Asellus aquaticus removal of unfertilized fish eggs and possible use in aquaculture as a biological control organism

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Abstract

The aim of the present experimental study was to better understand the foraging behavior of Asellus aquaticus. Different numbers of A. aquaticus were introduced into different experimental setups of unfertilised eggs, viable eggs, hatched larvae of Danio rerio. The number and time of A. aquaticus significantly affected the ratio of consumed non-fertilised eggs in each experimental cycle (MANOVA, $p<0.05$). A. aquaticus belongs to the aquatic saprophytes and no predatory behavior was observed during the experiments. They were able to distinguish between the dead eggs and those containing living embryos. Additionally, zebrafish larvae were not harmed by the A. aquaticus, even when there was not an alternative food source. The results help to understand the potential sanitary role of these crustaceans in natural waters and provide new insight into their possible application as a biological control organism in aquaculture hatcheries. Finally, our results indicate that there is a potential for A. aquaticus application against pathogens by reducing bacterial and fungal growth substrates.

Introduction

Hatching success of fish eggs depends on many factors in nature and in commercial fish producing systems as well. Among many other genetic compatibilities, maternal and ova characteristics, spermatozoa motility and sperm/egg ratio play a significant role in fertilization success and hatching rate of fish embryos [1,2,3]. Besides the internal biological features, environmental factors such as optimal temperature, salinity, predators, chemical pollutants, and diseases are all crucial factors for sufficient hatching rate [4,5,6,7].

Bacterial and fungal infections are a primary concern during the incubation period of fish eggs, especially in aquaculture hatcheries [8,9,10,11]. Harmful pathogens such as *Saprolegnia* sp., *Flavobacteraum* sp. or *Pseudomonas* sp. are commonly and naturally found in water systems. The mucous layer of eggs appears to be a good substrate for adhesion and colonization by many of these pathogens [12].

It is generally observed that the appearance of pathogens, such as bacteria and fungi, correlates with the proportion of unfertilized eggs [13,4]. However, it is possible that increased growth of bacteria and fungi is a result of and not necessarily a cause of increased dead fish eggs. On the other hand, dead eggs may promote the spread of horizontally transmitted diseases. Prior studies have shown *S. parasitica* to initially colonize dead eggs and the hyphae from these then infect the surrounding live eggs [14]. Smith et al. [13] showed that only dead eggs are infected by zoospores, whereas both live and dead eggs were infected by hypheal growth from adjacent infected eggs. In contrast, it has also been observed that *S. diclina* from fungus-infected salmonids can directly infect live eggs without any need for prior colonization of the dead eggs [15].

In addition to the possible transmission of disease between eggs, there may be other negative effects from a high number of unfertilized eggs in a water system. Dead and ruptured eggs may provide considerable nutrients for heterotrophic bacteria by leaching [16]. Large numbers of bacteria can have

high oxygen requirements [17] produce metabolic by-products [18] or toxins [19]. Proliferation of heterotrophic bacteria not only influence egg survival rate but has negative impact on a recirculating aquaculture system (RAS) by the competition of nitrifying bacteria [20].

The physical barrier of the chorion and membranes can supply defense against invading pathogens [21] after hatching the exposure to pathogens dramatically increases. Colonized dead eggs also create a route for pathogen transmission towards the freshly hatched embryo.

Various techniques such as egg disinfection have been applied to control egg pathogens [8, 10] in commercial aquaculture systems. Chemical treatments have been used to control disease transmission with varying levels of success. However, the elimination of dead eggs, with their potential as a substrate for disease and infection in fish, has been overlooked. This is due, in part, to the manual removal of these eggs being labour intensive and time consuming.

Thus, biological control methods can serve as effective alternatives to chemical or labour-demanding mechanical disease management. The concept of the biological control of pests is getting more and more focus as the harmful effects of agrochemicals comes to light. Grass carp *Ctenopharyngodon idella* is one of the best-known examples of a biological control “tool” to manage invasive aquatic plants [22]. Several species of wrasse (*Labridae*) are used as cleaner fish to remove salmon lice (Lepeophtheirus salmonis) from farmed Atlantic salmon (*Salmo salar*) [23]. The first application of macro invertebrates as a biological control of fungal disease was reported by Oseid [24]. He has demonstrated that grazing on the mycelia of oomycetes infected eggs by *Asellus militaris* and *Gammarus pseudolimnaeus* increased the hatching rate of walleye embryos (*Sander vitreum*). However, the application of crustaceans as a biological control in fish hatchery has not widespread.

A. aquaticus is a native detritivore abundant in most European freshwater ecosystems. High densities can be found in waters with organic pollution or in relatively clean water with high levels of naturally occurring organic matter [25]. Several studies have concluded that *A. aquaticus* selectively feed on detritus colonized by microorganisms in particular fungi [26,27]. We do not have the proof that fish egg non-infected by pathogen fungus do attracts *A. aquaticus*. The aim of the current study was to investigate that *A. aquaticus* is able to eliminate or reduce the number of unfertilized eggs before the spread of pathogen fungus, without harming live eggs or freshly hatched larvae. We implemented three experimental cycles investigating the foraging preferences of *A. aquaticus* using different ratios of fertile and unfertile eggs and freshly hatched larvae of zebrafish (*Danio rerio*).

