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Frogs as bioindicators of chemical usage and farm practices in an irrigated area

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FROGS AS BIOINDICATORS OF CHEMICAL USAGE AND FARM PRACTICES IN AN IRRIGATED AGRICULTURAL AREA

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Frogs as bioindicators of chemical usage and farm practices in an irrigated agricultural area

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Introduction

Dramatic declines and extinctions in frog populations worldwide (Houlahan et al. 2000) and high frequencies of physical or reproductive abnormalities in frogs (Ankley et al. 2004) may be directly or indirectly related to agricultural activities among other factors, particularly the use of agrochemicals such as pesticides (Fellers et al. 2004). The causes of the declines in amphibian populations remain unresolved, but recent studies suggest six main hypotheses to explain them: (1) habitat destruction; (2) chemical contaminants, particularly pesticides; (3) increasing UV radiation; (4) climate change; (5) introduced exotic predators; and (6) disease, particularly the pathogenic chytrid fungus (Marsh and Pearman 1997; Berger et al. 1998; Ankley et al. 2004; Fellers et al. 2004). Frogs are likely to act as bioindicators of environmental quality, and could be used to assess the impact of human activities in specific areas.

The herbicide atrazine, for instance, has been linked to adverse effects on tadpole development of *Rana pipiens* and *Xenopus laevis*. Several recent laboratory studies in North America have demonstrated that atrazine, at relatively low environmental concentrations in the range 0.1 to 25 µg/L, is an endocrine disruptor that may affect testicular development in male tadpoles (Carr et al. 2003; Hayes et al. 2003). Male tadpoles given a 48-h pulse exposure to atrazine just prior to gonadal differentiation had significantly reduced testicular development (Tavera-Mendoza et al. 2002).

The Southern Bell Frog (*Litoria raniformis*, Keferstein) was once common throughout south-central New South Wales (NSW), Australia, but has disappeared from most of the region. Presently, it has colonised rice bays in the Coleambally Irrigation Area (CIA), where it is currently abundant and widespread. This frog also occurred in the Murrumbidgee Irrigation Area, but is apparently extinct in that area now. Both of these irrigation areas draw water from the Murrumbidgee River, and it is reasonable to expect the initial water quality to be similar in the two irrigation areas. However, different crops are necessarily associated with different suites of pesticides and other farm practices, i.e. water usage. It should be noted that in irrigation-based agriculture the peak application of pesticides corresponds with the breeding periods and highest activity of frogs. The occurrence of the Southern Bell Frog in the CIA, a predominantly rice-growing area, and its disappearance from the nearby Murrumbidgee Irrigation Area where in addition to rice, soybeans, sorghum, maize, citrus, grapes and peaches are common summer crops, suggests a possible link between crop types, their associated pesticides and habitat changes with the decline of this frog from the Murrumbidgee Irrigation Area.

Aims

This study investigated the status of four frogs and their tadpole populations in rice bays within the CIA, and focused in particular on the Southern Bell Frog because it is considered an endangered species (Tyler 1997). This species seems more susceptible to human impact as its breeding occurs in water bodies that are either ephemeral or fluctuate significantly in water level (Pyke 2002). Our research aim was two-fold: (1) to determine whether or not there are links between specific agricultural chemicals used in the two main irrigated crops of the area, rice and corn, and the presence/absence of frogs in that area, since the only suitable frog habitat is anthropogenically created on irrigated farms; and (2) to assess the abundance, growth and abnormal development of tadpoles in the area in the light of such farm practices, which include the use of atrazine among other herbicides. In addition, the rate of detection of chytridiomycosis was evaluated, since diseases often follow stressors, and pesticides are known to affect frog survival due to synergistic stress (Relyea 2004). Although the decline of Californian frog species has been correlated with drift of agrochemicals (Fellers et al. 2004), no field study has yet demonstrated clearly a direct link with them for lack of pesticide residue data. This study aims at providing such data for a specific agricultural area in which farm practices, crops and associated pesticides are known.

Study sites

The Coleambally Irrigation Area (CIA) covers an approximate area of 79,000 ha of intensive irrigation supported by the Murrumbidgee River in the south-eastern corner of Australia (Figure 1). To facilitate more effective management, the CIA was divided up into four areas with a definitive separation between the north and south resulting from two roads that run horizontally through the middle of the CIA. The four areas are Boona (33,713 ha) and Coly (23,585 ha) in the north and Argoon (26,118 ha) and Yamma (22,613 ha) in the south. The main summer crops (November – April) are rice, corn and soybeans while main crops grown over winter (May – October) include wheat, oats and barley.

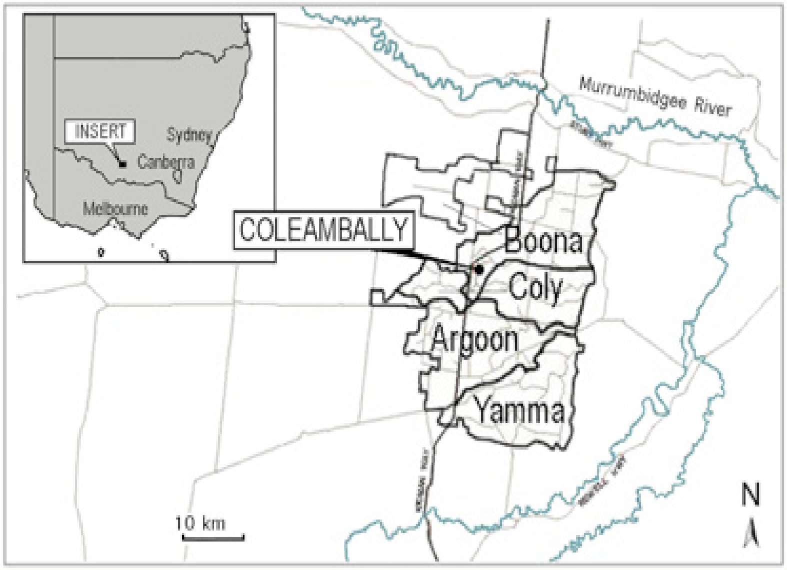

Figure 1. Map of the Coleambally Irrigation Area and its four regions. Source: www.colyirr.com.au

Survey design

The study farms were selected with a stratified-random design based on two general criteria being (1) farm type based on the crop(s) being grown (whether rice-only or rice plus corn growing adjacent to the rice bays) and (2) the location (northern or southern region) within the CIA. All study farms were privately owned and operated and therefore the selection of sites was conditional on the granting of permission to enter the properties. Sixty farms were surveyed over the 2005/06 and 2006/07 rice growing seasons during this study, 30 in the north (15 growing rice-only and 15 growing rice and corn) and 30 in the south (15 rice-only and 15 rice and corn). Among them, 10 farm sites were sampled during the two seasons, whereas the remaining 50 farms were selected as study sites only once in either season. All farms were considered as separate sites because crops were rotated on the properties, providing entirely new habitats to that of the previous season.

