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Size-susceptibility of *Cornu aspersum* exposed to the malacopathogenic nematodes *Phasmarhabditis hermaphrodita* and *P. californica*

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ABSTRACT

Slugs and snails are agricultural pests usually controlled by chemical bait pellets however an alternative method is the commercially produced malacopathogenic nematode *Phasmarhabditis hermaphrodita*, which is sold across northern Europe. *P. hermaphrodita* can kill several slug species but is unable to affect larger pestiferous snails. Therefore, we examined whether the closely related species *Phasmarhabditis californica*, isolated from the U.K. could kill neonate or adult common garden snails (*Cornu aspersum*). In our first experiment, neonate *C. aspersum* were exposed to 1000 nematodes per tube containing three strains of *P. californica* (designated DMG0017, DMG0018 or DMG0019) (as well as the commercial formulation of *P. hermaphrodita* DMG0001) and feeding inhibition and survival were monitored over 14 days. All nematodes apart from *P. californica* (DMG0017) killed the snails and caused feeding inhibition. In a follow up experiment we exposed adult *C. aspersum* to two doses (30 and 90 nematodes per cm^2) of *P. californica* (DMG0019) and *P. hermaphrodita* (DMG0001) and monitored survival, feeding inhibition and changes in weight over 21 days. Neither nematode species affected survival, feeding inhibition or weight of the adult snails. In summary, *P. californica* (DMG0018 and DMG0019) are as pathogenic as *P. hermaphrodita* (DMG0001) and could be used to target neonate snails, however, the use of malacopathogenic nematodes to control large adult snails continues to be problematic.

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1. Introduction

Many species of slugs and snails are highly pestiferous and affect crop production across the world (South, 1992). They are usually controlled by chemical bait pellets, such as metaldehyde, but due to its toxicity to non-target organisms (Homeida &

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Cooke, 1982) it has been banned in many European countries. An alternative to chemical control is the biological control agent *Phasmarhabditis hermaphrodita*, a parasitic nematode that can infect and kill several slug and snail species which has been formulated into a product called Nemaslug® available from BASF Agricultural Specialities (Wilson et al., 1993). *P. hermaphrodita* is applied to soil where it seeks slugs through attraction to mucus and faeces (Rae et al., 2006) and penetrates through a pore at the back of the mantle and kills the host in 4–21 days (Tan & Grewal, 2001a; Wilson et al., 1993). It is thought the nematodes vector the bacterium *Moraxella osloensis*, inside the slug where it proliferates and produces a lipopolysaccharide, which causes death to the slug (Tan & Grewal, 2001b, 2002). The nematodes reproduce on the cadaver and when the bacterial food supply is exhausted new dauer juveniles are produced that go in search of more hosts. *P. hermaphrodita* has been successfully shown to protect an array of crops from slug damage such as lettuce (Wilson et al., 1995), asparagus (Ester et al., 2003a), hostas (Grewal et al., 2001) and orchids (Ester et al., 2003b) (to name but a few).

P. hermaphrodita is not the only member of the 13 species (Tandingan De Ley et al., 2017) that encompass the *Phasmarhabditis* genus which can kill slugs. As well as *P. hermaphrodita, P. papillosa* (Laznik et al., 2020; McDonnell et al., 2020; Pieterse et al., 2017; Tandingan De Ley et al., 2020), *P. neopapillosa* (Hooper et al., 1999), *P. tawfiki* (Azzam & Tawfiki, 2003), *P. safricana* (Ross et al., 2018) and *P. californica* (McDonnell et al., 2020; Tandingan De Ley et al., 2020) have been shown to infect and kill slugs and snails. There is also a short report stating *P. bohemica*, *P. bonaquaense* and *P. apuliae* can also kill slugs (Nermut et al., 2019). One of the most frequently isolated species from the *Phasmarhabditis* genus is *P. californica*. It has been isolated from the U.K. (Andrus & Rae, 2019), the U.S.A. (McDonnell et al., 2020; Tandingan De Ley et al., 2016), New Zealand (Wilson et al., 2016) and recently Canada (Brophy et al., 2020). U.S. strains of *P. californica* have been shown to kill the grey field slug (*Deroceras reticulatum*) (McDonnell et al., 2020) and snails (*Theba pisana*) (Tandingan De Ley et al., 2020), however, there is nothing known about the pathogenic ability of *P. californica* isolated from the U.K. Three strains (designated DMG0017, DMG0018 and DMG0019) were isolated from a single snail (*Oxychilus draparnaudi*) in Pembrokeshire, Wales (SM809057) and have been kept in culture since 2014 on White traps (White, 1927) using rotting slug (*Limax flavus*), see Andrus and Rae (2019) for further details. Therefore, in order to investigate the pathogencity of these strains we exposed *P. californica* (DMG0017, DMG0018 and DMG0019) to neonate and adult *Cornu aspersum*, a notorious pest species found across Europe and America (Guiller et al., 2012), and compared their effectiveness at causing mortality to the commercial strain of *P. hermaphrodita* (DMG0001). We also assessed whether the nematodes caused feeding inhibition in the snails (a common symptom of *P. hermaphrodita* infection in slugs – Glen et al., 2000) and would subsequently affect the growth and development of the snails by assessing differences in weight at the start and the end of the experiment. If *P. californica* killed *C. aspersum* it could be developed as a biological control agent to target pestiferous snails, rather than *P. hermaphrodita* which struggles to cause mortality to larger snails and slugs (Grimm, 2002; Speiser et al., 2001).

