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Variation in Southeast Asian anurans

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Variation in Southeast Asian Anurans

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in
Biology

by

Jennifer A. Sheridan

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2008

The dissertation of Jennifer A. Sheridan is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2008

DEDICATION

I dedicate this dissertation to three women in science: Dawn Lawrence, my high school biology teacher who was my first woman in science mentor; Satie Airame, my undergraduate ecology TA and one of my first employers, who showed me the magic and beauty of Southeast Asian rainforests and frogs; and Carol Horvitz, my graduate ecology professor, who taught me that theoretical and field ecology can be easily mastered with the right instructor. Each of them has helped make me the scientist I am today, and for that, and so many other reasons, I thank them.

Pag may tiyaga may nilaga

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PUBLICATIONS

Sheridan, J.A., D. Bickford, K.S. Feng, and R. Meier. An examination of call and genetic variation in three wide-ranging Southeast Asian anuran species.? *In reveiw.* Raffles Bulletin of Zoology

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ABSTRACT OF THE DISSERTATION

Variation in Southeast Asian Anurans

by

Jennifer A. Sheridan
Doctor of Philosophy in Biology
University of California, San Diego, 2008
David Holway, Chair

This dissertation reports studies of variation in Southeast Asian frog species as it affects their ability to respond to environmental change. First, using museum specimens of Bornean anurans, I found that clutch size did not increase with altitude, as expected from studies of other ectotherms. Second, a comparative study of genetic, call, and morphological data in two distant populations (Singapore and Thailand) of each of three wide-ranging species, showed that the distant populations of two (*Microhyla heymonsi* and *Rana erythraea*) are conspecific, but the third (*Polypedates leucomystax*) has diverged in call and morphology, indicating that they may represent incipient cryptic species. Third, comparing reproduction in these three species in Singapore (where frogs breed year-round) with that in central Thailand (where reproduction is limited to six months) I found that clutch size varied significantly between sites, indicating that these three species compensate for shortened breeding time by producing more offspring in a given breeding event. Fourth, I report the results of field experiments to test the ability of tadpoles to respond to drying of their aquatic habitat; their inability to develop more rapidly when faced with desiccation suggests they may not adapt well to some climate change associated with global warming. Finally, I report field observations on the

reproductive behavior and larval development of two species of Thai anurans.

The initial goal of these studies was to examine patterns of reproductive variation in wide-ranging tropical species as a first step towards predicting how species may respond to climate change. Collectively, these results show that there is potential for some species to adjust reproductive parameters in response to climate change (different clutch sizes at different latitudes), but that adjustments may not occur as predicted from studies on temperate anurans (e.g., an inability of tadpoles to speed up development in response to desiccation) and that trends observed in other ectotherms (clutch size variation across altitude). Clearly, much remains to be learned about the evolutionary ecology of tropical anurans.

CHAPTER 1

Clutch size variation across elevation: an examination of trends in tropical anurans

Abstract

Ectotherms generally have smaller clutch sizes at higher latitudes and elevations. I tested this generalization in tropical anurans sampled at different elevations between 110–2200 m above sea level on the island of Borneo. Using museum specimens of gravid females I examined clutch size variation within seven species (*Ansonia leptopus*, *A. longidigita*, *A. hanitschi*, *Meristogenys whiteheadi*, *M. kinabaluensis*, *Philautus hosii*, *Rhacophorus gauni*). I also looked for trends within six genera involving 15 species (*Ansonia*, *Leptobrachium*, *Leptolalax*, *Meristogenys*, *Philautus*, and *Rhacophorus*), and within families across genera (Megophryidae, Rhacophoridae). I corrected for maternal body size differences in each case to determine whether elevation-related factors, or body size, drives observed differences in clutch size. The majority of species exhibited no significant relationship between clutch size and elevation. Comparing species within genera (six cases), a significant negative relationship held in five cases between clutch size and elevation before body size was accounted for, but not after. Both families had weak associations between clutch size and elevation, but not after accounting for body size. Combining these observations with results from the literature shows that anurans may not always have fewer offspring at higher elevations. Future life history strategy analyses must control for maternal size, ovum size, frequency of egg-laying, and environmental factors that influence maternal resources.

Introduction

Geographic variation in egg size and number is influenced by biotic and abiotic factors and evolutionary history. Ectothermic organisms tend to lay larger eggs at lower temperatures as larger ova are more efficient at retaining heat than smaller ones (Atkinson 1994, Yampolsky and Scheiner 1996, Angilletta et al. 2006). Life history strategies involve trade-offs between egg size and number (Stearns 1992), so because egg size is larger at colder temperatures, clutch sizes of ectotherms are predicted to be smaller in areas with lower temperatures (e.g., higher elevations). Such decreases in clutch size with increases in elevation have been documented for invertebrates (Wilhelm and Schindler 2000, Lardies et al. 2004), lizards (Mathies and Andrews 1995, Rohr 1997), and amphibians (e.g., Howard and Wallace 1985, Gollman and Gollman 1996, Lai et al. 2003).

Patterns of elevational variation in clutch size in amphibians and the generalization that clutch size decreases with elevation deserve further examination for three reasons. First, it is unclear whether amphibian clutch sizes generally decrease with elevation because exceptions exist (Bruce 1972, Licht 1975, Ryser 1996). Second, in many cases, whether elevational changes in observed clutch size are correlates of changes in maternal body size is unknown because females are not often observed with their clutch. When maternal body size was taken into account, at least in some cases, clutch size decreased with elevation (Pettus and Angleton 1967, Bruce 1972, Berven 1982b). Third, few studies have examined trends across higher taxonomic levels. The reduction of clutch sizes at higher elevations as a result of lower temperatures should hold true within genera across species: evolutionary constraints within a clade should produce similar clutch sizes under similar environmental conditions, but congeners should exhibit clutch size variation across elevations.

To address these three topics, I examined whether clutch size, and maternal body size-corrected clutch size, were related to elevation within seven species of Bornean anurans. Next, I analyzed the relationship between clutch size and elevation across species within genera (15 species in six genera) and across genera within families (four genera in two families). If these species follow predicted trends, there should be larger clutches at lower elevations within species, genera, and families. If the trend is independent of maternal body size, then it should also exist after factoring out maternal body size. This analysis is one of the first to examine life history trait changes across an environmental gradient in Southeast Asian frogs.

Materials and Methods

I examined museum specimens of 16 anuran species from Borneo for which gravid females were available from different elevations between sea level and 2200 m (see Table 1; Appendix 1). All individuals in this study were collected in the East Malaysian states of Sabah and Sarawak and were collected between 1° 19’ N and 6° 12’ N. For species with large elevational ranges and for those that are montane, all specimens were collected within 200 km of one another. The climate at each collecting site is similar in terms of seasonality, with all sites receiving rain year-round. All species studied are native to Borneo, and thus are presumed to have existed at their present elevations long enough to adapt to local climate conditions. Collections were made between 1937 and 2007, and no bias existed in elevations sampled in a given year. Some collections were made during or in the year following an El Niño Southern Oscillation (ENSO) event, but clutch sizes in these years did not fall outside of the range of those collected in other years.

For the three genera with three species each represented in this study, I examined a low elevation species, a montane species, and a species that spans low and high elevations. For three other genera, only two species were available, representing two of these three elevational distributions. Species examined represent a variety of reproductive modes, but the majority of genera (*Ansonia*, *Leptobrachium*, *Leptolalax*, and *Meristogenys*) are stream breeders. *Rhacophorus* create foam nests overhanging water, and the species in this study often lay their eggs over streams. *Philautus* are direct developers, and the species studied tend to lay their eggs on the underside of leaves of trees or shrubs.

Gravid females were measured for snout-vent length (SVL) and all eggs were extracted and counted. I counted yolked ova as a surrogate for clutch size. Females of the rhacophorid species contained eggs of two to three size classes, and I used only the number of eggs in the largest class as a measure of clutch size as I have found that in a related Southeast Asian rhacophorid (*Polypedates leucomystax*), only the largest eggs are laid at a given time Chapter 3. Preserved eggs are not spherical so it was not possible to accurately measure egg diameter or to calculate total clutch volume. Coarse measures of egg size were made for 10 of the 15 species for a rough comparison of egg size differences among species within genera.

I analyzed the relationship between elevation and clutch size for the seven species with $n \geq 9$ by performing regression analyses using every individual as a datum. I also regressed clutch size on maternal body size, and used the residuals to examine the relationship between body-size-corrected clutch size and elevation. Next, I analyzed the relationship between elevation and clutch size for the six genera and two families by regressing clutch size on elevation, with and without correcting for maternal body size to determine whether patterns observed at the species level also exist at higher taxonomic levels.

In order to compare the results with published observations, I calculated expected temperature differences between low and high elevations for the study species, and for published studies where data were available. Temperature data at the collection sites were not readily available, so I approximated these based on elevation. For the seven species in this study for which intra-specific clutch size differences were examined, I calculated expected temperature differences, converting °F to °C and feet to meters as necessary, between the minimum and maximum elevation based on the formula: Temperature (°F) = 80 – 0.0026*(elevation in feet) (http://edweb.sdsu.edu/wip/examples/temps/). This formula gave results that were concordant with published mean temperatures for Kota Kinabalu, Sabah (0 m, 27° C; expected temperature, 26.7° C) and Sabah Parks Headquarters (1560 m, 20° C; expected temperature, 19.4° C). For the six genera examined, I calculated temperature differences based on the mean elevation for the lowest and highest ranging species.

I summarized the existing literature on anuran clutch size variation across elevation, and calculated expected temperature differences between lowest and highest elevations for each study where latitude was given based on the formula: Temperature

(°F) = -0.988*(latitude) + 96.8 – 0.0026*(elevation in feet) (http://edweb.sdsu.edu/wip/examples/temps/).

Results

Trends within species.—No clear association existed between clutch size and elevation. Regression analyses showed five of the seven species with $n \geq 9$ had no significant relationship between clutch size and elevation (Table 2). One species (*M. whiteheadi*) had a negative relationship between clutch size and elevation, and one species (*M. kinabaluensis*) had a positive relationship. However, after correcting for maternal body size, these two species plus *Ansonia leptopus* all had significant relationships, with clutch size decreasing with elevation for *M. whiteheadi* and *A. leptopus* but increasing with elevation for *M. kinabaluensis*. For the other nine species with sample sizes < 9, sample sizes were too small to permit robust conclusions, but only one species had a significantly negative relationship between uncorrected clutch size and elevation (*L. abbotti*, $R^2 = 0.56$, $F_{1,5} = 6.31$, $P = 0.054$). When maternal body size was accounted for, none of these species had a significant relationship between clutch size and elevation ($P > 0.07$ for all).

Trends within genera.—Regression analyses with species pooled for the six genera showed mean clutch size decreased with increased mean elevation for four genera (*Ansonia*, *Leptobrachium*, *Meristogenys*, and *Philautus*; Figure 1, Table 3). In *Leptolalax* the trend was not significant, and in *Rhacophorus* clutch size increased with increased elevation. When maternal body size was factored out, the relationship between clutch size and elevation became negative for *Rhacophorus*, and this was the only genus which had a significant relationship between maternal body size-corrected clutch size and elevation. Expected mean temperature differences between the low and high elevation samples in each genus ranged from 7.8–11.8° C (Table 3).

Coarse measures of egg size were made for the representative species of *Leptobrachium, Leptolalax, Philautus*, and *Rhacophorus*. For *Leptolalax* (eggs of both species were 1.5–2 mm diameter) and *Rhacophorus* (eggs of all species were 2–4 mm), there were no obvious differences in egg size across species. In *Leptobrachium*, egg diameter of *L.* cf. *montanum* (montane) was larger (1.5–2.5 mm) than for *L. abbotti* (low-high, 1–1.5 mm). In the direct developer *Philautus*, the maximum egg diameter of *P. petersi* (montane, 1–3 mm) was smaller than that for *P. hosii* (lowland, 2–4 mm).

Trends within families.—Both families analyzed (Megophryidae and Rhacophoridae) had a nearly significant ($P = 0.07$) relationship between clutch size and elevation before maternal body size was factored out, but the relationship for both was weak ($R^2 < 0.14$; Table 4). When clutch size was corrected for maternal body size, neither family had a significant relationship between clutch size and elevation.

Discussion

Does anuran clutch size generally decrease with elevation?—Clutch size decreased with increased elevation for only one species, and the opposite pattern was observed in another congener. A literature search revealed that despite the negative relationship between clutch size and elevation in other taxa such as invertebrates (Wilhelm and Schindler 2000, Lardies et al. 2004), and lizards (Mathies and Andrews 1995, Rohr 1997), only in two anuran species did clutch size decrease with elevation

(Table 5). One anuran (*R. temporaria*) gave different results (smaller clutch size or no change at higher elevations) in different studies, so it remains unclear how clutch size changes over elevation for this species.

To validate the comparison of tropical and temperate species, it is necessary to examine expected temperature differences across elevation. The expected temperature difference across elevation for the tropical species in this study (2.17 ± 0.63 °C, range = 0.28–4.26 °C) are not significantly lower than those for temperate species (4.53 ± 1.76 °C, range = -1.61–10.56° C; ANOVA $F = 1.59_{1,12}$, $P = 0.23$). This indicates that, in terms of expected temperature differences across elevation, the tropical and temperate studies examined here are comparable. In two studies, the higher elevation populations of *Rana temporaria* (Cummins 1986, Ryser 1996) were actually at a warmer temperature due to their lower latitude; this latitude and temperature difference may explain the lack of relationship between clutch size and elevation in one of these studies (Ryser 1996).

The relatively small temperature differences (2–5° C) between high and low elevation populations may partly explain the lack of observed trends. Such temperature differences are the result of studies conducted mostly across elevation ranges of 60–1120 m (Tables 2, 5). Studies on other taxa which have reported a correlation between elevation and life history have sampled elevation ranges of 1000–3455 m. It is possible that life history changes across elevation are not easily detectable across elevation ranges of less than 1000 m. Thus, the smaller sample elevation and temperature ranges of amphibians compared to other taxa may explain the lack of observed trends in life history traits across elevation.

Collectively, these data indicate that clutch size variation across elevation does not vary in a predictable manner in anurans. Furthermore, no relationship exists between taxonomic clade and observed response: not all members of a given genus (e.g., *Rana*, *Meristogenys*) show the same trend in clutch size across elevation, so the response (or lack thereof) is not likely correlated with reproductive mode (e.g., stream breeders, foam nesters, direct developers).

Whenever possible, it is important to examine variation in egg size in conjunction with clutch size across elevation. The observational nature of this study precluded an examination of the ratio of offspring mass to maternal mass as has been done in several studies on reptiles (e.g., Iverson and Smith 1993, Rohr 1997, Zuffi et al. 2007). Interestingly, nearly all published studies on anurans that found no relationship between clutch size and elevation also found no change in egg size across elevation (six species, Table 5). Conversely, studies that found differences in clutch sizes across elevation consistently found differences in egg size produced by females of a given size across elevation (four studies, three species, Table 5). These observations indicate that when egg size differs across elevation, so does clutch size. Thus, cases where clutch size does not change across elevation within species in the present study may also be cases where egg size does not differ across elevation.

Are any observed patterns in clutch size variation across elevation independent of maternal body size?—In this study, only three species showed a significant relationship between clutch size and elevation independent of maternal body size. Interestingly, one of these species did not show a significant trend before correcting for maternal body size; this species also had a relatively small sample elevation range, highlighting the fact that variation in life history traits across elevation may be masked by differences in maternal body size. Of the two other species exhibiting a significant relationship between clutch size and maternal body size, one had a positive relationship and one had a negative relationship, as they did before factoring out maternal body size. The remaining four species showed no significant change in maternal body size-corrected clutch size across elevation.

Few published studies on clutch size variation across elevation correct for maternal body size differences. Studies that have corrected for maternal body size and those that report female body size and clutch size show that two species (Pettus and Angleton 1967, Berven 1982b) have smaller maternal body size-corrected clutch sizes at higher elevations. However, most species show no difference in maternal body size-corrected clutch size across elevation (Morrison and Hero 2003). Combining these data with results from this study, the majority of species (10 of 14) show no trend in maternal body size-corrected clutch size across elevation, three species have smaller clutches at higher elevations, and one species has larger clutches at higher elevations. Accounting for differences in maternal body size made a difference in the interpretation of results for only three species (Pettus and Angleton 1967, Berven 1982b, present study). These data indicate that accounting for maternal body size may change the observed relationship between clutch size and elevation for some taxa, but not for the majority of species that have been studied.

Are any patterns present across higher taxonomic levels?—Interestingly, although there was a significant relationship between clutch size and elevation (without correcting for maternal body size) in only two species, there was a significant relationship in five of the six genera examined. Four of these genera had a negative relationship between clutch size and elevation, but one genus had the opposite relationship. The positive relationship between clutch size and elevation in *Rhacophorus* became negative when maternal body size was accounted for. Further, all the genera that had a significantly negative relationship between clutch size and elevation before maternal body size was corrected for lost this relationship when maternal body size was factored out (Table 3). In the five genera whose relationship between clutch size and elevation changed when maternal body size was accounted for, maternal body size differences, and not abiotic effects associated with elevation, appear to be driving the observed relationship between uncorrected clutch size and elevation. This highlights the importance of factoring out maternal body size when doing such studies, despite the results from this study at the species level. If maternal body size is not accounted for, relationships between clutch size and elevation may simply be due to differences in maternal body size, which may or may not be driven by elevation. At the family level, both Megophryidae and Rhacophoridae had weak relationships between clutch size and elevation, but no such relationship between maternal body size-corrected clutch size and elevation.

The reason for examining trends across higher taxonomic levels is that if the influence of the environment is strong on the selected life history trait, it may outweigh phylogenetic constraints on clutch size, for example, and there may be reduced clutch sizes at higher elevations across species within genera. If patterns do not exist across genera, then evolutionary constraints are likely stronger than the effect of environment. For the genera in this study, there are no published molecular phylogenies so their evolutionary history is not yet clear, and it is possible that the species selected are not each other's closest relatives. Thus, the lack of relationship between maternal body size-corrected clutch size and elevation could be due to evolutionary constraints in some genera.

Other factors affecting clutch size variation.—It is important to note that there are other factors being selected for across elevation that were not measured in this study. As noted above, because colder temperatures at higher elevations select for larger ova, there should be a reduction in offspring number when offspring are larger (Stearns 1992). Coarse measures of egg size were made for the representative species of four genera, and for two genera there were no obvious differences in egg size across species. In *Leptobrachium* maximum diameter of the montane species was larger than for the low-high ranging species, and this pattern is what would be predicted by life history theory (Stearns 1992). In *Philautus*, the maximum egg diameter of the montane species was smaller than that for the lowland species, which is contrary to the basic life history prediction that higher elevation ectotherms should produce larger offspring at higher elevations. This anomaly may be due to the fact that *Philautus* are direct developers, so they may be under different selection pressure than genera with free-swimming tadpoles. It is important to remember, however, that these differences were examined in preserved females; thus egg sizes are approximate. Because females were preserved at different stages of egg development, it is possible that eggs may be larger when fully developed.

Looking at the three questions posed at the outset of the study, we see: (1) there are few significant relationships between clutch size and elevation within anuran species, (2) factoring out maternal body size may change the interpretation of results, and (3) there is evidence for trends in clutch size variation across taxa, but that these trends rarely exist after maternal body size is factored out. These results challenge the assumption that anurans follow patterns of clutch size variation across elevation seen in other ectotherms. As a group, anurans may represent an exception to the general pattern of clutch size variation across elevational gradients. The reason for the lack of trends in clutch size variation across elevation in anurans remains unknown and deserves further attention. If specimens are collected along a short geographic gradient, from sea level up to 2000 m along the slopes of Mount Kinabalu for example, patterns in clutch size variation may be more apparent and easier to interpret. Future studies should also examine changes in offspring size, time to metamorphosis, and variation in oviposition sites across elevation for these species, as these differences may also affect clutch size variation.

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Table 1.1. Clutch size, female body size, and elevation for 16 species of Bornean anurans. Clutch size and SVL are means ± SE.

Family	**Genus**	**Species**	**Category**	**Species Elevation Range (m)**	**Sample Elevation Range (m)**	**n**	**SVL (mm)**	**Clutch Size**
Bufonidae	*Ansonia*	*leptopus*	Lowland	100-700	150-245	9	51.33 ± 1.05	3737.56 ± 144.67
Bufonidae	*Ansonia*	*longidigita*	Lo-Hi	150-2200	400-1300	12	54.83 ± 1.75	2282.83 ± 305.49
Bufonidae	*Ansonia*	*hanitschi*	Montane	750-1850	900-1700	12	30.73 ± 0.94	115.75 ± 11.80
Megophryidae	*Leptobrachium*	*abbotti*	Low-High	100-1000	150-410	7	76.43 ± 1.59	3995.86 ± 373.06
Megophryidae	*Leptobrachium*	cf. *montanum*	Montane	1000-1520	1300-1520	4	68.00 ± 1.73	1261.00 ± 89.98
Megophryidae	*Leptolalax*	*gracilis*	Lowland	100-500	110-150	6	43.83 ± 1.58	290.17 ± 26.83
Megophryidae	*Leptolalax*	*pictus*	Low-High	500-1540	600-1350	6	41.17 ± 0.60	299.83 ± 22.41
Ranidae	*Meristogenys*	*phaeomerus*	Lowland	100-175	120-135	4	70.75 ± 0.85	2566.75 ± 194.15
Ranidae	*Meristogenys*	*whitheadi*	Low-High	100-1500	340-900	9	80.78 ± 1.36	2294.80 ± 126.48
Ranidae	*Meristogenys*	*kinabaluensis*	Montane	650-2000	700-1350	10	92.90 ± 0.87	959.45 ± 53.10
Rhacophoridae	*Philautus*	*hosii*	Lowland	100-600	125-185	14	59.21 ± 1.05	35.79 ± 2.11
Rhacophoridae	*Philautus*	*petersi*	Montane	1000-2200	1150-2200	4	31.05 ± 2.10	15.75 ± 5.12
Rhacophoridae	*Rhacophorus*	*gauni*	Lowland	100-750	110-250	20	33.30 ± 0.66	18.55 ± 1.46
Rhacophoridae	*Rhacophorus*	*angulirostris*	Low-High	100-1400	1230-1400	5	51.00 ± 1.10	58.60 ± 7.76
Rhacophoridae	*Rhacophorus*	*everetti*	Montane	1100-1800	933-1455	3	49.00 ± 3.21	38.33 ± 10.90

Table 1.2. Results of regression of clutch size, and maternal body size-corrected clutch size on elevation. Positive or negative R^2 indicates the respective relationship between clutch size and elevation. Δ T is low elevation temperature minus high elevation temperature.

Species	**Clutch size**			**Maternal body size-corrected clutch size**			**Δ T**
	R^2	**F_{df}**	**P**	**R^2**	**F_{df}**	**P**	
A. leptopus	-0.23	$2.14_{1,7}$	0.18	-0.42	$5.10_{1,7}$	0.058	0.45
A. longidigita	-0.18	$2.25_{1,10}$	0.16	-0.03	$0.32_{1,10}$	0.58	4.26
A. hanitschi	0.00	$<0.01_{1,10}$	0.94	-0.20	$2.55_{1,10}$	0.14	3.79
M. whitheadi	-0.69	$15.90_{1,7}$	<0.01	-0.55	$8.41_{1,7}$	0.02	2.65
M. kinabaluensis	+0.48	$7.47_{1,8}$	0.03	+0.48	$7.36_{1,8}$	0.03	3.08
P. hosii	-0.14	$2.03_{1,12}$	0.18	+0.01	$0.14_{1,12}$	0.71	0.28
R. gauni	+0.005	$0.10_{1,18}$	0.76	+0.005	$0.09_{1,18}$	0.77	0.66

Table 1.3. Results of regression of clutch size and maternal body size-corrected clutch size on elevation for six SE Asian frog genera. Positive or negative R^2 indicates the respective relationship between clutch size and elevation. ΔT is the expected temperature difference between the highest and lowest sample elevation.

Genus	**Clutch size**			**Maternal body size-corrected clutch size**			**Δ T**
	R^2	**F_{df}**	**P**	**R^2**	**F_{df}**	**P**	
Ansonia	-0.72	$78.16_{1,31}$	<0.001	-0.005	$0.14_{1,31}$	0.71	9.06
Leptobrachium	-0.92	$50.87_{1,9}$	<0.001	-0.008	$0.07_{1,9}$	0.79	10.18
Leptolalax	0.00	$0.00_{1,10}$	0.98	+0.005	$0.05_{1,10}$	0.82	7.82
Meristogenys	-0.58	$29.55_{1,21}$	<0.001	-0.002	$0.03_{1,21}$	0.86	8.63
Philautus	-0.54	$18.91_{1,16}$	<0.01	0.00	$0.004_{1,16}$	0.95	11.79
Rhacophorus	+0.30	$11.17_{1,26}$	<0.001	-0.45	$4.55_{1,26}$	0.04	9.08

Table 1.4. Results of regression of clutch size and maternal body size-corrected clutch size on elevation for two SE Asian frog families. Positive or negative R^2 indicates the respective relationship between clutch size and elevation.

