CHAPTER 11
Chlamydial disease

11.1 Introduction

During the disease survey (Chap 10) *Chlamydia pneumoniae* was identified in the lungs of an immunosuppressed *M. iteratus* with chronic pneumonia. This case was described in detail as it is significant for a number of reasons. Although chlamydiosis has been described from frogs in other countries (Newcomer et al., 1982; Mutschmann, 1998), the recent changes in the taxonomy of *Chlamydia* may mean that the identification of *C. psittaci* in these cases needs to be re-examined. *C. pneumoniae* is an important human pathogen that had previously been found only in humans, koalas and a horse (Storey et al., 1993). Since we published this report, *C. pneumoniae* was identified in captive *X. tropicalis* in the USA (Reed et al., 2000) and may be more common in amphibians than previously realised.

11.2 My role in the paper

I did the pathology and electron microscopy that led to the diagnosis of chlamydiosis, while co-authors from Queensland University of Technology identified the species using culture, PCR and immunofluorescence. Rick Speare made the initial presumptive diagnosis of chlamydiosis based on histology.

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Chlamydia pneumoniae in a Free-Ranging Giant Barred Frog (*Mixophyes iteratus*) from Australia

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The koala biovar of *Chlamydia pneumoniae* was identified in lung tissue from a sick, free-ranging giant barred frog (*Mixophyes iteratus*) by using electron microscopy, *C. pneumoniae*-specific fluorescent-antibody staining, cell culture, and sequencing of the *ompA*, *ompB* and 16S rRNA genes. This is the first report of a chlamydial strain infecting both a homeotherm and a poikilotherm and only the fourth host (in addition to humans, koalas, and horses) to be naturally infected with this species of *Chlamydia*. The frog had severe, chronic, mononuclear pneumonia and nonregenerative anemia and pancytopenia.

Chlamydia species are important pathogens infecting birds, humans, and a wide variety of other mammals (6, 18). Until 1988, there were only two species recognized in the genus, *Chlamydia trachomatis* and *Chlamydia psittaci*. In 1988, members of the TWAR group, formerly *C. psittaci*, were assigned to the new species *Chlamydia pneumoniae* (2), and then in 1992, the ruminant strains of *C. psittaci* were assigned to the fourth chlamydial species, *Chlamydia pecorum* (4). Members of the species *C. pneumoniae* are recognized as important human pathogens causing respiratory infections which may be asymptomatic or result in sinusitis, bronchitis, or pneumonia (7). In addition, they have recently been linked to coronary heart disease (1, 20). Apart from humans, the only other hosts naturally infected by *C. pneumoniae* are koalas (6, 24) and horses (based on a single report [22]).

Chlamydia infections have been reported previously in captive amphibians, causing moderate to high mortality rates in various species, including African clawed frogs (*Xenopus laevis*) in the United States (10, 17, 26), eyelash leaf frogs (*Ceratobatrachus guentheri*) in Canada (9), and *Bufo maculatum* and a *Pachytriton* sp. in Germany (16). There have also been a few case reports of *Chlamydia* infections in captive and wild reptiles (8, 12, 13). In all cases, however, the chlamydial species was either unknown or assumed to be *C. psittaci*. Here we report the first isolation of *Chlamydia* from a frog in Australia and demonstrate that it is identical to the *C. pneumoniae* strain that infects koalas.

A male, 60-g, giant barred frog (*Mixophyes iteratus*) with a snout-to-vent length of 7.8 cm, free-ranging in the Orara East State forest, New South Wales, was observed to be behaving abnormally, i.e., sitting unprotected during the day and in a lethargic state. At the laboratory the next day, the frog was found to be in poor nutritional condition and moribund and died soon after arrival. Hematological analysis of heparinized blood collected just before death revealed nonregenerative anemia (packed cell volume [PCV] = 18%) and a low total protein level (15 g/liter). The aspartate aminotransferase level was greatly increased, to 6,080 U/liter. The total white cell count was very low (0.36×10^9/liter), and examination of a Giemsa-stained blood smear showed that 85% of the leukocytes were large lymphocytes or monocytes that were difficult to differentiate. No neutrophils were detected.

During postmortem, organ samples were collected into 10% buffered neutral formalin, dehydrated, and embedded in paraffin wax or were frozen at −80°C for bacterial and chlamydial culture. Upon gross examination, the lungs were thickened and did not fully collapse after sectioning, and the spleen appeared small. Six-micrometer-thick histological sections were cut and stained with hematoxylin and eosin (H&E), Gram stain, or Perl's stain (3). On histological examination, the spleen and bone marrow were found to be depleted of hemopoietic cells, although no cause of the immunosuppression was apparent. Severe chronic mononuclear pneumonia with marked thickening of the septae due to the inflammation was present, and the airspaces contained masses of free monocytes, lymphocytes, erythrocytes, and plasma cells (Fig. 1). Many mononuclear cells had swollen cytoplasms with fine basophilic stippling (Fig. 2). A large proportion of renal tubular epithelial cells contained round intracytoplasmic deposits of light brown pigment which did not stain for iron with Perl's stain.

For electron microscopy, formalin-fixed lung tissue was postfixed in 2.5% glutaraldehyde and then 1% osmium tetroxide, dehydrated, embedded in Spurr's resin, sectioned at 70 nm, stained with lead citrate and uranyl acetate, and examined with a Hitachi 7000 transmission electron microscope. Typical chlamydial particles were frequently observed within membrane-bound inclusions in the cytoplasm of mononuclear inflammatory cells (Fig. 3) and also occasionally in alveolar epithelial cells. The chlamydial particles included dense elementary bodies (314 ± 39 nm in diameter [mean ± standard deviation; n = 12]), intermediate bodies (371 ± 44 nm [n = 12]), and numerous dividing reticulate bodies (537 ± 130 nm [n = 12]) (Fig. 4). The round elementary bodies had eccentric nuclei and a narrow or nonexistent periplasmic space. Mitochondria were not associated with the inclusion membrane. These ultrastructural characteristics are consistent with those of *C. pneumoniae* (14).

Cultures of bacteria from frozen lung, liver, and kidney samples were attempted on horse blood agar and MacConkey agar incubated at 37°C. A heavy growth of *Flavobacterium* species was obtained from lung sample cultures, and a much lighter growth was observed on cultures of bacteria from kidney and liver samples.

The presence of a *Chlamydia* sp. in lung tissue was initially

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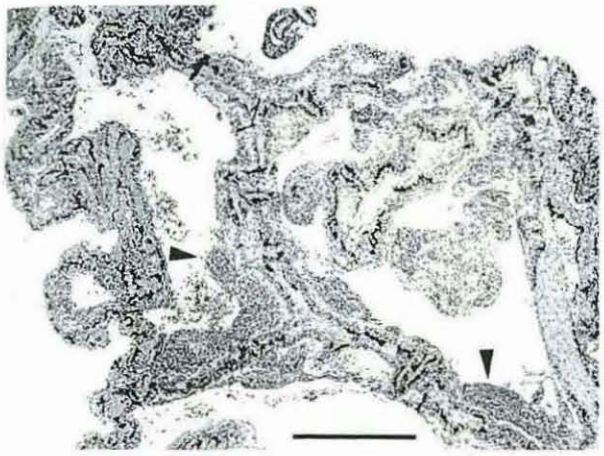

FIG. 1. Histological section of lung tissue from a giant barred frog with severe, chronic, mononuclear pneumonia. The septae, which are normally covered by a thin epithelium, are here markedly thickened by a layer of mononuclear inflammation (arrowheads). H&E stain. Bar, 500 μm.

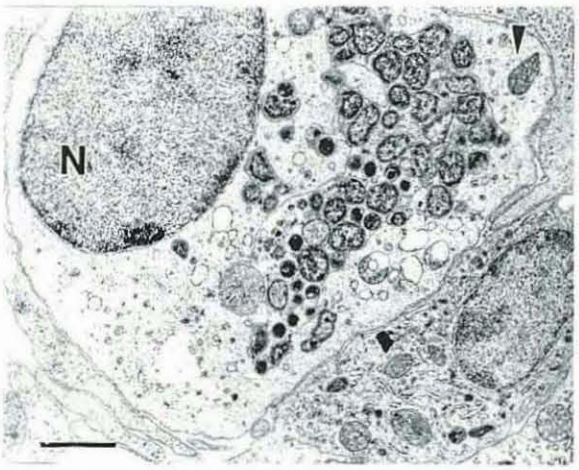

FIG. 3. Transmission electron micrograph of infected mononuclear cell with chlamydial particles at various stages present within a membrane-bound cytoplasmic inclusion. N, nucleus. The arrowhead indicates a mitochondrion. Bar, 1,500 nm.

confirmed by a strong reaction with the genus-specific Clearview lipopolysaccharide (LPS) antigen detection assay (Oxoid). The chlamydial species was identified as *C. pneumoniae* by positive staining of an impression smear with the Cellabs (Sydney, Australia) Chlamydia-TWAR fluorescent monoclonal antibody. *C. pneumoniae* was subsequently cultured from the frozen lung tissue in HEp-2 cells by using the polyethylene glycol pretreatment method (23). Infection levels were observed to be high in this cell line (with more than 75% of cells containing inclusions) when stained with either genus-specific (Cellabs Chlamydia-LPS monoclonal) or *C. pneumoniae* species-specific (Cellabs Chlamydia-TWAR monoclonal) antibodies.

The genus, species, and biovar designations of the frog isolate were confirmed by sequence analysis of the chlamydial 16S rRNA gene (235-bp fragment, positions 566 to 801) (19), the *ompB* gene (392-bp fragment) (25), and the *ompA* gene (279-bp fragment, variable domain 4 region) (24). The 16S rRNA gene sequence of the frog isolate was identical to both the horse N16 and koala strain sequences but was different by one nucleotide from the sequence of human strain TW-183. *ompB* sequence analysis indicated that the frog isolate was identical to the koala biovar of *C. pneumoniae* but its sequence was different by two nucleotides from that of the horse N16 isolate and by four nucleotides from that of human strain TW-183. *ompA* variable domain 4 sequence analysis indicated that the frog isolate was again identical to the koala biovar of *C. pneumoniae* but its sequence differed by 25 nucleotides from that of the horse N16 isolate and by 5 nucleotides from that of human strain TW-183.

This case report is significant not only for our understanding of frog diseases but also for expanding the range of hosts infected with *C. pneumoniae*. We suspect that the *Chlamydia*

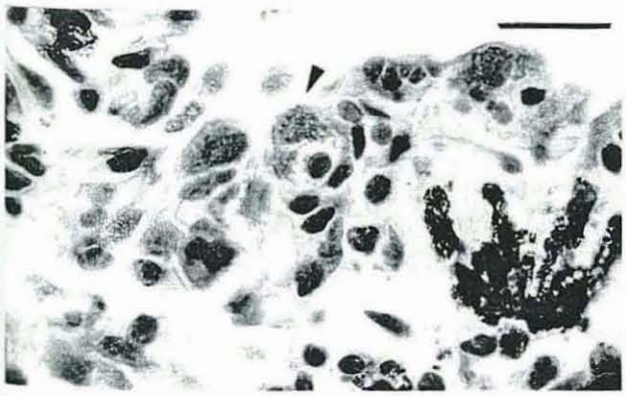

FIG. 2. Histological section of lung tissue with an infected mononuclear cell (arrowhead). It is important to note the swollen cytoplasm containing chlamydial organisms, visible as fine stippling. H&E stain. Bar, 20 μm.

