Mapping the spread of invasive cane toads (*Rhinella marina*) in southern Australia

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Submitted as part of the requirements for completion of the degree of Master of Research

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)_____ Date: 11th November 2020

Lincoln Finley Macgregor

The work for this thesis was conducted in accordance with animal ethics approval (ref: 2019/040) from the Animal Ethics Committee (AEC) at Macquarie University.

Note to Examiners

This thesis is written in the form of a journal article for the journal **Biological Invasions**, except for the format requirements from the Department of Biological Sciences, Macquarie University.

Candidate's statement about the impact of COVID-19 changes on the thesis

Dear Examiner,

Many of our HDR candidates have had to make changes to their research due to the impact of COVID-19. Below you will find a statement from the candidate, approved by their Supervisory Panel, that indicates how their original research plan has been affected by COVID-19 restrictions. Relevant ongoing restrictions in place caused by COVID-19 will also be detailed by the candidate.

Thesis Title: Mapping the spread of invasive cane toads (*Rhinella marina*) in southern Australia

Candidate Name: Lincoln Finley Macgregor

Department: Department of Biological Sciences

Statement:

At the beginning of this year, my project was to compare the effectiveness of different methods to detect cane toads in invasion-front areas; specifically, to compare eDNA-based methods to other types of surveys. After the first session of fieldwork had been completed from January to February, the COVID-19 pandemic forced the University to cancel all travel, rendering my planned fieldwork impossible. As a result, the nature of my project had to change dramatically, and I had to devise a study based on information I could obtain without going into the field. I decided to focus on mapping the historical range of cane toads in NSW to answer questions about their dispersal. Results from my first and only fieldtrip have been included in this thesis as a pilot study.

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Acknowledgements

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I would like to thank my primary supervisor, Rick Shine, for this exciting research opportunity you provided for me, and for all your help in overcoming the obstacles thrown at me throughout the year. Your infinite knowledge, constant support and help in improving this project and this document have been incredibly valuable and I am forever grateful. I would therefore like to express my deepest appreciation for the mentorship you have given me throughout this entire journey.

I would also like to thank my co-supervisor, Mark de Bruyn, for providing invaluable help and guidance throughout the past year, in and out of the lab. You have helped me immensely in refining my lab skills and the eDNA techniques used in this study and have made me feel welcome from the very start.

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Abstract

By fatally poisoning apex predators, invasive cane toads (*Rhinella marina*) have modified ecosystems across much of Australia. Most research on their invasion has been conducted in the tropics, with the southern front of their range expansion largely overlooked. To address that knowledge gap, I assembled multiple datasets to characterise the historical spread of toads through north-eastern New South Wales in order to clarify factors that might influence routes and rates of dispersal. My pilot studies at the range edge suggest that visual and acoustic surveys are as effective as eDNA-based surveys. Expansion of the toads' range in NSW has occurred through the establishment of satellite populations and through growth of the range core. Overall rates of spread have been far lower than on the tropical front, and in some decades the area occupied by toads appears to have declined rather than expanded. Rates of spread have accelerated to the south, but westward expansion has slowed. Toad range expanded most rapidly in decades with dry, warm weather conditions, suggesting that predicted future changes to climate could increase or decrease rates of toad spread. Understanding the historical patterns of toad invasion in NSW may clarify priority areas for monitoring and control programs.

Introduction

Invasive alien species pose a major threat to ecosystems and the persistence of native biodiversity throughout many regions of the world (Mack et al. 2000; McGeoch et al. 2010; Bellard et al. 2016). Among the impacts of invasive species are the capacity to alter ecosystem processes and food webs (Doody et al. 2006; Doody et al. 2009; Doody et al. 2013), act as potential vectors for pathogens (Hulme 2014; Soto-Azat et al. 2016) and disturb agricultural activities (Walsh et al. 2011; González-Bernal et al. 2013; Goergen et al. 2016; Silvester et al. 2019). Once an invasive species becomes established, it can be difficult to control and may spread rapidly, either on its own or through human-assisted dispersal (Wittenberg and Cock 2005; Chapple et al. 2012).

One of the most fundamental issues for invasive species managers is to know the rates and routes of population expansion for a species. Information on these topics can enable managers to predict when, or if, they need to deploy control strategies and which areas should be given priority. Accurate predictions may be difficult to make, however, because of the possibility of spatial and temporal heterogeneity in invasion dynamics. For many invasive species around the world, information on their spread often is available only at one invasion front, preventing detailed comparisons of spatially independent invasions (Grosholz and Ruiz 1996). If the dynamics of population expansion differ across the species' range-front, available information may be inadequate for predicting patterns of dispersal at other locations. From a management perspective, control strategies based on invasion patterns in a different region may fail if local populations of the invader respond differently to abiotic and biotic cues. Indeed, geographically distinct populations of some invasive species have been found to disperse at different rates and with different patterns of dispersal (Lubina and Levin 1988; Grosholz 1996; Urban et al. 2008). Thus, to understand the spread of an invasive species in a certain location, we need to collect data from that specific region.

In Australia, the spread of the invasive cane toad (*Rhinella marina; Bufo marinus* in earlier literature) has been intensively studied throughout tropical Australia, such that patterns and rates of dispersal are now well understood in this landscape (Urban et al. 2008). The species has expanded its distribution across over one million square kilometres of Australia since being introduced in 1935 (Urban et al. 2007). Due to their lethal toxins, these toads can severely disrupt ecological communities by killing native predators and

indirectly influencing species at other levels within the food web (Doody et al. 2006; Doody et al. 2009; Doody et al. 2013). The negative effects associated with toad arrival have stimulated the development of control methods which hold the potential to buffer the toads' impact on ecosystems, slow their spread and eradicate small, localised outbreaks of toads – though largescale eradication is not yet possible (Tingley et al. 2017). Understanding the toads' current range and their rates and patterns of dispersal is an important step towards predicting their patterns of movement to inform management strategies in the future.

In contrast to the extensive research in tropical Australia (see Shine 2010 for a review), only a few studies have examined the toads' southward spread along the east coast into New South Wales (NSW; Van Beurden and Grigg 1980; Seabrook 1991). As a result, questions regarding their current rates of invasion and the factors driving their dispersal remain largely unanswered. Previous surveys have concluded that the range front of toads in NSW expanded at 3 km/yr from 1965-1978 (Van Beurden and Grigg 1980) and then at a slower rate (maximum of 1.3 km/yr) from 1978-1990 (Seabrook 1991). Given that the last in-depth analysis of range expansion rates by cane toads in NSW was conducted nearly three decades ago (Seabrook 1991), this topic represents an obvious gap in our current knowledge. Over recent years, the number of records of cane toad presence in this region has increased dramatically due to efforts by government agencies (e.g., Landcare NSW and the NSW Department of Primary Industries), citizen scientists (e.g., via FrogID) and researchers. I collated and analysed these records to clarify the dynamics of a biological invasion.

By mapping the historical spread of cane toads in NSW, I hoped to answer four main questions:

(1) Do visual and acoustic surveys provide reliable information on the location of toad invasion fronts?
(2) How does environmental and climatic variation influence spatial and temporal variation in the rates of spread of cane toads?
(3) How does overall rate of spread of toads in NSW compare to that seen in the tropics?
(4) How is predicted climate change likely to affect rates of toad spread at the southern front?

To address (1) I conducted a pilot study along transects through the NSW invasion front to compare rates of detection using traditional methods (visual and acoustic surveys), to a more complex assay with environmental DNA (eDNA). Under some circumstances, eDNA surveys can achieve higher detection rates than can other methods (Dejean et al. 2012; Biggs et al. 2015; Smart et al. 2015; Torresdal et al. 2017), especially when the target species is at low abundance (as is likely to be true at an invasion front: Phillips et al. 2010b). The detection rates of each survey method were also compared to those of sniffer dog surveys conducted along some of the same transects.

To address (2) I mapped reports of cane toad occurrence through time using a combination of exact (GPS-coordinate) toad-presence records and historical range maps. I then used GIS layers to compare spatial and temporal variation in cane toad spread rates to a number of ecologically relevant abiotic factors.

To address (3) I compared the rates of spread over time generated in (2) to rates of spread reported in studies of the toad invasion in tropical Australia.

To address (4) I combined predicted values for climatic parameters with empirical relationships between those parameters and rates of spread of toads over recent decades.

In short, I integrated information from a variety of sources to document rates and patterns of expansion of the cane toad invasion in New South Wales, and to compare those rates and patterns to abiotic conditions and to results from studies on the toad invasion in tropical Australia.

Materials and Methods

Study species

Cane toads are large and highly toxic anurans, native to South America but translocated to many other parts of the world in attempts at biocontrol of insect pests of commercial agriculture (Lever 2001). Negative effects of cane toads on some species of native Australian predators, including death by lethal toxic ingestion of toads (Lever 2001; Letnic et al. 2008; Doody et al. 2009; Shine 2010), have stimulated intensive efforts to control

toad abundances and rates of spread (Tingley et al. 2017; Greenlees et al. 2020). Expansion of the main invasion front is due primarily to dispersal by adult toads, with radio-tracking studies suggesting that rates of spread are enhanced by warm, wet conditions and the availability of open linear corridors such as roads (Brown et al. 2006; Brown et al. 2011; Pettit et al. 2017). Substantial shifts in toad morphology, physiology and behaviour in the course of the Australian invasion have been interpreted as adaptations that facilitate more rapid dispersal (Phillips et al. 2006; Alford et al. 2009; Brown et al. 2014; Hudson et al. 2016; Gruber et al. 2017b; Kosmala et al. 2018), though some trait changes may also be non-adaptive (Gruber et al. 2017a). Studies on captive-reared progeny of toads from multiple locations within Australia have revealed significant heritability of many of these phenotypic changes, consistent with the hypothesis that rapid evolution is occurring along their expanding invasion fronts (Phillips et al. 2010a; Brown et al. 2014). In Australia, cane toads call and breed year-round in the tropics (Brodie et al. 2020), but along the southern front breeding occurs only in warmer months (NSW Department of Primary Industries 2020).

Since being released along the eastern Queensland coast in 1935, the toads have spread rapidly westwards across tropical Australia, and more slowly southwards into New South Wales (Urban et al. 2008). Individuals from the expanding population in Queensland were also intentionally translocated and released near Byron Bay in the 1960s, spawning a satellite population that eventually coalesced with the expanding southern front of the main invasion (Van Beurden and Grigg 1980; Seabrook 1991). Many other extralimital incursions have been recorded also, with toads sometimes dispersing long distances from the current main range by ‘stowing away’ on vehicles, typically in waste materials and landscaping supplies (White and Shine 2009). Some of these isolated populations fail to persist, but a few self-sustaining populations have been established by accidentally translocated toads (e.g., in the suburbs of Sydney, 500 km south of the main front: Greenlees et al. 2018).