Methods

Tadpole and frog-call surveys

Sampling periods coincided with the rice season and covered the austral summer of 2005/06 and 2006/07. The rice-growing season also coincides with the peak breeding season of the four frog species surveyed (*Limnodynastes tasmaniensis*, *Limnodynastes fletcheri*, *Litoria raniformis* and *Crinia parinsignifera*) and is therefore the optimum time for species richness surveys (Anstis 2002, Jansen and Healey 2003).

The qualitative tadpole surveys (i.e. presence/absence) for the 2005/06 season consisted of a subset of 20 farms surveyed on two occasions (November and December) selected using the criteria outlined above and 20 supplementary farms chosen at random on a separate occasion in November. Out of these 40 farms, 20 were located in the north (10 rice-only, 10 rice and corn) and 20 in the south (10 rice-only, 10 rice and corn). Repeated tadpole surveys for the 2006/07 season were undertaken on another subset of 20 farms, 10 in the north (five rice-only and five rice and corn) and 10 in the south (five rice-only and five rice and corn) on three occasions (October, November and December).

Tadpoles were sampled during the day from the banks of rice bays using a trapezoid-shaped (45 x 35 x 25 cm) net frame covered with a 2 mm mesh net of 30 cm depth. Ten sweeps were taken at each site in accordance with a standard sweeping technique used in similar tadpole surveys (Jansen and Healey 2003; Williamson and Bull 1999). Net sweeps were taken randomly at known microhabitats (Jansen and Healey 2003) so that tadpoles that may have been seeking shelter from the mid afternoon heat were not missed. Specific examples of the microhabitats sampled include the levees banks around each rice bay, the deeper waters in and around water gates, around emergent vegetation and where applicable amongst algal mats. The water quality parameters of temperature, dissolved oxygen, pH and conductivity were measured on each sampling occasion using a multiprobe meter (Hydrolab, Austin, TX, USA) using standard operating procedures (NSW EPA 2004). Tadpoles were held in a sorting tray and identified to species level where possible or otherwise to genus level (Anstis 2002) and each tadpole was staged in accordance with Gosner (Gosner 1960). Tadpoles of the two *Limnodynastes* species could only be identified to species level at Gosner development stage 38 or later.

In addition to tadpoles, nocturnal surveys for calling frogs were incorporated into the three surveys of the 2006/2007 season (3 x 20 farms) to complement the assessment of the distribution of the species, since each species had a unique call. The frog-call surveys were undertaken after dusk by two individuals standing at a reference point (Hazell et al. 2001), which in this case was a point adjacent to the pesticide passive samplers (see below). The surveyors waited 10 minutes for the initial disturbances caused by the vehicle to subside before survey time was commenced and the frog calls were identified.

Collection of frogs

During the 2005/06 season one nocturnal collection of 275 frogs was undertaken in January 2006 from six of the study sites (three in the north and three in the south) for sex determination and histological examination. The six farms were selected using the criteria of location, farm type and the presence of all four tadpole species from the subset of 20 farms previously surveyed twice for the presence of tadpoles. The frogs were euthanized in 1% chlorotone anaesthetic and then placed in phosphate-buffered formaldehyde fixative for two to three days, prior to storage in 70% ethanol.

During the 2006/07 season one nocturnal frog collection (n = 67) from eight of the 20 study sites (four in the north and four in the south, both farm types) was also undertaken in October 2006. The collected frogs were swabbed for chytrid fungus pathology, as described below, and released afterwards.

Time-weighted average pesticide concentrations

A range of insecticides and herbicides are used every year to protect the predominate rice and corn crops grown in the farms of the CIA in the austral summer. Among these, residues of six herbicides and three insecticides are commonly found in irrigation waters of this agricultural area. Thiobencarb, molinate, clomazone, chlorpyrifos and fipronil are applied only to rice crops (Lacy and Stevens 2003), metolachlor is only used in corn, and atrazine, diuron and endosulfan are used in corn as well as other crops but not in rice.

Passive sampling devices offer an effective procedure for measuring time-weighted average sampling of analyte concentrations in the aquatic environment, such as pesticides. Two types of solvent-based passive sampling devices were used in this study. The device for measuring herbicide concentrations consisted of pre-stained cellulose membrane tubing containing a binary mixture of the solvents 1-dodecanol and 2,2,4-trimethylpentane as the sequestering medium; this device is referred to as the CIDS sampling device (Hyne and Aistrope 2008). The other device, referred to as the TRIMPS device, consisted of polyethylene membrane tubing containing 2,2,4-trimethylpentane as the sequestering medium and was used to determine the presence of chlorpyrifos and endosulfan (Hyne et al. 2004).

Two CIDS and two TRIMPS devices were deployed three times during the 2006/07 season at each of the 20 study sites where tadpole surveys were undertaken, near a water gate of the rice bay adjacent to the site. They were recovered after a 14-d period and replaced with new devices on site visits that coincided with the tadpole surveys. The sequestering media from the passive samplers were analysed using gas chromatography to determine the analytes present as described in detail previously (Hyne et al. 2004; Hyne and Aistrope 2008). The sampling rates reported previously for atrazine (10.0/day), diuron (7.8/day), metolachlor (12.6/day), and molinate (9.6/day) by the CIDS device (Hyne and Aistrope 2008), and for endosulfan (48/day) and chlorpyrifos (67/day) by the TRIMPS device (Hyne et al. 2004) were applied to derive time-weighted average (14 d) water concentrations in the rice bays. Sampling rates for thiobencarb, clomazone and fipronil by the CIDS device of 11.6/day, 10.8/day and 8.6/day, respectively, were used to derive their time-weighted average water concentrations in the rice bays.

The relationship between the amount of an analyte in the receiving phase of the device and in the waterway after an exposure time during this linear or kinetic sampling phase has been formulated:

$$Cw(t) = CsVs/Rst \qquad (1)$$

where $C_W(t)$ is the analyte concentration in the aqueous environment (ng/ml), C_S is the concentration of analyte (ng) accumulated by the sorbent of the device at exposure time *(t)*, V_S is the volume of the sorbent (ml), R_S is the sampling rate (ml/day) of the device (Huckins et al. 1999) and is exposure time (day).

In the present study, equation (1) has been reformatted in terms of a concentration factor [*CF*] as follows:

$$CF(t) = C_S/C_W = R_S t/Vs \qquad (2)$$

where a plot of *CF* versus time was used to derive the sampling rate (R_S/V_S) normalised to the volume of solvent (10 ml) in the passive sampler devices. In this study the time-weighted average water concentration of each target pesticide for the period of deployment of the passive samplers could be determined from its concentration in the solvent of the device using the above sampling rates.