Materials & Methods

Ethics statement

According to the European Directive on the protection of animals used for scientific purposes (2010/63/EU) zebrafish embryos/larvae are not subject to ethical regulations until they reach

independent feeding stage (120 hpf) [28]. There were not involved independently feeding zebrafish larvae (older than 120 hpf) in our experiments.

Zebrafish maintenance

A wild zebrafish strain (AB) was used for the experiment. This line has been bred for several years in the Zebrafish Laboratory of the Hungarian University of Agriculture and Life Sciences (HUALS). Fish were maintained in 3 L polycarbonate tanks in a recirculated system (ZebTEC, Tecniplast S.p.a., Italy) through an upwelling bead filter at 25 ± 2 °C, and fed three times per day with commercial flakes (Sparos Zebrafeed, 400 – 600 µm) and live Artemia larvae grown from cyst (Ocean Nutrition > 230000NPG). The photoperiod was set to 14h light: 10h dark. Guidelines from the good laboratory practice (GLP – Organization for Economic Co-operation and Development (OECD)) and Institutional Animal Care and Use Committees of Hungarian University of Agriculture and Life Sciences were followed for animal care. The protocols of fish propagation and the template informed consent forms contained in Appendix (Scientific Ethics Council for Animal Experimentation; XIV- 001 – 2306 – 4/2012) have been reviewed and approved by the National Food Chain Safety Office, Animal Health and Animal Welfare Directorate of Government Office of Pest County with respect to scientific content and compliance with applicable research subject regulations.

Broodstock of *A. aquaticus*

A. aquaticus specimens were collected from an experimental recirculating aquaculture system (RAS) of HUALS designed for fish broodstock housing. Three 5000 L fish tanks were connected and operated as a freshwater RAS, containing a drum filter, moving bed bio reactor and aeration. Daily water exchange varied between 2 – 6 % of the total volume depending on the biomass kept in the tanks. The system has been in operation for over five years without shut down. The following fish species were kept in the system: common carp (*Cyprinus carpio*) – continuously, European catfish (*Silurus glanis*) – for 22 months and European eel (*Anguilla anguilla*) – for seven months. Commercial carp and catfish feed was used (Haltáp Kft, Hungary) in 0.6 – 2.1 kg daily portion depending on actual biomass. Eel were fed frozen baitfish (2 kg/week). For broodstock we offered live zooplankton as supplementation. We assume that *A. aquaticus* appeared in the system owing to this practice as zooplankton was collected from natural waters. The starting population then multiplied on the organic particles in the effluent water. A. aquaticus was collected from the effluent water with a plastic sieve in a weekly quantity of approximately 200 – 1500 individuals. Specimens for the experiments were chosen randomly, the only criteria were that adult individuals (> 6 mm) were used.

Propagation, egg collection and incubation prior to the experiment

During propagation, five zebrafish females with five males were introduced into each breeding tank (n = 6). According to the protocol of zebrafish fertilization and embryo isolation (http://www.zfic.org/common%20techniques/mating.pdf) all fish were released to spawn. 1.7 L breeding tanks (ZebTEC, Tecniplast S.p.a., Italy) were used in all experiments. Water conditions were: temperature

25°C; pH 7.0 ± 0.2; average conductivity 525 µS. Eggs were collected from every tank into Petri dishes (Ø 100 mm) 2 hours after the onset of light. Eggs were incubated in a thermostat (25.5 °C, photoperiod was set at 14 h light: 10h dark) with daily water changes. After 24 h incubation period, all eggs were checked and separated into two batches: unfertilized eggs (white) and fertilized eggs (transparent with living embryos).

General experimental design

The experiments were carried out in sterile, flat-bottom, non-treated, multiwell-plates with lid; 6 – well format, Vtotal = 5 mL, Vwater = 4 mL. In the first two experiment (E1 and E2) *A. aquaticus* individuals (total body length 4 – 5 mm) were added to some of the wells in different numbers (1, 3 or 5 per well; Table 1.). Prior to the experiments, *A. aquaticus* specimens were starved in each well for 24 hours. Eggs (unfertilised and fertilised) were introduced into the wells in differing ratios at 24 hours post-fertilization (hpf) (Table 1.). Every well contained one swimming biological filtration media (AQ-09KL 436 m^2/m^3, protected surface = 4942/m^3, Ø/m^3 = 9/7 mm, 165 kg = m^3 Auacultur GmBH), which provided hiding / hanging place for *A. aquaticus*. In order to provide identical experimental conditions, the control wells without A. aquaticus contained the same media. The number of fertilised and unfertilised eggs were checked every five hours till the end of the experiment. In the third experiment (E3), eggs at 24 hpf of age (unfertilised and fertilised), freshly hatched larvae (in non-feeding stage) and *A. aquaticus* (total body length 4 – 5 mm) were introduced into the wells at different ratios (see Table 1.). The number of larvae and unfertilised eggs were checked every 10 hours till the end of the experiment. Water temperatures were 24.5 °C – 25.0 °C (E1), 27.4 – 27.8 °C (E2) and 24.5 – 24.7 °C (E3) respectively. Photoperiod was set at 14h light: 10h dark without water changes during the experimental cycles. Summarized and detailed data about the experimental design can be seen in Table 1.