2. Materials and methods

2.1. Source of invertebrates

Neonate *C. aspersum* (0.16 ± 0.05 g, $n = 150$) and adult *C. aspersum* 'gros gris' (Supplementary Figure 1(A)) (mean weight 21.7 ± 0.33 g, $n = 180$) were purchased from online suppliers H and RH Escargot. Snails were kept at room temperature in terraria (35 × 23 × 22 cm) with ventilated lids containing moist coconut husk and fed lettuce and cucumber *ad libitum*. Snails were kept in moist conditions by liberally spraying tap water over the coconut husk every 2 days for 8 days prior to the experiment. *P. hermaphrodita* (DMG0001) were supplied by BASF Agricultural Specialities. Three strains of *P. californica* (DMG0017, DMG0018 and DMG0019) (Supplementary Figure 1(B)) were grown to the infective dauer stage using White traps (White, 1927) of dead *L. flavus* using methods by Andrus and Rae (2019).

2.2. Assessing the susceptibility of snails to P. hermaphrodita and P. californica

To assess the susceptibility of neonate snails a protocol by Cutler and Rae (2020) was adapted. Briefly, a piece of cotton wool was added to the bottom of fifteen 20 ml universal tubes. Nemaslug® (1–2 g) containing dauer stage *P. hermaphrodita* (DMG0001) was added to 45 ml of tap water, shaken vigorously for 2 min and then the numbers of alive *P. hermaphrodita* (DMG0001) in 20 µl were quantified three times. This process was repeated with *P. californica* (DMG0017, DMG0018 and DMG0019) though samples were removed from the surrounding water from fresh White traps where dauer stage nematodes were present. One thousand dauer stage *P. hermaphrodita* (DMG0001) (in 1 ml of water) were added to five tubes using a 1000 µl pipette and this process was repeated for *P. californica* (DMG0017, DMG0018 and DMG0019). Five tubes had the equivalent volume of water (1 ml) and no nematodes added and acted as the control. Two *C. aspersum* were added to each tube and the tubes were loosely sealed and stored in an incubator at 15°C. After 5 days exposure to the nematodes the snails were removed and allowed to feed on a 3.5 cm diameter piece of lettuce. Feeding was quantified on days 8 and 14 using methods by Cutler and Rae (2020). Briefly, remnants of the leaf discs were traced onto 1 × 1 mm^2 graph paper and their sized quantified by counting the number of 1 mm^2 squares. Survival of the snails was monitored every 2–3 days for 21 days. The experiment was repeated three times in total with 30 snails being exposed to each treatment.

To test the susceptibility of adult *C. aspersum* to *P. hermaphrodita* and *P. californica* we added approx. 100 g of pre-moistened coconut husk was added to 18 boxes (35 × 23 × 22 cm) to a depth of approx. 2 cm. To three boxes the recommended rate for field application (30 nematodes per cm^2) of *P. hermaphrodita* (Rae et al., 2007) was applied. The same dose was used for three boxes for *P. californica* DMG0019 application. We chose the DMG0019 strain of *P. californica* as it consistently produces large numbers of dauer juveniles under lab conditions (data not shown). This set up was also repeated to examine the susceptibility of *C. aspersum* to a higher dose (90 nematodes per cm^2) of *P. hermaphrodita* or *P. californica*. The other six boxes received water (10 ml) and acted as the control. Five discs of lettuce (3.5 cm diameter) were added to each box and the amount eaten was quantified every 2–3 days. The snails were weighed before

the start of the experiment and five *C. aspersum* were added to each box and stored at 15° C. The survival of the snails, as well as the amount eaten was quantified every 2–3 days for 21 days. After each time point the amount of food eaten was quantified it was replaced with fresh lettuce discs. The snails were weighed at the end of the experiment and the experiment was repeated twice.

