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# Thirty years of slug control using the parasitic nematode *Phasmarhabditis hermaphrodita* and beyond

Robbie Rae,<sup>\*</sup>  Laura Sheehy and Kerry McDonald-Howard

## Abstract

Several slug species are highly pestiferous and threaten global sustainable agriculture. Current control methods rely heavily on metaldehyde pellets, which are often ineffective, harm nontarget organisms and have been banned in some countries. A viable alternative is the parasitic nematode *Phasmarhabditis hermaphrodita* (and recently *P. californica*), which has been formulated into a biological control agent (Nemaslug<sup>®</sup>) to control slugs across northern Europe. Nematodes are mixed with water and applied to soil where they seek out slugs, penetrate behind the mantle and kill them in 4–21 days. *Phasmarhabditis hermaphrodita* has been on the market since 1994 and since then there has been ample research on its use. Here we review the research carried out on *P. hermaphrodita* over the last 30 years since its development and release as a commercial product. We provide information on life cycle, worldwide distribution, history of commercialisation, gastropod immunity, host range, ecological and environmental factors that affect its success in the field, bacterial relationships, and summarise results of field trials. Finally, we suggest future directions for *P. hermaphrodita* research (and other *Phasmarhabditis* species) to enhance its use as a biological control agent to control slugs for the next 30 years.

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**Keywords:** nematodes; slugs; snails; biocontrol; gastropods

## 1 INTRODUCTION

Several slug species are highly pestiferous and pose a significant global threat to agriculture, horticulture and floriculture.<sup>1</sup> Slugs cause crop damage by eating seeds, stems, growing points and leaves, leading to a reduction in growth.<sup>2,3</sup> They can be a major pest throughout the lifecycle of field vegetables and in extreme cases, whole fields have to be re-sown resulting in economic losses.<sup>4</sup> Contamination of the harvested crop also occurs from slug mucus and faeces, resulting in poor product quality.<sup>5</sup> It is estimated that a lack of slug control for crops such as oilseed rape and wheat would lead to £43.5 million a year in loss of product in the UK alone.<sup>6</sup> In Europe, wheat and oilseed rape suffer greatly from slug damage;<sup>7</sup> for example in 2010 it was reported that 22% of winter wheat crops suffered damage from slugs, and if left untreated by chemical molluscicides a 5% decrease in yield would be expected.<sup>8</sup> As well as causing damage in agriculture, slug-feeding can affect plant community diversity and richness<sup>9</sup> with preferential feeding on native species aiding in exotic plant growth.<sup>10</sup> Furthermore, slug-feeding reduces conservation efforts such as forest regeneration<sup>11</sup> and threatens endangered species such as lichens.<sup>12</sup> Slugs also can transmit plant pathogens such as *Phytophthora*<sup>13</sup> and parasites,<sup>3,14</sup> including the rat lungworm, *Angiostrongylus cantonensis*, the causal agent of eosinophilic meningitis, which is recognised as an emerging tropical and subtropical zoonotic disease.<sup>15</sup>

Slugs are commonly controlled by chemical bait pellets containing metaldehyde. In the past methiocarb was used, yet it is toxic to beneficial invertebrates and other nontarget organisms<sup>16,17</sup> and was banned in the UK in 2014.<sup>18</sup> Metaldehyde pellets are used globally.<sup>19</sup> For example, from 2008 to 2014 an estimated 1640 t metaldehyde was used in the UK alone.<sup>19</sup> Slugs feed on the pellets and exhibit symptoms such as increased levels of mucus secretion and paralysis, and die within several days from water loss.<sup>20,21</sup> Although effective, these bait pellets also cause harm to nontarget organisms including canines and other vertebrates.<sup>22</sup> Additionally, metaldehyde also is now considered an important emerging pollutant of concern as a consequence of leaching into watercourses<sup>23</sup> caused by its high mobility in soil.<sup>24</sup> Furthermore, in parts of the UK metaldehyde concentrations in water bodies have exceeded the European Union's regulatory drinking water standard for pesticides.<sup>24</sup> An alternative slug pellet (Ferramol<sup>®</sup>) is composed of iron III phosphate or ferric phosphate and is registered for use in many European countries.<sup>25</sup> Although it has been used to control slugs such as *Arion ater*, studies have

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shown that high doses can lead to mortality and reduced activity in earthworms.<sup>26</sup>

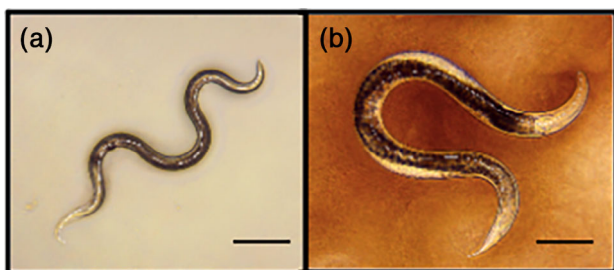
In agriculture, trapping, drilling at a greater depth, ploughing, crop rotation, increasing crop diversity and firm seedbed preparation also can help to limit slug damage, although some practices such as direct drilling and minimal tillage can result in an increase in pest slug populations.<sup>27</sup> Drilling to depths of 25–45 mm has been shown to provide the most effective protection against slug damage<sup>28</sup> and ploughing, and firm seedbed preparation reduces slug numbers by disrupting their normal surface activity patterns.<sup>29</sup>

In gardens and glasshouses, damage by gastropods can be limited by cultural control methods such as the use of copper (Cu) tape, garlic and mulch, although they are inefficient for larger scale agricultural use.<sup>30</sup> The use of Cu tape or Cu-impregnated matting has been shown to act as a barrier and reduce the velocity of pest slugs, possibly as a result of irritation.<sup>31</sup> In choice experiments, Cu was seen to repel slugs and they nearly always avoided mulch as it dries out quickly.<sup>31</sup> However, these methods are time-consuming, expensive and not always effective. An effective alternative for slug control is the gastropod parasitic nematode *Phasmarhabditis hermaphrodita* (Fig. 1) (for key diagnostic features see Stock and Hunt<sup>32</sup>), which has been formulated into a biological control agent (Nemaslug®) produced and sold by BASF Agricultural Specialities (Littlehampton, UK).<sup>33</sup> *Phasmarhabditis hermaphrodita* (strain DMG0001) is sold in 15 different European countries<sup>34</sup> and has been on the market since 1994; it also is available as a product called SlugTech® sold by Dudutech (Naivasha, Kenya; [www.dudutech.com/products/slugtech-sp/](http://www.dudutech.com/products/slugtech-sp/)).

Over the last 30 years, *P. hermaphrodita* has been successfully used to reduce slug damage in agriculture, floriculture and horticulture to levels comparable to those in crops treated with metaldehyde.<sup>35</sup> Here we describe the research that has been carried out on *P. hermaphrodita* since the first publication outlining its potential as a biocontrol agent of slugs in 1993,<sup>33</sup> and provide information on the *Phasmarhabditis* genus, host range and interactions, bacterial associations, nematode and gastropod behaviour, results of field trials, and suggest future research to enhance the use of *P. hermaphrodita* (and other *Phasmarhabditis* species) in the field.

### 1.1 Slug parasitic nematodes and the genus *Phasmarhabditis*

There are 108 nematodes associated with slugs and snails<sup>14</sup> used as definitive, intermediate or necromenic hosts.<sup>34</sup> Forty-seven species of nematode, belonging to eight families, use molluscs as a definitive host,<sup>14,34</sup> yet the only nematodes that can kill slugs and snails are those from the genus *Phasmarhabditis*.<sup>33</sup> There are some reports of mortality being caused by *Alloionema*



**Figure 1.** Dauer stage *P. hermaphrodita* (a) seek out slugs in soil and then penetrate inside. Once the slug dies the nematodes exit the dauer stage and grow to young adult nematodes (b) and reproduce on the cadaver. Bars, 100 µm.

