with respect to the immune component of its investigation.

Much of the pioneering research into reptilian immunity took place in the nineteen sixties and seventies. These early investigations were often controversial, which may have been the result of inconsistent and/or inappropriate application of experimental procedures such as differing ambient temperature, nature, dosage, route and protocol for antigen administration, as well as insufficient testing of the immune response (Cohen 1971). However, the absence of evidence for an immune response was not accepted as evidence of absence and later workers found reptiles capable of responding to antigenic stimuli by means of both cell mediated (Tam, Reddy, Karp and Hildemann 1976) and humoral immune factors (Kollias 1984), as well as complement (Day, Good, Finstad, Johannsen and Pickering 1970). Likewise, with repeated exposure to the antigen, reptiles were found to mount immune reactions in a fashion characteristic of the primary and anamnestic responses in mammals (Borysenko 1978).

The class Reptilia consists of 4 orders which include the Chelonia (turtles and tortoises), the Rhynchocephalia (the only living representative being the tuatara), the Squamata (lizards and snakes) and the Crocodylia (crocodiles and alligators) (Lawrence 1989). With the information available I will review the following categories of the reptilian immune system: lymphoid organs, humoral immunity, complement, cell mediated immunity, inflammation and factors affecting the response. I will also attempt to place certain immune functions of reptiles into context, by comparing them to other vertebrate classes for which much more knowledge is available.

2.1.1 Lymphoid organs

Increased complexity of the lymphoid organs in vertebrates appears to correlate with their phylogenetic placement (Tizard 1995). Reptilian lymphoid tissues are widely distributed throughout their body, the most prominent and best developed being the thymus and the spleen (Borysenko 1978). There is, however, a large variation among species with respect to the anatomical position of the lymphoid structures.

2.1.1.1 Thymus

Marchalonis, Ealey and Diener (1969) investigated the immune system of the tuatara (*Sphenodon punctatum*), a relict species of reptile. The only lymphoid organ that they could clearly identify was the spleen, but they found no evidence of a thymus or lymphoid aggregates. The tuatara was able to produce antibodies to inoculated antigen, but the question of a cell mediated immune response originating from non-thymus cells could not be experimentally addressed due to the rarity and protected status of these reptiles (Cohen 1971). Apart from the tuatara, the thymus remains a central organ in the ontogeny of immunity throughout the vertebrate classes (Borysenko 1978).

The thymic nodules of reptiles develop from the dorsal epithelium of pharyngeal pouches (Du Pasquier 1973). Turtles have a distinct single lobe on each side of the neck at the bifurcation of the common carotid arteries, each lobe is subdivided into a number of partial lobes. The thymus of crocodilians resembles that of birds: an elongated "string of pearls"-like structure beginning at the base of the skull and extending the length of the neck, reaching almost to the level of the heart (Borysenko 1978). Involution of the thymus occurs as the reptile ages or temporarily during disease and winter (Jurd 1994).

The turtle is not immunologically mature at hatching and requires several months to become completely immunocompetent (Borysenko 1978). Both the thymus and the spleen are fully differentiated at hatching. Over the first several months the thymus remains the same size while the spleen enlarges considerably, thus immunological competence may be gradual and may involve further maturation in the spleen.

2.1.1.2 Spleen

Red and white pulp of the spleen is clearly defined in the tuatara, turtles and snakes (Marchalonis et al. 1969; Borysenko 1976), but not in certain lizards (Kanakambika and Muthukkaruppan 1973). Intracoelomic injection of carbon is deposited in the red pulp of lizards, indicating the presence of phagocytic cells in that location (Ambrosius 1976). Germinal centres are absent in immunised reptiles (Cohen 1971). Nodules with germinal centres are associated with a secondary response in mammals (Thorbecke, Romano and Lerman 1974), but these responses occur in reptiles without such structures (Lerch, Huggins and Bartel 1967; Ambrocius, Hemmerling, Richter and Schmike 1970; Wetherall and Turner 1972).

The reptilian spleen plays a central role in both the cell mediated and the humoral immune system (Sidky and Auerbach 1968; Borysenko and Tulipan 1973; Borysenko 1975). Although functional B and T cell regions have not been clearly defined in this organ, turtles stimulated with keyhole limpet haemocyanin (KLH) show a prompt and strong proliferation in white pulp sheaths, followed by migration and proliferation of lymphoblasts into antigen secreting plasma cells in the red pulp (Borysenko 1976). In the turtle *Chelydra serpentina*, the inner lymphocyte sheath consists predominantly of T cells, while the outer zone appears to be mostly B cells (Borysenko 1978). Considering that plaque forming cells are found in the spleen and blood, but not in the thymus or other lymphoid structures, and that after splenectomy, lizards cannot produce antibody against sheep red blood cells (Rothe and Ambrosius 1968; Kanakambika and Muthukkaruppan 1972a), it appears that the spleen is the main antibody producing organ in reptiles.

2.1.1.3 Bone marrow

A functional bone marrow is found in species with a suitable bone structure, thus anuran amphibians, reptiles, birds and mammals all possess lymphopoietic tissues in their bone marrow (Jurd 1994). In young *C. serpentina* turtles extrathymic lymphopoiesis takes place in the liver, heart and mesonephros; however, as the bone marrow matures it becomes the source of all lymphoid cells (Jordan and Flippin 1913).

2.1.1.4 Intestinal lymphoid aggregates

Lymph node-like structures have only been found in snapping turtles, *C. serpentina* (Cohen 1971), other reptiles investigated do not posses true lymph nodes (Good and Papermaster 1966). Diffuse lymphoid tissue in the walls of blood vessels and perivascular lymphatics, however, show a superficial resemblance to lymph nodes of birds and could be interpreted as precursers to lymph nodes (Tam et al. 1976). Further support for this possibility comes from the lymphoid plexuses, such as the lymphoepithelial tonsils in the pharyngeal region of the alligator, which occur at sites where mammals have nodes (Good and Papermaster 1966).

Ectothermic vertebrate immune functions are less centralised than their avian and mammalian counterparts, and occur to a greater extent in connective tissue such as the lamina propria of the gut (Good, Finstad, Pollara and Gabrielsen 1966). Although big variations exist between species, diffuse lymphoid aggregates rather than Peyer's patches (Borysenko and Cooper 1972)

can be found in the lungs (Borysenko 1978), at strategic sites such as the urinary system and the bladder (Hussein, Badir, El Ridi and Akef 1979), and associated with all regions of the intestinal tract. Gut-associated lymphoid accumulations are abundant and distributed throughout the lamina propria (Borysenko and Cooper 1972). Subepithelial lymphoid aggregate can also be found in the bladder of *Pseudomys scripta* (El Ridi, El Deep and Zada 1980). Confusion persists as to whether these structures are permanent or a result of transient local infiltration into areas of gut epithelium infection (Borysenko 1978). The size and prominence of such systems varies according to season in Egyptian snakes and lizards, with a decline in winter (Hussein, Badir, El Ridi and Akef 1979a; Hussein, Badir, El Ridi and El Deep 1979b).

Due to the anatomical resemblance, the multiple aggregates of lymphoid tissue in the cloacal region of alligators and at least two chelonians were initially thought to be the reptilian equivalent of the bursa of Fabricius in birds (Sidky and Auerbach 1968; Tam et al. 1976). With further investigations, the cloacal complex could not be demonstrated to be an analogue or a homologue to the bursa of Fabricius. Rather, it was determined to be a secondary lymph tissue similar to the other gut-associated tissues (Solas, Leceta and Zapata 1981).

2.1.2 Leukocytes

Most of the reptilian leukocytes resemble those found in higher vertebrates (Frye 1991). Parts of the leukocyte population are involved in the humoral immune system (B lymphocytes), others in cell mediated immunity or mediating between the two (T lymphocytes). The actions of the granulocytic series, however, may be the primary line of defence in protecting the body against invading organisms (Saint Giron 1970).

2.1.2.1 Granulocytes

As the name implies, the granulocytes all posses cytoplasmic granules and additionally, they have a lobulated, irregular nucleus (Tizard 1995). Granulocytes originate in the bone marrow, but may migrate to the spleen for maturation. In reptiles, both the neutrophils, eosinophils, heterophils, and thrombocytes have been observed to be phagocytic (Frye 1991). The basophil cells provoke inflammation and a cell surface immunoglobulin have been implicated in histamine release (Sypek and Borysenko 1988). During bacterial infection and cellular necrosis an increase in neutrophils can be observed, but unlike its mammalian counterparts, the ability of the reptilian neutrophil to respond to an invasion of pathogenic micro-organisms tends to be

relatively small compared to other reptilian leukocytes. Thrombocytes form an integral part of the clotting system and can transform into erythrocytes (Frye 1991).

Crocodiles and turtles have two types of acidophilic granulocytes: heterophils and eosinophils. Snakes and lizards possess only heterophils (Montali 1988). For the most part, normal reptile heterophils and eosinophils have an eccentric nucleus that range from round to irregular (bilobed). In snakes the nucleus is generally round (E Jacobson, University of Florida, pers. comm., 1997). Both heterophils and eosinophils are acidophilic, but in smears, heterophils can be seen to have fusiform granules in the cytoplasm, where eosinophils have rounded granules. In section, the granules in both cell types appear rounded, making it difficult to distinguish between the two types (Montali 1988). As the heterophils are the more abundant granulocyte in reptiles, all acidophilic granulocytes are referred to as "heterophils" in sections (Frye 1991). There is a distinct seasonal variation in both heterophils and eosinophils. Elevated numbers of eosinophils have been observed during the winter hibernation period, whereas the maximum number of heterophils occur in the summer months (Duguy 1970). Reptilian heterophils appear to be homologous to mammalian neutrophils and play an important role in the inflammatory response to both parasitic and microbial invasion (Duguy 1970; Glassman and Bennett 1978).

2.1.2.2 Macrophages

Circulating, phagocytic macrophages (monocytes) may constitute over 20% of the unfixed leukocytes in certain snakes (Pienaar 1962), and do not show seasonal variation in their numbers (Duguy 1970). They are often seen in inflammatory and granulomatous reactions to parasitic (Wolke, Brooks and George 1982) and bacterial infections (Evans 1983). Macrophages also play an active role in giant cell formation (Evans 1983). "Histiocyte" is an interchangeable term for a fixed or tissue-based macrophage (Frye 1991).

2.1.2.3 Lymphocytes

The majority of leukocytes in the peripheral blood of reptiles is generally made up of lymphocytes (Ryerson 1949; Pienaar 1962). Their size and number may vary according to sex (Duguy 1970), age (Pienaar 1962), nutritional status (Borysenko and Lewis 1979), infection history and season (Duguy 1970; Hussein, Badir, El Ridi and Akef 1978; Hussein, Badir, El Ridi and Charmy 1979; Hussein et al. 1979a,1979b), with higher numbers occurring in the summer months. Ontogenically, lymphocytes are first formed in the yolk, then in the foetal spleen and

finally in the bone marrow of the mature lizard (El Deeb, Zada and El Ridi 1985). B-lymphocytes are thought to differentiate in the embryonic liver of reptiles (El Deeb and Saad 1990). T-lymphocytes appear to mature first in the thymus, then in the spleen (El Deeb, El Ridi and Zada 1986).

Lymphocyte heterogeneity has been confirmed for lizards (*Calotes versicolor*) by tne use of differential response to mitogens and migration inhibition studies (Manickasundari, Selvaraj and Pitchappen 1984; Manickasundari and Pitchappen 1988). It still remains to be ascertained that such populations of lymphocytes are functionally analogous or homologous to mammalian lymphocyte populations (Jurd 1994). Plasma cells can be identified in stained blood smears and in the spleen, and their numbers may be substantially increased in case of immunogenic stimulation (Evans 1963b; Frye 1991). The presence of memory cells can be deduced from the ability of tested reptiles to raise a secondary immune response. T-helper cell factors have been implied from studies of the cellular kinetics of the immune response in the lizard *C. versicolor* (Muthukkaruppan, Pillai and Jayaraman 1976a). Likewise, the presence of suppresser T-cells has been suggested (Cuchens, McLean and Clem 1976; Muthukkaruppan, Pitchappan, Ramila 1976b; Pitchappan and Muthukkaruppan 1977), as has natural cytotoxic cell activity in the snake, *Psammophis sibilans* (Sherif and El Ridi 1992).

2.1.3 Humoral immunity

Antibody mediated immunity in reptiles was first confirmed by Metchnikoff in 1901, when he detected a specific antibody response in crocodiles after experimental infection with tetanus and cholera toxins. Even the most primitive immune system of a living reptile, found in the tuatara, *Sphenodon punctatum*, can produce specific antibodies to *Salmonella adelaide* (Marchalonis 1969) and this ability to respond to antigenic stimulation by producing specific antibodies has since been established in all reptile orders (Kollias 1984).

2.1.3.1 Immunoglobulins

All reptiles tested possess at least two classes of immunoglobulins: an IgM molecule of approximately 18S sedimentation coefficient and an approximately 7S non-IgM (Evans 1963b; Lykakis 1968; Marchalonis 1969; Jurd 1994). In addition, some turtles have a 5.7S immunoglobulin (Leslie and Clem 1972).

The macroglobulin of reptiles is similar to IgM and is composed of five subunits (Leslie and Clem 1972). It is the major serum immunoglobulin of reptiles (Salanitro and Minton 1973) and appears unaltered in all vertebrate classes (Atwell and Marchalonis 1976). This degree of conservative evolution, with respect to IgM, may be because its major function, as lymphocyte surface receptor for antigen, has not changed through evolutionary time (Atwell and Marchalonis 1976). IgM may also act as a secretory antibody, a function analogous to mammalian IgA, with whom it shares marked structural similarities (Portis and Coe 1975). In fishes, where the blood vessels are permeable to the serum macroglobulins, IgM is the sole antibody and that may be sufficient, because it can penetrate into most fish tissue fluids, lymph and mucus in addition to plasma (Litman 1976). However, in terrestrial vertebrates, where there is a need for a more efficient vascular system, the junction between vascular endothelial cells is tighter, which favours the production of immunoglobulins with a low molecular weight (LMW) and subsequent access to the extravascular spaces (Atwell and Marchalonis 1976; Tizard 1995).

The 7S immunoglobulin has been termed IgY and appears to be the reptilian (and amphibian and avian) equivalent to IgG in mammals (Warr, Magor and Higgins 1995). Molecular cloning and functional parallels have recently identified IgY as the evolutionary ancestor to not only mammalian IgG, but also IgE. Like IgG, IgY is the major serum LMW antibody and thus serves a major role in the defence against systemic infections (Magor, Higgins, Middleton and Warr 1994), however, IgY is also capable of mediating anaphylactic reactions (Faith and Clem 1973), a function usually carried out by IgE in mammals. The entire IgY molecule has a molecular weight of 180 kDa, but this molecule can occur in a truncated form, IgY (Δ FC) which lacks the two terminal domains of the heavy chains and has a molecular weight of 120 kDa and a sedimentation coefficient of 5.7S (Magor et al. 1994). Thus, the LMW immunoglobulin identified in lungfishes, originally called IgN (Atwell and Marchalonis 1976), is likely to be an IgY (Δ FC) molecule. IgY (Δ FC) has the ability to effectively neutralise viruses (Warr et al. 1995).

2.1.3.2 Succession of immunoglobulins in the immune response

Following repeated immunisations, reptiles exhibit a prolonged high molecular weight antibody response: IgM, replaced only slowly by LMW antibodies: IgY and IgY (Δ FC) as the response matures (Grey 1963 1966; Lykakis 1968; Ambrosius 1976). In contrast, mammals have a short macroglobulin (IgM) response followed quickly by a long lasting, high titred LMW antibody (IgG) response (Cohen 1971; Salanitro and Minton 1973). In the case of repeated inoculations with

pig serum proteins, four different types of antibodies appeared sequentially in the serum of the turtle *Testudo hermanii* (Ambrosius 1976)

2.1.3.3 Dynamics of the antibody response

Some studies reveal a slow appearance and low titres of antibodies after experimental antigenic stimulation (Maung 1963; Lykakis 1968; Marchalonis et al. 1969), while other investigations show an immediate and vigorous response (Evans 1963b; Sidky and Auerbach 1968; Kassin and Pevnitskii 1969). This disparity may in part be the result of variations in the immune systems of the species investigated; in particular in the mechanisms involved in recognising, trapping and processing antigen (Cohen 1971). Alternatively, these kinetic variations may be caused by the use of different antigens, dose, route of administration, ambient temperature and method for detecting the antibody response. Salanitro and Minton (1973) inoculated colubrid snakes with two different antigens, bovine serum albumin (BSA) and keyhole limpet haemocyanin (KHL). Only LMW antibodies were detected against BSA and only IgM immunoglobulins against KLH, indicating that different immunoglobulin classes are employed towards different antigens. Likewise, Wright and Schapiro (1973) detected only LMW antibody in response to KLH, and recorded a secondary response in *Dipsosaurus dorsalis*. Evans (1963a) had previously investigated the same lizard species, but had not detected a typical anamnestic response.

2.1.3.4 Primary versus secondary response

Although Maung (1963) did not detect a secondary response to immunisation in the turtle *Testudo ibera*, there are later reports of reptiles that can raise an anamnestic response after repeated vaccinations (Lykakis 1968; Ambrosius et al. 1970; Wright and Schapiro 1973; Borysenko 1978). A typical secondary response is only elicited by protein antigens which are capable of inducing a true primary response, and only after a period sufficiently long to allow for the maturation process in the immune system (Ambrosius 1976). The secondary immune response in reptiles is quantitatively higher and qualitatively different from the primary response (Wright and Schapiro 1973; Borysenko 1978). In contrast to LMW antibodies, IgM shows little maturation in affinity after repeated exposure to an antigen. This strong difference in the affinity maturation of IgM and the succeeding LMW antibodies suggest both a different regulation of IgM and the other immunoglobulins and a very early separation of cell clones for the production of the different antibody isotypes (Ambrosius 1976).

2.1.4 Complement

The presence of haemolytic complement activity in snakes was first demonstrated in 1901 by Flexner and Noguchi. Immune haemolytic systems involving complement in turtles, snakes and lizards have since been identified (Dessauer 1974). The lytic activity appeared to be analogous to the complement system of mammals as it was heat labile, temperature dependent, potentiated by antibody and inhibited by EDTA (Day et al. 1970). Serum from the lizard *Tiliqua rugosa* performed complement-mediated bacteriolysis of several different species of bacteria (Schwab and Reeves 1966). Both the haemolytic and the bactericidal complement systems were temperature dependent.

2.1.5 Cell mediated immunity

The action of T lymphocytes in cell mediated immunity can be studied by means of graft rejection (Borysenko 1970). These can be either *in vivo*, skin or organ grafts, or *in vitro*, mixed leukocyte culture (Tam et al. 1976). When viable lymphocytes from unrelated individuals of the same species (allogenic graft) are cultured together *in vitro*, they respond by stimulating each other to rapid proliferation and differentiation into blast cells. The magnitude of response of one lymphocyte population is measured by inhibiting the DNA synthesis of the other population by subjecting it to sublethal doses of radiation or mitomycin treatment (Tam et al. 1976).

Such *in vitro* models show that maturity in terms of histocompatibility in turtles was reached at 6 months after hatching (Sidky and Auerbach 1968). Newly hatched snapping turtles (*C. serpentina*) injected with adult allogenic spleen cells succumbed to acute graft versus host reactions at 30°C. A chronic reaction with less mortalities was observed at 20°C. Four months old turtles were considered immunologically mature and were not affected by allogenic nor xenogenic spleen inocula (Du Pasquier 1973).

First set grafts appear to be rejected as a result of a temperature-sensitive response as recorded in turtles, snakes, crocodilians and lizards (Maslin 1967; Borysenko 1969, 1970; Cooper 1968, 1969; Manickavel and Muthukkaruppan 1969). Thus, the variation in graft rejection times among different reptile species is influenced by ambient temperature and degree of immunological maturation at the time of grafting. The response initially involved lymphocyte infiltration and macrophages dominated later (Terebey 1972). Anamnesis can be demonstrated

by the accelerated rejection of second set grafts (Hildemann and Thoenes 1969; Borysenko 1978).

Graft rejection appears to be predominantly chronic in most reptiles as opposed to acute in mammals (Cohen 1971), however, in *Tarantola annularis*, the kinetics of localised graft-versus-host reactions resemble those in mammals (Badir, Afifi and El Ridi 1981). Chronic or slow cell mediated reactivity to foreign tissue seems to provide as effective an immunosurveillance for reptiles as the promptly mobilised cell mediated reactions provide for birds and mammals. If anything then, reptiles are less susceptible to cancer induction than are birds and mammals (Tam et al. 1976).

2.1.6 Inflammation

Fish, reptiles and birds react to a wider spectrum of infectious agents with production of granulomas than do mammals (Jortner and Adams 1971; Wolke and Stroud 1978; Frye 1981). The two major types of granuloma recognised in reptiles are the heterophilic granuloma and the histiocytic granuloma. As the names imply, the predominant cells in the early phase of the respective lesions are either heterophils or histiocytes.

The exudate derived from an accumulation of heterophils usually forms a yellow/white caseous mass consisting predominately of degenerated or degranulated heterophils. The early phase of a heterophilic granuloma is characterised by an accumulation of heterophils with a necrotic centre. The outer cells are degranulated and the marginal ones intact. The necrotic heterophils or the released granules may act as "foreign bodies" and stimulate a strong macrophage response. At the late stage, the heterophils undergo necrosis and become surrounded by macrophages and, in some cases, by giant cells (Montali 1988).

Histiocytic granulomas induced by intracellular pathogens such as mycobacteria, may resemble a final stage heterophilic granuloma. Histiocytic granulomas induced by metazoan parasites tend to be chronic, fibrosing and usually calcify. Early lesions are formed from organised collections of foamy macrophages, which eventually becomes necrotic at the centre. Occasionally a few heterophils or giant cells may be present. The final lesion consists of caseo-necrotic masses with extracellular bacteria or tubercles (Montali 1988).

Heterophilic granulomas may be confused with mycobacterial lesions. The major difference between the two types lies in their necrotic centres: with either heterophil or histiocyte remnants. This difference, however, can not be distinguished in the final stage of the lesions (Montali 1988).

2.1.7 Factors affecting the immune response of reptiles

As discussed briefly in the previous sections there are a multitude of factors influencing the immune response in reptiles. Some are directly related to the experimental protocol such as nature of antigen (Ambrosius 1976), use of adjuvant (Wetherall 1969), dosage, route of inoculation and vaccination protocol (Hildemann 1962). Others are more related to the environment in which the animal under investigation is kept. Environmental factors which may influence the immune response of a reptile include the ambient temperature in the pen (Avtalion, Weiss and Moalem 1976), season of year (Zapata, Varas, Torroba 1992), availability of a suitable food source (El Ridi, Zada, Afifi, El Deeb, El Rouby, Farag and Saad 1988), social, health and reproductive status of the individual (Mahmoud, Guillette, MsAsey and Cady 1989; Aguirre, Balazs, Spraker and Gross 1995; Warwick, Frye and Murphy 1995) and other stressors such as predator or human activity (Warwick et al. 1995). These environmental factors exert some type of stress on reptiles, which in turn modifies most of the steroids that affect the immune system (Warwick et al. 1995). The developmental stage of the reptile is also important as a fully functional immune system may not be acquired until the animal is several months post hatching (Sidky and Auerbach 1968).

2.1.7.1 Temperature

Numerous reports exist concerning the influence of temperature on the immune response in reptiles (Metchnikoff 1901; Bisset 1948; Evans and Cowles 1959; Sirotinin 1959; Maung 1963; Evans 1963b). This differential response according to ambient temperature, led Cohen (1971) to pose the question of how reptiles survive at all, in a world of constantly fluctuating temperatures. Instead of viewing poikilothermic animals as less fortunate organisms than homeotherms, it may pay to consider them as more economic in their energy consumption (Bartholomew 1982). Reptiles living under natural conditions can, through their tolerance of hypo- and hyperthermia, selection of micro-habitat, diurnal activity rhythms, posture and other behavioural patterns, obtain a much more desirable body temperature for combating pathogenic organisms than the homeotherms. Attaining an increased body temperature by

means of certain activities is termed "behavioural fever" and is a common phenomenon in infected reptiles (Hutchison and Dupre 1992). It should also be remembered that the pathogens infecting poikilotherms are similarly faced with the challenge of an environment of fluctuating temperatures, which in turn may impair their pathogenicity.

Based on observations by Bisset (1948), Maung (1963), and Evans (1963a, 1963b), Avtalion et al. (1976) demonstrated experimentally that in the immune response of reptiles, a temperature sensitive event occurred between the third and the fourth day after primary stimulation. Events occurring prior to that critical period were not temperature sensitive, neither were the ones succeeding it. Priming of immuno-sensitive cells and cells involved in tolerance was still effective at suboptimal temperatures. However, while the animals were kept at the low temperature, the maturation of the immune response was arrested and the activation of T helper cells inhibited (Avtalion et al. 1976; Wright, Eipert and Cooper 1978). Upon transfer to optimal temperatures the inhibition was removed and the normal development of an immune response proceeded promptly. Imposing low temperatures on animals after the critical period had passed and the lymphocytes were already activated, did reduce the response as expected for a physiological reaction, but it did not entirely halt it (Wright et al. 1978). The secondary immune response does not appear to be affected by temperature other than kinetically.

2.1.7.2 Season of the year

Seasonal fluctuations occur in both the structure and function of the reptilian immune system (Muthukkaruppan, Borysenko and El Ridi 1982; Zapata et al. 1992). These include lymphoid organ structure, number of rosette and plaque forming cells, cell viability, proportion of T and B cells, antibody titres, response to mitogens and mixed leukocyte reactions (Saad 1988). In contrast, macrophage levels appear to be constant throughout the year (Sypek and Borysenko 1988).

A transient regression in lymphoid tissues has been recorded during the mating period and winter for many reptiles (Ambrosius 1976; Borysenko 1978; Frye 1991; Zapata et al. 1992), and correlates well with functional variations in the immune response. The concentration of circulating steroid hormones is inversely correlated to immune function and a winter immune system scenario can be induced in lizards during summer months by administering synthetic corticosteroids, thereby showing evidence for this steroid as having a major role in immunosuppression (Saad, El Ridi, El Deep and Soliman 1987). While keeping in mind that the

hormone is not the cause of certain manifestations, but merely a vehicle with a message, its increased release in winter may be a response to components of the circadian or circumanual rhythms.

2.1.7.3 Other stressors

According to Warwick et al. (1995) the "stress response" is the combination of responses mounted by an animal toward such a stressor that involve increased activity of the adrenal gland. The major source of circulating corticosteroids in reptiles is the adrenal gland (Saad et al. 1987) and the increase in production of these steroids may be brought about by several factors.

Crocodylus porosus hatchlings maintained at elevated temperatures had significantly increased plasma corticisterone levels (Turton et al. 1996). Malnutrition in the turtle *C. serpentina* appeared to suppress immune tissue morphology as well as immunocompetence (Borysenko and Lewis 1979). Dominant adult male *Anolis carolinensis* reinforce social hierarchies through aggressive behaviour (Greenberg, Chen and Crews 1984). Subordinate animals in the group, displayed a significantly elevated plasma corticosterone concentration, indicating that an animal's social status may be determinant in its ability to function immunologically. Green turtles, *Chelonia mydas*, afflicted with fibropapillomas were chronically stressed and immunocompromised as opposed to turtles without the disease (Aguirre et al. 1995). Testosterone and other pregnancy related hormones can also suppress the immune response in reptiles (Saad and El Deeb 1990).

The effects of captivity and handling on reptiles are hard to assess, because they are inbuilt factors in controlled experiments that require sampling of blood for testing of plasma hormone levels. However, analysis of disease and mortality data from captive reptiles shows that there is an abnormally high incidence of pathologic conditions associated with opportunistic pathogens, which implicates a suppressed immune competence (Warwick et al. 1995).

2.1.7.4 Maturity/ontogeny

Vertical transmission of both antigen and antibody can take place between mother and eggs in reptiles (Grasset and Zoutendyk 1931). This transfer of circulating immunoglobulins from the mother to the yolk occurs in the ovaries and constitutes a passive immunity equivalent to one sixteenth of the titre recorded in the mother at the same time (Maung 1963). Antibody titres of

more than 1:320 as determined by immunodiffusion was recorded in certain eggs (Maung 1963). Only the very LMW antibodies (5.7S) of the mother can be transferred across membranes into the yolk of the eggs (Leslie and Clem 1972; Ambrosius 1976; Borysenko 1978).

In snapping turtles (*C. serpentina*) the ability to make haemagglutinating antibodies, elicit or resist graft versus host reactions and to reject skin allografts is not fully developed before three months post hatching (Sidky and Auerbach 1968). This period is often spent in hibernation and possibly the antibodies, obtained via maternal transfer *in ovo*, are degraded slowly during this time, thus providing humoral protection for the hatchling until its own system is fully functional (Cohen 1971). In contrast, hatchlings of the lizard, *Calotes versicolor*, possess a fully developed humoral immune system upon hatching (Kanakambika and Muthukkaruppan 1972b), which re-enforces the view that reptiles are a collection of diverse species which react to immune stimulation with great variability.

2.2 Viruses Of Reptiles

Extensive research has been carried out on mammal, bird and fish viruses because of the commercial importance of these vertebrates. Although there are many reptilian viruses, little evidence exists of disease associated with such infections without prior stress of some kind. Signs indicative of viral infection are typically inclusion bodies in erythrocytes, hepatocytes, neurons, gastric mucosal cells, renal and pancreatic epithelial cells. Mononuclear perivascular cuffing around blood vessels serving the brain, can also occur (Frye 1991). In reviewing the viruses of reptiles I will, whenever the information is available, cover their characteristics with respect to host, behaviour and other gross signs associated with infection, histological changes, *in vitro* growth and cytopathic effect (CPE), morphology as revealed by electron microscopy (EM) studies and possible transmission pathway.

2.2.1 Adenoviruses

Adenovirus infections have been diagnosed in crocodiles, (Jacobson, Gardiner and Foggin 1984; Huchzermeyer, Gerdes and Putterill 1994), snakes (Heldstab and Bestetti 1984; Jacobson and Gaskin 1985; Schumacher, Jacobson, Burns and Tramontin 1994) and lizards (Jacobson and Gardiner 1990; Frye, Munn, Gardner, Barten and Hadfy 1994; Jacobson, Kopit, Kennedy and Funk 1996). Infections can be accompanied by lethargy, neurological disorder, hepatitis or

gastroenteritis (Heldstab and Bestetti 1984; Frye et al. 1994). An adenovirus was isolated from a Corn snake and cultured *in vitro*. The subsequent cytopathic effect (CPE) observed in cell cultures included intranuclear inclusion bodies and finally cell lysis (Ahne and Juhasz 1995). Electron microscopic studies revealed that the virus was not enveloped, possessed icosahedral symmetry, measured 60-80 nm in diameter and multiplied within the nucleus of infected cells (Jacobson et al. 1996).

2.2.2 Arboviruses

Arboviruses are arthropod borne viruses that multiply in both the arthropod vector and the vertebrate host (Shortridge and Oya 1984). Many are pathogenic to humans, but reptiles may represent an alternative host in which the virus does not produce overt disease, but may overwinter in hibernating reptiles. Two types of arboviruses have been identified in reptiles: the togaviruses and the rhabdoviruses.

2.2.2.1 Togaviruses

This group of viruses includes members such as Eastern equine encephalitis, Western equine encephalitis, Venezuelan equine encephalitis, Japanese encephalitis, St. Louis encephalitis and Tick-borne encephalitis (Shortridge and Oya 1984). Although infection by these viruses appears to be common according to antibody surveys, which detected antibodies against the viruses in 25 species of snakes, 14 species of lizards, 12 species of turtles and one crocodilian (Hoff and Trainer 1973; Lunger and Clark 1978), there is little evidence of them causing disease in the reptile hosts. Rather, reptiles may function as reservoir hosts due to their low metabolic rate and subsequent reduced immune response in winter (Lunger and Clark 1978).

Experimental infection of snakes and tortoises shows them to be highly susceptible (Hayes, Daniels, Maxfield and Wheeler 1964) with viraemia lasting from 3 to 105 days post infection (Doi, Oya and Telford 1968) depending on temperature (Bowen 1977). The antibody response to an infection is variable. Doi et al. (1968) reported little detectable antibody response, while Hayes et al. (1964) and Lee (1968) found that neutralising antibody persisted for at least 44 days after the viremia had subsided. Higher ambient temperatures during the experimental trials appeared to raise the titre of antibodies against the virus and reduce the duration of an infection (Bowen 1977).

Isolation of togaviruses from reptiles has been attempted predominantly from blood samples (Shortridge and Oya 1984).The variable results, ranged from 44% (37/84) for Western Equine Encephalitis Virus (WEEV) in garter snakes (*Thamnophis sirtalis* and *T. elegans*) collected in Utah in May to July (Gebhardt, Stanton, Hill and Collett 1964) to less than one percent for Japanese encephalitis virus, which was isolated from only two of 747 snakes (*Elaphe rufodorsata*) collected from Korea (Lee, Min and Lim 1972). This may be due to the cyclical nature of viremias (Burton, McLintock and Rempel 1966). Rosenbusch (1939) was able to isolate WEEV from the brain of *Bothrops alternata*, but not from the blood, showing that blood may not be the best organ for viral isolation, and that the virus may replicate in other organs during periods of low or no viremia.

One incidence of feeding by an WEEV-infected mosquito (*Culex tarsalis*) was sufficient to transmit the infection to a garter snake (Gebhardt, Stanton and De St Jeor 1966). Viremia lasted 70 days post hibernation in snakes which were bitten by infected mosquitoes before hibernation (Thomas, Eklund and Rush 1959). Conversely, 31% of mosquitoes became infected after feeding on snakes with low level viremia. This is an example of how a human pathogen can be transmitted to, harboured in, and recovered from reptiles with the aid of an arthropod vector. Vertical transmission between infected mothers and offspring has also been documented for WEEV in garter snakes (Gebhardt et al. 1964)

2.2.2.2 Rhabdoviruses

Chaco, Timbo and Marco rhabdoviruses were isolated from the lizard *Ameiva ameiva* (Causey, Shope and Bensabath 1966). All could be propagated in a Green monkey kidney (Vero) cell line and in reptilian cell lines after passage through suckling mice brain or Vero cells (Monath, Cropp, Frazier, Murphy and Whitfield 1979). Optimum temperature for propagation was 30°C. While most other arboviruses do not produce CPE in cell culture, a subtle change was noted in cultures infected with Marco virus (Pudney, Varma and Shortridge 1973). Electron microscopy studies on virus from infected cell cultures revealed that Chaco and Timbo viruses were cylindrical virions (202 nm long) that only bud from intracytoplasmic membranes. The Marco virions were conical (180 nm long) and budded both from plasma membranes and from the endoplasmic reticulum. No inclusion bodies were observed for any of the three rhabdoviruses studied (Murphy 1979). Despite their classification as arboviruses, it still remains unclear if Chaco, Timbo and Marco viruses are able to create sufficient viremia to serve as a source for arthropod infection (Cropp 1984).

2.2.3 Calicivirus

Sixteen isolates of calicivirus was obtained from four species of poikilothermic animals in a zoological collection (Smith, Anderson, Skilling, Barlough and Ensley 1986). Eight Aruba Island rattlesnakes (*Crotalus unicolor*) were asymptomatic and the isolation was obtained by rectal swab. The other eight isolates were obtained at necropsy of animals found dead in their cages. These included four Aruba Island rattlesnakes, two Bell's horned frogs (*Ceratophrys orata*), one rock rattlesnake (*C. lepidus*) and one eyelash viper (*Bothrops schlegeli*). Histopathology revealed a variety of inconsistent lesions in the necropsied animals. The isolates grew in Vero cells at 37°C and were identified by physicochemical characteristics as belonging to the Caliciviridae. The 16 isolates were antigenically indistinguishable and the strain was designated reptilian calicivirus *Crotalus* type 1.

2.2.4 Herpesviruses

Herpesvirus infections appear to manifest as acute signs which may turn latent and be quiescent for the rest of the animal's life, or until the host becomes sufficiently stressed for the virus to reappear as a disease (Hoff and Hoff 1984). In reptiles, both epidermal and systemic infections have been reported.

2.2.4.1 Green Turtle Fibropapilloma virus

Although many bacteria, leeches and mites are associated with Green Turtle Fibropapilloma of wild turtles (Aguirre, Balazs, Zimmerman and Spraker 1994), a herpesvirus has been implicated as the etiological agent of the disease. The cause of the papillomas has been determined to be a filterable and infectious agent, and even though the herpesvirus can not consistently be identified in lesions, it is suspected to operate under certain environmental conditions and in synergy with immune system modulators which may influence the persistence and severity of the lesions (Jacobson, Buergelt, Williams and Harris 1991; Herbst 1994).

Grey Patch Disease virus

A fibropapilloma disease affecting green turtle hatchlings (*Chelonia mydas*) was reported to cause mortalities in 5 - 20% of the severely afflicted animals (Rebell, Rywlin and Haines 1975). Circular papular skin lesions coalescing into diffuse grey skin lesions with superficial epidermal necrosis affected 90-100% of all hatchlings. The lesions were characterised by hyperkeratosis

and hyperplasia with acanthosis. Epidermal cells displayed basophilic intranuclear inclusions and marginated chromatin. Intranuclear enveloped particles of 160-180 nm with an electron dense core of 105-120 nm were visualised by EM. The intranuclear replication site and morphology of the particles placed the virus in the herpesvirus group (Rebell et al. 1975).

Transmission is thought to be vertical or water-borne (Rebell et al. 1975). Sudden changes in water temperature could bring about the onset of symptoms in tank reared turtles (Haines 1974). Low water temperatures lead to a longer lasting but less severe disease than high water temperatures (Haines and Kleese 1977). This is not surprising in a poikilothermic animal because its physiological processes, and thereby the immune response and the viral host cell metabolism, are temperature dependent.

Green lizard papilloma virus

A herpesvirus was identified in Green lizard papillomas. It was accompanied by two other viruses (Raynaud and Adrian 1976), and may have been an incidental finding of an otherwise latent infection.

2.2.4.2 Cobra venom virus

A herpesvirus was observed by EM in commercial preparations of cobra venom from *Naja naja* and *Bungarus fasciata* cobras. The virus measured 100-125 nm in diameter, but could not be isolated *in vitro* (Monroe, Shibley, Schidlovsky, Nakai, Howatson, Wivel and O'Conner 1968). Degeneration and focal necrosis of columnar glandular epithelial cells occurred in the venom gland of Siamese cobras (*Naja naja kaouthia*) with reduced venom production. The lesions were accompanied by infiltrating inflammatory cells. Naked and enveloped virions of herpesvirus morphology were visualised in necrotic cells (Simpson, Jacobson and Gaskin 1979).

2.2.4.3 Herpesvirus-associated infections of freshwater turtles

A herpesvirus was implicated in terminal hepatitis in the turtles *Clemmys marmorata* (Frye, Oshiro, Dutra and Carney 1977), *Chrysemus picta* (Cox, Rapley and Barker 1980) and *Graptemys sp.* (Jacobson, Gaskin and Wahlquist 1982b). Histological examination revealed an acute hepatic necrosis with intranuclear inclusions and marginated chromatin, mostly in the liver, but also in the kidney and the spleen of affected animals. Icosahedral intranuclear particles of 100nm with either empty or dense cores could be visualised by EM as well as mature

enveloped particles of 140nm in the cytoplasm of infected cells. Herpesvirus-like particles were also observed in necrotic stomatitis lesions from Greek and Argentine tortoises (*Testudo graeca* and *Geochilone chilensis*) (Cooper, Gschmeissner and Bone 1988)

2.2.4.4 Iguana virus

Routine tissue explants from the spleen, kidney and heart of a normal adult *Iguana iguana* underwent spontaneous cell degenerations. The CPE was characterised by formation of multinucleated giant cells and intranuclear eosinophilic inclusions, which are typical of herpesvirus infection. Total destruction of the cell layer was the final outcome. The CPE varied at different temperatures. At 23°C, the viral propagation was slow, and 1% of the otherwise cell-associated virions were released into the supernatant. At 30°C the virus replicated faster, 1% of virions were released from the cells and multinucleated giant cells with up to 10 nuclei in each cell could be seen. At 36°C, the replication was still fast, but no virions were released from the cells and the multinucleated giant cells contained several hundred nuclei. The virus could also grow in the box turtle cell line TH-1, but not in other reptilian, amphibian, avian or mammalian lines (Clark and Karzon 1972).

Electron microscopic examination of infected cultures confirmed the agent to be a herpesvirus. Mature membrane bound virions measured 165-300 nm and naked capsids within the cell nucleus were 115 nm (Ziegel and Clark 1971). Transmission was achieved by inoculating snakes with infected cell culture supernatant. Organ explants were prepared 15 days post inoculation and CPE developed in 4/5 cultures (Clark and Karzon 1972).

2.2.5 Iridoviruses

On a few occasions has an iridovirus been reported to cause overt disease in a reptile, namely a spur-tailed Mediterranean land tortoise, *Testudo hermanni* (Heldstab and Bestetti 1982) and a gopher tortoise (*Bopherus polyphemus*) (Westhouse, Jacobson, Harris, Winter and Homer 1996). The disease in *T. hermanni* was terminal and associated with focal necrosis of liver, intestine and spleen. Intracytoplasmic inclusion bodies of hepatic and mucosal cells adjacent to lesions, were revealed as arrays of iridovirus virions by EM examination. In the gopher tortoise, lesions occurred in the respiratory tract and the pharynx region. Intracytoplasmic basophilic inclusion bodies were observed in necrotic epithelial cells. TEM revealed virions and cytoplasmic inclusions similar to those of the family Iridoviridae (Westhouse, Jacobson, Harris, Winter and

Homer 1996). An iridovirus has also been implemented in the death of four box tortoises (*Terrapene c. carolina*) (Dr E Green, Department of Agriculture, Maryland, USA, pers. comm., 1997). The virus was isolated from the only speciment necropsied. The animal displayed lesions int he mouth, respiratory tract and stomach. Another isolation from a Russian tortoise (*Testudo horsfieldi*) appears to be an incidental finding.

Iridoviruses most commonly encountered in reptiles are the ones causing viral erythrocytic necrosis (VEN). The Erythrocytic Virus of the Australian gecko (*Gehyra variegata*) is such an iridovirus. The intracytoplasmic inclusions found in erythrocytes were originally thought to be an intra-erythrocytic parasite, *Pirhemocyton*, which have been documented extensively in the red blood cells of reptiles, amphibians and fish (Stebhens and Johnson 1966; Walker and Sherburne 1977; Evelyn and Traxler 1978; Speare, Freeland and Bolton 1991; Alves de Matos and Paperna 1993; Smith, Desser and Hong 1994).

In severe cases the erythrocyte destruction associated with infection may induce anaemia in juvenile hosts; however, a viral erythrocytic necrosis (VEN) infection is not normally associated with mortality and ill health (Meyers, Hauck, Blankenbeckler and Minicucci 1986). Marginated chromatin in the nucleus of infected cells produce evidence of the initial phases of viral morphogenesis (Appy, Burt and Morris 1976; Reno et al. 1978). The acidophilic inclusion body in the erythrocyte cytoplasm is the assumed site of viral assembly (McMillan, Mulcahy and Landolt 1989). The viruses have not been propagated *in vitro* (Wolf 1988). Virion size and the presence of an envelope is variable (Evelyn and Traxler 1978; Reno et al. 1978).

Transmission could be waterborne, vertical or by haematophagous vectors (Wolf 1988; Gruia-Grey and Desser 1992). Considering that infections occur predominantly in the juvenile part of the population (Smail and Egglestone 1980), and that animals appear to recover from a VEN infection (Reno, Kleftis, Sherburne and Nicholson 1986), the viruses may induce protective immunity.

2.2.6 Paramyxoviruses

Several epizootics in snake collections have been attributed to the ophoid paramyxovirus. In Switzerland, this respiratory disease of farmed Fer-de-lance snakes (*Bothrops atrox*) caused up to 87% mortality in individual rooms. Other species on the farm were not affected (von Folsch and Leloup 1976). Subsequent outbreaks were reported in rock rattlesnakes (*Crotalus lepidus*)

and several viper species (Jacobson 1980) as well as non-viper species (Homer, Sundberg, Gaskin, Schumacher and Jacobson 1995).

Terminally ill snakes displayed neural symptoms such as prostration, loss of equilibrium, convulsions, head tremors, gaping and discharge from the mouth. Post mortem examination often revealed fluid filled lungs and body cavity (von Folsch and Leloup 1976; Lunger and Clark 1979a). Lesions were observed in the lungs and occasionally in the brain.

Virus could be isolation from lungs and brain of infected snakes by propagation in cobra eggs, viper heart, gecko embryo, rattlesnake fibroma and Vero cell lines (Clark, Lief, Lunger, Walters, Leloup, von Folsch and Wyler 1979; Jacobson 1980; Richter, Homer, Moyer, Williams, Scherba, Tucker, Hall, Pedersen, Jacobson 1996). The virus grew better at 28°C than at 37°C, produced syncytia and eventually destroyed the cell layer (Blahak 1995).

EM of *in vitro* propagated virus showed the virions to be pleomorphic, spheroidal or filamentous particles budding from plasma membranes or as mature enveloped particles in the cytoplasm. The particles have a peripheral fringe of haemagglutinin and an internal nucleocapsid of 150 - 160 nm. Mature particles measure 146 - 321 nm depending on the host cell system and the incubation temperature (Lunger and Clark 1979a, 1979b; Richter et al. 1996). An immunohistochemical survey of sections from suspected ophidian paramyxovirus infection showed that the lungs were the main target organ for the virus and that there was multifocal cytoplasmic staining of infected cells (Homer et al. 1995). Paramyxovirus have also been identified in faeces of farmed Nile crocodiles (Huchzermeyer, Gerdes and Putterill 1994). However, these virions could potentially have derived from infected chickens fed to the crocodiles, rather than from an active gastrointestinal infection of the crocodiles themselves.

2.2.7 Papovavirus

Two side-necked turtles (*Platemys platycephala*) exhibited symptoms of circular papular skin lesions on the head and forelimbs. Histological examination of the epidermis revealed hyperkeratosis and hyperplasia with acanthosis, but no inclusions were observed. Intranuclear crystalline arrays of hexagonal particles of 42 nm diameter were visualised by electron microscopy. The particles resembled papilloma virions similar to those seen in mammalian wart lesions (Jacobson, Gaskin and Clubb 1982a).

Papilloma-associated viruses were identified via electron microscopy of benign papillomas from Green lizards (*Lacerta viridis*). The virions were found only in the highly keratinised regions of the papillomas and displayed morphologies similar to papovavirus, herpesvirus and reovirus. These mixed viral infections were consistent in the three animals examined. None of the viruses were cultivated *in vitro* (Raynaud and Adrian 1976). Although the etiological agent of the papillomas could not be determined with confidence, the papovavirus was implicated because this group is often associated with papillomas in mammals (Youngson 1992). The other two viruses may have been incidental findings.

2.2.8 Parvovirus

The only parvovirus recorded in reptiles is the Dependovirus which requires the presence of an adenovirus infection for replication. This virus was found associated with an adenovirus in intestinal epithelium of a ratsnake, *Elaphe quatuorlineata*, and an Aesculapian snake, *Elaphe longissima* (Heldstab and Bestetti 1984) and in the liver of a bearded dragon, *Pogona vitticeps* (Jacobson et al. 1996). The virions were icosahedral and measured 18-28 nm in diameter.

2.2.9 Picornavirus

The only record of picornavirus in reptiles is by Heldstab and Bestetti (1984). A boa constrictor with signs of gastrointestinal disease and central nervous system disorder, displayed groups of necrotic cells with intranuclear inclusion bodies throughout the intestinal tract, the liver, pancreas and spleen. Perivascular cuffing was observed in the meninges together with leukoencephalopathy. Adenovirus virions were visualised by EM in the duodenum and spleen, as were picornavirus virions. The latter were small, 22 - 27 nm diameter, spheroidal and arranged in rows or lattice formation in the cytoplasm of necrotic cells (Heldstab and Bestetti 1984). An Aesculapian snake showed loss of appetite, abnormal faeces and regurgitation. Upon EM examination four different types of viruses were identified in its duodenum, one of which was a picornavirus (Heldstab and Bestetti 1984). In these mixed infections it is difficult to attribute certain pathological changes to a specific virus, and the picornavirus may merely have been an incidental finding of a non-virulent virus. It should however, be noted that other picornaviruses, the human and porcine enteroviruses, manifest first in the alimentary canal, then proceed to the brain, where they can cause encephalitis with subsequent neurological disorders (Heldstab and Bestetti 1984).

2.2.10 Poxvirus

Poxviruses have been identified in skin lesions of *Caiman sclerops* and *Caiman crocodilus fuscus* (Jacobson, Popp, Shields and Gaskin 1979; Penrith, Nesbit and Huchzermeyer 1991), *Crocodylus niloticus*, *C. porosus* and *C. johnstoni* (Foggin 1987; Horner 1988; Pandey, Inoue, Ohshima, Okada, Chihaya and Fujimoto 1990; Huchzermeyer, Huchzermeyer and Putterill 1991; Buenviaje, Ladds and Melville 1992). The lesions presented as brown raised ulcers on the ventral skin, the head region or in the oral cavity. Eosinophilic intracytoplasmic inclusions were observed within hypertrophied epithelial cells. At higher magnification (EM) the inclusions were seen to be viral arrays consisting of pox-like virions 100 - 200 nm in diameter, which is small compared to poxviruses of other vertebrates and insects. Reptilian poxvirus remains unclassified within the poxviridae family (Gerdes 1991).

2.2.11 Reoviruses

Reoviruses were isolated from the kidney, liver and spleen of a moribund python (*Python regius*) and from the brain of a rattlesnake which had exhibited neurological symptoms (Ahne, Thomson and Winton 1987; Vieler, Baumgaertner, Herbst and Koehler 1994). The isolates grew in IgH2 and Vero cells respectively and displayed a CPE of syncytical giant cell formation. The python isolate revealed spherical to icosahedral particles with a diamter of 50-70 nm and a double capsid layer (Ahne, Thomson and Winton 1987). A reovirus was one of three viruses associated with papillomas in the Green lizard *Lacerta viridis* (Raynaud and Adrian 1976).

2.2.12 Retroviruses

Retroviruses are often associated with tumors in snakes and are also called oncornaviruses. The retroviral DNA is incorporated into the host cell chromosome, where it may direct the production of virus particles or remain quiescent indefinitely, being passed on to the cell's progeny as a non-active pro-virus (Lawrence 1989).

