Multidimensional complexity of Communication Signals of a Model Anuran, *Litoria fallax* (Pelodrayadidae), amid Genetic, Ecological and Social Constraints

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A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Ethical Note

All fieldwork and experiments were conducted with approval from La Trobe University Animal Ethics Committee (AEC17-47 and AEC19-027). Work was conducted under permits provided by the Victorian Government (Department of Environment, Land, Water and Planning Permit Nos. 10008539, 10008787), the New South Wales Government (Office of Environment & Heritage permit No. SL101998) and the Queensland Government (Department of Environment & Science Permit No. WA0009378).

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General Abstract

Complex communication systems are widespread among animals. To fully understand their functional and evolutionary significance, we must examine each dimension of communication together with possible genetic, environmental and ecological constraints. With the extensive use of acoustic signals, anurans are considered as excellent model systems for studying the evolution of such communication systems However, there is a lack of detailed knowledge on the multi-level complexity of the communication systems of anurans compared to other taxa. Litoria fallax, or the eastern dwarf tree frog, is a native Australian pelodryadid with a broad distribution range along the east coast. I provide a detailed quantitative description of the acoustic repertoire of this species, describing multiple levels of complexity in their acoustic repertoire, comprised of three note types arranged into monophasic and diphasic calls with a graded arrangement. Using female-choice experiments on the most common diphasic call type, I identified that female frogs prefer the shorter note type over the longer. Call types in a call sequences of this species are generated in a unique pattern and I used information theory to consider complexity in relation with the conspecific acoustic environment. I then compared the advertisement notes across known genetic clades of the species along with a variety of other environmental and ecological factors. The results suggest that geographic variation observed among the populations is both shaped by phylogenetic and local selection pressures. Choice experiments with female frogs demonstrated that they are sensitive to the phenotypic plasticity of the population variability as they chose local acoustic cues over foreign ones. In my final empirical chapter, I examine multimodal signalling of the species to report on the use of visual signals during male-male agonistic interactions. Playback experiments reproducing the

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multimodal structure of signals suggested both male and female frogs were more likely to choose the multimodal signal over acoustic only stimuli. Taken together, my thesis provides novel data on the complex communication system of an Australian anuran. This is one of the very few studies that used Information theory on the communication system of an amphibian species indicating the importance of implementing such methodologies in disentangling the multiple factors effecting complex communication systems.

Author Contributions

This thesis is presented as a series of papers to be submitted to peer-reviewed journals. The work described in the empirical chapters benefited from the input of collaborators that will be named on the respective journal submissions. In each case, the candidate is the lead author.

Chapter 2: Complex acoustic repertoire of the eastern dwarf tree frog, Litoria fallax

Study designed by Bhagya Herath with contributions from David de Angelis and Richard Peters. Fieldwork assistance was provided by Jose Ramos, while Jonathan Salisbury provided assistance with the preparation of materials for the captive experiment.

Chapter 3: Geographic variation in advertisement note types of Litoria fallax

Study designed by Bhagya Herath with contributions from David de Angelis and Richard Peters. Fieldwork assistance was provided by David de Angelis, Richard Peters, Jose Ramos and Jonathan Salisbury.

Chapter 4: Study of multimodal signalling by Litoria fallax

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Chapter 1

General Introduction

1.1 Animal communication

Animal communication systems have always been in the spotlight among the scientific community because they provide insight into many critical fitness relevant behaviours. Composed of three components, the signaller, the signal, and the receiver, communication systems show diversity across animal species that is shaped by the selective forces acting upon them. A variety of sensory modalities have been used by animals for intra- and/or interspecific communication (Patricelli & Hebets, 2016). For example, visual signalling via brightly coloured dewlaps of the lizard *Anolis cristatellus* (Leal & Fleishman, 2004), songs and calls produced by birds (Kumar, 2003), chemical communication using pheromones by female silkworm moths, *Bombyx mori* (Sandler et al., 2000), and the use of tactile modalities by brush-legged wolf spider, *Schizocosa ocreata* (Gibson & Uetz, 2008). These signals function in diverse contexts from mate choice (Wells & Schwartz, 2007) and agonistic interactions (Bruski & Dunham, 1987) to antipredator behaviour (Rundus et al., 2007) and foraging (Schneider et al., 1986).

The study of animal communication necessarily requires consideration of environmental constraints acting upon the process and is now recognised as important for understanding adaptation and plasticity in animal signals that we observe today (Ord et al., 2010). Indeed, animal communication systems are under constant pressure from the social environment (Toledo et al., 2015), ever changing abiotic environment (Brenowitz, 1986; Vargas-Salinas et al., 2014), unintended eavesdroppers like predators or parasites (McGregor et al., 2000) and the sensory systems of receivers that must detect and process the signals (Wiley, 2006). A successful communication system would have strategies to overcome these challenging environments via adaptation to each important element. For instance, modifying signal structure (Halfwerk & Slabbekoorn, 2009), the use of different signal types (Dunlop et al., 2010), and adjusting the timing of signalling (Fuller et al., 2007) are all strategies that reflect behavioural responses to signalling conditions. Another common strategy utilised by animals to enhance the efficacy of communication is possessing complex communication systems.

1.2 Complex communication systems

It is difficult to define "complexity" in the context of communication systems (Edmonds, 1995). One of the methods in understanding this complexity is to decode animal signals by quantitative comparative analysis and playback methods (Reznikova, 2007). Complexity within a communication system is attained by different adaptations in animals (Patricelli & Hebets, 2016). The sensory stimuli can differ in terms of unit structure, where they possess different types of units for addressing one or more receivers (Pollard & Blumstein, 2012). Indeed, some species with complex acoustic communication systems have repertoires comprising a large number of acoustic units or call types, as with many avian species (Deoniziak & Osiejuk, 2019; Holt et al., 2017), but also anurans such as the old-world frog *Boophis madagascariensis*

(*Rhacophoridae*) with a repertoire of 28 call types (Narins et al., 2000). Each of these units, or group of units, could be assigned to a different function (Templeton & Greene, 2007). Greater complexity is also assumed when species can arrange signal units into different combinations. For example, some species of frogs are known to produce diphasic calls, where the two units of the call are targeting different audiences. In the diphasic advertisement call of the frog species *Geocrinia victoriana* one note component was more attractive to the breeding females while the other note is used in a territorial context (Littlejohn & Harrison, 1985). Increased complexity of communication systems is also achieved via graded signal repertoires. Here, complexity is achieved by varying both within and between signal characteristics (Fischer et al., 2017). For example, graded communication of rhesus macaques, *Macaca mulatta* (Le Prell et al., 2002), graded alarm signals of reed warblers, *Acrocephalus scirpaceus* (Welbergen & Davies, 2008), and graded aggressive signals of Blanchard's cricket frog, *Acris crepitans blanchardi* (Wagner Jr, 1989b).

Another strategy employed by animals is the simultaneous use of multiple sensory modalities (Hebets & Papaj, 2005; Partan & Marler, 2005). Since this is often more advantageous over the simple signals (Wilson et al., 2013), it is wide spread among animal species. For

example, wolf spiders of the genus *Schizocosa* use both seismic and visual signals in courtship displays (Uetz et al., 2009), or the elaborate courtship displays of blue-capped cordon-bleu, *Uraeginthus cyanocephalus*, in which the birds combine acoustic, visual and tactile stimuli (Ota et al., 2015). Multimodal communication has diversified among animal groups not only in conspecific mate attraction communication (Uy & Safran, 2013), but also in other important behaviours like defending a territory and escaping or avoiding predators (Partan & Marler, 2005). While signalling can be costly (Hasson, 1994), the functional significance of the evolution of multimodal signals should exceed the elevated cost by improved efficacy and accuracy of signal transmission (Hebets & Papaj, 2005).

The prevalence of multimodal signals is becoming clearer and several hypothesis regarding their evolution have been proposed that relate to various selection pressures (Hebets & Papaj, 2005). For instance, content-based selection hypotheses relate to the information content of the signal and the evolutionary forces acting upon the signaller and/or receiver (Hebets & Papaj, 2005). The two major content-based hypotheses proposed are the redundant signal hypothesis and the multiple signal hypothesis (Hebets & Papaj, 2005). The redundant signal hypothesis suggests that the different components of the multimodal signal convey the same information (Partan & Marler, 1999). In this way, multimodal signals serve to improve detectability and reliable transmission, thereby increasing the accuracy of receiver responses (Bradbury & Vehrencamp, 1998). Empirical support for the redundant signal hypothesis has been provided for different taxa, from primates (Cartmill & Byrne, 2007), birds (Jawor et al., 2004) and fishes (Wedekind et al., 1998). In contrast, the multiple signal hypothesis states that different information is conveyed via the different channels (Grafe & Wanger, 2007), resulting in the expansion of the collective information content (Partan & Marler, 1999).

Efficacy based hypotheses of multimodal signalling describe how the factors act upon the production, transmission and reception of the complex signals (Hebets & Papaj, 2005). Two primary hypotheses have been identified as efficacy trade-off and efficacy back-up (Hebets &

Papaj, 2005). The efficacy back up hypothesis has been applied to the multimodal courtship display of the wolf spider species, *Schizocosa retrorsa*, such that the seismic signals help male spiders to increase the number of copulations in the dark compared to daylight, while the visual signals aid in mating behaviour on substrates that dampen the vibratory signals (Hebets & Papaj, 2005). On the other hand, because of the acoustic signal degradation and attenuation in noisy environments, the visual signal generated by inflating the vocal sac provides extra information to female túngara frogs in detecting potential mates (Rosenthal et al., 2004) is an example of the efficacy trade off hypothesis. The third and least studied hypotheses (Grafe & Wanger, 2007), inter-signal interaction hypotheses, state that the presence of one signal component alters either the production of the other signal or a response to that subsequent signal (Hebets & Papaj, 2005). For instance, the inter signal interaction alerting hypothesis suggest that an initial olfactory signal alert female sticklebacks (*Culaea inconstans* and *Gasterosteus aculeatus*) of a potential mate before it can be seen (McLennan, 2003). In contrast, the inter-signal interaction attention-altering hypothesis (Fröhlich & van Schaik, 2018) suggest that the visual signal cues could facilitate conception of auditory cues of humans (Massaro & Palmer Jr, 1998).

Another strategy employed by signallers with different types of signal elements is to combine them into diverse sequences, which is common in bird species such as common nightingales, *Luscinia megarhynchos* (Weiss et al., 2014), and Carolina chickadees, *Poecile carolinensis* (Freeberg, 2008). Consequently, in describing complex communication systems it is crucial also to identify patterns associated with how the structural units are combined (Weiss et al., 2014). The concept of information has been identified to be useful as a method of quantify and compare complexity on these terms (Blumstein & Armitage, 1997). Several methods have been utilised to quantify sequential order, although Markov chain analysis (Briefer et al., 2010) is most common. Alternatively, the amount of information embedded in a call/note sequence can be analysed in terms of entropy values derived from information theory (Shannon, 1948).

consequently the complexity (Morais et al., 2012). Hence, a higher entropy value can be equated with higher complexity in that call sequence (Smith, 2014). Use of Information theory has been first used on human European languages, to understand that the length of the message is corelated with the amount of information it carries (Reznikova, 2007). This has been used in investigating the communication system of several groups of animals including invertebrates like ant species, *Fomica pratensis* (Reznikova & Ryabko, 2003), and several vertebrate taxa like, rock hyraxes, *Procavia capensis* (Kershenbaum et al., 2012), humpback whales, *Megaptera novaeangliae* (Miksis-Olds et al., 2006), and rufous-bellied thrush, *Turdus rufiventris* (Da Silva et al., 2000). However to my knowledge, information theory has been used only on the acoustic repertoire of one anuran species *Dendropsophus minutus* (Morais et al., 2012).

Rather than the classic approach of studying isolated modalities (Hebets & Papaj, 2005; Wilkins et al., 2015), most of the current studies in animal communication take a multidimensional approach to studying complex animal communication systems (Patricelli & Hebets, 2016). Novel approaches like visual representation of signal phenotype networks (Wilkins et al., 2015) and use of information theory (Kershenbaum et al., 2016), have been used in unwinding temporal patterning of the communication system design. However, these methods are rarely used on anuran communication systems (Morais et al., 2012).

1.3 Communication systems of anurans

Anurans are well known for their unique acoustic signalling behaviour (Wells & Schwartz, 2007). These signals were long postulated to be important elements of sexual selection by Charles Darwin (Darwin, 1871). After a long time of undertaking research on the acoustic signals and repertoires it has been confirmed that anuran acoustic signals are indeed shaped by sexual selection (Gerhardt & Huber, 2002). The vocal repertoires of anurans are species-specific and hence are important taxonomically for species recognition. Shaped by both physiological and external constraints, anuran repertoires can possess different types of calls with varied spectral and temporal properties (Toledo et al., 2015) that can be categorised according to functional significance. The most commonly emitted call type comprised of one or more units (Toledo et al., 2015) that is mainly used in attracting a conspecific female is known as the advertisement call (Wells, 1977). The quantitative description of this call type is widely discussed due to its taxonomic use for species recognition (Kohler et al., 2017). Nomenclature has been also introduced to some other call types that are related to reproduction behaviour of the species. These include courtship calls that are emitted by both males and females in the presence of each other, as well as amplectant calls, which are emitted by male frogs while engaging in amplexus (Toledo et al., 2015). Furthermore, aggressive call types of the repertoire function in the spacing of male anurans. The territorial call can be released in the presence or absence of a rival male, and hence act as a long distance aggressive signal, while the close range encounter call directly targets a rival male (Toledo et al., 2015). However, differentiating call types is difficult for anuran species with graded signalling systems (Wells & Schwartz, 2007) as aggressive and advertisement call components transition in a graded continuum. This complex vocalisation has been observed in multiple frog species including spring peeper, *Pseudacris crucifer* (Schwartz, 1989), and Blanchard's cricket frog (Wagner Jr, 1989a).

A wide range of multimodal communication strategies have been observed in anurans in which visual signals are used in conjunction with acoustic signals. For example, vocal sac movements by túngara frogs, *Physalaemus* (*=Engystomops*) *pustulosus* (Cummings et al., 2008; Rosenthal et al., 2004), and European tree frog, *Hyla arborea* (Gomez et al., 2009), as well as limb movements (foot flagging and foot tapping) by male and female torrent frog, *Staurois guttatus* (Grafe & Wanger, 2007) and Amazonian treefrog, *Hyla parviceps* (Amézquita & Hödl, 2004). Despite being a widely-used model system for studying acoustic communication (Gerhardt & Huber, 2002), visual signalling behaviour is comparatively less studied in anurans (Hödl & Amézquita, 2001). Interestingly, recent studies have observed a surprisingly large number of nocturnal anurans using visual signals, which was once thought to be an evolutionary advancement of the communication of diurnal species (Hödl & Amézquita, 2001). Studies on the

visual sensitivity of frogs conducted using moving prey items have shown that some anurans have the ability to identify objects below the human sensitivity threshold (Buchanan, 1998; Pedersen & Larsen, 1981). These field observations and accompanying physiological knowledge highlight the importance of undertaking further studies on the visual signalling behaviour of all anurans (Taylor et al., 2007).

Several experimental approaches have been used for explicating the functional significance of the added visual signal(s) in the communication of this, predominantly vocal, group of animals (Hödl & Amézquita, 2001). These experiments have focussed on three types of selective pressures acting upon the evolution of multimodality: content-based selection, efficacy-based selection and inter-signal interaction selection (Hebets & Papaj, 2005). Acoustic playback results suggest that in some species, such as *Staurois guttatus*, one signal component has the ability to alert receivers about the next (Grafe et al., 2012), whereas studies utilising video playback techniques (Rosenthal et al., 2004) or robotic frog models (Taylor et al., 2008) on túngara frogs suggest that the combination of audio and visual signals increases the detectability of the signaller to the receiver.

1.4 Studies of anuran communication in Australia

There is a long history of studies of the communication systems of Australian frogs. The Australian scientist Murray Littlejohn, regarded by many to be the "godfather of frog recordings", was a pioneer in the field and started making acoustic recordings of frogs in the 1950s with basic equipment that he adapted for use in the field. Since then, he has contributed to a great array of bioacoustic studies of Australian anurans. One of his earliest studies was on the mating call descriptions and differentiation of the *Crinia* species complex (Littlejohn, 1959), with information on variation between sympatric and allopatric populations. He also described the functional significance of call components and the complex vocal repertoires featuring diphasic advertisement calls of *Geocrinia victoriana* (Littlejohn & Harrison, 1985) and *Geocrinia laevis* (Harrison & Littlejohn, 1985). Since then, knowledge of the bioacoustics of anurans in

Australia has improved considerably; however, there remains too few detailed quantitative descriptions of the acoustic repertoires of frog species. Furthermore, multimodal signalling has been described for few Australian anurans in the genus *Litoria* (Meyer et al., 2012; Richards & James, 1992), but I am not aware of any experimental studies into this behaviour to date.

In Australia, phylogeographical variation due to the divergence of genetic structure within species is considered distinct from that of the northern hemisphere species (Schäuble & Moritz, 2001). The expansion and contraction of species, both fauna (Hewitt, 2000; Taberlet et al., 1998) and flora (Magri et al., 2006), in the geographic space during the Quaternary period in the northern hemisphere is mainly explained by the recurrence of glacial events that resulted in the expansion and contraction of large ice sheets (Milner et al., 2012; Schäuble & Moritz, 2001). In contrast, with its unique low level of topography and latitudinal positioning, mainland Australia experienced very few glacial formations (Barrows et al., 2002) during the last 2 million years (Milner et al., 2012). Genetic divergence has been identified in several Australian frog species including Litoria fallax (James & Moritz, 2000), Limnodynastes tasmaniensis and L. peronii (Schäuble & Moritz, 2001), Litoria aurea (Burns et al., 2007), Metacrinia nichollsi (Edwards et al., 2008) and Crinia signifera (Symula et al., 2008). Subsequently, some studies have followed the information generated from the phylogeographical studies in elucidating whether mating signals coincide with the divergence. For instance, geographic variation in acoustic signals of Crinia signifera (Littlejohn, 2005; Littlejohn, 2009) and Litoria bicolor were compared in a systematic study using the previously described genetic data (Menzies et al., 2009). Additionally, geographic variation of the acoustic signals of Australian species appear to be independent of genetic divergence and reflect other selective forces like morphological traits, geographical distance (L. booroolongensis; (Smith & Hunter, 2005); Neobatrachus sp.; (Roberts, 1997), and the effect of interspecific competition (Litoria verreauxii; (Smith et al., 2003).

1.5 The study species

My focus in this thesis is *Litoria fallax* (Figure 1.1), which has undergone several taxonomic revisions since it was introduced as *Hylomantis fallax* (Peters, 1880). The species was first changed to *Hyla bicolor glauerti* (Copland, 1957), representing a subspecies of *Hyla bicolor*.

(a)



(b)



(c)



Figure 1.1 | The study species eastern dwarf tree frog (*Litoria fallax*). (a) Mating pair in amplexus (b) Calling male *Litoria fallax* (c) Male *Litoria fallax* exhibiting the foot flagging behaviour

It was subsequently reclassified as *Hyla bicolor* (Moore, 1961), only to be later redescribed as *Hyla bicolor glauerti* (Straughan, 1969). Finally, in the last revision it was classified as *Litoria fallax* (Cogger, 1983) and remains unchanged to the present date. *Litoria fallax* is commonly known as either the eastern dwarf tree frog or eastern sedge frog and is currently included in a species complex with three other similar-looking species collectively known as the *bicolor* complex. Among them, *L. bicolor* (northern dwarf tree frog) is morphologically similar to *L. fallax* and possesses overlapping ranges in the Atherton tableland area of northern Australia (Knight & Tyler, 2020). The other two species of the complex, *L. olongburensis* (Olongburra tree frog) and *L. cooloolensis* (Cooloola tree frog) have restricted distribution ranges compared to the study species and are morphologically distinguishable at the overlapping sites.

Phylogeographical work has revealed two separate mitochondrial lineages of *L. fallax* with a large magnitude of sequence divergence (11.1–12.5%), separated mainly by the split running across the McPherson and Main ranges in northern NSW (James & Moritz, 2000). These are identified as 'northern' and 'southern' lineages on either side of the split. Another rather shallow phylogeographical split (a sequence divergence of 5%) also has been proposed to the northern lineage at the Burdekin Gap (James & Moritz, 2000), a dry belt of savanna that separates the coastal woodlands of the Atherton Plateau and central Queensland (Schäuble & Moritz, 2001).

The natural distribution of the study species originally ranged from Jervis Bay, south of Sydney, to the Atherton tablelands in northern Queensland (Cogger, 2000; Gillespie & Clemann, 2000). However, *L. fallax* has been accidently translocated and persisted with breeding colonies in both greater Melbourne (Bevelander, 2014; Gillespie & Clemann, 2000) and in the Pacific island of Guam (Christy et al., 2007; Eldredge, 1988). The greater Melbourne populations resulted in a nearly 600 km range extension of the species when first identified in 1999 (Gillespie & Clemann, 2000). Furthermore, a later study in 2014 (Bevelander, 2014) and the current study during years 2017-2020 have confirmed that a breeding and active population of *L. fallax* has

been successfully established as an invasive species in this area. The accidental translocation of this species together with some other species like the dainty tree frog, *L. gracilenta*, and the northern dwarf tree frog, *L. bicolor*, is assumed to have happened as a result of transportation of fresh food produce from both the east coast of New South Wales and Queensland and are thus known collectively as "banana box frogs". Among these, only *L. fallax* was able to permanently establish in Melbourne (Bevelander, 2014) despite *L. gracilenta* sharing the same native range as my study species (Robinson, 1993). Additionally, climate modelling to predict the fundamental niche of *L. fallax* suggests that the current species establishments in Melbourne occur within an area classified as low likelihood of occurrence (Bevelander, 2014), implying that factors other than climate variables have contributed to the invasive ability of this species. This might necessarily involve the above-mentioned genetic clades of the species where one genetic clade has the necessary characteristics compared with others to establish in new environments. Certainly, further research is vital in identifying the genetic composition of the species in the introduced habitats.

The acoustic repertoire of *L. fallax* was described as part of the original taxonomic revision (Straughan, 1969), which described the "mating call" of the species to be diphasic. Earlier work has also identified the use of visual signalling by this species (Meyer et al., 2012; Richards & James, 1992). However, detailed quantitative descriptions have not been conducted on either signal modality. Preliminary observations of the species suggest that only male *L. fallax* are acoustically active. Typically, a few males initiate chorusing before other individuals join in. Calling continues until they reach a peak of activity after which individuals drop out gradually and calling fades to silence. Additionally, when considering the local acoustic environment, male frogs tend to duet with the neighbouring conspecific individuals where they tend to emit their calls in an alternating manner while the other frogs are not acoustically active. *Litoria fallax* does not preserve a specific territory, although they are observed to maintain space from neighbouring males when they are acoustically active. It was also observed that even though

there are more than two individuals in close proximity, only two of them tend to emit acoustic signals at a time unless it is a mass chorusing event.



Figure 1.2 | Distribution of the *Litoria fallax* in Australia as shown in the green colour., The green dots indicate the study sites in the natural distribution of the species while the blue dot indicate the introduced population in Melbourne. The lines along the McPherson range and the Burdekin gap are used for indicative purposes in showing the separation of the genetic clades of the species. Map obtained from the Atlas of Living Australia

(https://bie.ala.org.au/species/urn:lsid:biodiversity.org.au:afd.taxon:d33f5b1c-df22-42ea-9347-58f229a53054)

1.6 Thesis overview

My overall objective was to describe and quantify the complex communication system of the Australian pelodryadid frog *Litoria fallax*. In particular, I wanted to quantify both the acoustic and visual signalling repertoire of the species with information on geographic variation of the signals along their broad distribution. Environmental, ecological, and social factors influencing signalling behaviour, acting on both signal modalities, were also considered. From the outset I

wanted my thesis to comprise a combination of fieldwork and captive playback experiments. In preparing this thesis, I decided that it was appropriate to combine work that was inherently linked such that each of my empirical chapters (Chapters 2, 3 and 4) are comprised of a fieldwork component and an accompanying experimental playback component that follows from knowledge gained from the fieldwork.