*appendiculatum* towards *Arion vulgaris* but not at levels considered suitable for a biocontrol agent.<sup>36</sup>

*Phasmarhabditis hermaphrodita* is in clade V of the Nematoda,<sup>37</sup> and along with other *Phasmarhabditis* species, are easy to isolate from slugs and snails,<sup>33,38–40</sup> with many different species isolated from around the world. Identification can be accomplished using standard genotyping methods using 18SrRNA primers,<sup>41</sup> species-specific primers and quantitative polymerase chain reaction (qPCR) methodologies for nematodes isolated from soil or hosts.<sup>42,43</sup> *Phasmarhabditis hermaphrodita* was first described from Germany by Schneider in 1859,<sup>44</sup> then in 1900, Maupas isolated *P. hermaphrodita* in Normandy, France,<sup>45</sup> and 50 years later in 1953 it was re-isolated by Mengert in Germany.<sup>46</sup> The species was found in the UK in the early 1990's from diseased grey field slugs (*Deroceras reticulatum*) at Long Ashton Research Station, University of Bristol<sup>33</sup> as part of a project to identify potential biocontrol agents of slugs.<sup>47</sup> Further research focused on finding a suitable bacterium for mass production<sup>48,49</sup> and proof that the nematode could be used to control slugs under field conditions.<sup>50,51</sup> This research carried out by Mike Wilson and David Glen was used as a blueprint to commercially produce *P. hermaphrodita* first by MicroBio, then Becker Underwood and now BASF Agricultural Specialities. Subsequently, interest in *P. hermaphrodita* grew, and it was subsequently found in France,<sup>52</sup> Chile,<sup>53</sup> Iran,<sup>54</sup> Czech Republic,<sup>55</sup> Egypt,<sup>56</sup> New Zealand,<sup>38,57</sup> Norway<sup>58</sup> and Belgium.<sup>59</sup> One of the biggest markets for slug control is the USA, but for years *P. hermaphrodita* was never isolated despite several surveys.<sup>60–62</sup> However, recently numerous strains of *P. hermaphrodita* and other *Phasmarhabditis* species have been found in North America, specifically California, Oregon<sup>63–66</sup> and Canada.<sup>67,68</sup> The US strains of *P. hermaphrodita* have been shown to kill neonate giant African snails (*Lissachatina fulica*),<sup>69</sup> and several other *Phasmarhabditis* species can kill *D. reticulatum*,<sup>70,71</sup> the snails *Succinea* spp.<sup>72</sup> and *Theba pisana*,<sup>73,74</sup> as well as the subterranean slug *Testacella haliotideae*.<sup>75</sup> As well as *P. hermaphrodita* it has recently been shown another three species in the genus (*P. bohémica*, *P. bonaquanense* and *P. apuliae*) can infect and kill slugs (*D. reticulatum*).<sup>76</sup> Interestingly, full mitochondrial analysis of European and US strains of *P. hermaphrodita*, *P. californica* and *P. papillosa*, (as well as the Nemaslug® product) implies that the commercial strain *P. hermaphrodita* DMG0001 was introduced to the US.<sup>77</sup>

Nematodes from the genus *Phasmarhabditis* are problematic to classify as there are some poorly described species, but currently 18 species have been isolated from terrestrial gastropods, including *P. apuliae*, *P. bohémica*, *P. bonaquanense*, *P. californica*, *P. circassica*, *P. clausilliae*, *P. hermaphrodita*, *P. meridionalis*, *P. neopapillosa*, *P. papillosa*, *P. safricana*, *P. akhaldaba*, *P. kenyaensis*, *P. thesamica*, *P. quinamensis*, *P. zhejiangensis* and *P. tawfiki*, and one species (*P. huizhouensis*) from rotting leaf litter.<sup>78–91</sup> There were another two *Phasmarhabditis* species including *P. nidrosiensis* (isolated from a marine habitat) and *P. valida* (isolated from littoral detritus),<sup>92</sup> but after revision they were moved to the genus *Buetschlinema*.<sup>93</sup>

It is clear from the numerous surveys carried out over the last 30 years that *Phasmarhabditis* nematodes are commonly found in many countries from diverse terrestrial gastropod hosts. Whether or not there is any specific host preference the nematode has to a particular slug or snail species is unknown, but from survey results it would seem that there is a looser association with numerous terrestrial gastropod species: *P. tawfiki* was isolated from the snail *Eobania vermiculata* and the slug *Limacus flavus* in

Egypt;<sup>78</sup> *P. bonaquaense* was found in the slug *Malacolimax tenellus* in the Czech Republic; *P. apuliae* was isolated from slugs *Milax sowerbyi* and *Milax gagates* from Italy;<sup>80,81</sup> *P. bohemia* from the Czech Republic was isolated from *D. reticulatum*;<sup>82</sup> *P. papillosa* was isolated from *D. invadens* (previously called *D. panormitanum*) and *Tandonia sowerbyi* from the UK and *D. reticulatum* in the USA.<sup>62,79</sup> and South Africa;<sup>94</sup> *P. neopapillosa* was isolated from *D. reticulatum*, *D. panormitanum*, *L. flavus*, *Arion ater* and *Arion distinctus* in Scotland and England.<sup>40,62</sup> A new species (*P. safricana*) was collected from *D. reticulatum* in South Africa.<sup>90,95</sup> *Phasmarhabditis californica* was isolated from the USA from numerous species including *D. reticulatum*, *D. laeve*, *Arion hortensis* and *Ambigolimax valentianus*,<sup>79</sup> as well as being found in *Geomalacus maculosus* in Ireland<sup>96</sup> and from the snail *Oxychilus draparnaudi* in Wales<sup>40</sup> and Germany;<sup>97</sup> it also was isolated from *Arion rufus* from Edmonton, Canada,<sup>67,68</sup> and along with *P. hermaphrodita* has been infecting *D. reticulatum* in New Zealand.<sup>57,77</sup> *Phasmarhabditis meridionalis* was described from snails (*Quantula striata*) in Vietnam<sup>85</sup> and in 2019, *P. circassica* and *P. clausiliae* were found in snails *Oxychilus* sp. and Clausiliidae sp., respectively, in Russia.<sup>86</sup> Therefore, *Phasmarhabditis* nematodes have a cosmopolitan distribution across the globe and can be easily isolated from a diverse range of slugs and snails.

There are several *Phasmarhabditis* species still awaiting description, including two *Phasmarhabditis* species in Japan,<sup>98</sup> and two species (called '*Phasmarhabditis* sp. SA3' and '*Phasmarhabditis* sp. SA4') isolated from slugs in nurseries in South Africa.<sup>99</sup> A possible *Phasmarhabditis* species was found reproducing on the earthworm *Lumbricus terrestris*,<sup>100</sup> and was described as being virulent towards earthworms, which is highly unusual for a *Phasmarhabditis* species. Finally, *Phasmarhabditis* sp. EM434 was discovered in North America<sup>101</sup> but there is only limited information on this species, which amounts to only a few DNA sequences in the National Centre for Biotechnology Information (NCBI) database.

Out of all the currently described species, *P. hermaphrodita*,<sup>33</sup> *P. neopapillosa*,<sup>102,103</sup> *P. tawfiki*,<sup>104</sup> *P. papillosa*,<sup>94</sup> *P. safricana*,<sup>90,95</sup> *P. bohemia*, *P. bonaquaense* and *P. apuliae*,<sup>76,105</sup> and *P. californica*<sup>73</sup> have been shown to kill slugs and snails. Taken together, these results demonstrate that pathogenicity towards terrestrial gastropods is not confined to one *Phasmarhabditis* species and appears to be a common trait across the genus.

### 1.1.1 Life cycle of *P. hermaphrodita*

*Phasmarhabditis hermaphrodita* is a facultative parasite, able to kill several species of terrestrial gastropods and grow and reproduce on a variety of organic matter.<sup>45,106,107</sup> (Fig. 2). It also is able to infect larger host species such as *A. ater* where it will remain until the host dies and reproduce on the cadaver, termed 'necromeny'<sup>108</sup> (Fig. 2). *Phasmarhabditis hermaphrodita* is a hermaphroditic nematode and the occurrence of males is extremely rare,<sup>92</sup> with one study finding just one male in 14 888 hermaphrodites.<sup>45</sup>

### 1.1.2 Chemoattraction of *P. hermaphrodita* to slug and snail host cues

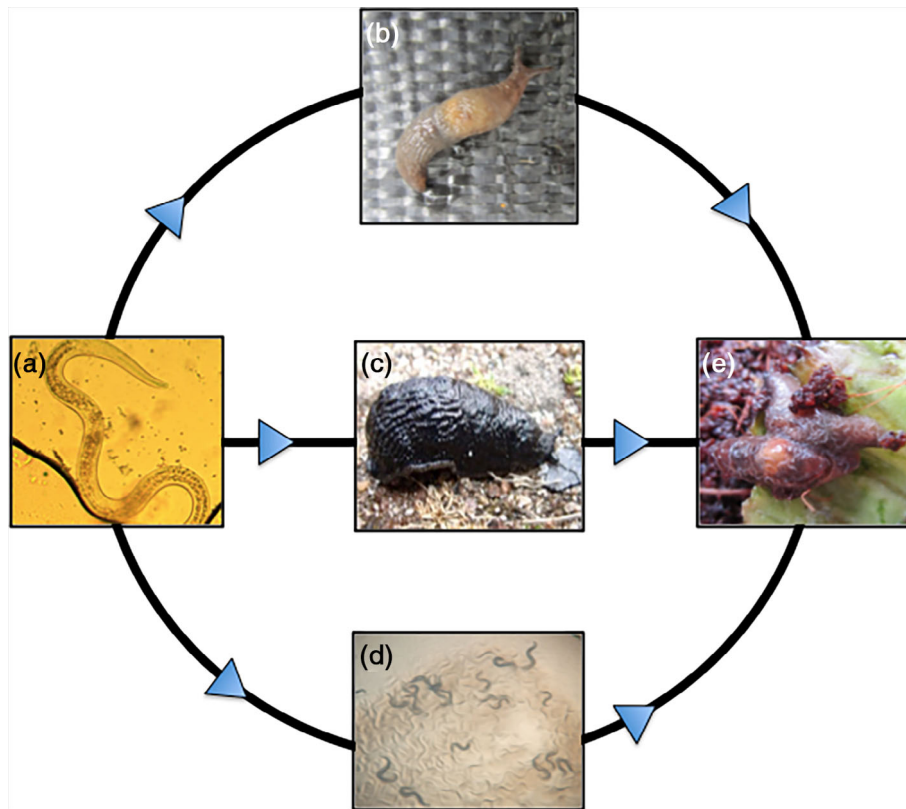
In order to locate hosts, *P. hermaphrodita* dauer stage nematodes seek out slugs in soil by following mucus, faecal and volatile cues.<sup>109–115</sup> Nictation (where entomopathogenic nematodes stand on their tail hoping to latch on to hosts passing by) and body waving has not been observed in *P. hermaphrodita*, potentially as a consequence of their long length.<sup>116,117</sup> Alternatively, these nematodes employ a 'cruiser'-based foraging strategy where they actively search for hosts following cues.

