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Assessing the invasion potential of non-native branchiobdellidans: experimental studies of survival, reproduction and competition

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Abstract – The impact of invasive species on the recipient ecosystem can be strongly influenced by the presence of associated symbionts. It is therefore important to evaluate the likelihood of co-introduced symbiont establishment, and this requires an understanding of their life history traits. Here, we investigate survival, reproduction and competition in two non-native branchiobdellidan ectosymbionts (*Xironogiton victoriensis* and *Cambarincola* aff. *okadai*) on invasive signal crayfish (*Pacifastacus leniusculus*). *In vivo*, *X. victoriensis* established viable infrapopulations within 10 weeks, whereas *C.* aff. *okadai* went extinct within 2 weeks. Both *X. victoriensis* and *C.* aff. *okadai* deposited cocoons *in vivo* that hatched in 10–27 and 10–11 days, respectively. *In vitro*, *X. victoriensis* and *C.* aff. *okadai* survived for over 13 and 15 weeks respectively, although both were negatively affected by increased temperature and nitrate, and were absent from kick samples taken in the field. Only *C.* aff. *okadai* deposited cocoons *in vitro*, and this larger species readily predated on *X. victoriensis* but not *vice versa*. Both branchiobdellidans possess traits associated with colonisation success, including a relatively fast reproductive rate and extended off-host survival. Given its survival *in vivo* and known detrimental effect on signal crayfish *X. victoriensis* is perhaps more likely to influence host invasion dynamics, although its persistence may be affected by the presence of co-occurring symbionts.

Keywords: invasive non-native species / invasive parasites / climate change / signal crayfish / *Xironogiton victoriensis*

Résumé – Évaluation du potentiel invasif de Branchiobdellidés non indigènes : études expérimentales de survie, de reproduction et de compétition. L'impact des espèces envahissantes sur l'écosystème récepteur peut être fortement influencé par la présence de symbiotes associés. Il est donc important d'évaluer la probabilité d'un établissement de symbiotes co-introduits, ce qui nécessite une compréhension de leurs traits d'histoire de vie. Ici, nous étudions la survie, la reproduction et la compétition de deux ectosymbiontes branchiobdellidés non indigènes (*Xironogiton victoriensis* et *Cambarincola* aff. *okadai*) sur l'écrevisse signal invasive (*Pacifastacus leniusculus*). *In vivo*, *X. victoriensis* a établi des infrapopulations viables en 10 semaines, alors que *C.* aff. o*kadai* s'est éteint dans les 2 semaines. *X. victoriensis* et *C.* aff. *okadai* ont déposé des cocons *in vivo* qui ont éclos en 10-27 et 10-11 jours respectivement. *In vitro*, *X. victoriensis* et *C.* aff. *okadai* ont survécu pendant plus de 13 et 15 semaines respectivement, bien que les deux aient été affectés négativement par l'augmentation de la température et du nitrate, et étaient absents des prélèvements prélevés sur le terrain. Seul *C.* aff. *okadai* a déposé des cocons *in vitro*, et cette espèce plus grande était prédateur sur *X. victoriensis* mais pas l'inverse. Les deux branchiobdellidés possèdent des traits associés à la réussite de la colonisation, y compris un taux de reproduction relativement rapide et une survie prolongée hors de l'hôte. Compte tenu de sa survie *in vivo* et de son effet nuisible connu sur les écrevisses signal, *X. victoriensis* est peut-être plus susceptible d'influencer la dynamique d'invasion de l'hôte, bien que sa persistance soit affectée par la présence de symbiotes co-présents.

Mots-clés : espèces envahissantes non indigènes / parasites envahissants / changements climatiques / écrevisse signal / *Xironogiton victoriensis*

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1 Introduction

Symbionts can be key in determining the outcome of biological invasions (Prenter *et al.*, 2004; Sargent *et al.*, 2014), one of the main causes of biodiversity loss (Gurevitch and Padilla, 2004; Didham *et al.*, 2005). The majority of introduced symbionts, however, fail to establish (MacLeod *et al.*, 2010), and predicting this is difficult as there is no single life-history strategy indicative of a successful invasion (Sol *et al.*, 2012; Lockwood *et al.*, 2013). Typically, invaders have a high dispersal ability and reproductive capacity, often involving hermaphroditism and/or asexual reproduction (MacArthur and Wilson, 1967). Having a broad environmental tolerance is also predicted to increase the likelihood of successful colonisation. For symbionts, host dispersal, host specificity and life cycle complexity are also important predictors of invasive status (Kennedy, 1994), with those infecting highly mobile hosts and having a simple direct life cycle being the most likely to successfully colonise.