Statistical analysis

The effects of time and the number of *A. aquaticus* was tested in the first and second experiment using a Multivariate Analysis of Variance (MANOVA), in which the 'number of fertilized eggs containing living embryos', the 'number of non-fertilized/dead eggs' and the 'number of hatched larvae' (only in the second experiment) was considered as dependent variables and 'hours' and the 'number of *A. aquaticus*' were factors. In case of the third experiment, there was no variances in larvae data, therefore a multiway factorial ANOVA was performed, in which 'number of unfertilized/dead eggs' was considered as dependent variable, and 'hours' and the 'number of *A. aquaticus*' were factors. Dependent variable data was transformed using log(x + 1) to satisfy the assumptions of MANOVA and ANOVA. All analysis was conducted in SPSS v25 statistical software.

Results

Experiment I.

In the first experiment, the number of unfertilised eggs in wells containing *A. aquaticus* continuously decreased after the first 5 hours. However, some unfertilized eggs disappeared from the wells of the control group as well. The number of remaining eggs was statistically different ($p < 0.05$) between *A. aquaticus*-containing and control wells, with the disparity between the two groups increasing with time (Table 2. and 3., Figure 1.).

Experiment II.

In the second experiment, the number of unfertilised eggs continuously decreased after the first 5 hours in *A. aquaticus* containing wells. The decreasing ratio of unfertilised eggs deepened according to the number of *A. aquaticus* specimens/well. At the first assessment time (5:00 hours), the number of eggs were statistically different ($p < 0.05$) between the experimental groups and this difference increased with time (Table 4. and 5., Figure 2.). The first larvae were hatched in the 30th hour in every experimental group, however the hatching process was more dynamic in the *A. aquaticus* groups compared to control groups.

Experiment III.

In the third experiment, similar to the previous experiments, the number of unfertilized eggs continuously decreased from the 5th hour in *A. aquaticus* containing wells. *A. aquaticus* did not harm the non-swimming free larvae and there was no natural mortality detected, thus all introduced larvae survived the experimental period independent from the number of coexisting *A. aquaticus* specimens (Table 6., Figure 3.).

Discussion

Fungal and bacterial infection of fish eggs is a common issue in aquaculture hatcheries, where the implementation of prevention measures are key factors in successful propagation. Despite preventive measures, the occurrence of fungi and bacteria on eggs is almost inevitable. To mitigate the harmful effects of the proliferation of saprophytic and potentially pathogenic microorganisms, the removal of dead eggs is essential. Mechanical picking of dead eggs of Chinook salmon (*Oncorhynchus tshawytscha*) at the eyed stage led to a decrease in bacteria numbers [4]. This process mainly could be applied in case of salmonids due to the relatively large size of the eggs, easier visibility and a non-whirling incubation medium. However, it has a significant time and labour demand. In other species, e.g., cyprinids or percids where egg size is much smaller or incubation takes place in whirling media or attached to surface [29], manual removal of dead eggs is difficult, ineffective, and sometimes impossible.

In the classical approach of biological control, predators and parasitoids are used which target the pathogens or pests themselves. The first attempts at chemical free control of oomycetes parasites using two invertebrates, *A. militaris*, and *G. pseudolimneaus*, were reported by Oseid [24]. According to their results, both invertebrates improved the survival rate of eggs by preventing fungal growth, however

Gammarus showed some predation on live eggs and larvae. Our knowledge is scarce about the presence and significance of this phenomena in natural fish stocks.

The scraping feeding strategy of *A. aquaticus* makes it possible to ingest mycelia selectively from the surface of dead organic materials [27]. Bloor [30] conducted feeding preference experiments with *A. aquaticus* and he found that they prefer detritus which is already partially digested by microbial communities and fungus. Possible causes could be that fungi can eliminate allelopathic chemicals of plants and through partial digestion they can make detritus more utilizable for the detrivores which can also use the fungal enzyme system to degrade organic materials. Fungi also enrich the feed with their own micro- and macronutrient content [31].

In this study, our theory on how *A. aquaticus* eliminates mycelia is three-fold. Firstly, direct grazing on the surface of the eggs as is known of their natural feeding strategy. Secondly, the consumption of infected and dead eggs is also observable. Thirdly, due to the movements and feeding activity of *A. aquaticus*, physical damage to egg shells can result in “disappearing” eggs as we observed in this study. We believe the chorion is harmed in these cases and fluid organic material rapidly diluted into the water, thus preventing the eggs from serving as a substrate for fungal growth. The results of our study show that these feeding habits of *A. aquaticus* mitigate infection and prevent fertilized eggs from being entangled in fungal mycelium. In contrast to classical biological control, we studied a different approach to biological prevention. Instead of measures, chemical and others, aimed at the elimination of pests or disease, our focus was on the preventive elimination of the substrate of saprophyte fungi or bacteria (Figure 4.).

