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Alternative Management Strategy Towards Weevils in White Clover Seed Production -Utilization of a Natural Enemy

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1. Forord

Projektet *"Alternativ bekæmpelsesmiddelstrategi af Snudebiller i Hvidkløver (ABSH) – Udnyttelse af snudebillens naturlige fjender"* J.nr. 667-00215, er gennemført med tilskud fra Miljøstyrelsens Program for Bekæmpelsesmiddelforskning, Tilskud til forskningsprojekter 2015-2018.

Projektet er gennemført af Institut for Agroøkologi, Aarhus Universitet og Institut for Plante og Miljøvidenskab, Københavns Universitet. Endvidere har frøfirmaerne DLF og DSV velvilligt stillet materiale til rådighed og deltaget i projektmøder.

I projektperioden har to studerende Lauriane Perrier og Kiri Miyaca Fløistrup deltaget. Førstnævnte har været involveret som led i et praktikophold. Sidstnævnte har arbejdet med snylthvepsekokonnerne i sit specialeprojekt.

Projektet er gennemført i perioden 15. maj 2015 til 31. oktober 2018

Projekttilskud 2.780.611 kr.

2. Sammendrag

Udbytterne i produktionen af hvidkløverfrø afhænger af bestøvning, skadedyr og høstbetingelser. Blandt skadedyrene er det især snudebiller af slægterne *Apion* og *Hypera*, hvis larver æder de umodne frø. I konventionel hvidkløver er det normal praksis med 1 eller 2 insekticidsprøjtninger, og udbyttereduktionen på grund af disse skadedyr i usprøjtede afgrøder anslås at være mere end 40%.

I Danmark er de vigtigste Hypera-arter kløvergnaveren *Hypera nigrirostris* (Fabricius) *og* kløverhovedgnaveren *H. meles* (Fabricius). Projektet har dokumenteret, at larver af begge arter snudebiller angribes af snyltehvepsen *Bathyplectes curculionis* (Thomson), som lægger æg i snudebillens unge larver i kløverblomsterne. Snyltehvepsens larve fuldender sin udvikling i snudebillelarven, og snyltehvepsens karakteristiske og synlige kokoner kan ved frøhøst findes i betydelige antal både i marken og i den høstede råvare.

Det foreliggende projekt har haft til formål at undersøge, om denne snyltehveps kan udnyttes til naturlig regulering af snudebillerne. Forudsætningen for dette er 1) at kokonerne kan sorteres fra det høstede materiale, 2) at kokonerne kan opbevares i vintersæsonen, 3) at kokonerne kan udsættes i de nye kløverfrømarker, 4) at klækningen af voksne snyltehvepse sker i tilstrækkeligt omfang og på det rette tidspunkt i forhold til snudebillelarvernes udvikling, samt 5) at de udsatte snyltehvepse reducerer enten snudebillens skade i udsætningsåret eller antallet af snudebiller, der klækkes og udgør det følgende års population.

Kløverfrø høstes ved skårlægning og efterfølgende tærskning eller ved direkte høst og opbevares derefter som råvare hos landmanden. Råvaren transporteres til frøfirmaets renseanlæg, hvor materialet sorteres i kløverfrø (renvare) og "frarens" bestående af ukrudtsfrø, planterester og andet affald. Snyltehvepsenes kokoner findes i denne fraktion og har på dette tidspunkt været udsat for skårlægning, tærskning, tørring og opbevaring på bedriften, transport samt mekanisk frørensning. Baseret på materiale fra økologiske hvidkløvermarker viser projektet, at det frarensede materiale som udgangspunkt kan indeholde mellem 70.000 og 140.000 kokoner pr. hektar høstet hvidkløver, dog med stor variation mellem avlere og år. Den samlede mængde kokoner består på dette tidspunkt af mange mindre, døde kokoner og få større, tungere kokoner indeholdende levende snyltehvepselarver. Resultaterne tyder på, at mindre end 5% af kokonerne var levende efter at have været igennem høst, opbevaring og rensning. I projektet er det undersøgt, hvordan de forskellige processer fra høst til udsætning i marken påvirker snyltehvepsene for dermed at kunne reducere dødeligheden. Det har ikke været muligt at følge en kohorte af kokoner gennem processen, men effekter af de forskellige delprocesser er undersøgt.
Figur 1 viser de påvirkninger, som kokonerne bliver udsat for, hvis de skal indsamles, lagres, oprenses og opbevares til næste års udsætning.

Sortering
I kokoner indsamlet fra blomsterhoveder i marken, dvs. inden de påvirkes af høst, tørring og sortering, er en betydelig andel af snyltehvepselarverne i live (figur 1A). I én mark, hvori der blev udtaget 21 kokoner, indeholdt 76% af kokonerne således levende larver. Tilsvarende blev der i 19 kokoner, ligeledes indsamlet fra blomsterhoveder før høst, ved dissektion fundet 79% levende larver. Ved dissektion af i alt 516 kokoner blev andelen af kokoner med levende larver fulgt fra det skårlagte materiale (40-50% levende) til 8-13% levende larver i kokonerne efter tærskning og tørring (figur 1B). Dette understøttes af, at der i en tilsvarende indsamling af 31 kokoner var levende larver i 10%.

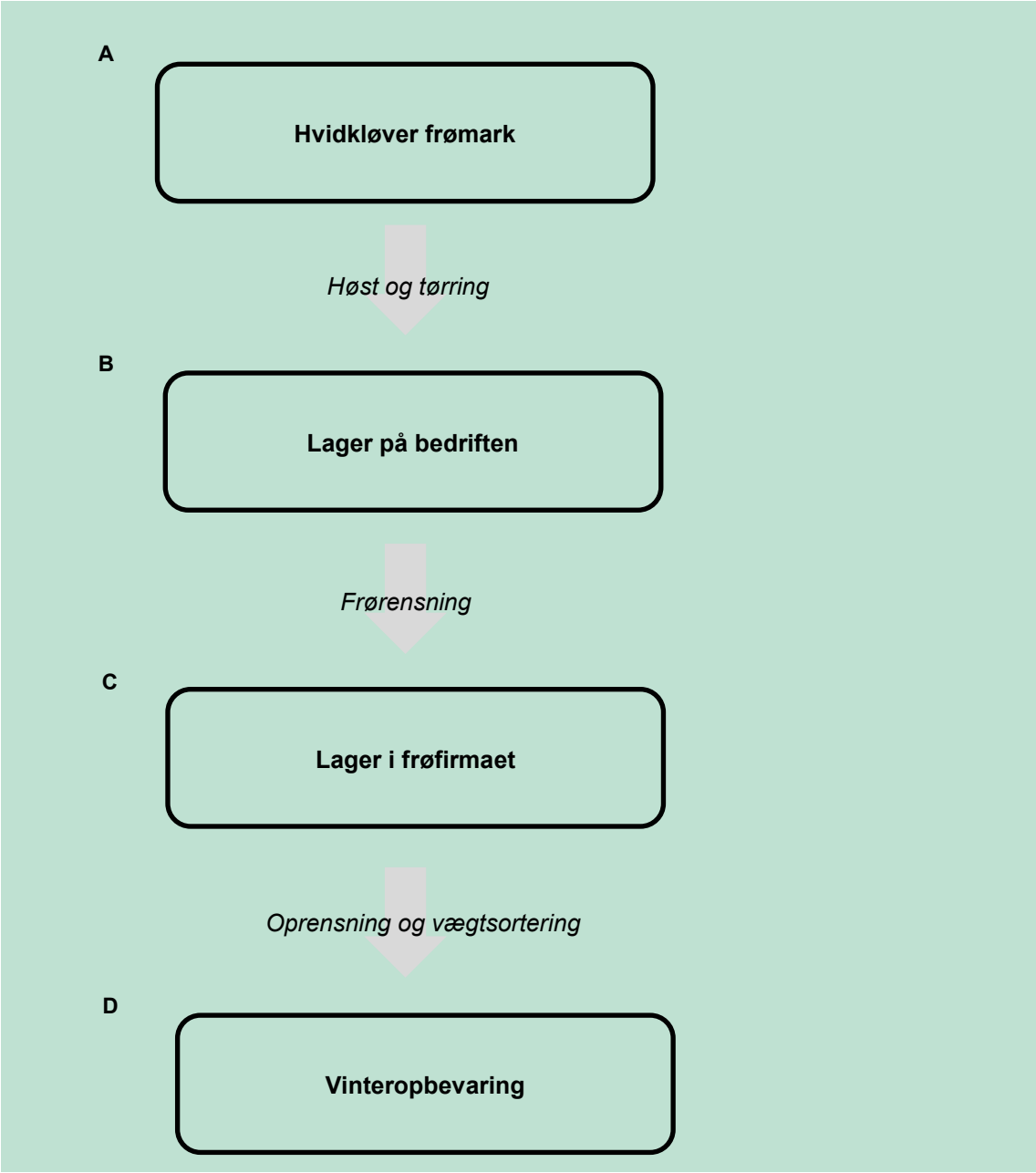

FIGURE 1. Påvirkninger, som kokonerne bliver udsat for fra høst til vinteropbevaring.

Effekten af selve renseprocessen blev undersøgt ved at sammenligne andelen af levende snyltehvepselarver i kokoner, indsamlet i den høstede råvare hos landmanden (figur 1B) og i den frarensede fraktion efter rensning i frøfirmaets renseanlæg (figur 1C). Kokoner udtaget fra råvaren efter transport til lageret, men inden rensning, viste ved undersøgelse af 215 kokoner fra fire bedrifter stor forskel på andelen af levende larver i kokonerne (2.5–28.9% levende larver). Endelig tyder indsamlinger af kokoner i det frarensede materiale på, at andelen af kokoner med levende larver i dette materiale er nede på 3-4%.

Kokoner med levende larver viste sig i gennemsnit at være tungere end kokoner med døde larver, og det frarensede materiale (figur 1D) kunne derfor oprenses yderligere, således at små, lette kokoner med få levende larver blev frasorteret. Det betyder, at der ved passende sortering kan opnås et slutprodukt, der indeholder en langt større andel kokoner med levende

larver end det oprindelige frarensede materiale og derfor er det velegnet til brug ved udsættelse af snyltehvepse i marken. Eksempelvis blev frarenset (figur 1D), vægtsorteret til et renere materiale (figur 1D) indeholdende 21% (2016) og 48% (2017) kokoner med levende larver.

Opbevaring

Fra høst indtil ultimo december/primo januar opbevares kokonerne i praksis i råvaren på bedriften og efter oprensning i frarens hos frøfirmaet. Betydningen af forskellige opbevaringsbetingelser på kokonernes dødelighed blev undersøgt ved at eksponere kokoner i 8-10 uger, dels til konstante temperaturer i klimakammer (hhv. +5 og –5°C), dels til svingende temperaturer i en uopvarmet bygning eller udendørs i et hegn. De anvendte kokoner var vægtsorteret med henblik på at opnå et materiale med høj gennemsnitsvægt og dermed høj andel af levende kokoner, og andelen af kokoner med levende larver var uændret efter 10 ugers opbevaring (57% før og 58% efter opbevaring). Det blev bekræftet af, at andelen af levende larver i 2017 var højere efter de 8 ugers opbevaring end før (67% og 84%). Snyltehvepsens larve ser derfor ikke ud til at være følsom over for opbevaringsbetingelserne i vinterperioden.

Klækning

Ved vinterens slutning udvikler snyltehvepsens larver sig til pupper og derefter til voksne i de overvintrende kokoner. Klækning af voksne snyltehvepse styres af varmesummen, hvorved synkroniseringen med værtsskadedyret sikres. I 2016 og 2017 blev 2400 og 2880 kokoner, hvoraf hhv. 57% og 84% var fundet at indeholde levende snyltehvepselarver, sat til klækning i marken i marts måned. Af disse klækkede hhv. 17% (9-26%) og 12% (5-23%) efterfølgende. Kokoner fra samme materiale (2017), men klækket indendørs ved 25°C, producerede voksne snyltehvepse fra 25% af kokonerne.

Resultaterne viser samlet, at kokonerne kan tåle opbevaring ved temperaturer mellem -5 og 5°C, uden at det påvirker deres overlevelse, vægt eller fedtindhold. Opbevaring af råvaren på bedriften samt af de frasorterede kokoner hos frøfirmaet sker derfor bedst ved temperaturer mellem -5°C og 2°C. Substratet har ikke stor betydning, men skal sikre luft omkring kokonerne samt at kokonerne ikke tørrer ud.

Effekt af snyltehvepsen på kløverhovedgnaveren

Snyltehvepsen antages at kunne begrænse udbyttetabet forårsaget af kløverhovedgnaveren på to måder: 1) ved at reducere skaden af kløverhovedgnaverens larve på frøafgrøden samme år, som snyltehvepsen angriber larven, enten ved at larven dør for tidligt, eller ved at den æder mindre, 2) gennem en reduktion af det antal voksne kløverhovedgnavere, der klækker og udgør det følgende års skadevoldende population. Begge dele er undersøgt i projektet i laboratorie, bur- og markforsøg.

Fra tidligere forsøg er det rapporteret, at parasiterede snudebillelarver æder mindre end ikke parasiterede. Dette kunne ikke eftervises, da tilvæksten af parasiterede snudebillelarver ikke var forskellig fra ikke-parasiterede larver.
I burforsøg med udsatte kløverhovedgnavere (6 hunner og 3 hanner/m^2 (2016) eller 12 hunner og 6 hanner (2017) blev det undersøgt, om et stigende antal snyltehvepse (0, 3, 6, 9, 12 pr. m^2 i 2016 og 0, 6, 12, 22, 44 pr. m^2 i 2017) havde effekt på skadedyrenes skade i blomsterhovederne eller på antallet af overvintrende kløverhovedgnavere. Skaden blev opgjort ved dissektion af 20 småblomster fra hver af 5 blomsterhoveder pr. bur. Småblomsterne blev kategoriseret som intakte og bestøvede, beskadigede og bestøvede samt ikke-bestøvede, og antal frø i hver småblomst blev opgjort.
Resultatet viste, at andelen af intakte, bestøvede småblomster var signifikant positivt påvirket af antallet af snyltehvepse og steg fra godt 60% til 80-85% begge år med stigende antal indsatte snyltehvepse. Tilsvarende faldt andelen af beskadigede småblomster med stigende antal snyltehvepse fra 29% til 6% i 2016, mens den tilsvarende reduktion var fra 19% til 8% i 2017. I

alle behandlinger udgjorde ikke-bestøvede småblomster 4-19% og var uafhængig af antallet af snyltehvepse.
Omregnes disse forskelle til potentielle udbyttetab med anvendelse af fundne frøudbytter fra intakte og skadede småblomster, fundne antal småblomster/hoved, gennemsnitlig tusindkornsvægt af hvidkløverfrø samt gennemsnitligt hovedantal pr. ha, tyder resultaterne på, at det potentielle udbytte øges med over 300 kg/ha ved en effektiv parasitering med *B. curculionis*. En sådan potentiel udbyttegevinst kan ikke umiddelbart overføres til markforhold pga. høsttab osv., men indikerer, at parasitering kan være en vigtig faktor.
Antallet af kløverhovedgnavere, der klækkede fra hovederne, og som vil udgøre det følgende års population, var derimod ikke påvirket af antallet af snyltehvepse udsat i burene.

Udsætning i marken
Snyltehvepse blev udsat i førsteårs frømarker i 2016 og 2017 ved placering af kokoner i markkanten af 4 forskellige marker begge år. Undersøgelsen af effekten af udsatte snyltehvepse i marken blev besværliggjort af, at der i alle marker potentielt allerede var en population af både snudebiller og snyltehveps, dvs. der var ikke kontrolmarker uden snyltehvepse. I 2016 blev der på grund af mangel på tilgængelige kløverfrømarker udsat snyltehvepse i alle 4 forsøgsmarker. Den fjernest beliggende ende af marken fungerede som kontrol (2016). I 2017 blev der udsat snyltehvepse i 4 marker, og 4 marker fungerede som kontrol. Indsamlinger af snyltehvepse viste, at de var jævnt fordelt i marken og sandsynligvis spredte sig hurtigt efter at være blevet udsat. Der var signifikant flere snyltehvepse tilstede i de marker, hvori de var udsat, end i kontrolmarkerne. Kontrolmarkerne havde imidlertid et væsentligt større antal kløverhovedgnavere. Indsamling af kløverhovedgnavere og snyltehvepse i blomsterhoveder tæt på høst viste, at parasiteringsgraden var 24% i marker med udsatte snyltehvepse, mens kun 13% af kløverhovedgnaverne var parasiteret i kontrolmarkerne. Disse resultater tyder på, at udsætning af snyltehvepsene kan øge parasiteringsgraden betydeligt, men også at der allerede findes en population derude, der måske kunne aktiveres.

Projektets resultater viser, at kløverhovedgnaverens snyltehveps *B. curculionis* har potentiale til at indgå i en fremtidig strategi for øgede frøudbytter og reduceret insekticidanvendelse i hvidkløverfrøproduktionen.

Det er vist, at der kan indsamles et højt antal kokoner fra hvidkløverfrømarken, men efter høst, tørring og frørenseprocessen er andelen af levende larver i kokonerne lav. Den andel kan øges gennem en vægtsortering af kokonerne, og andelen af levende larver kan bestemmes ved multispektral billedanalyse (Shrestha et al. 2018). Det er lykkedes at oprense kokonerne fra andet affald f.eks. ukrudtsfrø, så de kan udsættes til anvendelse i det kommende års hvidkløverfrømark.

Projektet identificerer imidlertid en række barrierer, som skal overvindes, hvis dette potentiale skal realiseres, gennem:

- udvikling af skånsomme indsamlings-, håndterings- og opbevaringsmetoder
- undersøgelser af, hvorfor og hvor i processen larverne dør
- undersøgelser af, i hvilket omfang kokoner, der bliver liggende i marken, indgår som ressource i næste års hvidkløverfrømark

Ud over de ovenfor afrapporterede resultater er det endvidere uden succes undersøgt,

- om og hvordan snyltehvepse kan kombineres med insekticider,
- om høstmetoden påvirker larvernes dødelighed

3. Summary

Yields in the production of white clover seeds depend on pollination, pests and weather conditions during harvest. Among the pests the insect larvae and in particular weevil larvae belonging to the genera *Apion* and *Hypera*, eat the unripe seeds. In conventionally grown white clover it is common practice to apply insecticides once or twice. Yield reduction due to weevil pests in non-sprayed seed crops is estimated to be ≥ 40%.

In Denmark, the most important *Hypera* species are the lesser clover leaf weevil *Hypera nigrirostris* (Fabricius) and clover head weevil *Hypera meles* (Fabricius). This project documents that both species are parasitized by the parasitoid *Bathyplectes curculionis* (Thomson), parasitizing the young weevil larvae in the clover heads. The larvae of the parasitoid ends its development in the weevil larvae and its characteristic and visibly distinct cocoon can be found in great numbers both in the field and in the raw material at the time of the white clover seed harvest.

The current project studied if the parasitoid can be used to regulate the *Hypera* weevils. The prerequisites would thus be 1) the cocoons can be separated from the harvested raw material, 2) the cocoons can be stored throughout the winter season, 3) the cocoons can be released in the new clover seed field, 4) adequate numbers of the adult parasitoid can hatch at the right time related to the development of the weevil larvae and 5) the released parasitoids reduce the damage by the weevil within the year of release or reduce the number of weevils hatching, which constitutes the weevil population in the next year(s).

Clover seeds are harvested by swathing and threshing or by direct harvest. Following the harvested raw material is dried and stored for some months at the farm. The raw material is then transported to the processing facilities of the seed company, at which the clover seeds are separated from a debris fraction, consisting of weed seeds, crop residue and others. The parasitoid cocoons can be found in the debris fraction and have up to this point been subjected to the swathing, the threshing, the drying and the storing at the grower, transport plus the mechanical seed sorting process. Based on material from organically grown white clover seed fields, the project showed that the debris fraction contains between 70.000 and 140.000 cocoons per hectare harvested white clover, though with a large variation between grower and year. In the debris fraction, the total amount of cocoons consists of many cocoons containing light weight and dead parasitoid larvae and few cocoons containing heavier and live parasitoid larvae. The results suggest that less than 5% of the cocoons contained a live larva after the cocoons had been subjected to the harvest, drying, storage and seed processing. It was studied how the different processes from the harvest to the release in next year's field influenced the parasitoid. This was done with the interest of clarifying how mortalities could be reduced. It has not been possible to follow a cohort of cocoons through the processes; however, the effects of the different parts of the processes were studied.

Figure 2 visualize the influence on the cocoons, if the cocoons are to be collected, sorted and stored until released the following year.

Seed processing
Cocoons collected from white clover flower heads prior to harvest, drying and processing, contain a considerable portion of live parasitoid larvae (figure 2A). In one field, in which 21 cocoons were sampled, 76% of the cocoons contained a live larvae. Similarly, in 19 cocoons also collected in flower heads prior to harvest 79% per cent of the larvae were found alive. By dissecting 516 cocoons the portion of live larvae was followed from the time of swathing (40-

50% live) to 8-13% live larvae in cocoons found after threshing and drying (figure 2B). The later was supported by a similar collection of 31 cocoons containing 10% live larvae.

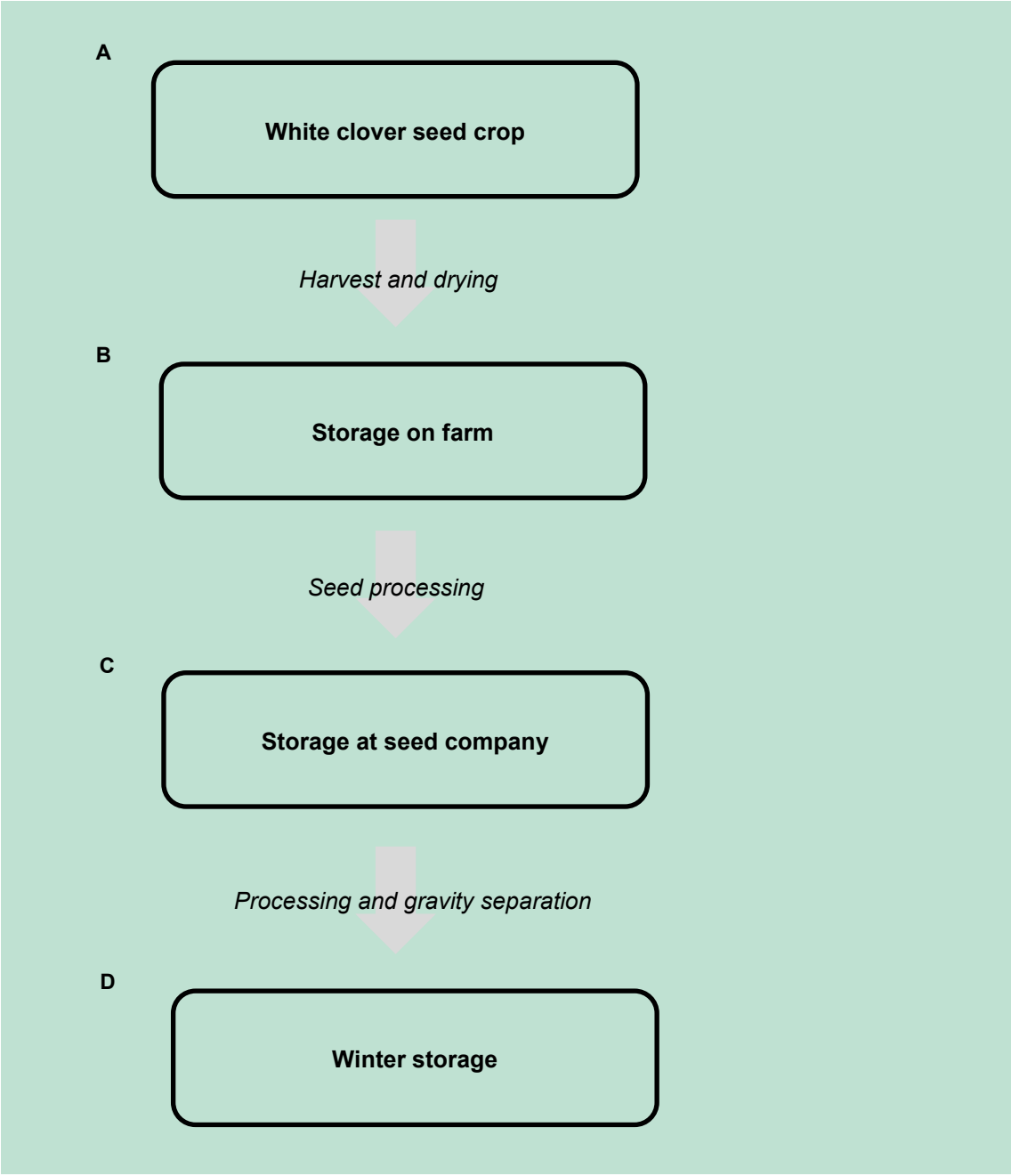

FIGURE 2. The processes influencing the cocoons from the harvest to the storing through the winter season.

The effect of processing alone was studied by comparing the portion of live parasitoid larvae in their cocoons collected in the harvested raw material. Sampling was done at the grower (figure 2B) and in the debris fraction from the seed processing facility in the seed company (figure 2C). Cocoons sampled from the raw material after transport to the storage of the seed processing facility and prior to the processing, showed large differences in the survival of the larvae. Of 215 cocoons obtained, survival ranged from 2.5 to 28.9%. Collected cocoons from the debris material showed that the portion of cocoons containing a live larva was 3 to 4%.

Cocoons of live larvae were found to have a higher average weight than cocoons harbouring a dead larva. It was therefore possible to further separate the lighter cocoons from the debris material (figure 2D). This suggests that by utilization suitable separation processes an end product containing a much higher fraction of live larvae than the original debris material. This fraction would therefore be suitable for introducing the parasitoid in next year's seed field. For example, the debris (figure 2D) was sorted by weight to deliver a cleaner material (figure 2D) containing 21% (2016) and 48% (2017) cocoons harbouring a live larva.

Hatching

At the end of winter the parasitoid larvae develop into pupae and following into an adult. The hatching of adult parasitoids is governed by the temperature sum, by which synchrony with the host pest is attained. In March of 2016 and 2017 2400 and 2880 cocoons, of which 57% and 84% were found to contain a live larva, were set out for hatching under field conditions. Of these 17% (9-26%) and 12% (5-23%) hatched subsequently. Cocoons from the same pool (2017), but hatched under constant temperature at 25°C, produced adult parasitoids from 25% of the cocoons.

In summary, results show that the cocoons can endure storing from -5 to 5°C without influencing survival, weight, or fat content. Storage of the raw material at the farmers and of the separated cocoons in the seed processing facility is best carried out at temperatures between -5 and 2°C. The substrate in which the cocoons are kept is not of great importance, but must safeguard ventilation and prevent desiccation of the cocoons.

The effect of the parasitoid on the clover head weevil

The parasitoid is assumed capable of reducing yield losses resulting from the weevils in two ways: 1) by reducing the damage caused by the weevil larvae on the seed crop within the same year of the parasitoid release, either as a consequence of an early demise of the weevil larvae or as a result of the parasitized weevil larvae eating less than normal. 2) through a reduction of the number of adult weevils which hatch and form the following year pest population. Both aspects have been studied in the project under laboratory, cage and field trials.

From earlier experiments it is reported, that parasitized weevil larvae eat less than non-parasitized larvae. This could not be confirmed, as the growth of parasitized weevil larvae was not different from non-parasitized larvae.

In cage trials with released clover head weevils (6 female and 3 male/m^2, 2016 or 12 female and 6 male, 2017) it was studied whether an increasing number of parasitoids (0, 3, 6, 9, 12 per m^2, 2016, and 0, 6, 12, 22, 44 per m^2, 2017) had an effect on the pests' damage on the white clover flower heads or the number of overwintering clover head weevils. The damage was estimated by collecting five flower heads per cage. From each flower head 20 florets were dissected. The florets were categorized as intact and pollinated, damaged and pollinated or non-pollinated. The number of seeds in each dissected floret was counted.

The result revealed that the number of intact and pollinated florets was significantly positively influenced by the number of parasitoids and increased from 60% to 85-85% in both years with an increase in the number of released parasitoids. Subsequently, the number of damaged florets dropped with the increase in the number of parasitoids added from 29% to 6% in 2016 while the reduction in 2017 was from 19% to 8%. Throughout all treatments, the non-pollinated florets ranged between 4 and 19% and was not dependent on the number of parasitoids released.

If the differences are recalculated to potential yield losses by utilizing the seed yields from intact and damaged florets, number of florets per flower heads, average thousand seed weight of white clover seeds combined with the average number of flower heads per ha, the results

suggest that the potential yield can be increased by over 300 kg/ha when with effective parasitization by *B. curculionis*. This potential yield gain is not directly applicable to field conditions where also harvest losses must be taken into account. However, it indicates that parasitism can be an important factor. The number of clover head weevils hatching from the white clover flower heads, which would constitute the following year's population was, however, not affected by the number of parasitoids released in the cages.

Field releases

The parasitoid was released in first-year seed fields in 2016 and 2017 by placing cocoons in the field margins in four different fields in both years. The study of the effect of the released parasitoid was hampered by the fact that all fields potentially already had a population of both the weevils and the parasitoid, i.e. the control fields were not without parasitoids, even though no parasitoid had been released. In 2016 parasitoid release was done in all experimental fields. This was due to the lack of available white clover seed fields. The part of the field furthest away acted as the control. In 2017, parasitoid release was done in four fields as previously stated and four additional fields acted as control fields. Collection of parasitoids showed an equal distribution in the fields with releases, and it is likely that the parasitoids spread quickly following the release. Significantly more parasitoids were present in the fields in which parasitoids had been released, compared to the control fields. However, the control fields had a larger number of clover head weevils. Samplings of the clover head weevils and parasitoids in 2017 from flower heads, close to harvest, showed that the parasitizing frequency was 24% in fields with released parasitoids while only 13% of the clover head weevils were parasitized in the control fields. These results suggest that the release of the parasitoid increase the parasitizatition frequency considerably, but also that a population of parasitoids already existed which perhaps could be activated.

The results from the project show that the parasitoid *B. curculionis* of the clover head weevil has the potential of being part of a future strategy to increase the seed yield and reducing the application of insecticides in white clover seed production.

It has been shown that a large number of cocoons can be collected from white clover seed fields; however, after harvest, drying and seed processing the number of live larvae in the cocoons is low. This amount can be increased through sorting the cocoons by weight, and the amount of live larvae can be determined by using multispectral image analysis (Shrestha et al. 2018). The sorting of the cocoons from other debris, such as weed seeds was successful; thus it will be possible to release the cocoons in next year's white clover seed field.