Family	**Clutch size**			**Maternal body size-corrected clutch size**		
	$\mathbf{R^2}$	$\mathbf{F_{df}}$	**P**	$\mathbf{R^2}$	$\mathbf{F_{df}}$	**P**
Megophryidae	-0.13	$3.51_{1,24}$	0.07	0.00	$0.11_{1,24}$	0.74
Rhacophoridae	+0.07	$3.44_{1,44}$	0.07	-0.06	$2.84_{1,44}$	0.10

Table 1.5. Summary of sample clutch size and egg size across elevation from published studies. ΔT is the expected temperature difference between the highest and lowest sample elevation.

Species	**Sample elevation range (m)**	**Clutch size at higher elevation**	**Egg size at higher elevation**	**ΔT (°C)**	**Source**
Geocrinia victoriana	180–1300	Decreases	Increases[1]	5.31	Gollmann and Gollmann 1996
Litoria chloris	80–600	Same	Same	N/A	Morrison and Hero 2002
L. leseuri	20–1000	Same	Same	N/A	Morrison and Hero 2002
L. pearsoniana	60–1000	Same	Same	N/A	Morrison and Hero 2002
Mixophyes fasciolatus	100–7000	Same	Same	N/A	Morrison and Hero 2002
M. iteratus	40–600	Same	Same	N/A	Morrison and Hero 2002
Pseudacris triseriata	590–2818	Same	Increases	10.56	Pettus and Angleton 1967
Rana pretiosa	70–2600	Same	N/A	9.55	Licht 1975
R. sylvatica	43–1100	Increases	Increases	5.01	Berven 1982b
R. temporaria	1–100	Decreases	Increases	-0.86	Cummins 1986
R. temporaria	200–1000	Decreases	Increases	N/A	Kozlowska 1971
R. temporaria	600–1930	Same	Same	-1.61	Ryser 1996

[1] Differences may not be significant: no statistical tests reported.

Table 1.6. Museum numbers of specimens used in this study. All specimens from Field Museum of Natural History except those with the prefix SP, from Sabah Parks Museum.

Family	**Genus**	**Species**	**Specimens examined**
Bufonidae	*Ansonia*	*leptopus*	194718, 194727, 194733, 194738, 194740, 194746, 222911, 240988, 248143
Bufonidae	*Ansonia*	*longidigita*	98527, 98534, 98544, 234542, 236886, 236918, 238152, 242541, 242564, 242566, 250896, SP02405
Bufonidae	*Ansonia*	*hanitschi*	235960, 236934, 236943, 236946, 236949, 236956, 242513, SP01536, SP02037, SP02326
Megophryidae	*Leptobrachium*	*abbotti*	222635, 222657, 231343, 236694, 241000, 242616, 244790,
Megophryidae	*Leptobrachium*	cf. *montanum*	236685, 236690, 250928, 250929
Megophryidae	*Leptolalax*	*gracilis*	156440, 222664, 222665, 222671, 222678, 222686
Megophryidae	*Leptolalax*	*pictus*	236558, 236560, 236576, 242628, 248178, 249803
Ranidae	*Meristogenys*	*phaeomerus*	220727, 220728, 220729, 220732
Ranidae	*Meristogenys*	*whitheadi*	234350, 234351, 234353, 248575, SP00234, SP00235, SP00657, SP01338
Ranidae	*Meristogenys*	*kinabaluensis*	237243, 237251, 237255, 237259, 237260, 237263, 237265, 248572, 237280, SP00653
Rhacophoridae	*Philautus*	*hosii*	76994, 137886, 146236, 146242, 146244, 195294, 195298, 195305, 195309, 195318, 196603, 240948, 240950, 240951
Rhacophoridae	*Philautus*	*petersi*	239304, 239310, 252390, SP26406
Rhacophoridae	*Rhacophorus*	*gauni*	145545, 221745, 239235, 245890, 245894, 245895, 245899, 245906, 245908, 245909, 245910, 245911, 245913, 245914, 245917, 245920, 245922, 245923, 245924, 245925
Rhacophoridae	*Rhacophorus*	*angulirostris*	109770, 239184, 239207, 239208, 250961
Rhacophoridae	*Rhacophorus*	*everetti*	51332, 239252, 250972

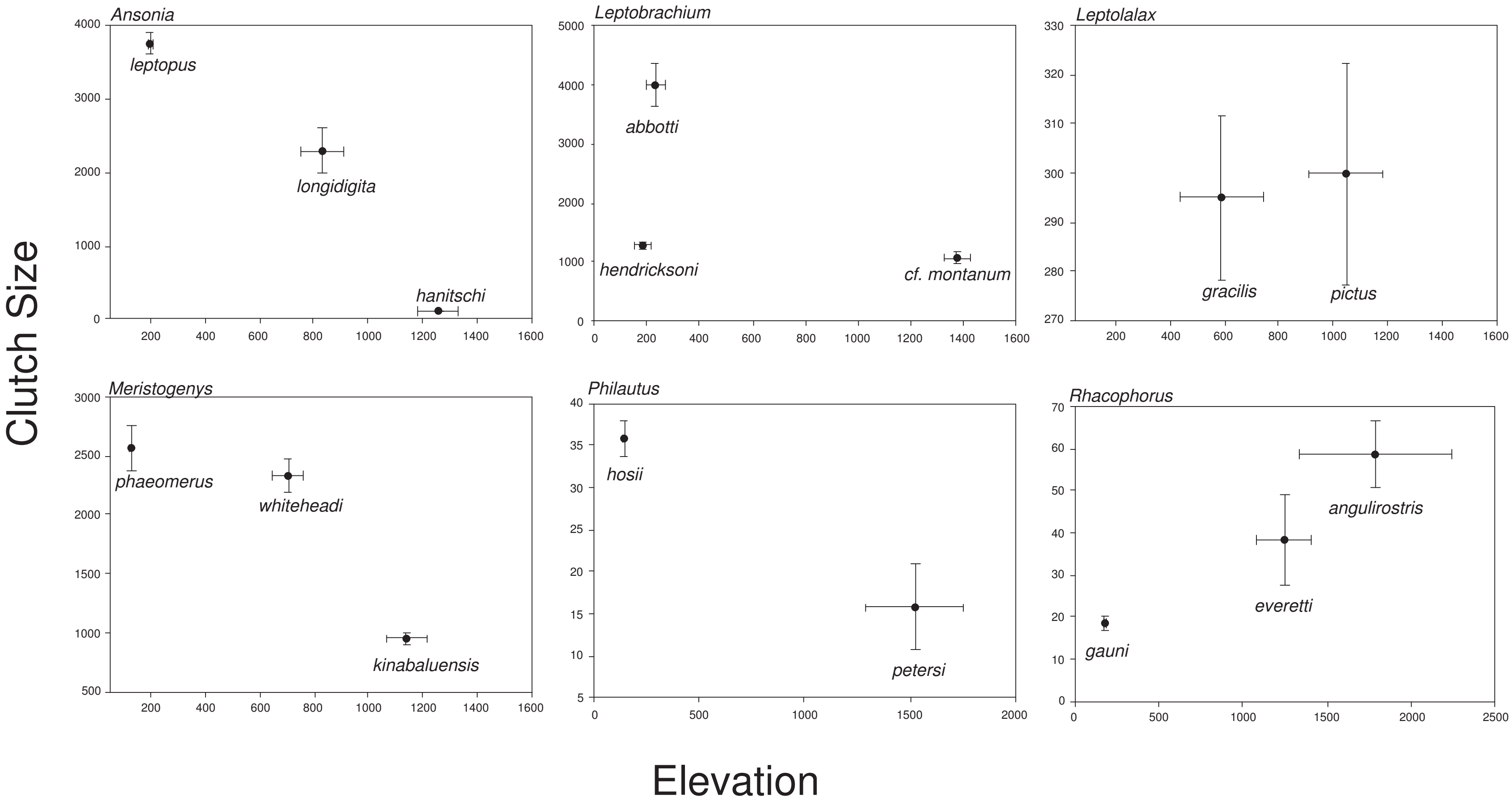

Figure 1.1. Clutch size and elevation for all study specimens. Points are mean values for sample clutch size (y-axis) and elevation (x-axis), vertical bars are standard errors for clutch size, horizontal bars are standard errors for sample elevation.

CHAPTER 2

An examination of call and genetic variation in three wide-ranging Southeast Asian anuran species

Abstract

We examined common male mating calls and portions of two mitochondrial genes (16S and cytochrome b) of three wide-ranging Southeast Asian anurans to determine whether populations separated by 1600 km are conspecific. For one species (*Polypedates leucomystax*), calls are significantly different, but genetic distances are relatively low. For the other two species (*Microhyla heymonsi* and *Rana erythraea*), calls do not differ significantly, and genetic distances are larger than those observed in *P. leucomystax*. We conclude that the study populations of *P. leucomystax* may represent different species, and that the populations of *Microhyla heymonsi* and *Rana erythraea* are likely conspecific. Further sampling of calls and genetics of geographically intermediate *P. leucomystax* populations will help resolve whether or not these populations are conspecific.

Introduction

Taxonomic identification of anurans was traditionally based on morphological variation and little attention was paid to geographic variation until John Moore's studies of *Rana pipiens* (Moore, 1944, 1949). In the 1960's it became clear that mating calls were more biologically meaningful in delimiting anuran species and that typically conservative morphological variation was often a poor indicator of species status (e.g. Ball and Jameson 1966, Littlejohn and Oldham 1968). Subsequently, genetic data became commonly used for delimiting species, either alone or in conjunction with morphological or call data (Goldberg et al. 2004, Lougheed et al. 2006, Stuart et al. 2006, Fouquet et al. 2007). Although different data sets may yield different answers, when examined individually, to the question of whether two individuals are conspecific, collectively they should give a reliable indication of species delimitations.

Morphologically cryptic frog species can be difficult to distinguish based on physical features, but they can often be distinguished based on call differences (Matsui et al. 1986, Matsui 1997, Boul et al. 2007), because calls may contribute to reproductive isolation through female discrimination and choice. However, despite the obvious advantages of using calls and DNA sequences for delimiting frog species, and the use of such data to delimit cryptic neotropical frogs (e.g., Pröhl et al. 2006, Boul et al. 2007), few researchers have examined call variation in conjunction with genetic variation in an attempt to discover cryptic Southeast Asian frog species (Narins et al. 1998, Brown et al. 2006). Here, we use a combination of call and genetic (16S and cytochrome b) data to examine whether distant populations of three common, widespread Southeast Asian frog species are conspecific (*Polypedates leucomystax*, *Rana erythraea*, and *Microhyla heymonsi*).

These three common species were sampled in Singapore and central Thailand, sites 1600 km apart. All three species range from southern China or northern Thailand to Indonesia (http://globalamphibians.org). The range of *P. leucomystax* extends west to India, but that of *M. heymonsi* and *R. erythraea* extends only as far west as Myanmar. The calls of all three species have been described elsewhere (see Table 1 for a summary of published call descriptions).

Currently, the study populations of these three species are considered conspecific, but *Polypedates leucomystax* is suspected to harbor cryptic diversity (e.g. Narins et al. 1998). *Polypedates leucomystax* is a medium-sized tree frog, whose snout-vent length (SVL) differs significantly between the two sites: males and females are significantly larger in Thailand (55.24 ± 0.25 mm and 80.94 ± 0.77 mm, respectively) than in Singapore (44.74 ± 0.18 mm and 64.11 ± 0.74 mm; ANOVA P-value < 0.001 for both; Chapter 3). Additionally, individuals in Thailand lack dorsal stripes, while those in Singapore have them (J. Sheridan, unpublished data). Snout-vent length of *M. heymonsi* does not differ between sites: males are 19.40 ± 0.08 mm and 19.53 ± 0.11 mm in Singapore and Thailand, respectively (ANOVA $F_{1,341} = 0.75$, $P = 0.39$) and females are 24.64 ± 0.39 mm and 24.98 ± 0.24 mm, respectively (ANOVA $F_{1,55} = 0.59$, $P = 0.45$; Chapter 3). Similarly, SVL of *R. erythraea* does not differ between the two populations: males are 42.76 ± 0.39 mm and 41.53 ± 0.64 mm in Singapore and Thailand, respectively (ANOVA $F_{1,86} = 2.69$, $P = 0.10$) and females are 70.91 ± 1.15 mm and 68.27 ± 1.06 mm, respectively (ANOVA $F_{1,46} = 1.64$, $P = 0.21$; Chapter 3). There are no obvious differences in morphology or color pattern of either *M. heymonsi* or *R. erythraea* between the two study sites. We would thus predict that geographic variation in calls and genetic markers will be higher for *Polypedates leucomystax* than for *Rana erythraea* and *Microhyla heymonsi*.

Materials and Methods

Study sites.—Observations were conducted in Thailand at Sakaerat Environmental Research Station (14º 30' N, 101º 55'E), and in Singapore at the Singapore Zoo and adjoining Mandai Orchid Garden (1º24' N, 103º47'E). These study sites lie approximately 1,600 km apart and the three species are found throughout the intervening area. The amphibian community size is similar (25 species at Sakaerat, 26 species in Singapore), and seven species are common to both sites (Lim 2002, Chan-ard 2003a). The study area in Thailand comprised secondary forest and cleared areas, and has a mean annual rainfall of 1,260 mm, with most of the rain falling between April and September. The study areas in Singapore were mainly cleared areas adjacent to secondary and primary forest with a mean annual rainfall of 2,345 mm distributed fairly evenly throughout the year.

Comparative analysis of calls.—We recorded calls of 7–9 individuals of each species from two populations of each species (total calls analyzed per species: 15–18). Recordings in Thailand were obtained between June and October 2006, and in Singapore between May 2006 and March 2007. Recordings were made either with a Sony WM D6C Professional Walkman Cassette Recorder and an Audio-technica condenser microphone or a Marantz PDM 660 digital recorder and a Sennheiser K6 microphone.

All analog recordings were digitized using Windows Sound Recorder at 22.0 or 44.1 kHz. Audiospectrograms and oscillograms were quantified with Raven Software 1.2 (Cornell Laboratory of Ornithology, Ithaca, NY, USA).

Because call type differed between species, we analyzed different call characters for each. For all three species, we quantified dominant frequency, call duration, and call rate. For *P. leucomystax*, we also quantified pulse number and pulse rate. For *M. heymonsi* we quantified those five parameters plus number of calls/bout. We tested for differences between the two populations using MANOVA in Statistica v. 6 (StatSoft, Inc. 2001).

Tissue collection.—Tissue samples from Sakaerat, Thailand were collected between April and September 2005 and from Singapore between February and May 2006. Tissues were obtained by extracting liver or calf muscle from a collected specimen or by clipping toes from an adult frog which was then released at point of capture. All tissues were preserved in 95% ethanol. Voucher specimens were deposited in the Natural History Museum at Chulalongkorn University, Bangkok, Thailand, the Raffles Museum of Biodiversity, Singapore, and the Natural History Museum and Biodiversity Research Center of the University of Kansas. Other tissue samples from Thailand were obtained via generous loans; species identifications, voucher numbers, localities (where available), and GenBank accession numbers are listed in Appendix 1. In addition to the 16S and cytochrome b sequences listed in Appendix 1, we downloaded 16S sequences for related species from GenBank:

DNA extraction, amplification, and sequencing.—Genomic DNA was extracted from tissue samples using the Qiagen DNeasy Tissue Kit. Approximately 500 base pairs from both the 16S and cytochrome b mitochondrial gene regions were amplified using the following primer sets: 16S: 16Sbi, 16Sar-L (modified from Palumbi et al. 1991); cytb, both designed for this study: tRNAf (GAAAARCTAYYGYTGWWAYTCAACTAC), cytbr (GTCWACTGARAASCCKCCTCAATTCATTG). For the PCR reactions, we amplified samples at 95° C for 3 minutes, followed by 40 cycles of 94° C for 1.5 minutes, 52° C for 1 minute, and 72° C for 1.5 minutes with a further extension at 72° C for 3 minutes. Samples were stored at 4° C until they were purified with Quick-Clean kits (BioLine, London, UK). Samples were then sequenced with the ABI BigDye terminator mix using fluorescent thermal-cycle sequencing followed by electrophoresis in an ABI 3100 automated sequencer (Perkin Elmer) following the manufacturer's instructions.

Phylogenetic analysis.—DNA sequences were obtained from 4-5 individuals of each species from each population (Singapore and Thailand). Each sample was sequenced in both directions and complementary sequences were aligned in Sequencher (Gene Codes Corp., Ann Arbor, MI), followed by multiple sequence alignment in Clustal X (Thompson et al. 1997) with some manual adjustments in MacClade 4.06 OS X (Maddison and Maddison 1989)**.** The dataset was subjected to Maximum Parsimony (MP) and Bayesian analyses. For MP, the sequences were analyzed in PAUP* 4.0b (Swofford 2000) under the MP criteria with all characters weighted equally, indels treated as missing data, and 1000 independent analyses using random-addition and TBR branch-swapping. Branch support was quantified using bootstrapping as implemented in PAUP* (1000 replicates analyzed with 100 random-addition runs each). MrModeltest 2.2 (Nylander 2004) was used to identify a substitution model, nucleotide frequencies, and optimal priors for the gamma parameter. We used the Akaike Information Criteria (AIC) to determine an appropriate model of molecular evolution. The Bayesian analysis was carried out in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with a Monte Carlo Markov chain (MCMC) length of 1 million generations. We sampled one out of every 100 generations, giving us a total of 10,000 samples. Of these, we discarded the first 2500 (burn-in), and used the remaining trees to calculate posterior probabilities for each branch. We consider a node well supported if its posterior probability is greater than 95%. We also ran a Bayesian analysis to find the best trees for these data. Trees obtained from Bayesian analyses matched well with those obtained from maximum parsimony analyses (see results of each species for details), and we present a single phylogram for each species with both bootstrap and posterior probability values for each node. Some nodes on the Bayesian tree lack bootstrap values (Figure 1) because these nodes were either not supported in the maximum parsimony analysis or the support was < 50. We also calculated F_{ST} values between our study populations using DnaSP 4.0 (Rozas et al. 2003).

Our phylograms are not meant to represent the complete phylogeographic history of the species, but rather to examine how the populations are related to one another and to closely related congeners. For *P. leucomystax*, two sequences downloaded from GenBank identified as *P. megacephalus* clustered within our *P. leucomystax* group. The sequences are represented by a single terminal on our tree because they were identical for our markers.

Results

We found the calls of *P. leucomystax* to be significantly different between the two locations, but genetic distances were relatively low. In contrast, the calls of *M. heymonsi* and *R. erythraea* were virtually identical in the two populations but genetic distances were nearly twice as high as those between populations of *P. leucomsytax*. The phylogenetic trees (Figure 1) indicate that individuals sampled for each species form well-supported monophyletic clades with respect to other sampled congeners.

Polypedates leucomystax.—The Singapore population of this species has two types of call. Both are found in the Thai population which also has at least four additional call types (Figure 2). In our population comparison, we compared the most common call consisting of a single note. MANOVA indicated significant differences in call parameters between the study populations (Wilks' Lambda = 0.32, F = 4.25, df = 5,10, P = 0.02), so we conducted ANOVA on the individual call components. Call duration is longer, pulse number is greater, and call rate is lower in Singapore than in Thailand (ANOVA P-value < 0.02 for each; Table 1). The dominant frequency was lower in Thailand, but the result was not significant (ANOVA $F_{1,14} = 4.05$, P = 0.06). Pulse rate did not differ between the two populations (ANOVA $F_{1,14} = 1.28$, P = 0.277). Uncorrected p-distances between the two populations of *P. leucomystax* were 2.95–3.57% and 6.99–7.46% for 16S and cytb, respectively, and ≤1.2% within a population. There were 149 parsimony informative characters in the 16S and cytochrome b data set (CI = 0.65, RI = 0.75, RCI = 0.49; Figure 1). The GTR + I + Γ was the best fit model and F_{ST} between the Sakaerat, Thailand and Singapore clades was 0.75. Two sequences (represented by a single node) downloaded from GenBank labeled as "*P. megacephalus*" cluster with our *P. leucomystax* sequences. We consider this to be a misidentification of the of the *P. megacephalus*, as other *P. megacephalus* sequences fell outside of our *P. leucomystax* group, and *P. megacephalus* is superficially similar to *P. leucomystax* (Matsui et al. 1986).

Microhyla heymonsi.—Standard call variables (dominant frequency, call duration, pulse number, pulse rate, call rate, and number of calls/bout) did not significantly differ between the two populations (Wilks' Lambda = 1.31, df = 6,10 P = 0.34; Table 1). Uncorrected p-distances between individuals across study populations of *M. heymonsi* were 4.29–4.56% and 11.90–13.08% for 16S and cytb, respectively, while distances between individuals within each population were less than 1%. There were 144 parsimony informative characters in the combined 16S and cytochrome b data set (consistency index, CI = 0.81, retention index, RI = 0.87, rescaled consistency index, RCI = 0.70; Figure 1). Bootstrap support was high for each population as well as for *heymonsi* relative to the outgroups in our data set. The GTR + Γ model was identified as the best fit for this species with these data, and the resulting phylogram is very similar to our most parsimonious tree. The F_{ST} between the Sakaerat, Thailand and the Singapore clades was 0.62.

Rana erythraea.—There was no difference in dominant frequency, call duration, and call rate between the two populations (Wilks' Lambda = 0.54, F = 1.92, df = 4,9, P = 0.19; Table 1). Uncorrected p-distances between the study populations of *R. erythraea* were 5.11% and 10.4% for 16S and cytb, respectively, while distances within each population were 0% (Figure 1). There were 94 parsimony informative characters for the combined 16S and cytochrome b data set (CI = 0.81, RI = 0.81, RCI = 0.66). As before, GTR + Γ was identified as the best fit model. F_{ST} between the Sakaerat, Thailand and Singapore clades was 0.93.

Discussion

We found that the calls of *P. leucomystax* were significantly different between the two locations, while the genetic distances were relatively small (Table 2). We found that although our populations are not each other's closest relatives, all sampled populations of *P. leucomystax* formed a well-supported monophyletic clade (Figure 1). Other studies of *P. leucomystax* calls (Table 1) indicate that there may be three or more cryptic species currently under that name across the range of *P. leucomystax*, and our call data support that hypothesis. In other widely distributed species, variation in calls decoupled from genetic divergence is not uncommon. Pröhl et al. (2006) found that differences in calls of *Physalaemus pustulosus* were better explained by geographic distance than by genetic distance, and that females preferred local males to foreign males in phonotaxis experiments. Although we attempted phonotaxis experiments to determine whether the differences in calls between our two populations contribute to prezygotic isolation, the response rate of females was very low (one in 18 trials), so we cannot determine the significance of the observed call variation.

Lougheed et al. (2006) found no geographic structure in the variation of 16S in *Hyla leucophyllata*, but calls varied significantly, similar to what we found in *P. leucomystax*. It is possible that our mtDNA markers do not accurately reflect reproductive isolation between populations, which would explain the observed differences in the call of *P. leucomystax* that was not accompanied by similar differences at our sampled genes. Our goal was to determine whether these two populations were conspecific, and for *P. leucomystax*, our data are unfortunately inconclusive. The calls indicate that the populations have diverged, and adult body size and time to metamorphosis are significantly different between the study populations (Chapter 4). However, the genetic distances are relatively small. These populations may be different species, but we refrain from formally describing them because we were unable to study the geographically intermediate populations. More exhaustive sampling across the range, and phonotaxis experiments would aid in determining whether these two populations of *P. leucomystax* are reproductively isolated and thus represent different species.

For *M. heymonsi* and *R. erythraea*, similar calls indicate that the two populations are conspecific, but the genetic distances (Table 2) are larger than those observed for *P. leucomystax*. These relatively large distances may only indicate that the populations have been separated for a long period of time, but long separation does not necessarily lead to speciation (Ferguson 2002). Other anurans are known to exhibit similar levels of conspecific genetic diversity. For example, Matsui et al. (2005) found distances of over 10% at the cytb marker across populations (but within species) of *M. okinavensis* and *M. fissipes*. Additionally, adults of *M. heymonsi* and *R. erythraea* are not significantly different in body size (Chapter 3) which indicates a lack of morphological divergence between the two populations. Call parameters for *M. heymonsi* have been examined for populations in N. Thailand and Taiwan (Table 1), but no populations intermediate between our study sites, so it is not yet possible to determine how call parameters vary across this species' range, and no data from other populations are available on the call parameters we examined for *R. erythraea*. Given that *M. heymonsi* and *R. erythraea* are probably nearly continuously distributed between our two study sites, the species may be effectively panmictic with minor genetic differentiation by distance. We thus conclude that our study populations of *M. heymonsi* and *R. erythraea* are conspecific. The study of call and genetic variation from isolated samples is unlikely to permit the identification of some species but the combination of these data sets coupled with comprehensive geographic sampling will eventually lead to the full characterization of these morphologically conservative animals.