Chytrid fungus pathology

The TaqMan PCR assay (Boyle et al. 2004) was used to determine the presence of DNA of *Batrachochytrium dendrobatidis*, which causes chytridiomycosis, in swaps of the frogs obtained for this purpose. A sterile swab (Medical Wire & Equipment, UK) obtained from bioMérieux Australia (Sydney, Australia) was used to sample the underside of the legs and 'drink patch' of each frog. Each frog was handled with a new pair of disposable gloves to prevent cross-contamination of individual frogs swabbed at the sites. Measures to reduce the possible spread of infectious pathogens such as the chytrid fungus between survey sites were implemented during all survey procedures in accordance with recommendations described by the NSW National Parks and Wildlife Service (NSW NPWS 2001).

Gonadal abnormalities and sex determination

Sex ratios of the two *Limnodynastes* species were determined by direct visual inspection of the frogs after dissection. The gonad-kidney complex of the frogs was isolated by dissection and photographed with a Leica DC100 camera fitted to a Wild Heerbrugg M3C stereo microscope using high intensity incident illumination. Images were processed using the digital image-analysis system Leica QWin V3.2.0 (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

For histology the gonads were stored in 70% ethanol and subsequently placed in agar prior to paraffin embedding. The small agar cube containing the gonad was embedded in wax. Tissue sections (8 µm thick) were routinely stained with hematoxylin and eosin. The criteria used for scoring anatomical gonadal malformations were individual frogs with multiple gonads, individual frogs with gonads anatomically in appearance of both sexes or the presence of intersex gonads, defined as the presence of oocytes in otherwise typical testicular tissue in the histological sections (Orton et al. 2006). Sex ratios of the two *Limnodynastes* species results were validated by histological examination of gonads from a sub-set of the visually sexed animals. Sex ratios of the *L. raniformis* tadpoles and metamorph frogs were only determined by histological examination of the gonads.

Statistical analysis

The probability for non-detection (α_{nd}) of *L. raniformis* during the presence/absence surveys was calculated using the following equation (Kéry 2002):

$$\alpha_{nd} = (1-p)^n$$

where n is the number of surveys of each site within each region and p is the probability of *L. raniformis* occurring at a site in either the northern or southern region of the CIA. The parameter p was calculated from the overall proportion of farm sites where *L. raniformis* was present on any site visit within each region. The data consisted of one group of 20 farm sites visited twice, another group of 20 farm sites surveyed once in the 2005/06 season and a group of 20 sites that were visited three times in the 2006/07 season (see sampling above). The parameter p was estimated for each of the three data sets for each region, which is equivalent to a maximum likelihood estimate of the parameter p of a geometric distribution.

Water quality parameters and pesticide residues between the rice-only and rice and corn farms were compared by *t*-tests, using log-transformed data when their values followed an exponential distribution.

A Principal Component Analysis (PCA) was undertaken to find out any possible relationship between the main pesticide variables (thiobencarb, molinate, clomazone, chlorpyrifos, atrazine, diuron and metolachlor only, since endosulfan and fipronil concentrations were about their respective limits of detection) and water quality variables (pH, conductivity, temperature and dissolved oxygen) on the distribution of *L. raniformis* across the 20 study sites during the three site visits of the 2006/07 season. In addition, a linear Discriminant Analysis (DA) was performed on the same set of variables to determine the best function describing the site occupancy by *L. raniformis* in the CIA.

Results

Abundance and distribution of species

Tadpoles were found to be more abundant across the study sites in the 2005/06 season compared to 2006/07 season within the CIA. Due to the difficulty of only identifying *Limnodynastes* tadpoles to species level at a late stage of development, only the tadpole survey data from the 2005/06 season was examined in detail. The mean abundance of *L. raniformis* tadpoles was significantly different (p = 0.028, Mann-Whitney) between populations in the north and the south (Figure 2).

A Kruskall-Wallis test found there is a highly significant difference (p = 0.016) between mean abundance of tadpole species within the northern region but not in the south. Similar trends were found in the tadpole abundance across the study sites in the 2006/07 season.

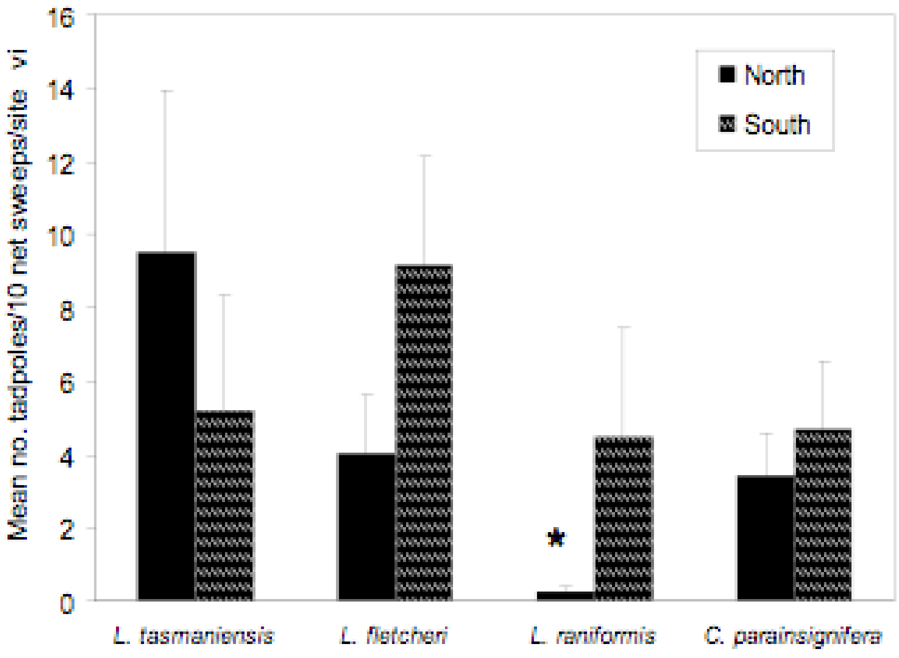

*Figure 2. Mean abundance of the four tadpole species assessed using 10 standardised net sweeps in rice bays at the farm study sites in the northern (n = 20) and southern (n = 20) region of the Coleambally Irrigation Area in the 2005 season. Error bars represent the standard error of the mean. * indicates significantly (p = 0.028, Mann-Whitney) lower abundance of L. raniformis tadpoles at the northern sites than at the southern sites.*

Since the frog distribution data from the 2006/07 season incorporated both tadpole and nocturnal frog-call surveys, it was a more comprehensive assessment of site occupation by each frog species. Examination of this data set showed that *C. parinsignifera* tadpoles and calling frogs were universally present across the study sites in rice bays both the northern and southern regions on both farm types (i.e. rice-only or rice and corn) throughout the three visits. There were also only four sites where *L. fletcheri* tadpoles and calling frogs were absent, and these were southern farms growing rice and corn on the first visit to the sites (October 2006). Similarly, the only site without *L. tasmaniensis* tadpoles or calling frogs was on a farm growing rice and corn on the first visit. However, *L. raniformis* tadpoles and calling frogs were rarely observed at the study sites in the northern region.

