The off-host survival and viability of a native and non-native fish louse (Argulus, Crustacea: Branchiura)

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Abstract

Fish ectoparasites are introduced to water bodies or aquaculture systems along with their hosts. Argulid fish lice can survive off-host for a given time, and in spite of quarantine measures might also be introduced via the transfer of water. However, they must cope with varying abiotic conditions without access to food. We investigated the effect of temperature (5 to 28°C) on the off-host survival time of Argulus japonicus, an invasive species which is apparently increasing its distribution, and compared this with the European A. foliaceus. A clear effect of temperature on the off-host survival time of all stages of both species was demonstrated. Larval and juvenile A. japonicus survived up to 9 days at 22°C and in the case of adults up to 13 days at 15°C. By comparison, larval A. foliaceus survived up to 5 days at 15°C; adults up to 14 days at 9°C and juveniles up to 7 days at 9°C and 15°C. Thus, A. japonicus is more resistant to starvation at higher temperatures under controlled off-host conditions and A. foliaceus is more resistant to starvation at lower temperatures. Infectivity of A. japonicus decreased linearly with the time spent off-host after 2 days for larvae and 4 days for adults. Temperature only had a significant effect on the infectivity of both developmental stages after 24 hours off-host between 13–23°C for larvae and 13–18°C for adults. In conclusion both species can potentially survive off-host periods in water transports for up to 13–14 days as adults. Their survival is shorter as larvae. This study demonstrates that transportation of water has the potential to introduce Argulus species [Current Zoology 57 (6): 828–835, 2011].

Keywords Argulus, Fish lice, Survival, Temperature, Off-host, Water transport, Introductions, Invasive species

Argulus species are ectoparasites of fish that have become widespread due to anthropogenic introductions of their hosts (ornamental and consumption fish) to which they are attached. They are the causative agents of the disease argulosis. Argulus species are also known to act as vectors for other pathogens (Bower-Shore, 1940; Stammer, 1959; Shimura, 1983; Ahne, 1985; Moravec, 1994) and increase the susceptibility of their host to secondary infections (Bandilla et al., 2006). Argulid parasites exhibit a low host specificity, and produce large numbers of eggs, including multiple clutches of eggs within one breeding season (Kollatsch, 1959; Stammer, 1959; Walker et al., 2008a, b). Consequently, when introduced to new environments these parasites have the potential to reach high densities quickly and spread throughout an entire fish community, even if the first introduction consists of just a few individuals. At very high fish densities, epizootics can occur resulting in fish kills as reported from lentic waters, fish hatcheries and aquaculture facilities, recreational fishing lakes, large public aquaria, home aquaria and outdoor ponds (Menezes et al., 1990; Taylor et al., 2006; Poly, 2008).

Argulus foliaceus is the most common and widespread native argulid in the Palaearctic and the most documented. This species has been reported as a threat to the culture of tilapia Oreochromis niloticus (Roberts and Sommerville, 1982; Paperna, 1991), rainbow trout Oncorhynchus mykiss (Menezes et al., 1990; Ruane et al., 1995) and common carp Cyprinus carpio (Jafari and Ahmed, 1994). Another widespread species, Argulus japonicus, is believed to have originated in Japan and China. It owes its current relatively ubiquitous distribution to the trade in ornamental fish such as goldfish Carassius auratus and koi carp Cyprinus carpio (Rush-тон-Mellor, 1992). Nowadays the species is known from every continent excluding Antarctica (Poly, 2008).
contrast to *A. foliaceus*, *A. japonicus* has also been reported from several locations in Africa (Rushton-Mellor, 1994) suggesting a greater tolerance for higher temperatures. *A. japonicus* also occurs in the Laurentian Great Lakes region where it is plausible that it was introduced via ballast water and sediments from ships (Reid and Hudson, 2008).

Larval lice hatch as free-swimming metanauplii from eggs that have been deposited on various substrates (e.g. plant stems and stones) (Kear, 2004; Walker et al., 2004). Once a suitable host has been located, argulid fish lice do not remain in contact with their hosts for the rest of their life. They may become dislodged by the fish or detach themselves for the purpose of mate location, egg deposition or to locate a more preferable host (Walker et al., 2004). The length of time spent apart from a host will ultimately depend upon the host location abilities of the parasite and the availability of suitable hosts. Reports regarding the survival time of adult lice during these off-host periods are fragmentary and sometimes conflicting. Factors such as timing of the parasites last meal, level of activity and temperature are likely to influence this time period.

