

## Influence of Some Environmental Parameters on Some Frog Populations and their Parasitic Fauna

Wahab A Rahman\* and Zatil Shakinah

School of Food Science and Technology, University Terengganu Malaysia, 21030 Kuala Terengganu, Malaysia

### Abstract

Wild animals, including frogs are usually infected with several species of parasites. Frogs are important for a variety of reasons. Firstly, they control populations of insects and secondly they can act as indicator species to the environment. Studies have shown that frogs can harbor many species of parasites. Worldwide, there were numerous researches carried out on frog parasites but few had been reported from Malaysia. From this study, 300 frogs from 14 species were collected from eight study sites: *Duttaphrynus melanostictus*, *Phrynooidis aspera*, *Hylarana erythraea*, *H. labialis*, *H. nigrovittata*, *Polypedates leucomystax*, *Fejervarya cancrivora*, *F. limnocharis*, *Limnonectes blythii*, *L. paramacrodon*, *L. ibanorum*, *L. ingeri*, *Microhylabutleri*, and *Kaloulapulchra*. Eleven (11) helminthic parasites (*Heterakis* spp., *H. vesicularis*, *Trichostrongylus* spp., *Pharyngodon* spp., *Ascaris* spp., *Oswaldocruzia* spp., *Rhabdias* spp., *Glypthelmins staffordi*, *Diplodiscusm sacculosus*, *Manodistomum* spp., and *Macracanthorhynchus* spp.), eight blood parasites (a rickettsia, *Hepatoozon* spp., *Haemogregarina* spp., *Lankesterella* spp., *Trypanosoma loricatum*, *T. rotatorium*, *T. chattoni*, and microfilaria) and one protozoa (*Nyctotherus* spp.) were recorded from frogs. There were correlations between the presence of helminthic parasites and distribution of rainfall, as well as between blood parasites and Biochemical Oxygen Demand (BOD) of water.

**Keywords:** Frogs; Peninsular Malaysia; Parasites; Correlations; Environmental parameters; pH; Rainfall; Biological oxygen demand

### Introduction

Taxonomists have described more than 5,000 species of frogs [1]. Disease has been a factor in the decline of amphibian populations worldwide, although other factors including habitat loss and fragmentation, chemical pollution, climate changes, introduction of exotic species, increased ultraviolet radiation, and natural pollution have also been responsible for the decline [2]. Malaysia is a country with an equatorial climate with high annual humidity ranging from 60% to 90% and rainfalls of 2000 to 3000 mm, resulting with a rich diverse biodiversity of wildlife [3]. Malaysia harbors about 165 species from six families of anurans of which more than 150 species are found in Borneo [4], 107 species in Peninsular Malaysia, including 26 species in Penang Island [5].

In Malaysia there had been some reports of helminthic parasites recovered from frogs. Some nematodes had been described from the intestines of frogs [6,7]. Other investigators had described several species of trematodes [8] and one species of cestode [9], also from the intestine of several species of frogs. In Malaysia, reports of blood parasites from frogs are scanty. Yong and Richards [10] reported a new trypanosome, *Trypanosoma hosei*, from a forest frog, *R. hosei*. Rahman reported the presence of micro filarial worms, and *Trypanosoma* spp. from the frog hosts of *B. melanostictus*. At the present time, the emergence and re-emergence of various parasitic organisms and pathogens are caused as a consequence of environmental changes in the environment. Therefore, the effects are determined by relationship between ecology, hosts and other organisms [11]. As reported by Ibrahim et al. [9], environmental factors can contribute to amphibians' parasitism. It is because the animals are exposed to the surrounding harboring this parasite. Population of amphibians worldwide are declining in the recent surveys, and generally it is because of environment changes [2,9,12]. The significant decline in amphibian populations has been observed all over the world [12]. Somehow, there is a familiar occurrence where the trends of losses appear to be

surpassing the usual population changeability [10,12]. Several causes of this problem have been recognized and the environment is the main factor contributing to decline in number of amphibians, for example, ultraviolet (UV) radiation, climate changes [13], pathogens [14], introduction of exotic species [2], pollution, and also pesticides [15].