The project has identified a range of barriers which must be overcome if the potential is to be realized. The following is to be undertaken:

- development of gentle collection, handling and storage methods
- studies of why and where in the process the larvae die
- studies on to what extent the cocoons remaining in the field can contribute as a resource in next year's white clover seed field.

Besides the above, the following studies were undertaken, but consistent results were not obtained:

- if and how the parasitoid can be combined with insecticides
- in the harvest method influence the mortality of the larvae

4. Introduction

A detailed description of the biology of the insects is found in appendix 1.

4.1 Pest management in white clover seed production

White clover (*Trifolium repens* L.) is a perennial crop. For seed production white clover is established undersown in spring barley and seeds are harvested in the following year. Denmark is the main producer of white clover seeds in Europe. White clover seed production is a certified production and carried out on contract with a seed company.

The yield potential in both organic and conventional white clover seed production is not realized (Boelt, 2005) and by comparison, organic white clover seed producers harvest half of what their conventional colleagues harvest (Hansen & Boelt, 2008, Branceudvalget for Frø, 2018). The low actual yields may result from pest damage due to lack of control of seed eating weevils, seed losses due to inadequate pollination and poor harvest conditions (Langer & Rohde, 2005, Hansen & Boelt, 2008 Topbjerg & Ytting, 2009).

Damage to unripe seeds is primarily caused by larvae of seed eating weevils. Two such species are the lesser clover leaf weevil, *Hypera nigrirostris* (Fabricius) (Coleoptera, Curculionidae) and the clover head weevil, *H. meles* (Fabricius) (Langer & Rohde, 2005, Hansen & Boelt, 2008 Topbjerg & Ytting, 2009). *H. nigrirostris* has been studied in Finland (Markkula & Tinnila, 1956). Little is known about *H. meles* under Scandinavian conditions. It is, however, likely that the lifecycle of the two weevils has similarities (Detwiler, 1923).

In conventional white clover seed production pests are controlled by insecticides. Traditionally one application has been performed just before flowering, but now it is more common with at least two applications. Earlier experiments have shown a yield reduction of 44 percent (average of 5 years experiments) when insecticides are not applied (Boelt, 2005). In general, seed growers are more aware of the occurrence of pests and hence risk of yield loss in white clover and during flowering they monitor the crop closely.

White clover is a forage crop and in the spring of the seed production year, the vegetative growth can be quite vigorous which may have negative effects on the development of inflorescences. Some seed growers will therefor defoliate the white clover before or when the first flower buds appear in the leaf axils. This “defoliation” may also be carried out by grazing sheep. In organic production, a late defoliation (2-3 weeks after the appearance of the first flower buds) is used as a pest management strategy although significant effects have not been verified in field experiments. Distance to the previous white clover seed crops is another management strategy to reduce the prevalence of white clover seed weevils (Langer & Rohde, 2005). For red clover (*Trifolium pratense* L.), evidence for a similar effect of distance to last year’s field has been found for seed pests belonging to the genus *Apion* (Coleoptera, Curculionidae) (Lundin, 2008).

A yet unexploited pest control tool in white clover seed production is the use of biological control agents. Indeed, in red clover integrated pest management guidelines have proven beneficial in the control of seed pests (Lundin et al., 2017) and parasitoids have shown potential as an effective biological control agent, see for example Gerald et al., (2011) and Billqvist & Ekbom (2001).

Biological pest control is mainly used in greenhouse production. In outdoor production systems utilization of beneficial organisms are appearing (Sigsgaard et al., 2011, Sigsgaard, 2015). Outdoor usage is largely restricted to *Bacillus turingensis* against lepidopteran species (Sigsgaard et al., 2011). However, in mainland Europe mass reared *Trichgramma* sp. (Hymenoptera, Trichogrammatidae) parasitoids have started to be used against *Ostrinia nubilalis* (Hübner) (Lepidoptera, Crambidae) (Sigsgaard et al., 2011). In northern America Hale and Elliot (2003) describes an increased demand in outdoor crops. However, development and production costs are barriers for implementation. Although costs are decreasing utilization are only slowly increasing Sigsgaard et al., 2011).

4.2 Potential of biological control of *Hypera* sp.

The starting point for this project was the observations of parasitoid cocoonin the harvested white clover seed. These cocoons has been noticed as far back as 1928 when Rostrup & Thomsen (1928) described the cocoons and linked them to the parasitoid *Bacthyplectes exigua* (Gravenhorst) (Hymenoptera, Ichneumonidae) previously found to parasitize *H. nigrirostris* (Detwiler, 1923). However, this species is very difficult to distinguish visually from *Bathyplectes curculionis* (Thomson) (Hymenoptera, Ichneumonidae) (Rockwood, 1920).

The parasitoid cocoons are harvested together with white clover seed. The crop is swathed and left to dry in the field for three to five days before the swathed material is combined. Clover seeds are small and the harvested material also contains weed seeds, plant debris, small stones and soil particles. The harvested material is dried to approximately 12% seed moisture immediately after harvest, and during the autumn or winter it is delivered to the seed company for seed processing – the separation into two fractions: White clover seed and debris. The previously mentioned cocoons are found in the debris fraction.

Based on these observations the project had the long-term goal to assess the potential of using the observed parasitoid to reduce weevil populations and in this way reduce insecticide application in white clover seed production. It addresses the following hypotheses: Cocoons of parasitized *H. nigrirostris* and *H. meles* can be collected during seed harvest; they can be extracted from debris, stored and distributed in next year's clover seed production field. The distribution of parasitized cocoons will reduce the number of *H. nigrirostris* and *H. meles* in the following year.

5. Part I. The separation of the parasitoid cocoons

The research conducted under part I is described in appendix 2 consisting of studies to separate the cocoons from debris, determination of parasitoid species and the state of the parasitoid larva within the cocoon.

5.1 Introduction

Previous investigations have shown that the harvested material of white clover seed may contain insect cocoons. These cocoons seem linked to a parasitoid on *H. nigrirostris* (Rostrup & Thomsen, 1928). The numbers of *H. nigrirostris* found in white clover seed fields are, however, low and *H. meles* seems to be the most abundant *Hypera* weevil (Topbjerg & Ytting, 2009). In the attempt to utilize the cocoons, firstly, the cocoons had to be separated from the debris, secondly, the parasitoid species had to be identified and thirdly, the fitness of the parasitoid larvae had to be evaluated.

5.2 Materials and methods

Cocoons were obtained from two seed companies (DLF and DSV) in Denmark. The material was provided after the processing of white clover seed, where we picked up the debris from the seed sorting procedure. AU-AGRO has seed processing facilities and trained personnel for seed processing. Through a series of sorting rounds, methods were optimized and the cocoons were separated from the debris. By obtaining the cocoons and hatching adult parasitoids a species determination was possible. Parasitoid mortality was high (97%), and the reason behind the high mortality was investigated by studying cocoon content. We monitored temperature and relative humidity in the swath under field conditions, and the influence of temperature and relative humidity on the parasitoid larvae. Furthermore, larval survival was monitored on-farm and temperature and relative humidity was monitored through the seed storage and seed sorting process. In the experimental plots in AU-Flakkebjerg, the impact of different combine settings during harvest was analysed in 2017. Further details can be found in appendix 2.

5.3 Results

Sorting of the cocoons

In 2015 debris was provided from more than 450ha and the following year from 160ha of organic white clover. It was possible to separate the cocoons from weed seeds, stone and soil particles in the debris. The sorting of the cocoons was improved over the course of the project with introduction of additional seed sorting equipment as experience and performance improved, constantly making the size of the final fraction smaller though containing more cocoons harbouring living parasitoid larvae.

The estimated number of cocoons was 26,000,000 in the first year and 23,500,000 cocoons the second year. When evaluating the number of living larvae within the debris only 3 percent were alive. After sorting, 21 percent (2016) and 48 percent (2017) of the cocoons harboured aliving larvae. This amounted to an estimated 890.000 living larvae the first year and 1.390.000 the second year. The amounts are difficult to compres primarily as the material comes from two different companies with different sorting processes. Therefore it is not possible to give an estimate of the number of cocoons per ha.

Species determination

The parasitoid species was characterized as *Bathyplectes curculionis* (Thomson) (Hymenoptera, Ichneumonidae) by the Zoological Museum, Section of Biodiversity and Environmental Science, University of Turku, Finland.

Parasitoid survuval
Survival rates of parasitoid larvae were between 3 and 29 percent when evaluating the survival of cocoons collected directly at seedgrowers. The high mortality was evaluated, thoug a unambiguous answer to why the mortality was so high, was not found.

Temperature measured in the swath during the harvest process was rarely found to be so high that they reach lethal values. Relative humidity in the swath was not found to decrease into the lethal range.
Subjecting the cocoons to nine different climatic conditions did not produce differences in larvae weight when comparing start weight with the weight after 110 hours.
Survival rate did not change over the time where the swathed material was drying up but it decreased significantly one day after harvest. Further, survival decreased after 18 days in the on-farm drying facility.
Immediately after combining, the drying of the seed is started, thereby avoiding temperature increases, which could reduce seed quality. The seed grower will dry the seed down to 12 percent seed moisture. Measurements of temperature and relative humidity in the harvested seed during the on-farm drying process showed average temperatures of 17 to 20°C and a relative humidity between 77 and 79%, but these values varies according to harvest conditions and outdoor temperature during drying. In the seed company the material was stored at around 19.5°C and 71.6%RH.
Testing different combine settings revealed that an average 18.6 percent of the cocoons showed signs of mechanical damage. However, as all cocoons in the harvested material were dead, the impact of the threshing on larval survival could not be established. This test was performed in 2017 where the weather conditions during harvest of white clover was very humid and the material was left for drying for an extraordinary long period.

5.4 Discussion

Sorting of the cocoons
Optimizing the sorting technique made it possible to separate the cocoons from debris. It was possible to find cocoons containing a live larva in an amount, which allowed for point releases in field in order to monitor the dispersal and influence of the parasitoid.

The amount of debris containing cocoons varied between the years according to production area but also according to harvest conditions and seed processing set-up. The provided material varied between almost 10t in 2015 and 2.3t in 2016. The content of cocoons per weight of provided material depended highly on the seed processing line, which varied between the two seed companies.

We observed a weight difference between living and dead larvae; this differences was used in the separation process. However, this does not provide a completely accurate separation and therefore we also tested other techniques. By using visible/near-infrared multispectral imaging such larvae can be separated from the living (Shrestha et al., 2017).

Dissection of cocoons showed 69 percent of the larvae to be alive (2017) though only 10.5 percent of the cocoons could produce an adult parasitoid. The differences between larval survival within the cocoon and number of adult parasitoids capable of hatching have been noticed previously as 25.6 percent of larvae within their cocoons were seen to be alive but only 9 percent of cocoons produced an adult (Pike & Burkhardt, 1974a). The high mortality rates of the larvae has been observed previously (Pike & Burkhardt, 1974a) and mortality factors such as temperature conditions, predations and hyperparasitation have been identified (Cherry et al.,

1976, Cherry & Armbust, 1975, Hama & Davis, 1983, Pike & Burkhardt, 1974a, Pike & Burkhardt, 1974b).

Observations on the influence of physical factors were performed later.
Sorting of the cocoons is necessary as cocoons only represent a small fraction of the provided material. The major part is primarily seeds from weeds, white clover and cover crop. Introducing this large amount of unwanted seeds in a farming system would be problematic.

Species determination
As far back as 1928 the cocoons in the white clover flower heads had been noticed. Rostrup & Thomsen (1928) described the cocoons and linked the cocoons to the parasitoid *Bacthyplectes exigua* (Gravenhorst) (Hymenoptera, Ichneumonidae) previously found to parasitize *H. nigrirostris* (Detwiler, 1923). The frequent occurrence of these cocoons suggested a link to a pest in white clover seed production. The present cocoons have been verified as cocoons of *B. curculionis.* It should be noted that the cocoons of *B. exigua* and *B. curcolionis* are difficult if not impossible to distinguish visually (Rockwood, 1920). This species has previously not been noticed on *H. meles* weevils.
Species of *Bathyplectes* have been found to parasitize *Hypera* species (Detwiler, 1923, Sechriest & Treece,1963) and have been utilized as classical biological control agents (Bryan et al., 1993, Dysart & Day, 1976). *B. curculionis,* a solitary koinobiont endoparasitoid is used as a classical biological control agent of important *Hypera* pests found in alfalfa (*Medicago sativa* L.) crops. The species has been collected throughout Europe and transported to the USA (Bryan et al., 1993, Dysart & Day, 1976). The parasitoid has been collected from neighbouring countries (Bryan et al., 1993); however, no records of its presence have been found for Denmark. The relationship of the parasitoid with *H. meles* has to the knowledge of the authors not previously been described. Sorting of the cocoons revealed cocoons similar in description to cocoons of *B. anurus* and *B. stenostigma* to occur in the provided material. Whether these parasitoids also inhabited the white clover seed fields was not tested. Future tests would be relevant as especially *B. anurus* seems to have a better functional response and has been seen to displace *B. curculionis* (Dowell & Horn, 1977, Harcourt, 1990).

Parasitoid survuval
When analysing cocoons in the material growers delivered to the seed company larval survival ranged between 3 and 29 percent. Compared to the average larvae survival percentage of 3 percent, the overall survival is astonishingly low and with large variations. Previously, winter survival has shown survival rates of 9 to 16 percent (Cherry & Armbrust, 1975, Pike & Burkhardt, 1974a), though on occasions, all diapausing larvae have been found dead (Armbrust et al., 1972, Pike & Burkhardt, 1974a).

Our results show that the harvest method of swathing and drying the material in the field did occasionally produce temperatures which could be seen as lethal to the parasitoid larvae.

Trials with different combine settings showed no differences between settings. Approximately 19 percent of the cocoons showed signs of mechanical damage.

Inspecting larvae in both the present set-up but also in trials on storage conditions (part II) showed diseased larvae primarily to be brown or blackened. Some having internal spots also seen on living larvae. Discolorations of intestines were also seen on living larvae. Previously, dissection of cocoons has revealed larvae to die of unknown causes (Pike & Burkhardt, 1974b) or host larvae to be infected by entomopathogenic fungi (Dysart & Coles, 1971). A likely explanation would be infection by an insect pathogenic fungi such as *Zoopthora phytonomi* (Zygomycetes: Entomophthorales). Infections have been found to occur on *H. postica* (Radcliffe & Flanders, 1998) and descriptions by Ben-Zeév & Kenneth (1980), Harcourt et al.

(1990) and Hassan (2013) of cadavers infected by *Z. phytonomi* resemble some of the observations in the present report. It was not established if *Z. phytonomi* is the cause of death, but the topic is relevant for future studies.

6. Part II. Storing the parasitoid cocoons

This part describes the work concerning the effect of storage conditions on parasitoid survival. Additional investigations of when free living weevils and parasitoids start their activity in spring were included and followed by monitoring population growth through the growing season. Further details can be found in appendix 3.

6.1 Introduction

After drying the harvested material are stored on farm until the seed company starts seed processing of white clover. In general, seed processing is carried out from August to March/April in the following year. The time span from harvest to seed processing is in average four months. The storage conditions in this period are not optimal for parasitoid survival. Temperature and relative humidity reflect conditions aimed at optimizing seed storage and is therefore above the minimum temperature for development for the parasitoids, reported to be 6.1°C (Eklund & Simpson, 1977).

Being able to predict the time of hatching of the adult parasitoid is beneficial when trying to release the cocoons in order for the adult parasitoid to appear when it is needed.
Storage is used for the time span from separating the cocoons from debris to the time of release. Therefore, the term storage is used for a short-term storage lasting no more than a couple of months.

6.2 Materials and methods

The minimum temperature for development and thermal needs of the parasitoid was estimated in two laboratory trials with three and five different temperatures. In addition, winter survival of the parasitoid was evaluated at four different temperatures and two different storage media. Later, stored cocoons were placed in the field for hatching. Field emergence of the parasitoid and weevil host was monitored through the spring of 2016 in a conventional white clover seed field , and the number of weevil larvae and parasitized larvae was monitored in a non-defoliated white clover seed crop. Further details can be found in appendix 3.

6.3 Results

Thermal needs for the parasitoid to end diapause
The thermal needs of the cocoons were found to be double of what was found previously with a basis temperature resembling previous findings i.e. basis temperature for development 6.5°C and a temperature requirements found in 2016 of 399°CDD and in 2017 476°CDD.

Storage of the parasitoid
For both years, the different storage conditions and material did not influenced larval survival. In 2016, prior to storage 56.6 percent of the cocoons contained a living larvae. After 10 weeks, this was 58 percent. Hatching the cocoons under field conditions showed a dramatic reduction in survival, as only 17.3 percent of the cocoons were able to produce a living adult parasitoid.

In 2017, 84 percent were determined to be alive after winter storage. Hatching under field conditions showed only 12.2 percent capable of hatching. When the cocoons were hatched at 25°C, the number of individuals capable of hatching was double of what had been found under field conditions.

The decrease in survival from winter storage to hatching might be linked to the physical state of the larvae as 27 percent of the living larvae were observed to have internal blackening spots.
Observations of when adult parasitoids emerge under field conditions seemed to correspond well with hatching under laboratory conditions.

Survey on the emergence and seasonal occurrence of the weevil and parasitoid
The majority of the *H. meles* weevils caught in weeks 15 to 16 in 2016 were at the border of the fields. Through the following weeks the dispersal of the weevils into the seed field could be followed.
Weevils were found in a first year field. The field was quite patchy thus, it did not provide an appropriate overwintering site for the weevils and earlier migration into the white clover patches was likely. It seems that *H. meles* migrate by walking and only occasionally migrate by flight. The first *B. curculionis* parasitoids were caught in week 20 to 21.

The highest number of weevils and parasitoids were seen in weeks 26 and 27. The parasitation rate was seen to be highest in week 29.

6.4 Discussion

Thermal needs for the parasitoid to end diapause
In the current project, the thermal needs were around double of the findings by Eklund and Simpson (1977), i.e. 399 to 476 °CDD. Eklund and Simpson (1977) described the thermal needs for the parasitoid having a base temperature of 6.1°C and a thermal need of ~236°CDD.
The individuals studied by Eklund and Simpson (1977) derived from the releases made by the USDA and associates focusing on *H. postica* on alfalfa. These *B. curculionis* individuals originated primarily from collections in alfalfa fields situated in the Southern European climate, though collections in Sweeden was also carried out (Bryan et al., 1993, Chamberlin, 1924, Chamberlin, 1926, Dysart & Day, 1976).
Evidently *B. curculionis* having *H. postica* as a host would derive from a warmer climate region and target a host having a different behavior than *H meles* on white clover.
The temperature needs of the parasitoid would seem to be dependent on hosts preferences and origin of collection. For *B. anurus*, also introduced to control *H. postica*, Moore (2014) found genetic variations correlated to temperature.
Adult parasitoids were used in a range of experiments. Hatching the parasitoids under laboratory conditions corresponded well with the observed thermal needs.

Storage of the parasitoid
Larva survival was not significant diferent between the storage materials and storage conditions. However, storage would preferable be done at the lowest temperatures and preferable in the buckwheat husks.
Storage at positive degrees accelerates the development of the adult parasitoid.
Previously, storage of the parasitoid larvae within their cocoon has only been of interest for experimental purposes in the sense that field-collected cocoons were stored until needed. Storage for six weeks at 4°C had been found to be sufficiently for braking diapause (Dowell & Horn, 1978). The parasitoid larvae in its cocoon has been shown to tolerate 10°C for two months (Bartell & Pass, 1978) prior to hatching. The parasitoids larvae in its cocoon can also be expected to survive storing at 4°C for six months (Dowell & Horn, 1977) or 5°C for five to eight months (England, 1995).
The comparison of field hatching and hatching at constant temperatures showed differences in the number of parasitoids capable of hatching i.e. parasitoid larvae, collected in its cocoons, capable of developing into a adult parasitoid. The differences were seen across storage environment and storage material and suggest that a high number of parasitoid larvae died under

the conditions in the field hatching experiment, which could be linked to lethal temperatures in the hatching setup.

The discolorations on the larvae might be connected to fungal infection. The inspected dead larvae were brown or blackened. Some having internal spots, also recognizable on living larvae. Discolorations of the intestines was also seen. The presence of fungal infections was not tested but would be obvious for future studies. Descriptions by Ben-Zeév & Kenneth (1980), Harcourt et al. (1990) and Hassan (2013) of cadavers infected by *Zoopthora phytonomi* (Zygomycetes: Entomophthorales) seem to resemble some of the observations in the present study.

Survey on the emergence and seasonal occurrence of the weevil and parasitoid
The biology of *H. meles* and *H. nigrirostris* is thought to be similar (Detwiler,1923); thus references on the biology of *H. meles* include a number of *H. nigrirostris* references. Previously hibernating *H. nigrirostris* adults have been found in debris of clover plants (Detwiler 1923).
In mid-April, *H. meles* was found in high numbers in the field borders. In the following weeks the weevil appeared to be spreading into the white clover seed field. As these observations were made in a second year white clover seed field, it is plausible that the weevil had been overwintering at the field border. The observation is in agreement with findings on *H. nigrirostris* (Detwiler,1923, Markkula & Tinnila,1956). Weiss & Gillott (1993) found the majority of *H. nigrirostris* to hibernate within red clover (*Trifolium pretense* L.) and less in the surroundings of the field. Weevils were also caught in the first year white clover field next to the second year field. This first year field was not well established and therefore thought not to provide sufficient winter protection for the weevil. It seems likely that weevil activity started prior to week 15 as noted by Detwiler (1923) as hibernating weevils will be active when temperatures are adequate.
It seems like *H. meles* seldom fly but mainly migrate into nearby seed fields by walking, which is in agreement with findings of Sechreist & Treece (1963) on *H. nigrirostris*. *H. nigrirostris* eggs can be found from the start of June and through to mid-July in Saskatchewan, Canada (Weiss & Gillott, 1993), suggesting a long ovipositioning period.

Compiling observations from the current study and observations in insects tents, flight of *H. meles* was observed after 396°CDD, basis temperature 0.0°C. In tents, flight was observed after 481°CDD. Mating of *H. meles* was observed in early May after 470 to 480°CDD. For *H. nigrirostris* ovipositioning starts at mean daily temperatures of around 10 - 15°C (Markkula & Tinnila, 1956, Sechriest & Treece, 1963, Weiss & Gillott, 1993). Hansen & Boelt (2004) noted that ovipositioning starts at 12°C for *H. nigrirostris*. The ovipositioning period for *H. nigrirostris* lasts for about 30-47 days and within this time an average of 289 eggs (Markkula & Tinnila, 1956, Sechriest & Teece, 1963). Eggs are carefully positioned within the stalk or petiole (Detwiler,1923). Given the *H. meles* likeness with the weevil *H. nigrirostris* the second and third larval stage would appear after 13 days and 22 days respectively (Weiss & Gillott,1993), which is in accordance with findings of Chan et al. (1990) at 25°C on *H. meles*.

H. nigrirostris eggs can be found from the start of June and through to mid-July in Saskatchewan, Canada (Weiss & Gillott, 1993), suggesting a long ovipositioning period for *H. nigrirostris*. Markkula & Tinnila, (1956) reports an ovipositioning period of 47 days and a larval development period is from 14 to 20 days at 17 to 20.4°C. The preferred larval stages for ovipositioning of *B. curculionis* occur through June and into August. Compared to the hatching of *B. curculionis* under field conditions starting in May and ending at the start of July the parasitoid seems synchronized to the long ovipositioning period of its weevil host.

The largest number of newly hatched weevils was found from collected flower heads in weeks 26 and 27 in 2016. Only a weak correlation between newly hatched adult weevils and the number of parasitoid cocoons was found, suggesting non-density dependent host seeking seen earlier (Barney et al 1977, Yeargan & Latheef, 1976).

Species of *Bathyplectes* are adapted to prey on young larvae of *Hypera* weevils, which the parasitoid searched out in their concealed locations (Rockwood, 1920).
The first *B. curculionis* parasitoid was caught in the third week of May, which is the normal time point for the start of defoliation (weeks 21 to 23) representing the start of the white clover flowering season. The flower heads present at the main flowering also have the largest number of damaged seeds, i.e. weevil larval activity seems to be most abundant in these flower heads (Topbjerg & Ytting 2009).

Adult *B. curculionis* were caught in tents after 509°CDD, which would suggest the parasitoid to appear just after mating of the host weevil and the start of ovipositioning. In May the average day temperature is 10.8 °C (Danmarks Meteorologiske Institut, 2018). The temperature at which the parasitoid starts its activity is between 6 and 8°C (Barney et al., 1979). Host searching and oviposition can occur at temperatures above 10°C (Barney et al., 1977). The appearance of the parasitoid seems adapted to the ovipositioning period of the alfalfa weevil, (Schroder & Metterhouse, 1980). The synchrony found between *B. curculionis* and *H. postica,* seem also present between *B*. *curculionis* and *H. meles*.

7. Part III. The release of the parasitoid cocoons

This part concerns the release of the parasitoid in the field. The work evaluates the potential for utilizing the parasitoid as a biological control organism, that is, if the parasitoid influences the population of the weevil *H. meles* and if the cocoons can be utilised in combination with registered insecticides. The efficiency of parasitation was evaluated under laboratory conditions, in insect tents and under field conditions. Further details can be found in appendix 4.

7.1 Introduction

B. curculionis is native to Europe and found widely distributed through Europe and the Middle East (Chamberlin, 1924, Chamberlin, 1926, Dysart & Day, 1976, Gonzalez et al., 1980, Kares & Zarif, 1992). The parasitoid genus holds species having *Hypera* weevils as hosts (Aeschlimann, 1990, Dysart & Day, 1976, Hogg, 1994, Sechriest & Treece, 1963). Investigations on parasitoid effectiveness have primarily concerned strains of the alfalfa weevil (*H. postica*) (Dysart & Day, 1976; Radcliffe & Flanders 1998). The parasitation rate on the alfalfa weevil varies greatly (5 to 95%) (Dysart & Day, 1976, Flanders et al., 1994, Pike & Burkhardt, 1974a) and studies of different *Bathyplectes* species have shown others to be more effective (Dowell & Horn, 1977, Harcourt, 1990) though, effectiveness seems to depend on the strain of the host (Kingsley et al., 1993). The parasitoid has not been reported from Denmark. As collections has been made in Sweden (Bryan et al., 1993) it is highly likely that the species is present in Denmark. In white clover, Rostrup & Thomsen (1928) described the cocoons of the parasitoid but linked it to another *Bathyplectes* species. The cocoons of the two species are however impossible to distinguish visually (Rockwood, 1920).
The parasitoid should therefore have a natural presence in the white clover seed fields and have a *Hypera* species as hosts. To evaluate the effectiveness of the parasitoid work focusing on the effectiveness of the adult parasitoid was undertaken.

7.2 Material and methods

The experiments were a combination of laboratory, semi-field and field trials, with trials on insecticide tolerance and feeding of parasitized and non-parasitized weevil larvae being performed under laboratory conditions. Semi-field setups were used to evaluate parasitoid density and application of insecticides. Field releases were performed in first year organic white clover seed crops.
Density trials involved five levels with 0 and 12 parasitoids per m^2 the first year. The second year densities was increased up to 44 parasitoids per m^2. Number of weevils per tent was the first year six and the second 12 females per m^2.
Assay on the susceptibility of the parasitoid to the active compound in two insecticides were performed with 12 concentrations, with the highest being twice the allowed concentration.
Feeding experiments were performed first as an initial screening followed by an experiment on 242 field collected third and fourth instar weevil larvae. Used parasitoids were hatched under laboratory conditions and allowed to mate.
In the insecticide application trials, first a setup with pollinators and weevils were subjected to insecticides just prior to or after the release of the parasitoid. The second year insecticide application was done with and without the release of adult parasitoids.
To evaluate the between year effect of parasitoid release, parasitoids and weevils were released in a density of 1 per m^2 in a 6.25 m^2 tent. The tents were setup in a white clover seed crop in AU-Flakkebjerg and pollination was carried out by bumblebees.

Field releases was for both years done at four different locations. Fields with no release was available only in one year. Parasitoid activity and weevil density was evaluated at six distances to a central parasitoid release point. Per distance, sampling was done on seven transects laid out in a fan-shaped manor. Further details can be found in appendix 4.

7.3 Results

Release of the parasitoid in different densities

Per attacked floret, the weevil damage was not dependent on number of parasitoids present. A trend of increased seeds yield per flower head was seen as parasitoid numbers increased, revealing a beneficial effect of the parasitoid within year. Introducing excess parasitoids (22 and 44 per m^2) showed no additional benefit. For both years, the number of retrieved adult weevils and cocoons did not reveal differences between treatments.

Parasitized Hypera larvae damage to white clover seed

No overall difference in weight gain was seen between parasitized and non-parasitized weevil larvae, indicating that parasitized larvae do not reduce Hypera larvae food intake and thus their crop damage. The survival rate did not differ between non-parasitized and parasitized third and fourth instar larvae.

Parasitoid release combined with pesticide application

The bioassay revealed both registered insecticides (Karate and Biscaya) influence the parasitoid. At normal doses lambda-cyhalothrin (Karate) was lethal and thiacloprid (Biscaya) killed 18 percent of the parasitoids within 24 hours. At low doses lambda-cyhalothrin was also found leathal and thiacloprid introduced behavior changes.

In the semi-field evaluations, the number of hatched weevils and retrieved parasitoid cocoons were for both years too low for treatment comparisons. Yield components did not produce an overall conclusion, however it was seen that the utilisation of the parasitoid alone did produce similar seed yields per flower head as treatments including insecticides.

The pesticide Karate is known to have a repellent effect on bees. It seems, however, that the effect is more pronounced on bumblebees, as both flower heads with a brown stem and a green stem had a general below average percentage of pollinated florets.

Parasitoid release reduces Hypera seed damage the following year

The experiment could not with certainty establish if the parasitoid had an influence on the weevil population, as significant differences were not found and differences could have derived from other parameters than intended.

Effects of field releases of the parasitoid

Cocoons were released from a single point and transect sampling showed the parasitoid to disperse freely throughout the field. Parasitation did not vary with distance to release-point. Parasitism rate was measured in three years. In 2015 seven fields were sampled without release of parasitoids. In 2016 four fields were sampled with release of the parasitoid and in 2017 four fields with release of the parasitoid was compared with four fields without release of the parasitoid. All fields were first year seed crops, i.e. no field was sampled more than once. For the three years, it seems evident that the introduction of the cocoons increases parasitation. Prior to implementing releases, the parasitation was 7.09±1.6 percent. The next year parasitation was on average 62.3±2.9 percent across the four fields in the study. The third year, the difference between control and release fields showed a positive effect of the introduction with parasitation ranging between 22 to 27 percent when cocoons had been released compared with 13 percent in the control.

