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Tadpoles as dengue mosquito (*Aedes aegypti*) egg predators

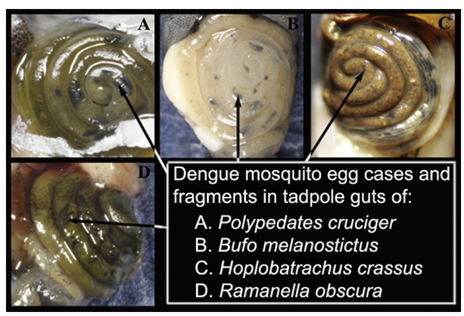
Gayan Bowatte, Piyumali Perera, Gayani Senevirathne, Suyama Meegaskumbura, Madhava Meegaskumbura *

Department of Zoology, Faculty of Science, University of Peradeniya, KY 20400, Sri Lanka

HIGHLIGHTS

- Dengue mosquito eggs help perpetuate the disease through seasons.
- Mosquitoes are attracted to lay eggs by tadpole cues.
- Tadpoles of all five genera tested, are dengue mosquito egg predators.
- Tadpoles live in various aquatic habitats that are often not accessible to fish.
- Tadpoles are important for biological control of mosquitoes than previously thought.

GRAPHICAL ABSTRACT



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ABSTRACT

Mosquito eggs survive drought conditions while harboring pathogens, perpetuating diseases through seasons. Biological control of mosquitoes using vertebrates mostly highlights the role of larvivorous fish. Here we show that tadpoles with different life-history characteristics predate on *Aedes aegypti* (vector mosquito of dengue virus) eggs. We did four experiments involving tadpoles of *Polypedates cruciger* to determine: (i) egg laying preference of female mosquitoes, in normal water, water in which tadpoles were previously present, and water in which tadpoles were present at the time of experiment; (ii) survivability of mosquito eggs in tanks in presence *vs.* absence of tadpoles; (iii) egg predation through direct observation (video-recording) and gut content analysis; (iv) if egg predation is prevalent among different species of tadpoles of four species of randomly selected genera, *Bufo, Ramanella, Euphlyctis* and *Hoplobatrachus* were subjected to experiment (iii) only. We show that mosquitoes have a preference to lay eggs in tadpole water and that tadpoles destroy such eggs laid. Direct observations substantiate that all tadpole species tested are mosquito egg predators. With about seven thousand frog species worldwide, living in a diversity of aquatic habitats where fish cannot reach, the role of tadpoles in biological control of mosquitoes can be significant than currently understood.

1. Introduction

Dengue fever (brain-hemorrhagic fever) is endemic to most tropical and subtropical countries (WHO, 2009a,b), but yet among vertebrates only fish have been highlighted in biological control of mosquitoes. The dengue virus is borne and spread by mosquitoes of the genus *Aedes*, annually affecting nearly 200,000 people, causing about 30,000 deaths worldwide (WHO, 2009a,b). Various means are used to control mosquitoes; among them, biological control methods, often involving predation, competition and disruption of physiological processes or a combination of these, are popular for their low ecological impact and reduced side effects on humans.

Various types of fishes are rightfully highlighted as excellent predators of mosquito larvae (Jenkins, 1964; Howard et al., 2007;

* Corresponding author.
E-mail addresses: gbowatte@gmail.com (G. Bowatte), piyumali22@gmail.com (P. Perera), gayani88s@gmail.com (G. Senevirathne), suyamam@pdn.ac.lk (S. Meegaskumbura), madhava_m@mac.com (M. Meegaskumbura).

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Chandra et al., 2008), but they could also cause damage when they are introduced into ecosystems from which they were previously absent. Larvivorous fishes (eg. *Poecilia reticulata* – Guppy and *Gambusia affinis* – mosquito fish) have been introduced in many parts of the world, just for this purpose (Bellini et al., 1994; Legner, 1995; Pyke, 2005; Chandra et al., 2008). Introduced mosquito controlling fish are often indentified as a threat to native aquatic fauna including amphibians and contributes to local extinction of amphibians (Goodsell and Kats, 1999; Komak and Crossland, 2000; Gillespie, 2001; Kats and Ferrer, 2003), highlighting the need to carefully consider the ecological cost of species introductions intended to perform mosquito control.

