國立臺灣師範大學生命科學研究所博士論文

翡翠樹蛙對環境中聲音訊息之行為反應
Behavioral response of acoustic information in *Rhacophorus prasinatus*

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致謝

就像大多數人一樣，問起碩士班畢業後是否會再繼續進修博士學位，回答都是:不可能的事。學術研究不是一件輕鬆的事，邏輯思維的要求、實驗設計的規劃、統計分析的學習，更別說還有那些永遠念不完也查不盡的文獻地獄，沒接觸過的人大概很難理解吧。除非未來有需要走相關的行業，別如此折磨自己。是的，何必折磨自己?就業多年才決定進修博士班，不管是在課堂考試、上山做實驗、下山熬夜撰寫報告，即便是論文即將送印時，心理的另一個聲音依然這麼問著自己。不過想著自己的研究回答了一些沒人探討的問題，也可能推著某些議題邁進了一小步，似乎就值得了。

在職進修博士班，首先得感謝林業試驗所黃所長的同意與鼓勵、林副所長的推薦、陳組長於業務上的調整與關心、同事玫玲沒有二話的請假代理，特別是瑞琴在工作上的細心協助，至少分擔了八成以上的工作壓力，沒有各位的幫忙絕對是畢業無望的。

有幸能在林思民老師指導下學習是一件很幸福的事，一個腦筋不好性格又頑固的大齡學生，竟然敢不顧老師的研究建議，一頭栽入莫名其妙的動物行為議題，實驗結果撲朔迷離，還得逼的老師不斷勞心、勞心又勞心的將結果導向重要的議題以及修改不知所謂的英文文法，最後，回饋老師的是一顆心血管支架。能獲此以自身健康指導學生的老師，不是區區感謝二字能夠表達的。

感謝關永才老師、李佩珍老師、陳怡惠老師以及黃淑萍老師在論文架構與內容上給予精闢的建議，讓議題尺度能夠更上層樓。除了修業課堂上的老師指導之外，特別要感謝廖培鈞老師/協長的支持，不管是學術議題的釋疑，檢討實驗邏輯上的漏洞，以及投稿文章的協助，都是畢業不可或缺的動力。謝謝賴俊祥-大砲學長帶領我走入兩棲類的領域，在進修碩士班以及博士班時給予不斷的鼓勵與建議，希望能像你一樣堅持的走下去。

師大

一個每週只來上課的大叔，說實在對實驗室提供不了甚麼幫助，頂多偶爾帶些小吃、冰棒止止大家的嘴饞，但是卻從實驗室夥伴以及同學身上獲得不成比例的助力。感謝致維學長、展蔚、閣桓、書書在實驗設計與統計分析上的協助，芳神、阿平、李昱幫忙花白工拉了一個隔年就被颱風摧毀的樣區，王蟲子、曾威、阿薇在實驗流程以及儀器設備的腦力激盪，阿寶、如如、A4、嘉偉、宇德、粹文、婉馨、致維、凱翔、處安、雨昕、鳳君、志和、敬家的各種 carry。也要特別感謝秉宏和一起於華林共患難的元誠，在課業與研究上的鼎力相助。

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實驗的過程總是比研究結果更讓人津津樂道，單槍匹馬在山裡晃蕩也讓我有許多難以言喻的經歷，山裡的槍響、恍如仙境的夜裡大霧、地震時滿山的樹木搖晃、迷路的獵犬、穿山甲的腳步聲以及褐貓遠方的鳴唱，感謝山神的接納與照應。

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中文摘要

訊息傳遞在兩棲類的繁殖中扮演非常重要的角色，不管是物種辨識、挑選配偶或建立領域，都仰賴從週遭環境中獲取並判斷不同形式的訊息，並依判讀的資訊做出進一步的決定。聲音訊號是無尾目物種最常用來傳遞訊息的模式，雄蛙於繁殖季節聚集至水邊，以鳴叫聲吸引雌蛙前來交配。然而，周圍同種及異種個體所發出的鳴叫聲，甚至自然環境產生的噪音，都對鳴聲的傳遞造成干擾。這些逆境對動物來說是必須克服的挑戰，並可能是促使物種演化的原動力。鳴叫對雄蛙而言是非常耗能的行為，在噪音干擾之下，雄蛙應該具有相對應的行為策略，減少訊號傳遞時受到的干擾，並提高鳴聲傳遞的成功率，以獲得繁殖的機會。不過相關研究多為人工環境下之測試，或探討人為活動所產生的噪音干擾，而在自然狀況下針對雄蛙對共域異種聲音訊號之行為反應實驗則非常缺乏。我的研究目的是了解雄蛙在繁殖季中如何克服噪音所造成的干擾，並且針對雄蛙”如何尋找適合鳴叫的地點”，以及”加入鳴叫集團後的策略”這兩個角度探討雄性翡翠樹蛙(*Rhacophorus prasinatus*)在噪音干擾下的行為策略。第 1 章首先探討環境中噪音的種類，以及前人研究中動物對噪音的因應方式。第 2 章則使用聲音回播的方式，測試雄性翡翠樹

蛙對不同回播聲音的趨聲偏好(Phonotaxis preference)。有別於前人在室內進行短距離的研究，我模擬翡翠樹蛙在自然環境的實際尺度，建置一個直徑 20 公尺的試驗場地進行測試，並且檢測以下 4 個問題:（1）雄蛙偏好加入大型或小型的合唱集團？（2）雄蛙偏好加入高品質或低品質的鳴叫同伴？（3）雄蛙是否利用異種鳴聲定位潛在的繁殖地點？（4）雄蛙是否會加入或避開具有異種鳴聲的混種合唱集團？結果顯示，雄性翡翠樹蛙偏好加入大型的合唱集團；低品質的雄性翡翠樹蛙傾向於接近高品質的雄性翡翠樹蛙，這可能屬於衛星雄性的潛行(Sneak)行為；此外，在沒有同種叫聲的狀況下，雄性翡翠樹蛙會利用生態棲位差異較大的腹斑蛙(*Babina adenopleura*)的叫聲尋找潛在繁殖地點，但不會接近使用生態棲位相似的布氏樹蛙(*Polypedates braueri*)叫聲，這也代表雄性翡翠樹蛙有能力透過鳴聲辨識共域物種。第 3 章我同樣使用聲音回播的方式，以分析雄蛙鳴聲反應的差異，探討雄性翡翠樹蛙對背景噪音干擾的因應策略，並且檢測以下 5 個問題:（1）雄蛙是否能夠調整節奏或鳴叫時機以避免鳴聲與噪音重疊？（2）雄性對不同音頻(Hz)噪音的反應為何？（3）雄蛙是否會加入具有相同音頻噪音的合唱集團？（4）不同品質的雄蛙在高強度噪音干擾下是否採取不同的鳴叫策略？（5）野外實際狀況

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下雄蛙的鳴叫反應為何？結果顯示，雄性翡翠樹蛙會利用噪音發出的空檔調整其鳴叫時機，避免鳴聲與噪音重疊；雄性翡翠樹蛙顯著避免於與自身鳴聲主頻率(~1500Hz)相同的中頻噪聲(1000Hz-2000Hz)時段鳴叫，並避開具有這類噪音的合唱集團；雖然在測試期間所有雄蛙其鳴叫率(call rate)均顯著下降，推測可能是為了在噪音干擾下節省自身能量，然而，與高品質雄性翡翠樹蛙相比，低品質量雄性的鳴叫率降低程度更為明顯，這也代表面對噪音干擾時所採取之鳴叫策略可能與雄性個體的身體狀況有關。在最後一個章節中，我針對研究結果中未來可延伸的議題進行探討，包括:物種鳴聲的主頻率可能為影響物種共存的另一種資源型態；噪音干擾可能促進訊號的演化；雄蛙在無法預測雌蛙出現的狀況下，將能量投資於繁殖場的出席率和投資於鳴叫展示，可能是權衡(trade-off)下的結果；物種的趨聲行為可應用在物種保育，以及外來種移除措施。本論文的研究結果不僅提供了第一個模擬自然尺度下的測試案例，亦同時檢測多物種的掩蔽效應(mask effect)，並顯示在野外實際狀況下，蛙類的群集合唱行為可能為包含訊號傳遞、感官靈敏度、合唱團物種組成以及與競爭者之間敵我評估的綜合策略。

Abstract

Acoustic communication plays a crucial role in the reproduction of most anuran amphibians, such as species recognition, mate choice and territory defense. For the species which aggregate in dense choruses, the high level of background noises would lead the frogs to face the interruption and further constrain their communication. Therefore, noise interference is a ubiquitous challenge for these animals. Signalers should behave to reduce masking by noise, and these strategies are presumably preferred by natural selection or sexual selection. The aim of my study was to explore how male frogs deal with background noises in the breeding season. By using broadcasting tests on a polyandrous choral treefrog *Rhacophorus prasinatus*, I examined how male frogs use conspecific and heterospecific signals to locate a chorus in Chapter 2 and tested the following questions: (1) Do male frogs prefer to join a large or a small chorus? (2) Do male frogs prefer to approach a high-quality or a low-quality male? (3) Do males utilize heterospecific calls to locate the potential breeding sites? (4) Do male frogs orient towards or avoid choruses containing heterospecific calls? My results indicated that male frogs prefer to join a large

rather than small chorus. Low-quality males tend to approach high-quality males, which might be explained as the sneaking behavior of satellite males. Furthermore, male frogs may use heterospecific calls delivered by a noncompetitive sympatric species (a ranid frog) to find potential breeding sites, but not by a sympatric competitor (another rhacophorid frog) that occupies a similar niche. On the other hand, I further examined the response of males with the interference from background noise after join a chorus in Chapter 3, and aimed to answer the following questions: (1) Are male frogs able to adjust their tempo or call timing to avoid overlap with noise? (2) What is the response of males to noises with different frequencies? (3) Would male frogs choose to join or keep away from a chorus with too much noise interference? (4) Do males with different body conditions apply different strategies when facing high-intensity interference? (5) What is the strategy of males applied in the wild? The results indicated that male frogs adjusted their calling pattern by using the spacing of the noises to avoid a direct temporal overlapping and avoided medium-frequency noises which obviously overlapped their own signal after join chorus. Although all the frogs showed a

significant decrease of call rates during broadcasting, possibly aiming to save their own energy; yet the level of decrease was more pronounced in low-quality males compared to high-quality ones. The results in my dissertation not only provided the first experimental test in natural condition which consider the multiple species masking effect in playback experiment, but also provided a guide for further approach, which should combine all relevant factors including signal transmission, sensory sensitivity, chorus joining decision, and alternative courtship strategy when testing the phonotaxis preference of anurans.

Keywords: Alternative mating tactics, Calling strategy, Energy constraint, Honest signal, Noise masking, Resource partition, Satellite males

Chapter 1

A brief introduction to acoustic communication, noise interference, and sexual selection

Signal transmission is critical in animal behavior, including predator or prey detection, communication, navigation and foraging (Smith and Harper 2003; Wiley 2006). Acoustic communication plays a crucial role in the reproduction of most anuran amphibians, such as species recognition, mate selection and territorial defense (Wells 2010). For species which aggregate in dense choruses, the high level of noises would lead the frogs to face the interruption from the background noises and further reduce their communication efficiency (Ryan et al. 1981; Gerhardt and Huber 2002; Velez and Bee 2010). Therefore, noise interference is a ubiquitous challenge for these animals.

Background noises is defined as a form of energy that is meaningless for signalers and receivers during their signal transmitting process (Luther and

Gentry 2013). Sources of acoustic background noises include: (1) abiotic noise, such as wind, stream and rain continuously produced by background energy; (2) biotic noise, produced by conspecific or heterospecific individuals which can result in acoustic interference; (3) anthropogenic noise, referring to various types of artificial sound attributable to an urban environment or a natural environment with human activity. These background noises not only interfere the transmission of signals between signalers and receivers, but also drive the evolution of signal to improve success in communication.

Communication between signalers and receivers comprises on two important parts: detection and recognition. Detection refers to the discrimination ability of a receiver whether a signal has occurred. For example, in many frog species, males aggregate to a shared breeding site and form a multi-species chorus. Females need to detect and recognize a conspecific male in order to avoid cross-species breeding (Gerhardt and Huber 2002). Interference from the background noises can constrain signal detection and recognition (Gerhardt and Klump 1988; Bee 2007; Amezquita et. al. 2011), and limit the ability of females to discriminate among species (Bee, 2008), ultimately reduce their fitness (Friedl and Klump 2005; Bee et al. 2013; Bleach et al. 2015; Neelon and Höbel 2017). Thus, background noises are expected to induce selection on behavioral plasticity which optimize individuals' fitness (Ryan et al. 1981). Since calling is one of the most energy-costly behaviors in anurans (Grafe and Thein 2001), producing calls that can be effectively evaluated by females should be favored by natural or sexual selection. Furthermore, males need to vocalize in order to attract females because most of the anuran mating system relies on females approaching the male after evaluation (Gerhardt and Huber 2002). Therefore, understanding how anurans respond to noise is particularly important with our increasing concern for anurans conservation (James et al. 2015).

Noise is a distress in animal communication. In order to response to ambient noise, some animals exhibit behavioral adjustments that minimize interference and improve signal transmission. Based on the review of Ruppe et al. (2015), five categories of behavioral response were defined as bellow:

Keep away from the source of noise:

To retreat from or to avoid the noisy habitat is a common way for animals to keep away from background noises (Höbel and Barta 2014; Schwartz et al. 2015). This behavior is thought to reduce the possibility of being interfered when the calling was inhibited by the interferences from conspecific (Wong et al. 2009) or heterospecific (Garcia-Rutledge and Narins 2001) species.

Increase the intensity of signal:

To increase the intensity of signal is a general solution when delivering signal in a noisy environment. Louder calls can be heard when exposed to noise, and could facilitate signal transmission as proposed by the Lombard effect proposed (Brumm and Zollinger 2011). Increase of energetic output allows real-time signal to noise ratio adjustment, thereby providing optimal signal detection, localization, and discrimination (Humfeld 2013; Luther and Gentry 2013; Halfwerk et al. 2016).

Extend the property of signal:

When exposed to the noise, the disruption is usually unpredictable. Therefore, repetitively producing signals may ensure the signal be detected by receiver under noisy circumstance (Toledo et al. 2014; Hanna et al. 2014). Signal detection theory has clear predictions about how can signaler deal with the background noise. Increasing the density of signal in transmission is the simplest way to improve signal detection and discrimination; it suggests that receivers and signalers might coevolve to increase communication efficiency (Wiley 2006).

Partition the species-specific frequency in calls:

Species should diverge in acoustic characters to allow receivers to discriminate between similar signals (Amezquita et. al. 2011; Mullet et al. 2017). Green frogs (*Rana clamitans*), for example, increase the peak frequency of their calls when exposed to loud noise (Cunnington and Fahrig 2010). Some frogs living beside noisy streams permanently produce ultrasonic calls, which prevents their frequency from overlapping with surrounding stream noise (Feng and Ratnam 2000).

Shift the timing of signal:

Temporal partition in signaling is applied to reduce noise interference (Garcia-Rutledge and Narins 2001; Potvin 2016). Silence-gap detection in the noise is a short-term signal adjustment to avoid overlapping with noise and increase the detectability and discriminability of their signals (Hanna et al.

2014). The ability of quickly producing a signal during the silence-gap of the noise should be beneficial, and has been reported for several species using acoustic communication system (Mönkkönen et al. 1997; Barber et al. 2010; Höbel 2014; Magrath et al. 2015).

In summary, signalers have a wide range of adaptations to reduce being masked by noise. However, some of these behavioral adjustments might increase the usual costs of signal production, such as energy expenditure or predator attraction (Brepson et al. 2012; Magrath et al. 2015). Plasticity in an individual's calling behavior may help counter for this variable ambient noise (Bee et al. 2013; Höbel 2014). Nevertheless, most of these studies were conducted in laboratory with low levels of background sound; whereas the background sounds in the field might be a combination of different choruses. Overlapping calls of rivals affect both the behavior of calling males and the preferences of females (Bee et al. 2013; Hanna et al. 2014). Determining how individuals respond to noise is therefore important for understanding how animals optimize communication in noisy circumstances. It is also important to understand how noise might affect populations and species distributions

(Kishi and Nakazwa 2013; Buxton et al. 2018).

The aim of my study was to explore how male frogs of *R. prasinatus* deal with background noises in the breeding season. I further aimed to discuss whether the conspecific and heterospecific vocal cue were used as the information of chorus selection, and how male frogs respond to background noise after joining a chorus. Although comprehensive discussion and further approaches were discussed in the last chapter.

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Chapter 2

Influence of conspecific and heterospecific cues on phonotaxis behavior in a polyandrous treefrog

Abstract

Although a large proportion of the literature has addressed the direction and preference of female choice in frogs, research about how males locate a breeding site and what strategies are involved in aggregation have been much less explored. By using broadcasting tests on a polyandrous choral treefrog *Rhacophorus prasinatus*, I examined how male frogs use conspecific and heterospecific signals to locate a chorus by asking the following questions: (1) Do male frogs prefer to join a large or a small chorus? (2) Do male frogs prefer to approach a high-quality or a low-quality male? (3) Do males utilize heterospecific calls to locate the potential breeding sites? (4) Do male frogs orient towards or avoid choruses containing heterospecific calls? Results indicated that males prefer to join a large rather than small chorus. Low-quality males tend to approach high-quality males, which might be explained

as the sneaking behavior of satellite males. Furthermore, males may use heterospecific calls delivered by a noncompetitive sympatric species (a ranid frog) to find potential breeding sites, but not by a sympatric competitor (another rhacophorid frog) that occupies a similar niche. Although the males did not show significant preference between conspecific chorus and mixed-specific chorus in the broadcasting tests, recapture records in the wild indicated that a high ratio of males would leave the mixed-specific choruses and move toward conspecific choruses in the next capture event.

Introduction

The co-occurrence of sympatric animal species in shared habitats is of interest in behavioral ecology, evolutionary biology, and community structure. Animals utilize conspecific and heterospecific cues to locate potential habitats (Sebastian-Gonzalez et al. 2010; Zeigler et al. 2011), and this scenario also exists in anuran species (Buxton et al. 2015; Madden and Jehle 2017). In forest environments where water resource is patchy and limited, suitable breeding sites are sometimes precious for anuran species. In this kind of habitat, competition at both intra- or inter-specific levels might be intense. Therefore, the choice of a breeding site should be a compromise after considering multiple clues of costs and benefits (Ryan et al. 1981; Kishi and Nakazawa 2013; Höbel 2014; Höbel and Barta 2014; James et al. 2015; Rehberg-Besler et al. 2016).

In anurans, males aggregate to perform their advertisement signals. This behavior, also known as “leks” or “choruses”, is thought to increase the fitness of males by attracting potential mates (Ryan et al. 1981; Stamps 1988). The “chorus attraction hypothesis,” reviewed by Gerhardt and Huber (2002), posits that anurans use conspecific choruses to locate new habitats. In addition to increasing the chance of encountering mates, it also helps in searching for a suitable habitat while using the lowest energy costs. For example, Bee (2007) broadcasted conspecific calls to male wood frogs (*Rana sylvatica*) in a testing room, and males showed positive phonotaxis toward the conspecific calls. Most studies suggested that sounds of a chorus allow frogs to locate the breeding aggregation in the breeding season (Bee 2007; Ursprung et al. 2009; Buxton et al. 2015, 2018).

Joining a suitable chorus affects the breeding success of a male. In a patchy environment, male frogs may have chances to move among different choruses, and the strategy of choosing choruses may maximize their fitness. Joining a high-quality chorus may result in a higher probability of encountering females; however, the trade-off is high male–male competition (Lucas et al. 1996; Friedl and Klump 2005; Fletcher 2009; Humfeld 2013; Höbel 2014). Therefore, the competitive strength of a chorus could be an important factor for males to evaluate. For example, high-quality and low-quality male frogs may strategically join different choruses to maximize their mating success (Lucas et al. 1996; Humfeld 2013; Höbel 2014).

In addition to conspecific calls, heterospecific cues are also used by frogs to locate suitable habitats. As addressed by the "heterospecific attraction hypothesis," the presence of other species could be signaled through visual, olfactory, or acoustic cues, which serve as a form of public information regarding the habitat's quality (Mönkkönen et al. 1997). In sympatric sites, species adapt to utilize different resources to reduce interspecific competition. Thus, recognizing heterospecific cues should be an ability required for optimal habitat choice. In previous studies, the marbled newt (*Triturus marmoratus*) and great crested newt (*Triturus cristatus*) showed the ability to use the calls of sympatric anuran species to locate a breeding site, even though they do not communicate with acoustic signals (Diego-Rasilla and Luengo 2004; Madden and Jehle 2017). Since mixed-specific choruses are often found in the wild, heterospecific calls could be recognized as an additional cue for habitat quality (Bee et al. 2013).

A considerable body of literature has focused on conspecific and heterospecific attraction in anurans. However, in the majority of these studies, frogs/toads were tested at a distance of less than three meters; this is vulnerable to the critique that such studies might only represent the situation “within” the chorus, not the choice “among” choruses (Beckers and Schul 2004; Bee 2007; Ursprung et al. 2009; Moreno-Gómez et al. 2015). Furthermore, most of these studies tested the response of females, whereas the male decision of joining a chorus was explored less thoroughly (Swanson et al. 2007; Bee and Riemersma 2008; Schwartz et al. 2015). More precise experiments, especially conducted with a larger spatial scale, are required to investigate male chorusing behavior.

In this study, *Rhacophorus prasinatus* (Rhacophoridae), an Oriental treefrog, was used to test the aggregative behavior of frogs. In Taiwan, this frog has a prolonged breeding season almost all year round, except when the temperature is lower than 10°C (Chen 1992). In the breeding season, males form choruses in bushes, and produce foam nests on vegetation near or above ephemeral and permanent water (Mou et al. 1983). Based on my observation, satellite males tend to join the copulation process and lead to polyandrous mating and extra-pair paternity in this frog. *Babina adenopleura* (Ranidae), *Polypedates braueri* (Rhacophoridae), and *Rhacophorus taipeianus* (Rhacophoridae) are often sympatrically distributed with *R. prasinatus*. *Babina adenopleura* usually breeds at permanent water in March through October and produces an egg mass in the water (resource noncompetitor), whereas *P. braueri* uses ephemeral and permanent water to breed in March through October and produces a foam nest on vegetation (resource competitor). *Rhacophorus taipeianus* also produces foam nests but always in a mud cave underground, and it breeds in the winter from November through

February (competitor with temporal partition). A recent study reported a higher overlapping ecological niche breadth between *R. prasinatus* and *P. braueri* than that between *R. prasinatus* and the other two species (Lu and Chen 2012), thus providing a good opportunity to study the interspecific interaction of chorusing behavior.

To test whether male frogs utilize conspecific and heterospecific cues in aggregating behavior, I employed a hypothesis-testing methodology. I constructed a circular testing arena in an outdoor environment, and broadcasted different chorus sounds that varied in chorus size, the member-quality of male frogs, and the presence or absence of heterospecific frogs. Based on the assumption that males would maximize their fitness, I hypothesized that males would join the patch where more males aggregate, or join the high-quality males, which have a higher potential to encounter females. I further hypothesized that males do recognize heterospecific cues, and the aggregating response would vary according to competitive pressure. I predicted that the following: (1) male frogs would prefer to join a larger chorus rather than a small one; (2) male frogs would prefer to approach to a high-quality male than a low-quality male because of the higher potential to encounter females; (3) heterospecific calls would be used to locate potential breeding sites when conspecific calls are absent; (4) male frogs would avoid joining a chorus with noise from competitive heterospecific calls. The findings in the current study may enhance our knowledge on the potential ecological and evolutionary factors that influence animal aggregative behavior.

Materials and methods

Study system

The experiments were conducted from 2016 to 2017 in Hualin Experimental Forest (24°53’N, 121°33’E), a 92-hectare protected region located in New Taipei City, northern Taiwan. The environment consisted of secondary hardwood forest with some grassland patches. Natural and artificial pools were located within the forest in a patchy and mosaic pattern, and attracted choruses during the breeding season. In addition to *R. prasinatus*, the most abundant frogs in the study site are *Babina adenopleura* (Ranidae) and *Polypedates braueri* (Rhacophoridae) in the same breeding season, and

Rhacophorus taipeianus in a different breeding season (only in winter).

An individual marking program using toe clipping has been conducted in the experimental forest for years, which helped us with individual identification in this experiment. Captured frogs were weighed to the nearest 0.01 g using an electronic scale, and the snout-vent length (SVL) was measured to the nearest 0.01 mm using a digital caliper. Following Peig and Green (2010), the scaled mass index (SMI) was calculated from the body length and body weight according to the following equation: SMI = $M_i[L_0/L_i]^{bSMA}$, where M_i and L_i are the body weight and the SVL of individual i, respectively; bSMA was calculated from the standardized major axis regression of the log body weight on log SVL; and L_0 was the mean body length for the study populations.

Call recording and editing

Acoustic files for playback (Fig. 2. 1) were recorded from the focal species by an SM2 digital recorder (Wildlife Acoustics, Maynard, Massachusetts, USA) at the study site. The original files contained the chorus of only one species from each of the four frogs. For *R. prasinatus*, I further recorded calls from single individuals, which could be randomly selected in playback experiments.

In addition to original calls, I also synthesized or modified call files for usage in different experiments. *Rhacophorus prasinatus* produces calls consisting of a series of high frequency "A" notes, and a series of low-frequency "B" notes (Fig. 2. 1c). In this experiment, I considered calls with A+B notes as high-quality calls, and those with only A notes as low-quality calls based on the following several reasons: (1) In the wild, B notes are only recorded from high-quality males who persist in making continuous breeding calls. In contrast, B notes are much less frequently recorded from low-quality males who make calls in a punctuated manner. (2) Considering that courtship calls are extremely energy consuming (Wells 2001), those who produce more calls have been found to have better body condition (Sullivan and Hinshaw 1992; Prestwich 1994; Reichert and Gerhardt 2011; Rodríguez et al. 2014). (3) In a previous study on a closely related species *Rhacophorus moltrechti* which also produce two types of notes, the length and number of B notes was the cue for females to distinguish high-quality males (Chen 2002). Based on the results of these previous studies, I used the original A+B files as a high-quality stimulus, and erase the B notes to produce a new file as a low-quality stimulus. Finally, the mixed-specific calls were generated by mixing natural chorus of *R. prasinatus*, *B. adenopleura*, and *P. braueri* into a single file. The white noise stimulus (Fig. 2. 1b) was synthesized to 15 minute files using Audacity software (version 2.06, http://audacity.sourceforge.net/), containing two-second intervals of white noise (with different frequencies in different treatments), spaced by two-second silent gaps.

All digital acoustic files were recorded and broadcasted with a 44.1 kHz sampling rate, and 16-bit resolution WAV format; the amplitude of playbacks was set to 75 dB SPL measured at 1 M from the speakers using a sound pressure level meter (Lutron SL-4030; fast RMS, ‘C’ weighting).

Experimental design: conspecific tests

In order to investigate the aggregating behavior of males, I designed three conspecific phonotaxis tests.