*Phasmarhabditis hermaphrodita* is attracted to faeces, foot and mantle mucus of *D. reticulatum*.<sup>109</sup> As many slugs and snails display homing behaviour and return to the same location each night,<sup>118</sup> faecal attraction of *P. hermaphrodita* may be beneficial for infecting new hosts. Volatile host cues such as CO<sub>2</sub> were found to be the least attractive cues to *P. hermaphrodita*,<sup>109</sup> potentially owing to the vast quantities of CO<sub>2</sub> released by microorganisms in soil,<sup>119</sup> but also as a result of *P. hermaphrodita* entering the slug host through the back of the mantle and not the respiratory pore.<sup>33</sup> When *P. hermaphrodita* is exposed to *D. reticulatum* mucus, speed, movement, distribution of turning angles and the fractal dimension of nematode foraging trails significantly increase.<sup>111,112</sup> *Phasmarhabditis hermaphrodita* not only responds to mucus from *D. reticulatum*, but also is positively attracted mucus from a wide range of diverse slug and snail species.<sup>110,120</sup> Of the species tested, *P. hermaphrodita* showed a preference for slugs such as *Arion subfuscus*, *D. invadens* and the snail *Cornu aspersum* (even though the nematode finds it difficult to infect and kill this species). These hosts represent a range of parasitic and necromenic life cycles. *Phasmarhabditis hermaphrodita* was more attracted to slugs than earthworms (*L. terrestris* and *Eisenia hortensis*). Reproductive success of *P. hermaphrodita* was not greater on attractive slug species (compared to nonattractive species), and the reason for this preference to certain slug species is still unknown.<sup>110</sup> In a similar experiment recently<sup>121</sup> the chemotactic response of *P. papillosa* was recorded when exposed to mucus from a selection of species, of which *L. maximus* and *C. aspersum* were particularly attractive to compared to *A. vulgaris* and *D. reticulatum* (for reasons unknown). The pathogenicity of *P. papillosa* to these slug and snail species is unknown; therefore conclusions about the reasons for their attraction cannot be made.

All these studies have focused on using the commercial strain of *P. hermaphrodita* (strain DMG0001) that has been in culture since 1994. To gain more insight into how wild strains of *P. hermaphrodita* would behave, several wild isolated strains of *P. hermaphrodita*, *P. neopapillosa* and *P. californica* were exposed to mucus from seven different slug species.<sup>122</sup> The wild strains differed in their preference to the slug species tested with *P. neopapillosa* preferring *Arion* spp. In a similar study<sup>123</sup> the response of *P. hermaphrodita*, *P. neopapillosa* and *P. californica* to snail mucus was recorded. Surprisingly, the commercial strain of *P. hermaphrodita* DMG0001 showed little chemotactic response and remained at the point of application, whereas wild isolates of *P. hermaphrodita* and *P. californica* were attracted to mucus of *Cepaea nemoralis*, *Cepaea hortensis* and *Arianta arbustorum*<sup>123</sup> (even though they are all resistant to the nematode). There is little information about what the exact compounds in slug and snail mucus *Phasmarhabditis* nematodes are attracted to, but metal ions (e.g. MgCl<sub>2</sub>, FeSO<sub>4</sub>) and hyaluronic acid (an abundant component of slug mucus) play a role.<sup>123</sup> Furthermore, there is natural variation in the chemotactic response of wild strains of *P. hermaphrodita*, *P. californica* and *P. neopapillosa* to hyaluronic acid, suggesting that it must be an important component for host finding.<sup>124</sup>

The majority of chemotaxis experiments investigating the behaviour of *P. hermaphrodita* have been carried out on agar plates and therefore may not be applicable to their natural soil environment. A more realistic experimental design, where sand grains were placed on agar plates, found that the speed, turning angle distribution, fractal dimension and mean square displacement of *P. hermaphrodita* was reduced when in contact with mucus.<sup>112</sup> Furthermore, in soil olfactometers *P. hermaphrodita* was averted from dead slugs (which are usually attractive) leading





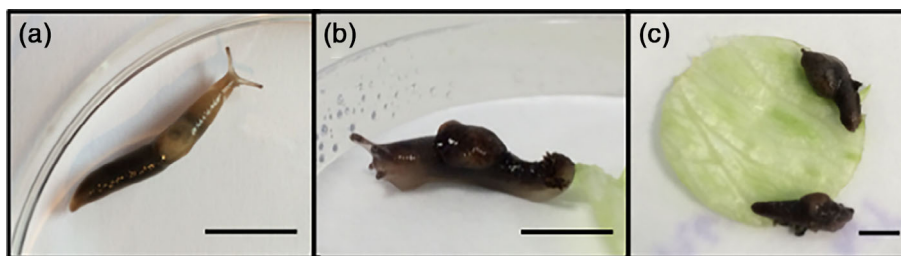
**Figure 2.** *Phasmarhabditis hermaphrodita* (a) can complete its life cycle in three ways. It can parasitise and kill susceptible hosts such as *D. invadens* (b), infect resistant slug species such as *A. ater* and wait for it to die (a ‘necromenic’ relationship) (c), or feed and reproduce on the bacteria that proliferate on decomposing organic matter (a ‘saprobic’ relationship) or can be kept under laboratory conditions on an agar plate with *E. coli* as a food source (d). In each case once the food supply has been depleted the nematode will develop to the dauer stage and move through soil to find more hosts to infect and kill (e).

the authors to hypothesise that the large variety of decay gases caused *P. hermaphrodita* to suffer from a lack of oxygen and move away.<sup>115</sup> In columns packed with different substrates *P. hermaphrodita* moved best through organic matter, uncompacted soil and soil containing large aggregates.<sup>107</sup> Dispersal of *P. hermaphrodita* was increased when placed in mineral soils with the earthworm *L. terrestris*. They also showed that the commercial strain of *P. hermaphrodita* was unable to move through the soil column, but a wild isolated strain from Norway dispersed significantly more.<sup>107</sup>

### 1.1.3 How *P. hermaphrodita* kills slugs—the questionable role of bacteria

When *P. hermaphrodita* locates a slug host it enters through the back of the mantle through a pore and migrates to the shell cavity.<sup>33,106</sup> Larvae then develop into self-fertilising hermaphrodites and start to reproduce. This produces characteristic signs of infection such as a swollen mantle and shell ejection (Fig. 3). Host death occurs 4–21 days after initial infection,<sup>33</sup> and nematodes feed and reproduce on bacteria proliferating on the cadaver. When the food source is depleted, dauer juveniles enter the soil to locate a new host. It is currently unknown how *P. hermaphrodita* kills slugs. Early research focused on a paradigm similar to entomopathogenic nematodes (EPNs) and their symbiotic relationship with bacteria. EPNs of the families Steinernematidae and Heterorhabditidae associate with *Xenorhabdus* spp. and *Photorhabdus* spp., respectively, that are responsible for killing host insects.<sup>125</sup> It was previously thought that *P. hermaphrodita* functioned in a similar way to EPNs and acted as a vector for the bacterium *Moraxella osloensis*, and the host died due to