Figure 1.3 | Examples of the different types of habitats inhabited by *Litoria fallax* and utilised in this dissertation. (a) Manmade permanent waterbody with anthropogenic disturbance (University of Wollongong, Wollongong, New South Wales). (b) Natural permanent waterbody with low anthropogenic disturbance (Brisbane, Queensland). (c) Temporary waterbody next to a busy road with anthropogenic disturbance (Townsville, Queensland). (d) Temporary waterbody away from anthropogenic disturbance (Townsville, Queensland).

Chapter 2 – Complex acoustic repertoire of the eastern dwarf tree frog, *Litoria fallax*

In Chapter 2 I quantify the acoustic signalling repertoire of the focal species. As the initial step in describing a species-specific acoustic repertoire it is important to identify common patterns in the recordings. This is a time-consuming process when the focal species has a complex and quite uncommon signal repertoire that is difficult to describe with conventional nomenclature. My preliminary findings for *L. fallax* were quite a puzzle as they did not match with previous descriptions of the different call types (Straughan, 1969). I chose to focus on the acoustic signals of male *L. fallax* from sites around the Brisbane area, which is within their natural distribution. A complex acoustic repertoire was identified, and I describe this in quantitative detail, separating the repertoire into note and call types. Furthermore, given the complexity observed in call sequences I computed first order transition matrices in different contexts to identify call production patterns. Moreover, information theory was used to determine whether complexity, in terms of the relative amount of information, has an affinity with the immediate conspecific acoustic environment.

One of my chief findings was the identification of monophasic and diphasic note types. One of the diphasic note types was assumed to represent an advertisement call and I used a phonotaxis playback experimental paradigm to consider whether females preferentially respond to one note over the other. Diphasic calls, which are comprised of two note types, might have evolved to communicate to two target receivers at the same time without compromising the information content required by each.

Chapter 3 – Geographic variation in advertisement note types of *Litoria fallax*

Widely-distributed species are usually exposed to different arrays of environmental and ecological factors, and can also be subjected to variability due to long term genetic drift. Comparisons between populations of such species can provide the opportunity to examine how behavioural characters have diverged and the relative contribution of the aforementioned factors in driving differences. *Litoria fallax* is one such species. In Chapter 3 I analysed the advertisement note characteristics of the species in previously described genetically different populations, from Townsville in the northern part of Queensland to Wollongong, south of Sydney, spanning a distance of over 2000 km and with multiple sites in between (Figure 1.2). I also examined an introduced population around Melbourne. The analysis has considered local environmental and ecological characteristics, that might have predicted variability in acoustic signal structure.

The divergence of communication signals by genetic distance could result in divergence in mate recognition differences. With knowledge that signals varied across their range, I designed a playback study to examine female preferences for 'local' and 'foreign' male frogs on the basis of information contained within acoustic signals. I was able to demonstrate that female frogs selected the local acoustic stimuli over the foreign alternative in all cases. Given that acoustic signals in anurans are important in mate choice and sexual selection, I speculate whether these findings are consistent with the hypothesis of the early stages of the speciation process.

Chapter 4 – Study of the multimodal communication by *Litoria fallax*

In Chapter 4 I focus on multimodal communication by *L. fallax*. I was able to confirm the occurrence of earlier descriptions but also observed new visual signalling behaviours that to my knowledge had not previously been described for this species. The use of visual signals is not common and I sought to determine whether its use can be explained by environmental and ecological factors. My results indicated that only the level of ambient noise was a significant predictor of the occurrence of visual signals. Field observations also revealed that visual signalling behaviour of *L. fallax* was associated with agonistic male-male interactions and graded acoustic signals. By quantifying the advertisement and aggressive calls, representing two ends of the continuum of graded acoustic signals, I was able to clarify how spectral and temporal properties of acoustic signals are altered as frogs' transition from advertisement to aggression.

The follow-up playback experiment sought to examine how male and female frogs would react to multimodal stimuli by comparing responses to a dynamic frog model combined with a relevant acoustic stimulus with a static frog model paired with the same acoustic stimulus. I also examined responses under two light levels. In order to complete this experiment, it was necessary for me to design a simple yet effective novel mechanism for moving the hind leg of a small sized model frog to reproduce the foot flagging movement (Figure 4.1b). Interestingly, the results revealed that both males and females were attracted to the multimodal stimulus featuring the dynamic foot flagging signal, and light level was not a key factor. My observations have opened up further opportunities for testing a variety of hypotheses as they relate to multimodal communication in anurans.

1.7 Summary

In this dissertation I have highlighted the importance of conducting systematic research on complex communication systems of anurans. Describing acoustic repertoires without considering the social and abiotic environment in which they are emitted can be misleading. Variability across its geographic range is consistent with genetic differences reported elsewhere (James & Moritz, 2000), but I have also identified the importance of habitat characteristics in the multimodal communication system of the study species. My empirical work has combined field recording sessions as well as experimental approaches for understanding some aspects of the complexity of the communication signals of the study species. However, the novel description of the graded acoustic signalling system of *L. fallax*, as one of the very few species studied in the anuran family Pelodrayadidae, have opened up several future experimental avenues that could aid in elucidating the evolutionary significance of such complex acoustic systems in comparison with its neotropical sister family Hylidae. More importantly, the use of information theory on the repertoire of this model frog species facilitates comparisons with the communication complexity of other taxa.

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Chapter 2

Complex acoustic repertoire of the eastern dwarf tree frog, *Litoria fallax*

2.1 Abstract

Much has been learned about animal communication systems from studying the species-specific acoustic repertoires of frogs. In many species of anurans, male frogs are more vocally active than females and have complex repertoires of vocalisations designed to efficiently communicate in different contexts. The complexity of the calling repertoire of animals can be considered at different levels such as the number of distinct acoustic units, how they vary, and how they are combined in complex sequences. Indeed, statistical description of the vocal repertoire of such species is important in elucidating the functional and evolutionary significance of complex communication systems. Here, I have described the vocal repertoire of a widespread Australian pelodrydidae, Litoria fallax, with information on multiple levels of complexity. Field recordings of acoustic signals of 32 individuals from Brisbane were obtained, along with the social environment at the time of recording considering both the number of, and distance to, calling conspecifics. The results revealed that males of this species produce three distinct, pulsatile note types, differing in both temporal and spectral properties. These note types were emitted as monophasic calls or arranged into diphasic calls varying with social context. Moreover, the acoustic repertoire of this species possesses graded signalling patterning, thereby adding another level of complexity. When the information content (entropy) of call sequences were quantified, complexity was found to be influenced by the number of calling conspecifics. I then used a choice experiment and identified preference by females toward the shorter of the two note types that represent the most commonly emitted diphasic call type, suggesting that this note might be particularly relevant to attracting potential mates. The work described herein highlights the importance of extensive analyses of complex acoustic repertoires in frog species, taking into account the acoustic environment. The newly described graded vocal repertoire of this species, one of the most complex of all Australian hylids (=pelodridids), will aid in future experimental studies in fully understanding the evolution of complex acoustic signals.

2.2 Introduction

The high degree of diversity of animal communication systems found throughout the animal kingdom reflects selection pressures acting upon one or more components of the system. These constraints are associated with the physical environment through which the signal travels (Richards & Wiley, 1980), the social context in which communication take place (Matos & Schlupp, 2005), and the effectiveness of the sensory systems of intended and unintended receivers (Endler, 1987). Animal communication systems have been observed to attain complexity in multiple dimensions in order to compensate for such constraints. Thus, adaptations are manifested by behavioural alterations, changing signal structure, altering the combination of distinct signals and/or by the way signals are implemented over time and space (Patricelli & Hebets, 2016).

The complexity of animal signalling systems has been considered in multiple ways. At a general level, complexity has been linked to repertoire size, whereby a larger repertoire is considered to be more complex (Freeberg et al., 2012). In some species, repertoire size has been increased by the use of multiple sensory modalities (Grafe & Wanger, 2007; Higham & Hebets, 2013; Smith & Roberts, 2003), for the initiation of a response or enhancement of an existing response from the receiver (Partan, 2004; Uetz et al., 2009). The production of different types of signal units in particular circumstances or social contexts is another correlate of complexity. For example, the structurally distinct alarm calls of birds and mammals within a given species have been demonstrated to code information about the nature of the threat such that functionally appropriate responses are made by receivers (Zuberbühler, 2000; Templeton et al., 2005). In anurans, the production of distinct short range encounter calls by male frogs in advertising their calling perch or territory ownership is directed only towards the intruder (Abrunhosa & Wogel, 2004; Toledo et al., 2014). However, in addition to distinct call types, some animals exhibit greater complexity by graded variation of signals, both within a given signal type and among the repertoire, allowing a gradual transition between signals that are purported to facilitate
communicating in a complex environment with several intended receivers (Freeberg et al., 2012; Nunn, 1999; Toledo et al., 2014; Wagner, 1989). Furthermore, when the repertoire of a species is comprised of multiple signal elements, construction of signal sequences from these individual elements is reported (Freeberg et al., 2012; Gerhardt & Huber, 2002; Kershenbaum et al., 2016). The order of presentation within the sequence might contain information potentially important to the receiver, and the uncertainty of which signal element follows provides an additional measure of the complexity of the sequence (Da Silva et al., 2000; Freeberg, 2008; Morais et al., 2012).

Fully elucidating the functional significance of a complex communication system necessarily involves consideration of the signaller, the signal and the intended receiver in the different contexts in which communication take place (Endler, 1993; Freeberg, 2008). An important part of this process is to quantify the physical characteristics of a signal. Acoustic signals have proved to be useful model systems in this respect as they can be recorded, quantified, and used in playback studies comparatively easily (Gerhardt & Huber, 2002). Furthermore, when combined with information of the abiotic (Brooke et al., 2000; Schwartz & Bee, 2013) and social environment (Toledo et al., 2015) within which the signal is produced, acoustic signalling systems have enhanced our understanding of the function and evolution of animal signals. Anuran acoustic systems have proven to be useful for understanding processes involved in the evolution of behavioural and morphological traits and a great platform for studying the evolution of sexual signals in animals, amid selective pressure from aspects of the social environment, both intended receivers (Bernal et al., 2009) and unintended eavesdroppers such as predators and parasites (Endler, 1984; Zuk et al., 2006).

As the primary communication mechanism, acoustic signals play a vital role in the social behaviour of anuran amphibians (Gerhardt & Huber, 2002; Wells & Schwartz, 2007). Vocalisations are more common in male frogs where it is used in the context of reproductive behaviour in attracting conspecific females (Gerhardt & Huber, 2002; Lemckert et al., 2013) and

in defending the territory from other male intruders by conveying information about their location and physical condition (Wells, 1988). Detailed bioacoustic studies with descriptions of both temporal and spectral call characteristics have provided the opportunity to fully understand the vocal repertoire of many anuran species and hence their reproductive and social behaviour (Bee et al., 2013; Chuang et al., 2016; Thomas et al., 2014; Wijayathilaka & Meegaskumbura, 2016a). Importantly, for species with complex communication systems, quantifying the signalling repertoire, which often vary in multiple dimensions, provides the foundation for future experimental approaches that can elucidate the functional significance of such systems in anuran communication. Furthermore, analyses can serve as a valuable tool in taxonomy, systematics and biodiversity research (Obrist et al., 2010). Therefore, it is somewhat surprising that only a small proportion of the more than 8200 amphibian species described worldwide (AmphibiaWeb, 2020), have detailed quantitative descriptions presented in the scientific literature. Given the rapid decline of amphibians (Lannoo, 2005; Semlitsch & Bodie, 2003), detailed acoustic analysis will become increasingly important. Accurate monitoring, documentation, preservation, and management on the basis of knowledge gained from acoustic signals has proven essential in conservation and preservation of impoverished biodiversity (Obrist et al., 2010). For example, these species-specific signals (Pavan, 2008) can be used as a powerful tool in identification and monitoring of species in complex environments like dense vegetation (Obrist et al., 2010). Anuran signals are also particularly important for differentiating cryptic anuran species that share a high degree of morphological similarities (Vieites et al., 2009). Consequently, bioacoustic data should be combined with morphological and molecular data for taxonomic description (Obrist et al., 2010; Pavan, 2017).

The acoustic repertoire of frogs more commonly comprises two or more distinct call types (Toledo et al., 2014; Wells & Schwartz, 2007) with terminology (Kohler et al., 2017) according to the different contexts in which they have been used (Wells & Schwartz, 2007). The most frequently produced calls by male anurans are regarded as the 'advertisement call' with intended receivers being conspecific gravid females (Kohler et al., 2017; Wells, 1977) and, in

some species, rival males (Littlejohn, 1977). While in most anuran species, the advertisement call is comprised of one structurally simple single note type (monophasic), some have diphasic calls comprising two structurally distinct notes in a single call that provides us the opportunity to segregate the functional significance of each component (Littlejohn, 1977; Littlejohn & Harrison, 1985). A note is often the main subunit of a call with short intervals between successive notes (relative to the call duration; (Kohler et al., 2017). Such behaviour is observed in the Puerta Rican *Eleutherodactylus coqui*, the Australian myobatrachid *Geocrinia victoriana*, and the African hyperoliid *Afrixalus brachycnemis*, where the first note of their two-part call conveys an aggressive signal and the latter is emitted toward the female frogs for reproductive purposes (Backwell, 1988; Littlejohn & Harrison, 1985; Narins & Capranica, 1978).

Species with two note diphasic advertisement calls tend to have a fixed temporal note order (Narins & Capranica, 1978), where the second component of the call is not given in isolation, is not used at the start of a call sequence and always follows the first component (Narins et al., 2000). For example, in túngara frogs, Physalaemus (=Engystomops) pustulosus the first component of the call, the "whine", is followed by a series of "chucks" and the number of chucks varies from zero up to seven (Bernal et al., 2007). The chuck component of the call is never emitted by itself (Ryan, 1985). This pattern of behaviour has been also observed in the Central American hylid Hyla ebraccata (Wells & Schwartz, 1984) and Australian Myobatrachid Geocrinia victoriana (Littlejohn & Harrison, 1985). The name "introductory note" is given to the first component of such diphasic calls because that component has the tendency to lead. A fixed temporal note order tends to be a distinctive characteristic of new world anuran species in the families of Hylidae and Leptodactylidae, with relatively fewer call types emitted as a selection from pre-decided packages (Littlejohn et al., 1985; Narins et al., 2000; Ryan, 1980; Wells & Schwartz, 1984). Furthermore, previous studies have shown that given a choice female frogs are preferentially attracted to the more complex call types over monophasic call types (Littlejohn & Harrison, 1985; Ryan et al., 2007). In contrast to this, anuran species in old world families like Rhachophoridae are known to produce a higher number of distinct call types in an unpredictable

Chapter2 – Complex acoustic repertoire of the eastern dwarf tree frog, *Litoria fallax* manner varying with context (Arak, 1983; Jehle & Arak, 1998; Matsui & Wu, 1994; Narins et al., 2000).

Apart from the commonly emitted advertisement calls, in the context of male-male conspecific and heterospecific interactions (Gerhardt, 1994), anuran vocal repertoires include aggressive call types such as the "territorial call", for managing individual spacing, and "encounter calls" in the context of interactions in close proximity to one another (Gerhardt & Huber, 2002). This differentiation of the distance is achieved either via distinct call types or by graded arrangement of calls (Narins et al., 2000). Courtship calls (Toledo et al., 2014; Wells, 1988) are also utilised by anuran species, which are emitted in the close presence of the conspecific females. Courtship calls could be a distinct call type (Wells, 1988) or a modified advertisement call (Toledo & Haddad, 2005; Toledo et al., 2014), that is rather a less observed and recorded call type because courtship behaviour often goes unnoticed in the natural context. Furthermore, comparatively fewer vocally active female anurans of some species are also known to produce distinct courtship calls influencing mating decisions (Vallejos et al., 2017; Wells & Schwartz, 2007). Both sexes are also known to emit 'distress', 'alarm' or 'warning' calls when they encounter predators or are in otherwise stressful situations (Toledo et al., 2014).

Typological classification of call types to the above-mentioned categories can be difficult in anuran species with a repertoire comprised of graded calls (Toledo et al., 2014). Describing a call repertoire of a species for taxonomic purposes, particularly the advertisement calls, without considering the graded patterns of the call arrangement might cause errors (Kohler et al., 2017). Such complex vocal repertoires have been previously identified and described for several Neotropical hylid species (Toledo et al., 2014; Vilaça et al., 2011). For example, *Dendropsophus minutus* has one of the most complex vocal repertoires identified in anurans (Toledo et al., 2014) with a long study history. This species has a multi-note vocalisation behaviour that is mainly comprised of three distinct note types (*A*, *B* and *C*; (Cardoso & Haddad, 1984) with different functional significance and the ability to arrange and use note types according to the social

context. Furthermore, the most emitted note, *Type A*, which is also known as the advertisement call, is used by isolated males only for the purpose of attracting females until more males join in the chorus, whereupon males then produce complex calls made of all three types of notes (Morais et al., 2012). Note *Type B*, which is identified to have a territorial function among male frogs (Haddad, 1987), tends to start the call notes in the presence of a rival male in close proximity, suppressing the introductory A notes and finally lead to only the emission of B notes with longer duration as an aggressive signal (Haddad, 1987). This sequence of acoustic signalling behaviour has been identified as a classic example of a graded communication system where the advertisement call has been gradually transformed into a territorial call (Toledo et al., 2014), but remains an aspect of anuran communication that is not fully understood (Grafe, 1995; Owen & Gordon, 2005; Schwartz, 1989). An additional driving force for the graded arrangement of advertisement calls of species might be the need to attract a potential mate (Bernal et al., 2009). Graded signals, therefore, provide an additional dimension to the communication system (Freeberg et al., 2012).

In this study I have quantified the acoustic repertoire of *Litoria fallax* (Peters 1880), commonly known as the eastern dwarf tree frog or eastern sedge frog (Figure 2.1). This relatively small frog is distributed in open forests and wetlands along the east coast and adjacent regions of Queensland and New South Wales (Anstis, 2013; Schäuble & Moritz, 2001). They are also found in southern Victoria, which is well south of their natural range, purported to be the result of accidental translocation (Gillespie & Clemann, 2000). *Litoria fallax* is a pond-breeding species known to breed throughout the year, though more commonly during the months of Austral spring and summer (Anstis, 2013). Male *L. fallax* emit a characteristic loud acoustic signal (Barker et al., 1995; Stewart, 1998) during the breeding months in order to attract conspecific females preferably from the emerging reeds surrounding the semi-permanent or permanent pools the species use in spawning (Anstis, 2013). After the introduction of the species as *Hylomantis fallax* (Peters, 1880), it has undergone several scientific nomenclature changes due to taxonomic revisions (Copland, 1957; Moore, 1961; Cogger et al., 1983). The advertisement call

has been previously described when the species was known as Hyla glauerti, from populations in Townsville (Straughan, 1969). The recordings for this description were undertaken in a habitat where L. fallax (H. glauerti) and one of its sister species, L. bicolor (H. bicolor) coexisted and the described calls were known as the mating calls of the species. However, preliminary field work pointed to a more complex communication system in the species with some unique characteristics compared to previously described anuran repertoires. Interestingly, this species is also reported to have a multimodal communication system, combining acoustic and visual signals (Hödl & Amézquita, 2001; Meyer et al., 2012). Neither the acoustic nor visual signal repertoires have been studied in detail. The aim of this study was to formally describe the acoustic repertoire of L. fallax, and to examine call characteristics in the context of variation of abiotic factors. In addition, I provide information on how signal complexity is achieved in different acoustic contexts. Furthermore, the basic understanding of the functional significance of the call note types is experimentally tested using the most frequent complex diphasic call in the repertoire. This work lays the foundation for more detailed analysis of field recordings across the geographic range of the species, and other playback experiments to examine signal function in different contexts.



Figure 2.1 | Male Litoria fallax.

2.3 Method

I recorded calling males on three consecutive nights during March 2019 in Brisbane (Queensland), which is within the natural distribution of *L. fallax*. I also wanted to minimise the effect of hormone fluctuation (Arch & Narins, 2009) of frogs on the call characteristics by sampling on consecutive days of the same field season. Various types of waterbodies were used, such as seasonal temporary waterbodies after heavy rain (27° 23' 10.9356"S 152° 51' 36.9504"E) and man-made ponds (27° 24' 3.8916"S 152° 50' 59.9496"E). Some of these study sites were close to human habitation, next to busy roads that feature artificial light and traffic noise, whereas some were away from likely anthropogenic disturbance.

2.3.1 Field recordings

Calling male frogs were located at night between 20:00 to 01:00 h by their characteristic call, and frogs were recorded from a distance under a red light to minimise disturbance. Approximately one minute of recording was obtained from each individual frog using a Sennheiser ME66 directional microphone with a foam windscreen connected to a Roland R-26 portable audio recorder using a sampling rate of 44.1 kHz (16 bits resolution). A recording distance of 0.5-1 m was maintained between the snout of the calling male and the tip of the microphone for all recordings. Gain settings of the microphone were adjusted according to the sound level of each environment and was not adjusted until the recording was completed. Abiotic factors such as temperature and humidity were recorded from a pocket weather meter (Kestrel 4000) immediately after each recording. The sound level of the habitat was recorded with a directional sound level meter one metre away from the individual, while notes on whether natural or anthropogenic noise, and the presence of heterospecific anurans were recorded.

The social environment and acoustic spatial arrangement potentially influencing the focal individuals was recorded as one of the following four social acoustic contexts:

- Chorus-close more than two male frogs were emitting calls and the focal individual was less than one metre away from other calling individuals
- Chorus-distant more than two individuals were emitting calls and the focal individual was more than one metre away from other calling individuals
- Duetting-close one other individual was calling alternatively with the focal individual from less than one metre away
- Duetting-distant one other individual was calling alternatively with the focal individual from more than one metre away

Non-calling conspecific individuals were not considered in the social context due to the inability to differentiate them as either a male intruder or a female given the lack of sexual dimorphism in this species. Close range male-male aggressive behaviours were observed, and acoustic signals were recorded in order to describe the potential encounter call.

2.3.2 Acoustic analysis

I examined all recordings, regardless of the social context, to identify unique call or note types. Three distinctive note types of *L. fallax* were assumed from visual inspection of the spectrographs of the calls (initially named as note *Types A*, *B* and *C* for identification purposes). I extracted spectral and temporal call characteristics for each of the three distinct note types using Raven Pro v1.5 software (Cornell Laboratory of Ornithology, Ithaca, NY USA). Six temporal properties of the notes (note duration, note rise time, note rise time as a percentage of the note duration, number of pulses per note, inter-pulse interval and pulse rate), two temporal properties of individual pulses (pulse duration and pulse rise time) and one spectral property (call dominant frequency by averaging spectrum over an entire call) were measured (Table 2.1). The pulse with the highest amplitude in a call was used in measuring the pulse temporal properties. PCA analysis was undertaken to confirm three distinct clusters using the function *prcomp* in the R statistical environment (R Core Team, 2018).

Call property	Description
Note duration (ms)	The duration of a single note within a call; measured from onset of the first pulse of the note to the offset of the last pulse of the note
Note rise time (ms)	Time between onset of first pulse and pulse of maximum amplitude
Pulses per call/note	Count of pulses (k)
Pulse rate (pulses/s)	(k– 1)/t, where t is the time between onset of first pulse and onset of last pulse
Pulse duration (ms)	Time between pulse onset and offset of the pulse with the maximum amplitude
Pulse rise time (ms)	Time between pulse onset and earliest maximum peak in the pulse waveform
Inter-pulse interval (ms)	Time between offset of the previous pulse and onset of the pulse with the maximum amplitude
Inter-note interval (ms)	The time period between the offset of the first note and the onset of the next note of a call
Dominant frequency (Hz)	Maximum frequency using Raven's selection spectrum function over the duration of the entire call

Table 2.1 | Glossary of terms used in the chapter to describe measured temporal and spectral acoustic properties.