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J. Avina, H. Betts, P. Bowles, R. Businello, A. Diesmos, B. Guha, K. Hesed, S. Ho, W.S. Hwang, J. Kee, K. Kee, S. Kutty, D. McLeod, L. Meijer, A. Ng, J. Ocock, R. Oon, H. Pong, M.R. Posa, P. See, G. Shimin, P. Valcarcel, and C. Yeong.

Table 2.1. Call features of *Microhyla heymonsi*, *Rana erythraea* “chirp” call, and *Polypedates leucomystax* “normal” calls. Values are means ± SE (range). For the Taiwan *M. heymonsi* info, we report what Kuramoto (1987) identified as the 3rd note of a 3-note call (by our terminology, this would be the third call in a calling bout). The study by Narins et al. (1998) recorded variables for two different morphs (striped and non-striped) in Kuala Lumpur that they determined to be different species based on allelic polymorphism. *Temperature for these specimens is water, not air temperature. †Values from this study are means ± SD.

Species and Location (n)	**Recording Temp (°C)**	**SVL (mm)**	**Dominant Frequency (Hz)**	**Call duration (ms)**	**Pulse number**	**Pulse rate (pulse/s)**	**Call Rate (calls/s)**	**Source**
M. heymonsi Manchou, Taiwan	24.5	20–23	3000	290 ± 20	10.1 ± 0.9	31.2 ± 1.9	N/A	Kuramoto (1987)
M. heymonsi Chiahsien, Taiwan	25.5	20–23	2800	410 ± 30	12.5 ± 0.7	28.3 ± 0.3	N/A	Kuramoto (1987)
M. heymonsi Chiang Mai, Thailand (5)	24–26	N/A	2490.4 ± 66.57	338.82 ± 8.63	13.1 ± 0.30	38.70 ± 0.68	N/A	Garcia-Rutledge and Narins (2001)
M. heymonsi Sakaerat, Thailand (9)	24.5 ± 0.44	19.52 ± 0.11	3292.11 ± 176.69	430.09 ± 20.89	9.63 ± 0.44	22.44 ± 0.28	0.87 ± 0.02	This study
M. heymonsi Sakaerat, Thailand (1)	28	N/A	1700–3000	480	11	23	N/A	Heyer (1971)
M. heymonsi Singapore (9)	26.5 ± 0.23	19.44 ± 0.08	3284.84 ± 82.75	461.99 ± 28.21	10.09 ± 0.53	21.98 ± 0.48	0.93 ± 0.06	This study
R. erythraea Sakaerat, Thailand (8)	24.63 ± 0.71	41.53 ± 0.64	2406.20 ± 126.96	19.90 ± 2.04	N/A	N/A	0.170 ± 0.03	This study
R. erythraea Singapore (7)	25.88 ± 0.59	42.71 ± 0.38	2701.84 ± 191.47	18.79 ± 4.09	N/A	N/A	0.206 ± 0.06	This study
R. erythraea Negros, Philippines	29–30	N/A	N/A	24.7 (16–33)	N/A	N/A	N/A	Alcala et al. (1986)
P. leucomystax Taiwan	25–27	N/A	N/A	153–628	2–5	7.78–11.90	N/A	Kuramoto (1986)

Table 2.1, continued.

Species and Location (n)	**Recording Temp (°C)**	**SVL (mm)**	**Dominant Frequency (Hz)**	**Call duration (ms)**	**Pulse number**	**Pulse rate (pulse/s)**	**Call Rate (calls/s)**	**Source**
P. leucomystax Ba Be, Vietnam (22)	26.2	N/A	1940	33	2–17	N/A	N/A	Trepanier et al. (1999)
P. leucomystax Pac Ban, Vietnam (2)	26.2	N/A	1950	64	4–16	N/A	N/A	Trepanier et al. (1999)
P. leucomystax Chiang Mai, Thailand (5)	24–26	N/A	N/A	48.22 ± 4.21	5.24 ± 0.51	108.43 ± 3.79	N/A	Garcia-Rutledge and Narins (2001)
P. leucomystax Sakaerat, Thailand (7)	24.17 ± 0.71	58.14 ± 1.52	1197.05 ± 183.50 (516.8–1778.7)	58.47 ± 5.43 (35.7–83.6)	4.18 ± 0.32 (2.7–5)	72.79 ± 4.22 (56.5–83.3)	0.09 ± 0.02 (0.03–0.21)	This study
P. leucomystax Sakaerat-a (2)	N/A	N/A	300–2600	230–380	4–5	N/A	N/A	Heyer (1971)
P. leucomystax Sakaerat-b (3)	N/A	N/A	1700–3100	120–250	2–4	N/A	N/A	Heyer (1971)
P. leucomystax Uthai Thani, Thailand (8)	ca. 26	N/A	660 ± 40	52 ± 15	4.3 ± 1.0	N/A	N/A	Christensen-Dalsgaard et al. (2002)
P. leucomystax KL, Malaysia Non-striped-A (5)	ca. 25	N/A	2336 ± 110.2	24.6 ± 1.4	1.0 ± 0.0	N/A	N/A	Narins et al. (1998)
P. leucomystax KL, Malaysia Striped-A (16)	ca. 25	N/A	2238 ± 43.2	174.3 ± 11.8	13.2 ± 0.8	N/A	N/A	Narins et al. (1998)
P. leucomystax Singapore (9)	26.21 ± 0.41	43.89 ± 0.92	1786.52 ± 214.54 (818.3–2562.5)	101.81 ± 12.21 (46–140.5)	7.97 ± 1.02 (4–12)	78.51 ± 3.03 (63.4–89.1)	0.03 ± 0.01 (0.01–0.07)	This study
P. leucomystax Negros, Philippines (17) †	28–30	41–52	1500–2500	192.7 ± 20.5	16.2 ± 2.3	81.4 ± 10.6	0.1–0.13	Brzoska et al. (1986)
P. leucomystax Kundasang, Sabah, Borneo (3)	21.5*	N/A	2250 (2100–2500)	158 (133–18.5)	11.3 (9–13)	N/A	N/A	Matsui et al. 1986
P. leucomystax Ranau, Sabah, Borneo (12)	27*	N/A	2390 ± 70 (1900–2700)	125 ± 5 (83–159)	12.3 ± 0.5 (8–16)	N/A	N/A	Matsui et al. (1986)

Table 2.1, continued.

Species and Location (n)	**Recording Temp (°C)**	**SVL (mm)**	**Dominant Frequency (Hz)**	**Call duration (ms)**	**Pulse number**	**Pulse rate (pulse/s)**	**Call Rate (calls/s)**	**Source**
P. leucomystax Sandakan, Sabah, Borneo (2)	27.5*	N/A	2550 (2400–2700)	144 (143–145)	13.0 (13)	N/A	N/A	Matsui et al. (1986)
P. leucomystax Sarawak, Borneo (3)	22–26	N/A	2623.7 (2398.9–2927.9)	143.5 (130.5–165.7)	13.4 (11.3–15)	94.0 (84.3–107.3)	0.06 (0.06–0.07)	Sanchezherriaz et al. (1995)
P. leucomystax Bali (6)	24.5–29	ca. 48	2550.1 (2320.6–2677.7)	218.5 (148–408.3)	16.5 (12.6–23.2)	84.7 (43.8–129.4)	N/A	Marquez and Eekhout (2006)

Table 2.2. Calls, morphology, and genetics of frogs from Sakaerat, Thailand and Singapore. “+” and “–“ symbols indicate greater and lesser values, respectively, in the Thai population compared to Singapore.

Species	**Call duration, pulse number, call rate**	**Genetic distance (%)**		F_{ST}	**Taxonomic conclusion by data type**	
		16S	**Cytb**		**Call**	**DNA**
M. heymonsi	Same	4.29–4.56	11.90–13.08	0.62	Conspecific	Ambiguous
R. erythraea	Same	5.11	10.4	0.93	Conspecific	Ambiguous
P. leucomystax	Lower, lower, greater	2.95–3.57	6.99–7.46	0.75	Cryptic	Conspecific

Table 2.3. Species, collection location (if available), and field tag numbers for specimens collected for this study, and GenBank accession number. TH, Thailand; SG, Singapore. Museum codes: KU, University of Kansas Natural History Museum and Biodiversity Research Center; CU, Chulalongkorn University Natural History Museum.

Species	**Locality info and field tag numbers**	**GenBank accession number**	
		16S	**cytb**
M. heymonsi	N/A	**AY458596**	**AY458596**
M. heymonsi	N/A	**NC006406**	**NC006406**
M. heymonsi	Sakaerat, TH; KU DSM1136	**XXX**	**XXX**
M. heymonsi	Sakaerat, TH; KU DSM1152	**XXX**	**XXX**
M. heymonsi	Sakaerat, TH; KU DSM1153	**XXX**	**XXX**
M. heymonsi	Sakaerat, TH; KU DSM1205	**XXX**	**XXX**
M. heymonsi	SG; N/A	**XXX**	**XXX**
M. heymonsi	SG; N/A	**XXX**	**XXX**
M. heymonsi	SG; N/A	**XXX**	**XXX**
M. heymonsi	SG; N/A	**XXX**	**XXX**
M. heymonsi	SG; N/A	**XXX**	**XXX**
M. heymonsi	SG; N/A	**XXX**	**XXX**
M. heymonsi	SG; N/A	**XXX**	**XXX**
P. megacephalus	N/A	**AY458598**	**AY458598**
P. megacephalus	N/A	**NC006408**	**NC006408**
P. leucomystax	SG; N/A	**XXX**	**XXX**
P. leucomystax	SG; N/A	**XXX**	**XXX**
P. leucomystax	SG; N/A	**XXX**	**XXX**
P. leucomystax	SG; N/A	**XXX**	**XXX**
P. leucomystax	SG; N/A	**XXX**	**XXX**
P. leucomystax	SG; N/A	**XXX**	**XXX**
P. leucomystax	Sakaerat, TH; CU HKV17473	**XXX**	**XXX**
P. leucomystax	Sakaerat, TH; CU HKV17474	**XXX**	**XXX**
P. leucomystax	Sakaerat, TH; CU HKV17475	**XXX**	**XXX**
P. leucomystax	Sakaerat, TH; CU HKV17496	**XXX**	**XXX**

Table 2.3, continued.

Species	**Locality info and field tag numbers**	**GenBank accession number**	
		16S	**cytb**
P. leucomystax	Sakaerat, TH; KU DSM1147	**XXX**	**XXX**
R. chalconota	SG; N/A	**XXX**	N/A
R. macrodactyla	Sakaerat, TH; KU DSM1308	**XXX**	N/A
R. erythraea	Sakaerat, TH; KU DSM1373	**XXX**	**XXX**
R. erythraea	Sakaerat, TH; KU DSM1147	**XXX**	**XXX**
R. erythraea	Sakaerat, TH; KU DSM1374	**XXX**	**XXX**
R. erythraea	Sakaerat, TH; CU HKV17473	**XXX**	**XXX**
R. erythraea	Sakaerat, TH; CU HKV17475	**XXX**	**XXX**
R. erythraea	SG; N/A	**XXX**	**XXX**
R. erythraea	SG; N/A	**XXX**	**XXX**
R. erythraea	SG; N/A	**XXX**	**XXX**
R. erythraea	SG; N/A	**XXX**	**XXX**
R. erythraea	SG; N/A	**XXX**	**XXX**

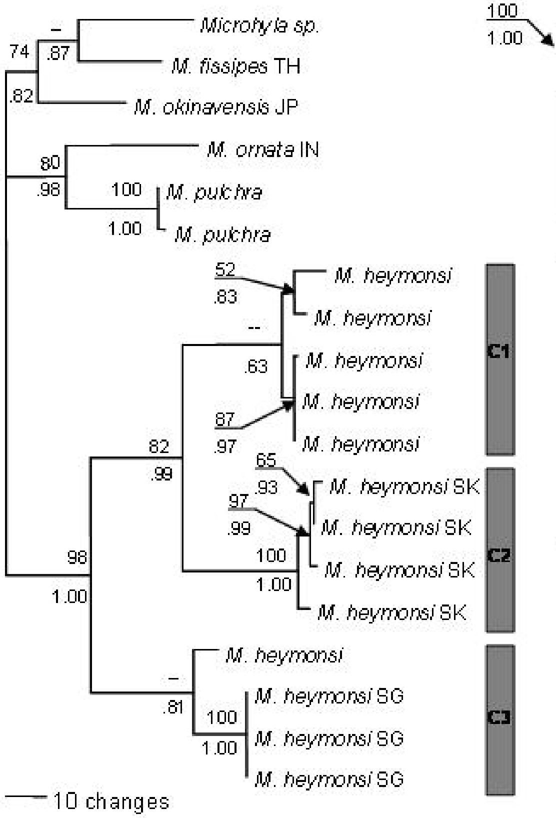
Table 2.4. 16S sequences downloaded from GenBank. Locality information was not available for the majority of these samples.

Species	**GenBank Accession Number**
Microhyla cf.	**AF215371**
M. fissipes	**AB201186**
M. okinavensis	**AB201184**
M. ornata	**AB201188**
M. pulchra	**AB201191**
M. pulchra	**AF285205**
M. heymonsi	**AF215372**
M. heymonsi	**AF285201**
M. heymonsi	**AF285200**
M. heymonsi	**DQ283382**
M. heymonsi	**AB201190**
Polypedates aff. leucomystax	**AF285224**
Polypedates aff. leucomystax	**AF285223**
P. colettii	**AF215354**
P. cruciger	**AY141845**
P. dennysii	**AF285219**
P. eques	**AY141846**
P. fastigo	**AY880518**
P. maculatus	**AF215358**
P. megacephalus	**AY880519**
P. mutus	**AY880521**
P. otilophus	**AF215356**
P. pleurostictus	**AY880522**
P. leucomystax	**AF215343**
P. leucomystax	**AF026368**
P. leucomystax	**AY141849**
P. leucomystax	**AF285220**
P. leucomystax	**DQ283048**

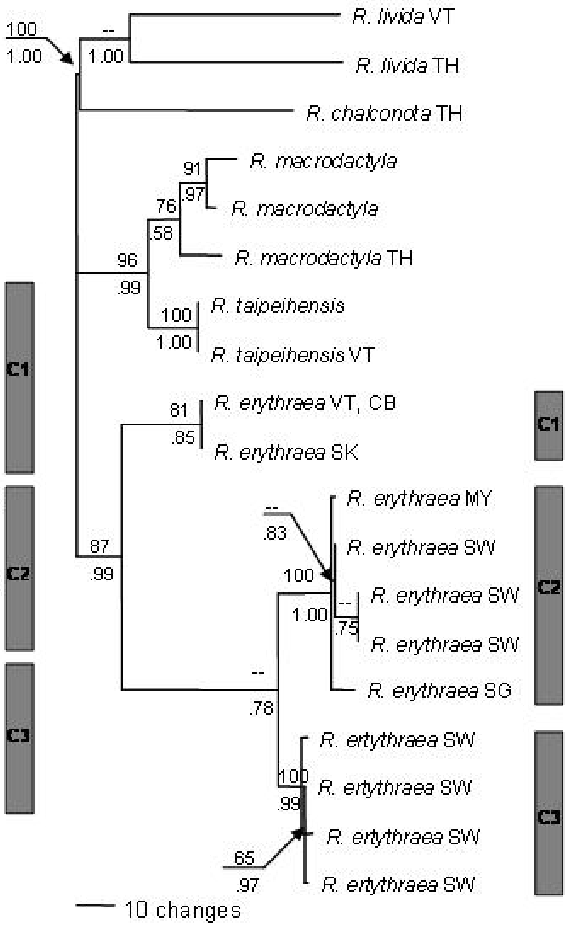
Table 2.4, continued.

Species	**GenBank Accession Number**
Rana livida	**AF206459**
Rana livida	**AB200955**
R. macrodactyla	**DQ360002**
R. macrodactyla	**AF206489**
R. taipehensis	**DQ283396**
R. taipehensis	**DQ360005**
R. erythraea	**DQ283138**
R. erythraea	**DQ835345**
R. erythraea	**DQ835340**
R. erythraea	**DQ835343**
R. erythraea	**DQ835342**
R. erythraea	**DQ835341**
R. erythraea	**DQ835339**
R. erythraea	**DQ835344**
R. erythraea	**DQ835346**
R. erythraea	**DQ835347**

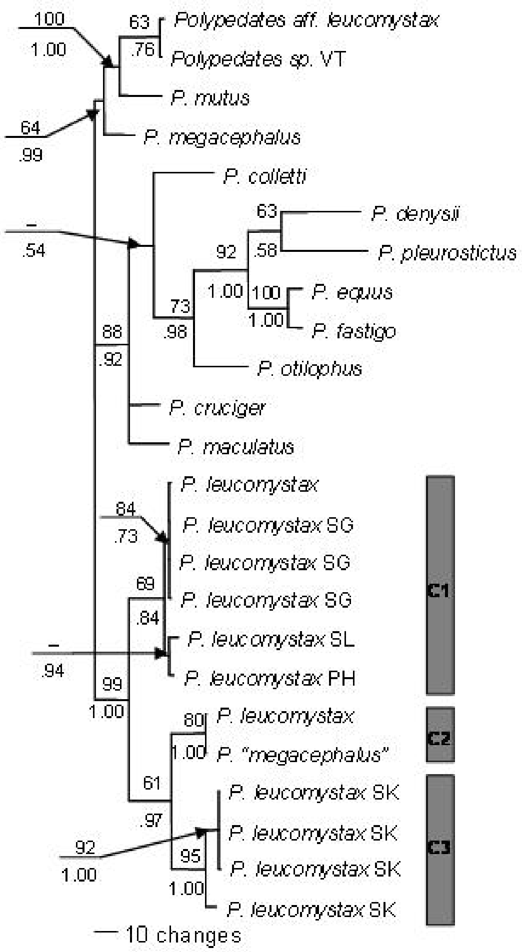
Figure 2.1. Phylogenies of *M. heymonsi*, *R. erythraea*, and *P. leucomystax*. Top numbers are bootstrap values from maximum parsimony analyses, and bottom numbers are posterior probabilities from Bayesian analyses. Locality codes: CB, Cambodia; IN, India; JP, Japan; MY, Peninsular Malaysia; PH, Philippines; SG, Singapore; SK, Sakaerat, Thailand; SW, Sarawak, Malaysia; SL, Sri Lanka; TH, Thailand; VT, Vietnam. Terminals sometimes represent multiple individuals with identical sequence data at our markers, i.e. all *R. erythraea* from Sakaerat, Thailand were identical at our markers and thus are represented by a single terminal on our phylogeny.



Population 1	Population 2	Fst
Clade 1	Clade 2	0.66
Clade 1	Clade 3	0.48
Clade 2	Clade 3	0.62



Population 1	Population 2	Fst
Clade 1	Clade 2	0.93
Clade 1	Clade 3	0.94
Clade 2	Clade 3	0.90



Population 1	Population 2	Fst
Clade 1	Clade 2	0.74
Clade 1	Clade 3	0.75
Clade 2	Clade 3	0.81

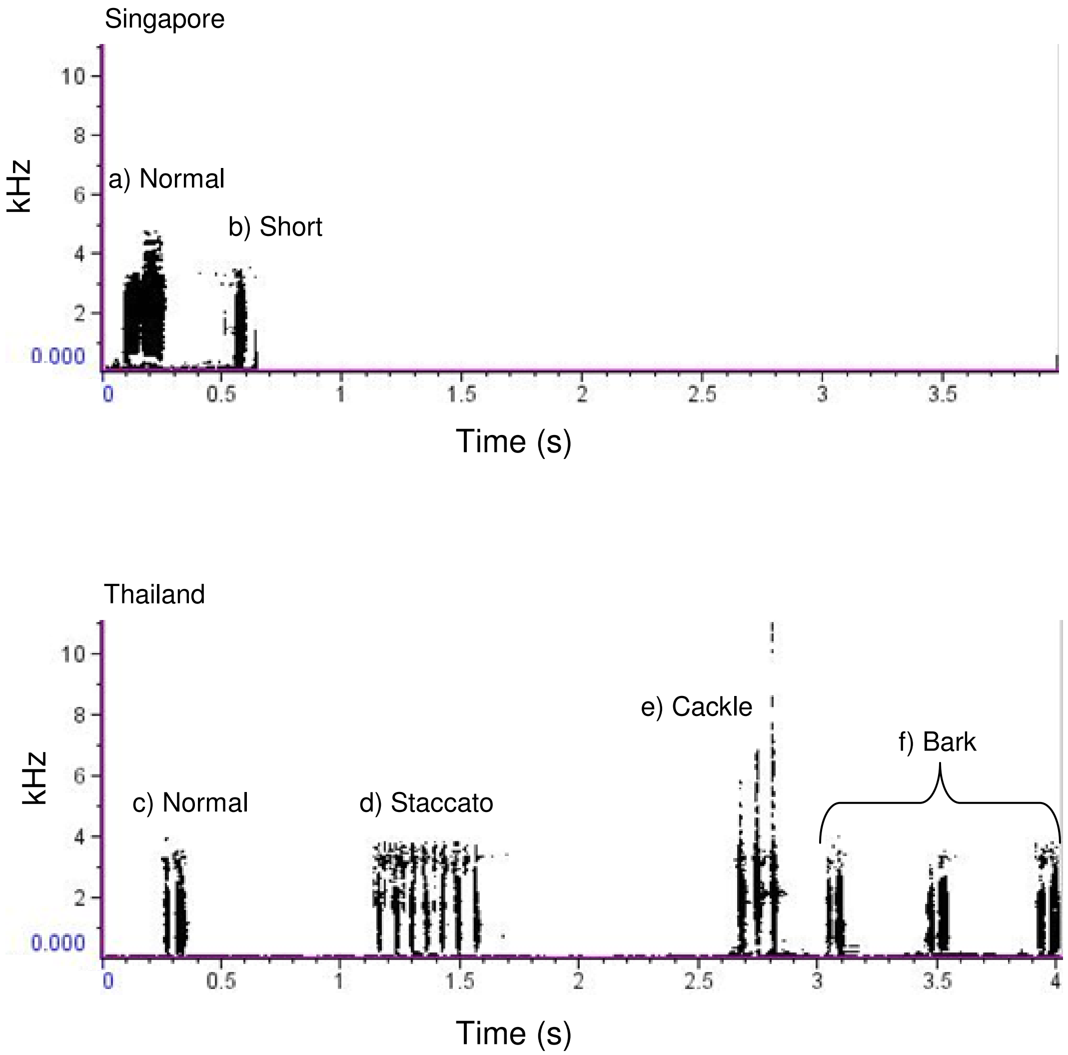


Figure 2.2. Spectrograms of *P. leucomystax* calls from Singapore (top) and Thailand (bottom). Calls are from a single individual in each population. For the Thai individual, call elements did not occur consecutively, but were cut and pasted from a 5 minute recording.

CHAPTER 3

Reproductive variation corresponding to breeding season length in three tropical anurans

Abstract

Several Southeast Asian anuran species can breed year-round near the equator (due to abundant rainfall in all months in Singapore, for example) but are limited to a six-month breeding season in other areas (including central Thailand). Their response or adaptation to changes in reproductive opportunity is informative as to how they, and possibly other species, may respond to on-going global climate change. Reproduction of three common species, *Polypedates leucomystax*, *Microhyla heymonsi*, and *Rana erythraea*, was compared in Singapore and Thailand over a two year period. All three species produce nearly twice as many eggs per clutch in seasonal environments compared to aseasonal environments, although annual fecundity may be similar in both environments. In *Polypedates leucomystax*, increased clutch size and volume are associated with larger female size, but in the other two species female size is unchanged with respect to latitude. In *R. erythraea*, the increased clutch size in Thailand was observed early in the breeding season; later clutches were not significantly different between sites. Collectively, these data indicate that the three widespread study species have the ability to adapt to the large difference in breeding season length, but it remains to be seen whether other tropical species possess this reproductive plasticity.

Introduction

Of the more than 1,000 anuran species in Southeast Asia, up to 40% may be at risk of extinction (Sodhi et al. 2004), and the two most significant drivers of this crisis are believed to be land use and climate change (Sala et al. 2000). With respect to the threat of global warming, it is difficult to make predictive statements about which species are at risk as little is known about the responses of Southeast Asian anurans to climate change. Southeast Asia provides anurans with tropical climates and habitats that have changed repeatedly during the last few million years. Some idea of the ways a species may respond to future climate change can be gauged by comparing conspecific populations living in different environments today. Geographic gradients exist with respect to temperature, altitude, and seasonality of rainfall across this region, and some species have successfully adapted to contrasting environments. Because rainfall, and not just the presence of water for breeding and larval development, is known to trigger oviposition in anurans (Duellman and Trueb 1994), it is of interest to examine how species have changed their behavior to live in areas with different rainy season lengths. A species' current adaptability (or lack thereof) may be useful in projecting its response to future changes in the seasonality of rainfall. Although many species of plants and animals show phenological responses to on-going climate changes (Penuelas et al. 2002, Parmesan and Yohe 2003), little is known about niche conservatism (Wiens and Graham 2005) or reproductive plasticity of tropical anurans. Morrison and Hero (2003) review life history variation in temperate and subtropical anurans across latitude and altitude gradients, but few studies have examined life history changes associated with rainfall gradients.