septicaemia.<sup>126</sup> When the first strain of *P. hermaphrodita* (DMG0001) was isolated an attempt was made to identify a bacterium that could be used for industrial production of these nematodes. Indeed, it is clear that bacterial diet, substrate and inoculation density can have dramatic effects on growth, lipid content and length of nematodes.<sup>48,49,105,127,128</sup> Initial studies focused on understanding the best bacterium that could be used to produce high numbers of consistently virulent nematodes. In these experiments *P. hermaphrodita* were fed a selection of bacteria that had been isolated from *P. hermaphrodita* infected slugs and from *P. hermaphrodita* dauer juveniles emerging from dead slugs.<sup>48,49</sup> Many different bacterial species were isolated and tested including: *Acinetobacter calcoaceticus*, *Aeromonas hydrophila*, *Aeromonas* sp., *Bacillus cereus*, *Flavobacterium breve*, *Flavobacterium odoratum*, *Moraxella osloensis*, *Providencia rettgeri*, *Pseudomonas fluorescens* (isolate no. 1a), *Pseudomonas fluorescens* (isolate no. 140), *Pseudomonas fluorescens* [isolate no. 141, *P. fluorescens* (pSG)], *Pseudomonas paucimobilis*, *Serratia proteamaculans*, *Sphingobacterium spiritocorum* and *Xenorhabdus bovienii*.<sup>48,49</sup> Successful feeding and growth of *P. hermaphrodita* also has been recorded on *Pseudomonas* sp. 1, *Bacillus* sp. 1, *Escherichia coli* OP50 and *E. coli* BR.<sup>40</sup> *Moraxella osloensis* was chosen as it produced consistently high yields of pathogenic nematodes.<sup>48,49</sup> It should be stressed that this bacterium was chosen for commercial production and does not reflect the natural tritrophic interactions that may be occurring between slugs, *P. hermaphrodita* and bacteria in the wild. Indeed, when *P. hermaphrodita* was grown on rotting slugs or emerging after parasitising slugs (*D. reticulatum*), there was no evidence of *M. osloensis* being present inside the



**Figure 3.** *Phasmarhabditis hermaphrodita* produces characteristic signs of infection when parasitising pestiferous hosts such as *D. invadens* (a). Nematodes infect the slug through a pore in the back of the mantle and reproduce, causing a swelling of the mantle area (b), this eventually leads to death in 4 to 21 days (c). Bars, 1 cm.

nematodes,<sup>129</sup> and, therefore, these nematodes do not vertically transmit this bacterium. Likewise, *M. osloensis* was lost after repeated culturing of *P. hermaphrodita* strain (DMG0001) over several generations on homogenised pig kidney.<sup>128</sup> However, research has shown that injection of 40- and 60-h cultures of *M. osloensis* into the haemocoel of *D. reticulatum* will kill slugs, with the 60-h cultures being more pathogenic than the 40-h cultures.<sup>126</sup> This is thought to be due to a lipopolysaccharide (LPS) which acts as an endotoxin,<sup>130,131</sup> and *ubiS* and *dsbC* genes that are upregulated by *M. osloensis* when infecting *D. reticulatum*.<sup>132</sup> *Moraxella osloensis* is only toxic to *D. reticulatum* when injected and showed no contact or oral toxicity to slugs.<sup>131</sup> The relationship between *M. osloensis* and *P. hermaphrodita* has been categorised as 'symbiotic' yet there are compelling reasons why this may not be the case. This is out with the scope of this review but see Wilson and Rae<sup>133</sup> for further details. What is clear is that *P. hermaphrodita* is a facultative parasite, able to grow on a multitude of different bacterial species which can dramatically affect the numbers of offspring produced and the nematode's pathogenicity. Whether or not the nematode relies on a strict symbiotic relationship with one bacterium is a matter of debate, but profiling the bacterial species wild *P. hermaphrodita* associate with in nature will provide insight. For example, a plethora of different bacterial species including *Acinetobacter* sp., *Alcaligenes faecalis*, *Bacillus cereus* and *Stenotrophomonas* sp. were identified from dauer juveniles of *P. hermaphrodita* DMG0001 and wild strains of *P. hermaphrodita*.<sup>128</sup> Likewise, by using 16SrRNA metagenomics the microbiome of wild *Phasmarhabditis* from California was profiled and the most predominant bacteria identified were *Shewanella*, *Clostridium perfringens*, Aeromonadaceae, Pseudomonadaceae and Actinetobacter,<sup>134</sup> however, the authors did not carry out any other experiments so it is difficult to come to any major conclusions about the role of bacteria in US strains of *Phasmarhabditis*. By contrast, a recent study<sup>135</sup> showed that *P. hermaphrodita* (wild and commercial strains), *P. californica* or *P. neopapillosa* dauer juveniles which had killed a slug harboured a plethora of bacterial species, including *M. osloensis* but in minute amounts. Furthermore, genotyping of the *M. osloensis* strains used by BASF Agricultural Specialities used to grow *P. hermaphrodita* revealed that the species was actually more closely related to *Psychrobacter faecalis*, and thus there seems to be limited use of *M. osloensis* in the pathogenicity process.<sup>135</sup>

## 2 REPRODUCTION

Upon host death, nematodes proliferate on the slug cadaver, and multiple factors can influence progeny dynamics. *Phasmarhabditis hermaphrodita* grown on tissue from different species of slugs

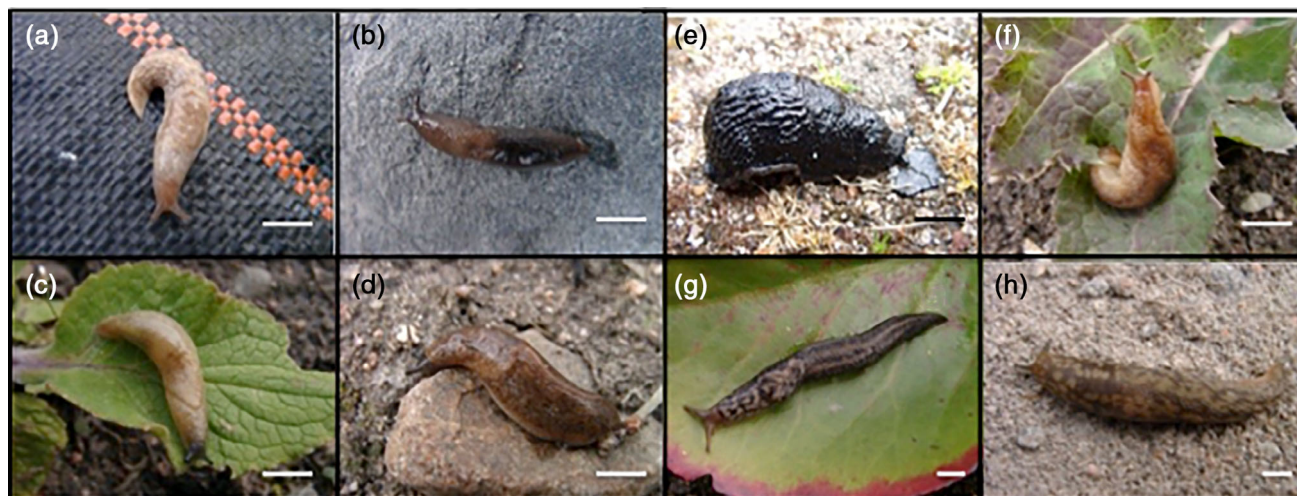
and snails yielded different numbers of offspring with *D. invadens* producing the highest number of progeny followed by *Limax marginata*, *M. gagates*, *C. hortensis* and *D. reticulatum*.<sup>110</sup> Development and quality of *P. hermaphrodita* can be severely affected by growing substrate:<sup>105,128</sup> it was able to successfully grow on multiple substrates including a mixture of homogenised pig kidney with different homogenised slug species (*Arion lusitanicus* and *D. reticulatum*) and homogenised moth (*Galleria mellonella*), faeces from *D. reticulatum* and *A. lusitanicus*, and leaf compost. The authors found the yield of *P. hermaphrodita* to be greater on invertebrate-based substrates, although the quality of *P. hermaphrodita* produced remained stable based on body size and lipid content.<sup>105,128</sup> Similar findings of dauer juveniles of *P. hermaphrodita* recovering and multiplying in slug faeces but not soil samples have been reported.<sup>106</sup> These results indicate that reproducing on an invertebrate can produce similar numbers of progeny as when the nematode kills a slug host and reproduces on it.<sup>128</sup> As well as *P. hermaphrodita*, other *Phasmarhabditis* species such as *P. bohémica*, *P. bonaquaense* and *P. apuliae*<sup>136</sup> can all be grown under laboratory conditions on dead slugs and have different generation times.