In 2016, the number of adult parasitoids hatched resulted in 0.3 parasitoids per m^2 and in 2017 0.11 parasitoids could be expected per m^2.

To evaluate which *Hypera* species was the most common the number of *H. meles* and *H nigrirostris* was counted. In collected flower heads from the three years, only 0.5 percent of the collected weevils were *H. nigrirostris*. The most common *Hypera* weevils in organic white clover seed crops are thus *H. meles*. As the collected number of *H. nigrirostris* was so low, no data on *B. curculionis* parasitation of this weevil were obtained.

7.4 Discussion

Release of the parasitoid in different densities and weevil larvae feed intake

The yield component analysis showed a tendancy to higher seed yields per flower head when increasing the number of parasitoids per m^2. This would suggest either that parasitized host larvae ate less seeds or was not present. Parasitized *H. postica* larvae has been shown to eat less than non-parasitized (Duodu & Davis, 1974a, Armbrust et al., 1970). This was not seen in the current study, as larva weight gain was not significantly different between parasitized and non-parasitized larvae. The seen correlation between seed yield per flower head and increasing parasitoid density must therefore be coupled to a reduction in numbers of weevil larvae. Although parasitation rate did not increase as numbers of parasitoids increased. Further, oviposition seems not to depend on host density (Barney et al 1977, Yeargan & Latheef, 1976) and ovipositioning seems random (Barney et al 1977).

Host defenses seems not capable to encapsulate the parasitoid egg or larvae. Such tendencies have been seen in other *Hypera* species towards *B. curculionis* (Salt & van der Bosch, 1967) thus it would be expected to find high numbers of parasitoid cocoons. Instead, only a low number of cocoons were found. This would point towards multi parasitism or perhaps host feeding. Host feeding of *B. curculionis* has though not been reported in the 77 years of work on biological control of the alfalfa weevil has been carried out by the UDSA and associates (Bryan et al., 1993).

Multiple punctures of the parasitoid *B. curculionis* increase mortality of the larvae of *H. postica* (Duodu & Davis, 1974b) and incedences of premature deaths in young larvae of *H. postica* is high (Bartell & Pass, 1978). As the parasitoid prefers younger larval developmental stages (Duodu & Davis, 1974b) It seems likely that the increase in seed yield per flower head is a result of weevil larvae death caused by multiple ponctuations of the parasitoid.

Parasitoid release combined with pesticide application

The bioassay revealed the both active compounds of the two insecticides registered in white clover for seed production to increase mortalities or severely influence parasitoid behaviour in normal doses. At low dose lambda-cyhalothrin was leathal and thiacloprid introduced shaking and behaviour changes. Combining insecticide and parasitoid would therefore be problematic. Neonicotinoids and pyrethroids are harmful to parasitoids (Kischik et al., 2007, Van de Veire & Tirry, 2003, Willow et al., 2019). This suggests that if the release of the parasitoid is to be combined with insecticide applications, the insecticide will have to be administered prior to the appearance of the adult parasitoids. Due to the half-life of both compounds being from 3 to 6 days (GLEAMS 2001, Fan et al 2013, Mukherjee & Gopal, 2000, Seenivasan & Muraleedharan 2009) it seems not possible to combine the two plant protection agents (insecticide and biological control).

The results of the bioassay showed that foliage ED_{50} values for lambda-cyhalothrin was reached after 40 to 48 days. For thiacloprid foliage ED_{50} values would be reached after 22 to 40 days, resulting in parasitoid behaviour changes and possible death of half of the released parasitoids.

As insecticides are applied at the onset of flowering to prevent weevil activity in the newly opened florets, parasitoid releases would have to occur late in the growing seasons and thereby reducing the effectiveness of the released parasitoid.

In the semi-field evaluations, the number of hatched weevil and retrieved parasitoid cocoons were in 2016 too low to be utilised for treatment comparisons. In 2017, the numbers increased,

though no treatment effect could be seen. Yield components did not produce an overall conclusion though tendencies to yield reductions were seen when applying Karate. Application of Biscaya compared to only release of the parasitoid showed similar seed yields per flower heads.

It was seen, that Karate increased the number of non-pollinated florets more than Biscaya, which was equivalent to non-pollinated florets found in treatments without insecticide application. Therefore, Karate influence the pollinators as previously reported (Ceuppens et al., 2015) indeed both classes of insecticides are harmful to pollinators (Sanchez-Bayo & Goka, 2014). To protect pollinators from lambda-cyhalothrin the applications has to be carryout at times when the pollinators are not in the crop. However as the product has a lasting effect (Miljøstyrelsen, 2019) the return of the pollinators will evidently expose them. Pyrethroids have a repellent effect lasting up to three days (Rieth & Levin, 1988, DLF, 2015). A major part of neonicotinoids is translocated to shoots and can be found in fruits (Alsayeda et al., 2008). This poses a risk of poisoning the pollinators as the compound can be found in bee collected pollen and nectar (Ellis et al., 2017).
The risk of poisoning the parasitoid through feed intake is also present. However, not through the white clover florets as they do not seem to be suited for parasitoid feeding. Instead weed flowers could be the source for a poisoning, as high numbers of parasitoids have be found on flowers belonging to the Cruciferous and Asteraceae family (Hogg et al. 2011) and *B. curculionis* is attracted to dandelion flowers (Jacob & Evans 2000). Aphid honeydew could also be a source of poisoning, as it has been found that honeydew extends the life of the parasitoid (England & Evans, 1997).

Effects of field releases of the parasitoid
Parasitation was not influenced by the distance to the release point. For the three years, it seems evident that the introduction of the cocoons did increase parasitation. In literature, the parasitoid has been described to disperse readily and within years of the release the parasitoid has been seen to travel long distances (Chamberlin, 1926). In the release experiments the parasitoid was evenly distributed across fields.
The recorded parasitation rates seems connected to the release of the parasitoid. Previous parasitation rates of *B. curculionis* on *H. postica* have been found to vary between 6.7 to 65 percent (Brewer et al 1997, Copley & Grant, 1998, Kuhar et al., 2000, Schroder & Metterhouse, 1980, Weaver, 1977), highlighting synchrony between the host and parasitoid (Schroder & Metterhouse, 1980). As there is a large year to year variation (Weaver, 1977), it is possible that the increased parasitation rates seen in the current project is due to such year variation. However, the mean numbers for parasitations do seem connected to parasitoid released i.e. in 2016 0.3 parasitoid per m^2 was released, in 2017 0.11 parasitoid in per m^2 was released. For the two years parasitation was on average 62.3 percent in 2016 and 24.5 percent in 2017.

Parasitoid release reduces Hypera seed damage the following year
The between year evaluation in cages could not establish if the parasitoid had an influence on the weevil population, as differences were not significant. Estimated seed yield per flower head did vary between treatments, though the variation was not significant different. For *H. postica* population declines have been seen to occur when parasitoids were introduced (Schroder & Metterhouse, 1980).

8. Discussion

In horticulture, the use of beneficial insects in augmented strategies has been successively implemented in greenhouse production. In open field agriculture the use of augmented biological control is not well integrated as it is hampered by the costs of producing the number of beneficial's necessary to establish control. However, in outdoor farming systems beneficial parasitoids have started to be applied (Sigsgaard et al., 2011) and an increased demand of beneficial insects for outdoor crops are seen, though development and production costs are holding back the implementation (Hale and Elliot, 2003).

In the USA a "novel" pest was recognized at the turn of twentieth century in alfalfa, the alfalfa weevil *H. postica*. Natural enemies were found in mainland Europe and released as classical biological control (Chamberlin 1924). One of the identified natural enemies was *B. curculionis*. Work with the classical biological control was conducted by the UDSA and related institutes over the last century from the first introductions in 1911 up to 1988. This work compile the state of knowledge on the parasitoid *B. curculionis* in connection to the alfalfa weevil.

The current work describes observations on *B. curculionis* in Denmark and the link of the parasitoid to *H. meles* as a host in white clover. No mention of the parasitoid on *H. meles* has been found until now. Rostrup & Thomsen (1928) reported cocoons found in harvested white clover seed material and linked them with the parasitoid *Bacthyplectes exigua* (Gravenhorst) (Hymenoptera, Ichneumonidae) previously found to parasitize *H. nigrirostris* (Detwiler, 1923). It might actually be observations of cocoons of *B. curculionis* or maybe both, as the cocoons of *B. exigua* and *B. curculionis* are difficult if not impossible to distinguish visually (Rockwood, 1920). This raises the question if both parasitoids are present in white clover. Further, as cocoons resembling those of *B. anurus* and *B stenostigma* has been found in the sorted material of white clover seed and as *B. stenostigma* has been identified in Denmark and neighbouring countries (Dysart & Coles 1971) it is likely that more than one parasitoid is present in the white clover seed crop having *H. meles* as a host.
Preliminary results of DNA barcoding of Mitochondrial subunit 16 S and Cytochrome C oxidase subunit I, suggest the presence of more than one *Hypera* species in the seed crops. A broader host range might also be suspected not just for *B. curculionis* but also for *B. anurus*, *B stenostigma* and *B. exigua,* if present.

The focus of the current work was to evaluate the possibility in using beneficial's towards weevils in white clover seed production.

As of now,

- the parasitoid species have been identified,
- a way to obtain large quantities of the parasitoid cocoons has been found, and
- the effect of short time storage on the parasitoid larvae survival rate has been evaluated.

Further, the parasitation rate of the weevil larvae have been evaluated and if the larvae is hampered by the presence of the parasitoid larva. Studies on releases of the parasitoid combined with insecticide treatment has been carried out and it has been evaluated how increasing numbers of parasitoids influence yield components. Finally, field releases was carried out and a within year benefit of release was seen. A year-to-year benefit of release could not be seen.

The major concern in the current project was the low survival rate of parasitoid larvae within their cocoons. Previously large variation in the survival rates of field collected parasitoid larvae has been found (Pike & Burkhardt, 1974a), though cocoons containing a living larvae seems to be in the area between 16 and 26 percent (Cherry & Armburst 1975, Pike & Burkhardt, 1974a). The variation of cocoons containing living larvae was clearly seen when comparing larva survival at four growers. For all years larval survival rates seemed to average three percent in the delivered material. Dead and living larvae had a weight difference which could be utilized in the sorting of the cocoons. Moreover, multispectral imaging could distinguish between cocoons containing living and dead larva (Shrestha 2018) and might be a future way to handle the sorting.

Studies of temperature and humidity in the swathed white clover material were carried out as well as studies of different combine settings during harvest. Our observations did not identify potential reasons for the low survival rates during these processes. The reason for the many dead larvae within the cocoons may be the drying process of harvested material.

The presence of hyperparasitoids was low compared to what could have been expected. See for example Rethwisch & Manglitz (1986) and Pike & Burkhardt (1974b). Previous predation on the cocoons has been seen to reduce larva survival (Cherry & Armbust, 1975, 1977). Signs of predation was not seen in the current study as opened cocoons would likely have been discarded through the cocoon sorting process or even prior to this, during seed processing.

Descriptions of cadavers infected by *Z. phytonomi* (Ben-Zeév & Kenneth 1980, Harcourt et al. 1990, and Hassan, 2013) seems to agree with findings of larval states in the present study. If the entomopathogenic fungi is present will have to be verified in future studies. If the cause of mortalities is connected to entomopathogenic fungi, determining the route of infection would be of importance in efforts of decreasing larvae mortality. Infections routes could happen through the host, when the parasitoid constructs its own cocoon outside the pupa of the host or perhaps at the harvest or the following processing.

The present study suggest that the survival of the larva is not hampered by short time storage. In a parallel study, this was also seen for storage up to 12 weeks (Fløistrup, 2017).

The parasitoid is synchronized with its weevil host *H. meles* in the white clover seed crop. The synchrony of the parasitoid and *H. meles* larvae seems similar to findings between the parasitoid and its host *H. postica* in alfalfa. However, differences in host larval feeding preferences and thermal needs of the adult parasitoid are present.

Feeding activity of parasitized *H. meles* larvae is not influenced by the presence of the *B. curculionis* larvae. This is different from what has been reported for *H. postica* larvae as parasitized *H. postica* larvae has been shown to eat less than non-parasitized (Duodu & Davis, 1974a, Armbrust et al., 1970). Yield component analysis indicates a higher seed yield per flower head due to less damaged seeds as parasitoids numbers per m^2 was increased. This suggests either that parasitized host larvae ate less seeds or was not present. The parasitation experiments showed no differences in food consumption between parasitized and non-parasitized larvae. It seems therefore likely that the increase in seed yield per flower head is a result of weevil larvae death caused by multiple ponctuations, as reported by Duodu & Davis (1974b)

Insecticide application and release of parasitoids showed overall not to be compatible. The half-lives of the insecticides meant that even if 50 percent parasitoid deaths could be accepted the time of parasitoid release would have to be late in the growing season. Of the two active compounds tested, the influence on the parasitoids were very different. One active compound killing the parasitoids whereas the other affecting the behaviour of the parasitoid.

The project does not deliver a complete management strategy towards weevils in white clover seed production, which requires studies over a much longer timespan. However, the project has established a strong base for further studies on the utilization of the parasitoid.

The study highlights the benefits of the cocoons as a source of value instead of just being part of the discarded debris. The idea of using beneficial's found in harvested seed material has been established and it is our hope that the idea can be expanded and in a longer perspective lead to a reduced pesticide use in white clover seed production.

9. Conclusion

The parasitoid species was found to be a well-known parasitoid *B. curculionis* known to parasitize species of *Hypera* and which previously has been released to control weevil pests.

It was possible to separate the cocoons from the delivered debris from seed processing to an extent where the final amount could be handled.

The parasitoid can be stored and temperature around zero does not influence the survival of the parasitoid under storage. Hatching of adult parasitoids seem to be influenced by storage condition.

Stored cocoons can be released when needed, and the release seems to have a “within year” effect on white clover seed production.

The parasitoid will parasitize *H. meles* larvae. The feeding by parasitized late instar larvae does not seem to be different from non-parasitized larvae. This is when feeding is examined as weight gain.

Combining the use of the parasitoid and application of insecticides needs to be considered. Insecticides impact the parasitoid in different ways.

No evidence of a between year effect of the parasitoid release on weevil population size was achieved.

The vast majority of *Hypera* weevils found in organic white clover seed fields are *H. meles*. No evidence of the parasitoid interaction with *H. nigrirostris* was found.

When releasing the parasitoid, it readily disperses throughout the field.

Parasitoid releases seemed to increase parasitation.

H. meles did not seem to be more abundant in specific parts of the field. It seemed to be well dispersed across the white clover seed field.

10. Perspective

Separating the cocoons and determining the species revealed the presence of a parasitoid previously utilized as a biological control agent towards *Hypera* species. The cocoons were found in vast numbers though the major part of the cocoons contained a dead parasitoid larva. The drying down of the seed seems to have killed a large portion of the parasitoid larvae, however the presence of a entomophagy fungus seems also present. To establish if such a pathogen would be present, work on the cause of larvae death would have to continue.

Although the majority of larvae was found dead the remaining portion was still large. The area on which the cocoons were collected could be envisioned to include both organic and conventional grown white clover. This would increase the area up to more than 4,500ha (Brancheudvalget for frø, 2018), resulting in a higher amount of parasitoids available for release. As insecticides are used in conventional production, the number of cocoons would per area be suspected to be less than what has been collected on the organically managed area. The collection of cocoons from conventionally grown areas and dispersing them in organically managed areas seems not limited by legislation (personal communication Andersen M. NaturErhvervstyrelsen, 2016). By including a larger collection area, it could be envisioned that the needed number of parasitoids per m^2 would be within reach, instead of the low numbers released in the present study.

The large amounts of debris would strain the current sorting process and handling the increase in material would need a processing setup. Fortunately, the seed companies are equipped with machinery capable of handling such amounts. Currently, sorting of the cocoons does not have a high priority and they might be stored for a long period. Storing conditions can be improved based on the present results.

The current end fraction would benefit from a more rigorous sorting. Techniques of such a process are available (Shrestha et al., 2018), and debris difficult to remove by mechanical sorting can be sorted by utilizing multispectral imaging.

The number of cocoons containing a living larvae could be considered to be higher in conventional white clover seed production if direct harvest principals was utilised. By doing so the seeds are matured on the flower stem and not in a swath followed by a drying of the raw-material. Utilizing such a technique might increase the survival of the parasitoid.

The utilized release techniques were not meant to be implemented in practice. The described setups were merely a practical solution to a problem. However, the release is considered possible as either 1) mechanically spreading portions containing 99 to 100 percent cocoons throughout the fields or 2) releasing in biodegradable or reusable sacks with netting allowing the adult parasitoids to venture out into the surrounding area.

During seed harvest, the cocoons are collected in the harvested material and later brought to the seed company. This allows for a field release in the following year.

If the present approach is appropriate can be debated. Other approached such as conservation biological control might prove to be better suited and management principals as no-tillage or introduction of crop strips not intended for harvest but for sustaining the number of beneficial insects might yield similar results. Farmland, in the proximity to groundwater access points and streams is not cultivated. Such areas could be a valuable resource for parasitoids given the seeding of white clover. If floral strips were to be sown in combination with a crop, it should

first be ascertained which floral plantings could support the parasitoid. As the parasitoid has been shown to disperse readily, it would be capable of following the migration of its hosts to new fields.

The fact that a large number of cocoons were found and could be collected and “purified” for future use, might stimulate further work with the perspective to reduce pesticide use in white clover seed production.

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Appendix 1. Biology of the insects

Appendix 1.1 The Clover Head Weevil *Hypera meles*

In Denmark, two *Hypera* species are found as weevil seed pests in white clover. These are the lesser clover head weevil *Hypera nigrirostris* (Fabricius) (Coleoptera, Curculionidae), and the clover head weevil *H. meles* (Fabricius) (Hansen & Boelt, 2008, Langer & Rohde, 2005, Topbjerg & Ytting, 2009). The *Hypera* weevils are, however, not the only seed pests to occur in white clover seed production as species of *Protapion*, primarily the white clover seed weevil *P. fulvipes* (Geoffroy) (Coleoptera, Curculionidae) are known to occur in great numbers (Hansen & Boelt, 2008, Langer & Rohde, 2005, Topbjerg & Ytting, 2009, Markkula & Myllykäki, 1964).

H. nigrirostris has been studied in Finland (Markkula & Tinnila, 1956). However, in Scandinavia little is known about *H. meles*. It is thought that the life cycle and habitats resemble those of *H. nigrirostris* (Detwiler, 1923). Of the two weevil species, it seems that *H. nigrirostris* is the most abundant on white clover (Markkula & Myllykäki, 1964) and on Trifolium in general (Hansen, 1965). Studies performed by Topbjerg & Ytting (2009) and observations in the current work reveal that the overwhelming majority of the found *Hypera* weevils have been *H. meles*. It is the prevalent species countrywide in investigated organic white clover seed fields, conventional white clover seed fields, and other areas in which white clover were found to flower, e.g. ditches and grasslands. It is therefore the opinion of the authors that it is not *H. nigrirostris* which today is the main *Hypera* seed pest in white clover but *H. meles*. How, why and when the shift from *H. nigrirostris* has occurred is unknown.

Adults of the two *Hypera* species *H. meles* and *H. nigrirostris* vary in their color scheme with the former being brown or black, whereas newly hatched individuals of the later are brown at emergence and change to green or blue-green within a few days. Size differences are also seen for the two. *H. meles* average a length of 4.2mm and *H. nigrirostris* is 3.7mm in length (Detwiler, 1923). However, separation of the two is most obvious when examining the shape of the prothorax (Chapin & Oliver, 1981), with *H. meles* having a broad prothorax being one and a half times as wide as its length with the lateral margins being much rounded. The prothorax of *H. nigrirostris* being more wide than long being, that is, one fifth to one sixth as wide as it is long with lateral margins being markedly rounded and without bulge as for *H. meles* (Detwiler, 1923).

Life cycle of Hypera meles

As information on *H. meles* is lacking, the following chapter will include information from observations on *H. nigrirostris* as their life cycles are reported to be comparable (Detwiler, 1923). Both weevils overwinter as adults. *H. meles* hibernate in white clover plant debris (Detwiler, 1923). Apart from white clover *H. meles* can feed on an array of clover species (Smith et al., 1975). Activity of adult *H. meles* in Louisiana has been recorded from the 10th of March to the 20th of September (Chapin & Oliver, 1981). For *H. nigrirostris* activity starts around late April where the weevils begin to feed, mate and oviposit (Weiss & Gillott, 1993). Ovipositioning starts at mean daily temperatures of around 10 - 15°C (Markkula & Tinnila, 1956, Sechriest & Treece, 1963, Weiss & Gillott, 1993,). In Denmark, Hansen & Boelt (2004) mention that ovipositioning starts at 12°C for *H. nigrirostris*. The ovipositioning period for *H. nigrirostris* lasts for about 47 days and within this time an average of 289 eggs are positioned with an average of six eggs deposited per day (Markkula & Tinnila, 1956). Sechriest & Teece (1963) report an

ovipositioning period of just under 30 days and observe that some of their experimental subjects do not oviposit at all. Detwiler (1923) describes how *H. meles* oviposit between one and more than 12 eggs at the time. Eggs are carefully positioned within the stalk or petiole and the female takes care to protect the eggs from desiccation, see Detwiler (1923), who also mentioned that eggs from the same oviposition differ considerably in hatching rates.

The first larval stage of *H. meles* is white and 1mm long. The larva goes through four larval stages, growing to a length of seven mm before reaching maturity. The development takes around 20 days with a complete larval period of approximately 23 days as the larva spends a few days within the cocoon before pupation. During the larval development, the larva become striped green to brown (Detwiler, 1923). The colouring of the larvae acts as an effective camouflage, making the larvae somewhat difficult to find in stands of white clover. Pupae of *H. nigrirostris* appear from late June until August, and new adults appear from mid-July (Weiss & Gillott, 1993).

The developing larvae of *H. meles* feed on florets and developing seeds (Gaynor & Skipp, 1987) sitting headfirst in the calyx, devouring the whole of the floret contents (Detwiler, 1923). For *H. nigrirostris*, the larvae eat both seeds and the green part of the plant, which can result in the destruction of the white clover shoot (Hansen & Boelt, 2008). In relation to adult *H. meles* feeding habits, the former somewhat mimics the feeding behaviour of *H. meles* as the adult primarily feeds on leaf material, especially the leaf stem. Detwiler (1923) found feeding by the adult *H. meles* to result in the separation of leaves from the petiole, and as the white clover starts to blossom *H. meles* adults were found sitting in the florets presumably feeding on pollen (Detwiler, 1923). Further, in the current and past surveys it has been noticed that the adult *H. meles* also feed on the stem of the inflorescence, and the feeding can result in the wilting of the inflorescence (personal observation). This behaviour of the adults represents a potent yield reduction factor.

Chan et al. (1990) evaluated the developmental stages of the H. meles larvae. By using constant temperatures, they found the developmental period from oviposition to adult emergence to differ between 174 days at 16.2°C and 16 days at 39°C. The optimum temperature for larval development lies between 25 and 35°C for egg and pupa, 25 and 37°C for the first, 30 and 37°C for the second and 25 and 37°C for the third and fourth larval instar stages. The percentage of eggs hatched was lowest at the extremes of the tested temperature range (12 and 37°C).

Damage during seed production

Earlier experiments have shown that if insecticide are not applied during seed production of white clover, yield is reduced by 44 percent (average of 5 years experiments) (Boelt, 2005) and even higher losses have been recorded (Hansen & Boelt, 2008). Studies show that the paramount of insect damage is caused by two seed weevils, *P. fulvipes* and *H. nigrirostris* (Hansen & Boelt, 2008, Langer & Rohde, 2005,). Between the two weevils. *H. nigrirostris* causes from 7 to 10 percent of the overall damage (Hansen & Boelt, 2004, Langer & Rohde, 2005,), which is in agreement with findings of Hansen and Boelt (2008) and Lundin et al. (2017). Hansen and Boelt (2008) found *Hypera* activity to reduce yields by 13.4 percent. By recalculation of the values in table 1 in Hansen and Boelt (2008), it was estimated that 15 percent of the damage caused to organic white clover seed yields is due to *H. nigrirostris*. Hansen & Boelt (2008) were able to estimate the economical threshold for *H. nigrirostris*, resulting in a threshold of 0.7 adult *H. nigrirostris* per m^2. Based on a literature review Topbjerg and Ytting (2009) estimated that a *H. meles* larva would damage 35 to 70 white clover seeds from 14 to 30 florets during its larval development stages.

Parasitoids of H. meles

The literature concerning the number of *Bathyplectes,* which have been found to parasitize *H. nigrirostris* is not clear. One or two parasitoids are mentioned. These are *B. exigua* Gravenhorst (Hymenoptera, Ichneumonidae) (Detwiler, 1923) and *B. exiguus* Gravenhorst (Hymenoptera, Ichneumonidae) (Sechriest & Treece,1963). Weiss (1989) noticed that, the two are of the same species. Weiss (1989) did not mention which of the two species was found to parasitize *H. nigrirostris*.

In the following chapter concerning *B. curculionis* it is argued that the cocoons of *B. curculionis* and the cocoons of *B. exigua* cannot be separated as they are visually indistinguishable (Rockwood, 1920). The relevance of distinguishing between cocoons is that one feature utilized to differentiate between *Bathyplectes* species, e.g. *B. curculionis*, *B. anurus* and *B. stenostigma,* is the appearance of the cocoon. Therefore, the parasitoids found on *H. meles* can be *B. exigua* or *B. curculionis* unless the adult parasitoid is hatched form the cocoon.

Appendix 1.2 The Parasitoid *Bathyplectes curculionis*

B. curculionis is a parasitoid on a number of *Hypera* weevils causing damage to agricultural important crops, primarily the alfalfa weevil *H. postica* (Gyllenhal) (Detwiler, 1923, Sechriest & Treece, 1963). At the start of the twentieth century, collection and releases of the parasitoid were done to control the alfalfa weevil (Chamberlin, 1924, Chamberlin, 1926). Cocoons were collected across Europe and shipped to the USA for release in areas where the alfalfa weevil were found as a novel pest. The parasitoid was utilized as a conventional biological control. Throughout the century, the US Department of Agriculture (USDA) released other parasitoids belonging to the genus *Bathyplectes* and parasitoids belonging to other families, e.g. Braconidae, in an effort to control the alfalfa weevil, see for example Dysart & Day (1976).

The *Bathyplectes* genus is a member of the family Ichneumonidae. The genus holds 30 species (Encyclopedia of Life 2019). Several *Bathyplectes* species parasitize *Hypera* spp. including *B. anurus* (Thomson), *B. carthaginensis* (Smits van Burgst), *B. corvinus* (Thomas), *B. curculionis* (Thomas), *B. exiguus* (Gravenhorst), *B. graecator* (Aubert) and *B. stenostigma* (Thomson) (Aeschlimann, 1990, Dysart & Day, 1976, Hogg, 1994, Sechriest & Treece, 1963). Species of *Bathyplectes* are adapted to prey on young larvae of *Hypera* weevils, which the parasitoid searched out in their concealed locations (Rockwood, 1920).
Of the abovementioned parasitoids *B. anurus*, *B. curculionis* and *B. stenostigma* has been introduced to the United States as classical biological control agents of the alfalfa weevil (*H. postica*) (Chamberlin, 1926, Dysart & Day, 1976, Radcliffe & Flanders, 1998). *B. anurus* was introduced in Japan also as a biological control agent towards the alfalfa weevil (Shoubu et al., 2005).

Life cycle of Bathyplectes curculionis
B. curculionis is a solitary koinobiont endoparasitoid on weevil larvae (Coleoptera, Curculionidae). The parasitoid is widely found across Europe and the Middle East (Chamberlin, 1924, Chamberlin, 1926, Dysart & Day, 1976, Gonzalez et al., 1980, Kares & Zarif, 1992). Studies of hosts have primarily focused at *H. postica,* and little to no attention has been given to other potential hosts. Adult parasitoids emerge in spring (Kingsley et al., 1993), having one completed generation per year with a partial second generation. The synchronization with *H. meles* is not known. However, from studies on *H. postica* it has been established that the *B. curculionis* population peaks 1 to 2 weeks before the hosts. A partial second generation is found 1 to 3 weeks after the peak of the host population (Dysart & Day, 1976). Barney et al. (1978) noted that the peak of the first parasitoid population coincided with the peak of the preferred second and third weevil larval instar stage. In the Midwestern states of the USA a delayed second or partial third or fourth generation might exist as adult parasitoids can be found through summer and fall (Dysart & Day, 1976). The thermal need of the species to end diapause and hatch as

an adult has been calculated. With a basis temperature of 43°F (6.11°C), the thermal requirements were 426.7°F degree days (°FDD) (~236°CDD) (Eklund & Simpson, 1977). The thermal needs were later confirmed by Barney et al. (1978).

The adult parasitoid

The adult parasitoid is 3mm long with a black robust body resembling *B. anurus*. Females of the two species can be distinguished by comparing the ovipositor as *B. curculionis* females have a longer ovipositor (Dysart & Day, 1976). Female parasitoids are sexually receptive for up to 48 hours after emergence from the cocoon (Dowell & Horn, 1978). The time of emergence was concentrated around a four-hour period starting at dawn (England, 1995). Studies of the occurrence of functional and non-functional ovaries have shown that on average almost 30 percent of the female *B. curculionis* have non-functional ovaries (Yeargan & Pass, 1978). Females with abnormal ovaries fail to oviposit. This failure has also been noted for females with morphologically normal ovaries (Yeargan & Pass, 1978); indeed, 40 percent of female *B. curculionis* have been found unable to oviposit (Salt & van den Bosch, 1967).

High temperatures increase ovipositioning and shorten the lifespan of the parasitoid (Yeargan et al., 1978). At 21°C, female longevity averaged 8 days. If the temperature fluctuates between 6.7°C (night) and 18.3°C (day), the average lifespan increased to more than 13 days (Yeargan et al., 1978). In the experiment by Yeargan et al. (1978) ovipositioning could be followed for 22 days compared to maximum 14 days at high temperatures. The total amount of eggs positioned was around 400 per female with 82 eggs deposited as the maximum number in one day (Yeargan et al., 1978). Peak ovipositioning occurs after three days after which it decreases (Barney et al., 1977, Yeargan et al., 1978). Interestingly, female *B. curculionis* not exposed to host larvae has a longer longevity than females exposed to hosts (Barney et al., 1977). This could indicate that extra energy is required during egg production.

Feed intake of the adult parasitoid

The longevity and lifetime fecundity is increased, when adult female parasitoids gain access to feed such as sugar, with increasing sugar concentration reflecting positively on longevity (Siekmann et al., 2001). Hunger has been shown to affect behaviour of female *B. curculionis* as unfed individuals are attracted to the floral odour of dandelion flowers (*Taraxacum officinale* Weber), whereas fed individuals prefer the odour of the host plants for *H. postica* (Jacob & Evans, 2001).