***Experiment I-1*—**Two unidirectional tests: phonotaxis preference toward *R. prasinatus* (conspecific) chorus calls, and phonotaxis preference of white noise as the control.

***Experiment I-2*—**Bidirectional test: phonotaxis preference of chorus calls of *R. prasinatus* compared to calls of a randomly selected single male in the opposite direction.

***Experimental I-3*—**Bidirectional test: phonotaxis preference between high-quality calls (A+B notes) and low-quality calls (A notes only) in the opposite direction.

Experimental design: heterospecific tests

In order to investigate the response of males to heterospecific calls, I designed two phonotaxis tests and one capture–mark–recapture experiment.

***Experiment II-1*—**Three unidirectional tests: phonotaxis preference toward chorus calls of *B. adenopleura* (resource noncompetitor), *P. braueri* (resource competitor), and *R. taipeianus* (temporal partition competitor).

***Experiment II-2*—**Bidirectional test: phonotaxis preference between artificially synthesized mixed-specific chorus calls comprising of *R. prasinatus*, *B. adenopleura*, and *P. braueri*, compared to conspecific *R. prasinatus* chorus calls in the opposite direction.

***Experiment II-3*—**Capture–mark–recapture records: The recapture process in the experimental forest helped us to locate the position of males in different capture occasions. By using translocated release into other choruses, this experiment tested the movement of the frogs after being released into conspecific or mixed-specific choruses.

Experimental protocol of phonotaxis tests

I conducted outdoor phonotaxis tests in a circular test arena (20 m diameter) to investigate the chorus aggregating behavior of male frogs (Fig. 2. 1a). This test arena was a deforestation zone > 100 m from the closest chorus, thus avoiding acoustic interference from other places. Acoustic signals were broadcasted by speakers facing the center, which were placed 15 cm above the ground along the arena's edge. In order to eliminate any possibility of a directional response bias in the arena, eight positions (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°) were defined on the edge of the arena (Fig. 2. 1a). For unidirectional experiments (I-1 and II-1), a speaker was randomly selected from one of the eight positions in each trail. For bidirectional experiments (I-2, I-3, and II-2), the position of the first speaker was randomly selected, and the second speaker was opposite (180°) from the first. The speakers used in this study were handmade following Cunnington and Fahrig (2013). In each set of equipment, a waterproof speaker (Pokka PK-505SPIP, Hylex, Taiwan) was connected to an amplifier and battery, and a digital recorder (Sony, PCM-M10, Sony Corporation, Tokyo, Japan) was used to broadcast the signal.

The tested frogs were caught about 1 hour after sunset (7:00 PM) from natural choruses in the study site. The frogs were kept separately in containers, and the phonotaxis tests were conducted one by one as soon as possible before the midnight. Before the process of the test, a 12 mm fluorescent rod was attached to the waist of the frog with a cotton thread and was detached immediately after the test. This fluorescent rod helped to monitor the movement of the frog without extra light sources. The weight of the fluorescent rod was about 0.2 g, which was less than 3% of the body mass for male *R. prasinatus*.

When a test started, the target frog was placed in a quadrate open blanket (50 cm × 40 cm × 33 cm) in the center of the arena (Fig. 2. 1a). The countdown began when the researcher left the frog free, and slowly moved away from the arena. Attracted by the acoustic signals, the frog would climb out the blanket to start making its choice, and was allowed to move freely in the arena. When the frog reached the edge of the testing arena within 15 min (defined as a successful test, comprising 81.7% of total trials), the approach angle was recorded in order to estimate the male's perception of sound location. The perimeter of the arena was divided into 16 arcs (each 22. 5°), and the approach angle was defined as one of 16 arcs (Fig. 2. 1a). For frogs that did not reach the edge within 15 min, the trial was considered as a nonresponse and was excluded from further analyses (18.3% of total trials).

In some occasions (48.9% among all cases), a frog was used in more than one experiments at the same night. Among these cases, 68.6% were used in two experiments, 25.4% were used in three, and 5.9% were used in four experiments. Under this situation, different experiments were spaced for more than 30 minutes and the experiments were randomly arranged. Based on previous literature, this treatment did not influence the performance of the frogs (Vélez and Bee 2010; Farris and Ryan 2011, 2017; Reichert et al. 2016). After the test(s) were accomplished, all the frogs were released into the experimental forest as soon as possible before midnight.

Movements among conspecific and mixed-specific choruses

Several water bodies exist in a patchy pattern within the experimental forest, which comprises a large zone of mixed-specific choruses in the middle (comprised of 18 small water bodies), and four conspecific choruses in the periphery. These choruses were spaced for 20 – 30 meters, with two farthest choruses spaced for 60 meters. This spatial arrangement allows all the males to move freely across all the choruses. A preliminary test indicated that the males did not represent homing behavior for a specific chorus (only 25.4% males returned to their original choruses after translocated release; and also only 36.4% males were found in their original choruses in the next capture after being marked).

After the arena experiments, 28 males were randomly chosen and released in conspecific breeding choruses (where only *R. prasinatus* exists), while 72 males were released in mixed-specific breeding choruses (containing *R. prasinatus*, *B. adenopleura*, and *P. braueri*). The routine capture–mark–recapture experiment, on average twice per week during the time that I conducted the experiments, helped us to locate the males in the next capture event.

Data analyses

In the phonotaxis experiments, the Rayleigh test was used to test whether approach angles were randomly distributed. The V-test determined whether phonotaxis responses were localized at a particular exit angle: 0° or 180° in bidirectional tests, and 180° in unidirectional tests.

In experiment II-2, for which the male frogs did not show a particular direction but preferred both 0° and 180°, a goodness of fit test with a post-hoc Bonferroni test was used to determine whether male frogs had a bidirectional preference. In contrast to the other bidirectional tests, the frogs in this experiment had phonotaxis preference, but could not distinguish between the two choices in the opposite directions. Therefore, I categorized the test arena into eight 45° arcs, with arcs in opposite angles grouped together as a set (e.g., the first arc grouped with the fifth arc, second arc grouped with the sixth, etc.) before multiple comparisons were made. A Bonferroni correction of α = 0.0125 was applied; it was derived from α = 0.05/4.

In experiment II-3, a Fisher's exact test was used to test the percentage difference of frogs moving to another habitat between the group which was released into conspecific sites, and that released into mixed-specific sites.

Circular statistic tests were performed with Oriana software (ver.4. Kovach Computing Services, UK). Other statistical tests were performed using JMP software (ver.7 SAS Institute Inc., Cary, NC, USA). All statistical tests were two-tailed and performed at α = 0.05 (except for Bonferroni correction at α = 0.0125).

Results

A total of 349 trails were conducted by using 140 identified males; each individual was tested once in each experiment. The body weight and SVL of male frogs (mean ± SD) were 58.89 ± 3.18 mm and 9.1 ± 1.55 g, respectively. In 81.7% of all the trails, the approach angle of the frog was acquired within 15 min and were thus defined as successful trials.

Experiment I-1 & I-2: males prefer to join a chorus rather than a single male

Experiment I-1 tested the validity of the experimental treatments. Male *R. prasinatus* showed a strong tendency to join a conspecific chorus (Fig. 2. 2a, *N* = 35). In contrast, the frogs did not show a phonotaxis preference to the white noise (Fig. 2. 2b, *N* = 23).

In experiment I-2, male responses for a chorus and a single male were compared. A total of 42 successful selections were acquired from 47 trials, yielding an 89.4% success rate. The mean angle was 163.7°, with an approach angle not randomly distributed (Fig. 2. 2c), and a significant trend toward 180°. This result supported my prediction that male frogs prefer to join a chorus that includes more males.

Experiment I-3: low-quality males tend to approach a high-quality male

I next compared male responses to high-quality and low-quality calls. A total of 45 successful selections was acquired from 51 trials, yielding an 88.2% success rate. The mean angle was 143.8°, and the approach angle did not show a particular tendency (Fig. 2. 2d). However, after I categorized the males into high- and low-quality groups using their SMI values, low-quality males showed a trend of approaching a high-quality male (Fig. 2. 2e, *N* = 12, mean angle = 156.8); which was not found for high-quality males (*N* = 26, mean angle = 49.5°; Rayleigh test: Z = 0.04, *p* = 0.965). I also divided the males into three groups by SVLs: small males showed a persistent tendency to approach high-quality males (*N* = 10, mean angle= 139.5°; Rayleigh test: Z= 2.77, *p* =0.059; V test: $V_{180°}$ = 0.40, *p* = 0.037), but neither medium- or large-sized males showed this trend (large males: *N* = 16, mean angle = 319.7°; Rayleigh test: Z = 0.02, *p* = 0.982; medium-sized males: *N* = 14, mean angle = 139.5°; Rayleigh test: Z = 0.12, *p* = 0.888). Results showed that low-quality individuals tend to join a high-quality male.

Experiment II-1: resource noncompetitors could attract male frogs

Here, I tested the response of male *R. prasinatus* to heterospecific calls. A total of 87 successful selections were acquired from 112 trials, yielding a 78.1% success rate (84.4% for *B. adenopleura*; 72.5% for *P. braueri*; and 77.5% for *R. taipeianus*). By broadcasting different stimuli from a single speaker at the 180° location, male frogs significantly approached *B. adenopleura*'s chorus calls (Fig. 2. 3a, *N* = 27, mean angle = 162.7°). In contrast, the approach angles showed a random distribution when calls of *P. braueri* and *R. taipeianus* were used (Figs 2. 3b and 2. 3c, *N* = 29 and *N* = 31, respectively). These results supported my prediction that male frogs may use the calls of sympatric, heterospecific, noncompetitor frogs to evaluate their breeding habitats.

Experiment II-2: males do not reject a mixed-specific chorus

In this experiment, males were provided a chorus with or without heterospecific calls. A total of 44 successful selections were acquired from 48 trials, yielding a 91.6% success rate. The result of the goodness of fit test with a post-hoc Bonferroni test demonstrated that male frogs significantly approached both mixed-specific and conspecific choruses (Fig. 2. 3d; goodness of fit test: $X^2_3 = 15.82$, $p = 0.001$; Bonferroni's post-hoc test: 1-2: $X^2_1 = 4.5$, $p = 0.039$; 1-3: $X^2_1 = 7.76$, $p = 0.005$; 1-4: $X^2_1 = 10.7$, $p = 0.001$; 2-3: $X^2_1 = 0.53$, $p = 0.47$; 2-4: $X^2_1 = 1.67$, $p = 0.19$; 3-4: $X^2_1 = 0.33$, $p = 0.56$); furthermore, there was no significant difference between the two types of choruses ($X^2_1 = 0.18$, $p = 0.669$).

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Experiment II-3: males prefer to stay in conspecific sites rather than mixed-specific sites

This experiment tested the movement of males after being translocated into a mixed-specific or conspecific site. Almost half of the males were recaptured after being released to a designated chorus (46/100). For these 46 frogs, the mean spacing of the next recapture was 12.54 ± 9.63 days (mean ± SD), providing sufficient time for movements among different choruses.

In the group of frogs that were released into sites with only *R. prasinatus* (conspecific chorus), 72.7% (8/11) individuals remained at the original chorus site for the next capture, while 27.3% (3/11) moved to mixed-specific sites (Fig. 2. 3e). In contrast, 68.6% (24/35) individuals moved to conspecific sites when they were released to mixed-specific choruses, and only 31.4% (11/35) remained at the original site. The ratio of movements was significantly different between the two treatments, thus suggesting that male *R. prasinatus* avoid remaining in mixed-specific choruses.

Discussion

Although a large proportion of the literature has addressed the direction and preference of female choice in frogs, research about how males locate a breeding site and what strategies are involved in aggregation have been much less thoroughly explored. This study is one of the first to test the orientation of males during breeding season, when the spatial scale in the experimental design is comparable to the real situation in the wild. The results indicated that both conspecific and heterospecific calls are used to locate potential breeding sites.

Strategy for joining a conspecific chorus

A typical breeding ground of *R. prasinatus* usually contains several water bodies and choruses, which provides different habitat patches for males to choose from and migrate among. This behavior was revealed by my relocated recapture results (Fig. 2. 3e). Past research on females (Neelon and Höbel 2017) indicated that the frogs could assess the information they received in previous choruses to make decisions when joining subsequent choruses; however, relevant evidence for males is scarce. For this experiment comparing multiple males and a single male (experiment I-2), most frogs chose to join the former (Fig. 2. 2c). Typically, a more crowded chorus indicates a higher strength of intra-sexual competition and a higher probability of attracting predators (Höbel 2014; Höbel and Barta 2014). Nevertheless, these disadvantages are compensated for by a higher probability of encountering females (Rehberg-Besler et al. 2016); this explains the aggregating behavior for most choral frog species (Ryan et al. 1981; Murphy 2003; Bee 2007).

In a polyandrous mating system like that in *R. prasinatus*, choruses contain a group of males with various qualities (Lucas et al. 1996; Friedl and

Klump 2005). In this kind of system, low-quality males can sneak into the spawning group or intercept a female who has been attracted by a high-quality male (Lucas et al. 1996). Therefore, the ability to recognize high-quality males may increase the fitness of low-quality males. The results of this study showed that low-quality males tended to approach the calls of high-quality males (Fig. 2e); these frogs could be described as “satellite males” and this behavior may be an alternative strategy depending on their own physical characteristics (Lucas et al. 1996; Humfeld et al. 2009; Brepson et al. 2012). This strategy further hints that *R. prasinatus* males might be able to evaluate themselves and their rivals. However, the weakness of the current test is that I only manipulated the presence or absence of low-frequency B notes, which oversimplified the evaluation process. Other clues in the acoustic signals, such as dominant frequency, call rate, call length, and the attendance of calling days, have also been reported as indicators of male quality (Toledo et al. 2014). Therefore, additional experiments are required to quantify these factors and further confirm the fitness of low-quality males when playing the role as a satellite male.

Heterospecific calls as supplementary clues

When conspecific calls were absent, male *R. prasinatus* frogs approached the calls of *B. adenopleura* (resource noncompetitor, Fig. 2. 3a), but not to *P. braueri* (resource competitor, Fig. 2. 3b). This behavior indicated that the calls of noncompetitors could serve as clues for breeding site allocation. The calls of heterospecifics may serve various functions in other species, including locating suitable habitats, avoiding predators, and identifying competitors (Magrath et al. 2015). For anurans, aggregating to limited breeding sites makes males face strong competitive pressure from conspecific and heterospecific individuals. Such competitive interactions are associated with access to food, calling space, ovipositional site, and the frequency channel of calls (Mullet et al. 2017). Therefore, avoiding choruses with heterospecific competitors, such as *P. braueri* in this study, may reduce competition (Miller et al. 2013). Furthermore, *R. prasinatus* showed no significant response toward *R. taipeianus* (Fig. 2. 3c). This could be explained by two alternative possibilities: (1) the breeding ground of *R. taipeianus* (usually in muddy caves underground near the water) does not represent a suitable habitat for *R. prasinatus*; or (2) *R. prasinatus* could not recognize the call because of their temporal partition in breeding season.

The last two parts of this study, experiments II-2 and II-3, represented a real situation in the wild. In experiment II-2, the mixed-specific calls were comprised of *R. prasinatus*, *B. adenopleura* (resource noncompetitor), and *P. braueri* (resource competitor) calls since I aimed to simulate the real situation in the wild. Male *R. prasinatus* in this test showed significant phonotaxis preference to both mixed-specific and conspecific choruses, but they did not show significant bias between these two ($X^2_1 = 0.18$, $p = 0.67$). This result indicates that when conspecific calls exist, *R. prasinatus* is attracted regardless of interference from heterospecific individuals. Nevertheless, the preliminary decision could be corrected by movements among the patches, as shown in experiment II-3 (Fig. 2. 3e). Recapture data from the real situation in the wild indicated that a large proportion of males (72.7%) left the mixed-specific chorus, and a large proportion of males (68.6%) remained in the conspecific chorus. This result agreed with my prediction that high inter-specific competition may force males to make alternative choices.

Just as in the case of conspecific aggregation behavior, there are several other factors that should be taken into consideration here. One is when the heterospecific call is regarded as background noise, which is known to interfere with or even cover acoustic signals of their same kind (Gerhardt and Klump 1988; Velez and Bee 2010; Halfwerk et al. 2016). Most anuran species are characterized by their unique dominant frequency in advertisement calls, where both males and females have evolved to represent the highest sensitivity toward their own frequency (Gerhardt and Huber 2002; Amézquita et al. 2011; Mullet et al. 2017). If strong overlapping (either temporal or in frequency) occurred among these species, the frogs would have had to overcome this effect by increasing energy output, increasing the signal duration, or switching communication channels (Wong et al. 2009; Luther and Gentry 2013; Bleach et al. 2015). All these responses could be energetic costly, and would not guarantee mating success. The phenomenon I observed in experiment II-3 might be a compromise between mating success and interference from other frogs; this will be more thoroughly studied in my future research.

In conclusion, the results of the current study confirmed both chorus attraction hypothesis and heterospecific attraction hypothesis. In conspecific tests, I conclude that male frogs could discriminate the components of chorus signals, and might have the ability to evaluate themselves and their rivals. In heterospecific tests, I conclude that the male frogs could utilize heterospecific cues, especially those from sympatric noncompetitors, as information for locating breeding sites. The results of this study help to clarify the potential interactions of frogs at both within-species and between-species levels, and further explain the formation of anuran aggregations coexisting around the limited water resources.

Figures

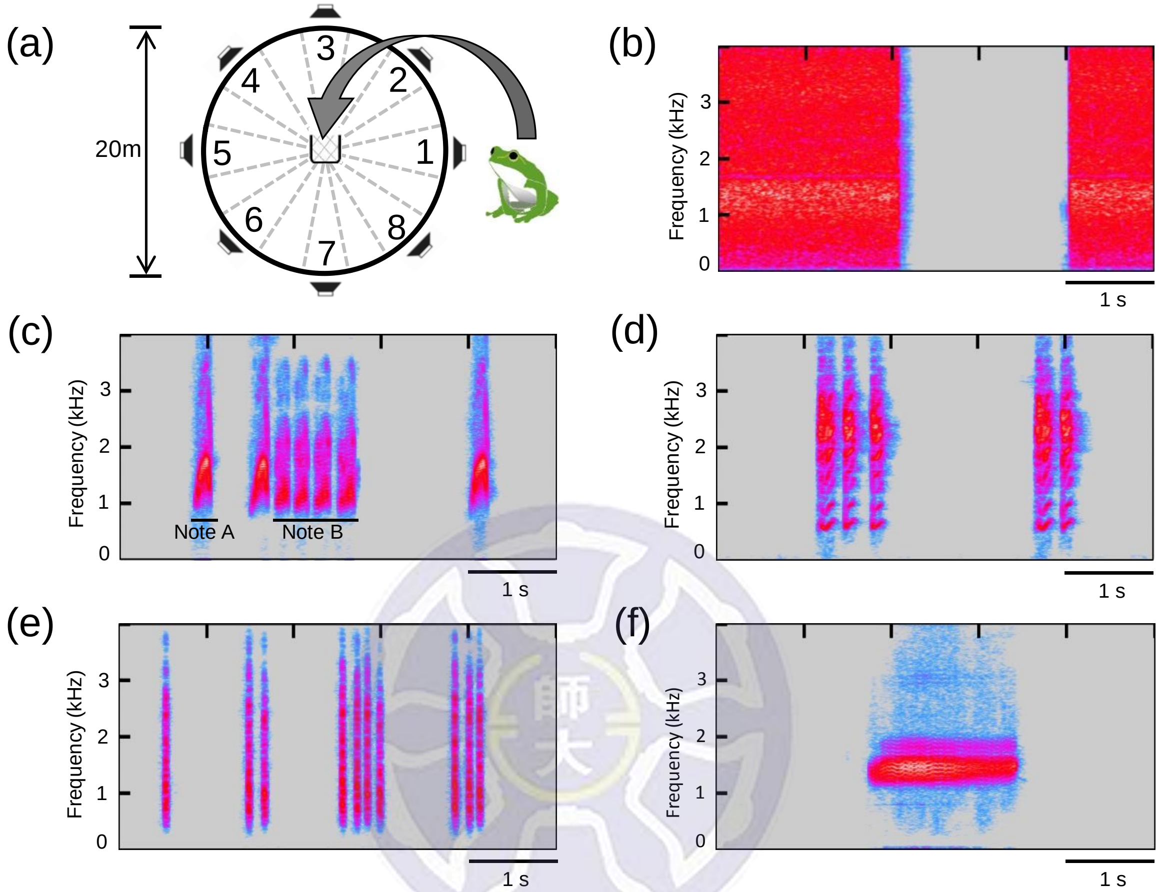

Fig. 2. 1

Experimental design. (a) Schematic diagram of the outdoor test arena used in the experiments, and spectrograms of (b) white noise, (c) *Rhacophorus prasinatus*, (d) *Babina adenopleura*, (e) *Polypedates braueri*, and (f) *Rhacophorus taipeianus*

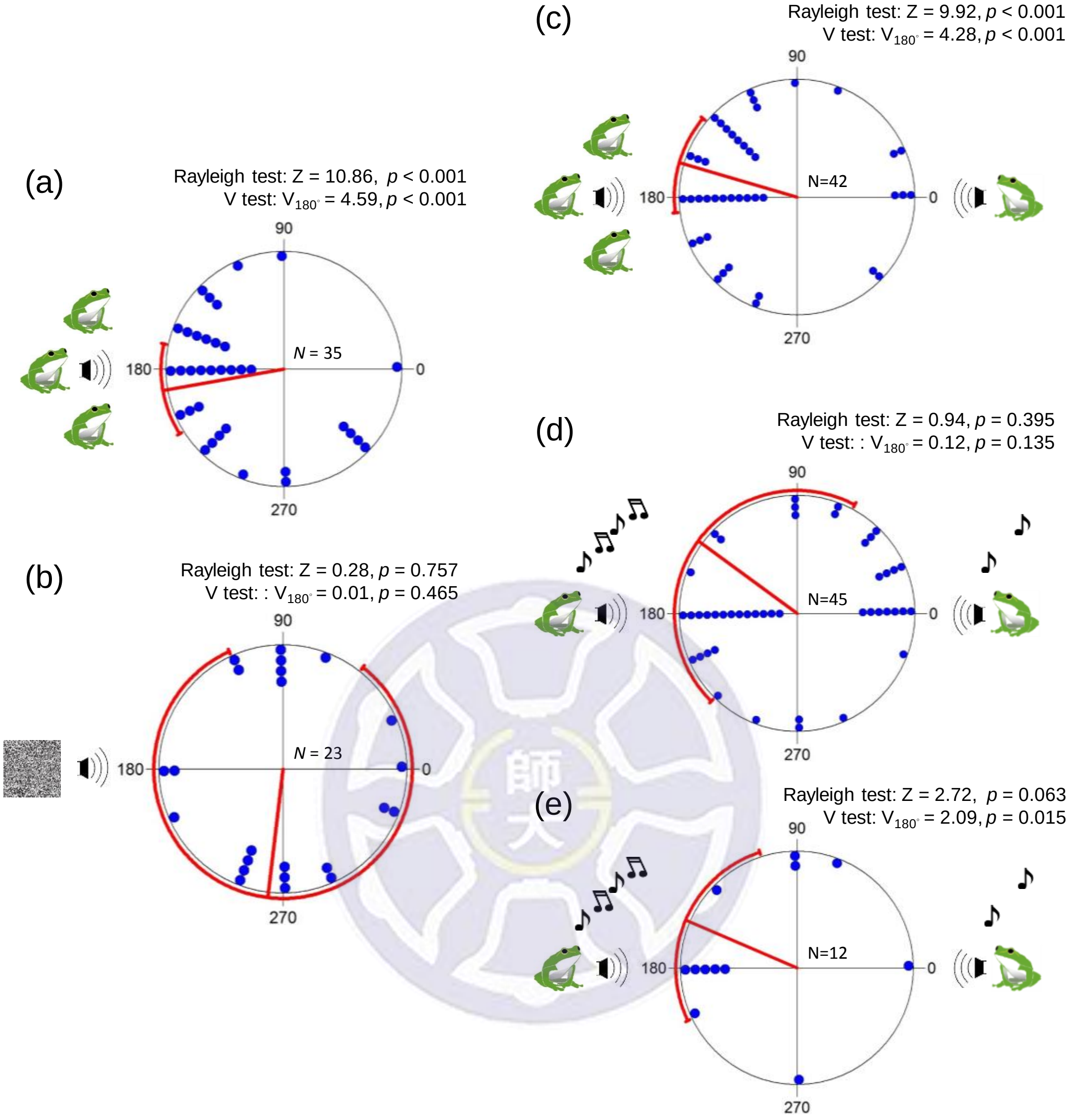

Fig. 2. 2

Conspecific experiments for aggregating behavior of male *Rhacophorus prasinatus*. The blue dots represent the approach angles of frogs, and the red line represents the mean angle with 95% confidence interval. (a) and (b) Experiment I-1: the males showed a strong tendency to aggregate for the conspecific chorus, but not for the white noise. (c) Experiment I-2: the males preferred to join to a chorus that aggregates more males. (d) Experiment I-3: no approaching tendency was observed for all males toward high-quality or low-quality males; however, (e) the males with low SMI (scaled mass index) values tended to join the high-quality males

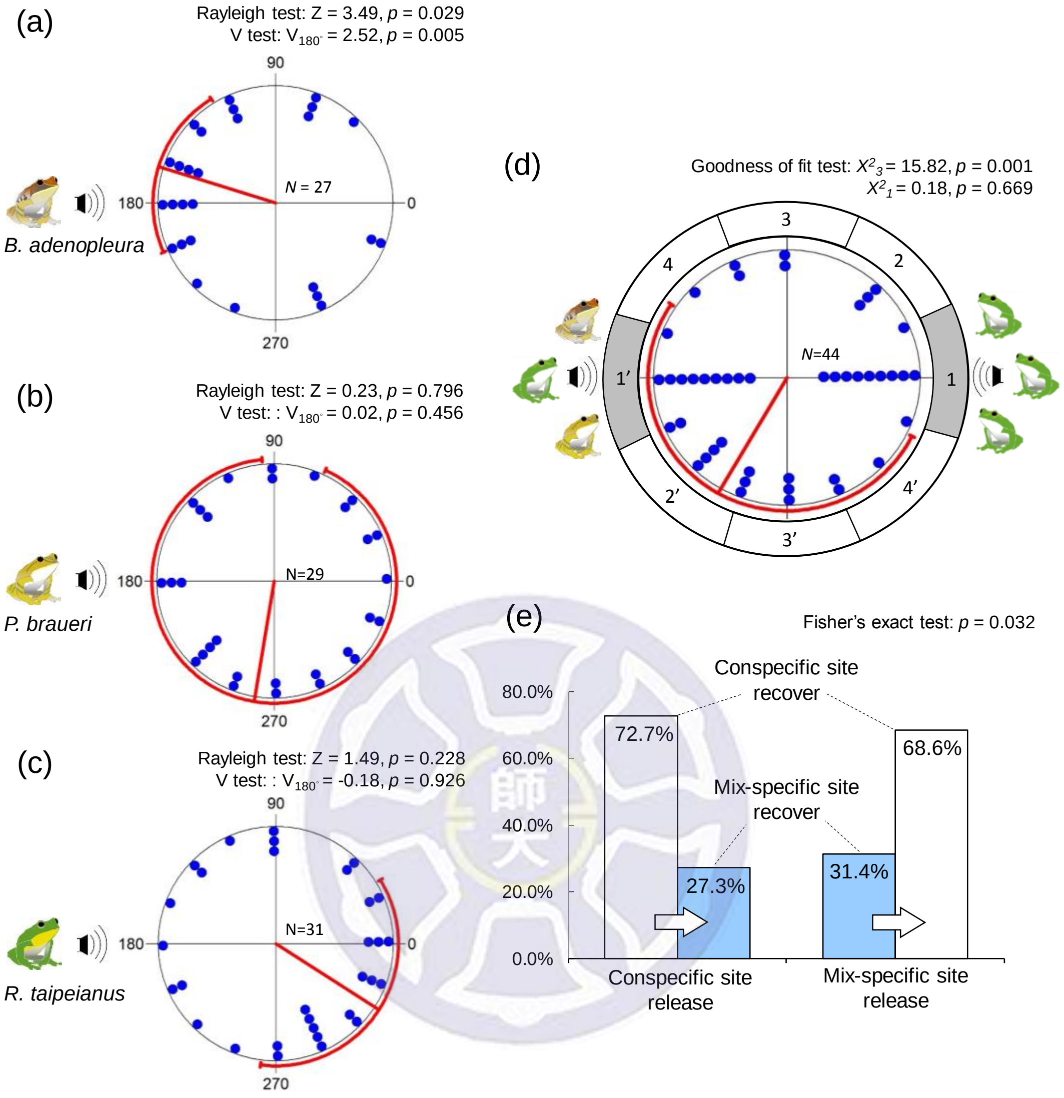

Fig. 2. 3

Heterospecific experiments for aggregating behavior of male *Rhacophorus prasinatus*. The blue dots represent the approach angles of frogs, and the red line represents the mean angle with 95% confidence interval. (a)–(c) Experiment II-1: the males showed a significant tendency to approach *B. adenopleura* (resource noncompetitor) but not *P. braueri* (resource competitor) and *R. taipeianus* (noncompetitor with temporal partition). (d) Experiment II-2: males aggregated to the signals in both directions, but did not show a significant difference between conspecific and mixed-specific choruses. (e) Experiment II-3: most males (72.7%; 8/11) released in conspecific choruses stayed in the original site, while most of those released in mixed-specific choruses (68.6%; 24/35) left.