Call recordings were then examined to identify the call types made from the previously identified note types. Monophasic calls, which are comprised of one note type, and diphasic calls made of two note types were identified and the above-mentioned temporal and spectral characteristics of each note type within each call were measured using Raven Pro v1.5 software. An extra temporal property of inter-note interval was measured in diphasic complex calls. Terminology for these characteristics follows previous literature (Bee et al., 2016; Wijayathilaka & Meegaskumbura, 2016b). Raven Pro's waveform display was used for measuring temporal properties, whereas the spectrogram function was used for measuring spectral properties by averaging the spectrum over the entire duration of a call (256 point fast fourier transform using a Hanning window). Similar note types in monophasic and diphasic call types were compared using principal component analysis (see Figure S2.1).

2.3.3 Statistical analysis

Descriptive statistics for each call property (mean, standard deviation, and coefficient of variation) were computed using Microsoft Excel 2010, with median and interquartile range calculated for indivisible characters (number of pulses per call). Principal component analysis was undertaken in the R statistical environment with the *prcomp* function using the following five variables: call duration, pulses per call, inter-pulse interval, pulse rate and dominant frequency. I then used linear regression to investigate the effects of temperature on the temporal properties of anuran vocalisations (Gerhardt & Huber, 2002). However, I could not assess the relationship of the call variables with SVL of the individuals because I could not obtain that data for all animals (see Appendix 1).

2.3.3.1 Call type patterning

Calling bouts comprise sequences in which the different call types outlined above are combined. To examine patterns in these sequences, I have considered 1 min recordings from 34 male *L. fallax.* Call groups were identified when sets of calls are separated by a silent period that is notably longer than the silent periods between calls within a call group. The call type sequence of each group was obtained by visual inspection of the waveform and spectrographic functions of Raven Pro v1.5. These sequences were then used to generate a first-order transition matrix that specifies the probability of each call type following each other call. I undertook this analysis using custom written functions in Matlab (Mathworks Inc.), and included the silence preceding and after the sequence ('start' and 'end' respectively) as categories to determine which notes start and end the sequence.

2.3.3.2 Effect of acoustic environment on call complexity

To consider whether call complexity varies with social context, I draw on information theory (Shannon & Weaver, 1949) and follow Morais et al. (2012) and (Da Silva et al., 2000) to compute

entropy (E) values for each calling male. This value provides us with information on the uncertainty of a call type to occur in a call sequence, consequently the complexity. Hence, a higher entropy value can be equated with higher complexity in that call sequence. Entropy is defined by

$$E = \Sigma(p.*\log_2 p)$$

where *p* is the probability of occurrence of each call type. For this I have used the *entropy* function in Matlab to compute *E* for each call sequence and averaged within each male (as recordings for a given male were in one context only). Entropy values as a function of acoustic context (chorus-close, chorus-distant, duetting-close, duetting-distant) were compared using a Kruskal-Wallis test, while entropy as a function of the number of calling males and ambient noise (natural and anthropogenic) were compared using the *Im* function in the R statistical environment.

2.3.4 Female phonotaxis experiments

I wished to examine how females responded to complex, diphasic calls, and selected *Type BA* as the most commonly emitted in our sample. Using a classic phonotaxis paradigm, I examined female preference for each component of the call.

2.3.4.1 Stimulus preparation

I chose to use a population average call to represent *L. fallax Type BA* diphasic calls. My design therefore tests whether females show a preference for each component of the average *Type BA* call. For this experiment, I used call recordings obtained from 30 male frogs in the Melbourne region, collected using the same method outlined above, and average call properties were calculated from ten acoustic variables. Following inspection of the distribution of call characteristics, I selected calls that closely matched the mean values of each call component of the call *Type BA* tabulated in table 2.3. I used the sound recording and editing software Audacity (version 2.3.3) to create partly synthetic stimuli of 20 s each, adjusting call characteristics, note

duration and the number of pulses. The sound clips were then tested in the experimental chamber (see below) via the amplifier to ensure consistent sound pressure level.

2.3.4.2 Study animals

Fourteen female frogs from Melbourne, Victoria, were captured for this study. Female frogs were hand captured wearing unpowdered vinyl gloves and held up against a light to examine eggs in the abdomen to confirm they are gravid. The sampling was completed over nine nights during December 2019 and March 2020 and transported to the research facility at La Trobe University, Bundoora, where they were placed in separate tanks until the subsequent evening when phonotaxis experiments were initiated. A maximum of two female individuals were tested per experimental night.

2.3.4.3 Procedure

Trials were conducted in an anechoic chamber (1.2m³) made of wood and covered in anechoic foam. The female frogs were given a choice of two stimuli broadcasted from two speakers (FR7-4 VISATON Germany) placed at 90° angles to the releasing point 1 metre apart from each other, which was fixed each time and contained within an acoustically transparent container I have designed. The conventional method of using a fully covered container to cover the animal and removing remotely did not work for my species as frogs tend to climb the walls of the container. My approach for the releasing apparatus is shown in Figure 2.2 and gave me the opportunity to open up the apparatus walls, releasing the animal on to the arena even if it had climbed up a wall. An acclimation period of five mins was allowed while the frog was constrained within the releasing apparatus, during which I commenced broadcasting the test stimuli antiphonally. The sound pressure level (SPL) from both speakers at the releasing point of the frog was maintained at 65 dBA (fast, high and linear weighting) using a sound level meter (QM-1589, Digitech), which was the mean value of the sound pressure level I have obtained from calling male frogs of similar distance in the field (N = 7). One stimulus was presented at each speaker, with speaker allocation randomised and counterbalanced across the cohort. The two stimuli of the two note components of Type BA (Type B and Type A) were presented in an alternating fashion, with the

order of presentation also randomised for a given individual and counterbalanced across the cohort. Each session was filmed using a Canon XA40 with infrared capability, which was fixed to the roof of the chamber and connected to an external monitor providing the opportunity to observe the sequence of behaviour from a distance.



Figure 2.2 | Anechoic chamber (1.2 m³) used in the phonotaxis experiment. *Top row*: Side and aerial views of the playback experiment arena showing the release point of the test animal (1) location of speakers and model frogs (2,4), camera with IR capability (3) and line painted on to the chamber representing 40 cm from each speaker. *Bottom row*: Releasing apparatus used in the present study.

2.3.4.4 Analysis

I reviewed video footage of each session and recorded the first phonotactic choice response of

each female, which I defined as approaching to within 40 cm of a given speaker. To facilitate this,

markings were painted on the arena (Figure 2.2). If a female frog remained motionless in the

release apparatus, or did not approach either speaker for 20 mins, they were considered

unresponsive and removed from the analysis. I used the binomial test in the R statistical environment to test whether females showed a preference for one call over the other.

2.4 Results

2.4.1 Note types

The Principal Component analysis, using five note property variables (Note Duration, Number of pulses, Inter-pulse interval, Pulse rate and Dominant frequency) separate out three note types in the PC space (Figure 2.3; Table S2.1). PC1 axis explains 51.3% of the total variation mostly by the note duration (factor score = 0.576) and PC2 axis is explained by the pulse rate mainly (factor score = -0.655), which explains 31.4% variance. Mean values, standard deviations (SD), ranges and the coefficient of variation (CV) values of both quantitative properties of each note type are presented in Table 2.2. All the note types of this species are pulsatile, with high variation observed in all the note characteristics within the population.



Figure 2.3 | Results of principal component analysis for the three note types produced by *Litoria fallax: Type A* (red), *Type B* (green) and *Type C* (blue).

Table 2.2 | Descriptive statistics of the three note types identified in *Litoria fallax*. Notes in both simple monophasic calls and complex diphasic calls are considered. N = 275 (*Type A*), 77 (*Type B*), 64 (*Type C*).

Call characteristic	Note type	Mean	SD	Range	CV ^a (%)
Note duration (ms)	А	37.0	16.0	13.0-102.0	41.7
	В	423.0	151.0	232.0-1327.0	35.7
	С	372.0	116.0	198.0-752.0	31.2
Note rise time (ms)	А	21.0	16.0	1.0-69.0	72.8
	В	391.0	154.0	176.0-1305.0	39.4
	С	331.0	126.0	13.0-707.0	38.0
(Note rise time/Note	А	51.8	25.0	3.0-99.0	48.0
duration)100%	В	92.0	7.0	60.0-98.0	8.0
	С	88.0	15.0	4.0-98.0	16.8
Number of pulses per note	А	5.0	2.0	3.0-11.0	30.8
	В	49.0	13.0	25.0-78.0	25.8
	С	19.0	6.0	11.0-43.0	34.0
Pulse duration (ms)	А	7.0	3.0	3.0-27.0	46.8
	В	6.0	3.0	3.0-16.0	46.2
	С	8.0	2.0	3.0-17.0	28.3
Pulse rise time (ms)	А	1.0	0.1	1.0-6.0	50.6
	В	1.0	0.5	1.0-4.0	41.2
	С	1.0	0.5	1.0-2.0	35.6
Pulse rate (pulses/s)	А	105.7	25.1	38.5-181.8	23.7
	В	117.0	20.8	46.0-147.0	17.6
	С	47.5	4.7	39.0-64.6	9.9
Inter-pulse interval (ms)	А	2.0	2.0	0-10.0	100.0
	В	3.0	2.0	0-7.0	62.0
	С	12.0	2.0	7.0-19.0	18.3
Dominant frequency (kHz)	А	4.3	0.3	3.4-5.0	6.7
	В	4.7	0.2	4.1-5.2	4.8
	С	4.6	0.2	4.1-5.1	4.4

^a Coefficient of variation

2.4.2 Call types

2.4.2.1 Monophasic calls

Monophasic call types comprise a single note type (*Type A, Type B* and *Type C*), while diphasic call types comprise two note types (*Type AA, Type BA* and *Type CA*). Monophasic calls made up 65% of calls (N = 902), with *Type A* being the most frequently used (50%). Each of the diphasic calls (*Type AA, Type BA* and *Type CA*) were used more often (16%, 13% and 6% respectively) than the other monophasic calls *B* and *C* (8.4% and 7.2% respectively). The characteristics of note types within diphasic calls were observed to vary with respect to their use as monophasic calls,

hence descriptive statistics of the note types were analysed and tabulated separately in monophasic call (Table 2.3) and diphasic call types (Table 2.4).

Call characteristic	Note type	Mean	SD	Range	CV ^a (%)
Note duration (ms)	А	37.0	9.0	23.0-57.0	24.0
	В	488.0	161.0	232.0-1327.0	33.0
	С	410.8	130.1	198.0-752.0	31.6
Note rise time (ms)	А	22.0	10.0	7.0-46.0	44.9
	В	453.0	165.0	209.0-1305.0	36.4
	С	363.1	137.9	93.0-708.0	38.0
(Note rise time/Note	A	58.0	19.2	17.0-93.0	32.9
duration)100%	В	92.2	7.40	66.3-98.9	8.0
	С	86.8	11.70	37.8-97.3	13.5
Number of pulses per note	А	5.0	1.0	3.0-7.0	18.9
	В	54.8	12.8	25.0-78.0	23.4
	С	20.5	6.8	11.0-37.0	33.1
Pulse duration (ms)	А	8.0	3.0	3.0-27.0	41.7
	В	7.0	3.0	3.0-16.0	47.5
	С	8.2	2.1	4.0-13.0	26.1
Pulse rise time (ms)	А	1.0	0.1	1.0-4.0	46.9
	В	1.0	1.0	1.0-4.0	49.4
	С	1.2	0.4	1.0-2.0	35.5
Pulse rate (pulses/s)	А	104	21.3	38.0-166.7	20.4
	В	116.3	22.8	46.7-138.6	19.6
	С	49.9	4.5	41.0-60.0	9.0
Inter-pulse interval (ms)	А	2.0	2.0	0-10.0	100.0
	В	3.0	2.0	0-7.0	77.2
	С	12.5	2.0	7-22.0	22.7
Dominant frequency (kHz)	А	4.3	0.3	3.4-4.8	6.7
	В	4.7	0.2	4.1-5.2	5.0
	С	4.6	0.2	4.1-5.0	4.1

^a Coefficient of variation

Note and call characteristic	Note type	Mean	SD	Range	CV ^a (%)
Complex call <i>Type AA</i>					
Note duration (ms)	A (30)	69	10	58.0-102.0	14.5
	А	19.9	1.7	16.0-22.0	8.9
Note rise time (ms)	А	49.7	11.3	22.0-69.0	22.7
	А	7.4	3.8	1.0-15.0	50.8
(Note rise time/Note duration)	A	72.1	12.90	31.8-88.2	17.9
100%	А	37.5	19.40	5.0-87.0	51.8
Inter-note interval (ms)		218.0	64.0	131.0-364.0	29.4
Number of pulses per note	A	7.2	1.8	4.0-11.0	26.0
	A	3.5	0.6	3.0-5.0	16.5
Pulse duration (ms)	A	12	4.8	5.0-20.0	40.0
	А	5.5	1.0	4.0-8.0	17.3
Pulse rise time (ms)	A	1.7	0.8	1.0-3.0	44.1
	A	5.5	1.0	4.0-8.0	40.6
Pulse rate (pulses/s)	A	90.2	24.6	39.2-131.1	27.2
	A	122.9	23.8	90.0-181.0	19.4
Dominant frequency (kHz)	A	4.5	0.2	3.9-4.8	4.9
	A	4.4	0.3	3.7-4.9	7.5
Complex call Type BA					
Note duration (ms)	B (51)	354.4	80.0	263.0-693.0	22.5
	А	22.0	4.0	13.0-34.0	19.2
Note rise time (ms)	В	319.4	80.6	196.0-670.0	25.2
	А	2.0	2.0	1.0-7.0	100.0
(Note rise time/Note duration)	В	90.20	8.40	61.0-98.0	9.3
100%	А	11.0	10.0	2.0-36.0	91.0
Inter-note interval (ms)		202.8	69.0	12.0-304.0	34.1
Number of pulses per note	В	42.1	8.5	15.0-63.0	20.3
	А	4.0	1.0	3.0-4.0	14.0
Pulse duration (ms)	В	5.9	2.2	3.0-14.0	37.4
	А	5.0	1.0	3.0-10.0	26.4
Pulse rise time (ms)	В	1.2	0.4	1.0-2.0	33.7
	А	1.0	1.0	1.0-6.0	100.0
Pulse rate (pulses/s)	В	120.8	19.7	118.5-135.7	16.3
	А	118.9	23.0	80-166.6	19.6
Inter-pulse interval (ms)	В	3.1	1.9	0.0-8.0	61.2
	А	0.2	0.2	0-3.0	100.0
Dominant frequency (kHz)	В	4.7	0.3	4.1-5.2	5.4
	А	4.4	0.2	3.9-4.8	5.3
				Continued r	next page

Table 2.4 | Descriptive statistics of the note types in complex diphasic call types of *Litoria fallax*.

Note and call characteristic	Note type	Mean	SD	Range	CV ^a (%)
Complex call <i>Type CA</i>					
Note duration (ms)	C (40)	331.2	59.8	243.0-499.0	18.1
	A	31.7	8.7	19.0-52.0	27.7
Note rise time (ms)	С	293.9	70.9	138.0-479.0	24.1
	А	9.0	8.0	1.0-33.0	85.9
(Note rise time/Note duration)	С	88.1	9.7	49.0-98.0	11.0
100%	А	29.0	20.5	2.0-68.0	70.5
Inter-note interval (ms)		347.6	77.3	202.0-534.0	22.2
Number of pulses per note	С	16.5	3.7	12.0-32.0	22.4
	А	5.0	1.0	3.0-7.0	19.9
Pulse duration (ms)	С	8.6	2.5	3.0-13.0	28.9
	А	6.0	2.0	3.0-11.0	30.9
Pulse rise time (ms)	С	1.3	0.5	1.0-2.0	35.8
	А	10.0	8.0	1.0-33.0	86.0
Pulse rate (pulses/s)	С	49.9	4.5	43.1-64.1	9.0
	А	113.0	27.0	50.0-182.0	24.0
Inter-pulse interval (ms)	С	13.0	2.5	9.0-20.0	19.0
	А	1.0	1.0	0-7.0	100.0
Dominant frequency (kHz)	С	4.7	0.3	4.1-5.2	5.3
	А	4.3	0.3	3.8-4.8	7.0

Table 2.4 | Continued ...

^a Coefficient of variation

The most common call type, *Type A* (Figure 2.4 and 2.5), was observed in all individual frog repertoires. The modified *Type A* calls that are used as the second note of the diphasic *Type AA, BA* and *CA* calls are not considered here. The call duration of the monophasic *Type A* call was the shortest of all the call notes $(0.037 \pm 0.016 \text{ s})$ and emitted with a "click" sound. This call consists of 3-9 pulses emitted at a high rate and with very little to nil inter-pulse interval. The highest amplitude of call *Type A* varied considerably throughout a call series for a given individual frog without any movement of our recording device. There was no pattern observed in the time for call *Type A* to reach maximum amplitude within the call, hence higher variation in the call rise time. The dominant frequency of the call was measured to be 4.344 ± 0.292 kHz and no frequency modulation was observed. When *Type A* calls were emitted as a series of the same call type, rather than being organised into call groups, the amplitude and call rate was reduced.





Figure 2.4 | Ten second segments of different call sequences of *Litoria fallax* with the indication of different call types involved.



Figure 2.5 | One second segments of the different call types of *Litoria fallax* showing the waveform (*left panel*) and the spectrogram view (*middle panel*) of the highlighted calls in Figure 2.4 *Right Panel*: Power spectrum of the call averaged over the duration of all notes depicted in the one second segment (256 FFT size, Hanning window).

The monophasic call *Type B* (Figure 2.4 and 2.5) is a ramp with much longer call duration compared to *Type A*, with a duration of 0.625 ± 0.211 s. Similar to *Type A*, *Type B* calls had compactly arranged pulses with short inter-pulse intervals. The call reaches the maximum amplitude near to the offset, with a higher call rise time (0.567 ± 0.208 s). There was no frequency modulation observed in this call type as well and the dominant frequency was $4.516 \pm$ 0.394 kHz. Call *Type B* was included in call groups as well as emitted as a call series when there is another individual calling in close proximity. Graded acoustic variation was observed in this call type when emitted repeatedly in a series (Figure 2.4). In particular, call duration was changed drastically (CV = 33%) at close range encounters with other calling male frogs suggesting a function of aggressive behaviour.

The monophasic call *Type C* (Figure 2.4 and 2.5) is similar to call *Type B* but with an extended longer duration (410 \pm 130 ms) ramp. However, the number of pulses in this call type is less than that of call *Type B* and arranged with a higher inter-pulse interval (12.5 \pm 2 ms) than that of both *Type A* and *Type B* call types. This call type has the lowest pulse rate (49.9 \pm 4.5 s) of all monophasic call types. There was no frequency modulation observed and the dominant frequency was 4.150 \pm 0.845 kHz. Similar to the monophasic call *Type B*, the call *Type C* was also observed to have high variability, particularly for call duration (CV = 31.6%), which also resulted in concomitant variation in other temporal characteristics. Monophasic call *Type C* is included in call groups as well as in call series of its own. Graded variation was observed with the call *Type C* when emitted as a call series (Figure 2.4) when a rival male is calling close by.

2.4.2.2 Complex calls

Diphasic calls were identified with a relatively smaller time gap between the two notes compared to the inter-call intervals between adjacent calls. Each diphasic call is comprised of two notes where the first note resembles one of the previously identified (monophasic) call types followed by a tail segment that is structurally similar to *Type A*, but with different temporal qualities. The diphasic note combinations that I identified are *Type AA*, *Type BA* and *Type CA* (Figure 2.4). After careful consideration of the structurally similar appearance of the tail clicks to the *Type A* notes in the spectrographic view and the overlapping PCA results (Figure S2.1) they are not considered as separate note types, rather treated as modified versions of note *Type A* and comparative quantitative measurements are given (Table 2.4). These modified calls are not considered as a note *Type A* in any of the further analysis.

Diphasic call Type AA

Properties of the two "A" notes in this complex call do not differ greatly from that of the monophasic call (Table 2.4). However, the call rise time was observed to be more static, whereas in the monophasic call the rise time had no pattern. The first A note of the diphasic call *Type AA* tended to reach its highest amplitude towards the offset of the note (note rise time/note duration = $72.1 \pm 12.9\%$) while the second note has a shorter rise time (note rise time/note duration = $37.5 \pm 19.4\%$). It was also observed that the highest amplitude of the second A note is most of the time higher than that of the first note. This arrangement enables the diphasic call to reach its highest amplitude in the middle of the call rather than the onset or offset as with the monophasic calls.

Diphasic call Type BA

This call was observed in all call recordings. The first note is structurally similar to the monophasic *Type B* note (Figure 2.4 and 2.5) with tightly arranged pulses and higher pulse rate (120.8 ± 19.7 pulses/s). However, note duration of the note in the diphasic call is more static (CV = 22.5%) compared to that of the monophasic call (CV = 33%). Note *Type B* in the diphasic call is not observed to have graded variation, hence, more static call temporal properties were achieved. There was no frequency modulation observed and the dominant frequency was 4.7 ± 0.3 kHz, which is similar to that of the monophasic call note. The second note of *Type BA* (Figure 2.5) in the diphasic arrangement is a modified *Type A* note which is shorter in duration (22 ± 4 ms), compared to the first call note. The main differences observed with the monophasic note *Type A* are the higher amplitude and shorter rise time towards the onset of the note (note rise time/note duration = $11 \pm 10\%$). There was no frequency modulation observed and the dominant frequency and the dominant frequency was 4.439 ± 0.228 kHz, which is similar to that of monophasic note *Type A*. The two units of this call are separated by 202 ± 69 ms.

Diphasic call Type CA

Call *Type CA* comprised of a note *Type C* as the first note and a modified *Type A* note as the second note (Figure 2.4 and 2.5). The first note is structurally similar to that of monophasic note

Type C with fewer pulses loosely arranged with higher inter-pulse intervals. No graded variation was observed in the note when arranged in the diphasic call and the note duration is more static (CV = 18.1%) compared to that of the monophasic calls (31.6%). There was no frequency modulation observed in the note *Type C* in the diphasic arrangement with dominant frequency of 4.7 ± 0.3 kHz which is similar to the monophasic call *C* as well. Similar to that of the *Type BA* call, a click sound follows immediately after the first note with a duration of 32 ± 9 ms and is a modified *Type A* note. This note also has a higher amplitude than the first note. There was no frequency modulation observed in this unit and the dominant frequency was 4.3 ± 0.3kHz, which is similar to that of the monophasic note *Type A*. The two units of this diphasic call is separated by 0.348 ± 0.077s.

2.4.3 Call type patterning

The highest probability to initiate a call group in all acoustic contexts was obtained by call *Type A* followed by call *Type AA* (Table 2.5). Call *Type B* and *Type BA* had lower probability of initiating a call group, while call *Type C* and *Type CA* were rarely observed to do so. To illustrate this graphically, I generated a kinematic graph summarising transitions between call types (Figure 2.6), while leaving transition probabilities unchanged from Table 2.5. Monophasic call *Type A* is most often repeated, while call *Type C* is most likely to follow call *Type B*, and *Type A* follows *Type C*. For diphasic calls, *Type AA* is mostly followed by itself, *Type BA* is mostly followed by *Type CA*, and *Type CA* is mostly followed by call *Type A*. Additionally, it was observed that both monophasic *Type B* and diphasic *Type BA* do not follow the call *Type C* or *Type CA*, while there is high probability of *Type CA* and *Type C* following *Type B* and *Type BA* (Figure 2.6, Table 2.5).

Table 2.5 First-order transition probability matrix of both simple and complex calls of <i>Litoria</i>
fallax (N = 34). The left-most column represents the preceeding call type and the adjacent
columns represent the following call type of the sequence or if it ended the call (End). The first
row represents the probability of each call type at the start of a call group.