By allowing female *B. curculionis* access to a honey-water solution their lifespan increased to above 20 days, access to dandelion flowers also increased parasitoid life span, when compared to either access to water, alfalfa foliage or flowers of *phacelia tanacetifolia* Bentham (Jacob & Evans, 2000). Foraging behaviour has been observed on dandelion flowers; however, the insect cannot gain access to the nectar of the flower (Jacob & Evans, 2000). Jacob and Evans (2000) further discuss how access to quality and accessibility to floral feed could enhance the lifespan of the parasitoid and thereby its effectiveness.

In an attempt to study the different sugars, representing potential feed for adult parasitoids Jacob & Evans (2004) found *B. curculionis* to benefit the most from glucose and fructose. In general, the sugars increased the potential longevity even when compared to aphid honeydew; see Jacob & Evans (2004) and England & Evans (1997). Access to honeydew increases the lifespan and the egg production of *B. curculionis* females, which points towards the beneficial influence of a moderate number of aphids in biological control (England & Evans, 1997).

As described, parasitoid longevity can be enhanced by allowing the insect access to sugars. An application technique could be a direct sugar foliage spray. A 15 percent (w/w) spray was tried by Jacob and Evans (1998). The spray resulted in an increased number of adult *B. curculionis* parasitoids when compared to water-treated control plots. However although the sugar

spray allowed for a ready food supply for the parasitoid, it attracted unwanted fungal growth of on the foliage.

The presence of aphids in a host crop may provide feed for a parasitoid such as *B. curculionis*. It may also increase the activity of predators such as species of Coccinellidae (Coleoptera). These predators is capable of handling larvae of *H. postica* as prey (Ouayogode & Davis, 1981), which in turn also means that ovipositioned eggs and larvae of parasitoids are eaten. In cage experiments, Evans & England (1996) showed that by introducing aphids, parasitism of *H. postica* larvae by *B. curculionis* increased 2.5-fold. If lady beetles (*Coccinella septempunctata* L.) were introduced, the parasitism dropped by 50 percent.

Parasitoid egg and larva
The egg of *B. curculionis* is 2mm long and 0.05mm wide, kidney-shaped with rounded ends. The egg is deposited directly into the host's hemocoel. Here the egg will float freely until logging onto the posterior end of the host's body (Bartell & Pass, 1978b). The preferred host stage is the early instars of the weevil larva (Dysart & Day, 1976, Kingsley et al., 1993). The female parasitoid does not seem to have a preferred oviposition site on the host larva (Bartell & Pass, 1978b).

Prior to hatching, the egg swells to near double in size during a four-day incubation period (Bartell & Pass, 1978b). By examining super-parasitized weevil larvae, Bartell & Pass (1978b) concluded that female parasitoids could return to a host and re-oviposit. When super-parasitism occurs, the parasitoid larvae competes for the host by actively puncturing and tearing the integument of competitors with their mandibles. In general, the first hatched larva will destroy the remaining eggs or attack other newly hatched larvae (Bartell & Pass, 1978b).

The required developmental time for a parasitoid larva to develop through its five instars is around 11 days at 22°C (Berberet, 1982) and 17 days at 21°C. The total developmental time ranges from 13 to 21 days (Bartell & Pass, 1978b). The larvae initial feeds on the hemolymph. As it grows, it shifts to consume fat bodies and other host tissue (Bartell & Pass, 1978b). Considerable differences in the developmental time has been found between non-diapausing and diapausing parasitoid larvae (Bartell & Pass, 1978b, Bartell & Pass, 1978a).

Per weevil host larva, one parasitoid larva can be supported (Hogg, 1994), which ultimately emerges to spin a white-banded brown cocoon. The number of eggs a female parasitoid can deposit per season lies somewhere between 95 and more than 400 (Barney et al., 1977, Barney et al., 1979, Hogg, 1994, Yeargan et al., 1978,), though the number of mature eggs seldom exceeds 140 (Barney et al., 1977). The egg laying activity is proportional to temperature, regardless of temperature regimes being constant or fluctuating (Barney et al., 1979). On average 19 eggs are deposited per day at 25°C, and at 7°C oviposition three eggs are positioned per day.

As the egg of *B. curculionis* is deposited, the defenses of the host larvae may encapsulate the egg (Dysart & Day, 1976). Thus, hindering the egg from hatching. The parasitism is often successful if super-parasitism occurs or if the host larva is in the early larval stages (Dysart & Day, 1976). By dissecting host larvae, Barney et al. (1978) concluded that super-parasitism occurs frequently in the later host instars stages compared to earlier instars. These findings were supported by Berberet (1982), who suggested that later weevil larva instars are parasitized even though they do not represent the preferred larva instar stage.

Parasitoid cocoon
As described earlier, attempting to control *H. postica* in alfalfa saw the introduction of three parasitoids belonging to the genus *Bathyplectes* genus: *B. anurus*, *B. curculionis* and *B. stenostigma*. All species form cocoons that are about 3.5 mm long, brown and having the

shape reassembling a cigar butt or an American football. *B. anurus* and *B. curculionis* form a white band around the middle of the cocoon (Hogg, 1994), whereas *B. stenostigma* does not (Dysart & Day, 1976). Based on the visual appearance of the equatorial white band of *B. anurus* and *B. curculionis* the two species can separated, as the band of *B. anurus* is raised and the band of *B. curculionis* is not. Furthermore, the cocoons of *B. anurus* have the capability of "jumping" when disturbed (Dysart & Day, 1976). The cocoons of *B. curculionis* are indistinguishable from *B. exigua*, a fourth parasitoid of *Hypera* weevils (Rockwood, 1920). In literature, this might have caused confusion in terms of determining parasitoid species as a recognizable key feature for separating the *Bathyplectes* species is the appearance of the parasitoid cocoon.

A fifth *Bathyplectes*, *B. exiguous,* has been found in combination with the Lesser Clover Leaf Weevil (*H. nigrirostris* Fabricius) (Sechriest & Treece, 1963).
B. curculionis has a facultative diapause to which the parasitoid larva can spin a diapausing or a non-diapausing cocoon. The cocoon of the parasitoid is constructed inside the weevil netlike woven cocoon. The coloring of the *B. curculionis* cocoon reveals if it contains a diapausing and non-diapausing larva. Cocoons containing a non-diapausing larva is light brown and more flexible than the thicker walled diapausing dark brown counterpart (Dysart & Day, 1976). Overwintering occurs on the soil surface or in alfalfa litter.

The structure of the *B. curculionis* cocoon has been described in detail by Cross & Simpson (1972). These authors describe that the cocoon to consist of three layers. Non-diapausing larvae need 24 to 30 hours to construct their cocoon and diapausing larvae need 32 to 42 hours (Cross & Simpson, 1972). Early in the season, the fraction of non-diapausing cocoons is higher than later in the season (Parrish & Davis, 1978).

For non-diapausing larvae pupation starts shortly after finalization of the cocoon. Emergence of the imagines happens after 14 to 21 days (Parrish & Davis, 1978). The diapausing larva remains in a pre-pupal stage for the rest of the summer and throughout the cold period. The development from larva to adult starts as the temperatures increase (Parrish & Davis, 1978).

In the current tested biological control strategy, only diapausing cocoons are of interest as only they can be stored during winter and released the following growing season. Both temperature and photoperiod affect the size of the fraction of larvae that enter diapause (Parrish & Davis, 1978). For *B. curculionis* adapted to *H. postica* population in Utah, a relative long dark phase during larval development resulted in less diapausing cocoons. The lowest percentage of diapausing cocoons (3.3 percent) was observed when the scotophase was 15 hours (temperature 7°C and in the light phase 25°C). A scotophase of less than 8h or more than 19h resulted in above 90 percent diapausing cocoons (Parrish & Davis, 1978). In addition, the temperature in the scotophase affected the fraction of diapausing cocoons. The lowest fraction of diapausing cocoons was found when the entire scotophase was kept at 7°C. If part of the scotophase was warmer, the fraction of diapausing cocoons increased (Parrish & Davis, 1978). In addition, the temperature, which the adult experiences in the period between emergence and mating, had an effect. Here it was found that at 27°C during photophase there were approximately 20 percent diapausing cocoons. This increased to 90 percent it the temperature in the photophase was above 29°C or less than 16°C (Parrish & Davis, 1978). Finally, the results by Parrish & Davis (1978) indicated that the age of the female parasitoid also affected the fraction of diapausing cocoons. Older females produced a higher fraction of diapausing offspring.

Using the information from Parrish & Davis (1978) with information on day-lengths and temperatures during July when the white clover flowers and the larval development of *H. meles* takes place, the fraction of non-diapausing *B. curculionis* cocoons in the harvested white clover seed can be assumed not to exceed 10 percent. During July in Denmark, the scotophase is be-

tween 7 and 8 h and the average temperatures during scotophase are around 11.5°C and average temperatures during photophase is around 19.8°C (Danmarks Meteorologiske Institut, 2018). From field studies by Abu & Ellis (1976) the partial second generation only involves few individuals. Indeed, field-collected cocoons revealed that between 33 and 100 percent entered diapause, which implies that the beneficial effects of the second generation towards *Hypera* weevils are minimal even if conditions would sustain a second generation.

Rate of dispersal of the adult parasitoid
As *B. curculionis* was introduced into the USA as a classical biological control agent, its establishment and dispersal could be monitored. Chamberlin (1926) describes how the parasitoid after one year was found 48 km from the release point, after four years the parasitoid had spread to 80 km from the release point and after six years, the parasitoid had travelled up to 370 km. The distribution of the parasitoid seemed to follow the weevil pest as the pest spread to new areas (Chamberlin, 1926). From the above, it is clear that the parasitoid can move over long distances relatively fast, which under Danish conditions implies the parasitoid to be ubiquitous.

Rate of parasitation
The effectiveness of *B. curculionis* to parasitize larvae of *H. postica* varies greatly and values between 5 and 95 percent have been seen (Dysart & Day, 1976, Flanders et al., 1994, Pike & Burkhardt, 1974a). The temperature at which the parasitoid starts its activity is estimated to be between 6 and 8°C (Barney et al., 1979), and host searching and oviposition can occur at temperatures ranging from 10 to 30°C (Barney et al., 1977). As mentioned earlier, *B. curculionis* and also *B. anurus* prefer to oviposit in the younger larval instars of *H. postica*. This behaviour increases premature death of young *H. postica* larvae. Premature death caused by *B. curculionis* and *B. anurus* is 24 and 29 percent respectively (Bartell & Pass, 1978a). Indeed, host mortality seemed to be correlated to some form of host injury made by *B. curculionis* (Yeargan & Latheef, 1976).

Through a growing season, Abu & Ellis (1976) monitored the population size *B. curculionis* and host larvae of *H. postica*. The emergence of the parasitoid coincided with the initial increase of the host larvae. The parasitoid population remained, however, low through the season. As the host population peaked, parasitation was between 6 and 33 percent; later parasitation rose to 68 percent when the host population decreased (Abu & Ellis, 1976), which would suggest parasitation to continue after the peak of the host population. It could also be envisioned that the findings were caused by a steady level of hatching adult parasitoids.

The density of hosts seems not to impact on the number of parasitations (Barney et al., 1977, Harcourt et al., 1977, Yeargan & Latheef, 1976), and the distribution of eggs seemed to be random regardless of host density (Barney et al., 1977). In a two year survey, Rand (2013) found in one year a density-dependent parasitism rate and in the next no influence of host density.

Host defenses
As the egg of *B. curculionis* is deposited, the defences of the host larvae may encapsulate the egg. This encapsulation reduces the effectiveness of the parasitoid (Dysart & Day, 1976). Compared to the other introduced *Bathyplectes* species, the encapsulation seems most pronounced for *B. curculionis* (Dysart & Day, 1976). Larvae encapsulation has been noticed primarily in larvae of *H. brunneipennis* and *H. punctate* (Salt & van den Bosch, 1967), of which *H. brunneipennis* is a second pest on alfalfa.
Observations on *H. brunneipennis* subjected to parasitism by *B. curculionis* have shown that the parasitoid can control up to 33 percent of the weevil population in coastal areas of southern California. However in desert valleys, the parasitation is insignificant, even though both parties was present (Van Den Bosch & Dietrick, 1959). The low parasitation rates was related

to immunity effects of the host weevil, effects which could encapsulated the egg of the parasitoid (Van Den Bosch & Dietrick, 1959). Further Van der Bosch & Dietrick (1959) write that there must be more than one strain of *B. curculionis* and that one of the strains is not fully adapted to *H. brunneipennis*. The authors also notes that if the weevil larvae had been subjected to super-parasitism the change for a parasitoid egg to enclose increased.

Berberet (1982) looked at how encapsulation was related to the immature larvae stages *of H. postica*. Experiments suggested that the risk for a parasitoid egg to be encapsulated increased with weevil larval age. Weevil larva in the first instar stage encapsulated three percent of found parasite egg, whereas weevil larvae in the third instar stage encapsulated 50 percent of the found parasitoid eggs.

A 28 year survey in Oklahoma have revealed, that the encapsulation of *B. curculionis* eggs by *H. postica* have decreases over the years (Berberet et al., 2003) and that encapsulation is more pronounced in the first parasitoid generation than in the second occurring generation (Berberet et al., 2003).

Mortality of diapausing Bathyplectes curculionis larvae
The parasitoid spends around 10 months in the cocoon (Parrish & Davis, 1978). During winter the mortality of diapausing larvae have previously been recorded to be around 70 to 84 percent (Cherry & Armbrust, 1975, Pike & Burkhardt, 1974a). However, records of up to 100% mortality have been noted (Armbrust et al., 1972). Some of the mortalities can be explained by predators and hyper-parasitoids, which will be described separately in the upcoming section.

Dissection of parasitoid cocoons have revealed large portion of cocoons to contain dead larvae with unknown cause of death (Pike & Burkhardt, 1974b). A reason for this seems coupled to the time when the parasitoid larvae starts diapause. Armburst et al (1972) found that the winter survival rate was higher for larvae entering diapause in early season while late season diapausing exhibited a low winter survival. A further explanation might be coupled to an indirect influence of insect pathogenic fungi as seen in studies by (Goh et al., 1989, Los & Allen, 1983, Parr et al., 1993). Indeed, Goh et al. (1989) found a reduced survival rate of the parasitoid of more than 90 percent. It is however likely that the fungal attack will not be expressed as a dead parasitoid larva in its cocoons, as the weevil larva would be destroyed by the pathogenic fungi prior to the formation of the cocoon. Harcourt et al. (1977) mention observations where the weevil host larva dies of a fungal infection after finalizing their cocoon. As *B. curculionis* emerges from the host larva at this same time, it is possible that the fungal infection travels to the parasitoid larva and later kills the parasitoid larva after it has finalizes its own cocoon within the net like cocoon of the weevil larva host.

Mortality due to high temperatures
Other factors, which could influence survival, would be temperature. In controlled experiments lethal temperatures for short time exposure (2 to 4 hours) have been recorded to be 60ºC (Cherry et al., 1976) and 50 percent mortality was seen after exposing non-diapausing cocoons to 43ºC for 1 hour (Hama & Davis, 1983). At 20 percent relative humidity and sub-lethal temperatures, mortality was increased when compared to a relative humidity of 70 percent (Hama & Davis, 1983). At temperatures above 43ºC the humidity did not impose additional constraints on the survival rate (Hama & Davis, 1983).

Mortality due to low temperatures
The diapausing larva in its hard-shelled cocoon can withstand recurring temperatures down to -20°C. It is therefore thought that winter survival under Danish conditions is not a thread to the survival of the diapausing parasitoid larva. Furthermore, it also suggests that storage can be carried out under conditions with freezing temperatures. Subjecting diapausing *B. curculionis*

larvae to freezing temperatures of 0 to -20°C showed that the parasitoid was able of withstanding such temperatures. Survival remained above 85 percent when subjecting the cocoons to six hours of the temperatures above -20°C and below 0°C. By decreasing the temperature to -20°C for 2 to 4 hours, survival decreases to a level above 60 percent at the first exposure. If the temperature recurrently was decreased, additional mortalities were not seen (Cherry et al., 1976). Exposure to -25°C for up to 30 minutes were, however, lethal (Cherry et al., 1976).

Studies on adult *B. curculionis* have shown that the mature stages of the parasitoid can endure freezing temperatures down to -9°C (Berberet et al., 2002). This implies that the adults can appear early in the year and that it can be present long before the first host larva starts to appear.

Predators and hyperparasites on Bathyplectes curculionis

The primary reason for mortalities seems to come from invertebrate predators. Indeed Cherry & Armbust (1975) reported that predation was the primary reason for mortality of diapausing *B. curculionis* larvae, and that this single effect was greater than the combined effect of weather, hyperparasitism and insecticide application.

During field studies and by hatching the cocoons of *B. curculionis* an array of hyperparasitoids has been found. These include species belonging to Chalcididae, Eupelmidae, Ichneumonidae and Pteromalidae (Caldwell & Wilson, 1975, Cross & Simpson, 1972, Dysart & Day, 1976, Ellsbury & Simpson, 1978, Pike & Burkhardt, 1974b, Rethwisch & Manglitz, 1986, Sorenson, 1934, Weaver, 1977). The hyperparasitoids include species, which oviposit in weevil larvae previously parasitized by *Bathyplectes* species. These are either solitary hyperparasitoids or gregarious hyperparasitoids. Some are always female and wingless as the species of *Gelis* (Dysart & Day, 1976). Combinations of different species belonging to Pteromalidae can develop from the same *B. curculionis* cocoons (Pike & Burkhardt, 1974b) and some hyperparasitoids, which oviposit in the weevil larvae, are able of determining if the weevil larvae previously have been parasitized by *B. curculionis* (Ellsbury & Simpson, 1978). Studies of *Mesochorus agilis* Holmgreen (Hymenoptera, Ichneumonidae) have shown that this hyperparasitoid can probe numerous times for a *B. curculionis* larva within the weevil larva. Testing *M. agilis* on young instars of *H. postica* larvae resulted in the death of the weevil larvae. Indeed, only 25 percent of the early instar weevil larvae survived the probing behavior of *M. agilis* (Ellsbury & Simpson, 1978). The results point towards hyperparasitoids as the reason for significant mortality of *B. curculionis* larvae, a mortality that is not recorded when evaluating the emergence of adult *B. curculionis* (Ellsbury & Simpson, 1978).

Evaluations of the amount of cocoons subjected to hyperparasitoids have shown that around 21 percent of *B. curculionis* cocoons are subjected to hyperparasitism (Pike & Burkhardt, 1974b) Under US conditions, the hyperparasitoids seem to limit the effectiveness of the parasitoid in the western states, whereas in the eastern states hyperparasitism appears to be less important (Radcliffe & Flanders, 1998).

As the cocoon of *B. curculionis* lies stationary for 10 to 12 months of the year, it is vulnerable to attacks by predators. Field studies reveal that predation on diapausing cocoons causes the greatest mortality of the parasitoid. Indeed, mortality rates due to predation were higher than the combined effect of weather conditions, hyperparasitism and insecticide application (Cherry & Armbrust, 1975). Two groups of predators have been found to consume the diapausing larvae: *Gryllus pennsylvanucus* Burmeister (Orthoptera, Gryllidae) and various species of Carabidae (Coleoptera). Predation was most severe throughout the months of September and October in which it likely reduced the population size of the parasitoid (Cherry & Armbrust, 1977). The predation intensity peaks in late summer and continues into the fall. The intensity decreased in the winter months and increased again as the temperature increased. The overall winter survival of *B. curculionis* was found to be 15.9 percent (Cherry & Armbrust, 1975).

With the large reduction of the parasitoid cocoons, Cherry & Armbrust (1975) drew attention to how the usage of predators in augmented biological control could influence parasitoid populations in field crops. The authors requests consideration of the utilization of invertebrate predators.
Further, in the absence of aphids, species of Coccinellidae (Coleoptera) have been found to predate on weevil larvae (Ouayogode & Davis, 1981), which would suggest the consumption of both weevil larvae and possible parasitoid larvae.

Efficiency of Bactyplectes *species on the control of* Hypera postica
In the efforts of controlling the introduced weevil pest *H. postica* in the USA, several species of natural enemies were introduced as classical biological control agents with European and Eurasian origin. Researchers have since the release of these natural enemies followed their distribution and effectiveness on *H. postica*. At least nine parasitoids and egg predators have been introduced (Radcliffe & Flanders, 1998) and even more species have been released but not re-observed (Dysart & Day, 1976). As numerous species of natural enemies have been released, a ranking of their effectiveness has been purposed. Within *Bathyplectes* it seems that *B. curculionis* is a more effective parasitoid in the western parts of the US, whereas *B. anurus* is the dominant larva parasitoid of in the eastern parts of the US (Kingsley et al., 1993). *B. anurus* seems to possess a greater reproductive capacity, is faster in its searching and host handling behaviour, has in general a more aggressive behaviour and is not subjected to egg encapsulation as seen for *B. curculionis* (Harcourt, 1990, Dowell & Horn, 1977). Thus, in situations where the *H. postica* larva is the intended host, it is evident that *B. anurus* has a higher functional response when compared to *B. curculionis*. If this is also the case when the intended host is *H. meles* is unknown.

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Appendix 2. Cocoons found in harvested material

Appendix 2 is divided into three main parts. The first part considers the sorting of the cocoons from delivered material. The second part describes the species determination and the third part describes work regarding survival of the larva within the cocoon.

Appendix 2.1 Sorting the cocoons from debris

To clarify which parasitoid and determine the potential hosts the first objective of the study was to separate the cocoons from the debris. Following, adult parasitoid should be hatched to determine the exact parasitoid species.

In the following the sorting of material from 2014 through to 2016 is described. The sorting began in 2015 with whatever material was available for starting of the adjustments of the seed cleaning instruments.
To separate the cocoons from the debris, numerous steps was utilized. Each step sorted more and more debris from the cocoons. Table 1 gives an overview of the utilized equipment. Testing the setups of the instruments started with the most important equipment, a laboratory small-scale air/screen seed sorting equipment (LA-LS, Westrup, Denmark). Later, as large amounts of material were delivered from the harvest in 2015 and 2016, sorting machinery capable of sorting tons of material was used for the initial sorting prior to using the LA-LS, the capacity of which is measured in kilos and not tons. In the final experimental year even finer sorting instruments were utilized on the product from the LA-LS.

Materials and methods

Equipment used for the separation of the cocoons

Table 1 gives an overview of which seed-sorting equipment was utilized through the experimental period. In the following tables the set points from the different equipment are given

TABLE 1. Utilized seed sorting equipment 2015 through 2016.

Year cocoons were delivered	Primary purpose	Position in cocoon sorting process			
		First	Second	Third	Fourth
2015	Equipment adjustment	small-scale air/screen seed sorting equipment (LA-LS)			
2015	Equipment adjustment	Large in-house built setup			
2015	Release 2016	Large in-house built setup	indented cylinder separator (LA-T)	small-scale air/screen seed sorting equipment (LA-LS)	

2016	Release 2017	Large in-house built setup	indented cylinder separator (LA-T)	small-scale air/screen seed sorting equipment (LA-LS)	Air stream separator (LA-Mini-Maxi-Blower)

The small-scale air/screen seed sorting equipment (LA-LS, Westrup, Denmark) includes a system of three sieves and two areas in which light objects can be discarded by suction. he primary collection area is placed after the material have passed through a adjustable light suction, two adjustable sieves and a second adjustable suction capable of removing heavier objects. Numerous settings are possible including flow speed, material amount, suction velocity and hole-shape and size of the sieves. Further, the settings depend on the composition of the delivered material i.e. content of soil, stones and weed seeds.

For the adjustment, white clover seed harvest material samples was collected from the seed company DLF in the summer of 2015 originating from the 2014 harvest. The material was processed in two steps on the small-scale air/screen seed sorting equipment with differences concerning the terminal airflow. The first step separated cocoons from debris containing both dead and living larvae. The second step separated the cocoons containing a dead or a living larva. Settings are given in table 2.

The small-scale air/screen seed sorting equipment is capable of handling 100 kg/hour. The actual debris amounts would be much larger. A larger in-house built setup with two sieves and two suction areas was utilized for the initial sorting. The arrangement discarded large quantities of weed seeds, plant parts, soil particles, small stones etc. The settings is shown in table 3 and established using 600kg of a debris fraction from the seed company DSV.

The in-house built sorting device left a large portion of awns from cereal crops and other monocots in the cocoons fraction. Such plant debris was removed on a laboratory scale indented cylinder (LA-T, Westrup, Denmark) capable of separating objects with different lengths and otherwise similar sizes. Settings are given in table 4.

The end product still contained a large amount of weed seeds. Thus the sorting of the 2016 white clover harvest included a fourth seed sorting instrument.
An small scale seed cleaning device the LA-Mini-Maxi-Blower (Westrup, Denmark). The equipment separates small sample volumes according to aerodynamic behaviour in an air stream. To maximize the quantity of cocoons containing living larvae, each sample was sorted twice. First, light material was discarded and secondly heavy objects were removed. Settings are given in table 5.

TABLE 2. The settings for the laboratory air/screen cleaner (LA-LS) for sorting out parasitoid cocoons from white clover seed harvest debris

	Set point	
	Run 1	Run 2
Flow regulator pre-aspiration	Position 2	Position 2
Flow regulator final aspiration	Position 5.75	Position 7
Flow regulator – air circulation	Position 1.5	Position 2
Sieve movement speed	357 rpm	357 rpm
Inlet feeder speed	Position 2.5	Position 2.5
Sieve position top	Ø2.75mm, hole shape round	Ø2.75mm, hole shape round

Sieve position middle	Ø2.5mm, hole shape round	Ø2.5mm, hole shape round
Sieve position bottom	Ø1.4mm, hole shape oblong	Ø1.4mm, hole shape oblong

TABLE 3. The settings for the in-house built seed sorter utilized for the initial removal of large and small debris objects in the sorting of the parasitoid cocoons

	Set points
Flow regulator pre-aspiration	closed
Flow regulator final aspiration	closed
Sieve position top	Ø4.5mm, hole shape round
Sieve position bottom	Ø1.5mm, hole shape oblong (length 20mm)

TABLE 4. The settings for the LA-T utilized for the removal of awn and small debris objects in the sorting of the parasitoid cocoons. The LA-T was utilized in the workflow after the in house built seed sorter and before the LA-LS air screen seed cleaner.

	Set points
Cylinder speed regulator	1.5
Position of trough collector within cylinder	4 to 5
Inlet feeder speed	3.5
Size of dents on the indented cylinder	5.5mm

TABLE 5. The settings for the LA-Mini-Maxi-Blower utilized for the final sorting of the cocoons. The LA-Mini-Maxi-Blower was utilized in the workflow after the LA-LS air screen seed cleaner.

	Set point	
	Run 1	Run 2
Fan speed regulator	Position 8	Position 9
Time of air stream	15 sec	15 sec
Dampers	Fully open	Fully open

Delivered material 2015-2016

In 2016 DLF delivered 2.5 tons of debris and an estimated 7.5 tons of debris were obtained from DSV. The material delivered by DLF contained enough material for the 2016 field experiments. The material from DSV was cleaned and kept as a backup. The material originated from more than 450ha of organic white clover seed crops. The composition of the delivered material reflected the different sorting strategies by the companies. The DLF delivered material was more compacted than what was delivered by DSV, containing a higher amount of monocot awns.

The material from DLF was delivered in large big bags and was stored in an unheated barn at AU-Flakkebjerg. Upon arrival, temperature and relative humidity were monitored with a data logger (Watchdog 450, Spectrum Technologies Inc., USA). In late winter 2015-2016 the material from DSV was delivered in large wooden crates. Upon delivery, the material was stored close to the seed sorting facilities. Temperature and humidity were not logged.

Separating the cocoons from debris began by utilizing the in-house built seed sorter, followed by sorting on the LA-T and final two rounds on the LA-LS. As previous described, the LA-LS has a high sorting accuracy, however, the speed of the LA-LS is slow. Therefore an initial coarse sorting of the material would be beneficial.

The final primary sorted cocoon product of the LA-LS was to be used in the field trials. A secondary cocoon fraction was collected and stored to supply parasitoids for the trials in which hatched parasitoids were needed. This secondary product originated from the collected material sucked up into the collector at the final LA-LS aspirational area. Cocoons at this secondary collection point were lighter than the primary product and suspected to have fewer cocoons containing a living larva, capable of producing a living adult parasitoid.

After cleaning, the parasitoid cocoons portion was stored in paper bags in a cold storage at 6±0.1 °C (±S.E) and a relative humidity of 85±1%.

Establishing the number of cocoons capable of hatching in the material delivered from DLF 2015-2016

The delivered material from DLF could not be treated within one day. Sorting on the in house built seed sorter took several days. Cocoons sorted at different days were kept separate. From four of these seven cocoon lots three 15g samples were taken from the final sorted portion. The 15 grams were obtained by first taking a portion of 3kg of a cocoon lot. The portion was transferred to a large plastic box and mixed and distributed evenly. Samples were taken with a 30ml plastic cup scooping up the material, ensuring to get cocoons from the entire layer. Per sample, cocoons were hand sorted, counted on a seed counter (Contador, Pfeuffer) using the feed container number 2 (Contador, Pfeuffer), and weighed per replicate on a 3-digit balance (Santorius ED623S-CW).

To establish how the estimated cocoon weight per replicate reflected individual cocoon weight, up to 20 individual cocoons per replicate were weighed on a 7-digit balance (Santorius MC5). Then the cocoons were returned to their replicate and set aside for hatching in 30 ml plastic cups with fitting lids. To allow ventilation the lids had a Ø1cm hole covered by a 0.5x0.5 mm iron netting. The hatching period lasted 51 days at room temperature.

Sorting of delivered material in 2016-2017

In the autumn of 2016, an estimated 2.3t of white clover seed sorting debris was obtained from DSV. The material originated from about 160ha. The material was delivered in seven portions of around 300 to 400kg. This year, DSV was the only seed producer having organic white clover seed production. On arrival the material was stored in an unheated barn at AU-Flakkebjerg. The material was delivered in either big bags or large seed containers.

Upon arrival, inspection of the material indicated that the cocoons were not evenly distributed among the delivered portions. As the sorting of the debris began, samples of 3 to 5kg from the top, middle and bottom of the delivered portions were collected from five of the delivered portions. Samples were mixed and three 140g sub-samples were taken. Cocoons within these sub-samples were picked by hand. The collected cocoons were weighed on a 3-digit balance (Santorius ED623S-CW) and counted on a seed counter (Contador, Pfeuffer) using the feed container number 2 (Contador, Pfeuffer). The collected cocoons were then sorted on the LA-Mini-Maxi-Blower utilizing the set points established previously (table 5). Cocoons were counted and weighed. Depending on the availability of cocoons, i.e. after the sorting on the LA-Mini-Maxi-Blower, up to 12 cocoons per subsample were weighed on a 7-digit microbalance (Santorius MC5) and dissected to establish if the larva was dead or alive.