Environmental changes would probably create an increase in vulnerability of the host in the parasites instead of direct lethal effect to amphibian populations. Previous studies specified that several kinds of stresses comprising the UV radiation were able to cause immunosuppression in mammals. A similar study was conducted on amphibians and the result showed that the ultraviolet radiation is not interrelated with immunosuppression in amphibians. But, high pH and coldness [16] were verified to initiate alterations in the immune system of frogs. Development of parasites is influenced by environmental factors. In association with this, temperature causes modified development of the parasites in host [17]. Besides, environmental stress has an effect on parasites as well. Primarily, rate of parasitism might increase by the cause of pollution either expanding the host vulnerability or increasing the profusion of intermediate hosts and vectors [18].

### Materials and Methods

#### Study sites

Penang Island, Peninsular Malaysia is located near the northwestern coast of Peninsular Malaysia (5°24.0'N, 10014.0'E). Samplings were

\*Corresponding author: Wahab A Rahman, School of Food Science and Technology, University Terengganu Malaysia, 21030 Kuala Terengganu, Malaysia, Tel: 09-6684995; E-mail: [arawahab@umt.edu.my](mailto:arawahab@umt.edu.my)

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carried out during May 2010 to May 2011 from eight (8) areas, randomly selected from various parts of the island.

### Sample collections

Collection of frogs was carried out at night between 2000-2300 h using torch lights and head lamps. Besides using butterfly nets, frogs were also caught by hand, especially for those frogs found inside rocks cervices. Captured frogs were kept alive in aquarium boxes and transported back to the laboratory. In the laboratory, the frogs were placed in the sink with running tap water to avoid dehydration of the frog's skin. Species of frogs were identified according to the descriptions of Inger and Stuebing [4]. Subsequently, frogs were killed by using low doses of chloroform. Frogs were then weighed using a top pan digital electronic balance (SHIMADZU Brand Japan).

### Parasite recovery

Each frog was examined for external abnormalities before the animal was euthanized. The stomach, intestines, rectum, heart and liver were recovered and examined for end parasites. Blood samples were drawn from the heart. The frogs were laid on the dissecting tray with belly facing upwards and their legs were pinned with pins. The first incision was done through the top layer of their skin by using a blade scalpel without damaging the organ. The incision was made vertically down the belly. Next, the incision was done laterally across the hind legs and across the throat as well. Then the two lateral incisions were connected together with a vertical incision down the belly. The flaps were opened and pinned down. Subsequently, the same incision was done on the last layer of frogs' skin by twisting the scissor to avoid cutting organ under the chest bones. The flaps were opened and all fats were removed from the organs. All stomachs, intestines and rectums were removed from the frogs and placed separately into different petri dishes. Later, the organs were opened longitudinally using a forceps and scissor. Next, 10 ml of water was poured into the petri dishes. The contents were observed under the dissecting microscope. Helminthes were picked and kept in 70% ethanol glass bottles and labeled.

### Identification of helminthes

All nematodes, trematodes and acanthocephalans were cleared in lactophenol. They were then killed in hot 70% ethanol. Nematodes were preserved in 70% glycerine alcohol, whereas trematodes and acanthocephalans were preserved in 70% ethanol. All helminthes were identified as according to the descriptions of Yamaguti [19].

### Environmental parameters

**pH of water:** Water samples in volumes of 500 ml were collected seven times from each sampling site and kept in different bottles. Samples were brought to the laboratory and analysis of pH was done within 24 h. The values of pH were obtained by using a probe and meter (Hach-SensIon6 Brand).

**Biochemical oxygen demand (BOD) and temperature of water:** A Portable Bench-top DO meter (YSI model) was used to measure BOD of water. Seven readings were taken each for each sample and a mean obtained. Water temperature was also obtained using the same apparatus.

**Rainfall distribution:** The rainfall distribution data was obtained from Meteorological Department Malaysian, Ministry of Science and Environment.

### Wet blood smear

A drop of fresh blood was put on a glass slide. Then the blood was

mixed using the tip of a cover slide and immediately observed under compound microscope.