Vertebrate predators of mosquito eggs (including the role of fish) have so far not been highlighted. Since the eggs of mosquitoes are minute, and in some species such as *Aedes aegypti,* only individual eggs are laid (as opposed to egg rafts in some mosquito species), propensity of egg predation is not apparent. However, studies of mosquito egg predators are important due to the ability of mosquito eggs to survive drought periods (Sota and Mogi, 1992) and hatch in the subsequent rainy season (Okogun et al., 2003; Rozilawati et al., 2007). It is also well known that transovarian transmission of dengue virus occurs from female mosquitoes to their eggs (Frier and Rosen, 1987; Joshi et al., 2002). These eggs under natural conditions can survive several months of seasonal drought (Trpis, 1972; Fontenille and Rohdhain, 1989; Juliano, 2002). Hence, dengue virus laden eggs that survive a drought can determine the starting mosquito population size and incidence of disease during the subsequent rainy season.

Management of natural egg laying habitats of mosquitoes is important. *A. aegypti* have the ability to lay eggs in a variety of aquatic habitats, this may range from water filled discarded plastic containers and tires, tree holes, wells, temporary and permanent pools and marshy areas, often close to human settlements (Becker et al., 2010; Weeraratne et al., 2013). Anthropogenic waste products that provide breeding sites such as discarded cans and tires can be removed from the environment, or the public could be made aware against creating such habitats, however, ecologically important tree holes, temporary pools, ponds and marshy areas are more difficult to manage, as they support a plethora of native organisms, and have important ecological roles. If these natural sites can be managed biologically, without introducing insecticides, larvicides or non-native predators, long-term ecological damage to ecosystems, including to humans, can be averted.

A factor that reduces the effectiveness of using fish for mosquito control is that most fishes (especially the species being highlighted as larvivorous fish) often need interconnected waterways for movement; frogs do not require this as they can move over land; but yet, mosquitoes theoretically are free to lay their eggs in any pool at their discretion. Often, many frogs, especially the common non-threatened forms that live in close association with humans, also have large distributions and are not impeded by absence of waterways to reach isolated pools to breed. So if tadpoles are actually predators of mosquitoes, their impact can be significant.

Information on effectiveness of amphibians in mosquito control is not adequately highlighted (Raghavendra et al., 2008). The role of tadpoles in disrupting early life history stages of mosquitoes, especially the egg stage, remains poorly studied. This is of utmost importance given that mosquito eggs with their ability to traverse dry periods, are the primordial elements that determine vector density and hence the difference between an epidemic and a low incidence of a disease in a given wet season.

While raising tadpoles of *Polypedates cruciger* and *Taruga eques* for various experiments in our lab (see. Meegaskumbura et al., 2010), we observed only a very few or no mosquito larvae in those tanks, although in tanks without tadpoles kept under similar lab conditions we observed many mosquito larvae. Given the fact that we also noticed sometimes a very few mosquito larvae even in tadpole containing tanks suggests that mosquitoes actually lay eggs in tadpole containing tanks (despite the presence of tadpoles) and that these tadpoles do not directly predate on mosquito larvae, hence we hypothesized that these tadpoles may be feeding on mosquito eggs or creating conditions that disrupt hatching.

Hence, to determine the mechanism and propensity of mosquito egg destruction by tadpoles, we tested five frog genera (*Polypedates, Bufo*, *Ramanella, Euphlyctis* and *Hoplobatrachus*) that are widely distributed and capable of laying eggs in a variety of aquatic habitats. As the experimental mosquito species, we use the medically important *A. aegypti*, whose eggs carry the dengue virus while surviving drought conditions.

We show using controlled experiments that egg laying mosquitoes are attracted to tadpole cues, and that tadpoles actively predate on mosquito eggs. Since most amphibians breed in isolated pools that are inaccessible to fish, the role of more than 7000 + species of amphibians living throughout the world need to be considered profoundly in the context of environmental management and biological control of mosquitoes.