2.3. Data analysis

Survival of neonate snails exposed to 0 or 1000 *P. hermaphrodita* (DMG0001), *P. californica* (DMG0017, DMG0018 and DMG0019) was analysed using a Log Rank test using OASIS (Yang et al., 2011). A Kruskal Wallis with Dunn's test was used to compare the amount eaten by neonate snails on day 8 and 14. The survival of adult snails exposed to 0, 30 or 90 *P. hermaphrodita* (DMG0001) and *P. californica* (DMG0019) per cm^2 on day 21 was compared using a Kruskal–Wallis test. The amount of lettuce eaten by adult snails exposed to 0, 30 or 90 *P. hermaphrodita* (DMG0001) and *P. californica* (DMG0019) per cm^2 on each recording day was compared using a Kruskal–Wallis test. The weight of the adult snails at the start and the end of the experiment was compared using a Student's *t* test. A One Way ANOVA was used to compare the mean weight of snails in each treatment on day 0 and 21. Normality was assessed using Kolmogorov–Smirnov tests. Statistical analysis was carried out using SPSS.

3. Results

3.1. Susceptibility of neonate C. aspersum to P. hermaphrodita and P. californica

There was a significant difference between the survival of untreated neonate *C. aspersum* compared to snails exposed to *P. hermaphrodita* (DMG0001) ($P = 0.0167$), *P. californica* (DMG0018) ($P = 0.0106$) and *P. californica* (DMG0019) ($P = 0.0105$) over 14 days (Figure 1). However, *P. californica* (DMG0017) ($P = 0.0809$) had no significant effect on the survival of neonate *C. aspersum*.

3.2. Feeding inhibition of neonate C. aspersum exposed to nematodes

After 8 and 14 days all nematodes had a significant effect on the amount of lettuce neonate *C. aspersum* ate compared to the untreated control ($P < 0.05$) (Figure 2(A,B)). There was no difference between the amount of lettuce eaten by *C. aspersum* exposed to *P. hermaphrodita* (DMG0001), or *P. californica* (DMG0017, DMG0018 and DMG0019) on day 8 or 14 ($P > 0.05$) (Figure 2(A,B)).

3.3. Susceptibility and feeding inhibition of adult C. aspersum to P. hermaphrodita and P. californica

After 21 days there was no significant difference between the survival of adult *C. aspersum* when exposed to 0, 30 or 90 *P. hermaphrodita* ($P > 0.05$) or *P. californica* DMG0019 ($P > 0.05$) per cm^2 (Figure 3(A,B)).