		Following call						End
		А	В	С	AA	BA	CA	
	Start	0.49	0.14	0.02	0.20	0.14	0.01	
	А	0.42	0.02	0.02	0.06	0.07	0.02	0.39
all	В	0.05	0.07	0.37	0.03	0.04	0.09	0.35
eding c	С	0.23	0.03	0.11	0.02	0.02	0.06	0.53
Prece	AA	0.07	0.01	0.00	0.19	0.11	0.00	0.62
	BA	0.24	0.05	0.12	0.03	0.09	0.27	0.2
	CA	0.47	0.04	0.00	0.02	0.04	0.04	0.39



Figure 2.6 | Kinematic graph representing first order transition probabilities between call types of *Litoria fallax* reported in Table 2.5 (excluding start and end). Arrow width is proportional to transition probability, while probabilities from 5-10% are represented in dotted lines and transitions with lower than 5% probability excluded.

2.4.4 Effect of acoustic social context on call sequence complexity

The total number of calls emitted per minute ranged from 9-87 (N = 34) across all contexts. Calls by single isolated males tended to have the fewest calls (14 ± 6), although this context was rare (N = 2). The mean number of calls obtained for other acoustic contexts is as follows: duetdistance 18 ± 6 (N = 6), chorus-distance 19 ± 6 (N = 11), duet-close 28 ± 12 (N = 10), and chorusclose 58 ± 26 (N = 5). Call complexity was computed as the mean non-conditioned entropy value per individual, which ranged 0-1.15. Excluding the two single isolated male recordings, I found no significant relationship between the entropy value and the total number of conspecifics calling (P = 0.53, Df = 32, adjusted R² = -0.018) and the environmental noise level (P = 0.74, Df = 32, adjusted R² = - 0.028). The highest mean entropy value was observed in the acoustic context of chorus-close (0.714), followed by chorus-distance (0.535), duet-distance (0.419), and duetclose (0.371). The entropy values showed a significant difference as a function of acoustic context (Kruskal-Wallis test, χ^2 = 8.0987, Df = 3, P = 0.044; Figure 2.7). However, pairwise comparisons using Wilcoxon rank sum test revealed that the significant difference of mean entropy values only occurs between the acoustic contexts of chorus-close and duetting-distance (P = 0.038) and between chorus-close and duetting-close (P = 0.038).



Figure 2.7 | Box plot of entropy as a function of acoustic context. Boxes represent the interquartile range, with the horizontal line in the centre representing the median value. The whiskers show the range of values that fall outside the middle 50% of data, while filled circles denote outliers.

2.4.5 Female phonotaxis experiments

Nine of the 14 female individuals tested with the two-choice experiment responded and were used in subsequent analyses. Of these, eight were attracted to the shorter note *Type A* of the complex call *Type BA* as the first choice suggesting a strong preference for this note (binomial test: P = 0.039). The individual initially attracted to *Type B* subsequently moved to note *Type A* and spent more time near to this speaker.

2.5 Discussion

Male *Litoria fallax* were identified to have a complex vocal repertoire that differs across social contexts. High acoustic variability has been achieved at three levels by this species. First, the species generated three temporally distinct note types with unique signal properties (monophasic signals). Two of these, the monophasic ramp call notes (*Type B* and *Type C*), can be described as graded vocalisations that provide increased variability at this level. Second, these note types can be combined into complex diphasic signals, in which some modification to the structure of these note types is apparent. The results of female-choice experiments are consistent with the hypothesis that these diphasic signals might target two different receiver categories. A third level of acoustic variability is achieved by the grouping of different call types (monophasic and diphasic) into composite call group units. To my knowledge, no other Australian Pelodrydid frog is identified with such multi-level complexity.

2.5.1 Note and call characteristics

The three note types I have identified here are in accordance with the earlier description of the "mating call" of the species (Straughan, 1969), with the identification of two extended ramp call/note types and a much shorter call/note type. However, the terminology used in the earlier study is different to mine, as is the case in other bioacoustic studies of anurans (Kohler et al., 2017; Toledo et al., 2014). Nevertheless, our results do not support the original description that the "mating call" is comprised of two diphasic ramp call components and the shorter click sound is just an occasional addition. Indeed, my results show that the *Type A* and *Type AA* calls are the

most prominent call types in the repertoire representing more than 60% of the total calls in my analysis. Furthermore, initial choice experiments on the phonotaxis of female *L. fallax* also suggest a higher preference towards the shorter click sound, and probably explains the more frequent use of that call type by the males. The previous description of the repertoire of the species was conducted in Townsville from populations that exist in sympatry with the sister species *L. bicolor.* It remains to be determined whether differences between my study and Straughan (1969) reflect alteration of the acoustic repertoire by interspecific acoustic interference/interaction (Gröning & Hochkirch, 2008; Littlejohn & Martin, 1969) of the two sister species, or if there are other contributing factors such as geographic variation (Nski & An, 1999).

2.5.2 Diphasic call arrangement

The second level of complexity that was identified in the species is the ability to organise all three note types into diphasic two-note calls. Diphasic call types of this species consist of two call units where a shorter interval is present between call units than that of adjacent calls. Hence, I have used the call centred approach in nomenclature following Kohler et al., (2007), to minimize the confusion of differentiating call types. Similar behaviour is observed in species such as E. coqui, Australian myobatrachid G. victoriana and African hyperoliid A. brachycnemis, where the first note of their two-part call is modified to convey an aggressive signal and the latter is used to attract female frogs in the reproductive process (Backwell, 1988; Littlejohn et al., 1985; Narins & Capranica, 1978). In some species with diphasic advertisement calls like Afrixalus brachycnemis and Dendropsophus nanus (Teixeira et al., 2016), the two units of the call are not permanently linked but are produced in association with the social arrangement of the habitat. Therefore, males might produce one component for a longer period (Backwell, 1988). When a complex call repertoire of a species is comprised of more than two call notes, which are mostly old-world species, they tend to arrange their calls in a graded manner (Smith & Roberts, 2003). Diphasic and graded calls might have evolved more than once from a monophasic call in different anuran groups, presumably functioning as an added advantage to the repertoire by

increasing efficacy of the information propagation (Backwell, 1988), where male frogs are capable of selectively modifying the proportion of each call component (Arak, 1983). Furthermore, the modified *Type A* note as the second note of the complex diphasic call types, *Type BA* and *Type CA* of *L. fallax* increases the total amplitude of the call by possessing a higher amplitude than the first note component similar to that of túngara frogs (Gridi-Papp et al., 2006). Further investigation using playback experiments of the species might reveal the importance of the modified *Type A* note.

The results of my choiceexperiment examining female preference for each part of the two-part call is in accordance with that of *E. coqui* and *G. victoriana* (Littlejohn & Harrison, 1985; Narins & Capranica, 1978) whereby female frogs are actively attracted to the shorter second part of the call (*Type A*) over the first part of the call. In contrast, the shorter second component of the call of male túngara frogs, the chuck component, is unable to elicit phonotaxis from females when presented separately to the introductory whine, whereas females will respond to an isolated whine component (Farris et al., 2002; Ryan, 1980; Ryan et al., 1990). Furthermore, females of this species maintain preference for the complex calls over the simple call of the whine, even in experiments where the whine is modified to increase the total energy (Ryan & Rand, 1990), or the duration is adjusted to be similar to that of a complex call (Wilczynski et al., 1999). It will be worthwhile to investigate how *L. fallax* females would react to the complex call types over simple monophasic calls.

2.5.3 Advertisement call types of *Litoria fallax*

The advertisement call of a species is generally defined as the most emitted call type of the repertoire of a frog species (Kohler et al., 2017; Wells & Schwartz, 2007). Most often produced by males and only occasionally by females (Wells, 1977), it typically possesses static call properties and as such can be used for taxonomic identification. As a species with graded call variation, it was challenging at first to define an advertisement call for *L. fallax*. My results suggest that variance in duration and pulse rate of note *Type B* and *C* is lower in the complex

diphasic arrangement when compared with the monophasic call arrangements, so the use of diphasic call arrangements might be more appropriate for taxonomic purposes for *L. fallax*. However, note *Type A* is modified in the complex arrangements and mostly emitted as monophasic calls in the repertoire. Therefore, the monophasic call *Type A* is more suitable to ascribe as the advertisement call of the species. Future playback studies on the other call types will be helpful in further confirming this.

2.5.4 Aggressive call types of *Litoria fallax*

Anuran species that have large breeding aggregations often defend their calling sites from their conspecifics using characteristic vocalisations (Wells & Schwartz, 2007). A territorial call emitted in the presence or absence of a rival male frog (Toledo et al., 2015) is one such aggressive vocalisation and is common in the repertoires of many species. The first notes of the two diphasic call *Type BA* and *CA* were more prevalent in chorusing, were common in the repertoire and at least the first note of call *Type BA* was unable to elicit phonotaxis from females in our choice experiment. Therefore, I speculate that the first note of call *Type BA* and *CA* are more likely functioning as a long-range territorial call. Being diphasic allows the calls to convey the aggressive information without compromising the emission of the note type that is important in attracting a potential mate. This is an advancement for a species with high frequency calls that have the tendency to trigger the emission of territorial calls even from a distance (Haddad, 1987) that might impact adversely on the reproductive behaviour.

The monophasic calls *Type B* and *Type C* of *L. fallax* are graded signals with similar structure to the first call notes of call *Type BA* and *Type CA*. However, the dominant frequency reduces, and the number of pulses and call duration increases in a continuous manner toward the creation of the monophasic *Type B* and *C* calls. Graded acoustic communication involving an increase in call note duration may play a role in spacing of neighbouring individuals and thus represents a short-range aggressive call emitted by male frogs when an intruder male comes too close to its calling perch (Toledo, 2015). This type of call is similar to the encounter call described

in some species (Toledo et al., 2014) in which the signal has a short range emission directly targeting the intruder individuals and which may display the physiological condition (Schwartz, 1994) or interrupt the calling signals of the intruder male (Schwartz et al., 2001). Although graded acoustic communication systems, such as in L. fallax, make it hard to categorise call types, it is worthwhile nonetheless to compare the characteristics of categorised signal systems with my data for L. fallax. I have observed the monophasic call Type B and C to be used in close range communication with intruder males. Several species of frogs are known to alter the dominant frequency in aggressive calling behaviour (Bee & Bowling, 2002; Perrill & Bee, 1996; Wagner Jr, 1992), and this has also been observed in species producing graded acoustic signals such as Leptodactylus albilabris (Lopez et al., 1988), Acris crepitans (Wagner, 1989), Hyperolius marmoratus (Grafe, 1995), and Rana clamitans (Owen & Gordon, 2005). I have observed similar behaviour when producing monophasic Type B and C calls of L. fallax in the presence of a rival male. The duration of the first note of both diphasic Type BA and CA calls progressively increased while inhibiting the emission of the secondary A notes when the two males get closer and the dominant frequency of the diphasic call is decreased significantly in the making of monophasic calls. Such behaviour is also observed in the Australian frog G. victoriana (Littlejohn & Harrison, 1985; Scroggie & Littlejohn, 2005) with the two-part advertisement call. These observations necessarily imply that the monophasic call Type B and C in L. fallax are functioning as close-range aggressive signals. Although rather than trying to establish a hard line between the aggressive encounter calls and the advertisement calls it is more suitable to consider the advertisement calls Type BA and CA and the encounter call Types B and C as being at opposite ends of a graded signal (Owen & Gordon, 2005). Graded transition from advertisement call into an aggressive call has been observed previously in A. crepitans (Wagner, 1989), H. marmoratus (Grafe, 1995) and R. clamitans (Owen & Gordon, 2005), but further work is needed to elucidate the context and possible internal and external factors of call type transition in anurans.

Given that the study species frequently inhabit urban areas, anthropogenic sound and light can have an influence over the communication signal production (Parris et al., 2009). Traffic

noise (Parris et al., 2009) and air plane sounds (Kruger & Du Preez, 2016) have been known to modulate temporal and spectral properties of frog call signals. These factors are therefore also important to consider in future studies of *L. fallax*.

2.5.5 Call type patterning of Litoria fallax

A fixed temporal order in the production of note types is observed in anurans that possesses multi-note calls (Larson, 2004; Littlejohn et al., 1985; Ovaska & Caldbeck, 1997; Toledo et al., 2015), as well as in other taxonomic groups such as birds (Freeberg, 2008). The transition matrix for L. fallax in the present study revealed that note Type B and Type C both in simple and complex call types is always in the direction of Type B to Type C. Additionally, the Type A note has the highest probability in both initiating and ending of call groups in complex settings (Figure 2.6). Considerable variability in call sequences without a fixed note order is usually common in old world anuran species with a larger number of call types, such as Boophis madagascariensis (Narins et al., 2000). Our focal species has a call patterning that is neither similar to hylids nor the old world species with complex yet ordered organisation. My data for L. fallax are more similar to the call patterning of the anuran northern leopard frog, Rana pipens, which is known as the species with the most complex advertisement calls of the temperate region (Larson, 2004). Specifically, male *R. pipens* also use three different note types in various combinations to generate unique call types. I have considered that the call types mentioned in the repertoire of this species is equivalent to the call groups that were described herein for L. fallax. My focal species has an additional component that enhances the complexity of their repertoire when compared to that of *R. pipens* by producing diphasic calls with the basic note types and then organising them into unique combinations. Whether the note order observed in species with complex call repertoires has a specific functional significance or a result of a physiological limitation is yet to be considered. Indeed, L. fallax might prove to be a useful model for understanding the evolution of complex call repertoires of anuran species.

2.5.6 Effect of acoustic context on complexity of the call sequences

Social context could influence the complexity of the repertoire of a species (Freeberg et al., 2012; Pollard & Blumstein, 2012), with call complexity partially driven by sexual selection (Kershenbaum et al., 2016). Anurans have a complex social environment with respect to group size and density. In my study, I have considered the social context in terms of the social unit size and the unit density, which have a profound impact on the calling of frogs. My results suggest that information content varied with the acoustic environment. Chorus-close context had the highest entropy value among all the contexts. *Litoria fallax* is a species with high density assemblages and as such their calling behaviour might be affected by both the number of conspecifics calling and the distance to the nearest neighbours. However, there was no significant difference obtained for the mean entropy values between chorus-close and chorus-distance, suggesting no influence of the spatial cue of the distance. Moreover, a similar result was seen in the duetting context, when two individuals communicate alternatively with each other (duetting-close and duetting-distance), no significant difference was found between the entropy values.

A significant difference was found between the mean entropy values of chorus-close context and both duetting acoustic contexts. This result suggests that the number of individuals involved in the process of communication is more important in determining the complexity of the acoustic sequence, than the spatial arrangement of the signalling individuals. My results are in accordance with a previous study on the Neotropical frog *Dendropsophus minutus*, which also possesses a complex acoustic repertoire but where there was no significant correlation between the entropy values and the distance to the nearby calling male (Morais et al., 2012). Even though studies on the relationship between social complexity and communicative complexity has been studied in several animal taxa (Pollard & Blumstein, 2012), such studies on amphibians are scarce. Future research on anurans must be mindful of the important role played by the social environment in influencing different aspects of the communication system. For example, The

size of the alarm call repertoire of yellow-bellied marmots, *Marmota flaviventris* depends on the social demographic complexity, while the individual complexity is a result of social group size (Blumstein & Armitage, 1997). I have used the categorical acoustic contexts in my study as I found it difficult to measure the distance to the nearby calling male given the smaller size and high-density aggregation of *L. fallax*. Since the social environment is known to be important in the call emission (Bosch et al., 2000; De la Riva et al., 1995; Wells, 1988), as well as the arrangement of call notes in anurans, description of the advertisement calls of species without considering the social context at which it is emitted might cause errors (Toledo et al., 2014).

2.5.7 Summary

I have confirmed three distinct note types of the focal species with novel information on three simple note types (*Type A*, *Type B* and *Type C*), three diphasic call types comprising two note types (*Type AA*, *Type BA* and *Type CA*) and graded variations of each. Furthermore, a speciesspecific note type order is identified in the call sequences of the species. The information content in the calling repertoires of *L. fallax* was found to be influenced by the social environment of the habitat. The choice experiments performed between the note types, *Type A* and *Type B* have reviled a stronger preference of female frogs of this species, towards the *Type A* the other. The current study highlights the importance of reviewing the described call repertoires of anuran species in different social contexts in order to identify the best suited call and note types in taxonomic and systematic purposes. The novel information generated from this study on the unique and complex vocal repertoire of *L. fallax* should stimulate future comprehensive studies on the evolutionary and functional significance of the complex vocal signals of anurans.

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2.7 Supplementary material



Figure S2.1 | (a) Principal component analysis of call characteristics between monophasic note *Type A* (red), note *Type A* of the diphasic call *Type BA* (green) and note *Type A* of the diphasic call *Type CA* (blue). (b) Principal component analysis of call characteristics between monophasic note *Type B* (red) and note *Type B* in the diphasic call *Type BA* (blue). (c) Principal component analysis of call characteristics between monophasic note *Type C* (red) and note *Type C* in the diphasic call *Type CA* (blue).

Note characteristic	PC1	PC2
Note duration	0.576	-0.189
Max frequency	0.424	-0.205
Pulse number	0.459	-0.484
Inter-pulse interval	0.431	0.510
Pulse rate	-0.303	-0.655

 Table S2.1 | PCA loadings differentiating the three note types of L. fallax.

Chapter 3

Geographic variation in advertisement note types of *Litoria fallax*

3.1 Abstract

Geographical variation in behavioural traits related to mate choice and sexual selection of species could lead to reproductive isolation and might reflect an initial stage of speciation. Species with broad distribution ranges relative to their dispersal ability tend to possess geographical variation in behavioural traits that are shaped by either or both gene flow and selection by local ecological traits. The Australian pelodryadid frog Litoria fallax is an excellent model species for understanding the driving forces of geographical variation of a behavioural trait, given its broad geographical distribution along the east coast of the continent. With the aim of investigating whether there is any geographic variation of the advertisement call characteristics of this species, in this study I have considered both the natural and invasive populations of the focal species. All three note types of the advertisement call have been analysed to see whether genetic grouping, environmental traits (temperature), ecological traits (canopy cover, waterbody type, noise level, presence of artificial noise), and acoustic social traits (number of actively calling sympatric species) predicted geographical variation of spectral and temporal characteristics. The results revealed that divergence of acoustic properties of the repertoire is significantly related to genetics, particularly dominant frequency and pulse rate of all three note types. Results also provided little support for influence of environmental conditions (temperature) and the number of sympatric species. However, relationships with ecological traits (waterbody type and canopy cover) were found to be effecting the note characteristics of all three notes. In addition, I conducted a playback study to examine whether female frogs from one population (Melbourne) showed preferences to local verses foreign (Townsville and Newcastle) advertisement call stimuli and found that females only approached the calls of local males, suggesting they are sensitive to population differences in call structure. In summary, this study demonstrated that geographic variation of advertisement calls of L. fallax is present and shaped by genetics and local selective pressures of the habitat. As females showed a clear preference for local calls over those from other populations, this study has

opened up several future study opportunities in investigating the reproductive isolation of this

species.

3.2 Introduction

Animal behaviour is widely used as a tool in species recognition (Curé et al., 2012; Ratciliffe & Grant, 1983; Thresher, 1976). However, behavioural traits often represent the interaction between genotypical variance and environmental factors (Hunt et al., 2003; Via & Lande, 1985). This is further complicated when wide-ranging species exhibit geographic variation (Amezquita et al., 2009; Barquero et al., 2015; Foster & Endler, 1999), which is driven by one or both of genetic composition and the environmental characteristics in which the behaviour is expressed. It has been suggested that geographical variation in a behavioural trait that is directly related to sexual selection, like the acoustic signalling of anurans, could be the initial step of speciation (Endler, 1977; Velásquez, 2014). Genetic drift alone is able to explicate geographical variation in some behavioural traits (Barquero et al., 2015; Päckert et al., 2004; Velásquez et al., 2006; Ruegg et al., 2006). In such cases, directional selection of sexual signals takes place under ecological constraints imposed by their local habitat and the requirement to maximise signal propagation to conspecifics (Pröhl et al., 2006; Ryan et al., 1990).

Animal signals are a useful system to explore phenotypic variation across geographic locations as they are often tractable to quantitative measurement and are locally consistent (Foster & Endler, 1999). The advertisement calls of anurans are widely used for species identification as they are species-specific (Gerhardt & Huber, 2002; Wells, 1977), and reportedly are governed by the genetics of the species (Gerhardt & Huber, 2002; Littlejohn, 2009). However, intraspecific variation in call characteristics of widely distributed species could be a consequence of both genetic drift and the adaptive characteristics developed in coping with the environmental variation (van Dongen et al., 2010; Vargas-Salinas et al., 2014).

Local environmental characteristics are known to influence acoustic signalling by ectothermic anurans (Gerhardt & Huber, 2002; Velásquez, 2014), although not for all call parameters. For example, pulse rate is highly dependent on ambient temperature (Schneider, 1977), but call duration and call rate is less affected (Gerhardt & Huber, 2002), and no relationship with temperature is observed for spectral parameters of anuran calls (Velásquez, 2014). The propagation of acoustic signals is also altered by vegetation structure (Ryan et al., 1991). For instance, forest and open habitat populations of Acris crepitans generate calls that differ in frequency, duration and pulse rate (Ryan & Wilczynski, 1991). Not only do habitats shape the structure of their calls, but also the neural apparatus that are used to detect them and filter environmental noise (Wilczynski et al., 1992) which can also be site specific. Habitats with vegetation cover also provide environmental heterogeneity, providing different levels of perching and calling sites to heterospecific frog assemblages rather than open habitats where species discrimination is solely depending on the advertisement calls of the species (da Silveira Vasconcelos & de Cerqueira Rossa-Feres, 2008; Duellman & Pyles, 1983). A key feature of the habitat for many species is the waterbody type, which can play a vital role in the behavioural adaptations of aquatic breeding anurans. If the conditions are suitable, there is the opportunity for an extensive period of calling, unlike the shorter window in the temperate region (Wells & Schwartz, 2007). Such a change in calling behaviour might be also manifested in fine scale acoustic structure.

Many tropical species of frogs with aquatic breeding behaviour (*Litoria chloris* and *Litoria xanthomera*; (Morrison et al., 2001) are known to form explosive breeding aggregations at temporary ephemeral rain pools, creating a high degree of competition among male frogs and could cause reproductive character displacement between species (Gerhardt, 1994; Higgie et al., 2000), including divergence in acoustic communication (Howard, 1993). This process can lead to diversification of signals within populations where sympatry occurs, compared with allopatric populations, in order to ensure reliable detection and processing by intended receivers (Lemmon, 2009). Furthermore, if call characteristics change due to inter-species competition, widely distributed species will encounter different species assemblages across their distribution and adjustments might vary in different ways.

It is important to consider not only measurable differences in acoustic structure, but also behavioural responses of receivers to these differences as variation may not be functional. In anurans, communication systems play an integral role in speciation by providing the opportunity to identify conspecifics in a mate choice context. Several studies have shown that inter population variation can be found in male reproductive signals (Boul et al., 2007; Masta & Maddison, 2002). Moreover, , long term divergence in sexual signals can lead to behavioural isolation and consequently lead to reproductive isolation when conspecifics from another population lose the ability to identify them as a potential mate (Coyne & Orr, 2004). Therefore, knowing that preference of female frogs could be influenced by geographical variation in call characteristics might indicate an early stage in the speciation process (Pröhl et al., 2006) as a result of the pre-zygotic isolation via sexual selection (Gray & Cade, 2000). However, inconsistent results have been obtained in experiments testing variation in mate recognition in species with geographic variation in sexual signals. In some studies, the choice made by females coincides with genetic variation (Pröhl et al., 2006) while in other studies no relationship is reported (Ryan et al., 1992). Studies that manipulated different components of the mating calls of túngara frogs, Physalaemus (=Engystomops) pustulosus, suggests that female frogs measure different components of the call unequally (Bernal et al., 2007; Ryan & Rand, 2003). Even with such diverged genetic lineages in L. fallax, no previous studies have focused on the communication system further in fully elucidating the possible correlation between the mating signals and the genetic or geographic distance.