Probability of detection of *L. raniformis*

Where detection of a species at a site is not certain, the probable rate of detection at a set of sites can be modelled. From each tadpole and frog-call combined survey, the estimated probability of detection of *L. raniformis* (*p*) for each site visit in the northern and southern region of the CIA was used to estimate an overall probability of detection after a given number of visits using a probability model (Kéry 2002). The likelihood of encountering *L. raniformis* was much higher in the southern regions of the CIA than in the north (Figure 3). Hence in the survey in the 2006/07 season, the number of visits to each farm site was increased from two to three to improve the probability of detecting the *L. raniformis*, particularly in the northern region.

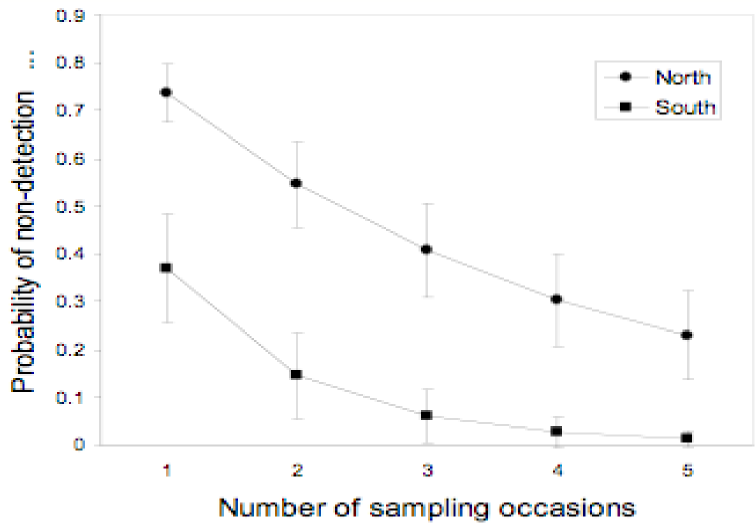

Figure 3. Comparison of the estimated probability of non-detection of Litoria raniformis at three groups of farm study sites located in either the northern (●) or southern region (■) of the Coleambally Irrigation Area in relation to the number of site visits. Error bars represent the standard error of the mean (n = 3).

Pesticide concentrations at the study sites

About half of the passive samplers deployed at the 20 study sites in the 2006/07 season contained residues of herbicides and fipronil, whereas the insecticides chlorpyrifos and endosulfan were detected in more than 85% of the samplers (Table 1). Chlorpyrifos is only applied to crops in the CIA occasionally when pest infestation is heavy but was detected at almost all sites (93%) at concentrations of 1.43 ± 0.70 µg/L (mean ± SE) over the three deployment periods, which exceed the water quality guideline trigger value of 0.01 µg/L for the protection of aquatic ecosystems (ANZECC, ARMCANZ 2000). Although the water quality guidelines trigger values do not apply to on-farm pesticide concentrations, comparison to the guideline values do give an indication of the amount of dilution that would be required before these farm waters would meet the guideline trigger values if these waters were released during a storm event. Concentrations of endosulfan and fipronil residues were very low (<0.05 µg/L) in all samples, and for endosulfan they never exceeded the trigger value of 0.03 µg/L, as it is not used in rice. Therefore, neither of the latter insecticides is included in further discussion in this study.

Table 1: Residues of pesticides µg/L) detected at the study sites

	Herbicides						Insecticides		
	Atrazine	Cloma-zone	Diuron	Metchlor	Molinate	Thioben-carb	Chlorpy-rifos	Endo-sulphan	Fipronil
Rice only									
Median	0.20	0.58	0.15	0.05	15.3	127	0.24	0.001	0.0014
Mean[a] ± SE	0.26 ± 0.08	3.53 ± 1.55	0.19 ± 0.05	0.08 ± 0.03	96.2 ± 43.5	204 ± 67	2.09 ± 1.34	0.001 ± 0.0002	0.0025 ± 0.0007
Detected	8	14	10	6	22	10	29	26	16
PAT (%)	0		40	83	77	100	100	0	
Rice & corn									
Median	0.29	0.12	0.04	0.21	1.4	71	0.35	0.003	0.0012
Mean ± SE	0.66 ± 0.15	0.37 ± 0.15	0.11 ± 0.04	0.39 ± 0.08	6.9 ± 3.4	185 ± 47	0.72 ± 0.15	0.007 ± 0.002	0.0016 ± 0.0004
Detected	29	16	16	29	11	27	27	26	15
PAT (%)	0		25	97	27	100	93	0	
Trigger value[b]	13	NTV[c]	0.2 (LR)[d]	0.02 (LR)	3.4	2.8	0.01	0.03	NTV
LOD	0.001	0.003	0.0001	0.001	0.022	0.001	0.005	0.001	0.0001
p (*t*-test)[e]	0.033	0.035	0.167	0.0003	0.04	0.862	0.615	0.010	0.247

[a] Mean of values at sites with a positive detection at each farm-type (rice-only or rice and corn).

[b] Water quality guideline values for the protection of aquatic ecosystems (ANZECC, ARMCANZ, 2000).

[c] NTV = no trigger value available.

[d] LR = low reliability provisional water quality guideline trigger value.

[e] Comparison between the two farm types using the log-transformed residue data.

Thiobencarb and molinate were the herbicides with the highest concentrations recorded. All thiobencarb concentrations were well above its water quality guideline trigger value of 2.8 μg/L. Fourteen out of the 20 sites (70%) had particularly high 14-d average thiobencarb concentrations in the first period of deployment of the passive samplers, which corresponds to the early application of this herbicide to rice crops (Lacy and Stevens 2003). Although a slight majority of these were rice and corn sites (9 or 64% of the 14 sites), average concentrations of this herbicide at these sites over the three deployment periods of 185 ± 47 μg/L (mean ± SE) did not differ significantly ($p = 0.862$, *t*-test) from those found at the rice-only sites of 204 ± 67 μg/L (mean ± SE). Molinate concentrations were particularly high also in the first period of deployment of the passive samplers: it was detected at a mean concentration of 96.2 ± 43.5 μg/L (mean ± SE) at rice-only sites (9 or 69% of 13 detections) whereas rice and corn sites had significantly lower ($p = 0.004$, *t*-test) concentrations of 6.9 ± 3.4 μg/L (mean ± SE). Concentrations of clomazone above 1 μg/L were mostly measured on rice-only farm sites.