Study area

My study is based on all of the records that I could locate of the occurrence of cane toads in New South Wales, with a particular focus on part of the North Coast region in the north-eastern part of the state, where cane toads were first established and are still most prevalent (Seabrook 1991). The North Coast region stretches from near Newcastle to just

beyond the Queensland border, with the climate predominantly ranging from sub-tropical to temperate (National Parks and Wildlife Service NSW 2003). Extending from shoreline zones to foothills and higher elevation areas associated with the Great Dividing Range (which runs almost the entire length of the Australian east coast), environments within this area are diverse and include temperate and sclerophyll forests, coastal dunes, farmland, estuaries and wetlands (National Parks and Wildlife Service NSW 2003).

Comparison of different methods to detect toads

I performed a pilot study to compare rates of detection of cane toads at the leading edge of their southern invasion using visual surveys, acoustic surveys and eDNA surveys. Because cane toads in this region often utilise anthropogenically-created waterbodies for rehydration and breeding (Semeniuk et al. 2007), my primary sampling units were farm dams at five sites along each of four transects at the southern edge of the toad invasion. Each transect extended from sites known to be occupied by toads to sites which were thought to not yet be invaded (Brooms Head to Minnie Water, Townsend to Shark Creek, Mongogarie to Upper Mongogarie, and Theresa Creek to Cambridge Plateau). In areas without dams, water-filled roadside culverts were used as substitutes. The distances between neighbouring sites along the same transect ranged from 0.9 km to 16.9 km, and all acoustic and visual surveys were conducted at night (the main activity period for adult cane toads; Pizzatto et al. 2008). All surveys were conducted by a team of three people from late January to early February 2020.

Acoustic surveys. As soon as we arrived at a site, we listened for calling toads for 10 minutes, standing still 20-30 metres from the waters' edge, without any lights on. The duration (10 min) was based on pilot studies of the duration needed to reliably detect toads on nights when they were calling.

Visual surveys. At each site we walked around the perimeter of the waterbody, using illumination from headtorches to search both water and land for adult toads and metamorphs, and to search the water for tadpoles and eggs. Visual surveys were always performed immediately after acoustic surveys.

Sniffer dog surveys. At eight of the sites where we sampled, subsequent surveys using sniffer dogs were conducted (from 28th February to 1st March 2020, data from Landcare NSW).

eDNA sampling. Water samples were collected in sterile bags either by day or at night, from 16 equidistant points around the waterbody edge. Water samples were filtered onsite through 0.45 μm Sterivex filter units (two samples per site) unless adverse weather conditions (e.g., rain) made this impractical (the Supplementary Material contains additional details), and filters were transported on ice to a freezer at -20°C. We aimed to filter 200 mL of sample water through each filter unit, but sediments sometimes caused clogging and resulted in as little as 24 mL being filtered. Contamination control samples were gathered using tap water.

eDNA extraction and ddPCR analysis. DNA was extracted from the filters using a method adapted from Tingley et al. (2019), using a QIAGEN DNeasy Blood & Tissue Kit. To remove potential PCR inhibitors, all samples were then cleaned using a Zymo Research OneStep PCR Inhibitor Removal Kit. Resultant sample template DNA was stored in 2 mL Eppendorf tubes at -20°C. All extractions were performed in a room dedicated to DNA processing, with all equipment and surfaces cleaned with a 10% bleach solution. Droplet Digital PCR (ddPCR) was performed on all 43 extracted samples, as well as five no-template control samples; two composed of 2 mL Buffer ATL and 20 μL proteinase K, and three composed of ultrapure water. The primers and probe used were developed by Tingley et al. (2019). Droplet generation was performed, and droplets were transferred to wells in a 96-well plate in a thermal cycler for amplification. The amplification occurred in conditions of 10 min at 95°C, followed by 30 s at 94°C and 1 min at 53.3°C for 40 cycles, followed by 10 min at 98°C, followed by a hold temperature of 4°C. Droplets were read within 24 h of amplification and readings were processed in QuantaSoft version 1.2.10.0 (Bio-Rad, CA, USA). The Supplementary Material contains detailed information about the methods and equipment used in this part of the study.

Mapping historical toad presence

Toad-presence records. I compiled toad-presence records from multiple sources to map the historical spread of cane toads in New South Wales. Records in the form of GPS coordinate points were obtained from the NSW Department of Primary Industries (via

ToadScan), NSW BioNet, FrogID (Rowley et al. 2019; Rowley and Callaghan 2020), Landcare NSW and Dr. Matthew Greenlees (personal records from field surveys). All of the records used to map toad distributions by Urban et al. (2007) were also included, as were nine records from my pilot study involving acoustic, visual and eDNA surveys (see above; one additional toad-positive site from this study was not included because eDNA results were not available at the time of map creation). One erroneous record (from Africa) was removed from the ToadScan dataset. Publicly available records of cane toad presence from the Atlas of Living Australia were not used for mapping because the vast majority of NSW records in this database (>4000) were listed as sourced from the Office of Environment and Heritage Atlas of NSW Wildlife, also known as NSW BioNet, from which we already sourced records directly. The validity of the remaining records from the Atlas of Living Australia database is unclear. Overall, I obtained 10,531 GPS coordinate points for Australia across all decades, of which 5,873 were within NSW. Most of the records were from 1971 to 2020 (N = 9,963 for Australia, N = 5,858 for NSW) so I focused on this period (Table 1).

Table 1. The number of GPS coordinate toad-presence records for each decadal period from 1971-2020 compiled for creating estimated toad range area maps for this study[1].

	1971-1980*	**1981-1990***	**1991-2000**	**2001-2010**	**2011-2020**	**TOTAL**
No. of Australia-wide toad-presence records in dataset	1244	100	475	2342	5802	9963
No. of NSW-only toad-presence records in dataset	464	62	446	2227	2659	5858

*Indicates that the estimated toad range area maps created for this decade were made using a combination of traced historical range maps from Seabrook (1991) and GPS coordinate records. NSW = New South Wales.

[1] A proliferation of records during the period 2011-2020 appears to be in-part the result of increased citizen science records. FrogID alone generated 1,791 Australia-wide toad records during the 2011-2020 period. The increase in records available from 2001-2010 may reflect the emergence of computational technology for GPS logging and/or increased survey effort.

To supplement the database for earlier periods when fewer GPS coordinate records were available, I took the NSW toad distribution map published by Seabrook (1991), traced it within Quantum GIS (QGIS) version 3.4 (Quantum GIS Development Team 2019), and thereby generated two historical range areas (one for 1978, another for 1990) to include within the maps generated with GPS coordinate points for this study.

Environmental and climatic layers. I obtained raster data layers containing the following climatic measurements from the Australian Bureau of Meteorology: average annual daily relative humidity at 1500 h (averaged from 1976-2005); average annual daily relative humidity at 0900 h (averaged from 1976-2005); average annual pan evaporation (averaged from 1975-2005); average annual rainfall (averaged from 1961-1990); maximum temperature annual 10-year average; minimum temperature annual 10-year average; rainfall annual 10-year average; and total annual rainfall for all years from 1971-2019 (from which I calculated average, maximum and minimum annual rainfall for each decade since 1971). The raster layers based on 10-year averages refer to the following year groupings: 1971-1980, 1981-1990, 1991-2000, 2001-2010, and 2010-2019 (records for 2011-2020 were not yet available). Because species distributions may respond to extremes in climate as well as changes in average conditions (Parmesan et al. 1999; Easterling et al. 2000), I chose data layers of maxima, minima and averages because they were readily available and provided simple measures describing such climatic changes which might affect toad distributions. Similar climatic measures have also been utilised in previous studies of the cane toad invasion in Australia to produce and analyse species distribution maps (Urban et al. 2007; Urban et al. 2008; Elith et al. 2010). Raster data layers for elevation and land cover measurements were obtained from Geoscience Australia in the form of the following datasets, respectively: GEODATA 9 Second DEM (DEM-9S) Version 3 (Geoscience Australia 2008) and the 2014-2015 layer of the Dynamic Land Cover Dataset Version 2.1 (Lymburner et al. 2015). Elevation raster data consisted of elevation above sea level across Australia, whereas the land cover raster data provided information on the locations of 23 different types of land cover. I combined some of these land cover categories to simplify analyses, such that four main land cover categories were recognised within the study area: waterbody (consisting of areas defined as ‘inland waterbodies’), pasture (consisting of areas defined as ‘rainfed cropping’, ‘rainfed pasture’, ‘irrigated cropping’ or ‘irrigated pasture’), open forest (consisting of areas defined as ‘shrubs and grasses – sparse-scattered’, ‘trees – open’, ‘trees – sparse’ or ‘trees – scattered’) and closed forest (consisting of areas defined as ‘trees – closed’).

Creating estimated decadal toad range areas. Kernel density estimation, a method of visualising the density of geospatial coordinate record points, has frequently been used to map species range areas based on presence records (Worton 1989; Seaman and Powell 1996; Fortin et al. 2005; Fleming and Calabrese 2017). Toad range areas for each decade were created in QGIS by producing heatmaps of datapoints using kernel density estimation with a 5 km search radius around each plotted toad-presence record point. I chose a 5 km search radius because more than 90% of the records of toad-presence in each decade were separated by less than 5 km from another record, and thus a 5 km search radius allowed for connectivity between most points when producing estimated toad range maps. Radio-tracked resident cane toads in this area have been reported to disperse between 29.4 m and 34.2 m on average per day during the summer period when toads are most active (Pettit et al. 2017), dispersal rates that generally support the biological relevance of a 5 km radius being used in this study (i.e., a toad could cover the 5 km distance within a year). Areas where the combined value of kernel density surfaces was greater than or equal to 1 were merged to form a toad range area for each decade, and areas where the kernel density was less than 1 were not used in creating the estimates of toad range. Historical range areas from Seabrook (1991) were traced for 1978 and 1989 and merged with the estimated toad range areas generated for 1971-1980 and 1981-1990, respectively. All of the estimated toad range areas were then cropped to the NSW state border. I then used QGIS to calculate the area covered by each estimated toad range area within NSW.

Criteria for designating areas as 'range core' or 'satellite population'. In order to quantify invasion rates in different regions of the toad range in NSW, I classified spatially separated areas of toad range as being either 'range core' or 'satellite population' areas. These zones were identified by first overlapping and merging all decadal toad range maps and then identifying all geographically distinct range areas on this merged map where toad ranges from at least two decades overlapped or connected. Overlapping ranges identified during this process did not need to be from consecutive decades. The largest of these geographically distinct areas was classified as the range core, and all smaller areas with interdecadal overlaps were classified as satellite populations. The range core and each individual satellite population area were all spatially separated (i.e., did not overlap or touch one another). Any geographically distinct areas which did not overlap or touch range areas from another decade could not be used to calculate rates of spread (because by definition, that calculation requires data from two time periods) and thus I did not quantify

invasion rates for these areas. Figure 1 shows the division of the cane toad's range in NSW into range core and satellite population components.