2. Materials and methods

2.1. Raising mosquitoes

A. aegypti mosquito larvae were collected from puddles at the University of Peradeniya, Sri Lanka. They were raised in water through pupation until emergence of adults; identification followed (Becker et al., 2010). These (both males and females) were kept in a fine mesh cage (size = $50 \times 50 \times 50$ cm), and were initially fed on a sugar and vitamin solution (Vit SP Total, s.p veterinaria, s.a Spain) for 2–3 days. Females were given a blood meal after this (males were continued on the sugar and vitamin diet); it was ensured that at least 15 female mosquitoes fed copiously. Experimentation started three days after blood meals were provided.

2.2. Raising tadpoles

Polypedates cruciger tadpoles, an endemic, non-threatened species, were collected from a pond and were raised in an aquarium. They were fed on a diet of boiled lettuce and fish food. Frequent water changes were made to prevent ammonia build up within the tank. At the start of the experiments they were between Gosner stages 30 and 38 (Gosner, 1960).

Bufo melanostictus tadpoles, a non-threatened; wide spread; species, were collected from a pond and raised in an aquarium. They were fed on a similar diet to that of *P. cruciger*. At the start of the experiment, they were between Gosner stages 28 and 35.

Ramanella obscura tadpoles, a non threatened species, were collected from a tree-hole, and raised in an aquarium. They were fed on a diet comprising of finely ground fish food (Tropical Flakes, Samyu Pets Crop, Taiwan) and boiled egg yolk. They were between Gosner stages 33 and 35.

Euphlyctyis cyanophlyctis tadpoles, a non threatened species, were collected from a shallow ephemeral pool and raised in an aquarium and fed on fish food and aquatic plants. They were between Gosner stages 30 and 33.

Hoplobatrachus crassus tadpoles, a non threatened species were collected from a deep ephemeral pool and raised in an aquarium and fed on fish food. They were between Gosner stages 33 and 35.

2.3. Female egg laying preference (experiment i)

This experiment was carried out to determine if dengue mosquitoes had a preference to lay eggs in normal water (NW), or water that previously contained tadpoles (but no tadpoles at the time of experimentation: TW) or water with tadpoles present (TP). Testing procedure was as follows: twelve containers that had a volume of 175 ml each were arranged randomly inside the mosquito cage. The source of water for all the experiments was the same (filtered aged tap water), which was later manipulated to mimic different conditions. Four of these containers had filtered aged tap water (normal water – NW, considered as the control); another four had filtered water from a tadpole (*P. cruciger*) containing tank (tadpole water – TW: this was taken from a tank which harbored 8 tadpoles in 800 ml of water for 3 days); the last four had filtered aged tap water and two tadpoles (Gosner stages 30–33; tadpoles present – TP). All containers had a 2 inch long piece of aquatic plant (*Hydrilla asiatica*), to simulate natural conditions and to reduce stress while also providing a continuous natural food source for tadpoles. Four replicates of this were done.

Three days after the containers were placed inside the mosquito cage (6 days after the blood meal was provided), the experimental trial was concluded, and the eggs in each container were counted under a microscope (Motic K6 6–50× zoom stereo microscope).

This experiment was replicated four times, each time using a different set of adult mosquitoes and a different set of tadpoles.

To determine if tadpole activity disturbs female mosquitoes laying eggs, a video was made of female mosquitoes laying eggs in TP.

2.4. Hatching patterns (experiment ii)

This experiment was done to determine the tadpole's propensity to feed on mosquito eggs. Eight glass aquaria measuring 8 × 33 × 18 cm were filled with filtered aged tap water. Four tadpoles (Gosner stage 35–38) were placed in each of these four tanks; the four aquaria without tadpoles were considered as controls. Using the same mosquito rearing setup, 25 mosquitoes were given a blood meal. A single container with aged filtered tap water was placed inside the cage 3 days after providing the blood meal. The container was retrieved after 24 h and the eggs were collected. Sixty-four *A. aegypti* eggs were placed in each of the experimental tanks. The larvae that hatched were counted at 5, 8, 10, 15, 20, 23 days. In the tadpole containing tanks, the tadpoles were fed with boiled lettuce, so that there was some lettuce in the tank at all times; control tanks also had a piece of boiled lettuce.

2.5. Statistical analyses

To test female egg laying site preferences, a one way within subjects ANOVA (SYSTAT ver. 11) was conducted to analyze variances in mosquito egg numbers laid in NW, TW and TP.