2.2.12.1 Russell's Viper Oncornaviruses

The Russell's Viper Oncornaviruses are rather cryptic and can only be observed after several subcultures of primary cell lines from original host (Ziegel and Clark 1969). One virus, VV-VSW, was detected after 48 passages of a cell line created from a snake tumor. The virus could not be

seen in the original tumor cells via EM. The other two, VV-VH2 and VV-VH3 were identified in late subcultures of a snake heart cell line from an apparently healthy snake (Ziegel and Clark 1971). When propagated *in vitro*, virions were produced within mitochondria and budded from the plasma membrane. "C-type" virions resembling retroviruses appeared to mature at plasmalemmal and vacuolar membrane sites. Virions measured 106 - 108 nm in external diameter, the core being 45 - 75 nm (Ziegel and Clark 1971; Lunger and Clark 1978)

2.2.12.2 Corn snake retrovirus

Electron microscopy studies of tumor cells from a corn snake (*Elaphe guttata*) demonstrated "C-type" virions (Lunger, Hardy and Clark 1974). Virions were also observed in the spleen both extracellularly and budding from plasma and vacuolar membranes. External diameter of virions approached 110 nm and the electron dense core 50 nm. Attempts to create a primary cell line from snake organs were unsuccessful and transmission in *Vipera*, *Gekko*, *Eublepharis* and hamster kidney cell lines were not possible. However, *in vitro* cultivation was achieved in rattlesnake fibroma and early passage of rattlesnake heart and/or kidney cells (Clark et al. 1979). Double immunodiffusion in agar gel shows two common antigenic components for Russel's viper and Corn snake oncornaviruses.

2.2.12.3 Inclusion body disease in Boid snakes

Affected snakes exhibited chronic regurgitation and neurological disorders. Eosinophilic intracytoplasmic inclusion bodies were found in epithelial cells of all major organs, and meningoencephalitis was apparent. A "C-type" retrovirus was visualised in the brain, pancreas and kidney. It was enveloped and measured 110 nm. Supernatant from primary cell cultures of the kidney from an infected boa constrictor (*Boa constrictor*) was inoculated into young Burmese pythons (*Python molurus bivittatus)* with a resultant retrovirus infection (Schumacher, Jacobson, Homer and Gaskin 1994). This disease is often found in snakes from collections with severe mite infestations (Jacobson 1993).

2.3 Iridoviruses of Fish and Amphibians

Members of the Iridoviridae are icosahedral, double stranded DNA viruses that assemble in the cytoplasm of the host cell (Aubertin 1991). Their nucleic acid is circular permutated and terminally redundant. Certain iridoviruses may be entirely host specific and will only be

propagated, if at all, in host-derived cell lines (Wolf 1988). Others, in contrast, can infect cell lines and hosts from different classes (Speare and Smith 1992; Moody and Owens 1994). Of the five Iridoviridae genera, *Chloriridovirus* and *Iridovirus* infect only invertebrates, mainly insects (Williams and Cory 1994). *Lymphocystivirus* infects connective tissue cells of teleosts (Lorenzen, des Clers and Anders 1991). Viruses belonging to the proposed Goldfish virus group include the goldfish viruses 1&2 which rarely cause overt disease in their natural hosts. Hosts of the *Ranavirus* genus appear to be limited to water-associated poikilothermic vertebrates. These viruses can infect a variety of cell types and tend to cause systemic infections.

2.3.1 Systemic iridoviruses

The viruses belonging to the genus *Ranavirus* are characteristically isolated from either amphibian or piscine hosts. However, one report of an iridovirus causing hepatitis in a tortoise (Heldstab and Bestetti 1982) is the first indication of systemic iridovirus infections in a reptile. The type species for *Ranavirus* is represented by Frog virus 3 (FV3), which was isolated from an amphibian host (Aubertin 1991).

Fish are of bigger economic importance than both reptiles and amphibians and their diseases have been studied to a greater extent. However, *Ranavirus* spp. infecting fish may have a very broad host range and in the case of the Bohle iridovirus, a virus first isolated from an amphibian host (Speare and Smith 1992), the same isolate has been reported to infect both amphibians and fish (Moody and Owens 1994). Thus, it may be of advantage to peruse the vast literature on systemic iridoviruses in fish, to gain insight into trends applicable to infections in reptiles.

Since 1980 iridoviruses causing systemic infections in finfish have become important pathogens on the world scene (Table 2.1). Certain strains have been responsible for epizootics, especially in the juvenile part of a population (Langdon et al. 1986; Ahne et al. 1989; Inouye et al. 1992; Pozet et al. 1992; Bovo et al. 1993; Kasornchandra and Khongpradit 1995).

The emergence of systemic iridoviruses may in part be the result of stress-induced manifestations of otherwise latent viruses in intensive fish culture. Alternatively, or additionally, the phenomenon may be attributed to the "jet-factor", which allows for swift and accessible transglobal transfer of infected material, be they mechanical carriers or actual hosts. Such an efficient "vector" system may to a large extent have enabled non pathogenic viruses access to naive and susceptible hosts. Of particular concern is the increased transfaunation associated

with the exotic pet trade, for which there is no adequate viral prophylaxis or quarantine procedures (Martinez-Picado, Blanch and Jofre 1993).

Table 2.1 A chronological listing of the reporting source, the year of publication and the country of original identification for 14 systemic iridoviruses, their name and propagation *in vitro*.

Source	Year	Country	Iridovirus	*In vitro*
#1	1980	USA	Ram cichlid	No
#2	1982	Japan	Japanese Eel	Yes
#3	1982	?	Tortoise	No
#4	1986	Australia	EHNV*	Yes
#5	1989	Canada	Chromide cichlid	No
#6	1989	Germany	Sheatfish	Yes
#7	1992	France	Catfish	Yes
#8	1992	Australia	Bohle	Yes
#9	1992	Japan	Red Sea Bream	Yes
#10	1993	Australia	Dwarf gourami	No
#11	1993	Denmark	Turbot	No
#12	1994	Singapore	Sleepy grouper	Yes
#13	1994	Israel	Tilapia larvae	No
#14	1995	Thailand	Nursing grouper	Yes

* Epizootic Haematopoietic Necrosis Virus (EHNV)

Sources:

#1: Leibovitz and Riis 1980; #2: Sorimachi 1988; #3: Heldstab and Bestetti 1982; #4: Langdon, Humphrey, Williams, Hyatt, Westbury 1986; #5: Armstrong and Ferguson 1989; #6: Ahne Schlotfeldt and Thomsen 1989; #7: Pozet, Morand, Moussa, Torhy and de Kinkelin 1992; #8: Speare and Smith 1992; #9: Inouye, Yamano, Maeno, Nakajima, Matsuoka, Wada and Sorimachi 1992; #10: Anderson, Prior, Rodwell and Harris 1993; #11: Bloch and Larsen 1993; #12: Chua, Ng, Ng, Loo and Wee 1994; #13: Avtalion and Schlapobersky 1994; #14: Kasornchandra and Khongpradit 1995.

Comparisons of the physical, chemical and serological properties, as well as pathogenicity, have been performed on many of the above viruses (Table 2.1), as well as iridovirus isolates from frogs. Those investigations suggest that the viruses are intimately related to FV-3, but are clearly individual strains of *Ranavirus* (Hedrick, McDowell, Ahne, Torhy and de Kinkelin 1992; Hengstberger, Hyatt, Speare and Coupar 1993; Ahne, Matasin and Bovo 1995). The great affiliation between the iridoviral pathogens in fish and amphibians indicates that these viruses are very adaptable and bound to have a broad host range, which further complicates the prophylaxis of such agents in aquaculture (Hedrick et al. 1992). The obvious absence of reptilian hosts, which may be both poikilothermic and water-associated, is conspicuous in this context.

2.3.2 Iridoviruses *in vitro*

Comprehensive characterisation of viruses is dependant on their successful isolation *in vitro*. Not all systemic iridoviruses can be propagated in cell culture (Table 2.1). This ability may be related to the mode of release from the host cell of individual virions and the acquisition of an envelope in that process. The envelope of iridoviruses appears to enhance the ability to infect host cells *in vitro*, but is not necessary for infectivity *in vivo* (Aubertin 1991).

The procurement of an envelope may be related to the host cell, because BIV for example was able to infect both amphibians, barramundi (*Lates calcarifer*) and tilapia (*Oreochromis mossambicus*), but could only be re-isolated from experimentally infected amphibians and barramundi, and not from tilapia (Speare and Smith 1992; Cullen, Owens and Whittington 1995; Ariel et al. 1997). This phenomenon seems to be related to an irreversible loss of envelope after passage through tilapia (Ariel, Owens and Moody 1995). Feeding BIV-infected tilapia homogenate to the highly susceptible barramundi, or inoculating them with chloroform-treated, non-enveloped BIV, induced typical BIV pathological changes and associated mortality in the barramundi (Ariel et al. 1995). However, in both instances, the virus was not re-isolated from the infected barramundi.

2.3.3 Bohle iridovirus

The Bohle iridovirus was isolated from moribund metamorphs of a native amphibian, the ornate burrowing frog (*Lymnodynastes ornatus*), in northern Queensland and could be propagated in a variety of mammalian and fish cell lines, but not in insect derived cell lines (Speare and Smith 1992). The upper temperature limit for growth is 32°C (Moody 1992). The virus shares physical, chemical and biological characteristics with viruses of both amphibian and piscine origin in the *Ranavirus* genus (Speare and Smith 1992). BIV is closely related to EHNV, another iridovirus isolated in Australia (Hengstberger et al. 1993).

In an attempt to identify native fauna at risk, several species of amphibians and fish as well as crustaceans, have been experimentally infected with BIV. In several cases the virus has been reported to affect juveniles more adversely than adults (Cullen et al. 1995; Ariel and Owens 1997; N Moody, Pers. Comm., 1997). Transmission of the virus was possible via natural routes in co-habitation experiments (Ariel 1992; Moody 1992; Cullen 1993; Speare, Owens and Spencer, Pers. Comm., 1997).

The freshwater crayfish *Cherax quadriquarinatus* and silver perch (*Bidianus bidianus*) were refractory to infection, whereas banana prawns (*Peaneus monodon*) could carry an asymptotic infection, with virus isolation in BF_2 cells possible up to 15 days post infection (Field 1993; Fry 1993). Experimental BIV infection of canetoads, *Bufo marinus,* and the native frogs *Limnodynastes terraereginae* and *Litoria latopalmata* caused lesions and mortalities in infected animals (Cullen 1993; Speare, Owens and Spencer, Pers. Comm., 1997). Re-isolation of the virus was possible from these amphibians, albeit not from every infected individual. Barramundi (*Lates calcarifer*) were found to be highly susceptible to BIV. Mortalities of 100% were experienced in infected populations at 10-14 days post exposure. Lesions were confined predominantly to the kidney and spleen, but were occasionally observed in the liver. Moribund fish exhibited loss of muscle co-ordination and viral isolation was possible in BF2 cells (Moody 1992). Tilapia were susceptible to BIV to varying degrees, possibly depending on age of the fish and the route of infection (Ariel and Owens, in Press). Lesions were mainly observed in the kidney and spleen, but over 400 attempts of re-isolating the virus *in vitro* were unsuccessful. The presence of infective virions in moribund tilapia were confirmed by employing barramundi in a bioassay (Ariel et al. 1995).

Recently, a trend has emerged to rename viruses according to the nearest settlement to the location of collection of infected specimens. In this manner the re-naming of iridoviruses from *Chloriridovirus* and *Iridovirus* genera has already been proposed (Williams and Cory 1994). This approach is especially applicable to *Ranavirus* because the viruses included in this genus may not be confined to the original host, but have a rather broad host range. Thus, naming a virus according to the host of the initial isolate is misleading. Bohle iridovirus is already named according to a geographic location and its scientific name, although not employed in general, would appear to be *Ranavirus bohlei*.

In Summary

So far BIV has proven to be infective and lethal to both amphibian and piscine hosts of the Australian fauna, making it a very versatile and hazardous pathogen. The prospects of BIV reaching commercial barramundi farms are daunting. Environmental implications have already become a reality, because the pathogen, attributed to have been instrumental in the decline of high altitude rain-forest frogs on the East-coast of Australia, is speculated to be a member of the *Ranavirus* group (Speare 1995).

Further investigations into the possible host range of BIV, especially reptiles, seem appropriate. Likewise, the development of a serological tool for rapid identification of past exposure to BIV in native animals, regardless of the ability to isolate the virus from these animals *in vitro* is essential, and is the central theme of the following chapters.

CHAPTER 3

PRODUCTION OF ELISA FOR DETECTION OF BIV ANTIBODIES IN REPTILIAN SERUM

3.1 Introduction

Bohle iridovirus has been shown to infect hosts ranging from fish to amphibian (Speare and Smith 1992; Moody and Owens 1994) and some of our native fauna is extremely susceptible. The main limiting factor of this virus seems to be its temperature limits for growth; above 32°C it is inactivated and thus its host range is limited to poikilotherms. Reptiles that come into contact with fish and amphibians could be at risk of infection with BIV. Serological tests for iridoviruses have mainly been directed towards antigen detection and characterisation. The viral isolates were predominantly from fish, but also from eels and amphibians. The primary aim of this study was to develop a test to monitor the antibody response in experimentally infected aquatic reptiles and to detect serological evidence of past exposure to BIV in wild populations.

Nakajima and Sorimachi (1995) used 20 monoclonal antibodies directed against Red sea bream iridovirus to investigate the antigenic relationship between 5 iridoviruses. Red sea bream iridovirus, Sea bass iridovirus and Japanese parrot fish iridoviruses all cross reacted, whereas Japanese eel iridovirus and Frog virus 3 did not react.

Catfish, sheatfish and EHN iridovirus have been compared to each other and to an iridovirus isolated from ornamental fish by cross immunofluorescence tests and Western blots (Hedrick, McDowell, Ahne, Torhy and de Kinkelin 1992; Hedrick and McDowell 1995). The viruses appear to share common antigens. The above tests were carried out on infected and subsequently fixed cell cultures using polyclonal antisera.

A series of antisera directed against different isolates and preparations of EHNV and one preparation of BIV were tested in an antigen capture ELISA developed for EHNV and BIV (Hengstberger, Hyatt, Speare and Coupar 1993). A high degree of cross reactivity between the two Australian iridoviruses was reported. The PCR test developed by Gould, Hyatt, Hengstberger, Whittington and Coupar (1995) also showed a 98% homology between the two viruses at the nucleotide level.

Whittington, Philbey, Reddacliff and MacGown (1994) described an antigen capture ELISA for EHNV which was used to detect EHNV antibodies in rainbow trout (*Oncorhynchus mykiss*) sera.

Reactive test sera attached to EHNV which was captured by rabbitαEHNV antisera. Rainbow trout immunoglobulins were detected indirectly by mouse monoclonal antibodies against rainbow trout IgM.

An ELISA tests for the presence of particular antigens or their antibodies in serum (Youngson 1992). The key component in ELISA is the enzyme indicator which is conjugated to an antibody (Burgess 1988). The assay is made up of a series of "steps" where reagents are added to wells of the ELISA plate, incubated for a set period after which unbound material is washed out. If reagents in subsequent steps are compatible with previously bound material, attachment will occur by means of antigen-antibody coupling. Binding of the antibody which is conjugated to an enzyme indicator will result in colour production with the addition of a chromogen substrate in the final step. If any two succeeding reagents are not compatible, the colour change will not take place.

The enzyme used in this study was horseradish peroxidase (HRPO). In the presence of this enzyme, the substrate 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) is converted to a green coloured product. The subsequent change in optical density (OD) of the solution is proportional to the enzyme activity which indirectly indicates the level of the immune reaction, i.e. the amount of conjugated antisera which attached specifically to the antigen. The final OD was quantified in an automated spectrophotometer.

Polyclonal antisera can be produced in quantities by collection of serum from vaccinated animals or by harvest of ascitic fluid in vaccinated mice. The titre and specificity of the serum will increase with repeated inoculations of pure antigen. However, antibodies directed towards other non-target antigens are invariably included. Polyclonal sera is directed against several epitopes on the same antigen and may cross react with closely related antigens, such as EHNV and BIV.

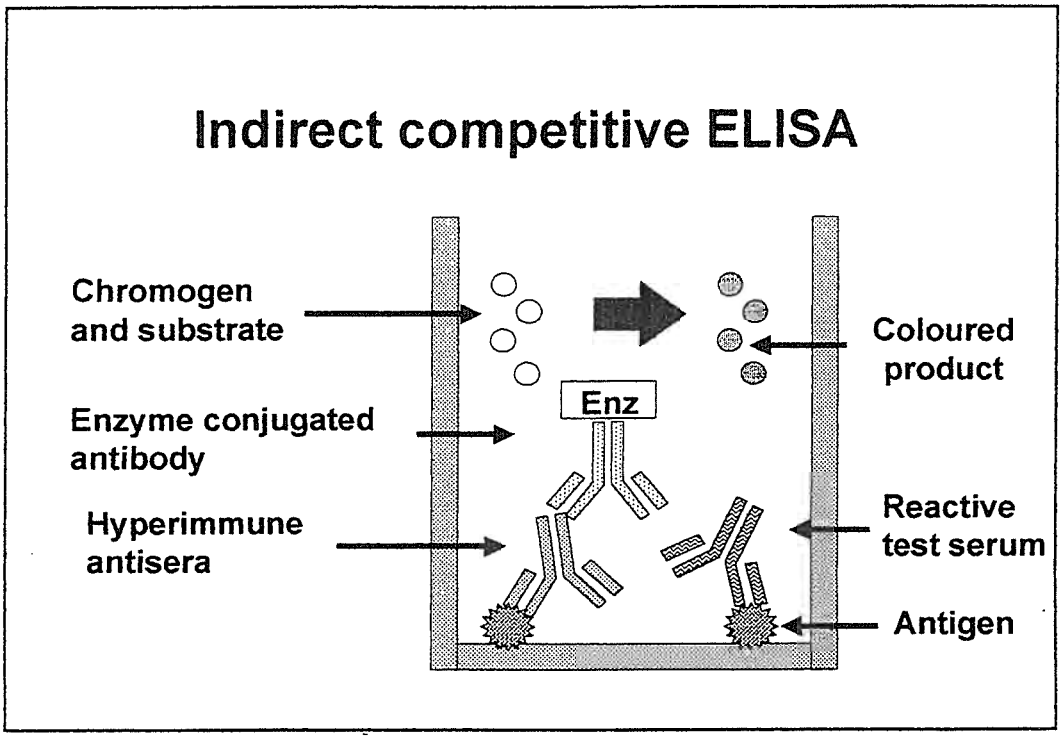
Monoclonal antiserum is the product of a clonal population of cells arising from a single B cell. The clone secretes identical antibodies to a single antigen recognition site (Harlow and Lane 1988). Cell fusions between individual B cells from vaccinated mice and mice myeloma cells produce hybrid cell lines (hybridomas), which are potentially immortal and produce homogenous antibodies (Smith 1988).

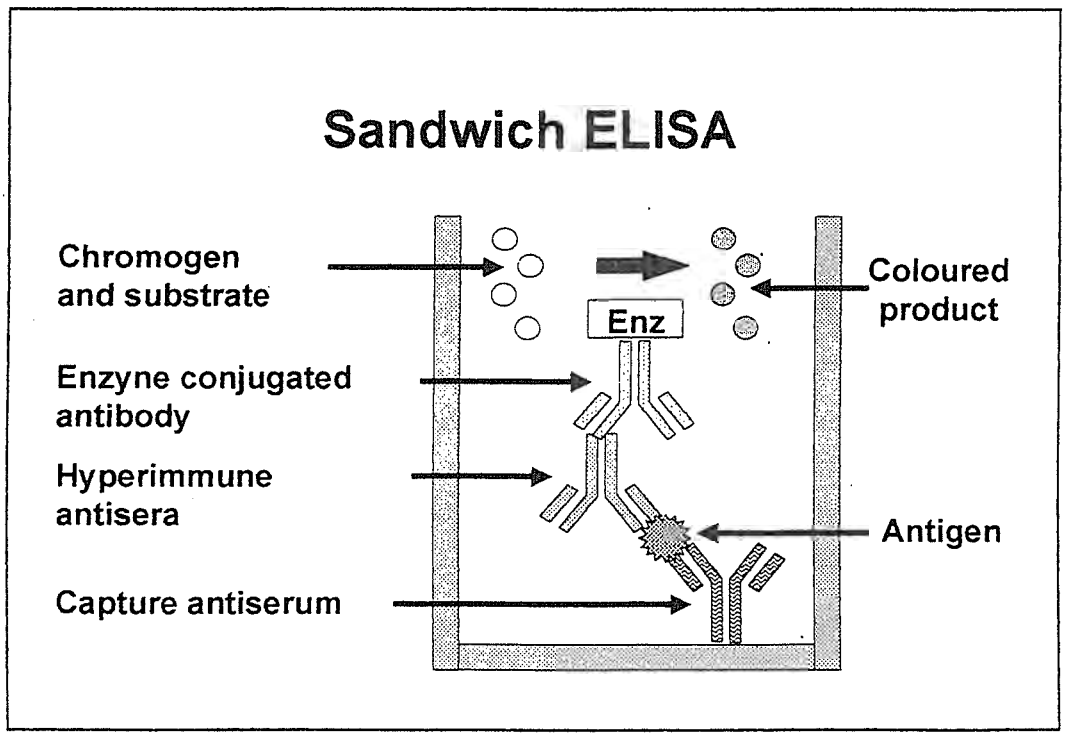
Conjugated antibodies are applied in indirect ELISA, where they detect immunoglobulins which have attached to a specific antigen. Certain conjugated antibodies are commercially available.

For example: antibodies produced in goats against purified rabbit immunoglobulins and conjugated to HRPO are termed goatαrabbit. However, antisera against reptile immunoglobulins are not commercially available and rather than detecting them directly, their presence in reactive serum can be assessed by their antigen blocking effect. In competitive ELISAs reactive test sera compete with experimentally produced hyperimmune antisera for epitopes on the antigen, thereby reducing the final OD of the system (Figure 3.1).

Alternatively, reptilian test sera can be applied as the capture antisera in a sandwich ELISA (Figure 3.2), where known amounts of antigen (BIV), hyperimmune antisera (rabbitαBIV) and enzyme conjugated antibody (goatαrabbit) are applied in sequence to the test serum. Given that the test serum is reactive, it would capture the antigen, present it to the rabbitαBIV in the following step and cause an increase in the final OD.

The objective of this study was to develop an indirect ELISA to screen reptilian test sera for the presence of antibodies to BIV. This was achieved by producing and comparing the configurations described above.


Indirect competitive ELISA
Chromogen and substrate
Enzyme conjugated antibody
Hyperimmune antisera
Enz
Coloured product
Reactive test serum
Antigen


Sandwich ELISA
Chromogen and substrate
Enzyne conjugated antibody
Hyperimmune antisera
Capture antiserum
Enz
Coloured product
Antigen

3.2 Materials and Methods

3.2.1 Culture and purification of Bohle iridovirus

The virus was propagated *in vitro* and purified for use as an antigen in ELISA and an immunogen for production of antisera in rabbits and mice. Polyclonal antisera directed against EHNV cross reacts with BIV to a high degree and was used to assess the yield of BIV from different cell lines undergoing different treatments.

3.2.1.1 Cell culture

Bluegill fry 2 (BF2) cells (Wolf, Gravel and Malsberger 1966) passage #116 to 160, and 3T3 mouse fibroblast cells (Todaro and Green 1963) from the BALB/c mouse strain (passage unknown) were cultured for viral propagation and re-isolation trials. The cells were cultured in 75 cm^2 flat bottomed polystyrene flasks (Corning Glass Works, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum (FBS) and 5% bovine donor serum (BDS) for BF2 cells and 20% BDS for 3T3 cells. Antibiotics (benzylpenicillin, streptomycin, polymycin B and kanamycin) and fungicide (Fungizone) were added at 1.0% and 2.6 μg/mL respectively. The BF2 cultures were incubated at 26°C and the 3T3 cultures at 30°C.

3.2.1.2 Viral stock

BF2 cells at 80% confluency in 150 cm^2 flasks were inoculated with 1.0 mL tissue culture infective dose ($TCID_{50}$) x $10^{5.3}$ of the original amphibian isolate (Speare and Smith 1992) of BIV and incubated until cell monolayers were completely destroyed, at approximately 48 hours post-infection. The suspension was frozen and thawed three times and centrifuged at 13 800g_{max} at 4°C for 20 minutes and the supernatant stored at -70°C.

3.2.1.3 Preliminary ELISA with rabbitαEHNV

To assess the different antigen (BIV) preparations in ELISA, samples were coated on to ELISA plates (96 well, U bottom, Micro test plate, Disposable Products, Adelaide) and detected indirectly by the hyperimmune antisera, rabbitαEHNV polyclonal antisera (kindly provided by Dr. Hyatt, Australian Animal Health Laboratory, Geelong) and subsequently by a commercial preparation of an enzyme conjugated antisera, goatαrabbit (BioRad, Australia Cat. No 170-

6515) and ABTS (Peroxidase substrate, Kirkegaard and Perry Laboratories, Maryland) (Figure 3.3). A preliminary indirect ELISA was optimized against the standard viral stock (Section 3.2.1.2), with respect to coating buffer, time and temperature. Primary (rabbitαEHNV) and secondary (goatαrabbit) antisera concentrations were kept at 1:1000 (the former on the recommendation of the supplier). The negative controls were buffer, and non-infected BF2 cells treated like the infected cultures.

TRIS, PBS and CBC buffers (Appendix 1) were compared in their ability to coat the antigen on to plates at 4°C, room temperature (RT) and 37°C for 1 hour and overnight (O/N). For each buffer, 50 μL of the sample was diluted two-fold in the buffer in rows down the plate. Each subsequent step in the ELISA was preceded by three rinses with TEN-T (Appendix 1) before incubation at RT for 1 hour. The primary and secondary antisera were diluted in TEN-TC (Appendix 1). The volume incubated for each step was 50 μL, except for the final step where 100 μL ABTS was added. The OD value of each well was measured by a microplate reader (Titertek Multiskan MCC, EFLAB, Finland) at dual wavelengths of 414 nm and 492 nm.

3.2.1.4 Optimizing viral yield from host cells

Virus was cultured for the production of a specific immune response when inoculated into an animal, and for coating onto ELISA plates. In order to get the viral inoculum as pure and concentrated as possible, the original technique (Section 3.2.1.2) was modified to reduce the amount of serum albumin in the sample, and to maximize the release of virions from the cell cytoskeleton. Both BF2 and 3T3 cell cultures were assessed. Uninfected cultures were used as negative controls and underwent identical treatment to the infected cultures.

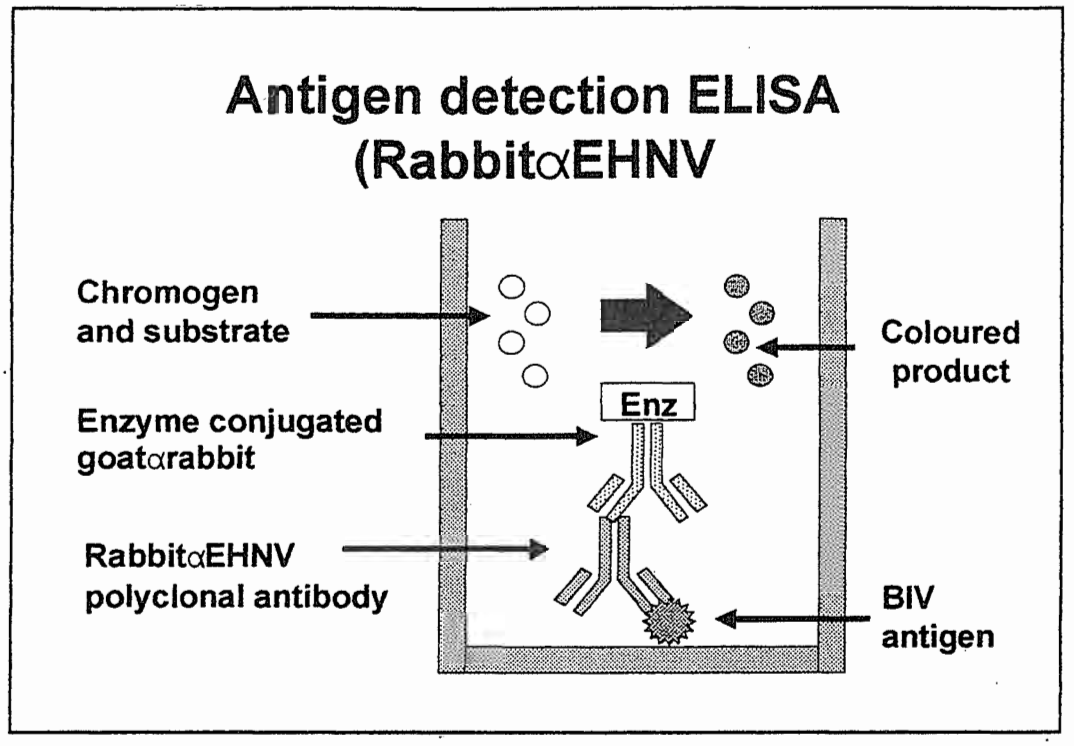


Figure 3.3 Schematic representation of an antigen detection ELISA. The virus is detected indirectly by the primary antibody rabbitαEHNV, which in turn is detected by the enzyme-conjugated secondary antibody goatαrabbit. In the presence of the enzyme horseradish peroxidase, the substrate ABTS changes colour.

To minimize the serum albumin in the sample the following procedure was adopted: At the first observation of a plaque in the infected cultures, which occurred approximately 16 hours after infection for BF2 and after three days for 3T3, the media was decanted from all flasks and the cells rinsed with serum free media. Cultures were then incubated in serum free media until most cells in the infected flasks displayed cytopathic effect (CPE). This occurred approximately 48 hours after inoculation for BF2 cells and after four days for 3T3 cell cultures. Cells were scraped off and the suspensions centrifuged at 800*g* for 10 minutes. The cells were then washed twice in sterile PBS, each time re-suspended in the original volume. Supernatant from each centrifugation was collected for testing in the preliminary ELISA. After the final wash the pellets were re-suspended in 10 mL centrifuge tubes with 2 mL sterile PBS or detergent or distilled water (dH_2O) where applicable.

Nine treatments for releasing virions from the cells were compared. Each one was carried out in duplicate. The first four treatments consisted of grinding the cells with a mortar and pestle with the addition of 2 mL of one of three different detergents or PBS. The three detergents were Sarcosine (Sigma, Australia), Triton x-100 (BDH Chemicals, Australia) and ND-40 (Sigma,

Australia). The fifth treatment involved vortexing (Vortex Mixer, RATEK Instruments, Boronia, Australia) the samples vigorously with three glass beads (3 mm diameter) in each tube for 5 cycles of 20 seconds. For the sixth treatment the cells were lysed by re-suspending the pellet in dH_2O. In the seventh treatment the cell suspension was exposed to three freeze-thaw cycles; in the eighth they were sonicated (Thomas Optical & Scientific Co. Pty. Ltd., Australia) for 3 minutes on wet ice at the automatic setting (amplitude: 9 microns), and lastly, the samples were homogenized by a hand-held Dounce homogenizer.

After each of these treatments the samples were centrifuged at 800*g* for 10 minutes, the supernatant was collected and the pellet re-suspended in equal volume of PBS. Viral re-isolation on BF2 monolayers in 24 well plates (Becton Dickinson Labware, New Jersey) was performed on all samples to test if the above treatments affected infectivity. All samples were tested in the preliminary ELISA (Section 3.2.1.4) and their resultant OD values compared. The treatment which provided the largest difference in OD between the final viral infected culture supernatant and the corresponding re-suspended pellet was selected. Both infected and un-infected cells were treated according to this procedure for use in ELISA as virus and control antigen respectively.

3.2.2 Production of polyclonal antisera against BIV

Polyclonal antisera to BIV was produced in New Zealand White rabbits (Walter Meadow, Melbourne) for use in the indirect detection of BIV. The viral antigen (Section 3.2.1) was further purified by Nick Moody (Department of Microbiology and Immunology, JCU, 1995) before vaccinating rabbits. The purification steps consisted of ultracentrifugation in a sucrose gradient (40-70%, 150,000 *g* for 2 hours at 15°C) followed by a potassium tartrate gradient (25-60%, 150,000 *g* for 16 hours at 4°C). The resultant hyperimmune sera was collected, purified and concentrated. Details are given below.

3.2.2.1 Protein estimation

Protein content of the BIV and control antigen preparations (Section 3.2.1.4) was estimated using a protein estimation kit (Pierce, Australia). The purple reaction product, formed by the interaction of two molecules of bicinchoninic acid with one cuprous ion (Cu^{1+}), is water soluble and exhibits a strong absorbency at 562 nm. This allows the spectrophotometric quantitation of

protein in aqueous solutions. The following procedure is adapted for microtitre plate from Pierce Instructions:

The working solution (A: 20 mL + B: 0.4 mL/plate) and two sets of protein standards in 1.5 mL microcentrifuge tubes (polypropylene, Treff AG, Switzerland) were prepared, the latter by diluting the stock bovine serum albumin (BSA) solution in distilled water to get 7 concentrations ranging from 0 to 1200 μg/mL. Ten μL of each standard or unknown sample were transferred into the appropriate wells in duplicate. Two hundred μL of working solution was added to each well and the plate tapped gently for 30 seconds to mix the samples, before covering and incubating it at 37°C for 30 minutes. The absorbency was read at 540 and 590 nm with a microtitre plate reader. A standard curve was prepared by plotting the net (blank corrected) absorbency versus protein concentration. Using this standard curve, the protein concentration for each unknown sample was determined.

3.2.2.2 Preparation of viral immunogens for inoculation

Equal volumes of BIV solution (250 μg/mL) and montanide adjuvant (Tall-Bennett, Australia) were mixed repeatedly with a 2.5 mL syringe and a 19 gauge needle until it reached a white, viscous state that did not revert to two phases (water/oil) upon standing. This emulsion was gently re-dispersed in an equal volume of an outer saline phase consisting of 2% Tween-80 (BDH Chemicals, England) in 0.85% NaCl. The final result was a less viscous fluid with a protein content of approximately 60 μg/mL.

3.2.2.3 Inoculation and blood collection

The rabbit was restrained by wrapping it up tightly in a lab coat. Inoculations were administered intra-muscularly with a 25 gauge needle and a 1 mL syringe in both thigh muscles (400 μL in each) and subcutaneously in the scruff of the neck (50 μL in each of four sites). For blood collection, the ear was shaved on the upper surface, sterilized with alcohol and the area of incision anaesthetized with Topical xylocaine (10% CFC-free spray, Astra Pharmaceuticals Pty. Ltd.). Noises were kept at a minimum to soothe rabbits because they can restrict circulation to their ears if frightened. For pre-treatment and other blood samples an 18 gauge needle was inserted into the median ear vein and the ear turned slightly downwards to let the blood flow into the collection tube. For testing of antibody titres, 2 mL of blood was collected and a maximum of 40 mL of blood was collected only when the titres reached a desirable level. Pressure was

applied to the hole with cottonwool until the bleeding stopped. After 10 minutes at RT the clot was loosened from the sides of the collection container with a sterile orange stick and the sample left for a further 1 hour at RT, then O/N at 4°C. Serum was collected from the clot and centrifuged at 1000*g* for 10 minutes at RT to pellet remaining cell debris.

3.2.2.4 Immunization schedule for rabbits

Rabbits were inoculated at intervals of 6 weeks. The first two inoculations consisted of double sucrose gradient (40-70%) purified BIV (kindly supplied by N. Moody) in multiple emulsion (Section 3.2.2.2). The third and subsequent inoculations consisted of virus without adjuvant. Samples of blood were collected at 7 days post-inoculation.

3.2.2.5 Ammonium sulphate precipitation

According to the recommendations by Herbert, Pelham and Pittman (1973) gamma globulin (IgG) was precipitated from the rabbit serum by three consecutive precipitations with 35% $(NH_4)_2SO_4$ (Sigma Chemical Co., Australia). Saturated ammonium sulphate solution was added drop-wise to the polyclonal rabbit serum over 30 minutes at 4°C whilst stirring. The solution was left stirring for a further 30 minutes, then centrifuged at 10000*g* for 20 minutes at 4°C. The pellet was re-suspended in half the original volume PBS and dialyzed against 5 L Affigel blue column starting buffer, buffer A (Appendix 1), at 4°C O/N.

3.2.2.6 Affigel blue column

The gamma globulins were separated from the serum albumin in an Affigel blue column (Bio-Rad, Australia, Cat. No. 732-2026) at 4°C according to manufacturer's instructions. Briefly, the column was washed with 40 mL of regeneration buffer (Appendix 1), then equilibrated with 40 mL of buffer A (Appendix 1). Antisera was loaded on to the column in 2 mL aliquots and 6 mL eluted fractions were collected while 25 mL of buffer A ran through. Buffer A was followed by 20 mL of buffer B (Appendix 1). The fractions were screened by a UV-monitor (Model 1327 Econo-recorder, Bio-Rad, Australia) and the fractions corresponding to the IgG peaks were collected and pooled.

3.2.2.7 Amicon filtration

The polyclonal antiserum was concentrated down to 1/5 of the volume collected from the Affigel blue column using a nitrogen pressurized Amicon filter unit (a Grace company, Massachusetts, USA). The membrane type was YM 30 (30 000 MW) and filtration took place at 4°C during continual stirring. Samples were stored at -70°C in 1.0 mL aliquots.

3.2.3 ELISA with rabbitαBIV polyclonal antisera

An ELISA with polyclonal antisera directed against BIV can detect virus and act as a standard when screening test serum for reactivity.

3.2.3.1 Optimizing the polyclonal ELISA

The ELISA configuration was similar to that depicted in Figure 3.1, except that rabbitαEHNV was substituted by rabbitαBIV (Figure 3.4). The assay was optimized with respect to coating buffers (PBS and CBC), incubation temperature (4°C, RT and 37°C), and coating period (O/N and 1 hour). The optimal concentration of antigen as well as the primary (rabbitαBIV) and secondary antisera (goatαrabbit) were chosen on the basis of checkerboard titrations (one variable diluted across the plate and the other down the plate). Negative controls were non-infected BF2 cells treated like the infected cultures (Section 3.2.1).

New batches of polyclonal rabbit serum were compared to previous ones, on an ongoing basis to ensure the selection of the most specific and high titred serum available.

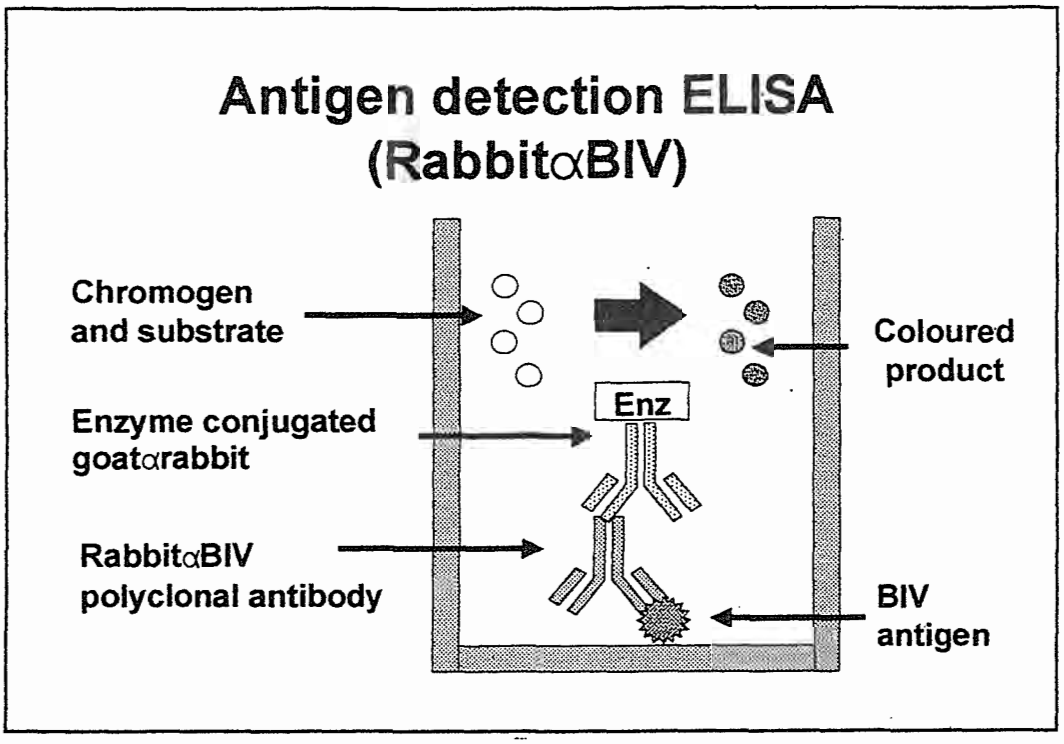


Figure 3.4 Schematic representation of an antigen detection ELISA. The virus is detected indirectly by the primary antibody rabbitαBIV, which in turn is detected by the enzyme-conjugated secondary antibody goatαrabbit. In the presence of the enzyme horseradish peroxidase, the substrate ABTS changes colour.

3.2.4 Production of monoclonal antibodies against BIV

Monoclonal antibodies are a more specific alternative to polyclonal antisera. Spleen B cells from vaccinated mice are fused with compatible myeloma cells (SP2/0) to produce a hybridoma with the B cell characteristic of producing homogenous antibodies and the myeloma cell attribute of being immortal.

3.2.4.1 Immunization schedule for mice

Ten weeks old female BALB/c mice were inoculated with BIV (Section 3.2.1.4) in multiple emulsion (Section 3.2.2.2). Each mouse received four intraperitoneal injections of 200 μL at intervals of three weeks. The immune response of the mice was monitored in ELISA (Section 3.2.3.1) to ascertain a high and specific response to the virus. For this purpose blood was collected from the mice via tail-bleeding onto filterpaper (Trop-Bio, Townsville, Qld). The filterpaper was air-dried, then one segment was soaked in 500 μL TEN-TC O/N to elute off antibodies. Such a procedure provided a 1:100 concentration of antibodies in solution. The last booster injection was administered four days before a fusion and consisted of virus without

adjuvant. In the first 15 fusions, virus was only administered intraperitoneally for the final boost. In the last four fusions, it was also administered intravenously by injecting 100 μL of 50 μg/mL virus into the tail vein with a 27 gauge needle and a 1 mL syringe.

3.2.4.2 Cell count

The concentration of viable cells in suspension was determined by counting cells in a haemocytometer slide (1/10 mm deep, 1/400 sq mm, W. Schreck, Hofheim/TS, Germany). Equal volumes of 0.14% Trypan blue and the cell suspension were mixed and examined under a microscope. The viable cells exclude the vital dye and stand out against the blue background for easier counting.

3.2.4.3 Preparation of spleen cells

Immediately prior to a fusion, the mouse was euthanized using CO_2. The following procedure was carried out under sterile conditions. After dousing the ventral surface in 70% ethanol, an incision was made into the peritoneal cavity to expose the viscera. The spleen was removed and transferred into 20 mL of CSL media (DMEM modified by CSL) at 37°C in a petri dish. Media was repeatedly injected into the spleen with a 5 mL syringe and a 25 gauge needle in order to purge out spleen cells. Finally, the remaining cells were gently massaged out with a pair of bent 18 gauge needles and the spleen capsule with other visible lumps was discarded. Concentration of lymphocytes in the solution was estimated using a haemocytometer (Section 3.2.4.2).

3.2.4.4 Culture of mice myeloma cells

Mice myeloma cells (SP2/0) were cultured in flat bottomed polystyrene flasks in CSL media supplemented with 20% BDS. Antibiotics (benzylpenicillin, streptomycin, polymycin B and kanamycin) and fungicide (fungizone) were added at 1.0% and 2.6 μg/mL respectively, and the cultures incubated at 37°C in a humid, 5% CO_2 atmosphere.

For cryopreservation of cell stock, the cultures, upon reaching 80% confluency in the 25 cm^2 tissue culture flasks, were aspirated off the plastic and the suspension centrifuged at 800*g* for 5 minutes. The cell pellet was re-suspended in 900 μL of CSL media with 10% Dimethyl sulfoxide (DMSO) and transferred into a 1 mL cryopreservation tube (NUNC, Denmark). Tubes were attached to a metal storage cane, which were inserted into a cardboard sleeve and left at -

70°C O/N. The following day the cane was placed in liquid nitrogen. One tube was thawed and cells checked for viability after four days.

Once thawed in 37°C water, the tube to be tested was wiped with 70% alcohol and the cell suspension transferred into 5 mL of 37°C CSL media. The solution was centrifuged at 800*g* for 5 minutes and the cells re-suspended in 10 mL pre-warmed media before transfer to a 25 cm^2 flask.

Three days prior to a fusion the flasks were passaged to provide an 80% monolayer on the day. Cells were aspirated off the growth surface with a plugged Pasteur pipette and their concentration estimated (Section 3.2.4.2). The volume of the suspension needed in the fusion was calculated to provide one myeloma cell for every 10 spleen cells. The myeloma cells were centrifuged at 800g for five minutes at RT. The supernatant was stored at 37°C for re-suspending the fused cells. The pellet was re-suspended in 5 mL CSL media and added to the spleen cell suspension.

3.2.4.5 Fusions

The first 15 fusions between vaccinated mice spleen cells and mice myeloma cells were performed as follows: Fusions were carried out under sterile conditions in a 37°C bath. One gram of sterile polyethylene glycol (Aldrich Chemical Company Inc. Milwaukee, Wisconsin; MW=4600) was melted in a microwave and mixed with 1.0 mL CSL media (37°C) and 100 μL 10% DMSO. The suspension containing the spleen and myeloma cells was centrifuged at 800*g* and the supernatant discarded. The pellet was loosened by gentle tapping and the Polyethylene glycol solution added over 1 minute while swirling the cell suspension. Then the solution was left for 1 minute to allow the fusion to take place. One mL of CSL media (37°C) was added over the next minute, followed by 20 mL over the subsequent 5 minutes all the while swirling the solution. The suspension of fused cells was centrifuged at 800*g* for 5 minutes at RT and the pellet re-suspended in the previously stored supernatant from the mice myeloma cells. The cells were incubated O/N in a growth media conditioned flask at 37°C in a humidified, 5% CO_2 cabinet. The following day the cells were aspirated off the growth surface and the solution was made up to 150 mL with CSL media supplemented with 20% NCS and 1% hypoxanthine aminopterin thymidine (HAT). The cell suspension was plated out into 96-well, flat-bottom tissue culture plates (Disposable Products, Adelaide) and incubated as before.

The last four fusions were performed according to a protocol described by Harlow and Lane (1988) and differed from the previous protocol in the following manner: Two hours prior to a fusion, 1% oxaloacetate pyruvate insulin (OPI) (Sigma, Australia) was added to the growth media of the myeloma cells. The fusion itself was performed at RT not in a 37°C water bath. Polyethylene glycol was added to the pellet containing both spleen cells and myeloma cells while stirring with a Pasteur pipette rather than swirling the suspension. Immediately after re-suspending the fused cells in 400 mL pre-warmed CSL media with 20% BDS, 1% HAT and 1% OPI, the preparation was dispensed into 96 well flat-bottomed cell culture plates in 100 μL per well.

3.2.4.6 Preliminary culture of hybridoma cells

Plates were examined under an inverted microscope for colonies after 7 days and every three to four days thereafter. Wells with colonies of large, round and refractory cells, were considered to contain a hybridoma. Culture media in these wells were exchanged with 100 μL new pre-warmed media (CSL media containing 20% CS, 1% antibiotics and 1% HAT) after plates were screened or routinely checked for growth.

3.2.4.7 Screening for BIV antibodies

Hybridomas were screened at 80% confluency for production of BIV antibodies. Fifty μL of supernatant were transferred into virus and control antigen coated plates, respectively. The ELISA was performed according to Section 3.2.3.1, except the enzyme conjugated secondary antisera was replaced with goatαmouse (Figure 3.5). Where the supernatants produced an OD of at least 0.3 units higher than the corresponding control well, the hybridoma was considered to be secreting BIV antibodies.

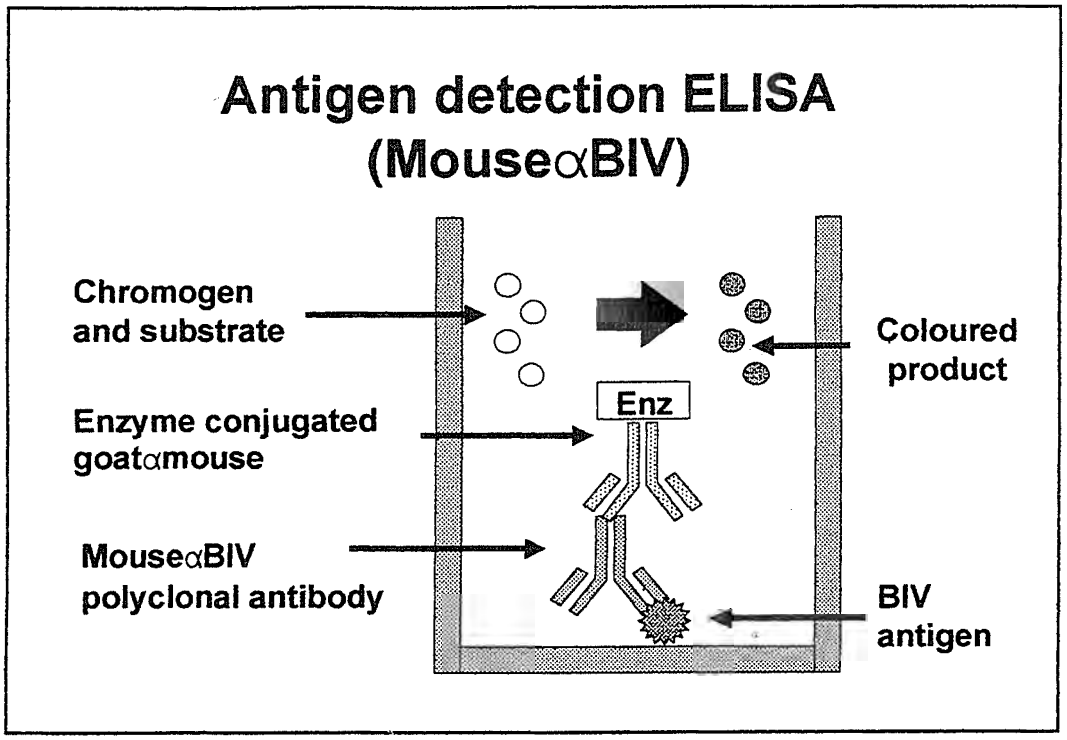


Figure 3.5 Schematic representation of an antigen detection ELISA. The virus is detected indirectly by the primary antibody mouseαBIV, which in turn is detected by the enzyme-conjugated secondary antibody goatαrabbit. In the presence of the enzyme horseradish peroxidase, the substrate ABTS changes colour.