The eastern seaboard of Australia exhibits a diversity of vertebrate fauna (Cogger, 1992; James & Moritz, 2000; Strahan, 1995). The vegetation structure and subsequent faunal distribution of this area has been affected by past climatic changes (Nix, 1981) as well as by the Pleistocene sea level fluctuations that resulted in the opening and closing of land bridges between New Guinea and the mainland (Chappell & Shackleton, 1986; Kikkawa et al., 1981). The east coast is comprised of a matrix of dry open forest lands and pockets of remnant wet rainforests (Byrne et al., 2011) that has resulted in two unique compositions of faunal diversity

shaped by the habitat structure. The closed wet forests are hypothesised to have been used as refugial grounds during drier periods of the Neogene and Pleistocene (McGuigan et al., 1998; Schäuble & Moritz, 2001). Hence, the diversification of restricted species in these isolated places tend to be independent from each other due to population fragmentation (James & Moritz, 2000). This has been observed in three wet rainforest dwelling frog species (*Litoria rheocola, Litoria nannotis* and *Litoria genimaculata*) isolated in north-eastern and southern Queensland separated around the Black Mountain Corridor, where they showed deep phylogeographical breaks among the two populations (Schneider et al., 1998) equivalent to that of palaeoclimatological modelling (Joseph et al., 1995). In contrast, the open forest or grassland dwelling species of the east coast have expanded their range during the major climatic changes, where the historical barriers along the region had a profound impact on population divergence (James & Moritz, 2000; Schäuble & Moritz, 2001). Therefore, there has been several phylogeographical studies of different taxa from this region on both rainforest and open forest species including the focal species of the present study *Litoria fallax* (James & Moritz, 2000).

Litoria fallax is a widely distributed open forest species found in wetlands along the eastern coast of Australia. This wide distribution provides a great opportunity to elucidate how natural barriers are acting upon the evolution of different genetic lineages, and how significant intraspecific genetic divergence (James & Moritz, 2000; Schäuble & Moritz, 2001) subsequently results in variation in behavioural characteristics (Foster & Endler, 1999). Previous phylogeographical research has revealed two separate mitochondrial lineages of *L. fallax* with a large magnitude of sequence divergence (11.1–12.5%), separated mainly from the split running across the McPherson and Main ranges in northern NSW, that is believed to have happened during the Pliocene period (James & Moritz, 2000). These are identified as 'northern' and 'southern' lineages depending on the side of the split, although some exceptions to this pattern have been observed suggesting gene flow via ancient or current dry forest corridors. Another phylogeographical split has also been proposed to the northern lineage at the Burdekin Gap (James & Moritz, 2000), a dry belt of savanna that separates the coastal woodlands of the

Atherton Plateau and central Queensland (Schäuble & Moritz, 2001). However, this is a rather shallow split relative to the McPherson range split, yet significant enough to yield a sequence divergence of 5% (James & Moritz, 2000).

Litoria fallax is therefore an ideal organism to investigate the relative contribution of genetic and environmental effects on signalling as it is a widespread species that shows genetic variability across its range and occupies varying ecological environments. Compared to other taxa, studies on how anuran signal diversity is influenced by environmental variation are scarce (Pröhl et al., 2006; Ziegler et al., 2011). My aims for this chapter were threefold:

- 1. To examine whether note characteristics of the complex advertisement call of the species varies according to the different genetic clades of the species. Here, I examine calls from two sites within the southern clade and separate the northern clade as per the proposed split at Burdekin Gap into two, sampling at Townsville and Brisbane. A fourth group were investigated for which their genetic alignment is unknown. These sites were in the greater Melbourne area and are outside the natural range of the species and have established due to accidental translocations.
- 2. To examine whether abiotic factors (temperature and humidity) and ecological factors of the habitat (waterbody type, vegetation cover, number of sympatric species present, noise level and artificial noise) predict variation in note characteristics.
- 3. To determine whether female *L. fallax* exhibit preferences for calls representing local or 'foreign' populations in a phonotaxis experimental paradigm.

Sexual signals are an important component of sexual attraction/mate choice and can provide initial information on the speciation process. Elucidating possible factors contributing to geographic variation in communication signals and how female choice is variable amid the divergence is important in predicting possible reproductive isolation of an animal species.

3.3 Methods

3.3.1 Field recordings

I recorded 115 calling male *L. fallax* from 18 study sites (Table 3.1, Figure 3.1) during the summer months of 2018 and 2019. Fourteen sites were within the natural distribution of the species and four sites were from greater Melbourne, which is outside the natural range of the species. Calling male frogs were located at night between 20:00 to 01:00 h by their characteristic call, and frogs were recorded from a distance under a red light to minimise disturbance. Approximately one minute of recording was obtained from each individual frog using a Sennheiser ME66 directional microphone with a foam windscreen connected to a Roland R-26 portable audio recorder using a sampling rate 44.1kHz (16 bits resolution). A recording distance of 1 m was maintained between the snout of the calling male and the tip of the microphone for all recordings. Gain settings of the microphone were adjusted according to the sound level of each environment and was not adjusted until the recording was completed. Abiotic factors such as temperature and humidity were recorded from a pocket weather meter (Kestrel 4000) immediately after each recording.

Each study site was categorised according to waterbody type (temporary or permanent), plant cover (with canopy cover or without canopy cover) and artificial noise (presence or absence; Table 3.1). Additionally, the noise level of the habitat one metre away from the recorded individual was noted using a sound level meter (QM-1589, Digitech) after each recording. Anthropogenic noise in our study sites was observed to be generated from several different sources such as traffic, occasional trains and airplanes, people in playgrounds, noise generated by people on walking paths and by heavy machinery in roadwork and in industrial areas. I categorised each site as either disturbed or undisturbed by anthropogenic noise. Sites deemed to be disturbed by anthropogenic noise implied noise was present and a major contributor to ambient noise levels at the time of recording. Seven sites were identified to be not disturbed by anthropogenic noise while 11 sites were affected by noise.

Genetic clade	Habitat no.	Waterbody type	Canopy cover	Sympatric species calling	Artificial noise	GPS coordinates
Townsville	1	Permanent	Absent	0	Present	19° 18.552' S 146° 45.999'E
	2	Permanent	Absent	7	Present	19° 10.547' S 146° 33.208' E
	3	Temporary	Present	4	Present	19° 10.580' S 146° 33.144'E
	4	Temporary	Absent	0	Present	19° 18.959' S 146° 49.419'E
	5	Temporary	Present	3	Absent	18° 58.646' S 146° 17.621'E
Brisbane	6	Temporary	Absent	2	Absent	27° 23' 10.9"S 152° 51' 37"E
	7	Permanent	Absent	1	Present	27° 24' 3.9"S 152° 50' 59.9"E
	8	Permanent	Present	0	Absent	27°24'1.5"S 152°50'41"E
Southern	9	Temporary	Absent	0	Present	29°39'20.6"S 152°56'06.1"E
	10	Permanent	Present	3	Absent	30°58'31.2"S 151°35'57.9"E
	11	Permanent	Absent	2	Absent	32°51'48.5"S 151°44'33.6"E
	12	Permanent	Absent	1	Present	32°53'13.7"S 151°42'19.6"E
	13	Permanent	Absent	0	Present	32°53'19.8"S 151°42'31.2"E
	14	Permanent	Absent	2	Present	34°29'36.6"S 150°47'16.1"E
Melbourne	15	Permanent	Present	0	Present	37°47'17.9"S 145°3'28.1"E
	16	Permanent	Absent	1	Present	37°44'49.9"S 145°5'8.52"E
	17	Permanent	Present	0	Absent	37°46'44.3"S 145°2'54.2"E
	18	Temporary	Present	0	Absent	37°46'47.6"S 145°2'59.1"E

Table 3.1	Details of stud	y sites visited to	record and quar	ntify acoustic sig	gnalling of Litoria	fallax.
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Figure 3.1 | Location of study sites visited to record and quantify acoustic signalling of *Litoria fallax*. Enlarged views are shown for the sampling sites in Townsville, Brisbane, Newcastle and Melbourne (Note: a single circle represents three separate sites in Newcastle). There was only one site in the Wollongong area.

3.3.2 Acoustic analysis

All three unique advertisement note types identified and described in Chapter 2 were analysed herein: namely the monophasic calls Type A and diphasic calls Type BA and CA. Notes Type B and Type C included in the diphasic calls of the species were used in the present analysis as I showed in the previous chapter that their properties were more static than that of the monophasic calls. Monophasic note Type A was analysed because of the static properties and the high frequency of its use. I extracted spectral and temporal call characteristics (see Table 2.1) for each of the three distinct note types using Raven Pro v1.5 software (Cornell Laboratory of Ornithology, Ithaca, NY USA). Three temporal properties of the notes (note duration, note rise time and number of pulses per note), three temporal properties of the pulses (pulse duration, inter-pulse interval and pulse rate) and a spectral property (note dominant frequency) were measured. Terminology for these characteristics follows previous literature (Bee et al., 2013; Wijayathilaka & Meegaskumbura, 2016). The pulse with the highest amplitude in a call was used in measuring the pulse temporal properties. Raven Pro's waveform display was used for measuring temporal properties, whereas the spectrogram function was used for measuring spectral properties by averaging the spectrum over the entire duration of a call (256 point fast fourier transform using a Hanning window).

3.3.3 Statistical analysis

Descriptive statistics for each call property (mean, standard deviation, and coefficient of variation) were computed using Microsoft Excel 2010, with median and interquartile range calculated for indivisible characters (number of pulses per call). I ran separate linear mixed models for each call property for each of the three note types using the *Ime* function from the *nIme* package (Pinheiro et al., 2018) in the R statistical environment (R Core Team, 2018). My predictor variables included genetic clade, waterbody type, canopy cover and artificial noise as categorical predictor variables, and temperature, noise level and number of sympatric species as continuous predictor variables. I performed all possible subsets regressions (Burnham &

Anderson, 2004) using the *dredge* function in the package *MuMIn* (Barton & Barton, 2015), and then used an information theoretic approach to determine the weight of evidence for the best model; variables featuring in models within 2 Δ AIC points of the best model were selected. The significance of predictor variables was determined using the *anova* function, while the *r.squaredGLMM* function from the *MuMin* package was used to asses variance explained by the model. The effects of significant continuous variables were explored graphically by plotting estimated marginal means using the *emmeans* function from the *emmeans* package (Lenth et al., 2019).

3.3.4 Female phonotaxis experiment

I wished to examine how females responded to local and foreign male advertisement calls using a classic two choice phonotaxis paradigm. The experiments were conducted in Melbourne hence the local acoustic stimuli were prepared with the population mean call characteristics of Melbourne frogs. Townsville and Newcastle populations were selected as the foreign call stimuli, representing both genetic groups for this study.

3.3.4.1 Stimulus preparation

I chose to use a population average call to represent *L. fallax* advertisement calls. As the local sound stimuli for this experiment I used call recordings obtained from 30 male frogs in the Melbourne region, collected using the same method outlined in chapter two with average call properties calculated from ten acoustic variables. Following inspection of the distribution of call characteristics, I selected calls that closely matched the mean values of each call component. I have used the sound recording and editing software Audacity (version 2.3.3) to create stimuli of approximately 15 seconds each, adjusting some call characteristics after broadcasting in the experimental chamber (see below) to ensure consistent sound pressure level. The same method was used for Melbourne, Townsville and Newcastle call recordings. Each stimulus was five seconds in duration and contained five *Type A* monophasic call types and the two diphasic calls containing *Type B* and *Type C* notes in the pattern A-A-A-A-BA-CA (this is the most common

note sequence in a call group, observed in the recordings of *L. fallax* while generating the transition matrix in chapter two). This was repeated three times in a loop to yield a 15 s stimulus for each call type.

3.3.4.2 Study animals

Fourteen gravid females from Melbourne, Victoria, were captured for this study. Female frogs were captured at night in the field during December 2019 and March 2020 and transported to the research facility at La Trobe University, Bundoora, where they were placed in separate tanks until the subsequent evening when phonotaxis experiments were initiated. All frogs participated in other experiments (Chapter 2, 4)

3.3.4.3 Procedure

Trials were conducted in the same anechoic chamber (1.5 m³) used in Chapter 2, with the two speakers (FR7-4 VISATON Germany) once again placed at 90° angles to the fixed releasing point. I used the same custom-designed releasing apparatus described in Chapter 2 (Figure 2.2), opening the walls after an acclimation period of five minutes during which time the test stimuli were broadcast antiphonally. As in the previous study, the sound pressure level (SPL) from both speakers at the releasing point of the frog was maintained at 65 dBA (fast, high and linear weighting). One stimulus was presented at each speaker, with speaker allocation randomised and counterbalanced across the cohort. Stimuli were presented in an alternating fashion, with the order of presentation also randomised for a given individual and counterbalanced across the cohort. One female frog was presented with the local, Melbourne call stimulus and one of the foreign call stimuli (Townsville or Newcastle) on day one. On day two, the same individual was presented with the alternate foreign call and the Melbourne call. Each session was filmed using a Canon XA40 with infrared capability, which was fixed to the roof of the chamber and connected to an external monitor for remote viewing.

3.3.4.4 Analysis

I reviewed video footage of each session and recorded the first phonotactic choice response of each female spent within 40 cm of a speaker using markings painted on the arena (see Figure 2.2). If a female frog remained motionless in the release apparatus, or did not approach either speaker for 20 mins, they were considered unresponsive and removed from the analysis. I used binomial tests to determine whether choices were significantly different to 0.5 (equally likely to choose either call).

3.4 Results

3.4.1 Field recordings

A summary of the results of regression analyses for notes *Type A*, *Type B* and *Type C* are presented in Tables 3.2, 3.3 and 3.4 respectively. In each case, the note characteristics in the final model are presented along with their relative significance values. The variance explained by the set of fixed effects, as well as the full model, are also provided. In the following sections, I examine the results separately for each note type.

Table 3.2	Outcome from	linear mixed	effects mode	ls for note	Type A.
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Note characteristic ^a	Predictor variable	Df ^c		Df ^c		F-ratio	P-value	Variance
	in final model ^b	Num	Den			explained ^a		
Dominant frequency	Genetics	3	109	17.3	<.0001	0.29 (0.71)		
	Waterbody type	1	109	9.6	0.0024			
	Artificial noise	1	109	13.8	0.0003			
Note rise time	Sympatric species	5	109	2.689	0.0248	0.035 (0.25)		
Number of pulses	Canopy cover	1	112	7.757	0.0063	0.03 (0.27)		
Note duration	Genetics	3	110	4.033	0.0092	0.07 (0.73)		
Pulse duration	(Intercept only)	1	161	25949.41	<.0001	0 (0.50)		
Inter-pulse interval	(Intercept only)	1	161	17837.77	<.0001	0 (0.76)		
Pulse rate	Genetics	3	108	13.19	<.0001	0.16 (0.51)		

^a Separate models constructed for each note characteristic

^b Full set of predictor variables before model selection: Genetics, Waterbody type, Canopy cover, Artificial noise, Temperature, Noise level, Number of sympatric species. See text for details

^c Numerator (Num) and denominator (Den) degrees of freedom

^d Variance explained by fixed effects and the full model (in parentheses). See text for details

Note characteristic ^a	Predictor variable	Df ^c		F-ratio	P-value	Variance
	in final model ^b	Num	Den			explained ^d
Dominant frequency	Genetics	3	72	19.8	<.0001	0.45 (0.85)
Note rise time	Waterbody type	1	78	3.2025	0.0774	0.02 (0.30)
Number of pulses	Genetics	3	72	7.869	0.0001	0.17 (0.54)
Note duration	Canopy cover	1	75	6.5826	0.0123	0.07(0.73)
Pulse duration	Genetics	3	73	0.840	0.4743	0.16 (0.47)
	Canopy cover	1	73	16.57	0.0001	
	Artificial noise	1	73	1.0875	0.3004	
Inter-pulse interval	Waterbody type	1	75	5.005	0.0282	0.45 (0.85)
Pulse rate	Genetics	3	73	11.60	<.0001	0.25 (0.57)
	Canopy cover	1	73	6.15	0.0916	

 Table 3.3 | Outcome from linear mixed effects models for note Type B.

(superscripts a-d as per Table 3.2)

 Table 3.4 | Outcome from linear mixed effects models for note Type C.

Note characteristic ^a	Predictor variable	Df	C C	F-ratio	P-value	Variance
	in final model ^b	Num	Den			explained ^d
Dominant frequency	Genetics	3	76	20.50	<.0001	0.43 (0.81)
Note rise time	Waterbody type	1	78	3.2025	0.0774	0.02 (0.30)
Note duration	Genetics	3	75	12.407	<.0001	0.25 (0.49)
	Waterbody type	1	75	6.37	0.0137	
Number of pulses	Canopy cover	1	77	9.50	<.0001	0.07 (0.72)
	Waterbody type	1	77	54.28	0.0029	
Pulse duration	Canopy cover	1	77	5.427	0.0224	0.07 (0.51)
	Waterbody type	1	77	4.516	0.0368	
Inter-pulse interval	Genetics	3	75	38.57	<.0001	0.54 (0.76)
	Artificial noise	1	75	21.03	<.0001	
Pulse rate	Genetics	3	74	56.86	<.0001	0.63 (0.81)
	Canopy cover	1	74	15.45	0.0002	
	Waterbody type	1	74	7.64	0.0072	

(superscripts a-d as per Table 3.2)

3.4.1.1 Note Type A

A summary of the results of mixed effects regression models for note *Type A* are shown in Table 3.2. Genetic clade was a significant predictor of dominant frequency, note duration and pulse rate. However, none of the call characteristics here showed clinal pattern. Brisbane frogs produced significantly higher dominant frequencies than each of the other genetic groups, while Townsville frogs produced significantly lower dominant frequencies than both southern and Melbourne frogs (Figure 3.2a). Townsville frogs produced significantly longer notes than frogs from each of the other clades (Figure 3.2b), while Brisbane frogs generated the lowest pulse rate and southern frogs produced the highest pulse rate (Figure 3.2c).

Waterbody type, artificial noise and canopy cover were also significant predictors of one note *Type A* call characteristic. Calls of frogs from permanent waterbodies and in the presence of artificial noise produced significantly higher dominant frequencies (Figure 3.2d, e respectively). The number of pulses produced by frogs in locations with canopy cover were significantly lower than by frogs without canopy cover (Figure 3.2f). I also found that note rise time varied depending on the number of calling sympatric species. The data suggest rise time is higher when there is higher species diversity (Figure 3.3).



Figure 3.2 | Estimated marginal means from mixed effects models for significant predictor variables of note characteristics for note *Type A*. (a) Dominant frequency, (b) note duration, and (c) pulse rate as a function of genetic group. Pairwise differences between levels of genetic group are applicable if letters above bars are different. Dominant frequency as a function of (d) waterbody type and (e) artificial noise conditions. (f) The number of pulses as a function of the presence or absence of canopy cover. Bars represent means and error bars are 95% confidence intervals.



Figure 3.3 | Estimated marginal means from mixed effects model for note rise time for note *Type A* as a function of the number of calling sympatric species. Significant pairwise differences are present if letters above bars are different. Bars represent means and error bars are 95% confidence intervals.

3.4.1.2 Note Type B

A summary of the results of mixed effects regression models for note *Type B* are shown in Table 3.3. Genetic clade was a significant predictor of dominant frequency, number of pulses and pulse rate. Brisbane frogs produced significantly higher dominant frequencies than each of the other genetic groups, while Townsville frogs produced significantly lower dominant frequencies than both southern and Melbourne frogs (Figure 3.4a). Townsville and Brisbane frogs produced significant more pulses and at higher pulse rate than both southern and Melbourne frogs (Figure 3.4a). Townsville and Brisbane frogs produced significant more pulses and at higher pulse rate than both southern and Melbourne frogs (Figure 3.4b,c respectively). Pulse rate also showed a clinal pattern of decreasing from Townsville to Melbourne. Canopy cover and waterbody type were also significant predictors of one or more note Type B call characteristic. The presence of canopy cover resulted in significantly longer note and pulse durations (Figure 3.4d,e), while calls of frogs from permanent waterbodies produced significantly shorter inter-pulse intervals (Figure 3.4f).



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Figure 3.4 | Estimated marginal means from mixed effects models for significant predictor variables of note characteristics for note *Type B*. (a) Dominant frequency, (b) number of pulses, and (c) pulse rate as a function of genetic group. Pairwise differences between levels of genetic group are applicable if letters above bars are different. (d) Note duration and (e) pulse duration as a function of canopy cover. (f) Inter-pulse interval as a function of waterbody type. Bars represent means and error bars are 95% confidence intervals.

3.4.1.3 Note Type C

A summary of the results of mixed effects regression models for note *Type C* are shown in Table 3.4. Genetic clade was a significant predictor of dominant frequency, note duration, inter-pulse interval and pulse rate. Brisbane frogs produced significantly higher dominant frequencies than each of the other genetic groups, while Townsville frogs produced significantly lower dominant frequencies than both southern and Melbourne frogs (Figure 3.4a). Townsville frogs also produced significant shorter notes than frogs from each of the other clades (Figure 3.4b). The inter-pulse interval of note *Type C* was significantly shorter in Townsville and Brisbane frogs compared with the other two genetic groups, while Melbourne frogs had significantly longer inter-pulse intervals than southern frogs (Figure 3.4c). The pulse rates of all groups were significantly different to each other (Figure 3.4d); higher in Townsville than Brisbane, southern and Melbourne frogs. Furthermore, pulse rate showed a clear clinal pattern reducing from Townsville to Melbourne.

Waterbody type, canopy cover and artificial noise were also significant predictors of one or more note *Type C* call characteristics. Calls from permanent waterbodies produced significantly fewer pulses, longer pulse durations, lower pulse rates and shorter note durations (Figure 3.5a-d respectively). The presence of canopy cover resulted in significantly fewer pulses, longer pulse durations and lower pulse rates than frogs calling in the absence of canopy cover (Figure 3.5e-g respectively). Finally, the absence of artificial noise resulted in significantly longer inter-pulse intervals (Figure 3.5h).

3.4.2 Female choice experiment

All frogs that made a choice of one speaker over the other chose the Melbourne stimulus (Figure 3.7a). Binomial tests revealed statistically significant preference to their local call over Townsville (p=0.002) and Newcastle (p=0.004) calls (Figure 3.7b).



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Figure 3.5 | Estimated marginal means from mixed effects models for significant predictor variables of note characteristics of note *Type C* as a function of genetic group: (a) Dominant frequency, (b) note duration, (c) inter-pulse interval, and (d) pulse rate. Pairwise differences between levels of genetic group are applicable if letters above bars are different. Bars represent means and error bars are 95% confidence intervals.





Waterbody type

Absent

Present

Artificial noise



Figure 3.7 | (a) Bar charts showing the proportion of frogs that selected the Melbourne (local) signal (blue), and the frogs that did not leave the releasing apparatus (white). Numbers indicate the number of frogs. Separate bars are shown for Townsville and Newcastle foreign stimuli. (b) The proportion of frogs that left the releasing apparatus who chose the local acoustic signal (filled circles), with error bars representing 95% confidence intervals obtained from binomial tests.

3.5 Discussion

Geographic variation in advertisement call properties of widespread Australian anuran species have been reported in *Litoria booroolongensis* (Smith & Hunter, 2005), *L. verreauxii* (Smith et al., 2003), *Crinia signifera* (Littlejohn, 2005; Littlejohn, 2009), and *Neobatrachus* sp. (Roberts, 1997). This study extends earlier work to demonstrate that all three note types of the eastern dwarf tree frog, *L. fallax*, varied across their wide geographical distribution, although the patterns of variation were different for each of the three note types (see Table S3.1 for a convenient summary). In my study, I was able to show that genetic drift seems to be a particularly important factor, with the spectral property dominant frequency and the temporal property pulse rate found to vary between genetic groups for all three note types. Furthermore, the considered ecological variables relating to waterbody type and canopy cover also were relevant to varying degrees for each of the note types. Interestingly, my single environmental variable, temperature, did not predict variation in any call characteristic of any of the note types.