Despite the knowledge that species such as *A. foliaceus* and *A. japonicus* have a relatively ubiquitous occurrence, and appear to be increasing their range (as is the case for *A. japonicus*), there is very little known about the effects of abiotic factors, including temperature, on these parasites, particularly with regard to their survival. This information could be vital in predicting the future spread of these potentially destructive lice, especially in light of global climate change. Besides attached to the host transport and introduction of these parasites can also occur by larvae and adults occurring off-host in water in which the host had been present as well as by eggs attached to hard substrates. Water can therefore be considered a vector for these parasites although the degree to which it acts so is dependent upon the off-host survival time of lice.

This paper reports on investigations into the effect of temperature and stage of development on the off-host survival of two argulid species, *A. japonicus* and *A. foliaceus*, with overlapping natural ranges, habitats and host species. The effects of these off-host periods and temperature on the host location capabilities of *A. japonicus* are also discussed.

1 Materials and Methods

1.1 Parasite culture

Populations of *A. japonicus* and *A. foliaceus* were maintained on common carp *Cyprinus carpio* (approximately 1 kg weight) in recirculation systems at 23°C (± 1°C) and with a 12:12 light: dark photoperiod. Nijmegen tap water (non-chlorinated) was used in all systems. Infestation intensities typically varied from 10 to 30 lice per fish. Eggs of *Argulus* were deposited on the glass sides and bottoms of the aquaria. Host fish were monitored regularly and parasite eggs were removed to control parasite numbers when infestation intensities appeared to be too heavy as indicated by host condition and host-behavioural changes e.g. lethargy and loss of appetite. All parasite populations had been established under these conditions for several generations prior to experimentation.

1.2 Off-host survival of *A. japonicus* and *A. foliaceus*: effect of temperature

Adult *A. japonicus* and *A. foliaceus* (males >3mm, females >4mm to ensure maturity) were collected from stock carp that had been anaesthetised in 2-phenoxyethanol (Sigma-Aldrich, St Louis, MO, USA) solution (dilution is 1:1000). Fish were considered anaesthetised once they displayed loss of equilibrium (i.e. turned ‘belly-up’) which took approximately 2 min. Parasites were subsequently removed using a set of blunt forceps and then held in beakers containing Nijmegen tap water at 23°C for 24 h prior to use in experiments. Previous studies have revealed that 2-phenoxyethanol does not affect the off-host survival or infectivity of either *A. japonicus* or *A. foliaceus* at the concentrations used here (*unpublished data*). During this time any eggs deposited by lice were collected and incubated in Nijmegen tap water at 23°C with daily refreshment of the water. Upon hatching (the difference between hatching rates was less than 10 h), larval lice were either held for approximately 24 h under the same conditions as adult lice or were allowed to attach to a group of juvenile common carp, maintained as stock carp, to develop for 12 d before being collected and treated in the same way as for adult lice. Adult *A. japonicus* and *A. foliaceus* are known to take approximately 25 d to reach maturity at 23°C (personal observation). Twelve day old lice are therefore juveniles (Rushton-Mellor and Boxshall, 1994). All lice used were taken from multiple egg strings and pooled prior to selection for subsequent experiments.

Lice from each of the developmental stages (*n* = 30–50 lice per trial depending on the number of lice available from cultures) were then placed into small clear plastic bags with 300 ml of Nijmegen tap water (23 ± 1°C) and acclimated to experimental temperatures...
for 30 min before being added to experimental aquaria. Experiments were conducted in three replicate small glass aquaria (10 L) containing Nijmegen tap water and maintained at 5, 9, 15, 23 or 28°C (±1°C) with a 12:12 light:dark photoperiod and without access to a host upon which they could feed. Aquaria were individually aerated and 10% of the water was refreshed daily after being adjusted to the appropriate temperature. Parasites were examined daily and the number of dead specimens recorded. Death was assumed when no limb movements could be observed after gently touching lice with a small metal seeker and then confirmed by examination under a binocular microscope.