Here, we assess the invasion potential of branchiobdellidan (Annelida: Clitellata) ectosymbionts, commonly found on crayfish throughout the Holarctic (Gelder, 1999). Previous studies indicate that the crayfish-branchiobdellidan association can vary from mutualistic to parasitic (reviewed by Skelton *et al.*, 2013). Therefore, these symbionts could influence the invasion success of non-native crayfish either beneficially or detrimentally. Relatively few reports exist of non-native branchiobdellidans in Europe despite the large number of crayfish introductions in recent decades (Souty-Grosset *et al.*, 2006; Holdich *et al.*, 2014; James *et al.*, 2014; Kouba *et al.*, 2014). This may be due to a lack of monitoring or because branchiobdellidans are poor invaders. Branchiobdellidans are sexually reproducing hermaphrodites, which deposit fertilised cocoons directly onto the host's exoskeleton (Govedich *et al.*, 2009). As monoxenous species, they have no requirement for an intermediate host (Taraschewski, 2006) and are directly transmitted between crayfish (Govedich *et al.*, 2009). Furthermore, branchiobdellidans appear to be generalists with respect to host species (Skelton *et al.*, 2013).

Branchiobdellidan survival, reproduction and environmental requirements are relatively poorly understood, perhaps with the exception of the genus *Cambarincola*. Existing *in vitro* studies indicate that survival can range from 46 days to over two months (Penn, 1959; Niwa *et al.*, 2014; Creed *et al.*, 2015), although this is likely to vary by species and limited data is available to quantify this. *In vivo*, branchiobdellidans of the genus *Cambarincola* produce cocoons that can hatch in 10–12 days, although this can take over 23 days (Creed *et al.*, 2015). These are short generation times compared to some other hermaphroditic freshwater annelids, including most predatory leeches (Hirudinida) that tend to reproduce annually or biannually, display semelparity and take up to 19 months to mature (Govedich *et al.*, 2009). Also, for some freshwater Oligochaeta (Lumbriculida) cocoons can take one to two months to hatch, and full maturity is not reached for around two years (Cook, 1969). In terms of environmental tolerance, again there is a paucity of information but some branchiobdellidans can survive in temperatures up to 33 °C (DeWitt *et al.*, 2013). Thus, branchiobdellidans possess some traits associated with successful colonists; although, this is likely to be species-dependant and information is sparse.

Two non-native species of branchiobdellidans, *Cambarincola* aff. *okadai* and *Xironogiton victoriensis*, were recently found for the first time on invasive signal crayfish (*Pacifastacus leniusculus*) in the UK (James *et al.*, 2015a). Signal crayfish are widespread across the UK (James *et al.*, 2014), and have altered the structure and function of recipient ecosystems throughout their non-native range (James *et al.*, 2015c). Under laboratory conditions, *X. victoriensis* reduces aggression and foraging efficiency of signal crayfish (James *et al.*, 2015b), which may alter host invasion dynamics in the field. Given the high frequency of co-infection between *C.* aff. *okadai* and *X. victoriensis* (see James *et al.*, 2015a), intraguild competition and predation may also predict the likelihood of either species persisting.

In a series of *in vivo* and *in vitro* experiments we assessed the potential invasion ability of *C.* aff. *okadai* and *X. victoriensis*. *In vivo* we monitored branchiobdellidan infrapopulation survival and individual worm reproduction on signal crayfish hosts. *In vitro*, we investigated branchiobdellidan survival and reproduction under altered temperature and nitrate conditions, and assessed competition between these co-habiting symbionts.

2 Methods

2.1 Collection and maintenance of animals

Crayfish, collected by trapping and manual searching in March–October of 2012–2014, were transported to the aquarium facility at Cardiff University. Signal crayfish (*P. leniusculus*) naïve to branchiobdellidan worms were collected from three sites in Powys, mid-Wales: Dderw Farm Pond, Llyswen (SO138375); Rhydlydan ponds, Painscastle (SO168457); and the River Bachowey, Painscastle (SO166457). All recipient host crayfish were sexed, weighed (blotted wet mass), measured (total carapace length: tip of rostrum to posterior margin of the carapace) and visually inspected for signs of disease. Any crayfish displaying signs of ill health, in the premoult stage, or missing chelae were excluded from the study. Given their tendency to moult frequently small crayfish (carapace length <28 mm) were also omitted from experiments. Signal crayfish harbouring branchiobdellidans (*X. victoriensis* and *C.* aff. *okadai* identified according to James *et al.* (2015a) were collected from the River Gavenny (SO308164), South Wales.