During our experiments none of the hatched larvae was harmed by *A. aquaticus*, even if no other feed source was provided for 48 hours. Other researchers found the same, that *A. aquaticus* specimens, as opposed to other crustaceans like *Gammaridae*, do not cause harm to young fish larvae [24,32]. However, it was also witnessed that hatching occurs faster in the presence of *A. aquaticus*. Most likely, the cause of this is the abrasion of egg shells due to the movements and feeding activity of crustaceans. This phenomenon did not correlate with the number of *A. aquaticus* per well. This could be beneficial in a way, as shortened incubation periods also reduce the chance of fungal infection. Though shorter incubation often results in smaller, weaker larvae, or limited hatching rate. We have observed this phenomenon without loss of larvae. This possible effect should be considered if implementing *A. aquaticus* into a water system, but its significance is dependent on species and water temperature.

Our results have shown that the elimination of dead eggs by *A. aquaticus*, in the case of large-scale incubation, is limited. Repeated inoculations of isopods containing large numbers of organisms are required at commercials hatcheries to replace manual egg picking. A more feasible application of *A. aquaticus* could be semi-natural spawning of endangered fish species e.g., *Misgurnus fossilis* and *Carassius carassius* or high value ornamental fish. Where egg incubation and larvae rearing take place in the same unit, and the breeders do not show cleaning and fanning behavior. Control of pathogens by

elimination of the substrate of bacterial and fungal growth could improve the hatching rate and larval survival as well as general hygienic measures.

Applicability in hatcheries should be tested in detail and circumstances of breeding and application of *A. aquaticus* should be optimized considering the effects of density, mating period, moulting and the different habits of sexes and life stages [32,33].

Conclusion

According to the results of the current study *A. aquaticus* can distinguish between fertile and unfertile eggs and are able to eliminate or reduce the number of 'dead eggs' which could be media for bacterial and fungal growth. We found that *A. aquaticus* do not harm eggs containing viable embryos and larvae even when alternative food sources are not available. We recommend the use *A. aquaticus* for biological prevention in special cases when small-scale egg incubation and larvae rearing are carried out at the same place (e.g., ornamental fish breeding or ex situ conservation).

Declarations

Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author Contributions

Conceptualization, Tamás Müller, Quyến Nguyễn Ngọc, Máté Havasi, Ádám Staszny, Béla Urbányi and Balázs Kucska; Methodology, Tamás Müller, Quyến Nguyễn Ngọc, Máté Havasi, Ádám Staszny, Károly, Vranovics, Béla Urbányi, Bence Ivanovics and Balázs Kucska; software, Ádám Staszny; validation, Tamás Müller, Máté Havasi and Balázs Kucska; formal analysis, Tamás Müller, Quyến Nguyễn Ngọc, Ádám Staszny, Béla Urbányi, Bence Ivanovics and Balázs Kucska; investigation, Tamás Müller, Quyến Nguyễn Ngọc, Károly Vranovics, Béla Urbányi; resources, Tamás Müller, Béla Urbányi and Balázs Kucska; data curation, Tamás Müller, Ádám Staszny and Béla Urbányi; writing—original draft preparation, Tamás Müller, Quyến Nguyễn Ngọc, Máté Havasi, Ádám Staszny, Károly, Vranovics, Jeffrey Daniel Griffitts, Béla Urbányi, Bence Ivanovics and Balázs Kucska; writing—review and editing, Jeffrey Daniel Griffitts, Máté Havasi; visualization, Tamás Müller, Máté Havasi and Balázs Kucska; supervision, Jeffrey Daniel Griffitts, Máté Havasi; project administration, Quyến Nguyễn Ngọc, Ádám Staszny and Béla Urbányi; funding acquisition, Tamás Müller and Béla Urbányi. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare that they have no known competing financial interests.

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Tables

Table 1 Summarised data about the experimental design for each experimental series.

	1 well contains			No of repetitions / treatment (n=)	duration (h)
	No of fertilized eggs (n=)	No of unfertilized eggs (n=)	No of *A. aquaticus* (n=)		
Experiment 1 (E1)	30	30	Ø	6	45
			5	5	
Experiment 2 (E2)	20	10	Ø	12	45
			1	12	
			3	12	
			5	12	
	1 well contains			No of repetitions / treatment (n=)	duration (h)
	No of fish larvae (n=)	No of unfertilized eggs (n=)	No of *A. aquaticus* (n=)		
Experiment 3 (E3)	5	5	Ø	12	40
	5	5	1	12	
	5	5	3	12	
	5	5	5	12	
	5	Ø	Ø	12	
	5	Ø	1	12	
	5	Ø	3	12	
	5	Ø	5	12	

Table 2 Summarised, detailed results of Experiment I.