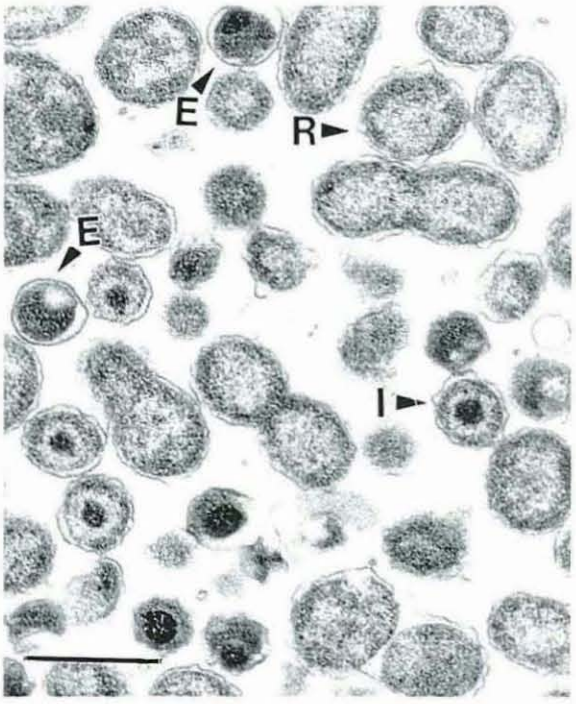

FIG. 4. Transmission electron micrograph of chlamydial particles at various stages in lung tissue. It is important to note the large reticulate bodies (R), which undergo binary fission, intermediate bodies (I), and condensed elementary bodies (E). Bar, 700 nm.

infection in the frog in this study was an opportunistic infection, secondary to immunosuppression of unknown cause. Previous reports of outbreaks of *Chlamydia* infection in captive amphibians have involved a number of animals, usually with fulminant, multisystemic infections, which is in contrast to the chronic pathology present in the free-ranging giant barred frog of the present study, where lesions were confined to the lung. *C. pneumoniae* in the other hosts that it infects, namely, humans, horses, and koalas, also usually causes respiratory disease. This might suggest that this species has a tropism for epithelial cells lining the respiratory tract. The human strains of *C. pneumoniae* have also recently been shown to be able to infect and grow in both peripheral blood and alveolar macrophages (15) as well as vascular endothelium and arterial smooth muscle cells (1).

The finding that the sequence of this frog isolate is identical to that of the koala biovar of *C. pneumoniae* raises interesting questions. We are confident that the gene analysis is reliable and does not represent laboratory cross-contamination. At this stage, however, we are unsure of the source of infection, although the Orara East State forest where the frog was found contains a significant population of koalas and reports indicate that *C. pneumoniae* infection is common in most Australian koala populations (11, 24). *Mixophyes iteratus* is a ground-dwelling fossorial frog with opportunity to come into indirect contact with koalas, who regularly walk on the ground to move between food trees. Despite recent investigations into diseases of wild amphibians in Australia (21), *Chlamydia* strains have not been identified previously in native frogs or introduced cane toads, although specific chlamydial tests for asymptomatic carriers were not done. Several studies have reported the very high genetic similarity of human *C. pneumoniae* strains (5) and also the clonality of koala *C. pneumoniae* strains (24), suggesting a possible recent divergence of these biovars. It is possible that the infection of a wild frog described in this report was an isolated incident, or alternatively, increased testing may show that amphibians are commonly infected with *C. pneumoniae* and that they are a natural reservoir for this species.

Nucleotide sequence accession numbers. The *ompA* variable domain 4, *ompB*, and 16S rRNA gene sequences have been deposited in the GenBank database under accession no. AF102830, AF102831, and AF102832, respectively.

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CHAPTER 12
Other fungal diseases

12.1 Introduction

Although systemic fungal infections in mammals are almost always associated with immunosuppression, many fungi apart from *B. dendrobatidis* are not uncommon primary pathogens in aquatic animals such as fish, crustaceans and amphibians (Reichenbach-Klinke and Elkan, 1965). The fungal infections reported in amphibians prior to this study have already been reviewed in Chapter 2. Most reports are about chromomycosis, which is caused by pigmented fungi, and concern captive amphibians. An *Ichthyophonus*-like organism was recently reported to cause fatal myositis in wild frogs and newts in Canada (Mikaelian et al., 2000).

In the survey of diseases in Australian frogs, a variety of fungal infections apart from chytridiomycosis were found in individual wild and captive amphibians and are listed in Chapter 10. Some were due to unidentified hyphae that infected the skin. Two diseases seemed to be primary and to have some epidemic potential. As they had not been previously well described, they were chosen for further investigation. This chapter consists of published reports on two specific fungal diseases where the organism was identified: aphanomycetosis in tadpoles of *B. marinus*, and mucormycosis in several species of amphibians. They warrant description and may prove to be not unusual in wild Australian amphibians.

12.2 My role in the papers

This work was done in collaboration with several scientists and has been published as four journal papers. During an outbreak caused by *Aphanomyces* in wild tadpoles of *B. marinus*, specimens were collected and their gross lesions described by Rick Speare. Annette Thomas performed the fungal culture. I attempted the virus isolation and examined specimens grossly, by histopathology and by scanning electron microscopy. Alex Hyatt directed the electron microscopy. For the cases of mucormycosis my role varied with the study. In the initial study on the pathology of mucormycosis in *B.*

marinus much of the basic work had been done by Rick Speare and my role was to finalise the data and produce the figures. In the case report of mucormycosis in an adult *L. caerulea*, John Humphrey assisted with the post mortem and Rick Speare assisted with the histological diagnosis, but I did the majority of the work and writing. My role in the report of mucormycosis in *L. adelensis* and *L. infrafrenata* from Perth Zoo was minor, and consisted of comment on the diagnosis and pathology, and input into the manuscript.

12.3 Aphanomycetosis in tadpoles of *B. marinus*

Berger, L., Speare, R., Thomas, A., Hyatt, A. 2001. Mucocutaneous fungal disease in tadpoles of *Bufo marinus* in Australia. Journal of Herpetology. 35: 330-335.
This paper describes two outbreaks of fungal disease in tadpoles of *B. marinus* near Townsville. The disease had an unusual appearance with tufts of fungi growing primarily on the head of tadpoles. When the disease was encountered a second time, more detailed investigations were undertaken. Although a range of fungi were cultured, all were considered to be secondary infections or contaminants except for an *Aphanomyces* sp. that is likely to be the primary agent. Mortality rate could not be determined, but the high prevalence (37%) and debilitating lesions show the disease would have impacted on the tadpole population. However, this would be unlikely to affect the adult population size as toads are phenomenally fecund. Also in normal circumstances tadpole survival has been reported to be very low (5%) due to density dependent predation (Calef, 1973). Attempts to find a disease for biological control of cane toads focussed on susceptibility of adults, as a very high mortality rate in tadpoles is required to have an impact on overall population levels (R. Speare and W. Freeland, pers comm.).

12.4 Mucormycosis

Speare, R., Berger, L., O'Shea, P., Ladds, P., Thomas A. D. 1997. Pathology of mucormycosis of cane toads in Australia. Journal of Wildlife Diseases. 33: 105-111.

Berger, L., Speare, R., Humphrey, J. 1997. Mucormycosis in a free-ranging green tree frog from Australia. Journal of Wildlife Diseases. 33: 903-907.

Creeper, J., Main, D., Berger, L., Huntress, S., Boardman, W. 1998. An outbreak of mucormycosis in slender tree frogs (*Litoria adelensis*) and white-lipped tree frogs (*Litoria infrafrenata*) at Perth zoo. Australian Veterinary Journal. 76: 761-762.

Mucor amphibiorum is a zygomycete fungus that causes systemic disease in frogs and toads. Although it exists as mycelia in soil and when cultured *in vitro*, in tissue it is only found as round bodies called "sphaerules". The fungus was described after causing deaths in captive anurans in Germany (Frank et al., 1974; Schipper, 1978) where it was suspected to have originated from an Australian *L. caerulea*. Mucormycosis was not recognised again until Rick Speare identified it during a survey of Australian cane toads (Speare et al., 1994). The disease is widespread throughout the distribution of cane toads and occurred as individual cases in 0.7% of toads. *M. amphibiorum* was subsequently identified as the cause of a fatal granulomatous, ulcerative disease of platypus in Tasmania (Obendorf et al., 1993).

The first paper presented here (Speare et al., 1997) is a description of the pathology in the original cases of cane toads from Speare et al., (1994). Chronic granulomas occurred in most organs and in some animals there was also necrotic mixed inflammation. Berger et al. (1997) is a case report detailing the pathology seen in the first wild frog found with mucormycosis, an adult of *L. caerulea* with a systemic infection that appeared to have originated in the nose. The third paper (Creeper et al., 1998) describes an outbreak of acute disease in Perth Zoo where 80% of a group of *L. adelensis* died over 30 days, and deaths also occurred in *L. infrafrenata*. Lesions were more necrotising and diffuse than the mainly circumscribed lesions in cane toads.

Since these publications, I have found cases of mucormycosis in three more wild frogs, one captive frog and three wild cane toads (Chap 10). A *Lim. peronii* from Rockhampton (99 1562/24) had multiple chronic granulomas in liver, spleen, kidney, heart, stomach, fat bodies and peritoneum. Two *L. caerulea* from Queensland that died

with chytridiomycosis had localised *Mucor* infections – a frog from Brassall (96 961/19) had a large inactive granuloma in the submucosa of the small intestine and a frog from Rockhampton (00 1645/3) had a few foci of granulomatous inflammation in the liver and large intestine. The captive frog, a *L. caerulea* from Adelaide (96 495/16) and the three wild cane toads from Queensland- from Sunny Bank (96 1429/3), Cordalba State Forest (99 951/2), and Enoggera State Forest (00 1266/14) - all had severe multi-systemic infections with chronic granulomas.

In summary, for the survey of sick amphibians (Chap 10), mucormycosis was found in 3/9 (33.3%) wild cane toads but just 4/243 (1.7%) wild frogs, two of which only had minor infections. It appears the disease is more prevalent in toads and may be less pathogenic to native frogs. If frogs have greater immunity, this adds support to the theory that the organism is endemic to Australia. Although outbreaks with high mortality occurred in captive amphibians, outbreaks were not found in the wild during our investigations. Therefore it appears that *M. amphibiorum* is not a highly pathogenic organism in frogs in nature. Experimental work on dose rates and transmission are lacking. More information on the distribution of *M. amphibiorum* would be useful in assessing the risks of its spread, particularly as cane toads are expanding their range and could act as vectors.

There are also many gaps in understanding the disease in Tasmanian platypus. It is interesting that in Queensland, the distributions of platypus and infected toads overlap but disease has not been seen in platypus. In northern Tasmania, mucormycosis has not been found in amphibians but occurs frequently in platypus (Connolly et al., 1999). Investigation of pollutants is underway to attempt to explain the susceptibility of Tasmanian platypus (Munday et al., 1998).

CHAPTER 13
Helminth diseases

13.1 Introduction

A large number of parasitic helminths have been described from Australian amphibians (Barton, 1994) but there are few reports of the pathogenic effects of these parasites. In the survey of diseases in Australian frogs (Chap 10), three helminth infections were suspected to have a significant impact on their hosts - *Rhabdias* sp., *Spirometra erinacei* and trematode metacercaria. Descriptions of the latter two infections comprise this chapter.

Although the lung nematode *Rhabdias* occurred commonly in frogs without evidence of ill effects, in three frogs heavy infections may have been involved in their deaths.

Spargana of the cestode *Spirometra erinacei* are known to be pathogenic in snakes with heavy burdens (McCracken, 1994), but there have only been some brief references to their potential to cause disease in amphibians (Flynn, 1973; Bennett, 1978; Mastura et al., 1996). Infections were detected in twelve free-living frogs and in at least seven of these cases the infection was considered to be pathogenic. The manuscript included below contains a description of the pathology seen in severe sparganosis.