All crayfish were maintained under a 16 h:8 h light/dark regime in aerated, filtered 180 L tanks filled with dechlorinated water (15 ± 1 °C), gravel substrates and refugia. The animals were fed every 24 or 48 h on Tetra Crusta flakes and weekly 50% water changes were performed in both stock and experimental tanks. Branchiobdellidan-naïve crayfish were maintained separately from infested crayfish, and no equipment was shared between the tanks, to ensure naïve crayfish had no exposure to branchiobdellidans prior to their use in experiments. Crayfish were given a minimum of 7 days to acclimatise to laboratory conditions before being used for experiments.

Worms were carefully dislodged from host crayfish using the edge of blunt forceps and placed in a glass dish of distilled water. Removal from the host did not cause any visible damage or behavioural change to the worms, and they readily re-

attached to the surface of the dish. Worms were examined under a dissecting microscope (×30) with fibre optic illumination and only apparently healthy, full-sized (>3.0 mm for *X. victoriensis*, >8.0 mm for *C.* aff. *okadai*), specimens were used in experiments. *C.* aff. *okadai* is less abundant than *X. victoriensis* in the Welsh population (James *et al.*, 2015a) and more difficult to maintain in the lab, and therefore could not be tested under all experimental conditions.

To determine whether branchiobdellidans living off the host could be found in the field, benthic invertebrate samples from the River Gavenny site ($n = 6$) were collected in September 2012 by kick-sampling for 1 min using a standard net (0.25 m × 0.25 m with a 0.5 mm mesh) and stored in 70% ethanol. Samples were subsequently examined under a dissecting microscope for the presence of any branchiobdellidans.

2.2 *In vivo* survival and reproduction

To investigate the persistence of worm populations on the host, naïve signal crayfish ($n = 40$) were experimentally infected with *X. victoriensis* worms. Infection intensities represented those naturally present in the field, based on crayfish size category (carapace length, mm): 28–31; 32–35; 36–39; 40–43; 44–48 infected with 21, 28, 65, 101 and 154 worms respectively (James *et al.*, 2015a). Host crayfish were maintained individually in aerated 15 L plastic tanks with a plastic refuge. Crayfish were screened and the number of worms counted each week for 10 weeks. At each screening, any lost worms were replaced with new ones to maintain worm numbers at natural levels and simulate branchiobdellidan transmission. If a crayfish moulted, this was recorded, and the moult was left in the tank for at least 24 h to allow worms to transfer back onto the crayfish.

To assess the time for cocoon deposition and hatching, branchiobdellidan-naïve signal crayfish were infected with a single adult worm (*X. victoriensis* $n = 18$, *C.* aff. *okadai* $n = 20$). Host crayfish were maintained individually in aerated 10 L plastic tanks with a refuge. Crayfish were inspected every 48 h for the presence of adult worms and cocoons. If a crayfish moulted, the exoskeleton was left in the tank for 24 h to allow the worm to move back onto the crayfish. Following cocoon deposition, the adult worm was removed from the host and the cocoon was examined every 48 h *in vivo* under a dissecting microscope to detect emergence of the juvenile. If the host moulted following cocoon deposition, the crayfish was removed from the tank so that cocoon development could be monitored on the exuviae until detection was no longer possible due to disintegration of the exuviae (ca. 2 weeks). The experiment was terminated when all cocoons had either hatched or there had been no change in the condition of the worm or cocoon for at least 30 days. We considered extending this study to native white-clawed crayfish (*Austropotamobius pallipes*) but it was not possible to gain ethical clearance to assess whether these endangered hosts were also susceptible to *X. victoriensis* and, or *C.* aff. *okadai*.

To assess the interval between cocoon deposition, the above procedure was repeated (using *X. victoriensis* only, $n = 16$) but adult worms were left on the host following deposition of the initial cocoon. Crayfish were examined every 48 h and the presence and location of worms and cocoons were recorded. Cocoons were examined under a dissecting microscope to determine when the juvenile worm had emerged. The experiment was terminated after 30 days.