More than 150 anuran species in Southeast Asia have geographic ranges extending from equatorial to subtropical zones (Iskandar and Colijn 2000). Such wide-ranging species can be especially useful in making projections about how amphibians in general might respond to climate change. Some of these species reproduce successfully in environments that receive rain year-round, and in environments that have limited seasonal rainfall. Numerous studies of amphibians across environmental gradients provide examples where species, mainly in the temperate regions, have responded to increases in altitude and latitude with changes in clutch size (Berven 1982b, Riha and Berven 1991b), time to metamorphosis (Howard and Wallace 1985, Gollman and Gollman 1996, Bury and Adams 1999), egg size (Pettus and Angleton 1967, Kaplan 1980b), and delayed onset of breeding (Highton 1962). Tropical species, despite their greater numbers and diversity, have not yet received comparable attention.

Within amphibian species, variation in clutch size is generally due to resource availability (e.g., higher net primary productivity provides more energy to females that can then produce more eggs), body size (larger individuals lay more eggs, Duellman & Treub 1994), or physiological constraints on egg size and a trade-off between egg size and number (e.g., larger and fewer eggs at colder temperatures, Berven 1982). Differences in breeding season length can also lead to differences in clutch size (Ritke et al. 1992). If a species' annual reproduction is the same across environments, then populations with restricted breeding seasons may lay fewer but larger clutches compared with populations that breed year round. In this paper I test how three common and widespread Southeast Asian anurans respond to differences in breeding season length and discuss the implications of my observations for other species in this biodiversity hot spot. The results of this study on shifts in phonological traits, if they occur, will be useful in projecting anuran ability to respond to further environmental change.

Materials and Methods

Study sites.—Observations were conducted in Thailand at Sakaerat Environmental Research Station (14º 30’ N, 101º 55’E), and in Singapore at the Singapore Zoo and adjoining Mandai Orchid Garden (1º24’ N, 103º47’E). These study sites lie approximately 1,600 km apart, and the three frog species occur throughout the intervening area. The study sites were chosen based on their rainfall patterns (six month rainy season in Thailand, year-round rain in Singapore), and presence of the three focal species. Amphibian diversity is similar (25 species at Sakaerat, 26 species in Singapore), and seven frog species are common to both sites (Lim 2002, Chan-ard 2003a). Further, the mean annual temperature for the two sites is the same (27 °C), and the long-term mean temperatures for the study months are similar as well (26.8–27.8 °C) (http://weatherbase.com). Thus, for the purposes of this study, the sites are roughly comparable, other than differences in rainfall as noted above. The study area in Thailand comprised secondary forest and cleared areas, and has a mean annual rainfall of 1,260 mm, with most of the rain falling between April and September. The study areas in Singapore were mainly cleared areas adjacent to secondary and primary forest with a mean annual rainfall of 2,345 mm distributed fairly evenly throughout the year. Rainfall patterns are shown in Figure 1.

For a complete description of the Thai site, see Sheridan (in press). *Polypedates leucomystax* and *M. heymonsi* were found on site at Sakaerat, and *R. erythraea* was found in nearby rice fields. In Singapore, *P. leucomystax* and *M. heymonsi* were found at the Mandai Orchid Garden, and *R. erythraea* was found nearby at The Singapore Zoo. Both the Mandai Orchid Garden and the Singapore Zoo are cleared areas surrounded by secondary forest, adjacent to primary forest. Although the Mandai Orchid Garden receives regular spraying with pesticides that may cause tadpole and adult mortality when rainfall is low, application of pesticides did not seem to affect adults or tadpoles when rainfall was frequent. When amplectant pairs were allowed to lay eggs in a basin in the laboratory, clutch size was within the range of clutch sizes seen in the field. Artificial breeding sites (shallow ponds and cisterns) were common in both Thailand and Singapore, but as for other anurans, it appears that standing water alone does not stimulate egg laying; breeding was triggered by rainfall combined with the presence of standing water (McCallum et al. 2004, K. Lovich, pers. comm., pers. obs.).

Study species.—I studied three common species: *Polypedates leucomystax* (Rhacophoridae), *Microhyla heymonsi* (Microhylidae), and *Rana erythraea* (Ranidae), in Singapore where the breeding season is 12 months and in central Thailand where the breeding season is six months. All three species range from Indonesia in the south to northern Thailand or southern China (http://globalamphibians.org). The range of *P. leucomystax* extends west to India, but that of *M. heymonsi* and *R. erythraea* extends only as far west as Myanmar. All three species require standing water to complete their 22–90 d larval phase (Alcala 1955, Chapter 4, Heyer 1973). Genetic sequence and call analyses of *M. heymonsi* and *R. erythraea* indicate that the study populations are conspecific (Chapter 2). *Polypedates leucomytax* is generally acknowledged to be a species complex (Narins et al. 1998), the complete phylogeny of which has yet to be published. Analysis of approximately 500 bp of the 16S mitochondrial gene sequence reveals that the two populations are only about 3% divergent (Chapter 2), a level of similarity normally associated with conspecific populations (Fouquet et al. 2007). For the purposes of this study, I regard the two populations to be conspecific until further evidence is available.

Surveys and analyses.—Observations were made sequentially at Sakaerat (21 and 17 weeks) and Singapore (12 and 14 weeks) between April 2005 and March 2007 as shown in Figure 2. Observations in 2005 were limited to only *P. leucomystax*; thereafter all three species were monitored. During each study period, study sites were searched for egg masses 3-6 times per week. Eggs of *P. leucomystax* are laid in a foam nest on vertical surfaces, usually overhanging water. *Microhyla heymonsi* and *R. erythraea* lay their eggs in a single layer on the surface of standing water. *Rana erythraea* lay their eggs among aquatic vegetation, rather than in open water, whereas *M. heymonsi* will lay their eggs in open water. Eggs were counted either in the field or in the laboratory, and were raised to tadpoles when necessary for species confirmation. Eggs of *P. leucomystax* lack a jelly capsule and can thus be measured with calipers to obtain egg diameter. If eggs of this species were found prior to Gosner (1960) developmental stage 13, the diameter of ten eggs was measured under a dissecting scope to the nearest 0.01 mm with digital calipers, averaged to give a mean egg size per clutch, and used to calculate clutch volume ($(2/3*3.14*(\text{radius of egg})^3)*\text{clutch size}$). Clutches that were large for a given study site were encountered on occasion, but all were double checked and determined to have been single clutches based on physical characteristics of the foam mass and the embryo's stage of development. Note that for *P. leucomystax*, not all clutches were used to calculate clutch volume as some clutches were found after eggs had passed Gosner (1960) stage 13. Clutch size, egg size, and clutch volume of *P. leucomystax* did not differ significantly between years at Sakaerat (ANOVA F 0.09–0.41, $P > 0.52$ for all), so data for both years were pooled for comparisons with Singapore. Except where noted, clutch size, egg size, and clutch volume did not differ significantly between sampling periods at a given site for any species, so data from both study periods were pooled for cross-site comparisons.

I analyzed the relationship between the number of eggs (clutch size) and rainfall in two ways: regressing mean weekly clutch size on rainfall of the week in which it was found, and regressing clutch size on rainfall of the day it was found plus the two previous days. Results were not different for these two methods, so I present just the results of the former analyses.

Areas were also surveyed weekly for 2–3 h at night to mark and measure adults. Adults were captured, sexed, measured, and marked via toe-clipping (Hero 1989) in order to estimate recapture rates, to determine how often individuals breed in a single season (Table 1). Following marking, adults were released immediately at point of capture.

In order to investigate whether females laid multiple clutches in a single breeding season, I collected amplectant pairs of *P. leucomystax*, and *M. heymonsi*, allowed them to lay eggs in a basin, preserved the females and counted any ova remaining in their body cavity. Amplectant pairs of *Rana erythraea* were never encountered in Thailand, and rarely in Singapore. Voucher specimens of adults were deposited in the Natural History Museum at Chulalongkorn University (Bangkok, Thailand) and the Raffles Museum of Biodiversity (Singapore).

Results

Polypedates leucomystax, *Thailand*.—In the 2005 sampling period, mean ± SE clutch size was 476.94 ± 20.72 eggs (range, 230–804), mean egg diameter was 1.79 ± 0.04 mm (n = 17), and mean clutch volume was 1527.37 ± 138.69 mm^3 (n = 17). Clutch size was not significantly related to egg size (regression $r^2 = 0.19$, $F_{1,15} = 3.52$, $P = 0.08$). Number of clutches found per week was 1.71 ± 0.42 (range, 0–6). Number of clutches found per week did not change predictably over the study period and was not dependent on recent rainfall (regression $r^2 = 0.01$, $F_{1,19} = 0.28$, $P = 0.60$). Between weeks there was no significant difference in clutch size (ANOVA $F_{20,15} = 0.14$, $P > 0.99$; Figure 2), egg size (ANOVA $F_{4,9} = 0.46$, $P = 0.77$), or clutch volume (ANOVA $F_{4,9} = 0.10$, $P = 0.98$).

In the 2006 sampling period, mean clutch size was 500.23 ± 19.19 eggs (range, 48–1242), mean egg diameter was 1.80 ± 0.01 mm (n = 62), and mean clutch volume was 1660.12 ± 93.29 mm^3 (n = 62). There was no relationship between clutch size and egg size (regression $r^2 < 0.01$, $P = 0.95$). Number of clutches per week was 6.65 ± 1.11, and was unrelated to recent rainfall (regression $r^2 = 0.14$, $F_{1,15} = 0.22$, $P = 0.65$). Clutch size (ANOVA $F_{13,93} = 1.88$, $P = 0.04$) and egg size (ANOVA $F_{13,48} = 2.25$, $P = 0.02$) varied between weeks, but there is no clear trend of an increase or decrease over time. Clutch size and egg size were unrelated to recent rainfall (regression $r^2 < 0.09$, $P > 0.30$ for both;

Figure 2). Clutch volume did not differ significantly between weeks (ANOVA $F_{13,48}$ = 1.15, P = 0.34).

In 8 of the 9 females found in amplexus and allowed to oviposit in the laboratory, no fully developed eggs remained, although they still contained many developing ova of about 25% and 50% the diameter of fully developed eggs. Overall recapture rate in Thailand was 55% for males and 12% for females, with 17.7 ± 6.6 d between recaptures for females.

Polypedates leucomystax, *Singapore*.—In the Feb–May 2006 sampling period, mean clutch size was 261.49 ± 12.30 eggs (n = 90), mean egg size was 1.76 ± 0.02 mm (n = 31), and mean clutch volume was 821.15 ± 56.35 mm^3 (n = 31). There was a significant difference in clutch size between weeks (ANOVA $F_{10,73}$ = 1.99, P = 0.047), but clutch size was unrelated to recent rainfall (regression r^2 = 0.12, $F_{1,10}$ = 1.40, P = 0.26). Rather, the significant difference in clutch size between weeks was due to a single large clutch in the first week of the study with 606 eggs. Between weeks there was no significant difference in egg size (ANOVA $F_{10,18}$ = 0.77, P = 0.77) or clutch volume (ANOVA $F_{10,18}$ = 1.82, P = 0.13). Mean number of clutches found per week was 7.33 ± 1.68 (range, 2–22) and was unrelated to recent rainfall (regression r^2 = 0.12, $F_{1,10}$ = 1.40, P = 0.26).

In the Nov 2006–Mar 2007 sampling period, mean clutch size was 229.27 ± 8.18 eggs (n = 51), mean egg size was 1.90 ± 0.03 mm (n = 23), and mean clutch volume was 894.57 ± 54.85 mm^3 (n = 23). None of these traits varied between weeks (ANOVA P > 0.44 for each). Clutch size (ANOVA $F_{1,139}$ = 3.39, P =0.07) and clutch volume (ANOVA $F_{1,52}$ = 3.17, P = 0.08) remained constant across the two sampling periods, but egg size was slightly smaller during the Nov 2006–Mar 2007 sampling period than in the Feb–May 2006 sampling period (ANOVA $F_{1,52} = 17.76$, $P < 0.001$). Number of clutches found per week was 2.81 ± 0.59 and was not related to recent rainfall (regression $r^2 = 0.07$, $F_{1,14} = 1.08$, $P = 0.32$).

In all five females discovered in amplexus that were allowed to oviposit in the laboratory, no fully developed eggs remained, although there were immature eggs present in all cases. As in Thailand, these eggs were of two size classes, the larger being yellow eggs about 33–50% the diameter of fully developed eggs, and the smaller being white and about 25% the diameter of fully developed eggs. The overall recapture rate in Singapore was 40% for males and 9% for females, with 100.3 ± 79.3 d between recaptures of females. However, two of the females were recaptured after 21 d and a third was recaptured after 259 d.

Polypedates leucomystax, *Thailand-Singapore comparison*.—Clutch size and clutch volume were significantly larger in Thailand than in Singapore (Table 1). Egg size in Singapore for the Feb–May 2006 sampling period was smaller than that in Thailand (ANOVA $F_{1,108} = 4.10$, $P = 0.045$), but during the Nov 2006–Mar 2007 sampling period, egg size was larger than that in Thailand (ANOVA $F_{1,100} = 15.98$, $P < 0.001$).

Females and males were over 23% larger in Thailand (80.94 ± 0.77 mm and 55.24 ± 0.25 mm, respectively) than in Singapore (64.11 ± 0.73 mm and 44.74 ± 0.18 mm, respectively; Table 1). Recapture rates were similar for the two study populations (arc-sin test of percentages $t = 0.07$, $n = 81$, $p > 0.20$). Number of days between recaptures was similar in the two populations (ANOVA $F_{1,7} = 2.45$, $P = 0.16$), despite different mean values, most likely due to large range in number of days between recaps for the Singapore population (21–259 d). In order to compare relative reproduction between the two sites, given the significant difference in SVL, I calculated the volume of the females as for a cylinder. By using published head widths for this species (Inger 1966) and by considering half the head width as the radius and SVL as the height of the cylinder, I estimated body volumes for each population, and then divided mean clutch volume (mm^3) by body volume (mm^3) to get a corrected measure of reproductive output. Using population mean values for these units, the reproductive output for Thai females was 0.037, for Singapore females it was 0.036.

The majority of individuals (8 of 9 in Thailand and 5 of 5 in Singapore) laid all fully developed eggs in a given oviposition event, but had smaller eggs already developing. The one exception was a Thai female who retained 316 fully developed ova. Although it is not known for either population how long it takes for these eggs to reach maturity, the presence of undeveloped eggs indicates that both populations are probably able to reproduce multiple times in a year or breeding season.

Microhyla heymonsi, *Thailand*.—From June–October 2006, mean clutch size was 402.42 ± 16.20 eggs (n = 161; Figure 2), and the number of clutches found per week was 9.47 ± 2.03 (range, 0–28). Clutch size did not differ between weeks (ANOVA $F_{15,144} = 0.70$, $P = 0.78$), and the number of clutches found per week (9.47 ± 2.03) was not related to recent rainfall (regression $r^2 = 0.01$, $F_{1,15} = 0.18$, $P = 0.69$).

Of the eight females captured in amplexus and allowed to lay eggs in the laboratory, none retained any fully developed eggs, but six had ova that were nearly full size, as well as some that were pigmented and about 50% the diameter of fully developed eggs, and some very small unpigmented ova. It is unknown how long it takes the remaining ova to become fully developed.

Microhyla heymonsi, *Singapore*.—In the Feb–May 2006 sampling period, mean clutch size was 236.25 ± 7.25 eggs (n = 105) and the number of clutches found per week was 8.75 ± 1.54. Clutch size did not differ significantly between weeks (ANOVA $F_{11,94} = 1.15$, P = 0.33) and the number of clutches found per week was not related to rainfall (regression $r^2 = 0.05$, $F_{1,10} = 0.53$, P = 0.48).

In the Nov 2006–Mar 2007 sampling period, mean clutch size was 246.25 ± 7.82 eggs (n = 137) and the number of clutches found per week was 7.33 ± 0.83. Number of clutches found per week was unrelated to rainfall (regression $r^2 = 0.08$, $F_{1,16} = 1.33$, P = 0.27). Clutch size varied across weeks (ANOVA $F_{17,117} = 1.81$, P = 0.03), but was unrelated to rainfall (regression $r^2 = 0.004$, $F_{1,16} = 0.06$, P = 0.80).

None of the ten females collected in amplexus and allowed to lay eggs in the laboratory had any fully developed eggs remaining, but all contained undeveloped ova, some of which were pigmented, and other smaller ones which were unpigmented.

Microhyla heymonsi, *Thailand-Singapore comparison*.—Clutch size was significantly larger in Thailand (402.42 ± 16.20) than in Singapore (241.79 ± 5.41; Table 3.1). Neither male nor female SVL differed significantly between Thailand and Singapore (Table 3.1). No females in either population had fully developed eggs left following oviposition, but in both populations, females had immature ova remaining, indicating that they may be able to lay multiple clutches in a single breeding season or year. Female recapture rates in the two populations were similar (arc-sin test of percentages $t = 1.63$, $n = 60$, $p > 0.10$), indicating that number of breeding events per female is similar in both locations.

Rana erythraea, *Thailand*.—From July–October 2006 mean clutch size was 1073.52 ± 73.00 eggs ($n = 52$; Figure 3). Clutch size decreased sharply after six weeks, and was nearly twice as large for the first six weeks (1421 ± 78.39 eggs) than for the remaining 9 weeks (775.68 ± 84.20 eggs; ANOVA $F_{1,50} = 30.75$, $P < 0.001$). Number of clutches found per week was 3.53 ± 1.01 and was unrelated to recent rainfall (regression $r^2 = 0.06$, $F_{1,13} = 0.76$, $P = 0.40$). Although there was no significant difference in number of clutches found per week when clutch size decreased (ANOVA $F_{1,13} = 0.13$, $P = 0.72$), it is notable that for six of the final 9 weeks, the number of clutches found was only 0 or 1 per week, while for the previous six weeks, this value was 2–7. Clutch size was inversely related to rainfall (regression $r^2 = 0.48$, $F_{1,10} = 9.41$, $P = 0.01$; Figure 3).

Rana erythraea, *Singapore*.—In the Feb–May 2006 sampling period, mean clutch size was 1072 ± 74.73 eggs ($n = 20$). There were 2.18 ± 0.30 clutches found per week. Clutch size did not differ significantly between weeks (ANOVA $F_{11,9} = 1.31$, $P = 0.35$), and number of clutches found per week was unrelated to rainfall (regression $r^2 = 0.23$, $F_{1,9} = 2.71$, $P = 0.13$).

In the Nov 2006–Mar 2007 sampling period, mean clutch size was 954.96 ± 51.76 eggs ($n = 48$). There were 3.22 ± 0.42 clutches found per week. Clutch size did not vary between weeks (ANOVA $F_{15,26} = 1.04$, $P = 0.45$) and number of clutches found per week was unrelated to rainfall (regression $r^2 = 0.02$, $F_{1,16} = 0.38$, $P = 0.54$).

Rana erythraea, *Thailand-Singapore comparison*.—Clutch size in Singapore (991.25 ± 42.78) was smaller than clutch size from the first six survey weeks in Thailand (1421 ± 78.39; Table 3.1). However, clutch size in Singapore is not significantly different from that of the entire study period in Thailand (ANOVA $F_{1,118} = 1.05$, $P = 0.31$). Number of clutches found per week seemed to decrease towards the end of the survey period in Thailand, but this did not seem to be the case in Singapore. Body size of the two populations was not significantly different for either males or females (Table 3.1).

Discussion

All three species have larger clutch sizes in the seasonal environment, suggesting an adaptation to shortened breeding season length.

Polypedates leucomystax.—Clutch size, clutch volume, and body size varied between Sakaerat and Singapore but egg size was not significantly different between the study populations. Clutch size was 1.8–1.9 times larger in Thailand (mean: 476–500 eggs) than in Singapore (mean: 261 eggs). However, the larger body size of the Sakaerat females likely contributes to the larger clutch size there compared with Singapore, as clutch volume scales with body volume. This suggests that reproductive output relative to body size is comparable in the two populations. In aseasonal Singapore, smaller females lay fewer eggs per clutch but may produce more clutches per year. This would result in similar numbers of offspring for the two populations, as the larger females in seasonal Thailand lay nearly twice as many eggs per clutch, but likely fewer clutches in a shorter breeding season. The different reproductive strategies (more eggs per clutch in the seasonal environment and presumably fewer clutches per year) should result in similar annual fecundity. This supports the hypothesis that populations in a seasonal environment make up for lost breeding time by producing more offspring in a given breeding event. The presence of undeveloped ova inside females post-oviposition in both populations, the similar female recapture rates (12% in Thailand, 9% in Singapore), and the overlap in the range of number of days between recaptures, suggest the females of both populations reproduce multiple times in a single season and return to the breeding sites periodically. Unfortunately, sample sizes for these measures are small, and my observations do not permit a quantitative comparison of iterparous behavior at the study sites, but yolking-up undeveloped ova will presumably take weeks or months. The recaptured individuals were found again after approximately 20 days in most cases, which may be the minimum time necessary for females to yolk up another clutch after oviposition. Accurate measures of iteroparity and time between oviposition events can be difficult to obtain, but are necessary to accurately interpret data on reproductive variation between populations. Future studies examining iteroparity in these populations will allow for a more complete interpretation of the results presented here. Nevertheless, it is clear that this species has adapted reproductively to the contrasting environments through adaptations in female body size, and the number of eggs produced per clutch.

Microhyla heymonsi.—Clutch size was 1.7 times larger in Thailand (seasonal) than in Singapore (aseasonal). Body size did not differ between the sites, so larger clutch size was not a result of larger body size. Females at Sakaerat produced more offspring in a single reproductive event than did females in Singapore that can breed year-round. This larger clutch size at Sakaerat would appear to be an adaptation to the shorter breeding season (6 months). Females in both populations contained undeveloped ova after oviposition, indicating that both populations may reproduce multiple times a year. Female recapture rate was higher in Thailand (5%) than in Singapore (0%), but this difference was not significant.

Rana erythraea.—The clutch size pattern in *R. erythraea* was similar to that in the other two species but more complex as clutch size decreased with time in the seasonal environment. Clutch size in the first six weeks of the breeding season in Thailand was significantly larger than that observed in Singapore. The difference decreased later in the season, however, as clutch sizes fell in Thailand but remained the same in Singapore. I interpret this to mean that in the seasonal environment the females lay most of their eggs at the first opportunity while those in aseasonal Singapore can breed repeatedly throughout the year. Although I did not examine the duration of the larval phase for either population, Alcala (1955) reports that for Philippine individuals reared in aquaria, the shortest time to metamorphosis was 66 days, and the mean time to metamorphosis was approximately 90 days. Thus, females in Thailand may be reproducing early in the breeding season in order to avoid exposing their offspring to desiccation in the larval phase late in the rainy season. Females in Singapore are not constrained in the same way, and are producing fewer offspring per breeding event, while females in Thailand may have fewer breeding opportunities per year. This suggests that females in the seasonal environment make up for lost larval developmental time with a greater fraction of their annual offspring production in a single reproductive event early in the rainy season. In this species, female body size did not vary between sites so the differences in reproductive output represent different strategies in contrasting environments.

Conclusions

All three species have adapted to the contrasting reproductive environments with larger clutches in the seasonally constrained environment. There is a difference in the clutch size of *R. erythraea* between weeks at the seasonal location (Sakaerat), and clutch size from the beginning of the sampling period at Sakaerat was significantly larger than that from Singapore, which did not change over time. In one of the three species, increased fecundity is associated with increased female body size, but in the other two species it is not. These data support the hypothesis that individuals in seasonal sites compensate for less breeding time by producing more offspring in a single reproductive event, and that adaptation involves a combination of morphological, behavioral, and other changes.

It is important to consider alternative explanations for the observed trends. Differences in clutch size between populations could also be due to differences in habitat productivity or predation pressure. Although I did not quantify larval predators in either population, there were no major differences in predator assemblages and I assumed that while the assemblages contain different species, functionally they are similar. Further, habitat was not quantified in either location, but each site occurred in disturbed forest or cleared areas adjacent to primary forest, and therefore were assumed to be functionally similar. Overall, I believe the study sites to be functionally similar, with the main difference being seasonality.

One abiotic factor that could explain differences in clutch size is net primary productivity (NPP). Higher NPP in Thailand would result in larger amounts of resources for females, who could then produce more eggs compared to females in Singapore. Actual evapotranspiration (AE) can be used as a proxy for NPP (Rosenzweig 1968), and this value is higher in Singapore (annual AE 1723 mm) than in Nakhon Ratchasima, the nearest major town to Sakaerat (1036 mm, Thornthwaite 1963). Further, AE is higher during the study months in Singapore (141 mm for Jan–May, 136 mm for Nov–Feb) than for the study months in Thailand (121 mm for May–Sep, 117 for Jun–Oct). This suggests that larger clutch sizes in Thailand are not due to higher resource levels there, and supports the hypothesis that larger clutch sizes are a response to seasonality differences.