Intraspecific competition for resources can influence *P. hermaphrodita* development; lipid content, yield and body length,<sup>127,128</sup> and nematodes may leave areas of dense populations to find other resources.<sup>127</sup> Also, the time it takes for new dauer juveniles to develop can differ with species. For example, *P. bohémica* had the shortest development cycle compared to *P. hermaphrodita*, *P. papillosa* and *P. kenyaensis* when grown on rotting slug (*D. invadens*), but it should be noted for industrial production that *P. hermaphrodita* is best as it is a hermaphrodite and not gonochoristic like the other species.<sup>136</sup> As well as differences between species, temperature also can severely affect the survival and growth of *P. hermaphrodita*. Survival dramatically decreases at 25 °C and 35 °C but there is no difference at 5, 10 and 15 °C,<sup>137</sup> with the optimum growth temperature for *P. hermaphrodita* at 17 °C.<sup>33</sup>

### 2.1 Susceptibility of terrestrial gastropods to *P. hermaphrodita*

There are currently 22 species of slug and 21 species of snail that have been tested for susceptibility to *P. hermaphrodita* under laboratory conditions (Fig. 4; Table 1). To date, 12 slug species and eight snail species can clearly be killed by *P. hermaphrodita*. There is little research into understanding how *P. hermaphrodita* is able to kill terrestrial gastropods and very little information about why there is this difference in susceptibility of different species. Some studies have shown that younger stages of certain slug species are susceptible to *P. hermaphrodita* whereas adults are not





**Figure 4.** *Phasmarhabditis hermaphrodita* can cause rapid mortality to the susceptible slugs *D. reticulatum* (a), *D. invadens* (b), *M. gagates* (c) and *T. sowerbyi* (d), but *A. ater* (e), *A. subfuscus* (f), *L. maximus* (g) and *L. flavus* (h) are resistant, for reasons unknown. Bars, 0.5 cm.

including *A. vulgaris* (previously known as *A. lusitanicus*)<sup>142–145</sup> and *A. ater*<sup>33,110</sup> (although it should be noted that *P. papillosa* can supposedly kill adult *A. vulgaris*<sup>155</sup>). It also has been recorded that *P. californica* can kill neonate *C. aspersum* but not adults,<sup>156</sup> similar to *P. hermaphrodita*.<sup>103</sup> Confusingly, studies that have carried out the same experiment have reported different results. For example, neonate stages of the giant African snail (*L. fulica*) can be killed by a wild strain of *P. hermaphrodita* from the US,<sup>69</sup> whereas the commercial strain *P. hermaphrodita* DMG0001 had no negative effect on juvenile stages of these snails.<sup>149</sup> Also the freshwater snail *Lymnaea stagnalis* was killed by *P. hermaphrodita*<sup>151</sup> but another study observed no mortality when the same experiment was repeated.<sup>150</sup> These differences could be to the result of using laboratory-reared or wild-collected nematodes or hosts. For example, in the former study<sup>151</sup> a laboratory strain of *L. stagnalis* was used whilst wild-collected *L. stagnalis* were used in the latter study.<sup>150</sup> Likewise, the commercial strain of *P. hermaphrodita* was exposed to *L. fulica* in the UK study<sup>149</sup> but a wild strain of *P. hermaphrodita* was used in the US study.<sup>69</sup> It is interesting to speculate why there are such differences; perhaps it could be a consequence of continuous laboratory culturing, which can have severe effects on the health of laboratory animals.<sup>157</sup> and possibly nematodes. For example, traits such as heat, UV light and desiccation tolerance, and reproductive potential have been shown to be reduced in *H. bacteriophora* through continuous culturing in *Galleria mellonella*.<sup>158</sup> The effect of continuous laboratory culturing in nematodes and hosts could therefore play a role in the differences found in these experiments

One common symptom of *P. hermaphrodita* infection is host feeding inhibition, which is strongly observed in slugs such as *D. reticulatum* and *D. invadens* but also has been observed in slug species that it cannot kill.<sup>33,110</sup> It has been suggested that slug control in field trials is probably from host-feeding inhibition as opposed to slug mortality.<sup>27,50,139</sup> Feeding inhibition may be a defensive behaviour of slugs to contract and reduce the numbers of nematodes penetrating inside.<sup>139</sup> Some species, however, are not killed by *P. hermaphrodita* and their feeding is not inhibited, such as *L. maculatus*.<sup>140</sup> Interestingly, it has been recently shown that as well as affecting feeding behavior, infection by *P. hermaphrodita* can alter the microbiome of the

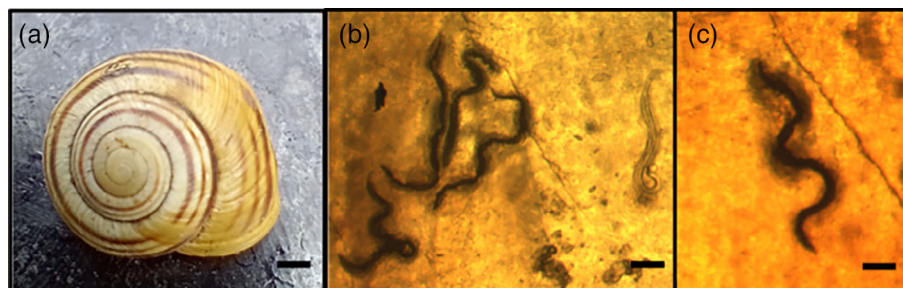
susceptible slug *D. invadens*, but has no effect on the bacterial communities of the resistant slug *A. valentianus*.<sup>159</sup>

In contrast to slugs, the effect that *P. hermaphrodita* has on snails has not been investigated in detail (although these nematodes have been isolated regularly from snails<sup>160</sup>). *Phasmarhabditis hermaphrodita* has been shown to cause high levels of mortality to snails (*T. pisana*, *Trochoidea elegans* and *Monacha cantiana*).<sup>52,73,161</sup> There are many snail species resistant to infection by *P. hermaphrodita* and one reason for this may be the snail shell. An observation during an infection experiment using *P. hermaphrodita* and *L. fulica* found nematodes trapped and encased in the inner layer of the shell.<sup>149</sup> Evidence of this process also has been shown in live *C. nemoralis*<sup>148</sup> (Fig. 5), *A. arbustorum*,<sup>146</sup> and in museum collections of *C. aspersum* and *H. pomatia*.<sup>162</sup> This process is remarkably well-conserved across the Stylommatozoa and has been thought to be present when the two major clades diverged 80–130 million years ago (Ma);<sup>163</sup> nematodes have even been observed in the vestigial shell of the slug *L. pseudoflavus*.<sup>140</sup> Nematodes have been infecting gastropods since the late Cambrian<sup>14</sup> and this evolutionary arms race has resulted in slugs and snails co-opting their shell to encapsulate and encase parasitic nematodes instead of just using the shell for shelter.<sup>163</sup> Interestingly, dark morphs of the snail *Cernuella virgata* were found to be more resistant to *P. hermaphrodita* than light morphs and this was not due to phenoloxidase levels;<sup>164</sup> those authors did not dissect the snails or examine the shells for nematodes, but perhaps this difference in susceptibility was due to the effectiveness of the shell morphs in encasing invading nematodes?

As well as the shell, the immune system of slugs and snails must play a role in combating infection, but this has been poorly researched. There have only been a couple of studies looking at the immune system of snails when infected by *P. hermaphrodita*.<sup>154,165</sup> Oxidative stress and cell metabolism were affected in the nematode-infected freshwater golden apple snails (*Pomacea canaliculata*)<sup>165</sup> and specifically *Pc-bpi*, a mammalian bactericidal/permeability increasing protein orthologue, was highly upregulated in the kidney and gills of the snail.<sup>154</sup> How abundantly upregulated this protein is and its role in combatting nematode infection in terrestrial gastropods is unknown.