Atrazine, which is applied to corn and cereal crops in the area, was also detected at the study sites. Its time-weighted average concentrations in the rice bays at each farm site during three 14-d deployment periods in both the seasons were below the water quality guideline trigger value of 13 μg/L. Atrazine average concentrations of 0.66 ± 0.15 μg/L (mean ± SE) in the bays of rice and corn farms in the 2006/07 season were significantly higher ($p = 0.033$, *t*-test) than the mean concentrations detected in the bays of rice-only farms 0.26 ± 0.08 μg/L (mean ± SE).

Metolachlor is applied in combination with atrazine to corn crops. During the 2006/07 season, 14 of the 20 farms studied had metolachlor concentrations above the provisional water quality guideline trigger value of 0.02 μg/L. The average metolachlor concentrations measured at sites growing rice and corn was 0.39 ± 0.08 μg/L (mean ± SE), which is significantly higher ($p = 0.0003$, *t*-test) than that of concentrations at rice-only sites 0.08 ± 0.03 μg/L (mean ± SE). Metolachlor was detectable at four rice-only sites on at least one occasion, of which 83% were over the provisional water quality guideline trigger value.

The herbicide diuron was also detected in 13 sites at very low concentrations 0.14 ± 0.03 μg/L (mean ± SE). Although diuron is not applied to rice crops, its residues were detected with equal frequency and concentration at either rice-only or rice and corn farms because it is a persistent herbicide commonly used in crop rotations in the area.

Water quality at the study sites

Water quality was measured in the rice bays at the 20 farm sites where the passive samplers were deployed on two sampling occasions in the 2005/06 season ($n = 40$), and in three occasions in the 2006/07 season ($n = 60$). In the latter season, one rice-only farm was found dry on the third visit because of the water restrictions that resulted in loss of the crop, so a total of 99 water quality values were recorded over the two seasons. The water temperature 24 ± 0.5°C (mean ± SE) and dissolved oxygen were very similar across all of the study sites. The pH of the rice-only farms of 8.14 ± 0.16 (mean ± SE) was slightly higher but not significantly different than that of the rice and corn farms of 8.01 ± 0.15 (mean ± SE). In contrast, the mean conductivity was found to differ significantly ($p < 0.001$, *t*-test on log-transformed data) between crop types, with rice and corn farms having a mean conductivity of 513 ± 71 μSi/cm (mean ± SE, $n = 50$), which is over three times higher than the mean value in rice-only farms of 150 ± 43 μSi/cm (mean ± SE, $n = 49$). Water conductivity over 400 μSi/cm was measured at 4 out of 49 (8%) rice-only farms and at 16 of 50 (32%) rice and corn farms over the two seasons.

Principal component analysis (PCA)

A PCA biplot (84% of the variance) showed four main groupings of the study sites, with a clear association between farm type and early use of the herbicides molinate and thiobencarb and such groupings (Figure 4). Higher concentrations of molinate are associated with the first visits to rice-only sites and high concentrations of thiobencarb with the first visits to the rice and corn farms. Later in the season these two groups in the PCA biplot moved closer together, indicating that they were becoming more similar as all sites had lower concentrations of all six pesticides. Higher water conductivity and temperature on the rice and corn farms may also be associated with the convergence shown by the farm types later in the season (Figure 4), although there was no correlation between the main components and conductivity ($r^2 < 0.19$) or temperature ($r^2 < 0.27$).

The sites occupied by *L. raniformis* are highlighted in the PCA biplot (Figure 4). Of the 28 sites occupied by *L. raniformis,* 20 out of 28 (71%) were in the southern region, but this analysis did not show any clear pattern of association with specific pesticide usage in those areas. The correlation between presence-absence of *L. raniformis* and farm type was marginally not significant ($p = 0.070$, Pearson's Chi-squared test with Yates' continuity correction), even though the rice-only sites had higher occupancy (57%) compared with rice and corn sites. During the first visit to the study sites, sites occupied by *L. raniformis* were predominately rice-only farms (Figure 4).

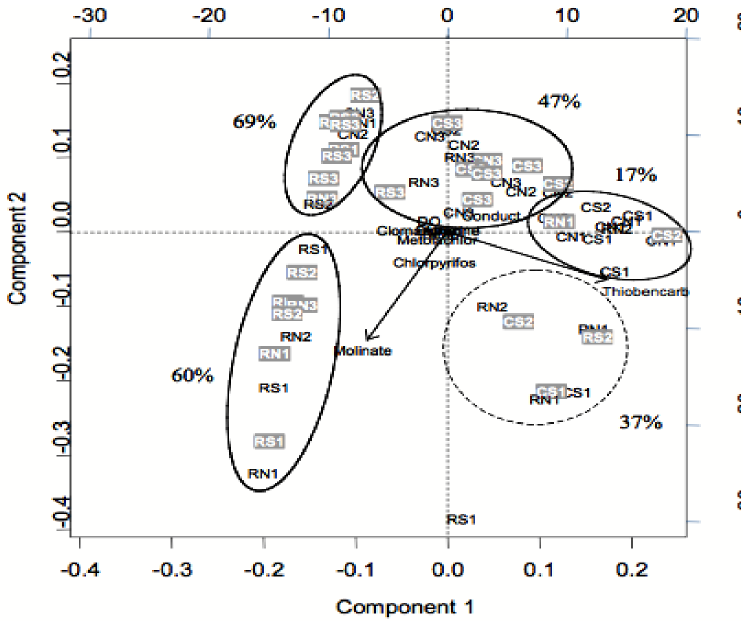

Figure 4. Principal components analysis for the 20 study sites during the three site visits in the 2007/07 season (n = 60) as determined by seven pesticides and four water quality variables. The first axis explains 57% of the variation while the second axis explains 27%. The site three unit codes read in the sequence left to right as follows: Farm type (R = rice-only or C = rice and corn), Location (N = north or S = south), Site visit (1,2 or 3). Sites shaded were those occupied by L. raniformis tadpoles or calling males, the percentage of which is also indicated for each group.

Discriminant Analysis (DA)

Since the PCA biplot grouped the sites according to farm type and time of pesticide application within the growing season, a DA was performed to determine how well the pesticide variables (thiobencarb, molinate, clomazone, chlorpyrifos, atrazine, diuron and metolachlor) and the water quality variables (pH, conductivity, temperature and dissolved oxygen) could be used to predict the presence or absence of *L. raniformis* on the two farm types. The first canonical correlation (r^2 = 0.84 and 76% of variance) was highly significant ($p < 0.001$) and distinguished well between rice-only farms and the rest (Figure 5).