2.3 *In vitro* survival, reproduction, cannibalism and intraguild predation

To assess survival and reproduction *in vitro*, individual worms were removed from their host and transferred to petri dishes (dia. 50 mm) containing 10 ml water. Petri dishes were kept under two or four different conditions for *C.* aff. *okadai* and *X. victoriensis*, respectively ($n = 20$ worms per treatment). Survival of both species was investigated at 15 ± 1 °C and 20 ± 0.5 °C. Survival of *X. victoriensis* was also assessed in low (<5 ppm) and high (100 ppm) nitrate water, but *C.* aff. *okadai* worms were only available for exposure to low nitrate water. Nitrate solutions were prepared by dissolving potassium nitrate (≥99.0% purity $K(NO_3)$, Sigma–Aldrich, USA) into dechlorinated tap water. Nitrate levels were tested with an API® Freshwater Master Test Kit. Worms were assessed weekly under a dissecting microscope to check for the deposition of cocoons and monitor condition until death occurred. When cocoons were detected they were transferred to a new petri dish under identical conditions and screened every 48 h using a dissecting microscope to assess hatching time and juvenile survival.

To investigate cannibalism and intraguild predation between co-habiting branchiobdellidans, five treatments were tested: inter-specific *C.* aff. *okadai* and *X. victoriensis* pairs, intra-specific *C.* aff. *okadai* or *X. victoriensis* pairs and two controls of a single *C.* aff. *okadai* or *X. victoriensis* ($n = 25$ for each treatment, except *C.* aff. *okadai* intra-specific pairs where $n = 18$ due to a lack of available specimens). These experiments were run *in vitro* to allow us to investigate cannibalism/intraguild predation without the confounding effects of host grooming. *X. victoriensis* and *C.* aff. *okadai* were placed into petri dishes filled with water from crayfish stock tanks to prevent starvation (general diet of branchiobdellidans includes diatoms, invertebrates and unicellular algae alongside crayfish exuviae; Govedich *et al.*, 2009) and the survival of each branchiobdellidan monitored over six weeks. To estimate the size difference between each pair (without fixing), branchiobdellidans were coaxed to naturally stretch out by disturbing the water. Video was recorded using a smart phone camera (Samsung Galaxy S4 Mini) and a still image taken when the branchiobdellidan was fully extended. Branchiobdellidan length was measured from these images using ImageJ (Rasband, 2017). Petri dishes were checked every day to record death or consumption of a branchiobdellidan.

2.4 Statistical analysis

All general linear models (GLMs) and generalised linear models were minimised by stepwise deletion of insignificant terms using analysis of variance. For all models, visual examination of data plots and Shapiro–Wilk tests were used to check standardised residuals for normal distribution and homogeneity of variance (Thomas *et al.*, 2013). In all tests, the level of significance was taken as $P < 0.05$. All statistical analyses were conducted in the R statistical package v2.15.1

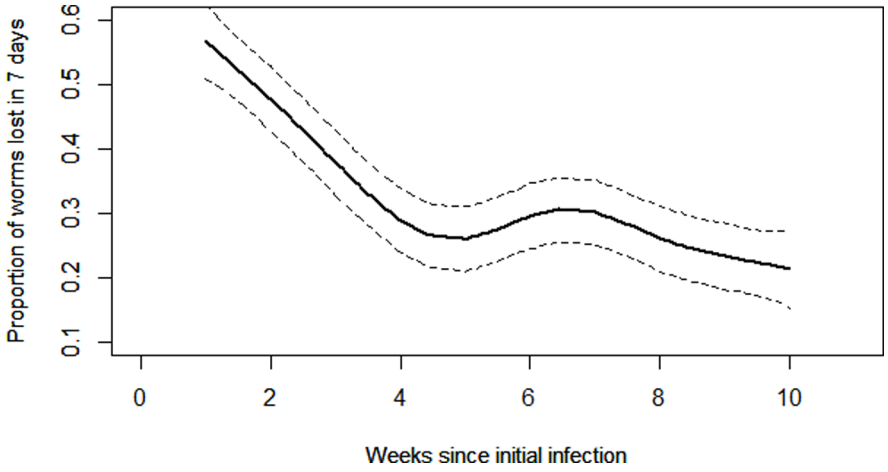

Fig. 1. Predicted proportion of *Xironogiton victoriensis* worms lost from initially naïve signal crayfish host each week, as a function of time since initial experimental infection. Additional worms were experimentally added to the crayfish each week to simulate transmission from host to host. Dashed lines represent 95% confidence intervals.

(R Development Core Team, 2012), with ASReml-R (version 3.0 package) used to conduct the generalised linear mixed model (GLMM) within the R interface.