Frogs are hosts for many species of trematode metacercaria (Flynn, 1973). Apart from reports of heavy experimental infections in tadpoles causing bloat (Cort and Brackett, 1938) and increased mortality (Johnson et al., 1999), the pathology caused by metacercaria in tadpoles has not been described. The heavily infected tadpoles seen in this survey appeared to be healthy but the bright orange cysts changed the appearance of the usually dark tadpoles and may have increased their chance of being predated. Although the metacercarial infection was striking, it appears not to be a significant disease. A short description is included below which may aid pathologists with diagnosis.

13.2 My role in the manuscripts

For the report on sparganosis, I performed the post mortems and histology on all cases, Lee Skerratt identified the parasites, and Rick Speare helped with interpretation. For the report on trematode metacercaria, I performed the post mortems and histology, Harry Hines collected the specimens and Di Barton identified the metacercaria.

13.3 Pathology of sparganosis

Berger, L., Skerratt, L., Speare, R. Pathology of sparganosis in Australian frogs. Australian Veterinary Journal. (in prep)

13.3.1 Abstract

Infection with plerocercoids of *Spirometra erinacei* occurred in 12/243 (4.9%) of sick, wild frogs in Australia. Infections were seen in the muscle and subcutis of adults of *Litoria caerulea*, *L. aurea*, *L. gracilenta* and *L. peronii*. Heavy burdens in seven frogs were associated with debilitating lesions, whereas infections in five sick frogs were considered to be incidental to other diseases. Concurrent diseases and parasites are described.

Key words: *Spirometra erinacei*, spargana, cestode, frog, *Litoria*, pathology, *Ophidascaris pyrrhus*, *Pleistophora*

13.3.2 Introduction

The adult stage of the cestode *Spirometra erinacei* inhabits the small intestine of carnivores such as dog, cat, fox and dingo. The procercoid stage occurs in copepods and the plerocercoid stage (spargana) is found in amphibians, reptiles and mammals that ingest infected copepods. Tadpoles are an important host in which the plerocercoid stage develops from the procercoid, and can transmit the spargana to other intermediate hosts (Sandars, 1953).

S. erinacei also exists in south-east Asia and is a public health problem as subcutaneous or intramuscular sparganosis occurs in humans (Mastura et al., 1996). Routes of infection include ingestion of incompletely cooked tadpoles and frogs, and drinking

water contaminated with procercoid stages. Ocular sparganosis also occurs, through using infected frog flesh as a poultice on the eye (Mastura et al., 1996).

In Australian amphibians, spargana have been reported in wild adults of *Bufo marinus*, *Litoria aurea*, *L. caerulea, L. nasuta* and *L. rubella* (Sastrawan, 1978; Barton, 1994), and experimental infections were produced in adults of *L. latopalmata* and *Limnodynastes tasmaniensis* and tadpoles of *L. latopalmata, L. caerulea* and *L. tasmaniensis* (Sandars, 1953; Bennett, 1978; Sastrawan, 1978). Sandars (1953) reports that about one quarter of the population of *L. caerulea* in the Brisbane area was infected with spargana. In a recent survey of Australian amphibians, spargana were found in 10/875 (1.1%) *B. marinus*, 2/6 (33.3%) *Lit. peronii*, 1/15 (6.6%) *L. caerulea* and 7/163 (6.1%) *L. inermis* (Barton, unpub). These animals, which had no signs of ill health, each had between one and seven spargana in thigh muscles.

In a group of 1000 *B. marinus* from Ingham, Queensland, 37 (3.7%) were found with infections of spargana provisionally called *S. mansoni* (Bennett, 1978). These cane toads had light infections - an average of 6.3 spargana per toad with 59% of spargana occurring in the thighs. There was a marked local inflammatory response in these toads and over half the spargana were dead. Immunodiffusion and immuno-electrophoretic tests in the toads revealed antibodies were produced to components of the spargana. Attempts to study the reactions in experimental *L. tasmaniensis* failed due to inconsistent infection rates and frequent deaths of infected frogs and tadpoles, which were thought to be due to the combined stress of parasitism and captive conditions (Bennett, 1978). Growth of experimentally infected *L. latopalmata* tadpoles was inhibited (Sandars, 1953). In a survey of 948 Malaysian frogs, 11.8 % were found infected with spargana, 57% of which had bleeding and/or swelling at infection sites (Mastura et al., 1996). The pathology of severe sparganosis has not been described in amphibians.

Infections in Australian snakes occur commonly in subcutaneous areas, intercostal muscles or the peritoneal cavity. Heavy infections can cause inappetence and weight loss, and are treated by surgical removal and antibiotics (McCracken, 1994). A variety of marsupials and the monotremes have been reported with sparganosis, with disease occurring in echidnas and antechinus (Beveridge, 1978; Whittington et al., 1992).

In our survey of 243 sick and dead wild frogs from around Australia, spargana were found in 12 (4.9%) frogs from New South Wales and Queensland. These spargana were identified as *S. erinacei* by sequencing of the cytochrome *c* oxidase gene, and the sequence results are reported in Zhu et al. (2001). Seven frogs had heavy infections that appeared to be causing disease. Lighter infections were seen in five sick frogs that were diagnosed with chytridiomycosis, opthalmitis or encephalitis. Here the pathology of sparganosis is described and details of concurrent parasites and diseases are recorded, as some of these are novel.

13.3.3 Methods

Between October 1993 and December 2000, 243 sick and dead wild amphibians from around Australia were collected and examined for the presence of pathology. Thirty-eight wild tadpoles were also examined. Dead frogs found by herpetologists were preserved in 10% formalin, 70% ethanol or were frozen. Sick frogs were identified by their unusual behaviour (e.g. lethargy, sitting unprotected during the day) or by the presence of lesions (e.g. reddening, ulcers, lumps). Sick frogs were either euthanased and preserved, or were couriered to the laboratory. If frogs were received live, blood was collected into heparinised tubes for haematology and biochemistry. Frogs were weighed and photographed and their snout-vent-length was measured. Parasites were collected and the following organs were placed in 10% buffered neutral formalin and prepared for histology: liver, kidney, spleen, lung, heart, stomach, intestines, pancreas, brain, spinal cord, vertebrae, feet, thigh muscle and skin. Small frogs (i.e. <15 mm) and tadpoles were not dissected but were cut in half and sectioned for histology. Tissues were dehydrated, embedded in paraffin, sectioned at 6 μm and stained with haematoxylin and eosin, Ziehl-Neelson or Gram's stain (Drury and Wallington, 1980).

In frogs with sparganosis, the number of worms were counted that were visible upon reflection of skin -i.e. in subcutis and on superficial muscles. A total count of spargana infecting each frog was not attempted, as this would involve dissection of all muscle bundles, which is incompatible with performing histopathology. Live spargana were relaxed in distilled water, then preserved in 70% ethanol or frozen at -80°C. As identification of spargana using morphological features is unreliable, specimens were identified by DNA sequencing of the cytochrome *c* oxidase gene, as reported in Zhu et al. (2001).

13.3.4 Results

Spargana were found in 12/243 (4.9%) sick wild frogs. All infected frogs were from New South Wales and Queensland. Prevalences in infected species were 1/2 *L. gracilenta*, 1/12 *L. peronii*, 2/8 *L. aurea*, and 8/73 *L. caerulea*. For details on other frogs examined see Berger (2001). Infections were not found in 38 wild tadpoles that were examined.

Between 3 and 11 spargana were seen in five sick frogs in which other diseases were diagnosed (Cases 1-5). Seven other frogs had heavy infections with over 16 spargana (Cases 6-12), and in these frogs the severe sparganosis was suspected to be the primary cause of illness. All frogs had plerocercoids in the muscle and subcutis of the thighs (Figs. 13.1-13.3), and nine were infected in additional locations such as subcutis and muscle of ventral abdomen (8 frogs), dorsal subcutis especially around the urostyle (7 frogs), lumbar muscle (3 frogs), peritoneal cavity (3 frogs), and muscle and subcutis of the forelimbs (2 frogs) (Table 13.1).

Spargana appeared as white flattened worms with transverse wrinkles. They were of variable length, up to about 50 mm. On histology they had features typical of cestodes, and had calcareous corpuscles, a thick cuticle and lacked a digestive tract.

Encapsulated spargana occurred within thin, membranous capsules or within thicker fibrotic capsules. In many frogs there was negligible cellular reaction, although granulomatous inflammation occurred within capsules and in interstitial areas of muscle in some frogs (Figs. 13.4 & 13.5). Unencapsulated spargana occurred free between muscle fibres, in subcutaneous spaces and in the peritoneal cavity.

Cases 1-5

The five frogs with light infections (three *L. caerulea*, one *L. peronii* and one *L. aurea*) are listed in Table 13.1. These frogs had between 3 and 11 spargana visible on reflection of skin. In addition to infection of the thighs, one frog had spargana in the subcutis of the caudal abdomen and dorsum, and one had infection around the urostyle. Both encapsulated and free spargana were observed. In three frogs spargana were examined histologically and a fibrous reaction was associated with infection in one, and in two

there was a moderate granulomatous response. In one of these the spargana were degenerate.

Case 6 - *L. aurea*, Homebush, NSW

A 25.1 gm frog at a brick pit in urban Sydney was severely lethargic with a slow righting reflex. It died in transit to the laboratory. Severe external lesions consisted of multiple ulcers and extensive patchy discolouration of the skin. The skin over the right tibia was haemorrhagic and ulcerated, and distally the limb became dark brown, shrivelled and gangrenous. Seven deep ulcers (2-5 mm) occurred on the right stifle, right tibio-tarsal joint, caudal right thigh, left tarsus, dorsal skin of back, right elbow and left ventral thigh. Most ulcers were surrounded by haemorrhage in the skin, and were associated with purpureal haemorrhages in the underlying muscle. Subcutaneous spargana were associated with some of these lesions and were present encapsulated or free in subcutaneous space and muscles of the thighs, head and lateral abdominal wall. Masses of unencapsulated spargana were present throughout the lumbar muscle associated with friable, haemorrhagic muscle. Spargana were also present in the abdominal cavity along dorsal peritoneum adjacent to the spinal chord. In total, over 35 worms were observed. None occurred in the forelimbs or distal to the stifles.

Histologically, spargana were seen between muscle bundles, either free or surrounded by thin membranes or thick fibrous capsules (Figs. 13.4 & 13.5). There was extensive myonecrosis, haemorrhage, fibrosis and interstitial granulomatous inflammation associated with spargana. A microsporidian identified as *Pleistophora* sp. by Frank Mutschmann (Tierarztpraxis, Berlin) occurred occasionally as clusters within fibrotic foci or in the centre of muscle fibres. Masses of short gram-negative bacterial rods occurred adjacent to spargana in the skin and muscle. Bacterial infection of the skin was associated with severe haemorrhage in the dermis.

The frog was heavily gravid, had no obvious fat bodies and the lungs, spleen and kidney were paler than normal. There was a severe septicaemia with bacterial colonies in blood vessels and parenchyma of the spleen, lung, kidney and spinal cord. Lymphoid tissue of the spleen appeared mildly depleted. Occasional renal tubules were composed of pyknotic cells. Encysted degenerate nematodes with fibrous capsules formed nodules in the bladder. The right foot had extensive gangrenous necrosis.

Bacterial cultures of the lung on horse blood agar and MacConkey agar grew *Aeromonas sobria* and *Sphingobacterium spiritivorum* in moderate numbers.