1.3 Attachment success of *A. japonicus*: Effect of starvation

Adult and larval lice were acquired using the methods above. Fifty adult and 50 larval lice were held in beakers containing Nijmegen tap water (23 ± 1°C) for varying periods of time (1, 2, 3, 4 or 5 days for larval lice and 1, 2, 3, 4, 5, 6 or 7 days for adult lice) without access to a host upon which they could feed. Parasites were then added to small (10 L), individually aerated aquaria containing five juvenile common carp and left for one hour to locate and attach to the host. After one hour all carp were removed and anaesthetised and the number of attached lice recorded. The number of lice remaining in the aquaria (those that did not successfully attach to a host) was also noted to check if host predation on the parasites had occurred. Experiments were performed in triplicate (i.e. 3 × 50 lice) for each period of time ‘off-host’.

1.4 Attachment success of *A. japonicus*: Effect of temperature

Adult and larval lice were acquired using the methods above. Sixty juvenile common carp were equally distributed between 12 small (10 L) individually aerated aquaria and acclimated to 13, 18, 23 or 29°C (with three replications of each temperature), for a period of two weeks prior to use in experiments. Fifty lice were then placed into small transparent plastic bags containing 300 ml Nijmegen tap water and allowed to acclimate to experimental temperatures for a period of 30 min before being released into the aquaria to search for a host. All fish were sampled after 1 h and thoroughly examined for any attached lice. The number of attached lice for each aquarium was then recorded and attachment success calculated as the percent of lice successfully attaching to a host.

1.5 Data analysis

Differences in off-host survival times for each developmental stage (larval, juvenile and adult) for each species (*A. japonicus* and *A. foliaceus*) were initially evaluated using Three-Way ANOVA with temperature, developmental stage and parasite species as factors. Data was then divided by species (*A. foliaceus* and *A. japonicus*) and evaluated using Two-Way ANOVA with temperature and developmental stage as factors. Data was further subdivided by developmental stage (larval, juvenile and adult) and evaluated with One-Way ANOVAs. When these tests indicated significant differences in the data set a Tukeys post hoc test was used to determine where off-host survival times differed. Between species comparisons were evaluated using 2-sample t-tests. Bonferroni-Holme corrections were applied to P-values where multiple pairwise comparisons were conducted within the same dataset.

Differences between the attachment success of larval and adult *A. japonicus* were evaluated with One-Way ANOVAs. When these tests indicated significant differences in the dataset Tukeys post hoc tests were used to determine where differences occurred. Differences in the attachment success of larval and adult *A. japonicus* at different temperatures were similarly evaluated using 1-way ANOVAs followed by Tukeys post hoc tests.

All data sets were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene’s test) to ensure that data met the assumptions of statistical tests (ANOVA, Tukeys test, T-test) prior to performing statistical tests. In all tests, significance was set at $P<0.05$.

2 Results

2.1 Off-host survival

Survivorship curves for *A. japonicus* and *A. foliaceus* larvae, juveniles and adults are shown in Fig. 1. The majority of curves exhibit a pattern somewhere between a type 1 and type 2 survivorship curve (after Deevey, 1947). However, the data for juvenile and adult *A. japonicus* at 28°C more closely resemble a type 3 survivorship curve (after Deevey, 1947).

Considerable variation is exhibited between parasite developmental stages and temperatures. Maximum off-host survival times varied from 3–9 d for larval *A. japonicus* and 3–5 d for larval *A. foliaceus*. Juvenile lice showed similar variation with maximum off-host survival times ranging from 5–12 d for *A. japonicus* and 5–7 d for *A. foliaceus*. Adult louse maximum off-host survival times ranged from 5–13 d for *A. japonicus* and 8–14 d for *A. foliaceus*.

The longest off-host survival times for larval lice
were at 22°C for \textit{A. japonicus} and 15°C for \textit{A. foliaceus}. Juvenile lice survived off the host for the longest time at 22°C for \textit{A. japonicus}, and 9°C and 15°C for \textit{A. foliaceus}. In the case of adult lice, \textit{A. japonicus} survived off-host for the longest time at 15°C and \textit{A. foliaceus} at 9°C. The mean off-host survival times of \textit{A. japonicus} and \textit{A. foliaceus} larvae, juveniles and adults at five different temperatures showed differences between developmental stages and at different temperatures (Fig. 2A and B).