**Table 1.** Susceptibility of slugs and snails exposed to *Phasmarhabditis hermaphrodita*

Gastropod	Family	Species	Susceptible to <i>P. hermaphrodita</i> ?	Relationship with host	References
Slugs	Agriolimacidae	<i>Deroceras reticulatum</i>	Yes	Parasitic	33,70,71,106,110
		<i>Deroceras invadens</i>	Yes	Parasitic	33,110,135,138
		<i>Deroceras laeve</i>	Yes	Parasitic	139
	Limacidae	<i>Limax maximus</i>	No	Necromenic	139
		<i>Limax maculatus</i>	No	Necromenic	140
		<i>Limax marginata</i>	No	Necromenic	110
		<i>Ambigolimax valentianus</i>	No	Necromenic	141
		<i>Arion ater</i>	Only juveniles	Parasitic/Necromenic?	33,110
	Arionidae	<i>Arion silvaticus</i>	Yes	Parasitic	33
		<i>Arion intermedius</i>	Yes	Parasitic	33
		<i>Arion distinctus</i>	Yes	Parasitic	33,142
		<i>Arion lusitanicus</i>	Only juveniles	Parasitic/Necromenic?	143,144
		<i>Arion subfuscus</i>	No	Necromenic	110,139
		<i>Arion hortensis</i>	No	Necromenic	139,142
		<i>Arion fasciatus</i>	Yes	Parasitic	145
		<i>Arion vulgaris</i>	No	Necromenic	145
		<i>Geomalacus maculosus</i>	No	Necromenic	96
		Milacidae	<i>Tandonia sowerbyi</i>	Yes	Parasitic
	<i>Tandonia budapestensis</i>		Yes	Parasitic	33
	<i>Milax gagates</i>		Yes	Parasitic	110,140
Testacellidae	<i>Testacella haliotideae</i>	Yes	Parasitic	75	
Veronicelloidae	<i>Leidyula floridana</i>	Yes	Parasitic	139	
Snails	Helicidae	<i>Cornu aspersum</i>	Only juveniles	Parasitic/Necromenic?	103,110
		<i>Arianta arbustorum</i>	No	Necromenic	145,146
		<i>Cepaea hortensis</i>	Yes/No	Parasitic/Necromenic?	110,147
		<i>Cepaea nemoralis</i>	No	Necromenic	147,148
	Thebaicidae	<i>Theba pisana</i>	Yes	Parasitic	52,73,74
		<i>Cochlicella acuta</i>	Yes	Parasitic	52
	Cernuelliidae	<i>Cernuella virgata</i>	Yes	Parasitic	52
		<i>Monacha cantiana</i>	Yes	Parasitic	147
	Succineidae	<i>Succinea</i> spp.	Yes	Parasitic	72
	Pomatiasidae	<i>Pomatias elegans</i>	No	Necromenic	147
	Oxychilidae	<i>Oxychilus helveticus</i>	No	Necromenic	147
	Clausiliidae	<i>Clausilia bidentata</i>	No	Necromenic	147
	Discidae	<i>Discus rotundatus</i>	No	Necromenic	147
	Achatinidae	<i>Lissachatina fulica</i>	No/Yes	Parasitic/Necromenic?	69,149
	Bithyniidae	<i>Bithynia tentaculata</i>	No	Necromenic	150
	Lymnaeidae	<i>Lymnaea stagnalis</i>	Yes/No	Parasitic/Necromenic?	150,151
	Physidae	<i>Physa fontinalis</i>	No	Necromenic	150
	Planorbidae	<i>Planorbarius corneus</i>	No	Necromenic	150
		<i>Biomphalaria pfeifferi</i>	Yes	Parasitic	152
		<i>Biomphalaria alexandrina</i>	Yes	Parasitic	153
	Ampullariidae	<i>Pomacea canaliculata</i>	Yes	Parasitic/Necromenic?	154



**Figure 5.** Snails such as *C. nemoralis* (a) can be infected with *P. hermaphrodita* under laboratory and field conditions and nematodes are trapped, encased and killed in the shell (b and c). Bars, 2 mm (a) and 100 µm (b and c).



Owing to its ability to kill snails *P. hermaphrodita* could be used to reduce snail populations that vector medically important parasites. Specifically, application of the nematode has been shown to negatively affect the freshwater snails *Biomphalaria alexandrina* and *B. pfeifferi* (under laboratory conditions), which could potentially result in a diminished transmission of schistosomiasis.<sup>152,153</sup>

The potential of these nematodes to control *Biomphalaria* snails warrants significant attention and could be highly promising.

## 2.2 Host avoidance and behavioural manipulation

Avoidance behaviour is the first strategy an organism can employ to reduce the threat of parasitism.<sup>166</sup> In order to reduce parasitism by *P. hermaphrodita*, slugs avoid areas where nematodes are present. Slugs such as *D. invadens* and *A. ater* are able to detect and avoid areas where *P. hermaphrodita* is present, and spend less time feeding and resting in such areas.<sup>167</sup> It could be presumed that slugs would avoid all parasitic *Phasmarhabditis* species, but this is not the case: *D. invadens* avoids *P. hermaphrodita* and *P. californica* but curiously is attracted to areas where *P. neopapillosa* has been applied.<sup>168</sup> The reasons for this are unknown but it has important ramifications for the use of other *Phasmarhabditis* species in the field.

Avoidance behaviour in slugs when exposed to *P. hermaphrodita* has been observed in several diverse slug species from three different families, yet snails (e.g. *C. aspersum*) do not avoid the nematodes.<sup>169</sup> Slugs specifically avoid *P. hermaphrodita* and not other nematodes such as the EPN *Steinernema kraussei* or the vinegar eelworm (*Turbatrix aceti*)—both of which are not parasites of terrestrial gastropods. Resistant slug species *A. subfuscus*, *A. hortensis* and *A. valentianus* avoid *P. hermaphrodita*, although *L. flavus* also is resistant to *P. hermaphrodita* infection but does not avoid the nematode.<sup>169,170</sup> Slugs do not avoid areas treated with the supernatant of a liquid suspension of *P. hermaphrodita* suggesting that the slugs are avoiding the mechanical stimulus of the nematodes probing the slug's body, rather than a chemical cue.<sup>169</sup> However, when a slug is infected with *P. hermaphrodita* the usual avoidance behaviour is abrogated and slugs are oddly more likely to be found on soil where *P. hermaphrodita* is present.<sup>170</sup> The exact reason why the nematodes are influencing slug behaviour is unclear, but it could increase chances for more successful infection and therefore reproduction.<sup>170</sup> It is unclear how *P. hermaphrodita* is able to manipulate slug behaviour, but it could be linked to neurotransmitter signaling. Uninfected slugs (*D. invadens*) fed fluoxetine or sertraline, which increase serotonin levels, were driven towards the nematodes, whereas infected slugs treated with cyproheptadine, which suppresses serotonin levels, were no longer attracted to the nematodes.<sup>170</sup> Uninfected slugs treated with apomorphine, which stimulates dopamine receptors, failed to avoid *P. hermaphrodita*, and infected slugs treated with a dopamine antagonist (haloperidol) no longer moved towards *P. hermaphrodita*.<sup>171</sup> This suggests that *P. hermaphrodita* is somehow able to influence levels of biogenic amines to alter slug behaviour.<sup>170,171</sup>

As well as the ability to alter attraction or avoidance behaviour in slugs, *P. hermaphrodita* has been reported to have caused other extreme effects on slug behaviour. For example, infected slugs eat less,<sup>27</sup> are slower,<sup>172</sup> are more likely to be found under refuge traps<sup>50</sup> and move underground to die,<sup>173</sup> and infected freshwater snails are more likely to be found outside the water.<sup>151</sup> Not only does *P. hermaphrodita* influence host behaviour, but also it has been suggested they exhibit an anti-feeding effect on scavenging beetles (*Carabus nemoralis* and *Pterostichus melanarius*) by

detering them from feeding on dead, infected slugs where the nematodes are reproducing.<sup>174</sup> Whether the nematode is actively manipulating the behaviour of the slugs or this is a by-product of infection of sick slugs warrants further investigation.

### 2.2.1 The effect of *P. hermaphrodita* on nontarget organisms

Concern has been raised about the use of *Phasmarhabditis* species on nontarget organisms,<sup>175</sup> particularly native snail populations, yet there has not been one observation of these nematodes significantly affecting the health or populations of nontarget slugs or snails in 30 years of use across northern Europe. Also, there has been unease about the potential spread of *M. osloensis* (an opportunistic human pathogen) used to grow *P. hermaphrodita*, yet the bacterium these nematodes are reared on is not *M. osloensis* but a species closely related to *P. faecalis*,<sup>135</sup> which poses no threat to humans, so the level of risk to nontarget organisms associated with the use of the these nematodes remains low. Nevertheless, the commercial strain of *P. hermaphrodita* has been tested against nontarget beneficial invertebrates. As expected for a parasite of gastropods, *P. hermaphrodita* has been shown not to harm several insect species including *Tenebrio molitor*,<sup>176</sup> *G. mellonella* or *Pterostichus melanarius*.<sup>33</sup> The earthworms *L. terrestris*, *Eisenia fetida*, *E. hortensis*, *E. fetida*, *E. andrei* and *Dendrodrilus rubidus* also are unaffected by the nematode as well as the platyhelminth *Arthurdendyus triangulatus*.<sup>177–179</sup> A *Phasmarhabditis*-like nematode that potentially killed earthworms (e.g. *L. terrestris*) has been reported<sup>100</sup> but there has been no subsequent research. This nematode was only identified morphologically and causing earthworm mortality would be highly unusual for a gastropod parasitic nematode. Another *Phasmarhabditis* species (*P. californica*) also has been exposed to earthworms (*L. terrestris* and *E. fetida*), as well as the insect larvae *T. molitor* and *G. mellonella*, with no mortality of any species tested observed.<sup>180</sup>

The effect of *P. hermaphrodita* on nontarget gastropods also has been investigated in the field. From seven snail species commonly found in hedgerows, *P. hermaphrodita* caused mortality to just two (*M. cantiana* and *C. hortensis*).<sup>147</sup> Also, over a 2-year field trial<sup>181</sup> there was no effect of *P. hermaphrodita* on the snail species *Ponentina ponentina* and *Oxychilus helveticus*, or on acarids, collembolans or earthworm populations. Therefore, the effect of *P. hermaphrodita* on nontarget organisms is limited in Europe but there are no data on nontarget effects in other parts of the world where *Phasmarhabditis* species have been isolated for example, South Africa, New Zealand, USA and Canada.