To visualize the relationships within the three experimental breeding water types, box plots depicting mean and standard deviations (STATISTICA 8.00.550) were made (Fig. 1).

2.6. Evidence for egg feeding (experiment iii)

To test if *P. cruciger* tadpoles actually feed on eggs, a tadpole was put in a 100 ml transparent plastic container in which mosquito eggs were placed. A video camera was set up to record the feeding activity of the tadpole. After 8 h, tadpole was euthanized using MS222 and was preserved in 10 percent formalin solution and the gut was observed for mosquito eggs and a macro photograph was taken (using a Canon 40D body, 100 mm Canon macro lens and Canon 24-MT twin-flash setup; Fig. 2).

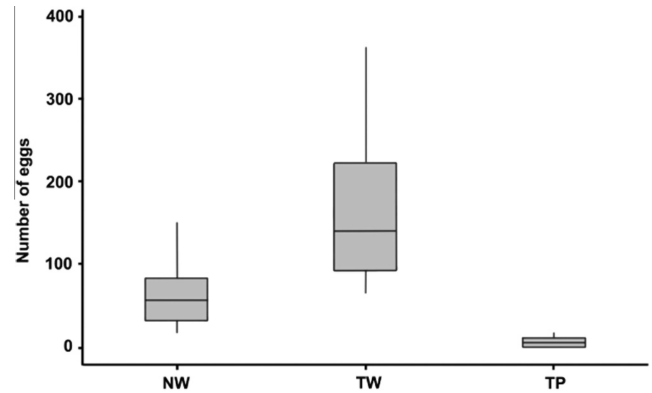

Fig. 1. Box and whisker plots depicting the median and spread of the number of eggs laid in Normal Water (NW), Tadpole Water (TW) and Tadpole Present Water (TP).

2.7. Do other amphibian species feed on mosquito eggs? (experiment iv)

To observe if other amphibian species also feed on mosquito eggs, *B. melanostictus*, *R. obscura*, *E. cyanophlyctis* and *H. crassus* tadpoles that live in several different aquatic habitat types were tested, experiment (iii) was repeated for these four species (Fig. 2).

3. Results

3.1. Female egg laying preference (experiment i)

One way ANOVAs showed significant differences between the three groups compared (F (2, 45) = 31.830, $p < 0.0001$). The Tukey post-hoc test showed that the female mosquito eggs laid in NW (mean = 63.2, SD = 36.9), TW (mean = 163.0, SD = 89.3) and TP (mean = 6.9, SD = 8.9) were significantly different from each other, with pair wise probabilities of NW – TW = 0.018; NW – TP < 0.0001; TW – TP < 0.0001. Hence, it is apparent that most of the eggs were laid in tadpole water instead (TW) of normal water (NW). The least number of eggs was observed in water that actually had tadpoles (TP) (Fig. 1). The reason for this presumably is even though the mosquitoes are significantly attracted to tadpole water, tadpoles fed on mosquito eggs. Video evidence highlights mosquitoes laying eggs even when tadpoles were active.

3.2. Hatching patterns (experiment ii)

Although an equal number of eggs (64 each) were placed in tadpole containing and tadpole absent tanks, the number of larvae that hatched in tadpole containing tanks were significantly less than in tanks that did not contain tadpoles (Figs. 3 and 4). Results of a two sample t-test, show that number of larvae that were present in tanks with tadpoles (mean = 6.00; SD = 4.08; SE = 2.0) is significantly different ($p = 0.023$) from those that did not have tadpoles (mean = 47.3; SD = 18.6; SE = 9.3; Fig. 3); suggesting that tadpoles were actually feeding on mosquito eggs.

The average hatching percentage of mosquito eggs from tadpole-absent tanks was 74.9 percent, and the hatching rate for tadpole present tanks was 9.4 percent (Fig. 4). Hence the overall destruction percentage attributable to tadpole predation is about 87.5 percent of the viable eggs.