Time	Fertilised egg with living embryo (%)	Unfertilised eggs (%)	Fertilised egg with living embryo (%)	Unfertilised eggs (%)
(h)	control		5 *A. aquaticus* / well	
00:00	100	100	100	100
05:00	95.6 ± 4.6	103.3 ± 5.2	96.7 ± 2.4	87.3 ± 10.1
10:00	93.3 ± 4.2	104.4 ± 5.8	96.7 ± 2.4	69.3 ± 4.3
15:00	92.2 ± 6.9	105 ± 8.1	94.7 ± 3.8	51.3 ± 8.4
20:00	87.8 ± 13.8	107.8 ± 16.7	94 ± 4.3	38.7 ± 13.5
25:00:00	87.2 ± 13.4	106.7 ± 17.3	90 ± 7.5	27.3 ± 15.5
30:00:00	86.7 ± 13.2	100 ± 16.3	89.3 ± 7.2	16 ± 11.4
35:00:00	86.1 ± 12.9	92.2 ± 23.3	84.7 ± 9	14 ± 11.4
40:00:00	86.1 ± 12.9	83.9 ± 28.3	80 ± 8.5	7.3 ± 8.3
45:00:00	86.1 ± 12.9	81.7 ± 29.6	76.7 ± 11.3	4.7 ± 4.5

Table 3 Summarised statistical analysis (MANOVA) of Experiment I. on the effects of time, the number of A. aquaticus and their combination on egg batches.

FECLE: fertilised eggs containing living embryos, NF: unfertilised/dead eggs

		df	Mean Squares	F	p
Hours	FECLE	9	0.01	3.994	< 0.001
	NF	9	0.923	13.041	< 0.001
A. aquaticus	FECLE	1	0.000	0.041	0.841
	NF	1	10.314	145.784	< 0.001
Hours × *A. aquaticus*	FECLE	9	0.002	0.648	0.753
	NF	9	0.645	9.117	< 0.001
Error	FECLE	90	0.003		
	NF	90	0.071		

Table 4 Summarised data of Experiment II. at the 45th hour.

Fertilised eggs and hatched larvae values are expressed in the percentage of the initial number of viable eggs. Values of unfertilised eggs are expressed in the percentage of the initial number. Data are presented as means and standard deviations (± SD). Different superscripts indicate statistical differences.

	Fertilised eggs with living embryo (%)	Unfertilised eggs (%)	Hatched larvae (%)
control	69.6 ± 18.9[a]	94.2 ± 7.9[a]	29.2 ± 18.2[a]
1 *A. aquatisus* / well	26.3 ± 29.2[b]	18.3 ± 15.9[b]	65.4 ± 25.4[b]
3 *A. aquatisus* / well	27.5 ± 16.3[b]	4.2 ± 6.7[c]	67.1 ± 14.2[b]
5 *A. aquatisus* / well	30.4 ± 26.2[b]	0	58.3 ± 22.1[b]

Table 5 Summarised statistical analysis (MANOVA) of the effects of time, the number of A. aquaticus and their combination, on egg batches in Experiment II.

FECLE: fertilised eggs containing living embryos, UF: unfertilised/dead eggs

		df	Mean Squares	F	p
Hours	FECLE	9	2.324	26.066	< 0.001
	UF	9	5.527	48.34	< 0.001
	Hatched larvae	9	16.148	221.206	< 0.001
A. aquaticus	FECLE	2	1.41	15.806	< 0.001
	UF	2	50.474	441.453	< 0.001
	Hatched larvae	2	3.293	45.112	< 0.001
Hours × *A. aquaticus*	FECLE	18	0.393	4.403	< 0.001
	UF	18	2.054	17.961	< 0.001
	Hatched larvae	18	0.67	9.172	< 0.001
Error	FECLE	330	0.089		
	UF	330	0.114		
	Hatched larvae	330	0.073		

Table 6 Statistical analysis (MANOVA) of the effects of time, the number of A. aquaticus and their combination on egg batches during Experiment III.

UF: unfertilised/dead eggs

		df	Mean Squares	F	p
Hours	UF	4	8.136	58.83	< 0.001
A. aquaticus	UF	3	6.298	45.54	< 0.001
Hours × *A. aquaticus*	UF	12	1.791	12.95	< 0.001
Error	UF	220	0.138		