Surprisingly few studies have examined clutch size variation across latitude in anurans, although some studies report egg size variation across latitude (Moore 1949, Kaplan 1980b, Riha and Berven 1991b, Gollman and Gollman 1996). Clutch size variation has been studied extensively across elevation (Kozlowska 1971, Koskela and Pasanen 1975, Howard and Wallace 1985, Beattie 1987, Elmberg 1991, Lai et al. 2003), which is generally assumed to mimic latitude in terms of temperature effects on clutch size and egg size. In the present study, mean temperatures are similar, but rainfall and seasonality differ. Many frog species in the tropics are known to breed year-round, while species in the temperate regions tend to breed only in the warm wet summer months (Duellman and Trueb 1994). Further, it has been shown that within temperate herpetofauna, populations at lower latitudes can reproduce more often in a given year than those at higher latitudes (Litzgus and Mousseau 2001). Spotted turtles reproduce as often as possible, and annual reproductive output is comparable in different populations, but clutch size varies among populations depending on breeding season length. Populations that breed multiple times a year have smaller clutches than those that reproduce only once (Litzgus and Mousseau 2001). The three species in the present study appear to show the same trend in that they adjust their clutch size in response to seasonality.

Anurans may be more sensitive to differences in breeding season length than to temperature (Parmesan and Yohe 2003) and future studies of the limits of adaptability in the species studied here should focus on this and the response of life history variables such as clutch size, frequency of oviposition, and time to metamorphosis. Further studies are needed to show whether life history traits aside from clutch size vary between populations, and whether differences in degree of iteroparity or life expectancy, for example, also co-vary with breeding season length.

This is one of the first studies of tropical amphibians to examine reproductive differences across large geographic ranges. I set out to test whether frogs that occur in both aseasonal and seasonal habitats adjust their reproduction in response to varying breeding opportunities. For these three common species, the data indicate that they can indeed adapt to different climates. I do not imply any directionality of adaptation (from aseasonal to seasonal environments, or vice versa.) as their phylogenetic histories are still unknown. In an evolutionary sense the process of adaptation could occur in either direction. With respect to the adaptation of these study populations to future climate change, both populations appear to have sufficient reproductive flexibility to respond successfully to projected changes associated with a doubling of atmospheric CO_2.

However, it is important to note that all three species are human commensal species, and they have had thousands to millions of years to adapt to their current environments. Thus, generalizations from these data must be made with caution. It is not clear whether other commensal species and species with restricted geographic ranges or restricted ecological niches will respond to environmental change in the same ways. Although amphibians as a group have shown enormous adaptability to climate change over millions of years, they are currently under multiple threats of extinction due to habitat alteration and infectious disease (Stuart et al. 2004). The survival of local endemics is by no means assured, even in the tropics, and their adaptability to environmental stress deserves more attention. Future studies along a latitudinal gradient could help confirm whether differences in clutch size are due to seasonality differences. The Thai-Malay peninsula has a seasonality gradient that ranges from year-round rainfall in most of Malaysia, to increasingly longer dry seasons at higher latitudes (Wells 1999). If seasonality is truly driving variation in clutch size, variations in clutch size between populations intermediate between the sites of this study should mirror variations in seasonality. Future studies should also include species with restricted ecological niches, as the majority of Southeast Asian anurans are forest-dwelling, and not human commensals like those studied here. Studies including controlled transplant and common garden experiments could determine how quickly these species can respond to climate change associated with global warming.

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Table 3.1. Clutches found, number of adults marked and recaptured, and SVL for three species at Sakaerat, Thailand and in Singapore.

Species	**Location**	**Sampling Period**	**Number of clutches**	**Clutch Size**	**Number of marked males**	**Number of male recaps**	**Male SVL (mm)**	**Number of marked females**	**Female SVL (mm)**	**Number of female recaps**
Polypedates leucomystax	Thailand	Apr – Sep 2005	36	476.94 ± 20.72	138	88	55.16 ± 0.30	23	78.83 ± 0.72	5
		Jun – Oct 2006	115	500.23 ± 19.19	103	45	55.35 ± 0.43	27	83.04 ± 1.08	1
	Singapore	Feb – May 2007	90	261.49 ± 12.30	90	26	45.11 ± 0.31	24	64.65 ± 0.85	2
		Nov 2006 – Mar 2007	51	229.27 ± 8.18	112	55	44.43 ± 0.21	9	62.71 ± 1.42	1
Microhyla heymonsi	Thailand	Jun – Oct 2006	161	402.42 ± 16.20	210	42	19.53 ± 0.11	42	24.98 ± 0.24	2
	Singapore	Feb – May 2006	105	236.25 ± 7.25	N/A	N/A	N/A	N/A	N/A	N/A
		Nov 2006 – Mar 2007	137	246.25 ± 7.82	132	33	19.40 ± 0.08	20	24.64 ± 0.39	0
Rana erythraea	Thailand	Jul – Oct 2006	52	1073.52 ± 73.00	56	16	41.53 ± 0.64	46	68.27 ± 1.06	16
	Singapore	Feb – May 2006	20	1072.00 ± 74.73	N/A	N/A	N/A	N/A	N/A	N/A
		Nov 2006 – Mar 2007	48	954.96 ± 51.76	47		42.76 ± 0.39	10	70.91 ± 1.15	0

Table 3.2. ANOVA F-values$_{df}$ and p-values for comparisons of Singapore and Sakaerat, Thailand populations. * indicates $P < 0.001$.

Species	**Female SVL**			**Male SVL**			**Clutch Size**			**Clutch Volume**		
	F	**df**	**P**	**F**	**df**	**P**	**F**	**df**	**P**	**F**	**df**	**P**
Polypedates leucomystax	235.89	1,73	*	1087	1,409	*	186.46	1,290	*	64.93	1,131	*
Microhyla heymonsi	0.59	1,55	0.45	0.75	1,341	0.39	119.61	1,406	*	N/A		
Rana erythraea	1.64	1,46	0.21	2.69	1,86	0.10	25.14	1,90	*	N/A		

Figure 3.1. Rainfall at Sakaerat, Thailand, and in Singapore. Open bars are long-term means for each month, and closed bars are monthly rainfall totals for the study years (http://weatherbase.com).

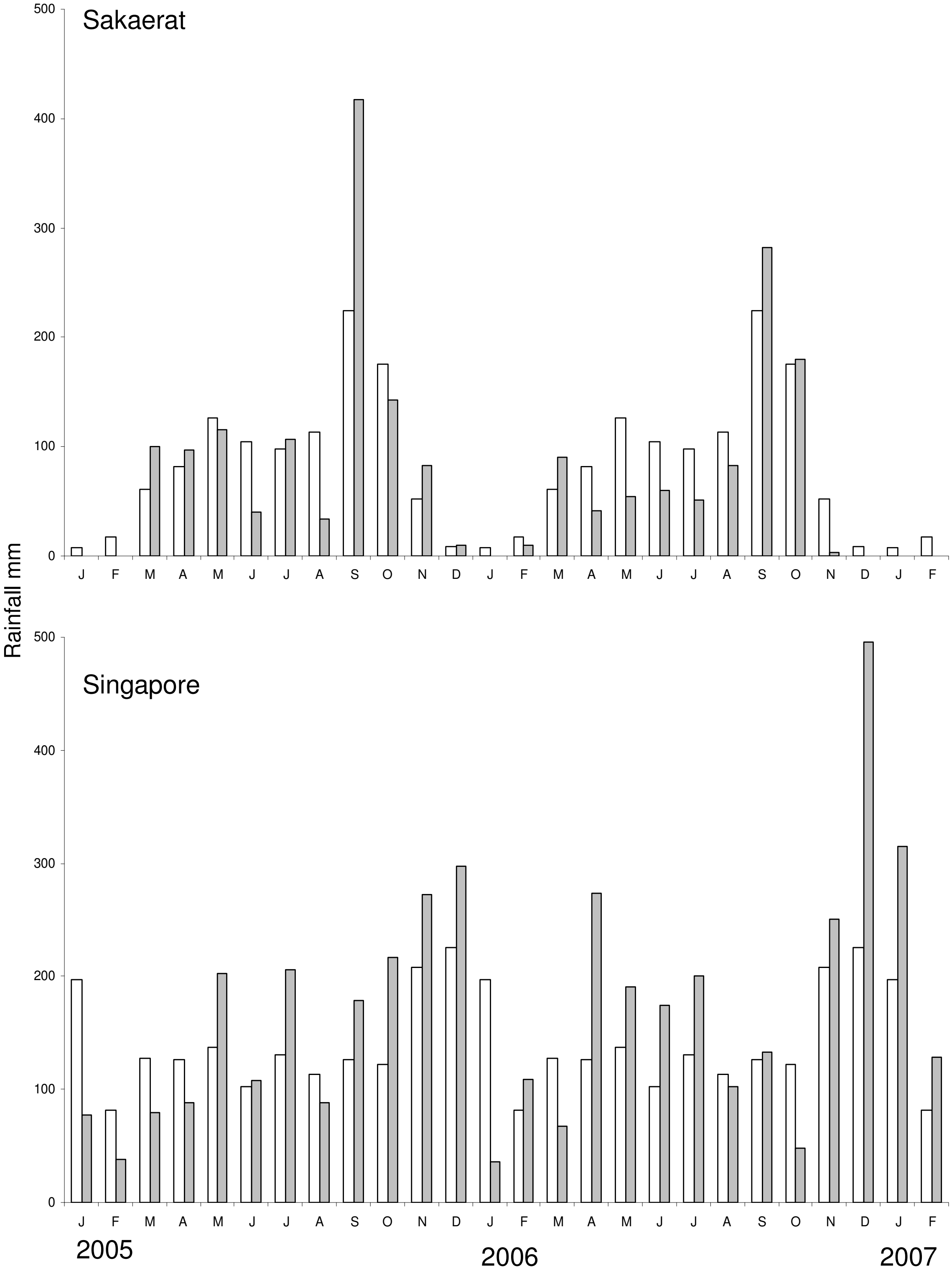

Sakaerat
Singapore
Rainfall mm
0
100
200
300
400
500
J F M A M J J A S O N D J F M A M J J A S O N D J F
2005
2006
2007

Figure 3.2. Clutch size for *Polypedates leucomystax* at Sakaerat and in Singapore (top panel). Thick bars indicate means ± SE, thin bars show ranges. Lower panels show rainfall for Sakaerat and Singapore during the study period (grey bars) and long-term average rainfall for those months (open bars).

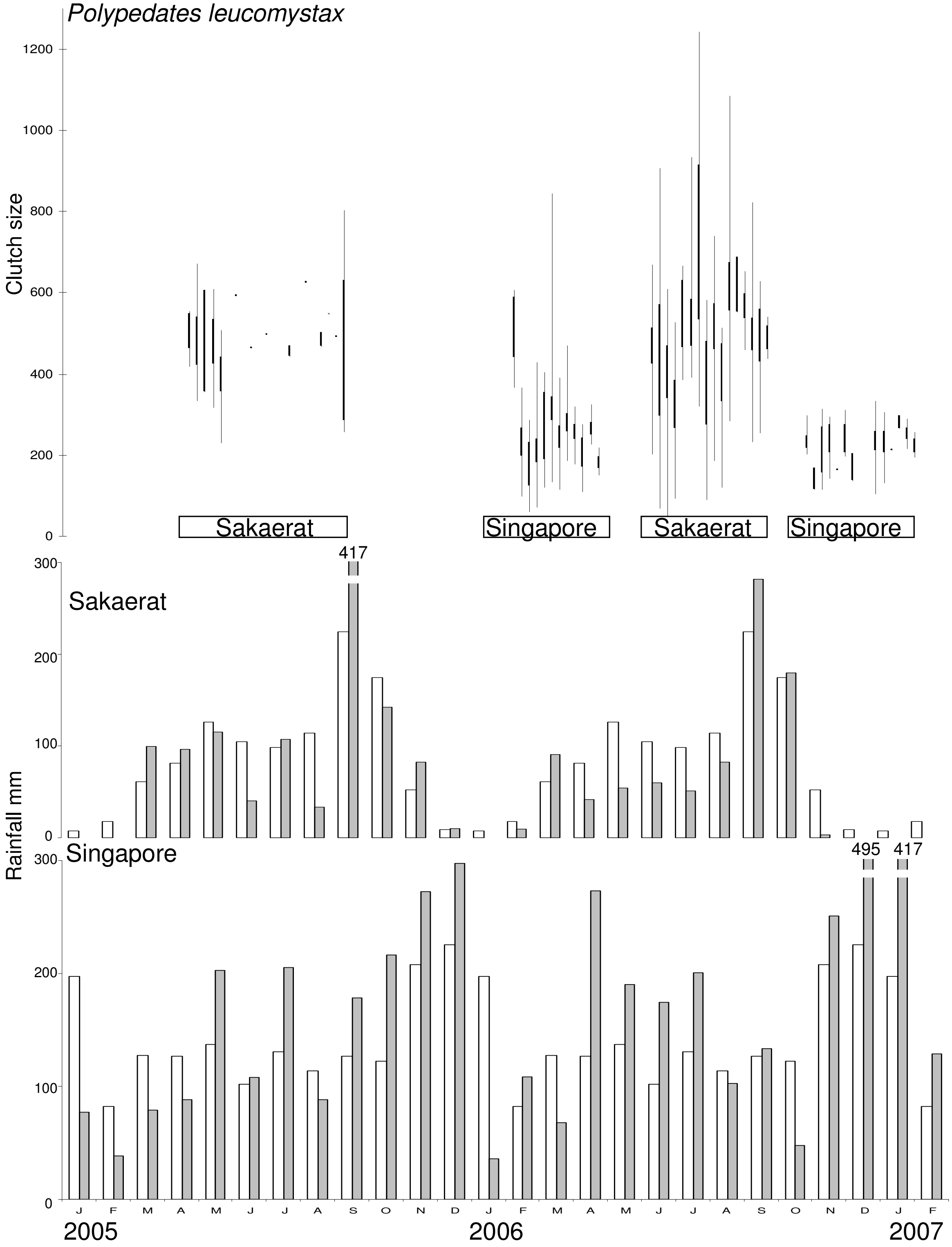
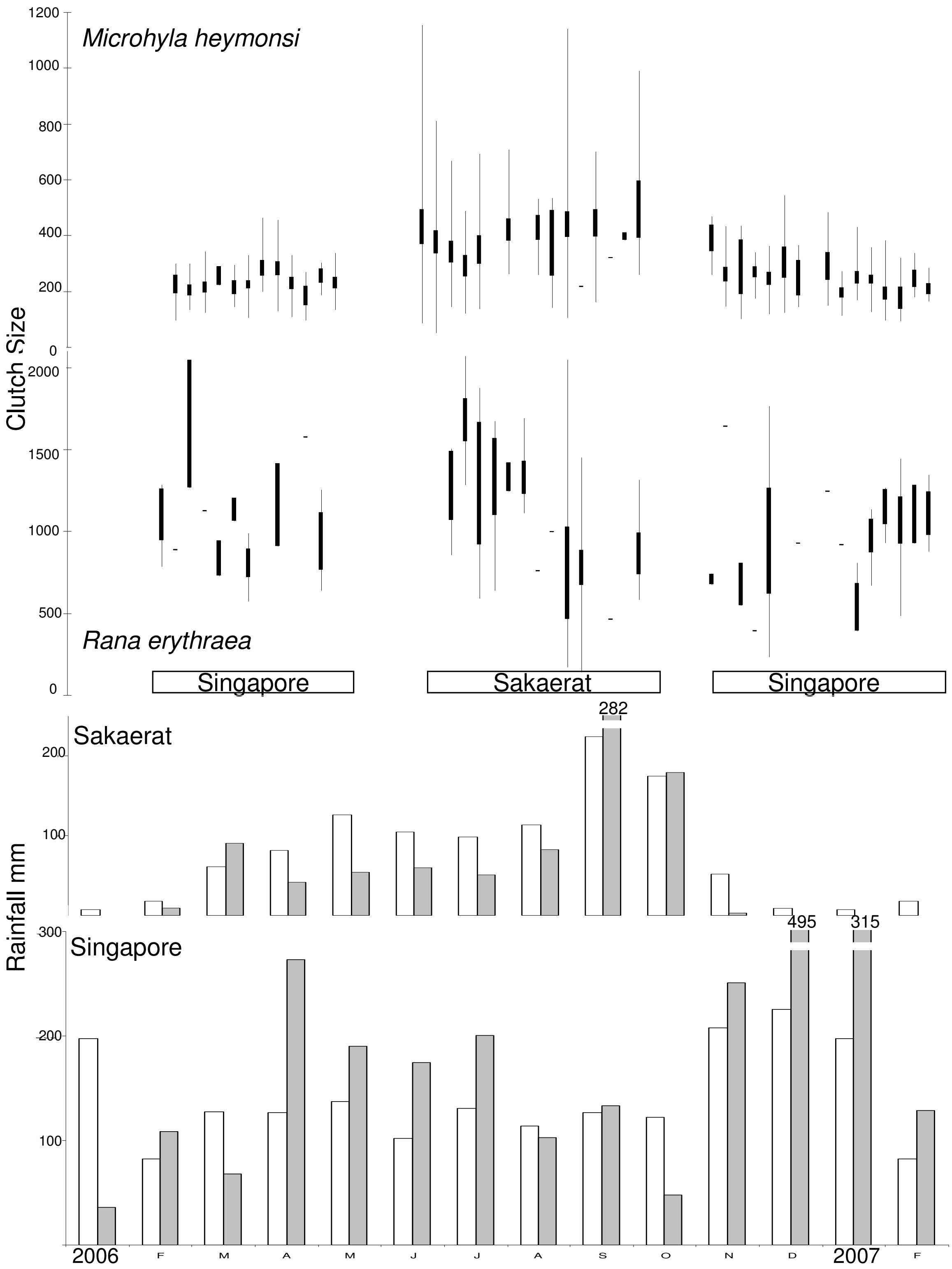

Polypedates leucomystax
Clutch size
1200
1000
800
600
400
200
0
Sakaerat
Singapore
Sakaerat
Singapore
Rainfall mm
300
200
100
0
417
Sakaerat
Singapore
495
417
J
F
M
A
M
J
J
A
S
O
N
D
J
F
M
A
M
J
J
A
S
O
N
D
J
F
2005
2006
2007

Figure 3.3. Clutch size for *Microhyla heymonsi* and *Rana erythraea* at Sakaerat and in Singapore (top two panels). Thick bars indicate means ± SE, thin bars show ranges. Lower two panels show rainfall for Sakaerat and Singapore during the study period (grey bars) and long-term average rainfall for those months (open bars).


Clutch Size
1200
1000
800
600
400
200
0
Microhyla heymonsi
2000
1500
1000
500
0
Rana erythraea
Singapore
Sakaerat
Singapore
Rainfall mm
Sakaerat
282
200
100
Singapore
495
315
300
200
100
2006
F
M
A
M
J
J
A
S
O
N
D
2007
F

CHAPTER 4

Failure of tadpoles to respond to reduced hydroperiod in two species of wide-ranging Southeast Asian anurans

Abstract

We examined the response of aquatic larvae of two wide-ranging Southeast Asian anurans (*Polypedates leucomystax* and *Microhyla heymonsi*) to desiccation of their larval habitat. Contrary to expectations from evidence for several temperate species, tadpoles of these species did not respond to habitat desiccation by increasing development rate. In experiments where tadpoles were raised in basins with decreasing water levels, mean time to metamorphosis either did not differ from that in control basins, or was longer. This indicates that these species may already have evolved to metamorphose as quickly as possible, and that the minimum observed time to metamorphosis is equal to that required for full development. The lack of larval response to habitat desiccation indicates that some tropical anurans may not be able to adapt to shorter hydroperiods or prolonged droughts associated with global climate change.

Introduction

Amphibians are the most severely endangered vertebrate group, with nearly one third of known taxa listed as “threatened”, compared with 25% of mammals and 13% of birds (IUCN 2006). Recent declines and extinctions have been attributed to habitat destruction, chytridiomycosis, and climate change (Pounds *et al.* 2006). Global warming, for example, has been implicated directly and indirectly in significant changes in breeding activity and the disappearance of some anuran species (Heyer et al. 1988, Crump et al. 1992, Pounds and Crump 1994, Beebee 1995). Although most research has focused on adult populations, less attention has been given to the embryonic and larval phases, which face a different set of challenges. Increased UV radiation, for example, causes increased mortality in some montane tadpoles (Belden et al. 2003, Blaustein et al. 2005). Additionally, factors such as reduced hydroperiod and increased drought duration can cause tadpoles to metamorphose at a smaller than average size (Newman 1989, Denver et al. 1998, Laurila and Kujasalo 1999, Ryan and Winne 2001); these changes can in turn lead to smaller size at maturity and lower fecundity (Berven and Gill 1983, Travis et al. 1985, Semlitsch et al. 1988, Merila et al. 2000b). Thus, a single drought event during the larval phase affects not just survival to adulthood, but the number of progeny produced in the next generation.

Research in this area has traditionally focused on temperate rather than tropical amphibians, and to date, only one study has been conducted on the response of tropical tadpoles to drying environments (Crump 1989). Tropical species that live in relatively aseasonal environments and breed year-round may be more adversely affected by global warming and increased climate variability compared to temperate species. In Southeast

Asia, for example, El Niño and La Niña events bring drought and heavy rainfall, respectively. If these ENSO's increase in severity as predicted (Timmermann et al. 1999, Tudhope et al. 2001), amphibian populations are likely to become stressed: long droughts mean lost breeding opportunities and floods increase mortality of eggs and tadpoles. Thus, studies examining the effects of decreased hydroperiod on tadpoles may clarify how species will be affected by climate change. The need for studies on tadpole responses to environmental variation is especially great in the Asian tropics. As a biodiversity hotspot subject to one of the world's highest rates of deforestation (Stuart et al. 2004, Sodhi and Brook 2006), efforts to determine how anurans will be affected by continued habitat loss and climate change should be prioritized. Studies indicate that with continued global warming, tropical species that have historically bred year round will experience increasingly long dry seasons (Manton et al. 2001, Li et al. 2006) and decreased hydroperiods for larval development. To date, no experiments on tadpoles' responses to drying environments have been reported for anurans of Southeast Asia.

Of the approximately 1000 known amphibian species in Southeast Asia, approximately 10 percent have large geographic ranges, extending from the equator to seasonal, sub-tropic latitudes (Iskandar and Colijn 2000). These wide-ranging species represent a useful system in which to study tropical species' responses to climate change. They naturally span drastically different environments: from warm and humid conditions year-round near the equator, effectively providing a 12-month breeding season, to increasingly longer dry seasons towards the north. In central Thailand (approximately 14° N), for example, the rainy season is reduced to six months, cutting in half the potential anuran breeding season compared with that in Malaysia or Singapore. By studying how wide-ranging species respond to drying of their larval environment at different points within their geographic range, we may be able to predict how they will respond to climate change. Further, we can test the response to habitat desiccation of conspecific populations from both seasonal and aseasonal environments. Those from seasonal environments should maintain the ability to respond to shorter hydroperiods, while those in an aseasonal environment may not. If however, neither population responds to shorter hydroperiods, then they may face disastrous consequences as a result of the increased frequency and duration of droughts associated with regional climate change. This study examines the effect of hydroperiod length on tadpoles of two common wide-ranging Southeast Asian anurans, *Polypedates leucomystax* and *Microhyla heymonsi*. Our goals were to determine: (1) whether tadpoles of these species can respond to desiccation by reducing time to metamorphosis, and (2) whether populations of these species in different environments respond similarly to habitat desiccation.

Materials and Methods

Experiments were conducted at Sakaerat Environmental Research Station, Thailand (14º 30’ N, 101º 55’ E), and in Singapore (1°17' N, 103°51' E).

Study organisms.—*Polypedates leucomystax* is a common, medium-sized tree frog that inhabits disturbed areas. It breeds in standing water such as natural ponds, ephemerally flooded open fields, cattle tanks, cisterns, and flower pots. For a review of the natural history of this species, see Appendix 1. With a geographic range extending from southern China to India and Indonesia, *P. leucomytax* is generally acknowledged to be a species complex (Narins *et al.* 1998) whose composition and phylogeny has yet to be published. However, one study has shown the two populations examined here to have low genetic divergence (Chapter 2) and may be considered conspecific. If future studies show the populations to be separate species, the interpretation of tadpole responses to desiccation within a population will remain unaffected. Adults of the Thai population are significantly larger than those in the Singapore population (male snout-vent length, SVL: 55.0 ± 0.2 mm vs 46.7 ± 0.2 mm; female SVL: 79.0 ± 0.7 mm vs 66.3 ± 0.6 mm; Chapter 3) but egg diameter does not differ significantly between the two populations (Chapter 3).