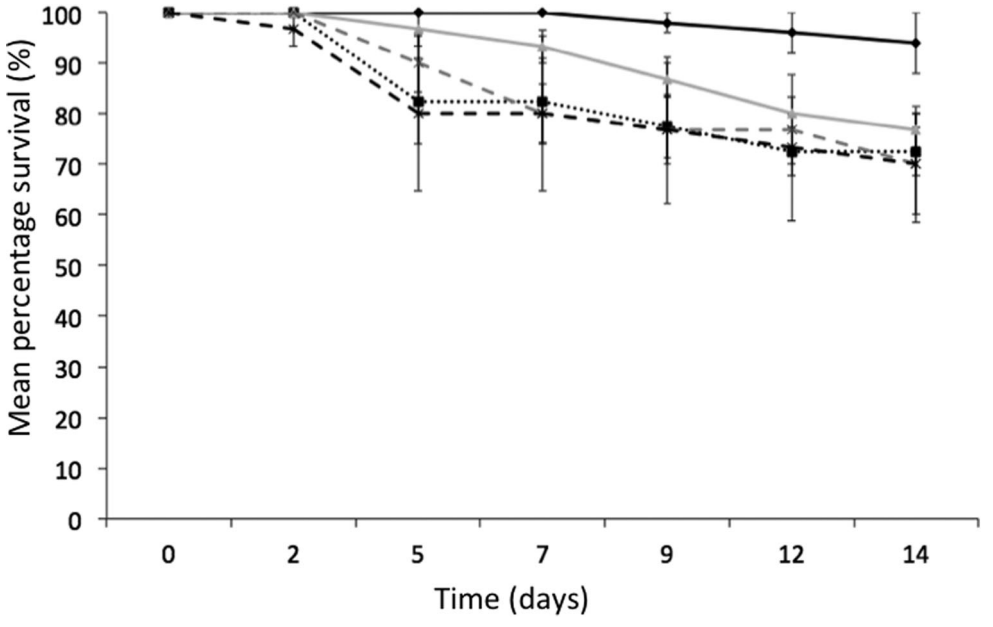

Figure 1. The mean percentage survival of neonate *Cornu aspersum* exposed to no dauer juvenile nematodes (black solid line), *Phasmarhabditis hermaphrodita* DMG0001 (black dotted line), *Phasmarhabditis californica* DMG0017 (grey solid line), *P. californica* DMG0018 (grey dashed line) and *P. californica* DMG0019 (black dash line) over 14 days. Bars represent ± one standard error.

Similarly, there was no significant difference between the amount of lettuce eaten when adult *C. aspersum* were exposed to 0, 30 or 90 *P. hermaphrodita* ($P > 0.05$) or *P. californica* DMG0019 ($P > 0.05$) per cm^2 on any of the days (Figure 4(A,B)).

3.4. Differences in weight of adult C. aspersum exposed to nematodes

The snails lost weight over the course of the experiment as there was a significant difference between the weight of *C. aspersum* on day 0 and 21 when exposed to 0, 30 and 90 *P. hermaphrodita* ($P < 0.05$) and *P. californica* DMG0019 ($P < 0.05$) per cm^2 (Figure 5(A, B)). However, both species of nematode did not induce feeding inhibition as there was no significant difference between the weight of snails on day 21 after exposure to 0, 30 or 90 *P. hermaphrodita* ($P > 0.05$) or *P. californica* DMG0019 ($P > 0.05$) per cm^2 (Figure 5(A,B)).

4. Discussion

We have shown both *P. hermaphrodita* and *P. californica* were able to kill neonate *C. aspersum* but not adults. The lack of effect on adult snails is perhaps not surprising as previous research has shown *P. hermaphrodita* has little effect on adult *C. aspersum* (Glen et al., 1996), however, this was only known for *P. hermaphrodita* and not *P. californica.* Differences in the susceptibility of different stages of terrestrial gastropod to *Phasmarhabditis* nematodes have been reported previously. Both Grimm (2002) and Speiser et al. (2001) showed *P. hermaphrodita* could kill juvenile *Arion lusitanicus* but not larger specimens. Similar results have been observed with experiments with the giant