Figures

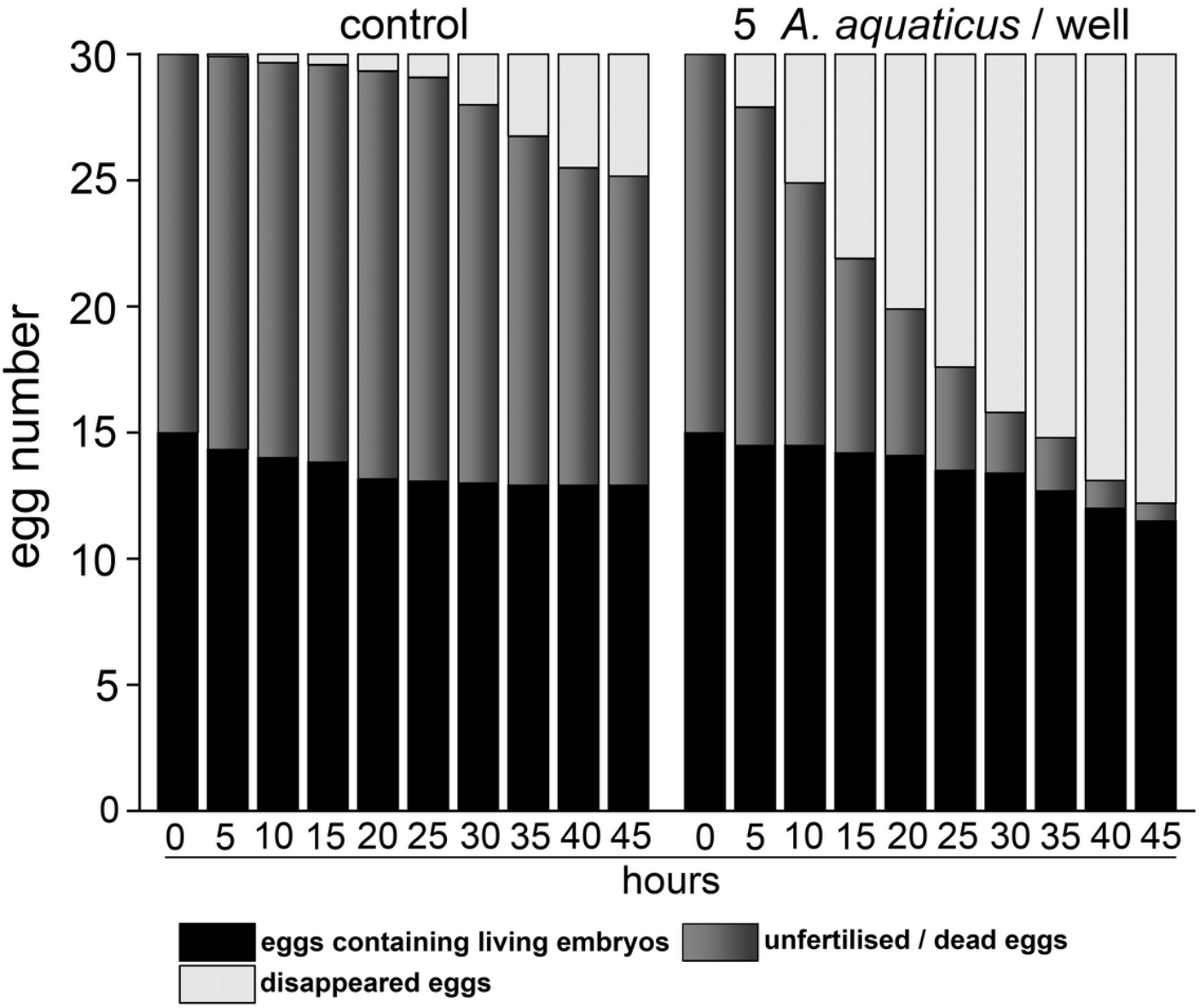

Figure 1

Average numbers of non-fertilised and fertilised eggs during Experiment I.