Prior to cleaning, EBI 20-TH data loggers (Ebro, Germany) were placed on top and at a depth of 50 cm into the white clover seed harvest lots in the DSV warehouse. At sorting, the loggers were removed.

Following the sorting of the parasitoid cocoons, the cocoon fraction was stored in an unheated barn. EBI 20-TH Data loggers were placed on top of the material. Following this, the material was sorted on the LA-Mini-Maxi-Blower. The final sorting reduced the volume of the material

so much that it was now possible to store the cocoons in a refrigerator at 5°C, relative humidity of 70%. The material was pooled in to three batches. Batch one contained cocoons from the original portions 1 and 7, batch two contained cocoons from the original portions 4, 5 and 7 and batch three cocoons from the original portions 2, 3 and 6. These were divided between nine 6.5 l plastic storage containers (18.5x18.5x19cm WxDxH) with perforated lids for ventilation.

Establishing the number of cocoons capable of hatching in the material delivered from DSV 2016-2017

To estimate the number of adult parasitoids capable of hatching, samples were taken from the three final batches. Per storage container three samples (top middle, bottom) of 40 to 70g were taken. The samples were mixed accordingly. From the mixes, 24 subsamples of 1g each were taken. After each sub-sampling the material was stirred. The cocoons within each final sample was sorted by hand, counted and weighed collectively. The cocoons in each subsample was arranged in 30ml plastic cups with perforated lids. The cups were placed in closed transparent polycarbonate boxes (22.4x31x12cm WxDxH). A saturated NaCl solution was added to the boxes to retain a relative humidity of around 70 to 78% at room temperature. After 79 days, the number of hatched adult parasitoids were counted.

The final fraction obtained after sorting on the LA-Maxi-Mini-Blower was to be used in the 2017 field releases. To supply cocoons for other 2017 experiments the fraction of 410g was set aside.

Testing of additional seed sorting instruments, testing the gravity separator

The gravity separator (LA-K, Westrup, Denmark) was tested on material from four of the seven delivered debris portions delivered by DSV in 2016. Settings are given in table 6. The material was taken out of the normal cocoon soring process at the second cleaning round on the LA-LS. For the gravity separator to operate properly the mantle has to be fully covered with material, hence the samples had to be of a certain size. The gravity separator sorts the material into five collection bins. The bins were named A to E, A being closest to the point where the material entered the mantle and E the most distant bin. Each bin had a divider on each side. The dividers were positioned according to how the material travelled on the mantle. Per debris portion (n=4) the cocoons in the collection bins were hand sorted. The number of cocoons per bin and their weight were noted.

TABLE 6. The settings for the gravity separator (LA-K) to test if the seed cleaner could optimize the number of cocoons containing a living larva.

	Set points
Inlet feeder speed	3.75
Sieve movement speed	470 rpm
Air flow	5.5
Tilt direction horizontal on collection bins	0.5
Tilt direction towards collection bins	1.5

Statistics

Data were analysed with the statistical software R (R Core Team, 2017) using the function lm() for linear regression. Pairwise comparisons was done using the emmeans package (Lenth, 2018). Significant levels of $P<0.05$, $P<0.01$ and $P<0.001$ are indicated as *, ** and ***

Results

The companies had two different seed processing lines. Therefore, the composition of the debris varied. The material from DLF were more densely packed than the material from DSV, which on the other hand contained a large amount of monocot awns.

Initially the laboratory air/screen cleaner (LA-LS) equipment was set up with debris material from the 2014 white clover seed harvest. At first, the debris material was cleaned once, revealing cocoons at one sampling point to be significant heavier that what was collected from other collection points (P<0.05 *). It was however evident that a second cleaning would be favourable. The second sorting revealed that cocoons collected at position 4 was significant heavier than what was collected at the other collection sites (3.78mg, P<0.05 *). However the cocoon weight was lower than what had been observed for cocoons harbouring a living larva previously (4.08mg). The average percentage of cocoons with living larva was at the fourth collection point was 50%.

Sorting of delivered material 2015-2016
The delivered 2.5 tons from DLF yielded a primary cocoon fraction of 208 kg. Based on the number of hatched individuals seen in 15g material, the 208kg was estimated to deliver more than 890.000 adult parasitoids. These cocoons were to be used for field releases.

This end fraction was kept in four portions. Inspecting the portions showed differences in the number of cocoons present, their weight and percent of cocoons harbouring a larvae capable of developing into an adult (Table 7). Thus mixing the portions thoroughly prior to the release would be necessary. In this sorted fraction 21.7±3.2 percent of the cocoons contained a living larva capable of developing into an adult parasitoid.

TABLE 7. Average number of cocoons per gram sorted material and calculated average cocoon fresh weight (FW) shown together with percent observed adult parasitoids hatched. Standard errors are shown. Letters represent significant differences P<0.05.

Portion	Cocoons per g material		Cocoons FW (mg)		% cocoons hatched	
04	1.2±0.07	A	3.49±0.23	B	26.4±3.4	B
09	1.2±0.33	A	3.16±0.5	AB	32.8±5.7	B
10	41.3±2.03	B	2.77±0.12	A	7.9±0.3	A
15	3.1±0.23	A	3.14±0.2	AB	19.8±3.1	AB

Cocoons for experiments other than field releases was obtained from the secondary product of the sorting process. Hatching cocoons from this product showed it to contain enough viable parasitoid larvae for the remaining experiments. The female to male ratio was for the primary fraction 70:30.

Sorting of delivered material in 2016-2017
The material was delivered in seven portions. Five of these portions were investigated for amounts of cocoons, cocoon weight and larva status (dead/alive). Cocoon numbers and fresh weight can be seen in table 8. Portions 1 and 4 contained 10 times as many cocoons as the remaining three portions. The average cocoon weight in portions 1 and 4 was significantly heavier than cocoons from portions 2 and 5.

Sorting of the cocoons revealed that only very few cocoons had the expected fresh weight for cocoons containing a living larva (>3.5mg); indeed only portions 1 and 4 could be expected to contain cocoons harbouring a living larva (table 8). The average weight of a cocoon containing a living larva was 3.85±0.11mg. The average weight of a cocoon containing a dead larva was 3.30±0.10mg. The differences between cocoons containing either a living or a dead larva was 0.55±0.15mg (P-value >0.001***). Combined estimations on the five fractions showed that 48 percent of the cocoons contained a living larva.

TABLE 8. Sub-samples (n=3) from five portions of delivered material 2016. Average number (No.) of cocoons found in 140g of the debris from the seed sorting process. Average cocoon

fresh weight (FW), number of cocoon obtained at the final sorting process. FW of the sorted cocoons. Standard errors are shown. Letters represent significant differences (P<0.05).

Portion	No. of cocoons		cocoon FW (mg)		Cocoons sorted		Sorted cocoon FW (mg)	
1	3812±112	C	2.20±0.03	BC	130±3	D	3.65±0.04	AB
2	189±4	A	1.90±0.03	A	0	A	-	
3	349±14	A	2.11±0.03	B	9±1	B	2.83±0.3	A
4	1967±30	B	2.23±0.01	C	98±2	C	3.66±0.03	B
5	272±12	A	1.95±0.02	A	1±1	AB	3.24±0.01	AB

Dissection of cocoons from the five portions only showed cocoons to contain a living larvae in portion 1 and 4.
The average cocoon weight was 2.08 mg for cocoons found in the initial delivered debris. The weight of the cocoons in the final sorted fraction averaged 3.35mg. On average 3.6% of the cocoons delivered would end up in the final fraction.

Combined the portions weighed 23.85 kg with an estimated 1.565.332 cocoons. As portion one contained a large amount of heavy cocoons capable of producing adult parasitoids only this portion were to be utilized for the field releases. Based on sub-sampling (n=24) of portion one; dividing the number of cocoons by the number of adult hatched, the estimated percentage of larva capable of developing into an adult parasitoid was 10.5±0.6 (S.E) percent.

In 2017, 49 ha with organic white clover seed production was chosen. Field size varied between 10 and 15 ha (n=4). By release of portion 1, an estimated 1.393.528 cocoons would be released. If 10 percent of the cocoons would produce a living adult parasitoid, the released amount would produce 0.28 parasitoid per m^2.

The weight of Cocoon material used in 2017 experiments besides the field release
The portions of cocoons picked out for experiments other than the 2017 field releases consisted of an estimated 64.574 cocoons with an average fresh weight of around 3.64mg. This amount would be more than sufficient for the trials conducted besides the field releases.

For the cocoon portion intended for other experiments than the field releases, the number of larvae capable of developing into adult parasitoids was estimated to 33.3±1.72 percent with a female to male ratio of 70:30 when hatched at 22.39±0.01°C and 73.81±0.02 % RH.

The weight of cocoons containing a living or dead larvae
Of 96 cocoons picked out to assessment if the larva within the cocoons was dead or alive, 68.8 percent was alive and 30.2 percent was dead. The average fresh weight of a cocoon containing a living larva was 4.03±0.07mg. Cocoons containing a dead larva weighed 3.54±0.09mg. Pairwise comparisons showed the fresh weights to be significantly different 0.49±0.12mg (P-value: <0.001***).

The living larva weighed 3.39±0.063mg and had a dry matter weight of 1.67±0.07mg. the water content was 50.13±2.01 percent. Dead larvae were not weighed due to their physical state of decay.

Based on the 96 cocoons, the percent of cocoons containing a living or dead larva is seen in figure 3. In the figure the cocoons are divided into eight bins depending on cocoon weight. Even though the majority of the cocoons contained a living larva, there is still a fraction of the cocoons containing a dead larva.

FIGURE 3. Percent of cocoons with either a dead (light brown) or a living (green) larva. Bin size is 0.5 mg.

Testing of additional seed sorting instruments, testing the LA-K

The used set points for the LA-K is seen in table 9. Positioning of the dividers per bin was changed according to how the material of the four samples behaved on the mantle of the LA-K; hence, no direct set points are given.

TABLE 9. The number of cocoons and calculated average fresh weight (FW) of the cocoons from the five LA-K collectors. FW was calculated by dividing the combined weight for the cocoons per replicate with the number of cocoons found per replicate. Standard errors are shown. Letters represent significant differences P<0.05.

Collector	Number of cocoons	Average FW of cocoons (mg)
A	106±5 C	2.31±0.09 A
B	67±13 BC	2.65±0.15 AB
C	52±14 AB	3.07±0.22 AB
D	18±12 A	3.29±0.29 B
E	-	-

In table 9 the number of cocoons collected is seen to be highest in collector A, representing the minimum traveling distance from the site of material entering the mantle. As the distance increases the number of cocoons decreases. Based on the weight, the majority of cocoons collected in collector D should harbour a living larva. The material in collector E was not included as it contained almost no cocoons. Weight differences in table 9 show differences between the cocoons ending up in collectors A and D. The separation between the collectors are around 0.3-0.4mg, which, compared to figure 3, highlights the benefits of utilizing the LA-K in future studies.

Discussion

In the provided harvested debris material the number of cocoons containing a living larva was around three percent. By using the sorting equipment the percent of cocoons harbouring a larva capable of developing into an adult was increased. The first year 21 percent of the cocoons for release were estimated to contain a larva capable of producing an adult parasitoid of which 70 percent were expected to be female. The male to female ratio was similar both

years, indicating a stable ratio. Previously, the ratio has been found to 64:36 female:male (England, 1995) suggesting a plausible female to male ratio.
The second year the number of cocoons containing a larva capable of producing an adult parasitoid was estimated to be 48 percent. Estimates of adults to release would be 890,000 and above 1,390,000 for the first and second year respectively. The differences in potential adults for release are different between the two years. Also, the area on which the material has been collected was very different. The first year material from more than 450 ha was provided. In the second, material from around 160 ha was provided. The differences in material and area of organically grown white clover would imply a non-steady supply of debris material and therefore parasitoid cocoons. The content of cocoons per weight of delivered material depended highly on the seed sorting process at the seed sorting facilities. An example would be the supplied by DSV, which between the two years changed their seed processing line. The first year the company provided around 7.5t of debris material and the second year 2.5t from a growing area largest in the second year.

When dealing with the actual release of the cocoons, it was realised that the numbers of adult parasitoids hatched was much lower than initial estimates. Differences could be thought to come from an inaccurate sampling strategy or mortality in the later parts of the diapause. When dissecting cocoons, larvae determined as alive often showed blackening discolorations of internal organs and/or containing white rounded bodies, which might reflect infections of some sorts leading to the demise of the larvae.

Dissection of 96 cocoons showed that 69% of the cocoons contained a living larva. However, compared to the number of cocoons capable of producing a living adult (10.5%), it is obvious that the transition from diapause through pupation and metamorphosis influence the parasitoid population. Previously, mortality of diapausing larvae of 70 to 100 percent has been noted (Armbrust et al., 1972, Cherry & Armbrust, 1975, Pike & Burkhardt, 1974a) and dissection of cocoons has revealed large portions of cocoons to contain dead larvae with unknown cause of death (Pike & Burkhardt, 1974b). Fungal infections have proved to reduce survival by more than 90 percent (Goh et al., 1989). Other reasons for parasitoid larval death could come from hyperparasitism, which has been found to be responsible for 21 percent of parasitoid larval death (Pike & Burkhardt, 1974b). Hyperparasitism would result in the present of an ectoparasitoid hyperparasitoid larva attached to the *B curculionis* larvae. Indeed hyperparasitoids have been found, though in a lower number (appendix 3) than what was observed by Pike & Burkhardt (1974b).

Whether the discoloration would originate from developing endoparasitic hyperparasitoids would be worth pursuing in future research. The thought of the discoloration to be linked to a fungal infection seems more appropriate and future efforts into identifying the reason of the discoloration would be beneficial. A potential entomopathogenic fungus could be *Zoopthora phytonomi* (Zygomycetes: Entomophthorales), which has been found in combination with *H. postica*, see for example Radcliffe & Flanders (1998) and a negative interaction between *B. curculionis* parasitism and fungal infections of *H. postica* has also been found (Morris et al, 1996). It would seem likely that a pathogenic fungi infecting the host larva would also be found in the internal organs of the parasitoid larva. However, susceptibility of the parasitoid larva is unknown.

A second string of inquiry relevant to the low survival rate of the parasitoid larva would be environmental condition around the time of harvest. Here conditions of high temperatures and low humidity could arise. In section 6.3 such differences were evaluated.

In a large release program towards the Alfalfa weevil, the Unites States Department of Agriculture released more than 78,000 *B. curculionis* adults from 1957 to 1988. Parasitoids were col-

lected in alfalfa fields either as parasitized host larvae or parasitoid cocoons. Collected parasitoids were either released in their larval stage or as adults (Dysart & Day, 1976, Radcliff & Flanders, 1998). Compared to the current study the number of obtained individuals seems low; however, as the efforts of the USDA concerned the alfalfa weevil feeding on the foliage of alfalfa, it seems appropriate to focus on parasitoid collection and distribution from alfalfa fields and to look for potential parasitoids in other leguminous crops. To the knowledge of the authors, *B. curculionis* has not previously been seen as a parasitoid of *H. meles* although the cocoons of *B. exigua*, a reported parasitoid on *H. meles* (Detwiler, 1923), and *B. curculionis* cannot be separated by visual clues (Rockwood, 1920).
Following this, the life cycles of the alfalfa weevil *H. postica* and *H. meles* are quite different with the larval stage of the former eating foliage and developing shoots and the later seeds of its host plant. The appearance and synchronization of pest and plant hosts must therefore reflect how the parasitoid is synchronized with its host. Later (appendix 3.1) observations on the accumulative temperature needs of *B. curculionis* found on larvae of *H. meles* are higher than what has been reported for *B. curculionis* on *H. postica*.

In both years the cocoons intended for field releases were at first kept as separated portions. When analysing the portions, differences in the number of cocoons, their weight and number of larvae capable of hatching as adults were seen. This evidently implies large differences in the density and survival rate of the parasitoids between the fields from where they originate. This could also point towards differences in post-harvest management and storage of the harvested material. The same tendency was seen when comparing samples from four growers showing larval survival from 3% to 29% (appendix 2.3). A stable supply of cocoons containing a larva capable of developing into an adult parasitoid might not be attainable.

Even though large efforts were spent on separating cocoons containing living larvae the presence of diseased larvae was obvious in the final sorted fraction. In figure 3 the weight distribution of living and dead larvae concealed in their cocoons is shown, and a clear weight overlap is shown. Sorting equipment capable of sorting out fractions as low as 0.3 mg such as the gravity separator (LA-K, Westrup, Denmark) would increase the percentage of cocoons containing a living larva, however, it would also discard a portion of cocoons containing living larva. Another technology, which seems promising in the separation of cocoons is visible/near-infared multispecteral imaging (Shrestha et al., 2018).

Appendix 2.2 Species determination

The identification of the pest and parasitoid started with individuals of the pests and parasitoids collected prior to the start of the project (2012) and continued with individuals found in the project in the delivered white clover seed harvest material from the growing season of 2014 to 2016. Furthermore, living individuals were collected in the 2015 growing season. Animals were collected from University of Copenhagen Campus Taastrup, Aarhus University - Flakkebjerg and at one organic white clover seed grower.

Identifications started by using insect identification keys. Specimens of the weevil pest were confirmed by the BioSystematics section, the Natural History Museum of Denmark for confirmation. The weevil pest was confirmed to be *Hypera meles* (Fabricius) (Coleptera, Curculionidae).

Using identification keys the genus of the parasitoid was determined as *Bathyplectes* (Hymenoptera, Ichneumonidae). The final identification of the species *B. curculionis* (Thomson) was done by Reijo Jussila at the Zoological Museum, Section of Biodiversity and Environmental Science, University of Turku, Finland. Later the species was reconfirmed by Lars Vilhelmsen from the BioSystematics section at the Natural History Museum of Denmark.

The vast majority of the collected cocoons resembled the cocoons of *B. curculionis*. However, from time to time hand sorting came across cocoons resembling the cocoons of *B. anurus* and *B. stenostigma*. However, if the host of all three parasitoids was *H. meles* is not further investigated and no effort was put into separating the cocoons based on the character traits of the three parasitoids. However, for future notice, it would be relevant to see is if especially *B. anurus* inhabits the white clover seed fields as this species has been found to possess a superior functional response compared to *B. curculionis* (Dowell & Horn, 1977, Harcourt, 1990).

Sorting of the cocoons revealed cocoons similar in description to cocoons of *B. anurus* and *B. stenostigma* to occur in the delivered material. If however these parasitoids also inhabits the white clover seed fields were not tested. However, it would be relevant as especially *B. anurus* seems to have a better functional response and has been seen to displace *B. curculionis* in the USA (Dowell & Horn, 1977, Harcourt, 1990).

Hyperparasitism of the parasitoid *B. curculionis* was observed. Several hyperparaistiod species were found. Of them, a wingless species was determined to be the genus of *Gelis* (Hymenoptera, Ichneumonidae, Cryptinae) by the BioSystematics section at the Natural History Museum of Denmark. Later the species was determined to be *G. kiesenwettri* (Foster) by Martin Schwarz at Biologizentrum, Austria. Besides *G. kiesenwetteri* two species, belonging to the family Pteromalidae (Hymenoptera, Calcidoidea) have been found. For genus and/or species determination specimens have been sent to the Stuttgart State Museum of Natural History. Unfortunately, no determination have yet been made on the two Pteromalids.

Previously, the primary *Hypera* species found in white clover seed production was the lesser clover leaf weevil *Hypera nigrirostris* (Langer & Rohde, 2005, Hansen & Boelt, 2008). *H. meles* has previously only once been found to be the primary *Hypera* weevil pest species (Topbjerg & Ytting, 2009). It is unknown when and if a shift in population distribution has occurred. In the current, the vast majority of found *Hypera* seed pests are *H. meles*.

It is also possible that a third *Hypera* species occurs in the white clover seed fields. Preliminary results of DNA barcoding of Mitochondrial subunit 16 S and Cytochrome C oxidase subunit I on collected *Hypera* adults seem to suggest a presence of *H. formicata* (Penecke) or a unknown *Hypera* species resembling *H. formicata* within the sampled material (personal communication M. Stockholm). Visual division of *H. meles* and *H. formicata* can be done based on the shape of the prothorax; however, the differences are much more subtle than what is seen between *H. meles* and *H. nigrirostris*.

In the current study, a third Curculionidae was numerous times observed at the time when *H. meles* started its spring activity. This third Curculionidae resembled the clover seed weevil *Tychius picirostris* (Fabricius) (Coleoptera, Curculionidae) (syn.: *Miccotrogus picirostris* (Fabricius)) and was found in hatching traps placed in white clover litter and in hedgerows of fields which the previous year were white clover seed fields. The weevil species has not been determined. Based on descriptions of Fabricius, Detwiler (1923) summarized its origin as being close to Copenhagen, Denmark.

Appendix 2.3 Cocoon content

As described previously the cocoons from the seed sorting facilities revealed the majority of cocoons to contain dead larvae. Cocoons were found to contain an unrecognizable dry mass rather than a live larva. The first delivered cocoons all contained contain dead larvae, later as the material from the 2015 harvest was delivered adult parasitoid could be hatched. However the larva survival rate was only three percent.
In an attempt to find the reason for the low survival different surveys were carried out these were: 1) Amount of cocoons containing living parasitoid larva. 2) Monitoring the temperature and relative humidity in the swathed material. 3) Examining the ability of the parasitoid larvae

to withstand high temperatures and low relative humidity. 4) Dissection of cocoons found in freshly harvested material and through the drying process at a farmers on-floor drying facility. 5) Temperature and relative humidity was monitored through the storage and drying of the white clover seeds at a grower and at the seed sorting facility. 6) The effect of different combine threshing settings on the survival of the larvae were to be investigated.

Materials and Methods

Cocoons containing living parasitoid larva

Samples were collected from the grower's seed storage and a seed cleaning facility. The cocoons in the two samples were hand sorted, weighed on a 7-digit microbalance (Santorius MC5) and dissected to establish if the larva was living or dead.

From the 2015 harvest 44 samples were delivered originating from 15 growers. To establish if growers had the same ratio of dead cocoons, 100 cocoons per sample were handpicked and weighed (100 cocoon fresh weight) on the 3-digit balance (Santorius ED623S-CW). Based on the weight, four samples were chosen, two samples with high cocoon weight and two with low cocoon weight. From these four samples 215 cocoons were picked out to establish the condition of the larvae within the cocoons. Cocoon weight was recorded followed by dissection to establish the state of the larva. Related to the analyses the four growers were contacted and interviewed concerning the harvest and later storage of the harvested produce.

Additional, two samples from conventional white clover seed production were delivered by the company DSV. Cocoons were sorted on the laboratory small-scale air/screen seed sorting equipment (LA-LS, Westrup, Denmark). All cocoons in the two samples were sorted from the debris and fresh weight recorded. Hereafter 32 cocoons were picked at random for dissection.

Monitoring the temperature and relative humidity of the swath

In the process of making the swath, the white clover heads are positioned in the upper part of the swath. Here, the flower heads are subjected to conditions which lead to a decrease in relative humidity. Thus fatalities among the parasitoid larvae could occur. At AU-Flakkebjerg, a white clover seed plot strip was used to determine possible temperatures and humidity in the swath. Different swathing dates were used to detect variation due to weather conditions. These were performed on July 20, August 11, August 18 and August 25 during 2016. The swathing was done using an experimental grass seed harvester (harvest width 1.55m) capable of collecting the cut material.

The harvested material was spread out in plots of 1x1m in layers representing 0.5, 1 and 2 times the amount of material harvested per m^2. The first swath had three replicates of each swath thickness. The later swaths had four replicates. Plots were randomly laid out shoulder by shoulder with the swath in the middle of the plot. The adjacent areas were left untouched.

To determine swath temperature and relative humidity a data logger (EBI-20 TH, Ebro, Germany) was placed in the upper 1-2 cm of the swathed plant material. Temperature of the surface was measured with an infrared temperature meter (TFI-54, Ebro, Germany) each day at 9am, 12pm and 3pm.

Capability of the parasitoid larva to withstand extreme conditions

In the start of august 2016, a large number of harvestable flower heads were collected from one of the growers participating in the field release experiment. The collected white clover flower heads were all examined for *B. curculionis* cocoons. Found cocoons were stored under outdoor conditions in 30ml plastic cups with perforated lids for ventilation. A total of 190 cocoons were retrieved from the collected flower heads.

The number of living larvae were determined by dissection on 19 individuals before the experiment. A living larva was defined as a larva with a yellow to white colour and moving when touched gently. The remaining cocoons were kept for up to 7 days in nine different treatments consisting of three temperature regimes: 15°C/15°C, 25°C/20°C and 40°C/20°C Day/Night and different relative humidity of: 20%, 70% and 100%. Daytime lasted 16 hours and nights lasted 8 hours.

Cocoons were kept separated in 96 well microtiter plates. The plates were covered with a thin layer of gauze held tightly with a rubber band. In total 15 cocoons were used per treatment. Climate chambers (KK 500, Pol-Eko Aparatura, Poland) were used to maintain the temperatures. To obtain the various relative humidities, glycerol-water solutions based on the calculations of Forney and Brandl (1992) were used. Per climate chamber, three closed transparent 6.5 l plastic storage containers (18.5x18.5x19cm WxDxH) were used to obtain the relative humidity. Per box 0.15ml of the calculated glycerol-water concentration (w/w%) was added. The microtiter plates were positioned above the glycerol-water solution by utilizing a galvanized iron plate (15.5x14.5cm) with 1.0x1.0cm square holes positioned on top of four 30ml plastic cups fixed to the iron netting by sticky tack. Temperature and humidity was logged (EBI-20 TH, Ebro, Germany).

The experiment lasted seven days. After 14, 38, 63, and 110 hours, larva tolerance to the treatments was evaluated by weighing and dissecting cocoons to establish if the larvae were alive (n=3).

Collection of flower heads and parasitoid cocoons at harvest
White clover flower heads were collected 3 and 7 days after the swathing. Later, as the harvested white clover seed material was transferred to the second grower's seed drying facility, samples of the harvested material were taken 1 and 18 days into the drying process. Sample size depended preliminary observations of how many flower heads would be necessary to obtain around 200 cocoons. Samples were transported in large 51x21x15.5cm (HxWxD) block bottom paper bags sealed with large paper clips and stored under outdoor conditions. For comparisons, harvestable flower heads were collected prior to swathing at a second grower. Samples were examined for parasitoid cocoons as quickly as possible. The weight of the cocoons were recorded on a 7-digit microbalance (Santorius MC5) and dissected to establish if the larva was alive. If alive, the weight of the larva was recorded. If possible, dead larvae were removed and weighed. The weight of a dead larva was only obtained under circumstances where the larva had either dried out or the skin of the larva was intact.

Monitoring the temperature and relative humidity when drying the white clover seed at the grower and at the seed sorting facility
At the seed drying facility of one of the participating growers, temperature and relative humidity loggers (EBI-20 TH, Ebro, Germany) were placed in the pile of harvested white clover seed material. Loggers were placed at a height of 30-40 cm and between 70-80 cm above floor level. The data loggers were fastened to a stick and driven into the seed pile. The pile had an estimated height of 90cm. The loggers recorded the temperature and relative humidity in the pile for 18 days. Unfortunately, the data loggers were disturbed after day 12.

After 18 days of drying, the seed crop was transported to one of the seed sorting facilities. At the time when the seed company had collected all the white clover seed harvest, six temperature and humidity loggers (EBI-20 TH, Ebro, Germany) were placed in three large containers containing the delivered material from two of the participating growers. Loggers were placed at a depth of 0 or 50cm measured from the surface of the seed pile. The loggers were left in the material until the seed sorting process (27 days), where after the loggers were transferred to two large containers containing debris from the seed sorting process. The containers were later shipped to Aarhus University-Flakkebjerg for the sorting of the parasitoid cocoons.

Identifications of harmful threshing settings

An experiment was performed to evaluate how different combine harvester settings affected the survival of the parasitoid larvae. Five combine settings were applied with three replicates. For the five treatments, the threshing drum was fully extended to minimize the clearance between the drum and the concave threshing surface. The settings are shown in table 10 where treatment 1 reflects the normal setting when harvesting white clover seeds.

TABLE 10. The combine harvester settings used to evaluate if the harvest influenced the survival of the parasitoid larva.

Treatment	Bars inserted in the concave threshing surface	Speed of threshing drum (rpm)
1	All present	1400
2	All present	1000
3	Second bar removed	1400
4	Second and second last bar removed	1400
5	Second last bar removed	1400

Two 5m wide white clover seed plot strips were used; a first year and a second year white clover stand. On May 31, the plot strips were defoliated and the swathing was performed on August 9 with the harvested on the August 17. At the swathing, 400 reflexed white clover flower heads with green stem were randomly collected in 26.5x8.0x4.5 (HxWxD) block bottom paper bags. The collection was repeated five days after the swathing. The samples were stored under outdoor conditions and examined through within a couple of days. Parasitoid cocoons were found by gently removing the florets from the flower heads. Swathing was performed with a Hesston 6500 (AGCO International GmbH EME, Switzerland) utilizing a swathing width of 2.5m. At this time point, the plot layout was constructed.

Each plot strip was divided into two lanes (width 2.5m). The length of the swath lanes were 60m for the first year crop and 70 m for the second year plot. After establishment of the swath lanes, treatments were randomly laid out in plots covering 5m of the swath. Plots were separated by a 1m strip in which the swath had been removed. Harvest was done with a Dronningborg 3800 (AGCO International GmbH EME, Switzerland).

Harvest followed a period of rainfall, which meant that the harvest was not performed under optimal conditions and a long drying period would be needed. Thus, the harvested material was dried for 30 days at the AU-Flakkebjerg on-floor drying barn, allowing outdoor air to pass through grates in the floor and move up through the harvested material. The harvested material was kept in cotton sacks (60x100cm WxH); visual inspection of the material was made regularly.

After the drying of the harvested material, the material was threshed using an in-house built cylinder brush thresher, capable of separating white clover seeds from dried white clover flower heads. Following the brushing, the cocoons were separated using a laboratory seed sorting device LA-LS (Westrup, Denmark) seed sorter. The set points of the LALS reflected the initial set points utilized to separate the parasitoid cocoons from the delivered debris material from the commercial seed sorting facilities. Separated cocoons were stored at 5°C until evaluation.