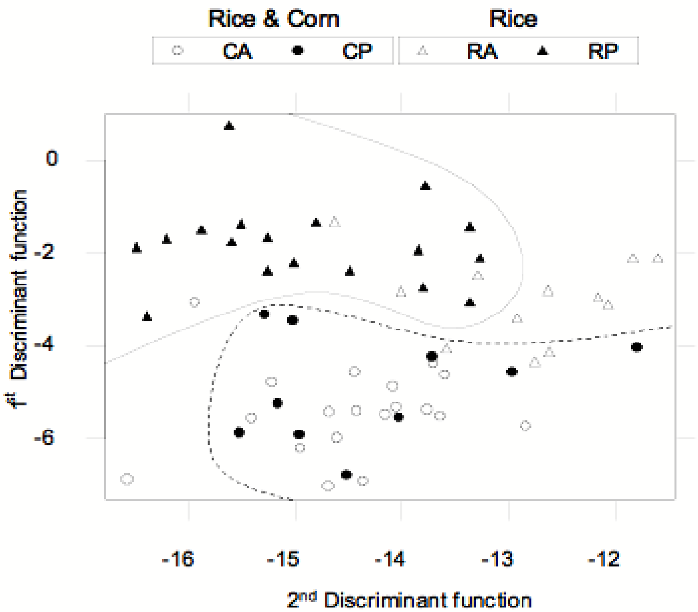

Figure 5. Classification of sites with (solid symbols) or without (empty symbols) L. raniformis on the rice-only or rice and corn farms as determined by the first two canonical functions of a discriminant analysis. Twenty out of the 28 sites were correctly classified. Correlation coefficients are 0.84 for the first function and 0.60 for the second, and together account for 94% of the variance. The ten variables used include seven pesticides and four water quality parameters, all natural logarithm transformed. Low metolachlor concentrations, high pH, low conductivity and high temperatures were the main factors accounting for the distribution of L. raniformis. CA = absence of L. raniformis at rice and corn sites; CP = presence of L. raniformis at rice and corn sites; RA = absence of L. raniformis at rice-only sites; RP = presence of L. raniformis at rice-only sites.

Low concentrations of the corn pesticide metolachlor (canonical coefficient -2.83) and alkaline pH (canonical coefficient 1.43) were the strongest predictors for the presence of *L. raniformis* at sites of both farm types. A third but minor predictor of the presence of *L. raniformis* was low water conductivity (canonical coefficient -0.95). However, to predict more accurately the presence/absence of *L. raniformis*, a second canonical function was required. This second function (r^2 = 0.60 and 18% of variance) was also significant (p = 0.036) and, in addition to pH and low metolachlor concentrations, it included higher water temperatures among the predictors for the presence of this species. The two discriminant functions could classify correctly 20 out of the 28 sites in which the species was found (Figure 5).

Chytrid fungus on frogs

Of the 67 frogs that were swabbed at eight field sites, only swabs from three frogs (4%) tested positive for infection with *B. dendrobatidis*. An additional four frogs (6%) were classified as indeterminate in the assay, because the samples returned a low number of zoospore equivalents in only one or two of three replicate wells. In addition, the results for 19 of the frog samples that gave a negative result in the assay had an internal control that showed that the samples had an inhibitor to the assay at a 1/100 dilution. Hence only 41 (61%) of the frogs gave an unequivocal negative result to the absence of infection with *B. dendrobatidis*. The results for the individual species are presented in Table 2.

Table 2. Screening for chytridiomycosis in frogs collected from rice farms in the Coleambally Irrigation Area in the spring of 2006.

Region	Frog species	No. of frogs	No. of negative detections	No. of samples with inhibitors [a]	No. of positive detections	No. of indeterminate detections [b]
North	*L. fletcheri*	17	11	4	1	1
	L. tasmaniensis	14	12	2	0	0
	L. raniformis	4	1	3	0	0
South	*L. fletcheri*	16	11	3	2	0
	L. tasmaniensis	13	6	4	0	3
	L. raniformis	3	0	3	0	0

[a] Internal control that showed that the samples had inhibitor to the assay at a 1/100 dilution.
[b] Samples returned a low number of zoospore equivalents in only one or two of three replicate wells.

Histology of frog gonads

Figure 6A shows the image of a pair of ovaries from a Gosner stage 40 *Litoria raniformis* tadpole with a "string of beads" appearance, which is similar in appearance to the image showing immature testes dissected from a juvenile *Litoria raniformis* frog (Figure 6C). Figure 6B shows a more developed pair of ovaries from a juvenile *Litoria raniformis* frog, but still sexually immature. All of the *Litoria raniformis* collected had recently emerged from the rice bays and were immature. In contrast, gonads dissected from those *L. tasmaniensis* and *L. fletcheri* frogs that had recently emerged from the rice bays were generally sexually mature in the same season that the tadpoles developed. Figure 6D shows the image of a pair of ovaries with mature oocytes and Figure 6E shows the image of a well-developed pair of testes, with both gonads dissected from two recently emerged *Limnodynastes tasmaniensis* frogs.

We also examined histological sections of the gonadal tissue of male frogs for frequency of intersex (see Materials and Methods for definition) and found it occurred in the two *Limnodynastes* species (Figure 6F) at a very low frequency (Table 3). The sex ratio of the frogs was also determined by examination of their gross gonadal morphology and the histology sections. Most species had a 1:1 sex ratio, but *L. raniformis* had a female biased 2:1 sex ratio, albeit based on a low number (17) of juveniles that were collected (Table 3).