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Chapter 3

Shift in calling timing and adjusted energy allocation of frog calls when facing background noises

Abstract

Condition-dependent strategies have been reported in various fields of behavioral ecology, but were less studied under the perspective of acoustic communication. In this study, I aimed to test the energy allocation and acoustic plasticity of a rhacophorid treefrog (*Rhacophorus prasinatus*) under noise interference, and compared the strategies between high-condition and low- condition males. I found that the frogs showed strongest avoidance to the medium-frequency noise that overlaps with their own signal; this noise also stopped the frogs from normal aggregating behavior. When broadcasting synthetic noise–silence cycles, males immediately adjusted their call tempo by using the silent gap to avoid signal overlap. Furthermore, males also showed a regulation ability, where they moved up their call starting time in the short silence gap, which helped them to deliver more notes within a limited period

of time. When broadcasting continuous noise, high- condition males maintained the original, intense call rates, which might be an honest signal of their high quality. In contrast, low- condition males ceased calling, possibly to serve as satellite males. The opposite strategies adopted by high- condition and low- condition males indicated that the strategy utilization could be condition-dependent. The final strategy seen in the wild is a compromise among several contradictory factors, such niche competitor, noise interference, and the frog’s own body condition.

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Introduction

Under sexual selection, females choose to mate with high-quality males who can effectively express their courtship signals. A high-quality signal is usually associated with preferable genetic characteristics and/or high body condition, and thus such a signal can be treated as an “honest signal” (Fisher, 1930; Williams, 1966; Zahavi, 1975; Dawkins, 1991; Stuckert et al. 2018). A courtship display requires messages to be effectively delivered to a receiver. In the real situation in the wild, however, signal senders have to face a variety of interferences from both intra- and inter-specific competitors (Ryan, Tuttle, & Taft, 1981; Hochkirch, Gröning, & bucker, 2007; Höbel & Gerhardt, 2007; Schrode & Bee, 2015; Lee et al., 2017). In anurans, different species usually aggregate at water sites during the breeding season and deliver their breeding calls simultaneously. Facing interference, males have to choose an optimal strategy that can maximize signal transmission to receivers but minimize energy costs.

One possible solution for males to overcome interference is to allocate more energy to signal transmission in noisy circumstances. This strategy can be achieved by increasing intensity, call rate, or the number of signals (Brumm, 2004; Ruppé et.al., 2015). In addition to prevent their signals from being masked by background noise, this investment also helps repel rivals and present good genetics/nutritional quality to potential mates. This can thus be considered an honest signal (Zahavi, 1975; Dawkins, 1991). However, males also face a challenge in producing high-quality signals because of energy constraints (Schwartz, 1993). To reduce unnecessary energy costs, temporal shifts in the timing of calls is a solution. This strategy can take many forms, from long-term adaptation for seasonal or timing partitions, to fine-scaled calling adjustment to avoid signal overlap. For example, a study on eight sympatric anuran species suggested that different species develop temporal differentiation at night (Garcia-Rutledge & Narins, 2001). The male strawberry poison frog (Dendrobates pumilio) reduces calling activity when exposed to cicada chorusing to prevent wasting energy (Paez, Bock, & Rand, 1993); meanwhile, a hylid frog (Dendropsophus microcephalus) adjusts its call timing according to their closest neighbor's calls to prevent signal overlap (Schwartz, 1993).

Although previous studies have indicated that an increase in signal intensity and reduction in signal overlap are both effective ways to overcome noise masking, these two choices are opposite behavioral strategies adopted in different situations. Determination of an optimal strategy might be condition-dependent, especially when the intrinsic condition of the males is taken into consideration (Humfeld, 2013; Crocker-Buta & Leary, 2018; Ferreira, Scheutz, & Boyd, 2018). Condition-dependent strategies have been reported in the field of animal behavior, mostly focused on phenotypic plasticity, parental care, courtship behavior, and mate choices (Jackson, 1992; Gross, 1996; Badyaev & Hill, 2002; Thomas, 2002). However, they have few been discussed in the field of acoustic communication. Could frogs exhibit behavioral plasticity, either in temporal or frequency characteristics, when facing noise interference? Is there behavioral plasticity within a single frog population?

In this study, I focused on the response of frogs when facing noise of different frequencies or different tempos, and I also took the body condition of the actor into consideration. *Rhacophorus prasinatus* (Rhacophoridae), an

Oriental treefrog, was used to test the calling strategy under noise interference. In the breeding season, *R. prasinatus* form choruses in bushes, and produce foam nests on vegetation above the water (Mou et al., 1983). In this courtship system, poor-quality males tend to join the copulation of high-quality males, which leads to polyandrous mating and extra-pair paternity (Chapter 2). *Babina adenopleura* (Ranidae) and *Polypedates braueri* (Rhacophoridae) are often sympatrically distributed during the breeding season, which might cause major acoustic interference. This system provides a good opportunity to study the responses of male frogs when heterospecific noise exists.

In order to test the response of the frogs, I conducted playback experiments in the wild by broadcasting different background noises that contained short silent gaps or different frequency bands, and measure the response of the males. I also examined the aggregating behavior of males when facing different types of interference, and further analyzed the real situation in natural choruses. Based on bodily energy constraints, I suspected that males would adjust the timing of calls to reduce being masked by background noise, and apply different strategies considering their own body condition. I aim to answer the following questions: (1) Are male frogs able to adjust their tempo or call timing to avoid overlap with noise? (2) What is the response of males to noises with different frequencies? (3) Would male frogs choose to join or keep away from a chorus with too much noise interference? (4) Do males with different body conditions apply different strategies when facing high-intensity interference? (5) What is the strategy of males adopt in the wild?

Material and methods

Study system

The experiments were conducted from 2016 to 2017 in Hualin Experimental Forest (24°53’N, 121°33’E), a 92-hectare protected region located in New Taipei City, northern Taiwan. Natural and artificial pools are located in the forest in a patchy and mosaic pattern; these ponds attract *R. prasinatus* during the breeding season. In this site, the frog produces courtship calls with a dominant frequency of 1576.06 ± 119.33 Hz (mean ± SD), with a variation between 1369.53 and 1858.32 Hz (Fig. 2. 1c). In addition to *R. prasinatus*, the most abundant frogs during the breeding season are *Babina adenopleura* (Ranidae, sympatric non-competitor) and *Polypedates braueri* (Rhacophoridae, sympatric competitor) (Chapter 2).

Experiment I: call adjustments in noise–silence cycles

To investigate the acoustic response of males when exposed to noises, I conducted three experiments by broadcasting the following stimuli for 30 minutes: (I-1) white noise, (I-2) chorus sounds of *B. adenopleura*, and (I-3) chorus sounds of *P. braueri*. The broadcasting files were edited to cyclic noise–silence repeats of 4-second cycles (repeats of 2-second noise and 2-second silence) and lasted for 30 minutes. In this 30-minute broadcasting period, I determined the total number of calls that overlapped with the noise intervals and the total number of calls that overlapped with silence intervals. The Wilcoxon rank sum test was used to compare the proportions of call allocation to the expected ratio of 0.5/0.5, which represents the random condition of no noise avoidance.

To further investigate if *R. prasinatus* has the ability to quickly adjust their call tempo, I categorized their calls into “single note” and “multiple notes” in the gaps of white noise stimulus. A total of 24 individuals, with sufficient sampling of both single and multiple notes (N > 3, Table 3. 1), were included in this analysis. I measured the starting time of the first note in the gaps, and compared differences between the two situations (Experiment I-4). The Wilcoxon rank sum test was used to test whether males moved up their start calling time when producing multiple notes during the silent gaps. All statistical tests were two-tailed and performed using SPSS software (ver. 23, SPSS Inc., Chicago, IL, U.S.A.) at $\alpha = 0.05$.

Experiment II: call adjustments under different noise frequencies

In order to test the avoidance of *R. prasinatus* in response to different noise frequencies, I broadcasted cyclic repeats of band-limited noises of high, medium, and low frequencies with no silence gap. The broadcasting file was edited to repeats of 6-second cycles (2 seconds for each frequency) and lasted for 30 minutes. For this 30-minute period, I determined the total number of calls of *R. prasinatus* that overlapped with each of the three different noise intervals. The Wilcoxon rank sum test was used to compare the observed ratio and the expected ratio (0.33/0.33/0.33); the latter represents no avoidance for a specific frequency (Experiment II-1).

In order to examine whether males can hear and avoid the frequency that they do not deliver (> 2 kHz or < 1 kHz), I further broadcasted high-frequency only (2 – 3 kHz) and low-frequency only (0 – 1 kHz) band-limited noise cycles (Experiment II-2 and II-3). The broadcasting files were edited as 4-second cycles (2-second noise and 2-second silence), as in Experiment I. The total number of calls in noise–silence intervals were compared to the expected 0.5/0.5 ratio, which represents the random condition of no noise avoidance.

Experiment III: phonotaxic response to chorus with noise

In my previous study, *R. prasinatus* showed phonotaxis, approaching a speaker broadcasting conspecific chorus sounds (Chapter 2). In order to test the interference of noise, I recorded the phonotaxic response of males when providing frog calls mixed with overlapping frequencies (medium-frequency noise, Experiment III-1) and frogs calls mixed with non-overlapping frequencies (high- and low-frequency noises, Experiment III-2). See Supporting Information for the details of phonotaxis experiments. The Rayleigh test was performed to test whether approach angles were randomly distributed, and the V test determined whether phonotaxic responses of male frogs were localized at the speaker (0°). Circular statistics were computed with Oriana software (ver.4. Kovach Computing Services, UK). All statistical tests were two-tailed and performed at $\alpha = 0.05$.

Experiment IV: energy allocation under noise interference

In this analysis, I aimed to test whether male frogs adjust their energy allocation before, during, and after the 30-minute broadcast. Thirty-minute recordings were acquired before, during, and after the broadcasting tests in Experiment I-1 (white noise–silence repeats) and Experiment II-1 (three frequencies repeats). From the three recordings (i.e., for the three time periods of before, during, and after broadcasting tests), call characteristics were analyzed and compared using Raven Pro 1.5 (Cornell Lab of Ornithology, Ithaca, NY). I analyzed the means of call duration, call rate, the number of calls, dominant frequency (Hz), and the intensity (dB) of A note (high-frequency) and B note (low-frequency) calls. Since some of the data in my measurements did not meet the assumptions of parametric statistics (normality or heterogeneity of variances), Friedman's ANOVA with post hoc tests were used to test whether these call characteristics were different across the three time periods.

Since call rate had a major difference across these three periods, I further tested the reduction of call rates in the broadcasting period (compared to the pre-test period) and its recovery in the post-test period (compared to the broadcasting period). The frogs' SVL (snout-vent-length, mm) and mass (g) were measured to assess their body condition index (BCI) using the residual index method (e.g. Denoël et al. 2002; Plăiaşu et al. 2010; Figure 3. 1, N = 40). Linear regressions of vocal responds (note difference per second) on BCI were produced in Experiment I-1 (white noise–silence repeats) and Experiment II-1 (three frequencies repeats). I further categorized the frogs into high-condition, medium-condition and low-quality males along with the BCI values, and T tests were performed to test the difference of A note between high-condition and low-condition males in Experiment II-1, which facilitated the comparison of strategy utilization by frogs with different conditions. All tests were performed using SPSS software at α = 0.05.

Experiment V: Energy allocation in natural choruses

In order to reveal the noise avoidance behavior in the real situation in the field, 30 chorus files (30 minutes each), comprising a variety of different levels of heterospecific background noises (some with a high noise ratio and others with a low noise ratio), were selected from soundscape recordings of the experimental forest. From each file, I first acquired the noise–silence ratio during the 30-minute recording. Then I counted the number of *R. prasinatus* calls that overlapped with the background noise and the number of calls that did not overlap with the background noise. If *R. prasinatus* individuals were not affected by the background noise, the overlapping ratio of their own calls would equal the ratio of background noise, which forms a regression line with a slope = 1. If the frogs behave to avoid the noise, the scatter plot would locate below this line, which means a reduced ratio of the calls overlapped with the noise. I used linear regression analysis to inspect the association between the overlapping ratio and the background noise ratio (observed slope) and to test whether the observed slope differs from 1 (i.e., from the expected slope).

Results

Experiment I: temporal adjustment to reduce noise interference

Male frogs showed a significant tendency to avoid delivering their calls synchronously with the noise inference. The ratio of calls overlapping with the noise was significantly lower than the expected 0.5 ratio in all three situations, including the broadcasting of white noise ($R_{\text{white noise}} = 26.3\%$; $Z = 4.45$, $N_{\text{white noise}} = 31$, $p < 0.0001$; Fig. 3. 2A), *B. adenopleura* ($R_{B.\ adenopleura} = 8.1\%$; $Z = 3.93$, $N_{B.\ adenopleura} = 21$, $p < 0.0001$; Fig. 3. 2B), and *P. braueri* ($R_{P.\ braueri} = 15.2\%$; $Z = 3.76$, $N_{P.\ braueri} = 21$, $p < 0.0001$; Fig. 3. 2C).

I further analyzed the tempo of calls in the silence gap. Among the 24 individuals used in this test, 23 expressed a shorter starting time for multiple notes (Table 3. 1). The Wilcoxon rank sum test revealed that "multiple notes" started significantly earlier than "single notes" ($Z = 4.53$, $N = 24$, $p < 0.0001$; Fig. 3. 2D). The early initiation of multiple notes helped the call deliverer accomplish signal transmission before the next noise inference.

Conversely, noise interference did not change other properties of the calls, including other frequency characteristics.

Experiment II: avoidance of the medium frequency when silence gap did not exist

When the three band-limited noises took turns, male *R. prasinatus* showed a significant tendency to avoid the medium frequency (1 – 2 kHz), which overlaps with the frequency of their own calls. The Wilcoxon rank sum test revealed a significant difference between the observed ratio and the expected ratio of 0.33 ($N = 25$; $R_{\text{high frequency}} = 54.6\%$, $Z = 4.29$, $p < 0.0001$; $R_{\text{medium frequency}} = 13.3\%$, $Z = -4.37$, $p < 0.0001$; $R_{\text{low frequency}} = 32.1\%$, $Z = -0.61$, $p = 0.545$; Fig. 3. 3A).

When broadcasting band-limited noise versus silence cycles, male *R. prasinatus* showed significant avoidance of both high-frequency noise ($R_{\text{high noise}} = 28.3\%$; $Z = -2.07$, $N_{\text{high noise}} = 9$, $p = 0.038$; Fig. 3. 3B) and low-frequency noise ($R_{\text{low noise}} = 34.3\%$; $Z = -2.80$, $N_{\text{low noise}} = 10$, $p = 0.005$; Fig. 3. 3C). These results indicated that while the frogs experience interference from high- and low-frequency noise, their first priority is to avoid the medium-frequency noise.

Experiment III: tolerance to non-overlapping frequency in phonotaxis tests

Male *R. prasinatus* showed a strong tendency to join a conspecific chorus that was mixed with noise of a non-overlapping frequency ($N = 36$, mean angle = 5°, Rayleigh test: $Z = 10.088$, $P < 0.0001$; V test: $V_{0°} = 0.527$, $p < 0.0001$; Fig. 3. 4A). In contrast, they did not show phonotaxic preference to a conspecific chorus mixed with noise of an overlapping frequency ($N = 30$, Rayleigh test: $Z = 0.266$, $p = 0.769$; V test: $V_{0°} = 0.042$, $p = 0.375$; Fig. 3. 4B). The result indicated that the frogs are tolerant to non-overlapping noise, and thus the aggregating behavior remained; meanwhile, noise with an overlapping frequency would stop the frogs from normal aggregating behavior.

Experiment IV: divergent strategies between low-quality and high-quality males

Friedman's ANOVA revealed a significant difference of call rates (note A) among pre-test, broadcasting, and post-test periods ($N = 31$, $\chi^2_2 = 11.09$, $p = 0.004$; $N = 25$, $\chi^2_2 = 7.28$, $p = 0.026$; Fig. 3. 5). Post hoc analysis on time effect indicated that call rate during the broadcasting period (0.236 notes/s) was significantly reduced compared to the pre-test period (0.283 notes/s) in Experiment I-1, and recovered in the post-test period (0.267 notes/s). A similar result was observed in Experiment II-1, which indicated a significant decrease from the pre-test period (0.304 notes/s) to the broadcasting period (0.248 notes/s), and recovery in the post-test period (0.287 notes/s).

In order to reveal the different strategies performed by males, I regressed vocal responds (note difference per second) against BCI and found that vocal responds were positively correlated with body conditions ($R^2 = 0.164$, $p = 0.055$) in Experiment II-1 (Fig. 3. 6C), but has no relationship between BCI and vocal responds in Experiment I-1($R^2 = 0.003$; $p = 0.772$; Fig. 3. 6A) when noise broadcasting. I further categorized the males into two groups (medium-condition group was excluded) based on their BCI values. High-condition males maintained the original call rate (-0.013 notes/s in broadcasting); therefore, they did not have a prominent recovery in the post-test period (+0.025 notes/s in post-test period). In contrast, low-condition males gave up calling by expressing a large reduction of calls (-0.115 notes/s in broadcasting), and a significant recovery in the post-test period (+0.1 notes/s of recovery). This result suggested that males of different conditions may have different calling strategies in noisy circumstances ($t_{14} = 2.38$, $p = 0.032$; Fig. 3.

7).

Experiment V: real situation in the wild

A significant association between the overlapping ratio and noise ratio was detected from the sampled recordings ($N = 30$, $F_{1,28} = 97.53$, $p < 0.0001$, $R^2 = 0.78$; with the 95% confidence intervals shaded in Fig. 3. 8), indicating a reasonable consequence that when background noise increased, the ratio of calls overlapping with the noise increased (coefficient: 0.6550 ± 0.0663). However, when compared to the expected slope (the broken line in Fig. 3. 8; slope = 1), the observed slope ($y = 0.665x – 0.0667$, $R^2 = 0.78$) showed a significant reduction ($t_{28} = -5.20$, $p < 0.0001$). The reduced slope (i.e., a reduced overlapping ratio) indicated that male *R. prasinatus* avoided calling synchronously with heterospecific sound.

Discussion

Call strategy could be condition-dependent

The most striking finding in this study is the condition-dependent call strategies of males (Experiment IV). To avoid being masked by noise, one possible solution for the signal sender is to adjust their transmission pattern either in time or frequency (Ruppé et. al., 2015, Roca et. al., 2016). This strategy was applied by all *R. prasinatus* males when the interference occurred in repeated noise–silence cycles. However, increased investment in signal delivery has been reported as a strategy employed in continuously noisy environments, or in ephemeral aggregating choruses, which are extremely noisy (Erdtmann, & Lima, 2013; Vélez, Schwartz, & Bee, 2013). In this study, this strategy was observed only in high-condition males, who maintained their original call rates under continuous noise interference. In contrast, low-condition males almost gave up calling under this stressful condition (Fig. 3. 7). Comparatively few experiments have been conducted to test the response of frogs over such a short time scale; therefore, it is worth noting that the strategy could be switched within only several minutes, which shows behavioral plasticity under different circumstances.

I propose that the strategy applied by high-BCI males is an honest signal (Dawkins, 1991; Brepson, Voituron, & Lengagne, 2012; Voituron et al., 2012).

Under the circumstance where background noise is unavoidable, high-condition males invest more to ensure their signals are successfully delivered. This strategy partially explains the aggregating behavior that was tested in Chapter 2, where I showed that the frog is attracted not only by conspecific signals but also by heterospecific choruses (but only from ecological non-competitive species). Here, I further propose that the background noise, either from conspecific or heterospecific calls, might serve as the selective agent for the evolution of honest signals.

The distinct retreat strategy from low-BCI males is another interesting issue that should be specially addressed in this mating system. In my previous study, low-condition males preferred to join the chorus of high-condition males (Chapter 2). This behavior was regarded as a strategy to become satellite males, which has been frequently observed in this polyandrous treefrog (Chen, 1992). To compete with the interference from continuous noise might be a less-efficient strategy for low-condition males in this highly male-biased population; they quickly switch their strategy from a signal deliverer to a sneaker in a harsh environment.

Tempo of calls could be adjusted quickly to avoid interference

The finding of this study proved that the frogs are able to adjust their call tempo to fit the tempo of the noise interference (Experiment I). Call adjustment in response to noise has been discussed in relevant studies (Gregory Byrne, 2008; Kaiser & Hammers, 2009; Goutte, Kime, Argo, & Argo, 2010; Llusia, Gómez, Penna, & Márquez, 2013; Höbel, 2014; Tárano & Hebets, 2015; Tárano & Carballo, 2016). The most common explanation for this is to prevent the signal from being masked (Vargas-Salinas & Amézquita, 2013; Tárano & Hebets, 2015; Gall & Wilczynski, 2016). In previous studies, male frogs and toads were reported to emit calls in silent gaps to increase signal detectability by females (reviewed by Bee (2012)). Interestingly, male *R. prasinatus* in my study not only detected and caught the gaps (Fig. 3. 2A, 3. 2B, 3. 2C), but also tended to move up their call starting time so that they could deliver more notes in the silent gaps (Fig. 3. 2D). In a previous study on *R. moltrechti*, a closely related congener of *R. prasinatus*, high-quality males were characterized for their length and number of multiple pulses after the first pulse (Chen, 2002); *R. prasinatus* was deduced to share this common feature (Chapter 2). Moving up the starting time provides a sufficient interval for frogs to complete their multiple pulses, which helps them declare their quality to potential mates. My findings suggested that male frogs have the ability to adjust their call tempo to fit the noise tempo, which could be beneficial in maintaining the integrity of signal transmission.

Overlap of noise frequency is the major interference

Based on the matched filter hypothesis, the auditory sensitivity of females matches the spectral energy distribution of conspecific males' signals (Capranica & Moffat, 1983; Moreno-Gómez, Sueur, Soto-Gamboa, & Penna, 2013; Sheehan et al., 2017). In mixed-species choruses, this might be an adaptation for receivers when facing heterospecific interference. This adaptation improves the capability to communicate under noisy environments, and also explains the observation that different species in the mix-species chorus tend to deliver non-overlapping frequencies (Amézquita et al., 2011; Villanueva-Rivera, 2014; Ruppé et al., 2015; Samplonius & Both, 2017;

Mullet, Farina, & Gage, 2017).

The third contribution of this study is that I verified frogs avoiding noise with an overlapping frequency (Experiments II and III). When exposed to continuous noise, the males chose to avoid the medium-frequency (1 – 2 kHz) noise, which seriously overlaps with their own calls (Fig. 3. 3A). The medium-frequency noise also ceased the original aggregating behavior of the males (Fig. 3. 4B). However, suitable habitats for breeding are limited, and the original aggregating behavior was maintained when non-overlapping noise existed (Fig. 3. 4A). Also in Chapter 2, *R. prasinatus* was proved to use *Babina adenopleura* calls (a non-competitive sympatric species) to locate potential breeding sites; however, they were not attracted by *Polypedates braueri* (resource non-competitor). Combination of the previous and the current studies indicated that the decision to join a chorus is a complicated process that takes both noise interference and inter-specific niche competition into consideration (Arlettaz, 1999; Costa-Pereira, Rudolf, Souza, & Araújo, 2018).

In conclusion, considering all my recent tests on *R. prasinatus*, I predict that the decisive strategy, which could be directly observed in the wild, might be a compromise considering multiple factors such as noise interference, existence of competitor, and the body condition of their own. Noise with an overlapping frequency presents major interference, which was shown by frogs ceasing their aggregating behavior. However, when the inference occurred in noise–silence cycles, all the frogs could quickly shift their call tempo to avoid overlap with the noise; this adjustment was frequently observed in choruses in the wild. The most interesting finding occurred under harsh noisy environments, which pushed high-condition and low-condition males to utilize divergent strategies — delivering an honest signal versus serving as satellite males or sneakers. The results of this study provide a guide for our future studies, which aim to combine all relevant factors, including signal transmission, sensory sensitivity, chorus joining decision, and alternative courtship strategy.

Tables

Table 3. 1

Comparison on the starting time between single note and multiple notes, calculated by the means of analyzable samples sizes from 24 *Rhacophorus prasinatus* (#1 – #24). n_s: analyzable sample size of single note in the silence gap; n_m: analyzable sample size of multiple notes in the silence gap; n: the sum of n_s and n_m.