Sampling

Prior to harvest, all cocoons found at the two time points were collected. At harvest, 30 cocoons per replicate were randomly selected. The number of parasitoid cocoons, condition of the cocoon: intact or signs suggesting the adult parasitoid had left the cocoon were noted. Also,, number of larvae, pupae and adult weevils were noted. The weight of the parasitoid co-

coons were registered on a 7-digit microbalance (Santorius MC5); then the cocoons were dissected. Living parasitoid larvae were weighed. The larva was determined as living if it had a yellow to white color and movement could be observed in a stereo microscope when gently disturbed.

Statistics
Data were analyzed in R (R Core Team, 2017) using the built in function lm. Pairwise comparisons were done by utilizing the functions in the emmeans package (Lenth, 2018).
Significant levels of P<0.05, P<0.01 and P<0.001 are indicated as *, ** and ***.

Results
Cocoons containing living parasitoid larvae
The two initial samples reflected a larva survival rate of 9.7% (n=31) at the growers seed storage and 4.0% (n=24) from the seed sorting facility. Cocoons containing a living larva weighed on average 4.46±0.32mg (±S.E.) (n=4) and dead larvae weighed 2.53±0.09mg (n=53). The difference 1.92±0.33mg (standard error of the pairwise comparison test) was significantly different (P-value <0.001***). In terms of percentage, a cocoon harboring a dead larva weighed 43% less than a cocoon with a living larva.
The average cocoon weight from the grower's seed storage was 2.85±0.13mg and cocoons found in the sample from the seed sorting facility weighed 2.35±0.14mg. A pairwise comparison of the cocoon weights showed a significant difference between the grower's seed storage and the seed sorting of 0.50±0.19mg (P-value <0.0113*).

The 44 samples collected from DLF showed an average 100-cocoon weight of 0.24 g ± 0.003. A multiple comparison test was set up to evaluate differences between growers. No differences were found. Analysis of 215 individual cocoons collected from four of the 44 samples showed weight of cocoons containing a living or a dead larva to be significantly different (P<0.001 ***) with living weighting on average 4.08mg±0.11 (n=23) and 2.34±0.04 for dead (n=192) larva. Within the four samples, the number of living larva ranged from 2.5% to 28.9%.The questionnaire did not give a clear answer on what could be the reason for the differences.

The additional two samples from DSV yielded 198 cocoons. The average cocoon fresh weight was 2.13mg ±0.04. The 32 randomly collected cocoons showed that only 3.1% of the cocoons harbored a living larva.

Monitoring the temperature and relative humidity of the swath
Measurements of temperatures and relative humidity on the surface and in the swath produced evidence of values around lethal temperatures; however, such occasions were quite rare.

To compare the continuous measurements in the three layers of swath, data were reduced to three time points 9.00, 12.00 and 15.00. By doing so, 973 measurements were available. Summarizing the four experimental setups and the three swath thicknesses gave an average relative humidity of 84.2 percent with a minimum of 40.8 percent and a maximum of 99.9 percent. The humidity range did not seem to vary into extremes.

Capability of the parasitoid larva to withstand extreme conditions
Comparisons between the nine climatic conditions did not reveal differences in cocoon weight. After 110 hours, larva weight was only found to be different between the initial larval weight and weight of larvae subjected to 15/15°C (Day/Night) temperatures at 100% RH. High temperatures and low relative humidity could not induce differences in larval weight.

The weight of the cocoon shell differed when comparing initial weights with cocoons subjected to 15/15°C, 100% RH and 25-15°C, 100% RH.

Collection of flower heads and parasitoid cocoons at harvest

Samples of the harvest material were taken at different time points at one grower white clover seed harvest in 2016 (figure 4). Unfortunately, it was not possible to collect a sample prior to the swathing. For comparison, a sample at another grower was obtained just before swathing. This sample showed 76.2±9.5% of the collected cocoons to contain a living larvae.

Figure 4 shows the decrease in number of living larvae found at four different time points around harvest. Compared to the sample prior to harvest, no different was seen up to 7 days after swathing ($P<0.05$). Numbers of cocoons found and studied at the four timepoints can be seen in table 11.

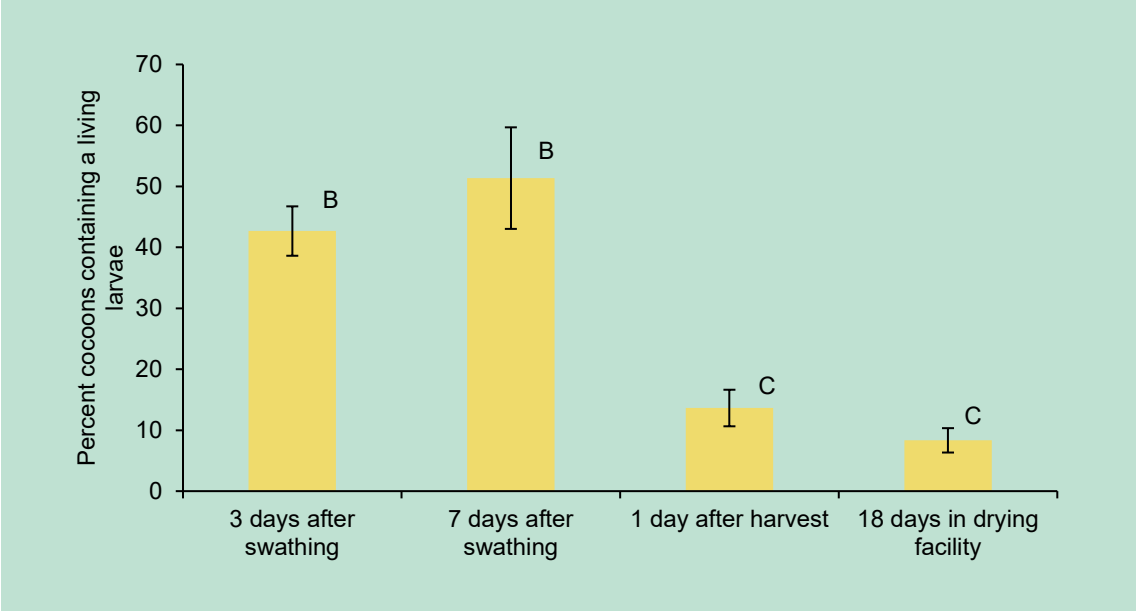

FIGURE 4. The percent of living parasitoid larvae obtained by dissection of found cocoons in harvest material at five time points. SE values are shown. Different letters display significant differences (P-value <0.05).

Analysis of the average larva weight did not identify differences between dead or living individuals.

The weight of the woven parasitoid cocoon increased with time. That is, cocoons found after 18 days in the drying facility had a heavier shell than the cocoons found prior to this time point. This weight gain was found for both cocoons containing a living or a dead larva; thus, the decrease in percent cocoons containing a living larvae seen in figure 4 cannot be explained by the increase in cocoon sheath weight. However, as the cocoon sheath weight increases, the larva must either be strengthening the cocoon as time passes or, alternatively, the construction of the heavier cocoons happens late at the time when the harvested material reaches the on-farm drying facility.

TABLE 11. The number of cocoons obtained from the four collections of harvestable material together with number of dissected cocoons and number of cocoons containing a living larvae.

Time of sampling	Cocoons found	Dissected	Number of living larvae
3 days after swathing	152	152	63

7 days after swathing	37	37	19
1 day in the drying facility	499	135	18
18 days in the drying facility	192	192	16

On numerous occasions, it was observed that dead larva would be scattered on the inner surface of the cocoon shell or the larva skin would be dissolving.

Monitoring the temperature and relative humidity when drying the white clover seed at the grower and at the seed sorting facility

In the growers no-floor drying facility no extremes was found in temperature and relative humidity. The temperature varied between 11.9°C and 31.3°C averaging 19.7±0.1°C at a height of 30 to 40cm. at 70 to 80cm above floor-level temperature varied between 8.6°C and 25.2°C averaging 17.4±0.1°C. The relative humidity averaged 77.3±0.2% varying between 61.0 and 87.2% relative humidity at 30 to 40cm above floor-level. At 70 to 80cm above floor-level the relative humidity averaged 79.9±0.1% varying between 64.5 and 95.3% relative humidity. Thus extreme values was not observed.

At the seed sorting facility temperature and relative humidity was logged at two heights (0cm and 50cm below surface) in large wooden boxes. The diurnal changes in relative humidity and temperature is seen in figures 5 and 6, where the measurements are compared to seasonal variations at soil level and 10cm above soil level in a hedgerow at the University of Copenhagen Campus Taastrup. In table 12 the average minimum and maximum temperature and relative humidity are shown for the four conditions.
Relative humidity was found stable. Temperature variations were more pronounced at the surface of the material compared to values measured 50cm below the surface.

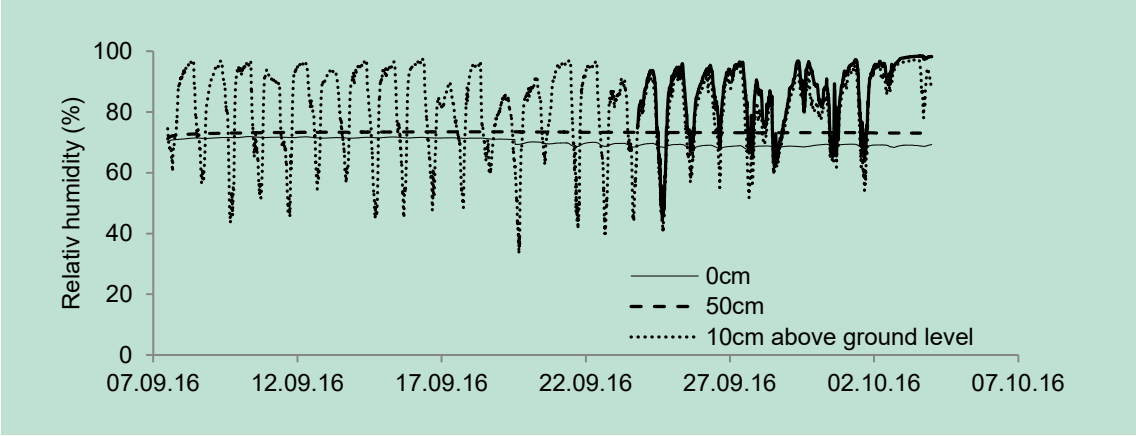

FIGURE 5. The relative humidity in the large wooden boxes used to store the harvested material from the white clover seed harvest prior to sorting of the seeds. Relative humidity measured at two heights 0cm and 50cm below material surface. Also seen is relative humidity measured at 0cm and 10cm above soil height in a hedgerow at the University of Copenhagen Campus Taastrup. Storage period lasted from September 7 to October 3.

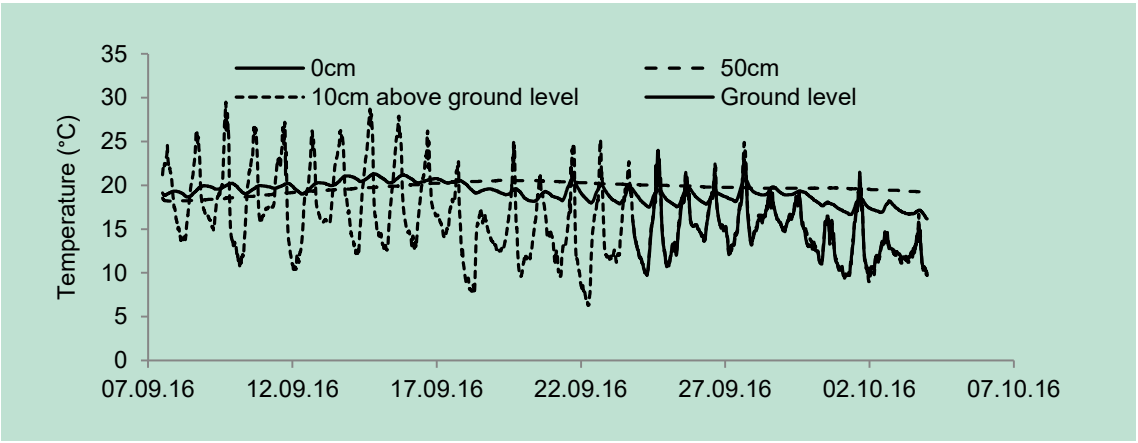

FIGURE 6. Average temperature in the large wooden boxes used to store the harvested material from the white clover seed harvest prior to sorting of the seeds. Temperature was measured at two heights 0cm and 50cm below material surface. Also seen are temperature measurements taken at 0cm and 10cm above soil level in a hedgerow at the University of Copenhagen Campus Taastrup. Storage period lasted from September 7 to October 3.

TABLE 12. Average, minimum and maximum relative humidity (RH) and temperature (Temp.) at two depths (0 and 50cm) in large wooden boxes used to store the harvested material shown together with measurements at 0cm and 10cm above soil level in a hedgerow at the University of Copenhagen Campus Taastrup. Storage period lasted from September 7 to October 3.

	Average RH (%)	Minimum RH (%)	Maximum RH (%)
0cm	70.1	67.6	71.9
50cm	73.2	71.1	73.5
Soil level	87.7	44.7	98.7
10 above soil level	81.6	33.3	97.3
	Average Temp. (°C)	Minimum Temp. (°C)	Maximum Temp. (°C)
0cm	19.2	16.2	21.3
50cm	19.7	18.2	20.6
Soil level	14.0	6.1	24.0
10 above soil level	15.7	5.3	29.5

Identifications of harmful threshing settings

The experiment did not go according to plan. Due to the wet conditions around harvest, the material had to be dried for a longer period than initially intended. The drying process killed the parasitoid larvae collected at harvest. Temperature and relative humidity was not measured in the barn sized on-floor drying facility, a drying facility in which outdoor air is blown through the material.

At swathing 400 flower heads were collected. From these heads 11 parasitoid cocoons were found, all containing a living larva. After the swathed material had been aerated for five days, 400 flower heads were again collected and examined. At this time point, six cocoons were found with five living larvae. After harvest, the harvested material was processed according to the processed previously described. After drying, 30 cocoons were randomly selected per sample. As all of these cocoons contained a dead larva, all remaining cocoons was sorted out across the five treatments. In total 1230 cocoons were found and all contained a dead larva, all showing signs of desiccation. It was further noticed that 85 percent of the larvae had either turned brown or black, 9 percent showed signs of a fungal infection, three percent had a reddish discoloration and two percent had been hyperparasitised. For comparison, when colleting parasitoid cocoons in 2016 prior to harvest, hyperparasitism was found in 15 percent of the

collected cocoons, and 4 percent of the parasitoid larvae showed signs of desiccation. Collecting cocoons at the seed sorting facilities showed hyperparasitsm to occur in 4 percent of the examined cocoons and 90 percent of the parasitoid larvae showed signs of desiccation.

Evaluation of the mechanical damage to the cocoons did not show differences between the five different settings to the combiner. The average percentage of mechanically damaged cocoons was 18.6±0.9 percent.

Discussion

Cocoons containing living parasitoid larvae

As found when sorting cocoons from provided white clover seed harvest debris, cocoons primarily contained dead larvae. This was found in samples from on-farm seed storage and at the seed cleaning facility. The survival rate of the parasitoid larvae varied between farmers ranging from below 3 percent to 29 percent. The variation prompted farmer interviews though no evident differences in handling of the harvest were found. Therefore, the differences in larval survival must primarily be connected to environmental conditions. Foremost temperature and humidity in the swath needed to be identified as high temperatures have been found lethal to the parasitoid larvae (Cherry et al., 1976, Hama & Davis, 1983) and low humidity also increases the mortality rate (Hama & Davis, 1983).

Monitoring the temperature and relative humidity of the swath

Temperatures averaged 23.8°C with a minimum of 15.1°C and a maximum of 39.8°C. Previous exposure of non-diapausing cocoons to temperatures of 43°C has been shown to produce a mortality rate of 50 percent after one hour of exposure, but mortality could also be achieved by exposing the cocoons to 38°C for 17 hours (Hama & Davis, 1983).

Looking at temperatures for the three time points, there is, however, only one measurement which shows temperatures above 38°C. This temperature was achieved in the treatment with the thinnest layer of swath material. The second and third highest temperatures observed were observed at the same time in the two other swath thicknesses, which would suggest that heating of the swath does not depend on the thickness of the swath. This was confirmed by model reduction (data not shown).

Temperatures known to be lethal to *B. curculionis* larvae have been observed in the swath; however, such temperature incidences are rare and did only occur with a frequency of 0.1 percent of the analysed time points.

Measurements of the surface temperature primarily revealed temperature differences to depend on when the measurement was taken: 9, 12 or 15 o'clock. The thickness of the swath was not seen to influence the temperature at the three time points. Temperature differences related to the swath thickness was observed when comparing the thinnest layer to the medium and thickest swath layer.

Capability of the parasitoid larva to withstand extreme conditions

Based on observations of larval survival at dissection the state of the larva was analysed, i.e. alive or diseased. The analysis did not reveal differences between temperature and relative humidity after 110 hours, which is in contrast to what has been found earlier (Hama & Davis, 1983). An issue in the current experiment has been establishing if a cocoon contained a dead or living larva. It was found that there is a weight difference between cocoons containing a living or dead larva; however, the weight distributions overlap, thus introducing possible errors.

Collection of flower heads and parasitoid cocoons at harvest

Overall, the number of cocoons containing a dead larva was seen to increase from the time of swathing to the time when the material was shipped off to the seed sorting facility. In addition, cocoons in the material delivered from individual growers contained different numbers of cocoons with a living larva, the span being from zero and up to 28 percent of the cocoons found, as seen earlier. The viability of field-collected diapausing cocoons of *B. curculionis* larvae has

previous been found to vary between 0 and 100% (Pike & Burkhardt, 1974a). Across sites and years, Pike & Burkhardt (1974a) found that an average of 25.9% of the collected cocoons contained a viable larva. Overwintering and hatching the cocoons showed that 9% of the larvae were capable of emerging as adult parasitoids, which suggests a mortality in the pupation stage of 16%. The differences could arise of suboptimal hatching conditions or winter storage.

It could, with reason, be speculated that an increasing number of larvae die as the white clover harvested material is stored and processed, first at the seed grower's storage and later in the processing of the harvested material at the sorting facility. However, as mentioned earlier, the debris fraction obtained from the seed sorting facilities contained about 3% cocoons containing a living larva and at the growers 10.7±2.1% of the cocoons should contain a living larva.

Monitoring the temperature and relative humidity when drying the white clover seed at a grower and at the seed sorting facility

When monitoring the temperature at 30 to 40cm above floor level an increase in temperature from 18°C to 31.3°C could be followed for the first three days. The steady incline was followed by a sudden drop in temperature. This development would suggest the activation of the large blower connected to the on-floor drying facility, reducing temperature by blowing air from outside through the pile of harvested material. At 70 to 80cm above floor level, the temperature increase was not seen. Temperature variation at this height followed the diurnal rhythm. Relative humidity decreased over time at 30 to 40cm above floor level and varied with the diurnal rhythm at 70 to 80cm above floor-level without large variation.

At the seed cleaning facility temperature and relative humidity was found to be stable both at a depth of 50cm and on top of the material. Temperature or humidity extremes capable of influencing the parasitoid larvae was not observed.

Identifications of harmful threshing settings

Although the experiment did not succeed, it did highlight the restraints on the parasitoid survival when drying down the white clover seed harvest. In order to sort out the white clover seeds and prepare the seeds for storage, the water content of the seeds needs to be below 11 percent (DLF-Trifolium, 2010). The drying of the seeds is normally done in two steps. Firstly, the crop is swathed and the swathed material is aerated in lanes. Secondly, the material is threshed and if needed, the harvested material is further dried in for example an on-floor drying facility.

In the present study, 90 percent of the larvae collected after drying showed signs of desiccation and every cocoon contained a dead larvae. When collecting cocoons form the seed sorting facilities 97 percent of the cocoons contained dead larvae and on-farm collected cocoons showed dead larvae to occur in 72 to 97.5 percent of the collected cocoons. Thus, it seems highly likely that the needed drying of the white clover seeds is the reason for the low survival rate of the parasitoid larvae.

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Appendix 3. Hatching depends on temperature and humidity

Appendix 3 is divided into three parts. The first part describes the temperature needs of the parasitoid larvae to end diapause. The next part describes how the parasitoid can be kept from the time point when the cocoon has been sorted from debris and to the point of release. The third part describes two field surveys: One on the spring appearance of the weevil and a second on how the populations progress through the growing season.

Appendix 3.1 Thermal needs for the parasitoid to end diapause

Hatching of the parasitoid is related to temperature. Eklund and Simpson (1977) described the thermal needs for the parasitoid and previously storage for six weeks (4°C) was found to be sufficiently for braking diapause (Dowell & Horn 1978) though the parasitoid have also shown to tolerate 10°C for two months (Bartell & Pass 1978) prior to hatching. Parasitoids can be expected to hatch after being kept at 4°C for six month (Dowell & Horn 1977).
Knowledge of how and when the cocoons should be kept and released was studied. Experiments were planned for 2016. In 2017 an additional experiment was set up to confirm results from 2016.

Materials and methods

Parasitoid larval temperature requirements 2016

In 2016, cocoons were hand collected from a fraction of the sorted white clover seed debris delivered from DLF in late 2015. The sorted cocoons were stored for eight weeks at 5°C in Ø 8.4cm 280ml plastic containers. For ventilation, fitting lids had a Ø3cm cut out closed off with a fine mesh netting (0.3x0.3mm mesh size). Storage relative humidity was not recorded.

After the eight weeks of storage at 5°C, the temperature needs of the parasitoid larvae were evaluated at three temperatures: 16, 20 and 25°C. The experiment was setup in three climate cabinets (MIR-554, Panasonic/Sanyo). Per temperature, 140 cocoons were placed individually in 30ml plastic cups. Lids had a Ø1cm hole covered by a finely meshed net to ensure ventilation. The cocoons were monitored regularly up to the time where the first imagines started to appear. Following the starting of the hatching, the cups were inspected daily.

Parasitoid larval temperature requirements 2017

In 2017 cocoons were stored for 13 weeks at 5°C and 75% RH (KG410LGDLL16W, Gram, Denmark) prior to the setup of the experiment. The cocoons were sorted by hand from a sub-fraction of the delivered harvest debris from DSV. This sub-fraction had previously been processed on seed sorting instruments with the aim to divide cocoons containing living larva from a dead larva.

The experiment involved five temperature set points: 16, 18, 20, 22 and 25°C. Relative humidity was set to 70 percent and day:night time was set to 12:12 hours. The used climate chambers were for 16 and 18 °C Refritherm 200 (Struers-Kebolab, Denmark), 20°C KK 500 (Pol-Eko Aparatura, Poland) and for 22 and 25°C IPC800 (Memmert, Germany).

In chambers used for 16, 18, 22 and 25°C temperature levels, humidity was controlled by pumping air through a water bath. The 20°C climate cabinet had an automated humidifying system. Per temperature 72 plastic cups each containing 10 cocoons were set up. Cocoons were separated into batches of 10 by using a seed counter (Contador, Pfeuffer) with the feed container number 2 (Contador, Pfeuffer) installed. Cups were randomly divided per treatment.

The cocoons were monitored regularly up to the time when the first imagines started to appear. Following the starting of the adult hatching, the cups were inspected daily. Hatched individuals were sex determined, freeze killed at -18°C and weighed (fresh weight) on a 7-digit microbalance (Santorius MC5). After weighing, the imagines were stored in microtiter plates at -18°C awaiting measurement of dry weight after drying at 55°C for 72 hours. The dried individuals were stored in an exicator. Water content was calculated as the difference between fresh and dry weight related to fresh weight.

Temperature and relative humidity were logged by having data loggers (EBI 20-TH, Ebro) in each climate cabinet.

Statistics
The relationship between temperature and developmental rate was calculated in accordance with Trudgill et al. (2005), determining the base temperature and the thermal constant from the linear relationship between temperature and the rate of development. Calculations were done in Excel (Microsoft, USA).

Results
In 2016, the three temperatures revealed the larvae to require 399°C degree-days (°CDD) at a bases temperature of 6.5°C. In 2017 the average temperature requirements were 476°CDD (minimum 243 maximum 592 °CDD) at a bases temperature was 5.6°C. The experimental lasted 51 days in 2016 and 58 days in 2017.

In 2017, the percentage of hatched individuals at 18°C was 5 percent compared to an average of 22 for the remaining four temperatures. In 2016, the average number of hatched individuals was 30 percent across the three temperatures.
Due to the low percentage of hatched individuals at 18°C the remaining cocoons were observed for additional 43 days at room temperature. It was seen that 15 percent of the cocoons previously kept at 18°C had hatched after the termination of the experiment. In addition, 10 percent of the cocoons kept at 20°C hatched and 3.8 percent of the cocoons kept at 22°C hatched. For the remaining temperatures (16 and 25°C) 0.1 percent hatched in the following 43 days. No differences were found in fresh weight, dry weight and water content of the parasitoids hatched at the five temperatures.

Discussion
The range of the basis temperature (5.6 to 6.5°C) seems acceptable when compared to the previous findings of Eklund and Simpson (1977), reporting a basis temperature of 6.11°C. The requirement of 399 to 476°CDD does, however, vary from the findings by Eklund and Simpson (1977), who found the parasitoid needing ~236°CDD.
Compared to the findings of Eklund and Simpson (1977) and Barney et al. (1978) the current thermal need of the parasitoid larvae is around twice as high. An even higher thermal need could be expected as storage temperatures at the seed sorting facility average above 19°C. It is, however, uncertain how the storage conditions at the sorting facility influence the thermal

needs as the initiation of the diapause is unknown. It is, however, believed that the diapause commences shortly after construction of the cocoon.
Furthermore, the collected *B. curculionis* released as a classical biocontrol agent against *H. postica* in the USA are from alfalfa fields primarily located in France, Italy, Switzerland, U.S.S.R and Sweden (Chamberlin, 1924, Chamberlin, 1926, Dysart & Day, 1976, Bryan et al., 1993). *B. curculionis* specimens used by Eklund and Simpson (1977) would therefore overall originate from a Southern European climate and be adapted to the life cycle of *H. postica*. Under such conditions *B. curculionis* parasitising *H. postica* would evidently emerge earlier than what is found for *B. curculionis* having *H. meles* as a host. The differences in temperature needs would point towards diffenences in the *B. curculionis* populations based on host preferences and origin of collection. However *B. curculionis* parasitising *H. postica* have also been collected in Sweden, which is near to the current study sites. No records on differences thermal needs from the released *B. curculionis* in the USA have been found.
For *B. anurus*, another classical biocontrol agent on *H. postica*, Moore (2014) found variations in the mithochondrial gene cytochrome oxidase subunit 1, which could indicate regional genetic variation correlated to temperature.

The parasitoid has a partial second generation. Under US conditions a partial third or fourth generation might exist as adult parasitoids can be found through summer and fall (Dysart & Day, 1976). Compared to the present observations this points towards the parasitoid having a large plasticity in its capability to follow the development of its hosts. In the current study, the parasitoid is present when the host *H. meles* starts its activities (section 7.3). Then the parasitoid is adapted to *H. postica*;, it seems that the thermal needs is adapted to this host (Eklund & Simpson, 1977, Barney et al.,1978). Parrish and Davis (1987) showed that it was possible to rear successive non-diapausing generations of the parasitoid.

It is known that temperature and photoperiod affect the number of larvae entering diapause. For *B. curculionis* adapted to *H. postica* populations in Utah, a dark period of less than 8 hours or more than 19 hours resulted in 90% diapausing cocoons. If the dark period was warm, this also contributed to an increase in diapausing cocoons (Parrish & Davis, 1978). Also the temperature that the adults experience between emergence and mating has an effect on the diapause (Parrish & Davis, 1978).

The experiment highlights when the cocoons would be able to hatch, i.e. the temperature dependent rate of development of the parasitoid larvae from the time of collection to the emergence of the adult parasitoid. The experiment does however, not provide direct information about the thermal accumulative needs of the parasitoid, as the utilized cocoons were obtained from the debris of the white clover seed sorting. It is more than likely that the larvae in the cocoons, prior to the experiment, had accumulated an unknown amount of their thermal needs as storage temperatures could have exceeded their lower developmental temperature (appendix 2.3) for an unknown time span.

The reason for the irregularities seen in the hatching of 2017 is unknown. A low humidity could be expected to change hatching conditions. However, logged humidity did not follow the imbalance in hatching performance as the lowest average RH was seen at 16°C (64%) followed by 22°C (68%), then 18°C (69%). Handling of cocoons prior to and in the experimental period did not differ between treatments. The differences in late hatching was therefore thought to depend on the climate chambers. The experiment was not repeated,, because of time restraints and as the primary objective had been achieved.

For future studies on the thermal need, it would be interesting to see if both the clover head weevil *H. meles* and the parasitoid *B. curculionis* would be present and how the thermal needs of the parasitoid have adapted in Oregon, where there is seed production of white clover and

alfalfa. Also, whether the parasitoid recognizes *H. meles* as a host and whether the parasitoid would have preferences for *H. postica*, the weevil which the parasitoid was intended to control.

Appendix 3.2 Storage of the parasitoid

The experiments evaluate the state of the parasitoid larva after storage under different conditions and the subsequent hatching of the adult parasitoids. The terminology storage is used for the time span from sorting of the cocoons and to the time of release. Therefore, the term storage is used for a short time storage lasting no more than a couple of month.

Prior to storing of the cocoons, they have been kept first at the seed grower within the harvested material, then at the seed processing facility and later in debris awaiting sorting as described earlier. The time span from harvest to the final separation of the cocoons was around four month. The storage conditions in this period aim to optimize seed quality (temperatures between 15-20° and low humidity), but they are not optimal for the parasitoid, as temperature is above the parasitoids minimum temperature demand for development.

Experiments were planned for 2015 and 2016. However, the experiment failed in 2015 as no parasitoids hatched from there cocoons. Following this, an additional experiment was conducted in 2017 to confirm results from the 2016 experiment.

Materials and methods

Table 13 displays an overview of the different time points for the experiment in the two years. The debris material was delivered in November of both years. As the sorting procedure did not produce a fraction only consisting of parasitoid cocoons, it was necessary to separate cocoons from debris by hand. After sorting out cocoons, the experiments begun by placing cocoons at different storage environments. The start occurred one month later in 2017 than in 2016. This influenced the storage time, which lasted 10 weeks in 2016 and 8 weeks in 2017.

TABLE 13. Dates for the storage experiments in 2016 and 2017.