Case 7 - *L. caerulea*, Emerald Beach, NSW

A 154 gm frog was found sitting unprotected on a garden fence during the day. It was lethargic and in poor body condition with loose lumpy skin over limbs, and had dark skin and dull eyes. On post-mortem 16 spargana were seen encapsulated on the superficial muscles and free in the adjacent subcutaneous spaces of the left ventral thigh, right forelimb and in the abdominal muscle. Spargana occurred within adhesions between the skin and the ventral abdominal wall. Histology revealed that much of the muscles of the thigh and forearm were replaced by spargana within fibrous capsules. There were occasional small interstitial granulomata in the muscle.

The frog had no visible fat bodies and a small (0.037 gm) spleen. A large seed (~35 x 25 mm) was in the stomach, similar to others present in the transport container. Examination of sections of internal organs showed trophozoites of *Myxidium* sp. were present in the gall bladder and a few protists occurred in the brain with no associated pathology.

Biochemical analysis of heparinised blood provided the following results: 15% packed cell volume (PCV), 42 g/l total solids, 25.4 mmol/l urea, 0.03 mmol/l creatinine, 170 u/l alkaline phosphatase, 190 u/l aspartate aminotransferase, 40 g/l total protein and 24 g/l albumin. Very few leucocytes were seen on a blood smear.

Case 8 - *L. caerulea*, Coffs Harbour, NSW

A 95 gm frog was collected as it had remained stationary on a window-sill for about a week. It was in moderate body condition and appeared slightly weak, sitting with the hind legs held loosely to the body. Many 3-10 mm nodules occurred in the skin of the caudal belly and ventral thighs, proximal tarsus, pectoral girdle and adjacent to the urostyle (Figs. 13.1 & 13.3). There were a few shallow 1 mm pink erosions/ulcerations on the ventral thighs. Bilateral hyperaemic patches occurred in the inguinal regions. Forty-one spargana were visible on reflection of skin over thighs and abdomen, with mild haemorrhage surrounding spargana in thigh muscles. Fibrotic adhesions had formed between the muscle and dermis, especially on the ventral abdomen. On

histological examination a few spargana were encapsulated by thick fibrous capsules around a layer of pyknotic inflammatory cells. Occasional muscle fibres were undergoing coagulative necrosis, and there were small granulomata replacing muscle. Under the dermis of the skin of the thigh and abdomen was a thick layer of granulomatous inflammation.

The frog had small fat bodies. Cream coloured 1-4 mm nodules protruded from the surface of the ventral liver and along the edge of lobes. On sectioning, the right and middle liver lobes had large central areas of white, fibrous spongy material. Nodules also occurred on serosa of spleen, mesentery, thickened bladder, kidney, testes and lungs. Histologically, these were chronic encapsulated granulomas with layers of eosinophils and they often contained larval nematodes that were consistent with spiruroids. The centre of the liver lobes contained masses of granulomatous inflammation with giant cells and colonies of gram positive bacterial rods. Biliary fibrosis and dilation suggested obstruction of the bile ducts had resulted due to the space occupying lesions. Some renal tubules were dilated with clear crystals in the lumen and flattened tubular epithelium.

Although a white cell count was not done, a differential white cell count showed a marked eosinophilia: 55% eosinophils, 32% lymphocytes, 11% neutrophils, 2% monocytes.

Case 9- *L. caerulea,* Korora, NSW

A 181 gm frog had remained stationary on the porch of a house for about a week. It had 3 x 4 mm bilateral reddened erosions in the inguinal regions and behind the stifles. Reflection of the skin over the thighs revealed excess subcutaneous fluid and adhesions between muscle and dermis. Forty-six spargana were present free in the subcutaneous space on the thighs and inguinal areas. There were also a few spargana around the caudal urostyle, on the dorsal head and in ventral abdominal muscles with over 60 visible spargana present in total. Many spargana that occurred freely in the subcutaneous fluid did not have a linear body and had a lobular appearance.

On histology, spargana were encysted between the dermis and muscle with moderate fibrosis and adhesions around worms with some focal degeneration of overlying

epidermis. Within the muscles of the thighs there was only a mild fibrous reaction to the worms and no inflammation.

The frog was in an early stage of vitellinogenesis, and had moderate fat bodies. An ascaridoid nematode encysted within a thin membrane was attached to the serosa of the small intestine, 52 mm distal to the pylorus. This was identified by John Sprent (Moggill, Queensland) as an adult of *Ophidascaris pyrrhus*. *Parathelandros* sp. were present in the cloaca.

Biliary fibrosis occurred in the liver which had an increased amount of melanin. Six pale nodules were present in the caudal bladder wall containing circular brown refractile objects on histology. These were suspected to be thorns that had attached during a bladder prolapse.

Haematological and biochemical tests performed on blood samples gave the following results: 28% PCV, 2.05×10^9 leucocytes, 2% metamyelocytes, 12% bands, 18% neutrophils, 44% lymphocytes, 18% monocytes, 6% eosinophils, 5 g/l total solids, 16.7 u/l urea, 0.02 u/l creatine, 129 u/l alkaline phosphatase, 196 u/l aspartate aminotransferase, 45 g/l total protein and 27 g/l albumin.

Case 10 - *L. gracilenta*, Kallangur, Queensland

The frog appeared lethargic and was collected and held in captivity for a week. Due to its inappetence and deteriorating condition it was euthanased with percutaneous lethabarb and preserved. It was emaciated and had markedly swollen, lumpy ventral thighs (Fig. 13.2). Masses of free spargana (53) were found under the skin of thighs and on the caudo-ventral abdomen. Spargana were also encysted in muscle of the thighs and comprised about half the mass of the limb. Histologically much of the thigh muscle was replaced by spargana surrounded by thin, loose capsules. Individual muscle cells appeared degenerate and there was some evidence of muscle regeneration with formation of sarcoblasts.

Fat bodies were not visible grossly and the frog was non-gravid. The left eye was sunken and the pupil constricted. The lens of the left eye was small with an irregular degenerate surface and loss of the lens capsule, although the retina was intact.

The liver appeared atrophic with a greater than normal amount of melanin. Numerous trophozoites of *Myxidium* sp. were present within the gall bladder which had a thickened fibrous wall. *Rhabdias* sp. were present in the lung. The spleen appeared inactive and bone marrow was depleted.

Case 11- *L. caerulea*, Dysart, Queensland

This frog was found dead and was emaciated with thin legs and abdomen, and a prominent urostyle and pelvic bones. A few small ulcers (about 3 mm diameter) occurred on dorsal skin and one larger one (5 x 3 mm) occurred dorsal to the right axilla. Over 23 spargana occurred free or encapsulated in muscles and subcutaneously on the thighs, caudo-ventral abdomen, lumbar muscle, dorsum, and also in the caudal peritoneal cavity. Histologically, spargana were surrounded by fibrosis and replaced much of the thigh muscle.

Gross examination of other organs revealed that there were no obvious fat bodies, the spleen was small and the gall bladder was enlarged. Moderate autolysis precluded useful histological examination of internal organs, but cutaneous chytridiomycosis was diagnosed.

Case 12- *L. caerulea*, Dysart, Queensland

This frog died soon after collection. It was emaciated with thin legs and a shrunken abdomen, and bones of the urostyle, pelvis, vertebrae and skull were prominent. Over 40 spargana occurred free or encapsulated in muscles and subcutaneously on the thighs, forearms, caudo-ventral abdomen, lumbar muscle, dorsum, and also in the caudal peritoneal cavity. On histology, worms were surrounded by fibrosis and comprised much of the thigh muscle.

Fat bodies were not obvious grossly, *Rhabdias* sp. occurred in the lungs, the gall bladder was large and the frog was non-gravid. There was moderate autolysis of internal organs but the skin appeared normal.

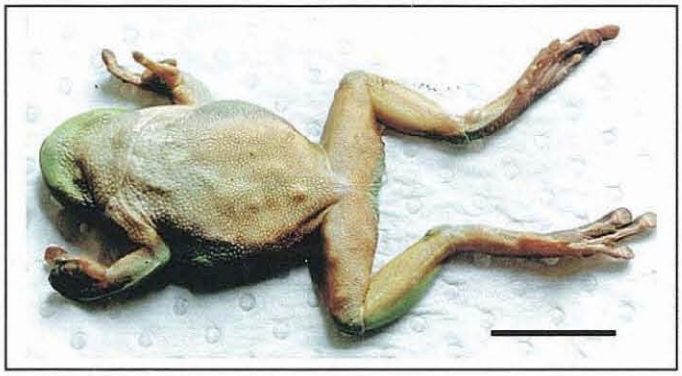

Figure 13.1 An adult of *Litoria caerulea* (case 8) with subcutaneous lumps due to spargana on the ventral abdomen and thighs and on the left tarsus. Bar = 30 mm.

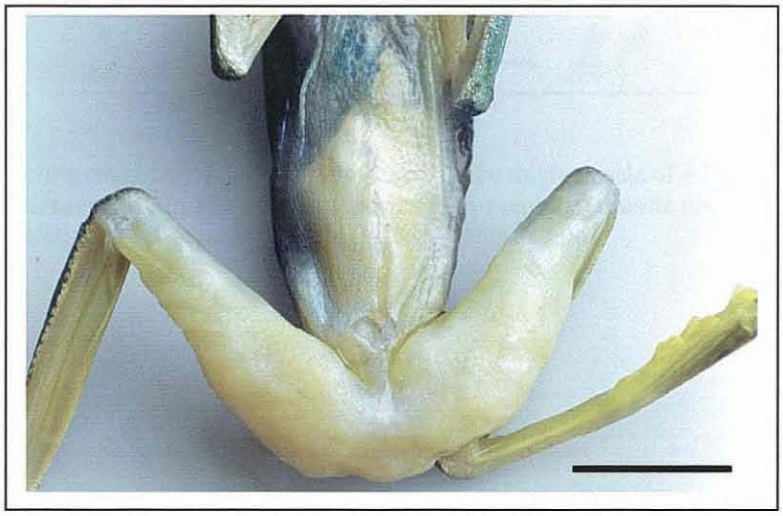

Figure 13.2 Ventral surface of a thin adult of *Litoria gracilenta* (case 10) with massive enlargement of the thighs due to a heavy burden with spargana occurring free under the skin. Bar = 12 mm.

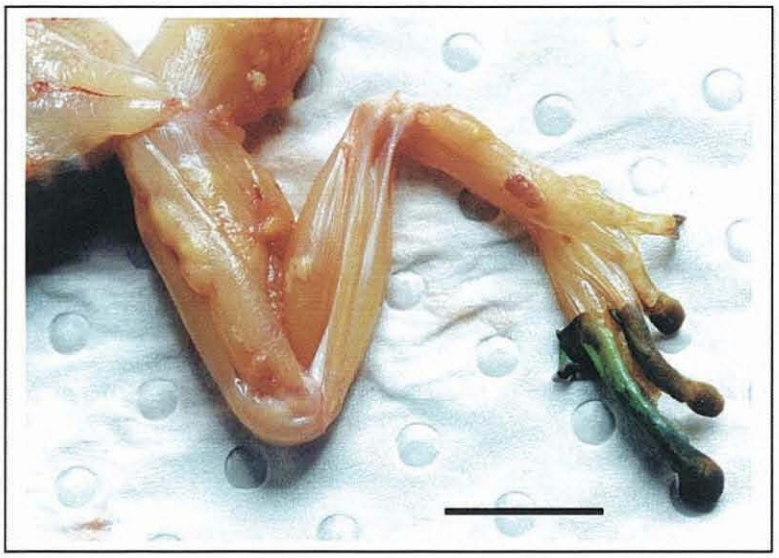

Figure 13.3 Ventral surface of the hind leg of an adult of *Litoria caerulea* (case 8) with encysted spargana in the muscles. Bar = 15 mm.