Measuring rates of invasion. I generated a series of North-South and East-West transects in QGIS to measure rates of linear spread of toads between successive decades. I performed these calculations separately for the range core and satellite populations, because of the possibility that rates of range expansion and contraction might differ between the two types of populations. Transects for each compass orientation were spaced 5 km from their neighbouring, parallel transects. For the range core and for each satellite population area, the locations of the furthest north, south, east and west intersecting toad range edge for each decade were marked along each transect. I then calculated the distances between these toad range edges between consecutive decades. This method allowed me to quantify range edge expansions and contractions along each transect between decades, separately along the northern, southern, eastern and western axes. I then used those estimates to calculate the average invasion rate of toads per year in each compass direction, separately for the core range area and for individual satellite populations. To the best of my knowledge, this method is novel and has not previously been used to measure invasion patterns of a range-shifting species. However, this method is loosely similar to the transect method used by Nuzzo (1999) to measure patterns and rates of invasion of the herb garlic mustard through forests; although my method differs in that I did not utilise quadrats as well as transects.

The method described above generated records of the location of toad range edges for each decade along each transect. I then extracted information on environmental and climatic values from raster layers for sites midway between the toad range edge points from consecutive decades.

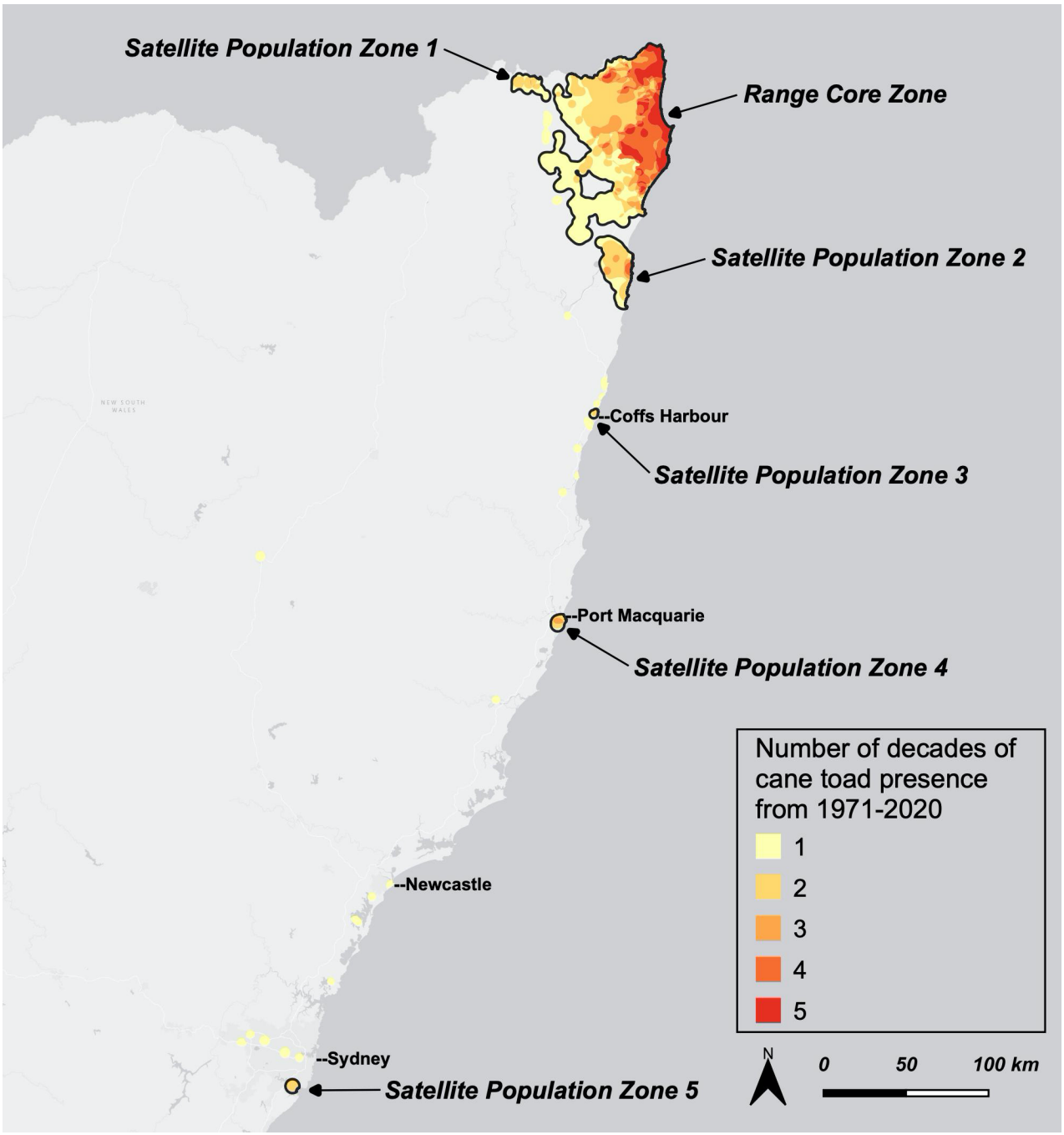

Figure 1. Map of New South Wales showing all areas where cane toad (*Rhinella marina*) range has been estimated to have occurred at some time since 1971, and the number of decades (not necessarily consecutive) from 1971-2020 it is estimated that toads have been present. The 'range core' and 'satellite population' zones are identified, and outline areas only where toad ranges from multiple decades overlap or touch. Estimated cane toad range areas were generated based on processed geospatial coordinate toad-presence records (total N = 5,858) and historical maps of range areas.

Statistical analyses. Data on rates of toad spread across decades were normally distributed, with homogeneous variances, and did not require transformation prior to statistical analysis. To evaluate changes in rates of spread of cane toads through time (decade) and space (satellite versus core populations), I used analysis of variance (ANOVA) with the average yearly invasion rate as the dependent variable. The factors were interdecadal period (as an ordinal variable) and type of population (range core vs satellite [nominal variable]). The ANOVA also included location (satellite populations 1 to 5, and range core, so N = 6 levels) as a random factor, to deal with pseudoreplication due to repeated measurements from adjacent transects.

To clarify rates of toad spread in the two main compass directions in which habitat was available (south and west), I then repeated the above ANOVA design after separating the dataset on rates of spread into these two compass directions. I did not include spread to the east (because this would take toads into the Pacific Ocean) or the north (because this would take toads into already-colonised areas). I examined frequency distributions for rates of spread to compare the degree of variation in spread rates between southern and western range-expansions and range-contractions.

To see whether or not rates of toad spread were affected by elevation (height above sea level) or type of land cover (waterbody/pasture/open forest/closed forest), I included these descriptors as factors in additional ANOVAs, again including location as a random variable to reduce any effects of spatial pseudoreplication.

To examine links between rates of toad spread and climatic conditions through time and space, I entered 14 measures of climatic conditions for each decade (see above) into a Principal Components Analysis (PCA) in order to identify underlying axes of variation, and deal with likely high intercorrelations among climatic variables. Having identified two major axes of variation corresponding to rainfall (PC1) and temperature (PC2; see below), I used these PCA scores as independent variables in linear regressions to compare climatic conditions to rates of spread of cane toads along both southern and western axes of range expansion. I included location (range core and 5 satellite areas) as a random factor to deal with spatial pseudoreplication.

To predict the likely impact of future climate change on rates of range core spread, I used linear regression to extrapolate empirical relationships between climatic variables and past

rates of spread (using only range core data) to generate predicted values under the altered climatic conditions. I used predictions for average maximum temperature and average annual rainfall for the NSW North Coast Region (State of New South Wales and Office of Environment and Heritage 2019) in the near future (2030) and far future (2070) to predict possible future rates of spread of cane toads in NSW. Compared to climate measurements from 1990-2009, maximum temperatures in this region are predicted to increase by 0.4 – 1.0°C in the near future and by 1.5 – 2.4°C in the far future, whereas annual rainfall is predicted to change by -8% to +11% in the near future and -6% to +31% in the far future (State of New South Wales and Office of Environment and Heritage 2019).

Results

Comparison of different methods to detect toads

I detected cane toads at 10 of the 20 sites surveyed, consistent with placement of the transects to include the range edge (i.e., such that toads occurred at some sites but not others). Visual surveys and acoustic surveys revealed the presence of toads at nine sites (including five sites with positive results from both survey types, two sites with positive results from the visual but not acoustic surveys, and two sites with positive results from the acoustic but not visual surveys), whereas eDNA detected toads at six sites (including five sites with positive results from the visual survey, three sites with positive results from the acoustic survey, and one site where neither visual nor acoustic surveys yielded positive records). At four sites where toads were detected and sniffer dog surveys were later conducted, toads were detected at two sites by dogs (Fig. 2).

In summary, rates of detecting toads were similar for visual, acoustic and eDNA methods, and lower for sniffer dogs. Sample sizes are too low to warrant statistical analysis of these data.