		Single note starting time			Multiple notes starting time			
No.	n	Mean (s)	n_s	%	Mean (s)	n_m	%	Time shifts (s)
#1	11	1.501	3	27.27%	0.414	8	72.73%	1.087
#2	26	1.576	12	46.15%	0.8	14	53.85%	0.776
#3	34	1.232	14	41.18%	0.511	20	58.82%	0.721
#4	13	1.531	3	23.08%	0.828	10	76.92%	0.703
#5	26	0.865	11	42.31%	0.198	15	57.69%	0.667
#6	49	0.939	42	85.71%	0.312	7	14.29%	0.627
#7	34	1.055	3	8.82%	0.467	31	91.18%	0.588
#8	35	0.717	23	65.71%	0.16	12	34.29%	0.557
#9	29	0.783	23	79.31%	0.244	6	20.69%	0.539
#10	21	0.804	5	23.81%	0.34	16	76.19%	0.464
#11	46	0.799	30	65.22%	0.337	16	34.78%	0.462
#12	20	0.683	15	75.00%	0.223	5	25.00%	0.460
#13	68	0.681	44	64.71%	0.239	24	35.29%	0.442
#14	47	1.046	31	65.96%	0.621	16	34.04%	0.425
#15	27	0.627	13	48.15%	0.218	14	51.85%	0.409
#16	53	0.581	29	54.72%	0.212	24	45.28%	0.369
#17	49	0.676	37	75.51%	0.316	12	24.49%	0.360
#18	10	0.905	7	70.00%	0.564	3	30.00%	0.341
#19	29	0.622	26	89.66%	0.327	3	10.34%	0.295
#20	45	0.507	35	77.78%	0.215	10	22.22%	0.292
#21	24	0.519	3	12.50%	0.236	21	87.50%	0.283
#22	18	0.652	15	83.33%	0.481	3	16.67%	0.171
#23	18	0.404	6	33.33%	0.259	12	66.67%	0.145
#24	7	0.606	3	42.86%	0.702	4	57.14%	-0.096

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Table 3. 2

Call characteristics of male *R. prasinatus* among three time periods in experiment I-1.

Characteristics (N = 31)		Per-Test				Broadcasting				Post-Test			
		Mean	SD	Median	Range	Mean	SD	Median	Range	Mean	SD	Median	Range
Duration (sec)		204.83	143.15	162.72	67.5-615.88	191.54	116.59	144.55	46.29-436.75	179.24	89.09	149.30	79.08-434.79
Dominant frequency (Hz)	Note A	1576.06	119.33	1576.24	1369.53-1858.32	1569.10	130.44	1591.31	1302.75-1847.06	1556.36	156.61	1562.23	1173.56-1901.38
	Note B	1103.20	70.40	1098.08	1000.32-1272.61	1088.42	60.60	1076.66	998-1212	1099.85	74.68	1096.04	967.8-1261.22
Power (dB)	Note A	70.66	8.15	67.97	55.66-96.24	69.47	8.62	66.97	56.61-94.13	67.08	7.38	65.17	53.91-83.35
	Note B	60.32	7.05	58.64	51.92-83.21	60.04	7.87	58.25	48-84	58.43	7.30	56.66	48.51-79.9
Call rate (calls −1/sec)	Note A	0.28	0.10	0.31	0.07-0.48	0.24	0.10	0.22	0.071-0.46	0.27	0.10	0.25	0.09-0.45
	Note B	3.99	2.14	4.27	0-8.58	3.40	1.87	3.55	0-7.21	4.63	1.75	4.54	0-8.6
Mean note number (A+B)		0.66	0.32	0.70	0.13-1.28	0.58	0.36	0.51	0.08-1.47	0.69	0.24	0.72	0.09-1.17

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Table 3. 3

Call characteristics of male *R. prasinatus* among three time periods in experiment II-1.

Characteristics (N = 25)		Per-Test				Broadcasting				Post-Test			
		Mean	SD	Median	Range	Mean	SD	Median	Range	Mean	SD	Median	Range
Duration (sec)		170.81	95.65	151.94	45.18-411.71	207.66	191.63	135.66	25.89-810.27	169.60	82.56	147.69	49.19-382.09
Dominant frequency (Hz)	Note A	1550.62	122.76	1548.26	1304.91-1810.95	1537.16	137.27	1563.32	1251.63-1864.78	1531.51	159.63	1539.65	1173.56-1813.1
	Note B	1096.06	62.60	1102.51	1000.32-1195.1	1101.26	78.90	1091.75	975.45-1296.31	1091.56	79.72	1085.27	967.8-1261.22
Power (dB)	Note A	68.86	9.61	67.32	46.42-96.24	66.67	6.40	66.57	53.61-79.8	67.34	8.11	64.28	52.1-83.35
	Note B	58.71	8.64	58.38	45.03-83.21	55.87	45.15	54.08	48.86-64.25	57.43	7.69	56.24	46.27-79.9
Call rate (calls −1/sec)	Note A	0.30	0.10	0.31	0.12-0.58	0.25	0.11	0.23	0.095-0.51	0.29	0.10	0.26	0.11-0.45
	Note B	3.80	2.35	4.16	0-8.58	4.60	2.15	5.06	0-7.57	4.88	1.63	4.79	1.16-8.6
Mean note number (A+B)		0.68	0.28	0.74	0.18-1.19	0.66	0.32	0.57	0.16-1.26	0.71	0.22	0.72	0.22-1.17

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Figures

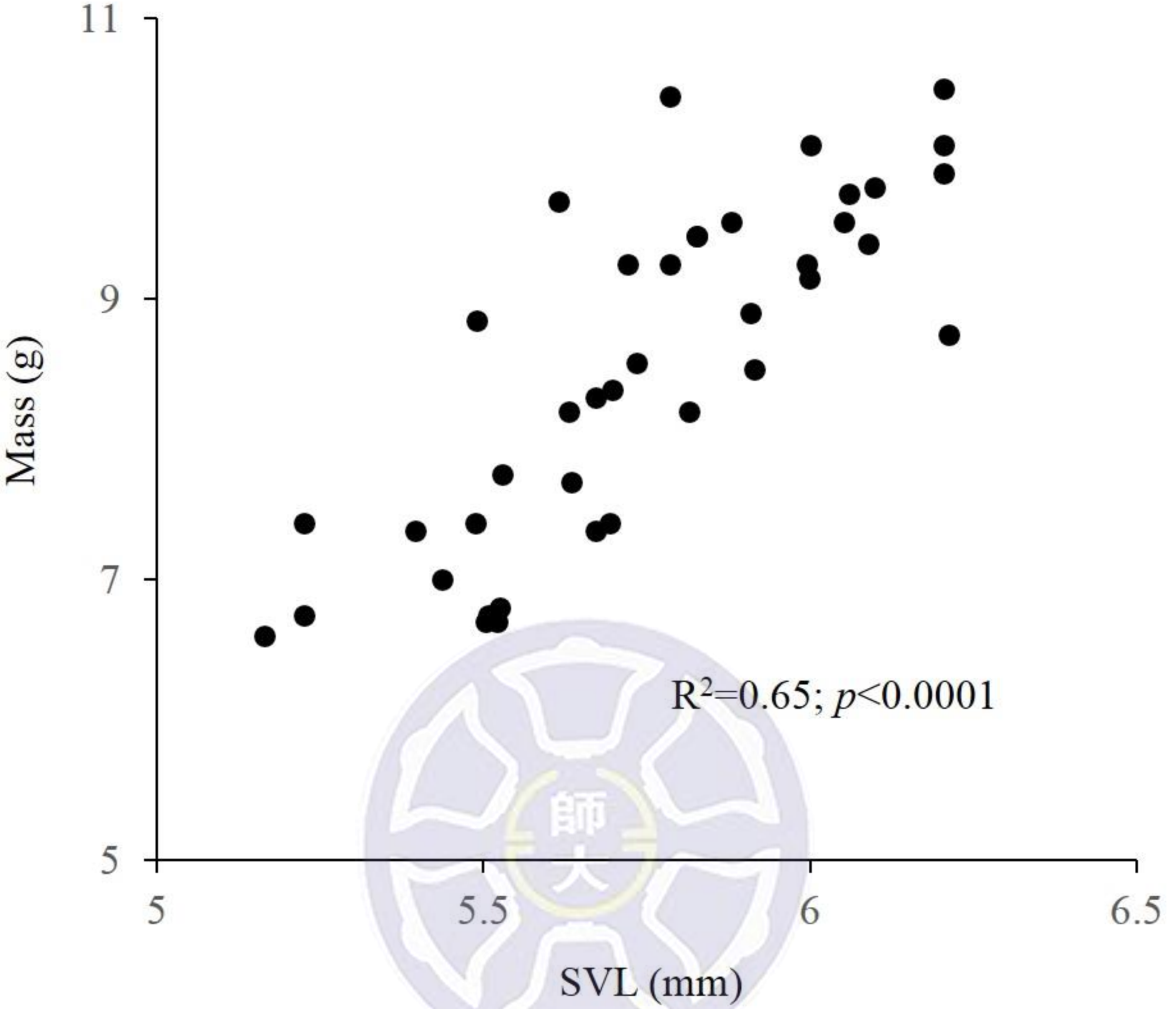

Fig. 3. 1

Scatterplot illustrating the relationships between SVL (snout-vent-length) and mass of 40 male *R. prasinatus*. The regression line ($y = 3.413x - 11.096$) was used to calculate the difference between observed and expected mass as a measure of body condition.

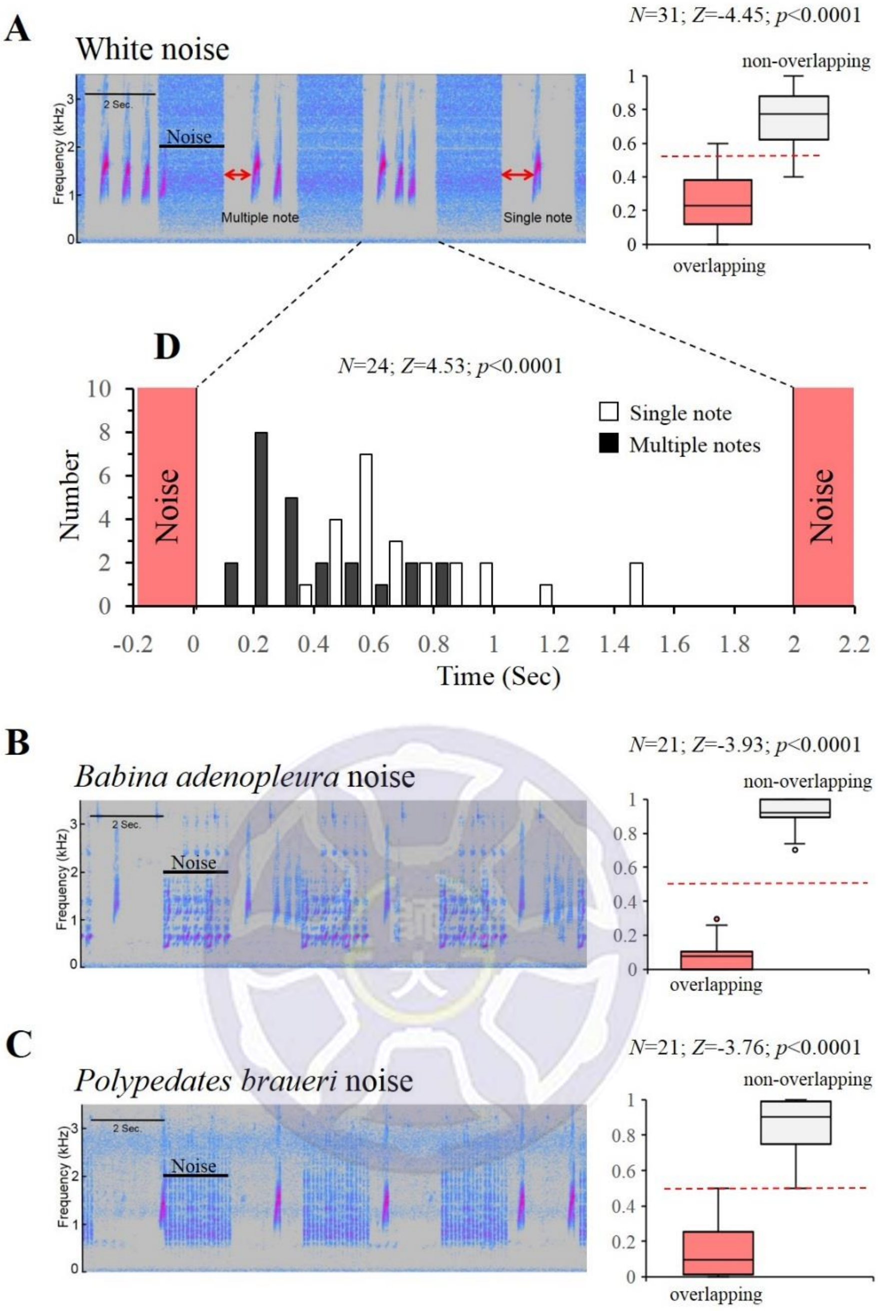

Fig. 3. 2

Call tempo adjustment of male *Rhacophorus prasinatus* (Rhacophoridae) when facing noise–silence inferences from (A) white noise, (B) *Babina adenopleura* (Ranidae), and (C) *Polypedates braueri* (Rhacophoridae). In each situation, *R. prasinatus* showed distinct trends to avoid making calls overlapping with the interference, which led to significant deviation from the expected 0.5/0.5 ratio if the calls were randomly delivered ($p < 0.0001$ in all cases). (D) Analysis of the starting time of calls within the 2-second silence, I found that the frogs are able to initiate the calls earlier if multiple notes were to be delivered.

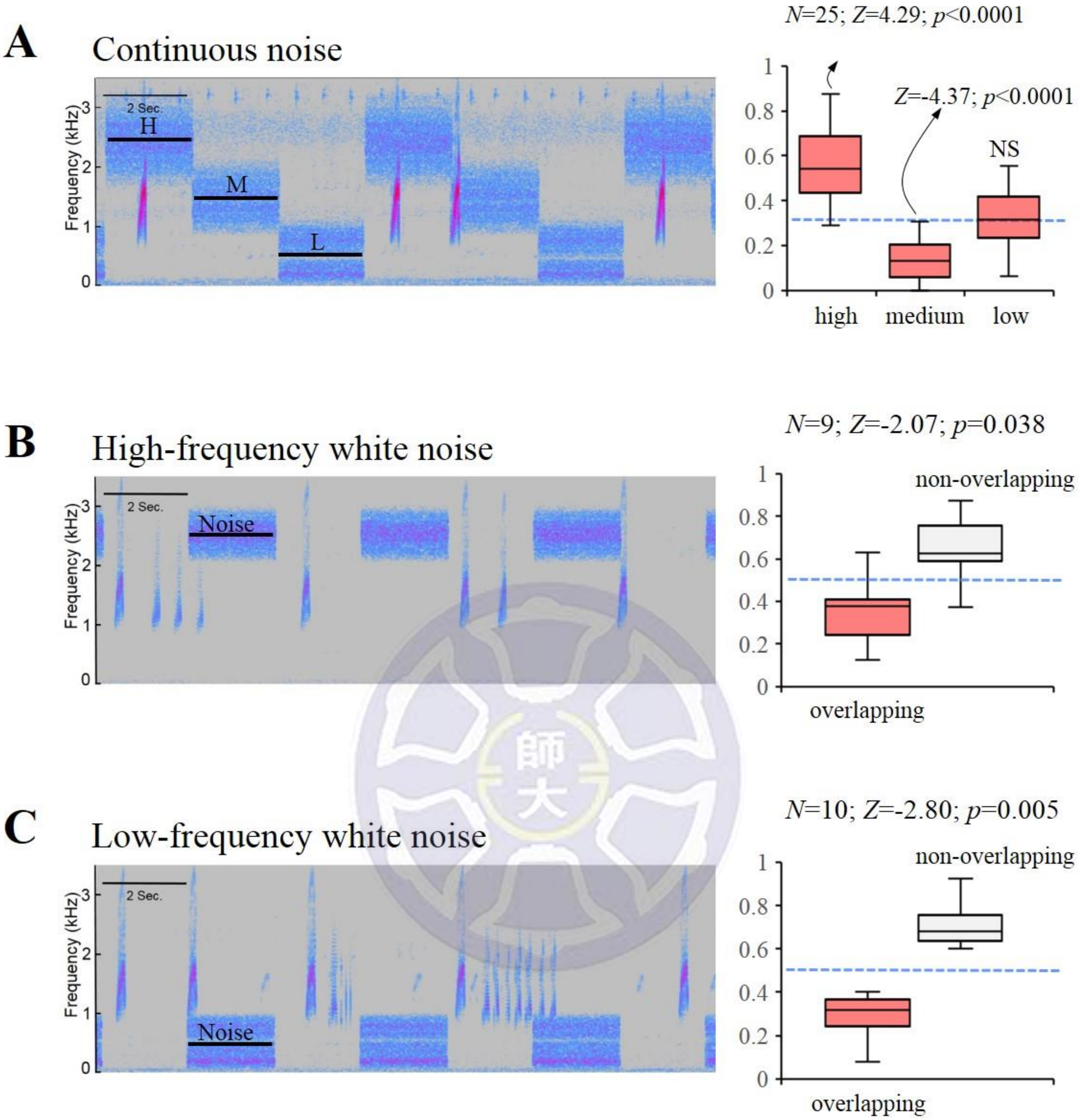

Fig. 3. 3

Call tempo adjustment of male *Rhacophorus prasinatus* (Rhacophoridae) when facing noise inferences from (A) continuous band-limited noise with high-, medium-, and low-frequency repeats; (B) noise–silence repeats of band-limited noise of high frequency (2 – 3 kHz); and (C) noise–silence repeats of band-limited noise of low frequency (0 – 1 kHz). In the first situation, *R. prasinatus* showed distinct trends to avoid making calls overlapping with medium-frequency noise (1 – 2 kHz), which led to significant deviation from the expected 0.33/0.33/0.33 ratio if the calls were randomly delivered ($p < 0.0001$). However, the frogs were proved to be able to hear high-frequency and low-frequency noises because they showed a significant response to avoid these noises (high-frequency noise: $p = 0.038$; low-frequency noise: $p = 0.005$).

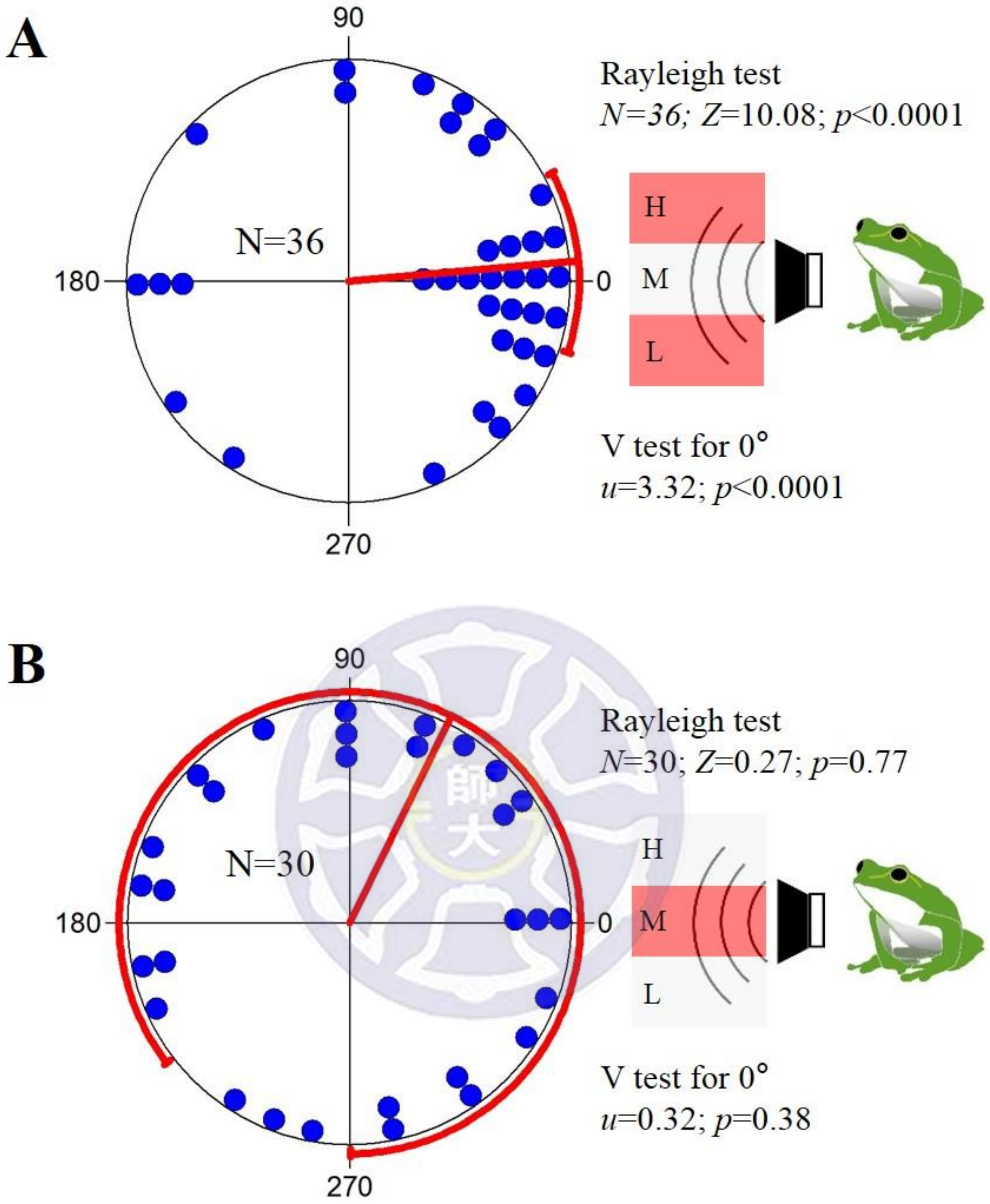

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Fig. 3. 4

The aggregating behavior of male *Rhacophorus prasinatus* changes in response to a conspecific chorus with (A) high- and low-frequency band-limited noise (non-overlapping frequency, > 2 kHz or < 1 kHz), and (B) medium-frequency band-limited noise (overlapping frequency, 1 – 2 kHz). The blue dots represent the approach angles of frogs, and the red line represents the mean angle and 95% confidence interval. The results indicated that the frogs are tolerant to non-overlapping noise, and thus the aggregating behavior was maintained; meanwhile, noise with an overlapping frequency stopped the normal aggregating behavior.

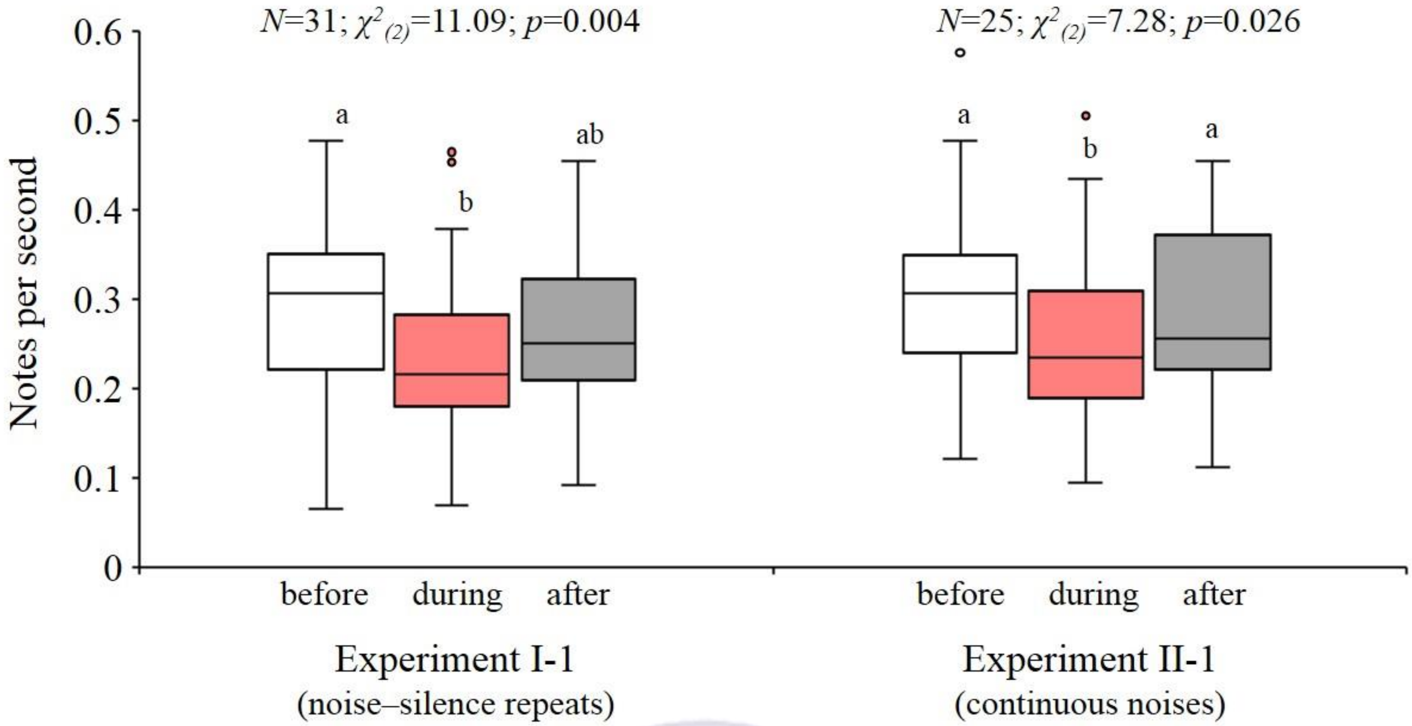

Fig. 3. 5

Comparison of call rates among pre-test, broadcasting, and post-test periods from Experiment I-1 (noise–silence repeats) and Experiment II-1 (continuous noise). In both experiments, the frogs had significant reduction in call rates during the broadcasting, and recovery in the post-test period. However, high-quality and low-quality males presented different responses to continuous and non-continuous noise.