A GLMM with a Gaussian error distribution and identity link was used to determine whether host size (carapace length), sex, moulting or time in the experiment had an effect on the weekly proportion of worms lost from crayfish artificially infected with natural branchiobdellidan intensities (Model 1). Interactions between sex and size and between sex and time were also included as fixed effects in the starting model. To detect any non-linear effects of time on worm loss, a spline was fitted to this variable and it was included as a random variable in Model 1. Assessment of the log-likelihood ratio was used to determine whether time was a significant random effect. Crayfish identification number was also included as a random factor in Model 1, to control for repeated measures. Following assessment of the random model, the fixed model was refined by stepwise deletions using the Wald statistic. For crayfish experimentally infected with individual worms, a Pearson's chi-squared test was used to assess whether the number of hosts that lost their branchiobdellidans differed between *X. victoriensis* and *C.* aff. *okadai*.

In vitro branchiobdellidan survival was analysed using two separate GLMs. Model 2a explored the effect of species, temperature and the interaction between these variables on survival, whilst Model 2b investigated the effect of temperature, nitrate and their interaction on *X. victoriensis* survival.

Intraguild predation and cannibalism among *X. victoriensis* and *C.* aff. *okadai* were investigated using a Generalised Linear Model with a binomial error distribution and logit link function. Treatment and size difference between branchiobdellidan pairs (mm) were controlled for as independent variables. A second order interaction between treatment and size difference was included as a fixed effect in the starting model, as there was a larger size difference among the interspecific pairs than the intraspecific pairs. Significant differences in survival between treatments were examined with a Tukey multiple comparisons of means using the package "multcompView" (Graves *et al.*, 2015).

3 Results

During the course of all experiments, it was evident that *X. victoriensis* tolerated laboratory conditions better than *C.* aff. *okadai*, surviving on crayfish hosts for >5 months compared to <2 weeks for *C.* aff. *okadai* cultures (15 ± 1 °C). Despite this, both species reproduced successfully *in vivo*. *In vitro* survival in water did not differ between the two species, but only *C.* aff. *okadai* deposited cocoons, which hatched successfully. *C.* aff. *okadai* readily predated on *X. victoriensis* and cannibalism was observed in both species *in vitro*. No worms of either species were found in the invertebrate samples from the River Gavenny where infected crayfish were collected. This finding is supported by previous extensive sampling of Welsh rivers over the last 35 years, in which no branchiobdellians were recovered from conventional benthic samples (Ormerod, personal communication).

3.1 *In vivo* survival and reproduction

Over 10 weeks, *X. victoriensis* infrapopulations on experimentally infected signal crayfish, which were re-infected weekly to maintain natural mean infection intensities, decreased by an average of 33.1% each week. There was, however, a significant, non-linear relationship between time and the proportion of worms lost ($F_{1,334} = 121$, $P < 0.0001$), with the high initial rate of loss (mean = 56.5% in week 1) reducing over time (mean = 21.5% in week 10; Fig. 1). The weekly percentage decrease in worm numbers was not affected by crayfish sex, size, nor host moulting in the preceding week (all $P > 0.05$).

For 32.4% (11/34) of crayfish experimentally infected with a single *X. victoriensis* worm, we were not able to detect the branchiobdellidan 48 h post infection. Of the 23 remaining worms, 95.7% deposited cocoons on their hosts within the 30-day experiment. The mean time to lay the first cocoon was five days (range 2–28) and the cocoons hatched after 10–27 days (mean 18.8) at 15 ± 1 °C. Six crayfish moulted before the