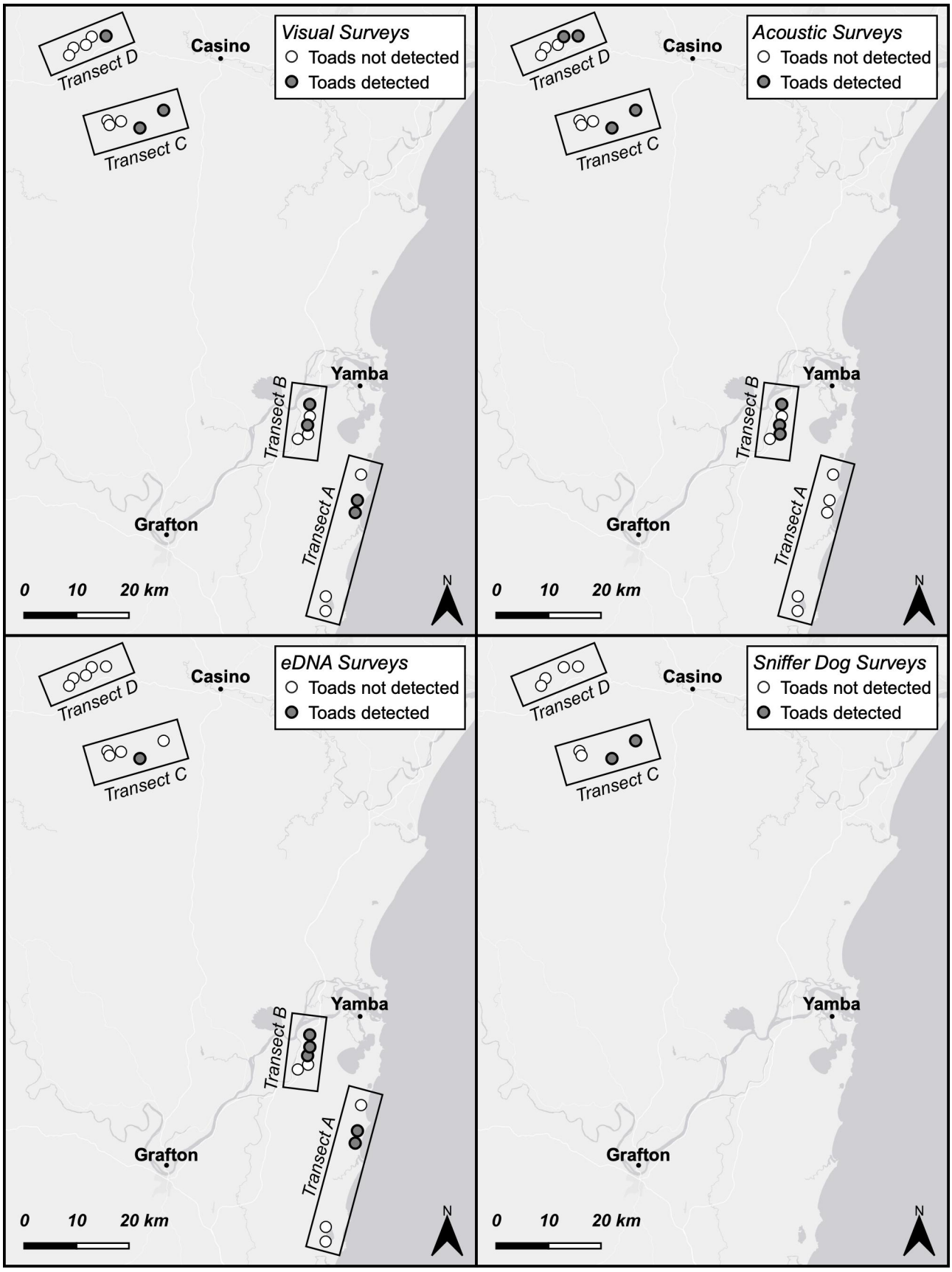

Figure 2. Results of each survey method used to detect cane toads (*Rhinella marina*) at waterbodies along four transects in northern New South Wales. Visual, acoustic and eDNA surveys were performed at twenty sites and sniffer dog surveys were performed at eight sites.

Historical toad range areas

The geographic distribution of cane toads in NSW has been highly dynamic over the period since 1971, which is close to when toads first arrived in this state (less than a decade earlier: Van Beurden and Grigg 1980). Notably, the toad's range has contracted over some interdecadal comparisons and expanded over others (Fig. 3). Areas in the extreme north-east of NSW have been occupied by toads consistently since the 1970s, whereas other patches appear to have expanded, fragmented, disappeared or fused with other patches (Fig. 4). Most of the cane toad's range in NSW remains restricted to the north-eastern part of the state, but with several isolated patches (presumably, founded by accidental translocations) inland as well as further south along the coast (Fig. 4).

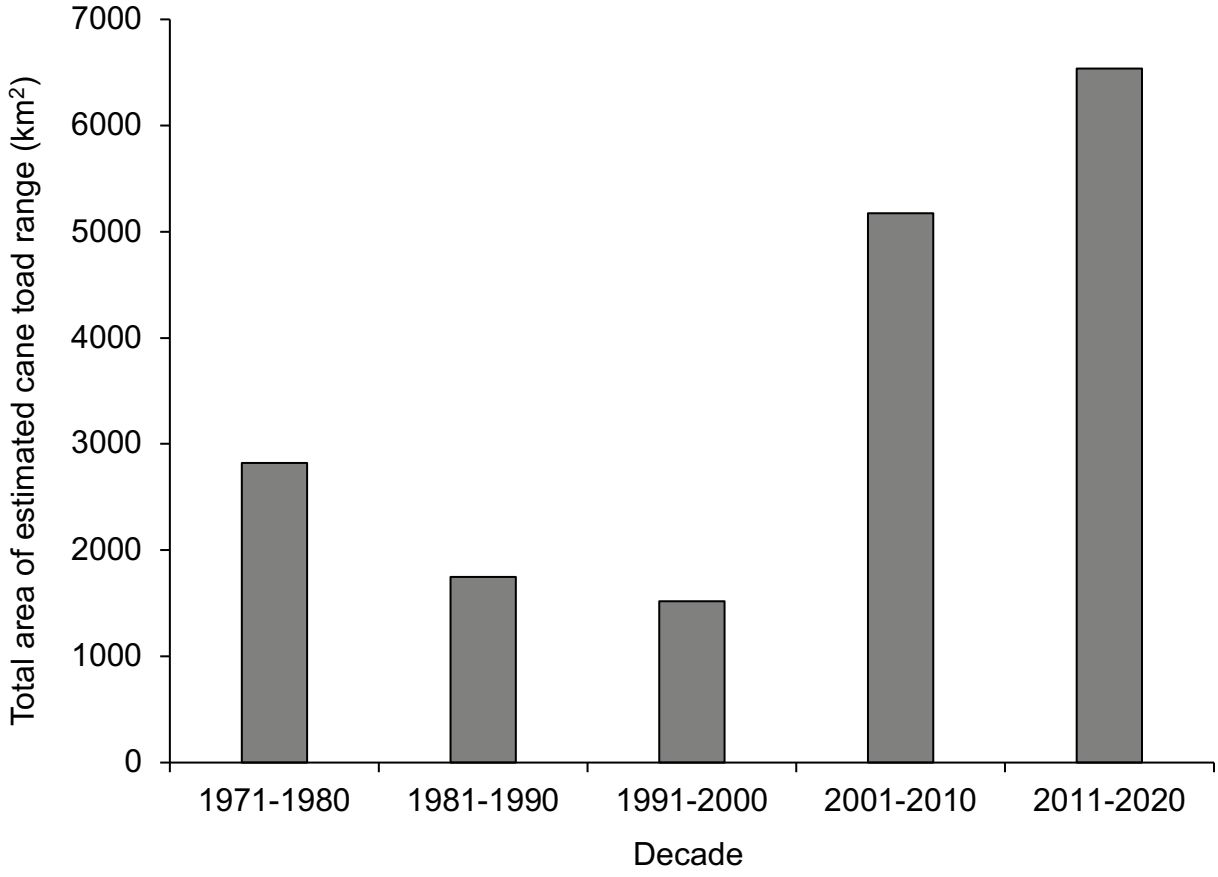

Figure 3. Total area of the estimated range of invasive cane toads (*Rhinella marina*) in New South Wales for each decade from 1971-2020 based on processed geospatial coordinate toad-presence records and historical range area maps.

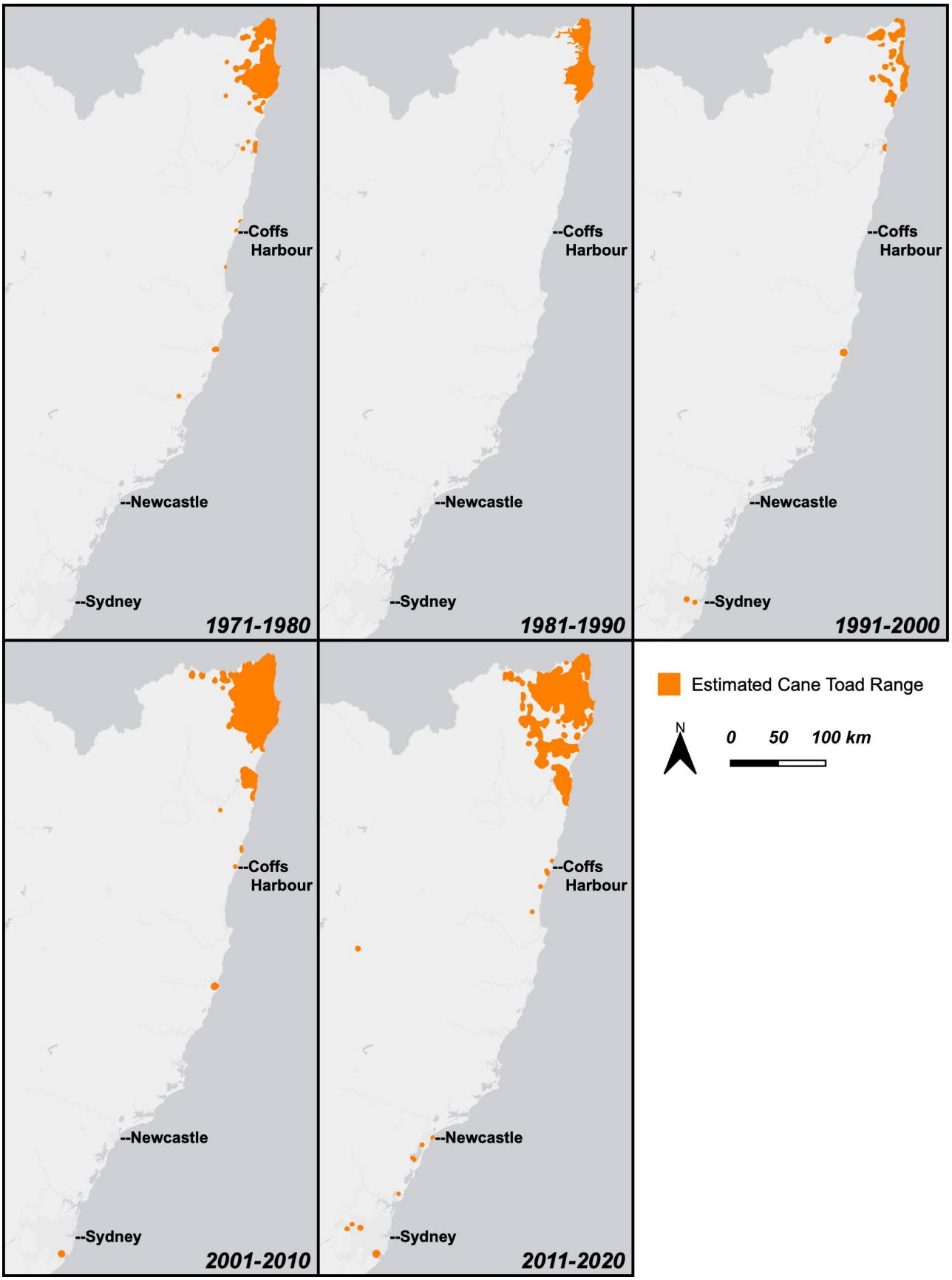

Figure 4. Estimated range of cane toads (*Rhinella marina*) in New South Wales for each decade from 1971-2020 based on processed geospatial coordinate toad-presence records (total N = 5,858) and historical range area maps. Areas with a low density of records are not shown.

Rate of spread of toads

The overall rates of invasion for cane toads in NSW (including all transects for both satellite and range core areas across all interdecadal periods) averaged 0.95 km/yr for the southerly advance, and 0.77 km/yr for the westerly advance. However, there was substantial temporal variation in these rates of spread (+8.7 to -7.9 km/yr for the southern edges of the toad range; +5.3 to -4.3 km/yr for the western edges of the toad range: see Fig. 5). Statistical analysis shows that the overall linear rate of spread of cane toads through NSW (averaging all compass directions) differed between decades for both satellite (ANOVA, $F_{1,57.01} = 6.56$, $p < 0.015$) and range core areas ($F_{3,272} = 20.71$, $p < 0.0001$; Fig. 6). Over the two interdecadal comparisons when data were available for both types of populations (1991-2000 vs 2001-2010 and 2001-2010 vs 2011-2020), rates of spread were higher for the range core than for satellite populations ($F_{1,214} = 5.03$, $p < 0.03$; Fig. 6).