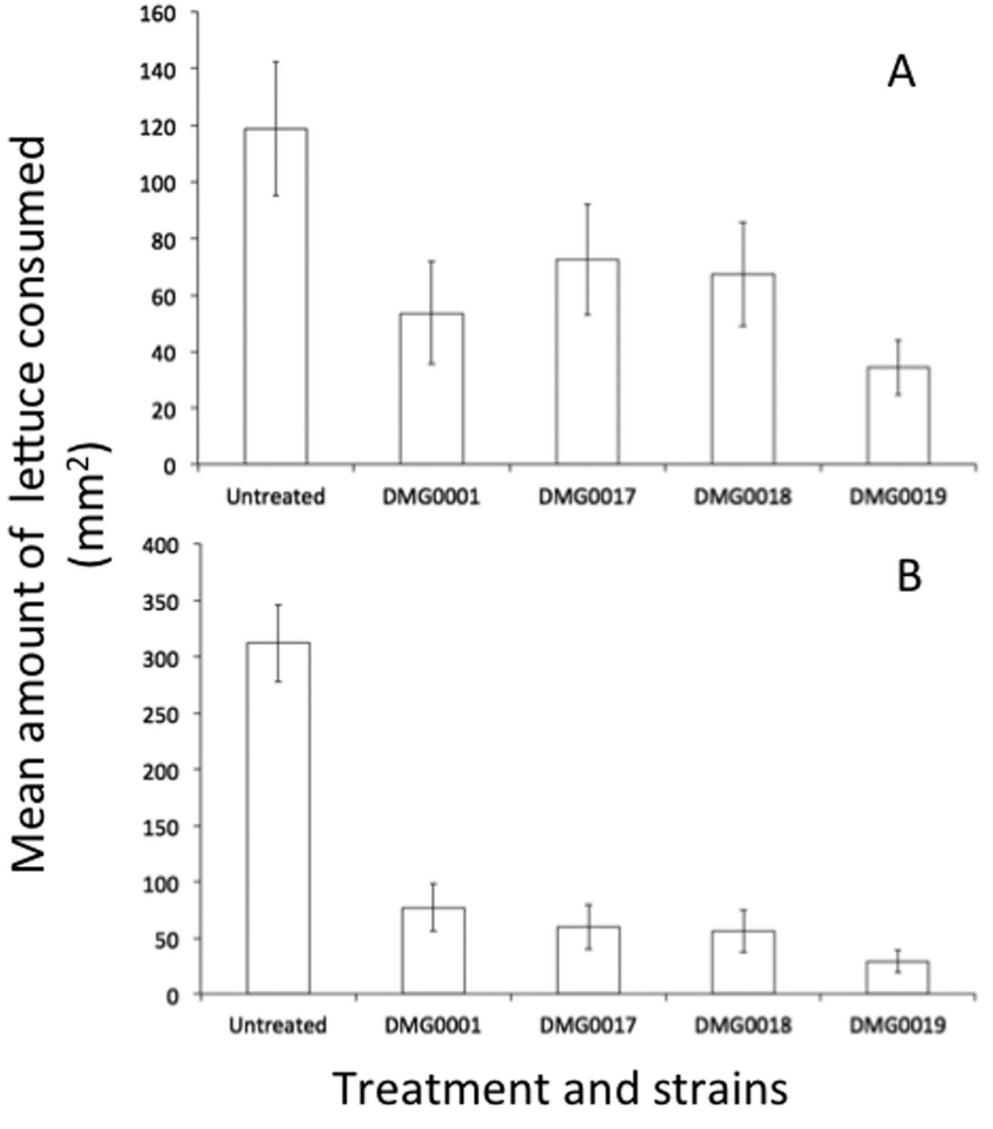

Figure 2. Feeding of neonate *C. aspersum* exposed to dauer juvenile *P. hermaphrodita* DMG0001, *P. californica* DMG0017, *P. californica* DMG0018 and *P. californica* DMG0019 on day 8 (A) and 14 (B). Bars represent ± one standard error.

African snail (*Lissachatina fulica*). Specifically, McDonnell et al. (2018) showed that a US strain of *P. hermaphrodita* could kill >80% of neonate *L. fulica* after 31 days whilst Williams and Rae (2015) showed the commercial strain of *P. hermaphrodita* could not kill larger snails. However, using other *Phasmarhabditis* species has yielded potential for controlling larger gastropods. For example, recently *P. papillosa* has been shown to kill young adult and large adult *Arion vulgaris* (Laznik et al., 2020). As these species are problematic to control due to their high reproductive rate (South, 1992) and their resistance to *P. hermaphrodita* (Grimm, 2002; Speiser et al., 2001), the use of other *Phasmarhabditis* species warrants further investigation.