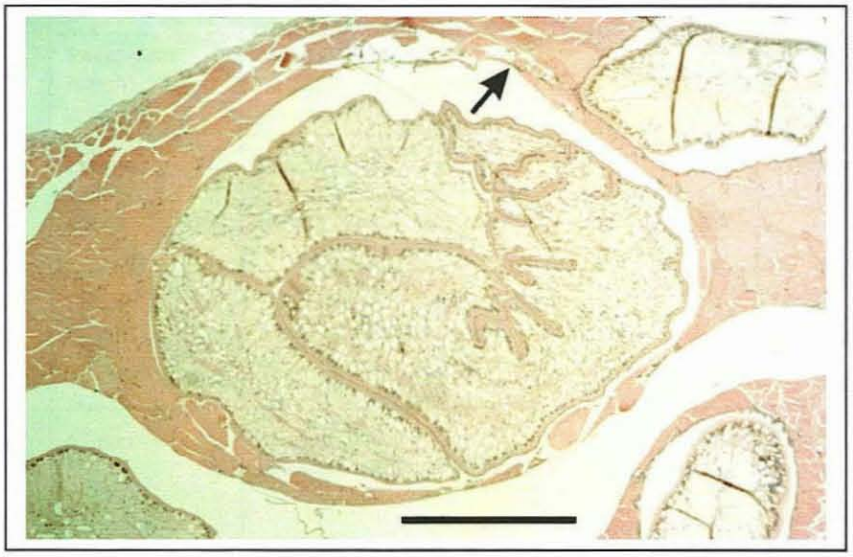

Figure 13.4 An encysted sparganum of *Spirometra erinacei* in the thigh muscle of a *Litoria aurea* (case 6) without associated tissue reaction. The cestode appears to have replaced muscle mass and there is degeneration and dissolution of the adjacent muscle (arrow). Bar = 380 μm.

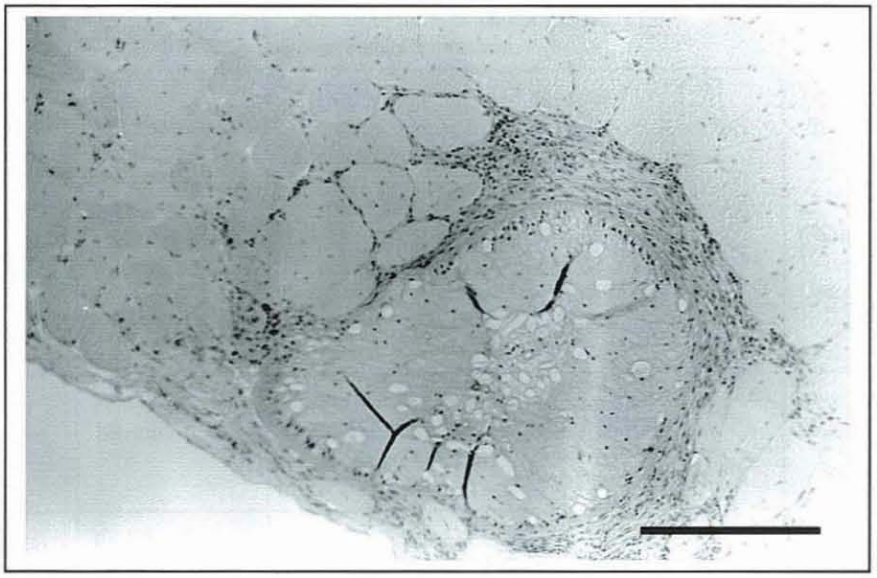

Figure 13.5 Sparganum in the muscle of a *Litoria aurea* (case 6) surrounded by fibrosis and granulomatous inflammation. Bar – 160 μm.

Table 13.1 Summary of the pathology and concurrent diseases for each frog with sparganosis.

Species **Location, Date** **Accession no.**	**Sex** **Snout-vent length**	**Sites of infection with spargana and associated pathology**	**Concurrent diseases and infections**
1. *Litoria caerulea* Yamanto, Qld July 1996 (96 961/11)	Female 126 mm	Thighs. Granulomatous foci in muscle and fibrous/granulomatous capsules around spargana.	Protistan encephalitis *Myxidium* sp.
2. *Litoria peronii* Bambree, NSW November 1998 (98 1469/11)	Female 82 mm	Thighs, dorsum, ventral abdomen. Ventral and dorsal adhesions in subcutis.	Mild chytridiomycosis
3. *Litoria caerulea* Qld November 1998 (98 1469/5)	57 mm	Thighs. Spargana not found on histology	Chytridiomycosis
4. *Litoria caerulea* Tully, Qld November 1998 (98 1469/13)	Female 96 mm	Thighs, urostyle. Spargana not found on histology	Opthalmitis *Myxobolus hylae*
5. *Litoria aurea* Hoskinstown, NSW May 2000 (00 782/11)	Female 52 mm	Thighs. Moderate granulomatous inflammation, degenerate spargana.	Chytridiomycosis
6. *Litoria aurea* Homebush, NSW March 1998 (98 320/14)	Female 66 mm	Thighs, ventral abdomen, dorsum, lumbar muscles, peritoneal cavity. Ulceration, fibrosis, haemorrhage, myonecrosis, interstitial inflammation.	*Pleistophora* sp. Septicaemia Encysted nematodes Intestinal ciliates
7. *Litoria caerulea* Emerald Beach, NSW April 1998 (98 320/16)	Male 122 mm	Thighs, ventral abdomen, forearm. Adhesions to skin, thick fibrosis, occasional foci of interstitial inflammation.	*Myxidium* sp. Protists in brain
8. *Litoria caerulea* Coffs Harbour, NSW August 1998 (98 871/28)	Male 94 mm	Thighs, ventral abdomen, tarsus, dorsum. Ulcers. Adhesions to abdominal skin, thin and thick capsules - some with pyknotic inflammation. Myonecrosis, granulomatous inflammation.	Cholangiohepatitis Larval spiruroids Gram +ve bacteria.
9. *Litoria caerulea* Korora, NSW August 1998 (98 871/29)	Female 126 mm	Thighs, ventral abdomen, dorsum. Excessive subcutaneous fluid. Adhesions to dermis, mild fibrosis.	*Ophidascaris pyrrhus* *Parathelandros* sp.
10. *Litoria gracilenta* Kallangur, Qld August, 1998 (98 1159/2)	Female 57 mm	Thighs, ventral abdomen. Fibrosis, myodegeneration.	Necrotic, shrunken lens. Immunosuppression *Myxidium* sp. *Rhabdias* sp.
11. *Litoria caerulea* Dysart, Qld September 1999 (99 1562/22)	Male 98 mm	Thighs, ventral abdomen, dorsum, lumbar muscle, caudal peritoneal cavity. Ulcers. Mild fibrosis.	Chytridiomycosis
12. *Litoria caerulea* Dysart, Qld August 1999 (99 1562/23)	Female 109 mm	Thighs, ventral abdomen, dorsum, forearms, lumbar muscle, caudal peritoneal cavity. Fibrosis.	*Rhabdias* sp. Oxyurids

13.3.5 Discussion

S. erinacei is the only species of pseudophyllidean cestode known to occur in Australia. Sequencing of the cytochrome *c* oxidase genes showed spargana from frogs were a different genotype to adults or spargana of *S. erinacei* found in dogs, fox, cats, tiger snake and a python (Zhu et al., 2001). The definitive hosts for this genotype are not known. The report of *S. erinacei* in an adult of *L. gracilenta* is a new host record.

In earlier stages of disease the main clinical signs were behavioural changes, frogs typically remaining stationary and often in the same unprotected site for about a week. Subcutaneous lumps over thighs and caudal belly were usually easily seen. Some frogs had ulcers of the skin. In more advanced stages, frogs were moribund. Some frogs had very heavy infections with widespread infection. All frogs had infections in the muscle and subcutis of the thighs and other commonly infected sites were the ventral subcutis, abdominal muscle, and dorsal subcutis especially around the urostyle. Less commonly infected sites were lumbar muscle, peritoneal cavity, and muscle and subcutis of the forelimbs.

Encapsulated spargana occurred within thin, membranous capsules or within thicker fibrotic capsules. Unencapsulated spargana occurred free between muscle fibres, in subcutaneous spaces and in the peritoneal cavity. Capsules containing spargana contained few or no inflammatory cells, and most spargana were alive. This contrasts with the toads studied by Bennett (1978) which produced a marked inflammatory response resulting in degeneration of the majority of spargana. Inflammation that was present in muscle in some of these frogs appeared to be stimulated by the presence of opportunistic bacterial or protozoal infections.

Coagulative necrosis of individual muscle fibres or more extensive myonecrosis occurred in some frogs. In heavily infected frogs spargana replaced muscle fibres, which appeared to have dissolved. Three frogs had dermal ulcers associated with infected muscle.

Although it was difficult to differentiate infection and disease in sick frogs infected with *S. erinacei*, we consider that the severe infections in seven of the frogs (58%) caused the

debilitation of these animals. The heavy burdens may have been a result of high levels of exposure to procercoids, or perhaps these frogs had underlying problems that depressed their immunity. In general, parasites are unevenly distributed between hosts - with most hosts harbouring few or no parasites and a few hosts harbouring many parasites. This phenomenon is known as overdispersion or aggregation (Poulin, 1998) and could be due to host differences, and to spatial and temporal changes in exposure. As almost all parasite populations fit this type of distribution, when heavily infected hosts are not found this is assumed to be due to parasite-induced host mortality (Poulin, 1998). As heavy infections with spargana were not detected in previous surveys of healthy frogs, this supports our pathological findings that spargana are pathogenic to frogs.

The concurrent diseases in three of the severely infected frogs may have contributed to their illness, whether as secondary or predisposing problems. The opthalmitis in the *L. gracilenta* (Case 10) may have interfered with its ability to catch prey. The nematode infections and bacterial hepatitis in the *L. caerulea* (Case 8) may have also been significant. The severe bacterial infection of the *L. aurea* (Case 6) appeared to be acute and was presumably secondary to the invasion of spargana.

The light infections with spargana in cases 1-5 were potentially significant in the pathogenesis of the concurrent diseases in these frogs, or may have been incidental.

Proteases occur in the region of the tegument of spargana of *S. erinacei* which are thought to assist in penetration of the intestinal wall of intermediate hosts (Kwa, 1972). These proteases may also help the spargana move through frog muscles and could contribute to pathogenesis.

Biochemical analysis of blood from two frogs did not reveal any abnormalities. Of three frogs from which a blood smear was examined, one had a significant eosinophilia. The eosinophils appeared to have been produced in response to a concurrent infection with encysted nematode larvae in the liver and on the surface of many internal organs.

Ophidascaris pyrrhus in a *L. caerulea* (Case 9) is a significant finding as it is the first adult ascaridoid found infecting an identified frog species (Sprent, 1988; Barton, 1994).

O. pyrrhus undergoes extensive growth and development in subcutaneous tissues of lizards which are the usual intermediate host, before being eaten by an elapid snake (the definitive host). It is possible frogs are a significant intermediate host of *O. pyrrhus* in wetter areas.

13.3.6 Acknowledgements

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13.4 Trematode metacercaria in tadpoles of *Mixophyes* sp.

Berger, L., Hines, H., Barton, D. Trematode metacercaria in tadpoles of *Mixophyes* sp. (in prep).