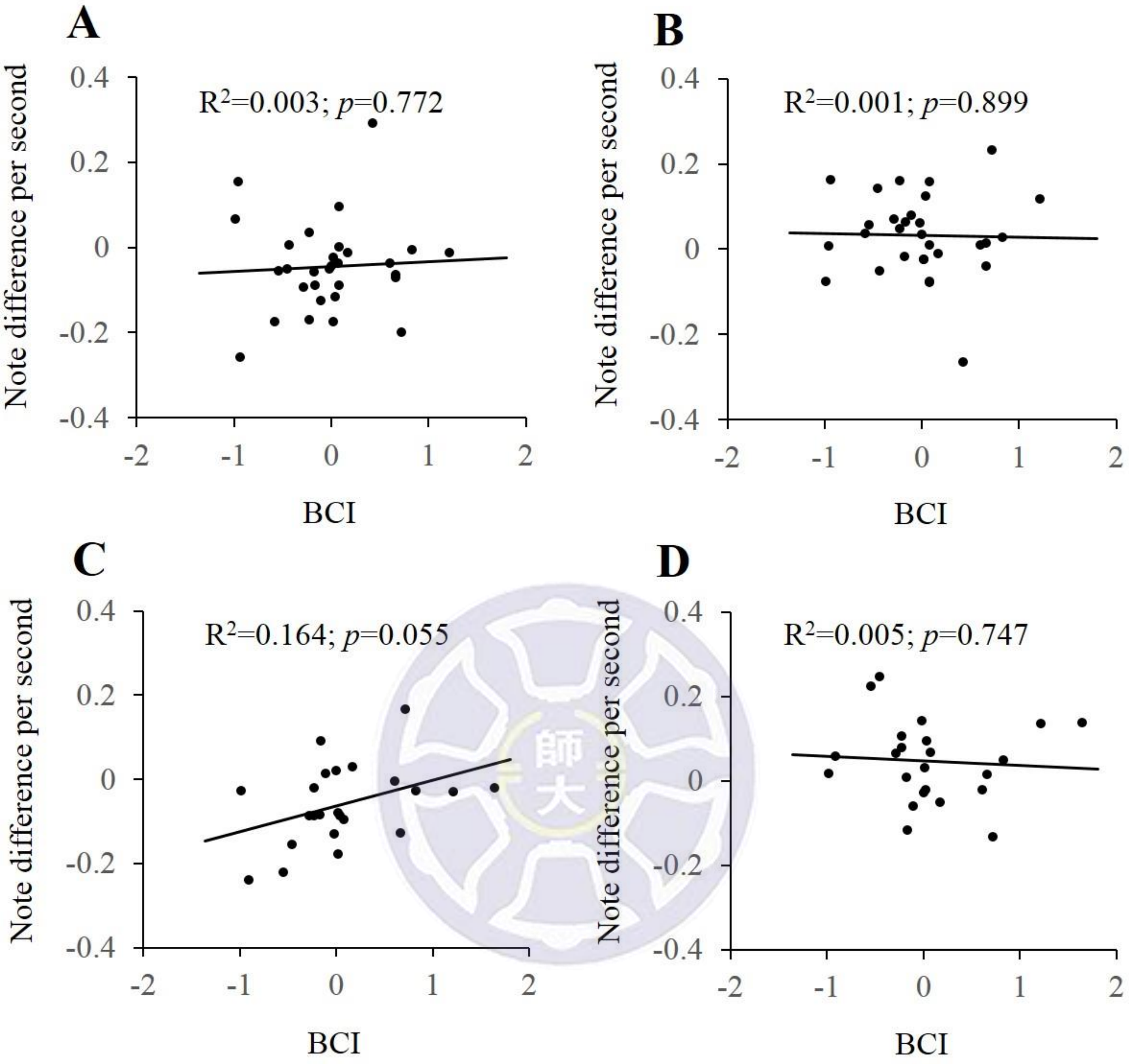

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Fig. 3. 6

Relationships between note difference and BCI in Experiment I-1 (noise–silence repeats; A, noise broadcasting; B, noise ceased) and Experiment II-1 (continuous noise; C, noise broadcasting; D, noise ceased).

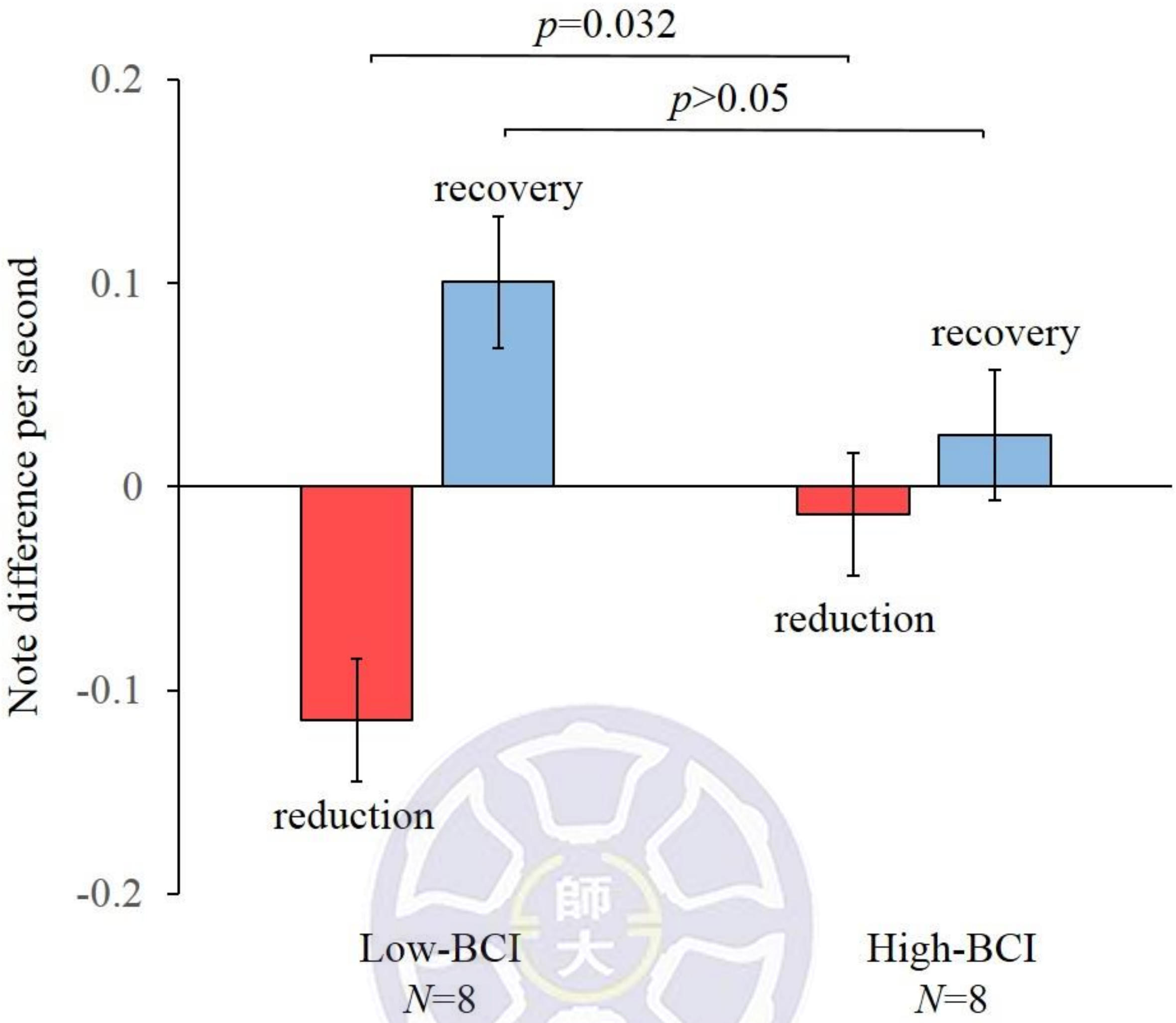

Fig. 3. 7

In Experiment II-1 (continuous noise), high-BCI males chose to maintain their call rate throughout the whole testing process while low-BCI males almost gave up their calling during the broadcasting period (T test: t_{14} = 2.38, p = 0.032). Red and blue bars represent the note difference of frogs when noise broadcasting and noise ceased, respectively.

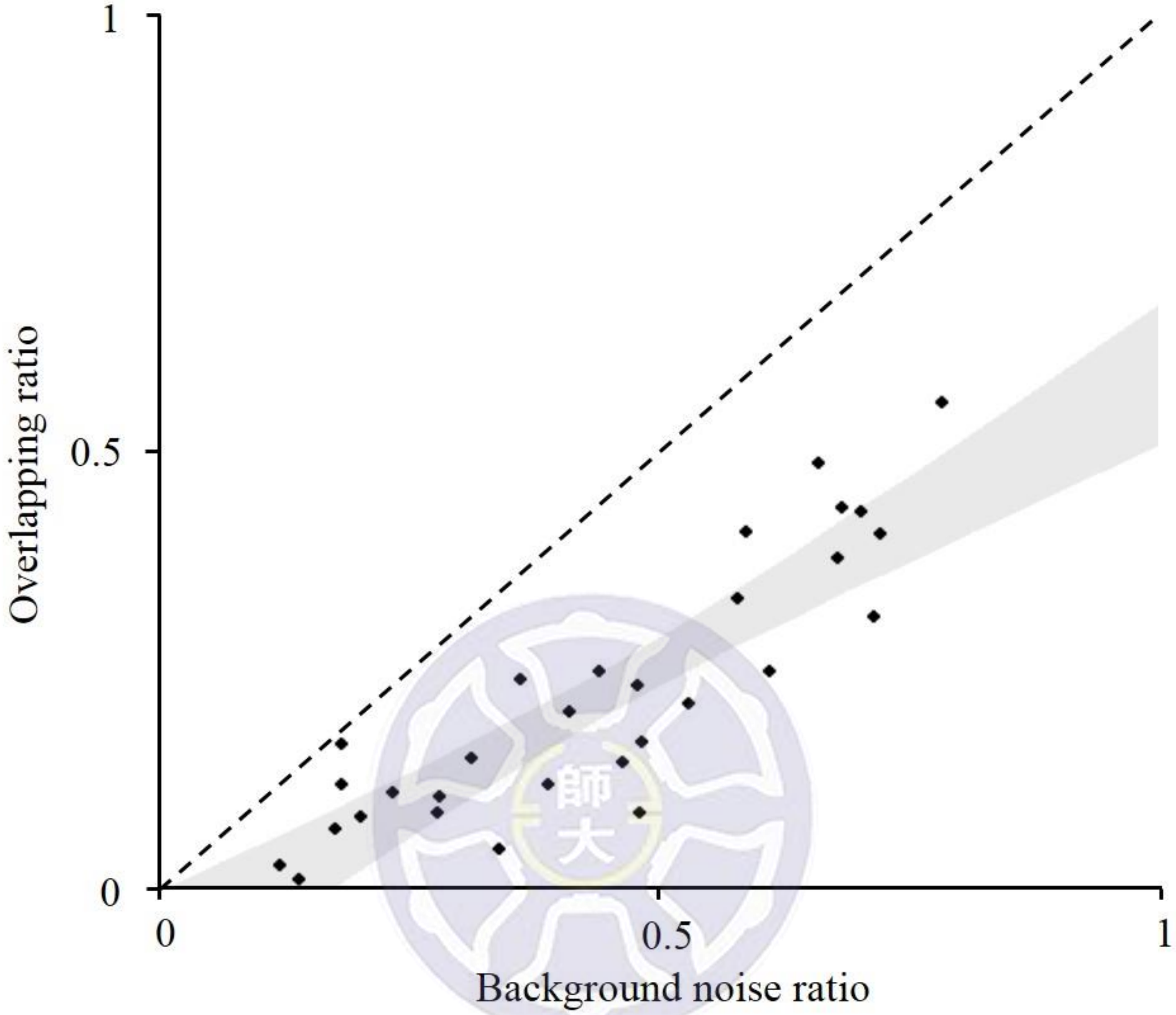

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Fig. 3. 8

In the real situation in the wild, most of the frogs avoid delivering calls overlapping with background noise produced by the chorus of other frogs. The overlapping ratio is suspected to be positively correlated to the background noise ratio (because when the background noise ratio increased, it was difficult for the frogs to avoid the noise). Nevertheless, the scatter plots formed a slope (solid red line; $y = 0.665x – 0.0667$, $R^2 = 0.78$) significantly lower than 1 (no avoidance; marked by the broken line), indicating that the frogs also avoid noise interference in the wild.

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Chapter 4

Comprehensive discussion and further approach

Increasing number of laboratory experiments have shown amphibian species that use conspecific or heterospecific cues to locate suitable habitats in recent years (see Chapter 2). Heterospecific signal may provide the information about the potential position of breeding sites. However, heterospecific signals also masks signal transmission. The results of my studies suggested that male frogs may have the ability to recognize the difference of signal and to avoid the signal which produced from heterospecific competitor. In Chapter 3, I further showed that males are able to adjust the tempo of calling to prevent their calls being masked by heterospecific signal in short distance. On the other hand, males' display may be condition-dependent when background noise is loud. The adversity from heterospecific signal on male frogs may provide a selective agent to differentiate the quality of male frogs for females; those who produce calls sustainably under this harsh environment may have higher quality than retreaters. Nevertheless, extending questions remain to be resolved, thus will be discussed below in this chapter.

Species-specific frequency in call may be another type of resource partition

The distribution of species is thought to be relevant to historical and environmental factors. However, biological factors also play a crucial role (Kishi and Nakazawa 2013; Cunningham et al. 2016). Competition and resource limitation among species restrict the distribution range. In addition of time, space and dietary, the opportunity to deliver signal and the electromagnetic spectrum of call in frequency bands are also considered as another resource about community (Henry and Wells 2010; Ruppe et al. 2015). The results in my study suggested that species-specific frequency in calls may be an important factor which represents a unique resource for amphibian species to recognize and discriminate among heterospecies. Frogs should have the ability to distinguish the signal produced by heterospecies and use it as the information of potential breeding sites. There are more and more researches showed that the unique signal used on frequency band may be the analogous resource used in activity time, breeding habitats and dietary types. These factors restrict species coexist with each other (Kishi and Nakazawa 2013; Vélez et al. 2013; Mullet et al. 2017).

The interference of background noise on sexual selection

Studies on the female preference and auditory discrimination ability were restricted on a relatively small number of species (Bee et al. 2013). We know little about the acoustic characters which represent male's quality and were preferred by female, especially in the field where background noise exists. In a newly conducted study on the effect of urbanization to túngara frogs, urban noise caused male túngara frogs (*Physalaemus pustulosus*) to adjust their call properties and drove rapid adaptive signal preference on female choice in the urban population (Halfwerk et al. 2019). It is interesting that although background noise masks the communication between signaler and receiver, the interference of noise seems to provide a selective pressure on sexual selection. In my study, those male frogs who maintained their efforts in calling under noise interference were also high-quality individuals. More works are needed on the perceptual consequences of call overlap (e.g. masking and interference) in species of frogs with different types of calls.

In most cases, adjustment in timing was a general behavioral response; however, amplitude modulation and frequency modulation were much less discussed. I did not investigate the frequency-modulation in calls in my study; it may enhance detectability by regulating their species-specific frequency in call in order to reduce interference from noise at the same frequency band (Rand 1985; Bosch and De la Riva 2004). Nevertheless, change of species-specific frequency may confuse the recognition of females, especially in the mixed-species chorus. However, it may be advantageous to regulate the frequency of calls in the circumstance of high intensity and continuous background noise, such as in a steam (Vélez et al. 2013; Goutte et al. 2016) or under anthropogenic noise (Kaiser et al. 2011; Roca et al. 2016).

Trade-off between chorus attendance and calling effort

In a lek or a chorus, performing vocal or dancing displays are time-consuming, energy-intensive and would engender high risk of predation. Thus, strong selective pressures act on males to modulate their competitive strategies according to abiotic and social contexts (Gerhardt and Huber

2002). Humfeld (2013) used starving experiment to investigate the call activity of male green tree frogs (*Hyla cinerea*). Males in high-food treatment maintained their call property, but those in low-food treatment reduced at the third day of experiment. These results indicated that energetic preservation would affect the mating success of males. Moreover, after ten days of starving experiment, male *Hyla arborea* in different conditions adopted different allocation strategies, suggest that this strategy shift might due to trade-off between investments in qualities and quantities (Brepson et al. 2012). Endurance rivalry hypothesis (Friedl and Klump 2005; Andersson and Simmons 2006) may explain why females have inconsistent preference on males' performance between field and laboratory experiments: females preferred the loudest or the fastest displays performed by males under laboratory conditions; however, those males who spent more time in chorus tend to sired more offspring in wild, instead of performing intensive display. Since males could not precisely predict the appearance of female at the breeding site, allocation of effort on chorus attendance or calling would be a more complicated issue.

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Application in conservative management

Amphibian species are facing population decline all over the world (Beebee and Griffiths 2005; Anderson 2019). One of the most serious threat is habitat lost and fragmentation (Cushman 2006; Anderson 2019). Natural habitats are rapidly replaced by city and farm. Although we have established conservative area and tried to protect the hot spots, low altitude species are inevitably losing their habitats. Playbacks of conspecific and non-competitor's signal may be used in attracting anurans to unoccupied but suitable habitat, and may be especially useful for seeking to restore or aggregating populations at newly created or restored wetlands (Buxton et al. 2018). This is now a valuable tool in avian management and conservation (Ward and Schlossberg 2004; Ahlering et al. 2010). Unless we figure out the detail about the migration, dispersal and aggregation of animals, we could not reduce the decline on species diversity. For instance, if we knew how animal find their way to breeding sites or potential habitats, we could avoid to using these habitats they headed to, or we could guide animal to a similar habitat nearby and reduce the effects from human activities. Even more, invasive species are the leading threat to native wildlife, one of recently developed method for population suppression is acoustic trap, Muller and Schwarzkopf (2017) used acoustic lure to trapping a highly invasive anuran, cane toad (*Rhinella marina*), in Australia, and suggested that acoustic trapping was more efficient than hand capture (7.1%–22.4% and 1.7%–6% of the estimated population per week, respectively) on controlling toads. Therefore, if we knew how animal choose their habitat when enter a newly environment, invasive species could be attracted or restricted to limited areas by signal and could remove them more effectivity (Schwarzkopf and Alford 2007; Tingley et al. 2017).

Conclusion

Not only conspecific signal, but also heterospecific signal provided information to individuals to make decisions in different situations including finding mates, competing rivals, evading heterospecific competitors, and searching potential habitats. On the other hand, background signal could also mask the signal transformation. However, it may provide a selective pressure for females to choose the males who have the ability to deal with the background signal. This selective pressure was not only from conspecific males or females, but also from heterospecific and environmental factors.

My experimental design explored the phonotaxis and vocal behaviors of male *Rhacophorus prasinatus* in the field. The results in my study not only provided the first experimental test in natural condition which considered the multiple species masking effect in playback experiment, but also provided a guide for further approach, which should combine all relevant factors including signal transmission, sensory sensitivity, chorus joining decision, and alternative courtship strategy. They could be applied to practical behavioral and conservation issues about how males deal with background noise. I conclude that:

(1) Male *Rhacophorus prasinatus* has the ability to identify the signals produced from sympatric anuran species.

(2) Conspecific and heterospecific cues could use as the information to selecting breeding site and may enhance the probability of mating success.

(3) Male *Rhacophorus prasinatus* adjust their timing of call to reduce masking by background noise.

(4) Strategy used by male frogs may be condition-dependent.

(5) The behavioral regulation before and after chorusing may be the mechanisms to reduce competition among heterospecies, and may be one of the explanations about how species coexist.

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Appendix A

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ORIGINAL ARTICLE

Influence of conspecific and heterospecific cues on phonotaxis behavior in a polyandrous treefrog

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Abstract

Although a large proportion of the literature has addressed the direction and preference of female choice in frogs, research about how males locate a breeding site and what strategies are involved in aggregation has been much less explored. By using broadcasting tests on a polyandrous choral treefrog *Rhacophorus prasinatus*, we examined how male frogs use conspecific and heterospecific signals to locate a chorus by asking the following questions: (1) Do male frogs prefer to join a large or a small chorus? (2) Do male frogs prefer to approach a high-quality or a low-quality male? (3) Do males utilize heterospecific calls to locate the potential breeding sites? (4) Do male frogs orient toward or avoid choruses containing heterospecific calls? Our results indicated that males prefer to join a large rather than small chorus. Low-quality males tend to approach high-quality males, which might be explained as the sneaking behavior of satellite males. Furthermore, males may use heterospecific calls delivered by a noncompetitive sympatric species (a ranid frog) to find potential breeding sites, but not by a sympatric competitor (another rhacophorid frog) that occupies a similar niche. Although the males did not show significant preference between conspecific chorus and mixed-specific chorus in the broadcasting tests, recapture records in the wild indicated that a high ratio of males would leave the mixed-specific choruses and move toward conspecific choruses in the next capture event.

Significance statement

Although a large proportion of the literature has addressed the direction and preference of female choice in frogs, research about how males locate a breeding site and what strategies are involved in aggregation has been much less explored. Our study represents one of the first to test the orientation of males during breeding season, when the spatial scale in the experimental design is comparable to the real situation in the wild. Our results supported the prediction that both conspecific and heterospecific cues are used as information for males to assess the quality of breeding sites. Heterospecific calls may play dual functions for the males, which might become interference when coexisting with conspecific calls but may also provide cues for a potential habitat when conspecific calls are absent.

Keywords Chorus attraction hypothesis · Heterospecific attraction hypothesis · Polyandry · Resource partition · Satellite males · Self-evaluation · Sneakers

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Introduction

The co-occurrence of sympatric animal species in shared habitats is of interest in behavioral ecology, evolutionary biology, and community structure. Animals utilize conspecific and heterospecific cues to locate potential habitats (Sebastian-Gonzalez et al. 2010; Zeigler et al. 2011), and this scenario also exists in anuran species (Buxton et al. 2015; Madden and Jehle 2017). In forest environments where water resource is patchy and limited, suitable breeding sites are sometimes precious for anuran species. In this kind of habitat, competition at both intra- or interspecific levels might be intense. Therefore, the choice of a breeding site should be a compromise after considering multiple clues of costs and benefits (Ryan et al. 1981; Kishi and Nakazawa 2013; Höbel 2014; Höbel and Barta 2014; James et al. 2015; Rehberg-Besler et al. 2016).

In anurans, males aggregate to perform their advertisement signals. This behavior, also known as "leks" or "choruses," is thought to increase the fitness of males by attracting potential mates (Ryan et al. 1981; Stamps 1988). The "chorus attraction hypothesis," reviewed by Gerhardt and Huber (2002), posits that anurans use conspecific choruses to locate new habitats. In addition to increasing the chance of encountering mates, it also helps in searching for a suitable habitat while using the lowest energy costs. For example, Bee (2007) broadcasted conspecific calls to male wood frogs (*Rana sylvatica*) in a testing room, and males showed positive phonotaxis toward the conspecific calls. Most studies suggested that sounds of a chorus allow frogs to locate the breeding aggregation in the breeding season (Bee 2007; Ursprung et al. 2009; Buxton et al. 2015, 2018).

Joining a suitable chorus affects the breeding success of a male. In a patchy environment, male frogs may have chances to move among different choruses, and the strategy of choosing choruses may maximize their fitness. Joining a high-quality chorus may result in a higher probability of encountering females; however, the trade-off is high male–male competition (Lucas et al. 1996; Friedl and Klump 2005; Fletcher 2009; Humfeld 2013; Höbel 2014). Therefore, the competitive strength of a chorus could be an important factor for males to evaluate. For example, high-quality and low-quality male frogs may strategically join different choruses to maximize their mating success (Lucas et al. 1996; Humfeld 2013; Höbel 2014).

In addition to conspecific calls, heterospecific cues are also used by frogs to locate suitable habitats. As addressed by the "heterospecific attraction hypothesis," the presence of other species could be signaled through visual, olfactory, or acoustic cues, which serve as a form of public information regarding the habitat's quality (Mönkkönen et al. 1997). In sympatric sites, species adapt to utilize different resources to reduce interspecific competition. Thus, recognizing heterospecific cues should be an ability required for optimal habitat choice. In previous studies, the marbled newt (*Triturus marmoratus*) and great crested newt (*Triturus cristatus*) showed the ability to use the calls of sympatric anuran species to locate a breeding site, even though they do not communicate with acoustic signals (Diego-Rasilla and Luengo 2004; Madden and Jehle 2017). Since mixed-specific choruses are often found in the wild, heterospecific calls could be recognized as an additional cue for habitat quality (Bee et al. 2013).

A considerable body of literature has focused on conspecific and heterospecific attraction in anurans. However, in the majority of these studies, frogs/toads were tested at a distance of less than 3 m; this is vulnerable to the critique that such studies might only represent the situation "within" the chorus, not the choice "among" choruses (Beckers and Schul 2004; Bee 2007; Ursprung et al. 2009; Moreno-Gómez et al. 2015). Furthermore, most of these studies tested the response of females, whereas the male decision of joining a chorus was explored less thoroughly (Swanson et al. 2007; Bee and Riemersma 2008; Schwartz et al. 2015). More precise experiments, especially conducted with a larger spatial scale, are required to investigate male chorusing behavior.

In this study, *Rhacophorus prasinatus* (Rhacophoridae), an Oriental treefrog, was used to test the aggregative behavior of frogs. In Taiwan, this frog has a prolonged breeding season almost all year round, except when the temperature is lower than 10 °C (Chen 1992). In the breeding season, males form choruses in bushes and produce foam nests on vegetation near or above ephemeral and permanent water (Mou et al. 1983). Based on our observation, satellite males tend to join the copulation process and lead to polyandrous mating and extra-pair paternity in this frog. *Babina adenopleura* (Ranidae), *Polypedates braueri* (Rhacophoridae), and *Rhacophorus taipeianus* (Rhacophoridae) are often sympatrically distributed with *R. prasinatus*. *Babina adenopleura* usually breeds at permanent water in March through October and produces an egg mass in the water (resource noncompetitor), whereas *P. braueri* uses ephemeral and permanent water to breed in March through October and produces a foam nest on vegetation (resource competitor). *Rhacophorus taipeianus* also produces foam nests but always in a mud cave underground, and it breeds in the winter from November through February (competitor with temporal partition). A recent study reported a higher overlapping ecological niche breadth between *R. prasinatus* and *P. braueri* than that between *R. prasinatus* and the other two species (Lu and Chen 2012), thus providing a good opportunity to study the interspecific interaction of chorusing behavior.

To test whether male frogs utilize conspecific and heterospecific cues in aggregating behavior, we employed a hypothesis-testing methodology. We constructed a circular testing arena in an outdoor environment and broadcasted different chorus sounds that varied in chorus size, the member quality of male frogs, and the presence or absence of heterospecific frogs. Based on the assumption that males would maximize their fitness, we hypothesized that males would join the patch, where more males aggregate, or join the high-quality males, which have a higher potential to encounter females. We further hypothesized that males do recognize heterospecific cues, and the aggregating response would vary according to competitive pressure. We predicted the following: (1) male frogs would prefer to join a larger chorus rather than a small one, (2) male frogs would prefer to approach to a high-quality male than a low-quality male because of the higher potential to encounter females, (3) heterospecific calls would be used to locate potential breeding sites when conspecific calls are absent, and (4) male frogs would avoid joining a chorus with noise from competitive heterospecific calls. The findings in the current study may enhance our knowledge on the potential ecological and evolutionary factors that influence animal aggregative behavior.

Materials and methods

Study system

The experiments were conducted from 2016 to 2017 in Hualin Experimental Forest (24° 53′ N, 121° 33′ E), a 92-ha protected region located in New Taipei City, Northern Taiwan. The environment consisted of secondary hardwood forest with some grassland patches. Natural and artificial pools were located within the forest in a patchy and mosaic pattern and attracted choruses during the breeding season. In addition to *R. prasinatus*, the most abundant frogs in the study site are *Babina adenopleura* (Ranidae) and *Polypedates braueri* (Rhacophoridae) in the same breeding season and *Rhacophorus taipeianus* in a different breeding season (only in winter).

An individual marking program using toe clipping has been conducted in the experimental forest for years, which helped us with individual identification in this experiment. Captured frogs were weighed to the nearest 0.01 g using an electronic scale, and the snout-vent length (SVL) was measured to the nearest 0.01 mm using a digital caliper. Following Peig and Green (2010), the scaled mass index (SMI) was calculated from the body length and body weight according to the following equation: $\mathrm{SMI} = M_i[L_0/L_i]^{\mathrm{bSMA}}$, where M_i and L_i are the body weight and the SVL of individual i, respectively; bSMA was calculated from the standardized major axis regression of the log body weight on log SVL; and L_0 was the mean body length for the study populations. It was not possible to record data blind because our study involved focal animals in the field.