	Material delivered by seed company	Storage experiment start date	Storage experiment end date where after cocoons were moved to hatching conditions
2016	04/11	21/12	03/03
2017	03/11	20/01	24/03

Storing of cocoons 2016

Cocoons were stored for 10 weeks under four storage conditions: At constant temperatures in climate chambers (MIR-554, Panasonic/Sanyo): -5, +5 and fluctuating temperatures in a barn at KU-Taastrup and in a hedgerow at AU-Flakkebjerg. For each storage condition two storage materiasl were used: husks of buckwheat and white clover harvest debris. Per storage condition and storage material, five replicates were used for destructive measurements at the end of the storage period. Each replicate consisted of 100 cocoons mixed into the storage material in a 1:9 w/w ratio storage material to cocoon respectively. Portions of 100 cocoons were made by using a seed counter (Contador, Pfeuffer) using the feed container number 2 (Contador, Pfeuffer). The mixture was placed in Ø8.4cm 280ml plastic containers. For ventilation, fitting lids had a Ø3cm cut out closed off with a 0.3x0.3mm iron netting. Additional three replicates per storage condition were used to establish how winter storage affected cocoon hatching under field conditions. To measure the temperature and relative humidity, data loggers (EBI-20 TH, Ebro) were placed at the four storage conditions within an empty 280ml plastic container.

To evaluate the bulk fresh weight of the cocoons before storage, five portions of 100 cocoons were set aside. From these, 20 cocoons per portion were randomly selected for dissection to evaluate the percentage of larvae alive within the cocoons.

Evaluation of storage condition

After 10 weeks of storage, the cocoons were separated from the storage material by hand. Per replicate eight cocoons were dissected to establish the number of living larvae. Later five cocoons with a living larva were chosen for cocoon and larval fresh weight, larval dry weight and larval fat free dry weight. Cocoons were kept at -18°C until weighing. Cocoon fresh weight was obtained by weighing the individual cocoon on a 7-digit microbalance (Santorius MC5) or similar. Afterwards the cocoons were dissected and living larvae were weighed. A living larva was determined by larval appearance, i.e. white to yellow skin colour and larval shape, no obvious punctures and suspicious formation of lava skin. Also, lava movement when physical touch revealed if a larva was alive. Individual larvae were kept in 96 well microtiter plates. Larva dry weight was obtained after drying at 60° for 48 hours followed by storage in an exicator. Fat free dry weight was obtained for five larvae per replicate, utilizing a Soxhlet equipment using petroleum ether as the solvent. The petroleum ether was heated to 35-40°C using a table hot plate with a magnetic stirrer. Cooling was provided by circling cold water through the condenser of the Soxhlet instrument and an ice cold water bath. To preserve the integrity of the larvae, individual larvae were placed in in-house made teflon cups with lids. The cups had 2mm holes in the bottom and lid to allow the solvent access to the specimen. Fat extraction lasted 48 hours. Afterwards the larvae were dried overnight at 60°C. Larva fat content was calculated as the difference between the start dry weight and the dry weight after fat extraction.

Evaluation of hatching frequency

The three replicates used for establishing hatching frequency were moved to the field at the same day as the 10 weeks storage ended and the destructive measurements were performed. The 280ml plastic containers were fitted with lids having a central Ø1.5cm opening. On top of the lid a Ø3cm cone made of overhead plastic was glued on. The top of the cone was removed to facilitate access. On top of the cone a 30ml plastic container was used for parasitoid collection. Lids for the collection container with a Ø3cm hole were used to secure a tight seal around the cone. The structure was held in place by a 1cm wide elastic band. Aluminium foil was wrapped around the 280ml plastic container to enclose the cocoons in darkness and facilitate newly hatched adult parasitoids to move up into the collection container. The containers were placed outdoor in a hedgerow facing south to north at ground level. To shield the plastic containers from precipitation and wind, the containers were collected in a transparent plastic box (72x35x14.5cm [LxWxH]). Ø2.9cm holes were made in the sides and bottom to enable ventilation and runoff of water. The south facing side of the box had a square opening 26x11cm [HxW] covered with an insect net mesh size 1x1mm).

Parasitoids began to hatch on May 11, where after the collection containers were inspected every second 2 day through the months of May and June. Hatching ended on July 4. Collected parasitoids were stored individually in 96 well microtiter plates at -18°C until weighing of fresh weight (Santorius MC5). Hereafter the parasitoids were freeze dried (Heto Drywinner 6 85, Thermo Fisher Scientific) for 48 hours and dry weight was established.

Temperature and relative humidity was measured by having data loggers (EBI-20 TH, Ebro) within the transparent plastic box in an empty 280ml plastic container setup to reflect the storage conditions of the cocoons. Furthermore, two data loggers were placed outside the transparent plastic box at soil surface level and at 10cm above the soil surface. To shield the outside loggers from warming up by solar radiation, the loggers were shielded by aluminium foil.

Storing of cocoons 2017

The experiment was repeated in 2017 with the storage period lasting 8 weeks instead of 10 weeks. Per replicate 120 parasitoid cocoons were used Instead of 100. Used cocoons came from a sub-fraction of the 2016 white clover harvest, delivered by DSV. The sub-fraction had been cleaned excessively allowing for a quick sorting of the cocoons necessary for the experiment.
Prior to storage five replicates of 20 larvae were weighed to obtain fresh weight and dry weight. Fat content was evaluated on five larvae. The storage treatments ended on March 24. Then five replicates per storage treatment were evaluated for storage effects and three replicates were set to hatching under the same outdoor conditions as in 2016.

Of the five replicates per storage treatment 20 randomly selected cocoons were used to evaluate storage conditions on larval fresh weight and dry weight. Fat free dry weight was measured on 5 dried larvae characterized as alive after leaving the storage conditions. Larval dry weights were obtained after drying in a drying cabinet at 55°C for 72 hours.

From each of the previous five replicate used to evaluate storage conditions 30 cocoons were transferred to 30ml plastic cups. The cocoons were placed in a climate cabinet at 25 °C and 70% relative humidity. For ventilation, the lids of the cups had a Ø1cm hole covered by a 0.3x0.3mm iron netting. Hatching started after 24 days and lasted 28 days. Cocoons were inspected daily.

From the three replicates per storage condition destined for adult hatching all hatched individuals were weighed when emerged (fresh weight); dry weight was registered after following the above procedure. Later, dried adult parasitoids (n=349) were analysed for fat content using the previously described procedure.

Hatching of the adult parasitoids started in 2017 under field conditions on May 26 and ended on July 10. The containers were inspected daily. Towards the end of the hatching period, inspection was only carried out every second day.

Statistics
The statistical program R (R Core Team, 2017) was used in for the statistical calculations. The built-in function lm() and the lme() function from the nlme package (Pinheiro et al., 2018) were used to analyze normal distributed data. The lme() function incorporates the possibility to include random effects in the statistical modeling. The function glm() was used for binomially distributed data respectively. Pairwise comparisons were done using the emmeans package (Lenth, 2018). The drc package (Ritz et al., 2015) was used to analyse emergence time expressed as time for 50 percent of the wasps to emerge as adult. For this, a three-parameter log logistic model was used. The model parameters represent the slope at the time of 50 percent emergence of adult parasitoids, the time point, in °C degree-days (°CDD), when 50 percent of adult parasitoids emerged and the total number of hatched adult parasitoids as a fraction of the number of cocoons per replicate which for 2016 was n=100 and 2017 n=120.

To estimate the number of °CDD accumulated by the parasitoid from the time of exiting winter storage to adult hatching, the lower developmental temperature was set to 6.5°C. This was the base temperature found from the 2016 experiment concerning thermal time. Significant levels of $P<0.05$, $P<0.01$ and $P<0.001$ are indicated as *, ** and ***.

Results
The first observed adult parasitoids were in 2016 seen 15 days earlier than in 2017. Table 14 gives an overview of the start and end dates for storage and hatching. Comparing the two years at the two additional time points, a decrease was visible in the time gap between years, i.e. the date on which the maximum number of parasitoids hatched and the date when the last parasitoid hatched decreased to 7 days.

TABLE 14. Specific dates for the storage experiments in 2016 and 2017.

	Hatching starting	Maximum hatching date	Hatching end date
2016	11/05	03/06	04/07
2017	26/05	10/06	10/07

Larval survival after winter storage

In 2016, the temperature and relative air humidity during winter storage differed for the four winter storage locations (table 15). The mean temperature was lowest at the -5°C location, followed by the barn and the field, both having an average temperature of 2.1°C. At the +5°C location, the average temperature was 6.3°C. The temperature in the field had a more extreme minimum and maximum compared to the barn. The absolutely lowest temperature measured was -7.6°C and the maximum was 12.1°C, both measured in the field. The relative humidity was lowest in the +5°C treatment and highest in the field.

Prior to winter storage 100 cocoons from five replicates were weighed revealing a 100-cocoon weight of 0.348±0.003g FW. After the 10 weeks of winter storage, the average 100 cocoon fresh weight was 0.341±0.002g FW

Just after the winter storage, *B. curculionis* larvae inside the cocoons did not seem to be markedly affected by the different winter storage treatments. For example, the fraction of living larvae in cocoons were the same as before treatment start and no difference in survival of larvae in cocoons was observed between the different locations or storage materials. Overall, the fraction of living larvae before winter storage was 56.5 percent, and after winter storage, it was 57.9 percent across the different treatments.

After the winter storage, the average weight of the cocoon containing a living larva was 4.06±0.06 mg FW (n=99). The living larvae had a water content of 57.8±0.2 percent and a fat content of 29.4±0.4 percent of the dry weight (n=204).

The number of the dead larvae differed among the treatments in an unsystematic manner. A moist content of the cocoons was found in most treatments except for cocoons stored among buckwheat shells at -5°C. Dead brown larvae were likewise found in most dead cocoons, but less often so in cocoons stored among plant debris at +5°C and in the barn and cocoons stored among buckwheat shells in the field. Shrunken dead larvae was found in most cocoons but less frequently among buckwheat shells at +5°C and in the field in and among plant debris in the barn and at -5°C. Fungi were only found in 9 out of 128 dead cocoons. Hyperparaitoids were only found in two out of 128 dead cocoons.

TABLE 15. Temperature and relative air humidity in the different winter storage treatments 2016. Data are from data loggers placed together with the cocoons inside the storage plastic container.

2016	-5°C	+5°C	Barn	Field
Temperature ºC (mean)	-4.6	6.3	2.1	2.1
Temperature ºC (minimum)	-4.8	3.5	-5.2	-7.6
Temperature ºC (maximum)	-3.2	9.3	10.8	12.1
RH % (mean)	61.5	49.9	83.3	90.3
RH % (minimum)	53.8	35.9	69.4	59.9
RH % (maximum)	78.7	64.6	89.9	98.0
2017				
Temperature ºC (mean)	-4.7	5.2	3.5	2.8
Temperature ºC (minimum)	-5.1	4.5	-4.0	-4.4
Temperature ºC (maximum)	-4.3	9.4	11.8	14.2
RH % (mean)	55.8	75.5	83.3	97.4
RH % (minimum)	50.5	41.8	53.5	67.4
RH % (maximum)	60.6	94.2	95.0	100.0

In 2017 the temperature and relative air humidity during winter storage differed for the four winter storage locations (table 15). Overall, temperature conditions follow the measurements in 2016 with the lowest mean temperature found at the -5°C location, followed by the field, the barn and finally the +5°C location. The temperature in the field had slightly lower minimum and higher maximum compared to the barn. The relative humidity was lowest in the +5°C treatment and highest in the field.

Prior to the onset of the winter storage 100 cocoons were dissected. The cocoons were randomly chosen from five subsamples, taking 20 cocoons per subsample. Of these, 67 percent of the found larvae were judged to be alive. No differences in cocoon fresh weights, water content and fat (dry weight) were seen between subsamples. The average weight of the cocoons containing a living larva was at the onset of winter storage 3.14±0.06 mg FW with a water content of 56.9±0.4 percent. The living larvae had a fat content of 21.0±1.7 percent of the dry weight.

After the winter storage, cocoons from each of the five replicates per storage condition were dissected. In total 100 cocoons were dissected per storage condition. The probability of a cocoon containing a living larva did not differ between treatments. Neither did cocoon weight, larval fresh weight, larvae water content and fat content. For the dissected cocoons, 84 percent contained a living larva with an average larval fresh weight of 3.0±0.02 mg, a water content of 58.3±0.1 percent and a fat content of 24.7±0.4 percent of the dry weight.

In 2017 dead larvae were found to be either brown or blackened, and a few showed signs of desiccation. 27 percent of the living larvae had white internal spots and 11 percent of the dead larvae had such spots. Furthermore, 13 percent of the living larvae had either black, brown or red internal colourings. Whether these colourings suggest diseases is un-certain as it has not been tested. Of the 800 cocoons dissected, none was found to contain hyper-parasitoids.

Hatching of adult parasitoids under field conditions
In 2016, 2400 cocoons was set up in three replicates for the eight winter storage conditions and left to hatch under field conditions. Of these 17.3 percent hatched (n=415).

During spring the cocoons was exposed to large fluctuations in temperature and relative air humidity. Most wasps emerged as adults between May 21 and June 4. Throughout the spring period, both temperature and relative air humidity fluctuated considerably in a diurnal pattern and the temperature exceeded 40°C on11 occasions. The relative air humidity was fluctuating between around 30%RH and up to approximately 95%RH, with a general drop throughout the period.

There were differences between the treatments with respect to the fraction of cocoons producing an adult and time to emergence of adults. Moreover, there seemed to be an interaction effect of storage location and storage medium (table 16). This was in contrast to the measurements of the larvae performed just after the winter, when no large differences in survival of larvae, weight and fat content of the *B. curculionis* larvae were seen.

The most successful winter location and material was among buckwheat shells in the barn. In this location the largest fraction of cocoons emerged as adult. When the cocoons overwintered among plant debris in the field, they had the lowest emergence frequency (Table 16). Moreover, the winter storage also affected the time to the emergence of the adult wasps. Fast emergence was found for cocoons overwintering at +5°C and slowest emergence was found for cocoons overwintering at -5°C. Cocoons stored in the field or in the barns had an intermediate rate of emergence. The difference in time to emergence between cocoons stored at 5°C and –5°C was approximately 65°CDD.
The fresh weight and dry weight of the hatched adult parasitoids were not different between the eight storage conditions (data not shown). Water content differed between the storing of the cocoons in buckwheat placed in the field and the two storage conditions with buckwheat stored in the barn and debris stored at +5°C. The differences were respectively 9.15±2.8 percent (P-value=0.26*) and 9.6±2.9 percent (P-value= 0.22*).

TABLE 16. Hatching of adult parasitoids under field conditions in 2016 (n=300) and 2017 (n=360). Parameters are derived from the three-parametric log logistic model. Time when 50 percent of the parasitoids have hatched and the percent of hatched parasitoids for the two storage material and four storage environments. Calculation of degrees-days °C (°CDD) is based on a minimum developmental temperature of 6.5°C. Standard errors are shown.

Year	Material	Environment	Time to 50 percent emerged (°CDD)	Percent hatched
2016	Debris	-5	454.3±7.9	14.3±2.0
		+5	388.1±10.6	19.4±2.3
		Barn	409.6±5.9	23.0±2.4
		Field	441.4±8.4	8.7±1.6
	Buckwheat	-5	463.3±5.0	21.3±2.4
		+5	401.6±10.0	13.7±2.0
		Barn	430.6±6.5	26.3±3.5
		Field	421.8±7.7	19.7±2.3
2017	Debris	-5	534.1±7.3	22.5±2.2
		5	420.6±17.6	4.7±1.1
		Barn	498.3±10.8	10.8±1.6
		Field	493.8±22.2	5.6±1.2
	Buckwheat	-5	549.8±7.1	19.7±2.1
		5	487.0±7.8	12.8±1.8
		Barn	514.8±11.3	13.7±1.8
		Field	517.0±18.4	8.1±1.4

In 2017, 2880 cocoons were set up in three replicates for the eight winter storage conditions and left to hatch under field conditions. Of the set out cocoons 12.2 percent hatched.

Table 16 shows the time in °CDD to when half of the hatched adult parasitoids appeared and the percentage of adult parasitoids capable of hatching of the n=120 cocoons set up per replicate.

To summarize the pairwise comparison of the eight storage conditions. Differences were seen to depend primarily on the environment and be less dependent on the storage material.

Storage material and storage environment were analysed separately. Storage material did not reveal differences when comparing the time when 50 percent of the adult parasitoids emerged. When comparing percent hatched, buckwheat produced the highest percentage of hatched parasitoids, 13.6±0.9 percent, compared to 10.9±0.8 percent for the debris material. However, the differences are quite small and it is speculated if the found differences are of importance.

When comparing the estimates in table 16 it can be seen that if the cocoons are stored at -5°C the percentage of hatched imagines is higher than if the cocoons are stored under other conditions. At -5°C, the developmental time to 50 percent emergence is increased, which would suggest that the larvae stored at the three other temperature regimes show indications of increased metabolic activity under winter storage. However, this has not been confirmed by the measurements of larval fresh weight or fat content after the eight weeks of winter storage (table 17).

TABLE 17. Hatching of adult parasitoids under field conditions previously stored under the eight winter storage conditions of two materials and four environments (2017). Adult parasitoid fresh weight (FW) mg, dry weight (DW) mg, water content of FW percent and fat content of DW percent. Standard errors are shown. Capital letters display differences of P<0.05.

Material	Environment	Fresh weight (mg)	Dry weight (mg)	Water content of FW (%)	Fat weight of DW (%)
Debris	-5	1.15±0.03 AB	0.46±0.01 A	60.2±0.9 A	26.1±1.0 A
	5	0.92±0.08 B	0.41±0.03 A	53.1±1.0 BC	23.1±2.2 A
	Barn	1.18±0.05 AB	0.48±0.02 A	59.5±1.3 AB	25.8±1.4 A
	Field	1.08±0.07 AB	0.48±0.03 A	52.7±1.8 C	23.4±2.0 A
Buckwheat	-5	1.19±0.04 A	0.48±0.01 A	59.5±0.9 AB	25.6±1.0 A
	5	1.16±0.05 AB	0.47±0.02 A	58.3±1.2 ABC	26.8±1.3 A
	Barn	1.25±0.04 A	0.49±0.02 A	59.2±1.1 AB	27.2±1.3 A
	Field	1.21±0.06 A	0.50±0.02 A	57.6±1.5 ABC	25.2±1.6 A

Analysis of the weights of the adult parasitoids showed differences in fresh weight based on the winter storage conditions with the wasps stored in debris at +5°C being the lightest. Water content was also seen to differ between the winter storage conditions; however, differences in water content do not reflect differences in fresh weight. No differences were seen in dry weight and fat content between the hatched adult wasps.

Cocoons from each of the five containers per treatment were picked out and transferred to 25°C. Table 18 shows the time in °CDD to when half of the hatched adult parasitoids appeared and the percentage of adult parasitoids capable of hatching. Few differences were found when making comparing the eight winter storage conditions. Most noticeable were the differences at 50 percent emergence between buckwheat at -5°C and buckwheat stored under field conditions (45.7±14.5, P-value 0.0016**). For the parameter: percent hatched, differences were found when comparing debris and buckwheat stores at -5°C to buckwheat stored at +5°C with

differences of respectively 13.4±6.3 percent, P-value 0.34* and 11.5±6.5 percent, P-value 0.39*.

TABLE 18. Hatching of adult parasitoids at 25°C in 2017 (n=150). Parameters are derived from the three-parametric log logistic model. Time when 50 percent of the parasitoids have hatched and the percent of hatched parasitoids for the two storage materials and four storage environments are shown. Calculation of °C degrees-days (°CDD) are based on a minimum developmental temperature of 6.5°C. Standard errors are shown.

Material	Environment	Time to 50 percent emerged (°CDD)	Percent hatched
Debris	-5	436.6±26.8	20.5±4.5
	+5	378.2±16.1	26.4±3.9
	Barn	391.2±12.9	23.0±3.6
	Field	408.9±20.7	23.8±4.2
Buckwheat	-5	419.9±8.3	22.4±3.5
	+5	388.7±15.5	33.9±4.4
	Barn	400.4±12.1	29.6±4.0
	Field	374.2±11.9	26.9±3.8

Discussion

Of the four storage conditions +5°C had the shortest time to when half of the adults emerged. This was seen in both years. When hatching under field conditions and at constant temperature, the longest emergence time were seen for cocoons kept at -5°C. Storage under field and barn conditions resulted in storage at average temperatures above zero and below the minimum acquirements for development.

When comparing the developmental time to the found temperature needs in 2016 (399°CDD) and 2017 (476°CDD) storage below the minimum acquirements for development seemed to fit the found thermal needs, which, however, is different from the findings by Eklund and Simpson (1977) (appendix 3.1).

In 2016 storage above an average 0°C resulted in thermal needs fitting the 399°CDD and so did the 2017 findings (476°CDD). This is seen when comparing average temperatures in table 15 with the time to emergence of half of the parasitoids (table 16). Between years, the time point of appearance is, however, different as mentioned before (399°CDD in 2016 and 476°CDD in 2017). In 2016 the thermal needs for half of the parasitoids to emerge were from 388 to 431°CDD. In 2017 thermal needs were between 420 and 517°CDD, which points towards differences in acquired thermal time prior to the experiments. This is also seen when comparing storage at -5°C revealing differences in thermal needs of 83°CDD. The parasitoid larvae would therefore have been accumulating thermal requirements earlier, and the total temperature needs are thus even higher than what was found in the experiment. Under Danish conditions, temperature requirements are therefore much higher than was originally found by Eklund and Simpson (1977).

The weight of individual living larvae in the cocoons was not affected by the winter storage environment. Storage material also had no effect on larval weight. Larvae stored at +5°C weighed slightly less than larvae stored in the barn; however, the differences were not significant.

The different storage conditions did not influence the survival of the parasitoid larvae. Survival prior to and after storage was close to 57 percent in 2016 and in 2017 survival after storage was 84 percent. This suggests that if the cocoons were to be stored for eight to ten weeks all of the above conditions would be recommendable. Fløistrup (2017) found no differences in larval survival when storing between -4°C and 8°C for 12 weeks.

The viability of field-collected diapausing cocoons of *B. curculionis* larvae has previous been found to vary between 0 and 100% (Pike & Burkhardt, 1974a). Field survival of diapausing cocoons has been estimated to 16 percent (Cherry & Armburst, 1975).
In field-collected cocoons Pike & Burkhardt (1974a) found an average of 25.9% cocoons containing a living larva. Overwintering and hatching the collected cocoons showed 9% capable of hatching an adult parasitoid. This relates to 16 percent of the living larvae dying in either the overwintering situation or prior to hatching.
In the present study, cocoons were set to hatch under outdoor conditions. Of the cocoons 17.3 percent hatched in 2016 and in 2017 12.2 percent of the parasitoids hatched. Hatching was also tried under constant temperature and relative humidity. This doubled the percentage of adults hatching (25.8 percent). Temperature in the hatching setup reached values found to be lethal (Hama & Davis, 1983). Compared to hatching under constant temperature, high temperatures could be seen as the reason for half of the found mortalities.

Diapausing parasitoid larvae have a high mortality and nonviable individuals can signify between 70 and 84 percent (Pike & Burkhardt, 1974a, Cherry & Armbrust, 1975). Higher numbers have also been found (Armburst et al.,1972). Some of the mortalities can be explained by predators and hyper-parasitoids though it also seems that the mortalities can be coupled to when the parasitoid larvae starts its diapause. Armburst et al., (1972) found that the winter survival rate was higher for larvae entering diapause in early season while late season diapausing exhibited a low winter survival. Predation of diapausing larvae is a major factor for reducing the parasite population from year to year (Cherry & Armburst, 1975). Having the cocoons in a hatching setup would reduce predation but mortalities would instead be induced by factors such as temperature. Later releases of parasitoid will be discussed in section III.

Dissecting the cocoon and inspecting the larvae showed diseased larvae primarily to be brown or blackened, some having internal spots, spots that also are recognizable on living larvae. A portion of the living larvae had discolorations of the intestine with black, brown or red coloring. Whether these discolorations suggested diseases is uncertain.
Previous dissection of cocoons has revealed larvae to die of unknown causes (Pike & Burkhardt, 1974b). A likely explanation would be infection by a insect pathogenic fungus as *Z. phytonomi*. Infections have been found to occur on *H. postica* (Radcliffe & Flanders, 1998) and host infections decreased parasitoid survival by decreasing the population of the host, thereby indirectly affecting the parasitoid population (Goh et al., 1989). Harcourt et al. (1977) observed weevil larvae to die from fungal infection after finalizing their cocoons. As the larvae of *B. curculionis* construct their cocoons after the finalization of the host cocoon, it is plausible that the fungal infection would also infect parasitoid larvae. Descriptions by Ben-Zeév & Kenneth (1980), Harcourt et al. (1990) and Hassan (2013) of cadavers infected by *Z. phytonomi* resemble some of the observations in the present study. Whether *Z. phytonomi* is the cause of death has not been established, but the topic is relevant for future studies.

Appendix 3.3 Survey on the emergence and seasonal occurrence of the weevil and parasitoid

Appendix 3.3.1 Spring emergence of the weevil and parasitoid

In 2016 a conventional second year white clover field it was investigated when the *H. meles* adults started their migration from the overwintering sites into the white clover field. As the field were to be grown for sees production two years in a row it would be likely that the *B. curculionis* overwintered in the field.
Next to the second year field lie a first year conventional white clover seed field. Emergence was surveyed in both fields mid-April 2016. Both fields were surveyed for one week, where after focus turned to the second year field. The survey ended as the grower initiated cropping maintenance procedures.

Materials and methods

Steel emergence traps (Ø39cm) lined with weed cloth underneath a nonwoven cloth were used to catch adult *H. meles* weevils and *B. curculionis*. Each hatching traps covered 0.12m^2.

Survey one

Emergence traps (n=28) were placed in four groups of seven traps. Two groups were assigned to both the first and second year seed field. One group was setup next to the field borders, in close proximity of a hedgerow. The other was placed 75m within the two fields. The individual hatching traps were setup 10m apart parallel to the hedgerow. The steel hatching traps were firmly secured into the ground by pounding the metal ring of the hatching trap 3 cm into the soil with a sledgehammer.

The survey lasted one week and traps was emptied twice three days apart. Traps were relocated when emptied.

Survey two

The second survey was performed in the second year white clover seed crop. The hatching traps were divided into four groups consisting of seven traps (n=28). The four groups were placed parallel to the field border at distances of 0, 2.5, 5 and 15m. Individual traps was placed 7m apart. The survey lasted four weeks. Trapped insects were collected twice a week. At each collection, the traps were moved 0.7m, keeping the distance to the hedgerow. Installation of the traps was as described previously. Additional to the hatching traps pan traps were placed besides the hatching traps in the second week of the survey. The pan traps consisted of an iron post secured firmly into the ground. On the post, two racks capable of holding three pans (Ø17cm) were placed 0.2 and 0.6m above ground level. Three pan colors were used: white, yellow and blue, randomly positioned in each rack. Pan traps were divided into three sets with two replicates and placed: 0, 15 and 50m from the hedgerow. The distance between each replicate was 63m, with 7m to the nearest hatching trap.

Statistics

Data analysis was done in R (R Core Team, 2017) by using the function glm and the negative binomial function glm.nb from the library MASS (Venables & Ripley, 2002). However due to the lack of statistical strength of the collected data only the percentage of caught insects is given.

Results

A total of 19 adult *H. meles* were caught in the initial survey. Of these, 79 percent were caught at the first collection, three days after the onset of survey one. At this time point, the majority (80 percent) were caught in the hatching traps closest to the hedgerow in the second year white clover seed field. In the first year white clover seed field 6 percent were caught at the hedgerow and 13 percent were caught within the field, 75m from the hedgerow.

Table 19 shows the number of caught adult *H. meles* per week. Overall 49 percent of the collected *H. meles* weevils were caught in the first week. Of these 91 percent were caught in the proximity of the hedgerow. In the second week, 60 percent were caught in the proximity of the hedgerow and 27 percent were caught 2.5m from the hedgerow. In week three and four no *H. meles* was found at the hedgerow; weevils were, however, encountered within the field. There is a tendency to a general movement and dispersal of the weevil away from the hedgerow. However, based on the scarcity of the data points this could be random observations.

TABLE 19. The number of adult *H. meles* caught in hatching traps at different distances from a hedgerow per week in survey 2. Numbers in brackets display the actual week number of the year. The total number of caught weevils per week and per distance is shown.

Week number	Distance from hedgerow (m)				Total per week
	0	2.5	5	15	
1 (16-17)	20	1	0	1	22
2 (17-18)	9	4	0	2	15
3 (18-19)	0	2	4	0	6
4 (19-20)	0	1	0	1	2
Total per distance	29	8	4	4	45

Within the two last weeks of the survey two *B. curculionis* adults were caught in the hatching traps. Both were caught within the field at 5m and 15m distance. Whether the two parasitoids had hatched at the capture location or flown in is uncertain.

The Pan traps did not capture *B. curculionis*. Three *H. meles* were caught; in a white (n=2, week 2) and in a blue pan trap (n=1, week 3). The distance to the hedgerow was for the individuals caught in the white pan traps 0 and 15m and for the individual caught in the blue pan trap 50m. Catching the weevils in the pan traps would suggest flight.

Discussion

The first survey revealed that the majority of the *H. meles* weevils were not within the field but still in or around the overwintering quarters. Previously, hibernating adults have been found in debris of clover plants (Detwiler, 1923). In the current study the majority of the weevils were found in the edges of old white clover seeds fields which concurs with findings on *H. nigrirostris* (Detwiler,1923, Markkula & Tinnila,1956). Weiss & Gillott (1993) found the majority of *H. nigrirostris*, to hibernate within red clover (*Trifolium pretense* L.) and less in the surroundings of the field.

The first year white clover seed crop was not well established. In some areas the distance between white clover patches were up to 0.5m due to its patchiness; the field did not provide an excellent overwintering quarter for the weevil. As weevils were caught in these patches, it is likely that activity started prior to week 15, which also has been noted by Detwiler (1923). However, as the weevil hibernates, activity can be expected when temperatures are adequate.

Based on the results from the first setup, attention shifted to the second year white clover seed field following the movement of the weevil into the second year seed field. The survey showed the weekly progression of the weevils into the seed field. As only few individuals were caught in the pan traps, it seems likely that *H. meles* migrate by walking and only occasionally migrate by flight into a seed field from nearby winter quarters, which is in agreement with findings of Sechreist & Treece (1963) on *H. nigrirostris*.

Compiling observations from the current survey number two (2016) and observations in insects tents (2017), flight of *H. meles* was observed after 396°CDD. In tents flight was observed after 481°CDD (basis temperature 0.0°C).