Metacercariae of various trematode species occur in tadpoles and frogs. The definitive host may be snakes, frogs, birds or mammals (Reichenbach-Klinke and Elkan, 1965). Sporocysts multiply in molluscs, the first intermediate hosts. Motile cercariae are released which encyst in tissues of amphibians (second intermediate host) where they reside until the amphibian is eaten by the definitive host. Usually encysted metacercariae are not pathogenic in amphibians and occur in skin and muscles, although some species infect vital organs such as eyes, heart, liver, lung, CNS or lateral line system where they can cause disease (Reichenbach-Klinke and Elkan, 1965; Flynn, 1973). Cort and Brackett (1938) report that heavy experimental infections of *Cercaria ranae* resulted in a fatal bloat disease in tadpoles with the unencysted diplostomula widely distributed in tissues. In America, metacercariae of *Ribeiroia* sp. encyst in tissues of the pelvic girdle and hindlimbs of tadpoles and are thought to cause deformities such as abnormal, missing, or extra limbs (Johnson et al., 1999). Infections also resulted in increased tadpole mortality. It appears that deformed frogs are more likely to be preyed upon, thus the induction of abnormalities by the parasite may be an evolutionary adaptation to complete its lifecycle (Johnson et al., 1999). Increased eutrophication and removal of snail predators can both result in an increase in snail numbers and in the incidence of parasitism (Johnson et al., 1999). Deformities have rarely been reported in Australian frogs. Frogs from Jabiru in the Northern Territory and Paralana Springs in South Australia had abnormalities that may be associated with naturally high levels of radon (Tyler, 1994). Metamorphs of *M. fasciolatus* at O'Reilly's plateau in southeast Queensland were seen with additional legs (H. Hines, unpub).

In Australia, metacercariae of *Neodiplostomum intermedium* and *Dolichoperoides macalpini* have been found naturally in various frog species (Barton, 1994). In experimental infections using trematodes obtained from molluscs, *Cercaria ellisi* was experimentally transmitted to tadpoles of *Crinia signifera* which became infected in the kidney, mesenteries, heart, and lung (Johnston and Simpson, 1944), and *C. natans* was transmitted to tadpoles of *L. tasmaniensis* resulting in infection of the kidneys and

adjacent peritoneum (Johnston and Muirhead, 1949). The definitive hosts for these parasites are not known.

Here we report on the pathology caused by metacercariae of *Fibricola* sp., a digenetic trematode that occurred within cysts in the subcutaneous tissue and muscles of tadpoles of *Mixophyes* sp. from Queensland.

Nine healthy tadpoles of *Mixophyes* sp. in good body condition were collected from Scrubby Creek in rainforest in the Conondale Ranges in June 1996. These were most likely to be tadpoles of *M. fasciolatus*, but members of this genus are difficult to differentiate as tadpoles. They were collected as they had multiple orange cysts over their bodies. Tadpoles were euthanased by bathing in benzocaine solution, and were weighed, measured and examined before preserving in 10% buffered neutral formalin.

Hind limb buds were present on two tadpoles; the others did not have limbs. The mean weight of the tadpoles was 1.24 gm (range 0.46-2.30) and the mean snout-vent-length was 19.7 mm (range 15-25).

Smooth, spherical cysts, less than 1 mm in diameter, occurred over the tail, and dorsal and lateral body, and elevated the normal skin and connective tissue overlying them (Figs. 13.6 and 13.7). One heavily infected tadpole also had lesions on the ventrum. Most lesions were orange with a paler centre but some were white or black. The mean number of subcutaneous cysts per tadpole was 25 and the following total numbers were present in individual tadpoles- 10, 11, 11, 13, 29, 29, 38, 47, and 66. Cysts from one tadpole were scraped off the skin and examined unstained under a light microscope. An oval metacercaria lay flat within each spacious cyst (Fig. 13.8). Cysts were squashed under a coverslip till they ruptured and released the metacercariae (Fig. 13.9). Based on morphology, the metacercariae were identified as *Fibricola* sp. (Diplostomidae, Digenea) (D. Barton, unpub). Cysts and metacercariae were measured with a calibrated eyepiece micrometer at 100x magnification, using fresh material. The mean length and width of the cysts was 653 μm (range 560-804) x 634 μm (range 578-784) (n = 20). The mean length and width of metacercariae was 369 μm (range 352-400) x 216 μm (range 160-272) (n = 20).

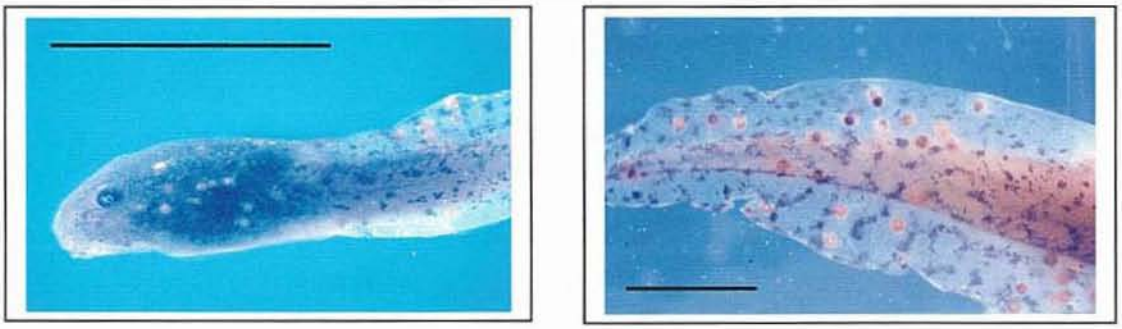
Six tadpoles were cut in half sagittally down the midline and embedded with cut surface downwards. These were processed routinely for histology and stained with haematoxylin and eosin. Of the 24 cysts available for histological examination on all tadpoles, 15 cysts occurred in the myxomatous tissue in the subcutis over the body and tail and 9 cysts occurred in the muscles adjacent to the notochord. In one tadpole with a heavy infection (47 subcutaneous cysts seen grossly) a cluster of thick-walled cysts was present in the peritoneal cavity adjacent to the gut. Cysts in the myxomatous tissue were consistently formed with smooth circular rings of about ten fibroblasts thick forming a layer about 48 μm wide, surrounded by a layer of about seven fibrocytes that was 50 μm wide (Fig. 13.10). Virtually no inflammatory cells were associated with the viable cysts and varying amounts of melanin were present in the outer edge of the fibrocyte layer. There were occasional foci of mixed inflammation in the myxomatous tissue assumed to be associated with the remains of degenerate cysts. Cysts within muscle were more irregular and smaller than those in the myxomatous tissues.

Concurrent infections with *B. dendrobatidis* occurred in three of four tadpoles where the mouth parts were seen histologically. Examination of internal organs revealed a *Goussia*-like coccidian within basal cells of the gut epithelium in four tadpoles, sometimes associated with ballooning degeneration of basal cells.

The infections in these tadpoles did not appear to have any pathogenic effects, although the bright orange cysts would have reduced the camouflage of the tadpoles and perhaps made them more vulnerable to predation. The ability of parasites to change the appearance of their intermediate hosts has evolved in other species, leading to a greater chance of the parasite completing its lifecycle (Poulin, 1998).

Fibricola is considered synonymous with *Neodiplostomum* (Cribb and Pearson, 1993). *N. intermedium* is the only species from this genus to be reported from tadpoles in Australia, and the lifecycle has been studied in detail (Pearson, 1961). The adult stage of this species has been found in the allied rat (*Rattus assimilis*) and the water rat (*Hydromys chrysogaster*), and the freshwater limpet *Pettancylus assimilis* can act as the first intermediate host (Pearson, 1961). At Mt Glorious, southeast Queensland, 110/161 tadpoles and frogs of *L. pearsoniana* were infected; however, only 1/102 tadpoles of *M. fasciolatus* was infected with a single diplostomulum, suggesting that *L. pearsoniana*

was the preferred host (Pearson, 1961). In tadpoles of *L. pearsoniana* the diplostomula were found encapsulated mostly between muscle fibres of the trunk muscles around the notochord, but in the tadpoles described here, many cysts occurred in the subcutis. During metamorphosis, diplostomula of *N. intermedium* in the resorbing tail were released from their capsules and were carried forward into the thighs to become re-encapsulated (Pearson, 1961). We did not follow the metacercarial infection in the tadpoles of *Mixophyes* sp. during metamorphosis, and this may be an interesting subject for future study.



Figures 13.6 & 13.7 Body and tail of a tadpole of *Mixophyes* sp. (96 570/7) with a heavy burden of orange and black subcutaneous cysts containing metacercariae. Bar in fig. 13.1= 20 mm. Bar in fig. 13.2 = 7 mm.

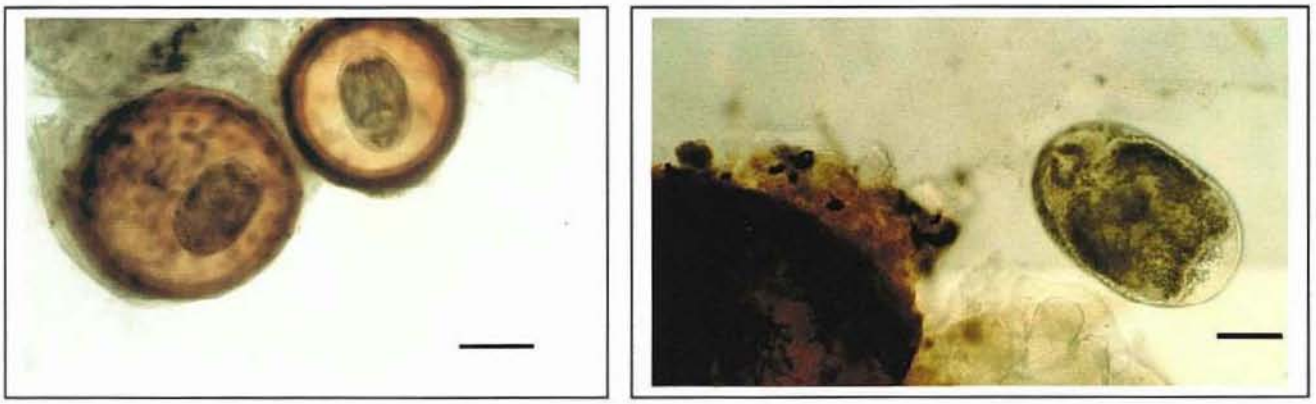


Figure 13.8 Whole cysts from skin of a tadpole. Bar = 200 µm.
Figure 13.9 Metacercaria of *Fibricola* sp. after the cyst has been squashed to release the organism. Bar = 100 µm.

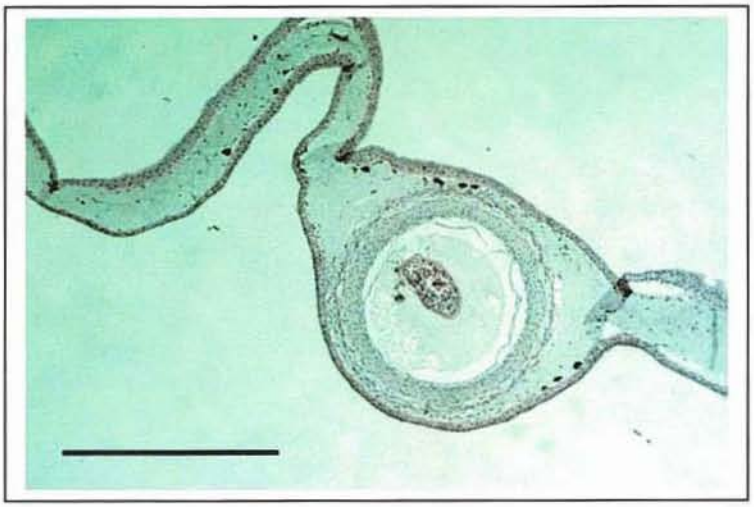


Figure 13.10 Histological section of an encapsulated metacercaria in the myxomatous tissue of the tail. The wall of the cyst is formed by smooth layers of fibroblasts and there is no associated inflammation. Bar = 650 µm.