### Thin and thick blood smear

Thin and thick blood smears were carried out. Thin blood smears were fixed in methanol and stained with Giemsa. For the thick blood smears, the slides were directly stained with Giemsa without the methanol. The slides were then examined under the compound microscope.

## Results

### Species composition of frogs

Fourteen species of frogs from the five families were identified: *Duttaphrynus melanostictus* and *Phrynooidis aspera* (Family Bufonidae); *Fejervarya cancrivora*, *F. limnocharis*, *Limnonectes blythii*, *L. paramacrodon*, *L. ibanorum* and *L. ingeri* (Family Dicroglossidae); *Microhyla butleri* and *Kaloula pulchra* (Family Microhylidae); *Hylarana erythraea*, *H. labialis* and *H. nigrovittata* (Family Ranidae); and *Polypedates leucomystax* (Family Rhacophoridae) (Table 1). *Duttaphrynus melanostictus* from Bufonidae family seemed to be the most dominant frog species in Penang Island, followed by *H. erythraea* from the Ranidae family. The least common species of frog was *L. ibanorum* from the Dicrosidae family.

### Composition of helminthes

A total of 8058 individual helminthes were collected comprising of 43.7% nematodes, 36.8% trematodes and 19.5% acanthocephalans (Table 2). The most common nematode species were *Heterakis* spp., *Heterakis vesicularis*, *Pharyngodon* spp., *Trichostrongylus* spp., *Ascaris* spp., *Oswaldocruzia* spp. and *Rhabdias* spp. The three species of trematodes were *Glypthelmins steffordi*, *Diplodiscus sacculosus* and *Manodistomum* spp. One species of acanthocephalan was collected and identified as *Macracanthorhynchus* spp. The frog species *Duttaphrynus melanostictus* seemed to be infected by almost all species of parasites

Frog Species	Total number of frog caught
<b>Family Bufonidae</b>	
<i>Duttaphrynus melanostictus</i>	90 (27.0)*
<i>Phrynooidis aspera</i>	47 (14.1)
<b>Family Ranidae</b>	
<i>Hylarana erythraea</i>	71 (21.3)
<i>Hylarana raniceps</i>	20 (6.0)
<i>Hylarana nigrovittata</i>	2 (0.6)
<b>Family Rhacophoridae</b>	
<i>Polypedates leucomystax</i>	16 (4.8)
<b>Family Dicroglossidae</b>	
<i>Fejervarya cancrivora</i>	10 (3.0)
<i>Fejervarya limnocharis</i>	25 (7.5)
<i>Limnonectes blythii</i>	25 (7.5)
<i>Limnonectes paramacrodon</i>	3 (0.9)
<i>Limnonectes ibanorum</i>	1 (0.3)
<i>Limnonectes ingeri</i>	4 (1.2)
<b>Family Microhylidae</b>	
<i>Microhyla butleri</i>	4 (1.2)
<i>Kaloula pulchra</i>	5 (1.5)
Total	333 (100)

\*Figure in bracket denotes percentage.

Table 1: Number of frogs caught in the eight study sites.

Species of helminthes	Total no. of Helminthes (%)	Host Species
<b>Nematode</b>		
<i>Heterakis</i> spp.	762 (9.4)	a,b,c,d,e,f,g,l,j,l,n*
<i>Heterakis vesicularis</i>	1345 (16.7)	a,b,c,d,e,f,g,l,j,l,m,n
<i>Pharyngodon</i> spp.	1021 (12.7)	a,b,c,d,f,g,l,j,l,m,n
<i>Trichostrongylus</i> spp.	77 (1.0)	a,c,f
<i>Ascaris</i> spp.	272 (3.3)	a,b,c,d,f,h,i
<i>Oswaldocruzia</i> spp.	31 (0.4)	a,b,c,d,f,h,j
<i>Rhabdias</i> spp.	13 (0.2)	a,b,n
Percentage	43.70%	
<b>Trematode</b>		
<i>Diplodiscus sacculousus</i>	144 (1.8)	a,c,j
<i>Glythelmins staffordi</i>	2601 (32.3)	a,b,c,d,f,j,k,l,m
<i>Manodistomum</i> spp.	214 (2.7)	e,f,j
Percentage	36.80%	
<b>Acanthocephalan</b>		
<i>Macracanthorhynchus</i> spp.	1578 (19.5)	a,b,c,d,e,f,j,k,m
Percentage	9.50%	
Total number of Helminthes	8058 (100%)	