## 2.3 Production of *P. hermaphrodita*

Consistent and efficacious pest control as well as low cost, storage, delivery, handling and marketing are required for any bio-control product (including nematodes) to become commercial.<sup>182</sup> *Phasmarhabditis hermaphrodita* has successfully been in production since 1994 by MicroBio, which was bought by Becker Underwood and then by BASF Agricultural Specialities. *Phasmarhabditis hermaphrodita* is grown in *in vitro* liquid culture with a bacterium closely related to *P. faecalis*<sup>48,49</sup> with upwards of 100 000 dauers mL<sup>-1</sup> being produced.<sup>183</sup> Monoxenic liquid culture of nematodes for mass production allows for more predictable and high virulent yields.<sup>48,49,184</sup> After monoxenic fermentation, dauers are harvested and the most effective dauer recovery methodology is using a combination of continuous phase density and flotation by adjustment.<sup>185</sup> The same authors also found that the introduction of an air supply to break apart and clear insoluble spent media was recommended. To separate

dauers and other life stages, the product can be sieved at an aperture size of 75–106  $\mu\text{L}$ <sup>186</sup> or by using vibrating membrane filtration.<sup>187</sup> Centrifugation and repeated washing also can be used.<sup>35</sup> After extraction, dauer juveniles are mixed with an inert gel polymer and packaged.<sup>34</sup>

### 2.3.1 Field use and application of *P. hermaphrodita*

*Phasmarhabditis hermaphrodita* is formulated into a water-dispersible formulation that can be suspended in water and applied to soil at a rate of  $3 \times 10^9$  dauer juveniles  $\text{ha}^{-1}$ ,<sup>35</sup> via spraying equipment<sup>183</sup> and irrigation lines.<sup>188</sup> As well as being applied to the soil surface, *P. hermaphrodita* can be incorporated into soil through cultivation to kill subterranean slugs though this has mixed results in terms of efficacy at reducing slug damage and slug numbers.<sup>189</sup> *Phasmarhabditis hermaphrodita* has been used to successfully control slug damage in an array of plants including lettuce,<sup>51,190</sup> winter wheat,<sup>50,189</sup> oilseed rape,<sup>191,192</sup> cabbage,<sup>192,193</sup> asparagus,<sup>194</sup> Brussels sprouts,<sup>195</sup> glasshouse orchids<sup>141</sup> and sugar beet.<sup>196</sup>

In general, there have been few field trials using *P. hermaphrodita* since 2009 but many before (see the complete list of field trials and results<sup>35</sup>). It has been largely successful at controlling slugs, yet there are reports of failures. For example, *P. hermaphrodita* was unable to reduce slug damage<sup>51</sup> or slug numbers<sup>181,197</sup> in some field trials. The most likely reasons for the lack of slug control are exposure to abiotic (e.g. UV light, desiccation, temperature) and biotic (e.g. predators) factors that the nematodes face once they have been applied or the presence of nonsusceptible slug species.

Other factors which may influence the efficacy of *P. hermaphrodita*, such as watering regime and earthworm activity, were investigated in comparison to chemical controls.<sup>198</sup> No effect on slug feeding or mortality was observed, but this could be a result of the presence of the slug *A. vulgaris*, which is known to be resistant to *P. hermaphrodita*.<sup>143,145</sup> It has, however, been suggested that failures could be avoided by following recommended protocols.<sup>39</sup>

The effect of treatments of crops before nematode application also has been investigated. When manure was applied before *P. hermaphrodita* dauer juveniles, the nematodes were rendered ineffective, possibly as a result of poor dauer survival, manure interfering with chemoreception or the manure attracting more slugs.<sup>181</sup> By contrast, there was no effect of cover crops or lupin on the ability of nematodes to control slugs in the next crop planted.<sup>199,200</sup>

Novel application strategies that improve efficiency and economic use of nematode biological control products will improve their attractiveness,<sup>201</sup> which have been investigated with *P. hermaphrodita*. For example, the most efficient control method of slugs in sugar beet utilised nematode application and methiocarb pellets in furrow treatment,<sup>202</sup> however, it has been found that methiocarb can reduce nematode survival but not infectivity.<sup>203</sup> In spite of this, there is limited scope for this combination as methiocarb affects nontarget organisms such as birds and has been banned in the UK and Europe.<sup>6</sup>

Multiple lower rate applications of *P. hermaphrodita* can sometimes offer better control,<sup>196</sup> or the same level of control as standard recommended broadcast rates,<sup>194,195,197,204</sup> but they require more time to achieve a reduction in slug damage.<sup>205–207</sup> Lower application rates and concentration could be beneficial for larger areas of crop, as *P. hermaphrodita* can be applied via irrigation lines,<sup>208</sup> instead of broadcast application. Nematodes also have

been applied in bands but offered no economic advantage over recommended broadcast application at the standard rate, possibly as a result of too few nematodes being applied.<sup>205,206</sup> Other application strategies such as dipping root plugs in a nematode/carboxymethyl cellulose solution also have been found to be successful, thereby providing protection against slugs using a lower number of nematodes and reducing the cost.<sup>197,209</sup> More targeted application methods have been proposed<sup>208</sup> including nematode application machinery (Wroot water Nemaslug xtra applicator) which injects nematodes onto irrigation water and aerates and agitates the nematode solution, allowing nematodes to be applied over a longer timescale. In plots of hostas, targeted application of *P. hermaphrodita* to slug shelters at a reduced application rate provided similar protection to that of uniform broadcast application.<sup>204</sup> Likewise, damage to oilseed rape by *A. lusitanicus* was reduced for 25 days by spraying *P. hermaphrodita* on the plants at a rate of  $2 \times 10$  nematodes  $\text{cm}^{-2}$ ,<sup>210</sup> rather than a broadcast spray. In order to optimise the numbers of *P. hermaphrodita* used for slug control several models have been developed.<sup>206,211–213</sup>

### 2.3.2 Persistence and environmental factors affecting the success of *P. hermaphrodita* in the field

In order for *P. hermaphrodita* to be successfully used as a biological control agent, it must persist in soil after application, but there is little research on this. Soil type can affect the movement of *P. hermaphrodita*<sup>42,107</sup> and its persistence has been monitored using real time qPCR techniques<sup>42</sup> showing that the *P. hermaphrodita* population declines sharply after 2 weeks.<sup>214</sup> However, in other studies survival of *P. hermaphrodita* has been recorded up to 5 months in wet sand, and even 8 months in garden soil and organic horticultural substrate.<sup>215</sup> In field trials *P. hermaphrodita* can survive up to 6 weeks in soil<sup>209</sup> and even up to 99 days.<sup>199</sup> Under laboratory conditions, the survival of *P. hermaphrodita* was best at 5, 10 and 15 °C, and osmotic desiccation in 10% glycerol could increase survival of the nematodes at temperature extremes.<sup>137</sup>

Unfavourable abiotic and biotic conditions including UV light, temperature and desiccation affect nematode survival and persistence.<sup>216</sup> This can be reduced by cultivating the land immediately after nematode application.<sup>189,205</sup> As well as abiotic factors, nematodes are killed by mites, collembolans and fungi.<sup>216,217</sup> DNA analysis has shown that mites and collembola including *Heteromurus nitidus* devour *P. hermaphrodita* under laboratory conditions and in the field,<sup>218–220</sup> and fungi have been speculated to affect the survival of these nematodes.<sup>215</sup>

With temperatures increasing in parts of the world due to climate change, the efficacy of *P. hermaphrodita* in controlling slugs may be affected; in particular slug-feeding was not reduced in infected slugs as temperatures increased from 14 °C to 24 °C.<sup>221</sup> It is thought that *P. hermaphrodita* is well adjusted to the cooler climate of northern Europe,<sup>222</sup> yet *P. hermaphrodita* could be used to reduce slug damage in warmer conditions in Spain, where the mean air temperature was  $19.8 \pm 2.6$  °C.<sup>207</sup> The impact of temperature on the efficacy of *P. hermaphrodita* also was investigated through field trials using predicted winter warming conditions.<sup>223</sup> They found that damage to plants and slug survival was much lower in the predicted wintering conditions than under normal wintering conditions. Therefore, *P. hermaphrodita* may perform better at controlling slug damage under winter warming conditions.<sup>223</sup>

### 2.3.3 Combining chemical and biological control methods with *P. hermaphrodita*

There is evidence to show that *P. hermaphrodita* combined with other methods could enhance slug control. In 2007 the efficacy of combining *P. hermaphrodita* infection with cadmium and *Bacillus thuringiensis* (BT) in the snail *C. aspersum* was investigated.<sup>224</sup> The growth rate of *C. aspersum* was reduced by both BT and cadmium and increasing doses of *P. hermaphrodita*.<sup>224</sup>

The repellent effect of Birch tar oil (BTO) has been examined and suggested for possible complementary use with *P. hermaphrodita* to control *A. arbustorum* and *A. vulgaris*.<sup>225</sup> The authors found that BTO repels *A. arbustorum* and *A. vulgaris* in confined heavily nematode-infested areas, and that repeated application of BTO over several weeks was required to deter *A. lusitanicus* with weekly treatments offering the best slug control.