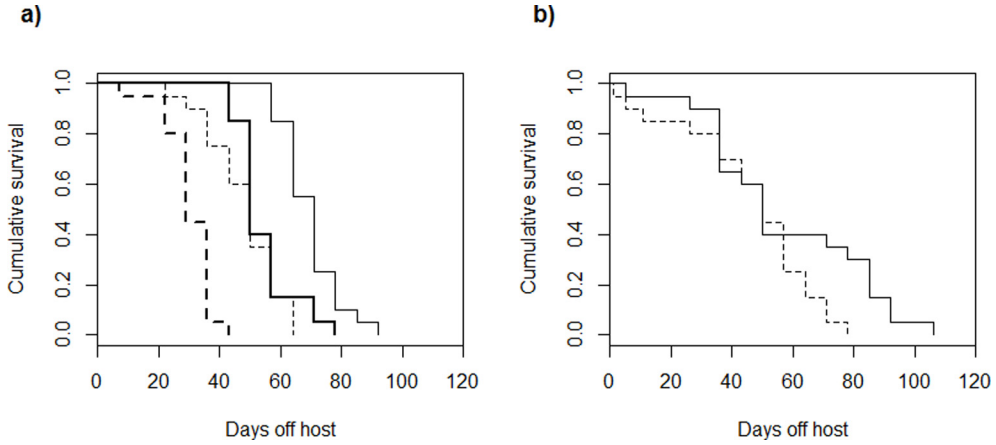

Fig. 2. Cumulative *in vitro* survival of branchiobdellidans at 15 °C (solid lines) or 20 °C (dashed lines) in low nitrate (light lines) or high nitrate (bold lines) water of (a) *Xironogiton victoriensis* and (b) *Cambarincola* aff. *okadai*.

Table 1. Mean (range) survival (days) of *Xironogiton victoriensis* and *Cambarincola* aff. *okadai in vitro* under various temperature and nitrate regimes ($n = 20$ per treatment).

Species	Temp. (°C)	Nitrate (ppm)	Mean (range) survival (days)
X. victoriensis	15	<5	69.6 (57–92)
	15	100	54.2 (43–78)
	20	<5	47.9 (22–94)
	20	100	30.4 (7–43)
C. aff. *okadai*	15	<5	57.4 (5–106)
	20	<5	46.4 (1–78)

cocoons had hatched: these cocoons remained attached to the exuviae but none subsequently hatched. When *X. victoriensis* worms were left on the host ($n = 16$) following deposition of the first cocoon, the mean number of cocoons laid over the 30-day period was 5.7 (range 2–9), or one cocoon every 6.5 days, although cocoon deposition was irregular. Often, multiple cocoons were laid over a period of a few days, but then laying did not resume for up to 14 days.

Of the 20 crayfish experimentally infected with a single *C.* aff. *okadai* worm, significantly more (80%) lost their branchiobdellidans within the first 48 h compared to *X. victoriensis* ($\chi^2 = 5.72$, df = 1, $P < 0.05$). Of the four remaining *C.* aff. *okadai* worms, three laid a cocoon (on days two, six and seven, respectively), while the remaining worm became detached after nine days. Of the three cocoons, two hatched (10 and 11 days later). All *X. victoriensis* and *C.* aff. *okadai* cocoons were laid on the ventral chelae or dorsal carapace, respectively, corresponding to the preferred host locations of the adult worms (James *et al.*, 2015a).

3.2 *In vitro* survival, reproduction, cannibalism and intraguild predation

Under control conditions (nitrate <5 ppm, temperature 15 °C) survival periods were up to 15 and 13 weeks for *C.* aff. *okadai* and *X. victoriensis*, respectively. There was no difference in the average survival time of *C.* aff. *okadai* and *X. victoriensis* under low nitrate control conditions ($F_{1,77} = 2.60$, $P = 0.11$), but both species survived significantly longer at 15 °C than 20 °C ($F_{1,78} = 14.48$, $P < 0.0001$; Fig. 2). Under the high nitrate treatment, the mean survival of *X. victoriensis* was also significantly higher at 15 °C than 20 °C ($F_{1,77} = 111.05$, $P < 0.0001$). For *X. victoriensis*, mean survival was significantly lower under conditions of high compared to low nitrate ($F_{1,77} = 58.11$, $P < 0.0001$; Tab. 1).

X. victoriensis did not lay cocoons *in vitro*, whereas 9 out of 20 *C.* aff. *okadai* worms maintained at 15 °C and 4 out of 20 at 23 °C deposited globular cocoons, each containing a single embryo. These cocoons were not attached to the petri dish, but some were attached to each other via a peduncle (Fig. 3a). Most worms (76.9%) deposited only a single cocoon, however, clusters of up to four were observed (Fig. 3b), and one worm laid six cocoons over 15 days. All cocoons were deposited within the first 23 days of the experiment. From a total of 19 cocoons, 47% (9) hatched. All living juveniles were transferred individually to a new petri dish containing dechlorinated water: four survived over 48 h and, of these, two survived 23 days *in vitro*.