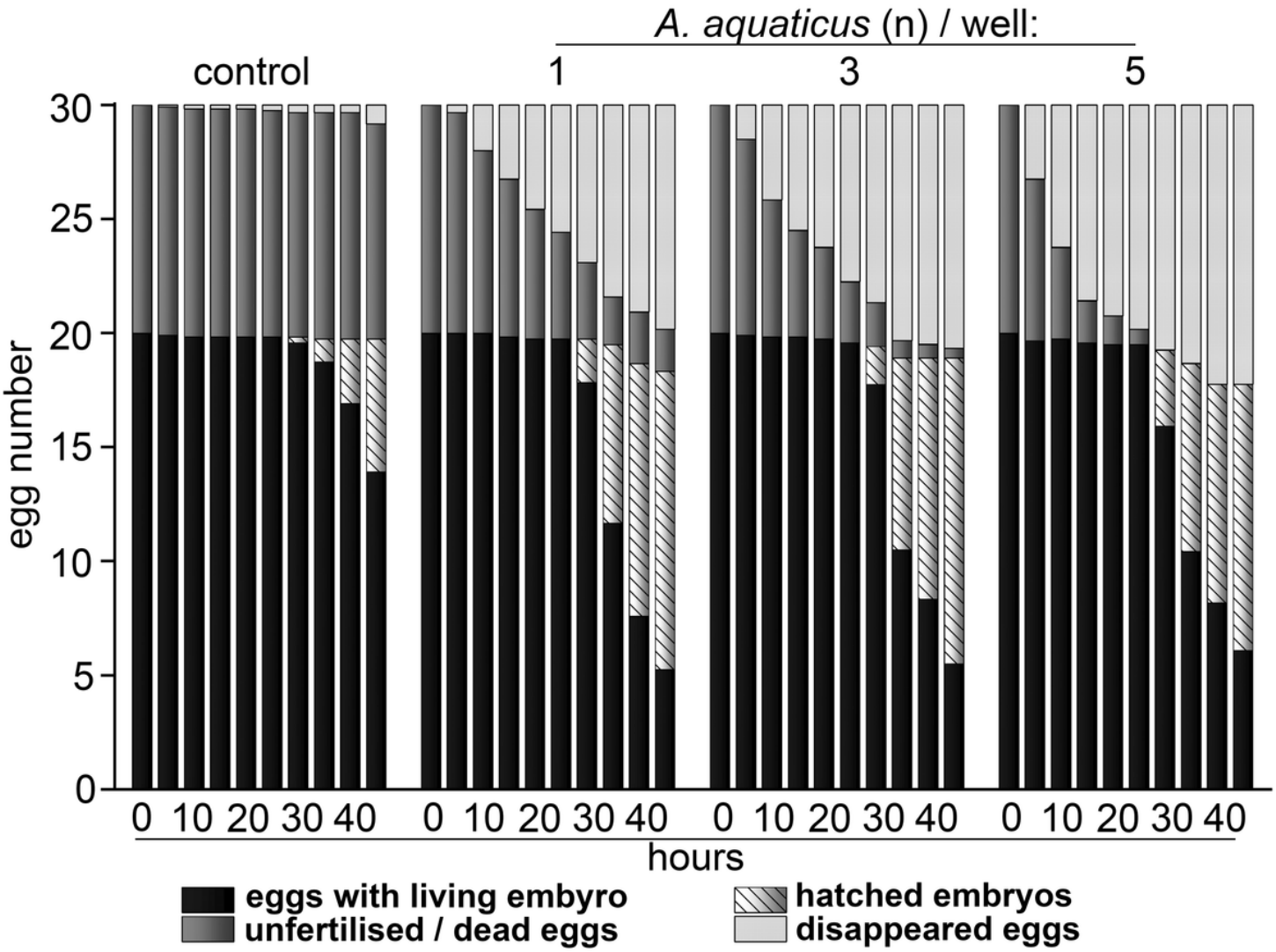

Figure 2

Average numbers of non-fertilised eggs, fertilised eggs and hatched embryos during the experimental period depending on the number of *Asellus* specimens.

