

# Predation by carabid beetles (Coleoptera, Carabidae) on the invasive Iberian slug *Arion lusitanicus*

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**Photographs front page:** one adult *Arion lusitanicus* (*sensu* Altena 1955) taken by Bjørn Arild Hatteland (top-left), one juvenile *A. lusitanicus* taken by Arild Andersen (top-right), eggs of *A. lusitanicus* taken by Solveig Haukeland (central picture), the carabid *Pterostichus niger* (bottom-left) and *Carabus nemoralis* (bottom-right) taken by Bjørn Arild Hatteland.

## **PREFACE**

This thesis is part of the fulfilment for the degree philosophiae doctor (PhD) and consists of a synthesis and four individual papers. The study was mainly funded by the Department of Biology, University of Bergen with additional funding by the Norwegian Research Council (project nr. 900233 and 803194). Most of the work was carried out at the Department of Biology, University of Bergen. However, some of the laboratory work was also performed at the School of Biosciences at Cardiff University as well as at the School of Biological Sciences, Aberdeen University.

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## ABSTRACT

Predator-prey and host-parasite interactions are essential in understanding the population dynamics of any species, including pest species. In fact, integrated pest management that incorporates biological control requires a thorough understanding of the ecology of the pest and their interactions with natural enemies. The Iberian slug *Arion lusitanicus* has been spread from southern Europe to many parts of northern Europe, and has become a pest species in gardens, horticulture and agriculture. One of the explanations or hypotheses for the success of this introduced species has been that it lacks natural enemies. In the present study this hypothesis was tested by investigating selected predatory beetles that are known to feed on gastropods; four carabid beetles and one staphylinid beetle. However, in order to differentiate *A. lusitanicus* in Norway from the closely related native species, such as *A. ater* and potential hybrids, its taxonomic status had first to be resolved. A combination of morphological and DNA-based methods were applied, which revealed that *A. lusitanicus* in Norway is a pest form, which is clearly separated from *A. ater* based on morphological characters. However, the mitochondrial DNA of some *A. ater* showed *A. lusitanicus* haplotypes, suggesting there may have been introgression. Further, many of the *A. ater* specimens tested showed clear hybridisation with *A. rufus* confirming the complexity of the large arionids. Using those taxonomic/systematic characters which reliably differentiate these species a diagnostic DNA-based method employing a multiplex PCR was developed. This was optimized further to allow detection of slug-DNA in predators such as carabid beetles. The abilities of the predatory beetles to kill and feed on eggs of *A. lusitanicus* as well as newly hatched slugs and juveniles were tested in the laboratory, where the carabids *Pterostichus niger*, *P. melanarius*, *Carabus nemoralis* and the staphylinid *Staphylinus erythropterus* all fed on eggs and newly hatched slugs, while the carabid *Nebria brevicollis* hardly consumed any eggs or slugs. In addition, *C. nemoralis* was able to consume juvenile slugs up to one gram fresh weight. This carabid beetle is very common and active in spring in northern Europe when *A. lusitanicus* is highly abundant as juveniles, and predation was found to be significant in meadows and strawberry patches until the slugs grew larger (2-10g fresh weight) in the beginning of summer. Furthermore, *C. nemoralis* showed no preference for any specific slug species either in the laboratory testing *D. reticulatum* versus *A. lusitanicus*, nor in the field as long as the slugs were less than one gram. Instead *C. nemoralis* switched to the most abundant prey, which in most cases was *A. lusitanicus*. Thus, this beetle seems to be an opportunistic slug predator feeding on the most abundant slug species present at a given time.

The nematode *Phasmarhabditis hermaphrodita* containing the bacteria symbiont *Moraxella osloensis* is being used as a biological control agent against slugs including *A. lusitanicus*, but the effect in the field is often short-lasting. Intraguild predation has been proposed as being part of the explanation for this, where predators may feed directly on nematodes or indirectly by consuming infected slugs. Hence a field experiment testing this latter part of the explanation was carried out. The non-pest slug *Arion silvaticus* and *D. reticulatum* were strongly affected by the applied nematodes, while *A. lusitanicus* was only moderately affected. Intraguild predation on nematode-infected slugs was low in the field compared to predation on uninfected slugs as indicated by 22% of the beetles being positive for slug-DNA while only 2% were positive for nematode-DNA. Thus carabid beetles are probably not a significant reason why the nematodes disappear from the field shortly after application. *Carabus nemoralis* may be used in conservation biological control of *A. lusitanicus* by facilitating set-asides and the cropped field for sustaining this species and other carabids in the area. Future studies should focus on manipulation experiments to find what densities of beetles are required to achieve a significant impact on slug populations, and how practicable this might be in commercial agriculture.

**Keywords:** Arionidae, *Carabus nemoralis*, *Deroceras reticulatum*, molecular methods, predator-prey interactions

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A handwritten signature in blue ink, reading "Bjørn A. Hatteland". The signature is written in a cursive style with a large initial 'B'.

Bergen, 15 April 2010



## LIST OF PAPERS

**1. Hatteland, B. A., Noble, L. R., Schander, C., Skage, M. & T. Solhøy.** Differentiation of the invasive *Arion lusitanicus* and the native *Arion ater* (Pulmonata, Arionidae) in Norway using morphology and genetics.

*Zoologica Scripta* (submitted)

**2. Hatteland, B. A., Grutle, K., Mong, C. E., Skartveit, J., Symondson, W. O. C. & T. Solhøy. (2010)** Predation by beetles (Carabidae, Staphylinidae) on eggs and juveniles of the Iberian slug *Arion lusitanicus* in the laboratory.

*Bulletin of Entomological Research* **100**, doi:10.1017/S0007485309990629

**3. Hatteland, B. A., King, R. A., Symondson, W. O. C. & T. Solhøy.** Molecular analysis of predation by carabid beetles (Carabidae) on the invasive Iberian slug *Arion lusitanicus*.

*Bulletin of Entomological Research* (to be submitted)

**4. Hatteland, B. A., Haukeland, S., Roth, S., Brurberg, M. B. & W. O. C. Symondson.** Spatiotemporal dynamics of predation by carabid beetles (Carabidae) on nematode infected and uninfected slugs in the field.

*Molecular Ecology* (to be submitted)

The published paper (referred thorough the thesis as “paper II) is reprinted with the kind permission of the publisher Cambridge University press.

## DECLARATION

**Paper I** – Differentiation of the invasive *Arion lusitanicus* and the native *Arion ater* (Pulmonata, Arionidae) in Norway using morphology and genetics

Bjørn Arild Hatteland – fieldwork, morphological and genetic analyses, statistics, writing, editing

Leslie R. Noble – fieldwork, morphological analyses, statistics, writing, editing, supervision

Christoffer Schander – genetic analyses, writing, editing

Morten Skage – genetic analyses, editing

Torstein Solhøy – fieldwork, editing, supervision

**Paper II** – Predation by beetles (Carabidae, Staphylinidae) on eggs and juveniles of the Iberian slug *Arion lusitanicus* in the laboratory.

Bjørn Arild Hatteland – fieldwork, feeding experiments, statistics, writing, editing

Kari Grutle – fieldwork, feeding experiments

Christian E. Mong – statistics

John Skartveit – writing

William O. C. Symondson – editing, supervision

Torstein Solhøy – fieldwork, editing, supervision

**Paper III** – Molecular analysis of predation by carabid beetles (Carabidae) on the invasive Iberian slug *Arion lusitanicus*

Bjørn Arild Hatteland – fieldwork, feeding experiments, genetic analyses, statistics, writing, editing

R. Andrew King – genetic analyses, editing

William O. C. Symondson – feeding experiment design, editing, supervision

Torstein Solhøy – field design, supervision

**Paper IV** – Spatiotemporal dynamics of predation by carabid beetles (Carabidae) on nematode infected and uninfected slugs in the field

Bjørn Arild Hatteland – fieldwork, feeding experiments, genetic analyses, statistics, writing, editing

Solveig Haukeland – fieldwork, nematode analyses, writing, editing

Steffen Roth – fieldwork, editing

May Bente Brurberg – genetic analyses, writing, editing

William O. C. Symondson – feeding experiment design, editing, supervision

# SYNTHESIS

## 1. Introduction

### 1.1 Trophic interactions – setting the scene

Trophic interactions are fundamental to ecology and spatial as well as temporal dynamics are essential to any understanding of such interactions (Powell *et al.*, 1995; Abrams, 2000). Predator-prey interactions have traditionally been explained by theories such as optimal foraging theory, involving handling and searching time of predators (MacArthur & Pianka, 1966; Krebs, 1978). Frequency-dependent predation, where predators switch to the most common prey, is predicted from foraging theory (Allen, 1988) and may often stabilize predator-prey dynamics (Abrams & Matsuda, 2003). Predators may show functional or numerical responses to prey abundance by increased consumption rates with higher density of prey or aggregating in patches with higher prey densities, respectively. However, anti-predator defences that increase handling times may lead to prey preferences instead of frequency-dependent predation. Prey may also use different kinds of refugia to avoid being eaten. Refugia may simply mean hiding, but in a more abstract way it may also mean growing to a large size to avoid size selective predators, or finding refuge in high numbers (Abrams, 2000). However, many factors (e.g. nutrient values, learning) often make predator-prey interactions quite complex. In addition, multiple links between predators, prey and parasites may exist, and even intraguild predation can occur, where predators exhibit cannibalism, or feed on other predators or parasites which feed on or parasitize a common prey/host (Rosenheim *et al.*, 1995).