3.2.4.8 Culture of BIV antibody secreting hybridomas

At 80% cell confluency, positive hybridomas were transferred from 96-well plates to 24-well plates. Cells were aspirated off the plastic surface with a plugged Pasteur pipette and all but one drop transferred to the new well. Hybridomas were cultured in media as in 96-well plates except aminopterin was omitted and only 1.0% HT used.

3.2.4.9 Cloning (limiting dilutions)

After four wells had been established to 80% cell confluency in the 24-well plate, cloning was performed according to the method of limiting dilutions on cells from the well with highest OD. A cell suspension of hybridoma cells was counted (Section 3.2.4.2) and diluted with culture media to reach a final concentration of 20 cells/ mL in at least 10 mL. A 96-well plate was divided into four sections, the first received 50 μL of culture media, the second 150, the third 200 and the fourth 225 μL per well. On to this, the cell suspension was added in aliquots of 200, 100, 50 and 25 μL to sections one, two, three and four, respectively. In theory, this would result in four cells per well in section one; two in section two, one in section three and half a cell per

well in section four. The plates were examined daily under the microscope and those with a single colony were marked and supernatant screened for antibody at 80% cell confluency.

3.2.5 ELISA for detection of antibody to BIV in reptilian serum

Polyclonal antisera directed against reptilian immunoglobulins was produced to detect reptile sera indirectly and the different ELISA configurations were optimized accordingly.

3.2.5.1 Affi-T purification of immunoglobulins from six species of reptiles

One millilitre of serum from three individuals were pooled for each of the following six reptilian species: Sawshelled tortoise (*Elseya latisternum*), Krefft's river tortoise (*Emydura krefftii*), freshwater crocodile (*Crocodylus johnstoni*), brown tree snake (*Boiga irregularis*), common tree snake (*Dendrelaphis punctulatus*) and keelback snake (*Amphiesma mairii*).

The reptile gamma globulins were attempted separated from other serum components in an Affi-T column (Kem-En-Tec, Denmark, Cat. No. 1340 D) at 4°C according to manufacturer's instructions. At high $(NH_4)_2SO_4$ concentrations immunoglobulins in human, mouse and rabbit serum will bind to the thiophilic matrix, and are eluted off by lowering the salt concentration in the washing buffer. Briefly, the column was equilibrated with 10 mL 0.75M $(NH_4)_2SO_4$ running only by gravity. Three mL of sample was adjusted to 0.75M $(NH_4)_2SO_4$ and loaded onto the column. The fractions were screened by a UV-monitor and the column washed with 0.75M $(NH_4)_2SO_4$ buffer until the baseline on the UV-monitor was reached. The bound immunoglobulins were eluted off the column with 0.05M TRIS, pH 9.0. Three mL fractions were collected until the baseline was reached again.

3.2.5.2 SDS PAGE gel

For each species, the fractions were pooled and a protein estimation was carried out (Section 3.2.2.1). To ascertain the efficacy of the Affi-T column to purify reptile immunoglobulins, raw serum and an Affi-T purified sample from each species were run in a 7.5% SDS-polyacrylamide gel (Appendix 1) according to the method of Laemmli (1970). The samples were diluted in non-reducing sample buffer (Appendix 1) to gain a final concentration of 3 μg/12 μL which was the volume loaded into each lane. The molecular weight standards were broad range 45 000 to 200 000 kDa (BioRad, Cat. No. 161-0317).

Gels were run in a Mini Protean BioRad apparatus at 200 volt until the tracking dye reached the bottom of the gel. The gels were gently removed from the plates and fixed in trichloroacetic acid (UNILAB, Sydney) for 10 minutes, before staining in Coomassie blue (Gradiopore™, Gradipore, Sydney, NSW) for 24 hours on an orbital shaker (BIO-LINE, Edwards Instrument Company, Sydney) and destained in dH_2O. Gels were scanned by a Gel Doc 1000 (BIO-RAD, Australia) and analysed by Molecular Analyst™ software, version 1.2.

3.2.5.2 Producing ascitic fluid in mice

Ascites fluid was induced in vaccinated mice according to the method described by Harlow and Lane (1988). Briefly, BALB/c male mice were vaccinated with 100 μg/mouse of reptile immunoglobulin (Section 3.2.5.1) or BIV (Section 3.2.1.4) in an equal volume of Freund's complete adjuvant (FCA) in the first vaccination. In the following three injections, which were administered at intervals of three weeks, Freund's incomplete adjuvant (FIA) was used. Five mice were inoculated for each type of reptile serum, another 5 mice were inoculated with BIV and the control group was given no inoculations. After the third inoculation, three mice from each group were tail bled (Section 3.2.4.1) and the samples pooled within groups. Fifty μL reptilian immunoglobulin was coated on to ELISA plates with CBC (65 μg/mL) and incubated O/N at RT. With the assumption that the serum had titres comparable to the ascitic fluid (Harlow and Lane 1988), an ELISA was carried out with the primary antibody being 1:100 mouse serum from vaccinated mice. The serum was also checked for cross reaction to the other immunoglobulin groups. Five days after the fourth and final booster injection all mice were given an intraperitoneal inoculation of 200 μL together with 10^5 cells/mL of SP2/0 cells in the exponential growth phase. The mice were observed twice daily for development of ascites which was harvested when the belly was extended to the point where the fur on the neck of the mouse would appear raised. These mice were anaesthetised with CO_2, doused with 75% alcohol and the ascitic fluid collected with a 23 gauge needle and a 5 mL syringe. The fur on the abdomen was grasped and lifted with a pair of forceps to cause a pooling of ascitic fluid above the organs. The needle was inserted just below the forceps and the maximum amount of fluid extracted in this manner, then the peritoneal cavity was opened up and the remaining fluid collected from between organs. The ascitic fluid was centrifuged for 5 minutes at 800g and stored at -20°C. Samples from within groups of mice were pooled and purified in an Affi-T column (Section 3.2.5.1). Pre- and post-Affi-T samples were run in a 7.5% SDS-acrylamide gel (Section 3.2.5.2).

3.2.5.4 *Comparing rabbitαBIV and mouseαBIV polyclonal sera*

Mice were vaccinated with non-gradient purified virus (Section 3.2.1.4) and had a high background against BF2 cells. To reduce the amount of antibodies against the cells the mouseαBIV ascitic fluid was incubated at equal volumes with BF2 cells [10^6/mL], which had been sonicated for 2 minutes. The mixture was incubated for 1 hour in a rotating mixer at RT, before centrifugation at 1000*g* for 10 minutes. The supernatant was tested by ELISA alongside non-treated mouseαBIV and rabbitαBIV.

3.2.5.5 *Optimizing the indirect ELISA*

The configuration of the indirect ELISA (Figure 3.6) involved coating BIV (1:50 in CBC, RT, O/N) on to the plates, followed by a blocking buffer to prevent non-specific attachment of reptile immunoglobulins in the next step. For each species of reptile, antisera was titrated against mouseαreptile in a checkerboard manner, and goatαmouse (BioRad, Australia Cat. No. 172-1011) added at 1:1000. Non-specific attachment of the reptilian serum to the plastic was addressed by trying different types of plates (Maxisorb™, Polysorb™ and Sarstedt) and adding various blocking buffers (Trop-Bio post-coating buffer, Cat. No. 05-004-04, TEN-TC and BSA) to the BIV coated plates for various times (2, 4, 8, 12 and 24 hours). Negative control plates were coated with non-infected BF2 cells treated like the infected cultures (Section 3.2.1).

3.2.5.6 *Optimizing the capture ELISA*

In this configuration the problem of non-specific attachment by the reptilian serum to the plates was circumvented by coating the test serum onto the plate. Reactive reptile test serum would capture BIV, which could then be detected with rabbitαBIV (Figure 3.7). Reptilian test sera were coated onto the plates with CBC at a concentration of 1:50 and incubated O/N at RT. Attachment of the reptile sera, irrespective of its reactivity, was confirmed by mouseαreptile (1:100 in TEN-TC) for individual species (Figure 3.8). To test that the section of the assay involving BIV and its detection was functional, BIV and control antigen were coated onto the plate with CBC at 1:50 O/N at RT.

The same principles were applied in the optimisation of the capture ELISA as those in Section 3.2.3.1.

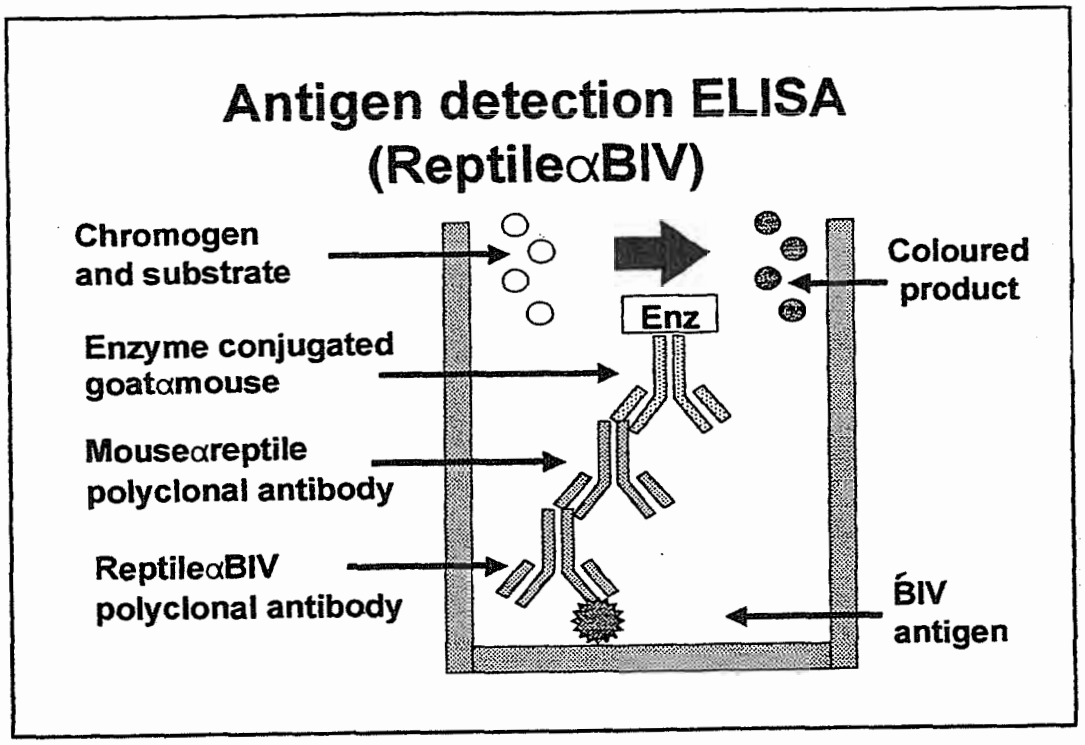
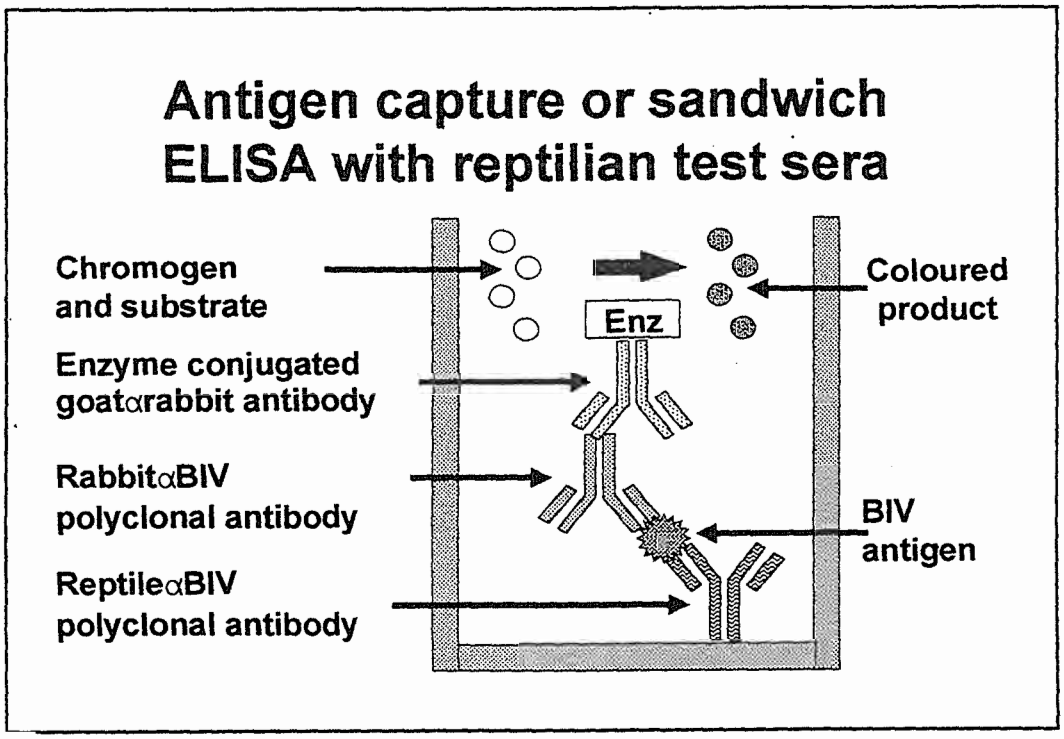
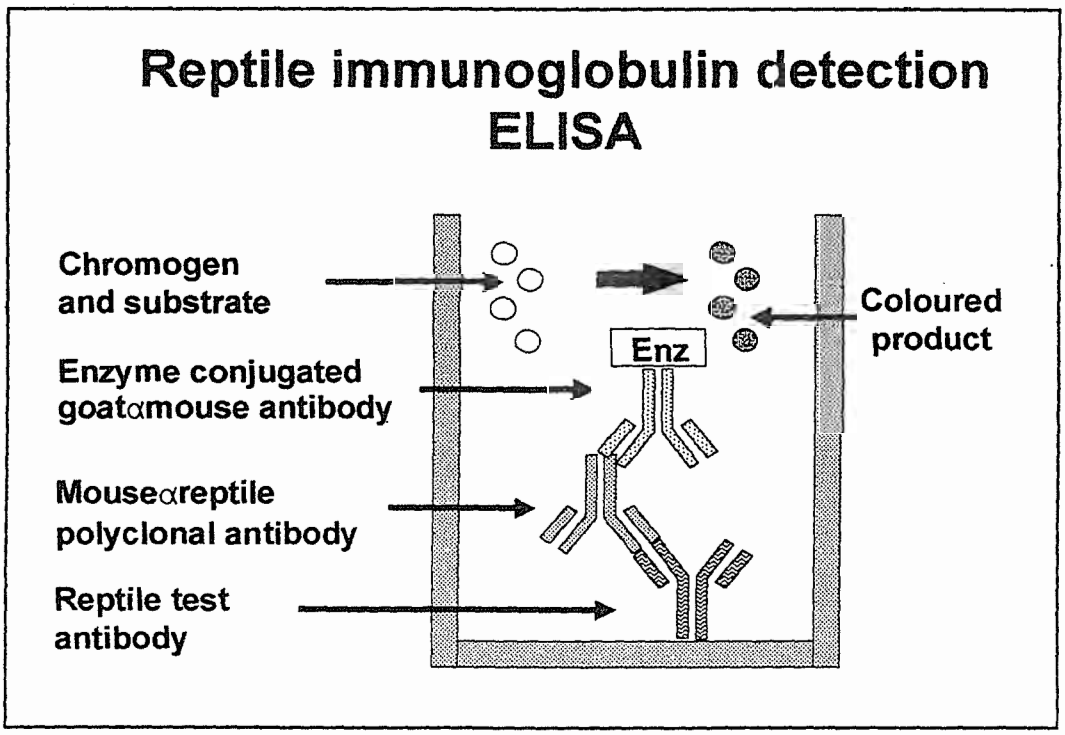


Figure 3.6 Schematic representation of an antigen detection ELISA. The virus is detected indirectly by reactive reptilian sera, which in turn is detected by hyperimmune mouseαreptile and the enzyme-conjugated goatαmouse. In the presence of the enzyme horseradish peroxidase, the substrate ABTS changes colour.


Antigen capture or sandwich ELISA with reptilian test sera
Chromogen and substrate
Enzyme conjugated goatαrabbit antibody
RabbitαBIV polyclonal antibody
ReptileαBIV polyclonal antibody
Enz
Coloured product
BIV antigen


Reptile immunoglobulin detection ELISA
Chromogen and substrate
Enzyme conjugated goatαmouse antibody
Mouseαreptile polyclonal antibody
Reptile test antibody
Enz
Coloured product

3.3 Results

3.3.1 Details of the preliminary ELISA with rabbitαEHNV

The optimal coating buffer, incubation time and temperature were determined to be CBC for 1 hour at RT. The readings were very low (around 100 × OD = 40) indicating a low concentration of antigen in the viral preparation.

3.3.2 Optimal viral yield from host cells.

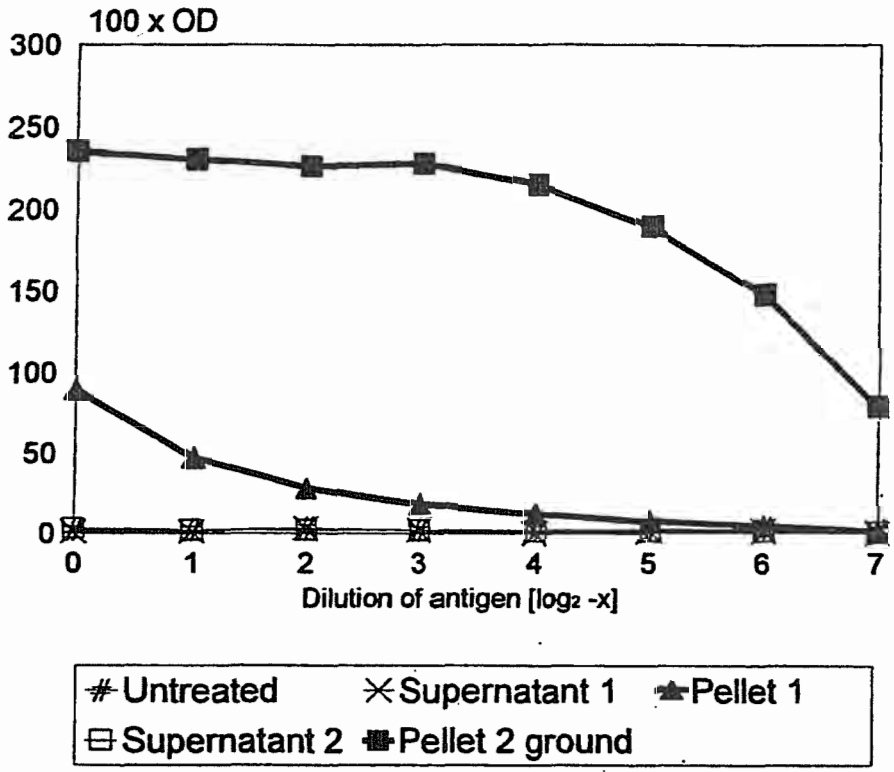
The BIV yield as measured by ELISA (Section 3.2.1.4) was consistently higher from BF2 cells than from 3T3 cells (Appendix 2). The OD readings in the ELISA, where BF2 cells where applied as control antigen were negligible for all the treatments (Appendix 3). In comparing the OD readings from washes of entire infected BF2 cells it was apparent that it was possible to wash the infected cells twice in PBS without losing much BIV (Figure 3.9a).

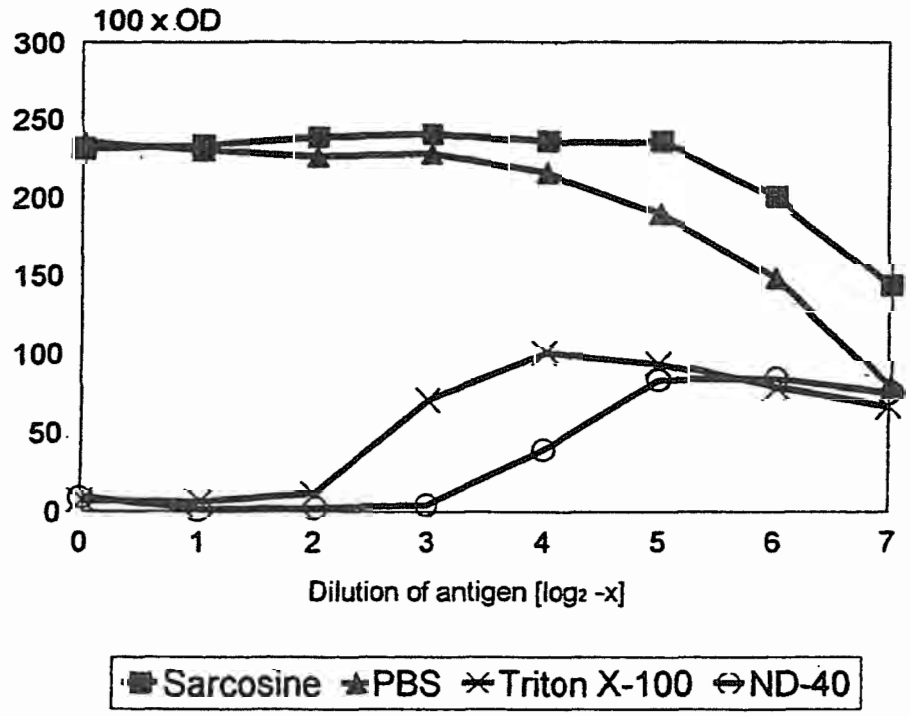
Grinding the infected cells with Sarcosine and PBS released more virus from host cells than grinding with Triton x-100 and ND-40 (Figure 3.9b). Although the Sarcosine treatment was slightly better than grinding the infected cells with PBS, all subsequent grinding with detergents was abandoned in order to avoid any unnecessary complications in the following procedures resulting from possible interference of the detergent with binding of the ELISA reagents.

This left 6 treatments to be considered: grinding with PBS, vortexing, lysing, freeze/thawing, sonicating and homogenizing (Figures 3.10.a-f). In choosing the superior treatment, the differentiation between the amount of virus in the pellet (square with thick line) and in the supernatant (filled square with thick line) is of importance, i.e. the less virus in the pellet and the more in the supernatant the better. Following these criteria:

Vortexing > sonicating > lysing > homogenizing > freeze/thaw > grinding

Virus was recovered in BF2 cell cultures from all treatments. The chosen treatment was a combination of re-suspending infected cells in dH_2O (lysing) followed by vortexing.


100 x OD
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
Dilution of antigen [log2 -x]
Untreated
Supernatant 1
Pellet 1
Supernatant 2
Pellet 2 ground


100 x OD
300
250
200
150
100
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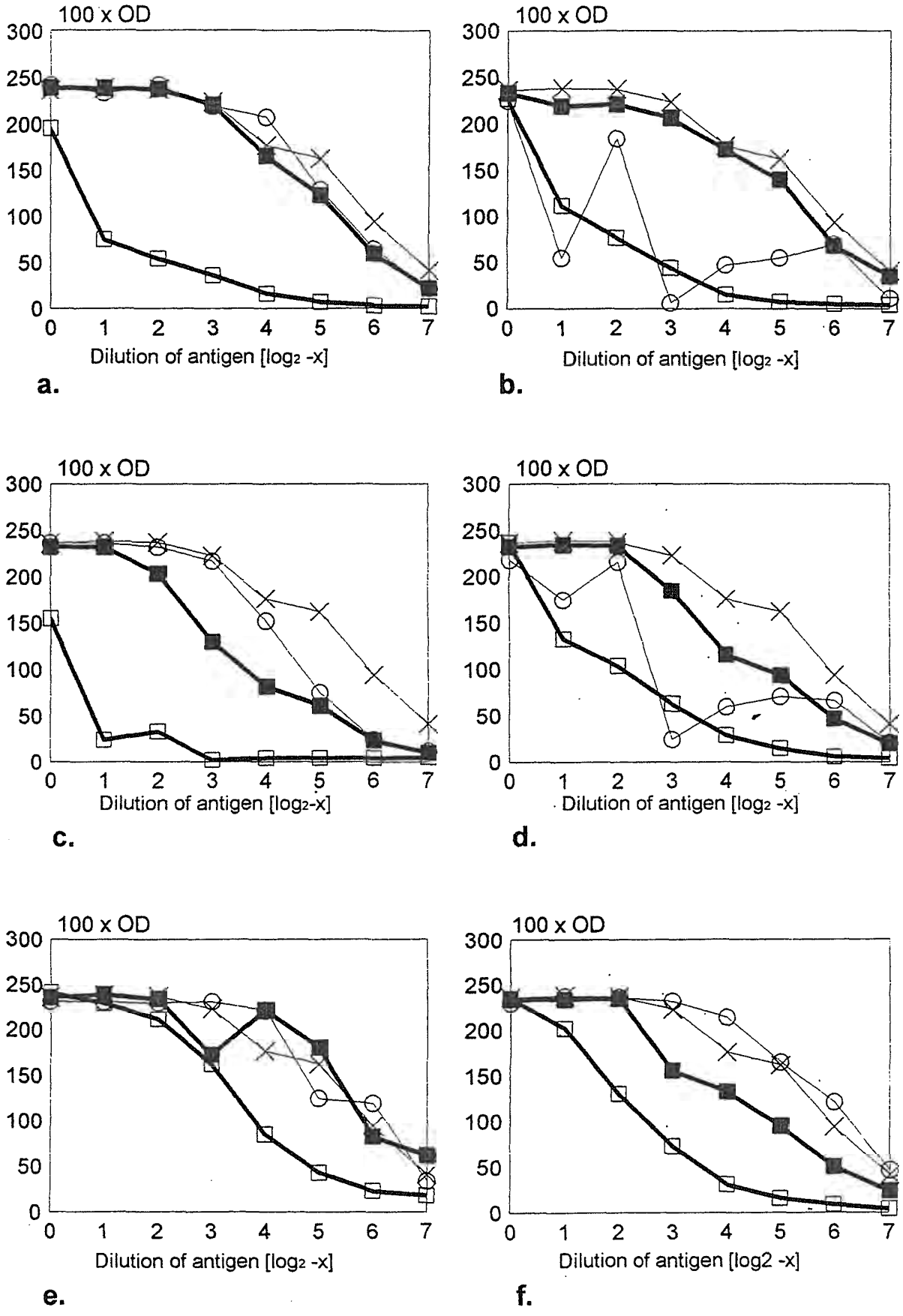
1
2
3
4
5
6
7
Dilution of antigen [log2 -x]
Sarcosine
PBS
Triton X-100
ND-40


100 x OD
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7
Dilution of antigen [log2 -x]
a.
b.
c.
d.
e.
f.
Untreated
Pre-spin
Supernatant
Pellet resuspended

3.3.3 Testing polyclonal antibody from BIV vaccinated rabbits

The antibody levels against BIV in different batches of serum from vaccinated rabbits were tested in ELISA on an ongoing basis (Figures 3.11.a-d). The batch with the highest titre of antibody against BIV and the lowest level of antibody against BF2 cells was finally batch # 4, which was subsequently used for ELISA purposes.

3.3.4 Optimal rabbitαBIV ELISA

When the ELISA initially was optimised batch # 2 of vaccinated rabbit serum was used. The results of the coating buffer trials are displayed in Figures 3.12.a to 3.12.i. CBC buffer was consistently superior to PBS in its ability to coat BIV onto the ELISA plates. Overnight incubation was also consistently better than 1 hour. Results of coating the sample with CBC O/N at three temperatures and performing the assay with 1:100 dilution of antibody is displayed in Figure 3.13. The antibody concentration of 1:100 was chosen, because it gave the highest reading under all conditions and is comparable to the dilutions obtained for test serum collected via filterpaper (Section 3.2.4.1). Under these conditions, the incubation at RT was found to be the best option. An acceptable antigen concentration was determined as 1:50, because that value was on the plateau of the graph. Antigen and the primary antibody (rabbitαBIV) were both titrated against the secondary antibody, goatαrabbit conjugate. A conjugate concentration of 1:1000 was chosen, because it gave the highest readings (Figure 3.14.a and b).

3.3.5 Fusions

In the 19 fusions performed, a maximum of 95% of wells seeded yielded hybridomas. Despite achieving such a high success-rate in certain fusions, and despite the mice having a good antibody response to BIV, only 12 out of approximately 4700 hybridomas were weakly positive (36-81 OD × 100, as opposed to 0-7 OD × 100 in the background) the rest did not respond at all. Following subculture and cloning, no further antibody activity could be detected in those 12 positive hybridoma cultures.

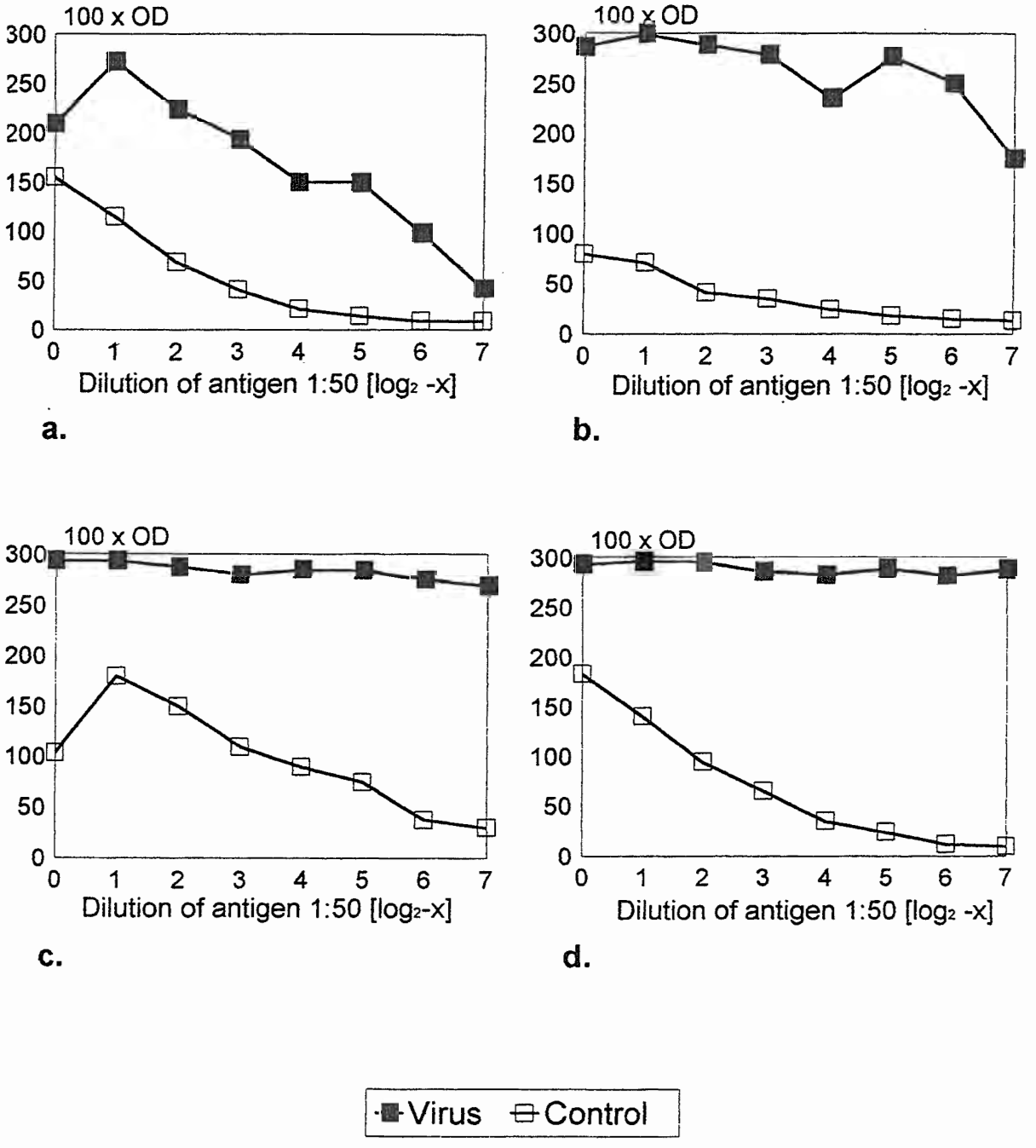


Figure 3.11 Absorbance values as measured by ELISA with 8 dilutions for four batches of serum (a to d) from a BIV-vaccinated rabbit. Plates were coated with BIV (virus) or BF2 (control).

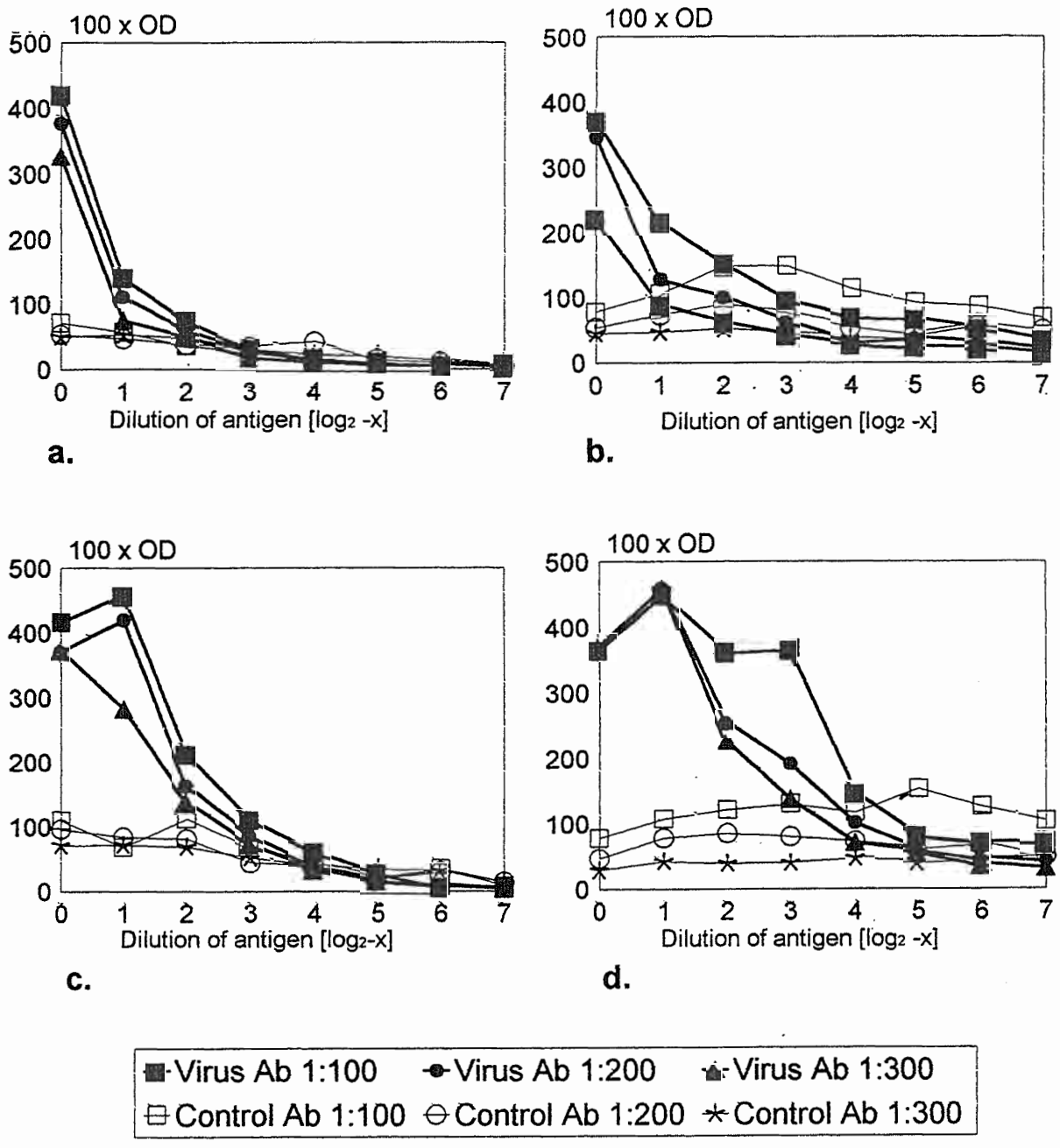
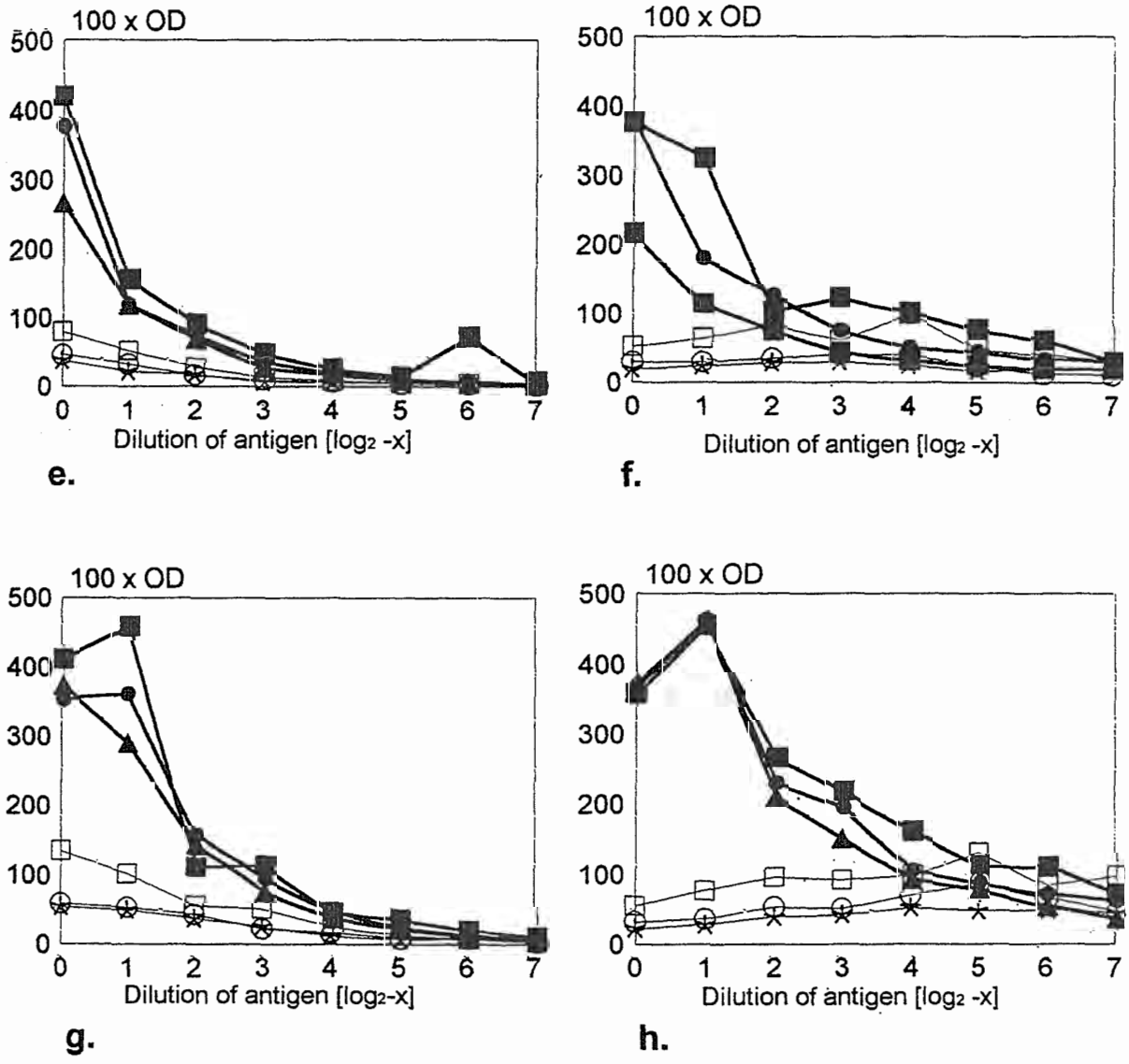


Figure 3.12 Absorbance values as measure by ELISA with 8 dilutions of antigen for different coating buffers (PBS and CBC), incubation temperatures (4°C, RT, 37°C), coating period (one hours and O/N) and detecting antibody dilutions (rabbitαBIV 1:100; 1:200 and 1:300). Plates were coated with BIV (virus) or BF2 (control).

a. Antigen coated on to plate with PBS for one hour at 4°C
b. Antigen coated on to plate with CBC for one hour at 4°C
c. Antigen coated on to plate with PBS O/N at 4°C
d. Antigen coated on to plate with CBC O/N at 4°C

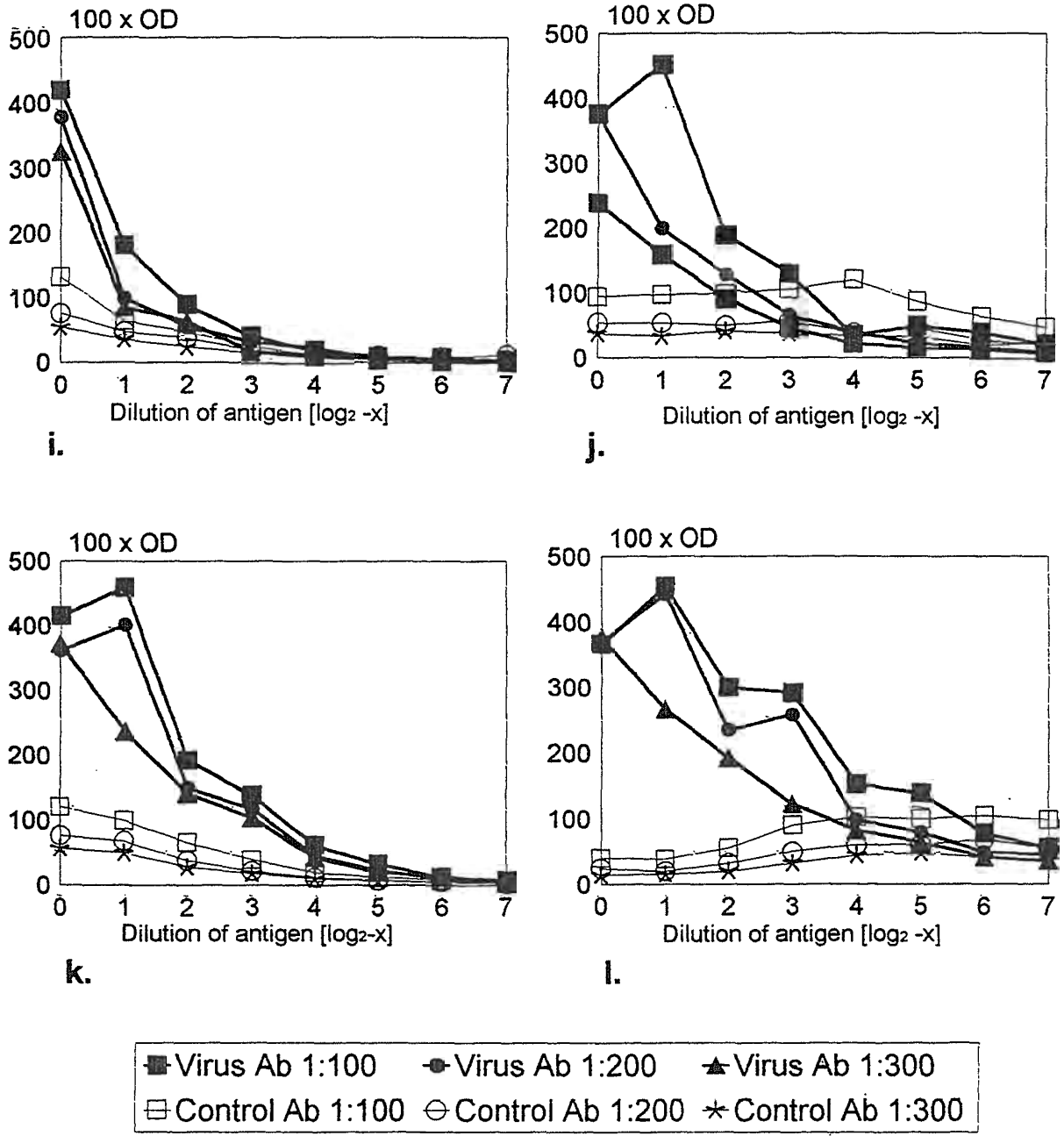


e. Antigen coated on to plate with PBS for one hour at RT

f. Antigen coated on to plate with CBC for one hour at RT

g. Antigen coated on to plate with PBS O/N at RT

h. Antigen coated on to plate with CBC O/N at RT



i. Antigen coated on to plate with PBS for one hour at 37°C

j. Antigen coated on to plate with CBC for one hour at 37°C

k. Antigen coated on to plate with PBS O/N at 37°C

l. Antigen coated on to plate with CBC O/N at 37°C

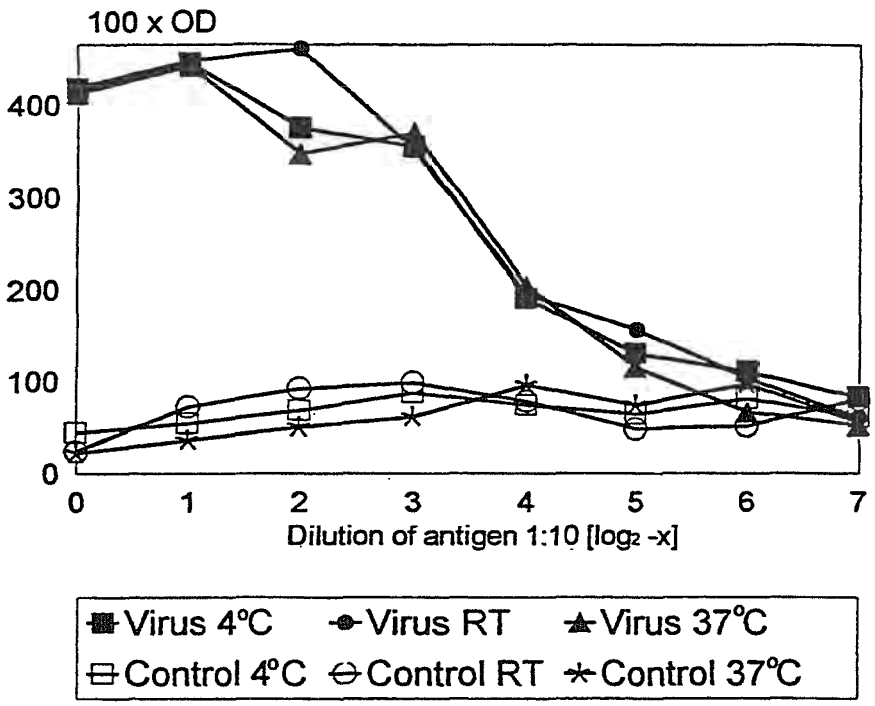
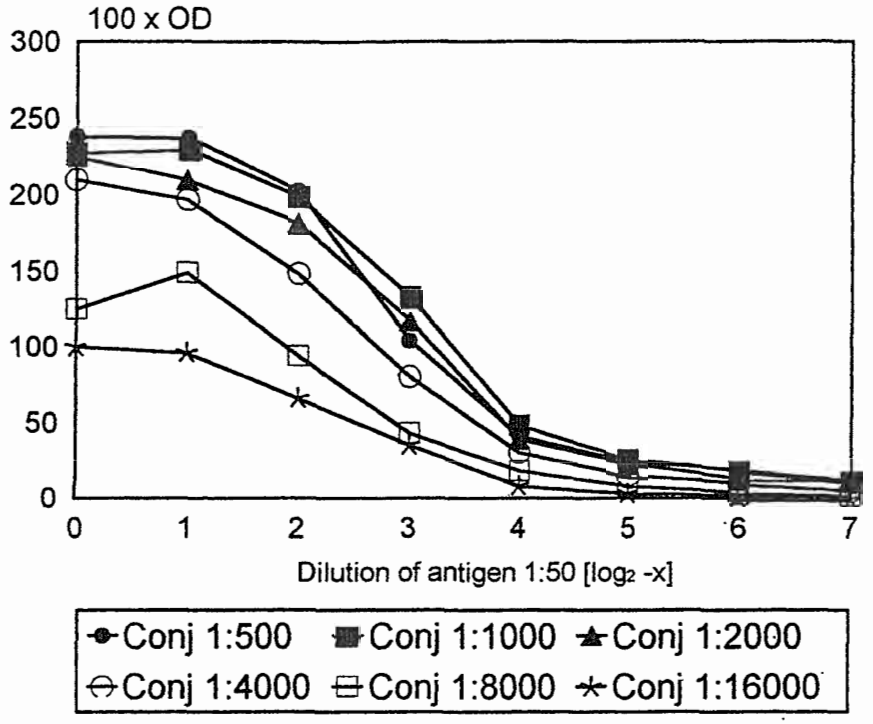
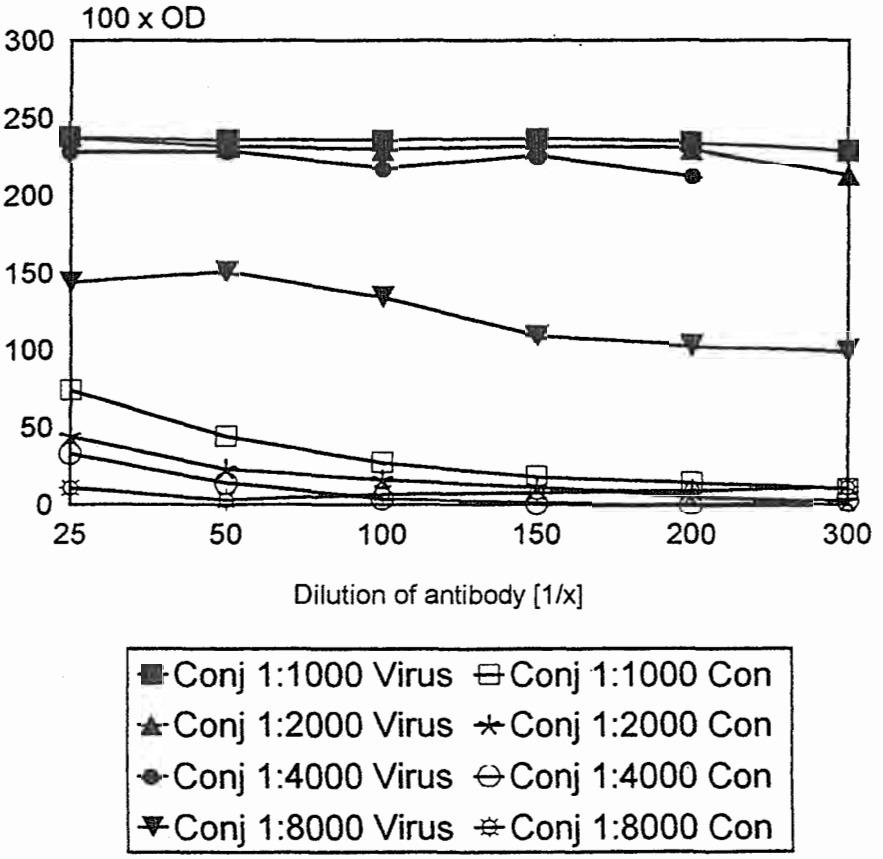


Figure 3.13 Absorbance values as measured by ELISA with 8 dilutions of antigen coated on to plates with CBC O/N at three incubation temperatures (4°C, RT, 37°C). Detecting antibody was rabbitαBIV at 1:100. Plates were coated with BIV (virus) or BF2 (control).