Dividing rates of spread into southern versus western directions, ANOVA shows a significant interaction between interdecadal period and orientation both for the main range ($F_{9,260} = 10.59$, $p < 0.0001$) and satellite populations ($F_{3,55.5} = 4.61$, $p < 0.001$; see Fig. 7). Notably, initial rates of expansion and contraction of the range core were similar along both compass orientations, but the most recent decade has seen expansion continuing more rapidly to the south rather than to the west (Fig. 7). A similar reduction in rates of expansion to the west (but not the south) is evident in satellite populations also (Fig. 7). The mean rates of invasion for the range core of cane toads in NSW for the latest interdecadal period (2001-2010 to 2011-2020) were 2.7 km/yr for the southerly advance and 1.6 km/yr for the westerly advance (Fig. 7).

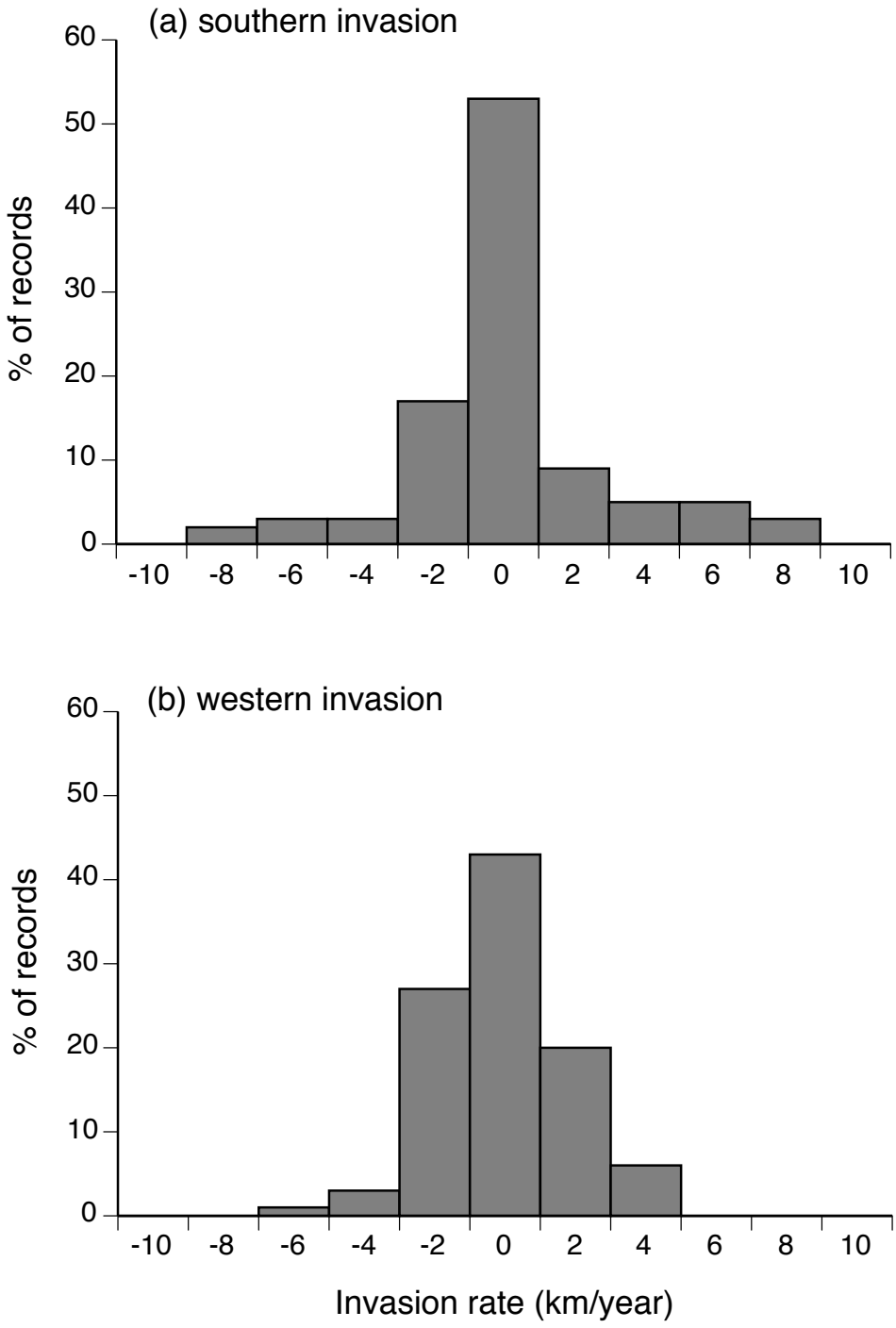

Figure 5. Frequency distributions for annual rates of linear range spread of cane toads (*Rhinella marina*) in New South Wales, comparing rates of spread to the south (a) and the west (b) across all interdecadal periods from 1971-2020.

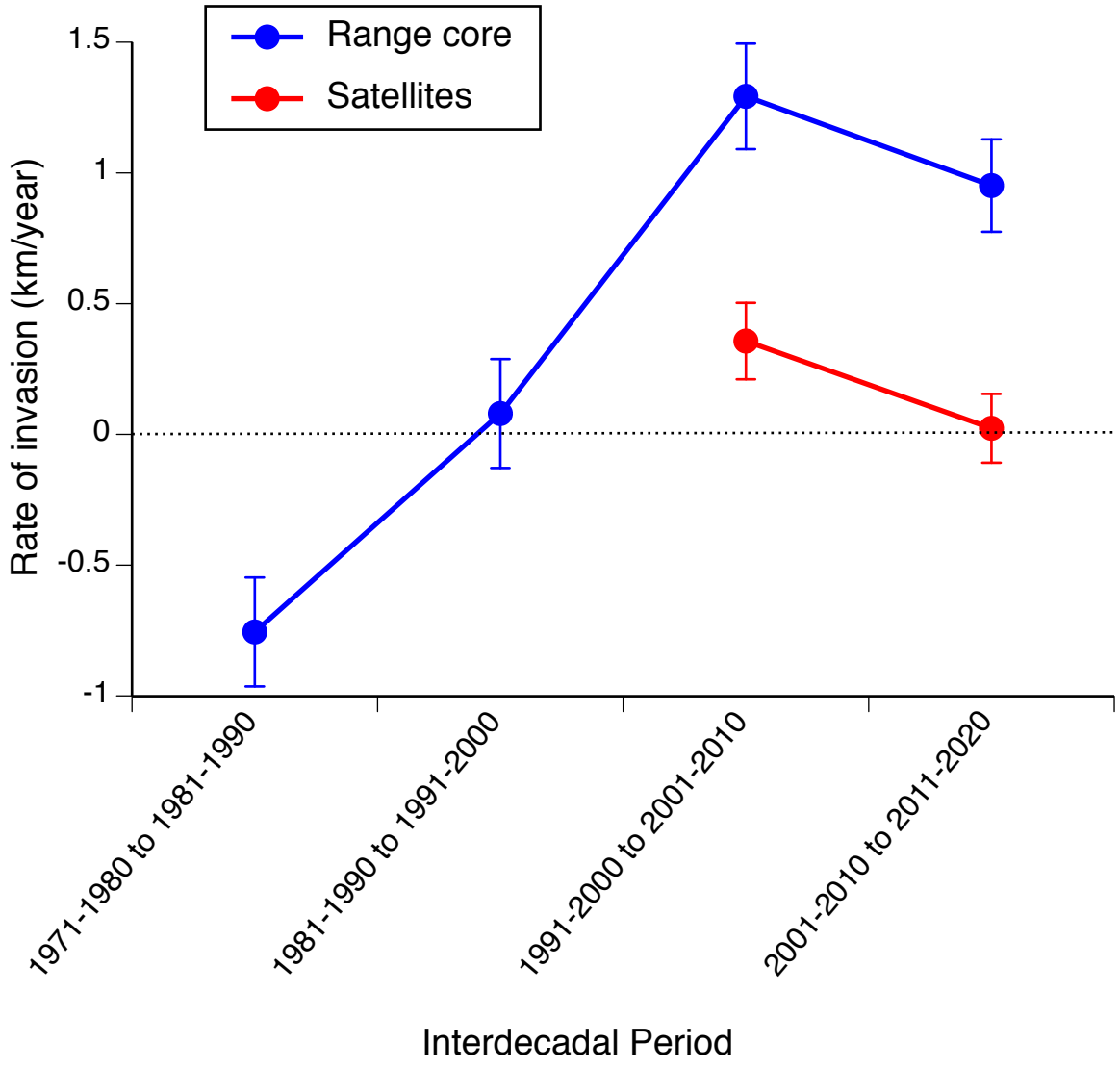

Figure 6. Average annual invasion rates of cane toads (*Rhinella marina*) in New South Wales. The graph shows the range core area and satellite populations during each interdecadal period from 1971-2020. Invasion rates (linear spread rates) were calculated by measuring interdecadal movements of toad range edges to the north, south, east and west.