This study used molecular techniques to study predator-prey interactions between slugs and beetles. These were studied by investigating frequency-dependent predation versus prey preferences, at different spatial and temporal scales in the field and in laboratory experiments. In addition, nematodes considered to be slug parasites were used to study intraguild predation by the same beetle species. Such work has implications for biological control of pest slugs in northern Europe, in particular invasive slugs such as the Iberian slug *Arion lusitanicus* and the grey field slug *Deroceras reticulatum*.

## 1.2 Terrestrial gastropods – biology, pests, chemical and biological control

### *General biology*

Terrestrial gastropods are one of the most successful and diverse groups of the terrestrial fauna with an estimated 35 000 species (Barker, 2001, 2004). Some species have been used as food, medicine, tools, personal ornamentation and currency in trade, while others have more negative associations, being crop pests and parasite vectors (Barker, 2001). However, most species go unnoticed, being important detritivores in many ecosystems (Barker, 2001). Many species are also negatively affected by human impact, some are even red-listed (Sneli *et al.*, 2006). Alternatively, many species are highly adaptive to environmental changes such as habitat degradations due to human impact and often become abundant in such modified habitats. Some of these species have been spread by human activity and are important pest species of contemporary agriculture.

Terrestrial gastropods are commonly divided into snails and slugs, snails having a fully developed external shell while slugs have a reduced and often vestigial internal shell. However, this distinction is arbitrary, since slugs are polyphyletic having evolved many times from divergent snail groups showing parallel evolution (Barker, 2002). In Norway, currently 98 species of terrestrial gastropods have been recorded, of which 20 are slugs, two of which have been recorded recently; *Milax gagates* (Sneli *et al.*, 2006) and *Arion rufus* (Roth, S., Hatteland, B. A., Solhøy, T., in prep.). *Arion rufus* has also been recorded previously in the 19<sup>th</sup> Century (Økland, 1922). A total of at least eight slug species are thought to have been introduced, and a few of these are pests such as *D. reticulatum* and *A. lusitanicus*.

### *Gastropods as pests*

Gastropods are regarded among the most important agricultural pests, affecting agricultural and horticultural crops in many parts of the world including Europe (Port & Port, 1986; South, 1992; Glen *et al.*, 1994; Barker, 2002). In Western Europe crops such as sunflower, maize, cereals, and oilseed rape as well as cabbage and strawberries have been severely affected by gastropods (Speiser & Andermatt, 1996; Barker, 2002; Hofsvang, 2003; Kaluski *et al.*, 2005; Kozłowski, 2007). Their importance as crop pests has increased significantly during the past 30 years, which may partly be explained by the expansion of closed-canopy crops (e.g. oilseed rape), change from spring to autumn sowing, leaving crop residues and the application of reduced tillage regimes (Glen & Moens, 2002; Henderson & Triebkorn, 2002).

In addition, heavy use of molluscicides may have led to resistance problems as well as having side-effects on non-target organisms (Bailey, 2002). Hence, integrated pest management incorporating biological control, may prove invaluable in the long term. However, according to Barker (2002), molluscs have been given minor focus in pest control literature, although gastropods have increased as significant pests in agriculture due to changes in modern agriculture (e.g. intensification, human trade, new crops). Climate change leading to increased precipitation and milder winters in Western Europe may also be part of the explanation why gastropods are increasing as pests (Sternberg, 2000; Glen & Moens, 2002; Willis *et al.*, 2006).

The most noticeable introduction of an exotic gastropod species in Norway has been the Iberian slug *A. lusitanicus* (von Proschwitz & Winge, 1994), which has become a serious pest in horticulture and agriculture (Frank, 1998b; Speiser & Kistler, 2002; Hofsvang, 2003). However, little is known of why this species is a pest in Norway and the current study mainly explores the hypothesis that it lacks natural enemies.

#### *Chemical control of gastropods*

Molluscicidal baits are the most commonly used chemical control against gastropods. The main problem has been to induce gastropods to consume a lethal dose of molluscicide (Barker, 2002). The commercial products normally contain metaldehyde or carbamates; methiocarb (e.g. sold commercially as the product “Mesuro<sup>®</sup>”) and thiodicarb (e.g. sold as “Skipper<sup>®</sup>”), which can be applied in different ways (Speiser, 1997). The products have often been either too weak so gastropods do not ingest a lethal dose before they are sated, or too strong, which means they reject the baits immediately. Molluscicides cause collateral damage to other organisms, especially metaldehyde which is poisonous for mammals such as dogs and hedgehogs (Bailey, 2002). However, metaldehyde and methiocarb have been found to have little or no effect on soil arthropod pests (e.g. tipulid larvae and isopods), collembolans and earthworms (Bailey, 2002). Molluscicides may also have negative effects on natural enemies of gastropods, although no adverse effects from metaldehyde has been found on carabid beetles (Kennedy, 1990) which are common natural enemies of gastropods. In fact, metaldehyde may even have a positive effect in integrated pest management since the common carabid beetle *Pterostichus melanarius* prefer slugs exposed to sub-lethal dosages of metaldehyde (Langan *et al.*, 2001; Langan *et al.*, 2004). However, Buchs *et al.* (1989) did find a negative effect on the large carabid *Carabus granulatus*, and further studies are needed in other common species. Furthermore, methiocarb-based baits have been shown to be toxic to carabids at the time of application, although the longer-term effects are negligible as indicated

by population recoveries (Purvis, 1996). However, this is dependent on hinterland rich in carabids, which often do not exist within agricultural areas. Preferentially, molluscicidal baits should be used prior to a pest outbreak of gastropods, but forecasting such outbreaks is still difficult and molluscicides are commonly applied after the outbreak has occurred (Hommay, 2002). However, although molluscicides such as metaldehyde are less effective in rainy weather and have negative effects on other organisms, they are still considered the only effective and economically valid option for many farmers.

#### *Natural enemies and biological control*

One possible reason for increased abundance of gastropods in agricultural fields is suggested to be a reduction in the impact of their natural enemies (Glen and Moens, 2002). The invasive character of some pest slugs has been attributed to “natural enemy release” (von Proschwitz and Winge, 1994), although this hypothesis remains to be addressed adequately in this group. Natural enemy release has mainly been applied to invasive plants and has often been used uncritically based on a lower diversity of enemies in the introduced range compared with native areas (Colautti *et al.*, 2004; Liu & Stiling, 2006). Natural enemies of molluscs have been reviewed in Barker (2004) and consist of a range of different organisms like nematodes, snails, centipedes, dipterans and beetles. Nematodes are used commercially as biological control agents against slugs, being applied as the product “Nemaslug<sup>®</sup>”, which contains the nematode *Phasmarhabditis hermaphrodita* and its bacteria symbiont *Moraxella osloensis* (Wilson *et al.*, 1993; Glen *et al.*, 2000a; Glen *et al.*, 2000b; Iglesias & Speiser, 2001; Rae *et al.*, 2007). This agent was developed mainly as a product against the grey field slug *D. reticulatum* but has recently been tested against other slugs like the invasive Iberian slug *A. lusitanicus* (Grimm, 2002). A significant effect has been reported against newly hatched and small juveniles but is much less effective against larger juveniles (Speiser *et al.*, 2001; Grimm, 2002). Furthermore, Nemaslug is more effective in rain and is vulnerable to desiccation in dry weather (Hass *et al.*, 1999). One way of solving this problem might be to mix the soil after application, covering the nematodes with soil to avoid desiccation (Glen & Moens, 2002).

Beetles have been reviewed as natural enemies of molluscs by Symondson (2004), covering carabid beetles (ground beetles, Carabidae), rove beetles (Staphylinidae), silphids (Silphidae), lampyrids (Lampyridae) and drillids (Drillidae). The main bulk of the existing literature focuses on carabid beetles which represent one of the largest, most diverse and well-known beetle families worldwide. Nevertheless, for using carabids in biological control a

better understanding of the interactions between these beetles and their prey, including gastropods, is needed.