3.5.1 Genetic determinants of call variation

The previously described population genetic structure of *L. fallax* has identified two main mitochondrial DNA lineages of the species (James & Moritz, 2000). The northern clade of this genetic split is represented here by the Brisbane population, while our southern populations match those of James and Moritz (2000). Variation of the advertisement call properties were in accordance with the genetic separation as eight out of the ten note properties that were significantly affected by the genetic factor showed differences between these two populations. In particular, dominant frequency and pulse rate of all three note types, and the number of pulses of note *Type B* and inter-pulse interval of note *Type C* were different among the two populations.

In this study I decided to follow the less prominent genetic divergence within the northern clade at the Burdekin Gap, thereby distinguishing the Townsville and Brisbane populations. My results revealed significant variation between Townsville and Brisbane frogs for six out of the ten note characteristics that showed genetic differences. Dominant frequency was significantly higher in the Brisbane population for all three note types. Although exhibiting genetic sequence divergence of only 5%, my data suggest that isolation by distance (Bernal et al., 2005) might be acting upon the *L. fallax* populations of Townsville and Brisbane. It is worth emphasising that the genetic divergence was between Atherton in northern Queensland and central Queensland (James & Moritz, 2000), while Brisbane is located in southern Queensland. A future study focussing on the differences of the advertisement call components of the northern genetic clade, including populations from central Queensland, could provide an excellent opportunity to understand fully how isolation by distance effect is acting within this genetic clade of the species.

As the Townsville population is from the northern genetic clade, the advertisement call was expected to be significantly different from the southern genetic clade. My data support this as the main note parameters considered (dominant frequency and pulse rate of all three note

types and note duration of note *Type A* and *C*) were significantly different between the two populations. Given the long distance between the above-mentioned *L. fallax* populations, both genetic divergence cause by natural barriers and isolation by distance might be responsible in the variation of the communication signals in this species.

The population in greater Melbourne is approximately 600 km away from its natural range and this species was first documented in the area in 1999 (Gillespie & Clemann, 2000). Litoria fallax belongs to a species complex (Litoria bicolor and Litoria gracilenta) that has been collectively known as "banana box frogs" (Bevelander, 2014) since they are known to be accidently translocated to Victoria from the east coast of New South Wales and Queensland along with fresh food produce (Gillespie & Clemann, 2000). However, a habitat generalist, L. fallax is the only one of these species known to establish populations and persist in Victoria, specifically in greater Melbourne (Bevelander, 2014). To my knowledge, genetic work on these populations of *L. fallax* has being not conducted, so I considered them here as a separate genetic group. Interestingly, when examining variation in the advertisement call of the species, strong similarities were observed between the Melbourne population and the southern genetic clade, while most of the call characteristics were found to be significantly different with both northern genetic clade populations. Although genetic composition analysis is necessary, my analysis of acoustic signal structure is consistent with the hypothesis that Melbourne populations of L. fallax show greater affinity to the southern genetic clade. Hence, frogs translocated from New South Wales, rather than Queensland, are thriving as an invasive population well adapted to the colder weather in Melbourne.

Overall, this study provides sufficient evidence for variation in the complex communication signals of *L. fallax* driven by genetic drift. Amphibians are typically philopatric (Waldman & McKinnon, 1993) with a low dispersal capability (Driscoll, 1998) and are habitat specialists (Klaus & Noss, 2016), so tend to possess high interpopulation genetic variation relative to other vertebrates (Avise & Markers, 1994; Bernal et al., 2005). Indeed, several studies

have found evidence that population genetic structure is the main driving force for geographic variation in advertisement vocalisations, including Microhyla fissipes, Hyla japonica, *Physalaemus pustulosus* and in the Australian anuran species *Crinia signifera* (Jang et al., 2011; Lee et al., 2016; Littlejohn, 2005; Pröhl et al., 2006). Clinal variation of call characteristics along a geographical transect has been described in anuran species (Baraquet et al., 2015; Laugen et al., 2003; Pröhl et al., 2007; Ryan et al., 1996; Tessarolo et al., 2016). Furthermore, several studies of geographic variation have found that variation in male body size result in having a negative correlation with the spectral note property dominant frequency (Bernal et al., 2005; Narins & Smith, 1986). The body size coincides with clinal variation of the moisture level and temperature which lead to the quality and food availability of the habitat resulting this (Tessarolo et al., 2016). Due to the lack of body size data from the Townsville sites, this was not incorporated in the analysis as a factor in this study. I have however used linear mixed effects models on the other three populations' data in order to see whether there is any correlation between the SVL and the dominant frequency of advertisement notes (Appendix 1). A significant negative effect of SVL on the call dominant frequency was only found on note Type B in the southern genetic population and note Type C in the Melbourne population (Table A1) suggesting a weaker relationship between the body size and the call spectral properties of this species. It would be worthwhile nonetheless to determine whether the dominant frequency of the advertisement note are affected by the body size of this species with data from northern habitats in Townsville in future studies (Bernal et al., 2005).

3.5.2 Ecological determinants of call variation

Different adaptations are used by animals to counter the potential for signals to degrade as they are transmitted through the environment. Consequently, the modifications we identify often reflect the environment in which they are produced (Bradbury & Vehrencamp, 1998; Forrest, 1994). Subsequently, the evolution of call properties can be explained by the acoustic adaptation hypothesis (Hansen, 1979), when some species alter call structure in response to habitat

characteristics (Patricelli & Blickley, 2006). This is also known as phenotypic flexibility, a form of phenotypic plasticity (Dingemanse et al., 2010). My objective was not to systematically analyse how various ecological features of a habitat are affecting the calling behaviour of *L. fallax*; rather, I wanted to identify ecological variables with a potential impact on the production of calls or their propagation through the environment (Velásquez, 2014). Temperature is an important environmental parameter because of its impact on cyclic muscle activity such as note or pulse repetition rate (Girgenrath & Marsh, 1997). However, despite recordings being obtained over a broad temperature range (17.9-28.4 °C) there was no significant effect of temperature on any of the note characteristics of the repertoire of *L. fallax*. This is particularly interesting given that the Melbourne population of *L. fallax* is outside of the typical climate zone; although climate modelling suggests that Melbourne does fall within the climatic range of *L. fallax* (Bevelander, 2014). My work in Melbourne was undertaken during warm summer months with high temperatures, so further research on the calling behaviour of the species during cooler evenings in Melbourne would be informative.

The waterbody type utilised by anurans is important as several studies have demonstrated that the rate of desiccation of a waterbody can impact phenotypic characteristics such as body size (Wells & Schwartz, 2007). Results of this study suggested that several call characteristics of the species are affected by the waterbody type, which I classified as permanent or temporary. Following Valásquez (2014), my data provide the impetus for future studies examining how waterbody drying time affects call characteristics of anurans. Similarly, the preliminary results obtained here suggest that vegetation cover might be another important habitat characteristic affecting the geographic variation of call properties in widespread anurans as it has the tendency to alter signal propagation, particularly with respect to spectral characteristics (Slabbekoorn & Smith, 2002). Signals with higher frequencies tend to be adversely affected due to reflection off multiple leaf surfaces in dense vegetation (Narins & Smith, 1986). Therefore, male frogs might be producing advertisement calls with lower

frequencies in environments with high vegetation cover to increase the active space of the signal (Narins & Smith, 1986).

Although my results do not suggest an influence of ambient noise levels shown in other species (Bleach et al., 2015; Lopez et al., 1988), the presence or not of anthropogenic noise was found to have an impact on aspects of calling behaviour. Anthropogenic activities can alter the complexity of the acoustic environment and lead to changes in signalling behaviour in multiple taxa (Brumm, 2013; Kunc et al., 2014). My results indicate an effect of anthropogenic noise on some call characteristics of *L. fallax*. The dominant frequency of note *Type A*, which is the most attractive note type to females (unpublished data), and potentially an important signal in mate attraction, has been significantly reduced in the sites that were exposed to anthropogenic noise. This result is in accordance with Pelophylax ridibundus and Boana bischoffi where they reduce the dominant frequency in sites disturbed by traffic noise (Caorsi et al., 2017; Parris et al., 2009). However, Litoria ewingii, a sympatric species of the southern population of our study species, has shown that traffic noise led to an increase in dominant frequency (Parris et al., 2009). In anurans, frequency alterations are limited to 300 Hz, (Cunnington & Fahrig, 2010; Kruger & Du Preez, 2016), and the reduction of the dominant frequency of L. fallax lies well within that (258 Hz). However, L. fallax has a high dominant frequency above 3 kHz, which is potentially the frequency where the energy of the traffic noise reduces (Caorsi et al., 2017). A study solely on the effect of the traffic noise on the communication of my study species would reveal whether the frequency drift observed is influenced by traffic noise. My study was not designed to fully understand how artificial noise is affecting the calling behaviour and possibly the breeding and sexual selection of L. fallax. However, significant differences in sites indicate an avenue for future research. This is important because anthropogenic noise could affect the behaviour of anurans to a level that adversely impacts the health of the animals by increasing the energy consumption of an already very costly activity (Wells & Schwartz, 2007). Hence, future efforts investigating the effect of anthropogenic noise on both spectral and temporal properties of L. fallax is vital. This would elucidate whether differences in call properties observed are innate

(Parris et al., 2009) or plastic (Caorsi et al., 2017; Cunnington & Fahrig, 2010), considering how long a frog population has been under the influence of traffic noise. The social environmental effect considered in this study (the number of sympatric species calling) had little effect on calling behaviour with the significant effect found for note rise time of note *Type A*. The lack of further differences suggests a low possibility of reproductive character displacement of this species (Howard, 1993). However, given that *L. fallax* has a complex acoustic repertoire (Chapter 2) and is highly invasive (Gillespie & Clemann, 2000), it is possible that this species might be implicated in the divergence of sexual signals of other species (Lemmon, 2009). I have not considered the conspecific chorus density of *L. fallax* for this study. However, it is worth considering this factor in future research as acoustic signals may get masked by both conspecific and heterospecific mating signals (Bradbury & Vehrencamp, 1998; Ziegler et al., 2011) resulting in socially induced plasticity (Bee & Bowling, 2002; Bee et al., 2000).

The results of the playback experiment revealed a strong preference by females to local calls over both the Townsville and Newcastle population calls. The two populations for the foreign sexual signal stimuli represent the northern and southern genetic clades. Even though the acoustic signals of the Melbourne population showed similarities with the southern genetic clade, there were still some significant differences when compared with the calls of Newcastle populations and this might be attributable to local adaptation. Regardless, it would appear that these were enough for females to discriminate the calls of local and 'foreign' frogs and could lead to reproductive isolation. Certainly, females attracted to the sexual signals of males from the local area might be in expectation of fitness advantages gained by the offspring in familiar ecological conditions (Kawecki & Ebert, 2004; Mortega et al., 2014). This observation is congruent with the behaviour of the túngara frog, *Physalaemus* (*=Engystomops*) *pustulosus* (Pröhl et al., 2006), whereby females preferred local calls over foreign calls that were notably different in call duration and call fall time (Pröhl et al., 2006). The dominant frequency and pulse rate of all note types were found to vary across the geographic range of the focal species of this thesis and should be the focus of future work to determine whether these are particularly salient

to female frogs. This would help understand whether variation of these characters result the female preference of local calls over foreign calls or whether there is a character displacement in the female preference. However, it is in contrast with the observation that female cricket frogs, *Acris crepitans,* preferred low frequency calls thereby disregarding the geographic location of the call origin (Ryan et al., 1992). Due to restrictions on my activities outside of my influence, I have only considered these two populations in elucidating female preferences. However, with evidence that the strength of female preference could vary among populations (Pröhl et al., 2006), consideration of additional populations would be helpful in fully understanding whether there is a correlation between the genetic distance and behavioural isolation of this species and how prominent is the variation in call characteristics for mate choice decisions.

3.5.3 Summary

This study revealed that the geographic variation of the introductory notes of *Litoria fallax* is driven possibly by the cumulative effect of genetic divergence as well as other local ecological selective forces. Furthermore, this study implies the importance of considering all advertisement call or note types in a signalling repertoire of a species with a complex communication system in order to fully understand how each component varied under the selective pressures. Even with a small sample size, my results also revealed that the female receivers of the study species have a preference towards local acoustic signals over foreign ones, indicating a possible local adaptation. Further experiments are vital in fully elucidating whether the divergence observed in the acoustic signals of *Litoria fallax* is a possible first stage of evolutionary divergence.

3.6 References

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3.7 Supplementary material

Table S3.1 | Summary of significant predictor variables from linear mixed models by note type and call characteristic for variation in acoustic structure of *Litoria fallax*. The shaded boxes indicate a significant correlation.

	Predictor variable						
Note type and call characteristic	Genetics	Temperature	Waterbody type	Canopy cover	Artificial noise	Noise level	Number of sympatric species
Туре А							
Maximum frequency							
Note rise time							
Number of pulses							
Note duration							
Pulse duration							
Inter-pulse interval							
Pulse rate							
Туре В							
Maximum frequency							
Note rise time							
Number of pulses							
Note duration							
Pulse duration							
Inter-pulse interval							
Pulse rate							
Туре С							
Maximum frequency							
Note rise time							
Number of pulses							
Note duration							
Pulse duration							
Inter-pulse interval							
Pulse rate							

Chapter 4

Study of multimodal signalling by *Litoria fallax*

4.1 Abstract

The use of multiple sensory modalities in signal production and perception is widespread among animal taxa. Investigating the information conveyed by each signal component is vital in understanding the evolutionary significance of such multimodal communication systems. Anuran amphibians are an excellent system in investigating how this multimodal communication is beneficial in sexual selection. The pelodryadid frog, Litoria fallax is used as a model organism here to observe whether any physical abiotic and biotic factor can predict the occurrence of the visual signalling behaviour comprised of limb movement like foot flagging, and foot flicking. The results revealed that the visual signalling behaviour of this species is more incidental and associated with agonistic interactions and a graded aggressive acoustic signals. In the playback experiments, both male and female frogs were tested for potential phonotactic and agonistic response to unimodal stimuli (acoustic+static frog model) and multimodal stimuli (acoustic+ dynamic frog model) under two different physical environments of light levels. Males demonstrated higher preference towards the multimodal stimulus regardless of the light level while females choose multimodal stimulus in higher light level than in the low. Results here indicate that despite being a nocturnal species visual cues play an important role in the mate attraction and intraspecific communication in the model species. Moreover, results of this study suggest that in a heterogenic habitat inter signal interaction of the signals is more prominent than the efficacy-based selection of the multimodal communication of the study species.

4.2 Introduction

Several taxa have evolved to increase the efficiency of signal transmission via the use of signals from more than one sensory modality (Grafe & Wanger, 2007). Such complex communication systems are diversified in vertebrates such as birds (Ota et al., 2015) and lizards (Vicente & Halloy, 2017), and in invertebrates like spiders (Uetz et al., 2009). Understanding the functional advantages of multimodal over unimodal systems is important in elucidating the evolutionary significance of the multimodal communication systems in animals (Gomez et al., 2011; Partan, 2004). For example, studies suggest that overcoming the cognitive constraints of the receiver might be driving the use of multiple sensory modalities as it can improve the detection of signals by the receiver (Kulahci et al., 2008).

Multimodal signals could involve multiple signals that have the ability to elicit a behavioural response on their own, or interactively with each other (Hebets & Papaj, 2005) whereby the two signals could be redundant and carry the same information, or carry different information for each modality (Partan & Marler, 1999). The type of signals in a such multimodal system can be either "fixed" or "free" depending on the strength of the relationship between them (Higham & Hebets, 2013). When several signal types are intrinsically emitted together, they are known as "fixed" (Partan & Marler, 2005), such as the concurrent occurrence in humans of visual signals, manifested through lip movements, while communicating with acoustic signals (McGurk & MacDonald, 1976). Conversely, "free" systems occur when signal types are not always tied together (Partan & Marler, 2005). For example temporary combinations of motion and seismic signals of the jumping spider *Habronattus coecatus* (Elias et al., 2012).

Several independent hypotheses have been proposed to explain the functional significance of each modality in a multimodal communication systems. Efficacy based hypotheses address the influence of the external environment on both signaller and receiver (Grafe et al., 2012). Specifically, the efficacy trade-off hypothesis suggest, that separate components of a communication system might have different functions, addressing the constraints imposed by transmission through the environment (Hebets & Papaj, 2005). For

example, in the nocturnal túngara frog, *Physalaemus* (=*Engystomops*) *pustulosus*), the vocal sac display increases the localisation of the receiver in a high density aggregation, while the acoustic signal facilitates long distance signal transmission (Hebets & Papaj, 2005; Rosenthal et al., 2004). While two modalities are working together, as mentioned above one signal could inevitably amplify the detection of the other (inter-signal interaction amplifier hypothesis) (Hebets & Papaj, 2005) by changing a receiver's detection threshold. For instance, several animals have conspicuous coloured pigments on the parts of the body that are used in dynamic visual signalling to increase detectability (Fleishman, 2000). This is consistent with attention altering explanations under the inter-signal interaction hypothesis as one signal component has the ability to alter the response of the receiver to the other component (Hebets, 2005). For example, in the courtship display of the wolf spider species *Schizocosa uetzi*, the vibratory signals alters the response of the female spiders to visual signals (Hebets, 2005).

Sexual selection and mate choice in anuran amphibians fundamentally depends on their species-specific acoustic signalling system (Duellman & Trueb, 1994; Toledo et al., 2015; Wells, 1977). Most anuran species have more than one call type in their repertoire in order to ensure effective communication to variant receivers in different contexts (Starnberger et al., 2014b; Wells, 1977). However, these might not be limited to acoustic signals as broader multidimensional studies have demonstrated that visual signalling behaviour is more common among the communication systems of anurans than previously known (Hödl & Amézquita, 2001). Diverse visual signalling repertoires have been described in diurnal (de Lourenço et al., 2020; Narins et al., 2003) and nocturnal frog species (Doris Gomez et al., 2009; Taylor et al., 2008). Indeed, female frogs of nocturnally active amphibians have been reported to both perform (Lipinski et al., 2012) and respond to visual signalling (Hartmann et al., 2004). Acoustic signals are an effective mode of communicating over both short and long distance (Gerhardt, 1994), while the evolution of visual signals, particularly in nocturnal anurans, might have arisen due to environmental constraints of locating mates in high density choruses (Hödl & Amézquita, 2001). With evidence of sensitivity towards night-time illuminations (Buchanan, 1993; Cummings et al., 2008) it is interesting to further investigate whether male and female receiver frogs reliably respond to these visual signals and to identify the potential function of multimodal signalling. Furthermore, variation of ambient light level due to vegetation cover or lunar cycle (Cummings et al., 2008) could affect the decision making in mate choice by female frogs (Cronin et al., 2019). While the higher ambient light level has been found to make a shift of the decision of female túngara frog, *Physalaemus* (*=Engystomops*) *pustulosus*) from the multimodal stimulus comprised of both acoustic and visual signals in to an acoustic only unimodal stimulus (Cronin et al., 2019), no influence of light level has been observed in the female choice of gray tree frog, *Hyla versicolor* (Underhill & Höbel, 2017).Therefore, it is important to consider the possible light level heterogeneity on visual communication systems in animals (Baugh & Ryan, 2010).

Multimodal signals comprising acoustic and visual components (bimodal signals) have been observed both in mate attraction and in agonistic interactions of several anuran species. Even though fundamental mate recognition and mate choice is based on the acoustic signals, some species of female frogs are more attracted to the multimodal cues in this process (Hödl & Amézquita, 2001). For example, females of the diurnal *Staurois guttatus* are more attracted to the bimodal signalling males that use foot flagging in combination with acoustic signals (Grafe & Wanger, 2007). In addition, females of some nocturnal species appear to be sensitive to the colour patterns of males as many such species are sexually dimorphic in colouration (Buchanan, 1994; D. Gomez et al., 2009). For example, the lateral stripe of the squirrel tree frog, *Hyla squirella* (Taylor et al., 2007), and the vocal sac colouration and pulsation of *E. pustulosus* (Rosenthal et al., 2004) are important in mate attraction. Even though visual signalling by males has been predicted to improve detectability by females of some nocturnal species that often inhabit above ground level (Hödl & Amézquita, 2001), there is little empirical work examining the role of foot flagging behaviour in mate choice.

In complex social contexts, male frogs tend to use their appendages and body movement in displaying their (resource holding) potential in territorial defence (Hödl &

Amézquita, 2001; Narins et al., 2003) while trying to actively avoid energetically expensive physical combats (Roberts, 1997).

This will necessarily involve two sensory modalities where the visual signals and aggressive calls convey the signal in a graded manner (Littlejohn & Harrison, 1985; Schwartz, 1989; Wagner Jr, 1992). Species like *Dendropsophus minutus* and *Hyperolius marmoratus broadleyi* shows gradual transition from advertisement call to an aggressive call that ultimately would lead to agonistic behaviour (Grafe, 1995; Toledo et al., 2015). These graded signals have the ability to alter the call characteristics, particularly dominant frequency, call duration and pulse rate, in a continuous fashion to convey the motivation level to fight along with body size information (Wagner Jr, 1989). Therefore, complex communication systems of anuran amphibians with both graded acoustic and visual signalling behaviour provide an opportunity to further investigate the functional significance of the multimodal communication in agonistic interactions (Grafe, 1995; Toledo et al., 2015).

Notably, visual signalling behaviour has being described in several tree frog species (Furtado, 2017; Hartmann et al., 2004; Hödl & Amézquita, 2001; Meyer et al., 2012), including species native to Australia. Several hylid (=pelodryadid) species of the genus *Litora* residing in the Australo-Papuan region are known to have a multimodal communication system including *L. nannotis, L. rheocola, L. serata, L. fallax* (Hödl & Amézquita, 2001; Richards & James, 1992), *L. pearsoniana, L. cooloolensis* and *L. iris* (Meyer et al., 2012). Here, I focus on *L. fallax* (Peters 1880), commonly known as the eastern dwarf tree frog or eastern sedge frog. This relatively small frog is often found in open forests and wetlands along the east coast of Australia. With a unique, complex graded acoustic repertoire, (described in chapter 2), visual signalling behaviour, and a broader distribution, this species is a great model system for elucidating the evolution and function of multimodal communication systems.

The first aim of the present study was to observe the visual signalling events of *L. fallax* in the field and to identify whether its use is predicted by relevant environmental and ecological factors. For example, visual signalling behaviour has been observed in species (*Staurois*

latopalmatus and Staurois parvus) inhabiting habitats with high levels of background noise, where it might be serving as an alternative or complementary function in communication (Grafe et al., 2012; Hödl & Amézquita, 2001; Preininger et al., 2009). Furthermore, the light level of the environment might affect detectability, which can change both signaller and receiver behaviour (Cronin et al., 2019). My second aim was to identify whether signals are combined with acoustic calls, and if so, to describe the acoustic signals that are associated with the visual signalling events of the species. As identified in a previous study (Meyer et al., 2012), the visual signalling behaviour of this species is expected to be associated with agonistic interactions and aggressive acoustic signals (Narins et al., 2003; Wagner, 1989), so I focus my effort on close range interactions in the field. Finally, I wanted to explore whether dynamic visual signals influenced the responsiveness of male and female frogs using multimodal playback studies: do males and females preferentially approach calling frogs that are also generating a visual signal? The inclusion of an additional signal modality in a multimodal signalling system can increase the attractiveness to the receiver (Narins et al., 2003; Taylor et al., 2007) by providing additional information about the signaller. As detectability might be constrained in certain environmental circumstances, I include a manipulation of ambient light levels to determine whether its use is influenced by such external factors.