\*Note: Denotes the respective frog species.  
a: *Duttaphrynus melanostictus*; b: *Phrynonidis aspera*; c: *Hylarana erythraea*;  
d: *Polypedates leucomystax*; e: *Fejervarya cancrivora*; f: *F. limnocharis*;  
g: *Microhyla butleri*; h: *Limnonectes blythii*; i: *L. ibanorum*; j: *H. labialis*;  
k: *L. paramacrodon*; l: *L. ingeri*; m: *Kaloula pulchra*, n: *H. nigrovittata*.

**Table 2:** Composition of helminthes recovered from the respective frog hosts.

except for the trematode *Manodistomum* spp. Unlike other parasite species, four in particular were confined to three frog host species: the nematodes *Trichostrongylus* spp and *Rhabdias* spp. and the trematodes *Diplodiscus sacculousus* and *Manodistomum* spp. The acanthocephalan, *Macracanthorhynchus* spp. seemed to be able to infect numerous species of frog hosts.

### Composition of blood pathogens

As shown in Table 3, there were 105 frogs infected with blood parasites. The highest infection of blood parasite was *Phrynonidis aspera* (29.5%) and the frog with the lowest blood infection was *Limnonecte sibanorum* (1%) and *Limnonectes kuhlii* (1 %). There were eight species of blood pathogens recovered from frogs. They comprised of *Aegyptianella* spp, *Haemogregarina* spp., *Hepatozoon* spp., *Lankesterella* spp. *Trypanosoma* spp. and a microfilaria species (Table 4). *Trypanosoma chattoni* had the highest blood parasite count, with the lowest being seen in *Lankesterellas* spp.

### Environmental factors

Table 5 shows four environmental factors: BOD, pH, and temperature of water and distribution of rainfall. Also, are shown the number of frogs caught and the respective helminthes, protozoa and blood pathogens obtained. From the data, the average mean of BOD was between 5.61 to 6.08 mg/L. The lowest mean reading was 5.61 mg/L and the highest mean reading was 6.08 mg/L. The lowest mean of pH was 6.16 and the highest 6.46. The lowest reading of water temperature was 26.6°C, while the highest 27.5°C. Conversely, the lowest rain was 2.64 mm and the highest 12.99 mm.

### Mean significance between frogs and parasites

One-way ANOVA analysis and LSD test on significant mean differences between groups of helminthes parasites and frogs for *H. vesicularis*, showed there was high mean significance (p<0.05) in *D.*

*melanostictus* (63.14 ±24.67), *P. aspera* (48.57±20.20) and *H. erythraea* (44.00±17.06) compared to the other frog species: *P. leucomystax*, *F. cancrivora*, *F. limnocharis*, *M. butleri*, *L. blythii*, *L. ibanorum*, *H. labialis*, *L. paramacrodon*, *L. ingeri*, *K. pulchra* and *H. nigrovittata*. For *Heterakis* spp., there was high significance (p<0.05) in *P. aspera* (33.14 ±27.03) and *H. erythraea* (33.43 ± 13.31). *Ascaris* spp. was the least number of parasites found in frogs. *Phrynonidis aspera* (29.71 ± 8.59) had a high significance at level (p<0.05). *Macracanthorhynchus* spp. in the frog *D. melanostictus* (120.42 ±93.51) had a high mean significance (<0.05) followed by *Phrynonidis aspera* (34.14 ± 18.51). Conversely, *D. sacculousus* numbers were also found to be low in frogs. But it has a high significance (p<0.05) in *D. melanostictus* (16.42 ± 5.93). *Glythelmins staffordi* had high significance (p<0.05) in *D.*