Call recording and editing

Acoustic files for playback (Fig. 1) were recorded from the focal species by an SM2 digital recorder (Wildlife Acoustics, Maynard, MA, USA) at the study site. The original files contained the chorus of only one species from each of the four frogs. For *R. prasinatus*, we further recorded calls from single individuals, which could be randomly selected in playback experiments.

In addition to original calls, we also synthesized or modified call files for usage in different experiments. *Rhacophorus prasinatus* produces calls consisting of a series of high-frequency "A" notes and a series of low-frequency "B" notes (Fig. 1c). In this experiment, we considered calls with A + B notes as high-quality calls, and those with only A notes as low-quality calls based on the following several reasons: (1) in the wild, B notes are only recorded from high-quality males who persist in making continuous breeding calls. In contrast, B notes are much less frequently recorded from low-quality males who make calls in a punctuated manner. (2) Considering that courtship calls are extremely energy consuming (Wells 2001), those who produce more calls have been found to have better body condition (Sullivan and Hinshaw 1992; Prestwich 1994; Reichert and Gerhardt 2011; Rodríguez et al. 2014). (3) In a previous study on a closely related species *Rhacophorus moltrechti* which also produce two types of notes, the length and number of B notes were the cue for females to distinguish high-quality males (Chen 2002). Based on the results of these previous studies, we used the original A + B files as a high-quality stimulus and erased the B notes to produce a new file as a low-quality stimulus. Finally, the mixed-specific calls were generated by mixing natural chorus of *R. prasinatus*, *B. adenopleura*, and *P. braueri* into a single file. The white noise stimulus (Fig. 1b) was synthesized to 15-min files using Audacity software (version 2.06; http://audacity.sourceforge.net/), containing 2-s intervals of white noise (with different frequencies in different treatments), spaced by 2-s silent gaps.

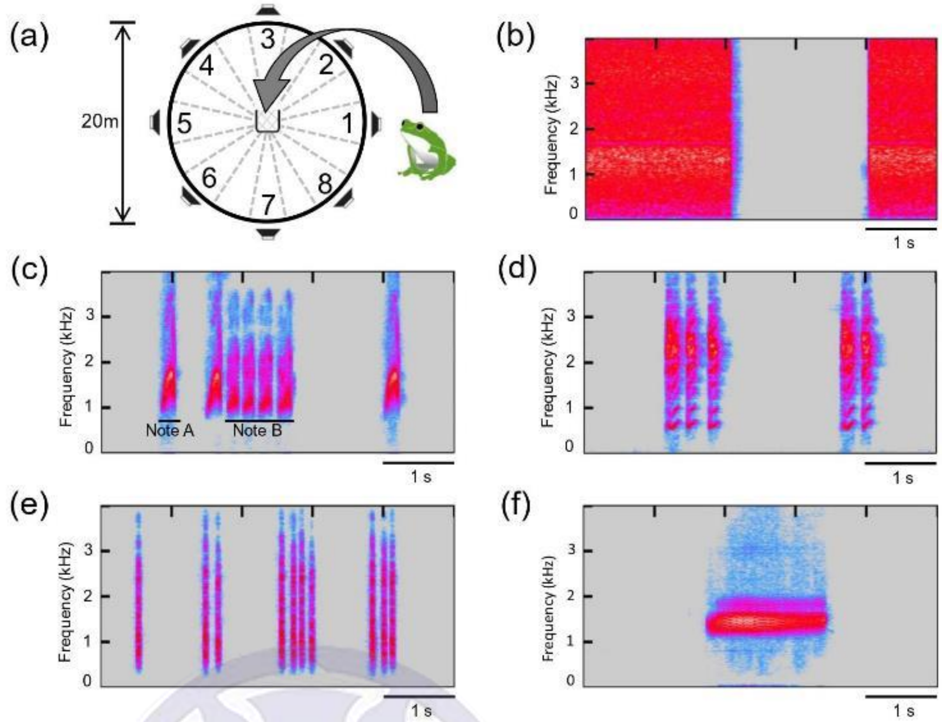
All digital acoustic files were recorded and broadcasted with a 44.1 kHz sampling rate, and 16-bit resolution WAV format; the amplitude of playbacks was set to 75 dB SPL measured at 1 M from the speakers using a sound pressure level meter (Lutron SL-4030; fast RMS, "C" weighting).

Experimental design: conspecific tests

In order to investigate the aggregating behavior of males, we designed three conspecific phonotaxis tests:

> *Experiment I-1*—Two unidirectional tests: phonotaxis preference toward *R. prasinatus* (conspecific) chorus calls, and phonotaxis preference of white noise as the control

Fig. 1 Experimental design. **a** Schematic diagram of the outdoor test arena used in the experiments. Spectrograms of **b** white noise, **c** *Rhacophorus prasinatus*, **d** *Babina adenopleura*, **e** *Polypedates braueri*, and **f** *Rhacophorus taipeianus*



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Experiment I-2—Bidirectional test: phonotaxis preference of chorus calls of *R. prasinatus* compared to calls of a randomly selected single male in the opposite direction

Experiment I-3—Bidirectional test: phonotaxis preference between high-quality calls (A + B notes) and low-quality calls (A notes only) in the opposite direction

Experimental design: heterospecific tests

In order to investigate the response of males to heterospecific calls, we designed two phonotaxis tests and one capture–mark–recapture experiment.

Experiment II-1—Three unidirectional tests: phonotaxis preference toward chorus calls of *B. adenopleura* (resource noncompetitor), *P. braueri* (resource competitor), and *R. taipeianus* (temporal partition competitor)

Experiment II-2—Bidirectional test: phonotaxis preference between artificially synthesized mixed-specific chorus calls comprising of *R. prasinatus*, *B. adenopleura*, and *P. braueri*, compared to conspecific *R. prasinatus* chorus calls in the opposite direction

Experiment II-3—Capture–mark–recapture records: the recapture process in the experimental forest helped us to locate the position of males in different capture occasions. By using translocated release into other choruses, this experiment tested the movement of the frogs after being released into conspecific or mixed-specific choruses.

Experimental protocol of phonotaxis tests

We conducted outdoor phonotaxis tests in a circular test arena (20 m diameter) to investigate the chorus aggregating behavior of male frogs (Fig. 1a). This test arena was a deforestation zone > 100 m from the closest chorus, thus avoiding acoustic interference from other places. Acoustic signals were broadcasted by speakers facing the center, which were placed 15 cm above the ground along the arena's edge. In order to eliminate any possibility of a directional response bias in the arena, eight positions (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°) were defined on the edge of the arena (Fig. 1a). For unidirectional experiments (I-1 and II-1), a speaker was randomly selected from one of the eight positions in each trail. For bidirectional experiments (I-2, I-3, and II-2), the position of the first speaker was randomly selected, and the second speaker was opposite (180°) from the first. The speakers used in this study were handmade following Cunnington and Fahrig (2013). In each set of equipment, a waterproof speaker (Pokka PK-505SPIP; Hylex, Taiwan) was connected to an amplifier and battery, and a digital recorder (Sony, PCM-M10; Sony Corporation, Tokyo, Japan) was used to broadcast the signal.

The tested frogs were caught about 1 h after sunset (7:00 PM) from natural choruses in the study site. The frogs were kept separately in containers, and the phonotaxis tests were conducted one by one as soon as possible before the midnight. Before the process of the test, a 12-mm fluorescent rod was attached to the waist of the frog with a cotton thread and was detached immediately after the test. This fluorescent rod helped to monitor the movement of the frog without extra light sources. The weight of the fluorescent rod was about 0.2 g, which was less than 3% of the body mass for male *R. prasinatus*.

When a test started, the target frog was placed in a quadrate open blanket (50 cm × 40 cm × 33 cm) in the center of the arena (Fig. 1a). The countdown began when the researcher left the frog free and slowly moved away from the arena. Attracted by the acoustic signals, the frog would climb out the blanket to start making its choice and was allowed to move freely in the arena. When the frog reached the edge of the testing arena within 15 min (defined as a successful test, comprising 81.7% of total trials), the approach angle was recorded in order to estimate the male's perception of sound location. The perimeter of the arena was divided into 16 arcs (each 22.5°), and the approach angle was defined as one of 16 arcs (Fig. 1a). For frogs that did not reach the edge within 15 min, the trial was considered as a nonresponse and was excluded from further analyses (18.3% of total trials).

In some occasions (48.9% among all cases), a frog was used in more than one experiments at the same night. Among these cases, 68.6% were used in two experiments, 25.4% were used in three, and 5.9% were used in four experiments. Under this situation, different experiments were spaced for more than 30 min and the experiments were randomly arranged. Based on the previous literature, this treatment did not influence the performance of the frogs (Vélez and Bee 2010; Farris and Ryan 2011, 2017; Reichert et al. 2016). After the tests were accomplished, all the frogs were released into the experimental forest as soon as possible before midnight.

Movements among conspecific and mixed-specific choruses

Several water bodies exist in a patchy pattern within the experimental forest, which comprises a large zone of mixed-specific choruses in the middle (comprised of 18 small water bodies) and four conspecific choruses in the periphery. These choruses were spaced for 20–30 m, with two farthest choruses spaced for 60 m. This spatial arrangement allows all the males to move freely across all the choruses. A preliminary test indicated that the males did not represent homing behavior for a specific chorus (only 25.4% males returned to their original choruses after translocated release, and also, only 36.4% males were found in their original choruses in the next capture after being marked).

After the arena experiments, 28 males were randomly chosen and released in conspecific breeding choruses (where only *R. prasinatus* exists), while 72 males were released in mixed-specific breeding choruses (containing *R. prasinatus*, *B. adenopleura*, and *P. braueri*). The routine capture–mark–recapture experiment, on average twice per week during the time that we conducted the experiments, helped us to locate the males in the next capture event.

Data analyses

In the phonotaxis experiments, the Rayleigh test was used to test whether approach angles were randomly distributed. The V test determined whether phonotaxis responses were localized at a particular exit angle: 0° or 180° in bidirectional tests and 180° in unidirectional tests.

In experiment II-2, for which the male frogs did not show a particular direction but preferred both 0° and 180°, a goodness of fit test with a Bonferroni post hoc test was used to determine whether male frogs had a bidirectional preference. In contrast to the other bidirectional tests, the frogs in this experiment had phonotaxis preference but could not distinguish between the two choices in the opposite directions. Therefore, we categorized the test arena into eight 45° arcs, with arcs in opposite angles grouped together as a set (the first arc grouped with the fifth arc, the second arc grouped with the sixth, etc.) before multiple comparisons were made. A Bonferroni correction of $\alpha = 0.0125$ was applied; it was derived from $\alpha = 0.05/4$.

In experiment II-3, Fisher's exact test was used to test the percentage difference of frogs moving to another habitat between the group which was released into conspecific sites, and that released into mixed-specific sites.

Circular statistic tests were performed with Oriana software (ver.4; Kovach Computing Services, UK). Other statistical tests were performed using JMP software (ver.7; SAS Institute, Inc., Cary, NC, USA). All statistical tests were two-tailed and performed at $\alpha = 0.05$ (except for Bonferroni correction at $\alpha = 0.0125$).

Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Results

A total of 349 trails were conducted by using 140 identified males; each individual was tested once in each experiment. The body weight and SVL of male frogs (mean ± SD) were 58.89 ± 3.18 mm and 9.1 ± 1.55 g, respectively. In 81.7% of all the trails, the approach angle of the frog was acquired within 15 min and was thus defined as successful trials.

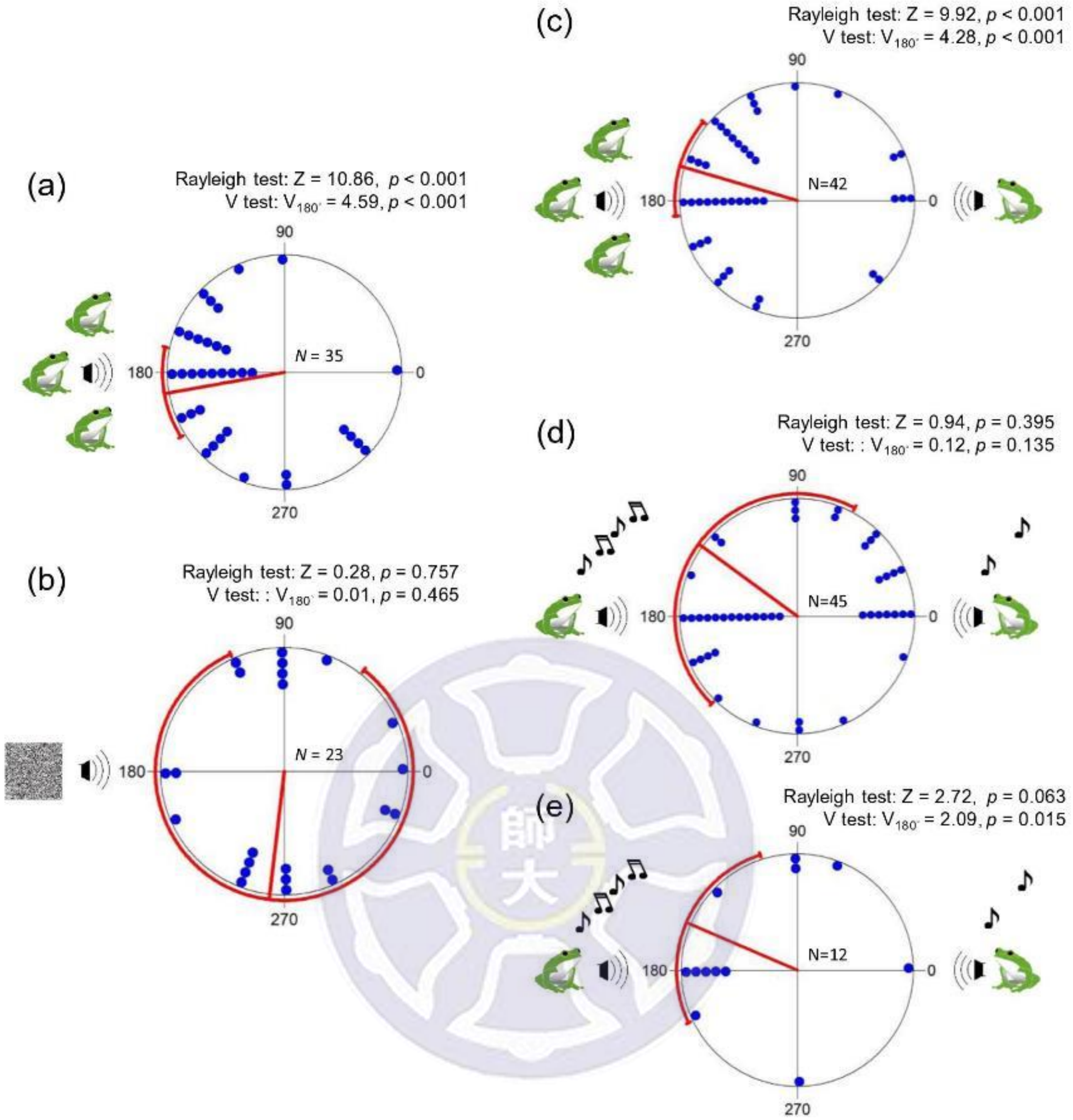

Fig. 2 Conspecific experiments for aggregating behavior of male *Rhacophorus prasinatus*. The blue dots represent the approach angles of frogs, and the red line represents the mean angle with 95% confidence interval. **a**, **b** Experiment I-1: the males showed a strong tendency to aggregate for the conspecific chorus, but not for the white noise. **c** Experiment I-2: the males preferred to join to a chorus that aggregates more males. **d** Experiment I-3: no approaching tendency was observed for all males toward high-quality or low-quality males; however, **e** the males with low scaled mass index (SMI) values tended to join the high-quality males

Experiments I-1 and I-2: males prefer to join a chorus rather than a single male

Experiment I-1 tested the validity of the experimental treatments. Male *R. prasinatus* showed a strong tendency to join a conspecific chorus (Fig. 2a, $N = 35$). In contrast, the frogs did not show a phonotaxis preference to the white noise (Fig. 2b, $N = 23$).

In experiment I-2, male responses for a chorus and a single male were compared. A total of 42 successful selections were acquired from 47 trials, yielding an 89.4% success rate. The mean angle was 163.7°, with an approach angle not randomly distributed (Fig. 2c) and a significant trend toward 180°. This result supported our prediction that male frogs prefer to join a chorus that includes more males.

Experiment I-3: low-quality males tend to approach a high-quality male

We next compared male responses to high-quality and low-quality calls. A total of 45 successful selections were acquired from 51 trials, yielding an 88.2% success rate. The mean angle was 143.8°, and the approach angle did not show a particular tendency (Fig. 2d). However, after we categorized the males into high- and low-quality groups using their SMI values, low-quality males showed a trend of approaching a high-quality male (Fig. 2e, $N = 12$, mean angle = 156.8), which was not found for high-quality males ($N = 26$, mean angle = 49.5°; Rayleigh test: $Z = 0.04$, $p = 0.965$). We also divided the males into three groups by SVLs: small males showed a persistent tendency to approach high-quality males ($N = 10$, mean angle = 139.5°; Rayleigh test: $Z = 2.77$, $p = 0.059$; V test: $V_{180°} = 0.40$, $p = 0.037$), but neither medium- nor large-sized males showed this trend (large males: $N = 16$, mean angle = 319.7°; Rayleigh test: $Z = 0.02$, $p = 0.982$; medium-sized males: $N = 14$, mean angle = 139.5°; Rayleigh test: $Z = 0.12$, $p = 0.888$). Results showed that low-quality individuals tend to join a high-quality male.

Experiment II-1: resource noncompetitors could attract male frogs

Here, we tested the response of male *R. prasinatus* to heterospecific calls. A total of 87 successful selections were acquired from 112 trials, yielding a 78.1% success rate (84.4% for *B. adeno pleura* 72.5% for *P. braueri* and 77.5% for *R. taipeianus*). By broadcasting different stimuli from a single speaker at the 180° location, male frogs significantly approached *B. adenopleura*'s chorus calls (Fig. 3a, $N = 27$, mean angle = 162.7°). In contrast, the approach angles showed a random distribution when calls of *P. braueri* and *R. taipeianus* were used (Fig. 3b, c, $N = 29$ and $N = 31$, respectively). These results supported our prediction that male frogs may use the calls of sympatric, heterospecific, noncompetitor frogs to evaluate their breeding habitats.

Experiment II-2: males do not reject a mixed-specific chorus

In this experiment, males were provided a chorus with or without heterospecific calls. A total of 44 successful selections were acquired from 48 trials, yielding a 91.6% success rate. The result of the goodness of fit test with a Bonferroni post hoc test demonstrated that male frogs significantly approached both mixed-specific and conspecific choruses (Fig. 3d; goodness of fit test: $X^2_3 = 15.82$, $p = 0.001$; Bonferroni post hoc test: one to two: $X^2_1 = 4.5$, $p = 0.039$; one to three: $X^2_1 = 7.76$, $p = 0.005$; one to four: $X^2_1 = 10.7$, $p = 0.001$; two to three: $X^2_1 = 0.53$, $p = 0.47$; two to four: $X^2_1 = 1.67$, $p = 0.19$; three to four: $X^2_1 = 0.33$, $p = 0.56$); furthermore, there was no significant difference between the two types of choruses ($X^2_1 = 0.18$, $p = 0.669$).

Experiment II-3: males prefer to stay in conspecific sites rather than mixed-specific sites

This experiment tested the movement of males after being translocated into a mixed-specific or conspecific site. Almost half of the males were recaptured after being released to a designated chorus (46/100). For these 46 frogs, the mean spacing of the next recapture was 12.54 ± 9.63 days (mean ± SD), providing sufficient time for movements among different choruses.

In the group of frogs that were released into sites with only *R. prasinatus* (conspecific chorus), 72.7% (8/11) of individuals remained at the original chorus site for the next capture, while 27.3% (3/11) moved to mixed-specific sites (Fig. 3e). In contrast, 68.6% (24/35) of individuals moved to conspecific sites when they were released to mixed-specific choruses and only 31.4% (11/35) remained at the original site. The ratio of movements was significantly different between the two treatments, thus suggesting that male *R. prasinatus* avoid remaining in mixed-specific choruses.

Discussion

Although a large proportion of the literature has addressed the direction and preference of female choice in frogs, research about how males locate a breeding site and what strategies are involved in aggregation has been much less thoroughly explored. Our study is one of the first to test the orientation of males during breeding season, when the spatial scale in the experimental design is comparable to the real situation in the wild. The results indicated that both conspecific and heterospecific calls are used to locate potential breeding sites.

Strategy for joining a conspecific chorus

A typical breeding ground of *R. prasinatus* usually contains several water bodies and choruses, which provides different habitat patches for males to choose from and migrate among. This behavior was revealed by our relocated recapture results (Fig. 3e). Past research on females (Neelon and Höbel 2017) indicated that the frogs could assess the information they received in previous choruses to make decisions when joining subsequent choruses; however, relevant evidence for males is scarce. For our experiment comparing multiple males and a single male (experiment I-2), most frogs chose to join the former (Fig. 2c). Typically, a more crowded chorus indicates a higher strength of intrasexual competition and a higher probability of attracting predators (Höbel 2014; Höbel and Barta 2014). Nevertheless, these disadvantages are compensated for by a higher probability of encountering females (Rehberg-Besler et al. 2016); this explains the aggregating behavior

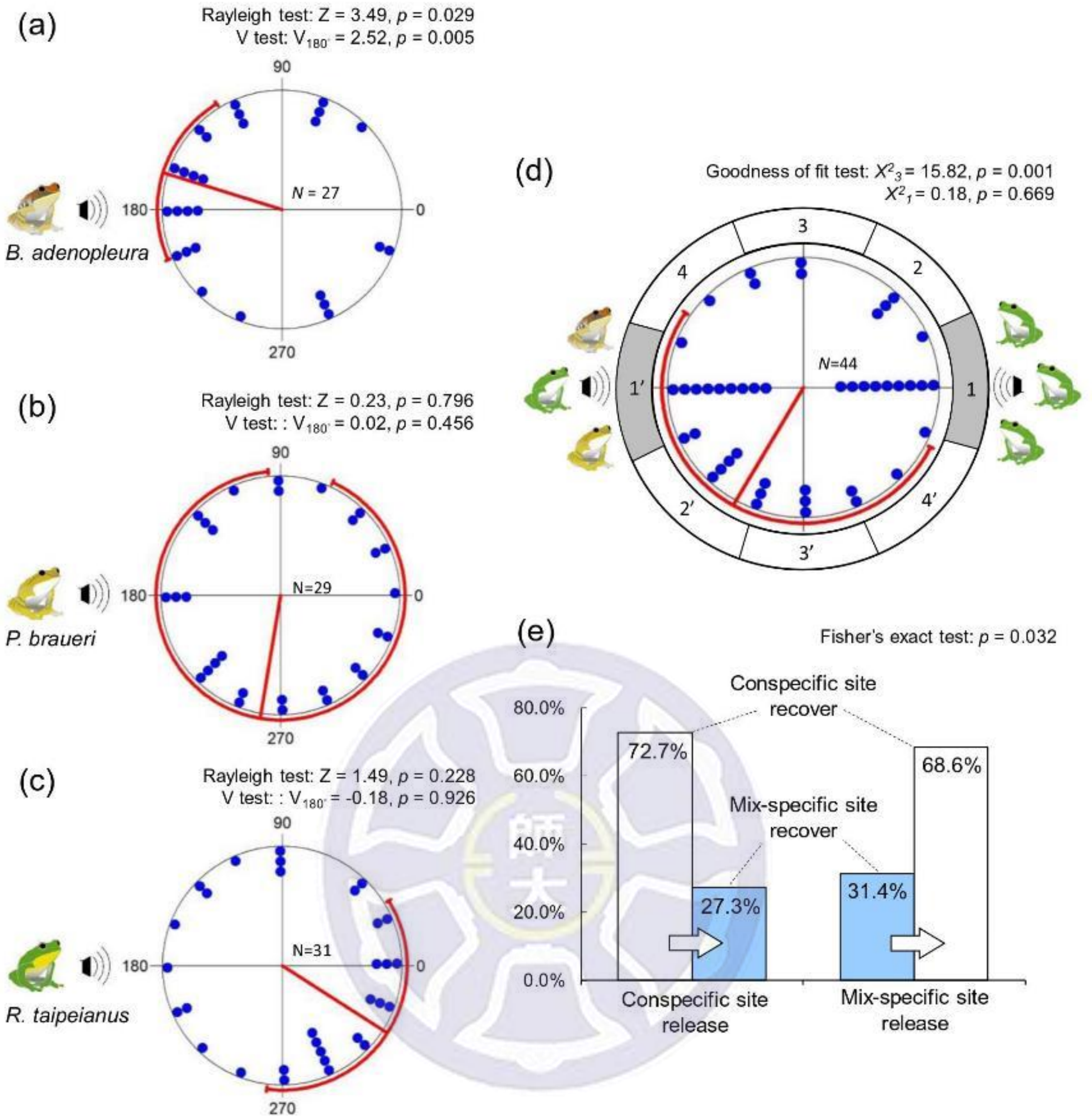

Fig. 3 Heterospecific experiments for aggregating behavior of male *Rhacophorus prasinatus*. The blue dots represent the approach angles of frogs, and the red line represents the mean angle with 95% confidence interval. **a**–**c** Experiment II-1: the males showed a significant tendency to approach *B. adenopleura* (resource noncompetitor) but not *P. braueri* (resource competitor) and *R. taipeianus* (noncompetitor with temporal partition). **d** Experiment II-2: males aggregated to the signals in both directions but did not show a significant difference between conspecific and mixed-specific choruses. **e** Experiment II-3: most males (72.2%) released in conspecific choruses stayed in the original site, while most of those released in mixed-specific choruses (68.6%) left

for most choral frog species (Ryan et al. 1981; Murphy 2003; Bee 2007).

In a polyandrous mating system like that in *R. prasinatus*, choruses contain a group of males with various qualities (Lucas et al. 1996; Friedl and Klump 2005). In this kind of system, low-quality males can sneak into the spawning group or intercept a female who has been attracted by a high-quality male (Lucas et al. 1996). Therefore, the ability to recognize high-quality males may increase the fitness of low-quality males. The results of our study showed that low-quality males tended to approach the calls of high-quality males (Fig. 2e); these frogs could be described as "satellite males," and this behavior may be an alternative strategy depending on their own physical characteristics (Lucas et al. 1996; Humfeld et al. 2009; Brepson et al. 2012). This strategy further hints that *R. prasinatus* males might be able to evaluate themselves and their rivals. However, the weakness of the current test is that we only manipulated the presence or absence of low-frequency B notes, which oversimplified the evaluation process. Other clues in the acoustic signals, such as dominant frequency, call rate, call length, and the attendance of calling days, have also been reported as indicators of male quality

(Toledo et al. 2014). Therefore, additional experiments are required to quantify these factors and further confirm the fitness of low-quality males when playing the role as a satellite male.