Both the size difference between the worms ($\chi^2 = 5.08$, df = 1, $P = 0.02$) and treatment ($\chi^2 = 11.26$, df = 4, $P = 0.02$) had a significant effect on branchiobdellidan survival in cannibalism/intraguild predation experiments (Fig. 4). Among treatments, *X. victoriensis*/*C.* aff. *okadai* inter-specific pairs had significantly lower survival than all other treatments

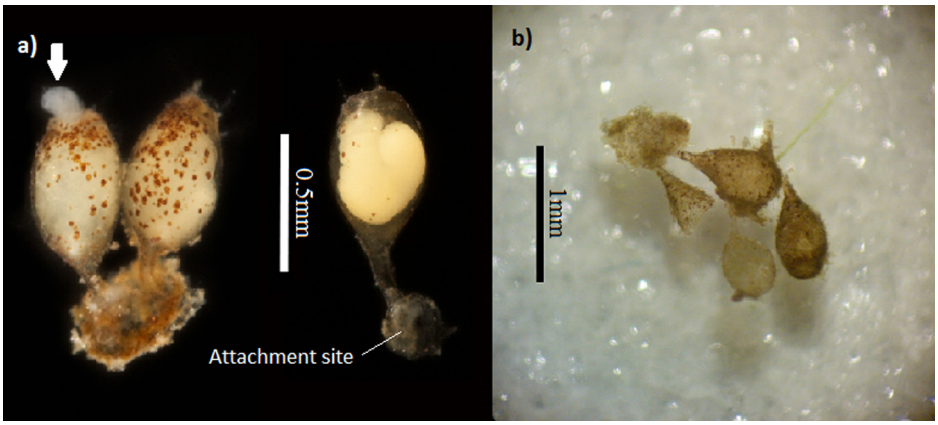

Fig. 3. (a) Transparent cocoons of *Cambarincola* aff. *okadai* containing larvae, juvenile worm (arrow head) emerging from the far left cocoon (image courtesy of Andy Mackie, National Museum Wales), and (b) empty cocoons.

($P < 0.001$ for all) with 92% of *X. victoriensis* consumed within six weeks. Both intra-specific pairs had significantly higher survival than inter-specific pairs ($P < 0.0001$) and significantly lower survival than both controls (*i.e.* individual worms of either species) with 27% of smaller *X. victoriensis* and 33% of smaller *C.* aff. *okadai* consumed ($P < 0.05$). Intra-specific pairs of *X. victoriensis* and *C.* aff. *okadai* did not significantly differ from each other, and neither did *X. victoriensis* and *C.* aff. *okadai* individual worm controls.

4 Discussion

The populations of *C.* aff. *okadai* and *X. victoriensis* in south Wales have been present for at least four years (James *et al.*, 2015a) suggesting that both species overcame any initial barriers associated with small founder population sizes. Whilst this may seem contradictory to the high initial decrease in *X. victoriensis* infrapopulations we observed *in vivo*, within 10 weeks these branchiobdellidans had established viable populations on all experimentally infected hosts. It is likely that the low initial survival of *X. victoriensis* on signal crayfish was a result of host grooming, which is known to be efficient in regulating branchiobdellidan numbers (Farrell *et al.*, 2014). Once *X. victoriensis* began reproducing on the host, however, the rate of worm infrapopulation increase is likely to have outweighed the number removed through grooming. Establishment of worm infrapopulations and further invasion will be facilitated by this relatively fast reproductive rate, compared to some other freshwater annelids (Cook, 1969; Govedich *et al.*, 2009). Furthermore, *X. victoriensis* laid cocoons (one every 6.5 days that hatched after 10–27 days) even when isolated on the host with no opportunity to mate, indicating the ability for internal sperm storage/self-fertilisation. Hermaphroditism and self-fertilisation are traits typically associated with successful invaders (Kennedy, 1994).

As observed in other branchiobdellidan species, we found that both *X. victoriensis* and *C.* aff. *okadai* were able to survive for extended periods off the host (Penn, 1959; Young, 1966; Creed *et al.*, 2015). Furthermore, *C.* aff. *okadai* deposited cocoons off the host, only the second time this has been documented for branchiobdellidans (Woodhead, 1950). These characteristics will broaden the opportunity for worm transmission in the event of host moulting/death, potentially