### **1.3 Carabid beetles as natural enemies of gastropods**

#### *General biology*

Carabid beetles have been one of the most popular insect groups to study due to their aesthetic appeal, ease of collecting and relative ease of identification (Holland, 2002b). Thus their biology and taxonomy is well known compared to that of other beetle groups, particular within Europe. In total, 32 561 species have been described covering 1859 genera (Lorenz, 1998), while the family has been estimated to include about 40 000 species (Luff, 2007), of which 2 700 and 269 are found in Europe and Norway, respectively (Ottesen, 1993; Wachmann *et al.*, 1995). However, in Norway only about 20 of these are common species in agricultural fields (Andersen, 1991). The first significant contributors to understanding their ecology, taxonomy and distribution were made by Carl Heinz Lindroth, who covered most of northern Europe as well as substantial parts of North America, but concentrated mainly on Fennoscandia (Lindroth, 1945a, b, 1949, 1985, 1986, 1992). Lindroth's work was followed by Thiele, who published a monograph in 1977 focused on ecology in natural and managed habitats. Den Boer (1990) has also been among the main contributors working largely on dispersal in heathlands. More recent reviews on agroecology in particular can be found in Lövei & Sunderland (1996), Kromp (1999) and Holland (2002a).

#### *Diets of carabids*

Carabid beetles are typically polyphagous predators, and their importance as predators in agricultural ecosystems as well as other ecosystems has often been the reason for studying them (Allen 1979). However, this general assumption has been criticized by Lindroth (1992) who pointed out the omnivorous character of many species. Furthermore, many species are also phytophagous and opportunistic (Lindroth, 1992; Hengeveld, 1980a; Allen, 1979). Lindroth (1992) considered only the genera *Calosoma* and *Cicindela* as true predators while the genera *Agonum*, *Bembidion* (partly), *Calathus*, *Carabus*, *Cychrus*, *Dyschirius*, *Elaphrus*, *Notiophilus* and *Pterostichus* (partly) consume mostly animal food. Nevertheless, food specializations do occur and have often been traced based on the morphology of mouth and head parts (Forsythe, 1983; Evans & Forsythe, 1985). Furthermore, larvae tend to be more



restricted in their food and are often more carnivorous than the adults (Lövei & Sunderland, 1996), as found for instance in larvae of *P. melanarius* (Thomas, 2002; Thomas *et al.*, 2009).

The diet of Carabidae has been reviewed in numerous books and papers (Thiele, 1977; Allen, 1979; Hengeveld, 1980a, b; Luff, 1987; Laroche, 1990; Lövei & Sunderland, 1996; Toft & Bilde, 2002). Thiele (1977) described three categories of feeding types while Hengeveld (1980a) only differentiated between specialists and generalists. More recently, Toft and Bilde (2002) suggested a number of feeding guilds to categorize the feeding mode of various species; generalist carnivores, generalist insectivores, mollusc specialists, microarthropod specialists, caterpillar specialists and granivores. Mollusc specialists have mainly been found in the tribe Cychrini (Tod, 1973; Digweed, 1993), which shows morphological adaptations to snail predation. In addition, other specialists such as *Notiophilus* have been studied (Ernsting & Mulder, 1981; Ernsting & Van der Werf, 1988), which are springtail specialists. In addition, supplementary feeding activity such as scavenging is important for many species (Toft & Bilde, 2002). Multiple questions can be addressed by studying the diets of carabids; their role in a given biological system can be defined, and how this relates to a specific prey species which is often a pest.

### *Slug predators*

Some large carabid beetles have been shown to feed readily on slugs (e.g. *Carabus* spp., *Abax parallelepipedus*, *P. melanarius*) with species in the tribe Cychrini regarded as predators of snails and slugs by many authors (e.g. Tod, 1973; Digweed, 1993; Laroche, 1990). However, *Cychrus caraboides* is the only Cychrini species found in Fennoscandia, and is uncommon in agricultural fields (Lindroth, 1985). Nonetheless, some *Carabus* (e.g. *C. violaceus*, *C. nemoralis*, *C. problematicus*) species have also been regarded as predominately mollusc predators (Scherney, 1959, 1961; Evans & Forsythe, 1985). Furthermore, chemoreceptors on the tips of the palps have been found to be associated with mucus trail detection in gastropod predators such as *Carabus* species (Symondson & Williams, 1997). Most studies on predation have focused only on adults as with much research on carabid beetles in general, but recently larvae have also been regarded as significant slug predators (Thomas, 2002; Thomas *et al.*, 2008). In total, 30 species of carabids are currently known to consume or predate slugs (Table 1).

A strong case for abundant generalist predators as potential biological control agents has been put forward by Symondson *et al.* (2002). Supplementary predation on slugs by such species may have a significant effect even without an aggregating response and low

individual predation rate. This is especially true when the pest species is in a vulnerable phase (e.g. juvenile). For example Lukasiewicz (1996) found that due to their much higher abundance generalists such as *Pterostichus nigrita*, *P. caerulescens* and *Staphylinus erythropterus* were equally as important in earthworm predation as the more specialized *C. granulatus*. Consequently, a guild of generalist predators may be more important in regulating pest numbers than a few specialist predators.

In the present work I focused mainly on the large carabid *Carabus nemoralis*, which is a common slug predator in man-made habitats of northern Europe (Lindroth, 1985). One could assume that this species includes slugs like *A. lusitanicus* in its diet, based on the fact that *Carabus* species are regarded as oligophagous feeders or at least as a predominant feeder on molluscs and earthworms. Nevertheless, neither field nor laboratory data exist to prove this assumption so far. The first authors to report slug predation by *C. nemoralis* was Glendenning (1952) and Tod (1973). The latter also showed that predation on gastropods was related to the size of carabid species. Furthermore, Tod (1973) found much higher proportions of gastropod remains (gastropod tissue detected with serological techniques) in large *C. nemoralis* and *C. violaceus* compared with other species of carabids. More recently, Ayre (1995) also found that *C. nemoralis* and *C. violaceus* as well as *C. caraboides* killed *D. reticulatum* slugs more effectively compared with the generalist species *P. niger* and *A. parallelepipedus*. The present study is the first to investigate the interaction between *C. nemoralis* and the invasive Iberian slug *A. lusitanicus*.

**Table 1:** Overview of carabid beetles found to feed on slugs.

Carabid species	Slug consumption in the field	Evidence for slug predation
<i>Abax parallelus</i>	slugs (5)	
<i>A. parallelepipedus</i> *	<i>Arion</i> sp. (11), slugs (7,3,6)	<i>D. reticulatum</i> L (19) F (22) slugs L (18)
<i>Amara aulica</i> *	slugs (3)	
<i>A. lunicollis</i> *	slugs (3)	
<i>A. similata</i> *	slugs (3)	
<i>Calosoma frigidum</i>		<i>A. ater</i> LF (17)
<i>Carabus granulatus</i> *	<i>A. lusitanicus</i> (14)	
<i>C. nemoralis</i> *	slugs (2)	<i>A. ater</i> (1)
<i>C. problematicus</i> *		slugs L (18)
<i>C. violaceus</i> *	slugs (3), <i>A. lusitanicus</i> (14)	<i>A. fasciatus</i> L (12), <i>D. reticulatum</i> L(12)
<i>C. violaceus</i> larvae*	<i>A. lusitanicus</i> (14)	
<i>Cychrus attenuatus</i>		slugs L (18)
<i>C. caraboides</i> *	slugs (3)	<i>A. fasciatus</i> L (12), <i>D. reticulatum</i> L (12)
<i>Cyclotrachelus alternans</i>		Limacidae L (20)
<i>Diplocheila striatopunctata</i>		<i>A. ater</i> LF (17)
<i>Harpalus aeneus</i>		<i>D. reticulatum</i> L (23)
<i>H. latus</i> *	slugs (3)	<i>D. reticulatum</i> L (23)
<i>H. rufipes</i> *	slugs (3,9)	
<i>Megadromus antarcticus</i>		<i>D. panormitanum</i> L (16), <i>D. reticulatum</i> L (16)
<i>Nebria brevicollis</i> *	slugs (3)	<i>D. reticulatum</i> L (23,24)
<i>Poecilus cupreus</i> *		<i>A. lusitanicus</i> eggs L (26); <i>A. lusitanicus</i> L (26)
<i>P. lucublandus</i>		Limacidae L (20)
<i>Pterostichus aethiops</i>	slugs (8)	
<i>P. madidus</i>	slugs (7,3)	<i>D. reticulatum</i> L (23,24)
<i>P. melanarius</i> *	slugs (4,3,8,9), <i>D. reticulatum</i> (25,27,28,29,30), slug eggs (10); <i>A. lusitanicus</i> (14)	<i>D. reticulatum</i> L (15,21), F (25), <i>A. lusitanicus</i> eggs L (26), <i>A. distinctus</i> L (28), <i>A. subfuscus</i> L (27), <i>L. marginata</i> L (27), <i>M. tenellus</i> L (27)
<i>P. melanarius</i> larvae*		<i>D. reticulatum</i> LF (31)
<i>P. niger</i> *	slugs (3,8)	<i>A. fasciatus</i> L (12), <i>D. reticulatum</i> L (12)
<i>P. nigrata</i> *	slugs (3)	
<i>P. oblongopunctatus</i> *	slugs (8)	
<i>Scaphinotus marginatus</i>		<i>D. reticulatum</i> L (13)
<i>Scarites anthracinus</i>		<i>D. reticulatum</i> L (32)