Heterospecific calls as supplementary clues

When conspecific calls were absent, male *R. prasinatus* frogs approached the calls of *B. adenopleura* (resource noncompetitor, Fig. 3a), but not to *P. braueri* (resource competitor, Fig. 3b). This behavior indicated that the calls of noncompetitors could serve as clues for breeding site allocation. The calls of heterospecifics may serve various functions in other species, including locating suitable habitats, avoiding predators, and identifying competitors (Magrath et al. 2015). For anurans, aggregating to limited breeding sites makes males face strong competitive pressure from conspecific and heterospecific individuals. Such competitive interactions are associated with access to food, calling space, ovipositional site, and the frequency channel of calls (Mullet et al. 2017). Therefore, avoiding choruses with heterospecific competitors, such as *P. braueri* in this study, may reduce competition (Miller et al. 2013). Furthermore, *R. prasinatus* showed no significant response toward *R. taipeianus* (Fig. 3c). This could be explained by two alternative possibilities: (1) the breeding ground of *R. taipeianus* (usually in muddy caves underground near the water) does not represent a suitable habitat for *R. prasinatus*, or (2) *R. prasinatus* could not recognize the call because of their temporal partition in breeding season.

The last two parts of this study, experiments II-2 and II-3, represented a real situation in the wild. In experiment II-2, the mixed-specific calls were comprised of *R. prasinatus*, *B. adenopleura* (resource noncompetitor), and *P. braueri* (resource competitor) calls since we aimed to simulate the real situation in the wild. Male *R. prasinatus* in this test showed significant phonotaxis preference to both mixed-specific and conspecific choruses, but they did not show significant bias between these two ($X^2{}_1 = 0.18$, $p = 0.67$). This result indicates that when conspecific calls exist, *R. prasinatus* is attracted regardless of interference from heterospecific individuals. Nevertheless, the preliminary decision could be corrected by movements among the patches, as shown in experiment II-3 (Fig. 3e). Recapture data from the real situation in the wild indicated that a large proportion of males (72.7%) left the mixed-specific chorus, and a large proportion of males (68.6%) remained in the conspecific chorus. This result agreed with our prediction that high interspecific competition may force males to make alternative choices.

Just as in the case of conspecific aggregation behavior, there are several other factors that should be taken into consideration here. One is when the heterospecific call is regarded as background noise, which is known to interfere with or even cover acoustic signals of their same kind (Gerhardt and Klump 1988; Velez and Bee 2010; Halfwerk et al. 2016). Most anuran species are characterized by their unique dominant frequency in advertisement calls, where both males and females have evolved to represent the highest sensitivity toward their own frequency (Gerhardt and Huber 2002; Amézquita et al. 2011; Mullet et al. 2017). If strong overlapping (either temporal or in frequency) occurred among these species, the frogs would have had to overcome this effect by increasing energy output, increasing the signal duration, or switching communication channels (Wong et al. 2009; Luther and Gentry 2013; Bleach et al. 2015). All these responses could be energetically costly and would not guarantee mating success. The phenomenon we observed in experiment II-3 might be a compromise between mating success and interference from other frogs; this will be more thoroughly studied in our future research.

In conclusion, the results of the current study confirmed both chorus attraction hypothesis and heterospecific attraction hypothesis. In conspecific tests, we conclude that male frogs could discriminate the components of chorus signals, and might have the ability to evaluate themselves and their rivals. In heterospecific tests, we conclude that the male frogs could utilize heterospecific cues, especially those from sympatric noncompetitors, as information for locating breeding sites. The results of this study help to clarify the potential interactions of frogs at both within-species and between-species levels and further explain the formation of anuran aggregations coexisting around the limited water resources.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All the treatment procedures in this study followed the animal use protocols approved by Institutional Animal Care and Use Committee (IACUC), National Taiwan Normal University (license no. 107023), and Chinese Culture University (CCU-IACUC-104008). All these protocols were further reviewed and certificated by the Forest Bureau, Council of Agriculture, Taiwan (license no. 1041701403-105-002; no. 1061700773-106-026) and were performed in accordance with the local law of the Wildlife Conservation Act in Taiwan.

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Appendix B

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BMC Microbiology

RESEARCH ARTICLE

Open Access

Changes of diet and dominant intestinal microbes in farmland frogs

Chun-Wen Chang[1,2†], Bing-Hong Huang[1†], Si-Min Lin[1], Chia-Lung Huang[1] and Pei-Chun Liao[1*]

Abstract

Background: Agricultural activities inevitably result in anthropogenic interference with natural habitats. The diet and the gut microbiota of farmland wildlife can be altered due to the changes in food webs within agricultural ecosystems. In this work, we compared the diet and intestinal microbiota of the frog *Fejervarya limnocharis* in natural and farmland habitats in order to understand how custom farming affects the health of in vivo microbial ecosystems.

Results: The occurrence, abundance, and the numbers of prey categories of stomach content were significantly different between the frogs inhabiting natural and farmland habitats. In addition, differences in the abundance, species richness, and alpha-diversity of intestinal microbial communities were also statistically significant. The microbial composition, and particularly the composition of dominant microbes living in intestines, indicated that the land use practices might be one of factors affecting the gut microbial community composition. Although the first three dominant microbial phyla Bacteroidetes, Firmicutes, and Proteobacteria found in the intestines of frogs were classified as generalists among habitats, the most dominant gut bacterial phylum Bacteroidetes in natural environments was replaced by the microbial phylum Firmicutes in farmland frogs. Increased intestinal microbial richness of the farmland frogs, which is mostly contributed by numerous microbial species of Proteobacteria, Actinobacteria, Acidobacteria, and Planctomycetes, not only reflects the possible shifts in microbial community composition through the alteration of external ecosystem, but also indicates the higher risk of invasion by disease-related microbes.

Conclusions: This study indicates that anthropogenic activities, such as the custom farming, have not only affected the food resources of frogs, but also influenced the health and in vivo microbial ecosystem of wildlife.

Keywords: Abundance, Custom farming, Diet, *Fejervarya limnocharis*, Gut microbiota, Richness

Background

The gastrointestinal tract is the primary site where microorganisms interact with the host species. The gastrointestinal microbiota maintains the functions of nutrient, immune, and development regulation and is important for host health [1–4]. The gut microbiota is commonly influenced by the host diet [3, 5]. The composition of the intestinal microbial community is suggested to result from natural selection operating at the host level to stabilize the gut environment and at the microbial level to promote functional specialization [6]. The relative abundance of symbionts and pathogenic microbes reflects the health status of the host species [7]. Microbial interactions, e.g., resource competition, represent a deterministic factor for the dominance of the gastrointestinal microbial community [8]. Diets serve as a source of gut microbes, which are further selected by the gastrointestinal environment.

The gastrointestinal microbes are composed of autochthonous components (residents) and allochthonous members (hitchhikers from ingested food and waters). Species composition of the gastrointestinal microbial community is different from that of environmental microbes [9]. Firmicutes and Bacteroidetes are the most abundant gastrointestinal microbial phyla [10–14] that were suggested to descend from the early colonists in mammalian gut evolution [6], while Proteobacteria was found to be most dominant in tadpoles [14], house sparrow [15], and fish [16]. The codominance of these phyla was suggested to be a consequence of niche partitioning and metabolic complementation [6]. A healthy gut

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microbial community occupied by native bacteria could establish a selective environment to prevent emerging pathogens from building up a necessary population size to cause disease. The native microbes can prevent other similar taxa for colonization by high density blocking, and this effect is like the "founder-takes-all" effect of the field of population ecology [17]. However, such "founder-takes-all" effect [17] could be vanished if fast colonization by abundant allochthonous microbes [18]. Here, host immunity not only plays a role as a selective pressure on intestinal microbes but also an object of natural selection by the emerging microbes.

Since the importance of relationships between the food web complexity and species composition in an ecosystem, and between the diet content and intestinal microbiome is emphasized in literature [19], we aimed to explore whether the change in ecosystems as a result of agricultural activities alters the intestinal microbial composition of wildlife. Agricultural activities affect the distribution of wildlife in the natural environment. Farmland biodiversity is typically lower than that in natural fields, which is reflected in a widespread decline of species richness and/or abundance of farmland wildlife [20–23]. Custom farming and agricultural intensification that rely on the use of fertilizers and pesticides recurrently create selective pressures not only on vertebrates, but also on invertebrates [24, 25], plants [26, 27], and soil microbes [28, 29]. The change in land use alters the nutrient cycling of soil, which affects the diversity and abundances of numerous environmentally important genes of microbes [30]. Changes in nutrient cycling affect the food webs of ecosystems [31], and changes in heterotrophic processes and diet diversity can further alter the digestive-tract microbiome of animals [32].

In this study, we used the rice frog *Fejervarya limnocharis* (Gravenhorst, 1829) (Amphibia, Anura, Ranidae) as a system to compare the food composition (diet) and intestinal microbiota between natural and farmland frog populations. *Fejervarya limnocharis* is widespread in East, Southeast, and South Asia. In Taiwan, it is one of the dominant amphibian species in lowland areas and is commonly found in farmlands. The Taiwanese *F. limnocharis* is phylogenetically inferred as a member of the core *F. limnocharis* group based on mtDNA and allozyme evidence [33, 34]. In this study, we profiled the diet and intestinal microbial composition of *F. limnocharis* in natural and farmland habitats. Comparisons of the stomach contents and dominant intestinal microbes were made to understand the changes of intestinal microbiota under the selective pressure of environmental change, such as the habitat variation due to agricultural activities. The multiple comparisons allowed us to address three questions: (1) Is there any dietary difference between frogs living in different habitats? (2) Does the intestinal bacterial diversity differ within and between frogs living in different habitats? (3) Does the compositional change in intestinal microbiota reflect the health of the *in vivo* microbial ecosystem in farmland frogs?

Results

Diet differentiation between habitats

A total of 26 *F. limnocharis* individuals (65 %, $n = 40$), of which 17 originated from natural habitats and 9 from farmlands, had stomach contents. A total of 63 individual prey items were identified to 12 orders (Table 1). The prey consumption rate was significantly different between the two sites ($\chi^2 = 7.03$; $P = 0.008$). In the natural habitat, Hymenoptera had the highest index of relative importance (*IRI*) score (1938.05), followed by Orthoptera (459.68). In farmlands, the highest and the second highest *IRI* scores were 2131.69 and 1310.41 in Orthoptera and Coleoptera, respectively (Table 1). The abundance and number of categories of stomach contents were significantly higher in natural habitat than that in farmlands ($Z = -2$, $P = 0.045$ and $Z = -2$, $P = 0.036$, respectively; Table 2), but the prey volume was not significantly different between habitats ($Z = -1.53$, $P = 0.12$; Table 2).

Sequencing depth and alpha-diversity of intestinal microbiota

The gut microbes of frog samples from the natural environment (individual labeled with N1 ~ N3) and farmland (individual labeled with F1 ~ F3) were used for exploring the influence of land use practices on intestinal microbial composition. We generated a microbial 16S rRNA dataset consisting of 115,580 filtered high quality, classifiable sequence reads from 133,819 raw sequence reads in total (86.37 %), with a mean number of sequences per frog sample 19,263 ± 6868 (85.42 ± 6.83 %) (Additional file 1: Table S1). The total number of microbial species (OTUs) of intestinal communities characterized using a criterion of >97 % sequence similarity was 1463, with an average length of 496 bps per sequence. The average number of OTUs of each intestinal community was 592 ± 220, ranging from 291 (sample N2) to 1011 (sample F2). The average coverage was 0.733 ± 0.061, ranging from 0.619 (sample F3) to 0.799 (sample N3). The rarefaction analysis indicated that the sequence samplings mostly reached the plateau phase, particularly for farmland frogs (Fig. 1). The microbial community richness and diversity were inferred based on the OTUs characterized using the Abundance Coverage-based Estimator (ACE) and Chao1 indices, which are nonparametric species richness estimator accounting rare species. The ACE of the intestinal microbiota of frogs from natural and farmland populations was 508.696 ± 114.491 and 1006.373 ± 250.663, respectively (significant difference in *t*-test, $t = 7.503$, df = 19, $p = 4.29\text{e-}07$, $p = 2.00\text{e-}05$ under

Table 1 Stomach contents of *F. limnocharis* in natural habitat and farmlands

	Natural habitat				Farmlands			
Prey category	*N*	%*F*	%*V*	*IRI*	*N*	%*F*	%*V*	*IRI*
Insecta								
Orthoptera	8	6.89	50.39	459.68	4	33.33	35.39	2131.69
Hymenoptera	27	31.03	7.36	1938.05	4	16.66	3.99	542.5
Coleoptera	3	6.89	7.21	91.83	3	25	30.99	1310.41
Blattaria	2	6.89	12.98	117.53	0	0	0	0
Hemiptera	1	3.44	0.09	7.34	0	0	0	0
Lepidoptera (Larvae)	2	6.89	0.9	34.29	0	0	0	0
Dermaptera	0	0	0	0	1	8.33	6.5	113.61
Chilopoda								
Scolopendromorpha	0	0	0	0	1	8.33	14.61	181.24
Malacostraca								
Isopoda	1	3.44	1.65	12.71	0	0	0	0
Arachnida								
Araneae	2	6.89	3.12	49.64	0	0	0	0
Gastropoda								
Stylommatophora	2	6.89	2.66	46.48	1	8.33	8.53	130.51
Oligochaeta	1	3.44	13.64	53.95	0	0	0	0
Total	49				14			

N number of prey, %*F* percentage of frequency of each prey item, %*V* percentage of prey volume, *IRI* index of relative importance

99,999 times permutation), whereas the Chao1 index was 522.050 ± 116.238 and 1006.272 ± 268.590, respectively (significant difference in *t*-test, *t* = 3.972, df = 19, *p* = 0.0008, *p* = 0.0009 under 99,999 times permutation). The intestinal microbial community diversities in frogs from both natural and farmland populations were significantly different in terms of Shannon index (585.711 ± 116.074 and 1105.978 ± 289.945, respectively, *t* = 4.559, df = 19, *p* = 0.0002, *p* = 0.0003 under 99,999 times permutation) but not significantly different in Simpson index (3.977 ± 0.660 and 4.438 ± 1.030 respectively, *t* = 0.9947, df = 19, *p* = 0.3324, *p* = 0.3297 under 99,999 times permutation). The detailed estimates of alpha-diversity are shown in Table 3.

Dominant intestinal microbial taxa in rice frogs

The abundance and richness are significantly different between intestinal microbial species richness of *F. limnocharis* from natural and farmland population. The microbes of *F. limnocharis* mainly belonged to the phyla Bacteroidetes, Firmicutes, and Proteobacteria: 35.43, 33.06, and 24.08 % in natural population, and 19.51, 46.02, and 25.22 % in farmland population, respectively (Fig. 2b). Figure 2b showed an obvious increase of abundance in Firmicutes and decrease in Bacteroidetes in farmland frogs compared with those in natural habitats. The three highest-richness phyla of intestinal microbes are Firmicutes, Bacteroidetes, and Proteobacteria (400 ± 60, 153 ± 21, and 79 ± 4 species in natural population; 511 ± 65, 207 ± 28, and 232 ± 51 species in farmland population, respectively, Fig. 2c). Obvious increase richness in Proteobacteria was shown in farmland frogs (Fig. 2c). We compared the identity and differences in microbial taxa between frog samples and between different habitats and found that the farmland frogs were composed of roughly 10 times more unique microbial

Table 2 Comparison of stomach contents of rice frog (*Fejervarya limnocharis*) between two habitats by Wilcoxon rank-sum test

		Mean	Std. Dev	Median	Range	Z value	*P*
Number of prey item	Natural habitat	2.82	2.48	2	1 ~ 10	−2	0.045
	Farmlands	1.33	0.71	1	1 ~ 3		
Number of prey category	Natural habitat	1.76	0.9	2	1 ~ 4	−2	0.036
	Farmlands	1.11	0.33	1	1 ~ 2		
Volume of prey(mm^3)	Natural habitat	134.1	140.04	71.2	2.09 ~ 396.05	−1.53	0.12
	Farmlands	38.77	26.2	32.98	0.78 ~ 78.54		

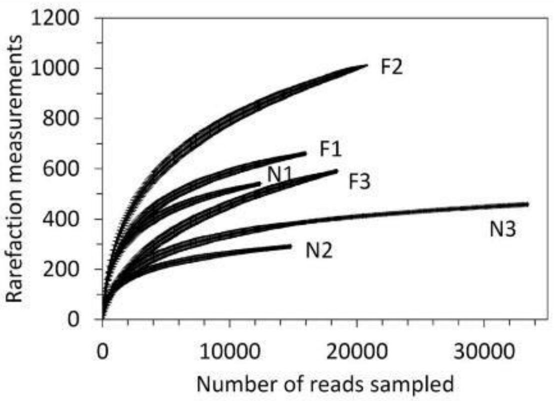


Fig. 1 Rarefaction curves for the intestinal microbial communities of rice frogs in natural habitats (N1 ~ N3) and farmland (F1 ~ F3) at a difference level of 3 %

taxa than the frogs from natural habitat, particularly in the sample F2 (Fig. 3, Additional files 2 and 3: Figure S1 and S2). In addition, there are several farmland-specific clades in Neighbor-Joining (NJ) trees of Proteobacteria, Actinobacteria, Acidobacteria, and Planctomycetes (Fig. 3), indicating that the farmland environments have created special ecological niches for these microbial groups; in contrast, most intestinal microbes of the natural population are commonly found in farmland frogs, indicating that these microbial species retain their ancestral traits. Most of the dominant microbes in frogs from natural habitats were common in farmland frogs. In contrast, many dominant microorganisms in farmland frogs were not found in frogs from natural habitats (Fig. 3, Additional file 1: Table S2). This comparison suggests a replacement of dominant intestinal microbes in farmland frogs. The phylogenetic analysis also showed consistent results that the main composition of intestinal microbes of the rice frogs was different between the natural and farmland habitats. The phylogenetic grouping of the top 10 microbes indicated an admixture of dominant microbes collected from the same habitat, but represented apparent sorting of microbes in different habitats. Taxa in the phyla Bacteroidetes and Firmicutes dominated the guts of frogs from the natural population (N1 ~ N3), whereas taxa from the phyla Proteobacteria and Firmicutes dominated the intestines of the farmland frogs (Fig. 4).

Classification of gut bacteria

Intestinal microbial organisms were classified into three categories—generalists, specialists, and too rare—based on the multinomial species classification method. Eight phyla were habitat generalists (Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetae, Lentisphaerae, Deferribacteres, Cyanobacteria, and Planctomycetes), ten were farmland specialists (Fusobacteria, Actinobacteria, Verrucomicrobia, Deinococcus-Thermus, Acidobacteria, Elusimicrobia, Synergistetes, Chloroflexi, Gemmatimonadetes, and one unclassified phylum), one was a natural-habitat specialist (Tenericutes), and ten phyla were too rare to be identified (Additional file 1: Table S3 and Additional file 4: Figure S3). At the species level, we found that 44 (8.1 %), 34 (6.3 %), 82 (15.2 %), and 381 (70.4 %) OTUs belonged to the generalist, natural-habitat specialist, farmland specialist, and too-rare types, respectively (Additional file 1: Table S4 and Additional file 4: Figure S3). This classification indicated that relatively low proportions of common gastrointestinal bacteria are found in both natural and farmland habitats and that approximately one-fifth of the bacteria differentiate the gut microbiota between habitats, while most microorganisms are rare in these habitats. We also performed pairwise comparisons of the gut microbial community of different host individuals and found a relatively high proportion of generalists and a lower proportion of specialists in gut bacterial communities of frogs within habitats (generalists: 13.97 % ± 1.59 %; specialists: 26.62 % ± 2.98 %) than those seen for frogs between habitats (generalists: 9.67 % ± 0.99 %; specialists: 30.83 % ± 1.28 %). However, these differences were not statistically significant, indicating that the individual effect cannot be neglected while explaining gut microbial diversity (Additional file 5: Figure S4).

Table 3 Alpha-diversity of intestinal microbiota of rice frogs (*Fejervarya limnocharis*) at the natural habitat (N1 ~ N3) and farmland (F1 ~ F3)

Sample	OTUs	Coverage	Community richness		Community diversity	
			ACE (95 % CI)	Chao1 (95 % CI)	Shannon index (95 % CI)	Simpson index (95 % CI)
N1	540	0.774	640.451 (609.752, 684.662)	653.554 (611.576, 720.152)	720.152 (4.850, 4.907)	4.907 (0.022, 0.025)
N2	291	0.753	361.315 (334.879, 403.679)	370.875 (334.723, 436.919)	436.919 (3.522, 3.583)	3.583 (0.085, 0.091)
N3	458	0.799	524.321 (501.485, 559.152)	541.720 (507.338, 600.063)	600.063 (3.398, 3.442)	3.442 (0.100, 0.104)
F1	660	0.765	797.248 (759.731, 848.877)	794.101 (749.071, 861.895)	861.895 (4.950, 5.001)	5.001 (0.018, 0.019)
F2	1011	0.687	1358.822 (1287.073, 1449.218)	1385.211 (1289.752, 1513.360)	1513.360 (5.275, 5.320)	5.320 (0.013, 0.014)
F3	590	0.619	863.048 (797.642, 949.057)	839.505 (766.514, 942.678)	942.678 (2.928, 2.993)	2.993 (0.148, 0.155)

ACE abundance coverage-based estimator

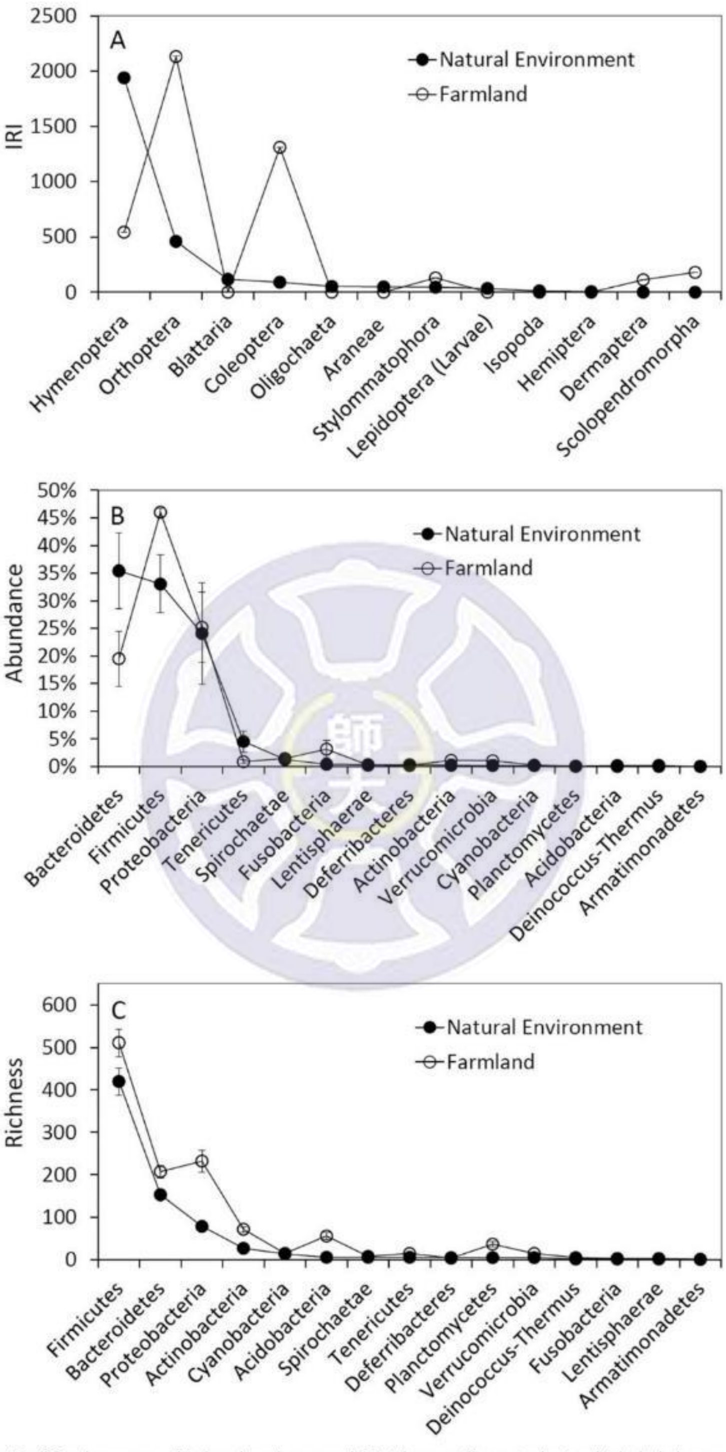


Fig. 2 Comparisons of (**a**) the *IRI* of food contents, (**b**) the abundance and (**c**) richness of intestinal microbial phyla between frogs of the natural habitats and farmlands