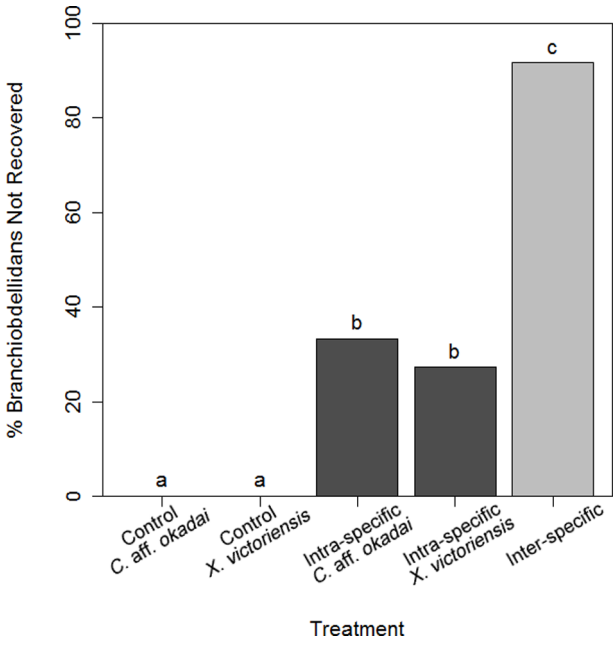

Fig. 4. Intraguild predation (proportion of branchiobdellidans not recovered) between *Xironogiton victoriensis* and *Cambarincola* aff. *okadai* (light grey bar) was higher than cannibalism (dark grey bars) when worms were maintained *in vitro* at 15 ± 1 °C and <10 ppm nitrate. Letters show statistical differences; treatments that do not share letters are significantly different.

increasing the invasion success of these symbionts. However, no free-living worms were found in the invertebrate samples collected from the River Gavenny, suggesting that it is either not common for branchiobdellidans to leave the host in the wild or they are vulnerable to predation.

In terms of environmental tolerance, both *X. victoriensis* and *C.* aff. *okadai* were able to survive for extended periods at temperatures ranging from 15 °C to 20 °C, and under nitrate concentrations of up to 100 ppm (twice the legal limit for UK waters, according to the 1991 Nitrates Directive 91/676/EEC). Whilst this will increase the likelihood of these branchiobdellidans persisting/spreading in the UK, the survival of both species was reduced at higher temperatures and nitrate concentrations. Mean summer surface water temperatures

(currently 22.2 °C in the UK; Orr *et al.*, 2010) are predicted to rise by up to 0.5 °C per decade (Johnson *et al.*, 2009), and nitrate levels as high as 100 ppm have already been reported in British waters (Davies, 2013). Therefore, the persistence of both species of branchiobdellidans in the UK may be influenced by temperature fluctuations and/or pollution incidents.

Signal crayfish in the River Gavenny are commonly (75%) co-infected with *X. victoriensis* and *C.* aff. *okadai* so the potential for intraguild predation is high. Indeed, we found that the larger *C.* aff. *okadai* regularly predated on *X. victoriensis in vitro* but not *vice versa*. Intraguild predation has previously been observed in congeneric branchiobdellidans, with *C. vitreus* being consumed by *C. chirocephalus*, which has a larger jaw size (Gale and Proctor, 2011). Intraguild predation is an important component of crayfish symbioses (Thomas *et al.*, 2016), however to date the presence of *C.* aff. *okadai* has not prevented the establishment of *X. victoriensis* in the River Gavenny, most likely because they occupy separate host micro-habitats (James *et al.*, 2015a). If niche overlap between these branchiobdellidans was forced, for example by removal of signal crayfish from the River Gavenny for control (reducing the number of available hosts), intraguild predation would likely become more important. Ultimately, this could impact the survival of competitively subordinate *X. victoriensis*, which are known to reduce the aggression and foraging success of infected crayfish (James *et al.*, 2015b).

Overall, it is likely that both branchiobdellidan species will persist in the UK considering their relatively fast reproductive rate, direct life cycle and fairly broad environmental tolerance. Considering its ability to reproduce *in vitro*, *C.* aff. *okadai*, is likely a facultative symbiont, whereas *X. victoriensis*, considering its dependency on the host to reproduce and the detrimental effect it is known to have on host behaviour (James *et al.*, 2015b), is an obligate symbiont that may be parasitic. Therefore, whilst both species have established in the UK and are predicted to spread with the dispersal of signal crayfish, *X. victoriensis* may have greater potential to influence ecosystem structure and function. The ability of *X. victoriensis* to persist/spread may, however, be influenced by the presence of competitively superior *C.* aff. *okadai* or other co-occurring symbionts. Overall, this study contributes towards understanding the symbiotic fauna of invasive non-native signal crayfish, and demonstrates the need to consider such co-introduced organisms as a "hidden cost" of biological invasion (Sherrard-Smith *et al.*, 2014).

Conflicts of interest. The authors declare that they have no competing interests.

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Author's contributions

JJ, KED and JC designed the study and drafted the manuscript. KED and RH conducted laboratory experiments. JJ and KED performed statistical analyses. All authors approved the final manuscript.

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