Microhyla heymonsi is a common, small frog (males SVL: 19.52 ± 0.11 mm; female SVL: 24.97 ± 0.23 mm) that inhabits disturbed areas and secondary forest. Like *P. leucomystax*, it breeds opportunistically in both temporary and permanent water bodies. Adults in the two populations are the same size (Chapter 3). This species ranges from southern China to Sumatra. The study populations are only about 4% divergent at the 16S mitochondrial gene and their calls are not significantly different, suggesting that the two populations are conspecific (Chapter 2).

Experiments.—In both locations, egg masses were collected from the field and placed in plastic basins to develop to Gosner (1960) stage 25 (7–10 d). At that stage tadpoles are free swimming with fully formed mouthparts. They were then transferred to experimental basins as described below. Each experimental replicate contained tadpoles from a single clutch, in order to eliminate confounding genetic effects on development rate, and in some cases an experiment was replicated with multiple clutches. Tadpoles were fed commercial aquarium fish flakes (*M. heymonsi*) or fish pellets (*P. leucomystax*) *ad libitum*. Water was replaced 1–3 times per week, depending on water clarity; when water in any basin turned cloudy, water in all basins of a given experiment was replaced. For all experiment types, when tadpoles reached Gosner stage 42 (forelimb emergence), they were moved to small holding basins which provided them with both water to swim in and a dry surface to climb on. Individual SVL, mass, and time to metamorphosis were recorded when all but the last 1 mm of tail was resorbed. Both species naturally breed in water bodies that are both exposed to direct sunlight and shaded, but at our study sites the majority of available breeding sites were shaded, so all basins, except where noted, were kept in an outdoor enclosure, protected from direct sunlight and rainfall. Experimental set-ups are summarized in Table 1.

Experiment A was a comparison of *P. leucomystax* tadpole responses to constant high, decreasing, or fluctuating water levels. The goal of this experiment was to determine whether tadpoles respond to shortened hydroperiod by decreasing time to metamorphosis and to fluctuating water levels by varying development rate. For this experiment, we seeded 11, 7 L basins per treatment with 13 tadpoles each. This experiment was only conducted once in Thailand, starting in July 2005.

Control basins maintained constant water volume (approximately 7 L) throughout the experiment. Basins with decreasing water levels had water removed on a daily basis such that water levels were reduced by 700 mL per week. This dried the basins in approximately 70 d. Basins with fluctuating water levels were reduced by 100 mL, five days per week, and one day per week received a “plus” or a “minus” treatment, determined randomly for each basin. Basins receiving the “plus” treatment were refilled to 7 L, and those receiving a “minus” treatment were reduced by 200 mL.

Experiment B was a comparison of responses to constant high or decreasing water levels over a reduced hydroperiod compared to Experiment A. Upon finding that 50 d was the approximate time to metamorphosis in Experiment A, we adjusted the protocol of Experiment B so that drying basins had a hydroperiod of 42 d. We used smaller basins (3.8 L) because of space limitations, and we seeded ten basins per treatment with 19–20 tadpoles each in Thailand in June 2006 and seven basins with 12–14 tadpoles each in Singapore in April 2006. Fewer basins per clutch were necessary in Singapore due to smaller clutch sizes (averaging 265 in Singapore and 475 in Thailand).

No prior studies have estimated time to metamorphosis for *M. heymonsi* in Singapore, so we initially guessed it to be approximately 42 d. Tadpoles were held in 1 L basins and control basins remained full during the course of the experiment. Water was removed from drying basins three times per week such that water levels were reduced by 165 mL per week. This dried the basins in approximately 42 d. We seeded 6 basins per treatment with 19–22 tadpoles each in April 2006. Tadpoles metamorphosed in about 30 d in Singapore. Subsequent attempts to repeat the experiment at this location with a shorter hydroperiod failed due to high mortality of tadpoles within 2–3 d of collection, for unknown reasons. In Thailand, water levels in control basins were held constant and water levels in drying basins were reduced by 225 mL per week to dry the basins in approximately 28 d. Heyer (1973) estimated minimum time to metamorphosis for this species as 22–25 d at Sakaerat. Since this was the minimum time to metamorphosis, and based on our observations in Singapore, we chose to use a 28 d hydroperiod for the drying basins at Sakaerat, Thailand. For these basins, once the water reached a depth of 5 mm (75 mL), no further water was removed as this remaining volume was sufficient to just cover the tadpoles. Due to larger clutch sizes in Thailand (402 ± 16.20) than in Singapore (241.79 ± 5.41), we seeded ten basins per treatment with 13 tadpoles each in September 2006.

Experiment C was a comparison of *P. leucomystax* responses to constant high or constant low water levels. Because we failed to get a response to decreasing water levels in Experiments A and B, we tested whether consistent low water levels would elicit an increase in development rate. For this experiment, control basins held 2 L of water at a depth of 13 cm and low water basins had 250 mL of water at a depth of 1 cm. This experiment was conducted in Thailand only, and we seeded ten basins per treatment with 18–21 tadpoles each in August 2006.

Experiment D was a comparison of the effects of temperature and drying regime on tadpoles of *P. leucomystax*. There were two temperature treatments, representing the mean ambient temperature in Singapore (outdoor greenhouse, mean water temperature 29.8 °C) and that of Sakaerat, Thailand (an indoor holding room on a 12 h light:12 h dark cycle, mean water temperature 23.0 °C). At each temperature, we had four water level treatments: control, slow drying, moderate drying, and fast drying. Control basins had constant high water levels (depth: 6 cm), and slow drying basins had water reduced by 925, 500, and 200 mL during weeks 2–4, respectively; by 1.5, 2.25, and 1.5 cm in depth each of those weeks. Water in moderately drying basins was reduced by 1200 mL at the beginning of week 2, then by 425 mL for week 3; by 3.75 cm and 1.5 cm, respectively. Water in the fast drying basins was reduced by 1625 mL at the beginning of week 2 which reduced it by 5.25 cm. For all drying basins, water was reduced until it reached a depth of 7.5 mm, from which point no further reductions occurred. The slow, moderate, and fast drying basins reached this depth at four, three, and two weeks, respectively. There were 10 tadpoles per basin, 2–3 replicates per treatment within each clutch, and this experiment type was repeated for six clutches found in June and July 2006. Due to logistical constraints (the lack of a temperature-controlled environment), we were unable to repeat this experiment in Thailand.

All tadpoles in all experiments developed without any noticeable behavioral or developmental abnormalities under these experimental conditions.

Statistics.—We recorded time to metamorphosis to the nearest day, and SVL at metamorphosis to the nearest 0.5 mm. Mass of each *P. leucomystax* metamorph was recorded to the nearest 0.05 g using a Pesola scale, except for experiments noted in Table 2. For all analyses of all variables, we used the mean value for each basin and treated each basin as a single data point. Data were analyzed using Excel and Statistica software, and are reported as means ± SE.

Results

Polypedates leucomystax, Thailand.—In Experiments A and B, there was no significant difference in time to metamorphosis or size at metamorphosis between treatments. In Experiment C, tadpoles in control basins reached metamorphosis significantly faster (48.32 ± 0.29 d) than tadpoles in low water level basins (59.52 ± 0.86 d, ANOVA $F_{1,14} = 223.78$, $P < 0.001$). Tadpoles in control basins were also significantly larger at metamorphosis (20.01 ± 0.12 mm) than tadpoles in low water level basins (19.13 ± 0.17 mm, $F_{1,14} = 18.52$, $P < 0.001$). There was no significant difference in mass at metamorphosis between the two treatments ($F_{1,14} = 3.63$, $P = 0.08$).

Polypedates leucomystax, Singapore.—In Experiments B and D, there was no difference in time to metamorphosis between control and drying basins, but tadpoles in drying basins were smaller at metamorphosis than tadpoles in control basins (Table 2 and 3). In Experiment D, metamorphs in drying basins were lighter at metamorphosis than those in control basins.

Tadpoles in control basins metamorphosed sooner in the high temperature treatment (43.33 ± 0.73 d) than in the low temperature treatment (56.37 ± 0.88 d; $F_{1,10} = 57.02$, $P < 0.001$). Tadpoles in the low temperature treatment were heavier (0.44 ± 0.01 g) than those in the high temperature environment (0.37 ± 0.01 g; $F_{1,10} = 13.18$, $P < 0.005$), but there was no difference in SVL ($F_{1,10} = 1.60$, $P = 0.24$).

Polypedates leucomystax, Singapore-Thailand comparison.—We compared results of Experiment B control basins from Singapore and Thailand to determine whether tadpoles in the two populations differed in their developmental characteristics. Tadpoles in Thailand took 8.5 d longer to reach metamorphosis ($F_{1,13} = 156.79$, $P < 0.001$), and metamorphosed at a larger size ($F_{1,13} = 135.14$, $P < 0.001$) than did tadpoles in Singapore. An important consideration in this comparison is the amount of maternal investment (egg size). In general, larger egg size contributes to larger size at metamorphosis (Kaplan 1992), but in these study populations, egg size does not differ significantly (Chapter 3).

We plotted time to development and water temperature for control basins from Thailand Experiments B and C and Singapore Experiment D (Fig. 3). Data represent two clutches from each location, and show large variation between clutches within a given temperature treatment. The regression ($R^2 = -0.39$, $F_{1,29} = 18.76$, $P < 0.001$) indicated a significant effect of temperature on time to metamorphosis.

Microhyla heymonsi, Thailand.—Tadpoles in control basins reached metamorphosis in 45.61 ± 0.51 d, which was not significantly different than for tadpoles in drying basins (44.83 ± 0.69 d, $F_{1,18} = 0.82$, $P = 0.38$). However, tadpoles in control basins were larger (7.64 ± 0.14 mm) than those in drying basins (6.71 ± 0.10; $F_{1,18} = 30.57$, $P < 0.001$).

Microhyla heymonsi, Singapore.—Tadpoles in control basins reached metamorphosis in 34.10 ± 1.07 d at 7.41 ± 0.11 mm. There was no significant difference in time to metamorphosis ($F_{1,9} = 3.81$, $P = 0.08$) or size at meatamorphosis ($F_{1,9} = 3.46$, $P = 0.10$) between the two treatments.

Microhyla heymonsi, Singapore-Thailand comparison.—Tadpoles in the control basins in Thailand took longer to reach metamorphosis ($F_{1,13} = 124.77$, $P < 0.001$) than those in Singapore, but metamorphosed at the same size ($F_{1,13} = 1.17$, $P = 0.30$).

Discussion

Based on numerous studies on temperate anurans (e.g., Denver et al. 1998, Laurila and Kujasalo 1999, Ryan and Winne 2001) and a single study in the Neotropics (Crump 1989), we expected tadpoles to respond to reduced hydroperiod by decreasing time to metamorphosis, and also to metamorphose at a smaller size. Merila *et al.* (2000b) found that tadpoles in drying treatments metamorphosed, on average, at a smaller size compared with those in constant water treatments, even after controlling for variation in development time. However, in all instances, for both species, where there is a difference between control and experimental basins, the tadpoles in our control basins reached metamorphosis faster, and with a longer body length than those in drying treatments. Though uncommon, this pattern has been observed in *Rana arvalis* and *R. temporaria*, (Loman 2002) and has been attributed to crowding.

In closed systems tadpoles interact with one another's development, and it is reasonable to ask whether our experimental design may have contributed to our unexpected results. "Crowding" effects are generally food competition, waste accumulation, or chemical suppression of growth of others by a single or a few tadpoles. For example, Merila *et al.* (2000a) studied *R. temporaria* tadpoles exposed to drying and found that reduced tadpole survival in high food treatments may have resulted from high ammonium or nitrate concentrations in the water. In our study, crowding and waste accumulation were unlikely to account for the inability of tadpoles in drying basins to reach metamorphosis faster than those in control basins. Our tadpoles were fed *ad libitum* and water was changed regularly to eliminate the effects of food competition and waste accumulation. If tadpoles were suppressing growth of basin mates, then when one tadpole reached metamorphosis, another one should then speed up development and metamorphosis should be spread out over several days or weeks for each basin. Although there was variation in the time to metamorphosis within a given basin, we found no such evidence of serial development suppression.

Heyer (1973) reported minimum times to metamorphosis for these two species at Sakaerat that are significantly shorter than our findings of the mean times to metamorphosis: 22–25 vs. 33 d for *M. heymonsi* and 28–33 vs 33 d for *P. leucomystax*.

However, his observations did not begin at the time of hatching so he may have underestimated the full duration of the larval period.

In *P. leucomystax*, the 21% longer time to metamorphosis in seasonal Thailand (26 ºC) compared with aseasonal Singapore (30 ºC) is likely partially due to lower ambient water temperatures. This hypothesis is supported by the fact that in Singapore, *P. leucomystax* developed 30% slower at 23 ºC than at 30 ºC. Several studies have shown that, in general, tadpoles take longer to reach metamorphosis at lower temperatures than at higher ones (Moore 1939, Bachmann 1969, Harkey and Semlitsch 1988). The difference in time to metamorphosis between 23°C and 30°C in our study was very similar to what Harkey and Semlitsch (1988) found for *Pseudacris ornata*: tadpoles metamorphosed about 10 d (25%) later at 20°C than they did at 30°C. Although the effect of temperature on time to metamorphosis is significant (Fig. 3), there are large differences in the control basins between clutches, suggesting strong maternal effects.

The two *P. leucomystax* populations also differed significantly in size at metamorphosis with Thai frogs being larger. This might be due to the longer larval period in Thailand but tadpoles in the low temperature treatment in Singapore took longer to reach metamorphosis than those in the high temperature treatment but were not significantly larger than those raised at higher temperatures. Additionally, they were 12% smaller at metamorphosis than Thai tadpoles. This indicates that although temperature may affect larval period within a given population, temperature alone cannot explain differences in larval period across populations. Similarly, the difference cannot be explained by differences in egg size (larger eggs lead to larger size at metamorphosis), because egg size in the two populations does not differ significantly (Chapter 3). Finally, although *P. leucomystax* is likely a species complex (Narins *et al.* 1998) and our study populations were 1600 km apart, preliminary analyses of mtDNA sequences indicate that the two populations are less than 4% divergent at the 16S gene (Chapter 2). This low level of divergence suggests that they are similar enough to be considered conspecific. Genetic isolation by distance is not a likely explanation for observed differences in time to metamorphosis.

For *M. heymonsi*, we did not find any differences in time to metamorphosis for tadpoles in control or drying basins, in either seasonal Thailand or aseasonal Singapore. One possible reason for this is that the drying treatments may not have been severe enough to elicit a response. Like *P. leucomystax*, *M. heymonsi* take longer to reach metamorphosis in Thailand than in Singapore, which could partially be explained by cooler mean water temperatures there (24.4°C) compared with Singapore (31.9°C; ANOVA F-value > 682, $P < 0.001$). This does not, however, result in larger body size of the Thai population. The similar size at metamorphosis is not surprising, given that the adults of the two populations do not differ significantly in size (Chapter 3).

Another anuran species whose tadpoles have been examined across a latitudinal range is *Rana temporaria* (Laurila and Kujasalo 1999, Merila et al. 2000a, Laugen et al. 2003). Within a population, tadpoles decrease time to metamorphosis in response to drying environments (Laurila and Kujasalo 1999). In common garden experiments, northern populations of this species metamorphose faster than those from the south and low temperatures result in longer time to metamorphosis and larger size at metamorphosis (except at high food levels) for tadpoles from all populations (Merila *et al.* 2000a). Laugen *et al.* (2003) found that time constraints at higher latitudes, rather than temperature, are the causal agents selecting for faster development in the north compared to the south. Although we did not conduct common garden experiments, we did raise tadpoles from our southern population (Singapore) at temperatures that mimic those of the northern population (Thailand). We found, like Merila *et al.* (2000a), that tadpoles from seasonal Thailand metamorphosed faster (41–51 d) than those from aseasonal Singapore (56 d) raised at temperatures comparable to those in Thailand. Thus, differences in the development time for the two populations could be due to adaptations to hydroperiod constraints in nature rather than temperature differences alone.

It is important to note that within each species, there is considerable variation in time to metamorphosis between clutches. In control treatments, time to metamorphosis in our experiments with *P. leucomystax* ranges from 41–51 d in Thailand, and from 33–43 d in Singapore. Further, tadpoles in low temperature control treatments in Singapore metamorphosed at 56 d, which is longer than the time to metamorphosis recorded for any of the Thai tadpoles in control basins, despite similar temperatures and the larger size at metamorphosis of the Thai individuals. Thus caution should be used when comparing results from different clutches. Variation in time to metamorphosis between the study populations also indicates that although tadpoles from a single clutch may develop slower at lower temperatures, there may be no difference in time to metamorphosis between populations when the effects of temperature are removed.

Regardless of the limitations in comparing our results from different populations, it remains notable that no population of either species responded to experimentally shortened hydroperiod length by decreasing time to metamorphosis. It is possible that these species are already developing as fast as possible in each population, and that they lack the ability to respond to drying conditions by increasing development rate. However, the considerable variation in time to metamorphosis between clutches indicates that selection for those that develop quickly may be strong if global warming increases as expected and hydroperiods shrink. Thus, despite the apparent inability of tadpoles from the same clutch to increase their development rate in response to experimentally shortened hydroperiod, we may see decreased larval period for these species in response to climate change over the next decades. Additionally, the effects of temperature on larval development may prove stronger than the effects of hydroperiod alone. For the majority of our experiments, temperatures in control and experimental basins were similar. In nature, water bodies that are drying out often warm as water levels go down. This temperature difference may be the driving force in affecting development rate changes. Our experiments were kept shaded from direct sunlight to mimic what happens to tadpoles in forested areas, but because these species also breed in open, exposed areas, where they may experience higher temperatures in drying water bodies. Thus, these tadpoles may develop faster in exposed environments in response to higher temperatures associated with decreasing water depth. Ironically, this indicates that tadpoles in forested areas may not adapt to changing rainfall patterns, but those in deforested areas might. If these results are generalizable to other tadpoles in the region, species that restrict their breeding to forested areas may be hit hardest by increased seasonality because they are unable to increase development rates when water bodies dry out. Without the ability to respond to reduced hydroperiod, anuran larval mortality will increase and population numbers will decline.

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Table 4.1. Summary of experiment types in Thailand (T) and Singapore (S) for *Polypedates leucomystax* and *Microhyla heymonsi*.

Exp't Type	**Water level treatments**	**Temperature treatments**	**Basin size (L)**	**Replicates/ treatment**	**Tadpoles/ basin**	**Trials/ location**	**Species**
A	Control, drying, variable	Ambient	7	11	13	1	*P. leucomystax*
B	Control, drying	Ambient	3.8	10 (T), 7 (S)	19-20 (T), 12-14(S)	1	*P. leucomystax*
B	Control, drying	Ambient	1	6 (T), 10 (S)	13 (T), 19-22 (S)	1	*M. heymonsi*
C	Control, constant low	Ambient	2	10	18-21	1	*P. leucomystax*
D	Control, slow drying, moderate drying, fast drying	Ambient (29.8° C), Cool (23.0° C)	2	2-3	10	6	*P. leucomystax*

Table 4.2. Mean values ± SE of key variables measured for hydroperiod experiments. Note that for experiment Type C, “drying basin” = constant low water levels. Each experiment type was conducted on a single clutch except for Experiment Type D, which was conducted on five clutches of P. leucomystax, and the mean for those clutches is given here.

Species	**Location**	**Exp’t**	**Temp, control (°C)**	**Temp, drying (°C)**	**Time to met., control (d)**	**Time to met., drying (d)**	**Size at met., control (mm)**	**Size at met., drying (mm)**	**Mass at met., control (g)**	**Mass at met., drying (g)**
P. leucomystax	Thailand	A	N/A	N/A	51.35 ± 1.02	50.63 ± 1.40	16.13 ± 0.32	15.95 ± 0.42	N/A	N/A
P. leucomystax	Thailand	B	24.95 ± 0.08	24.73 ± 0.05	41.83 ± 0.47	41.73 ± 0.44	19.35 ± 0.17	18.37 ± 0.18	0.58 ± 0.01	0.49 ± 0.01
P. leucomystax	Singapore	B	29.48 ± 0.31	30.44 ± 0.17	33.15 ± 0.24	32.71 ± 0.42	16.33 ± 0.15	15.67 ± 0.17	N/A	N/A
P. leucomystax	Thailand	C	25.68 ± 0.03	22.88 ± 0.83	48.32 ± 0.29	59.52 ± 0.86	20.01 ± 0.12	19.13 ± 0.17	0.59 ± 0.01	0.55 ± 0.02
P. leucomystax	Singapore	D-high	29.75 ± 0.05	29.92 ± 0.02	43.33 ± 0.73	42.09 ± 0.42	16.92 ± 0.14	14.83 ± 0.16	0.37 ± 0.01	0.28 ± 0.05
P. leucomystax	Singapore	D-low	23.02 ± 0.02	22.84 ± 0.03	56.37 ± 0.88	59.68 ± 0.91	17.32 ± 0.15	15.37 ± 0.94	0.44 ± 0.01	0.30 ± 0.005
M. heymonsi	Thailand	B	24.64 ± 0.04	24.64 ± 0.05	45.61 ± 0.51	44.83 ± 0.69	7.64 ± 0.14	6.71 ± 0.10	N/A	N/A
M. heymonsi	Singapore	B	31.75 ± 0.43	32.14 ± 0.17	34.10 ± 1.07	37.18 ± 1.13	7.41 ± 0.11	7.17 ± 0.08	N/A	N/A

Table 4.3. Results of ANOVA comparisons showing significant differences between tadpoles in control and drying basins.

Species	**Location**	**Experiment**	**Variable**	**F**	**df**	**P**
P. leucomystax	Thailand	B	Size at metamorphosis	16.51	1,19	<0.001
P. leucomystax	Thailand	B	Mass at metamorphosis	23.47	1,19	<0.001
P. leucomystax	Thailand	C	Time to metamorphosis	223.78	1,14	<0.001
P. leucomystax	Thailand	C	Size at metamorphosis	18.52	1,14	<0.001
P. leucomystax	Singapore	B	Size at metamorphosis	7.38	1,10	0.02
P. leucomystax	Singapore	D-high	Mass at metamorphosis	8.56	3,20	<0.001
P. leucomystax	Singapore	D-high	Size at metamorphosis	13.65	3,20	<0.001
P. leucomystax	Singapore	D-low	Mass at metamorphosis	14.86	3,20	<0.001
P. leucomystax	Singapore	D-low	Size at metamorphosis	20.69	3,20	<0.001
M. heymonsi	Thailand	B	Size at metamorphosis	30.57	1,18	<0.001

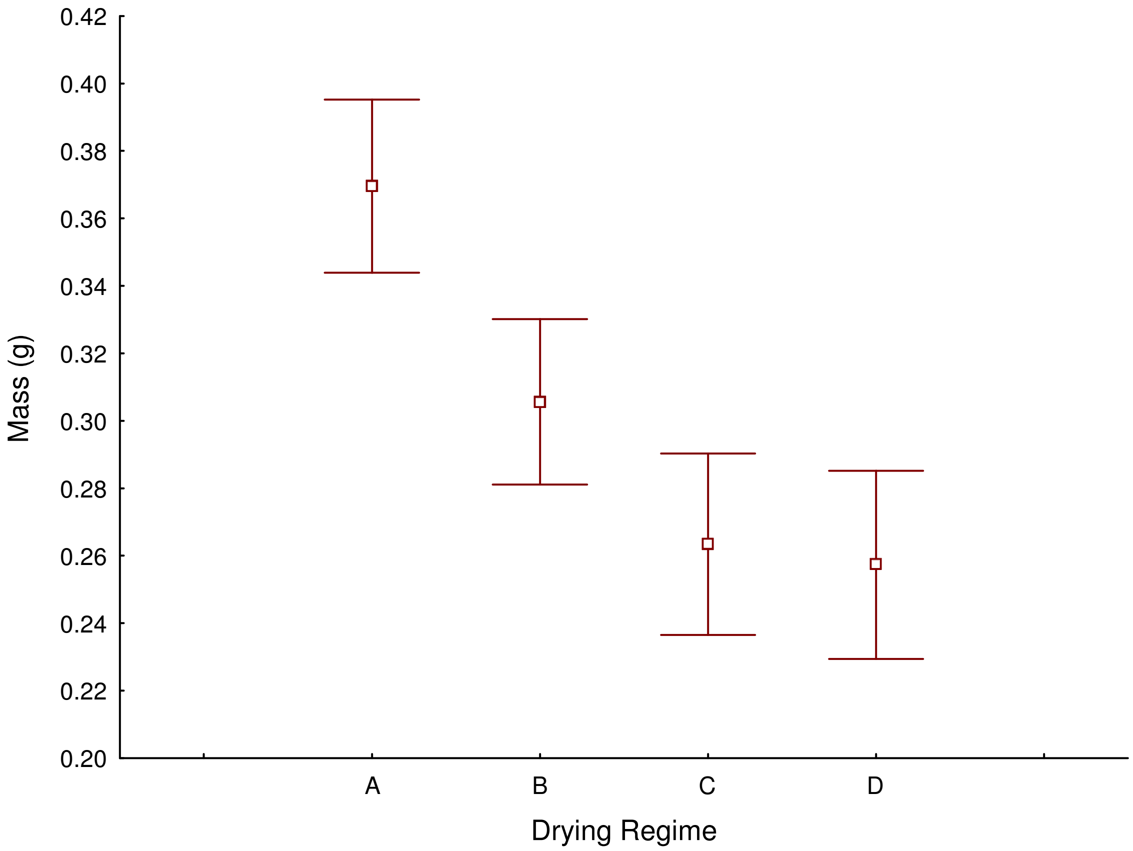

Figure 4.1. Mass (g) of *P. leucomystax* tadpoles in control (A) and drying (B, C, D) basins at high temperatures (29.75 °C) in Singapore. Boxes are means, bars are 95% confidence intervals.