3.3. Evidence for egg feeding (experiment iii)

The video clearly shows a tadpole of *P. cruciger* taking mosquito eggs into its mouth (video clip available as supplementary

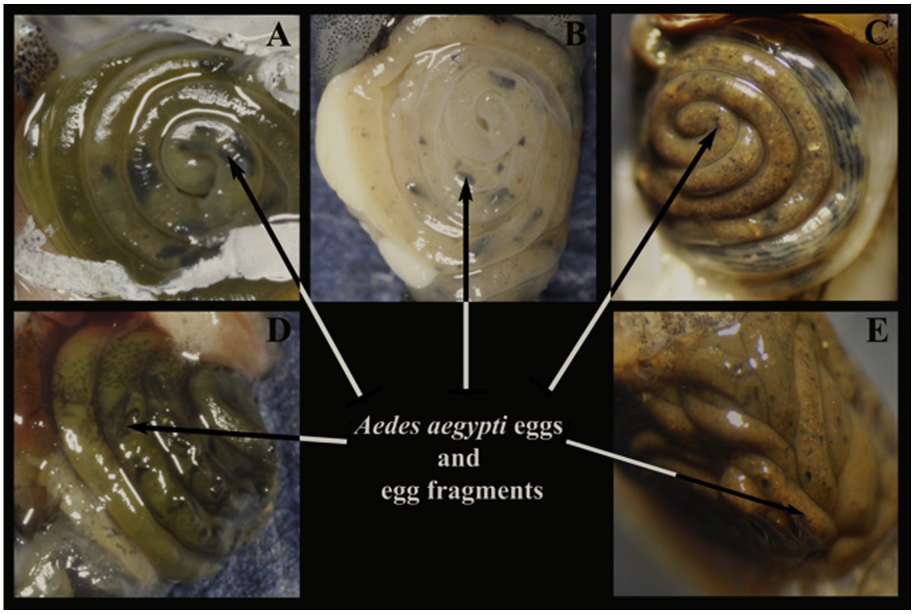

Fig. 2. Mosquito egg case fragments found within the five species of Tadpoles: A – *P. cruciger*, B – *B. melanostictus*, C – *H. crassus*, D – *R. obscura*, E – *E. cyanoplyctis*. Egg cases remain intact in *R. obscura*, but not others.

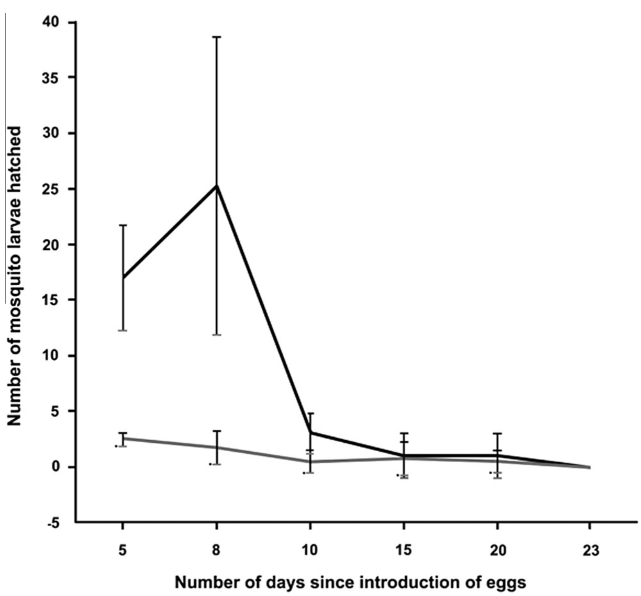

Fig. 3. Out of the 64 eggs initially introduced, the mean ($n = 4$) number of eggs hatched in tanks with tadpoles (light line) and tanks without (dark line) against the number of days since the introduction of eggs, error bars represent standard deviations.

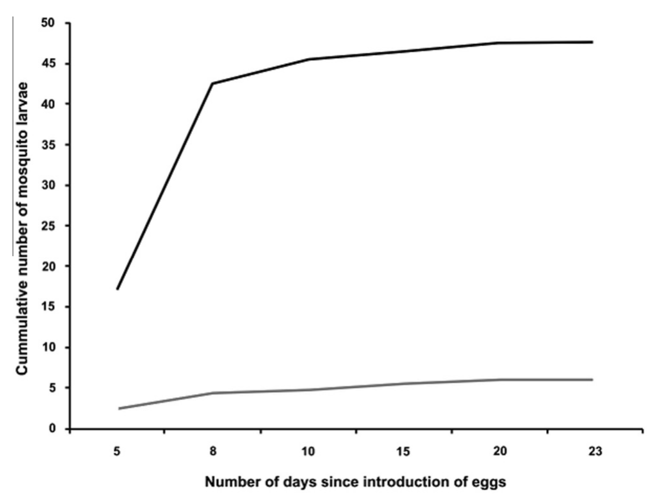

Fig. 4. Cumulative mean number of mosquito larvae that hatched, in tanks with tadpoles (light line; $n = 4$) and in tanks without tadpoles (dark line; $n = 4$).