Results of the Three-Way ANOVA showed that the interaction variable Temperature (T) × Developmental Stage (DS) × Species (S) was significant ($F=216.719$, $P<0.001$, $df=8$). Therefore data were divided by parasites species and further evaluated by 2-way ANOVAs. The interaction variable T × DS was significant for both the \textit{A. japonicus} and \textit{A. foliaceus} Two-Way ANOVAs (\textit{A. japonicus} $F=2763.23$, $P<0.001$, $df=8$; \textit{A. foliaceus} $F=305.35$, $P<0.001$, $df=8$). Therefore data were subdivided by developmental stage and further analysed by One-Way ANOVAs.

ANOVA showed significant differences between temperatures for the off-host survival times of larval \textit{A. japonicus} ($F=22588.75$, $P<0.001$, $df=4$). Tukeys pairwise comparisons revealed that these differences were significant between all temperatures except between 9°C and 28°C and 15°C and 22°C ($P=0.3316$ and 0.3316, respectively). ANOVA also showed that off-host survival times of juvenile \textit{A. japonicus} were significantly different between temperatures ($F=20644.05$, $P<0.001$, $df=4$). Tukeys test confirmed that differences in off-host

\begin{figure}
\centering
\includegraphics[width=\textwidth]{survivorship_curves.png}
\caption{Survivorship curves for three developmental stages of \textit{A. japonicus} (larvae, A; juveniles, B; and adults, C) and \textit{A. foliaceus} (larvae, D; juveniles, E; and adults, F) at five different temperatures
\textbullet 5°C; ■ 9°C; ▲ 15°C; × 22°C; ○ 28°C. Larvae used in experiments were 1 day post-hatch, juveniles were 12 days post hatch and adults > 24 days post hatch.}
\end{figure}
Mean off-host survival time (in days) of A = A. japonicus and B = A. foliaceus, larvae (1 day post hatch), juveniles (12 days old) and adults (> 24 days old) at five different temperatures

Survival times were significant between all temperatures for juvenile A. japonicus. A similar result was obtained for adult lice with ANOVA showing significant differences between the off-host survival times of adult A. foliaceus at different temperatures (F=10738.32, P<0.001, df=4) and Tukey's test confirmed that these differences were significant for all temperatures.

For A. foliaceus, ANOVA showed significant differences between off-host survival times of larval lice at different temperatures (F=1109.45, P<0.001, df=4). Tukey's test showed these differences were significant in all cases for larval A. foliaceus except when comparing 9°C and 22°C (P=0.4619). ANOVA also showed significant differences between off-host survival times of juvenile A. foliaceus at different temperatures (F=329.21, P<0.001, df=4) and between off-host survival times of adult A. foliaceus at different temperatures (F=329.21, P<0.001, df=4). Tukey's tests confirmed differences were significant between all temperatures for juvenile and adult A. foliaceus.

A. japonicus and A. foliaceus adults typically survive off-host for the longest periods and larvae for the shortest, irrespective of temperature. Larval and juvenile A. japonicus generally survive longer off-host than A. foliaceus. For adult stages however the trend is reversed as A. foliaceus survives longer off-host at 5°C, 9°C and 22°C. A. japonicus adults survived longer than A. foliaceus adults at 15°C and 22°C however. Off-host survival times were significantly different between A. japonicus and A. foliaceus for all developmental stages at all temperatures.

2.2 Attachment success of A. japonicus: Effect of starvation

The two developmental stages differed in the relative numbers of lice successfully locating and attaching to a host within the one hour time frame of this experiment (Fig. 3). A maximum of 98% of adult lice successfully located and attached to a host within one hour whereas for larvae the maximum value was 74%. ANOVA showed significant differences in the attachment success rates of adult A. japonicus following different starvation periods (F=2080.81, P<0.001, df=6). Tukey's test shows that the attachment success of adult A. japonicus started to significantly decline daily after approximately four days of starvation. For larval A. japonicus ANOVA showed significant differences in the attachment success after different starvation periods (F=946.50, P<0.001, df=5). Tukey's tests showed that attachment success of larval A. japonicus started to significantly decline daily after approximately two days of starvation.