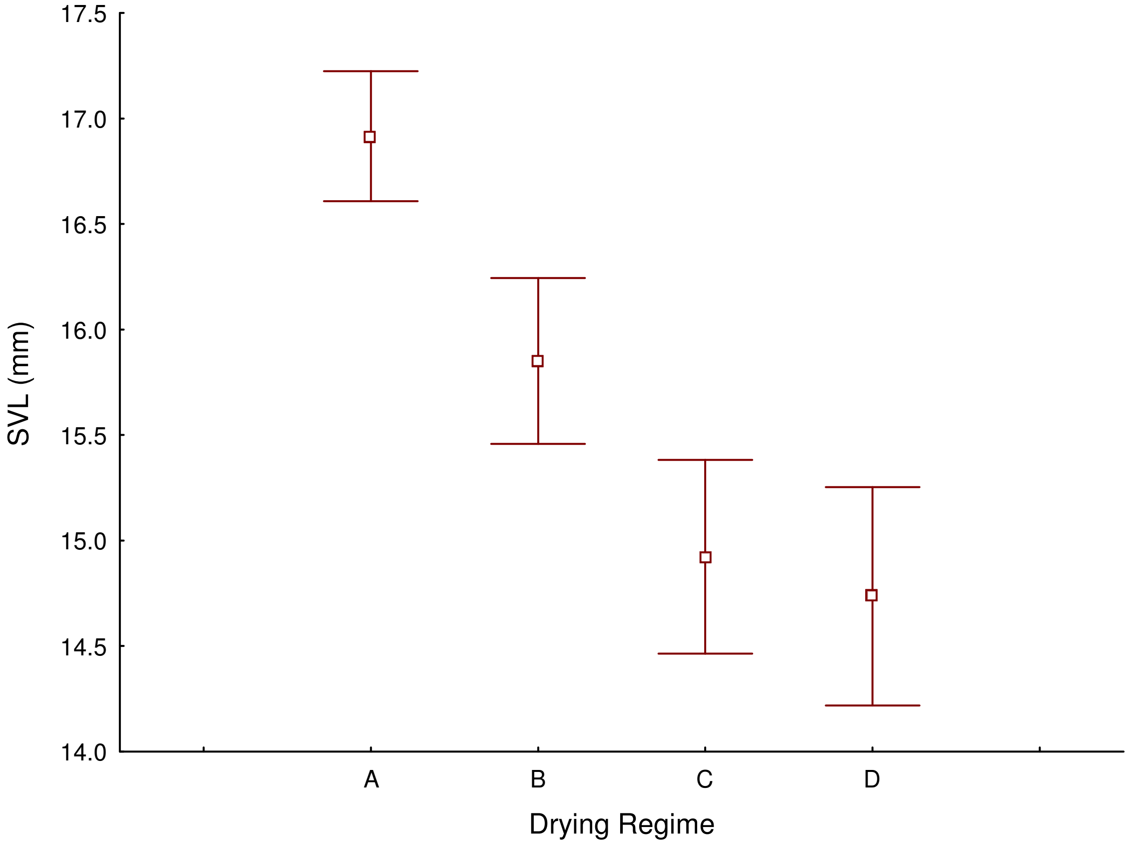

Figure 4.2. Size (mm) of *P. leucomystax* tadpoles in control (A) and drying (B, C, D) basins at high temperatures (29.75 °C) in Singapore. Boxes are means, bars are 95% confidence intervals.

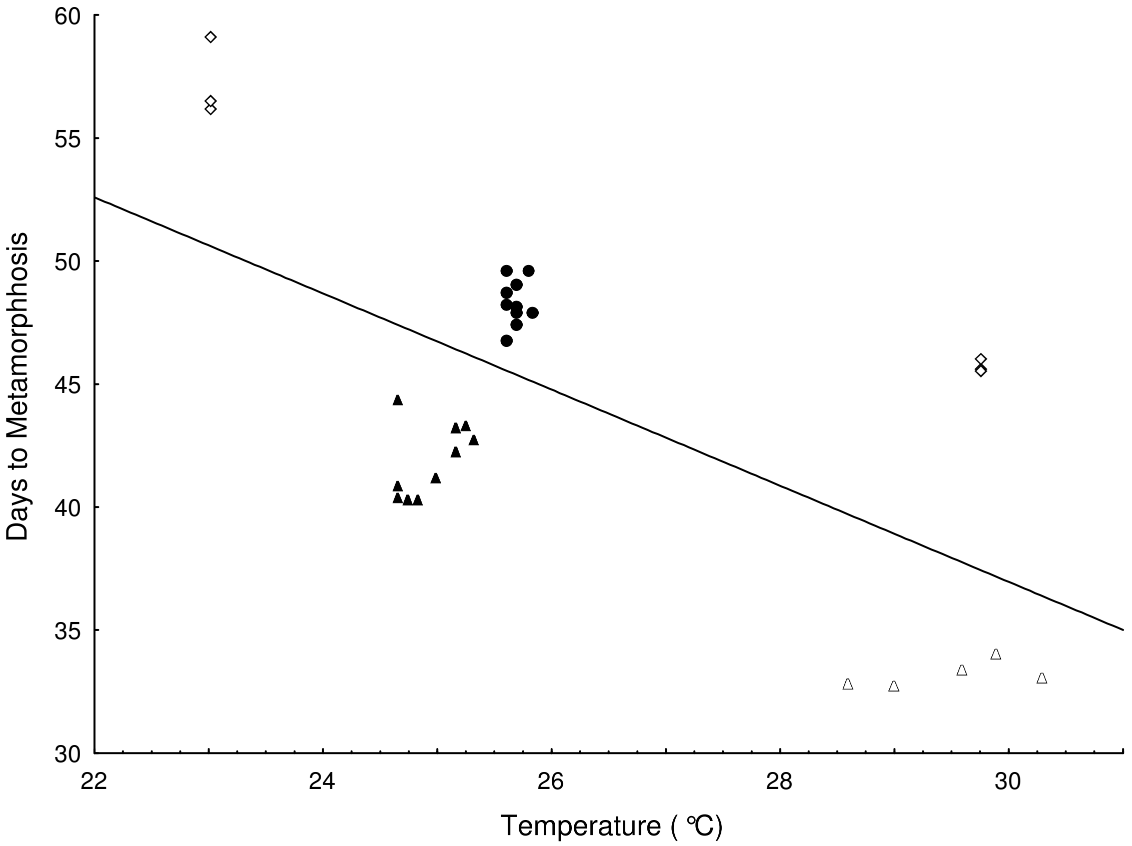

Figure 4.3. Time to metamorphosis for *P. leucomystax*. Each dot represents the mean value per basin. Symbols represent basins from a single clutch; open symbols are Singapore clutches, closed symbols are those from Thailand.

Appendix I

Ecology and behavior of *Polypedates leucomystax* (Anura: Rhacophoridae) in northeast Thailand

Abstract

Clutch size, egg size, time to metamorphosis, and numbers of marked adults are described for a population of the common rhacophorid *P. leucomystax* in a seasonal habitat in northeast Thailand. Body size is found to be larger than previously reported for this species on Borneo and in Peninsular Malaysia while the range of clutch sizes is smaller than those reported from Borneo and the Philippines. Clutch size varied little over the course of the season, whereas previous studies on other species in seasonal tropical environments show a significant reduction in clutch size over time. Mean time to metamorphosis for this population was longer than previously reported for this site. Potential reasons for geographic variation are discussed, including the likelihood that this taxon is actually a complex of cryptic sympatric and allopatric species.

Introduction

Polypedates leucomystax (Rhacophoridae) is among the most common of the nearly 800 species of anurans in southeast Asia (Iskandar and Colijn 2000), and among the most widespread, ranging from southern China south to Indonesia, and from India to the Philippines. Compared to most anurans of tropical southeast Asia, there are several reports on its habitat (Inger and Steubing 1997, Narins et al. 1998, Garcia-Rutledge and Narins 2001, Malkmus et al. 2002), calls (e.g., Christensen-Dalsgaard et al. 2002) and reproductive habits (Yorke 1983, Feng and Narins 1991, Malkmus et al. 2002). Despite this, relatively little is known of its population size, sex ratio, and variation in reproduction. In this paper, I present a detailed study of reproduction (clutch size and variation therein), time to metamorphosis, adult body size, and male site fidelity at a seasonal site (Sakaerat, Thailand) to allow for an evaluation of variation in these traits across the broad range of *P. leucomystax*. My observations permit the exploration of the adaptations seen in other tropical anurans that range from aseasonal to seasonal environments.

Polypedates leucomystax is a medium-sized tree frog (male SVL 37–64 mm, female SVL 57–89 mm) common in disturbed areas. It breeds in standing water such as natural ponds, cattle tanks, cisterns, and flower pots. Males form calling groups around standing water and females create foam nests above water in emergent vegetation or other suitable substrate (e.g., cistern wall). In some cases, multiple males will clasp a single female during a given breeding event (Feng and Narins 1991; personal obs.). In Singapore, eggs have been found in January, February, April, August, and September, and females had enlarged oviducts in all months in which they were captured (all months except May, July, and November, Berry 1964). Zeller (1960) reported that they can breed throughout the year but are inhibited by dry conditions in western Java. Yorke (1983) noted that near Kuala Lumpur, Malaysia, the females deposit 100–400 eggs in foam masses measuring about 10 cm in length on vegetation above ephemeral ponds. In Sabah, Malaysia, Malkmus et al. (2002) found larger clutch sizes, 150–900 eggs. Time to metamorphosis was reported as 4 weeks at Sakaerat, northeast Thailand (Heyer 1973), and 7 weeks in Kuala Lumpur (Yorke 1983).

Polypedates leucomystax is not a well-defined species and is probably a complex of cryptic sympatric and allopatric taxa. Narins et al. (1998) reported significant differences in calling habits of two genetically distinct sympatric morphotypes of *P. leucomystax* near Kuala Lumpur, but did not propose new nomenclature. Within the Sakaerat, Thailand, population, I found no significant differences in calls, and analysis of 500 bp of the 16S gene indicated variation of less than 1% (Chapter 2). Thus, all individuals encountered at Sakaerat are likely conspecific, but further studies of call, genetic, and morphological variation are necessary to determine how this population of *P. leucomystax* is related to others.

Materials and Methods

I conducted this study from April to September 2005 (except for call recordings as noted below) at Sakaerat Environmental Research Station (14.5º N, 101.92º E), Thailand. This forested region is 60 km south of Nakhon Ratchasima and 250 km NE of Bangkok on the northeastern slope of the central highlands at the edge of the Korat Plateau. I monitored five areas during the rainy season between 25 April and 4 September

2005. Annual mean rainfall is 1240 mm and there is a marked dry season from November through March. The 78 km^2 area is 70% dry dipterocarp and dry evergreen forest, with the remaining area comprised of grasslands, bamboo, and plantation forests (Heyer 1973, Lynam et al. 2006). Elevation ranges between 280 and 762 m above sea level at the site, but all study areas were below 600 m. I selected areas in different habitat types, including dry dipterocarp forest, dry evergreen forest, two types of pond systems, and a cleared area (Figure 1). For logisitical reasons, two areas (Tam Jong An and Tai Yee Pun) were added to the study at 3 and 5 weeks, respectively. For a full description of each study area, see Appendix 1.

Each area was surveyed 1–3 times per week between 19:00 h and 0:00 h. Frogs were detected by eye-shine and vocalizations. All adult *P. leucomystax* encountered were measured for snout-vent length (SVL) using a ruler, individually marked according to Hero (1989), and released at point of capture. Sex was determined by size, calling behavior, or presence of male nuptial pads. In this species, females are larger than males, so any individual between 35 and 60 mm, heard calling, or with nuptial pads was assumed to be a male. All individuals above 70 mm were assumed to be female (no individuals measuring 65–70 mm SVL were found). Voucher specimens were deposited in the Natural History Museum of Chulalongkorn University, Bangkok.

Areas were searched in the morning at least every other day for foam-covered egg masses. Within 36 hours of discovery, eggs were staged (Gosner 1960) and counted. If eggs were at or below stage 13, diameter of ten eggs was measured under a dissecting scope to the nearest 0.01 mm with digital calipers, averaged to give a mean egg size per clutch, and used to calculate clutch volume ((2/3*3.14*(radius of egg)^3)*clutch size). Eggs of this species lack jelly capsules, making it easy to obtain ovum size.

Time to metamorphosis for *P. leucomystax* was determined in 7 L round plastic basins, 35 cm in diameter. A single clutch was divided among eleven basins with thirteen tadpoles each when tadpoles were at developmental stage 25 (Gosner 1960). Tadpoles were given algae water once a week and fed commercial aquarium fish food (flakes and pellets). Basins were kept in a shade house with plastic roofing to prevent exposure to rainfall and direct sunlight. Nighttime water temperatures in cement basins in the forests of Sakaerat were 26.0 ± 0.2º C during the study period and that of the experimental basins was assumed to be similar. Mean daytime air temperature fell from 35.4º C in April to 30.0º C in September.

Calls were recorded between 5 July and 30 September 2006 using a Sony WM D6C Professional Walkman Cassette Recorder and an Audio-technica condenser. Calls were digitized using Windows Sound Recorder at 44.1 kHz. Audiospectrograms and oscillograms were produced and quantified by Raven Software 1.2.

Results

I marked 225 *P. leucomystax* (174 males, 31 females, and 20 juveniles) over 150 search nights. Males were commonly encountered and were heard calling even when no females were observed. Across weeks, the mean ± SE number of observations per night was 9.35 ± 0.87 (range = 3.5–17, n = 19). Mean ± SE male SVL was 55.05 ± 0.24 mm (range, 43–65 mm) and that for females was 79.0 ± 0.69 mm (range, 71–89 mm). Overall recapture rate during the study period was 75% for males and 29% for females (Table 1).

Most of the locations where males were encountered were spaced such that determining site fidelity to an accuracy of 20 m was possible for 98 of the 130 recaptured males. Of these 98, 72 (73 %) were only ever encountered within 20 m of their original capture point, and 26 (27%) were encountered at least one time more than 20 m from their original capture point. Rainfall did not influence total observations or new individuals per search night (regression $R^2 < 0.01$ for both; see Figure 2). After the first week of surveys, the number of new individuals per search night each week was 1.94 ± 0.28 (n = 19) which represented 23.3 ± 3.7% of the total observations per search night each week.

I counted eggs of 76 *P. leucomystax* clutches. Number of clutches found per week ranged between 1–15 (mean ± SE = 3.07 ± 0.76) and differed between weeks ($\chi^2 = 52.9$, $P < 0.01$). However, this variation between weeks is due to a single week in which 15 clutches were found, and was the week in which an additional area (Tai Yee Pun) was added to my searches. Number of clutches found in a given week did not change predictably over the study period and was not dependent on rainfall (regression $R^2 = 0.01$). An additional 10 clutches were not included in the analyses because ova could not be counted accurately due to their late stage of development or the presence of insect larvae which appeared to have eaten a large number of eggs. Mean ± SE clutch size for the entire study period was 454.45 ± 12.41 (range, 230–804; n = 76), mean ± SE egg diameter was 1.81 ± 0.02 mm (n = 37), and mean ± SE clutch volume was 1494.79 ± 75.11 mm^3 (n = 37). Not all clutches were used to calculate clutch volume because some clutches were found after eggs had passed developmental stage 13 (Gosner 1960). Dissections of preserved females and from females collected immediately after oviposition indicated that females contain eggs at different stages of development at any given time, but that all eggs of a given size class were oviposited at once (Chapter 3). (Sheridan 2008)

There was no difference in clutch size (ANOVA F-value = 0.76, $P > 0.70$) or clutch volume (ANOVA F-value = 0.60, $P > 0.85$) between weeks (Figure 3). Clutch size and clutch volume were unrelated to rainfall in a given week (regression $R^2 = 0.01$ and 0.02, respectively). Time to metamorphosis for tadpoles in basins with constant water levels was 41.66 ± 0.35 days and size (SVL) at metamorphosis was 19.40 ± 0.16 mm (mean ± SE).

I recorded several different call types. The most common was a single note ("normal") followed by 0 - 3 lower notes. Males also produced a "wreh-eh-eh" akin to a drawn out croak ("staccato"), a cackling sound ("cackle"), and a chuckle or laughing sound ("bark"; Figure 4). These corresponded to call elements recorded in western Thailand (Christensen-Dalsgaard et al. 2002). All call elements were heard to be combined in various ways to create a complex call repertoire (Figure 4). I analyzed call parameters of only the most common type of call, since this is believed to be the mating call (Christensen-Dalsgaard et al. 2002). Dominant frequency was 1197.1 ± 183.5 Hz, call duration was 58.5 ± 5.4 ms, pulse number (pulses/call) was 4.2 ± 0.3, and pulse rate (pulses/second) was 72.8 ± 4.2.

Discussion

I report several new findings regarding the reproductive demography and behavior of *P. leucomystax* from Sakaerat, Thailand. First, both the mean and the maximum SVL of *P. leucomystax* at Sakaerat were larger than the maximum SVL of *P.*

leucomystax in Borneo (Inger and Steubing, 1997: male maximum SVL, 50 mm; female maximum SVL, 75mm). The larger body size at higher latitudes is not surprising since many amphibians have been shown to follow Bergmann's Rule (Ashton 2002). Second, recapture rate for males was high, as was site fidelity (percentage of males only encountered within 20 m of their original capture point). This could indicate that males of this population are territorial. Christensen-Dalsgaard et al. (2002) frequently observed vocal interactions between males, in one case leading to wrestling between males. Although I never observed such interactions in the Sakaerat population, such interactions may also be indicative of territoriality. The number of new individuals found per search night was relatively low, but represented nearly a quarter of the total observations per search night. One possibility is that these individuals were present but not found during previous searches, but it is also possible that new individuals were constantly entering the local population.

Geographic variation in clutch size across the range of this species is uncertain. Although Although most reports on clutch size of *P. leucomystax* give only ranges and not mean values, Berry (1964) reported a mean clutch size of 315 (range 270–373) in Singapore, which is smaller than that found at Sakaerat (454). However, the range of clutch sizes at Sakaerat (230–804) overlapped with clutch size ranges from Borneo (150-900, Malkmus et al. 2002) and the Philippines (150-900, Taylor 1921, Villadolid and del Rosario 1930, Alcala 1962), indicating that within-site variation may swamp variation between sites.

Lack of variation in clutch size over time is surprising, as other frogs in seasonal tropical environments have shown decreasing clutch sizes over the course of the rainy season (Williamson and Bull 1995, Spieler and Linsenmair 1997, Lips 2001, Lampert and Linsenmair 2002). This could be due to the different variabilities, durations, or severities of the rainy seasons of each study location. For studies showing a decrease in clutch size as the rainy season progresses, rainfall generally decreases over the course of the season. At Sakaerat, the rainy season typically has five months of consistent rainfall (about 100 mm/month April - August) and then one to two months of extremely heavy rain (400 mm/month in September and October). Given the relatively short time to metamorphosis (about 42 d in basins kept in a shade house at ambient temperature), this 5–6 month rainy season may allow tadpoles to reach metamorphosis before larval habitat dries, even if eggs are laid in September or October. Temporal variation in breeding times of *P. leucomystax* at this site may reduce competition for resources among tadpoles and increase survivorship to metamorphosis.

Time to metamorphosis (42 d) was one and a half times longer than the 28 d previously recorded at Sakaerat (Heyer 1973) but nearly the same as the 49 d in Kuala Lumpur, Malaysia (Yorke 1983). It is important to note that Heyer's (1973) values are from non-experimental settings, and differences may be due to water temperature, food availability, and food type. Time to metamorphosis in my study was faster than the 70 to 119+ days reported for Philippine populations (Alcala and Brown 1956), and size at metamorphosis was slightly larger than the 14–17.5 mm reported for Philippine frogs (Alcala and Brown 1956). As with body size and reproductive measures, these differences in time to metamorphosis and size at metamorphosis could be due to temperature and rainfall differences between study sites, or could reflect the unrecognized taxonomic differences within this species.

Call types and diversity are similar to those reported from western Thailand (Christensen-Dalsgaard et al. 2002). Multiple call types also have been reported from northern Thailand (Garcia-Rutledge and Narins 2001) and Vietnam (Trepanier et al. 1999) but calls of *P. leucomystax* in Peninsular Malaysia, Borneo, and Bali appear to be less diverse (Matsui et al. 1986, Sanchez-Herriaz et al. 1995, Marquez and Eekhout 2006). A detailed summary of known call parameters from across the range of this species is given in Chapter 2.

Differences in life history variables between central Thailand and other populations of *P. leucomystax* are not surprising. Several studies on temperate amphibians show variation in clutch size, egg size, time to metamorphosis, and size at metamorphosis across a species' range (Meeks and Nagel 1973, Kaplan 1980a, Berven 1982a, Riha and Berven 1991a, Bury and Adams 1999). However, no consistent trends of increases or decreases in these traits across latitude emerge from published data on temperate species and in general, we are still unable to predict variation in reproduction across latitudes for tropical species. Detailed studies on the reproductive ecology and behavior of tropical species such as *P. leucomystax* illustrate variation in reproduction across the range of a tropical species, and provide a baseline against which future changes can be measured.

Acknowledgements

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Supplemental Information

Detailed Description of Study Areas

Dry dipterocarp forest.—This area consisted of 20 cement cisterns located 1–5 m from the main road in the deciduous dipterocarp forest at Sakaerat. These cisterns are round, 0.75 m in diameter, 0.32 m deep, and held water at depths of 0.1–0.3 m during the study period. This area occurs between km 1 and km 2.7 of the station road, with km 0 located at the junction of Highway 304 and the station road (Figure 1).

Dry evergreen forest.—This area consisted of 34 cement cisterns, 0.75 m in diameter, in the evergreen forest at Sakaerat. Water depths were not less than 0.25 m. Cisterns were 1–5 m from the main road, except for two cisterns located 10 and 20 m from the road. The evergreen forest extends west from km 3 along the station road (Figure 1).

Tam Jong An (Cobra Cave Pond) .—This area comprised 70 m of an ephemeral stream that runs parallel to the main road through Sakaerat, about 700 m to the north of the main road in the evergreen forest. The western end of the area was a semi-permanent pool of water at the base of a 3 m waterfall. As the two years prior to the study year were drier than normal, rainfall was quickly absorbed by the ground, the stream was not flowing during the study period, and the pool shrank from 4 x 20 m, to 3.5 x 12 m. Water in the remaining 65 m of stream bed was restricted to small ephemeral pools in rock crevices. The stream was bounded on the north and south by steep banks about 6 m apart. Note that the first date this area was sampled was 9 May 2005.

Dam Pond.—This area was an ephemeral pond covering approximately 75 m^2 created by a 5 m dam located approximately 100 m north of the main road near the km 5 marker (distance measured from Highway 304 along the main road through Sakaerat) in evergreen forest. The bottom of the pond was covered with herbaceous vegetation during this study period and contained standing water on only 2 survey nights. No eggs were ever found on this transect, but amplectant pairs were found twice. This is not the same area as the “dam stream pond” referred to in Heyer (1973).

Tai Yee Pun (Thai Japanese ReAfforestation Project) .—This area was separated from those detailed above by approximately 7 km, and was a cleared area used as a plant nursery, approximately 120 m on a side. There were 20 cement cisterns 0.8 m in diameter along the road, and four 15 x 25 m nurseries covered by shade cloth. Two nurseries contained 6 rectangular 0.8 x 2 m cisterns 1m deep, with variable volumes of water. Depth of water in these cisterns varied between 0.1–1 m. A third nursery had four of these cisterns and the fourth nursery had four standard 0.75 m round cisterns. Note that the first day this area was sampled was 25 May 2005.

Table A1.1. Overall recapture rate for adult *Polypedates leucomystax* at Sakaerat, Thailand, between 25 April and 4 September 2005.