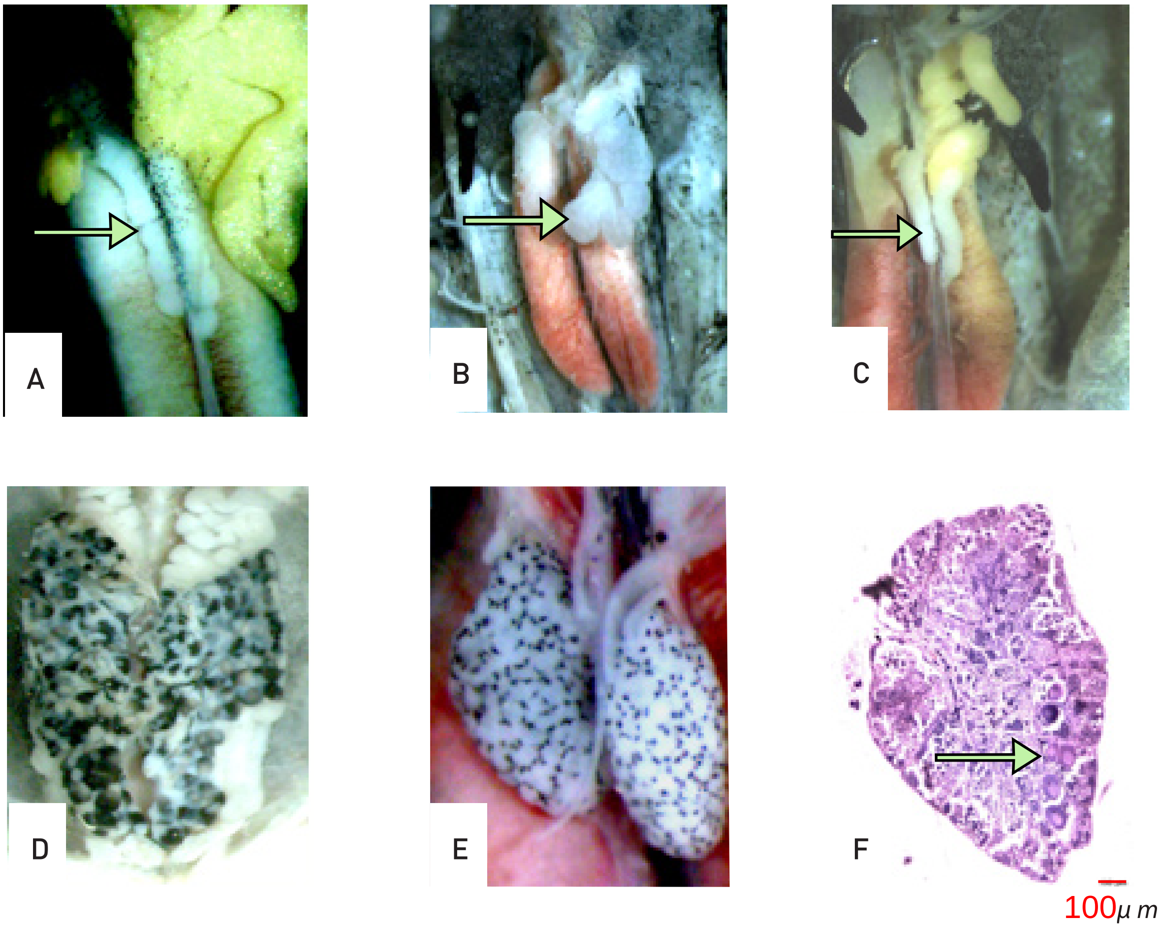

Figure 6. Gonadal morphology of (A) a pair of ovaries from a Gosner stage 40 Litoria raniformis tadpole with a "string of beads" appearance (arrow); (B) more developed, but still sexually immature, pair of ovaries (arrow) from a recently emerged Litoria raniformis frog; (C) pair of immature testes (arrow) from a recently emerged Litoria raniformis frog; (D) a pair of ovaries with mature oocytes from a recently emerged Limnodynastes tasmaniensis frog; (E) a pair of mature testes from a recently emerged Limnodynastes tasmaniensis frog; and (F) a histological section of testis from a Limnodynastes fletcheri frog with normal morphological appearance, showing intersex with developing oocytes in otherwise normal testicular tissue (bar = 100 μm).

Table 3. The no. of intersex and sex ratio of 216 metamorph frogs collected from rice farms in the Coleambally Irrigation Area 2005/2006 growing season.

Region	Frog species	Total no. of frogs	No. of male frogs	No. of intersex male frogs	Sex ratio female : male
North	*Limnodynastes fletcheri*	41	16	1	61:39
	Limnodynastes tasmaniensis	43	20	0	53:47
	Litoria raniformis	0	0	0	--
	Limnodynastes fletcheri	61	25	0	59:41
South	*Limnodynastes tasmaniensis*	54	24	1	56:44
	Litoria raniformis	17	6	0	65:35

Discussion

Distribution of frog species across sites

In conjunction with the presence/absence surveys of the tadpoles during the day, this study also incorporated nocturnal surveys of frog calls at the study sites during the season 2006/07. Frog call surveys have been widely used in various studies as a relatively rapid assessment of adult frog presence at a particular site (Zimmerman 1994, Driscoll 1998, Hazell et al. 2001). The only weather conditions encountered in this study described as adverse by Hazell et al. (2001) for frog calling were gusty winds and temperatures dropping below 5 degrees. These conditions were only encountered on two out of the 12 occasions the frog-call surveys were undertaken.

The four species of frogs studied here usually breed in temporary water bodies such as ponds, swamps, farm dams and irrigation channels. *C. parinsignifera* tadpoles and calling frogs were found to be universally present at the study sites. *C. parinsignifera* is an opportunistic breeder and is heard calling all year round but calling and breeding intensifies during late winter to spring, and after summer or autumn rains (Tyler 1994, Anstis 2002). *L. tasmaniensis* and *L. fletcheri* were also frequently found in most sites during the study. *L. tasmaniensis* and *L. fletcheri* are opportunistic breeders that are capable of reproducing all year round (Tyler 1994, Anstis 2002). Calling and breeding is most common after rain events, but breeding typically peaks in summer and autumn (Anstis 2002).

In contrast to the other three frog species, *L. raniformis* tadpoles and calling frogs were rarely observed at the farms of the northern region, compared to their more frequent site occupancy in the southern regions of the agricultural irrigation area. *L. raniformis* breeding typically occurs during the warmer months of spring and summer and breeding events usually follow a dramatic rise in water level (Pyke 2002; Wassens et al. 2008). In the study area, the *L. raniformis* frogs retreat to the main supply channels during winter, and disperse when the rice bays are flooded for growing the new rice crop which is linked to the peak breeding period for this species (Wassens et al. 2008).

L. raniformis distribution and pesticide usage

The presence of *L. raniformis* was mostly associated with farms that had lower concentrations of the herbicide metolachlor, slightly higher pH and lower conductivity. In fact, rice-only farms had higher occupancy by *L. raniformis* than farms that grew rice and corn because, among other things, this herbicide is not applied to the rice crop. Metolachlor was, according to the DA analysis, the strongest predictor of site occupancy by this species on the two farm types, whereas pesticides such as atrazine and chlorpyrifos were not because they are used on a variety of crops in addition to corn, and were found in most sites irrespective of the crop type. The 14-d average metolachlor concentrations that the *L. raniformis* tadpoles were exposed to at the study sites (0.05-0.73 μg/L) were four to five orders of magnitude lower than its 96-h LC50 of 13.6 mg/L for embryotoxicity to the frog, *Xenopus laevis* (Osano et al. 2002). Assuming there is not much difference in sensitivity between these species, this suggests that metolachlor may not cause direct adverse effects on *L. raniformis*, but rather it is an indirect indicator of agricultural practices such as pesticide usage, which in turn results from the different crop types being grown in the area.

The PCA showed that the herbicide molinate was associated with early sowing of the crop on rice-only farms, while thiobencarb was associated with the early establishment of rice on farms growing predominately both rice and corn. Clomazone is also used frequently in the CIA in rotation with molinate to reduce weed resistance (Lacy and Stevens 2003). None of these rice herbicides, regardless of their relatively high residues (Table 1), appear to influence the presence or absence of *L. raniformis* on farms.