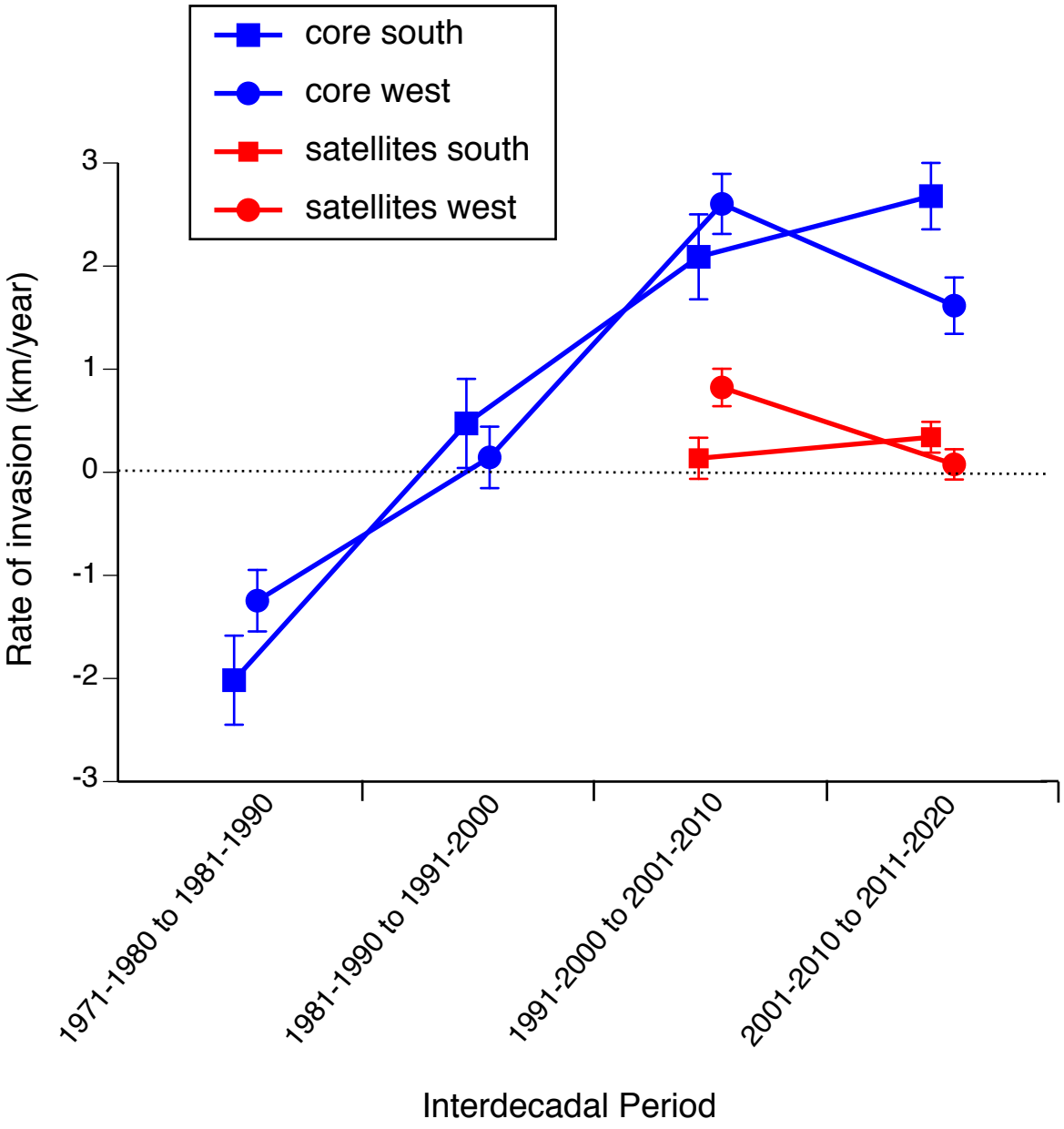

Figure 7. Average annual invasion rates of cane toads (*Rhinella marina*) in New South Wales, separated into southward versus westward components of range expansion. The graph shows southern and western invasion rates for the range core area and satellite populations during each interdecadal period from 1971-2020. Invasion rates were calculated by measuring interdecadal movements of toad range edges.

Correlates of the rate of spread of toads

Elevation and land cover. ANOVAs that included these variables did not reveal any significant impacts of either elevation ($F_{4,329} = 0.85$, $p = 0.49$) or land cover ($F_{1,329} = 1.63$, $p = 0.20$) on the rate that the range of cane toads expanded. Thus, I did not include these variables in subsequent analyses (below).

Climate. Many of the climatic variables were highly intercorrelated (several had $r > 0.90$) and PCA produced two orthogonal (uncorrelated) axes to summarise variation in the original dataset. The first PC axis (explaining 59.6% of variation in the original data) loaded strongly on rainfall measures (e.g., PC1 vs mean annual rainfall within a decade, $r = 0.94$; vs maximum annual rainfall within a decade, $r = 0.95$; vs minimum annual rainfall within a decade, $r = 0.95$), with high values of PC1 indicating unusually wet decades. In contrast, PC2 (explaining 16.6% of total variation in climatic data) loaded heavily on thermal measures (e.g., PC2 vs mean annual temperature within a decade, $r = 0.96$; vs maximum annual temperature within a decade, $r = 0.83$; vs minimum annual temperature within a decade, $r = 0.58$), with high values of PC2 indicating unusually warm decades. As expected, intercorrelation between these two PCA axes was zero, and PC1 scores were not closely related to temperature while PC2 scores were not closely related to precipitation (all $r < 0.30$). Thus, I interpret PC1 as indicative of rainfall levels per decade, and PC2 as indicative of temperatures.

In linear regressions, interdecadal variation in rates of spread was significantly associated with both rainfall and temperature PC axes (Fig. 8). Along both compass orientations, the toad front expanded further in years with low values for PC1 (i.e., in drier years) and high values for PC2 (i.e., in hotter years). In each case, these relationships were statistically significant (location included as random factor; PC1 vs southern spread $F_{1,61.74} = 29.52$, $p < 0.0001$; PC1 vs western spread $F_{1,102.1} = 24.37$, $p < 0.0001$; PC2 vs southern spread $F_{1,86.1} = 6.05$, $p < 0.04$; PC2 vs western spread $F_{1,86.72} = 4.61$, $p < 0.04$).

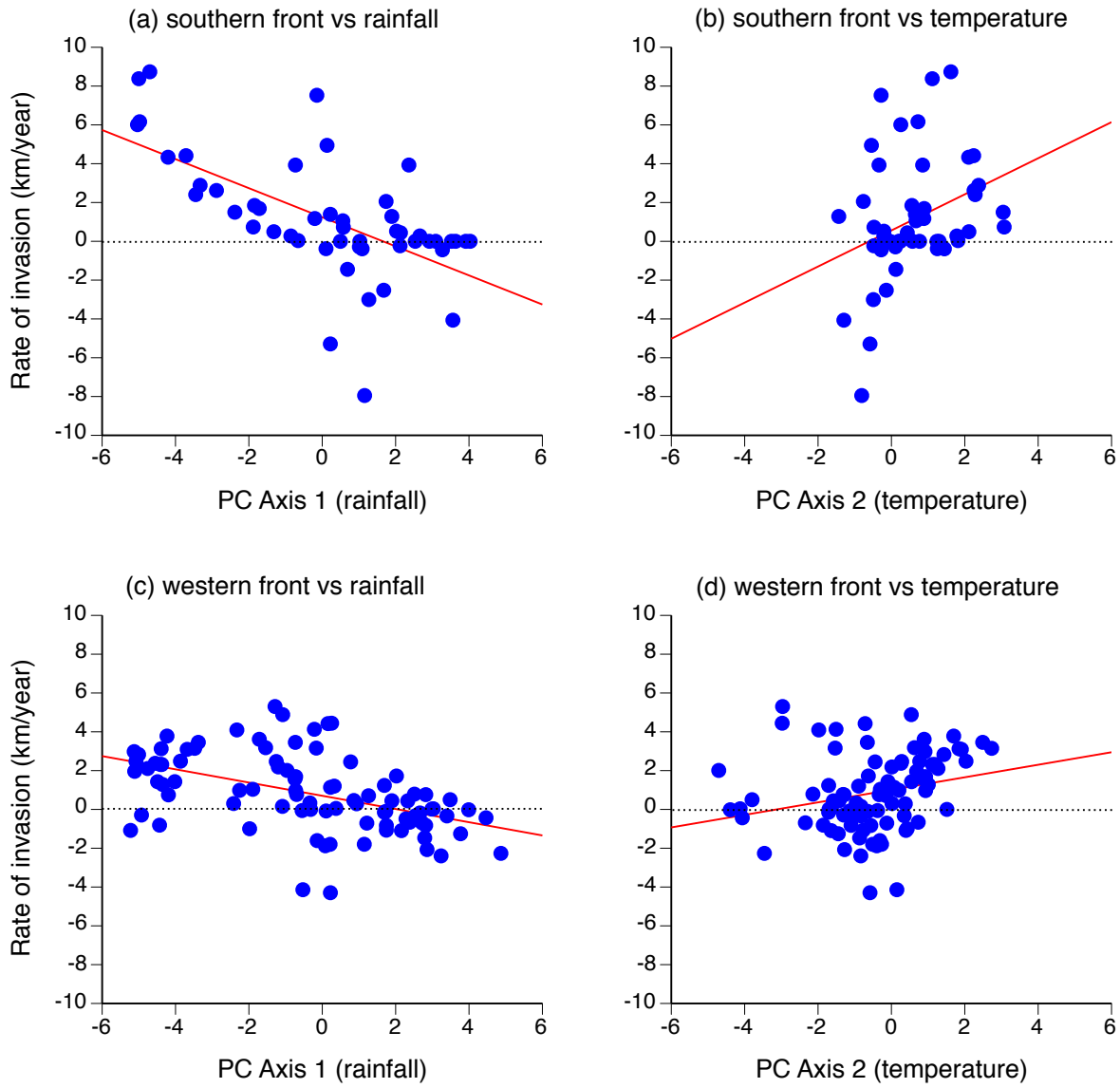

Figure 8. Relationships between linear rates of spread of cane toads in New South Wales and climatic conditions per decade. The left-hand panels compare rates of spread to the south (a) and west (c) to a rainfall-related axis from Principal Components Analysis, whereas the right-hand graphs compare rates of spread to the south (b) and the west (d) to a temperature-related axis. For PC Axis 1, high values represent wetter decades. For PC Axis 2, higher values show hotter decades.

Predictions for future rates of spread of toads

Empirical relationships between climatic variables and past rates of spread (Fig. 8) suggest that every increase of 1°C in annual maximum temperature increases rate of spread of the range core by approximately 1.6 km/yr to the south, and 0.4 km/yr to the west. Using those estimates, under future climate projections we would predict rates of southerly spread for the range core to increase and reach around 3.3 to 4.3 km/yr in the near future (2030) and 5 to 6.5 km/yr in the far future (2070). Following a similar pattern, westerly rates of spread for the core range would be predicted to reach around 1.8 to 2 km/yr in the near future (2030) and 2.2 to 2.5 km/yr in the far future (2070). Based on rainfall patterns, for every 100 mm increase in average annual rainfall the rate of spread of the range core would decrease by around 0.9 km/yr to the south, and 0.4 km/yr to the west. Based on those approximations, we would predict rates of southerly spread for the range core to increase or decrease under future climate predictions, reaching around 1.3 to 3.7 km/yr in the near future (2030) and -1.3 to 3.4 km/yr in the far future (2070). Westerly rates of spread for the core range would also be predicted to either increase or decrease, reaching around 1.1 to 2 km/yr in the near future (2030) and 0 to 1.9 km/yr in the far future (2070).