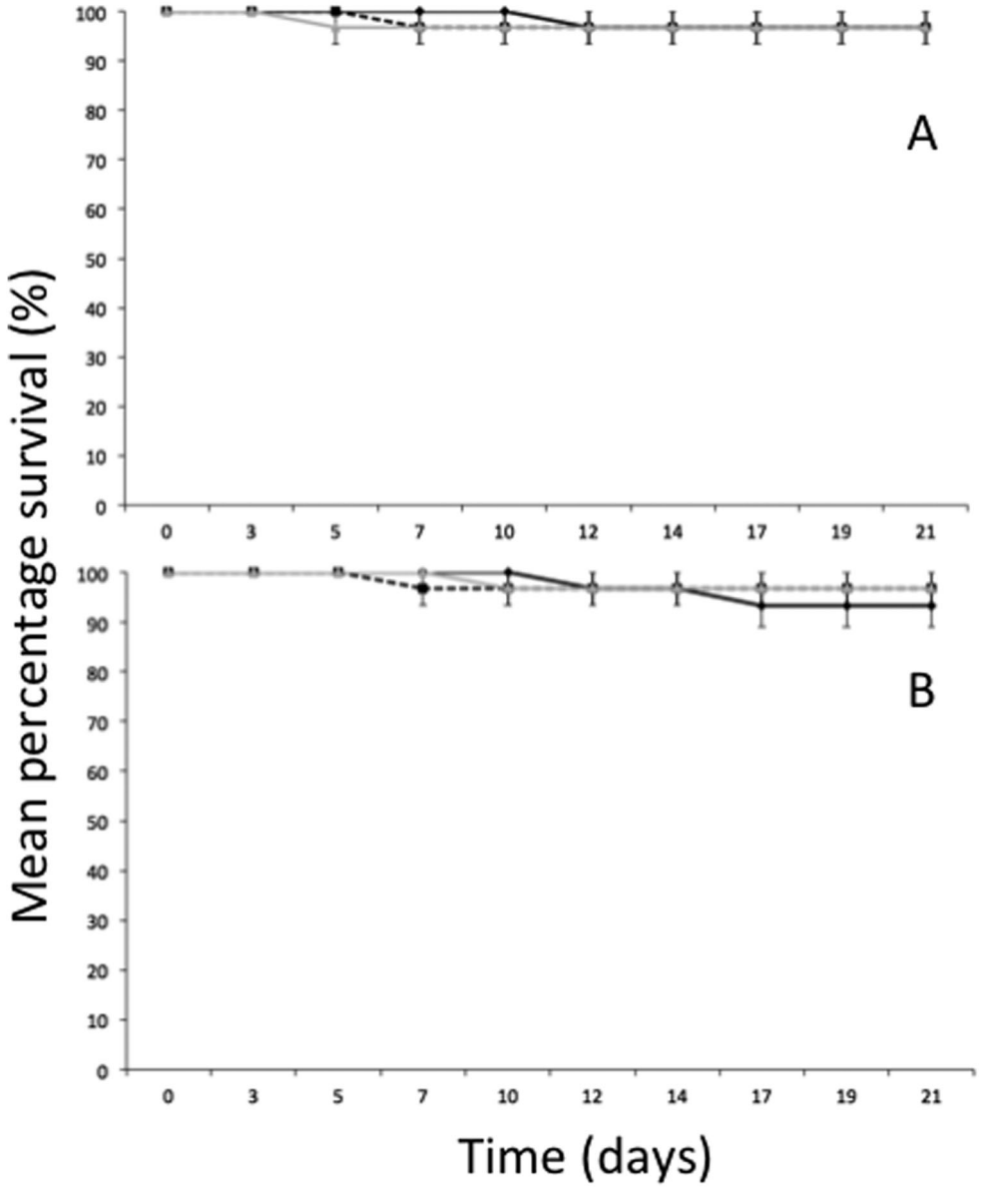

Figure 3. The mean percentage survival of adult *C. aspersum* exposed to 0 (black line), 30 (black dashed line) or 90 dauer juvenile *P. hermaphrodita* (grey line) (A) or *P. californica* DMG0019 (B) per cm^2 (grey line) over 21 days. Bars represent ± one standard error.

In general, the pathogenic potential of *P. californica* is poorly researched. There has been no research on the pathogenic potential of *P. californica* isolated from the U.K. however, Tandingan De Ley et al. (2020) showed *P. californica* from the U.S. could kill the snail *T. pisana* (albeit with high doses of 150 nematodes per cm^2) and was equally as pathogenic as *P. hermaphrodita* and *P. papillosa*. McDonnell et al. (2020) tested the infectivity of *P. hermaphrodita*, *P. californica* and *P. papillosa* to *D. reticulatum* and