4.3 Methods

4.3.1 Fieldwork

4.3.1.1 Field observations

I undertook field observations at nine field locations in Townsville, Wollongong and Melbourne (Table 4.1), with calling male frogs located at night between 20:00 to 01:00 h by their characteristic call. The criteria for inclusion in this study was the occurrence of two actively calling male frogs within approximately 40 cm of each other, resulting in 19 sessions across three field seasons. Behavioural observations were undertaken using focal sampling for one to two hours until one of the frogs moved away. Frogs were recorded from a distance of 1 m under a red light using a Canon XA40 with infrared capability, with a Sennheiser ME66 directional microphone providing audio input to the recording at a sampling rate of 44.1 kHz (16 bit resolution). Abiotic factors were recorded at the time of observations. These included temperature and humidity obtained using a Kestrel 4000 weather meter, the environmental sound level using an omnidirectional sound level meter (QM-1589, Digitech) and the light level of the habitat (Unihedron Sky Quality Meter with a TAOS TSL237S sensor). My light level meter provide measurements as low as 0.0002 lux and is thus is well suited to measuring light levels at night where full moonlight is typically around 0.2 lux. I recorded whether or not a visual signal was performed during the interaction. Visual signalling was identified considering previously reported types of visual signals including foot flagging and foot flicking as well as leg stretching and vocal sac displays in between calls (Table 4.2).

Date	Noise (db)	Temperature (C°)	Humidity (%)	Light level (lux)	Visual signalling	Distance between frogs (cm)		
Melbourne – Wilson Reserve								
02.10.2017	72.2	22.0	74.1	-	yes	10.00		
19.01.2018	73.2	23.2	77.5	0.0145	yes	30.00		
19.01.2018	70.1	23.0	78.0	0.0145	no	30.00		
19.01.2018	70.2	22.8	77.2	0.0145	no	20.00		
09.02.2020	71.2	26.2	48.0	0.0160	no	10.00		
Melbourne – V	Varringal	Parklands						
18.01.2018	70	24.2	60	0.0900	yes	10.00		
18.01.2018	70	24.2	60	0.2000	no	10.00		
Melbourne – H	lays Padd	ock						
27.02.2018	70.2	20.9	53.8	0.0134	no	20.00		
27.02.2018	71.4	20.9	53.9	0.0134	no	30.00		
27.02.2018	70.2	20.9	53.8	0.0134	no	20.00		
Melbourne – Wonga Park								
05.02.2020	75.0	18.0	58	0.0130	yes	10.00		
05.02.2020	75.0	18.0	58	0.0130	no	10.00		
NSW – University of Wollongong								
05.11.2018	74.2	24.6	80	0.0600	yes	10.00		
Queensland – Townsville – Bluewater SW								
20.10.2017	73.4	22.8	88.8	0.0140	yes	10.00		
20.10.2017	73.5	22.6	88.5	0.0140	yes	20.00		
21.10.2017	74.0	23.7	80.9	0.0135	yes	20.00		
20.10.2017	73.0	22.6	88.5	0.0140	no	10.00		
21.10.2017	74.0	23.7	80.9	0.0135	no	20.00		
Queensland – Townsville – Ross River								
19.10.2017	65.3	24.1	91.7	0.0135	no	10.00		

 Table 4.1 | Summary of fieldwork sessions investigating the use of visual signals by Litoria fallax

Visual signalling behaviour	Description
Foot flagging	Raising one or both hind legs extending then slowly out and back in an arc above the substrate level and returning it to the body side. At maximum extension feet vibrate and toes are spread and outstretching (Meyer et al., 2012; Richards & James, 1992)
Hind limbs flicking	Rapid flicking movement of hind limbs lasting for only a fraction of a second (Meyer et al., 2012) in the absence of any tactile stimulus such as an insect on the body of the frog
Leg stretching	Stretching a single leg at the substrate level and it keeping it extended for some time
Vocal sac display	Vocal sac remains inflated between two calls while the male does not make any acoustic signal

 Table 4.2 | Visual signalling displays observed in Litoria fallax

4.3.1.2 Statistical analysis

I wished to determine whether any of the environmental or ecological factors predicted the occurrence of a visual signal in these interactions. I created a generalised linear model using the *glm* function in the R Statistical Environment (R Core Team 2018) with presence or absence of a visual signal as the dependent variable and a binomial error distribution. I used waterbody type (permanent, temporary) as a categorical fixed effect and noise level, distance between frogs at the time of signalling, temperature and humidity as continuous fixed effects. I assessed the significance of fixed effects using the *anova* function. Regardless of significance, I provide bar plots of the means and standard errors for each variable grouped by whether or not visual signalling occurred (Figure 4.5).

4.3.1.3 Acoustic signal associated with visual signalling

Acoustic signals associated with visual signals were extracted from the video recordings of four individuals in the Melbourne population for further analysis. To compare that with the other call types identified in the repertoire of *L. fallax,* the diphasic state of the note *Type C* in advertisement calls and monophasic *Type C* calls were analysed from ten individuals of the

Melbourne populations. To obtain this comparison dataset, calling male frogs were located at night between 20:00 to 01:00 h by their characteristic call, and frogs were recorded from a distance under a red light to minimise disturbance. Approximately one minute of recording was obtained from each individual frog using a Sennheiser ME66 directional microphone with a foam windscreen connected to a Roland R-26 portable audio recorder using a sampling rate of 44.1 kHz (16 bits resolution). A recording distance of 0.5-1 m was maintained between the snout of the calling male and the tip of the microphone for all recordings. Gain settings of the microphone were adjusted according to the sound level of each environment and was not adjusted until the recording was completed. I used Raven Pro's (Cornell Laboratory of Ornithology, Ithaca, NY USA) waveform display to measure temporal properties, whereas the spectrogram function was used for measuring spectral properties by averaging the spectrum over the entire duration of a call (256 point fast fourier transform using a Hanning window).

4.3.2 Multimodal playback experiments

I sought to determine whether frogs would be attracted to multimodal signals over unimodal signals, and used a similar phonotaxis experiment to those described in Chapters 2 and 3. However, here one of the stimuli I presented involved a dynamic visual stimulus, comprising only foot flagging (Table 4.2), which I paired with simultaneous presentation of an acoustic signal. Stimulus frogs were given separately to male and female frogs under low and high light levels in the anechoic chamber used in Chapters 2 and 3.

4.3.2.1 Visual signal

I created a 3D model frog and a custom-designed mechanism to move its leg to replicate a foot flagging display. Five adult male *L. fallax* were captured to obtain morphometric measurements (body and limb length and width) before photographing them from multiple viewing angles. I computed mean values of morphometric data to help with the creation of a 3D wireframe model in Autodesk Maya 2015 animation software (Figure 4.1a). Two versions of the model were 3D printed to scale in white soft flexible resin (SLA) in which one model featured a frog with leg

extended (Figure 4.1a *left*) and the other a crouching frog (Figure 4.1a *right*). The models were painted to match real frogs. I captured three live adult male *L. fallax* in the field and the reflectance spectra of the head, dorsal abdomen, deflected vocal sacs and ventral abdomen and the bright coloured inner thighs were obtained using a bifurcated fibre optic probe connected to a portable Jazz spectrometer with inbuilt pulsed xenon light source (Ocean Optics). All spectra were generated by holding the probe at an angle of 45° to the surface. Since the vocal sacs are deflated when a frog is captured, spectra of inflated vocal sacs of live frogs were made from photographs and may not be accurate representations of actual reflected spectra. Enamel paint colours were selected by trial-and-error to match the reflectance spectra of the relevant body part.

I used flexible wire and water-filled syringes to achieve the required leg movement (Figure 4.1b). The flexible wire was attached near to the ankle of the extended leg and also to the plunger of a syringe that was inserted into the syringe barrel, which was filled with water. A rubber tube attached to the tip of the syringe was then attached to the tip of a second syringe. The second syringe was also filled with water and with the plunger inserted. The set up was designed in a way that each time the operator would push or pull on the plunger of the second syringe, a corresponding movement in the first syringe caused the frog's leg to move. Using a specific amount of water, measured by the syringe scale markings I have decided the amount of movement needed to achieve the desired movement of the frog's leg, which was repeatable and consistent across trials. The frog model was installed on a wooden perch inside the anechoic chamber, with the wire and first syringe also located inside the chamber and painted completely black. The rubber tube from the tip of the first syringe was passed through a small whole in the wall of the chamber so that I could control it from outside while watching the waveform of the acoustic signal on the computer screen to make sure the timing of visual signal movements were synchronised with the acoustic component. The joints of the extended leg of the models were flexible but additional support was provided by applying a small amount of transparent silicon prior to painting. The crouching frog model was painted in a similar way to above, positioned on

a matching wooden perch and placed in the anechoic chamber next to the speaker on the opposite side to the dynamic frog model.

4.3.2.2 Acoustic signal

The acoustic stimulus was based on the calls associated with the visual signalling behaviour of Melbourne populations of *L. fallax.* I used call recordings obtained from four male frogs that displayed foot flagging displays in the Melbourne region (see above) and computed mean call properties from ten acoustic variables. Following inspection of the distribution of call characteristics, I selected calls that closely matched the mean values of each call component and used Audacity (Version 2.3.3) to create stimuli of 20 seconds each, adjusting some call characteristics after broadcasting in the experimental chamber to ensure consistent soundpressure level.

4.3.2.3 Experimental arena

Trials were conducted in an anechoic chamber (1.2 m³) made of wood and covered in anechoic foam to improve sound quality (Figure 4.1c). To prevent females from leaving odorant scents, the foam was covered with wire netting and floor with a removable cloth rinsed in water between any two tests. During trials, female or male frogs were given a choice of two stimuli broadcasted from two speakers (FR7-4 VISATON Germany) placed at 90° angles to the releasing point, which was fixed each time and contained within an acoustically transparent container we designed (Figure 3a), and connected to the computer via an amplifier (Stageline LSP-102). We used the same releasing apparatus described earlier in this thesis (Figure 4.1c). The sound pressure level (SPL) from both speakers at the releasing point of the frog was maintained at 65 dBA using the approach described in Chapter 2.

The two light levels in the test arena were achieved using a white LED bulb (3.2 V White LED 5mm Through Hole, Nichia NSPW510DS) controlled by a LED dimmer via a BC547 transistor to control the brightness. This provided 0.01 lux as the low light level and 0.2 lux as the high light level. This light level range includes all the observations we have observed on the visual

signalling events of the species in both natural and artificial light. The higher margin we used also did not exceed the maximum illumination level that occurs during full moon (0.05-0.2 lux(Kyba et al., 2017) and the lower margin coincides with the illumination produced on a clear quarter moon day.

4.3.2.4 Experimental procedure

Both male and female frogs were used in the experiments. Frogs were placed in the releasing apparatus for a five minute acclimation period, during which we commenced broadcasting the acoustic stimuli antiphonally. One stimulus was presented at each speaker, with speaker allocation randomised and counterbalanced across the cohort. The two stimuli comprised unimodal (acoustic signal + stationary frog model) and bimodal (acoustic signal + dynamic foot flagging frog model). Stimuli were assigned to one side of the box, with random allocation for a given individual and counterbalanced across the cohort. Stimulus presentation alternated throughout the trial with the order of presentation also randomised for a given individual and counterbalanced across the cohort. Light and dark trials were randomised for a given individual as well. Each session was filmed using a Canon XA40 with infrared capability, which was fixed to the roof of the chamber and connected to an external monitor providing the opportunity to observe the sequence of behaviour from a distance. I tested a total of 32 male frogs and 14 gravid female frogs (sample size for females was impacted by lockdown restrictions during the COVID-19 pandemic).

4.3.2.5 Statistical analysis

For each trial, I identified the first stimulus approached to within 40 cm. In the event that a frog left the releasing apparatus but did not proceed to within 40 cm of either stimulus, I recorded the trial as a 'neutral response'. I ran binomial tests in R separately for males and females in low and high light conditions (4 tests). Initially, I excluded trials in which frogs did not leave the releasing apparatus such that the total number of trials in the test was the sum of unimodal + bimodal + neutral. I then repeated the analysis with the neutral responses excluded from the total.



Figure 4.1 | (a) 3D reconstruction of *Litoria fallax* showing the extended limb and crouched postures. (b) Schematic illustration of the mechanism for generating foot flagging. A flexible wire (1) was connected to the ankle of the model frog and the plunger of a syringe (2). A rubber tube is attached to the tip of the syringe (3) and connected to the tip of a second syringe. The plunger of the second syringe (4) is moved in and out to generate foot flagging movements in the model attached allowing the foot of the model to move accordingly. See text for full description. (c) Schematic illustration of the anechoic chamber configuration showing the stimulus perches and releasing apparatus (speakers not shown).

4.4 Results

4.4.1 Fieldwork

Visual signalling was observed in eight of the nineteen sessions and involved each of the leg movements described in Table 4.2 (Figure 4.2). Interestingly, leg stretching (Figure 4.2c) occurred in isolation and not as the initial stage of foot flagging as described by Richards & James (1992). Additionally, the orange colour throats of the two calling males were inflated and appear larger when emitting longer duration monophasic encounter calls. The throats never went back to the fully deflated position throughout the visual signalling period (unless disturbed such as in more serious agonistic interactions) allowing the bright colours to be visible more than when they emitted advertisement calls (Figure 4.2d).

Mean (±SE) values for the predictor variable as a function of whether or not a visual signal was generated during the session are shown in Figure 4.3. The result of the generalised linear model suggested that only noise level was found to significantly predict the occurrence of visual signalling events of *L. fallax* (Table 4.3), with sessions resulting in visual signals characterised by lower levels of environmental noise (Figure 4.3a). However, the mean difference in SPL between the two was small (73.2 and 71.0 dB).

When a rival male frog is calling close by, the individuals tend to progressively change from diphasic advertisement calls, *Type BA* and *Type CA* to producing monophasic *Type B* and *Type C* while omitting the note *Type A*. The note duration of the monophasic calls are increased as the distance between the two individuals are reduced. However, visual signals were generated only in combination with the monophasic note *Type C* (Figure 4.4). Visual signalling behaviour was observed both simultaneously, and with a variable delay with the monophasic *Type C* note in an irregular manner. Relative to other times, the multimodal note Type C was longer in duration, incorporated a higher number of pulses but at lower pulse rate (Table 4.4). Furthermore, the inter-pulse interval, which is important for differentiating notes *Type B* and *Type C* was greater in the multi-modal context (Table 4.4).



Figure 4.2 | Visual signalling behaviour observed in *Litoria fallax*. (a) Foot flagging while in an agonistic interaction. (b) Foot flagging while the rival male was within 40 cm. (c) Foot stretching and (d) vocal sac display without vocalisation.

Predictor Variable	Df	Deviance	AIC	LRT ^a	P-value
Waterbody	1	16.724	28.724	1.6980	0.19255
Noise	1	21.330	33.330	6.3047	0.01204
Humidity	1	16.766	28.766	1.7400	0.18713
Temperature	1	15.112	27.112	0.0865	0.76874
Light level	1	15.349	27.349	0.3236	0.56948
Distance	1	15.689	27.689	0.6634	0.41535

Table 4.3 | Summary of the results of generalised linear model predicting the occurrence of visual signals by *Litoria fallax*.

^a Likelihood ratio test



Figure 4.3 | Mean values of (a) ambient noise and (b) light levels, (c) temperature and (d) humidity and (e) distance between rival frogs as a function of whether or not the session resulted in the occurrence of a visual signal by either frog. Error bars are 95% confidence intervals. *significant relationship with the occurrence of the visual signals

Table 4.4 | Descriptive statistics of the note *Type C* in the diphasic and monophasic contexts. Monophasic contexts are split further into encounter calls and calls associated with visual signals (multimodal).

Note	Note	Mean	SD	Range	CV
characteristic				(min-max)	(%)
Note duration (ms)	Diphasic	366.2	84.60	222-666	23.1
	Monophasic encounter	408.8	127.10	198-752	31.1
	Monophasic multimodal	1139.5	300.13	666-1804	26.3
Note rise time (ms)	Diphasic	292.5	99.70	83-590	34.1
	Monophasic encounter	359.2	123.10	130-705	34.3
	Monophasic multimodal	1008.9	283.70	558-1709	28.1
(Note rise	Diphasic	79.3	17.11	25-95	21.6
time/Note	Monophasic encounter	85.8	11.00	36.8-95.8	12.8
duration)100%	Monophasic multimodal	88.3	5.20	74-99	6.0
	Diphasic	13.3	2.90	9-22	21.9
Number of pulses per note	Monophasic encounter	19.5	6.00	10-32	30.8
	Monophasic multimodal	40.0	9.00	26-56	21.7
Pulse duration (ms)	Diphasic	8.0	3.10	4-18	36.3
	Monophasic encounter	8.1	2.60	4-14	32.1
	Monophasic multimodal	5.3	1.60	2-9	30.9
Pulse rate (pulses/s)	Diphasic	49.9	4.50	43.1-64.1	9.0
	Monophasic encounter	49.9	4.50	41-60	9.0
	Monophasic multimodal	35.8	5.00	20.4-43.7	13.9
Inter-pulse interval (ms)	Diphasic	13.0	2.50	9-20	19
	Monophasic encounter	12.5	2.00	7-22	22.7
	Monophasic multimodal	25.2	6.00	18-50	22.3
Dominant	Diphasic	4.5	0.30	4.1-4.9	6.6
frequency (kHz)	Monophasic encounter	4.4	0.20	4.1-5.0	4.5
	Monophasic multimodal	4.1	0.20	3.8-4.7	5.2



Figure 4.4 | *Top*: A 20 s waveform of a duetting call sequence between two individuals during agonistic and visual signalling display. *Bottom*: Multiple 1s waveforms of the transition of note *Type C* from diphasic advertisement call *Type CA* (left panel) to monophasic *Type C* encounter call (middle) to the long duration *Type C* call (right) associated with the visual signalling of male *Litoria fallax*.

4.4.2 Playback experiment

The responses of frogs to the two stimuli by sex and light level is shown in Figure 4.5a. A higher proportion of frogs approached the multimodal stimulus compared with the unimodal stimulus. However, when neutral responses are included, whereby frogs left the releasing apparatus but did not reach the 40 cm threshold for making a choice, the only significant preference for the multimodal stimulus was by females in high light levels (Figure 4.5b; p=0.04). However, if we restrict the analysis to those frogs that made a choice, then males can be regarded as having a significant preference for the multimodal stimulus in both low (p=0.04) and high (p=0.01) light levels.



Figure 4.5 | (a) Stacked bar charts showing the proportion of frogs that selected the visual + acoustic signal (blue), the acoustic only signal (orange), those that moved from the releasing apparatus but remained in a neutral location (grey), and the frogs that did not leave the releasing apparatus (white). Separate bars are shown for males and females in high and low light conditions. N = 32 and 14 for males and females respectively. (b) The proportion of frogs that left the releasing apparatus who chose the visual + acoustic signal (filled circles), with error bars representing 95% confidence intervals obtained from binomial tests. Black circles and error bars are when only responses that resulted in a choice were included.

4.5 Discussion

This study has confirmed the use of visual signals by *L. fallax* and found that it is used simultaneously or in a varying time gap only with a modified version of a specific monophasic call (note *Type C*). The use of visual signals is not always performed during interactions at close proximity, and does not appear to be strongly linked to environmental or ecological conditions. However, both male and female frogs are more likely to approach signallers that perform such visual signals than to those who do not.

My field observations of the visual signalling behaviour of *L. fallax* were in accordance with previous descriptions of the limb movement of the species (Meyer et al., 2012; Richards & James, 1992) on the foot flagging and foot flicking observations. Additionally, leg stretching movement was observed more frequently than anticipated and potentially allows the usually concealed bright colouration of the thighs to be exposed (Haddad & Giaretta, 1999). Furthermore, the brightly coloured vocal sac was observed to be partially inflated even while the males were not emitting acoustic signals. This exposes additional colour patches to receivers and might represent an additional visual display by the species. The action was similar to the vocal sac display described in other frog species like Boana albopunctata, Dendropsophus nanus and Hypsiboas raniceps (de Lourenço et al., 2020; Furtado et al., 2017). Although throat display visual cues are described elsewhere as involving pulsation of the sac in the absence of acoustic signals (Hödl & Amézquita, 2001), recent studies have identified that the visual aspect of the vocal sac combined with the acoustic signals is important in providing the receiver with a more complete sensory cue (Partan & Marler, 2005; Starnberger et al., 2014a). The importance of vocal sac inflation as a visual cue has been observed in other species like Micrixalus kottigeharensis (Preininger, Boeckle, Freudmann, et al., 2013) and Phrynobatrachus krefftii (Starnberger et al., 2014a), which also has dynamic visual signalling repertoires during agonistic male-male interactions. The playback experiments in this study were not designed to focus on the function of vocal sac in the visual display, and both stationary and foot flagging frog models were made with half inflated vocal sacs. It is intriguing to consider the possibility that the multiple male and

the single female frogs that approached the stationary frog model might be responding to the vocal sac. Future studies solely targeting this newly documented behaviour might aid in fully elucidating whether the *L. fallax* vocal sac has a functional significance in communication.

Previous research has investigated environmental and ecological predictors of visual signal use in anurans. However, my investigations were unable to relate visual signal use with any of these factors. The only significant result suggested that visual signals could be predicted by ambient noise level, although surprisingly in the opposite direction to what might be expected. Rather than high levels of ambient noise prompting visual signalling (Grafe & Tony, 2017), the sessions in which leg movements of L. fallax occurred were characterised by low ambient noise levels. Noise generated by conspecifics might be more important to the aggressive acoustic signal component of the multimodal signal, as frogs have a higher tendency to emit aggressive calls while more males are engaged in the same behaviour in high density choruses (Grafe, 1995). Furthermore, playback studies have documented a reduction in aggressive calling by male frogs in higher background noise (Schwartz & Wells, 1984), and an increase under low background noise (Grafe, 1995). This could suggest that they possess a higher threshold for aggressive calling in higher levels of background noise (Grafe, 1995). However, although statistically significant, I am hesitant to conclude that my result reflects a biologically meaningful effect as the difference in noise level was extremely small (2.2 dB) in this study.

Unlike the widely used acoustic signals, visual signals are often used in short distance communication and light level/illumination of the environment could be a limiting factor for visual signalling (Hödl & Amézquita, 2001). During agonistic interactions, the distance between the two individuals is important (Preininger, Boeckle, Sztatecsny, et al., 2013) but I did not detect an effect of distance in this experiment. Of course, I limited sessions to those already in close proximity so might have masked any effect. Nevertheless, the results of this study are congruent with the previous observations of the visuals signalling behaviour of *L. fallax* being independent from the light/illumination level of the environment(Meyer et al., 2012). While the previous

study has related behaviour to moon phases, I was more specific here and measured light level on site. Consequently, I was able to capture both natural and artificial light, which is likely to be important for this species as it is more adapted to the urban environment. At this point I speculate that the use of visual signals is more related to the specific individuals involved rather than a function of environmental and ecological conditions. As such, efficacy-based explanations may be less applicable in explaining multimodal signalling in this species.

I suggested in Chapter 2 that the long-range territorial calls of *L. fallax* are represented by the first notes of the advertisement call Type BA and CA. In the close-proximity encounters observed in the present study, I recorded only monophasic call Type B and Type C notes, with only the latter accompanying visual signals. I found no evidence of the rapid clicking sounds described by Richard and James (1992) that the authors suggest are potential encounter calls. The advertisement calls and aggressive calls of this species are at opposite ends of a continuous graded arrangement (Grafe, 1995; Owen & Gordon, 2005) of both temporal and spectral properties. This is consistent with findings for other species producing graded acoustic signals such as, Leptodactylus albilabris (Lopez et al., 1988), Acris crepitans (Wagner, 1989), Hyperolius marmoratus (Grafe, 1995) and Rana clamitans (Owen & Gordon, 2005). It was interesting to find drastic variation in both the temporal and spectral note characteristics when producing the note Type C that is associated with the visual signalling behaviour. The spectral property note dominant frequency is reduced with the increased aggressive behaviour of the species. Such dominant frequency reduction in aggressive calling behaviour is also observed in other Australian frog species such as *Geocrinia victoriana* (Littlejohn & Harrison, 1985; Scroggie & Littlejohn, 2005) and several other species (Bee & Bowling, 2002; Bee et al., 1999; Lopez et al., 1988; Wagner Jr, 1992). Variation of the dominant frequency can provide honest information on the fighting ability of the signaller (Burmeister et al., 2002), which can shape the response of the receiver (Wagner Jr, 1992). Note duration, pulse rate and inter-pulse interval are among the temporal characteristics that showed drastic changes in the transition from the advertisement call to aggressive calls. In particular, the note duration increased almost threefold from the

diphasic note to the monophasic note used in agonistic interaction, while the pulse rate is decreased. This is in concordance with behaviour observed in other tree frogs with complex graded acoustic repertoires like *Dendropsophus sanborni*, *Dendropsophus nanus* (Toledo et al., 2015) and other species like *Acris crepitans* (Wagner 1989), *Hyperolius marmoratus* (Grafe, 1995) and *Rana clamitans* (Owen & Gordon, 2005). Unlike the spectral changes of the calls, the temporal changes are advertising the intention for an attack in graded aggression levels (Wagner Jr, 1989) but advertise little about the body size of the signaller (Burmeister et al., 2002; Robertson, 1986). It is still to be determined whether individual *L. fallax* are advertising their fighting ability via changes in acoustic signalling, which the literature suggests would help to avoid physical contests (Burmeister et al., 2002) and increase the time spent in attracting a mate (Perrill & Lower, 1994). Importantly, I have considered average changes across four individuals rather than differences at an individual level, which is what would be needed to make more definitive conclusions.