Discussion

Results from my pilot study (which I was unable to follow up due to COVID-19 lockdown) suggested that visual and acoustic surveys were about as sensitive at detecting cane toads as were eDNA surveys. A combination of all three methods may produce the most reliable results where detection is critical. Sniffer dogs appear to be less effective at detecting toads, but further studies are needed to increase the sample sizes (and thus, statistical power) of these comparisons. My conclusion that eDNA is no more sensitive than visual and acoustic surveys stands in contrast to several previous freshwater studies that have reported higher sensitivity for the eDNA approach than traditional methods (Jerde et al. 2011; Dejean et al. 2012; Biggs et al. 2015; Smart et al. 2015; Wilcox et al. 2016). However, most of that work has focused on secretive, small or cryptic creatures (such as newts) that are difficult to detect using conventional surveys. In contrast, cane toads are relatively obvious in their environment: adults are large and typically select open habitats for foraging and calling, males produce a loud call, and metamorphs and tadpoles

are usually abundant when present and easily seen in and around waterbody margins (e.g., Hagman and Shine 2006; Child et al. 2008; Greenlees et al. 2018). As a result, the potentially higher sensitivity of eDNA-based surveys for this species may not be enough to offset the higher expenditure of time and money needed to implement this method. Regardless, the clear conclusion from my (small) sample of standardised surveys is that visual and acoustic surveys should be adequate to document the presence of cane toads. If eDNA had proven to be far more sensitive, my mapping of toad distributions (based largely on visual and acoustic data) might have substantially underestimated the area colonised by this species. Hence, it is reassuring to know that simple methodologies such as visual and acoustic surveys likely are adequate for detecting the presence of cane toads.

My estimates of the rate of linear spread of cane toads in NSW are broadly similar to those reported by Seabrook (1991) but span a wider range of dates and include substantially more detail. Based on her maps of toad distribution over the period 1978 to 1990, Seabrook (1991) concluded that toad ranges were expanding fastest in a southerly direction at about 1.3 km per year. That approximation is close to the overall mean value that I have calculated in a southerly direction (0.8 km per year from 1971-2020), but my analysis shows strong temporal and spatial variation in rates of expansion – and also reveals a marked contraction in overall range in one interdecadal comparison (between the 1970s and 1980s; Fig. 3). However, I note that caution is necessary when interpreting these estimated cane toad range areas. Specifically, the search radius I used to estimate kernel density was fixed, when in actuality such dispersal kernels may be flexible and change over time (evolve) or vary with environmental conditions (Urban et al. 2008). Inaccuracies in the search radius chosen thus may generate errors in estimated cane toad range maps.

Most of the satellite populations were well-separated from the main range (Fig. 1), suggesting that these small populations were founded by stowaway dispersal rather than main-range expansion. Although small sample sizes reduce confidence in population trends within satellite population zones, it appears that some of these isolated groups were extirpated (perhaps by climatic conditions, or an insufficiently low number of founders) whereas others persisted and, in some cases, expanded. Cane toads appear to have been eradicated from at least one site in the Sydney region after years of focussed control

efforts (Greenlees et al. 2018), but it remains unclear whether such efforts have also eradicated other recent satellite populations in NSW.

While it is unclear whether all of the satellite population zones identified (Fig. 1) represent self-sustaining populations of toads or simply 'hotspots' for incursions, these zones may at least warrant monitoring for toad activity in the future. If populations of toads have become established in these areas, hand-collecting ("toad-busting") techniques may offer a viable way of eradicating such populations (Greenlees et al. 2020); however, eradication may only be feasible while populations remain small and isolated (Greenlees et al. 2018). Nonetheless, evidence suggests that "toad-busting" techniques may still be effective in reducing the spread of toads along the expanding invasion front of the NSW range core (Greenlees et al. 2020). Combining the most recent estimated range core zone and the two northern-most satellite population zones from my maps (Fig. 1) would produce a map that closely corresponds to the NSW cane toad biosecurity zone recognised by the NSW Department of Primary Industries (NSW Department of Primary Industries 2020). Based on the current location of this zone and the continued advancement of the southern and western invasion fronts in NSW, priority areas for monitoring and control can be identified. If national parks are chosen as priority areas for the conservation of biodiversity, those apparently facing the most immediate risk of toad invasion (or currently under invasion) are Toonumbar National Park, Richmond Range National Park, Mallanganee National Park, Everlasting Swamp National Park and Yuraygir National Park. Identifying such areas will be crucial for producing targeted plans to control the spread of toads within NSW.

Low and variable annual rates of change in the area colonised by invasive cane toads in NSW, and the spatially heterogenous nature of toad range expansion, provide a strong contrast to reports from tropical Australia. Near the current north-western edge of the toad invasion, in the Kimberley region of Western Australia, the toad front has moved fairly uniformly across the landscape (rather than in a spatially heterogeneous fashion) and has progressed at approximately 50 km per year (Shine et al. 2018). More generally, an historical analysis of rates of toad spread through Australia calculated annual spread rates of around 10 to 15 km per year in the years after toads were first released in north-eastern Queensland, accelerating to > 50 km per year as the toads travelled westwards through western Queensland and the Northern Territory (Urban et al. 2008). My data suggest a consistent but modest acceleration in the southward spread of cane toads in NSW, but a

decreasing rate of spread within the past decade as the toads have dispersed westwards into higher elevations in the Great Dividing Range (see Fig. 7).

The relatively slow and patchy spread of cane toads into north-eastern NSW might reflect several factors (or a combination of those factors). Among the possible influences on rates of dispersal are:

(1) Ambient temperature. The climate in this region is cooler than in the cane toad's native range, and in most other areas colonised by invasive populations of the species (Tingley et al. 2014; Kosmala et al. 2017; McCann et al. 2018). Low temperatures constrain locomotor activity in anurans, including cane toads (Kearney et al. 2008; McCann et al. 2014). Comparisons among invasive cane toads from a range of climates have revealed a greater tolerance for activity at low temperatures in individuals from cool-climate populations in NSW and Hawai'i (McCann et al. 2018). Those physiological shifts suggest that toads cannot exploit relatively cool areas without adapting (e.g., via phenotypic plasticity or shifts in gene frequencies) to the challenges of low-temperature locomotion (McCann et al. 2018). Plausibly, these thermal challenges may have slowed down the rate at which cane toads have been able to penetrate further into NSW. Consistent with that constraint, my analysis showed that toads have expanded their range most rapidly in periods of warmer weather (Fig. 8). Arguing against this hypothesis, however, radio-tracking has shown that toads in this region are capable of dispersing long distances about as rapidly as are tropical toads (Phillips et al. 2007; Pettit et al. 2017); and a satellite population successfully established within the suburbs of Sydney, 500 km south of the current main range (Greenlees et al. 2018).

(2) Unsuitable habitat. Because toads move more slowly through dense vegetation than through more open areas (Brown et al. 2006), some of the vegetation assemblages in NSW may somehow impede toad dispersal (Seabrook 1991). However, my analysis detected no significant impact of land cover on rates of population expansion. Massive recent wildfires in this region have altered vegetation communities dramatically (Nolan et al. 2020), such that further study could usefully examine rates of toad spread into the open areas created by those fires.

(3) Parasite load. Research in the tropics has shown that parasitic lungworms (*Rhabdias pseudosphaerocephala*) can curtail rates of toad dispersal (Finnerty et

al. 2018; but see Brown et al. 2016). Such parasites appear to occur more commonly in cane toads at the slowly spreading front in north-eastern NSW than at the invasion front in tropical Australia (Lettoof et al. 2013).

(4) Management. North-eastern NSW has been a focus of intensive efforts in toad control via "toad-busting", a management technique that appears to be far more effective at reducing toad abundances in this area than in the tropics (Greenlees et al. 2020).

(5) "Genetic backburn". Radio-tracking and morphological analyses of toads from different regions in Australia suggest that the evolution of a highly dispersive phenotype has been the key to the toads' accelerating rate of spread across tropical Australia (Phillips et al. 2006; Alford et al. 2009; Hudson et al. 2016). Hence, introducing toads from long-colonised areas (where individuals are sedentary) to the invasion front (where individuals are dispersive) has been suggested as a way to break apart the dispersive genotype through interbreeding (Phillips et al. 2016). High rates of accidental translocation of cane toads along the coast of Queensland and NSW (because of high rates of vehicular traffic) may frequently achieve exactly this aim, bringing toads from long-colonised areas to the invasion front. If so, that admixture may have prevented the evolution of a highly dispersive phenotype at the southern invasion front. However, that result depends upon the numbers and timing of translocation events (Kelly and Phillips 2019); if rates of translocation of cane toads along the coast are low, then the number of toads being introduced to the front may not achieve a significant "genetic backburn" effect.

My data do not enable me to test these alternatives. The strongest patterns observed from my datasets are that rates of spread overall are slow and variable, but tend to be faster in warm, dry years. As noted above, the impact of warmer years may relate to relaxing the impediments on locomotion enforced by cool conditions. Also, hotter years may increase the duration of breeding activity and expedite larval development (Kearney et al. 2008; Wijethunga et al. 2016). At first sight, faster spread during drier rather than wetter years is counterintuitive, as rainfall facilitates toad dispersal in tropical Australia (Brown et al. 2011). However, drier years may induce toads to move further to locate water sources; or cessation of flow in streams may create isolated ponds that are ideal breeding sites for toads (these animals rarely breed in flowing water: Hagman and Shine 2006; Semeniuk et al. 2007).

Lastly, we can use the empirical relationships between climatic conditions and rates of toad spread to predict what may happen under a changing climate. Such an attempt is weakened by the possibility that these links (e.g., Fig. 8) are not causal; and wide scatter of points around the regression line mean that any extrapolation has to be done with caution. Correlative-based projections of species distributions under future climate scenarios can perform poorly for range-shifting species for many reasons; most notably because predictions assume that a species is at equilibrium with the environmental factors influencing its distribution (Dormann 2007; Elith et al. 2010). Nonetheless, the exercise presented here may give some indication of what we might expect to happen if toads continue to exhibit the same sensitivity of spread rate to climatic factors. Broadly, my calculations suggest that the predicted shift (to the year 2070) in average maximum temperatures (1.5 – 2.4°C increase) and average annual rainfall (6% decrease to 31% increase) will result in rates of spread to the south for range core populations to range from -1.3 to 6.5 km/yr. Westerly rates of spread are predicted to range from 0 to 2.5 km/yr. In comparison, the current average rate of spread for the range core is 2.7 km/yr for the southerly advance and 1.6 km/yr for the westerly advance. In practical terms the difference between current and predicted future rates of spread ranges from minor to substantial, but at the maximum future rate of invasion of 6.5 km per year the main core range of cane toads would likely reach the Sydney region (approximately 500 km away) within about 76 years, or close to the turn of the 22nd century (as opposed to 185 years at present rates of dispersal). In practice, long-distance accidental translocations may represent a greater threat to such areas, as exemplified by frequent media reports of toads being found far south of their current range (e.g., Sanda 2019; Messenger 2020; South Coast Register 2020). Awareness of toads by the general public will continue to be important in detecting these sporadic, long-distance translocations of toads.

In summary, my data emphasise that invasions can be highly variable both in space and time. The intensively studied invasion of cane toads in tropical Australia, with vast numbers of animals travelling in a continuous wave across a range of habitat types, bears little similarity to the slow and patchy southwards spread of the same species. Few biological invasions have been studied in enough detail, with replication across multiple invasion fronts, for us to obtain a true picture of the extent and pathways by which local conditions modify invasion dynamics. At least in the case of the cane toad in Australia, it seems that temporal and spatial variation in rates of expansion are so profound as to warrant further close examination.