Frog Species	Number Infected (%)
<i>Hylarana erythraea</i>	23 (21.9)
<i>Hylarana raniceps</i>	6 (5.7)
<i>Hylarana nigrovittata</i>	2 (1.9)
<i>Limnonectes ibanorum</i>	1 (1.0)
<i>Limnonecte skuhlii</i>	1 (1.0)
<i>Limnonectes ingeri</i>	4 (3.8)
<i>Limnonectes paramacrodon</i>	2 (1.9)
<i>Limnonectes blythii</i>	2 (1.9)
<i>Fejervarya cancrivora</i>	2 (1.9)
<i>Fejervarya limnocharis</i>	5 (4.7)
<i>Polypedates leucomystax</i>	3 (2.9)
<i>Phrynonidis aspera</i>	31 (29.5)
<i>Duttaphrynus melanostictus</i>	23 (21.9)
Total (n)	105 (100)

**Table 3:** Species of frogs infected with blood parasites and their rates of infection.

Species of blood pathogens	Total number of parasites (%)
<i>Aegyptianella</i> spp.	387( 2.0)
<i>Haemogregarina</i> spp.	655( 3.4)
<i>Hepatozoon</i> spp.	2696 (13.9)
<i>Lankesterella</i> spp	93 (0.01)
<i>Trypanosom achattoni</i>	11770 (60.6)
<i>Trypanosoma rotatorium</i>	3262 (16.8)
<i>Trypanosoma loricatum</i>	131 (0.7)
Microfilaria	425 (2.2)
Total (n)	19,419 (100)

**Table 4:** Total number of blood pathogens in various species of frogs.

Sampling Times	No. of Frogs/ sampling	No. of helminthes/ sampling	No. of protozoa and blood parasites/ sampling	Factors (mean)			
				BOD (mg/L)	pH	Temp °C	mean
Rainfall (mm)							
1	31	819	1232	5.95	6.46	27.5	2.64
2	53	827	4845	5.63	6.31	26.8	6.38
3	32	1518	3471	5.74	6.23	26.7	4.26
4	36	1510	5315	5.62	6.16	26.9	11.97
5	32	1112	2483	5.46	6.19	27.2	2.47
6	50	1110	4939	5.61	6.21	26.6	12.9
7	66	1162	3503	6.08	6.31	26.7	5.21

**Table 5:** Number of frogs caught, number of helminthes, protozoa and blood pathogens collected per sampling, environmental factor measurements of Biochemical Oxygen Demand (BOD), pH, temperature of water and rainfall distributions.

*melanostictus* (259.85 ± 213.66). *Lankesterella* spp. has significance with *Duttaphrynus melanostictus* (47.14 ± 21.21) at level  $p < 0.05$ , ( $F(13, 84) = 3.769, p=0.000$ ). Three frogs had high mean significances for *Trypanosoma chattoni* ( $p < 0.05$ ). They were *Phrynowidia aspera* (769.28 ± 429.55), *Hylarana erythraea* (341 ± 112.37) and *D. melanostictus* (332.00 ± 171.13).

### Correlation between environmental factors and composition of parasites

The results are presented in a matrix such that, as can be seen, the correlations were replicated (Table 6). There was a low correlation between helminthes parasites and rainfall distribution, that the Pearson correlation coefficient,  $r$ , was 0.321 and was statistically significant ( $p < 0.01$ ). Also, there were negative correlations between parasites and pH (-0.713) at level  $p < 0.01$ . From Table 7, there was moderate correlation between numbers of blood parasites and levels of BOD, that the Pearson correlation coefficient,  $r$ , was 0.526 and statistically significant ( $p < 0.01$ ).

### Discussion

There were 300 frogs from 14 species captured in eight localities in Penang Island, Peninsular Malaysia. Different species of frogs inhabit different habitat areas. Therefore, the distribution of frogs found in every sampling site was different. There were 300 frogs from 14 species captured in eight localities in Penang Island, Peninsular Malaysia. They were *D. melanostictus*, *P. aspera*, *H. erythraea*, *H. labialis*, *H. nigrovittata*, *L. ingeri*, *L. paramacrodon*, *L. blythii*, *L. kuhlii*, *F. cancrivora*, *F. limnocharis*, *P. leucomystax*, *M. butleri* and *K. pulchra*. These species had similarly been reported to occur in other parts of Malaysia as well [4,5,9]. The highest number of frogs collected was 90, which was from the species *D. melanostictus*. This species was found in most habitats in disturbed rather than stable areas. *Phrynowidia aspera* was commonly found on rocks and often found in clusters rather than individuals. *Oswaldocruzia* spp. has not been previously recorded to occur in frog in Malaysia. A previous study discovered *Oxysomatium* spp. and *Rhabdias* spp. From *D. melanostictus* and *F. limnocharis* *Batrachonema synaptospicula* and *Paracosmocera* spp had also been described in *Ranamacrodon* spp.