Other more novel strategies have been investigated. *Phasmarhabditis hermaphrodita* has been used in combination with wasp venom from *Pimpla hypochondriaca* to kill and inhibit feeding of *D. reticulatum*.<sup>226</sup> The authors concluded that together with *P. hermaphrodita* the venom can be more effective than *P. hermaphrodita* on its own and is more successful at causing slug fatality and significantly reducing slug-feeding. One of the suggested strategies for future studies is to genetically engineer *P. hermaphrodita* to express individual venom factors<sup>226</sup> for slug control.

More recently the behaviour and feeding of *Tetanocera elata* fly larval, (a parasitoid and predator of slugs) and its potential for use with *P. hermaphrodita* have been explored.<sup>227</sup> The results demonstrate that *T. elata* larvae suffer in development and pupariation if feeding from an infected slug with only 20% pupating. Oddly, however, the larvae did show a preference for slugs previously infected with *P. hermaphrodita*. Ultimately further work is needed to examine if they can provide a consistently efficient synergistic level of slug control.

## 3 FUTURE DIRECTIONS AND CONCLUSIONS

Over the 30 years since *P. hermaphrodita* was first developed as a biological control agent, interest in this nematode has slowly increased as chemical usage is being reduced. However, compared to other nematodes used in biological control such as EPNs, the number of researchers investigating *P. hermaphrodita* is low<sup>133</sup> and subsequently, there are still many unanswered questions about the use and basic biology of *P. hermaphrodita*. Here we outline several research avenues which we think could improve the use of *P. hermaphrodita*: an appreciation of co-evolution between host and parasite; genetic improvement and genomic understanding of *P. hermaphrodita* and other *Phasmarhabditis* species; and investigating new application strategies of *P. hermaphrodita* in the field.

### 3.1 The importance of understanding the co-evolution between host and parasite

Nematodes and slugs have been co-evolving in an arms race for 540 Ma.<sup>14</sup> The geographical mosaic theory of co-evolution predicts genetic variation in the ability of hosts to combat parasites as well as pathogenicity of parasites.<sup>228</sup> There is little information on natural variation in pathogenicity of *P. hermaphrodita* strains, with only one study<sup>138</sup> recently demonstrating several wild strains of *P. hermaphrodita* that were more virulent than the commercial strain DMG0001 to *D. invadens* and other strains poor at killing slugs. Also there is no information on whether local and global

populations of specific slug species differ in their susceptibility to the nematode. It seems highly likely that there would be genetic variation in both host defence and pathogenic potential of the parasite, which has been observed in other animals. For example, there is considerable variation in the resistance of the fruit fly *Drosophila melanogaster* to the fungal pathogen *Entomophthora muscae*<sup>229</sup> and in wild populations of *Daphnia magna* exposed to the bacterial pathogen *Pasteuria ramosa*.<sup>230</sup> For *Phasmarhabditis* nematodes this has only been investigated at the interspecies level (see the 'Susceptibility of terrestrial gastropods to *P. hermaphrodita*' section and Table 1), where species such as *A. ater* are resistant, and *D. invadens* and *D. reticulatum* are highly susceptible.<sup>33,106,110</sup> There are limited data on host susceptibility to *P. hermaphrodita* at the intraspecies level. The only evidence comes from two studies focused on the snail *C. hortensis* where a population from Bristol, UK was found to be resistant to *P. hermaphrodita*,<sup>147</sup> yet *C. hortensis* from Aberdeen, UK were susceptible to the nematode.<sup>110</sup> This has important ramifications for gastropod control. If different populations have evolved resistance to *P. hermaphrodita* then application of the current strain (DMG0001) for control of resistant populations will be futile. Therefore, we propose that mechanistic understanding of how different populations of slugs and snails overcome parasitism and infection by *P. hermaphrodita* would be beneficial. Furthermore, as well as examining the pathogenic potential of wild *P. hermaphrodita* strains, variation in beneficial traits also should be examined. This approach is commonly used in EPN research; for example, wild strains of *Steinernema* and *Heterorhabditis* have been isolated and screened for superior virulence,<sup>231</sup> host finding and stress tolerance for example, heat, desiccation<sup>232</sup> and longevity<sup>233</sup> (to name but a few traits). This approach has never been utilised for *P. hermaphrodita*, however, as researchers tend not to keep their wild isolated strains in culture. Therefore, natural variation of different traits has not been investigated in great detail for *P. hermaphrodita* apart from tolerance to extreme pHs and temperature,<sup>40</sup> as well as chemotactic response to slug and snail mucus and hyaluronic acid.<sup>122–124</sup>

### 3.2 Genetic tools and genomic sequencing of parasitic nematodes

Coupled with the isolation of wild strains, the development of genetic techniques could enhance the efficacy of *P. hermaphrodita* in the field. This also is inspired by approaches used in EPN research. There have been numerous successful examples of selection of different advantageous traits using EPNs for example, high responsiveness to foraging cues,<sup>234</sup> heat tolerance and low-temperature activity,<sup>235</sup> which could potentially increase their viability as biological control agents. Other techniques such as inbreeding, hybridisation and mutagenesis have been employed to improve oxidative stress tolerance and longevity in *H. bacteriophora*.<sup>236,237</sup>—methods that also could be employed for *P. hermaphrodita*. More sophisticated genetic techniques have been shown to work in EPNs, such as RNAi in *S. carpocapsae*<sup>238</sup> and *H. bacteriophora*,<sup>239</sup> and even transgenic techniques in *H. bacteriophora*.<sup>240</sup> Although *P. hermaphrodita* has been proposed as a model nematode to understand the genetic mechanisms of parasitism,<sup>241–244</sup> development of techniques for genetic manipulation are in their infancy.<sup>40</sup> With the subsequent sequencing of the genome ongoing (Sheehy, Rae, unpublished data), the unravelling of the genetic blueprint of *P. hermaphrodita* may aid in the development of molecular tools. As seen with *C. elegans* and parasitic helminths, genomic investigations can lead to valuable



insights regarding the evolution of these organisms<sup>245–247</sup> as well as the development of beneficial online resources such as WormBase and WormBase ParaSite. The availability of genomic data would enable the identification of key genes such as those for pathogenicity, dauer formation, longevity and chemoattraction as well as their manipulation, which could lead to improvements in the use of *P. hermaphrodita* as a biological control agent. In terms of genomics, research on EPNs is well ahead of *P. hermaphrodita* with the genomes and transcriptomes of several *Steinernema* species including *S. carpocapsae*, *S. scapterisci*, *S. monticolum*, *S. feltiae* and *S. glaseri* already sequenced<sup>248</sup> as well as *Heterorhabditis bacteriophora*<sup>249</sup> and their bacterial symbionts *Xenorhabdus* and *Photorhabdus*.<sup>250</sup>

### 3.2.1 Novel application strategies of *P. hermaphrodita*

Novel application strategies can reduce the cost of using nematodes and increase attractiveness to the consumer.<sup>201</sup> Instead of standard broadcast spraying, these techniques include dipping roots of plants into adhesive mixtures containing nematodes, using lower, more frequent applications of nematodes as well as applying infected cadavers or applying nematodes to slow-release bags. Some of the techniques have been shown to work well in field trials for example, mixing *P. hermaphrodita* with carboxymethylcellulose to adhere to root plugs and smaller more frequent doses of nematodes to control slug damage in Chinese cabbage.<sup>197</sup> However, methods such as using already infected hosts, gels and slow-release tea bags have not received commercial or research attention using *P. hermaphrodita*. Another promising method is encapsulating nematodes in alginate beads providing a more targeted approach, which has been shown to work with EPNs to control *Diabrotica balteata* larvae.<sup>251</sup> These methods also could be combined with others to allow synergistic slug control for example, using essential oils, such as clove bud oil that kills snail eggs,<sup>252</sup> and spearmint and thyme oil that kill slugs<sup>253</sup> (*P. hermaphrodita* is unaffected by several essential oils that kill gastropods<sup>254</sup>), or combining with other biocontrol agents such as the fly *T. elata*.<sup>227</sup>

## 4 CONCLUSION

With the discovery of *Phasmarhabditis* nematodes from slugs and snails in many countries across the world,<sup>35</sup> including North America<sup>65</sup> there is ample opportunity for expansion of the Nemaslug® product across the globe. Ultimately, we hope by focussing on the approaches that we have suggested previously, *P. hermaphrodita* (and other *Phasmarhabditis* species) could be developed and used as successful biological control agents of slugs for the next 30 years. In fact, at the time of writing BASF have announced that a new *Phasmarhabditis* product (Nemaslug 2.0®) will be launched for use in gardens in spring 2023 containing not *P. hermaphrodita* but *P. californica*, owing to its pathogenicity towards slugs,<sup>135</sup> snails<sup>156</sup> and its lack of effect on nontarget organisms.<sup>180</sup>

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## DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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