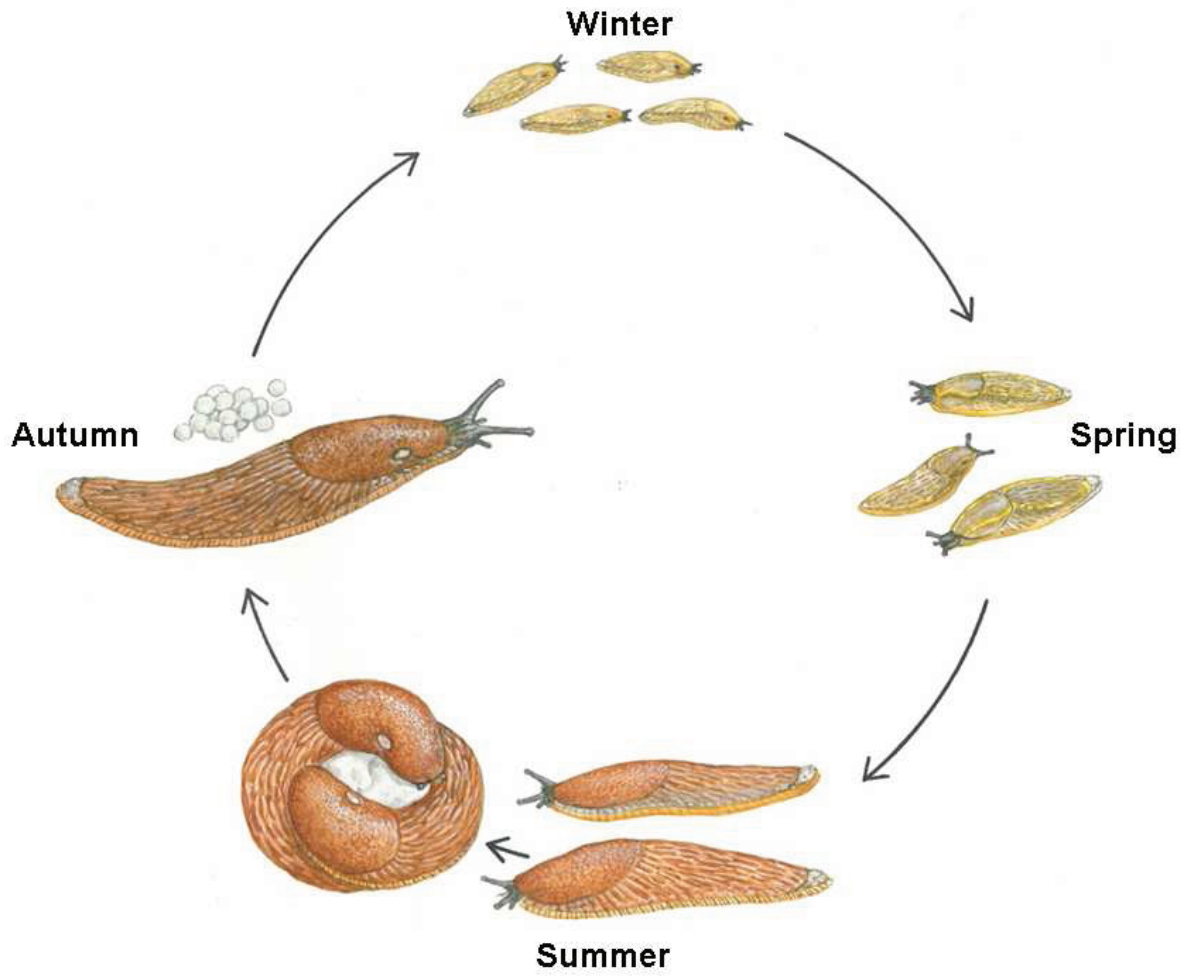
**Key to Table 1:** Table modified from Sunderland (2002). L = laboratory data, F = field data, \* = species recorded in Norway. 1: Glendenning (1952), 2: Tod (1973), 3: Ayre & Port (1996), 4: Symondson *et al.* (1996), 5: Loreau (1983b), 6: Loreau (1983a), 7: Symondson & Liddell (1993), 8: Sergeyeva & Gryuntal (1990), 9: Cornic (1973), 10: Pollet & Desender (1985), 11: Walrant & Loreau (1995), 12: Pakarinen (1994), 13: Digweed (1993), 14: Grimm *et al.* (2000b), 15: Symondson & Liddell (1996), 16: Chapman *et al.* (1997), 17: Poulin & O'Neil (1969), 18: Loreau (1984), 19: Symondson (1989), 20: Best & Beegle (1977), 21: McKemey *et al.* (2001), 22: Symondson (1993), 23: Ayre (2001), 24: Mair & Port (2002), 25: McKemey *et al.* (2003), 26: Oberholzer & Frank (2003), 27: Foltan (2004), 28: Dodd *et al.* (2005), 29: Foltan *et al.* (2005), 30: Read (2007), 31: Thomas *et al.* (2009), 32: Tulli *et al.* (2009).

## 1.4 The invasive Iberian slug *Arion lusitanicus*

The invasive form of *A. lusitanicus* (*sensu* Altena 1955 and also regarded as *A. vulgaris* Moquin-Tandon 1855 (Anderson, 2005)) probably originated on the Iberian Peninsula, hence it is referred to as the Iberian or Lusitanian slug, and has spread to many parts of Europe including Scandinavia during the last few decades (von Proschwitz, 1992; Dolmen & Winge, 1997). The Iberian slug was first recorded in Norway in 1988 (von Proschwitz & Winge, 1994) and *A. lusitanicus* has since become locally very abundant, in particular along the western coast. It has even been recorded north of the Polar Circle in Bodø (Solhøy, T., pers. obs.) and Finnsnes close to Tromsø (Roth, S., pers. obs.). Considerable damage caused by *A. lusitanicus* has been reported in gardens, vegetable, strawberry and grass fields (von Proschwitz & Winge, 1994; Dolmen & Winge, 1997; Grimm *et al.*, 2000a; von Proschwitz, 2008). In addition, it is possible that *A. lusitanicus* may have a negative effect on native slug species like *Arion ater* L.; the numbers of *A. ater* fall following invasion of an area by *A. lusitanicus* (Davies, 1987; von Proschwitz, 1997). It is currently unknown if this is due to competition, egg predation or other factors (e.g. introgression).

The pest nature of this slug has been explained by a high reproductive rate, catholic feeding habits, high survival rate and large body size (von Proschwitz, 1992; Kozłowski, 2007). In addition, it is hypothesised that *A. lusitanicus* lacks natural enemies, or at least has fewer enemies (von Proschwitz & Winge, 1994; von Proschwitz, 2008), perhaps due to its heavier, more sticky mucus secretion when disturbed or attacked (Solhøy, T., pers. comm.). According to published information and field observations (e.g. Kozłowski, 2007; Dirks *et al.*, in prep.) this species follows an annual cycle, although some individuals live for two years (Davies, 1987). Most of the adults die after egg laying in the autumn followed by hatching of eggs, overwintering as juveniles and maturation in the following spring and summer (Fig. 1). Thus, through most of the year the species is potentially prone to predation by carabid beetles.

**Figure 1:** Lifecycle and phenology of *Arion lusitanicus*. The figure has been modified from the drawing by H. Karlsen in Hofsvang *et al.* (2008).



## 2. Aims of this study

The main goal of this study was to investigate the potential role of carabid beetles as natural enemies of the invasive slug *Arion lusitanicus* in agricultural and rural areas. However, first it was necessary to make a taxonomic survey of the large arionid slugs of Norway, due to the complexity of different species, forms and potential hybrids. Furthermore, different species of beetles needed to be tested in the laboratory to know to what degree they were able to kill and consume *A. lusitanicus*. Based on these results the overall aim was to investigate predation on this introduced pest in the field. Assuming that some beetles are able to feed on *A. lusitanicus* in the field, to what degree do these beetles predate *A. lusitanicus* compared to other slugs? Another objective of this study was to analyze predation on slugs according to time of year and if beetles switch to the most abundant slug species according to density and size of slugs. Furthermore, I wanted to test the prediction that carabid beetles prefer other slug species due to the stickier mucus of *A. lusitanicus*. I also wanted to investigate potential indirect intraguild predation by carabid beetles on a slug-parasitic nematode (*Phasmarhabditis hermaphrodita*) when feeding on nematode-infected slugs. Lastly this study discusses the potential of using carabid beetles as agents of biological control.

This study aimed to address the following objectives:

1. Determine the taxonomic status of *Arion lusitanicus* in Norway, and determine how to separate this introduced pest species from the native and closely related *A. ater*, as well as potential hybrids of *A. ater* aggr. (*sensu stricto* Cain & Williamson 1958).
2. Investigate the ability of several beetle species to kill and consume egg and juvenile stages of *Arion lusitanicus* in the laboratory.
3. Develop a molecular detection method to analyse predation by carabid beetles on *Arion lusitanicus* in the field, while at the same time detecting predation on other common slugs.
4. Determine prey and size choice in some selected carabid beetles feeding on slugs both in the laboratory and in the field.
5. Investigate intraguild predation by carabid beetles on nematode-infected slugs in the field, following application of the slug-parasitic nematode *Phasmarhabditis hermaphrodita*.
6. Discuss the potential for biological control of *Arion lusitanicus* using carabid beetles.