13.4.1 References

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CHAPTER 14

Proliferative diseases

Although the focus of this project has been on infectious diseases, three malignant tumours diagnosed during the disease survey are described in detail. Virally induced tumours have been found in amphibians in other countries (Asashima et al., 1987), but the tumours reported here showed no evidence of a viral aetiology. These tumours are significant as they are the first reported from Australian frogs. A description of a skin lump due to osseous metaplasia is included here as an interesting pathological finding.

14.1 My role in the manuscript

I dissected the frogs and examined them histologically. Rick Speare assisted with the post mortem in Case 1 and advised on the histopathology in Cases 1 and 2. Deborah Middleton commented on the histopathology of all cases.

This chapter is comprised of an unpublished manuscript:
Berger, L., Speare, R., Middleton, D. Spontaneous proliferations in four Australian tree frogs: a basal cell tumour, a squamous cell carcinoma, a possible intravascular lymphoma and osseous metaplasia.

14.2 Introduction

Many tumours of amphibians have been described (Schlumberger and Lucké, 1948; Balls and Clothier, 1974; Asashima et al., 1987) but none have been reported from an Australian species. A large proportion of reported cases of anuran tumours are epithelial and many originate in the skin. Amphibian tumours include squamous cell carcinomas, adenomas, adenocarcinomas, papillomas and epitheliomas (van der Steen et al., 1972; Asashima et al., 1987). Basal cell tumours have not been reported, although similar tumours may have been classified as adenocarcinomas. Most reports of spontaneous "lymphosarcomas" are now considered to be mycobacterium-induced granulomas (Asfari, 1988). Osseous metaplasia is a common response to tissue injury in mammals (Jubb et al., 1992) but has not been reported in amphibians.

We describe the histopathology in a wild adult of *L. caerulea* with a basal cell tumour, a captive adult of *L. infrafrenata* with a squamous cell carcinoma, a captive adult of *L. caerulea* with an intravascular lymphoma and a captive adult of *L. peronii* with dermal osseous metaplasia. The three neoplasms comprised 0.93% (3/322) of cases in our pathologic survey of sick, post-metamorphic amphibians in Australia.

14.3 Methods

Post-mortems were conducted on 322 fresh, formalin-fixed or ethanol-fixed ill or dead frogs collected from the wild or in captivity in Australia (Chap 3). Frogs were weighed, measured and photographed. Samples of the proliferative lesions and a range of organ samples including liver, kidney, heart, spleen, lung, brain, spinal chord, thigh muscle, stomach, intestines and skin were processed routinely for histology, sectioned at 6 μm and stained with haematoxylin and eosin, periodic acid-Schiff (PAS) or Ziehl-Neelsen (Luna, 1968).

14.4 Results

Case 1: Squamous cell carcinoma in an adult of *L. infrafrenata*

A captive gravid female of *L. infrafrenata* with a snout-vent length of 93 mm died in Adelaide in 1995 and was preserved whole in 10% formalin. On the skin were five well-demarcated raised grey/brown, rough, circular plaques between 8 and 14 mm in diameter (Fig. 14.1). These occurred along the dorsal edge of the right tympanum, on the flexor surface of the right elbow, on the dorsolateral back 10 mm anterior to the right hindlimb, below the cloaca extending to the right, and on the ventral surface of the right stifle. Plaques on the elbow and stifle were ulcerated. On the left side of the central abdomen was an ill-defined area of roughened skin about 4 x 12 mm, with an elevated grey surface.

Histologically, there were rounded pegs of epidermal cells proliferating downwards and invading the dermis, or thicker lesions comprised of multiple solid or cystic balls of epidermal cells (Figs. 14.2 & 14.3). These ranged between 56 and 400 μm in diameter and cystic structures were lined by layers of about 5-8 cells. The cells were well differentiated with frequent formation of keratin pearls. The moderately pleomorphic

cell nuclei were oval or kidney-shaped with one or two very prominent nucleoli and there were about two mitoses per high power field. Intercellular bridges were seen. Cells had abundant eosinophilic cytoplasm. Necrosis of individual cells was common with ballooning degeneration and formation of microvesicles. The surfaces of lesions were hyperkeratotic and focally ulcerated. Interstitial fibrosis and mixed inflammation occurred between epidermal structures in ulcerated areas and infiltrated the entire thickness of the dermis in the diffuse ventral lesion.

The frog had moderate sized fat bodies and the internal organs appeared normal. A greater than usual amount of melanin was present in the liver.

Case 2: Basal cell tumour in an adult of *L. caerulea*

A 110 gm female of *L. caerulea* with a snout-vent length of 97 mm was found free-ranging at a high school in Lismore, New South Wales in May 1998. The frog was lethargic on collection and died in transit to the laboratory. Thirteen nodules, 3-21 mm diameter, were present on the dorsal skin of the body and limbs (Fig. 14.4). Most larger nodules were ulcerated to expose the cream coloured, bleeding, friable surface of the tumour. Three large nodules (21-24 mm diameter) grew on the skin over the urostyle together with two smaller ones about 5 mm wide. One of the larger nodules covered with intact skin had ruptured through the dermis, and a few adhesions to underlying muscle fascia had formed. All other cutaneous nodules were contained above the deeper connective tissue of the dermis. Smaller nodules (3-10 mm in diameter) were present on dorsal skin caudal to the head, on the lateral edge of the right hind foot, dorsal skin of the right forearm, under the left eye, and three occurred in a cluster caudal to the left parotid gland. One large smooth encapsulated nodule 10 x 20 mm was embedded under the superficial muscles behind the right tympanum but was easily shelled out.

Histologically, the nodules in the dermis were composed of epithelial cells in various stages of differentiation. The complex tumour formed lobules ranging from 343 to 2525 μm wide surrounded by fibrous stroma (Fig. 14.5). Most cells had dark, ovoid nuclei about 6.1 x 4.2 μm, and eosinophilic cytoplasms. The mitotic rate was low and there was little variation in cell and nuclear size. The bulk of the tumour was comprised of solid or glandular acini. There were areas undergoing squamous metaplasia forming

cystic structures filled with keratin pearls (400 - 2000 μm) or necrotic red blood cells. Scattered throughout were small ducts formed by a single layer of about eight cuboidal cells with pale round nuclei. Larger dilated ducts were also present and some contained necrotic cell debris. Occasional clusters of cells in solid acini had large round cytoplasmic vacuoles that did not stain with PAS. Large areas of haemorrhage and necrosis occurred in the centre of lobules or were associated with surface ulceration.

At necropsy, internal organs appeared to be normal. The frog had moderate-sized fat bodies and was heavily gravid. *Rhabdias* sp. occurred in the lungs.

Histological examination of internal organs did not reveal any neoplastic lesions. A non-suppurative cystitis was present with large colonies of bacterial rods and foci of inflammation. There was a greater than normal amount of melanin in the liver.

Case 3: Possible intravascular lymphoma in an adult of *L. caerulea*

An adult female of *L. caerulea* with a snout-vent length of 73 mm became lethargic after 7 years in captivity in Gladstone, Queensland, and was euthanased with halothane and preserved whole in formalin. It had sunken eyes and pale skin mottled brown and cream.

On histology of the lung, solid masses of tumour cells filled many large blood vessels. Cells with dark oval nuclei and sparse eosinophilic cytoplasm grew in a whorled pattern. Cell nuclei were regular in size and shape (mean 6.3 x 3.5 μm) (n=10) and had a low mitotic rate. The tumour appeared to be attached to blood vessel walls but did not invade. In the heart masses of tumour cells, some with central pyknotic foci, occurred in some blood channels of the ventricular wall (Fig. 14.6). Blood appeared to be flowing normally in other areas of the heart. In an attempt to identify the cell type, a CD 3 lymphocyte immunoperoxidase stain was performed (Hemsley et al., 1998), and was negative. A Ziehl Neelsen stain for mycobacteria was also negative.

The dermis of the feet contained bacteria associated with haemorrhage and congestion that occurred in the dermis and between underlying muscle fibres. Dermal glands in the toes appeared hyperplastic.

Other organs appeared normal and a primary tumour was not found. The frog had small fat bodies and a small dark brown liver of which a large proportion consisted of melanomacrophages. Large egg masses filled the abdomen and compressed the organs.

Case 4: Osseous metaplasia in an adult of *L. peronii*

An active 11 gm wild-caught captive gravid female of *L. peronii*, with a 57 mm snout-vent length had an enlarging lump on the left lateral abdomen 11 mm caudal to axilla (Fig. 14.7). The frog was euthanased with MS 2222 (tricaine methanesulphonate, Ruth Consolidated Industries, Annandale, Australia). The lesion was round and smooth with a darkened apex. It was 10 mm in diameter at the base and protruded outwards 7 mm. It did not extend through the dermis and was visible from underneath the skin as a smooth white circle. The cut surface consisted of three distinct layers - a thin white layer on the base, then a grey layer comprising the bulk of the lump, and the apex which was brown and friable.

Histologically, the lump was a chronic, inflammatory metaplastic lesion comprised of five layers including bone. Adipose tissue formed the base, then layers of ordered connective tissue, swirls of fibrous tissue with some distension and occasional pyknotic cells, a layer of healthy trabecular bone, and the superficial layer was comprised of mixed inflammatory cells and fibrosis with a few remnants of serous glands (Fig. 14.8). The epidermis had ulcerated over the apex of the lump leaving the fibrous tissue exposed.

Abnormalities were not observed in internal organs.

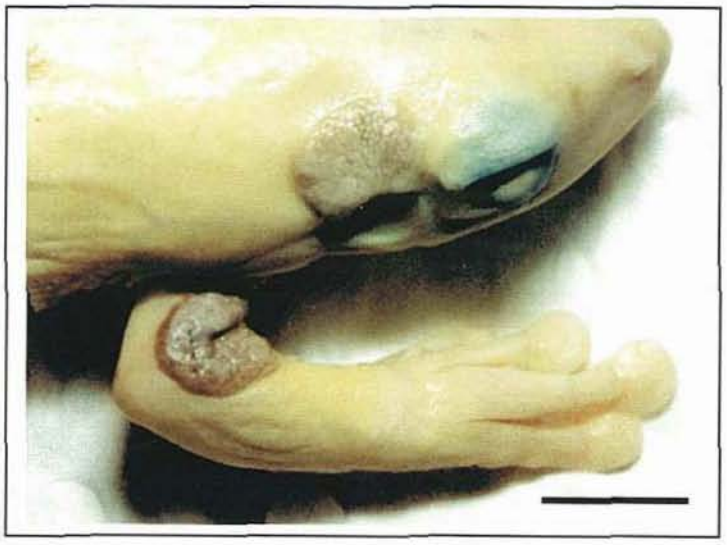

Figure 14.1 Adult of *Litoria infrafrenata* with dermal papules formed by a squamous cell carcinoma. Bar = 8 mm.

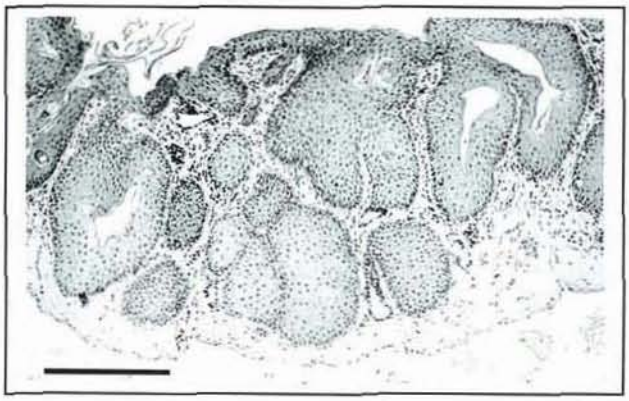

Figure 14.2 Section of skin through the squamous cell carcinoma with rounded epidermal pegs invading the dermis. Bar = 250 μm.