material). Dissection of tadpole's gut shows eggs contained within; in four species, *P. cruciger*, *B. melanostictus*, *E. cyanoplyctis*, and *H. crassus*, fragments of mosquito eggs were observed, however, whole eggs were observed within *R. obscura* guts (Fig. 2).

3.4. Do other amphibian species feed on mosquito eggs? (experiment iv)

Other species tested, *B. melanostictus*, *R. obscura*, *E. cyanophlyctis* and *H. crassus* tadpoles also have mosquito eggs contained within their gut, showing that these species also feed on mosquito eggs (Fig. 2).

4. Discussion

Presence of a few eggs in TP not only confirms that mosquitoes lay eggs even in the presence of tadpoles, but also that most eggs that are laid do get destroyed by tadpoles. Video documentation (of feeding) and tadpole gut content observations conclusively show that active egg predation (and not a change in conditions caused by presence of tadpoles) is the cause of egg destruction for all five species considered in this study.

Specific reasons for mosquitoes laying a larger number of eggs in TW when compared to NW are beyond the scope of this work. However, many studies show that mosquito species use a combination of cues, biological, chemical and physical, to find egg-laying sites (Beehler et al., 1994; Zhao et al., 2006; Ferrari et al., 2007; Kesavaraju et al., 2007; Ponnusamy et al., 2008; Zahiri et al., 1997; Mokany and Shine, 2003a; Wilton, 1968; Ahmadi and McCelland, 1983; Ponnusamy et al., 2008; O'Gower, 2004). Since *Aedes* mosquito larvae show detritus feeding behavior (Sota and Mogi, 1992), the female mosquitoes may be correlating tadpole cues with abundance of detritus. It has also been shown that although *Aedes* requires clear water it necessarily does not need to be clean water (Lee, 1990).

Mokany and Shine (2003a) have suggested that frogs introduced into segregated mosquito larval breeding habitats such as ponds, puddles, tanks, etc., may prey on larvae and subsequently reduce vector population and vector born disease burden. However no study has revealed the fact that tadpoles feed on mosquito eggs.

The form of mosquito eggs within guts suggests that four of the species, *P. cruciger*, *B. malanostictus*, *E. cyanophlyctis* and *H. crassus* crushed eggs prior to swallowing. Of these, *H. crassus* had well crushed fragments of eggs, suggesting that they chewed most; but complete eggs were found within *R. obscura* tadpoles. The reason for this could be the presence of horny teeth in the four species that can be used to crush mosquito eggs and the absence of horny teeth in *R. obscura* (they only have a horny ridge on their upper lip).

Tadpoles of *P. cruciger* feed on mosquito eggs even despite the presence of the alternative food source within the experimental set up suggest that the tadpoles have preference for mosquito eggs. Since *P. cruciger* tadpoles fed on mosquito eggs despite the presence of an aquatic plant (*H. asiatica*), it can be presumed that these tadpoles were not forced to feed on mosquito eggs due to the lack of any other food source within the setup. However, more experimentation is needed to determine the preference of mosquito eggs when compared to many other alternative food sources that are available to a tadpole in the wild.