### **3. Material and methods**

#### **3.1 Pitfall trapping of carabid beetles**

Carabid beetles as well as other ground active arthropods have mainly been sampled by pitfall traps, first reported by Barber (1931). The main reason for this is the convenience of using these traps as they are rather inexpensive, labour-efficient, and yield high numbers of many species, making data from this approach suitable for rigorous statistical analyses (Spence & Niemelä, 1994). Furthermore, most carabids are ground and night active and either lack flying abilities (brachypterous) or have limited flying ability (macropterous). Thus pitfall traps are an effective means of catching many individuals of those species occurring in a particular habitat. However, pitfall catches are influenced by a range of different factors which complicates data interpretation. Firstly, pitfall traps obviously measure activity; hence many authors refer to “relative-density” or “activity-density” when interpreting results. Thus, species catches are very much a function of density, activity and trapability (Thiele, 1977; Adis, 1979; Luff, 1982, 2002; Halsall & Wratten 1988). Generally, larger species are more mobile and therefore get more easily caught compared with smaller ones (Luff, 2002). In addition, catch numbers are influenced by abiotic factors such as temperature and moisture (Ericson, 1979; Honek, 1988, 1997) as well as biotic factors like surrounding vegetation (Greenslade, 1964) and hunger levels (Wallin & Ekbohm, 1994). Species assemblages may therefore be difficult to compare between different habitats or areas. Furthermore, the type and size of pitfall traps, preservative and arrangement of traps are important (Luff, 1975; Adis, 1979).

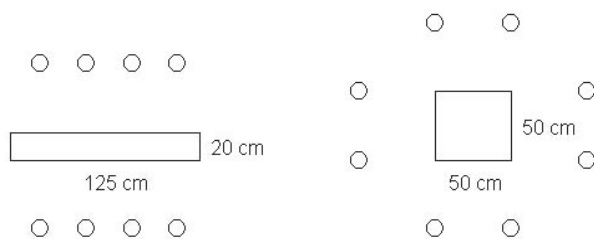
Fortunately, various evaluations of pitfall trapping have been carried out and recommendations about additional sampling techniques, as well as various pitfall trap design and sampling intensity with or without enclosures and fences have been given (Luff, 1975; Baars, 1979; Desender & Maelfait, 1986; Luff, 1986; Niemelä *et al.*, 1986; Niemelä *et al.*, 1990; Spence & Niemelä, 1994; Mommertz *et al.*, 1996). As with all sampling in general, the design of pitfall trapping should be decided in light of the working hypotheses/questions. Integral to the questions we intend to address are whether a spatial or temporal sampling design should be used, since the ideal combination is often too laborious and expensive to implement. For instance, if the aim is to cover phenologies of all species, one needs to sample continuously throughout the year, or at least from spring to autumn.

In the present study I aimed for beetle responses to slug densities and therefore used high numbers of traps (88-375 per site) to achieve spatial resolution, as well as a sufficient sample size for predation analyses (see design Fig. 2).

In general, 5-10 small (6-8 cm diameter) plastic pots should be spaced out not less than 2 m apart according to Luff (1996). The pitfall traps used in the present study were dry plastic pots of 6.5 cm in diameter and 9.5 cm in depth (Fig. 3), which have been found to be effective in catching large carabids (Luff, 1975). A metal cover of 11x11 cm was placed approximately 3-4 cm above each trap.

The frequency of emptying the traps is also an important issue, since the traps may become filled with debris or disturbed in other ways, which often means less effective sampling. The spacing is important to decrease the effect of local depletion (Digweed *et al.*, 1995), and catch should be pooled for each set of traps to minimize the effect of any small-scale local heterogeneity in trap efficiency (Luff, 1986). This kind of design should be sufficient for catching the most common species, but if spatial responses to, for example, prey are the aims of the study one clearly needs many more traps.

**Figure 2:** Sampling design for the strawberry field site (left) and meadow site (right). Circles represent pitfall traps and squares quadrat sampling for molluscs.



**Figure 3:** Pitfall traps with covers removed at the strawberry field site at Askøy, outside Bergen.





### 3.2 Density estimates of slugs

The density of slugs can be measured by various methods (Bishop, 1977; Archard *et al.*, 2004), such as searching fixed areas, soil washing, mark-recapture and with baited traps. The latter method measures relative densities and is highly activity-dependent and biased in favour of species or stages in the respective species lifecycles that are attracted to the traps (Archard *et al.*, 2004). Thus baited traps make it hard to compare densities of different species. Attempts have been made to estimate slug populations using mark-recapture techniques (Richter, 1976; Grimm, 1996), but these have major disadvantages in that small slugs cannot be marked easily by transponders and the method is quite laborious. In addition, some of the marking methods used are likely to influence the survival of the marked individuals (Hogan & Steel, 1986). However, recent work using UV-dye on *A. lusitanicus* and *D. reticulatum* has shown promise, being cheaper and less detrimental to slug survival (Foltan & Konvicka, 2008).

Soil washing and flooding techniques yield absolute densities, having the desirable property of including slugs lying below the soil surface down to the depth sampled (Bishop, 1977; Glen *et al.*, 1992). However, this technique is very laborious (Crawford-Sidebotham, 1972) and disturbs the study area to a large extent because soil and vegetation are removed. The latter consideration is important when numerous repeated samples are needed from a rather small area as in this study. However, this method is probably more accurate but still underestimates small specimens. Quadrat searches of randomly or regularly selected, fixed areas give straightforward estimates of density, but care must be taken to locate all slugs within the sampled area, and a certain underestimate for smaller specimens in particular is likely (Frank, 1998a). However, Grimm (2001) did not find any significant difference between hand searching and the other methods used; refuge traps and mark-release-recapture. Besides, I believe this to be a minor problem in the areas I have investigated in western Norway, since the youngest slugs which would be most difficult to find have a pale colour which makes them stand out clearly against soil and herbage (Dirks *et al.*, in prep., Solhøy T., pers. obs.). Adult slugs are normally more colourful but these larger slugs are easy to see anyway. My searches did not include slugs buried beneath the soil surface, except on one sampling date where soil samples were taken in the strawberry site. However, most slugs probably inhabit the vegetation layer and the soil surface, since mosses and other vegetation offer sufficient hiding places in meadows and set-asides in my study sites. Furthermore, a thick and compact organic layer beneath the vegetation makes the soil less permeable to

slugs. Previous studies in the same meadow site used in the present work have shown that only a few slugs (less than 10%) may be found below the soil surface (Tomasgård, 2005). Searches were made of 50x50 cm plots in the meadow field, and of 125x20 cm plots at the strawberry field site, including strawberry patches and surrounding set-asides to fit the strawberry rows (Fig. 3). All sampling events included species identification, counting and weighing of slugs in the field (Fig. 4).

**Figure 4:** Mollusc sampling in quadrats (top picture), and weighing of live slugs using Pesola scales (picture below).



### 3.3 Molecular methods as diagnostic techniques to reveal carabid diets

Predation in the field is often very difficult to study without disturbing the species involved, which is especially true with smaller animals such as carabid beetles. Many previous studies of carabid diets have instead been based on laboratory experiments where pest arthropods or slugs were fed to starved beetles without any alternative prey (Basedow *et al.*, 1976; Allen, 1979). This gives an indication of what kind of prey beetles are capable of killing and consuming but it does not provide any idea of potential predation in the field. Traditional methods of analysing carabid diets have also been based on morphological traits (e.g. prolonged head parts in *C. caraboides* to drag snails out of their protective shells) and gut dissections, identifying hard parts such as the radula (Ingerson-Mahar, 2002). Based on these methods Larochelle (1990) was able to list dietary information of 1054 species and food preferences for about 50 species of carabids. However, these results are biased towards prey with solid parts which are recognisable in the guts of some carabid beetles. In addition, some carabids (e.g. *Calosoma*, *Carabus*, *Cychrus*) have pre-oral digestion, leaving hardly any recognisable particles in their guts. Clearly an approach to dietary analysis relying upon other reliable taxonomic characteristics surviving digestion is required.