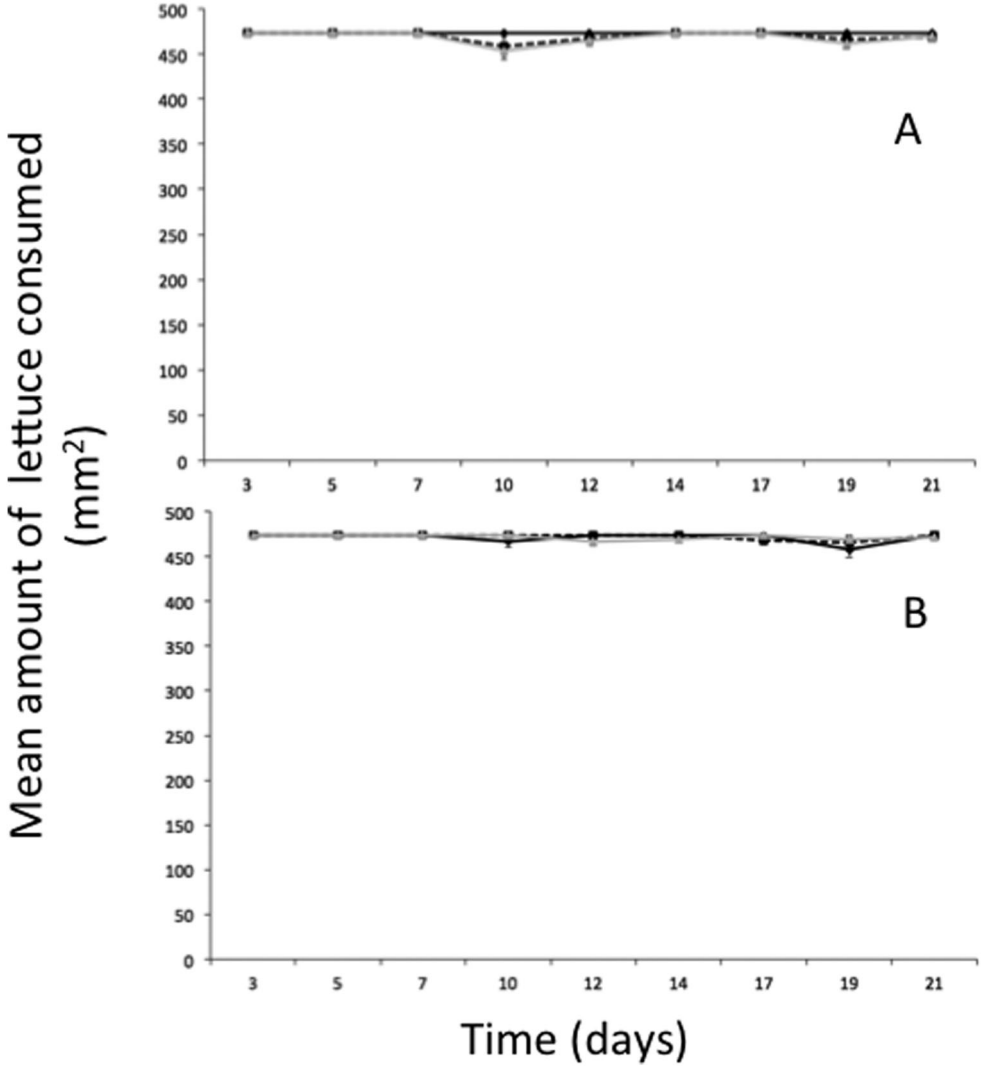

Figure 4. Feeding of adult *C. aspersum* exposed to 0 (black line), 30 (black dashed line) or 90 dauer juvenile *P. hermaphrodita* (grey line) (A) or *P. californica* DMG0019 (B) per cm^2 (grey line) over 21 days. Bars represent ± one standard error.

found *P. papillosa* was the most pathogenic. From our research it is clear that *P. californica* is pathogenic to neonate stages of *C. aspersum* but this is strain dependant. Specifically, both *P. californica* DMG0018 and DMG0019 were pathogenic to neonate *C. aspersum* but *P. californica* DMG0017 was not. Natural variation in pathogenicity of *Phasmarhabditis* nematodes towards other terrestrial gastropods has been reported. For example, Cutler and Rae (2020) reported that several wild strains of *P. hermaphrodita* were more virulent to slugs (*Deroceras invadens*) than the commercial strain and other wild strains. It should be noted that these *P. hermaphrodita* strains were isolated from separate slugs from different areas around Liverpool and are genetically distinct (Howe et al., 2020). The three strains of *P. californica* used in this study were