Competition between tadpoles and mosquito early life history stages probably extends beyond egg predation by tadpoles. There is a substantial body of knowledge highlighting that competition between tadpoles and mosquito larvae results in poor growth or death in either the tadpoles, mosquito larvae or both (Blaustein and Margalit, 1994, 1996; Mokany and Shine, 2002, 2003a,b; Mokany, 2007, Hagman and Shine, 2007). They compete for food and change water quality (Mokany and Shine, 2002). We have not evaluated mosquito larval development in our study. However, for *P. cruciger*, since eggs are laid out of water, on an overhanging foam nest (Meegaskumbura et al., 2010), in which early development takes place from which Gosner stage 23 tadpoles fall into water, the effect of mosquitoes larvae predating on early embryonic tadpole stages can be considered to be negligible. However, since *P. cruciger* tadpoles have a large body size, with numbers up to about 400 eggs per nest, coupled with their communal breeding behavior, they can cause severe competition to mosquitoes utilizing a given breeding pool. Hence the control of mosquitoes by tadpoles probably extends beyond egg predation and, for this reason, it cannot be conclusively stated that egg predation is totally responsible for the reduced number of mosquito larvae in tadpole present pools when compared to pools without tadpoles, however, results of this study suggests that mosquito egg predation could be a major contributor to reduce the density of mosquito larvae.

Since eggs are known to survive dry spells especially those of *Aedes* (*A. triseriatus*) (Bradshaw and Holzapfel, 1988), predation on eggs is important to attenuate the continuation of diseases by reducing starting population size of a disease-carrying vectors.

Other methods (chemical methods) of control have a severe impact on aquatic stages of tadpoles (Cooke, 1972, 1973; Rayjohnson, 1980). Globally, currently, many frog populations are in decline due to various causes. Some of these causes are introduced invasive species, and pollution of water bodies through biocides. Hence, in disease management, the potential benefits in terms of biological control, such as the effects of egg predation by tadpoles in isolated water bodies, should be considered.

Previously egg predation may have been considered not important for several reasons. It is usually thought that mosquito eggs hatch within a few days; however there is great variation in this. Sometimes depending on the temperature, they can take as much as a month or more to hatch (Rueda et al., 1990). Depending on the time taken to hatch, the chances of a tadpole finding and preying on mosquito eggs will increase. Moreover, given that all five tadpoles studied so far feed on mosquito eggs, factors such as the breeding site, size of tadpoles, and tadpole density could be important factors that could determine the importance of incidence of destruction of mosquito eggs. Determination of these factors requires further studies.

All five frog species that are considered in this study are commonly found among human habitations. *P. cruciger* lay eggs as foam on walls or other objects overhanging wells, water tanks, manmade ponds, temporary pools and swamps. *B. melanostictus* are in fact rare in primary forest habitats. Since *A. aegypti* have a distinct preference for human blood, and hence live close to human dwellings, the role of the frog species highlighted could be extremely important in reducing the populations of vectors around human dwellings. Species such as *B. melanostictus* lay thousands of eggs in large ponds and pools. Often tadpoles of this species could be observed swarming (Wassersug, 1973; Khan, 2008). The effect of such a group feeding on mosquito eggs can be extremely significant. *Ramanella obscura* exploit smaller water puddles and tree holes that most other species of amphibians do not find attractive as breeding sites (Bowatte and Meegaskumbura, 2011). These tadpoles are medium sized, and feed on the substrate, water column and the surface. Usually, only a few tadpoles are present in a given pool.

Since most frog species lay eggs with the start of the rainy season, and mosquitoes also breed during the rainy season, the interaction between tadpoles and mosquitoes can occur as soon as the mosquitoes start breeding. For instance, each year in Sri Lanka two peaks of dengue fever occur annually in conjunction with the southwest monsoon in June–July and the north-east monsoonal rains during October–December. (Wijesinghe et al., 2009); these two monsoon season are in fact also the breeding seasons of most of the frogs, including explicitly the five species used in this study.

Given that all five species used by us for this experiment feed on *A. aegypti* mosquito eggs, it is possible that the phenomena is more widespread. With more than 7000 species of frogs, many of which are characterized by free-living tadpoles, with many species of medically important mosquito species throughout the world, the importance of tadpoles in controlling mosquitoes may be very significant.

Here, we have highlighted the role of amphibians in reducing mosquito populations through destruction of mosquito eggs. Since amphibians having different life histories and different breeding sites can predate on mosquito eggs (that have the potential to traverse dry periods) laid in a variety of isolated habitats, when environmental management policy decisions are made, such as application of biocides or introduction of predatory fish, the role of amphibians in control of mosquito populations should be considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2013.10.005.

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