100 x OD
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
Dilution of antigen 1:50 [log2 -x]
Conj 1:500
Conj 1:1000
Conj 1:2000
Conj 1:4000
Conj 1:8000
Conj 1:16000

a.


100 x OD
300
250
200
150
100
50
0
25
50
100
150
200
300
Dilution of antibody [1/x]
Conj 1:1000 Virus
Conj 1:1000 Con
Conj 1:2000 Virus
Conj 1:2000 Con
Conj 1:4000 Virus
Conj 1:4000 Con
Conj 1:8000 Virus
Conj 1:8000 Con

b.

3.3.6 SDS-PAGE

Running the eluted fractions alongside serum on SDS-PAGE demonstrated that the Affi-T column can at least partly purify reptile immunoglobulins. The albumin fraction (50 000 kDa) was completely separated and immunoglobulin in purified samples consisted of between 45 to 79% of the fraction in undiluted serum. Fractions corresponding to IgY and IgY(ΔFc) could be discerned at 180 and 120 kDa, respectively (Figure 3.15) (Magor, Higgins, Middleton and Warr 1994).

3.3.7 Ascitic fluid

The mice were harvested between day 12 and day 22 after inoculation with SP2/0 cells. The average amount of fluid from each mouse was approximately 6 mL after debris were pelleted by centrifugation. Cross reactivity between tortoises antisera and among snakes antisera were strongest, however, each preparation of ascitic fluid had the highest affinity for the specific immunoglobulin with which the mouse had been vaccinated (Figure 3.16 a to f).

3.3.8 RabbitαBIV or mouseαBIV

Incubation of mouse ascitic fluid with a sonicated preparation of BF2 cells reduced the level of antibodies to BF2 cells and induced an apparent rise in the level of antibodies to BIV (Figure 3.17a). Nevertheless, rabbitαBIV was still superior to mouseαBIV (Figure 3.17b) and was therefore maintained as the primary antibody in the ELISA.

3.3.9 Indirect ELISA

Despite testing a range of different makes of ELISA plates, blockers and their incubation times, it remained impossible to overcome the problem of non-specific attachment of the reptile serum to the ELISA plates. Background versus real signal could not be deduced by an ELISA with this configuration, because the reptile serum attached non-specifically. There may have been a real signal, but it would have been completely masked by the high background and therefore this configuration was abandoned.

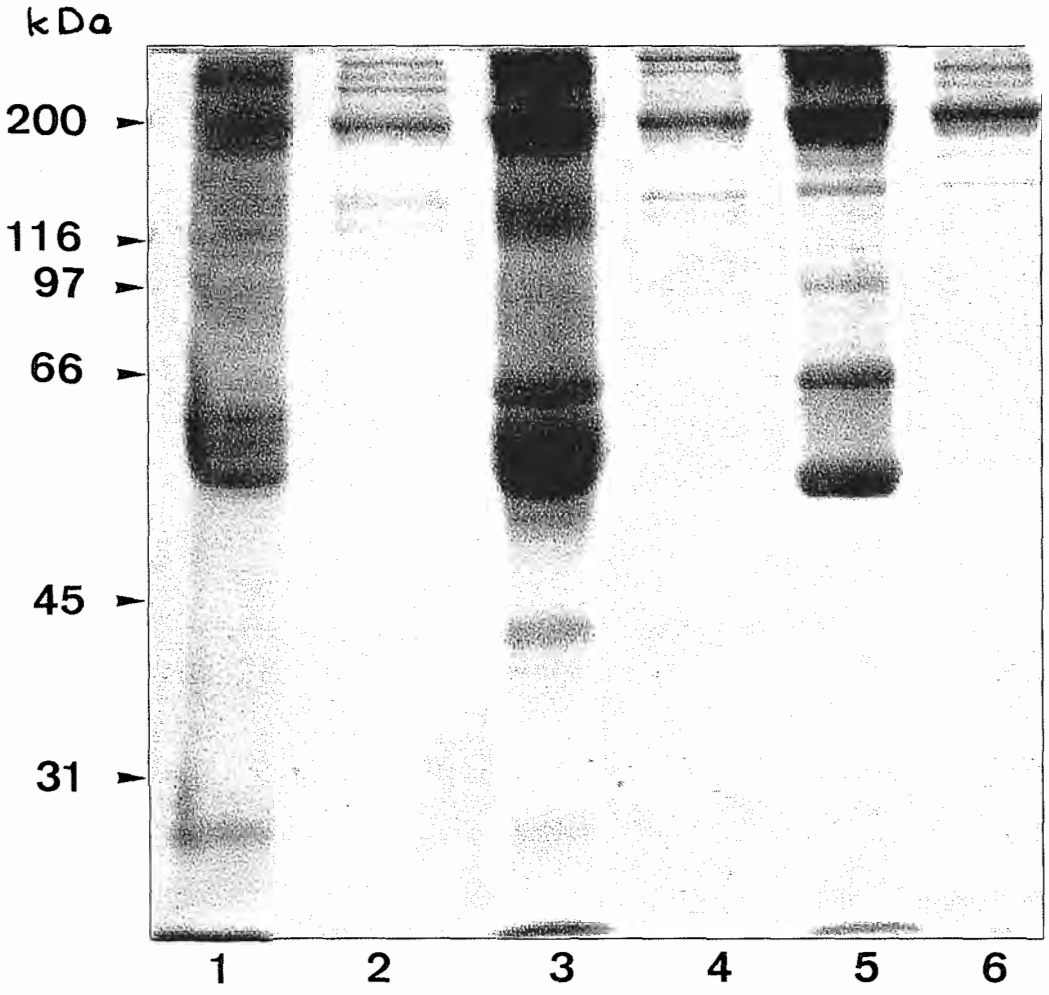


Figure 3.15a SDS-PAGE gel of affi-t purified and unpurified serum from *E. krefftii*, *E. latisternum* and *C. johnstoni*.
Lane 1: Unpurified serum from *E. krefftii*. Lane 2: Affi-t purified serum from *E. krefftii*. Lane 3: Unpurified serum from *E. latisternum*. Lane 4: Affi-t purified serum from *E. latisternum*. Lane 5: Unpurified serum from *C. johnstoni*. Lane 6: Affi-t purified serum from *C. johnstoni*.

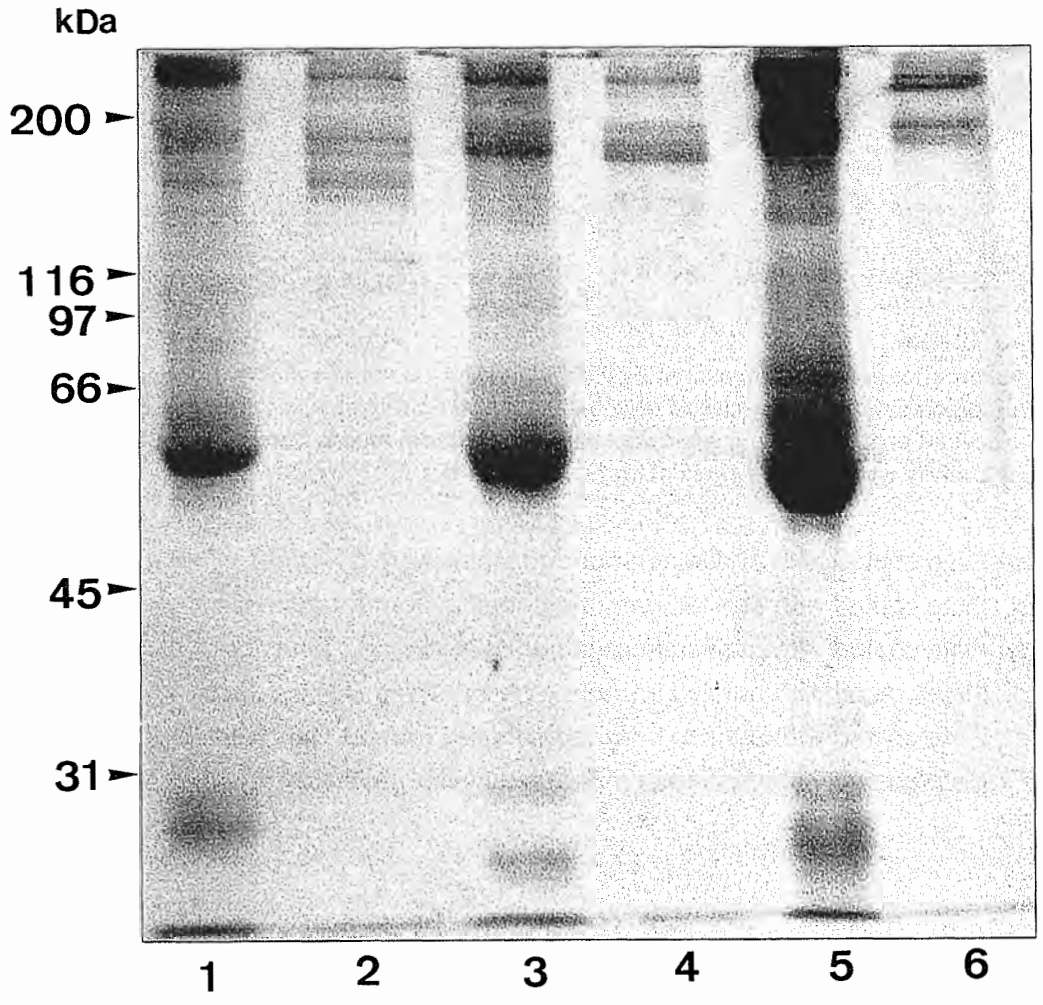
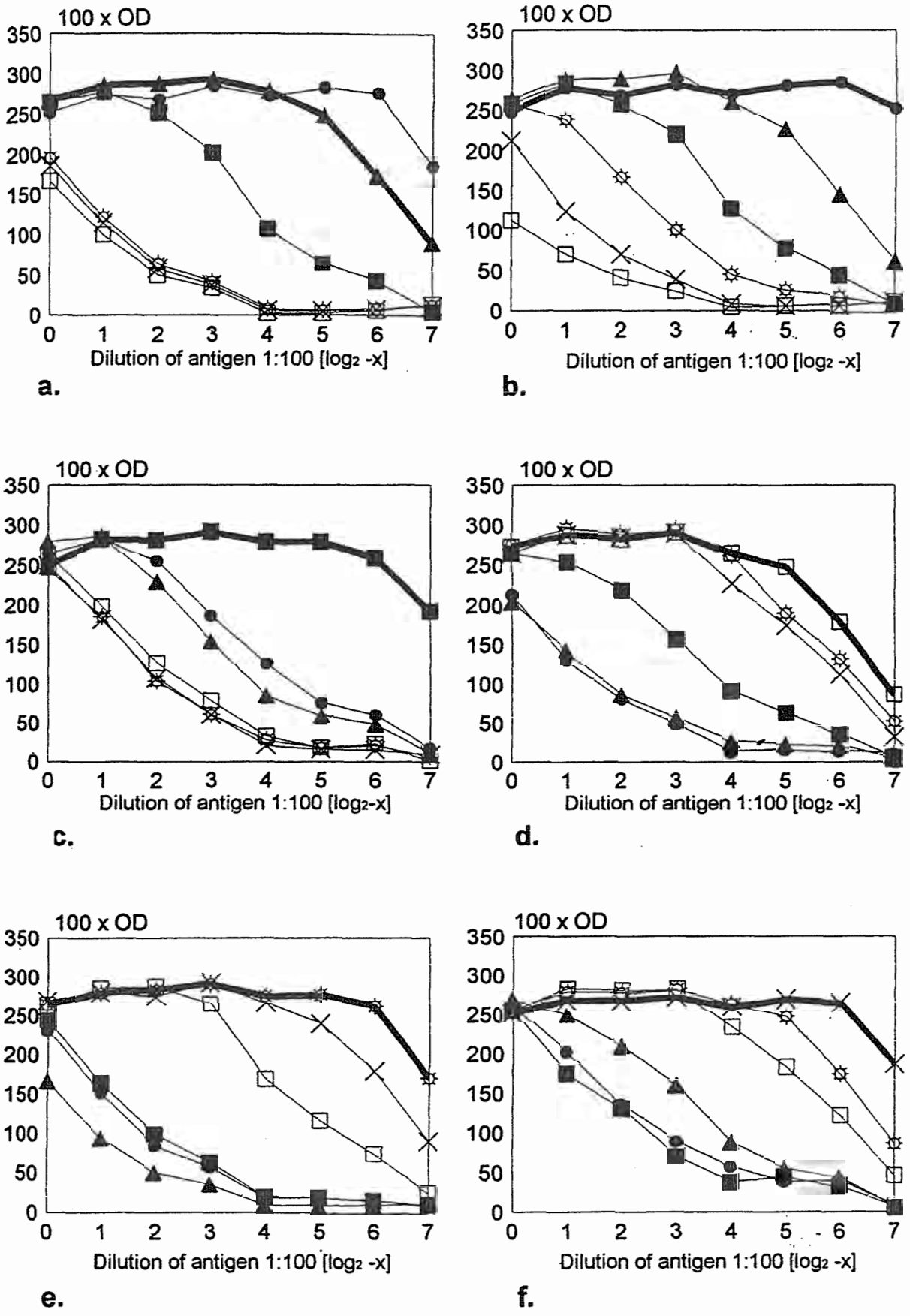
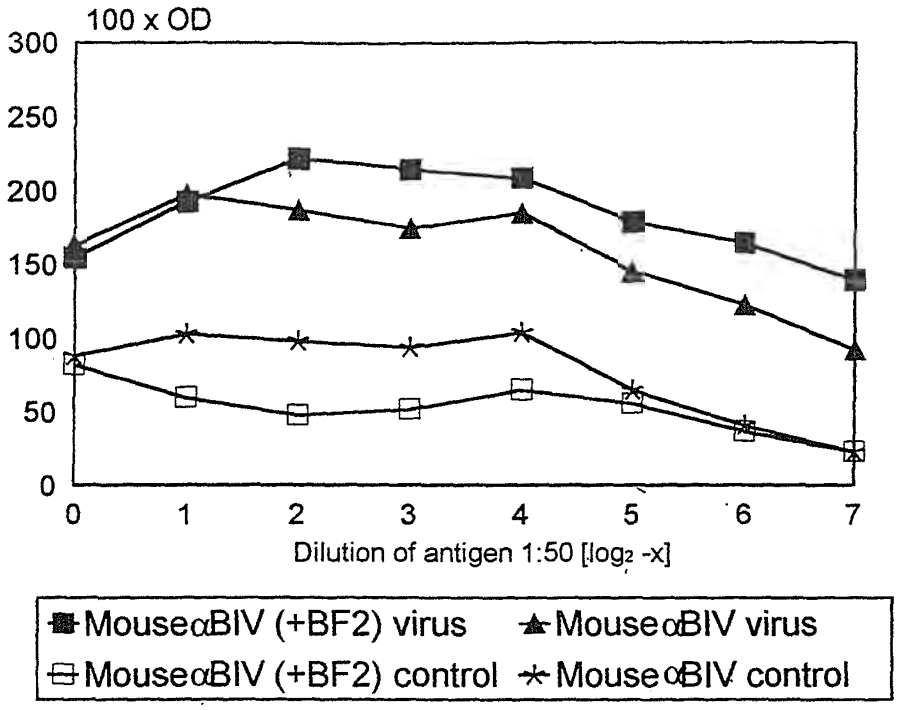
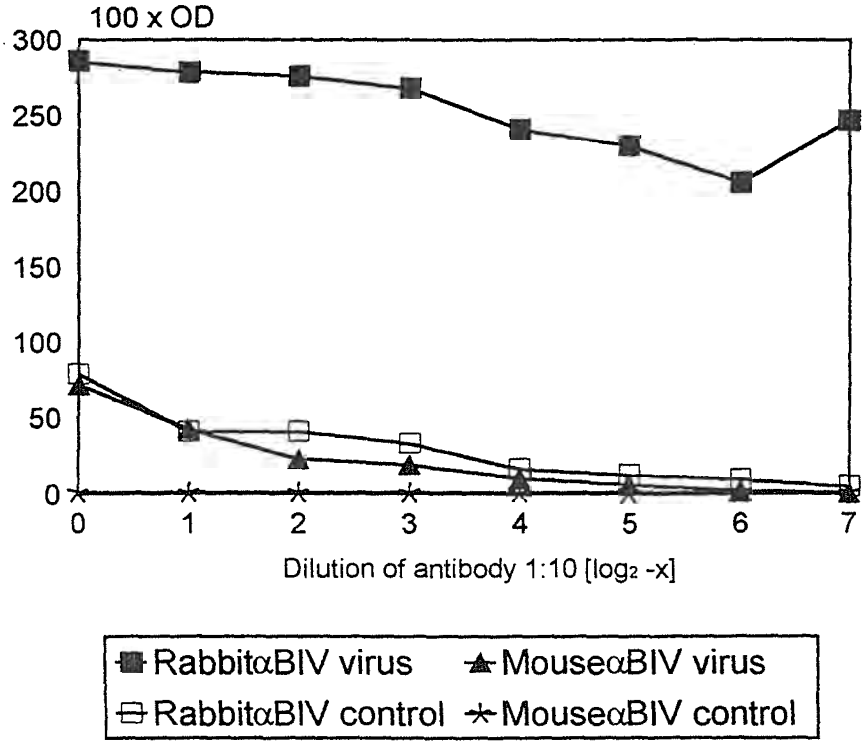


Figure 3.15b SDS-PAGE gel of affi-t purified and unpurified serum from *A. mairii, D. punctulatus* and *B. irregularis*.
Lane 1: Unpurified serum from *A. mairii*. Lane 2: Affi-t purified serum from *A. mairii*. Lane 3: Unpurified serum from *D. punctulatus*. Lane 4: Affi-t purified serum from *D. punctulatus*. Lane 5: Unpurified serum from *B. irregularis*. Lane 6: Affi-t purified serum from *B. irregularis*.


100 x OD
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7
Dilution of antigen 1:100 [log2 -x]
a.
b.
c.
d.
e.
f.
Mouse αE.k.
Mouse αE.l.
Mouse αC.j.
Mouse αB.i.
Mouse αD.p.
Mouse αA.m.


100 x OD
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7
Dilution of antigen 1:50 [log2 -x]
MouseαBIV (+BF2) virus
MouseαBIV virus
MouseαBIV (+BF2) control
MouseαBIV control

a.


100 x OD
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7
Dilution of antibody 1:10 [log2 -x]
RabbitαBIV virus
MouseαBIV virus
RabbitαBIV control
MouseαBIV control

b.

3.3.10 Capture ELISA

Reptilian sera attached well to the plates and there was minimum non-specific attachment in the remaining steps. The optimal protocol was determined by checkerboard titrations as follows: Test serum was diluted 1:50 in CBC and 50 μL coated onto plates at RT O/N. To duplicate control wells were added (1) 50 μl reptile serum alone, (2) with control antigen, and (3) with BIV, all in dilutions of 1:50 CBC (see Figure 7.5 wells H7 & 8, H9 & 10, and H11 & 12 respectively). All succeeding incubation steps were incubated for 1 hour at RT. Prior to each step the wells were washed with TEN-T. The following day, 50 μL of BIV at 1:50 in CBC was added to each well. In the next step, 50 μL of rabbitαBIV (1:200 in TEN-TC) was added to all wells except the test serum control wells, to which 50 μL mouseαreptile species (1:100 in TEN-TC) were added. In the succeeding step goatαrabbit (1:1000 in TEN-TC) and goatαmouse (1:1000 in TEN-TC) were added to the respective wells. Lastly 100 μL ABTS were added to all wells.

3.4 Discussion

In this study an ELISA was developed for the detection of anti-BIV serum proteins in the serum of selected reptiles. The test provides the means for monitoring the antibody response in experimentally infected reptiles. It may also enable the detection of serological evidence of past exposure to BIV in wild populations.

In the configuration of the sandwich ELISA described here, reactive test serum captures BIV and presents it to the hyperimmune antisera, rabbitαBIV. This is then detected by the enzyme conjugated goatαrabbit which resulted in the colour change with the addition of the ABTS substrate.

In this ELISA format a false positive reaction may be caused by non-Ig serum proteins binding to the virus and not immunoglobulins in the reptile serum. The ELISA can detect relative changes in BIV reactivity in the serum of experimental animals over a time period. In surveys, however, where an animal is only tested once, a positive result may be due to either adaptive or innate immunity in that animal.

Propagation and release of BIV from host cells was optimized using an ELISA which utilised polyclonal rabbitαEHNV, which cross reacts to a high degree with BIV (Hengstberger et al. 1993). Iridoviruses are closely associated with the cytoskeleton of their host cells (Aubertin

1991), and a series of treatments for BIV-infected cells, were tested to determine how best to optimize the viral yield from cells of two different host cell lines. The BIV thus produced, was used as the antigen in the sandwich ELISA, an immunogen for vaccinating mice, and in a further purified form, acted as an immunogen to vaccinate rabbits for the purpose of producing hyperimmune antisera to BIV. An optimal method for the release of EHNV from infected fish tissue was determined by testing the yield of eight different protocols in an antigen capture ELISA (Whittington and Steiner 1993). The most efficient method of antigen release was manual grinding of tissues followed by vortexing. Cell culture material is more fragile than organ material and to release BIV from *in vitro* cultured cells, resuspension of cells in distilled water followed by vortexing with glass beads was determined to be the most efficient method for release of BIV.

Polyclonal antisera from hyperimmunised rabbits was highly specific and yielded high titres. Ascitic fluid produced in BIV vaccinated mice had a high background titre to BF2 cells which was likely due to host cell contaminants in the inoculum. The background titre was reduced considerably after incubating the ascitic fluid with a preparation of sonicated BF2 cells. An increase in the level of antibodies directed against BIV coincided with the reduced background and may be explained by the removal of αBF2 antibodies which could have masked the BIV antigen and prevented attachment, and thereby detection, of αBIV antibodies.

Several attempts were made at producing monoclonal antibodies to BIV. The technique seemed to function, because vaccinated mice responded to vaccination by producing serum antibodies, and hybridomas resulted from fusions. However, only a very small proportion of the hybridomas produced antibodies towards BIV, and the subculture and cloning of those hybridomas were not successful.

Nakajima and Sorimachi (1995) produced a panel of monoclonal antibodies against Red sea bream iridovirus. They inoculated mice with whole infected cells and rather than purifying the antigen, they excluded in their screen those hybridomas which produced antibodies against host cells. In the present study, partly purified antigen was used to vaccinate mice before fusions and induction of ascitic fluid. The procedure described by Nakajima and Sorimachi (1995), may be an avenue worthwhile exploring in future fusion, alongside the use of out-bred or different strains of mice for a better immune response (Harlow and Lane 1988).

Partially purified immunoglobulins from 6 species of water-associated reptiles (*E. krefftii*, *E. latisternum*, *C. johnstoni*, *B. irregularis*, *D. punctulatus* and *A. mairii*) induced a

Figure 4.1 *Elseya latisternum*, the saw-shelled tortoise. Note serrated posterior edge of juveniles.

Figure 4.2 *Emydura krefftii*, Krefft's river tortoise.

CHAPTER 4

PATHOGENESIS OF BOHLE IRIDOVIRUS IN ADULTS AND HATCHLINGS OF THE FRESHWATER TORTOISES *ELSEYA LATISTERNUM* AND *EMYDURA KREFFTII*

4.1 Introduction

Australian tortoises are aquatic or semi-aquatic turtles in the family Chelidae (Cogger 1992). Their head and neck are folded to one side under the carapace and the limbs are jointed with webbed feet and clawed digits. Tortoises inhabit a variety of freshwater environments and are found throughout mainland Australia (Cann 1978). They are capable of migrating cross country to new water sources (Legler and Georges 1993). Tortoises forage in the water, bask on logs and rocks and lay eggs in excavated borrows on land. Hatchlings are adapted to aquatic life from the time they emerge. Most species are opportunistic omnivores (Legler and Georges 1993), however, scavenging on carrion is also common (Cann 1978). In turn, juvenile tortoises may be eaten by snakes, fish, eels, birds, or crocodiles (Cann 1978; Tucker, Limpus, McCallum and McDonald 1996).

The saw-shelled tortoise, *Elseya latisternum,* (Figure 4.1) dwell in rivers all along the Queensland coast from New South Wales to the Northern Territory (Cogger 1992). The serrated posterior edge of the shell gives rise to the common name of this tortoise. This tortoise is carnivorous, taking tadpoles, frogs, fish, aquatic insects and even cane toads (Ehmann 1992).

Krefft's river tortoise, *Emydura krefftii,* (Figure 4.2) are characterised by a yellow/green stripe along the head which extends back from the eye (Cogger 1992). This species inhabits rivers and larger billabongs in the floodplains of both the eastern and western drainage systems throughout the state of Queensland. Hatchlings and juveniles are carnivorous, whereas adults prefer an omnivorous diet (Ehmann 1992).

Under natural conditions, the ecology of these tortoises would expose them to fish and frog viruses, like BIV. Therefore, it was considered important to determine the impact of a BIV infection on native tortoises. Additionally the study aimed to confirm whether tortoises were able to produce antibodies directed against the virus. In which case, a cut-off level had to be selected to differentiate between BIV-reactive and non-reactive sera. The effects of exposure to live BIV infection were investigated for freshwater tortoise adults and hatchlings of the species *E. latisternum* and *E. krefftii* with respect to pathogenesis and serum antibody levels.

4.2 Materials and Methods

4.2.1 Source of animals

Animals were collected from the wild under permit (Scientific Purposes Permit, No. H0/000096/95/SAA) from the Queensland Department of Environment (QDOE), which also included their offspring. Experiments were covered by permit from the Experimental Animal Ethics Committee, James Cook University (Ethics Approval No. A302).

4.2.1.1 Adults

Elseya latisternum were collected during a field trip to Mount Surprise (latitude: 17°47'S to 18°2'S; longitude: 144°21'E to 144°5'E), in conjunction with Dr. Colin Limpus, QDOE. Sections of Fossilbrook and Elizabeth Creeks were dragged by nets (Figure 7.3) and 8 adult tortoises were captured from each creek system. Six of the captured animals were mature females, the other 10 were males. Their weights ranged from 112 to 2395 g. Seventeen adult *E. krefftii* were likewise captured from shallow billabongs in the Townsville region (latitude: 19°19'S; longitude: 146°42-46'E). The 9 female and 8 male *E. krefftii* from the Townsville region weighed between 408 and 2146 g.

4.2.1.2 Hatchlings

Juvenile tortoises were hatched from eggs obtained from captive females (Section 4.2.1.1). The presence of hard-shelled eggs in a female was determined by palpating the viscera in the area posterior to the hind legs, while holding the animal in a vertical position (J. Miller, QDOE, Pers. Comm., 1995). In one case, this method was verified by X-ray (Figure 4.3) according to the technique reported by Whitfield Gibbons and Greene (1979).

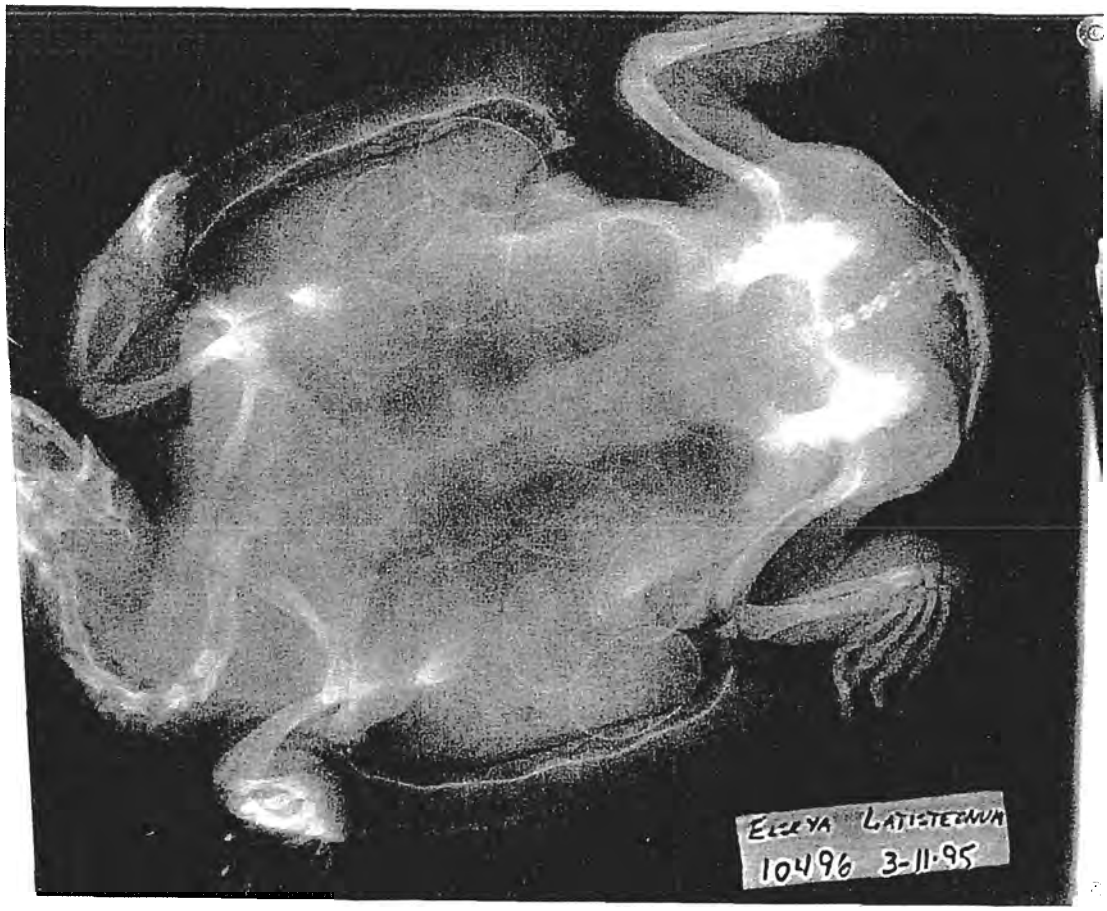


Figure 4.3 X-ray of female *Elseya latisternum* showing the outline of 16 eggs.

Female tortoises, of both species, with hard shelled eggs were induced with oxytocin (10 international units/mL, Heriot AgVet, Australia) to lay eggs in shallow water according to recommendations by Ewert and Legler (1978). The dosage was 3 international units per 100 g body weight, half of which was administered intramuscularly in each hind leg. Eggs were promptly removed from the water, dried, labelled and measured before placement in moist, sterile vermiculite in humid atmosphere incubators at 30°C. A fungus, growing in the vermiculite and the eggs of the first batch of *E. latisternum* eggs, was cultured and identified as a *Humicola* sp. (W. Shipton and S. Bowden, Department of Microbiology and Immunology, James Cook University, pers. comm., 1996). Eradication of the fungus was attempted unsuccessfully by treating the eggs with 1% iodine before transfer to newly sterilised vermiculite. Eggs which developed shells during the experiment, were collected at necropsy of the experimental animals and incubated as described above.

Fertile eggs from both species of tortoises could be identified by the chalking process in the egg (Ewert 1985). A white spot on the upper most part of the shell, which developed into an

equatorial band became apparent over the first few days of incubation (Figure 4.4). Viability of the developing embryo was determined after two months of incubation by candling the egg, while taking care not to tilt it in any direction, which may result in the detachment of the embryo and vitelline membrane from the shell (Ewert 1985). Hatchlings emerged after approximately 70 days of incubation (Figure 4.5).

Eight *E. latisternum* eggs collected at necropsy of a female were successfully hatched. The first batch of 72 eggs ceased development at different stages. The rapidly growing *Humicola* sp. probably invaded the eggs post mortem as did other saprophytic fungal and bacterial species (S. Bowden, Department of Microbiology and Immunology, JCU, Pers. Comm., 1996). Sixty-four *E. krefftii* eggs were collected and 51 hatched. None of which were infected with fungus. Data on weights of tortoise hatchlings was not available.

4.2.2 Experimental design

4.2.2.1 Adults

Sixteen *Elseya latisternum* adults were tagged with monel web tags (National Band and Tag Company, Sydney) in the skin flap between the two outer toes of their webbed hind feet. Seventeen *Emydura krefftii* adults were identified by cutting a notch into one of their marginal scales with an angle grinder. Scales were designated a letter each in alphabetical order starting from the scale to the right of the central and posterior nucal scale and proceeding in a clockwise direction along the periphery of the shell.

Adult tortoises were ranked according to weight with the lightest animal being assigned number one, the second lightest number two and so on, with the heaviest tortoise getting the highest number in the group. Each animal was then allocated to one of three treatments: control, inoculation with live BIV or co-habitation with another BIV-inoculated animal, according to the experimental design with two animals in each pen (Table 4.1). During the four weeks of the experiment, blood was collected twice weekly from every individual to monitor serum antibody levels against BIV by ELISA. At the termination of each experiment, animals were humanely killed nd samples collected and processed for serology, viral isolation, histological and parasitological examination (Section 4.2.5).

Figure 4.4 Batch of eggs from *Emydura krefftii* (tortoise U) showing non-developing eggs (#21, 22, 23 and 26) and eggs undergoing chalking (#24, 25 and 27). Only #24 and #25 hatched.

Figure 4.5 *Emydura krefftii* hatchlings emerging from the eggs after approximately 70 days of incubation.


U25
U26
U24
U27
U23

Table 4.1 Design for experimental BIV-infection of 16 *Elseya latisternum* and 17 *Emydura krefftii* adults. Animals were ranked according to weight and distributed sequentially into the three treatment groups with two animals per pen (indicated by lines).

Treatment	Animal Number in Ascending Weight Rank											
Control	Pen A	1		4	Pen B	7		10	Pen C	13		16
Inoculated	Pen D	2	Pen E	5	Pen F	8	Pen G	11	Pen H	14	Pen I	17
Co-habitation		3		6		9		12		15		18

Control animals were housed in a quarantine area and were given intraperitoneal placebo inoculations of 500 μL PBS at multiple sites. The others were kept in the Aquatic Disease Facility (JCU) and animals in the "inoculated" treatment given 500 μL BIV ($10^{4.5}$ $TCID_{50}$) intraperitoneally at multiple sites. Animals in the "co-habitation" treatment were given PBS injections similar to the control animals and shared a pen with a BIV-inoculated animal.

4.2.2.2 Hatchlings

Elseya latisternum

Five of the eight *E. latisternum* hatchlings were inoculated with 50 μL BIV (10^3 $TCID_{50}$) intraperitoneally and kept at the Aquatic Disease Facility (JCU); the other three received 50 μL PBS and stayed at the isolation unit. The experiment ran for four weeks. Whenever an infected hatchlings died or became moribund, a control animal was sacrificed to provide an ontogenic reference for histological purposes.

Emydura krefftii

A larger number of *E. krefftii* eggs hatched, some of which were released to the wild, while 24 hatchlings were used for experimental purposes (12 inoculated and 12 controls). The hatchlings were sampled in the same manner as *E. latisternum* hatchlings; additionally, for *E. krefftii* hatchlings, one control and one infected animal was killed and processed for serology, viral isolation and histological examination on days 4, 8, 11, 16, 18, 22, 25 and 29 post-inoculation (pi).

Experimental animals were chosen from four clutches. The central scale of each hatchling was marked with nail-polish in colours according to their clutch of origin. A month after hatching, animals were assigned a number each. For each clutch, 6 numbers were chosen at random from a hat, and the corresponding animal placed in one of 6 ice-cream containers. This scheme resulted in 6 ice-cream containers with four non-siblings in each. Three containers with hatchlings were randomly picked as controls while the other three containers of hatchlings were

transferred to the Aquatic Disease Facility (JCU) and the animals subsequently inoculated with 50 μL BIV (10^3 $TCID_{50}$) intraperitoneally. The control animals received an intraperitoneal placebo inoculation of 50 μL PBS.

4.2.3 Husbandry

Control animals were always attended to before the experimentally exposed animals on each day. This procedure was meticulously followed to prevent accidental exposure of the control animals to BIV.

4.2.3.1 Adults

Adult tortoises were kept at approximately 25 °C in glass tanks (40 × 60 × 90 cm^3) filled with town-water to a depth of 15 cm. Half of the tank was covered with newspaper to minimise visual disturbance to the tortoises by human activity in the area. Under the covered area, a platform of "weather rite" board placed on bricks, provided a dry area for the animals. The tortoises were fed a diet of diced beef and freshly chopped tomatoes three times weekly. A complete water change was carried out on days subsequent to feeding. Two days prior to termination of an experiment, food was withheld from the tortoises to ease post mortem examination of the gut for parasites.

4.2.3.2 Hatchlings

Hatchlings were maintained at approximately 25 °C in ice-cream containers with 500 mL of town-water. The ice-cream containers were tilted to create a dry area and a pool for the hatchlings. A central piece of the lid was cut out to allow for air circulation, and mosquito netting inserted under the lid to protect the hatchlings from predators (Figure 4.6). The hatchlings were fed on boiled lettuce, Wardley Reptile TEN pellets (Wardley Corp., New Jersey) and finely chopped tomatoes. The water was changed daily.

Figure 4.6 Experimental setup for hatchlings.

4.2.4 Serum collection

Twice weekly, blood was collected from the femoral vein on alternating sides of adult tortoises (Dessauer 1970), using a 25 gauge needle and a 1 mL syringe (Figure 4.7). Hatchlings were too small for repeated bleeding and blood was only collected at necropsy.

The sample of approximately 100 μL, was deposited into a 1.5 mL microcentrifuge tube. After one hour at RT the clot was dislodged from the plastic with a wooden tooth-peg and left at 4°C O/N. The following day the serum was collected with an automated pipette, clarified in a Beckman microfuge E at 15000 RPM for two minutes at 4°C, and stored at -20°C until tested in ELISA (Section 3.3.10).

Within treatment groups the mean of the OD values from individual tortoises was graphed for each day of sampling.

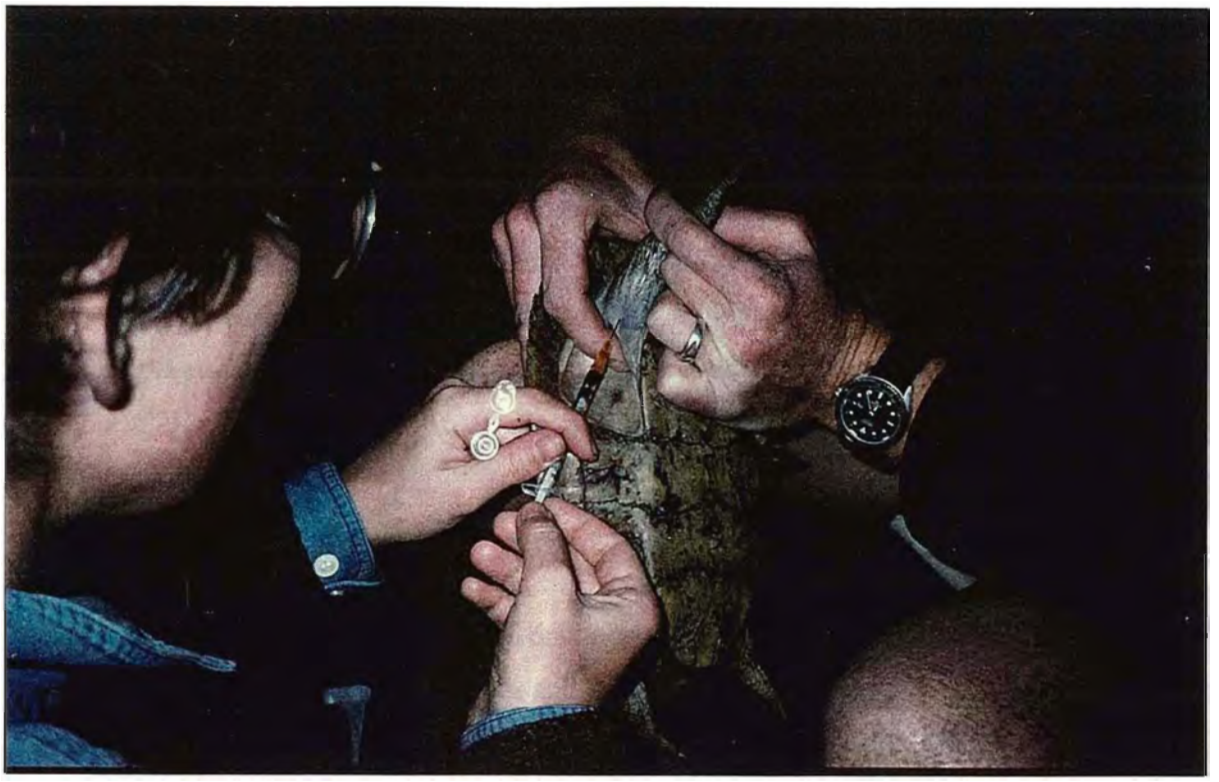


Figure 4.7 Blood sampling from femoral vein of tortoises.

4.2.5 Post mortem processing of samples

Animals were checked every morning. Some hatchlings died before sampling. An animal was designated dead if its limbs were extended and limp and there was no eye-reflex. Moribund hatchlings had only a very weak response to touch and made hardly any resistance when head or limbs were being extended. Hatchlings in such a state were immediately killed and necropsied.

In selecting a method for killing the experimental animals, minimum trauma to the animal and swiftness were paramount. It was also important to collect a large volume of blood for immunoglobulin purification (Section 3.2.5) and to obtain organs in a suitable state for histological processing.

Due to the ability of diving reptiles to endure prolonged anoxia and breath-holding (Frye 1991), euthanasia by means of gassing is not effective. In killing reptiles for necropsy, intracardial injection of the barbiturate "Lethabarb" was found to be a slow, albeit pain-free process, which interfered with the histological quality of organs in the vicinity of the injection site (personal

observation). Additionally, euthanasia arrests the heart beat, which results in blood being trapped in the tissues and thus being unavailable for collection.

Decapitation was followed by injection of 10% neutral buffered formalin (NBF) into the brain of adults, and pithing of hatchlings. Immediately upon severance of the spinal cord there was no eye reflex. This method was humane, swift and enabled drainage of blood by gravity and heart pumping. It is considered to be humane and cause immediate unconsciousness in crocodiles (Hutton 1992).

4.2.5.1 Serology

Decapitated animals were tilted to aid the drainage of blood from the main arteries. Blood from adults was collected in plastic petri dishes and transferred into 10 mL clotting tubes (Disposable Products, Adelaide). After one hour the clot was loosened with an orange stick and left to contract further O/N at 4°C. The following day, serum was separated and clarified at 900 *g* for 10 minutes, before storage at -20°C. For hatchlings, blood was collected directly from the body into a microcentrifuge tube and treated as blood collected repeatedly from adults (Section 4.2.4).

Just prior to screening in ELISA, the sera were defrosted at room temperature and diluted 1:50 in CBC. Fifty μL of each diluted sample was placed in triplicates in wells of a 96 well ELISA plate. The remainder of the ELISA was carried out according to Section 3.3.10.

For each sample the mean of the OD from the triplicate wells was recorded. Although no attempt was made to find a conversion factor between antibody titre and OD value, it is assumed that there is a strong correlation between the two and therefore the OD values presented here, are taken to represent levels of antibody. The data from the ELISA was graphed in Harvard Graphics 2.1 for Windows according to site and year for both species.

Unfortunately, no other tests for BIV exists that are 1) biologically independent of this ELISA, and 2) give an accurate indication of infected and non-infected individuals. Pathogenesis trials were conducted on wild animals, in order to calibrate this test to an independent standard, namely that of experimentally exposed and unexposed individuals. Obviously, SPF stock for this purpose could not be obtained from the wild, and antibody levels of experimentally infected animals had to be assessed in relation to their initial levels and to that of the control animals in the

experiment. The OD cut-off level for non-reactive animals was selected for each species as the 95% confidence limit based on the mean OD of all non-inoculated animals +/- two standard deviations. For this purpose 86 *E. latisternum*, 53 *E. krefftii*, 63 *C. johnstoni* and 76 snakes (19 *B. irregularis*, 18 *D. punctulatus*, 9 *A. mairii*, 7 *Liasis childreni*, 21 *Morelia spilotes* and two *Liasis fuscus*) were tested.

4.2.5.2 Viral isolation

Samples from selected organs (liver, kidney, spleen, heart, brain, muscle, lung, gonad, pancreas, stomach and small intestine) of adults and from the liver of hatchlings, were collected in stomacher bags (Seward Medical, Stomacher Lab-blender, Model '80', Cat. No. BA6040) and stored at -20°C until further processing. After thawing, 1 mL dH_2O was added to each bag and the samples homogenised. The resultant liquid homogenate was transferred to microcentrifuge tubes and subjected to three freeze/thaw cycles before clarification by centrifugation (Beckman microfuge E) at 15000 RPM for 15 minutes at 4°C. Fifty microlitres of supernatant from each tube was added to BF2 cell monolayers in duplicate wells of a 24 well tissue culture plate. The plates were incubated in a humid, 5% CO_2 rich atmosphere at 26°C for one week and checked for BIV CPE daily. Two blind passages were performed for each sample at weekly intervals, by transferring 50 μL of cell culture supernatant from inoculated wells to corresponding wells with new, non-infected BF2 cell monolayers in a separate plate.

4.2.5.3 Histology

Any observable lesions, as well as a range of tissues (Section 4.2.7) from adults were preserved in 10% NBF. To avoid confusing ontogenic changes with BIV-induced lesions, histological sections from inoculated hatchlings were compared to quarantined hatchlings of the same age. Because of their small size the whole viscera of hatchlings was preserved. Trimmed tissues were processed and embedded in paraffin wax. Sections were cut at a thickness of 5 μm, stained with haematoxylin and eosin and mounted using routine methods (Culling, Alison and Barr 1985).

4.2.5.4 Photomicrography

Photographs were taken of representative lesions in histological sections with a Leitz Orthoplan Universal large field microscope fitted with a Leitz Vario-Orthomat camera system (Ernst Leitz

Wetzlar GMBH, D-6330 Wetzlar, Germany) for automatic photography. EPY-64 Kodak Ektachrome professional tungsten film was used.

4.2.5.5 Parasitology

External and internal organs of adults were examined macroscopically for parasites. The intestinal tract, lungs, trachea and gall bladder were opened along their full length, placed in saline and examined under a dissecting microscope within 30 minutes of collection, or stored at 4°C until examined (up to 12 hours post mortem). Observations were made of any gut content, lung nodules and cysts. Parasites were removed, counted and stored in 70% ethanol (nematodes) or 10% NBF (pentastomes and trematodes). Identification of parasites was carried out by Dr. Gareth Hutchinson, Department of Microbiology and Immunology, James Cook University.

4.3 Results

Captive animals were active and fed vigorously when offered food. Seven of 8 *E. krefftii* males lost weight during the captive period, in contrast to all breeding females which gained body weight in addition to producing eggs. These fluctuations were thought to be related to natural rhythms during and after the breeding season rather than diet. The diet and husbandry conditions were satisfactory for egg development. A sub-adult *E. latisternum* tortoise acquired an infection in the tag site. *Providencia rettgeri* bacteria was isolated in pure culture from the lesion (S. Bowden, Department of Microbiology and Immunology, JCU). After removal of the tag and treatment of the area with iodine the lesion healed.

4.3.1 Serology

The maximum level for non-reactive serum was determined at 95% confidence limit to be OD 1.1 (110 OD × 100) for adults of both species of tortoises. The present ELISA can detect relative changes in anti-BIV serum proteins in reptilian sera. A constant level of reactivity in certain individuals may be due to either adaptive or innate immunity, but any fluctuations in reactivity are probably caused by changes in levels of antibodies to a BIV-like antigen. Levels of serum antibody against a BIV-like antigen during the experiment are displayed for the three treatment groups for adult *E. latisternum* and *E. krefftii* (Figures 4.8 and 4.9 respectively) and for *E. krefftii* hatchlings (Figure 4.10). A pre-inoculation bleed, 60 days prior to onset of the

experiment was included for *E. krefftii* adults. Non-reactive sera is below the cut-off level of 110 (OD × 100), medium reactive levels are between 110 and 200 (OD × 100) and highly reactive sera are in the 200 to 300 (OD × 100) category.

Elseya latisternum

In the inoculated group, tortoises #90 and #95 exhibited a decrease in levels of anti-BIV serum proteins during the first 4-7 days of the experiment (Figure 4.8a). Following inoculation, the level for tortoise #90 stabilised well above the cut-off value, and for # 95 the decline was succeeded by an increase in levels of antibody against BIV. In the rest of the group the levels of serum proteins reactive to BIV remained below or just above the cut-off value for the duration of the experiment.

Co-habitation and control animals showed no noticeable change in levels of BIV-antibody during the experimental period (Figure 4.8b, c). Numbers 96 and 97 in the control group displayed remarkably high OD levels throughout the experiment.

Emydura krefftii **adults**

In all animals which initially displayed high OD values, a decline was noted in the levels of antibody against a BIV-like antigen during the sixty day holding period in a quarantine area prior to the experiment (Figure 4.9a-c). In some tortoises the levels reached baseline (non-reactive), others stabilised well above the cut-off value.

Following inoculation with live BIV, the levels of BIV-like antibody in one animal (O) with initial high levels continued to decline until day 7 post-inoculation, when a gradual increase to high levels started (Figure 4.9a). Another animal (F) with high levels at 60 days prior to experimental exposure showed a decline to and stabilisation near the cut-off value, even after inoculation. Other individuals displayed a general trend of increase in BIV-like antibodies during the experimental period. After four weeks, 5 out of 6 inoculated animals had antibody levels above the cut off limit.