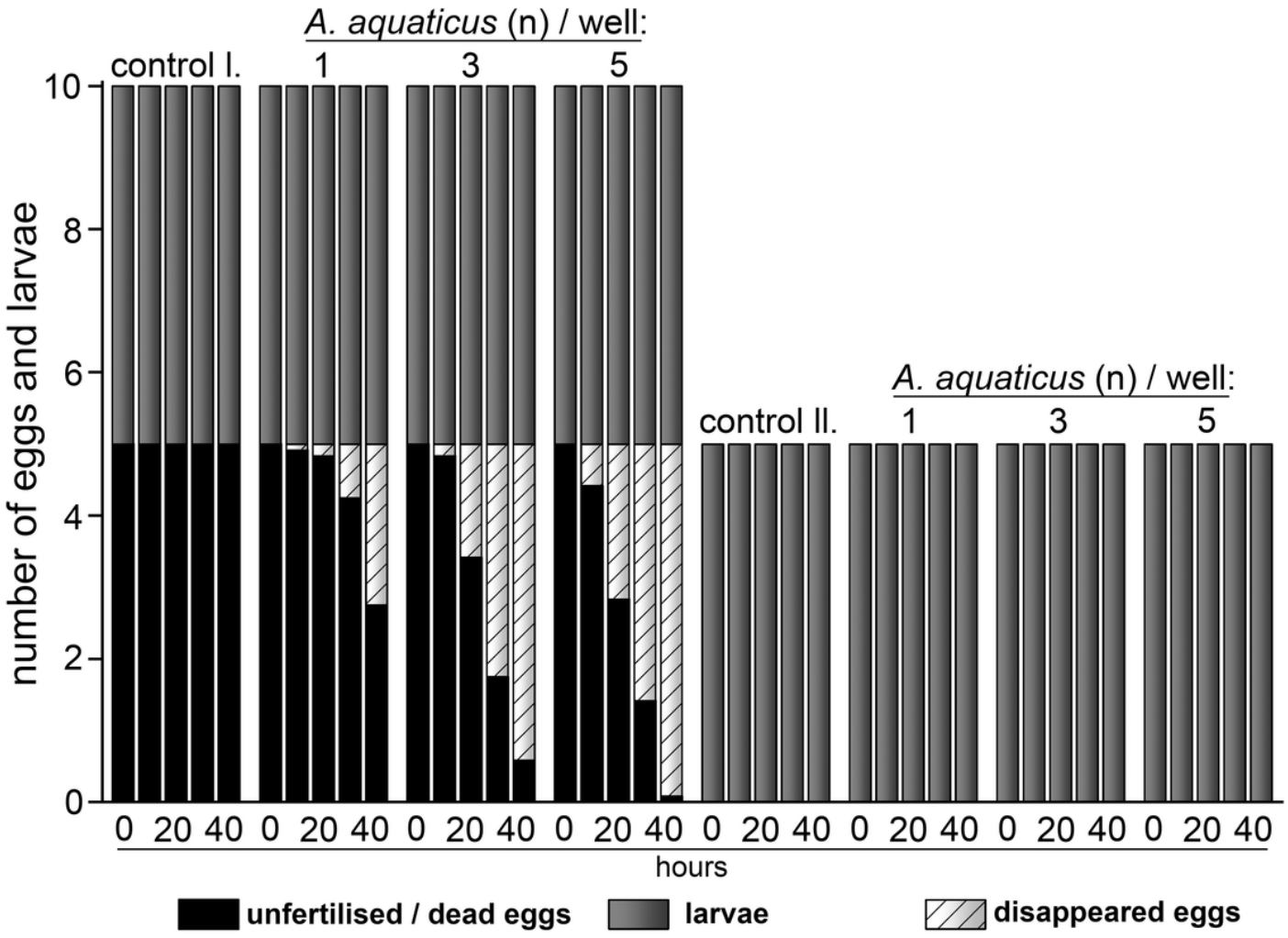

Figure 3

Average numbers of non-fertilised eggs, fertilised eggs and hatched embryos during the experimental period depending on treatments.

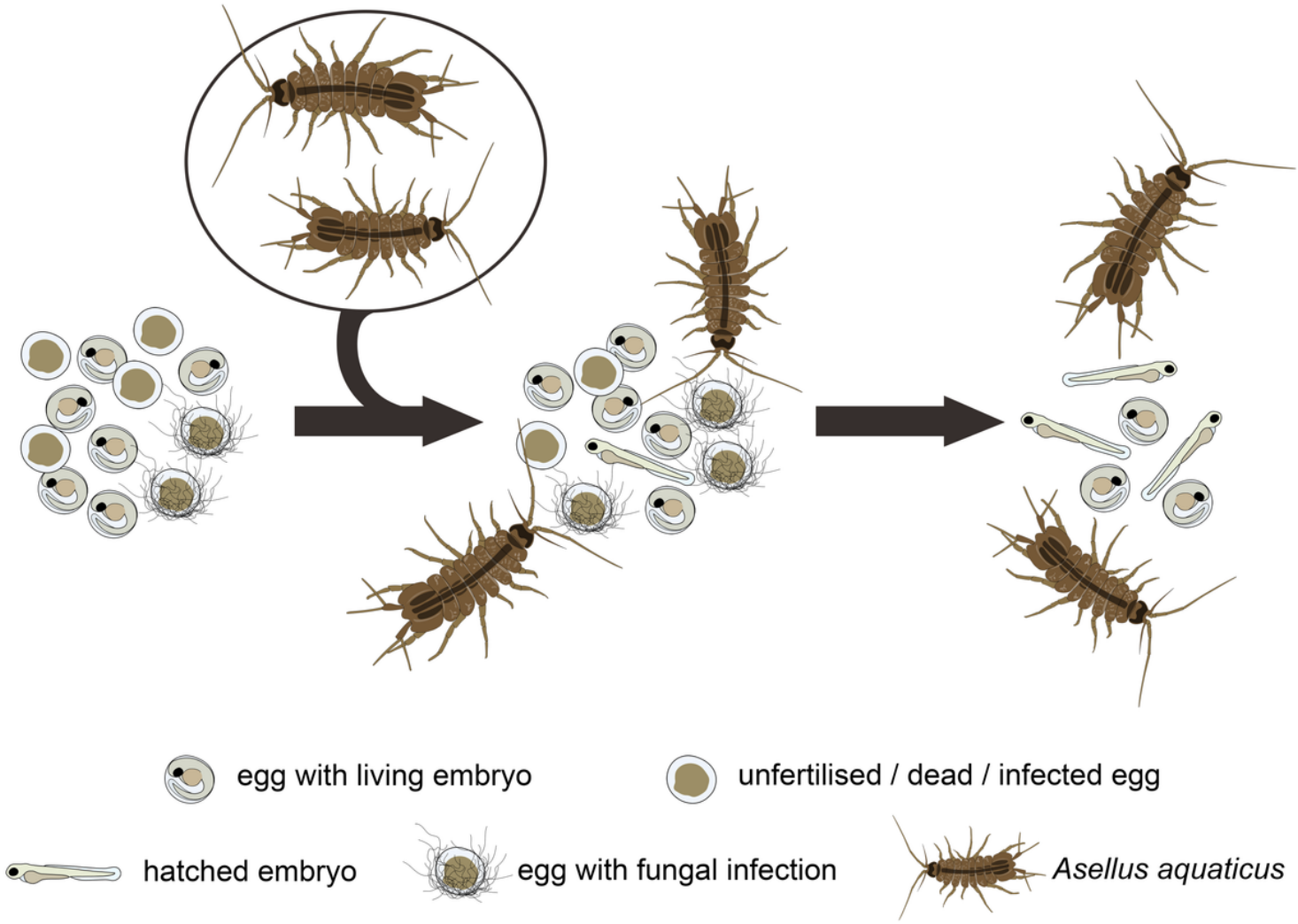

Figure 4

Average numbers of non-fertilised eggs, fertilised eggs and hatched embryos during the experimental period depending on treatments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- RawDataKucskaetal..xlsx