The results of the playback experiment revealed a group of male frogs that responded to the stimuli yet did not choose one stimulus over another, while appearing to spend time observing both speakers from a distance. This might imply that the stimuli lack sufficient information for the frog to make a choice. In anuran species with a similar graded aggressive call system, such as the spring peeper, *Pseudacris crucifer*, and African painted reed frog, *Hyperolius marmoratus* stimulus duration and intensity were found to significantly influence the response (Grafe, 1995; Schwartz, 1989). Since the stimulus used in this study was constructed from average calls emitted by individuals and maintained at the average natural sound pressure level it might have lacked an element that is vital in initiating a more aggressive response in the test animals. In addition, the relationship between the dominant frequency and the body size of the anurans was not considered in this study. In some anuran species, dominant frequency exhibits a negative relationship with body size (Burmeister et al., 2002; Gerhardt, 1994; Meuche et al., 2012). In physical contests, receiver males are less likely to respond to low frequency calls (Burmeister et al., 2002; Robertson, 1986) and more likely to respond with agonistic behaviour

to higher frequency calls (Wagner Jr, 1992; Wagner, 1989). Given that the acoustic stimulus we have incorporated in this study with the visual signalling was based on the average frequency of such calls it might have limited the response of some male frogs with more clear agonistic behaviour. Future playback experiments should consider this more explicitly.

Certainly, male frogs that responded did approach the multimodal signal more than the acoustic only stimulus, which I consider to be equivalent to that of an aggressive approach (Bee et al., 1999) or an initial behavioural indication of the male's tendency to involve in an agonistic interaction (Steffen et al., 2000). In general, multimodal signals usually are expected to elicit equal or enhanced responses than the unimodal signals alone (Partan & Marler, 2005; Preininger, Boeckle, Sztatecsny, et al., 2013). In the absence of any efficacy based explanations, I suggest that my data are consistent with inter-signal interaction hypothesis (Hebets & Papaj, 2005) by providing greater opportunity to discriminate signallers.

It was interesting to see that female frogs have a tendency to get attracted to the foot flagging model with significant results at high light level. However, my results contradict early work on túngara frogs, where females prefer a unimodal cue in high light levels, probably given the enhanced predator risk (Cronin et al., 2019). Unfortunately, the sample size used in my study was lower than intended (due to interruptions caused by the COVID-19 pandemic). It is worth emphasising that nocturnal female frogs are capable of acquiring and reacting to visual signalling of the males even with poor illumination levels (Gomez et al., 2011; Taylor et al., 2007). While mate recognition in a highly dense chorus is challenging, female frogs may be able to detect other cues like motion (Taylor et al., 2007). For example, female túngara frogs are known to show more attraction towards a moving vocal sac than a deflated static one (Rosenthal et al., 2004). The attraction of *L. fallax* females to the foot flagging stimuli in the results here could be the same, hence it serves as an important additional behavioural modulation (Caldart et al., 2020) in mate recognition of the species. Further experimental data is needed to understand whether female choice has driven the evolution of multimodal signalling in male *L. fallax*.

This study provides the first description of the association between graded agonistic acoustic signalling behaviour and visual signalling behaviour of Litoria fallax. Visual signals were not associated with environmental and ecological factors except ambient noise levels, which included that produced by conspecifics. Even though I did not consider the density of conspecifics in the environment, the results obtained with respect to noise level suggest the importance of considering this factor in the future studies as the species is a dominant feature of the soundscape. Both male and female frogs showed a tendency to choose multimodal stimuli over the acoustic-only stimuli in multimodal playback experiments. The use of multimodal communication in the territorial context has been observed in other taxa like pied currawongs (Strepera graculina) (Lombardo et al., 2008) where the multimodal cues enhanced the ability of assessing the level of the challenge created by the intruder. Future experimental approaches should consider the spacing of individuals during interactions, while alterations to the details of signalling, including the frequency component of calls and movement of the limb might help in fully elucidating the functional significance of multimodal signalling on the communication of L. fallax. The results also highlight the importance of studying different signal components of a multimodal communication system in conjunction, rather than individually, in better understanding the complex signal evolution.

4.6 References

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Chapter 5

General Discussion

5.1 Thesis overview

The communicative signals of the eastern dwarf tree frog or eastern sedge frog (*Litoria fallax*) had been described previously, however, careful and systematic investigation described in the three empirical chapters of my thesis have revealed a level of complexity that exceeds what was previously assumed for the species. I have also been able to obtain insight into the functional significance of some of this complexity. I was able to achieve this through a combination of fieldwork and experimental playback studies on captive frogs. My results are highly informative despite various constraints outside of my control that were imposed on my activities. In this chapter, I review the empirical results I have obtained and outline drawbacks in the methodology used. I conclude with remarks on the implications of my research for future opportunities.

Among the communication signals of animals, acoustic signals are the most widely studied because they are relatively easy to detect, record and quantify. In Chapter 2 I have redescribed in quantitative detail the vocal repertoire of *L. fallax* and provide descriptions of the multiple levels of complexity. In particular, I was able to identify three distinct types of notes in the repertoire, which are used both in advertisement and aggressive contexts, albeit with some modifications in each context. Furthermore, I have found that the acoustically active conspecific social environment has a great influence on the complexity of the call sequence produced by *L. fallax*. The topic of signal complexity has been debated over the years in the areas of mathematics, physical sciences and also in the social and life sciences resulting in various approaches to define and measure complexity (Kershenbaum et al., 2016). Indeed, identifying the complexity of a vocal repertoire is easy, but defining and quantifying it is a difficult process (Edmonds, 1995; Kershenbaum et al., 2014). Certainly, this discussion raised the question of how to classify the call components of *L. fallax* at the beginning of the study. Ultimately, I decided to make use of Shannon's equation of entropy (E; Shannon & Weaver, 1949) as a measurement of the information encoded in a call sequence. Specifically, larger entropy values imply higher
structural diversity along the call sequence (Da Silva et al., 2000; Freeberg & Lucas, 2012), and higher uncertainty of each structural element to occur in the sequence (Morais et al., 2012). I have made the assumption that each element/call type in the sequence is produced independently of all the other elements in order to use the Shannon entropy values (Kershenbaum et al., 2014). This method has been widely used in quantifying bird song sequences and I have highlighted its relevance to complex anuran call sequences.

The advertisement note types of the species that I identified in Chapter 2 were used in Chapter 3 in which I investigated geographic variation of acoustic signals by *L. fallax*. I was able to identify geographic variation in call characteristics of all three advertisement note types across the distribution, which I suggested is mainly driven by genetic drift. However, my analyses also suggested that there is likely to be some influence of local ecological conditions in the divergence of note characteristics. I was not able to pursue this line of enquiry and recommend that future studies systematically address in greater detail how ecological constraints are contributing to geographical variation in acoustic signals of *L. fallax* over and above genetic influences. My findings also show that the call characteristics of the invasive population of this species established in Melbourne has more affinity to the southern genetic group than with northern genetic clades. This too needs further investigation. Defining the genetic structure of the invasive population will be important in order to determine whether similarities I have described herein were a result of genetic similarities or defined by the similar local conditions.

In my third empirical chapter (Chapter 4), I changed focus from acoustic-only communication by the study species to acoustic-visual bimodality. With previous knowledge on the occurrence of visual signalling of the species (Meyer et al., 2012; Richards & James, 1992), my main objective was to investigate a possible relationship between the two signal modalities of this behaviour. Admittedly, the frequency at which the visual signalling behaviour occurred during field observations was lower than I anticipated. However, despite the limited number of observations, I was able to describe new observations of limb movement and vocal sac displays

to the visual signalling repertoire of the species. Furthermore, I was able to identify the acoustic signals associated with the visual signalling and agonistic behaviour. This call was emitted as the maximum end of the graded acoustic signal continuum that was identified in Chapter 2.

My field data on visual signalling suggested the occurrence of the behaviour was not strongly predicted by external ecological factors. The noise level of the habitat was implicated, however, I speculated that the difference in sound levels was small and might be a spurious result. Certainly, future experimental approaches solely focussing on how noise level of the habitat is affecting the visual signalling behaviour and the signal propagation by the potential receivers is required. These observations encourage a more detailed analysis of the external environment when studying visual signalling behaviour of nocturnal animals.

The results of the playback experiment revealed a higher rate of attraction by both male and female frogs toward the multimodal stimuli, suggesting that visual signals added functional significance. However, since these signals are always associated with agonistic interactions, and not predicted by ecological and environmental factors, there is the possibility for these to represent displacement activities (Furtado & Nomura, 2013). In particular, unintentional behaviours may be elicited as a biproduct of an ongoing conflicting activity like agonistic fighting behaviours (Maestripieri et al., 1992). Future experiments with visual signal obstructed and nonobstructed stimuli might aid in answering this question. During planning for this chapter I had hoped to be able to consider variation in the use of visual signalling along the species' broad geographical distribution. Although I did observe this behaviour to occur in each of the locations studied in the chapter, the sample size did not allow me to assess whether geographical variation occurs. The use of visual signals is known to vary across populations within species in other taxonomic groups such as lizards (Ramos & Peters, 2017). It would therefore be worthwhile to examine more closely the visual signals of *L. fallax* to know whether geographical variation is incorporated into the visual signalling of the species just as it has for the acoustic signals (Chapter 3).

5.2 Methodology

During field observations, I followed previously established methods of amphibian surveys and quantitative analysis of acoustic signals. Similarly, my captive playback experiments were designed to match as closely as possible recommended protocols. However, various factors beyond my control meant that aspects of each of these components of my work could be improved.

Given the small size of the study species and the thick ground level vegetation of the habitats, it was hard to capture all individuals to obtain body measurements. Furthermore, a major flooding event occurred in Townsville during 2019 that disrupted my fieldwork and prevented me from obtaining body size data for the frogs in the area. This followed on from permit limitations that prevented me from catching animals during the first fieldwork effort in this location. Pool breeding Australian hylids (=pelodryadids) are known to possess a higher dominant frequency compared to their body size (Hoskin et al., 2009), which is in accordance with the results I obtained in the habitats I was able to sample. Although I found weak evidence for the classic negative correlation of body size (SVL) and the dominant frequency of the note types (Gerhardt & Huber, 2002); Appendix 1), it is of course unfortunate that I could not include this factor in statistical modelling described in Chapter 3, and this should be incorporated into future work of this kind.

In constructing stimuli for playback experiments it is necessary to ensure that the stimuli represent the phenomena of interest and the statistical analysis is appropriate (i.e. not pseudoreplicated). The acoustic stimuli I have used in each of the three empirical chapters represent the population average, and the same exemplar for each type was presented to all subjects in the experiment. An alternative strategy would have been to use unique exemplars from each stimulus category for each subject. I did not have sufficient exemplars with planned sample sizes to adopt this approach. However, in my experiments, I asked specifically whether there is a preference for the population average of stimulus A compared with the population

average of stimulus B. The design and explicitly stated research question, as well as the interpretation of statistical outcomes in terms of population averages are thus entirely appropriate (McGregor, 2000). My approach has reasonable external validity so long as the stimuli are sufficiently different. However, I concede that generalising further – concluding that stimulus A is preferred to stimulus B – is not appropriate with my approach and would require the alternative strategy.

Another aspect of the experimental approach that was less than ideal was the placement of frogs in the anechoic chamber. In my experiments, the position of frog models and the release point for live frogs were at ground level for practical reasons. Limiting the vertical dimension, which is important for tree frogs in their natural environment, may have inadvertently constrained frog behavioural responses. For this reason, I kept my response measure simple but consistent with published literature. It would be worthwhile in future studies to replicate more accurately the ecology of focal species when considering responses to multimodal signals, particularly when more subtle questions are asked that might require consideration of behaviour at a finer scale.

Planning an indoor playback experimental arena for a tree frog species was challenging as the preliminary studies revealed the focal animals favour vertical substrates. This meant that the conventional method of covering the test animal with a release box and removing it distantly with string was not a successful strategy for my study species given their propensity to climb and cling to the vertical surfaces of the release apparatus. It was surprising that comparable work on tree frog species such as *Hyla cinerea* (Vélez et al., 2012) and *Hyla arborea* (Gomez et al., 2011) did not encounter this issue. However, after adopting a trial-and-error approach, the design I created (Figure 2.2) was a success in releasing the animal without disturbing its natural behaviour. I have also designed a new mechanism for recreating the foot flagging movement of the species (Chapter 4) as the method used in previous literature (Preininger et al., 2013) was unable to incorporate the *L. fallax* leg movement because of its small body size and the indoor

experimental setup. It is also worth emphasising that recreating foot flagging movements in frog multimodal phonotaxis studies is rare compared to recreating colour pattern (de Luna et al., 2010; Gomez et al., 2011) or vocal sac movement (Narins et al., 2005; Narins et al., 2003; Taylor et al., 2008) of frog models, probably given the difficulty in designing such a setup. The simple yet effective method of moving the leg which I implemented in Chapter 4 (Figure 4.1) might give some insights into future experimental approaches in similar species with a smaller body size.

5.3 Future implications and closing remarks

During this project I was able to identify that *L. fallax* has achieved greater levels of signal complexity in their communication repertoires, which has highlighted several follow-up research opportunities. For instance, female preference was examined for only one diphasic call type (*Type BA*) given the time limit for completing this work. I was not able to test female preference of each note type in the *Type CA* diphasic call. Results described in Chapter 4 subsequently revealed that visual signalling behaviour of the species is associated with the extended monophasic *Type C* note, which can elicit female phonotaxis towards it in combination with the visual signalling component. It would be interesting to know whether this preference of female frogs is consistent with different components of note *Type CA* in their advertisement call state as well. Even though females have demonstrated preference towards the note *Type A* (shorter click sound) when given a choice, the functional significance of the diphasic call formation in mate attraction is still to be elucidated and would require presentation of diphasic and monophasic calls in a similar styled choice experiment.

The *bicolor* species group comprises my study species (*L. fallax*), as well as *L. bicolor*, *L. olongburensis* (Olongburra tree frog) and *L. cooloolensis* (Cooloola tree frog or the northern dwarf tree frog). These species are sympatric in parts of their ranges and as such inter-species interactions offer interesting avenues of future research. With previous knowledge of the systematic analysis of the acoustic repertoire of *L. bicolor* (Menzies et al., 2009) and the novel knowledge generated in this study it would be useful to undertake a detailed acoustic

description of the other two species. Given that the distribution of *Litoria fallax* coincides with *L. bicolor* (Knight & Tyler, 2020) and the other two species of the complex, this might be a great opportunity for comparing acoustic signalling behaviour of the sympatric and allopatric populations of the study species with other members of the complex.

The results identified in Chapters 3 and 4 encourage more field work because I have only just scratched the surface on the potential geographic variation in the communicative behaviour of *L. fallax*. As stated above, this would necessarily involve both visual and acoustic signals and further investigation on how local habitat characteristics may be involved in the variation process. Furthermore, how sexual preference of female *L. fallax* varies toward the observed geographical variation among populations is yet to be tested in the populations away from Melbourne. In this analysis I have only considered the previously descried genetic variation instead of the geographic distance of *Litoria fallax*. An interesting avenue for future research would be to consider the populations within these genetic groups in determining how geographic distance acts upon the divergence of call characteristics.

The graded call patterning identified in the study species has opened up new experimental research opportunities for understanding the agonistic and visual signalling behaviour of the species. Consequently, whether the male frogs evoke more aggression towards the model frogs when given different dominant frequency values of the graded continuum should be examined. The attraction of female frogs towards the multimodal stimuli of *L. fallax* is one of the most interesting findings generated in this study and warrants further investigation. Given that the experimental setup has already been established, manipulating each signal component stimuli in further experiments will be relatively straightforward with a view to elucidating the functional significance in the multimodal signals in mate choice of the species. Furthermore, this would also generate additional information on whether the complex communication signals of the species are facilitating the higher invasive ability compared to closely related species (Bevelander, 2014). I suggest *L. fallax* to be an excellent model anuran species for understanding the evolution of complex communication systems.

An initial objective of my project was to study further the multimodal communication of *L. fallax* building upon earlier work (Meyer et al., 2012; Richards & James, 1992) including previous descriptions of the acoustic repertoire (Straughan, 1969). However, it was quite apparent early on that the acoustic repertoire of the species comprises greater complexity than previously thought. Subsequently, I diverted my research focus to the task of describing the vocal repertoire of the species and varying levels of complexity. The conventional nomenclature of separating signal types of anurans could not be followed with this species mainly due to the graded signal patterning. However, the step wise approach of note and call type separation employed in Chapter 2 has not only generated new knowledge but also provided the foundation for further studies.

Long term studies of anuran communication systems in other parts of the world have focused on model anuran species, such as the Nearctic species the gray tree frog, *Hyla versicolor* (Gerhardt, 1978; Reichert & Gerhardt, 2012), the Nearctic species with graded aggressive calls the spring peeper, *Pseudacris crucifer*, (Doherty & Gerhardt, 1984; Parris, 2002; Schwartz, 1989), and the Neotropical species with a multidimensional complex communication system the túngara frog, *Physalaemus* (*=Engystomops*) *pustulosus* (Gridi-Papp et al., 2006; Ryan, 1985). Long term studies of this nature are rare for frogs in the Australian region despite the high anuran diversity and potential study systems. I would like to suggest that *L. fallax* could be *the* model system for further studies of anuran communication in Australia, given the newly found complex communication system and wide and common distribution. Refining of traditional techniques and the development of innovative methods for studying visual communication behaviour provides great opportunity for future research of other Australian anurans with multimodal signalling (Meyer et al., 2012). I hope that my study serves to encourage other researchers to consider undertaking detailed investigations of the wonderful and complex communication systems of Australian anurans.

Moreover, this study highlights the importance of integrating multiple levels of analysis in investigating how different components of communication signals function across contexts,

variable receivers and environmental conditions. Specially the use of information theory in studying communication systems could provide insight into important properties of animal intelligence (Ryabko & Reznikova, 2009). This serves as a common language for cross-taxon comparisons of the complex communication system designs (Reznikova, 2007). I encourage others to consider incorporating the information theory when studying the evolution of complex, multicomponent courtship phenotypes as the amount of information plays an integral part in receiver preference (Hebets et al., 2016). Furthermore, this approach has been previously used in other taxa, when investigating how different attributes of social demographic complexity explains the evolution of complex communication systems (Pollard & Blumstein, 2012). My work has contributed to this database of information, which ultimately could help in better understanding of the relationship between the social environment and the evolution of complex communication signals (Doyle et al., 2011; Kershenbaum et al., 2016; McCowan et al., 1999).

5.4 References

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Appendix One

Introduction

Allometry has been identified as the correlation of body size to physiological, anatomical and behavioural traits of animals (West et al., 2000). This has been observed in variety of taxonomic groups (Tonini et al., 2020) including the call characteristics change of anurans. Body size of the anurans have been identified to constraining the spectral property dominant frequency of the call characteristics (Gerhardt, 1994), which is in turn effecting the sexual selection. Furthermore, the temporal characteristics are typically known to not get effected by the body size of the anurans (Hoskin et al., 2009). Given this relationship, it is worth analysing the correlation between the note dominant frequency and the SVL of the individuals while comparing the call characteristics between populations.

Methods

During fieldwork and after recording calls from a male, I captured frogs to measure body size. Snout vent length (SVL) of calling males were measured in situ to the nearest 0.01 mm using a digital Vernier calliper. Frogs were released back to their natural habitat after all measurements were completed.

I examined recordings of calls from frogs in northern (Brisbane), southern (Newcastle and Wollongong) and Melbourne populations and extracted monophasic notes *Type A*, *Type B* and *Type C*. I used Raven Pro's spectrogram function identify the dominant frequency (256 point fast fourier transform using a Hanning window).

Linear mixed effects models were used to consider whether dominant frequency was related to body size (SVL). I used the *lme* function from the *nlme* package (Pinheiro et al., 2018) in the R statistical environment (R Core Team, 2018), fitting snout-vent length as a single predictor variable and individual identity as a random effect to account for multiple observations of the same individual. Separate models were constructed for each genetic group (Brisbane, Southern, Melbourne groups) within each of the three note types.

Results

Results of linear mixed effects models are shown in Table A1 and the respective model estimates are shown along with raw data for note *Type A* (Figure A1), *Type B* (Figure A2) and *Type C* (Figure A3). A significant effect of SVL was found for note *Type B* for the southern population (Figure A2 *middle panel*), and for note *Type C* for the Melbourne population (Figure A3 *bottom panel*).

Discussion

The results obtained in this study is mostly aligned with the previous literature of call dominant frequency having a negative relationship with the body size of the frogs (Bee et al., 1999; Hoskin et al., 2009; McClelland et al., 1996). Although the trend of negative correlation with the body size is mostly consistent in *L. fallax*, the relationship does not appear to be strong with only two significant results of negative correlation. However, it is surprising to see a positive correlation of the body size and the dominant frequency of note *Type B* and Type C in the northern, Brisbane populations, while note Type A of the same population follows the common pattern of the negative correlation. This might be an error occurred due to lack of sampling size. On the other hand, since note *Type B* and *Type C* are used in territorial behaviours the positive results might have influenced by the social factors. It is worth nonetheless to sample more frog calls and body size data of this area to see fully understand this variation.

		^a DF _{num}	^a Df _{den}	F-value	p-value
Note Type A					
Brisbane	(Intercept)	1	72	4464.18	<.0001
	SVL	1	12	2.32	0.1534
Southern	(Intercept)	1	303	11138.18	<.0001
	SVL	1	303	1.58	0.2098
Melbourne	(Intercept)	1	188	5471.86	<.0001
	SVL	1	188	2.92	0.0892
Note <i>Type B</i>					
Brisbane	(Intercept)	1	24	5842.86	<.0001
	SVL	1	11	2.20	0.166
Southern	(Intercept)	1	49	21742.22	<.0001
	SVL	1	21	15.92	<.0001
Melbourne	(Intercept)	1	41	5853.32	<.0001
	SVL	1	17	0.03	0.8583
Note <i>Type C</i>					
Brisbane	(Intercept)	1	23	3618.29	<.0001
	SVL	1	23	2.67	0.1156
Southern	(Intercept)	1	45	11237.81	<.0001
	SVL	1	45	1.98	0.1659
Melbourne	(Intercept)	1	24	16973.51	<.0001
	SVL	1	19	6.17	0.0225

Table A1 | Results of linear mixed effects models predicting variation in dominant frequency of note *Type A*, *Type B* and *Type C* as a function of snout-vent length (SVL).

num = numerator; den = denominator



Figure A1 | Relationship between snout-vent length and dominant frequency for note *Type A* for frogs from the northern (top row), southern (middle row) and Melbourne (bottom row) populations. Black circles are raw data, red circles are estimates from the model and the red shaded region represents 95% confidence interval of the model estimate.







Figure A3 | Relationship between snout-vent length and dominant frequency for note *Type C* for frogs from the northern (top row), southern (middle row) and Melbourne (bottom row) populations. Black circles are raw data, blue circles are estimates from the model and the blue shaded region represents 95% confidence interval of the model estimate.

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