The use of molecular techniques in ecology has increased enormously during the last decade, making it possible to study trophic interactions. Molecular analysis of predation has been reviewed by Symondson (2002), Sheppard and Harwood (2005) and King *et al.* (2008), and these papers also show the change from protein-based to DNA-based methods which has taken place in the last 20 years or so. Protein-based techniques, such as enzyme electrophoresis and immunological approaches (polyclonal and monoclonal antibodies), are still being used, but have been largely replaced by DNA-based methods, in particular the polymerase chain reaction (PCR). PCR has many advantages being rather fast and cheap compared with other diagnostic methods and requires very small quantities of tissue (Harris & Jones, 1997). Furthermore, most contemporary biological laboratories contain PCR equipment and apply these methods routinely. Temperature and denaturing gradient gel electrophoresis may be used to examine predator responses to prey diversity (Harper *et al.*, 2006), while diagnostic methods using species-specific primers can be applied to analyse feeding ratios on specific species (Agusti *et al.*, 2003; Harper *et al.*, 2005; Read *et al.*, 2006). Multiplex PCR incorporates multiple primers making it possible to analyse predation on several prey species simultaneously (Harper *et al.*, 2005). However, these methods also have some major limitations, mainly due to the time critical window for DNA detection in the guts

of predators (King *et al.*, 2008). Misinterpretation of results is also often a major issue due to false positives because of cross-amplification of non-target DNA. Furthermore, these methods do not discriminate between predation and scavenging, hence the results are effectively prey consumption (Foltan *et al.*, 2005). Additionally, it is not possible to discriminate between primary and secondary predation (Sheppard *et al.*, 2005). A predator of interest may have fed on another predator which has eaten the target prey. Moreover, contamination problems are major issues in PCR due to its exquisite sensitivity. With few exceptions (Troedsson *et al.*, 2007) the field of molecular ecology normally yields only qualitative data, due to the difficulties of quantitative calibration of field data. King *et al.* (2008) reviewed the most recent findings touching upon the problems mentioned earlier and suggest best practices to avoid these problems, involving issues such as feeding experiments to analyse the detection of prey-DNA in the guts of the predator (Fig. 5-6), cross-reaction tests on non-target prey and how to avoid contamination problems.

In the present work I designed and optimized one multiplex PCR to study predation by carabid beetles on *Arion lusitanicus*, *A. ater* and *A. rufus*. I also used a previously designed multiplex PCR to incorporate other slug species such as *Deroceras reticulatum* and *Arion distinctus* (Dodd, 2004; Harper *et al.*, 2005), making it possible to analyse potential prey preferences in the field. Prior to all PCR screenings the beetle foregut was dissected out either by cutting through the sclerotic abdomen (Fig. 7) or twisting off the pronotum from the abdomen to release the foregut, which was then used for DNA analyses.

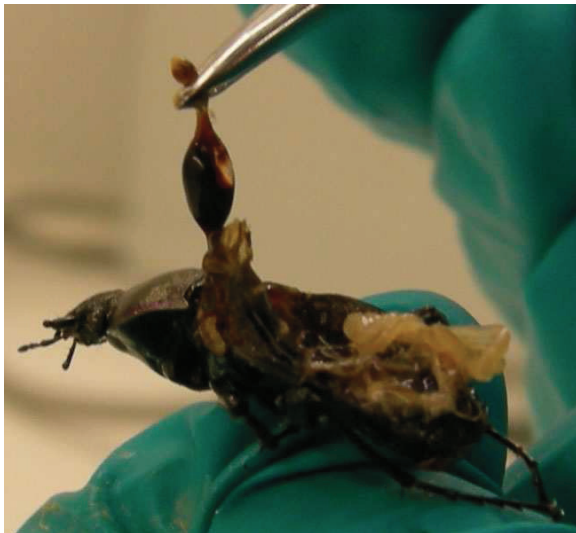
**Figure 5:** Overview of a feeding experiment carried out in Petri dishes with *C. nemoralis* served *A. lusitanicus* juveniles on moist filter paper.



**Figure 6:** One male *Carabus nemoralis* feeding on a juvenile *Arion lusitanicus* (0.1g fresh weight) in a feeding experiment carried out in Petri dishes.



**Figure 7:** Dissection of the carabid beetle *Carabus nemoralis* prior to DNA analyses. The foregut is suspended using a pair of forceps.

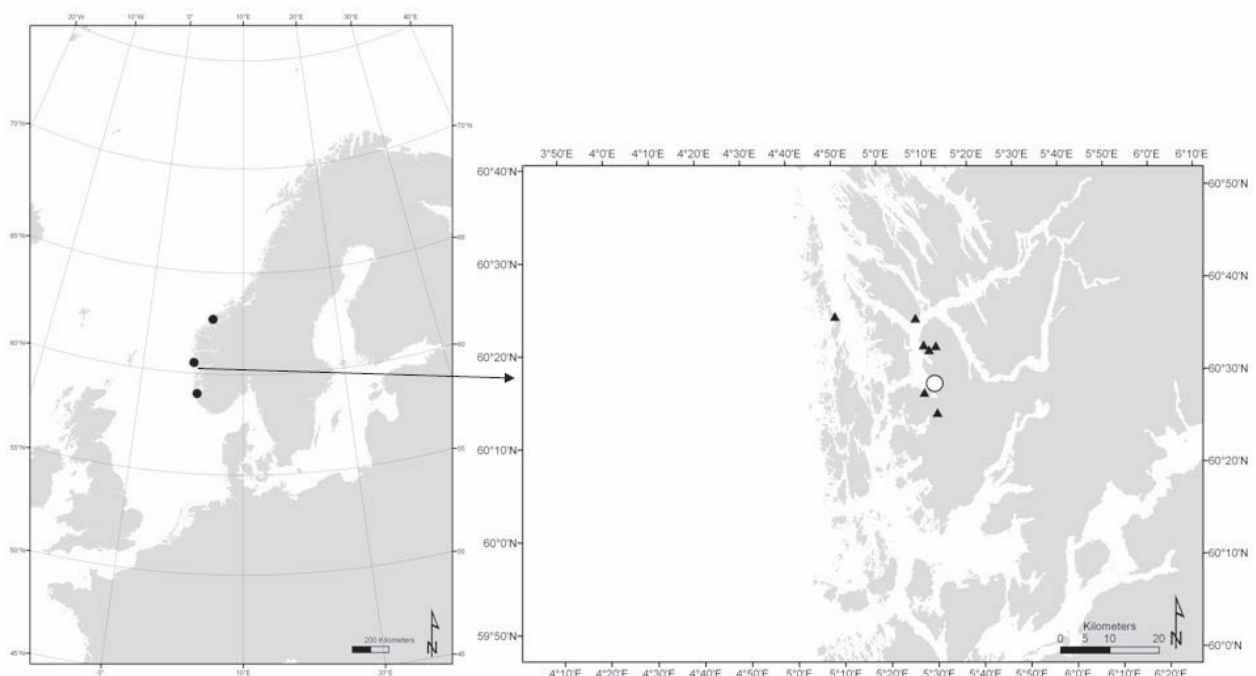


### 3.4 Study sites

Sampling was carried out in different parts of western Norway for taxonomic analyses of the large arionids; *Arion ater* and *A. lusitanicus* (Fig. 8). Several sites in the county of Hordaland were used; the island of Toftøy in Øygarden, Ask on the island of Askøy and Frekhaug in Meland, as well as several sites in the city area of Bergen; Damsgård, Sletten and Åsane (Morvik and Ulset). In addition, one site in Klepp in the county of Rogaland, south-west Norway, and one at Draget on the island of Bolsøya in the county of Møre og Romsdal, north-west Norway.

Samples for predation studies were mainly taken in the vicinity of Bergen, in Åsane and Askøy, respectively. The Åsane site is an abandoned meadow in a rural area outside Bergen, (60° 38' N, 5°34' E) close to small patches of deciduous trees, and is mowed once a year (Fig. 9). The site in the island of Askøy (60° 28' N, 5°12' E) consisted of patches of strawberry cultures and surrounding set-asides consisting mainly of grassland (Fig. 10).

**Figure 8:** Maps of the sites used for taxonomic analyses. Sites shown as filled black circles and triangles, while the city of Bergen is shown as a white circle with a black border.



**Figure 9:** The study site in an abandoned meadow in Haukedalen, Åsane, Bergen. Photo: Solveig Haukeland (top picture). Dominant grass species: *Deschampsia cespitosa*, *Holcus lanatus*, *Cardamine pratensis*, *Rumex acetosa*, *Alchemilla vulgaris* coll., *Epilobium angustifolium* and the moss species *Rhytidadelphus squarrosus*. Top picture taken in May and the picture below taken in August.



**Figure 10:** The study site with strawberry patches and surrounding set-asides at Ask, Askøy. Main vegetation in the set-asides: grasses, mosses, *Anemone nemorosa*, *Ranunculus acris*, *R. ficaria*, *Alchemilla* sp., *Trifolium* sp., *Rumex* sp., *Taraxacum* sp., *Cardamine pratensis* and *Epilobium* sp. Top picture taken in April and the picture below taken in September.