In the co-habitation group (Figure 4.9b), tortoise L exhibited a strong trend of increase in levels of serum antibody against BIV following exposure to inoculated animals. Two tortoises (D and I) displayed initial low levels which increased slightly over time, but at the end of the experiment 4

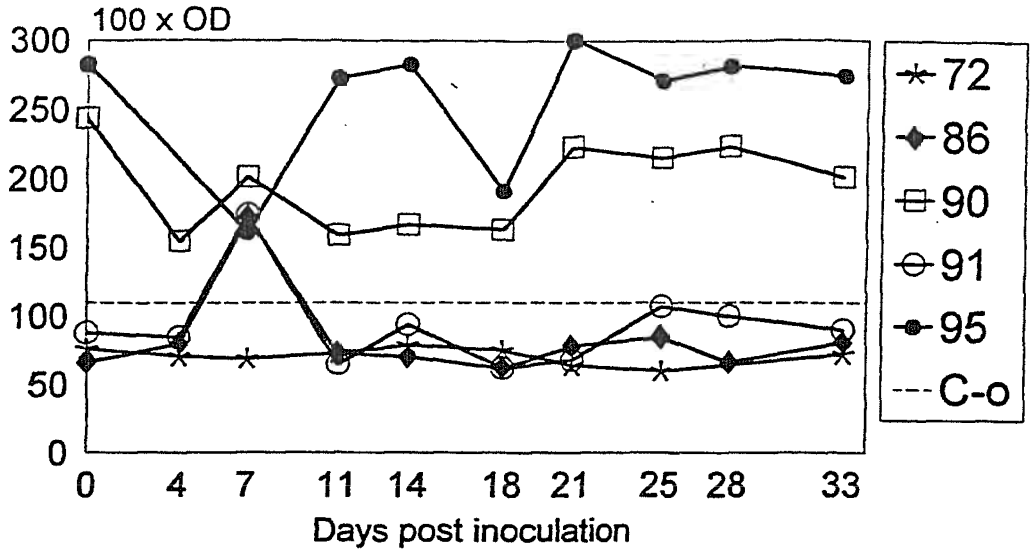
out of 5 animals were in the vicinity of the cut-off level, and only tortoise U, which had consistent high levels both prior to and during the experiment, was well above the rest.

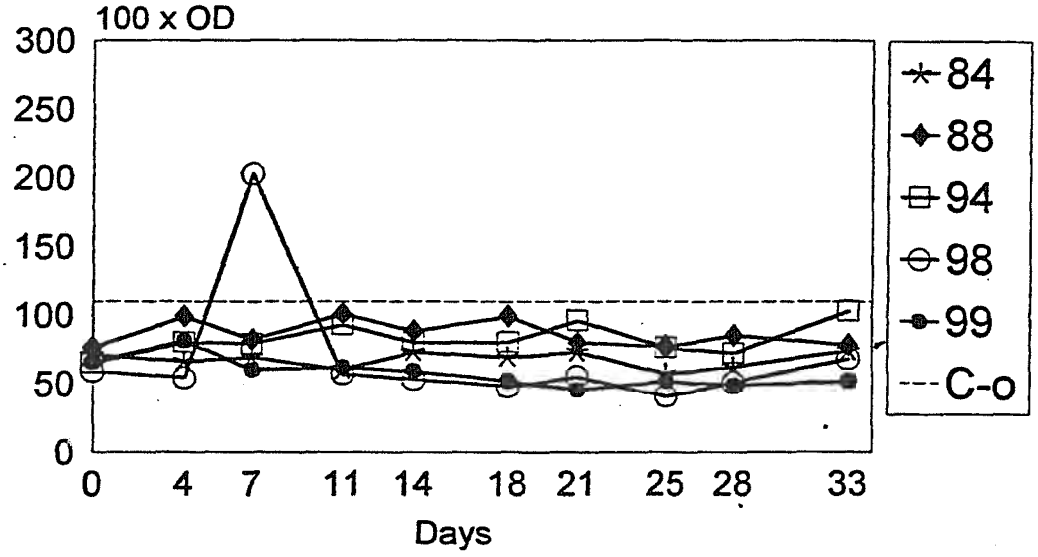
In the control group (Figure 4.9c), three animals had titres above the cut-off value for reactive serum when first tested. They all displayed a decline in BIV-like antibody levels over the 60 day quarantine period, two fell below the level of the cut-off value for reactive serum, while one with very high initial levels declined and stabilised at medium reactive levels. No increase in BIV-like antibody levels was detected for any of the control animals.

Figure 4.8a Levels of antibody directed against BIV in the inoculated group of *Elseya latisternum* following experimental infection for tortoises number 72, 86, 90, 91 and 95. Cut-off level between reactive and non-reactive serum is indicated by a broken line.

Figure 4.8b Levels of antibody directed against BIV in the co-habit group of *Elseya latisternum* during the experimental period for tortoises number 84, 88, 94, 98 and 99. Cut-off level between reactive and non-reactive serum is indicated by a broken line.

Figure 4.8c Levels of antibody directed against BIV in the control group of *Elseya latisternum* during the experimental period for tortoises number 82, 87, 89, 92, 96 and 97. Cut-off level between reactive and non-reactive serum is indicated by a broken line.


100 x OD
300
250
200
150
100
50
0
0 4 7 11 14 18 21 25 28 33
Days post inoculation
72
86
90
91
95
C-o


100 x OD
300
250
200
150
100
50
0
0 4 7 11 14 18 21 25 28 33
Days
84
88
94
98
99
C-o

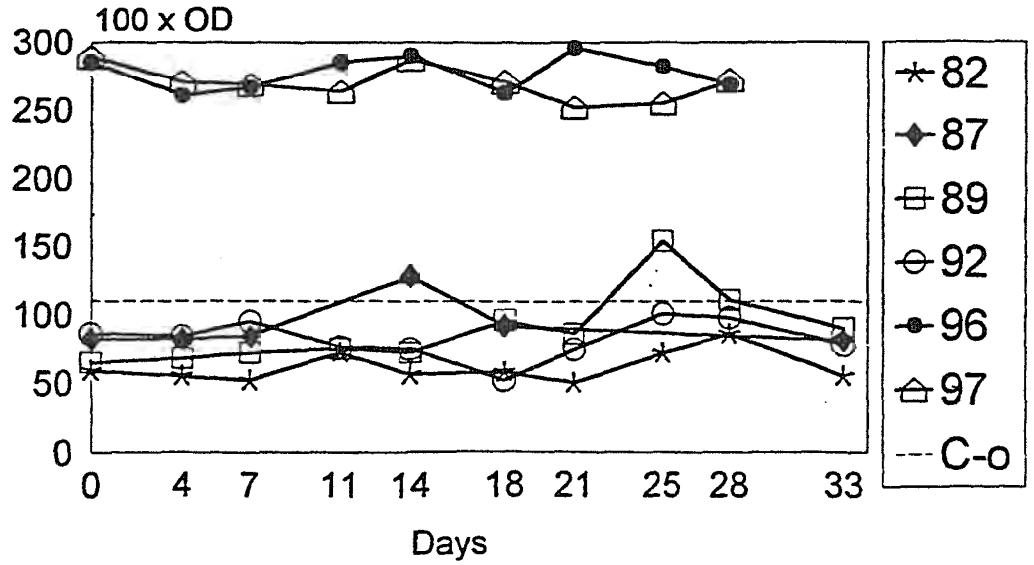
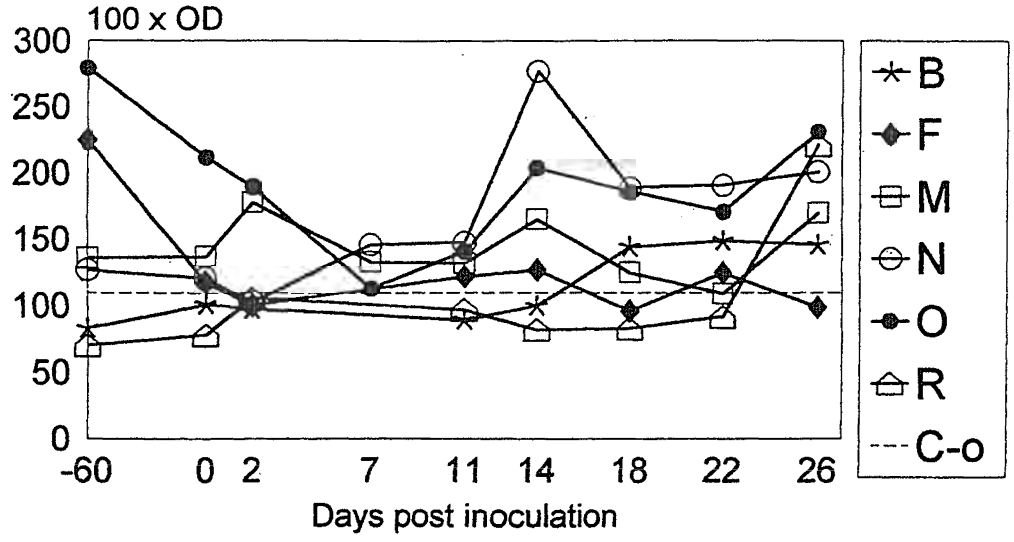
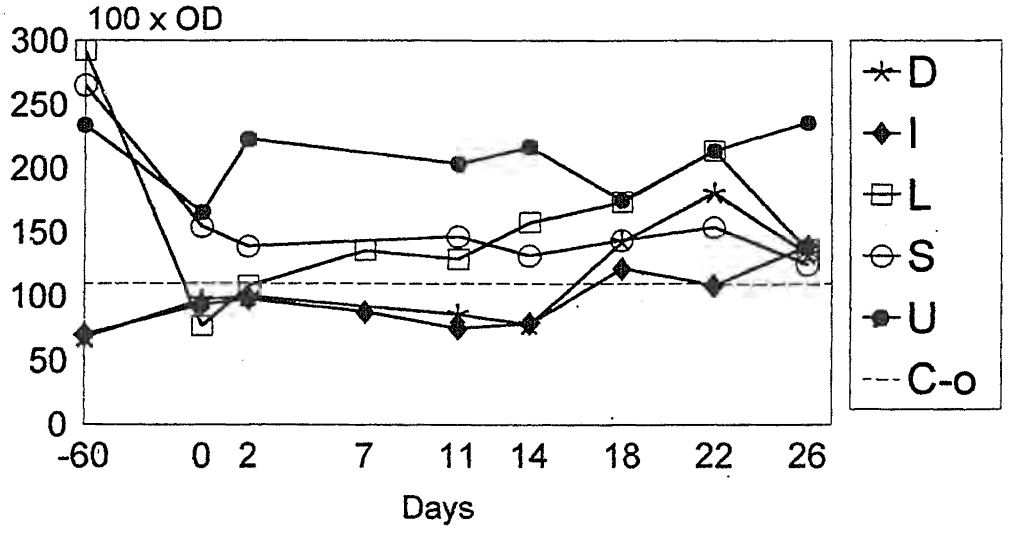

100 x OD
300
250
200
150
100
50
0
0 4 7 11 14 18 21 25 28 33
Days
82
87
89
92
96
97
C-o

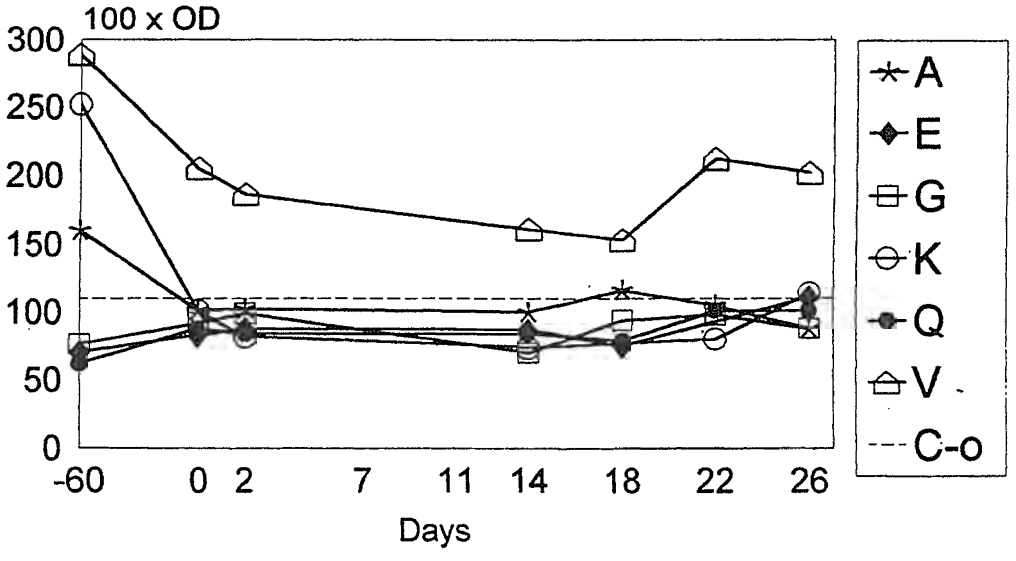
Figure 4.9a Levels of antibody directed against BIV in the inoculated group if *Emydura krefftii* following experimental infection for tortoises B, F, M, N, O and R. Cut-off level (C-o) between reactive and non-reactive serum is indicated by a broken line.

Figure 4.9b Levels of antibody directed against BIV in the cohabit group of *Emydura krefftii* during the experimental period for tortoises D, I, L, S and U. Cut-off level (C-o) between reactive and non-reactive serum is indicated by a broken line.

Figure 4.9c Levels of antibody directed against BIV in the control group of *Emydura krefftii* during the experimental period for tortoises A, E, G, K, Q and V. Cut-off level (C-o) between reactive and non-reactive serum is indicated by a broken line.


100 x OD
300
250
200
150
100
50
0
-60
0
2
7
11
14
18
22
26
Days post inoculation
B
F
M
N
O
R
C-o


100 x OD
300
250
200
150
100
50
0
-60
0
2
7
11
14
18
22
26
Days
D
I
L
S
U
C-o


100 x OD
300
250
200
150
100
50
0
-60
0
2
7
11
14
18
22
26
Days
A
E
G
K
Q
V
C-o

Emydura krefftii **hatchlings**

The antibody response to BIV in inoculated *E. krefftii* hatchlings during the trials remained below the cut-off level for reactive serum as established for adults (Figure 4.10).

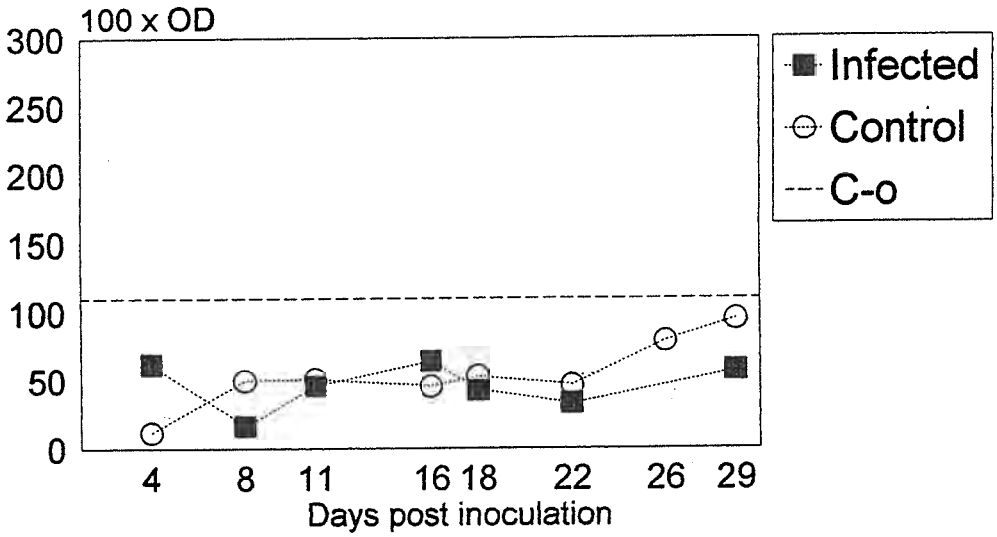


Figure 4.10 Levels of antibody directed against BIV for individual inoculated and control *Emydura krefftii* hatchlings "killed" at various days post-inoculation. Cut-off level between reactive and non-reactive serum for adults is indicated by a broken line.

4.3.2 Mortalities and viral isolation

None of the adult tortoises died during the experiments and there were no mortalities in the control group of hatchlings from either species of tortoises. Despite there being no external signs of life in some hatchlings designated 'dead', a heartbeat was noted in two cases at necropsy. Virus was re-isolated from experimentally infected hatchlings only. Attempts at virus isolation from control animals and from experimentally infected adults were unsuccessful. Cases of BIV re-isolation are represented below alongside mortalities encountered in the hatchling experiments (Table 4.2).

All of the inoculated *E. latisternum* hatchlings died before the four week trial was over, two died on day 10 pi and three on day 20 pi. Virus was isolated in culture from two of those hatchlings. Five of the12 inoculated *E. krefftii* hatchlings died during the experiment and BIV was re-

isolated from three of those and from one hatchling sampled on day 8 pi as part of the experimental schedule.

Table 4.2 Natural mortality and cases of successful viral isolation for *Elseya latisternum* and *Emydura krefftii* hatchlings following experimental inoculation with live BIV.

	Natural mortality (days pi)	BIV re-isolated
Elseya latisternum	10	yes
	10	no
	20	yes
	20	no
	20	no
Emydura krefftii	(8)*	yes
	16	no
	22	yes
	24	no
	25	yes
	29	yes

* Infected hatchling killed and processed as part of scheduled sampling.

Two batches of eggs were collected at necropsy from females in the control group, one had 100% hatching success the other 0%. The batch collected from a BIV-infected female produced two hatchlings from 7 eggs. One of those hatchlings appeared normal, the other was pale, had external egg yolk and a folded shell which had not straightened out one week post-hatching (Figure 4.11).

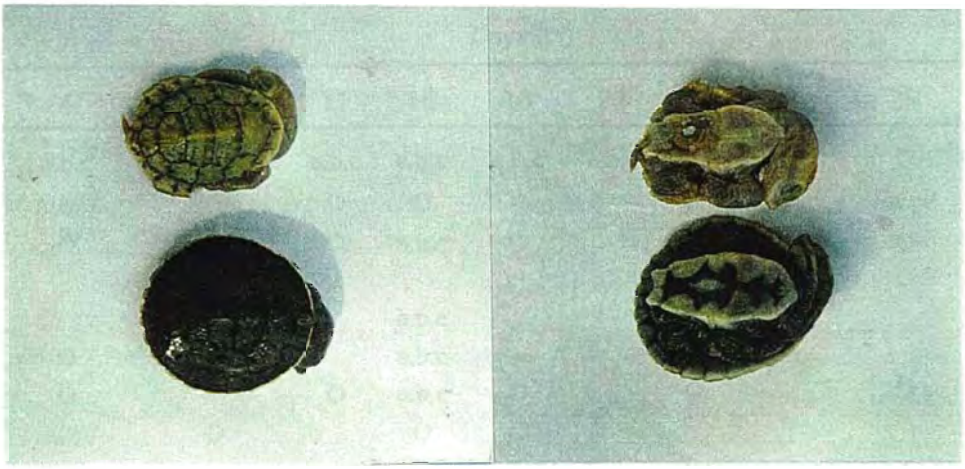


Figure 4.11 The only two surviving hatchlings in a batch of seven collected post mortem from a BIV-infected female *Emydura krefftii*. The dark hatchling emerged after 70 days incubation, the yellow hatchling 12 days later.

4.3.3 Histology

For both species of adult tortoises, there was no obvious evidence of histological changes which could be attributed to a BIV infection. It was impossible to distinguish between viral lesions and post mortem changes in the *E. latisternum* hatchlings, despite two hatchlings being necropsied immediately after death.

Not all *E. krefftii* hatchling organs were consistently available for examination. Because of the small size of the animals, the whole viscera of each individual was mounted in a paraffin block and subsequently sectioned. The organs present in a section was dependent on the orientation and level of the section. Table 4.3 lists histological changes in examined organs from *E. krefftii* hatchlings following inoculation with live BIV (Table 4.3a) and placebo inoculation with PBS (Table 4.3b). No evidence of bacterial (apart from gut flora) or parasitic infection were noted in histological sections.

Table 4.3a Histological changes in organs from *Emydura krefftii* hatchlings following experimental inoculation with live BIV. Organs examined with no lesions (O), with elevated numbers of eosinophilic granular cells (//////) or other distinct lesions (a,b,c).

	Days (pi)									
Organ	4	8	11	16	18	22	25	29	29	29
Liver	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c		O	O
Lung	/////////	/////////	O	O			O	O	/////////	O
Gut	O	O	O	a,b,c	O		a,b,c	a,b,c	O	
Kidney							a,b,c	a,b,c	O	
Spleen	/////////			a,b,c						
Pancreas	O			a,b,c						
Muscle	O		O	a,b,c	O		O		O	
Heart	/////////	O		O	O					
Thymus	/////////				/////////					
Thyroid										
Yolk	O	O	O	O						

a = karyorrhexis of cells b = haemorrhage
c = infiltration of eosinophilic granular cells and macrophages

Table 4.3b Control hatchlings. Histological changes in organs from *Emydura krefftii* hatchlings in the control treatment. Organs examined with no lesions (O), or with elevated numbers of eosinophilic granular cells (//////).

	Days (pi)									
Organ	4	8	11	16	18	22	25	29	29	29
Liver	O	O	O	/////////	O			O	O	O
Lung	O	O	O	O	O			/////////	O	O
Gut	O	O	O	O	O			/////////		O
Kidney	O				O			O		
Spleen	O		/////////							
Pancreas	O	O			O			O		O
Muscle	O	O	O		O					
Heart	O	O	O	O						
Thymus	O	O		/////////					/////////	
Thyroid	O									
Yolk	O	O	O							

Distinct lesions occurred in organs of infected *E. krefftii* hatchlings which were absent in non-infected animals (Table 4.3). These included areas of focal necrosis with karyorrhexis of cells, haemorrhage, infiltration of eosinophilic granular cells and macrophages, which were most prominent in the liver (Figure 4.12), and submucosa of the gut (Figure 4.13), but individual animals also had lesions in the kidney (Figure 4.14), spleen (Figure 4.15), pancreas and/or muscle. Professor Philip Ladds (JCU Pathologist, pers. comm., 1996) examined the sections. There were no difference in lesions between animals that died by their own account and those that were killed.

4.3.4 Parasites

Nematodes (*Camallanus* sp.) and trematodes (*Aspidogastrea* sp.) were frequently found in the stomach, small intestine and large intestine of *E. latisternum* (Appendix 4). Cysts with nematodes were collected from the oesophagus of two tortoises. Additionally, pentastome (unidentified) in cysts were encountered in the lungs of one individual and in the stomach of another.

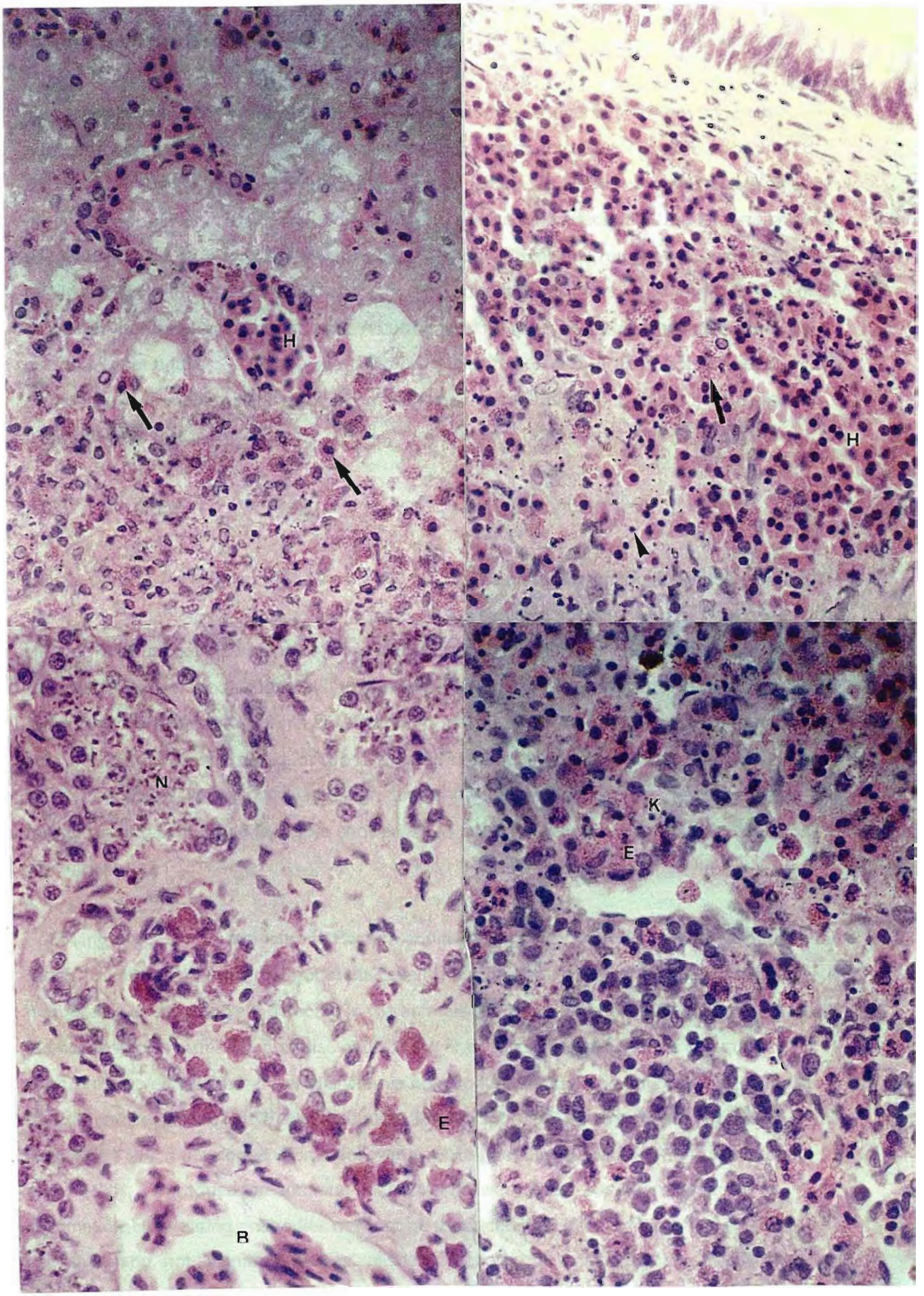
Parasites were found in only three of the *E. krefftii* tortoises, they were all nematodes (*Camallanus* sp.). In tortoise A, one male and one female nematode were found in the lining of the stomach, with their heads buried in a crater-like ulcer containing calcified material. Some haemorrhage was associated with this lesion. In tortoise Q, two small female nematodes were present at the junction of the stomach and the small intestine. One very small red, immature nematode was found in the small intestine of tortoise V.

(Top Left)
Figure 4.12 Necrosis in the liver from a BIV-inoculated *E. krefftii* hatchling. The necrotic area in the top half of the picture is characterised by karyorrhexis, eosinophilic material and macrophages. An elevated number of eosinophilic granular cells (arrows) are evident especially along the margin of the necrotic area. Haemorrhage (H) can also be seen. (Haematoxylin and eosin, × 482).

(Top Right)
Figure 4.13 Haemorrhage (H) and necrosis in the submucosa of the large intestine of a BIV-inoculated *E. krefftii* hatchling. A large amount of eosinophilic material (arrow) is present and karyorrhexis (small arrow) is exhibited in the necrotic area. (Haematoxylin and eosin, × 516).

(Bottom Left)
Figure 4.14 Necrosis (N) and eosinophilia (E) in the kidney of a BIV-inoculated *E. krefftii* hatchling near a blood vessel (B). (Haematoxylin and eosin, ×654).

(Bottom Right)
Figure 4.15 Eosinophilia (E) associated with cells undergoing karyorrhexis (K) in the spleen of a BIV-inoculated *E. krefftii* hatchling. (Haematoxylin and eosin, ×620).


H
H
N
K
E
E
B

4.4 Discussion

This study showed that Bohle iridovirus infected and was susceptible following parenteral administration to hatchlings of *E. latisternum* and *E. krefftii* tortoises. Thus the host range of BIV includes not only fish and amphibians, but also reptiles. *E. latisternum* and *E. krefftii* adults produced detectable levels of antibody to BIV and survived the infection. A bio-accumulation of BIV is likely to occur in tortoises because they feed on animals that may be infected. These qualities make adult *E. latisternum* and *E. krefftii* good indicator species for the presence of BIV in their environment in the recent past. A cut-off level for reactive and non-reactive serum was established for adults of both species. The above information and the ELISA described previously, now enables a survey of serum proteins reactive to BIV in tortoises from Australian freshwater systems.

4.4.1 Serology

Hayes, Daniels, Maxfield and Wheeler (1964) reported neutralizing antibodies in the serum of a box turtle (*Terrapene carolina*) for 18 months after a single inoculation with Eastern encephalitis virus. In contrast, rainbow trout experimentally exposed to EHNV only raised an antibody response after four inoculations with the antigen (Whittington et al. 1994). Serum antibodies to BIV can reveal a past exposure in *E. latisternum* and *E. krefftii* tortoises for at least one month after the event. This was noted for two *E. latisternum* adults which were kept in isolation. Two *E. krefftii* tortoises, which initially had high titres showed a decline to below detection over a two month holding period and therefore showed up as false non-reactors at the onset of the experiment.

Following experimental exposure to Eastern equine encephalitis, viremia developed in 6 spotted turtles (*Clemmys guttata*), but not in the seventh (Smith and Anderson 1980). Subsequently, neutralizing antibodies were detected in the animals with viremia, whereas the single turtle which did not develop viremia did not raise neutralizing antibodies. Another species of turtle (*Chrysemys picta*) did not develop a viremia nor produce neutralizing antibodies after similar experimental exposure (Smith and Anderson 1980). Out of 16 Texas tortoises (*Gopherus berlandier*) which developed viremia following subcutaneous inoculation with Western equine encephalitis, 11 produced neutralizing antibodies (Bowen 1977). Based on the above information, neutralizing antibodies may not be produced if an infection does not establish, on the other hand, not all animals with established infections produce neutralizing antibodies.

In the present study, the ELISA does not differentiate between antibodies that do or do not neutralize BIV. However, the general antibody response may mimic that of the neutralizing response described above.

Not all tortoises displayed a noticeable rise in specific antibody levels following inoculation with live BIV. Under the conditions applied, infection may not have established in some inoculated adults and therefore they did not respond by producing antibodies (Smith and Anderson 1980). Alternatively, the four week trials were perhaps too short a period for consistent detection of a response, which under certain circumstances is mounted much later in reptiles than a typical mammalian response (Avtalion et al. 1976). Hatchlings probably did not live long enough and adults not followed long enough to demonstrate a significant immune response (E Jacobson, University of Florida, pers. comm., 1997). Similar arguments could apply for hatchlings, but additionally an immature immune system may account for the absence of a detectable immune response as described for snapping turtle hatchlings (Sidky and Auerbach 1968)

The ELISA values obtained for hatchlings may have been affected by the method with which serum was obtained (E Jacobson, University of Florida, pers. comm., 1997). Decapitation and tilting may result in contamination of the sample by other body fluids.

Some animals may have been naive to BIV and did not produce detectable levels of antibody during their first exposure (Borysenko 1978). Extremely high levels of reactivity, which were encountered in some individuals, could have reflected a high degree of innate immunity or, if adaptive, such levels may have required multiple exposure to BIV or exposure via alternative pathways.

The decline in levels of BIV antibody observed in two *E. latisternum* adults in the initial phase of the trial, followed by an increase or stabilisation two weeks after inoculation with live BIV may be explained by a new stimulation of an already hyper-immune animal (Ambrosius 1976). The same pattern was noted for certain *E. krefftii* adults.

PCR and viral isolation identify the antigen in question, which means that the infection must be either active or latent. The viral isolation technique identifies only a fraction of animals with an active infection, and PCR for BIV has not yet been optimised for viral detection in tissues. Any follow up work should include Western blots to demonstrate that specific antibody is present against specific viral protein. Specific antibodies persist after the infection recedes and their

detection gives evidence to the past exposure to the antigen (Shortridge and Oya 1984). The cut-off level in ELISA between BIV reactive and non-reactive tortoise serum was determined by monitoring BIV reactivity in the serum of quarantined and BIV-inoculated tortoises over time.

All experimental adult animals were obtained from the wild and their history of past exposure to BIV, if any, was unknown. No independent tests were available for determination of their immune status to BIV, so the ELISA could not be calibrated in any other manner. The cut-off level between reactive and non-reactive serum was selected as the level below which included 95% of the assumed non-exposed control animals.

4.4.2 Pathogenesis

Adult *E. latisternum* and *E. krefftii* were not adversely affected in the short term by inoculation with live BIV. Virus could not be re-isolated from inoculated individuals and no lesions were observed which could be related to a BIV infection.

In contrast, hatchlings of both species appeared to be highly susceptible to BIV. Infection was lethal over a period of 4 weeks and BIV could be re-isolated from 1/3 of inoculated individuals. Infected hatchlings developed lesions in several organs, although mainly the liver was involved.

Viral isolation *in vitro* from BIV infected animals is an accurate, but not a very sensitive technique for detecting an active BIV infection. Past inoculation trials for frogs and barramundi yielded only 1/6 and 1/4 re-isolations from inoculated animals, all of which succumbed to the infection and displayed typical BIV lesions microscopically (Cullen et al. 1995; N. Moody, DPI, Townsville, Pers. Comm., 1996). BIV could not be recovered on cell culture from inoculated adult tortoises, indicating that either (1) the infection had not established, or (2) it had been overcome by the immune system, or (3) virions were present at levels below detection by this technique.

In tested frog and fish species, juveniles were found to be more susceptible to BIV than adults (Cullen et al. 1995; Ariel and Owens 1997; N Moody, Pers. Comm., 1997). This pattern is clearly also repeated for the tortoises *E. latisternum* and *E. krefftii*. It is also likely to be the case under natural conditions and may explain why BIV has only been isolated once from the wild (Speare and Smith 1992). Because tadpoles, fish fry and tortoise hatchlings occur only during short seasons, and the progression of the infection from initial exposure to death occurs relatively

rapid (6 days to 4 weeks), the window of opportunity to isolate BIV from these animals is very small.

In addition to a more mature immune system, adult tortoises show basking behaviour which may elevate their body temperature to above the viral inactivation temperature of 32°C. Hatchlings tend to be more cryptic, and consequently colder in their first year when they are most susceptible.

Frye (1991) describes typical lesions associated with systemic viral infection in reptiles as mononuclear perivascular cuffing and inclusion bodies in infected cells. Neither of these lesions were obvious in the infected tortoise hatchlings. However, generalised necrosis and inflammation was observed in several organs indicating a systemic agent. There was no evidence of parasitic or bacterial cause for these lesions and they were absent in animals from the control treatment, which strongly suggests that BIV was the aetiologic agent. Due to time constraints, TEM and immunohistochemistry were not performed on the sections. Such methods would clearly prove the association of BIV with lesions.

Hatchling tortoises were found to be highly susceptible to BIV under experimental conditions and should be considered at risk in the wild during outbreaks of BIV. However, the adult tortoises were able to mount high levels of antibody directed against BIV, and survived BIV infection to provide the future with evidence of the viral presence in their environment. Susceptible animals die and leave no evidence of the virus, where indicator species, such as these adult tortoises live to 'tell the tale'.

Figure 5.1 The Australian freshwater crocodile, *Crocodylus johnstoni*. Note the long slender snout, the smooth skin covering the head and the enlarged post-occipital scutes (arrow) in a single row, close to the parietal region of the head.

CHAPTER 5

PATHOGENESIS OF BOHLE IRIDOVIRUS IN THE AUSTRALIAN FRESHWATER CROCODILE (*CROCODYLUS JOHNSTONI*)

5.1 Introduction

In Australia there are two species of crocodile: the freshwater crocodile, *Crocodylus johnstoni*, and the estuarine crocodile, *Crocodylus porosus* (Cogger 1992). This chapter is concerned with the former species.

The head of *C. johnstoni* is covered by smooth skin and the length of the snout, from its tip to a point midway between the eyes, is longer than twice the width of the head at the level of the eyes (Figure 5.1). Post-occipital scutes are present in a single row and are separated from the smooth parietal region of the head by less than 8 granular scales. Males may grow to a maximum length of 3 m (Cogger 1992).

Freshwater crocodiles inhabit inland billabongs, creeks, rivers and associated flood-plains of Northern Australia. During the dry season they congregate in permanent billabongs. *C. johnstoni* always occur upstream from *C. porosus* and there is only minimal overlap in habitat (Webb and Manolis 1989).

Crocodylus johnstoni hunt in the shallows primarily at night. During the day they may bask on the banks or rest on the bottom of the water-body or in excavated borrows with underwater entry (C. Limpus, QDOE, Brisbane, Pers. Comm., 1995). During the nesting season in September/October, a typical female *C. johnstoni* digs a hole in suitable soil or a sandbank and deposits approximately 20 eggs (Cogger 1992). The incubation time of the eggs averages 75 days in the field (Webb and Manolis 1989). Newly hatched young will call from within the nest and the female will attempt to excavate the nest to aid the young. Hatchlings stay together in a creche and are usually accompanied by a female for a short period after hatching. The young vocalise when disturbed, which may illicit aggressive behaviour from the female (Webb and Manolis 1989).

Like other species of crocodiles (Cott 1961), it appears that there is a progressive shift in diet as the freshwater crocodile increases in size (Tucker et al. 1996). The young crocodiles eat a larger number of smaller prey items, whereas the larger animals eat a smaller number of larger prey

items. The overall diet of *C. johnstoni* consists of insects, crustaceans, frogs, fish, small snakes, lizards, tortoises, birds and small mammals (Webb and Manolis 1989; Tucker et al. 1996). Carrion is also taken.

Like the tortoises (Section 4.1), wild freshwater crocodiles may encounter fish and frog viruses in their diet and habitat. The aim of this experiment was to assess the pathogenicity of BIV in *C. johnstoni*, the ability of the crocodiles to produce antibodies, and consequently determine the ELISA cut-off level for reactive and non-reactive sera.

5.2 Materials and Methods

5.2.1 Source of animals

Animals were donated from captive populations and kept under permit (Scientific Purposes Permit, No. H0/000096/95/SAA) from QDOE. Experiments were covered by permit from the Experimental Animal Ethics Committee, James Cook University (Ethics Approval No. A302).

Yearling *C. johnstoni* were kindly donated by Hartley's Creek Crocodile Farm, Cairns (latitude 16°55'S; longitude 145°45'E). Two shipments were received, the first consisted of 20 animals and after one month a second batch of 8 arrived to replace some of the animals that had died in the first batch. The 16 crocodiles that underwent experimental BIV infection measured from 41 to 52 cm in total length and weighed between 165 and 427 g.

5.2.2 Experimental design

Crocodiles were individually marked by removing tail scutes according to the crocodile marking code described by QDOE (1995). Animals were then ranked according to weight and allocated to one of three treatments (control, inoculation with live BIV or co-habitation with inoculated animals) according to the experimental design (Table 5.1). Pre-experimental mortalities and intercurrent disease were thoroughly investigated as part of the monitoring of the well-being of experimental animals, but also to determine if these incidental findings were in some way influenced by the pathogen studied, or vice versa.

Table 5.1 Experimental design for 16 *C. johnstoni* infections with BIV. Animals were ranked according to weight and distributed sequentially into the three treatment groups. Control animals were in one pen (A), inoculated and co-habitation in two (B and C): Pen B: animals from clear spaces; Pen C: animals from shaded spaces.

Treatment	Animal Number in Ascending Weight Rank															
Control	Pen A	1			4			7			10			13		16
Inoculated	Pen B	2	Pen C	5		Pen B	8			11		Pen C	14			
Co-habitation		3			6			9			12		Pen B	15		

Control animals were housed together in one pen (A). Inoculated and co-habitation animals were housed together in two recyclable pens (B and C). Pen B consisted of animals ranked into the clear spaces of the design table (rank 2, 3, 8, 9 and 15), pen C of animals ranked in the shaded spaces (rank 5, 6, 11, 12 and 14). All three pens were kept in a secure area with limited access. The pen with control animals was separated from the other two by a 2 m tall plastic sheet wall to reduce cross infection via aerosols. The walled off control area had separate access from outside the building. Control and co-habitation animals were given intraperitoneal inoculations of 500 μL PBS. Animals in the inoculated treatment were given 500 μL BIV ($10^{4.5}TCID_{50}$) intraperitoneally. The experiment was conducted over 5 weeks. Blood samples were collected twice weekly from every individual and the serum stored frozen at -20°C for later screening in ELISA for anti-BIV antibody levels. At the end of the experimental period, animals were killed by decapitation (Section 5.2.5) and samples collected for serology, viral isolation, histological, parasitological and bacteriological examination.

5.2.3 Husbandry

In handling and maintaining the experimental animals, the control treatment was allocated separate handling, feeding and maintenance equipment and was always attended to before infected animals. This procedure was meticulously followed to prevent accidental cross contamination via handling.

5.2.3.1 Pens

The crocodiles were housed in oval (2 m × 1 m) 1 tonne Reln™ bins which were tilted to provide a dry area at one end and a pool at the other. The water was heated to 29°C by two submersible heaters, which were positioned inside Besser™ concrete blocks to stop the animals from direct contact and possible burns. Electrical cords were hidden inside PVC pipes. Hide

areas were provided by placing "weather rite" board over the Besser blocks, leaving 2 cm between the board and the water level for breathing (Figure 5.2). The crocodiles were contained in the bins by a sheet of plywood over the wet area and netting over the remainder. Pens were drained and cleaned three times weekly and the water replaced with heated town water.

5.2.3.2 Handling

Individual crocodiles were gently pinned down by the neck and after assuring that no other animals were within striking range, the pinned animal was picked up by the neck and pelvic area. A rubber band was tied around the snout and the animal placed in a flannel bag while the pen was cleaned and the pen-mates processed. After blood sampling and/or feeding each animal was released back into the clean pen.

5.2.3.3 Feeding

Crocodylus johnstoni are notorious for being nervous and stop eating in response to stress (Peter Freeman, Hartley's Creek Crocodile Farm, Pers. Comm., 1995). The experimental *C. johnstoni* failed to eat voluntarily following translocation and after several mortalities, they were fed manually four times weekly, by placing a food bolus directly into their stomach via a perspex plunger. The animals were encouraged to open their jaws by pulling down gently on the skin directly under their head (Figure 5.3). A piece of garden hose with a large hole drilled through it at a perpendicular angle, was then placed in the mouth of the crocodile to keep the jaws open (Figure 5.4). A blended mixture of 75% kangaroo meat and 25% chicken heads as well as crocodile pellets supplied by Steve Peucker (Queensland Department of Primary Industries), was placed in the perspex plunger (kindly donated by Mark Read, Department of Zoology, University of Queensland). The plunger was then inserted through the hole in the hose and into the oesophagus (Figure 5.5), where its content was deposited. The plunger was cleaned and rinsed in 10% Dettol in hot water before feeding each animal.

Figure 5.2 Pen design for *Crocodylus johnstoni* young. Electrical cords for the submersible heaters inside the blocks, were protected in the white PVC pipe. A weather rite board over the blocks provided a hiding area alongside the heated blocks.

Figure 5.3 A gentle method for opening the jaws of crocodiles. While lifting the head off the substrate, the skin of the chin was pulled down until the jaws opened.

Figure 5.4 A piece of garden hose with a hole through it, kept the jaws open while permitting insertion of the plunger.

Figure 5.5 The plunger was inserted through the hole in the hose and into the stomach to deposit the food.

5.2.4 Serum collection

Crocodiles were restrained manually in a dorsal position on the upper surface of a tilted board to allow blood to drain to the tail by gravity. Blood was collected twice weekly with a 25 gauge needle and a 1 mL syringe from the ventral caudal vein (Figure 5.6) (Jacobson 1964). Samples were treated as described in Section 4.2.4.

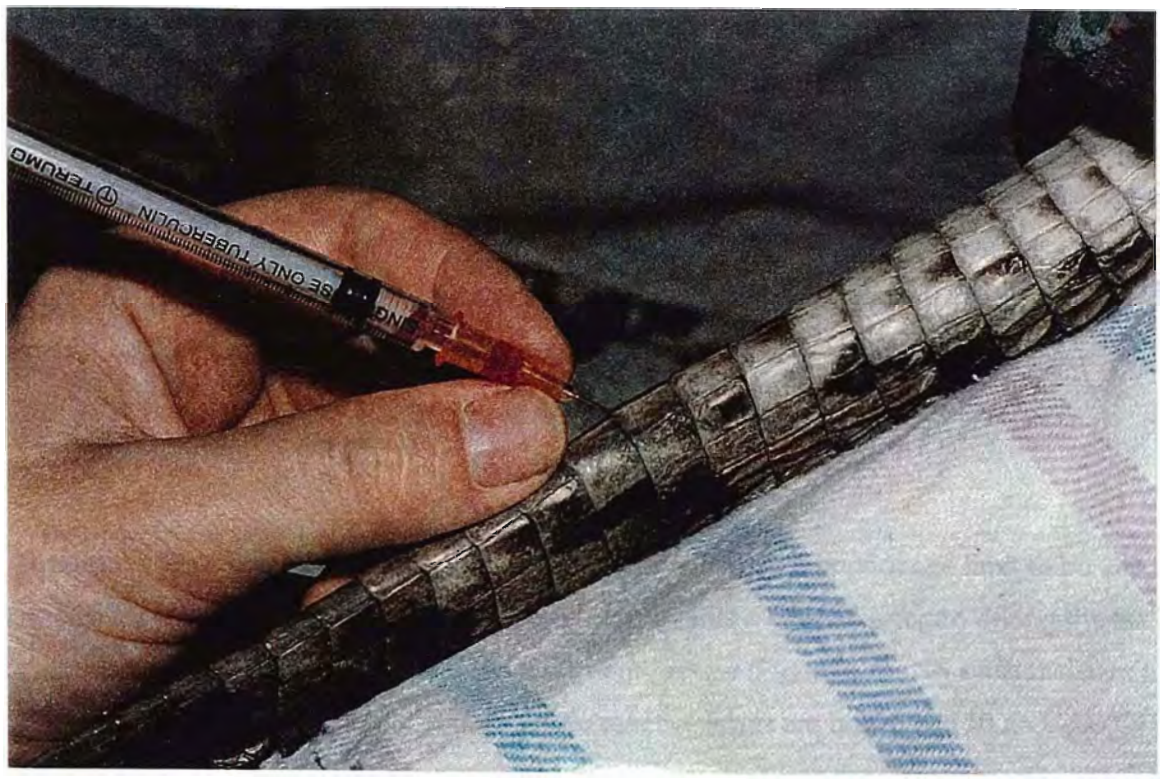
5.2.5 Post mortem procedures

Animals were checked every morning and any fatalities were necropsied immediately. At the end of the experimental trial animals were killed in the same manner as described for tortoises (Section 4.2.5). All dead, euthanised or killed animals underwent standard post mortem examination (Section 4.2.5). Exceptions were the three carcasses which had undergone prolonged exposure to the warm water in the pen and reached an advanced stage of deterioration. These animals were examined macroscopically, but tissues were beyond use for histological analysis.

Just prior to the experiment, one animal developed abnormal neurological behaviour accompanied by dehydration and opaque eyes (Figure 5.7). The condition was treated with 2.5 mg gentamycin per kg body weight (Shield 1991) and subcutaneous injection of saline for three days. The animal did not improve and was subsequently euthanased and examined post mortem. Bacterial isolation was attempted from the brain and kidney of the crocodile. Briefly, immediately post mortem, samples of kidney and brain were collected with sterile instruments and transferred to stomacher bags. The samples were homogenised with dH_2O streaked onto blood agar and MacConkey agar plates. The plates were incubated in 5% CO_2 at 28° and 37°C for 24 hours and colonies identified by Ms. J. Bradley, Department of Microbiology and Immunology, JCU.

Figure 5.6 Blood was collected from the ventral caudal vein with a 25 gauge needle and a 1 mL syringe.

Figure 5.7 Keratitis observed in this crocodile, together with signs of neurological disorder is indicative of meningitis.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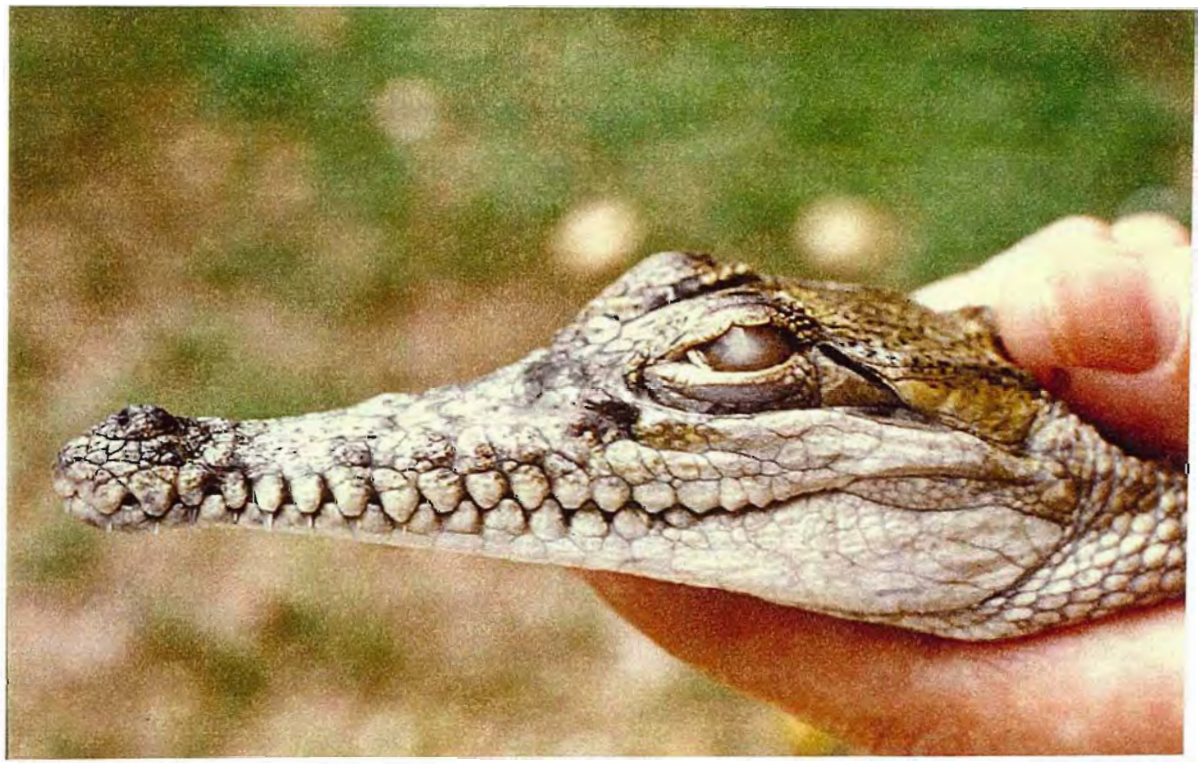


5.3 Results

From the time manual feeding was commenced, there were no spontaneous mortalities. Nor did any of the animals inoculated with BIV die. Over the course of the experiment the crocodiles became progressively more aggressive towards people and certain individuals vocalised when handled. Animals moved freely between dry land and the water, but were most frequently observed alongside the heated Besser blocks, under the "weather rite" cover.