	Number of recaptures				
	0	1–2	3–5	6–10	11+
Males	44	52	39	27	12
Females	22	8	0	1	0

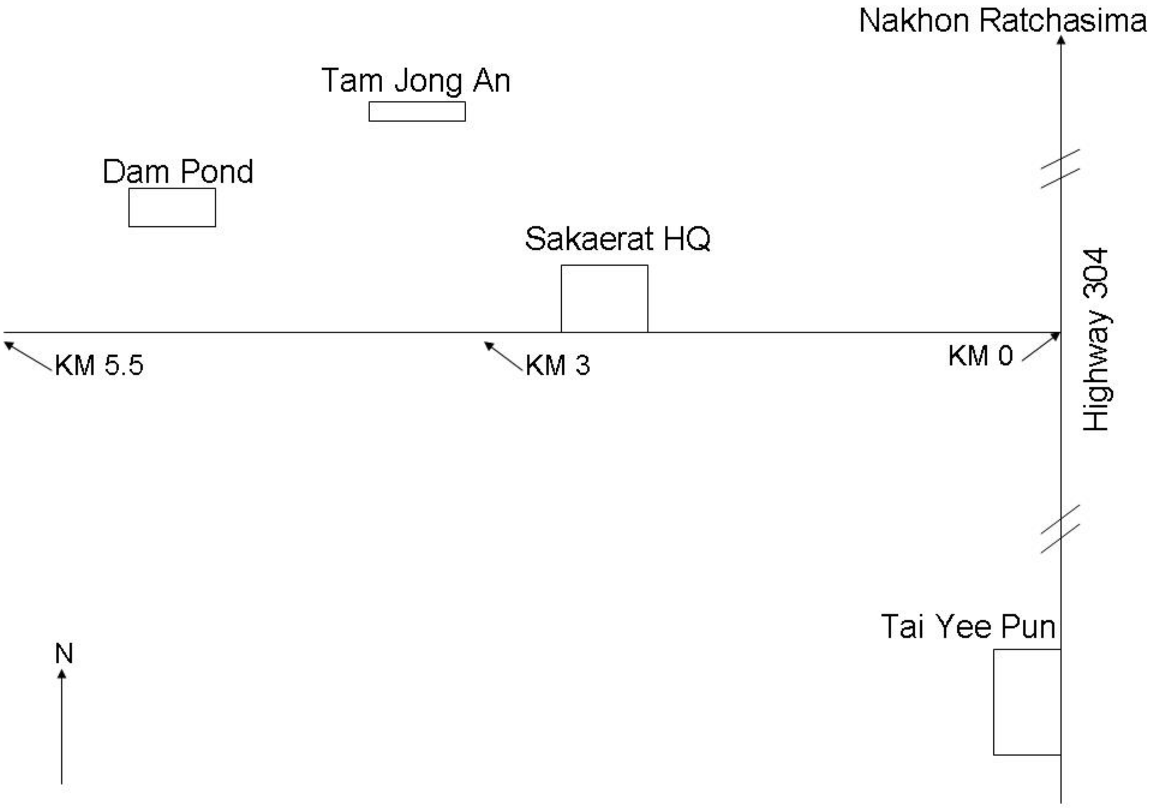


Fig. A1.1. Schematic diagram of study areas of *Polypedates leucomystax* breeding at Sakaerat, Thailand. Image not to scale. Tai Yee Pun is 7 km south of Sakaerat headquarters.

Fig. A1.2. Number of individual *Polypedates leucomystax* observed per search night (solid line and squares) and number of new individuals per search night (solid line and triangles) at Sakaerat, Thailand. Rainfall (mm, dashed line and diamonds) is also shown.

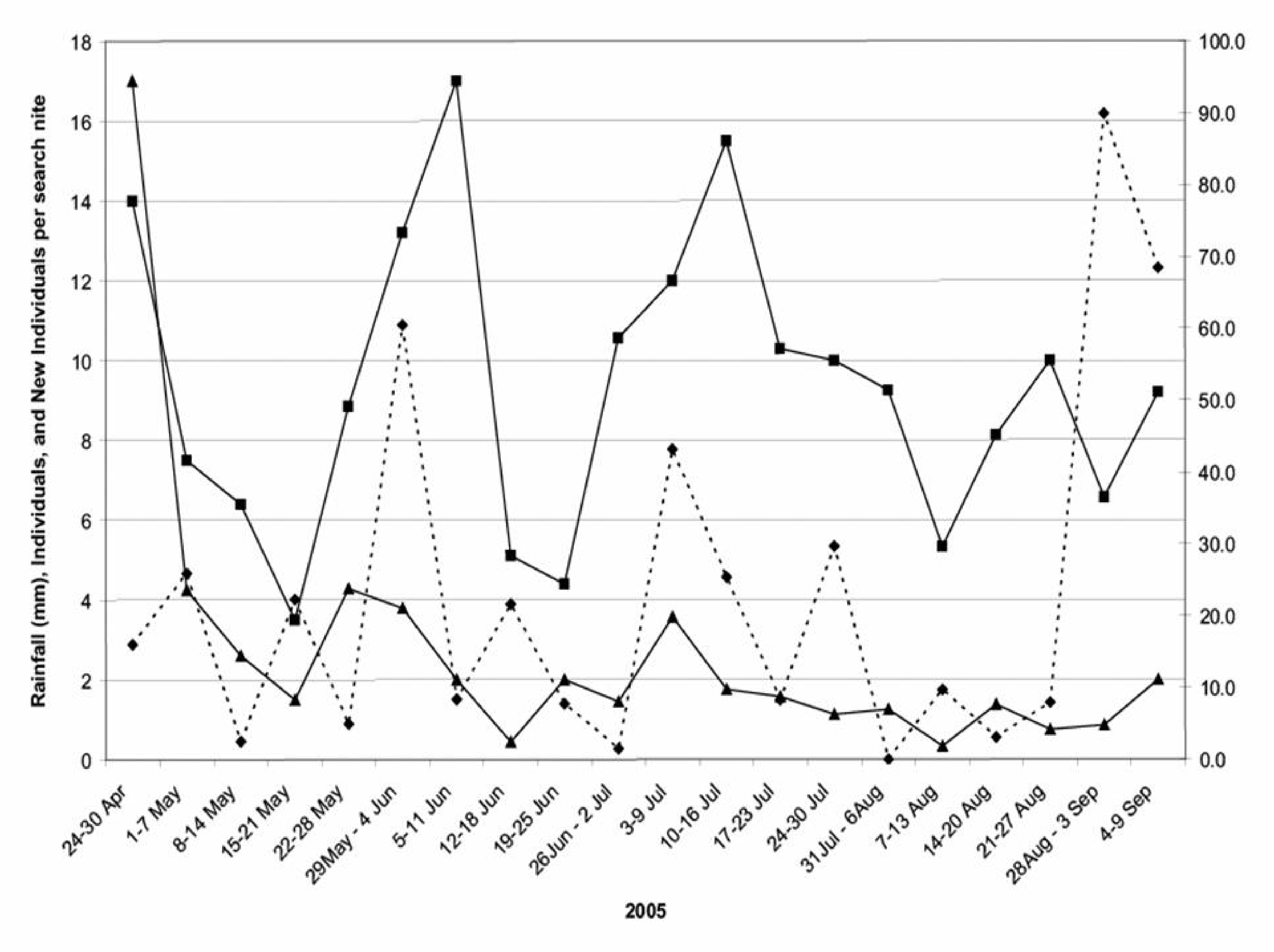
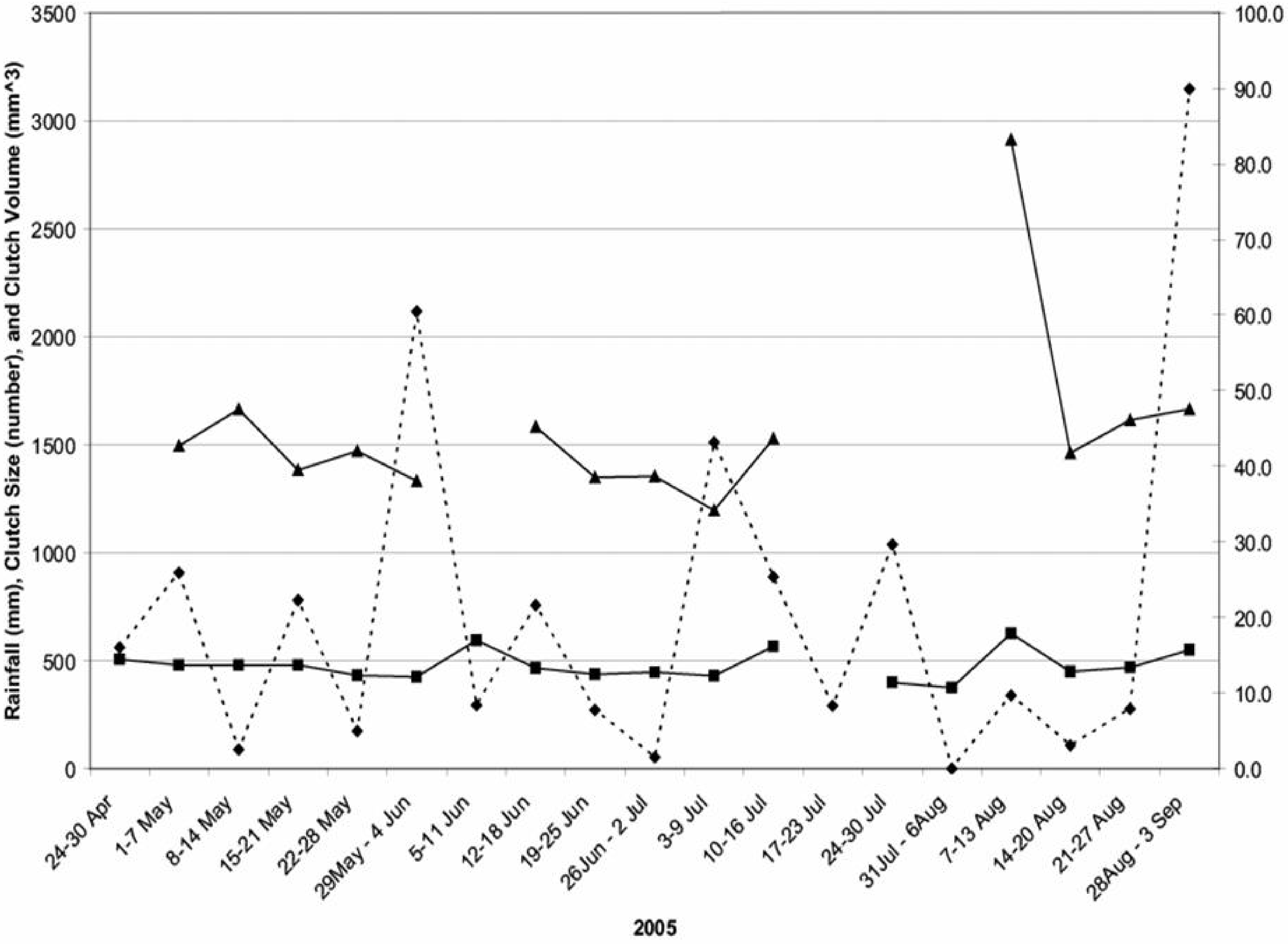

Rainfall (mm), Individuals, and New Individuals per search nite
18
16
14
12
10
8
6
4
2
0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
24-30 Apr
1-7 May
8-14 May
15-21 May
22-28 May
29May - 4 Jun
5-11 Jun
12-18 Jun
19-25 Jun
26Jun - 2 Jul
3-9 Jul
10-16 Jul
17-23 Jul
24-30 Jul
31 Jul - 6Aug
7-13 Aug
14-20 Aug
21-27 Aug
28Aug - 3 Sep
4-9 Sep
2005

Fig. A1.3. Mean clutch size (number of eggs, solid line and squares) and mean clutch volume (mm^3, solid line and triangles) of *Polypedates leucomystax*, and rainfall (mm, dashed line and diamonds) at Sakaerat Environmental Research Station, Thailand.


Rainfall (mm), Clutch Size (number), and Clutch Volume (mm^3)
3500
3000
2500
2000
1500
1000
500
0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
24-30 Apr
1-7 May
8-14 May
15-21 May
22-28 May
29May - 4 Jun
5-11 Jun
12-18 Jun
19-25 Jun
26Jun - 2 Jul
3-9 Jul
10-16 Jul
17-23 Jul
24-30 Jul
31Jul - 6Aug
7-13 Aug
14-20 Aug
21-27 Aug
28Aug - 3 Sep
2005

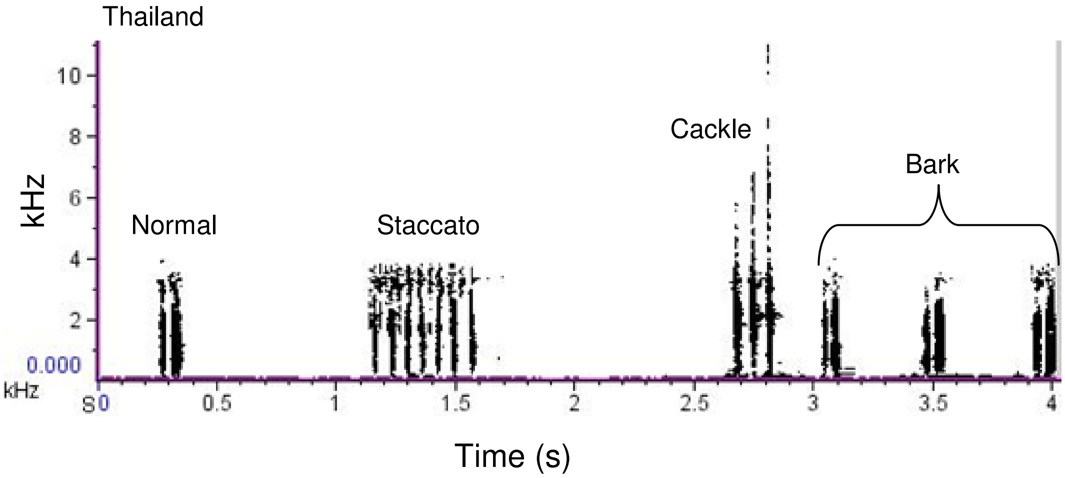


Figure A1.4. Spectrogram of calls made by *Polypedates leucomystax* at Sakaerat, Thailand. Call elements are from a single individual but did not occur consecutively; call elements were cut and pasted from a 5 minute recording.

Appendix II

Parental Care in *Chiromantis hansenae* (Anura, Rhacophoridae)

Abstract

We report a new case of parental care in a *Chiromantis* species from Northeast Thailand. This is the first report of parental care in this genus. Our observations indicate that *C. hansenae* adults sit on the clutches approximately 72% of the time, both day and night. This clutch attendance may protect eggs from desiccation, parasitic flies, or fungal infection, but we were unable to conduct removal experiments to test these hypotheses. In no cases did we observe adults actively guarding against known predators such as skinks. This species is unusual amongst arboreal breeders with parental care in having a high rate of egg attendance, relatively large clutch sizes (235.75 ± 19.78, $n = 4$), and a short period before hatching (4–6 d).

Introduction

Of more than 5500 species of anurans, only about 10% are known to exhibit parental care (Duellman and Trueb 1994, Crump 1995, Reynolds et al. 2002), which has evolved multiple times in 17 of the 27 traditionally recognized anuran families (Crump 1996). Common modes of anuran parental care include egg attendance, egg transport, tadpole attendance, tadpole transport and tadpole feeding (Crump 1996). Egg attendance, the most common form of parental care, occurs in 14 families and 49 genera (Lehtinen 2003) and requires the attending adult to remain in direct physical contact with the egg mass at a fixed location. This is usually the oviposition site, which may be terrestrial or aboreal (Crump 1995, Lehtinen and Nussbaum 2003). Egg attendance can reduce egg mortality by protecting against predators, desiccation, pathogens, and fungal infections (Green 1999, Bickford 2004).

In mainland Southeast Asia there are approximately 50 known genera of frogs (Iskandar and Colijn 2000), only one of which has been recorded exhibiting parental care *(Philautus, Kanamadi et al. 1996)*. Within this genus, reports of species exhibiting parental care remain uncommon (1 of 141 species), and it remains unknown whether parental care is widespread: the only reported case comes from India. Here, we report the first record of parental care in a mainland Southeast Asian species, *Chiromantis hansenae*.

Chiromantis hansenae is a tree frog whose known range encompasses eastern Thailand and Cambodia (Chan-Ard 2003b, but see Stuart et al. 2004). This species breeds in small pools or ponds in lowland forests (Taylor 1962, personal obs.), and green eggs in a gelatinous mass are deposited on vertical surfaces of plants and boulders (personal obs.). Egg masses are usually deposited on plants and boulders above lentic water bodies, but are occasionally deposited above dry ground that fills with water after heavy rain. Around Gosner (1960) developmental stage 25, the egg capsule breaks open and tadpoles drop into the water to complete development (Mode 18; Duellman and Trueb, 1994). This species was formerly in the genus *Chirixalus* but was recently moved to *Chiromantis* (Frost et al. 2006), and there is a suggestion that it is a junior synonym of *C. vittatus* (Wilkinson et al. 2003, Stuart and Emmett 2006). However, we follow Frost et al. (2006) in continuing to recognize *C. hansenae* as a valid species.

Materials and Methods

Observations were made on the parental care behavior of *C. hansenae* from July to October 2006 during the rainy season at Sakaerat Environmental Research Station (SERS), Thailand (14º 30' N, 101º 55'E). This forested region is 60 km south of Nakhon Ratchasima and 250 km NE of Bangkok on the northeastern slope of the central highlands at the edge of the Korat Plateau. Mean annual rainfall is 1240 mm and there is a marked dry season from November through March. The 78 km^2 research station is 70% dry dipterocarp and dry evergreen forest, with the remaining area comprised of grasslands, bamboo, and plantation forests (Heyer 1973, Lynam et al. 2006). Elevation ranges from 280–762 m above sea level, but our observations were all made below 600 m.

We initially discovered a *C. hansenae* sitting on a clutch of eggs attached to the vertical surface of a tree trunk overhanging a pond in the Tam Jong An area (Cobra Cave Pond) of SERS on 4 July 2006. Later that month, several more clutches were found attached to the vertical or near-vertical sides of boulders above water. In September, when rainfall greatly increased, we observed numerous clutches attached to vegetation in ephemeral pond areas that filled with water over the course of several weeks and we conducted observations on 23 clutches for 1–5 days each at a nearby area known as the Dam Pond (Appendix 1).

We conducted visual surveys of the pond area, noting the position of egg clutches and the presence or absence of an attending adult. We determined the sex of the adult visually by its size, females being slightly larger (25 mm) than males (20–22 mm), and in all cases, it appeared to be females attending the clutches. However, since males lack nuptial pads or vocal sacs (which would make them easily distinguishable from females) and we never confirmed the sex of the attending adults by dissections, we present this as a report of parental care and refrain from calling it maternal care until further confirmation is available. Upon detection of the clutches, we began regular observation periods of up to five hours, but usually two hours each, during which clutches were checked at least every fifteen minutes for the presence and behavior of the adult. All observations took place between the hours of 06:00 and 01:00 h. Observations continued daily until the tadpoles hatched, or the clutch was abandoned or submerged by rising water. Binoculars and a telescope were used during the day to observe the clutches, and at night presence or absence was noted with a quick scan of the clutch using a 6 V headlamp. Observations were made from a distance of 10 m to prevent disturbance, but closer approach was sometimes necessary to accurately locate and identify the clutch.

For our analyses, we used only clutches for which an adult was actually seen on the clutch at some stage and for which we had more than five hours of observation time.

Five hours was chosen as the cut-off point, as it seemed sufficient observation time to determine patterns of clutch attendance. Observation times below five hours ranged from 2.25–4.25 h, and only two nests never had an adult present. Because we could not ascertain why these two nests did not have an attending adult, we omitted them from our analyses of clutch attendance. As the number of hours of observation varied for each clutch, we calculated the proportion of observation hours the adult spent on the clutch for each clutch and pooled the data to get a mean and standard error of rate of egg attendance for the population.

Results

We observed a total of 23 clutches between 4 July and 19 September 2006. Thirteen were seen to have an adult in attendance at some stage and were observed for a total of more than 5 hours. These 13 clutches were used to calculate proportion of time adults attended the eggs. Of the remaining 10 clutches, two were never observed to have adults in attendance, and eight clutches were observed for less than five hours due to being eaten ($n = 1$), submerged by rising water levels ($n = 3$), disappearing from one observation period to the next ($n = 2$), or discontinued observations due to lack of manpower ($n = 2$).

Attending adults were observed to sit with the posterior half or third of their ventral surface in contact with eggs (Figure 1). This usually covered the top half or third of the egg mass, but occasionally all the eggs would be covered by the entire ventral side of the adult. Adults spent 72 ± 7 % (range = 50–100%) of observed hours on the clutch. The percentage of time on the nest was lower during daylight hours (64 ± 10 %; range =

0–96%) than at night (80 ± 10 %; range = 0–100%), but this difference is not significant (arcsin test of percentages t = -0.91, df = 144, P = 0.36). In no cases were adults observed to attack potential predators such as reptiles, arachnids, or other invertebrates, and in one case, we observed a skink (Scincidae) eating the eggs while the adult sat nearby. We have no data on what adults eat during this time, or what they do when they are off the clutch.

Of the 13 clutches observed, three (23%) were observed to hatch, nine (69%) were submerged by rising water before the tadpoles hatched, and one fell off the vertical rock face and observations were discontinued. We regularly observed the adult returning to the clutch after moving off when people, skinks, or spiders approached. Eggs placed in basins in a nearby shade house developed normally without an attending parent. Clutch size at this location was 235.75 ± 19.78 (n = 4) and the period before hatching was 4–6 d.

Discussion

We speculate that one function of egg attendance in *C. hansenae* is protection against desiccation. *Chiromantis hansenae* clutches are often laid on vegetation and rocks exposed to hours of direct sunlight daily. While no experiments tested the effect of removing the attending adult, a clutch found without an adult dried up within 24 hrs. By contrast, two unattended clutches collected and left in a shade house, not exposed to direct sunlight, developed normally with a high success rate. As eggs developed normally in the shade house, parental care is unlikely to be necessary for development, but likely helps keep the eggs moist during hours of direct sunlight in the natural habitat. Adults often moved off of the clutch when another organism (e.g. skink, spider) approached so clutch attendance was not observed to serve a significant anti-predator function.

The purpose of the nocturnal attendance remains unknown, but could provide protection against parasitic fly infestation. In a sympatric rhacophorid with arboreal clutches (*Polypedates leucomystax*), infestation of foam clutches by parasitic flies is extremely common (J. A. Sheridan, unpubl.). The presence of the adult on the clutch may also prevent fungal infection. Other anurans are known to attend their eggs to prevent fungal infection (Salthe and Mecham 1974, Simon 1983), which may be occurring with *C. hansenae*, but we were unable to test this hypothesis.

Bickford (2004) suggested desiccation is a selective force shaping parental care behaviors in arboreal habitats. The eggs of an undescribed *Oreophryne* species (an arboreal breeder) suffered from desiccation when the parent was removed, and the eggs were visibly larger (more hydrated) after the parent had been in contact with them overnight (Bickford 2004). Similarly, some centrolenids and eleutherodactylids in Central America and the Caribbean are known to provide a hydrating, rather than an antipredator function (Taigen et al. 1984, Savage 2002). As with *Eleutherodactylus coqui* in Puerto Rico (Taigen et al. 1984), *C. hansenae* adults were observed to occasionally move their limbs over the clutch and to alter their body position, suggesting that one function of the egg attendance was prevention of desiccation. This could also serve to coat the eggs with antifungal secretions, as has been shown in both salamanders (Stebbins 1954, Salthe and Mecham 1974) and frogs (Salthe and Mecham 1974, Simon 1983).

New reports and descriptions of parental care are valuable in bettering our understanding of its evolution as well as its costs and benefits (Jungfer and Weygoldt 1999, Vences et al. 2003, Bickford 2004). Parental care in anurans is rare, but widespread across taxa (Duellman and Trueb 1994). Egg attendance is the most basic and the most common form of parental care (Bickford 2004) and is usually associated with terrestrial breeders with small (< 100) clutch sizes and prolonged embryonic periods (> 20 d) (e.g., Woodruff 1977, Duellman and Trueb 1994, Lehtinen 2003). The clutch sizes of several other arboreal egg-layers with parental care do not exceed 75 (e.g., *Eleutherodactylus spp.*, *Dendrobates spp.*, Savage, 2002; *Hylophorbus rufescens*, *Oreophryne sp.*, Bickford, 2004; *Philautus variabilis.*, Kanamadi et al., 1996) and most species with parental care that produce more than 200 eggs have small aquatic eggs (Crump 1995). *Chiromantis hansenae*, however, is an arboreal breeder with a comparatively large clutch size (235.75 ± 19.78, n = 4), and a short duration of the egg stage before hatching (4–6 d). Additionally, *C. hansenae* displays extensive egg attendance (day and night) over the short time to hatching, leaving only for short periods of time, presumably to forage and rehydrate. Other arboreal frogs with parental care exhibit irregular attendance (Savage 2002) or show exclusively nocturnal attendance (Bickford 2004). These features of the reproductive biology of *C. hansenae* make it unusual among anurans exhibiting parental care. Further observational and experimental studies are necessary to fully investigate the sex of the attending adult and the function of egg attendance behavior in *C. hansenae*.

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Figure A2.1. *Chiromantis hansenae* female attending eggs.

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