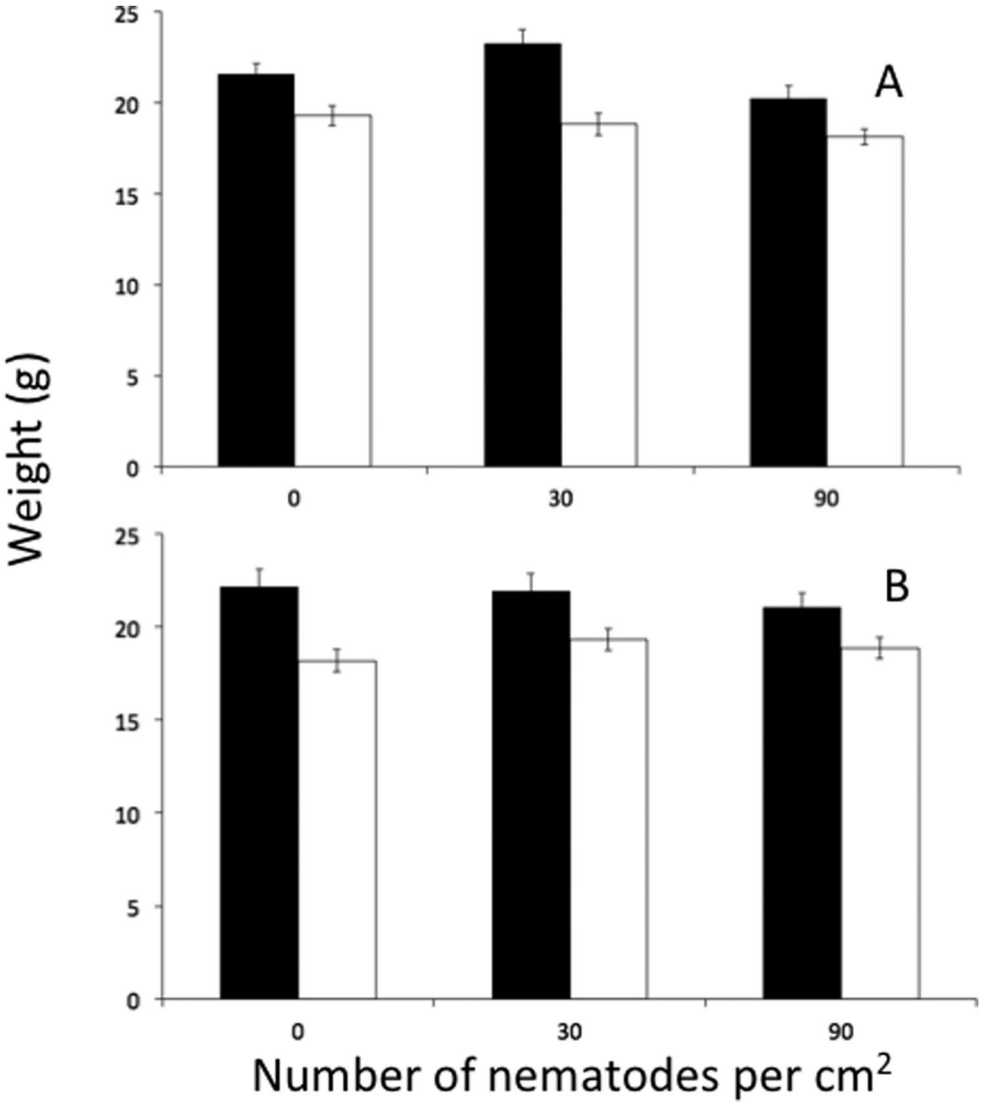

Figure 5. Weight of adult *C. aspersum* exposed to 0, 30 or 90 dauer juvenile *P. hermaphrodita* (A) or *P. californica* DMG0019 (B) per cm^2 on day 0 (black) and 21 (white). Bars represent ± one standard error.

isolated from one single snail (*O. draparnaudi*) (Andrus & Rae, 2019). This highlights how strains isolated in close proximity to each other and phylogenetically similar (Howe et al., 2020) differ in their pathogenic abilities (Cutler & Rae, 2020) as well as host seeking abilities (Andrus et al., 2018). Therefore, in order to produce and develop a suitable biological control agent for slugs and snails, intraspecific variation of strains should be investigated.

It is clear that the ability to kill terrestrial gastropods is an evolutionary conserved trait across the genus *Phasmarhabditis* as from the 13 species *P. neopapillosa* (Hooper et al., 1999), *P. tawfiki* (Azzam & Tawfiki, 2003), *P. safricana* (Ross et al., 2018), *P. bohemica*,

P. bonaquaense and *P. apuliae* can also kill slugs (Nermut et al., 2019) (as well as *P. hermaphrodita* and *P. californica*). As these nematodes are easy to isolate, maintain (Andrus & Rae, 2019) and capable of killing a range of pestiferous gastropods, there is ample opportunity to explore the pathogenic potential of all these other species for commercialisation and formulation to make superior biological control agents. To begin with *P. californica* (particularly strains DMG0018 and DMG0019) would be worth investigating further as they show a clear pathogenic effect to neonate *C. aspersum*.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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