5.3.1 Serology

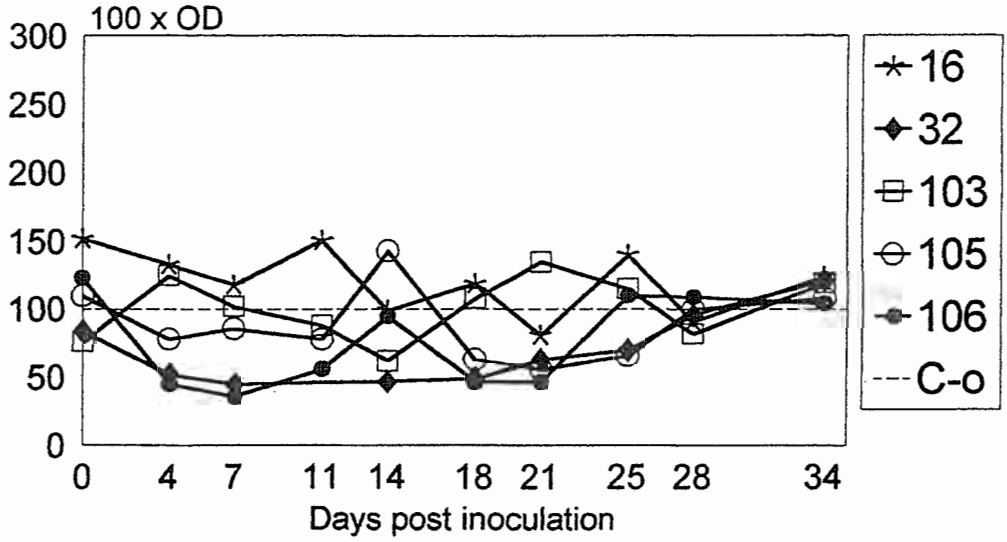
The maximum level for non-reactive serum was determined at 95% confidence limit to be OD × 100 = 110 for *C. johnstoni*. Levels of serum antibody against a BIV-like antigen during the five week experiment are displayed for the three treatment groups (Figure 5.8). Non-reactive sera are below the cut-off level of 110 OD × 100, medium reactive levels are between 110 and 200 OD × 100 and highly reactive sera are in the 200 to 300 OD × 100 category.

The levels of serum antibody in all inoculated animals were just above the cut-off point for non-reactive sera at the end of the experiment (Figure 5.8a). In the co-habitation treatment, the serum antibody levels for all animals were fairly stable throughout the experiment (Figure 5.8b). Two were consistently above the cut-off limit for BIV, whereas the others were below. Most animals in the control group exhibited decreasing levels of serum antibodies directed against BIV (Figure 5.8c). An exception was crocodile #101 which displayed an increase in levels of serum antibody directed towards BIV.

Figure 5.8a Mean levels of serum BIV-antibody in the inoculated group of *Crocodylus johnstoni* (crocodiles #16, 32, 103, 105 and 106) following inoculation. The cut-off (C-o) level between reactive and non-reactive sera is indicated by a broken line.

Figure 5.8b Mean levels of serum BIV-antibody in the co-habitation group of *Crocodylus johnstoni* (crocodiles #12, 13, 24, 25 and 108) during the experimental period. The cut-off (C-o) level between reactive and non-reactive sera is indicated by a broken line.

Figure 5.8c Mean levels of serum BIV-antibody in the control group of *Crocodylus johnstoni* (crocodiles #22, 28, 29, 101, 104 and 107) during the experimental period. The cut-off (C-o) level between reactive and non-reactive sera is indicated by a broken line.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Days post inoculation
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105
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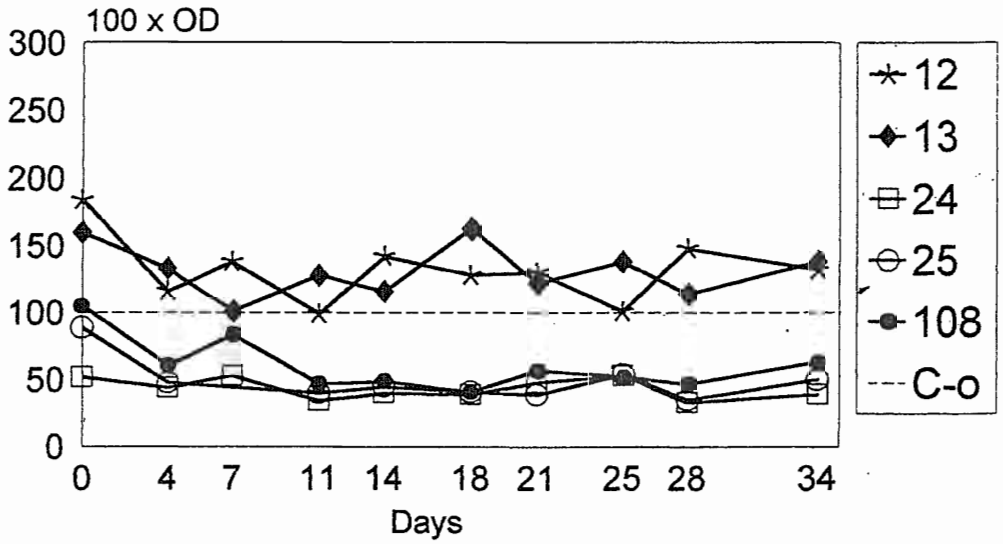
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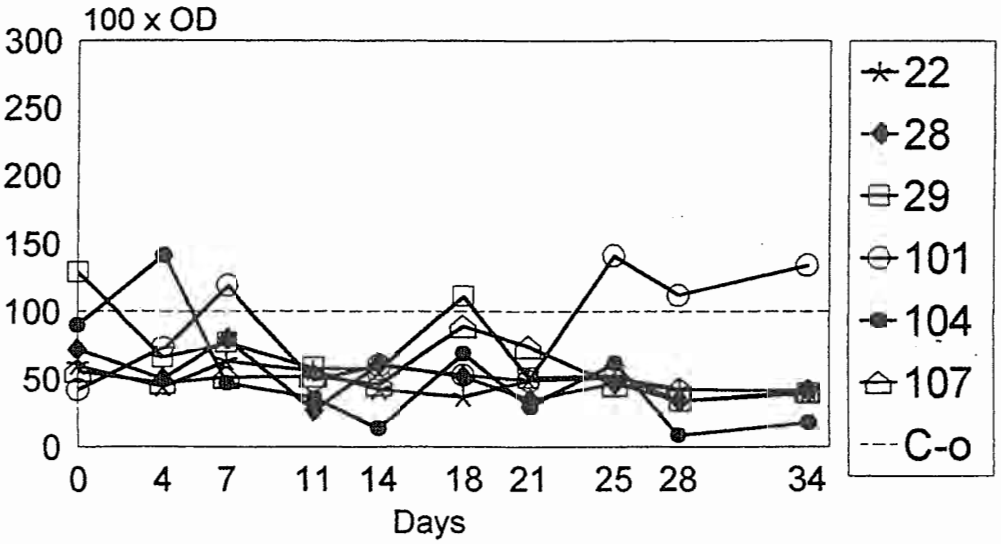
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5.3.2 Histology

There were no specific lesions limited to the experimentally infected or co-habitation group of *C. johnstoni* yearlings which could be ascribed to BIV infection. Other disease conditions became apparent either prior to, during or after the experimental period.

Twelve animals died prior to the experiment. Three were in a condition that could not be evaluated histologically and the cause of death is unknown. Gram-negative organisms were observed microscopically in the meninges of the brain from the animal which displayed signs of neurological disorder. A pure culture of *Providencia rettgeri* was isolated from the brain (Ms. J. Bradley, Department of Microbiology and Immunology, JCU). The isolate was resistant to tetracycline, but sensitive to gentamycin.

The remaining eight animals that died in the three weeks prior to the experiment, had lesions indicative of concurrent gout and hypovitaminosis A (Ariel, Ladds and Buenviaje 1997). During the necropsy examinations soft accumulations of white granular material were observed throughout the kidneys of all the animals and around the knee joints of one individual. White flecks of similar material were occasionally found on the serosa of liver, gastric mucosa, peritoneum and covering the entire pericardium. Pale brown nodules measuring up to 5 mm in diameter were present on the epithelium of the dorsum of the tongue of one individual (Figure 5.9).

Microscopically gout tophi were observed in sections of kidney, liver, spleen, stomach and heart tissues. Three apparently progressive stages of tophi development were noted in the kidneys (Figure 5.10).

Although samples processed for routine histology did not polarise light, the same fixed tissues cut with a cryotome, displayed birefringent particles in renal tubules. Squamous metaplasia and hyperkeratosis of the epithelium in the large collecting ducts of the kidney were concurrent with the tophi lesions (Figure 5.11).

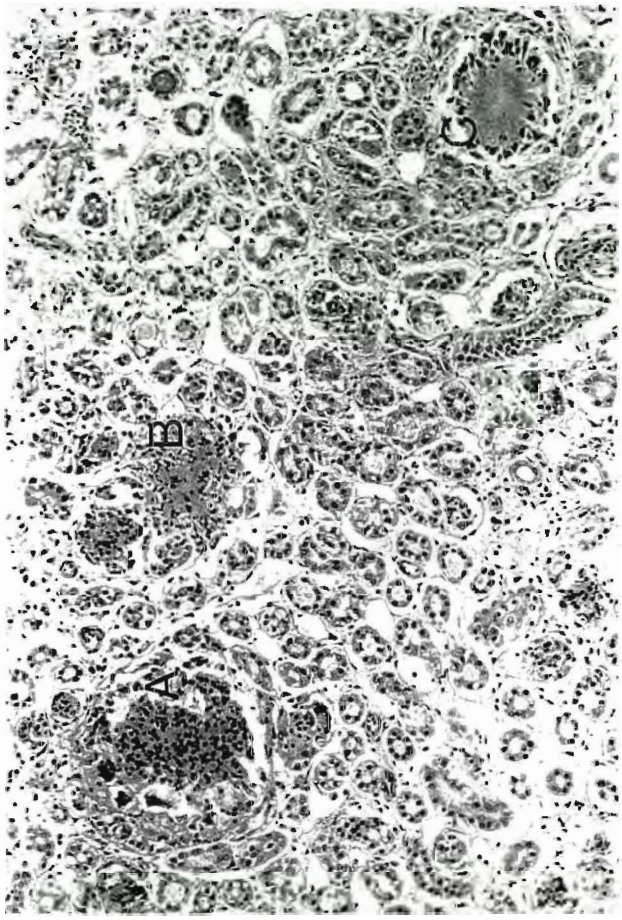
Diffuse pyelonephritis caused by bacterial infection was noted in six of nine *C johnstoni*. The lesions were contained within the renal tubules and consisted of eosinophilic debris, gram negative bacterial colonies and an intense infiltration of heterophils.

Figure 5.9 Macroscopic lesions in a *Crocodylus porosus* hatchling displaying numerous, predominantly pale raised nodules on the dorsum of the tongue.

Figure 5.10 Three presumed progressive stages of gout in the kidney of a *Crocodylus johnstoni* hatchling. The initial lesion (A) is an eosinophilic hyaline mass with a heavy infiltration of macrophages. The intermediate stage (B) is less hyaline and the macrophages more dispersed. The final type (C) shows the characteristic tophus and margination of macrophages (H&E ×140).

Figure 5.11 Concurrent lesion of squamous metaplasia (long arrow) and tophus (short arrow) in the kidney of *Crocodylus johnstoni* (H&E ×180).

Figure 5.12 Tongue lesion from *Crocodylus porosus* showing squamous metaplasia (long arrows) of glands and marked hyperkeratosis (short arrows). (H&E ×55).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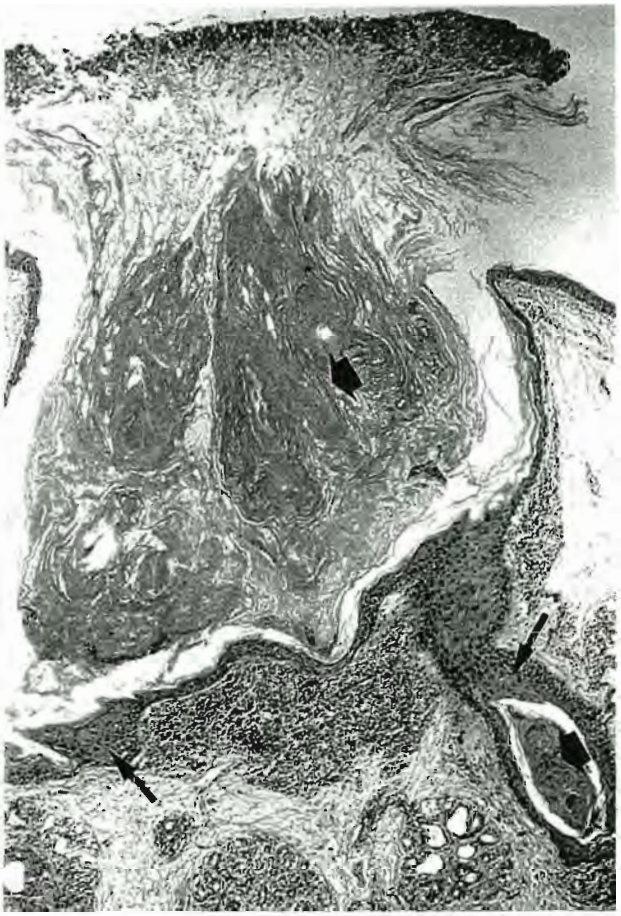
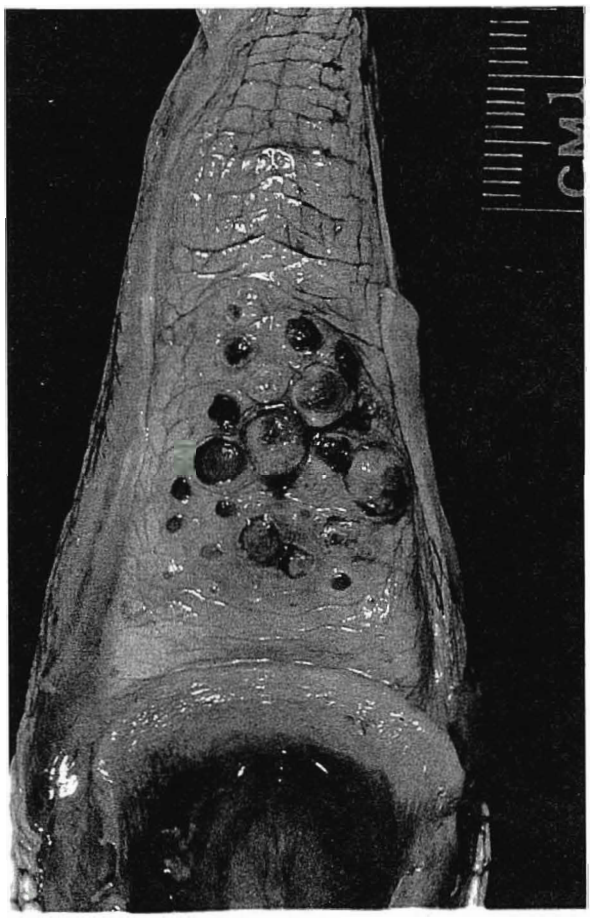
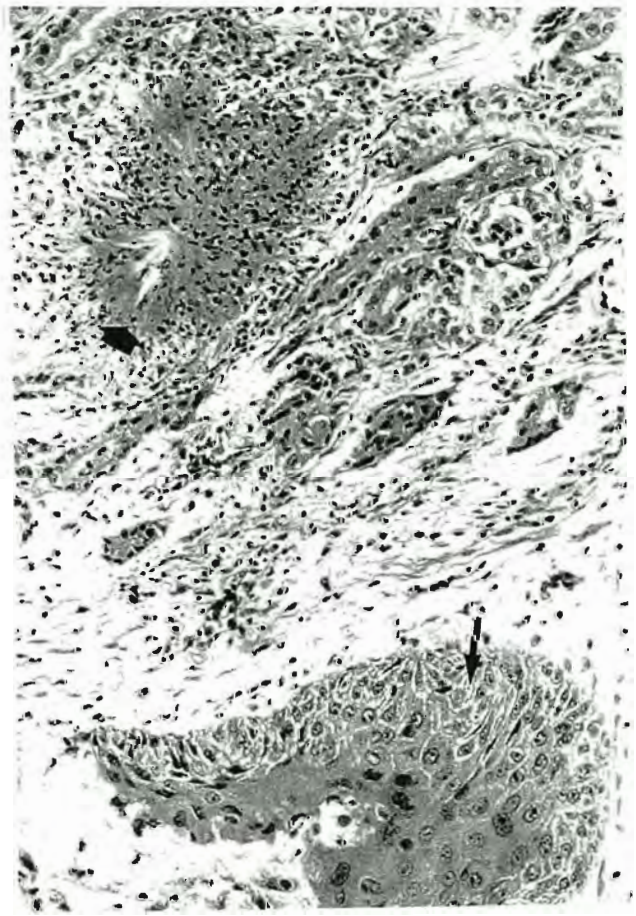



CM1



The tongue lesions consisted of squamous metaplasia with much keratin trapped in the affected glands (Figure 5.12) Opportunistic pathogens, including mycotic elements, and occasionally, filamentous organisms (*Dermatophilus* like) were present within the keratin. This type of tongue lesion was also noted in a crocodile (# 101) in the control group of the experiment.

Analysis of feed obtained from the farm revealed total protein content of 69% on a dry matter basis and a vitamin A content of 125 IU/kg dry matter (Yeerongpilly Veterinary Laboratory). Serum uric acid concentration in a hatchling diagnosed with gout and in the apparently healthy hatchling were 1248 mmol/L and 309 mmol/L, respectively (Consultant Pathologists, Townsville).

During routine post mortem examination of experimental animals, 12 cases of mycobacteriosis were identified in animals from all 3 treatment groups, including the original and subsequent shipment of crocodiles (Ariel, Ladds and Roberts, submitted). Macroscopically, raised white nodules were apparent on the surface of organs including lungs (Figure 5.13), liver, kidney and/or spleen. Multifocal, coalescent granulomas could be observed in sections of the above mentioned organs (Table 5.2) They were characterised by central zones of eosinophilic debris surrounded by multinucleated giant cells and histiocytes (Figure 5.14).

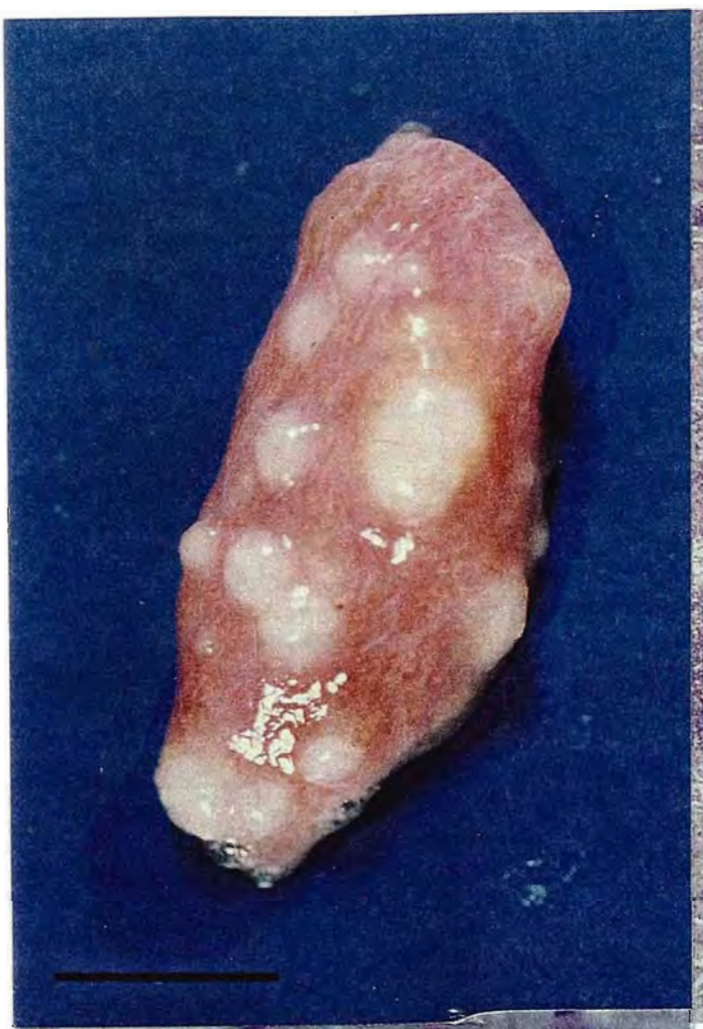
No causal organisms were observed in sections stained by the Gram or PAS method, but in Ziehl-Neelsen stained sections many large irregularly-shaped acid-fast deposits were seen within the eosinophilic zones noted above and within giant cells. Occasionally, strongly acid-fast bacilli ($\sim$ 1.8 μm) with a beaded appearance were seen within the cytoplasm of giant cells (Figure 5.15). These rods although never numerous were identified in 20 of the 24 tissues with granulomas. They were widely dispersed and could not be isolated *in vitro* on blood and MacConkey agar incubated at 28°C in a CO_2 incubator. The diagnosis of mycobacteriosis was therefore confirmed by the Polymerase Chain Reaction (PCR) technique using a universal primer for mycobacteria (Hance, Granchamp, Levy-Frebault, Lecossier, Rauzier, Bocart and Gicquel 1989). Frozen samples of liver, kidney, spleen and lung, in which acid-fast rods had been identified were tested. Two samples, spleen and lung, were positive for mycobacteria using PCR (Figure 5.16).

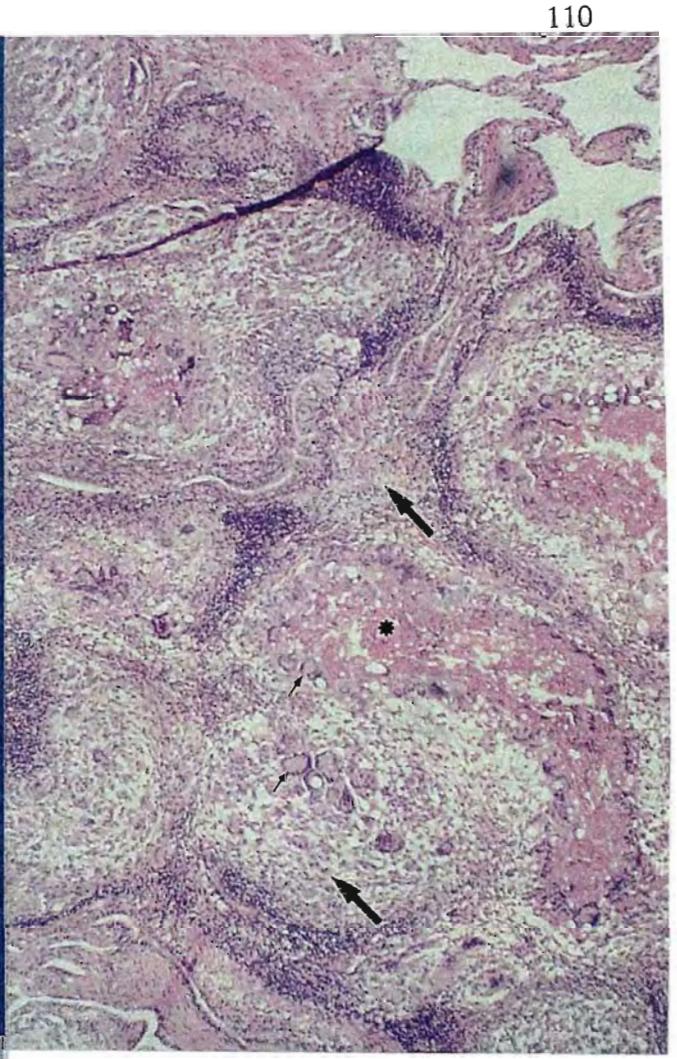
(Top Left)
Figure 5.13 Lung of *Crocodylus johnstoni* juvenile. Numerous white protruding nodules are evident beneath the pleura. Scale bar = 1 cm.

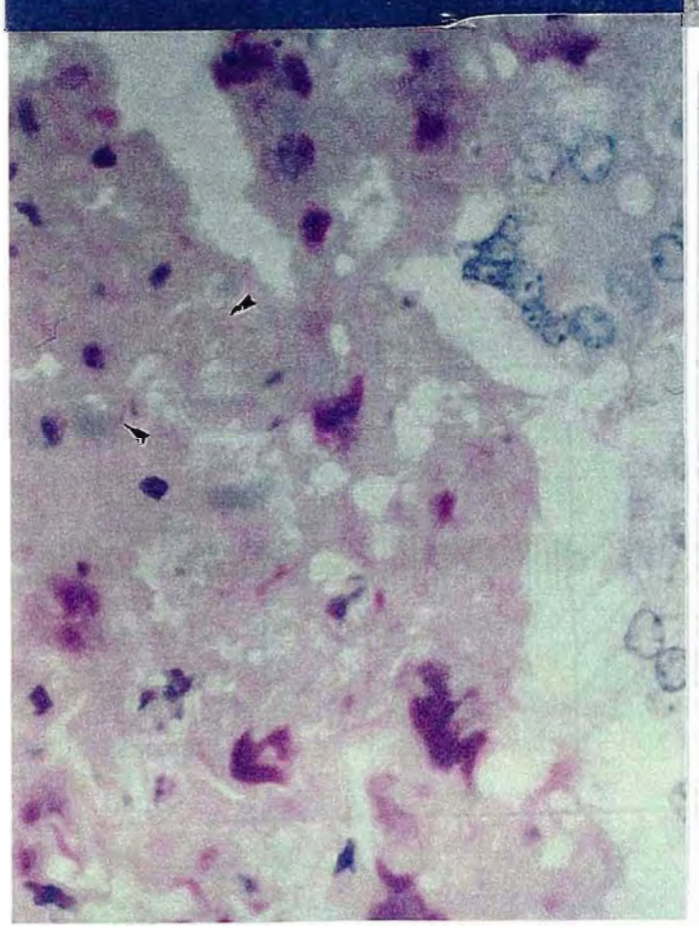
(Top Right)
Figure 5.14 Photomicrograph of hepatic lesion showing coalescence of several small granulomas with central zones of eosinophilic amorphous debris (*) bordered by multinucleated giant cells (small arrows). Interspersed within the granulomatous mass are numerous histiocytes (large arrows). Haematoxylin and eosin, ×116.

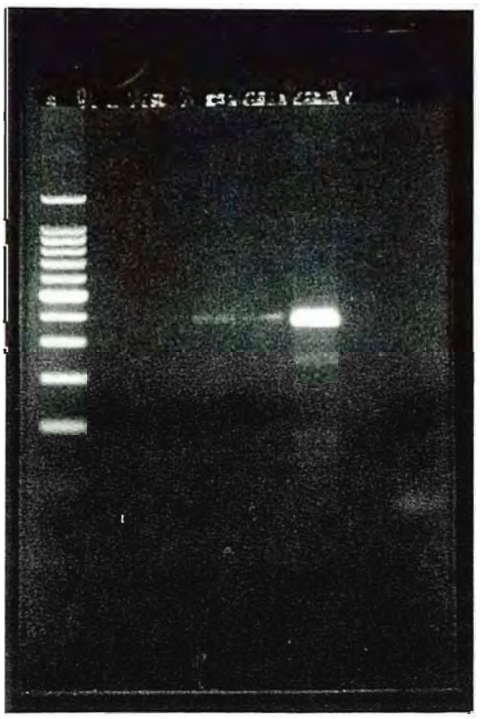
(Bottom Left)
Figure 5.15 High magnification of giant cell cytoplasm revealing acid-fast rods (arrowheads). Ziehl-Neelsen, ×180.

(Bottom Right)
Figure 5.16 PCR of nucleic acid purified from infected *Crocodylus johnstoni* organs producing a positive product of 384 base pairs.
Lane 1 Molecular weight standards
Lane 2 Live
Lane 3 Kidney
Lane 4 Spleen
Lane 5 Lung
Lane 6 Lung tissue spiked with *Mycobacterium ulcerans* DNA
Lane 7 -ve control
Lane 8 -ve control.





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A single animal (# 105) had swollen areas of gum in the vicinity of almost every tooth (Figure 5.17). Teeth were frequently lost from those areas. The cause of this condition was not determined.

Foci of cellular alteration was noted in the livers of 2 control animals, 4 infected and 4 from the co-habitation group (Table 5.2a, b). The foci were characterised by an apparent loss of 3-dimensional cellular arrangement (Figure 5.18).

Table 5.2a Lesions in examined tissues from *Crocodylus johnstoni* yearlings in the control group. Cross striations = multifocal granulomas; vertical lines = micronodular regeneration; solid spaces = focal necrosis or other lesions.

TREATMENT	CONTROL					
PEN	A					
Croc. No.	22	28	29	101	104	107
Liver						
Lung						
Spleen						
Kidney						
Tongue						

Table 5.2b Lesions in examined tissues from *Crocodylus johnstoni* yearlings in the BIV-inoculated and co-habit groups. Legend as for Table 5.2a.

TREATMENT	INFECTED			CO-HABIT						
PEN	B			C					B	
Croc No.	32	106	103	16	105	13	24	12	25	108
Liver										
Lung										
Spleen										
Pancreas										
Gums										

Figure 5.17 *Crocodylus johnstoni* with 'bubble gums'. The gums around almost every tooth are swollen and teeth were frequently lost.

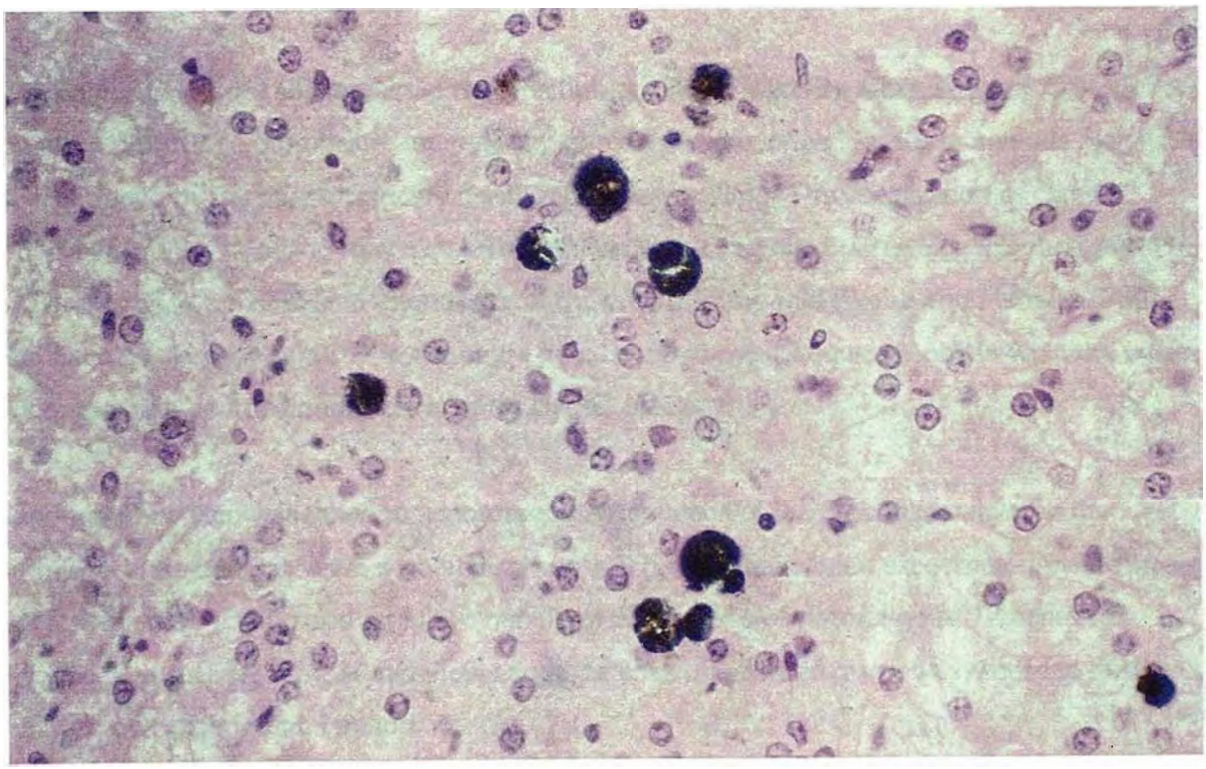


Figure 5.18 Liver of *Crocodylus johnstoni* showing micronodular regeneration. Haematoxylin and eosin. ×430

5.3.3 Viral isolation and parasites

Viral isolation from organs of experimental animals was not successful. Examination of animals post mortem did not reveal any parasites externally, or internally.

5.4 Discussion

The freshwater crocodiles in this group of experimental animals did not appear to be adversely affected in the short term by intraperitoneal exposure to live BIV. A clearer picture of an immune response to inoculated BIV may have been obtained had the experiment been extended over a longer time period. There were no definite trends in the response of inoculated animals. However, certain animals appeared to produce antibodies to inoculated BIV over the course of the experiment, and some individuals displayed pre-existing moderate levels of antibodies directed against a BIV-like antigen throughout the experiment. None of the lesions in experimental animals could be ascribed to a BIV infection, and virus was not re-isolated from any animals. The experiment should ideally have been extended over a longer time period in order to demonstrate a significant immune response.

Crocodylus johnstoni are very timid towards humans in the wild (Dr. Colin Limpus, QDOE, Pers. Comm., 1995). The progressive increase in aggression experienced for these animals may have been induced by the stress associated with repetitive handling. The vocalisation of certain individuals during handling may also be a manifestation of stress. Such a signal is thought to be a distress call to the female in attendance of the crèche in the wild (Webb and Manolis 1989). The animals did move around in the entire pen, but were most frequently observed in the water, under the hide board, alongside the warm Besser blocks.

Although every step was taken to accommodate the needs of the crocodiles while on the premises, there certainly were several diseases which could have been exacerbated by the stress involved in translocation and repetitive handling. Eight cases of visceral gout, 12 cases of mycobacteriosis, one case of meningitis and one case of inflamed gums was recorded during the five week experimental period.

The investigations of the feed, lesions and serum of the gout afflicted crocodiles, strongly suggest a causal link between the lesions and the diet which was high in protein and deficient in vitamin A. Relocating the group of freshwater crocodiles (*C. johnstoni*) hatchlings appeared to

hasten the onset of a subclinical condition resulting in mortalities. The hatchlings had been fed a diet containing 125 IU vitamin A/kg dietary dry matter, considerably less than the suggested requirement of 12000 IU/kg (Staton and Vernon 1991). The level of protein in the feed was 69%, as opposed to the recommended dietary allowance of 45% per kg dietary dry matter (Staton and Vernon 1991). A diet rich in animal protein, which contains purine and pyrimidine, both precursors of uric acid, can be expected to exacerbate a gout condition (Foggin 1987; Frye 1991).

Yolk derived vitamin A deposits in the liver may initially have compensated sufficiently for a diet low in vitamin A (Frye 1991). Continual depletion of such stores without replenishment, however, would eventually lead to hypovitaminosis A. Vitamin A deficiency affects mucus secreting epithelial tissues, which undergo squamous metaplasia. Metaplasia and hyperkeratosis of renal tubules in turn decreases renal clearance of urates (Harrison and Harrison 1986). Subsequently, reduced renal perfusion induces hyperuricaemia and accumulations of precipitated uric acid crystals (tophi) occur throughout the body (Frye 1991). If the condition progresses, obstruction of nephrons by tophi will eventually cause metabolic failure (Cotran, Kumar and Robbins 1994).

The serum uric acid concentrations of a crocodile with lesions of gout and hypovitaminosis A was four fold higher than that determined in a crocodile of similar age and size which had none of these lesions. Although the reference range of uric acid from *C. johnstoni* is not known, the value for the presumed healthy crocodile (309 mmol/L) is within reference ranges for other species of crocodiles: 83 to 446 mmol/L serum for *C. niloticus* (Foggin 1987), 155 to 339 mmol/L serum for *C. siamensis* (Siruntawineti, Ratanakorn and Homswat 1993) and 250 to 550 mmol/L for *Alligator mississippiensis* (Hernandez and Coulson 1983).

Renal ducts with squamous metaplasia are especially prone to bacterial infection (Foggin 1987), a condition relatively prevalent in these animals, which in turn could lead to reduced clearance of uric acid and thus potentiating gout. This observation supports the finding that 76% of crocodiles with renal gout also displayed pyelonephritis caused by gram negative bacteria (Buenviaje, Ladds, Melville and Manolis 1994).

The tongue lesions with excessive keratinised epithelium observed in this study, appear to provide useful clinical means for the diagnosis of hypovitaminosis A. To identify the urate crystals consistently by microscopical examination with polarised light, the tissues must be fixed

in absolute alcohol (Marcus 1981), as an aqueous solution will tend to dissolve the crystals. As described above, early stages of tophi development can possibly be identified in histological sections, prior to an appearance of the characteristic star burst lesion pathognomonic of gout (Marcus 1981). Serum uric acid concentrations should be evaluated in a number of normal and affected *C. johnstoni* to determine whether it is a useful index of gout in this species.

This investigation reinforces the notion that visceral gout in crocodiles can develop from hyperuricaemia through high protein consumption. However, additionally, it suggests a role for hypovitaminosis A in producing visceral gout by the mechanism of reduced renal excretion of uric acid through tubular squamous metaplasia and the subsequent predisposition to bacterial infection.

Mycobacteria are ubiquitous organisms which are commonly recovered from both diseased and apparently healthy reptiles (Brownstein 1984). Saprophytic and commensal mycobacteria may cause disease in compromised hosts, which seems to be the case for amphibians and reptiles in particular. The source of infection is contaminated soil, water or food (Thoen and Schliesser 1984).

Only two reports on crocodilian mycobacteriosis have been published in the last 40 years. Zwart (1964) observed 'tubercle bacilli' associated with renal granulomas in a *Caiman sclerops*, and *Mycobacterium avium* complex was cultured from granulomatous lesions in a crocodile (Thoen, Karlson and Himes 1981).

Although there are few published reports of mycobacteriosis in crocodilians, six cases of presumed mycobacteriosis have been diagnosed histopathologically in this department in addition to those in the outbreak now documented (Ariel, Ladds and Roberts, submitted 1997). Three of the previous cases were *C. porosus* and three were *C. johnstoni*. Five of the six animals were hatchlings or juveniles and in these the mycobacterial granulomas were in the sub-cutis – presumably originating from cuts or abrasions and in one case associated with interdigital subcutaneous emphysema ('bubble foot') (Turton et al. 1996).

The remaining case was a sub-adult *C porosus* with multiple disseminated caseating granulomas, similar to those in the present case, as well as fibrino-haemorrhagic lesions in the spleen, liver and lung (G Buenviaje, Pers. Comm., 1997). *Aeromonas hydrophila* was isolated by the Department of Primary Industries Oonoonba Veterinary Laboratory from each of the

above organs and both gram-negative and acid-fast bacteria were prevalent histologically. Additionally, a *Mycobacteria* sp. was isolated from lung but not from liver and spleen.

In the past, the general consensus was that mycobacterial infections in reptiles could be separated into either pulmonary or cutaneous/hepatosplenic types (Wallach 1969). The first sort of infection was assigned to chelonids and the latter to lizards, snakes and crocodilians. However, Rhodin and Anver (1977) reported atypical chelonian mycobacteriosis in the skin, liver and spleen of a *Phrynops hilari*. Our case of mycobacteriosis in crocodiles involved both pulmonary and visceral organs, but not the skin.

The *C. johnstoni* juveniles in the present study were presumably stressed by repetitive handling or winter temperatures even though pens were heated. Stress derived immunosuppression, in turn, could have facilitated the proliferation of an otherwise commensal organism. Alternatively a pathogenic strain may have been present.

Mycobacterial strains isolated from poikilothermic animals have often been reported to be resistant to antibiotics (Wallach 1969; Thoen, Richards and Jarnagin 1977; Brownstein 1984; Thoen and Schliesser 1984; Colorni 1992; Knibb, Colorni, Ankaoua, Lindell, Diamant and Gordin 1993). Further compounding a treatment is the lack of a suitable drug regime for reptiles (Thoen, Richards and Jarnagin 1977; Brownstein 1984) and recommendations at present are to destroy infected individuals and sterilise pens (Marcus 1971; Cooper and Jackson 1981; Frye 1991). Such drastic measures make prevention and prompt diagnosis especially important to large populations of captive animals, such as commercial crocodile farms.

Given the difficulty and length of time necessary for culture of mycobacteria from infected organs, and at times the scarcity of the organism in section, PCR may become the method of choice for diagnosing mycobacteria in the future, especially since tests can be carried out on samples obtained by biopsy as well as at post mortem. The mycobacteriosis reported here for Australian crocodiles was based on the diagnosis by conventional histopathology and confirmation by PCR.

Ladds, Bradley and Hirst (1996) reported three annual outbreaks of meningitis in captive *C. porosus* hatchlings. Animals displayed signs of nervous disorder and in some cases they had unilateral conjunctivitis. *Providencia rettgeri* was isolated in pure culture from the brain of an affected crocodiles. The crocodiles in this present study originated from a different farm. Like

the *C. porosus* isolate, the *P. rettgeri* isolate from *C. johnstoni*, was resistant to tetracycline, but sensitive to gentamycin. However, treatment with gentamycin and subcutaneous injection of saline did not improve the condition in the *C. johnstoni* yearling. *P. rettgeri* was also isolated from a lesion on the foot of a captive tortoise, and it may be part of the normal crocodile flora or a prevalent opportunistic bacteria in the reptile environment. This is the first report on *P. rettgeri* induced meningitis in *C. johnstoni*.

Micronodular regeneration

This chapter set out to investigate the short term pathogenesis of BIV in freshwater crocodile yearlings. However, there does not appear to be any pathological effects attributable to BIV under the experimental conditions applied. However freshwater crocodiles are able to produce antibodies to BIV, which became evident during the experiment. Some animals had consistently elevated levels of serum antibody against BIV, which is indicative of past exposure. Extrapolating from the pattern of antibody responses to BIV for fish, frogs and tortoises, where the very young are highly susceptible, it would be of great interest to determine the effects of exposure to live BIV in hatchling crocodiles.

Foci of cellular alterations, similar to the lesions observed in some of the *C. johnstoni* in this study, are believed to be precursors to hepatic tumors in rats (Popp and Goldsworthy 1989). Long term experimental exposure (18 months) of rats to hepatocarcinogens induce an increase in both size and number of foci, prior to tumor development. The foci observed in *C. johnstoni* reported here were neither associated with BIV-exposure, gout, vitamin-A deficiency or mycobacterial lesion. The cause of these lesions in *C. johnstoni* is unknown at present.

Figure 6.1 Brown tree snake (*Boiga irregularis*).

Figure 6.2 Common green tree snake (*Dendrelaphis punctulatus*).

CHAPTER 6

PATHOGENESIS OF BOHLE IRIDOVIRUS IN THE BROWN TREE SNAKE (*BOIGA IRREGULARIS*), THE COMMON GREEN TREE SNAKE (*DENDRELAPHIS PUNCTULATUS*) AND THE KEELBACK SNAKE (*AMPHIESMA MAIRII*)

6.1 Introduction

The brown tree snake (*Boiga irregularis*), the common green tree snake (*Dendrelaphis punctulatus*) and the keelback or freshwater snake (*Amphiesma mairii*) belong to the subfamily Colubrinae within the family Colubridae (Ehmann 1993). Each of these species have enlarged ventral scales in a single row, the tail is cylindrical and the anal scale divided. The head is distinct from the body and loreal shield is present between the pre-ocular and the nasal scales (Cogger 1992). These snakes occur throughout the northern and eastern coastal areas of Australia (Cogger 1992).

Boiga irregularis is characterised by its broad head on a long slender neck and large golden eyes with a narrow vertical pupil (Cogger 1992). The colour morph most commonly found in the Townsville region is light brown with many darker, irregular cross-bands (Figure 6.1). *Boiga irregularis* can be found in more arid habitats than the other snakes in the Colubridae family. This is thought to be facilitated by its nocturnal life which exposes it to cooler and less desiccating conditions (Ehmann 1993).

Dendrelaphis punctulatus is a slender, agile snake with a whip-like tail and smooth scales (Ehmann 1993). The colour varies greatly between individuals from "yellow-bellied black" (Figure 6.2) to a drab olive green. *Dendrelaphis punctulatus* is diurnal and basks in the canopy (Gow 1976).

Amphiesma mairii is a small, robust snake with strongly keeled scales (Cogger 1992). Colour varies between geographic locations, from red to black to grey with narrow irregular cross-bands (Figure 6.3). This snake may be either diurnal or nocturnal (Gow 1989), and basks on the ground or on the surface of shallow warm water (Ehmann 1993). It is the only known snake capable of dismembering its tail when caught, in a manner similar to lizards (Gow 1976).

Figure 6.3 Keelback snake (*Amphiesma mairii*).

Boiga irregularis is mildly venomous and rear-fanged, the venom aiding to subdue prey and initiate digestion. *Dendrelaphis punctulatus* and *A. mairii* are solid toothed and non-venomous (Cogger 1992). Both *B. irregularis* and *D. punctulatus* are arboreal, but can frequently be found foraging on the ground. *D. punctulatus* is also a competent swimmer (Gow 1976; Cogger 1992). *Amphiesma mairii* is semi-aquatic and always found near freshwater (Gow 1989).

The diet of *B. irregularis* consists mainly of birds, lizards, small mammals, and occasionally frogs. *Dendrelaphis punctulatus* and *A. mairii* eat tadpoles, frogs, fish and lizards (Gow 1976).

These three species of snakes were chosen for BIV-pathogenesis studies, because they were likely to eat frogs which are potential hosts of BIV. *Limnodynastes ornatus*, the ornate borrowing frog, is very common in the Townsville region and was the host for the original BIV-isolate (Speare and Smith 1992). In addition to the three treatments used in the previous infection studies of tortoises and freshwater crocodiles (Chapters 4 and 5), the treatment of feeding infected frogs to the snakes was attempted to determine whether this could be a possible route of infection.

6.2 Materials and Methods

6.2.1 Source of animals

Snakes were collected and kept under a scientific purposes permit (Number H0/000096/95/SAA) from the Queensland Department of Environment (QDOE). Experiments were covered by permit from the Experimental Animal Ethics Committee, James Cook University (Ethic Approval Number A302).

Boiga irregularis and *D. punctulatus* were kindly made available (courtesy Mike Pople, QDOE) in several batches through the QDOE snake relocation program for Townsville (19°15'S; 147°47'E). *Amphiesma mairii* were caught during a field trip to the Giru area south of Townsville (19°37'S; 147°5'S), with the help and expertise of Dr. Marc Hero (Department of Zoology, James Cook University). The sex of the snakes could not be determined with confidence. The 10 *B. irregularis* used in the experiment measured between 130 and 165 cm in total length (snout to tail tip) and weighed between 187 and 449 g. The 6 *D. punctulatus* measured from 94 to 163 cm in total length and weighed between 42 and 314 g. The seven *A. mairii* in the experiment measured between 55 and 85 cm in total length and weighed from 44 to 153 g. Despite cooperation with QDOE, Dr Hero and numerous volunteer collectors, the group size of snakes remained regrettably small.

6.2.2 Experimental design

For each species, individual snakes were designated a number (1 to 10) and the corresponding ventral scale, counting anterior from the anal scale (=0) was marked by scale clipping (Figure 6.4) according to the method by Fitch (1987).

Within each batch of a particular species, snakes were ranked according to weight and allocated to one of four treatments (control, inoculation, co-habitation and feeding of infected frogs) according to experimental design (Table 6.1).

Figure 6.4 Scale clipping. Half of a ventral scale is removed to identify the snake. The anal scale is indicated by arrow.

Table 6.1 Experimental design for infection of *Boiga irregularis*, *Dendrelaphis punctulatus* and *Amphiesma mairii* with Bohle iridovirus. For each species, snakes were ranked according to weight within batches and distributed sequentially into the four treatment groups according to design with two or three animals per pen (indicated by dotted lines).

Treatment	Rank/Pen		
Control	1	5	
Inoculated	2	6	9
Co-habitation	3	7	
Feeding	4	8	10

Experiments were run sequentially according to availability of animals. Control animals were held in a quarantine area; the other treatment groups were housed in the Aquatic Disease Facility at JCU. Snakes in the control and co-habit group were given placebo inoculations of 500 μL PBS intra-peritoneally. Animals in the BIV-inoculated group were given 500 μL of live BIV ($10^{4.5}TCID_{50}$) by the same route. For each of the snakes in the feeding group, two newly metamorphosed frogs (froglets) were inoculated intraperitoneally with 50 μL live BIV ($10^{3.5}$

$TCID_{50}$), and fed to the snakes at four days post inoculation. At this BIV dose rate, frogs die between 6 and 10 days after inoculation (B. Cullen, JCU, Pers. Comm., 1996). Snakes in the feeding treatment were fed infected frogs only once. The first day of the experiment was counted on the day the infected frogs were eaten by the snakes in the feeding experiment. Each experiment was conducted over four weeks.