## **4. Results and discussion**

### **4.1 Differentiation of the invasive *Arion lusitanicus* and related species**

The invasive Iberian slug *Arion lusitanicus* was separated from the closely related *A. ater* based on the overall genital morphology, morphometrics of the genitalia and mitochondrial DNA (**Paper I**). COI-sequences of the mitochondrial DNA were used to design one diagnostic multiplex PCR applying species-specific primers (markers) to separate between the two species as well as the closely related *A. rufus*. The coloration of adult *A. lusitanicus* varied from red-brown to dark brown or almost black; hence the latter colour morphs are difficult to differentiate from adult *A. ater*, which also has dark brown and black colour variants. Juveniles of the large arionids are easier to separate based on coloration; *A. lusitanicus* has two distinct dark lateral bands, while *A. ater* and *A. rufus* have faint bands or are almost colourless with a greenish hue. Furthermore, based on the form of the ligula (distal part of the genitalia), all *A. lusitanicus* sampled belonged to the pest or “Scandinavian form” (*sensu* Altena 1955). This is also found in Central and northern Europe and is different from *A. lusitanicus s.s.* Mabile, which is only found in the Iberian Peninsula. The Mabile taxon has the distal genital characteristics of the Scandinavian form, but a different ligula. Thus the pest *A. lusitanicus* should still be considered a form of *A. lusitanicus* and not *A. vulgaris*, as also concluded by Quinteiro *et al.* (2005) due to limited documentation for giving this taxon a species in its own rights. A large proportion of *A. ater* collected at most sites was introgressed with *A. rufus* based on the morphology and morphometry of the distal genitalia (ca. 50%), and mitochondrial DNA (27%). Based on mtDNA, two specimens even showed evidence of introgression with *A. lusitanicus*. This study emphasizes the importance of integrating several methods in taxonomy to reliably differentiate invasive species from closely related native species before implementing any biocontrol or other pest eradication measures.

### **4.2 Capabilities of some selected beetles to kill and feed on *Arion lusitanicus***

All the studied carabid beetles; *Pterostichus niger*, *P. melanarius*, *Carabus nemoralis* except *Nebria brevicollis*, were able to feed on eggs of *A. lusitanicus* as well as subdue and feed on newly hatched juveniles (**Paper II**). In addition, the staphylinid beetle *Staphylinus*

*erythropterus* killed and fed on these size classes of *A. lusitanicus*. Both *P. melanarius* and *P. niger* also destroyed *A. lusitanicus* eggs and hatchlings under conditions emulating those in the field. However, the ability to kill larger juveniles (>0.1g fresh weight) were fairly limited for all species, except *C. nemoralis* which was able to consume slugs up to a size of 2-3 cm in length (1g fresh weight). On some occasions, *C. nemoralis* even fed on two or all of the three slugs presented to it during the two hour duration of the experiment. Nonetheless, *P. niger* consumed slugs up to 0.5g, while *S. erythropterus* was restricted to slugs smaller than 0.1g, a finding which has been confirmed for *P. melanarius* (Paill, 2004). Thus, the capabilities of beetles to kill and feed on *A. lusitanicus* were clearly related to the size of the beetle, which is in accordance with previous studies comparing various carabid species within genera such as *Carabus*, *Cychrus* and *Pterostichus* (Tod, 1973; Kaiser *et al.*, 1993)

#### **4.3 Molecular detection of predation on *Arion lusitanicus* and *Deroceras reticulatum***

The diagnostic multiplex PCR used in the first paper was optimized to detect predation by carabid beetles on *A. lusitanicus*, *A. ater* and *A. rufus*. The primers were sufficiently sensitive and specific for predation analyses. The most significant finding was that mtDNA of *A. lusitanicus* could be detected for up to 40 hours in foreguts of *C. nemoralis*, with median detection times (the time at which 50% of samples tested positive) of 22h (**Paper III**) post-feeding. In addition, a diagnostic multiplex PCR using genus and species-specific primers was applied to separate between 12S sequences of the mtDNA of the slugs *D. reticulatum* and *A. distinctus*. The detection period of *D. reticulatum* was also up to 40h, with a median detection time of 20h (**Paper III**). Thus the detection of predation by beetles on these slugs were nearly identical, hence feeding-ratios in the field could be compared directly using these genetic markers. Predation of all target slugs in the field was successfully detected, the most significant being feeding by *C. nemoralis* on *A. lusitanicus* during spring (**Paper III and IV**). However, slug-positive beetles were also found among *P. melanarius* and *P. niger*, the former being positive only for *D. reticulatum* while *P. niger* tested positively for all target slugs except *A. distinctus*. Thus, predation in the field accords with the results from the feeding experiments; the ability to feed on slugs is related to the size of the beetle.

#### 4.4 Size and prey choice of carabid beetles feeding on slugs

The intensity of predation by carabid beetles on slugs was negatively related to the size of the slugs, where *C. nemoralis* preferred *A. lusitanicus* less than one gram with a tendency for choosing slugs less than 0.4g fresh weight (**Paper II and IV**). Hence, predation on *A. lusitanicus* decreased at the beginning of summer when most of the slugs were larger than one gram. Further, *C. nemoralis* showed no preference for *D. reticulatum* over *A. lusitanicus* in the laboratory tests (**Paper II**), and seems to lack any preference for specific slug species in the field based on slug-DNA positive beetles (**Paper III and IV**). Instead, these beetles seem to feed opportunistically on slugs according to the densities and sizes of these slugs. *Carabus nemoralis* is known as predominantly a gastropod and earthworm feeder (Toft & Bilde, 2002), thus according to foraging theory one might expect that this beetle switches to the most abundant prey within its range. The same pattern was found by Barker (1991), suggesting that predation on slugs by carabids may be density dependent rather than prey specific.

Further, the predation intensity by *C. nemoralis* was spatially associated with its slug prey, where foregut mass and activity of beetles were related to the density of slugs when using a spatial point sampling design (**Paper IV**). The strongest association was found between *C. nemoralis* and *A. lusitanicus* which was the most abundant slug species. Hence, our original hypothesis that *C. nemoralis* prefer slugs other than *A. lusitanicus* due to less sticky mucus did not hold. This is probably due to *C. nemoralis*' way of attacking the slugs compared to generalist species like *P. melanarius*. *Carabus nemoralis* directed their attacks at the posterior end of the slug, in most cases killing it in the first attack. The other beetle species tested did not direct their attacks to any specific body part and often gave up after repeated attacks due to the increasing amount of mucus.

#### 4.5 Intraguild predation by carabid beetles on nematode-infected slugs

A diagnostic primer-pair designed by Read (2007) was applied for detection of intraguild predation on nematode-infected slugs using the nematode *P. hermaphrodita*. The primers were sufficiently specific while the detection time of nematode-DNA was up to 12h, which is rather short compared to the target slug species (**Paper IV**). However, less than half of the slugs used in the feeding experiment were actually infected and just moderately so ( $5.1 \pm 1.4$  nematodes per slug), which means that the detection method was very sensitive. The number

of infected slugs in the field after applying “Nemaslug” containing *P. hermaphrodita* varied according to slug species, with the highest incidence of infection in *D. reticulatum* and *A. silvaticus*. *Arion lusitanicus* (mean fresh weight;  $0.7\pm 0.1\text{g}$ ) was only moderately infected (**Paper IV**). Furthermore, the slugs *D. reticulatum*, *A. silvaticus* and *A. distinctus* were negatively associated with nematodes spatially. No nematodes in the soil or infected slugs were found four months after the treatment. Intraguild predation by *C. nemoralis* was low with only three PCR-positive beetles out of 130, suggesting this is not an explanation of why the effect of “Nemaslug” is short lasting in the field. However, this does not necessarily suggest any preference for non-infected slugs, since these slugs may be less available to beetles after being infected, hiding away to die (Glen & Wilson, 1997). Predation may be an important factor in the decline in nematode numbers in the soil following application, but this is more likely the result of consumption by microarthropods (Read *et al.*, 2006).

#### **4.6 Potential for biological control of *Arion lusitanicus* using carabid beetles**

##### *Slug control by carabid beetles*

In general, there are few studies showing carabids depress pest populations to an economically useful level in certain crops (Kromp, 1999; Sunderland, 2002; Symondson *et al.*, 2002). Many studies have shown various interactions and correlations between carabids and slugs (**Paper III and IV**), which indicate their potential as biological agents but remain unsolved in terms of direct impact on pest numbers and crop loss. However, Symondson (1993) demonstrated that the mortality of *D. reticulatum* increased significantly in plots of lettuce in a polythene tunnel when *A. parallelepipedus* was added (six adult specimens per  $\text{m}^2$ ). Similar results were also obtained by Asteraki (1993), who found that the effect of *A. parallelepipedus* (four beetles per  $\text{m}^2$ ) and *Pterostichus madidus* (eight beetles per  $\text{m}^2$ ) on *D. reticulatum* was as strong as the molluscicide methiocarb. Furthermore, Barker (1991) also found a significant mortality of *D. reticulatum* and *Arion intermedius* when carabid beetles were added to microplots. In addition, Buckland and Grime (2000) found that *P. melanarius* significantly reduced herbivory of *D. reticulatum* in microcosms due to higher mortality rates of slugs, while McKemey *et al.* (2003) showed that the same beetle reduced numbers of *D. reticulatum* in outdoor miniplots irrespective of slug size within the range of 2-100mg. Furthermore, Chapman *et al.* (1997) found a reduction of *D. panormitanum* as well as *D.*

*reticulatum* in presence of the carabid *Megadromus antarcticus* in New Zealand. Moreover, larvae of *P. melanarius* have been shown to be capable of reducing numbers of *D. reticulatum* as well as *A. intermedius* (Thomas, 2002). However, alternative prey can potentially divert beetles from feeding on pest slugs as shown by Symondson *et al.* (2006), although this is highly taxon-specific depending on diversity and dietary qualities.