2.3 Attachment success of A. japonicus: effect of temperature

Attachment success was similar for adult and larval lice at different temperatures (Fig. 4). However, there were significant differences in the attachment success of larval A. japonicus at different temperatures (F=16.53, P<0.001, df=3). Tukey's test showed that this difference was only statistically significant when comparing the 13°C and 23°C treatments (T=6.948, P=0.0003, df=1). For adult A. japonicus, ANOVA showed that there were significant differences in attachment success at different temperatures (F=16.53, P<0.001, df=3). Tukey's test however confirmed that the difference was only significant when comparing the 13°C and 18°C treatments (F=5.964, P=0.008, df=1).
Wilson (1902) observed yolk in the digestive system of larval argulids but there is no published data available on how long this yolk can sustain them. Reports vary with respect to the off-host survival times of newly hatched argulids but typically, state between 2 and 4 days (Mikheev et al., 2003), or even as little as one day in the case of *A. japonicus* (Tam and Avenant-Oldewage, 2006). Our data for *A. foliaceus* differ only marginally from those reported by Mikheev et al. (2003). The average off-host survival times determined by these authors ranged from 2.10–3.26 days with a maximum off-host survival time of 5 days (at 15°C). Shafir and Oldewage (1992) stated that *A. japonicus* perished after only 3 or 4 days off-host without mentioning the developmental stage of these lice. In our study, the mean off-host survival time of larval *A. japonicus* ranged from 2.39 days to 5.17 days (depending upon ambient temperature) which appears to be in rough agreement with the observations of Shafir and Oldewage (1992). However, larval *A. japonicus* in our study survived for a maximum period of 8 or even 9 days off-host in a few cases. Most of these animals appeared incapable of locomotion and could have been easily mistaken as dead, which may account for the differences between our data and those reported by Shafir and Oldewage (1992). Many of the reports regarding off-host survival times of argulids were based on observations under unspecified conditions regarding parasite developmental stage, time since parasite collection/hatching, light regimes and temperature.

We showed that the mean off-host survival time of adult *A. japonicus* ranged from 2.46 to 9.43 days with a maximum off-host survival time of 13 days (at 15°C). Similar results were obtained for *A. foliaceus* although here mean survival times were higher than those of *A. japonicus* for 3 out of 5 of the temperatures and the maximum off-host survival time was 14 days (at 9°C).

In general, adult *A. foliaceus* survived longer at lower temperatures than *A. japonicus*. Larval *A. japonicus* appeared to be able to survive longer than larval *A. foliaceus* at temperatures in the mid-range of those studied here. *A. foliaceus* is native to Europe whereas *A. japonicus*, although widespread in Europe for many decades, is believed to have been introduced from the orient via the trade in ornamental fish varieties (Rush- ton-Mellor 1992). These two species may have evolved in climates experiencing different temperature regimes which have determined their temperature tolerance. Temperature has the greatest influence on off-host survival times at the two extremes of the parasites tolerance range but is most striking at higher temperatures. *A. foliaceus* appears more adapted to survival at lower average temperatures typical of northern climates than its relative from the orient. *A. japonicus* is reported as widespread across much of southern and western Europe (Walker et al., 2004). We propose that the further spread of *A. japonicus* will be restricted by its reduced ability to survive at lower temperatures although global warming may ultimately extend its range. Several reports on *A. foliaceus* showed overwintering as adults at temperatures lower than 5°C. Such reports are lacking for *A. japonicus* or *A. foliaceus* larval and juvenile stages. Our experimental data demonstrate that all developmental stages of *A. foliaceus* and *A. japonicus* are
probably also able to over-winter although their survival rates may be limited in comparison with adult *A. foliaceus* due to a shorter off-host survival time at lower temperatures. For both species high temperatures seem to be detrimental in terms of off-host survival times. They both originate and persist in regions where such high water temperatures are not frequently experienced.