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SUPPLEMENTARY MATERIAL

Materials and Methods

Comparison of different methods to detect toads

Establishing sampling sites. Within their established range, cane toads (*Rhinella marina*) frequently use farm dams as sites for rehydration and breeding (Semeniuk et al. 2007). Farm dams were chosen as sampling sites for toad-presence surveys for this study as they: (1) are also expected to be utilised by toads at the edge of their expanding range in New South Wales (NSW); (2) represent a relatively uniform sampling site type that allows for consistent toad-presence survey methods to be used on them; (3) can be easily pinpointed on satellite maps for survey planning; and (4) are often easily accessible after gaining permission to access properties. Sites were chosen after first identifying the regions in NSW where the toad invasion front was likely to exist. This was achieved by consulting with local toad control groups and experts who had recorded sightings of toads in north-eastern NSW and were able to provide information on their best approximation of where the toad invasion front was located. Having identified the areas where toads were likely to be present and absent, four draft transects for sampling were outlined in Google Earth Pro on a satellite map spanning the southern-most regions of the suspected range core of the toad invasion in NSW. Each transect extended from sites already occupied by toads to sites which were suspected to be toad-free.

Potential dam sampling sites within the transects were marked on a satellite map using Google Earth Pro. Additional sites were found by talking to Landcare NSW staff who advised of properties where toads had been found previously and provided contact details for these properties. Where possible, property owners were contacted by phone to ask for permission to access their dam as a sampling site; however, many sites chosen for sampling required doorknocking at properties and asking for permission to access dams. All property-owners who we interacted with were happy to let us perform surveys on their dams. Where property-owners were unable to be contacted to gain access to their dam, alternative sampling sites nearby had to be located. In areas where dams were not present or access to a dam was not possible, water-filled roadside culverts were used as substitute sites for surveys. Such sites were only chosen when they were judged to be able to hold water for a sufficient period of time and were unlikely to accrue runoff from

long distances which could impact eDNA survey results. Due to the natural heterogeneity of the areas surveyed and the presence of some inaccessible areas along proposed transects, spacing between neighbouring sites chosen along the same transect varied considerably (0.9 km to 16.9 km).

Precautions for visiting sites. Prior to and after conducting surveys at each site, gumboots of all survey participants were sprayed with a 10% bleach solution as a precautionary measure to reduce the likelihood of eDNA transmission between sites. Participants avoided making any contact with the waterbody prior to performing eDNA sampling procedures.

Acoustic surveys. If cane toads were heard during an acoustic survey, the remainder of the survey for that site was terminated and the presence of toads was recorded.

Visual surveys. All visual surveys were conducted by walking within approximately 2 metres of the waters' edge, except where dense vegetation prevented access.

eDNA sampling. All participants who performed eDNA sampling procedures wore nitrile gloves. Samples were collected following a strict protocol to prevent the improper handling of samples and equipment that could lead to eDNA contamination.

When performed at night, eDNA surveys were performed after both the acoustic and visual surveys had been completed. Water samples were collected from each site by walking around the perimeter of the waterbody and collecting scoops of water in a Whirl-Pak bag (Nasco, United States) from approximately equidistant points around the waterbody edge. A total of 16 scoops of water were collected at each site using a sterile 532 mL (18 oz) Whirl-Pak bag with scoop, causing the bag to be nearly entirely filled. The collected sample was then filtered through two individual 0.45 μm Sterivex-HV Durapore PVDF (Merck, Germany) filter units. Up to 200 mL of water was forced through each of the filters by hand with a sterile 50 mL luer-lock syringe. Water samples were filtered onsite unless adverse weather conditions (e.g., rain) made this impractical, in which case the Whirl-Pak bag containing the sample was placed in two zip-lock bags, stored in a portable cooler with ice and then filtered in an appropriate location less than 12 hours later. Waterborne sediments appeared to cause filters to clog prior to having 200 mL water pass through them, in which case the remaining unfiltered liquid was discarded. Air was pumped

through the Sterivex filters to expel residual water before approximately 2.3 mL of Buffer ATL (QIAGEN) was pipetted into each Sterivex unit. Sterivex units were then sealed with parafilm at each end and individually double-bagged in plastic zip-lock bags. Bagged samples were placed on ice in a portable cooler, before being placed into a freezer upon arriving back from the field. All samples were transported in a portable cooler on ice to a DNA processing lab where they were stored in a freezer at -20°C.

Two negative control samples were also gathered by using the aforementioned procedure to filter tap water collected in a Whirl-Pak bag, rather than dam water, through two separate Sterivex filter units. A total of 200 mL was filtered for each of these negative controls.

eDNA extraction and processing. DNA was extracted from the Sterivex filters using a QIAGEN DNeasy Blood and Tissue Kit (using the spin column protocol) using a method adapted from Tingley et al. (2019). 20 μL proteinase K was added to each Sterivex filter unit. Filter units were then sealed with parafilm and incubated overnight in a hybridisation oven (XTRON HI 2001, Bartlett Instruments) at 56°C while under constant rotation. The lysis solution was extracted from each filter unit using sterile 5 mL luer-lock syringes and transferred to 2 mL Eppendorf tubes. The remainder of each extraction was performed by following the manufacturer's instructions with the following adjustments: 500 μL Buffer AL, 500 μL ethanol, and final elution using 50 μL Buffer AE for each sample. Some samples contained small amounts of sediment which caused minor clogging of DNeasy Mini spin columns, in which case they were centrifuged for additional periods of time until all of the sample passed through the spin column. The resultant sample template DNA was stored in 2 mL Eppendorf tubes at -20°C. As a result of improper handling, the contents of one of the Sterivex filter units from one site was split into two samples. The first sample was obtained by: (1) Pushing air through the filter to force the liquid contents into Eppendorf tubes; (2) adding 20 μL proteinase K to the tubes and incubating overnight as per the aforementioned procedure; and (3) performing the remainder of the extraction in the same manner as for all other samples. The second sample was obtained by adding approximately 2.3 mL of Buffer ATL to the drained Sterivex filter unit, adding 20 μL proteinase K and performing the remainder of the extraction in the same manner as for all other samples. All extractions were performed in a room dedicated to DNA processing, with all equipment and surfaces cleaned with a 10% bleach solution, and gloves changed between all steps and samples.

ddPCR processing and analysis. Droplet Digital PCR (ddPCR) was performed on all 43 samples extracted, as well as five other no-template control samples; two composed of 2 mL Buffer ATL and 20 μL proteinase K, and three composed of ultrapure water. Trials were performed to refine the ddPCR methods used for these samples and temperature gradient PCR was used to identify the optimal annealing temperature (53.3°C). The primers and probe used were developed by Tingley et al. (2019) and were ordered from Integrated DNA Technologies. Prior to setting up samples for droplet generation, primers, probe and Bio-Rad ddPCR Supermix for Probes (No dUTP) were all diluted according to manufacturer's instructions. A PCR Master Mix was generated by mixing the supermix, forward primer, reverse primer, probe and ultrapure water in a ratio of 100 : 18 : 18 : 5 : 11. This equated to the following quantities per reaction: 12.5 μL supermix, 2.25 μL of each primer, 0.625 μL probe and 1.375 μL ultrapure water. For each sample, a 25 μL reaction for droplet generation was prepared containing 19 μL Master Mix and 6 μL of sample DNA template solution. 20 μL of each 25 μL preparation was pipetted into droplet generator cartridges for ddPCR droplet generation and the remainder of the solution was disposed. Droplet generation was performed using a Bio-Rad QX200 Droplet Generator. Droplets were transferred to wells in a 96-well plate that was then sealed with foil and placed in a Bio-Rad T100 Thermal Cycler for amplification. The amplification occurred in conditions of 10 min at 95°C, followed by 30 s at 94°C and 1 min at 53.3°C for 40 cycles, followed by 10 min at 98°C, followed by a hold temperature of 4°C. Droplets were read with a Bio-Rad QX200 Droplet Reader within 24 hours of amplification and readings processed in QuantaSoft version 1.2.10.0 (Bio-Rad, CA, USA).

Analysis of ddPCR droplet reader results was performed in QuantaSoft Analysis Pro version 1.0 (Bio-Rad, CA, USA) and wells containing samples from the same sampling site were merged for analysis using this software. If a sample produced less than 6000 droplets in a well, it was discarded from further analysis and was not merged with another well containing sample from the same site. A standard threshold was set for all merged wells, with the lowest possible threshold value being selected such that the droplets from all negative controls and no-template controls tested negative for cane toad eDNA. Under this threshold, site samples were only classified as positive for cane toad eDNA when at least three droplets in a merged set of wells produced fluorescence readings higher than the specified threshold. Samples that generated less than 6000 droplets were re-tested once and, if droplet count was sufficient, were classified as positive or negative using the

same criteria. Threshold values for classifying samples as positive or negative for cane toad DNA were determined after consulting with ddPCR specialists at Bio-Rad.

Because toad DNA remained undetected in certain samples taken from sites where toads were visibly present during field surveys, PCR inhibitors were suspected to be present in some of the samples. To remove potential PCR inhibitors, all 43 field samples were cleaned using a Zymo Research OneStep PCR Inhibitor Removal Kit. Droplet Digital PCR and analysis was then performed once more on all cleaned samples using the aforementioned procedures.

MACQUARIE University

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2019/040 **Date of Expiry:** 20 February 2021

Full Approval Duration: *20 February 2020 to 30 November 2022*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

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In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Animal Welfare Officer: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **The ecology of cane toads on the southern front**

Purpose: 7 - Research: environmental study

Aims: To understand the ecology of cane toads in the south of their range and the factors that both enhance and hinder their continuing invasion to inform the management of the spread and impact of toads in New South Wales.

Surgical Procedures category: 3- minor conscious intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex/Weight/Age	Total	Supplier/Source
24 - Amphibians	Cane Toad - *Rhinella marina*	Male/85g/Any	480	Wild
		Female/85g/Any	480	
		Any/Any/Any	20,000	
		TOTAL	**20,960**	

Location of research:

Location	*Full street address*
In-Situ	The vicinity of Sandon road and Bosche's waterhole road, Brooms Head; Angourie Blue Pool, Angourie; Bundjalung National Park, the vicinity of Big Marsh firetrail, Woombah; Whiporie StateFforest, the vicinity of Bungawalbin-Whiporie Road, Whiporie; Bungawalbin National Park, the vicinity of Neilley's Lagoon Road, Bora Ridge; Camira State Forest, the vicinity of Old Tenterfield Road, Whiporie; Knox Park, Brisbane street Murwillumbah; The vicinity of Cudgera Creek, Pottsville; Summerland Way, Wiangaree, Jabiru Wetlands, Queensland Road, Casino, NSW

Amendments approved by the AEC since initial approval: N/A

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

A/Professor Nathan Hart (Chair, Animal Ethics Committee) **Approval Date:** 20 February 2020