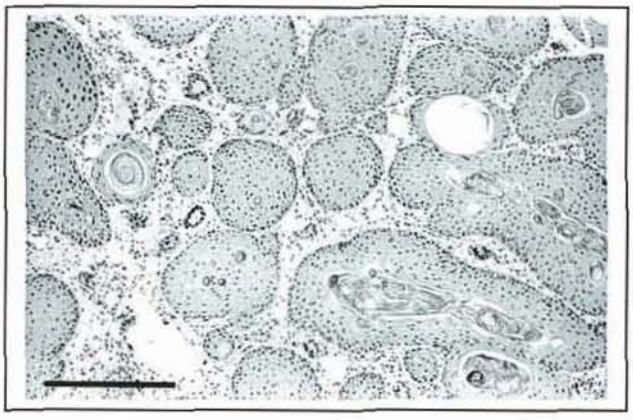

Figure 14.3 Section of the squamous cell carcinoma showing keratin pearls and interstitial fibrosis. Bar = 200 μm.

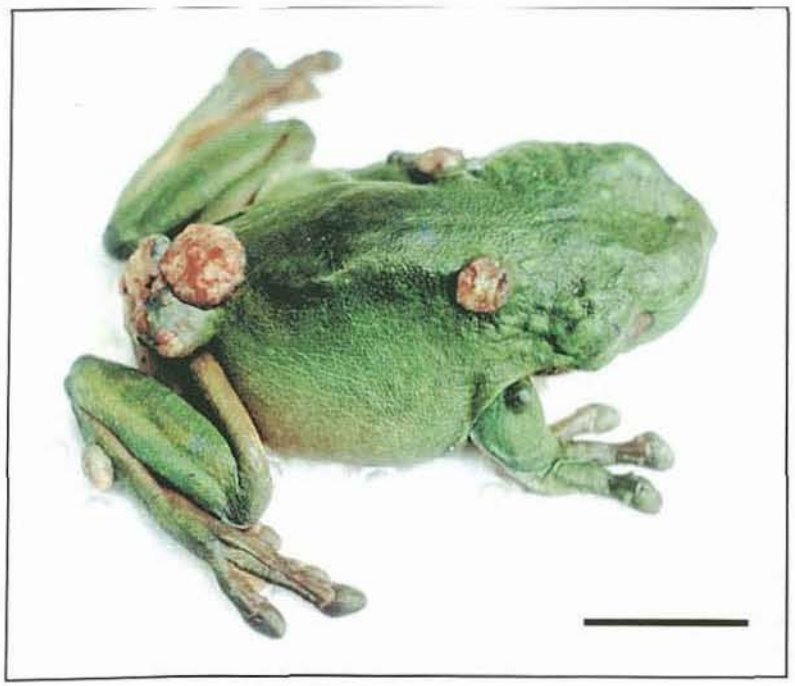

Figure 14.4 Adult of *Litoria caerulea* with large, ulcerated, haemorrhagic nodules on dorsal skin, formed by a basal cell tumour. Bar = 25 mm.

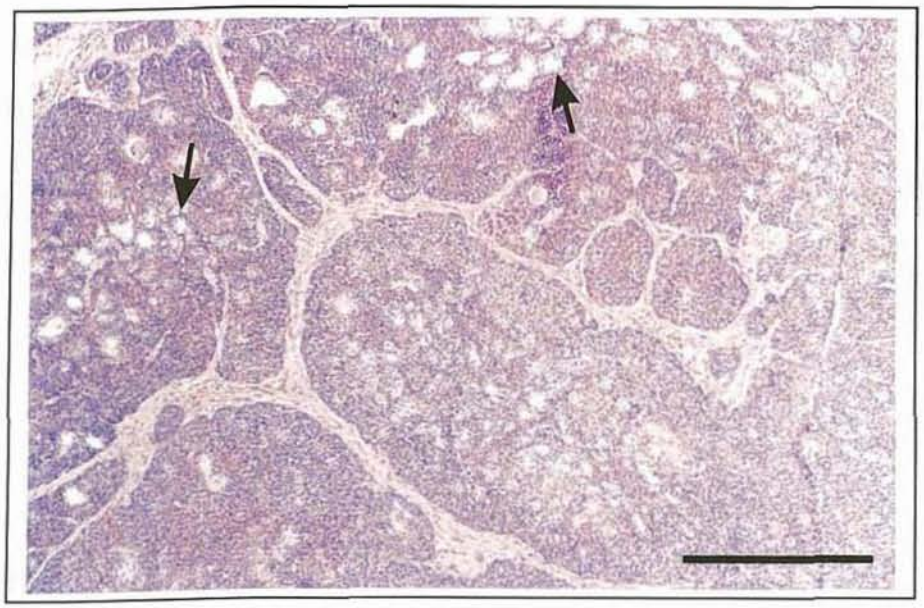

Figure 14.5 Section of basal cell tumour with lobules of solid acini surrounded by fibrous stroma. Many pale ducts (arrows) are present in this image. Bar = 400 μm.

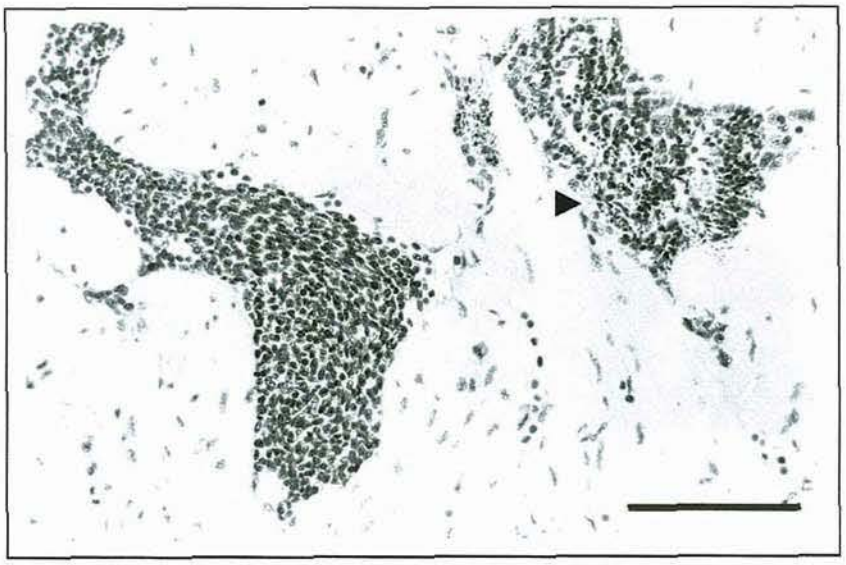

Figure 14.6 Intravascular lymphoma in the ventricular myocardium of an adult of *Litoria caerulea*. Some tumour cells are necrotic (arrow head). Bar = 80 μm.

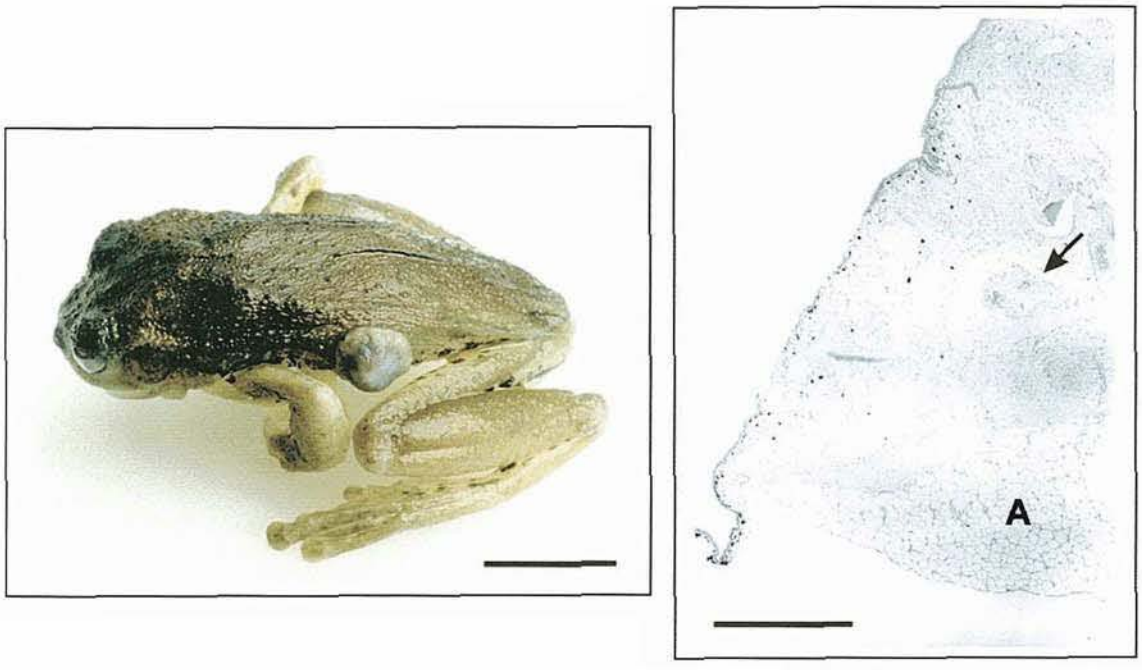

Figure 14.7 Adult of *Litoria peronii* with a dermal lump due to osseous metaplasia. Bar = 15 mm.

Figure 14.8 Section through the edge of the lump, containing a thick layer of adipose tissue at the base (A), trabecula bone (arrow) and covered by epidermis. Bar = 800 μm.

14.5 Discussion

The two epidermal neoplasms were considered to be fast growing and moderately malignant. Although they were mostly contained and had a low mitotic rate, features of malignancy included ulceration, necrosis, haemorrhage and multiple sites of occurrence. It is possible the basal cell tumour and squamous cell carcinoma metastasised along the dorsal cutaneous blood vessels or dorsal subcutaneous lymph sinuses, but perhaps it is more likely they arose spontaneously due to a predisposing factor. In mammals, squamous cell carcinomas are usually slow to metastasise and basal cells tumours rarely metastasise (Stannard and Pulley, 1978).

The diagnosis of intravascular lymphomas is complicated, and in the past they were thought to be malignant angioendotheliomas. Intravascular lymphomas are rare tumours previously reported in humans, dogs and a cat, and usually affect blood vessels of the brain and lung (Lapointe et al., 1997). The tumours may be comprised of B or T lymphocytes, and immunohistochemical stains are needed to confirm the diagnosis. Although the diagnosis of lymphoma in the *L. peronii* was not confirmed by immunostaining, it had a typical histological appearance of a lymphoma.

Osseous metaplasia can develop from fibrous connective tissue formed in response to chronic inflammation or tissue degeneration (Jubb et al., 1992). In the *L. peronii* there was no evidence of the cause of inflammation, which was possibly traumatic.

Compared with mammals, birds and fishes, reports of neoplasia in amphibians are uncommon. This may be due to neoplasms being over-looked and not reported, or alternatively, amphibians may have some resistance to neoplasms (Asashima et al., 1987). The prevalence of neoplasms in our study of Australian frogs (0.93%) is similar or less than that seen in other countries. Higher prevalences occur with the infectious tumours (i.e. Lucké renal tumour and newt papilloma) where the same tumour occurs in many individuals of the same species, and incidence varies seasonally (Asashima et al., 1987).

14.6 Acknowledgements

We thank Don Nichols for advice on diagnosis of the basal cell tumour, Lee Skerratt for performing the CD3 lymphocyte stain, Megan Braun for histology, and Craig Taylor, Mike Tyler, Gerry Marantelli, John Clarke and Harry Hines for collection of specimens.

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