Comparison of methods for controlling *Saprolegnia*-like infection in the egg sacks of Asiatic salamanders (*Hynobius*)

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ABSTRACT - Asiatic salamanders, genus *Hynobius*, deposit paired egg sacks rather than individual or clumped uncovered spawn. This oviposition mode means that clutches are vulnerable to water mould infections of non-viable ova. I trialled five methods of arresting mould infections in the egg sacks of *Hynobius dunni*, in pairwise comparisons with control sacks. Chemical treatment using methylene blue was unsuccessful in controlling infections. Mechanically removing infected eggs or parts of egg sacks was more successful. Open sacks were successfully resealed using nylon fishing line, and individual eggs were successfully incubated outside of the sack. These methods may be particularly useful to captive breeders and experimenters working with these salamanders in laboratories and the field.

INTRODUCTION

Salamanders of the family Hynobiidae, and especially the genus *Hynobius*, are notable in their reproductive biology for exhibiting external fertilisation and for depositing paired egg sacks (Fig. 1), rather than individual ova. The egg sacks consist of a tough external membrane containing numerous ova within individual jelly envelopes. At eclosion, larvae break through the individual jelly envelopes and swim down the sack, leaving via an opening at the tip.

Male *Hynobius* exhibit scramble competition and release sperm over the egg sacks shortly after oviposition (Hasumi, 1994; Park & Park, 2000). It is very rare for all ova to be fertilised (Hasumi, 2001) and it is common for infertile ova to become opportunistically infected with ubiquitous *Saprolegnia*-like water moulds, which rapidly spread to and kill healthy embryos (Wallays, 2002). Additionally, clumps of dead, water mould infected embryos and ova can block the exit of healthy larvae when they are ready to hatch, leading to death (pers. obs.). In this way entire or partial clutches may be lost.

In captivity, a number of *Hynobius* species are routinely reproduced, largely in private hands (Rafaelli, 2013). *Hynobius dunni* Tago 1981, from lowland central Japan, has long been established in captivity in Europe. A single genealogical line initially propagated by Henk Wallays in the 1990s is still in existence (Wallays, 2002; Rafaelli, 2013) and likely represents the vast majority of *H. dunni* in captivity today. I have repeatedly reproduced this species and line in captivity, and trialled several techniques to reduce the number of embryos lost to *Saprolegnia*-like infection; the results are presented here.

METHODS

Trials took place in the springs of 2015, 2016 and 2017; egg sacks were deposited between mid-March and mid-April depending on prevailing weather conditions. Techniques trialled were methylene blue (a chemical available over-the-counter to treat fungal infections in a variety of aquatic organisms) baths and injections, removal of infected ova via syringe, and removal of parts of egg sacks with and without resealing of the sack. I also trialled incubation of eggs removed from the external sack membrane, with and without methylene blue baths. The different techniques are detailed in Fig. 1.

After deposition, pairs of egg sacks were used as control and trial sacks, with one sack in each pair exposed to each treatment, and the other allowed to develop naturally with no interventions, unless otherwise stated in Fig. 1. All egg sacks were incubated in substrate-less glass aquaria in the absence of adult salamanders with water (alkalinity c. 180mg/l at tank setup) at 10-15°C, taken from the breeding aquarium.

RESULTS AND DISCUSSION

The results of different trialled methods are reported in Fig. 1. I report median percentages of the total number of eggs that were fertile and that hatched successfully, rounded to the nearest 5%, across the indicated number of trialled egg sacks. Sample sizes (usually 2, no more than 3 replicates) were not large enough for statistical analysis.

Results indicate that treatment of sacks with methylene blue, either through addition to the incubation medium or injection directly into the sack itself is not very effective at controlling *Saprolegnia*-like infections. Methylene blue at similar concentrations is used with success to treat fungal and fungal-like infections, including saprolegniasis, in post-hatching amphibians (Maruska, 1994; Wright & Whitaker, 2001; Crawshaw, 1992; Raphael, 1993; Smith, 2007), but is apparently of little use against such infections in *Hynobius* egg sacks.

The egg sacks and the ova contained therein proved to be relatively robust against interference and tolerated partial or complete opening of the sack, removal of non-viable ova and even incubation once separated from the sack for the duration of development. Methods involving such manipulation of the sacks were much more successful in