L. raniformis distribution in relation to farm type and water quality

L. raniformis tadpoles and calling frogs were found to occur less frequently in the northern sites, despite suitable habitat in the form of rice bays available in both regions. Examination of the land area, based on water allocations, used for the different summer crops in the 2005/06 and 2006/07 seasons (www.colyirr.com.au) indicated that there was very little difference in the rice acreage that was available as potential tadpole habitat between the northern and southern region (rice acreage ratio 45:55). In contrast, there were more row crops (corn/soybeans) grown in the northern region compared to the southern region of the CIA. Since more corn is grown in the northern region, this results in land used for growing rice becoming more dispersed and hence the region would offer less suitable habitat for *L. raniformis*. Australia is a drought-prone country and a drought period in the CIA region started in 2003 and is still ongoing in 2008. In this context, the difference in water availability in the present study between 2005/06 and 2006/07 seasons was only a matter of degree. Water was still being delivered through the main supply channels for stock and domestic purposes to the farms throughout the year during both seasons that provided refuge for the wintering frogs.

The DA showed that after metolachlor concentrations, water pH was the strongest predictor of site occupation by *L. raniformis* on the two farm types. The mean pH of sites occupied by *L. raniformis* in the 2006/07 season was 8.4 irrespective of farm type and was significantly different to the pH of sites unoccupied by *L. raniformis* ($p < 0.01$, *t*-test). The pH of sites where *L. raniformis* was absent was 7.6 for rice-only farms and 7.8 for rice and corn farms. Alkaline pH at specific sites during the day (when the samples were taken) is likely to result from the growth of algae (Abdullah et al. 1997, Dodds 2003), which is the preferred food of tadpoles in the rice bays, and it is therefore another indirect indicator of the preferred habitat conditions chosen by the recruiting *L. raniformis* frogs.

The presence of riparian and emergent vegetation has been strongly correlated with the presence of frogs in many studies of habitat in altered landscapes (Hazell et al. 2001, Hamer et al. 2002, Jansen and Healey 2003, Wassens 2005). Among the farms that grew rice and corn, *L. raniformis* was more likely to be present in the southern region. Sites in the southern region are dominated by traditional rice farmers with small land holdings, which utilise minimal machinery for the construction of rice bays and levees. This results in well-vegetated rice bay levees on the farms and terrestrial environment adjacent to the irrigation supply channels from which the frogs disperse. In contrast, a feature of the northern irrigation region is the preponderance of larger land holdings, which in summer grow other crops such as corn and sorghum in addition to rice. This has lead to more mechanised farm practices on the larger holdings including more water recycling which results in higher conductivity. Associated with the water recycling are less vegetated on-farm water channels particularly at the beginning on the rice season, which may also discourage frog recruitment. Limited recruitment early in the rice growing season would be particularly disadvantageous for *L. raniformis* because of its more restricted breeding period, compared to the other anuran species.

Chytrid infection and hermaphroditism

Apart from the above factors that seem to determine site occupancy by *L. raniformis* in the CIA agricultural area, other possible causes were also evaluated. The incidence of chytrid infection was examined in frogs collected in early austral spring, when the water temperatures in the region were low and typically ranged from 10ºC to 20ºC. Chytrid infection was low in mature frogs for both *Limnodynastes* species and mature *L. raniformis* frogs across both regions of the CIA (Table 2). Since the chytrid fungus does not survive for long periods in temperatures above 30ºC (Woodhams et al. 2003), it is unlikely that chytrid infection may be affecting any frog population in this area to a significant extent, as water temperatures in the rice bays during the rice-growing summer season typically exceed 30ºC during the day. Nevertheless, the second function of the DA indicates that higher water temperature within the study sites is a predictor, albeit minor, for the presence of *L. raniformis*.

Examination of the histological sections of the gonadal tissue of frogs collected adjacent to the rice bays in late summer showed that the two *Limnodynastes* species had often reached sexual maturity. Newly metamorphosed *L. tasmaniensis* and *L. fletcheri* juvenile frogs take approximately 12 weeks to reach sexual maturity and their breeding is regular (Davis 1992, Horton 1982). In contrast, *L. raniformis* frogs do not reach sexual maturity until two years old (Pyke 2002) and we observed all juvenile

L. raniformis frogs collected to be sexually immature. We also examined histological sections of the gonadal tissue of both *Limnodynastes* species and *L. raniformis* male frogs for the occurrence of hermaphroditism or intersex gonads and found intersex gonads occurred in both *Limnodynastes* species, but only at a very low frequency (below 2%) which is considered to be the natural frequency of hermaphroditism or intersex gonads in wild frog populations (Pettersson and Berg 2007). Furthermore, we found no evidence of increased hermaphroditism or intersex gonads in late stage tadpoles or juvenile *Limnodynastes* species and *L. raniformis* frogs at sites with relatively high atrazine concentrations (0.16-1.67 μg/L) over six weeks during the period they would be undergoing metamorphosis. This contrasts with the study by Hayes et al. (2003) that found an increased occurrence of intersex gonads of male frogs in agricultural areas growing corn that was attributed to their exposure to concentrations of atrazine higher than 0.1 μg/L.

Conclusions

In the Coleambally Irrigation Area, Australia, the occurrence of four tadpole and frog species in rice bays on farms growing either rice-only or both rice and corn was studied over two seasons. In addition to analysis of species occurrence, gonadal histology and assessment of *Batrachochytrium dendrobatidis* infection rates were performed. The rice acreage available as potential tadpole habitat was extensively distributed throughout the irrigation area but more corn was grown in the northern region compared to the southern region. The mean abundance of *Litoria raniformis* tadpoles was significantly lower in the northern sites compared to southern sites.

In contrast, tadpoles of *Limnodynastes fletcheri*, *Limnodynastes tasmaniensis*, and *Crinia parinsignifera* had a uniform distribution across all the study sites. A principal components analysis showed there was a relationship between farm type and the rice herbicide applied when the crops were initially sown, with sites occupied by *L. raniformis* in the beginning being predominately rice-only farms. A discriminant analysis showed that low concentrations of the corn herbicide metolachlor and increased pH were the main variables studied that determined site occupation by *L. raniformis*. This suggested that farms growing only rice (and not corn) with high algal production were preferred sites. The rate of chytrid infection and gonadal malformations was low across both regions. Histology of the gonads of metamorphs showed that *L. raniformis* gonadal differentiation is slow compared to the two *Limnodynastes* species.

We concluded that farm practices associated with increased corn cropping in the northern region, rather than any direct effect of corn herbicides, determine the reduced presence of *L. raniformis* in the northern region.

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