6.2.3 Husbandry

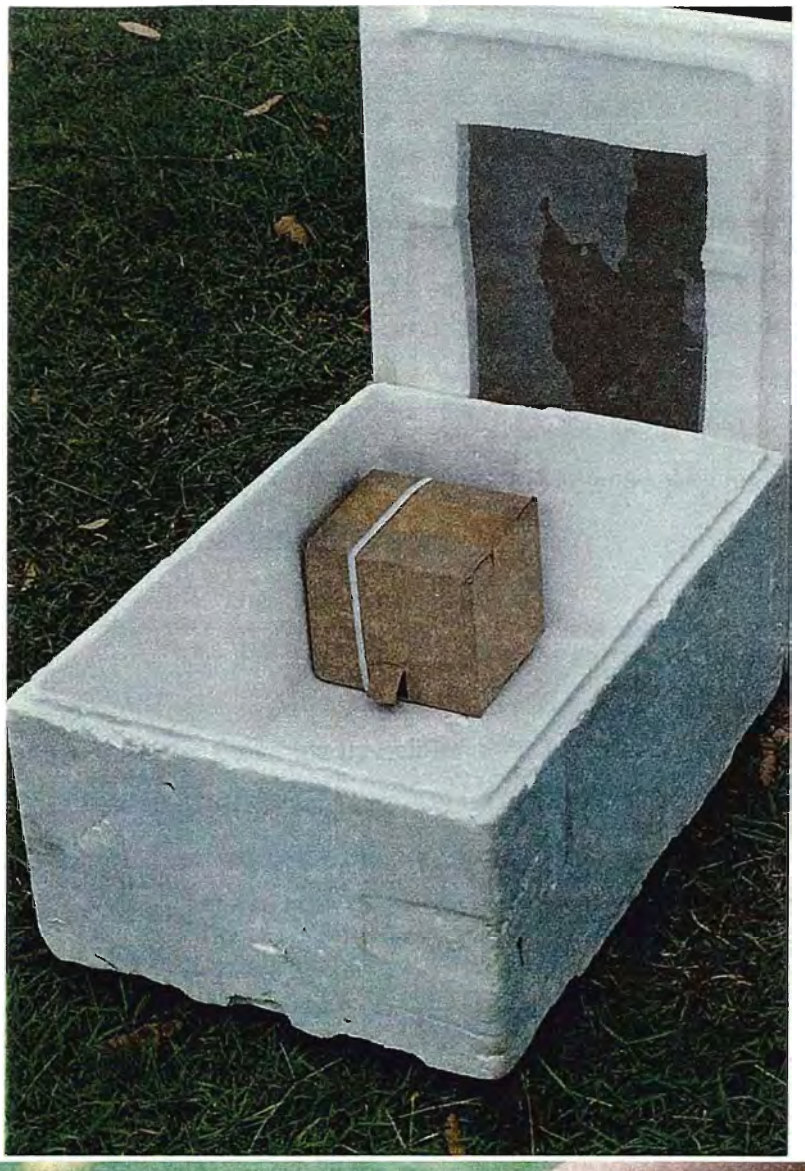
Although snakes were either non-venomous or only mildly venomous, it was a high priority to create a husbandry situation which was safe for the handler, while providing adequate care for the snakes. Snakes in the control treatment were attended to daily before the snakes in the other treatments.

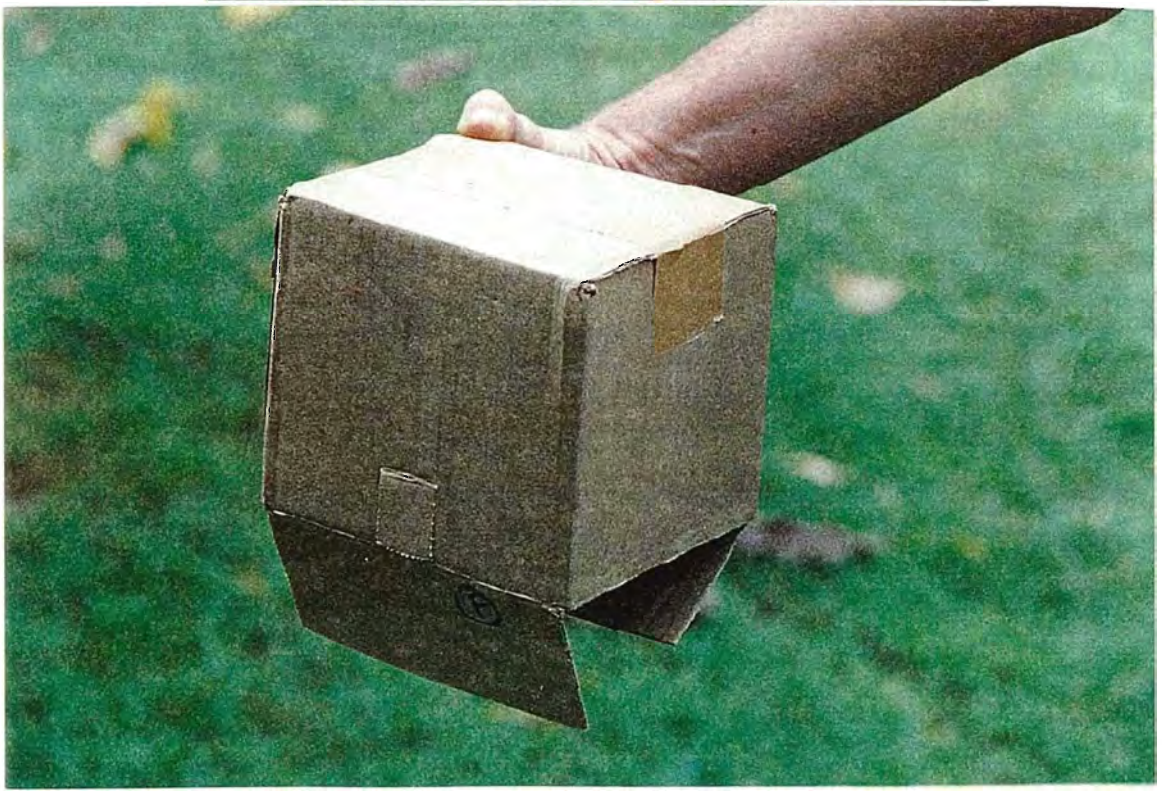
6.2.3.1 Pens

Snakes were housed in pairs or in groups of three in polystyrene broccoli boxes (35 × 60 × 20 cm). These were placed on bricks in 1 tonne Reln™ bins containing 10 cm of water, thus creating a moat around the boxes to prevent ants from entering the pens and harassing the snakes. Elasticised mosquito-netting was extended over each box, under a tight-fitting lid which had a large central piece cut out (Figure 6.5). Bricks were placed on top of the lids to prevent snakes from escaping. This provided a secure enclosure, while allowing for air circulation through the mosquito-net. Each box had newspaper bedding, a water-bowl, and a hide for each snake. Snake hides were made from small card board boxes, featuring a small "cat-flap" for the snake to enter and leave the hide freely, and a large trapdoor taking up most of the floor of the hide (Figure 6.6).

Figure 6.5 Broccoli boxes served as pens for the captive snakes. A central piece of the lid was removed for air circulation, and mosquito netting prevented the snakes from escaping through the hole.

Figure 6.6 Snake hides featured a small "cat-flap" through which the snake entered and left the hide at will. Once the hide with inhabitant had been placed in a large bag the trap door in the floor of the hide was released to drop the snake into the bag.





6.2.3.2 Feeding

Tadpoles of *Limnodynastes ornatus* were obtained from temporary puddles in suburban Townsville just prior to the puddles evaporating, and raised in captivity on a diet of boiled lettuce. These frogs occur in abundance in and around Townsville waterways. In addition to the infected froglets which were fed to the snakes in the feeding treatment at the beginning of the experiment, non-infected froglets were fed twice weekly to the snakes at a ratio of two per snake. Particular snakes were not fed additional froglets until the previous froglets had been eaten. The remaining froglets were released back to the wild.

6.2.3.3 Handling

Snakes were encouraged to enter their hide by holding their head through the "cat-flap". They would then slither out of the handler's hands and curl up inside. When handling the snakes for blood collection, the handler reached inside the polystyrene box, closed the "cat-flap" gently and placed the whole hide into a large linen bag. Once the bag was tied, the trapdoor was released by manipulation through the bag, and the snake shaken into the bag. The head of each snake, was identified by placing the bag flat on the floor and observing and palpating through the bag. Snakes were separated inside the bag by manipulating one head into a corner, tying off the corner loosely with a piece of string and feeding the rest of the snake into the corner before tying it off tightly. With only one snake left in the main body of the bag, its head was held firmly while the bag was opened and the hide removed. The tail was then drawn out of the bag, the snake identified according to the scale marking, and a blood sample taken as described below.

6.2.4 Serum collection

Animals were confined in a bag with only the tail protruding. A string was tied firmly around the bag and tail to prevent the rest of the snake from emerging. Blood was collected twice weekly for four weeks from the ventral caudal vein (Frye 1991) with a 27 gauge needle and a 1 mL syringe. The needle was inserted centrally between two ventral scales, at least 7 scales down from the vent (Figure 6.7). Samples were treated as described in Section 4.2.4 and anti-BIV antibody measured by ELISA (Section 3.3.10).

Figure 6.7 Blood sampling from the ventral caudal vein.

6.2.5 Post mortem procedures

At the termination of the experiment, snakes were bagged and placed on ice for 10 minutes prior to killing them by decapitation for autopsy. See Sections 4.2.5 for serum collection at post mortem, serology, viral isolation, histology and parasitology procedures. Parasites were identified by Dr D. Barton, Department of Zoology, JCU, and Dr. G. Hutchinson, Department of Microbiology and Immunology, JCU.

6.3 Results

Boiga irregularis are nocturnal and were only observed out of their house during nightly inspections of the snakes. *Dendrelaphis punctulatus* were often observed out of their house during the day, but would quickly retreat at the approach of a human. The *A. mairii* were "curious" and often came out of their house to inspect passers-by; they seemed the least bothered by handling. All of the *D. punctulatus* and the *A. mairii* fed eagerly on the froglets. Individual *B. irregularis* would also take the froglets occasionally. There were no mortalities in any of the three species during the experiment.

6.3.1 Serology

The maximum level for non-reactive serum was determined at 95% confidence limit to be OD × 100 = 70 for snakes. Levels of serum antibody against a BIV-like antigen during the experiment are displayed for the three species (Figure 6.8). Non-reactive sera is below the cut-off level of 70 OD × 100, medium reactive levels are between 70 and 200 OD × 100 and highly reactive sera are in the 200 to 300 OD × 100 category.

Irrespective of their treatment group, none of the *Boiga irregularis* showed much variation in levels of serum antibody directed towards BIV during the experimental period (Figure 6.8a). They all had non-reactive levels of BIV antibody in sera.

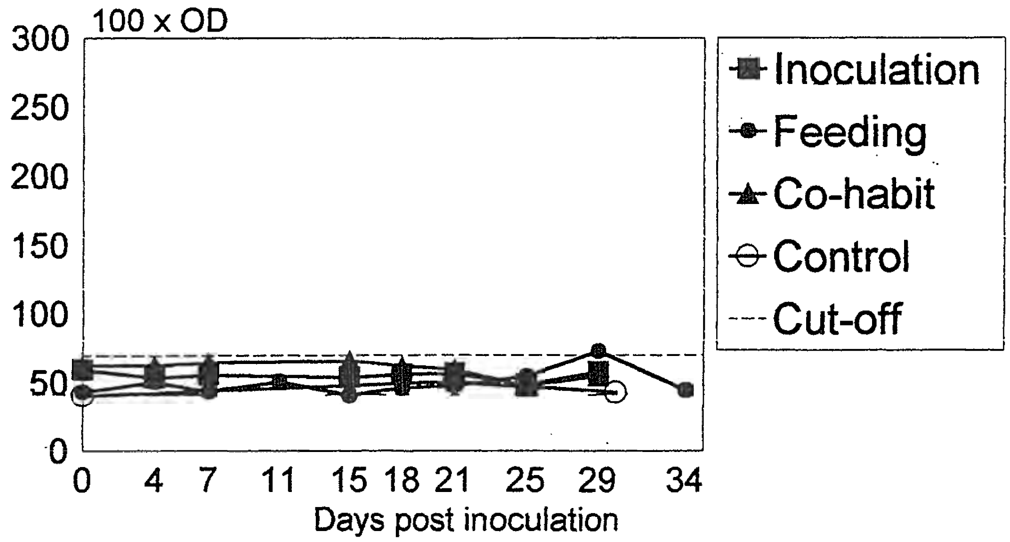
This was also the case for *D. punctulatus* in the inoculated, co-habitation and control treatments. However, the *D. punctulatus* from the group which was fed infected frogs displayed a steady and continuous increase in levels of serum antibody directed against BIV (Figure 6.8b). At the onset of the experiment, their serum was above the cut-off level and therefore already reactive.

No change in the level of serum antibody against BIV was observed in the *A. mairii* control treatment. In contrast, *A. mairii* from all other treatment groups exhibited an increase during the course of the experiment (Figure 6.8c). Like the *D.* punctulatus, many of the *A. mairii* sera were already reactive at the beginning of the experiment.

Figure 6.8a Mean levels of serum antibody against BIV for *B. irregularis* in the treatments of control (two snakes), inoculation (three snakes), co-habitation (three snakes) and feeding (two snakes) over the four week experimental period.

Figure 6.8b Mean levels of serum antibody against BIV for *D. punctulatus* in the treatments of inoculation (two snakes), co-habitation (one snake) and feeding (three snakes) over the four week experimental period. Control levels were constructed from the mean of 15 non-reactive *D. punctulatus* in the serum survey.

Figure 6.8c Mean levels of serum antibody against BIV for *A. mairii* in the treatments of control (one snakes), inoculation (two snakes), co-habitation (two snakes) and feeding (two snakes) over the four week experimental period.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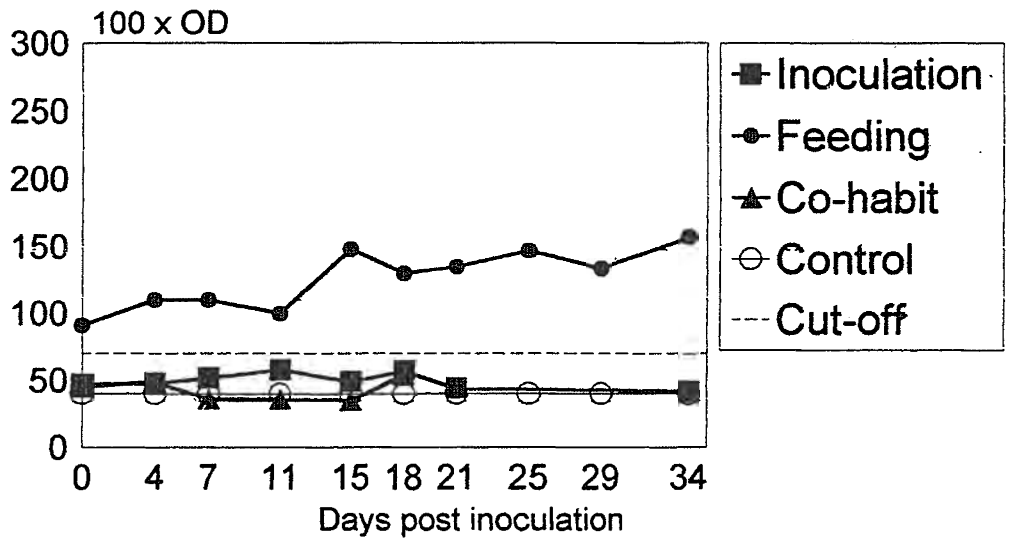
Days post inoculation
Inoculation
Feeding
Co-habit
Control
Cut-off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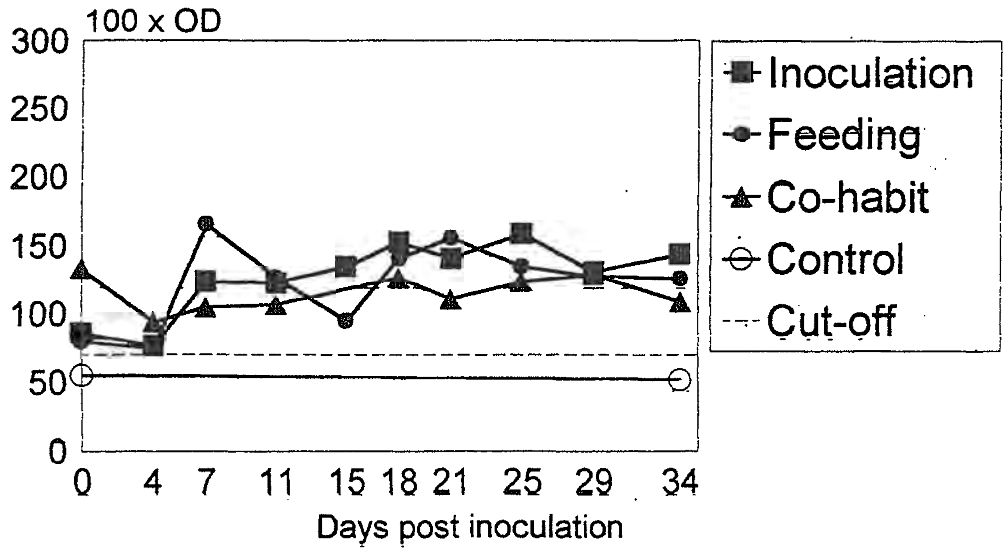
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Days post inoculation
Inoculation
Feeding
Co-habit
Control
Cut-off

6.3.2 Virus isolation

Virus was only re-isolated *in vitro* from a single *B. irregularis* which had been inoculated with BIV four weeks previously. None of the other tissue homogenates from the remaining experimental snakes caused viral CPE in culture.

6.3.3 Parasites

Pentastomes (*Waddycephalus punctulatus*) were encountered within the lungs of two *D. punctulatus* (Figure 6.9), in 6 out of 7 *A. mairii* and a single *B. irregularis* contained *Raillietiella* sp. Spargana stages of *Spirometra erinacei* tapeworms were observed as lumps under the skin in four *B. irregularis* and one *D. punctulatus*. Encysted tapeworms (presumably *S. erinacei*) were also found in the serosa of visceral organs of those snakes.

6.3.4 Histology

Microscopical examination of selected organs revealed a multitude of parasites in all three species of snakes, which masked any lesion that may have been caused by a BIV infection. Adults and embryonated larvae of pentastomes were observed in the lungs of *D. punctulatus* and *A. mairii* (Figure 6.10). A trematode cyst was found in the kidney of *A. mairii* and an adult larvae in the pancreas of the same snake (Figure 6.11 and 6.12). Apart from the parasites themselves, necrotic areas could have been either migration tracks or caused by the virus.

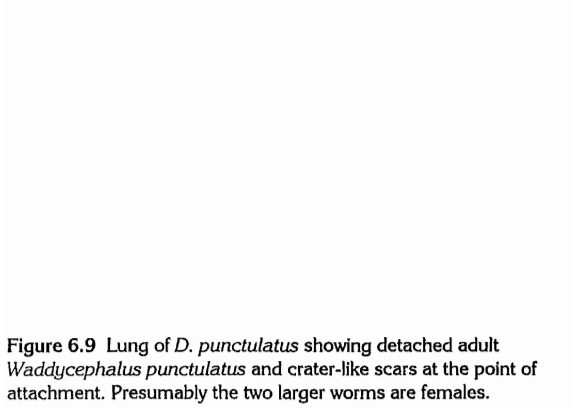


Figure 6.9 Lung of *D. punctulatus* showing detached adult *Waddycephalus punctulatus* and crater-like scars at the point of attachment. Presumably the two larger worms are females.

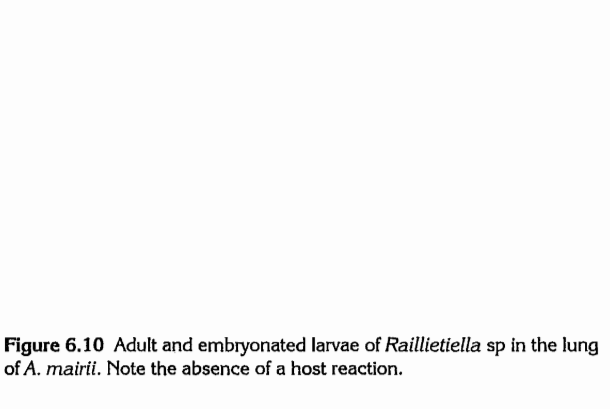


Figure 6.10 Adult and embryonated larvae of *Raillietiella* sp in the lung of *A. mairii*. Note the absence of a host reaction.

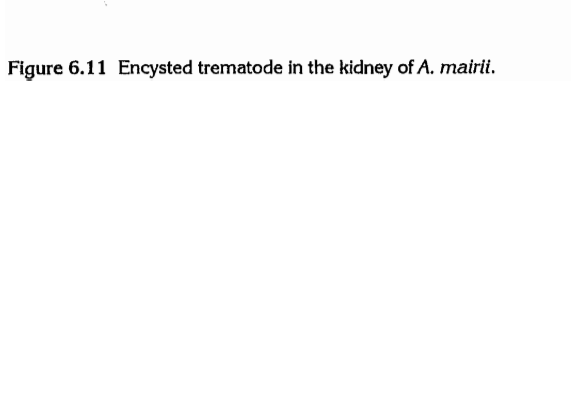


Figure 6.11 Encysted trematode in the kidney of *A. mairii*.

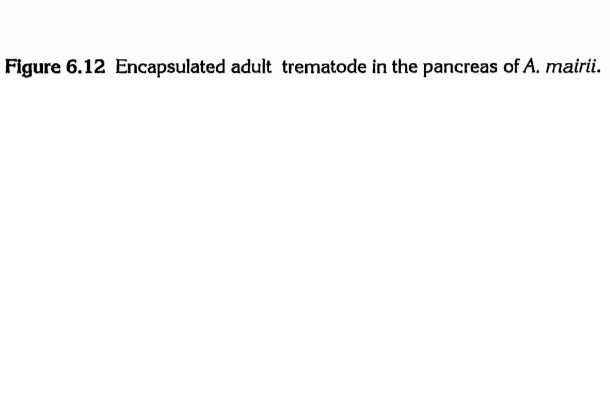
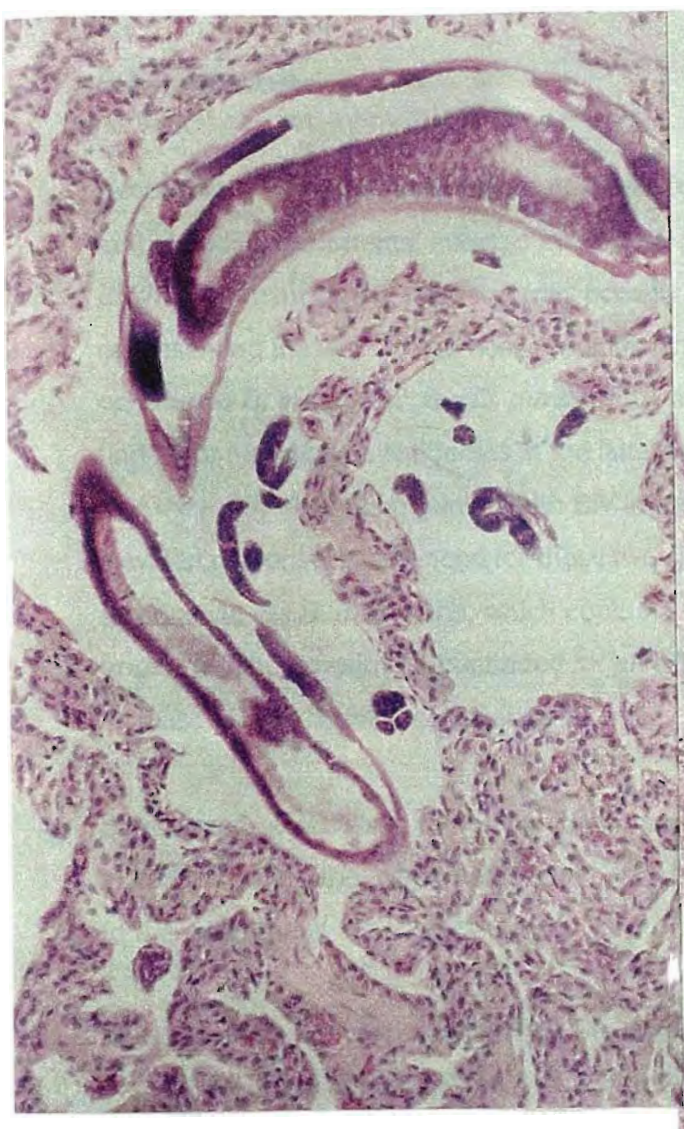
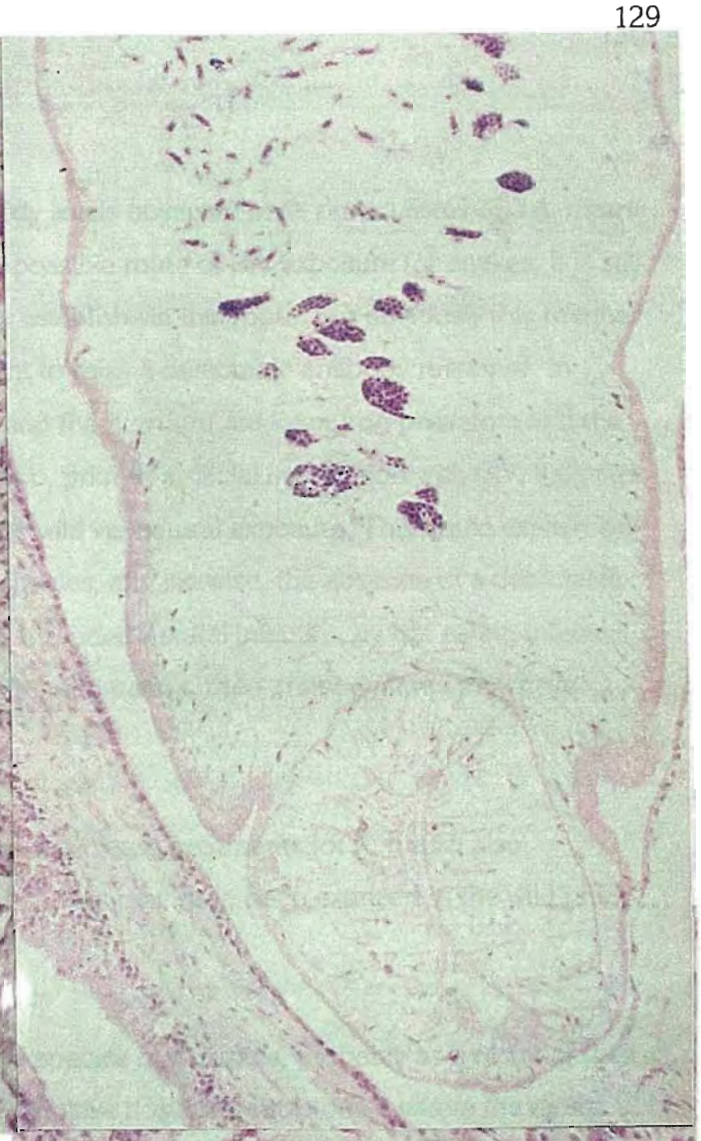
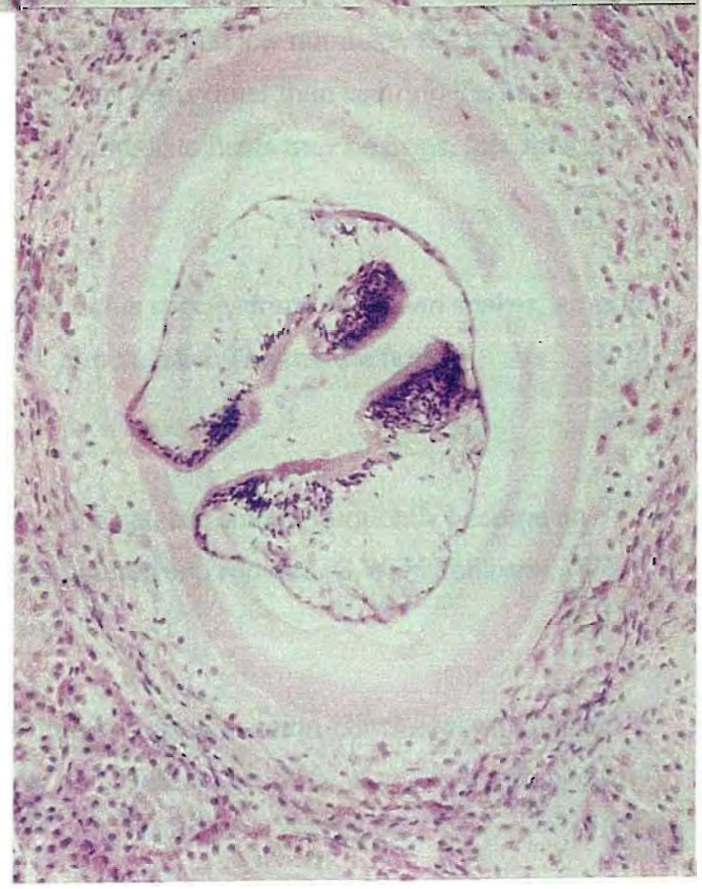


Figure 6.12 Encapsulated adult trematode in the pancreas of *A. mairii*.







6.4 Discussion

According to the increase in serum BIV-antibody levels observed in *D. punctulatus* and *A. mairii* in the feeding treatments, infected frogs are a possible route of BIV exposure for snakes. It is still unknown whether a BIV infection may actually establish via this route, but obviously this manner of presenting the virus to the host was sufficient to raise a detectable antibody response. In contrast to *B. irregularis*, the *D. punctulatus* and the *A. mairii* are keen frog predators and the finding of measurable antibodies in the latter two, prior to artificial inoculation with BIV, indicates that they may have been primed with BIV in the wild via natural exposure. This could explain the elevated response experienced for those two species, and likewise, the absence of a detectable response in the *B. irregularis*, which could have evaded natural infection by not eating infected frogs. Had the experiment continued for an extended period, then antibody titres may have increased in the *B. irregularis* group as well.

Serum antibody levels in the co-habitation and inoculated treatments for *A. mairii* also increased during the experiment. Again, these snakes may have been exposed in the wild prior to the experiment.

All the snakes were infected with one or several species of parasites, probably as a result of their live diet. Reptiles, particularly snakes, are the definitive host for pentastomes where the adults worm occupy the lungs and trachea of the host (Frye 1991). In low numbers, the adult form normally co-exist with the host without causing clinical signs other than scarring the lungs at the point of attachment and feeding (Stolch 1993). Intermediate hosts may be frogs, fish, lizards or rodents (Marcus 1981; Frye 1991).

Riley and Self (1981) described several *Waddycephalus* species from Australian snakes, among which was *Waddycephalus punctulatus* from *D. punctulatus*. The mature female *W. punctulatus* is larger than the male.

The definitive host of the tapeworm *S. erinacei* is the cat, but snakes frequently become an alternative intermediate host via their diet of frogs, lizards and rodents (G.W. Hutchinson, JCU, Pers. Comm., 1996).

Histological examination of tissues from experimental snakes was non-conclusive with respect to BIV pathogenesis because of the interference by parasites. Eosinophilia associated with necrosis

may have resulted from the parasite infection. In short, parasites and associated changes masked any possible lesions caused by BIV.

The only successful re-isolation of the virus was from an infected *B. irregularis*. Diurnal snakes like *D. punctulatus* and *A. mairii* are active in the warmer parts of the day and being poikilothermic, they could possibly approach temperatures during basking that would be detrimental to the virus (>32°C). *Dendrelaphis punctulatus* and *A. mairiis* may also have been exposed via their diet in the wild and overcome the infection by means of a previously primed immune system, as evidenced by the measurable antibody titres in this group. Being nocturnal and not a habitual frog eater, *B. irregularis* would have had less of a chance to rid itself of an infection than the diurnal frog predators, *D. punctulatus* and *A. mairii.* Possibly that is why BIV was still active after four weeks, to the extent that it could be re-isolated. The limited number of snakes in each treatment, combined with a low sensitivity virus isolation assay may also explain why virus was only re-isolated from a single snake. Alternatively, the isolated virus is a chance finding of a reptilian virus which causes CPE similar to BIV. Sequencing or immunoperoxidase staining of infected cell cultures to compare the different isolates could prove interesting.

There was no dramatic short term effect of BIV in *D. punctulatus* and *A. mairii*. *Boiga irregularis* did not appear to suffer any ill-effects during an active BIV infection, at least not in the four week period the experiment ran, which makes them potential carriers of the virus between naïve populations.

Figure 7.1 Captured *C. johnstoni* by the Lynd River 1995, secured and lined up for measurements and blood sampling before release back to the wild.

CHAPTER 7

SERUM SURVEY OF AQUATIC REPTILES IN NORTH QUEENSLAND FOR ANTIBODIES TO BOHLE IRIDOVIRUS

7.1 Introduction

The most recently recorded host class for BIV infection is the Reptilia. The status of reptiles as suitable hosts for a BIV infection has been determined for six species of reptiles by experimental infection under laboratory conditions (Chapters 4, 5 and 6). In other classes (fish and amphibians) tested, the impact of an experimental infection varies considerably among species and age groups. The most detrimental effects of the virus have been recorded in juvenile native frogs (Cullen et al. 1995), juvenile fish (Moody and Owens 1994; Ariel and Owens 1997) and now also juvenile tortoises, where significant levels of mortalities have been reported (Chapter 4).

Of great concern is the ecology of this virus in the wild and to what extent it is present in and poses a threat to Australian native fauna. In the past, the only technique available to test for the presence of BIV was viral isolation, which is not very sensitive. Experimental work shows that a viral infection can kill juveniles within 6 to 10 days of exposure (Moody and Owens 1994; Cullen et al. 1995; Section 4.3.2). Additionally, juveniles are only present during certain seasons, which makes the window of opportunity for isolating the virus minute. Despite repeated attempts at viral isolation from sick and apparently healthy amphibians from across the state by Mr Brad Cullen (Pers. Comm., 1997), BIV has only been isolated from the wild on a single occasion (Speare and Smith 1992). Recently an ELISA was developed for the detection of antibodies against iridoviruses in the serum of the amphibian *Bufo marinus* (Whittington, Kearns and Speare 1997). When applied in a survey of toads from the area where BIV was originally isolated, three out of 21 toads had antibodies against a BIV-like antigen.

The previous chapters outlined how an ELISA was developed for the detection of BIV-reactive serum proteins in reptiles (Chapter 3), and how it was applied in ascertaining that selected reptiles (tortoises, freshwater crocodiles and snakes), living in or near an aquatic habitat, were capable of producing antibodies to BIV (Chapters 4, 5 and 6). The aim of this chapter was to carry out an ELISA-based serum survey of wild populations of these species and other squamate fauna encountered in the region of Townsville and Mount Surprise, north Queensland. The species investigated were the tortoises *Elseya latisternum* and *Emydura krefftii*, the freshwater

crocodile, *Crocodylus johnstoni*, plus any non-venomous snakes captured by Mr. Tim Oswin and rangers at QDOE as part of the snake relocation program for Townsville.

7.2 Materials and Methods

7.2.1 Source of animals

Animals were captured from the wild in the region of Townsville and Mount Surprise (Figure 7.2) and most were released once a blood sample had been obtained. Others were kept for experimental purposes after the sampling (Chapters 4, 5, and 6). Six *E. latisternum* and 13 *C. johnstoni* were caught and sampled from the Lynd River during QDOE field-trips in August 1994. In August 1995, 72 *E. latisternum* and 34 *C. johnstoni* were caught and sampled in the Lynd River and 8 *E. latisternum* in Elizabeth Creek (Section 4.2.1.1). Entire sections of the waterways were screened off by a series of gill-nets. The marginal nets were then dragged along the bottom of the creek and along the banks towards the adjacent intermediate set of nets (Figure 7.3). Any animal caught in the net was immediately removed and in case of *C. johnstoni*, their snout was secured with a rubber band before it was taken ashore for measurements and blood sampling.

Another 16 *C. johnstoni* were made available from Hartley's Creek Crocodile Farm, Cairns during September 1995. Fifteen *E. krefftii* were netted in the spill-way below Ross River Dam (19°54'S; 146°30'E) in July 1994; 16 in August 1995, 5 and 16, respectively, in the billabongs at the Palmetum and Willows Golf Course, Townsville, in September 1995 (Section 4.2.1.1). This capture method excluded very small tortoises because they passed through the mesh in of the net.

Permission was kindly granted by Mr. M Pople (QDOE) to take blood samples from the snakes in the QDOE snake relocation program for the Townsville area, while they were being held prior to release in remote locations. Total of 76 snakes were tested: 19 brown tree snakes (*Boiga irregularis*), 18 green tree snakes (*Dendrelaphis punctulatus*), 9 keelback snakes (*Amphiesma mairii*), 7 children's pythons (*Liasis childreni*), 21 carpet pythons (*Morelia spilotes*) and two water pythons (*Liasis fuscus*).

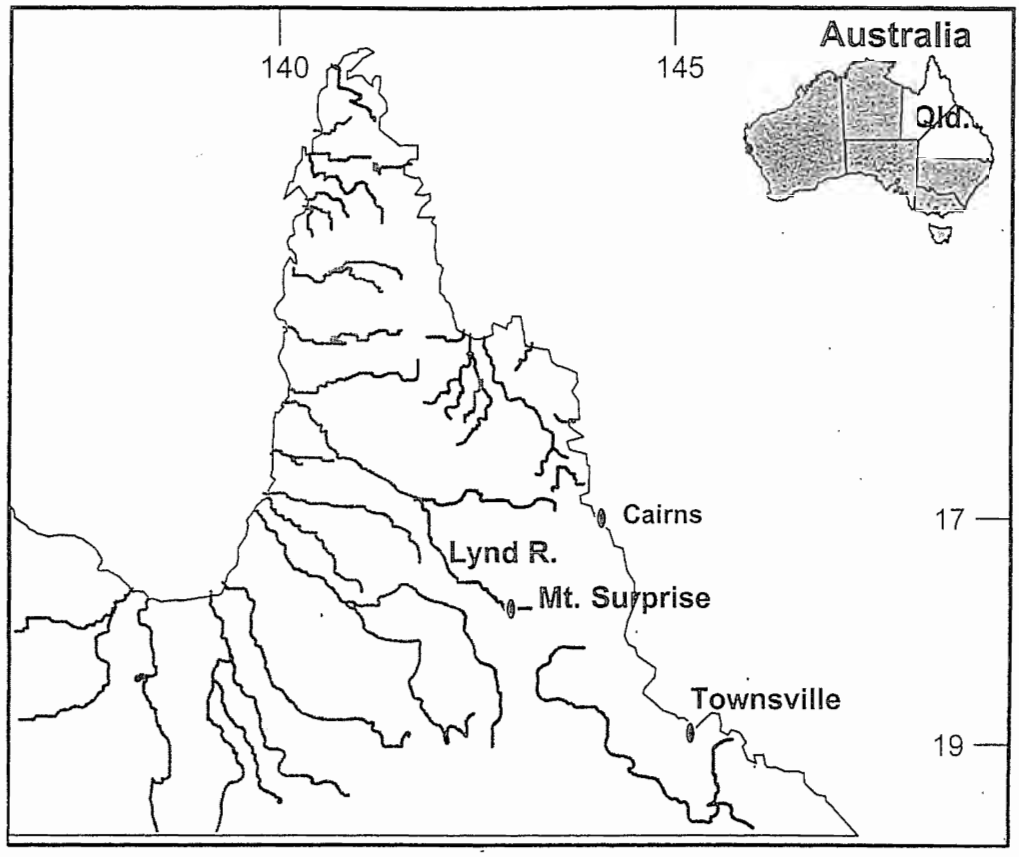


Figure 7.2 A map of north Queensland. The sites are indicated where sera were collected from wild populations of water-associated reptiles.

Figure 7.3 Line of wet-suit clad people dragging the gill-net towards the next intercepting set of nets. Lynd River 1995.

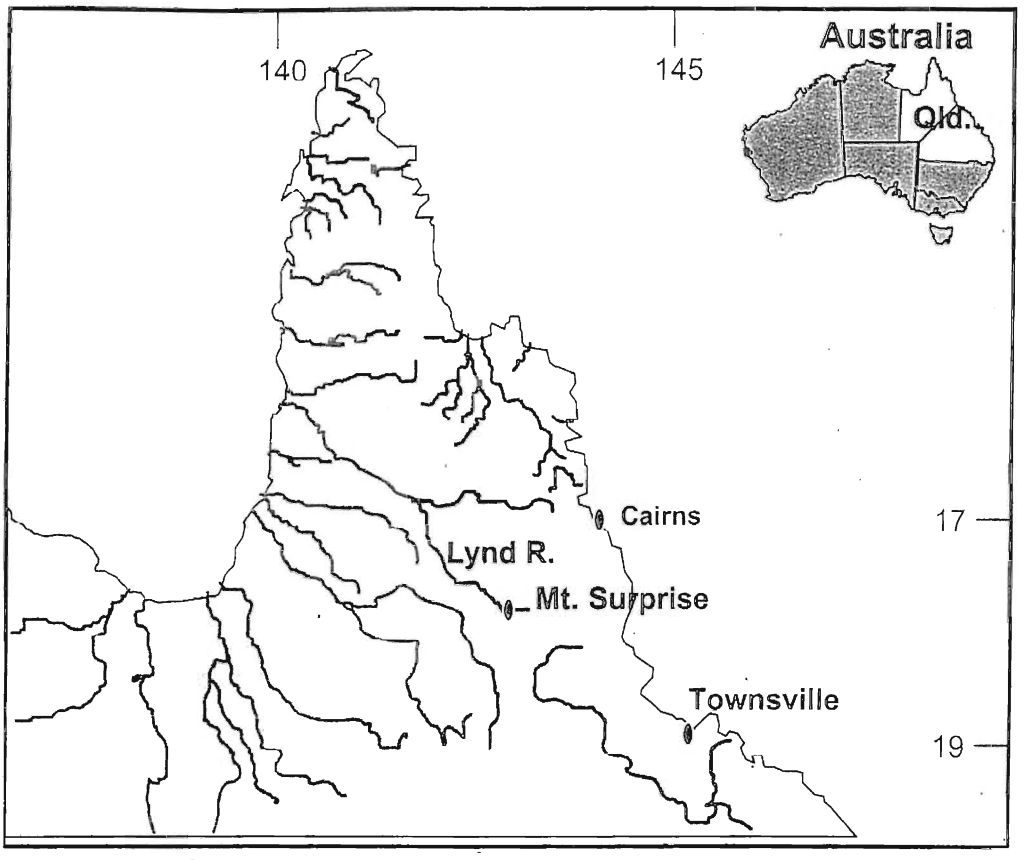


Figure 7.2 A map of north Queensland. The sites are indicated where sera were collected from wild populations of water-associated reptiles.

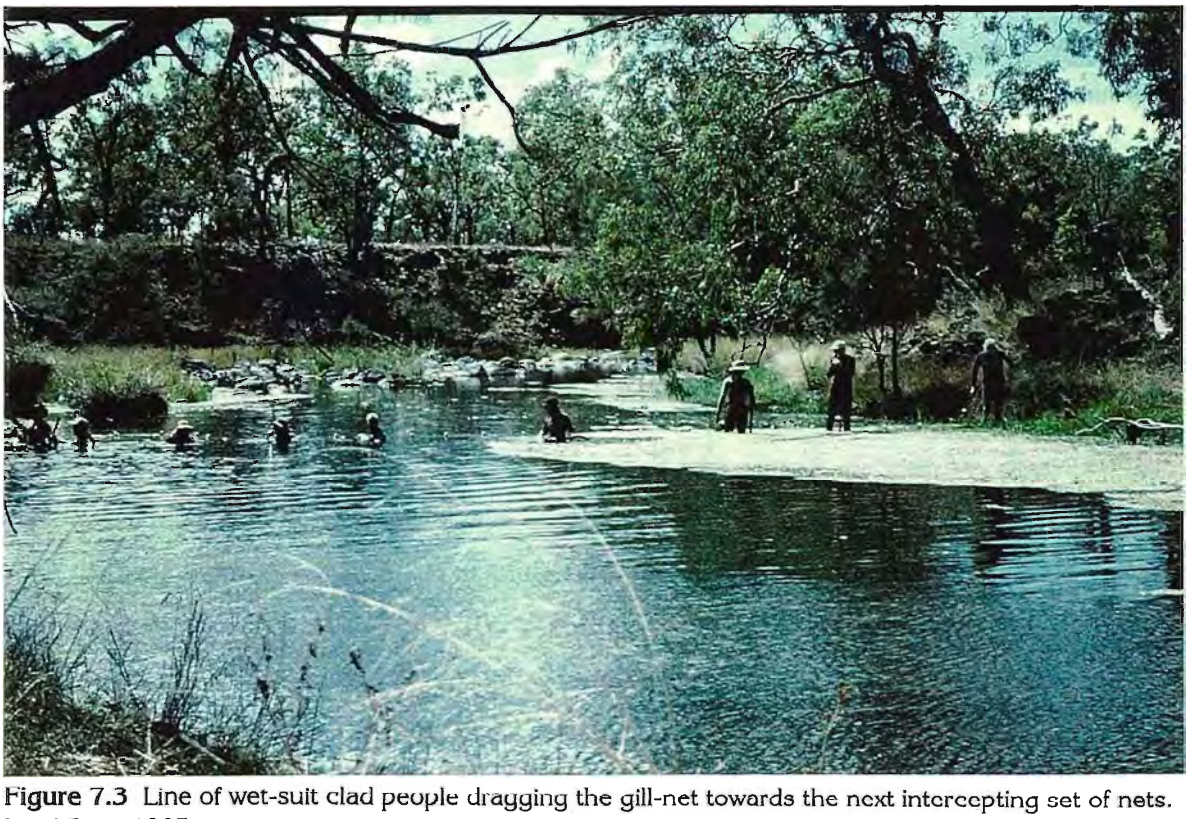


Figure 7.3 Line of wet-suit clad people dragging the gill-net towards the next intercepting set of nets. Lynd River 1995.

7.2.2 Measurements

Weight of reptiles fluctuates according to food availability and season, whereas length is a more stable parameter (Cann 1978). Straight carapace length from anterior border of nuchal scale to notch between the two most posterior marginal scales (Peters 1964) was measured with callipers for each tortoise. The snout to vent length was measured for crocodiles and the total length of snakes was measured from snout to tail tip.

7.2.3 Serum collection and processing

Blood samples from tortoises were obtained from the femoral vein (Section 4.2.4). Crocodiles were bled from the ventral caudal vein (Section 5.2.4) or via heart-puncture with a 5 mL syringe and a 21 gauge needle (Figure 7.4). Blood samples from snakes were collected from the ventral caudal vein (Section 6.2.4). Samples were collected into 1.5 mL microcentrifuge tubes. Following clotting, the sera were stored at -5°C for up to one week when in the field, before transfer to -20°C upon return to the laboratory. The sera was then tested in the antibody capture ELISA as described in Sections 3.3.10 and 4.2.6.

The cut-off level between non-reactive and reactive sera was determined from the experimental pathogenesis studies for each of the 6 species of reptiles. For both species of tortoises the cut-off value was optical density (OD) × 100 = 110, for *C. johnstoni* it was OD × 100 = 100 and for snakes it was OD × 100 = 70 (Sections 4.3.2, 5.3.2 and 6.3.2).

7.2.4 Serological data analysis

Optical density values from the ELISA were processed according to procedures described in Section 4.2.6 and graphed using Harvard Graphics 2.0 for Windows according to site and year for all species, and according to size of animals for tortoises and crocodiles. Reactive sera were classed into medium reactive (OD × 100 > cut-off - <200) and highly reactive (OD × 100 >200). Two way ANOVAs without replication were performed between OD values and animal size for each species using Microsoft Excel (Version 5.0a).

Figure 7.4 Blood sampling via heart puncture from a juvenile *Crocodylus johnstoni* in the field.

7.3 Results

The difference between strongly reactive and non-reactive serum was often quite obvious before reading the plates in an automated plate-reader (Figure 7.5).

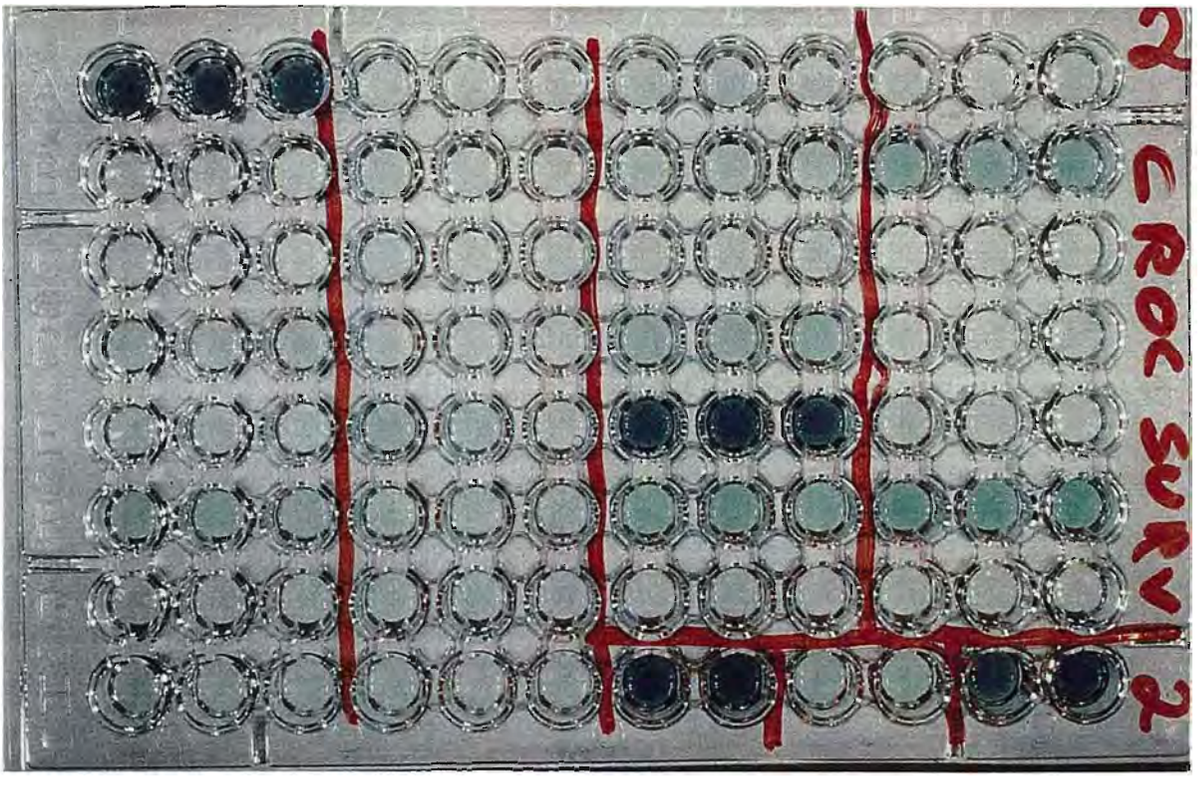


Figure 7.5 Example of ELISA results for reactivity to BIV in serum from surveyed wild *Crocodylus johnstoni*. Each sample was tested in triplicate wells, thus the two samples in wells number A1, 2, 3 and E7, 8, 9 were reactive, the rest were non-reactive. Controls were included for the attachment of reptilian sera (H7, 8), non-specific attachment of the detecting antibody, rabbitαBIV (H9, 10) and a positive control for BIV (H11, 12).