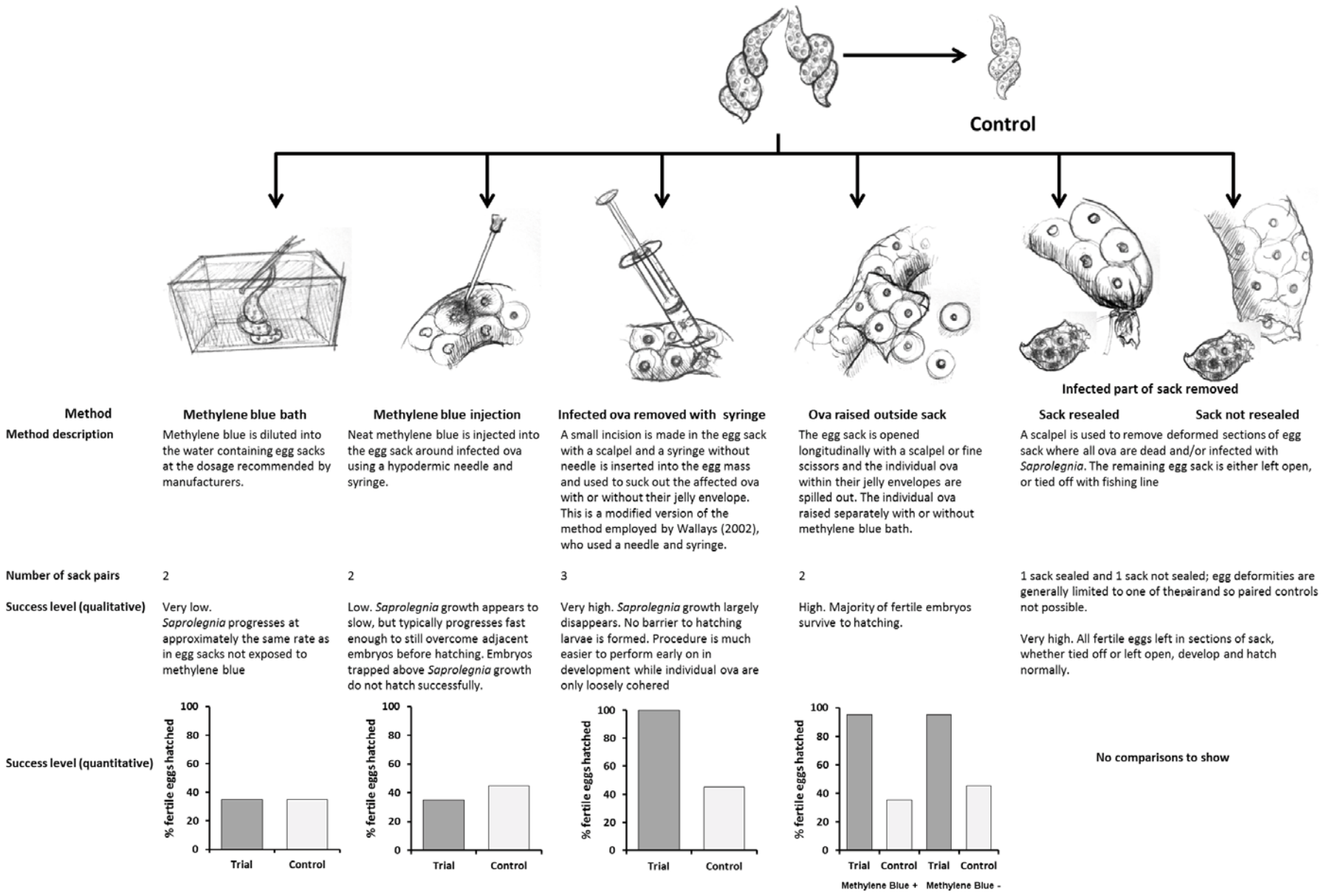

Figure 1. Methods, qualitative and quantitative results of techniques trialled to address water mould infection of non-viable eggs in the egg sacks of *H. dunni*. Each paired egg clutch was split between control and trial conditions; graphs show median percentage of fertile eggs that survived to hatching across the number of trials indicated by 'number of sack pairs'.

controlling saprolegniasis and hatch rates were very close to total fertility rates; once non-viable ova were removed as a food source for water moulds, the pathogen was apparently unable to infect developing embryos. There was no observed difference in proportion of fertile ova that hatched between eggs kept in methylene blue solution (made up from aquarium water) and those in standard aquarium water after being separated from the egg sack. Re-closing an opened egg sack with nylon fishing line was trialled with success (Fig. 1), but this does not appear to be necessary to ensure further development and needed to be removed in order to allow larvae to escape at eclosion.

The data presented here are not well replicated, but the combination of the use of a split clutch design and the magnitude of apparent positive effects suggests that they are likely to be realistic.

These results are for the egg sacks of *H. dunni* only and data are not available for other *Hynobius*. However, it seems likely that such techniques could be transferred to other species as egg sack structure is very similar. These techniques are likely to be limited to use in captivity only, as the tough outer membranes of the egg sacks of *Hynobius* are important in protecting ova from mechanical damage and from predation. Opening the egg sack, especially early in development, will also allow individual ova to fall out. The use of nylon fishing line to reseal egg sacks may allow egg sacks to retain protective function and so extend some of the methods trialled here to be used in wild sites, but repeat visits to free larvae trapped in the sack at hatching would be necessary. Given the threatened status of some *Hynobius* (including *H. dunni*; Kaneko & Matsui, 2004), these data may have relevance conservation projects, both for application in the field to improve recruitment, and in captivity to maximise the output of captive breeding programmes.

The tolerance for egg sacks being cut open and the possibility of incubating eggs successfully to hatching outside of the egg sacks may be useful beyond controlling the spread of *Saprolegnia*-like infection. Several *Hynobius* species are used as model systems in laboratories for, inter alia, developmental, evolutionary and ecological work (e.g. Michimae & Wakahara, 2002; Nishihara, 1996; Moriya, 1982; 1983; Wakahara, 1994). The production of egg sacks makes it difficult to use hynobiids for studies requiring clutches to be split into groups before hatching or for embryos to be clearly visualised or manipulated. Therefore, opening egg sacks early in development may allow for a greater variety of experimental work to be performed on this group.

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