Augmentative biological control (i.e. release of natural enemies) of slugs may be possible with some species (e.g. *A. parallelepipedus*), but conservation biological control is the most realistic option for most carabids due to their cannibalistic behaviour at larval stages (Symondson, 2004). The provision of refugia is especially important since hiding places may increase the number of beetles (Altieri *et al.*, 1982).

#### *Biological control of Arion lusitanicus*

The potential high impact of *C. nemoralis* on *A. lusitanicus* in spring (**Paper III and IV**) is probably affected by the use of molluscicides both negatively (Buchs *et al.*, 1989) and positively (Langan *et al.*, 2001). Thus I recommend minimizing the use of chemical control that has a negative effect (methiocarb especially) to only focus on heavily infested patches in spring. The use of molluscicides should at least be kept to a minimum along field edges and semi-natural areas surrounding the crop fields, where *C. nemoralis* is an abundant species (**Paper III**). *Carabus nemoralis* as well as some other carabids have been found to disperse along linear hedgerows (Gruttke, 1994) and replace arable field species (e.g. *P. melanarius*) along the edges of such hedgerows (Sustek, 1994). Semi-natural habitats are also overwintering places for other slug predators such as *P. melanarius* and *P. niger* that may diffuse into arable fields during summer, but may also be used as hiding places for slugs during daytime (Langan *et al.*, 2001; Langan *et al.*, 2004). Types of cultivation are also important where smaller patches with set-asides probably have a positive effect on beetle densities compared with large monocultures (Chapman *et al.*, 1999; Holland *et al.*, 2005). Furthermore, cultivation impacts such as deep ploughing often have a negative effect on both carabid numbers (Cárcamo, 1995; Symondson *et al.*, 1996; Kromp, 1999) and slug numbers (Barker, 2002). However, the timing of cultivation should be limited in spring to decrease the effects on active carabid beetles. Thus, *C. nemoralis* may have potential in conservation biological control in arable fields surrounded by semi-natural habitats, such as hedgerows and woodlands.

## 5. Conclusions

The invasive Iberian slug *Arion lusitanicus* is proposed to be a different form of the species than the topotype described by Mabilie (1868), hence I refer to it as the “pest form” or the “Scandinavian taxon” (also regarded as *A. vulgaris* Moquin-Tandon 1855). It has many of the same morphological characteristics as other *A. lusitanicus* taxa, but is differentiated by genital morphology (i.e. the ligula). The species can be differentiated easily from the closely related *A. ater* and *A. rufus* by genital morphology, morphometrics and genetic analyses.

The hypothesis that the invasive nature of *A. lusitanicus* is due to lack of natural enemies does not hold. The selected carabid beetles *P. niger*, *P. melanarius*, *C. nemoralis*, and the staphylinid beetle *S. erythropterus* all preyed on eggs and newly hatched *A. lusitanicus* in the laboratory. In addition, *C. nemoralis* killed and consumed juvenile slugs up to a size of ca 1 gram, while the other species were much less successful with larger slugs. Furthermore, the carabid beetles *P. niger* and *C. nemoralis* fed on *A. lusitanicus* in the field based on molecular analyses of gut contents. Predation by *C. nemoralis* in spring was the most significant, since *A. lusitanicus* is mainly abundant as juveniles in spring and *C. nemoralis* is spring active and a predominant gastropod and earthworm feeder. Predation by *C. nemoralis* seemed to be related to density; feeding on the most abundant slug species as long as slugs were less than one gram. Hence, no obvious preference for a particular slug species was found. Thus there is some potential to use *C. nemoralis* to suppress high densities of *A. lusitanicus* in spring by conservation biological control facilitating agricultural areas to foster higher numbers of these beetles. Further, this carabid beetle seems not to feed significantly on nematode-infected slugs, which means that nematodes can be used along side carabid beetles as biological control agents. All in all this study show that the “enemy release hypothesis” is highly questionable in the case of *A. lusitanicus*.

## 6. Future studies

Integrated taxonomic approaches can be used to differentiate between invasive and native species (such as *Arion* slugs) that are closely related. Future studies should include larger number of specimens and larger sampling ranges within Europe (including southern France, Spain and Portugal) to verify the potential species status (e.g. *A. vulgaris*) of the pest form of *A. lusitanicus*. Furthermore, population studies on *A. lusitanicus*, *A. ater* and *A. rufus* involving appropriate genetic markers (e.g. allozymes, microsatellites) may further disentangle the complexity of these taxa and possible unsuspected introgressions. It may also shed light on how *A. lusitanicus* has been spread through northern Europe, and once population-genetic markers have been developed, how it may affect the genetic architecture of other species. However, such studies should also use mitochondrial DNA, genital morphology and morphometrics to strengthen these analyses.

Carabid beetles are important predators in agricultural ecosystems feeding on pest insects and molluscs. Studies are, however, often hampered due to the inability to confirm a significant pest reduction solely by the action of predators operating in a complex ecosystem, where many factors can affect pest population dynamics. The studies on slug predators are also limited by the difficulty of producing quantitative data. It also implies that the potential economic advantage of taking measures to increase the abundance of predators like carabid beetles (e.g. weed strips, hedgerows, and reduced tillage) is often unknown. However, strips of grass or other non-crop vegetation have been shown to have a positive effect as a refuge for carabids and other natural enemies of slugs (Lee *et al.*, 2001; Bommarco & Fagan, 2002). Furthermore, provision of other kinds of shelters and overwintering sites, lowering the amount of indiscriminate pesticides used as well as manipulating carabid activity by semiochemicals, may all have potential (Kromp, 1999; Halaj *et al.*, 2000; Symondson *et al.*, 2002). The present study has shown that some carabids, in particular *C. nemoralis*, are important natural enemies of slugs like the invasive Iberian slug *A. lusitanicus*. It is therefore vitally important to follow up these results by analysing the direct effect of these beetles on slugs. Future studies should carry out manipulations within semi-natural enclosures, to analyze potential effects of the presence of particular carabid species, as well as the consequences of generally enhancing beetle abundance.

Predation by *Pterostichus* larvae is probably more significant on newly hatched slugs compared with adult beetles since the larvae are found primarily below or on the soil surface sharing the same habitat with smaller slugs and eggs at the same time of year in late autumn

and early spring. Alternatively, carabid larvae are highly cannibalistic and predation has been found to be affected by density-dependent intraspecific competition (Thomas *et al.*, 2009). Future studies should investigate the role of carabid larvae such as certain *Pterostichus* species as natural enemies of *A. lusitanicus*.

The application of nematode-infected insect cadavers in the field has been found to be far more effective than spraying nematodes on the soil and vegetation in controlling pest weevils (Shapiro-Ilan *et al.*, 2001; Shapiro-Ilan *et al.*, 2003). The exposure to stress in form of desiccation, UV light and extreme temperatures is much lower for the nematodes, and the spreading of nematodes is quite effective due to the scavenging behaviour of slugs. Furthermore, intraguild predation by micro-arthropods such as springtails and mites (Read *et al.*, 2006) feeding directly on nematodes prior to finding a host may also be lower using nematode-infected carrions. Thus this approach should be tested on *A. lusitanicus* in semi-field experiments in comparison with the conventional method of spraying the nematodes on the ground.

The laboratory results of Read (2007), Foltan *et al.* (2008) as well as the field results from the present work may suggest that beetles feed less on infected slugs compared to uninfected slugs. However, this should be followed up using semi-field experiments with infected and non-infected slugs to test if this is due to preferences of the respective beetles or the availability of slugs.

Significantly, *C. nemoralis* and other slug-predators such as *C. violaceus* are also found in the northern parts of the Iberian Peninsula along the Atlantic coast (Turin *et al.*, 2003), from where *A. lusitanicus* is suggested to have originated. Thus, studies of these predators as well as other natural enemies (e.g. microsporidia) in these areas may shed new light on the reasons why *A. lusitanicus* is a pest in northern Europe but not in the Iberian Peninsula. A parasitic release in terms of nematodes has been shown in European slugs being invasive in the USA (Ross *et al.*, 2010), and a similar pattern may also exist within Europe. Climatic differences may also account for the reasons why *A. lusitanicus* is not a pest in Spain or Portugal, where the climate is generally dryer than in Western Europe. However, the whole explanation is probably that the pest form is not abundant in the Iberian Peninsula at all but has its origins elsewhere (e.g. southern France). It might be a hybrid of other as yet poorly characterized *A. lusitanicus* taxa, which are only now beginning to spread north from the Iberian region, as was first proposed by Noble & Jones (1996) and Campbell (2000).



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