We observed that one or two days before they perished both argulid species became very inactive. Hakalahti et al. (2005) reported similar observations for larval *A. coregoni* (metanauplii) and attributed this to a possible state-dependent behavioural modification (Fenton and Rands, 2004). In our study, the attachment success of larval *A. japonicus* was optimal for approximately two days post hatching and for approximately 4 days off-host for adults. Attachment success in relation to the time spent without access to a host upon which to feed confirmed that lice become very lethargic after a certain period of starvation. In fact, several days before they die as a result of starvation, their ability to locate and infect a host is significantly diminished and they do not appear to possess sufficient energy reserves to locate and attach to a host and can be considered not viable/infective. We estimate this time frame to be after 4 days for larval *A. japonicus* and after 6 days for adult *A. japonicus* at 23°C. The difference in maximum numbers of lice successfully locating and attaching to a host fish suggests a difference in host location abilities of the different developmental stages. Adults are more successful at locating a host than larvae. Several factors may cause this difference including the level of development of the sensory organs, swimming ability, morphological differences in attachment organs or, speculatively, an ability of lice to ‘learn’ to recognise a host more quickly as they mature.

Kollatsch (1959) stated an optimal temperature for locomotion of 18–23°C for *A. foliaceus*. Therefore, we expected temperature to have a significant effect on host location and attachment success of *A. japonicus*. From our data it appears that the optimum temperature for host location and attachment success for *A. japonicus* is probably similar to that of *A. foliaceus* as recorded by Kollatsch (1959). A parasite would be minimally affected by temperature in terms of its ability to successfully locate and attach to a new host. Dislodgement or death of the host can occur during any season and therefore at any temperature, and any reduced ability to locate a host could be lethal to this parasite. Intermittent parasites need to retain their ability to mobilize themselves to locate and attach to a host under a range of environmental conditions and because >50% of parasites successfully located and attached to a host at each temperature, it appears that *A. japonicus* accomplishes this with respect to temperature.

Argulids modify their host-searching behaviour in order to best-utilize energy reserves in relation to the likelihood of host encounters, i.e. they switch from sit-and-wait to active searching strategies connected with the changes from light to dark conditions (Mikhnev et al. (2003) and literature therein). Hakalahti et al. (2005) propose a second type of host-searching behavioural modification that is state dependent i.e. dependent upon the state of the parasite's energy reserves. We observed that at a certain point however these mechanisms apparently fail and parasites lose the ability to successfully locate a host resulting in mortality of the parasite.

In conclusion, we have shown that temperature and developmental stage have a clear influence on the off-host survival time of *A. japonicus* and *A. foliaceus* under experimental conditions. Off-host survival time increases as lice mature. There were significant differences in the survival times of the two parasite species, each with its own optimum temperature range, which also differs between developmental stages. We have demonstrated that, like the native European *A. foliaceus*, *A. japonicus* can probably overwinter as adult stages, increasing the potential number of generations produced in subsequent breeding seasons. We have shown that off-host periods significantly influence the infectivity of *A. japonicus*, with lice becoming less viable the longer they have been without food. Temperature does not appear to greatly influence the infectivity of *A. japonicus* within the broad temperature range tested although there may be a small increase in infectivity between the optimum temperatures of 18°C and 23°C. We found that adult lice can survive in water for significantly longer periods without a host than larval lice.

Control strategies must account for the fact that *A. foliaceus* and *A. japonicus* can probably survive as both egg stages and adult stages during the winter months and this poses a much larger threat in terms of the number of potential infective lice appearing at the start of each breeding season as water temperatures in temperate areas such as western Europe tend to rise as a result of global warming and suggests a further spread of this non-indigenous species. This study also demonstrates the ability of these parasites to survive for several days in water without access to a host. These observations
have implications regarding the potential for these lice spreading to new water bodies via ships’ ballast water or pumping of untreated water between reservoirs.

Acknowledgements The authors would like to acknowledge T. Spanings for assistance with fish husbandry, sampling and anaesthesia of fish. Thanks are extended to members of the PARITY EU training network for advice and comments during informal presentations of these data. P. D. Walker and I. Russon acknowledge the financial support provided through the European Community’s Improving Human Potential Programme under contract (HPRN-CT-2001-00214), (PARITY).

This is Centre for Wetland Ecology publication No. 485.

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