7.3.1 Location and antibody levels

E. latisternum

At both locations (Lynd River and Elizabeth Creek), and in both years of sampling (1994 and 1995) at the Lynd River location, individual *E. latisternum* tortoises displayed very high levels (>250 OD × 100) of serum antibody directed against a BIV-like proteins (Figures 7.6). Sera from a large part (39 - 50%) of the sample, for each of the three data sets, remained non-reactive (<110 OD × 100). None of the tortoises from the 1995 Lynd River sample were recaptures from the previous year.

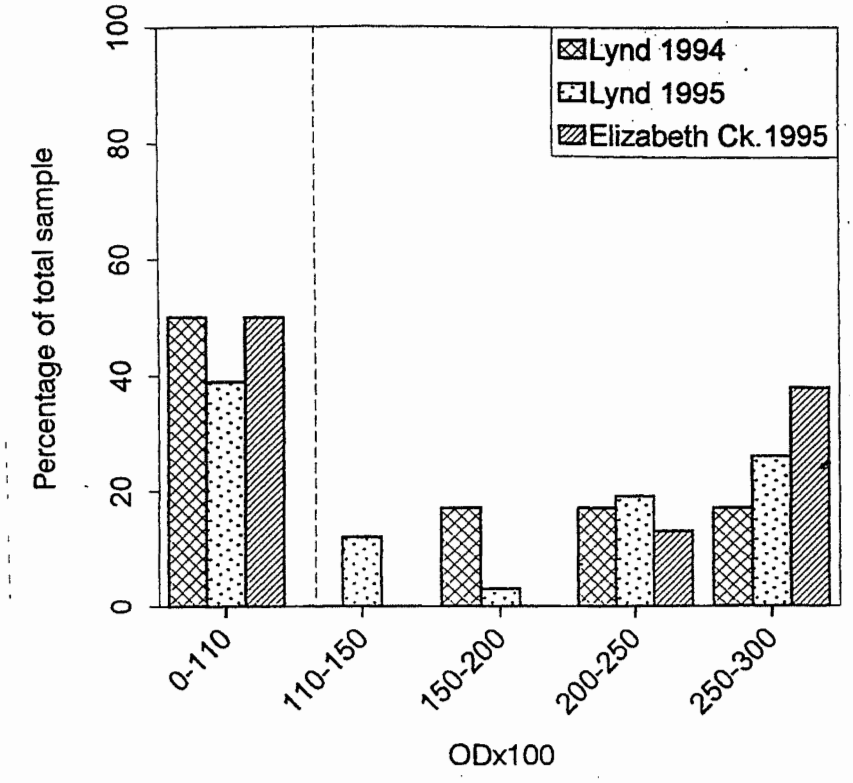


Figure 7.6 Frequency distribution of BIV-reactivity levels in serum, as measured by ELISA in *Elseya latisternum* captured in the Lynd River 1994 (n=6) and 1995 (n=72), and Elizabeth Creek 1995 (n=8). The cut-off level between BIV-reactive and non-reactive sera (OD × 100 = 110) is indicated by a broken line.

E. krefftii

In the 1994 sample from Ross River Dam spill-way, a few *E. krefftii* tortoises (8%) had very high levels (>250 OD × 100) of BIV-reactive serum (Figure 7.7). Only two tortoises were recaptured in the 1995 sample and they were non-reactive in both years. No high levels of BIV-reactive serum proteins were recorded in the sampled tortoises from Ross River Dam spill-way in the 1995 sample. Most sera (82%) were non-reactive (<110 OD × 100) and a few (18%) displayed medium reactivity (110-200 OD × 100). In both the Palmetum and the Willows Golf Course samples from 1995, individual *E. krefftii* tortoises (40 and 31%) had highly reactive sera (OD × 100>250), as well as low level (110-200 OD × 100) and non-reactive (<110 OD × 100).

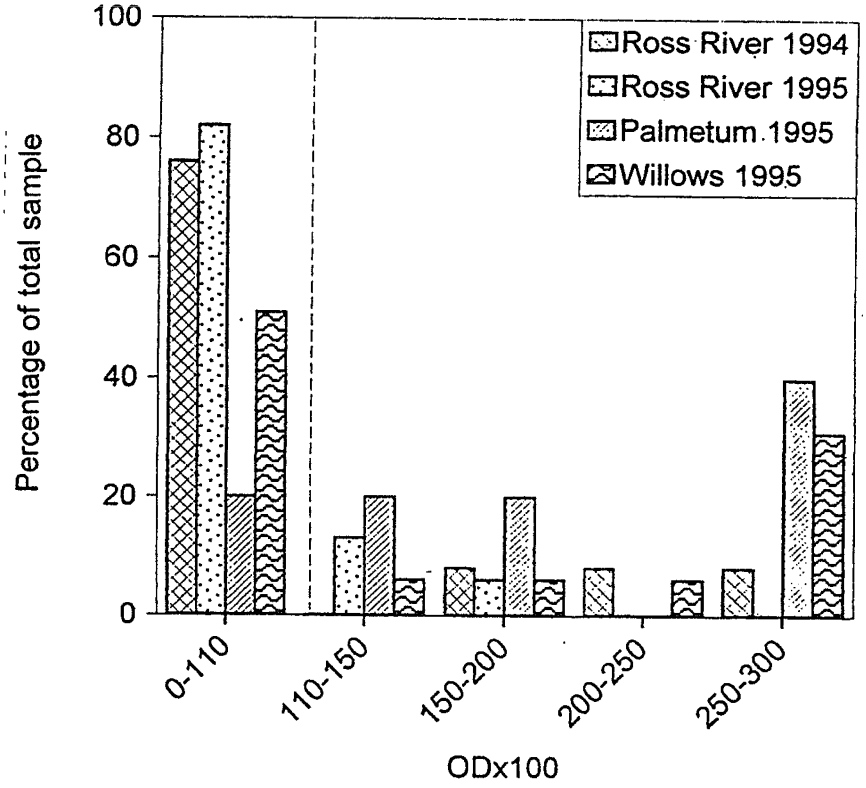


Figure 7.7 Frequency distribution of BIV-reactivity levels in serum, as measured by ELISA in *Emydura krefftii* captured in Ross River Dam spillway 1994 (n=15) and 1995 (n=16), the Palmetum (n=5) and Willows Golf Course billabongs 1995 (n=16). The cut-ff level between BIV-reactive and non-reactive sera (100 × OD = 110) is indicated by a broken line.

C. johnstoni

In both years when sampling from the Lynd River, certain *C. johnstoni* (15 and 10%) displayed very high levels of reactive serum (>250 OD × 100) (Figure 7.8). However, the majority (85 - 86%) of the samples had non-reactive sera (<100 OD × 100). Only 5% of the sample in 1995 displayed intermediate reactivity (100-250 OD × 100) and there were no crocodiles recaptured. In the Hartley's Creek sample all crocodiles were of known age and classified as yearlings, and although most (56%) had non-reactive sera (<100 OD× 100), some (25 and 19%) displayed low level or moderate reactivity (100-150 and 150-200 OD × 100).

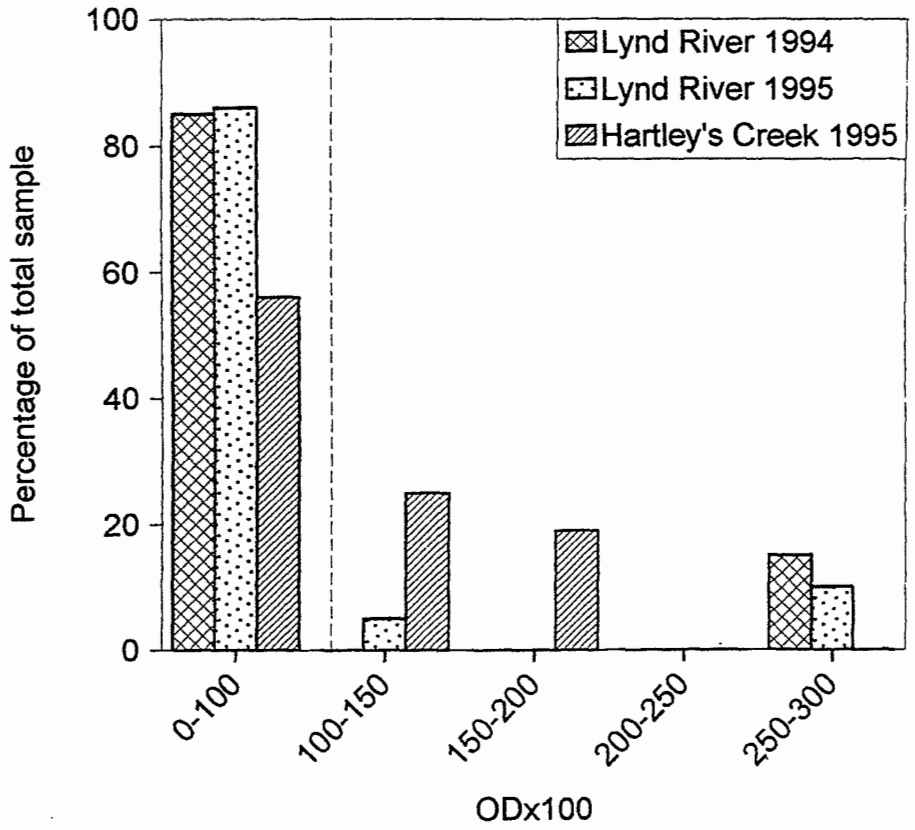


Figure 7.8 Frequency distribution of BIV-reactivity levels in serum, as measured by ELISA for *Crocodylus johnstoni* captured in the Lynd River 1994 (n=13) and 1995 (n=34) and from Hartley's Creek Crocodile Farm 1995 (n=16). The cut-off level between BIV-reactive and non-reactive sera (100 × OD = 100) is indicated by a broken line.

Snakes

Representatives of most the sampled snake species displayed sera reactive to a BIV-like antigen (Figure 7.9). The exception was the water python (*L. fuscus*) with two non-reactive individuals. In the category 110-150 OD × 100, there was a single snake (*M. spilotes*). Two snakes (*A. mairii* and *L. childreni*) had sera in the moderately reactive category of 150-200 OD × 100. Only one *M. spilotes* displayed highly reactive serum against a BIV-like antigen at OD × 100 levels of 245.

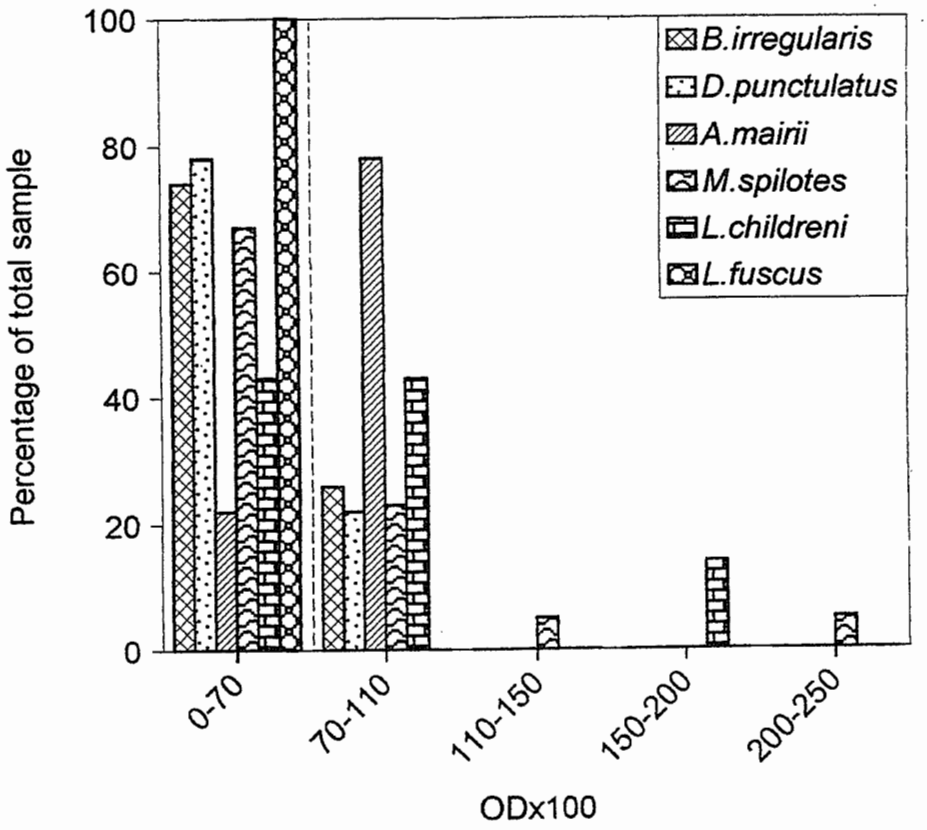


Figure 7.9 Frequency distribution of BIV-reactivity levels in serum, as measured by ELISA for *Boiga irregularis, Dendrelaphis punctulatus, Amphiesma mairii, Morelia spilotes, Liasis childreni* and *Liasis fuscus* captured in the Townsville region during 1995 (n=78). The cut-off level between BIV-reactive and non-reactive sera (100 × OD = 70) is indicated by a broken line.

7.3.2 Size of animals and antibody levels

Sufficient data were available for *E. krefftii* and *C. johnstoni* to statistically analyse the parameters of animal size and corresponding OD level. For *E. krefftii* tortoises hyper-immune sera was more commonly encountered in larger animals (Figure 7.10). A two-way ANOVA without replication showed there was a significant difference in levels of BIV-reactive serum proteins between size classes [$F_{(4,12)}=7.89$; $P=0.0023$]. None of the juvenile tortoises (<12 cm) had BIV-reactive sera. In the sub-adult category (12 - 16 cm), only a single individual (n=10) had reactive serum (200 - 250 OD × 100). In the small adult category (16-20 cm) 25% (n=16) of the sampled animals displayed reactive sera at various levels (110-300 OD × 100). In the largest size class (20+ cm) approximately half of the animals (n=21) showed non-reactive sera, but a large proportion of the sample (38%) had hyper-immune sera (250-300 OD × 100).

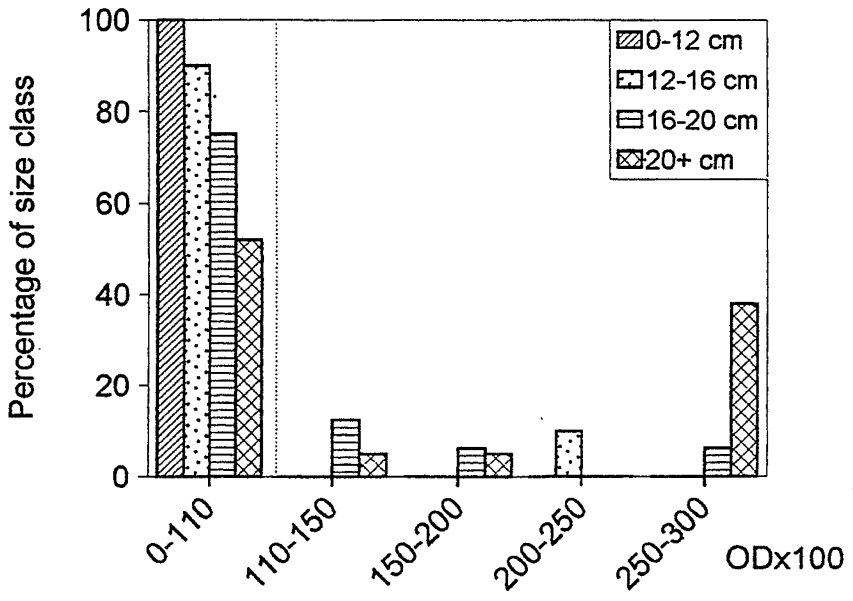


Figure 7.10 Frequency of each of four *Emydura krefftii* size classes (0 - 12 cm, 12 - 16 cm, 16 - 20 cm and 20+ cm straight carapace length) present in each of five OD categories (0 - 110, 110 - 150, 150 - 200, 200 - 250 and 250 - 300 OD). Cut-off level for reactive sera (100 × OD = 110) is indicated by a broken line.

The same bimodial pattern is evident for *C. johnstoni*, but is more pronounced (Figure 7.11). The animals are either non-reactive or highly reactive, with very few in the intermediate categories. Hyper-immune sera was only recorded for animals in the larger size class, especially from the 80+ cm class. All the reactive animals were female. The two-way ANOVA without replication showed that there was no significant difference in levels of BIV-reactive serum proteins between size classes of *C. johnstoni* males [$F_{(1,3)}=72$; $P=0.0034$], but there was a significant difference in levels of BIV-reactive serum proteins between the size classes of *C. johnstoni* females [$F_{(1,3)}=0.85$; $P=0.422$].

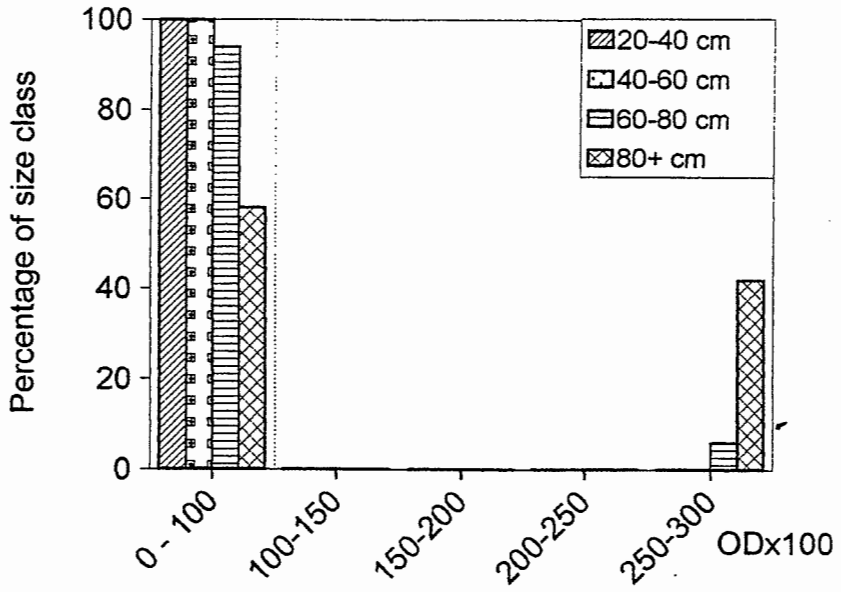


Figure 7.11 Frequency of each of four *Crocodylus johnstoni* size classes (20 - 40 cm, 40 - 60 cm, 60 - 80 cm and 80+ cm snout to vent length) present in each of five OD categories (0 - 100, 10 - 150, 150 - 200, 200 - 250 and 250 - 300 OD). Cut-off level for reactive sera (100 × OD = 100) is indicated by a broken line.

7.4 Discussion

High BIV-reactivity levels in serum of native reptiles surveyed at several sites in north Queensland, indicate they have had exposure to a BIV-like antigen. BIV-reactivity was found in two tortoise species, freshwater crocodiles and 5 snake species. Provided the serum proteins were directed against BIV and not a different antigen with similar epitopes, then this survey shows that exposure of native water-associated reptiles to BIV is a common event in north Queensland.

Other published studies, which involved surveying the sera of reptiles for antibodies against a virus, are the United States based surveys for Eastern and Western encephalitis antibodies in a variety of vertebrates including tortoises and snakes, and a survey of three Mediterranean chelonians for evidence of infection by Sendai virus and pneumonia virus of mice. The techniques used were serum neutralisation tests, haemagglutination inhibition titration and complement fixation (Jackson and Needham 1983; Shortridge and Oya 1984). Presently, ELISAs are commonly applied in detection of both specific antigens and antibodies. Whittington et al. (1994), for example, reported the use of an antigen capture ELISA for diagnosing and analysing an outbreak of EHNV in farmed rainbow trout (*Oncorhynchus mykiss*).

Optical density values for test sera obtained using the ELISA developed in this study (Chapter 3) may include false non-reactive sera, because certain animals exposed under experimental conditions do not develop a detectable antibody response (Chapters 4, 5 and 6). The infection may not have established, or the antibodies were produced at levels below our defined cut-off level of detection. In other cases, animals which had very high titres (reactive) displayed a decline over several months to below detectable levels (non-reactive). The high BIV-reactivity levels detected in some animals in each species int he survey, could be due to innate immunity, or they could be caused by an adaptive antibody response. The high values were observed in individuals from all age-classes. Such a pattern is more consistent with an adaptive Ig immune response than a physiological component of the serum and this indicates that the ELISA in fat does detect antibodies to a BIV-like antigen, and not innate non-specific serum proteins with an affinity for BIV.

The survey shows a trend towards larger animals being more likely to have high levels of BIV-like antibodies, than the smaller animals in the population. Considering the higher susceptibility to BIV among juvenile amphibians and fish (Cullen et al. 1995; Ariel and Owens 1997), young reptiles that became infected may not have survived and therefore were not sampled. However, this does not explain why intermediate sized *C. johnstoni,* did not have reactive sera int he wild populations. Animals in this size class do not appear to be susceptible under experimental conditions (Chapter 5) and therefore would be expected to survive a BIV infection and produce specific antibodies against the virus. In contrast the yearlings from the captive population at Hartley's Creek Crocodile Farm did include small animals with reactive sera indicating a past exposure to the virus while in captivity.

Alternatively, this pattern may be related to an ontogenic diet shift, which has been described for *C. johnstoni* (Tucker et al. 1996), where larger animals include larger prey in their diet (e.g. tortoises and fish) that may harbour a BIV-infection, whereas the smaller prey such as insects and crustaceans eaten by the smaller *C. johnstoni* may not. If this trend is diet related, that may mean that susceptible juveniles do not eat potentially infected animals. In crocodiles, the diet shift is very distinct at around 70 cm snout-vent length (A. Tucker, University of Queensland, Pers. Comm., 1997) and could account for the sudden increase of individuals with reactive sera in the larger size group.

For the tortoises, the trend of larger animals having higher levels of BIV-antibodies is more gradual than for *C. johnstoni*. It may be related to a progressive diet shift as the tortoises grow, and/or it could be a matter of maturation of the immune system with time. The very small juvenile tortoises (<7 cm straight carapace length) were not sampled from the wild as a result of capture bias.

In experimental infections BIV can be transmitted to non-reptile groups via aerosols, water (Moody 1992), faeces (Speare, Owens and Spencer, Pers. Comm., 1996) and predation (Ariel et al. 1995). Living in the same water body would expose individual animals equally to the first three methods of transmission, irrespective of their size. Based on the pattern of reactors in the survey, I propose that infection is selectively imposed on the larger size classes by means of some behavioural bias, for example prey type. Within the larger size classes there may be individual prey preference, which could explain why some get exposed often enough to produce anti-BIV antibodies in large quantities. For *C. johnstoni*, the only individuals with highly reactive sera were large females. This is not the case with tortoises, where there were no differences between the sexes in the frequency of high antibody levels. This may be a coincidence, or it may be related to prey preference, since female *C. johnstoni* tend to inhabit different habitats from males within the river (Webb and Manolis 1989).

The snakes surveyed were not a homogenous group. In a sample of 78 snakes of different species with varying prey preferences and sizes, it is difficult to identify a trend in BIV-antibody frequencies. However, some individuals were certainly reactive and must have experienced some exposure to a BIV-like antigen.

The number of animals surveyed for each species was limited by their availability, accessibility and time dedicated to the survey. The results presented here are therefore not fully definitive of

the BIV-antibody status of north Queensland reptiles. However, it does clearly and strongly show that there is a presence of a BIV-like antigen in the reptile populations sampled. Sample size, sites and especially season would have to be carefully considered if more specific issues regarding the ecology of the virus had to be addressed on the basis of serological evidence. Possibly, the tortoise *Elseya dentata* could be surveyed because it is distributed further across northern Australia than both *E. krefftii* and *E. latisternum*. The ecology of BIV in wild populations is still an elusive subject, but in light of the serum survey of cane toads by Whittington et al. (in press) and this study, it appears that BIV infections are not an uncommon occurrence out there beyond the laboratory.

CHAPTER 8

GENERAL DISCUSSION

It is commonly known that the Australian freshwater ecosystem is under threat from human associated degradation such as land degradation of bordering areas, pollution with fertilisers and biocides from adjacent agricultural pastures, dams and introduction of exotic species (Lake and Marchant 1990). However, the impact of disease on the survivorship of native fauna has long been overlooked.

A pathogen with the characteristics of BIV is clearly suited to an opportunistic existence in a dynamic freshwater ecosystem. Most of the inhabitants are poikilothermic and thus do not consistently exceed the critical temperature of 32°C that inactivates BIV. Having such a broad host range spanning over fish, amphibians and reptiles, BIV can be transferred from one host to another via the food web or via the water.

The susceptibility of juveniles to BIV in a population of frogs and fish appears to be higher than in more mature animals (Cullen et al. 1995; Ariel and Owens 1997; N. Moody, Department of Microbiology and Immunology, JCU, Pers. Comm., 1997) which is parallelled in tortoises investigated here. This phenomenon is probably caused by the gradual maturation of the immune system (Borysenko 1978), and could result in declining numbers of susceptible species.

The efficiency of the reptilian immune system is directly related to temperature and decreases with a reduction in ambient temperatures. Basking may increase the body temperature of reptiles to above 32°C and thereby rid the host of a BIV infection. Basking is more common in adult tortoises and crocodiles (Dr. J. Miller, QDOE, Pers. Comm., 1996) which further increases their chances of surviving an infection. In contrast, juveniles tend to be more cryptic and hide among the weed. Winter temperatures would also be more suitable for BIV replication, and hence more detrimental to poikilothermic hosts than summer temperatures.

The experimental infection of snakes via BIV-infected frogs indicates that BIV can be passed from prey to predator under natural conditions. A similar conclusion was reached for barramundi eating BIV-infected tilapia (Ariel et al. 1995). Thus, BIV can cycle through the food-web in the freshwater ecosystem.

Adult tortoises are capable of producing detectable levels of BIV-antibodies and they survived experimental infection without any obvious ill-effects. Tortoises are abundant in the Australian freshwater environment, they prey on frogs and fish known to become infected with BIV and they are able to survive outbreaks of BIV in the wild. These qualities make tortoises good indicator species for a past presence of BIV in the environment.

In the experimental infections of snakes reported on here, there appeared to be a trend for habitual frog-eating snakes to have higher levels of reactivity towards BIV, than those species of snake (eg. *Boiga irregularis*) which prefer homeothermic prey. Although virus could not be re-isolated from inoculated individuals of the two frog-eating species, it was re-isolated in culture from the liver of a *Boiga irregularis*. Whether this was a coincidence and is merely a reflection of the inadequacies of the viral isolation test, or an indication that *Boiga irregularis* is more susceptible (or less immune) than the other species, is unknown. What it does show, however, is that *Boiga irregularis* can carry an active BIV infection for at least four weeks without any obvious distress. Therefore, this species of snake is a potential carrier of BIV into naive populations.

The development of the ELISA for detecting BIV-reactivity in reptilian sera, with the establishment of a cut-off level between BIV-reactive and non-reactive sera in the experimental studies, enabled a preliminary survey of the north Queensland water-associated reptilian fauna. Although the current format of the ELISA cannot differentiate between innate and adaptive immunity towards a BIV-like antigen, the pattern observed in the survey indicates that the ELISA mainly detects serum antibodies and not non-specific innate factors which have previously been implicated in binding antigens (Ingham 1980). In several locations sampled, evidence of past exposure to BIV was found in the sera of at least part of the population. Furthermore, in *Emydura krefftii* tortoises and freshwater crocodiles, *C. johnstoni*, a trend was apparent for larger animals to be more likely to have BIV-hyperimmune sera than the smaller animals in the sample. This may be explained by differential susceptibility, where all the BIV-exposed young ones had died. However, an ontogenic diet shift from non-infected prey to potentially BIV-infected prey may equally account for the pattern.

Future research in this field should encompass a more widely based serum survey for BIV antibodies in tortoises, to map the presence of BIV in Queensland and the Northern Territory, both behind and beyond the advancing line of the canetoad, *Bufo marinus*, which are potential hosts for BIV. Freshwater crocodile hatchlings also need a more thorough investigation with respect to their susceptibility to BIV. Following the pattern for amphibians, fish and tortoises, *C. johnstoni* hatchlings are potentially highly susceptible to a BIV infection, which could have

serious ramifications to both wild populations and captive stock held at farms. Other areas of interest are (1) the possible transfer of maternal anti-BIV antibodies to the eggs and hence young of hyperimmune female tortoises, and (2) the effects of artificially imposed high temperature regimes on BIV-infected hatchlings, to test the theory that a BIV-infection may be overcome by increasing the body-temperature of infected reptiles. Clearly there is still much to be learned of the importance of BIV in the ecology of the native poikilothermic fauna of northern Australia.

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APPENDIX 1

Reagents for the preparation of components in the ELISA

1.1 PBS 'A' (x 25)

dH_2O	1.00 L
NaCl	200.00 g
KCl	5.00 g
K_2HPO_4	4.00 g
Na_2HPO_4	16.00 g

Adjust to pH 7 - 7.5 at working dilution.

1.2 Carbonate/Bicarbonate Buffer

dH_2O	1.00 L
$NaHCO_3$	3.11 g
Na_2CO_3	1.38 g

Dissolve each reagent separately in half the water, then mix together. If necessary, adjust to pH 9.6 with HCl. Store at 4°C.

1.3 TEN-Tween 20 casein buffer (TEN-TC) (x 10)

dH_2O	1.00 L
Tris	60.55 g
EDTA	3.70 g
NaCl	87.70 g
Tween 20	5.00 mL
Casein	20.00 g

Add all ingredients except casein to the water while stirring. Adjust to pH 8.5 at working dilution. Add casein slowly, and leave stirring O/N at R/T. (Casein is acidic and should lower the pH to approximately 8, adding HCl after casein will make it clot in solution) Store stock in 100 mL aliquots at -20°C, working dilution at 4°C. Do not autoclave!

1.4 Tris Buffer

dH_2O	1.00 L
Tris	6.05 g
NaCl	8.78 g

Adjust to pH 9 with HCl. Store at 4°C

1.5 ELISA substrate solution

Solution 1:

dH_2O	750.00 mL
Citric Acid	15.75 g

Solution 2:

dH_2O	500.00 mL
Na_2HPO_4	14.20 g

While stirring, adjust pH of solution 2 with solution 1 to pH 4.2. Autoclave (solution 1+2) and store at 4°C.

Solution 3:

dH_2O	10.00 mL
ABTS (2,2'-azino-bis-3-ethylbenthiazoline-6-sulphonic acid)	0.28 g

Store as stock at room temperature in a foil covered bottle.

Solution 4:

dH_2O	5.00 mL
H_2O_2 (30%)	63.00 µL

Make fresh stock daily.

Just before use mix:

Solution (1+2)	10.00 mL
Solution 3	200.00 µL
Solution 4	200.00 µL

1.6 TEN-T (x 10)

dH_2O	10 L
Tris base	605.5 g
EDTA	37 g
NaCl	877 g
Tween 20	50 mL

Dissolve in dH_2O while stirring O/N at R/T. Adjust to pH 8 at working solution.

1.7 DEAE Affi-Gel blue column (Biorad)

Buffer A (Loading buffer)

dH_2O	1 L
K_2HPO_4	3.48g

This gives a 0.02 M solution. Adjust to pH 8.0 with HCL. Store at 4°C.

Buffer B (Elution buffer)

Buffer A	1L
NaCl	81.81g

Store at 4°C.

1.8 SDS-PAGE gel

Stock solutions:

Acrylamide/BIS

dH_2O	300 mL	
Acrylamide/BIS (37.5:1)	90 g	(Bio-Rad, Cat. No. 161-0125)

1.5 M Tris-HCl (pH 8.8)

dH_2O	60 mL
Tris base	18.15 g

Adjust to pH 8.8 with 1N HCl and make to 100 mL with dH_2O. Store at 4°C.

0.5 M Tris-HCl (pH 6.8)

dH_2O	60 mL
Tris base	6 g

Adjust to pH 6.8 with 1N HCl and make to 100 ml with dH_2O. Store at 4°C.

10% SDS

dH_2O	100 mL
SDS	10 g

Dissolve SDS in water with gentle stirring and bring to 100 mL with dH_2O.

10% APS

dH_20	1 mL
Ammonium persulphate	0.1 g

Make up daily.

Buffers

SDS non-reducing sample buffer

dH_2O	4 mL	
0.5 M Tris-HCL pH 6.8	1 mL	
Glycerol 98%	0.8 mL	
10% SDS	1.0 mL	
0.05% bromophenol blue	0.2 mL	(Bio-Rad; Cat. No. 161-0404)

Dilute the sample at least 1:4 with sample buffer.

Electrode running buffer (x5)

Tris base	15 g
Glycine	72 g
10% SDS	5 g

Take to 1 l with dH_2O. Store at 4°C and warm to R/T before use. Dilute 70 mL stock with 280 mL dH_2O for one electrophoretic run.

Gels

Separating gel

dH_2O	4.85 mL	
1.5 M Tris-HCl pH 8.8	2.5 mL	
10% SDS stock	100 μL	
Acrylamide/BIS stock	2.5 mL	
10% APS stock	50 μL	
TEMED	10 μL	(PROGEN Industries, Qld, Australia; Cat. No. 200-0166)

Stacking gel

dH_2O	3.0 mL
0.5 M Tris-HCl pH 6.8	1.25 mL
10% SDS stock	40 μL
Acrylamide/BIS stock	0.75 mL
10% APS stock	20 μL
TEMED	10 μL

1.9 HAT (x 100)

Thymidine	0.038 g
Hypoxanthine	0.136 g
Aminopterin	0.0018 g
PBS 'A'	100 mL

Hypoxanthine is dissolved alone by adding a few drops of 1 M NaOH. The resulting solution is then made up to 100 mL with PBS and the other components added. Filter and store at -20°C in 10 mL aliquots, covered in foil.

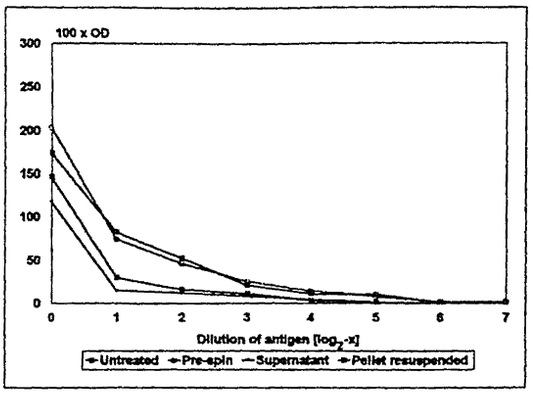
1.10 HT (x 100)

As for HAT without the aminopterin

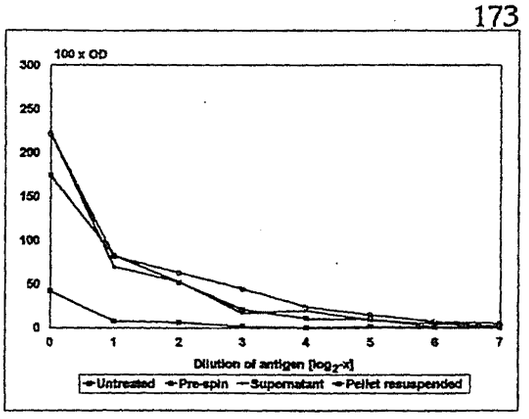
APPENDIX 2

Appendix 2a - f Absorbance values as measured by ELISA with 8 dilutions of supernatant from, or resuspended pellet of 3T3 cells exposed for different treatments (a to f) to release virions from host cell. The detecting antibody in the ELISA was rabbitαBIV. Cells were treated as follows:

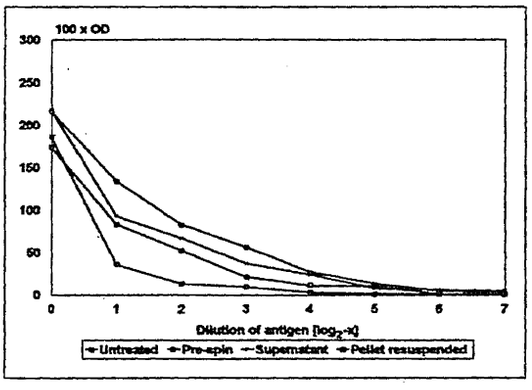
a. Vortexing for 3 minutes
b. Sonicating for 3 minytes
c. Lysing with dH_2O
d. Dounce homogenizing
e. Three freeze/thaw cycles
f. Grinding with pestle and mortar



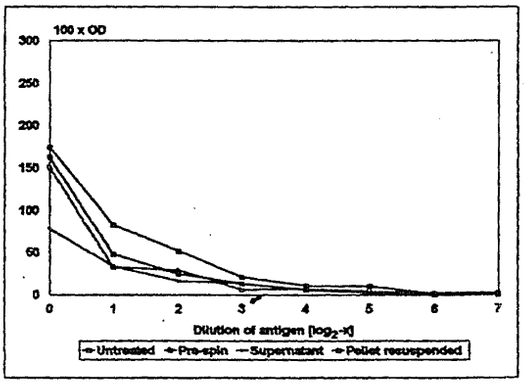
Appendix figure 2.a BIV infected 3T3 cells vortexed for 3 minutes



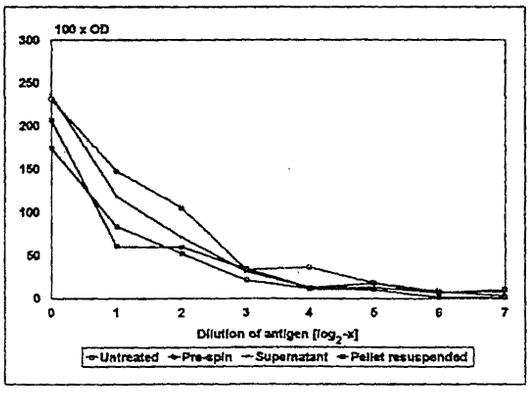
Appendix figure 2.b BIV infected 3T3 cells sonicated for 3 minutes



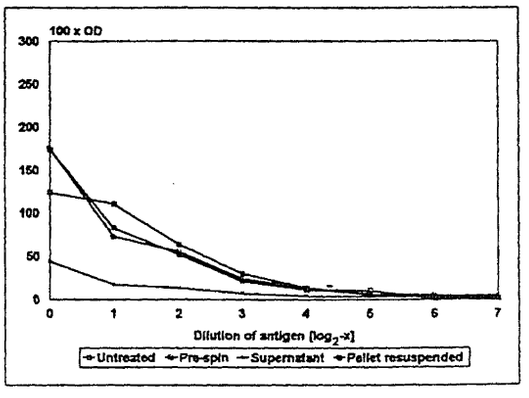
Appendix figure 2.c BIV infected 3T3 cells lysed with distilled water



Appendix figure 2.d BIV infected 3T3 cells Dounce homogenized



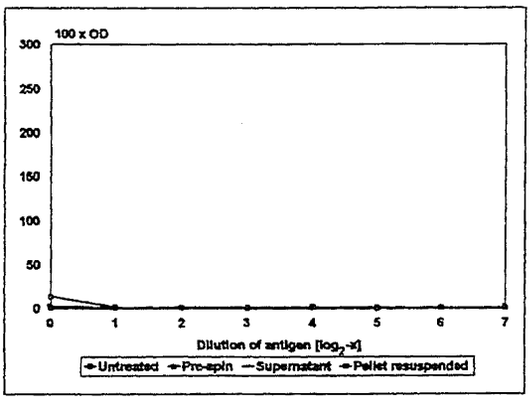
Appendix figure 2.e BIV infected 3T3 cells freeze / thawn 3 times



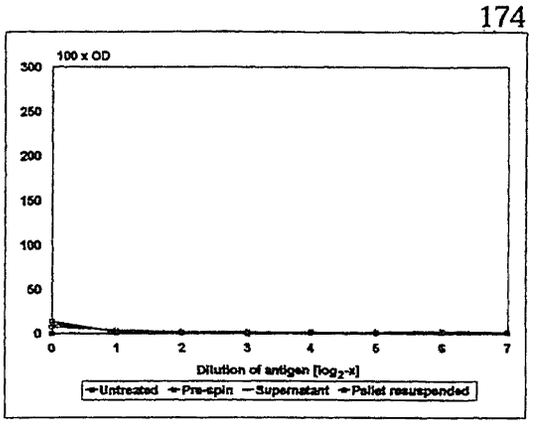
Appendix figure 2.f BIV infected 3T3 cells ground with pestle and mortar

Appendix 2g - l Absorbance values as measured by ELISA with 8 dilutions of supernatant from, or resuspended pellet of non-infected 3T3 cells exposed for different treatments (a to f) to release virions from host cell. The detecting antibody in the ELISA was rabbitαBIV. Cells were treated as follows:

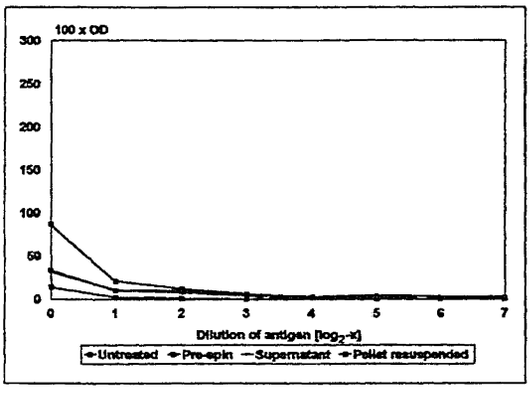
g. Vortexing for 3 minutes
h. Sonicating for 3 minytes
i. Lysing with dH_2O
j. Dounce homogenizing
k. Three freeze/thaw cycles
l. Grinding with pestle and mortar



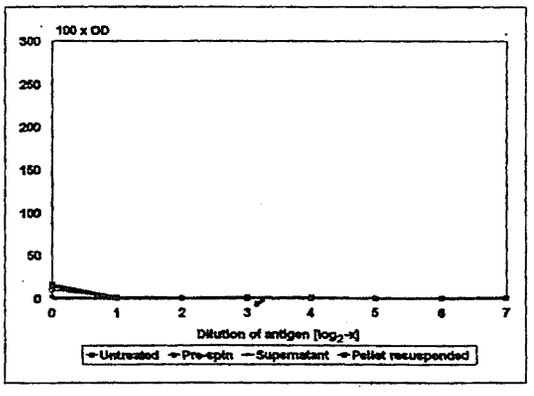
Appendix figure 2.g Non-infected 3T3 cells vortexed for 3 minutes



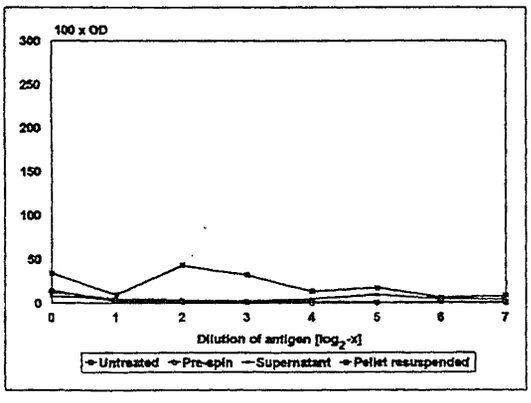
Appendix figure 2.h Non-infected 3T3 cells sonicated for 3 minutes



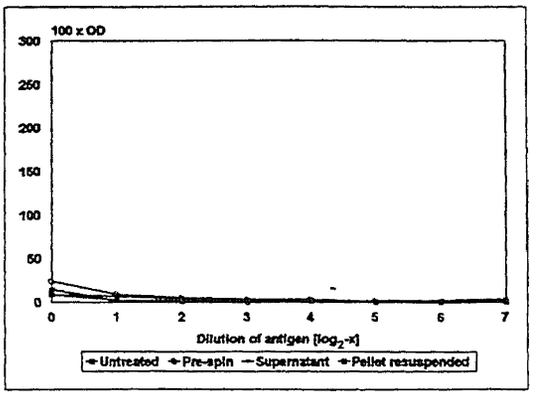
Appendix figure 2.i Non-infected 3T3 cells lysed with distilled water



Appendix figure 2.j Non-infected 3T3 cells Dounce homogenized



Appendix figure 2.k Non-infected 3T3 cells freeze / thawn 3 times

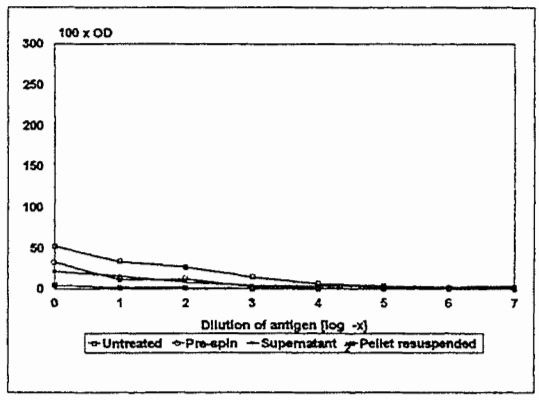


Appendix figure 2.l Non-infected 3T3 cells ground with pestle and mortar

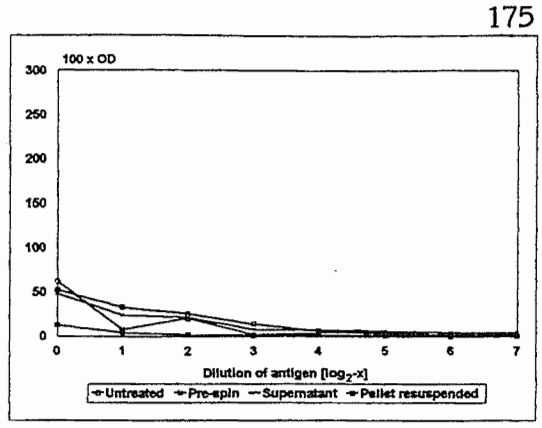
APPENDIX 3

Appendix 3a - f Absorbance values as measured by ELISA with 8 dilutions of supernatant from, or resuspended pellet of non-infected BF2 cells exposed for different treatments (a to f) to release virions from host cell. The detecting antibody in the ELISA was rabbitαBIV. Cells were treated as follows:

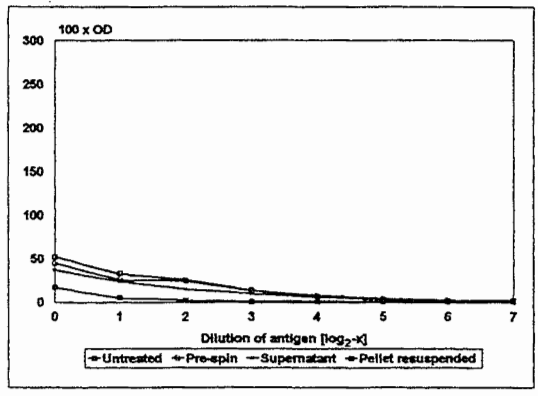
a. Vortexing for 3 minutes
b. Sonicating for 3 minytes
c. Lysing with dH_2O
d. Dounce homogenizing
e. Three freeze/thaw cycles
f. Grinding with pestle and mortar



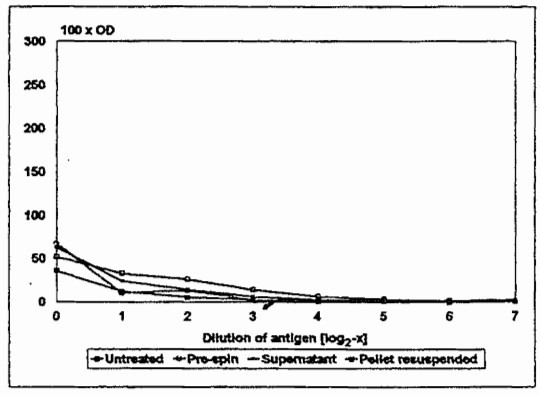
Appendix figure 3.a Non-infected BF2 cells vortexed for 3 minutes



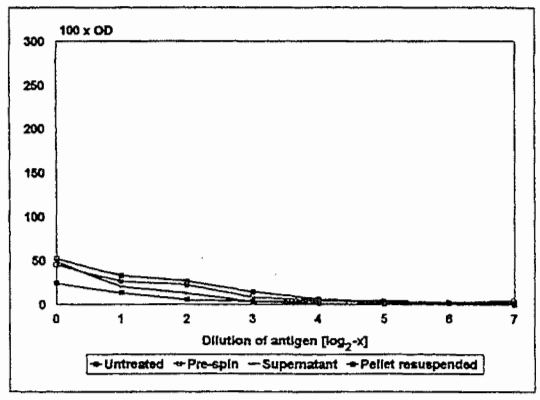
Appendix figure 3.b Non-infected BF2 cells sonicated for 3 minutes



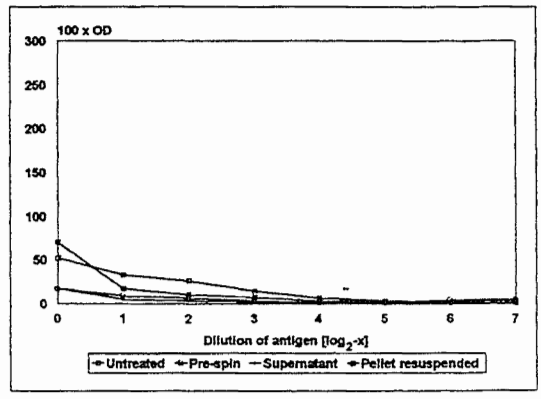
Appendix figure 3.c Non-infected BF2 cells lysed with distilled water



Appendix figure 3.d Non-infected BF2 cells Dounce homogenized



Appendix figure 3.e Non-infected BF2 cells freeze / thawn 3 times



Appendix figure 3.f Non-infected BF2 cells ground with pestle and mortar

APPENDIX 4

PARASITES FOUND IN *ELSEYA LATISTERNUM* TORTOISES

Turtle #	72	82	84	86	87	88	89	90	91	92	94	95	98	99
Bile duct / gall bladder	NE	NE	NE	NE	NE	NE	–	NE	NE	NE	NE	NE	NE	NE
Trachea	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lungs	–	–	–	cyst with pentastomes	–	–	–	–	–	–	–	–	–	–
Oesophagus	1 cyst + nematode	–	–	–	6 cysts with nematodes	–	–	–	–	–	–	–	–	–
Stomach	2 nematodes	3 nematodes head buried	Nematodes buried in wall	12 nematodes	–	–	–	–	–	–	–	2 nematodes	6–8 encysted pentastomes	1 large nematode head buried
Small Intestine	8 nematodes 3 trematodes	–	Many red nematodes buried in nodules on serosal side	7 red nematodes	–	1 trematode 2–3 nematodes	–	–	1 juvenile nematode	1 nematode	Few cysts with nematodes	2 nematodes	3 red nematodes	3 red nematodes
Large Intestine	–	6–10 red nematodes 3 trematodes	–	7 red nematodes	2 red nematodes	–	–	–	–	–	–	–	–	–