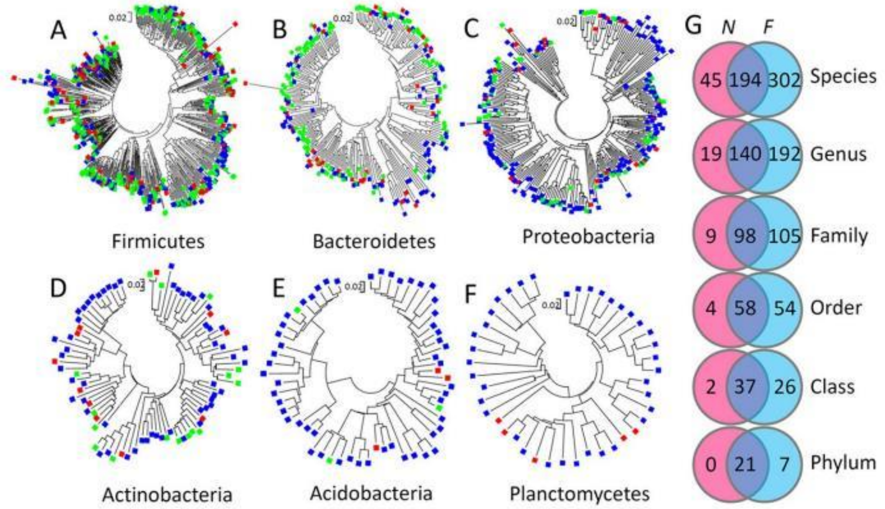


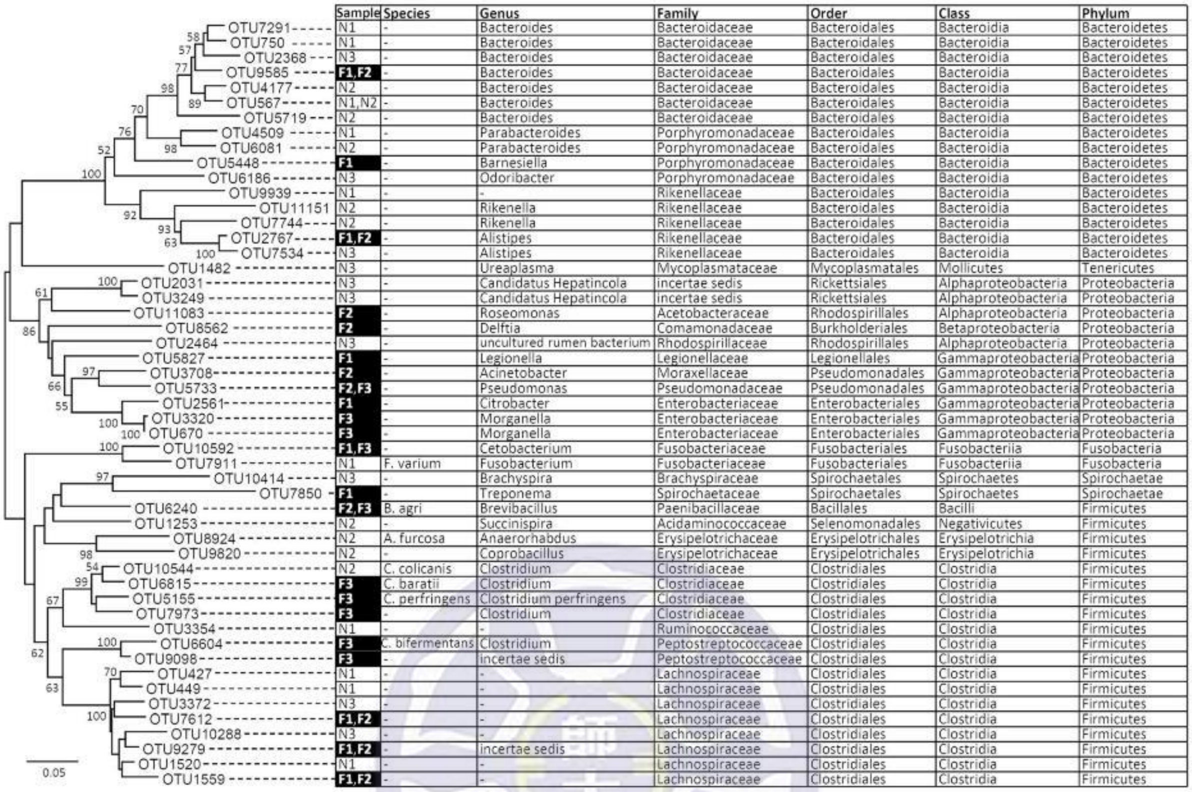
Fig. 3 Microbial composition revealed in neighbor-joining trees and Venn diagram. **a–f** The phylogenetic analyses revealed that the microbes of phyla Bacteroidetes and Firmicutes are mostly found in both habitats, while several microbes of phyla Proteobacteria, Acidobacteria, Planctomycetes, and Actinobacteria are only found in farmland frogs. This result suggests that the special intestinal environment leads assemblage of unique microbes with higher phylogenetic niche conservatism in farmland frogs. The red, blue, and green dots indicate the microbes found in frogs of natural population only, farmland population only, and both natural and farmland populations, respectively. **g** Venn diagram representing the species diversity of the intestinal microbial taxa in frog intestines. The numbers in circles represent the number of taxa. As can be seen, a higher number of unique microbial taxa were found in farmland frogs (for which the individual F2 made the highest contribution). These results revealed that the anthropogenic interference have altered the microbial phylogenetic distribution and species composition

Discussion

The differences in stomach contents of rice frogs may reflect the changes in faunal species composition. There were long-term anthropogenic disturbance such as the use of fertilizers and pesticides in custom farming. These anthropogenic activities may alter the faunal species composition in farming habitat although the invertebrate fauna of both natural and farmlands were not directly investigated. The diet analysis showed that the food volume of rice frogs was not significantly different between the natural and farmland habitats, but the stomach content (abundance and number of prey categories) in farmland frogs showed lower diversity than that in frogs from natural habitats. Such dietary alteration is probably ascribed to agricultural activities. The increase of soil life (700 % increase of megafauna, 2500 % increase of nematodes, and 70 % increase of soil microorganisms) in 14-year of monitoring conservation agriculture and organic farming indicated the harmful effects of pesticides and fertilizers on the terrestrial ecosystem [35]. In this study, the decreased number of prey categories in the guts of farmland frogs did not only point to the presence of simplified invertebrate and plant communities in farmlands and the decreased *IRI* of Hymenoptera (Fig. 2a), but also reflected the reduction of pollinator demand in the plant community [36]. In contrast, the increase in the *IRI* of Orthoptera (Fig. 2a) often suggested an increase in monocot abundance, particularly of the Poaceae [37–39]. The dietary analysis suggested the occurrence of dramatic changes in the farmland ecosystem due to agricultural activities.

Conventional agriculture or custom farming may strongly alter the species composition in many ways [35]. For example, habitat destruction during farming may reduce diet resources and subsequently alter gastrointestinal microbiome composition in the howler monkey (*Alouatta pigra*) [40]. Habitat degradation following temperature change [41] and water pollution [42] can also subsequently alter amphibian gut microbiota. Research on the microbial composition surrounding the forest soils revealed similar dominant microbes among different forest types with slight difference in microbiota [43]. This is similar to our findings that the dominant gut bacteria are habitat generalists and most gut microbes belong to the "too-rare" type bacteria. However, we found that approximately one-fifth of the gut bacteria were habitat specialists (Additional file 1: Table S3 and S4), indicating that the varied habitat indeed altered the gut microbial composition.

Our results suggest that the estimated intestinal microbial species richness in frog guts varied within the same order of magnitude as that in the human gut, as estimated based on fecal analysis [10]. The gut microbiota is mainly determined by the environmental conditions where the host species reside [44]. Although relatively few frog samples were chosen to represent the



	Sample	Species	Genus	Family	Order	Class	Phylum
OTU7291	N1	-	Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU750	N1	-	Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU2368	N3	-	Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU9585	F1,F2	-	Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU4177	N2	-	Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU567	N1,N2	-	Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU5719	N2	-	Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU4509	N1	-	Parabacteroides	Porphyromonadaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU6081	N2	-	Parabacteroides	Porphyromonadaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU5448	F1	-	Barnesiella	Porphyromonadaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU6186	N3	-	Odoribacter	Porphyromonadaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU9939	N1	-	-	Rikenellaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU11151	N2	-	Rikenella	Rikenellaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU7744	N2	-	Rikenella	Rikenellaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU2767	F1,F2	-	Alistipes	Rikenellaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU7534	N3	-	Alistipes	Rikenellaceae	Bacteroidales	Bacteroidia	Bacteroidetes
OTU1482	N3	-	Ureaplasma	Mycoplasmataceae	Mycoplasmatales	Mollicutes	Tenericutes
OTU2031	N3	-	Candidatus Hepatincola	incertae sedis	Rickettsiales	Alphaproteobacteria	Proteobacteria
OTU3249	N3	-	Candidatus Hepatincola	incertae sedis	Rickettsiales	Alphaproteobacteria	Proteobacteria
OTU11083	F2	-	Roseomonas	Acetobacteraceae	Rhodospirillales	Alphaproteobacteria	Proteobacteria
OTU8562	F2	-	Delftia	Comamonadaceae	Burkholderiales	Betaproteobacteria	Proteobacteria
OTU2464	N3	-	uncultured rumen bacterium	Rhodospirillaceae	Rhodospirillales	Alphaproteobacteria	Proteobacteria
OTU5827	F1	-	Legionella	Legionellaceae	Legionellales	Gammaproteobacteria	Proteobacteria
OTU3708	F2	-	Acinetobacter	Moraxellaceae	Pseudomonadales	Gammaproteobacteria	Proteobacteria
OTU5733	F2,F3	-	Pseudomonas	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria	Proteobacteria
OTU2561	F1	-	Citrobacter	Enterobacteriaceae	Enterobacteriales	Gammaproteobacteria	Proteobacteria
OTU3320	F3	-	Morganella	Enterobacteriaceae	Enterobacteriales	Gammaproteobacteria	Proteobacteria
OTU670	F3	-	Morganella	Enterobacteriaceae	Enterobacteriales	Gammaproteobacteria	Proteobacteria
OTU10592	F1,F3	-	Cetobacterium	Fusobacteriaceae	Fusobacteriales	Fusobacteriia	Fusobacteria
OTU7911	N1	F. varium	Fusobacterium	Fusobacteriaceae	Fusobacteriales	Fusobacteriia	Fusobacteria
OTU10414	N3	-	Brachyspira	Brachyspiraceae	Spirochaetales	Spirochaetes	Spirochaetae
OTU7850	F1	-	Treponema	Spirochaetaceae	Spirochaetales	Spirochaetes	Spirochaetae
OTU6240	F2,F3	B. agri	Brevibacillus	Paenibacillaceae	Bacillales	Bacilli	Firmicutes
OTU1253	N2	-	Succinispira	Acidaminococcaceae	Selenomonadales	Negativicutes	Firmicutes
OTU8924	N2	A. furcosa	Anaerorhabdus	Erysipelotrichaceae	Erysipelotrichales	Erysipelotrichia	Firmicutes
OTU9820	N2	-	Coprobacillus	Erysipelotrichaceae	Erysipelotrichales	Erysipelotrichia	Firmicutes
OTU10544	N2	C. colicanis	Clostridium	Clostridiaceae	Clostridiales	Clostridia	Firmicutes
OTU6815	F3	C. baratii	Clostridium	Clostridiaceae	Clostridiales	Clostridia	Firmicutes
OTU5155	F3	C. perfringens	Clostridium perfringens	Clostridiaceae	Clostridiales	Clostridia	Firmicutes
OTU7973	F3	-	Clostridium	Clostridiaceae	Clostridiales	Clostridia	Firmicutes
OTU3354	N1	-	-	Ruminococcaceae	Clostridiales	Clostridia	Firmicutes
OTU6604	F3	C. bifermentans	Clostridium	Peptostreptococcaceae	Clostridiales	Clostridia	Firmicutes
OTU9098	F3	-	incertae sedis	Peptostreptococcaceae	Clostridiales	Clostridia	Firmicutes
OTU427	N1	-	-	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes
OTU449	N1	-	-	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes
OTU3372	N3	-	-	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes
OTU7612	F1,F2	-	-	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes
OTU10288	N3	-	-	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes
OTU9279	F1,F2	-	incertae sedis	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes
OTU1520	N1	-	-	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes
OTU1559	F1,F2	-	-	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes

Fig. 4 Neighbor-joining analysis for the top 10 species of intestinal microbes of rice frog in natural (N1 ~ N3) and farmland (F1 ~ F3) field ecosystems using the 16S rRNA gene. Taxonomic hierarchies of the identified microorganisms are listed after the NJ tree. The symbol "-" indicates unclassified

gut microbiota of rice frogs, the gut environments of different frog individuals from the same habitat may be similar because frogs from the same habitat might share similar niches. The digestive fluid secreted from some specialized cells in the epithelium of the small intestine, which maintains a stable environment in the gut [45, 46]. Therefore, the dominant gut bacteria could be similar among frogs with similar habitats because the coexistence of these dominant bacteria reflects the consequence of long-term selection in the gut environment. We also found that both dominant gut bacterial species in the natural habitat (Candidatus Hepatincola, uncultured α-Proteobacterium, total of 21.02 %) and farmland habitat (*Morganella* sp., total of 10.95 %) were habitat specialists, indicating that the external environment plays a major role in the gut-dominant bacteria in frogs.

On comparing the microbial species composition, a certain proportion of common microbial taxa (approximately 1/3 ~ 1/2) was similar between frogs from natural habitats and farmlands, revealing the host species specificity of microbes (Additional file 3: Figure S2). The same pattern of similar taxa in different microbial communities of diet-differentiated, geographically distant host populations was also found in wild and laboratory *Drosophila* [47] and *Cylindroiulus fulviceps* (Diplopoda) with different feeding treatments [48]. However, the higher proportion of bacterial habitat specialists than habitat generalists suggested that external factors such as diet, geographic variation, and the environment still play major roles in determining the gut microbial composition (Additional file 4: Figure S3). Differences in dominant taxa, revealed by different clusters in phylogenetic analysis (Fig. 4), suggested strong selectivity of the microbial community in the gut environment [49].

The microbial abundances between frogs of natural habitat and farmland are different, especially in Bacteroidetes and Firmicutes. These two phyla are the most prevalent bacteria in digestive tracts of terrestrial animals [50, 51]. In natural population, Bacteroidetes are the mostly abundant microbes in rice frogs' intestines followed by Firmicutes, but the abundance of Bacteroidetes is lower than Firmicutes in farmland population (Fig. 2b). The Bacteroidetes of the frogs of natural habitats were mostly composed of the order Bacteroidales (Fig. 4). Bacteroidales are known as symbiotic bacteria essential for the digestive activity of several organisms

[1, 16, 25, 48, 52, 53]. However, the abundance of Bacteroidetes decreased in farmland frogs and was replaced by Firmicutes (Fig. 2b). Higher Firmicutes-to-Bacteroidetes ratio could improve the efficiency of calories uptake from food [54]. These Firmicutes microbes were almost composed of Ruminococcaceae and Lachnospiraceae, which digest cellulose and ferment glucose and xylose to obtain nutrients and are the prevalent bacterial families in herbivore's digestive system [55]. The ecological meaning of the increased abundance of Ruminococcaceae and Lachnospiraceae in farmland frogs' intestine is unknown yet, but the high Firmicutes-to-Bacteroidetes ratio suggests that the alteration of food composition might change the intestinal environments and microbial community in farmland frogs.

The differences in dominant intestinal microbial taxa between natural habitats and farmlands revealed in NJ analysis indicated that the frogs acquired different bacteria due to the changes in environmental conditions. In contrast to the high frequency of common microbial taxa in frogs from natural habitats, the intestinal microbial communities of farmland frogs were characterized by higher ratios of unique microbes (Additional files 2 and 3: Figure S1 and S2). These unique microbes mostly belonged to Proteobacteria, Actinobacteria, Acidobacteria, and Planctomycetes (Fig. 3). From the analysis of the top 10 microbial organisms of every frog intestinal samples, we found that the Classes of γ-Proteobacteria in farmland frogs have an obvious increase in abundance (19.75 %) than the frogs of natural habitats (0.05 %). Although γ-Proteobacteria is common in guts of diverse taxa including amphibians [e.g., House Sparrow (*Passer domesticus*) [15], leopard frog (*Lithobates pipiens*) [14]], most of the γ-Proteobacteria in farmland frogs belong to the order Enterobacteriales (72.91 %) (Fig. 4). Several intestinal bacteria that are dominant in the farmland frogs, such as species in the genera *Treponema* (Spirochaetes), *Roseomonas* (α-Proteobacteria), *Clostridium* (Firmicutes) and genera *Legionella*, *Acinetobacter*, *Pseudomonas*, *Citrobacter*, *Morganella* of γ-Proteobacteria (Fig. 4), were probably infectious disease-related pathogens. These bacterial genera are clinically proven to cause emerging infectious diseases (EID) not only in humans (e.g., *Legionella* [56, 57]; *Acinetobacter* [57–59]; *Pseudomonas* [60, 61]; *Citrobacter* [62, 63]; *Morganella* [64, 65]; *Treponema* [66, 67]; *Roseomonas* [68–70], and *Clostridium* [71, 72]), but also in amphibian (for example, *Acinetobacter* would cause ulceration and necrosis in *Rhinoderma darwini* [73], *Pseudomonas* and *Citrobacter* might induce immune-response in *Rana pipiens* [74, 75], and so did the *Morganella* in *Andrias davidianus* [76].)

These bacteria mostly belong to the Proteobacteria. Several Proteobacteria have pathogenic or antipathogenic functions in amphibians, for example, *Janthinobacterium lividum* in amphibian guts can inhibit the growth of lethal amphibian fungi [77, 78]. Certain antifungal bacteria from the genus *Pseudomonas* that were discovered on the skin of amphibians, that is, the salamander (*Plethodon cinereus* and *Hemidactylium scutatum*) [79, 80] and the frog (*Rana muscosa*) [81]), were also found in leopard frogs (*Rana pipiens*) [82]). The intestines of amphibians could serve as the reservoir for these antifungal bacteria via uptake of invertebrates that have come into contact with these bacteria in the soil or by eating their own shed skin [77]. The dynamics of these bacteria may be an indicator of host susceptibility to these lethal fungi [83]. These Proteobacteria were previously found in smaller amounts than Bacteroidetes and Firmicutes in healthy adult mammals [19] and human gastrointestinal samples [6] and in the frogs from natural habitats investigated in this study.

In addition to the phylum Proteobacteria, certain bacterial genera, such as *Flavobacterium* in phylum Bacteroidetes and *Actinobacterium* and *Bacillus* in phylum Firmicutes, that are chytrid-resistant or have probiotic capabilities were mostly found in the gut of frogs in the farmland habitat (sequence reads = 21, 59, and 4 in *Flavobacterium*, *Actinobacterium*, and *Bacillus*, respectively) but rarely in the natural habitat (sequence reads = 1, 4, and 0, respectively). The higher proportion of pathogen-resistant bacteria found in farmland frogs reflected the higher occurrence rate of harmful pathogens in the farmland habitat. The intestinal microbial composition in frogs from farmlands was possibly under a high risk of infectious diseases. Because stomach content is a proxy of food source in the natural environment, the differences in intestinal microbiota between farmland and natural habitats did not only explain the dietary alteration, but also reflected the risk of disease in farmland wildlife due to the ecosystem alterations as a result of anthropogenic activities.

Intestinal microbiota can possibly reflect the state of the immune system and health of the host species [2]. Different microbial composition in frog guts may be related to pathogen resistance, for example, differential antifungal bacterium composition between populations may be important in preventing chytrid fungus infections [84]. Differentiation of gut microbial composition could even reflect overall body healthy of frogs. We found lower food diversity but higher intestinal microbial richness in farmland frogs than in natural habitats. This can possibly be explained by the recurrent selective pressures from agricultural activities simplifying the farmland fauna and increasing the risks of infectious disease in frog predators. Simplified food sources could also weaken the immunity of wild animals and lead to higher pathogen invasion [85]. Disturbance in gut microbial composition and microbial and ecological dynamics can lead to an increased risk of disease outbreaks in wildlife

[86], or provide less anti-pathogen reservoir for preventing infection of skin pathogen [84]. Anthropogenic ecosystem alteration and pathogen or vector movements via human or natural agencies could give rise to EID in wildlife [87, 88]. In this study, we showed that anthropogenic interference in ecosystem (such as agricultural activities) and a change of "vectors" (food contents) can weaken frog immunity, which is reflected in the change of intestinal microbial climax.

Conclusions

Alterations in diet and intestinal microbial composition in farmland *F. limnocharis* indicate that custom farming influences the intestinal ecosystem of wildlife. Food may not only play a resource role but also be one of the factors serve as a vector of infectious microbes. The intestinal microbial composition reflected the result of intestinal environmental selection by both extrinsic (environment, such as habitat disturbance, temperature, and food) and intrinsic (immune system) factors. In the farmland habitat, less diverse food content and more diverse intestinal microbiota in frogs were found than in natural habitats. This result suggests that the in vitro ecosystem changes in vivo ecosystem. The intestinal microbiota of *F. limnocharis* was determined by both environmental factors and host species, whereas the dominant intestinal microbes were more easily affected by external environmental conditions than were the rare microbes. The increased numbers of Proteobacteria suggested that pathogenic invasion was affected or will be affected by the weakened immunity of farmland frogs, which is probably caused by the heavy use of pesticides and fertilizers in farmland. The current study revealed the change in food resources and intestinal microbial diversity in farmland wildlife, and also suggested that outbreaks of disease-related bacteria within the gut microbial community can reflect the damage of in vivo and in vitro ecosystem health due to agricultural interference. It should be notified that the small sample size of frogs in this study may not be sufficient to draw strong conclusions, but could be indicative of changes across habitats. Even though, this research still represents different microbial composition between habitats and provides a reference for future studies regarding the amphibians' gastrointestinal microbiota.

Methods

Sampling system

Fejervarya limnocharis is a medium-sized frog with an average body length of 4.3 cm and 5.5 cm in males and females, respectively [89]. This species is widespread in lowland areas at altitudes below 1500 m in Taiwan. *F. limnocharis* generally aggregates around breeding ponds during the breeding season from spring to summer. *F. limnocharis* is tolerant to human disturbance, and therefore is commonly found in farmlands. Forty frog samples were collected from Hualin experimental forest (the natural habitat, 24°53′N, 121°33′E) and farmlands in Quchi Community (24°55′N, 121°32′E) in Xindian (Dist., New Taipei City, Taiwan) on September 27th, 2012. The distance between these two sites is less than 3 km. All frog samples were brought to the laboratory of Nation Taiwan Normal University for immediate profiling of diets and intestinal microbial composition.

Diet analyses

Frog samples were sacrificed and preserved in 70 % ethanol. Stomach content of frogs (prey) was collected via dissection and stored in 70 % ethanol. The length and width of the stomach content items were measured using cartesian papers and the prey items were identified to order level under a stereomicroscope. The volume of prey items was estimated using the formula proposed by Dunham [90]. For assessing the importance of each consumed prey category, the *IRI* was calculated based on the formula $IRI = \%O\ (\%N + \%V)$ [91], where $\%O$, $\%N$, and $\%V$ are the percentages of the occurrence, relative abundance, and measured volume of each prey category, respectively, in all stomachs. The intake rate (which describes the prey eaten by *F. limnocharis*) between habitats was compared using the Chi-square test. The numbers, categories, and volumes of stomach contents were compared between habitats using Wilcoxon rank-sum test. All statistical analyses were conducted using JMP 7.0.

Intestinal microbiota

The gut microbes of six frog samples from the natural environment (N1 ~ N3) and farmland (F1 ~ F3) were used for exploring the influence of food composition on intestinal microbial composition. Intestinal microbial metagenomic DNA was extracted based on a protocol described by Sharma et al. [92]. For each sample, we amplified the V4 hypervariable 16S rRNA region using the primer set 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′). The DNA library was constructed according to the Roche GS FLX Titanium emPCR kit (Roche Applied Science). Pyrosequencing was carried out by Welgene Biotech Co., Ltd. (Taipei, Taiwan) using a Roche 454 FLX titanium instrument and reagents following the manufacturer's instructions. The V4 sequence fragments shorter than 200 bp, without barcodes, with polyN or polyA/T, and the reads with $< Q25$ were removed. Sequences with $>97\ \%$ identity were treated as the same species and as an operational taxonomic unit (OTU). Each OTU was classified by annotating to the SILVA database. The alpha-diversity of the gut microbiota was estimated using community richness indices, i.e., the ACE [93], the Chao1 index [50, 94], and community diversity indices, i.e., the Shannon index and Simpson index. Indices ACE, Chao1, Shannon index and Simpson index between habitats were compared. Because the small sample size could result in non-normal distribution, we regenerated 20 normalized pseudodata based on the mean and standard deviation from the observed data, and performed 99,999 times permutations for a one-sample randomization test on differences of values between habitats. Validation of *t*-test was given by similar *p*-values before and after permutations. Rarefaction analysis was executed to measure how the gut microbial composition of the rice frog varied depending on the sample size.

After determination of the microbial diversity, the differences in the gut microbial composition of rice frogs between the natural and the farmland populations was examined by phylogenetic analysis. The neighbor-joining (NJ) method was used for constructing a genetic-distance tree to elucidate the genetic distribution patterns of intestinal microbes between different habitats. Nucleotide sequence alignments were performed using the Clustal W program. The evolutionary distances were computed using the *p*-distance method. The NJ tree was constructed using the program MEGA v. 5.05 [51]. The NJ relationships of six dominant microbial phyla Bacteroidetes, Firmicutes, Proteobacteria, Planctomycetes, Actinobacteria, and Acidobacteria were reconstructed separately for revealing the grouping patterns of intestinal microbes between different habitats. In addition, every top 10 microbial OTUs of frogs (yielding a total 51 OTUs) were used to construct a genetic-distance tree using the NJ method for clearly representing the systematic positions of the dominant intestinal microbes of frogs in natural habitats and farmlands, with the following settings: the composite likelihood substitution model [95], the uniform rate among sites, the heterogeneous rates among lineages and complete deletion of gaps. A 1000 bootstrap replication was used to evaluate the supporting values for lineage grouping.

We also used the multinomial species classification method (CLAM) [96] to classify the bacteria of generalists and specialists in two distinct habitats with the vegan package in R. CLAM is a kind of two-group species classification method. The supermajority rule that uses the specialization threshold value 2/3 was adopted for determining the bacteria of habitat specialists. Under the supermajority rule, the minimum abundance for classification (i.e., coverage limit) was estimated and the taxa that had abundance below the coverage limit were considered as "too rare". CLAM was also used for pairwise comparison between host individuals within habitats and between habitats. Comparisons of the proportions of generalists and specialists between "within habitats" and "between habitats" aid in understanding the degree of individual effect on gut microbial diversity.

Availability of data and materials

The 16S rDNA sequences identified in this study have been deposited in the NCBI GenBank under the Bioproject PRJNA279212 (Accession number: SRX965751). Frog samples were stored in the specimen room of Department of Life Science, National Taiwan Normal University.

Ethics statements

Protocols of this study and the animal use were reviewed and approved by the Ethics Committee of National Pingtung University of Science and Technology (NPUST) (Approved No. NPUST-101-079) and the Institutional Animal Care and Use Committee of National Taiwan Normal University (Approved No. 101024).

Additional files

Additional file 1: Table S1. Summary of sequence reads used in this study. **Table S2.** Percentages of top 10 microbial taxa of every rice frog (*Fejervarya limnocharis*) sample. **Table S3.** List of categories of the habitat generalists, specialists and too-rare types at the phylum level. **Table S4.** List of categories of the habitat generalists, specialists and too-rare types at the species level. (DOCX 129 kb)

Additional file 2: Figure S1. Venn diagram representing the species diversity of the intestinal microbial taxa in frog intestines. The numbers in circles represent the number of taxa. As can be seen, a higher number of unique microbial taxa were found in farmland frogs (for which the individual F2 made the highest contribution). (TIF 17137 kb)

Additional file 3: Figure S2. The Neighbor-joining tree reveals the phylogenetic position of the intestinal microbes of *Fejervarya limnocharis* in farmland only (blue), in natural habitats only (red), and in both environments (green). The phylogenetic analysis showed that roughly 1/3 clades of microbial species are found in the farmland frogs only. (TIF 15121 kb)

Additional file 4: Figure S3. Categories of gut bacteria between habitats classified by multinomial species classification method (CLAM). Classification in phylum level and species level are shown. Values in plots indicate the number of bacterial taxa (i.e. phyla and species) of each category. F and N in the x- and y-axes indicate the farmland and natural habitat, respectively. (TIF 156 kb)

Additional file 5: Figure S4. Pairwise comparison of gut bacterial categories of individual hosts within habitats and between habitats. All three comparisons of generalists, specialists, and too-rare type of bacterial communities within habitats and between habitats are non-significant different. (TIF 3668 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PCL conceived the study. CWC conducted the dissection experiments and BHH conducted the molecular experiments. CWC identified the food contents of frog samples. CWC, CLH and PCL analyzed the data. PCL wrote the paper. CWC, BHH, SML, and PCL critically reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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