(currently *L. paramacrodon*) [6,8] and *Paracapillaria malayensis* from *D. melanostictus* [7]. Also, previously, frog trematodes had been recovered from *Haematoloechus singaporensis* from the lungs of *Rana cancrivora* (currently *F. cancrivora*), *D. sacculosus* from the rectums of *R. erythraea* (currently *H. erythraea*) and *G. staffordi* from the intestines of *R. cancrivora*, *R. erythraea* and *R. macrodon*. There was only one species of acanthocephalan, i.e. *Macracanthorhynchus* spp. that was isolated. This species was abundantly found in the intestines of various species of frogs. Most of the parasites were at the larvae stage. A previous study stated that this species was found in pigs and thus the medium of infection mode is unclear. The most common adult form in amphibians is *Acanthocephalu sranae* [1] as well as *A. bufonis* [20]. However, so far, not much has been reported on *Macracanthorhynchus* spp. and knowledge of this species is still lacking. There was no cestode recorded from this study although the study by Ibrahim et al. [9], reported a species of cestode, *Nematotaenia* spp. in *F. cancrivo* and *F. limnocharis* frogs in northern Peninsular Malaysia. *Phrynowidia aspera* and *H. erythraea* were mostly exposed to water and the chance to get infected by helminthes is higher comparing to other species. As reported by Brooks et al. [21], parasites require water for transmission when the frogs feed on aquatic intermediate hosts or when the parasite is swimming from onehost to another host. Besides that, type of habitat also contributes to the parasite infection. A previous study by Kusriani et al. [22] reported that the higher prevalence in nematode infection is mostly in terrestrial frogs compared to dwelling frogs. Since *D. melanostictus* is a terrestrial frog, thus explaining that *D. melanostictus* was infected with *H. vesicularis*.

With regards to blood pathogens, 105 of 300 frogs were infected with at least one species of *Lankesterella* spp. and *T. chattoni*. There were significant differences in the means of parasitism among frog species, with blood parasite detected in only 2 of 8 species. *Trypanosoma chattoni* was the most common blood parasite. Other studies had also reported that *Trypanosoma* spp. *Lankesterella* were the most common parasite observed in frogs [23]. This study found that significant differences in the means of blood parasite among frog species, possibly reflecting the different habitat preferences of the host species and consequently different vector abundance and interaction rates. Environmental factors did not influence the infection rate of parasitism in frogs except for rainfall. According to Brooks et al. [21], parasites require water for transmission, either when the frog utilizes aquatic intermediate hosts like trematodes, or when the parasite is swimming from one host to another like in mono geneans, thus explaining the high number of parasites during the rainy season [24-27]. There was no relationship between pH and parasite numbers since pH values remained unchanged throughout the sampling period. BOD and temperature did not influence the number of parasites although the reason for this is not clear.

Correlation value (r-value)	Environmental factors			
	BOD	pH	Temperature of water	Rainfall
-0.223	-0.713*	-0.439	0.321**	
1	0.62	0.315	-0.608	
0.62	1	0.524	-0.52	
0.315	0.542	1	-0.573	
-0.608	-0.52	-0.573	1	

Table 6: Result of Pearson's correlation analysis between compositions of helminthes in frogs.

Correlation value (r-value)	Environment Factor			
	BOD	pH	Temperature of water	Rainfall
0.526*	-0.076	-0.446	0.143	
1	0.62	0.315	-0.608	
0.602	1	0.524	-0.52	
0.312	0.524	1	-0.573	
-0.608	-0.52	-0.573	1	

\*Correlation is significant at the 0.01 levels (2-tailed)/moderate correlation.

Table 7: Result of Pearson's correlation analysis between compositions of blood pathogens in frogs.

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