Mating of *H. meles* was observed in early May, after 472°CDD in 2016 and 481°CDD in 2017. It is striking that the temperature sums are only 9°C apart between years. In connection with this, the first of two adult *B. curculionis* was caught after 509°CDD, which would suggest the parasitoid to appear just after mating of the host weevil and the start of ovipositioning. The ovipositioning of *H. nigrirostris* occurs as temperatures increase above 12 - 13.3°C (Weiss & Gillott, 1993, Hansen & Boelt, 2004). Barney et al. (1978) found that *B. curculionis* prefer the second and third larvae stage of *H. postica*. Related to *H. meles* and given the weevils' likeness with the weevil *H. nigrirostris* the second and third larval stage would appear after 13 days and

22 days respectively. This is when summarizing findings at constant 22°C, 70% relative humidity and a photoperiod of 16L:8D (Weiss & Gillott,1993), which is in accordance with findings of Chan et al. (1990) at 25°C on *H. meles*.
H. nigrirostris eggs can be found from the start of June and through to mid-July in Saskatchewan, Canada, suggesting a long ovipositioning period for *H. nigrirostris*. Markkula & Tinnila, (1956) reports an ovipositioning period of 47 days and a larval development period is from 14 to 20 days at 17 to 20.4°C. The preferred larval stages for ovipositioning of *B. curculionis* occur through June and into August. Compared to the hatching of *B. curculionis* under field conditions starting in May and ending at the start of July the parasitoid seems synchronized to the long ovipositioning period of its weevil host. The lifespan of the parasitoid at fluctuating temperatures, 6.7°C night and 18.3°C day, average 13 day with individuals capable of ovipositioning in up to 22 days (Yeargan et al., 1978). Longevity and lifetime fecundity increased if adult females have access to a sugary diet (Siekmmann et al., 2001) and honey-water increasing the lifespan to more than 20 days (Jacob & Evans, 2000).
The parasitoid has been found to deposit between 95 and 400 eggs (Barney et al., 1977, Barney et al., 1979, Hogg, 1994, Yeargan et al., 1978). The weevil *H. nigrirostris* oviposit around 290 eggs (Markkula & Tinnila,1956). It therefore seems likely that one parasitoid can cover the ovipositioning of one *H. meles* weevil. However, as the ovipositioning period of the weevil is longer than the lifespan of the parasitoid, parasitoids need to emerge for an extended period, which has also been seen found in the present study.

Appendix 3.3.2 Population development through the growing season
To follow how the numbers of parasitoid and weevil developed over the growing season, reflexed white clover flower heads were collected on a weekly basis at one organic white clover seed grower.

Materials and methods
The population dynamics of the weevil and frequency of parasitation with *B. curculionis* were studied in an organic white clover seed field in Zealand. The field was located > 400 meters from the *B. curculionis* release point and was not defoliated during the flowering period. Each week from the beginning of the white clover flowering (week 24) and through to seed harvest (week 31) flower heads were collected from the field. Flower heads were collected along a 250m transect with 20 sampling points approximately 10m apart. At each sampling position, 50 flower heads were collected. All sampled flower heads were at the same flowering stage, having reflexed florets and a green stem. This stage was chosen as it contained either weevil larvae in the final instar stages or newly spun weevil cocoons. The flower heads were stored in 520ml plastic cups. For ventilation, the lids had a Ø2cm hole covered by a finely meshed iron net.

The plastic cups were stored in large outdoor insect cages, 75x68x83cm (LxWxH) with a fine mesh on three sides (mesh size 0.2x0.2mm) and a glass top and door. The cages were protected from direct sunlight by a west to east facing wall with an overhang. To the north, trees shaded the site. Storage lasted at least 8 weeks, allowing weevil larvae either to develop into an adult weevil or letting the parasitoid construct its cocoon. Afterwards the flower heads were gently threshed and found adult weevils and parasitoid cocoons were recorded.

At the same time as the sampling of flower heads, the development of the flowering was recorded. This was done by counting the number of flower heads in different developmental stages at 10 locations along the transect in quadrants of 10x10cm. The flower heads were divided into six developmental categories: 1) Buds, 2) At least one floret open, 3) All florets open or senesced, 4) All flowers senesced but not harvestable, 5) Harvestable flower heads reflexed with green stem and 6) Harvestable flower heads reflexed with brown stem.

Statistics

The statistical program R (R Core Team, 2017) was used in for the data analysis utilizing the built-in functions lm(). Pairwise comparisons were done using the emmeans package (Lenth, 2018).

Results

No collection was performed in week 25 due to limited number of harvestable flower heads fulfilling the requirements. Due to heavy rainfall the flower heads collected week 31 were later discarded due to fungal infection.

In figure 7 the progression of the flowering is followed over the growing season. Figure 8 displays the number of collected adult weevil and parasitoid cocoons per week in the growing season. Comparing the two figures, it is possible to visualize how the number of weevil larvae are more abundant early in the season and decreases later in the season as the florets mature. The number of parasitoid cocoons collected does not seen to be strongly correlated (R^2 value 0.133) with the number found newly hatched adult weevils.

The two figures indicate that the highest numbers of weevils and parasitoids could be gathered if sampling were to be performed in week 26 to 27 in non-defoliated fields. The average number of weevil larvae parasitized throughout the season was 12±1.5 percent with the largest number seen in week 29 (18.3±3.3 percent) and the lowest number of parasitized weevils occurring in week 28 (4.5±3.4 percent) in the collected material.

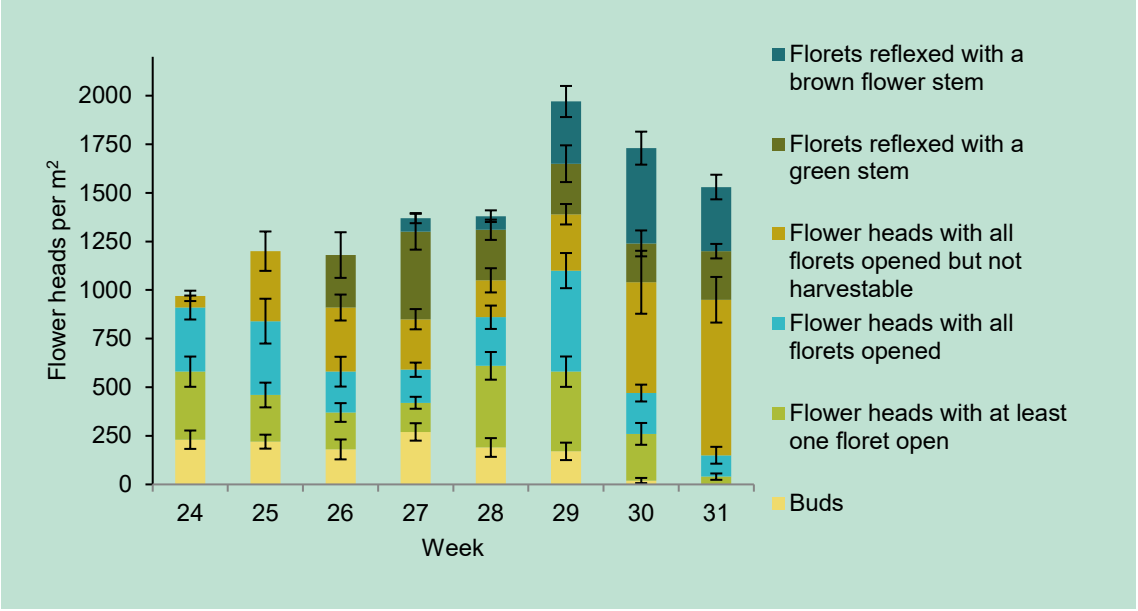

FIGURE 7. Number of flower heads per m^2 based on 10 counts in the non-defoliated organic white clover seed field during the 2016 growing season. The number of flower heads belonging to the six categories are displayed with different colours described in the figure legend above. Standard errors are shown.

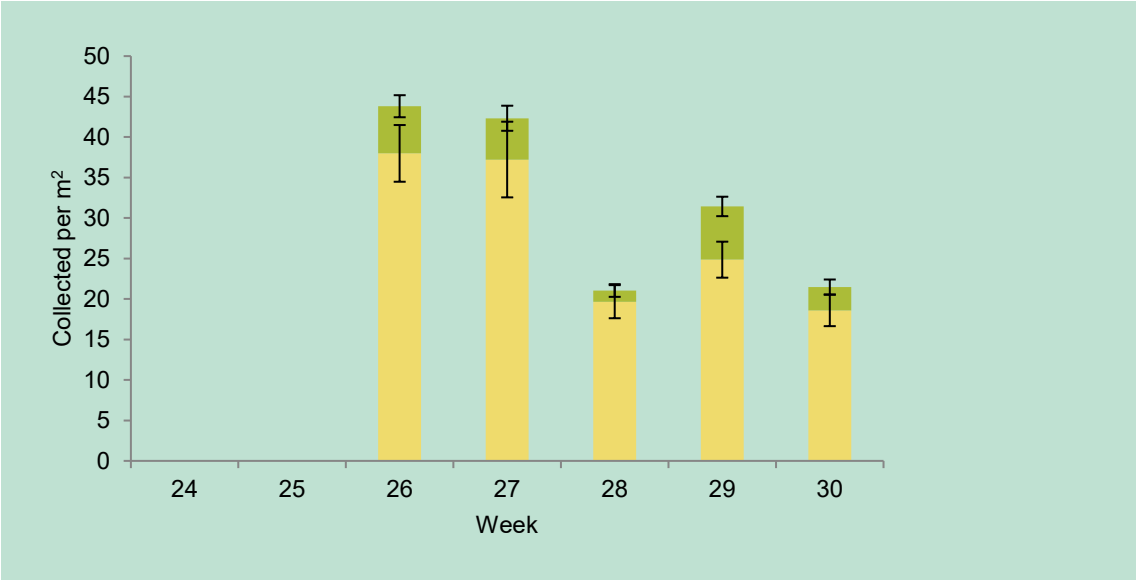

FIGURE 8. Number of insects per m^2 hatched from collected flower heads of the category reflexed with a green stem. Flower heads were collected in a non-defoliated organic white clover seed field during the 2016 growing season. Number of hatched *H. meles* (light brown) and *B. curculionis* cocoons (green) are shown. Standard errors are given.

Discussion

Figure 7 shows the flower heads with a brown stem to appear week 27 and increasing in numbers throughout remaining period. In week 27 the weevil population is at its highest with more than 40 individuals per m^2 of which 15.3 percent has been parasitized. From here on the number of weevil larvae producing either an adult weevil or parasitoid cocoon decreases (figure 8)

The parasitation rate of field collected *H. postica* has been found to be more than 60 percent (Schroder & Metterhours, 1980) though rates between 5 and 95 percent were observed (Dysart & Day, 1976, Flanders et al., 1994, Pike & Burkhardt, 1974a). Therefor the 15 percent parasitation lies in the lower end of what would be expected.

Earlier it has been found that the parasitoid does not oviposit in a host density depending manner (Barney et al., 1977, Harcourt et al., 1977, Yeargan & Latheef, 1976). Therefore the found decoupling between collected number of parasitoid cocoons per m^2 and number of newly hatched weevils is in line with earlier findings. Contrary to this, Rand (2013) found ovipositioning to be host density dependent in one year of a two year study.

Given the reported dispersal rate of the parasitoid (Chamberlin, 1926, Dysart & Puttler, 1965) of readily more than 48km per year and the previous mentioned non host density-dependent parasitism rate. It is possible that set out parasitoids (>400m away) will have contributed to the found 12 percent average parasitation rate. A survey in 2015 (section 8) of seven fields showed an average parasitation of seven percent. Indeed, without the added parasitoids, it seems that the presence of *B. curculionis* in the studied area is low when compared to what is found when studying parasitation of *H. postica* in North America, where the parasitoid was introduced as a classical biological control agent.

However, due to the dramatic decrease in the availability of organic white clover seed fields in 2016, the present location was the only possible site in which this survey could be performed.

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Appendix 4. Release of the parasitoid cocoons

Appendix 4 is divided into five parts. Part one deals with the release of the parasitoid tested in a semi-field setup. Part two describes behaviour changes in parasitized weevil larvae. The third part focuses on the combination of the parasitoid release and timing of insecticide application. Part four describes how the release of the parasitoid will effect weevil population the following year. The final part, describes work in relation to the field releases of parasitoid.

Appendix 4.1 Release of the parasitoid in different densities

The aim was to evaluate how different densities of *B. curculionis* affected the parasitation of *Hypera* weevils. This was done in two similar set-ups in 2016 and in 2017.

Materials and methods

Experimental setup

In 2016 and in 2017 two similar setups were used to study how densities of the parasitoid influenced the parasitation of *Hypera* weevils.

In 2016, 20 tent shaped soil emergence traps (BT2007, Bugdorm) measuring 60x60x60cm (LxWxH) were used. The “tents” were bottomless and placed in first-year white clover stands. Insects were added to each tent in the spring. The weevil density was the same in each tent, but the parasitoid density varied with five different densities.

The experiment was conducted as follows: tents were placed in one row in an organically maintained experimental white clover strip. A shallow trench, 8-10cm in depth, was dug around the cages to allow burial of the flaps on the lower parts of all four sides. The tents were held down by four tent spikes in the corners. The spikes were likewise buried in the trench. Soil were compacted to prevent insects from escaping. After installation, the white clover within the tent were swathed to a height of 12-15cm and treated with lambda-cyhalothrin with the intent of killing white clover seed pests enclosed in the tents. Lambda-cyhalothrin was applied in the form of 0.3kg/ha Karate 2.5 WG (Syngenta Nordics A/S) diluted in 200 l water and dispersed using a backpack sprayer. Insect releases started 20 days after insecticide application when sufficiently new white clover flowers were present to sustain the ovipositioning and larval development of *H. meles*.

To supply *H. meles* larvae, collected adult weevils were in 2016 set out at a density of six female and three male weevils per m^2, released 20 days after the insecticide treatment. Prior to release male and female weevils had been stored together to encourage mating.

Five treatments with four replicates were set up in 2016. Parasitoids were released in the tents eight days after the release of the weevils. For the five treatments the densities of female parasitoids were, 0 (control), 3, 6, 9, 12 female parasitoids per m^2. Parasitoids male: female ratio was 1:2.

Feed for the parasitoids was provided in the form of a 15 percent w/w sugar solution in a 6.5ml test tube closed with a Ø1.5cm, length 2.0cm dental cotton roll. The test tube was placed at

the same height as the white clover canopy and positioned horizontally to insure wetting of the cotton roll.

Collecting insects for the experiment
Adult weevils were collected throughout the spring of both years by netting and by setting up hatching traps in surrounding organic white clover strips and in commercial white clover seed fields. Until release, the weevils were held in transparent polycarbonate cages (22.4x31x12cm LxWxH) with ventilated lids. Weevils were fed fresh organic white clover leaves, which were replaced when needed.

B. curculionis cocoons were obtained by sorting debris from seed processing facilities in DLF and DSV. The sorting procedure has been described previously. *B. curculionis* adults were hatched under laboratory conditions in transparent polycarbonate boxes (22.4x31x12cm LxWxH) with a central Ø6.4cm hole in the lid. Per hatching box, adult parasitoids were hatched from 600 grams of the sorted material. The cages were wrapped in black plastic to encourage hatched imagines to move through the central hole in the lid. Collection of the imagines was done by having a Ø8cm cone made of overhead plastic on top of the 6.4cm hole. The top of the cone was removed to facilitate access of the parasitoids. On top of the cone a Ø8.4cm 280ml or 520ml plastic container was used for parasitoid collection. The container, placed top down, was held in place with rubber bands. In the collection container, imagines were offered a 15 percent w/w sugar solution. The solution was administered by dripping the solution onto a dental cotton roll (Ø0.7cm 3.6cm in length). The dental roll was inserted through a Ø0.7cm hole in the lower part of the plastic container. As the imagines appeared in the collection container, they fed freely on the cotton roll, on which the sugar solution was administered one to two times a day, depending on the number of imagines in the collection container. As more and more imagines emerged, the collection container was replaced with another collection container. Male and female parasitoids were stored together to encourage mating.

Pollination of the experimental tents was done by releasing bumblebees (*Brombus terrestris* L., Hymenoptera: Aphidae) (cat.nr. 207, BioProduction EWH). Previously, in a pre-test, it had been found, that bumblebees were able to perform pollination in the tents. Regular inspection secured that at least two living bumblebees were present in each tent at all times.

Sampling of weevils and cocoons in the tents
Parasitized and non-parasitized weevil cocoons were collected by collecting white clover flower heads in the tents. In 2016, white clover flower heads were sampled in late July, 40 days after the release of the parasitoids.

The final harvest consisted of collection of flower heads at two growth stages: reflexed flower heads with a green stem and reflexed flower heads with a brown stem. Previously differences between intact florets have been observed between the two categories (Topbjerg & Ytting, 2009) with the reflexed flower head with a brown stem significant higher numbers of insect damaged florets.
Per growth stage 25 (2016) and 20 (2017) flower heads were picked. The flower heads were kept in brown paper bags (8.0x26.5cm WxH) and stored outside in 75x68x83cm cages (LxWxH). The cages had a fine mesh on three sides (mesh size 0.2x0.2mm) and a glass top and door. Storage lasted at least eight weeks to allow weevil larvae to pupate and hatch as adult weevils or the development of the parasitoids cocoon. Registration of weevil: larvae, cocoons, adult females and males together with parasitoid: cocoons, adult females and males) were done for each tent per flower collection date. Collecting and counting were done manually by gently threshing the flower heads by hand.

Additionally, on the last collection date, 10 flower heads of the two growth stages were collected from the tents and used for the analysis of yield components. The flower heads were

stored in brown paper bags (8.0x26.5cm WxH) at -20°C until analysis. In the tents, the density of flower heads (heads per m^2) were assessed on an area of 10x10cm and divided into five categories: 1). Buds, 2). At least one floret open, 3). All florets open or senesced, 4). All flowers senesced but not harvestable, 5). Harvestable flower heads reflexed with green stem and 6). Harvestable flower heads reflexed with brown stem. Of the ten flower heads collected, five were randomly selected and number of florets counted. To lower workload, 20 florets per flower head were randomly selected and number of intact florets, number of insect damaged florets and number on non-pollinated florets were counted. Number of seeds in intact florets and number of seeds in insect damaged florets were counted in five florets selected from the pool of intact florets and insects damaged florets respectively.

In 2017 the weevil density was increased to 12 females and six males per m^2 released nine days after insecticide treatment of the cages. Five treatments with four replicates were set up in 2017. Parasitoids were released in the tents five days later than the weevils. Densities of female parasitoids were for the five treatments 0, (control), 6, 12, 22, 44 per m^2 respectively.

The experiment lasted 36 days. The earlier harvest was necessary as an increase in aphids threatened to destroy the experiment. In both years, release of field collected hoverflies (Diptera: Syrphidae) and green lacewings (*Chrysoperla carnea*, Stephens) (Neuroptera: Chrysopidae) (BioProduction EWH) were used in an attempt to control the buildup of aphid populations. Besides the final harvest, two additional sampling time points were introduced: 16 days and 28 days after the release of the parasitoids. Per sampling time point, 20 flower heads of the two flower head categories: reflexed flower heads with a green stem and reflexed flower heads with a brown stem were collected.

Yield components
Based on the count data, percentage of pollinated and intact florets, pollinated and damaged florets and non-pollinated florets was calculated based on the total number of florets evaluated. The number of seeds was counted for up to five florets, which enabled the calculation of the average number of seeds in intact and damaged florets.

Florets per head was noted, which enabled the estimation of the number of intact florets and damaged florets per flower head. Further, it also enabled the calculation of seeds per head from intact florets and seeds per head from damaged florets. In turn, this would be used to estimate the yield per flower head using a 1000 seed weight of 0.7 (Langer & Rohde, 2005) together with the estimated seed contribution from intact florets and from damaged florets.

The number of flower heads per m^2 was registered. As the density of ripe white clover flower heads are known to vary greatly yield per hectar has not been give as such estimations will be surrounded with large uncertainties.

Statistics
Data were analysed with the statistical software R (R Core Team, 2017) using the function lme() from the nlme package (Pinheiro et al., 2018).

Yield components were analysed with tent as the random effect. The different yield components were analysed as a two-way ANOVA having treatment and floret category as the fixed effects and tent number as a random effect. Multiple comparisons were made by using glht() from the package multcomp (Hothorn et al., 2008). For multiple tests, P-values were adjusted to control Type-I error by using the multivariate T-distribution (Bretz et al., 2008).

In cases of different significances occurring between the ANOVA analysis and the multi comparisons, boot strapping was applied on the final model to re-evaluate the archived P-values.

Model evaluation was done by residual plots and plotting the sample quantiles against the theoretical quantiles (qqplot). Evaluation of the residuals of the random effect was done by including the random effect in a linear model lm(). The qqplot of the random effect was evaluated as a plot of the predicted random effects (EBLUPs) of the end model.

Hatched adult weevil and found parasitoid cocoons were analysed with the built in function glm() for generalized linear models. Pairwise comparisons were done using the emmeans package (Lenth, 2018).

Statistics on the flower head category data were analysed with lm() in the statistical program R. Treatment were set as the fixed variable and the flower head categories were set as the response variable. The data were analysed using a one way ANOVA and pairwise comparisons were calculated using the glht() function. Significant levels of $P<0.05$, $P<0.01$ and $P<0.001$ are indicated as *, ** and ***.

Results

Yield components

In 2016 treatment differences were in general not present. Yield components showed differences between the flower head categories. Statistics on the different yield components are not depicted in the result but available in supplementary data 1.

The overall difference in flower head category is seen as an outcome of florets subjected to the weevil larvae. Higher damage rates were seen for the flower heads reflexed with a brown stem compared to those with a green stem, which indicates that the oldest flower heads were the most damaged. See figure 9A and 9B.

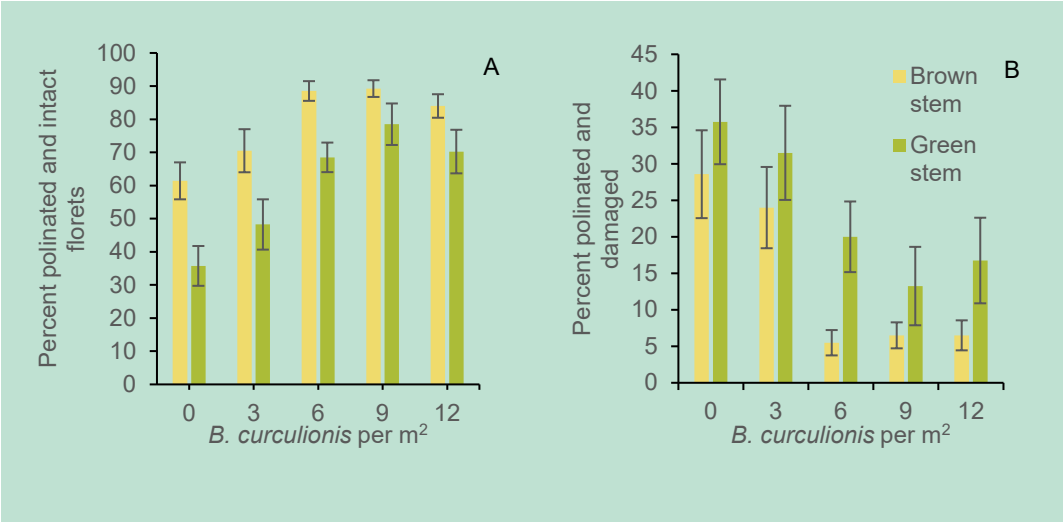

FIGURE 9. A. Percent florets pollinated and intact florets in 2016 for the two flower head categories fully reflexed with a brown stem (light brown) and fully reflexed with a green stem (green) for the five treatments. B. Percent pollinated and damaged. Average values with standard errors are shown.

When estimating the percentage of pollinated and intact florets, pollinated and damaged florets, intact seeds per intact floret and intact seeds per intact floret, no differences between treatments were found (supplementary data 1) differences were only seen between flower head category. This would suggest that the damage by *Hypera* larvae per floret attacked does not depend on the number of parasitoids present.

Scaling up to estimate seeds per flower head, no differences in florets per flower head were found between flower head category or density of *B. curculionis*. The overall average number

of florets per flower head was 85.5±2.3. Assuming there is about 900 flower heads per m^2 (Nordestgaard, 1986; Langer and Rohde, 2005) this will relate to around 76.500 florets per m^2.

Per flower head it was estimated that the average number of seeds from intact florets was 170±14 per flower head and that the damaged florets contributed with an average 18.4±3.4 seeds. Differences between flower head category and parasitoid density was not obvious although influences of the two fixed effects was seen.

By utilizing a 1000 seed weight of 0.7g a yield per flower head was calculated. Differences in seed yield (g) from intact florets depended on flower head category and treatment. Yield from damaged seeds was not dependent on the two fixed effects. Figure 10 shows the yield per flower head. Although no differences could be observed, figure 10 does show a steady increase in the seed yield as the parasitoid density increases up to six female parasitoids per m^2, after which the seed yield levels out and decrease. The increase suggested that experiments with higher concentrations of the parasitoid would be relevant thus in 2017 the density of the parasitoids was increased.

FIGURE 10. Seed yield per flower head in 2016 for five *B. curculionis* densities shown for the two flower head categories: Flower head fully reflexed with a brown stem (light brown) and fully reflexed with a green stem (green). Average values with standard errors are shown.

In 2017 observations on percent pollinated and intact florets, percent pollinated and damaged florets follow the general trends of what was observed the year before. However, the expected increased benefit of additional parasitoids was not found. For the seed yield per flower head, no differences were found with an average seed yield of 0.11±0.01g seeds per flower head. Comparing the seed yield per flower heads between years, the two years produced similar yields per floret (supplementary data 1).

Collection of adult pests and parasitoid cocoons from collected flower heads
In 2016, flower heads were collected at harvest and stored until either the adult weevil emerged or a parasitoid cocoon was spun. In total, across all treatments, 229 individual adult weevils were found together with 33 cocoons of the parasitoid. The number of weevils found was analysed per collection (25 flower heads) and per flower head using the binomial distribution. Number of found animals were compared to the total number of collected insects. For both approaches, no differences were found between flower head category and the five densities of the parasitoid.

In 2017, flower heads were collected at three collection dates: July 6, July 19 and at harvest on July 26. Per tent, 20 heads were picked. Across all parasitoid densities, 14 parasitoid cocoons or hatched adult parasitoids were found together with 123 adult weevils. Of the collected adult weevils 21 percent (n=26) were collected at two samplings. The number of hatched weevil adults and cocoons spun by the parasitoid was analyzed for the five densities and the three collection times. No differences were found for both number of collected weevil adults and parasitoid cocoons.

Total number of collected weevils and parasitoid cocoons were bulked across collection time. This did not produce differences between the five parasitoid densities.

Flower head categories
The treatments did not display differences in the flower head categories in 2016 and 2017.

Discussion
For both years, trends based on the density of the parasitoid was seen. Comparisons of treatment and flower head category did not reveal differences.

In both years, yield components showed that the density of *B. curculionis* influenced positively on the percentage of pollinated and intact florets and decreased the number of damaged seeds. The major part of the seed yield originates from the earlier flower heads (fully reflexed with a brown stem) as seen previously (Topbjerg & Ytting, 2009). These are also the ones subjected to the largest pest pressure. Crop protection should therefore focus on these flower heads.

Comparing the overall strategy of reintroducing collected parasitoid cocoons and the results from the yield components, it is clear that a direct control is achieved. As the parasitoids only have one and a partial second generation per year, the direct control cannot originate from the inoculate interaction but must be linked to an inundative interaction between parasitoid and host.
The decrease in damage must be derived from the parasitoid influence on weevil larvae, meaning that the larvae are killed by the parasitoid (Bartell & Pass, 1978a, Duodu & Davis, 1974b). Parasitation do not seem to decrease feeding (current study) though the opposite has been found previously for *H. postica* (Bartell & Pass, 1978a).
Previously, the injuries caused by the parasitation process led to a direct control of *H. postica* by *B. curculionis*, as such injuries seemed to cause host mortalities (Yeargan & Latheef, 1976) and superparasitisms were known to occur in the later larval stages of *H. postica* (Barney et al., 1978, Berbert, 1982). Superparasitism might also happen to *H. meles* larvae killing off early instars of the weevil larvae. The current findings are, however, an indirect observation on the parasitoid killing of the weevil larvae, as they are based on differences in yield components. The direct parasitoid to host larvae observation is lacking.

As the density of hosts previously has been seen not to impact the number of parasitations (Barney et al., 1977, Harcourt et al., 1977, Yeargan & Latheef, 1976) and the distribution of parasitoid eggs is random regardless of host density (Barney et al., 1977), a non-density dependent host seeking (Barney et al., 1977, Yeargan & Latheef, 1976) is likely. This suggests that several of the weevil larvae were not found by the parasitoid.

Another possibility could be linked to the lifespan of the weevil. The weevil has a longer lifespan than the parasitoid. Weevil larvae hatching late would therefore be free from the presence of the parasitoid. This seems a possible explanation of why the decrease in weevil damage did not change when increasing the parasitoid number per m^2 to more than three times the maximum released in the first year.

Other possible explanations of the decrease could be host feeding. However, as *B. curculionis* is a koinobiont endoparasitoid, this points towards it being proovigenic, whereby host feeding is less likely. Indeed in the year-long work (1911-1988) from the first introduction (Chamberlin, 1924, Chamberlin, 1926) through release programs of *B. curculionis* and related *Bathyplectes* species carried out by the USDA up to 1988 (Bryan et al., 1993), host feeding has not been mentioned, see for example Dysart & Day (1976) or Bryan et al. (1993). In a review on host feeding species belonging to Ichneumonidae are found to host feed (Jervis & Kidd, 1986) though the genus *Bathyplectes* was not included. Also, the biological parameters of *B. curculionis* (Dowell & Horn, 1977) do not suggest *B. curculionis* to be a host feeding parasitoid.

Appendix 4.2 Parasitized *Hypera* larvae inflict less damage to white clover seed

The aim was to evaluate if the parasitation of *Hypera* weevils by *B. curculionis* changed the feeding pattern of the *Hypera* weevil larvae. This was done by setting up a laboratory parasitation experiment measuring the weight gain of weevil larvae, which had or had not been parasitized by *B. curculionis*. In 2016, a preliminary experiment was set up with a total of 48 weevil larvae. In 2017, a larger experiment with 242 weevil larvae was set up.

Although the overall strategy depended on the recurrent release of the parasitoid, literature stated that there is a within year effect. The present experiments concerning the density of the parasitoid, try to evaluate this effect. The numbers of hatched individuals from collected flower heads should visualize the effect. However, an indirect effect should also be present on larval food consumption.

Materials and methods

The 2016 experiment was set up in two parts. This was done to establish how the larvae would respond to being handled and weighed. In part one larvae of stage three were handled every second day. In part two larvae of larval stages three and four were handled every day. The differences in handling times gave experience on how the weevil larvae would react to being handled. Also, these small experiments provided insight into how the larger experiment in 2017 should be conducted.

Parasitation experiments in 2016

Parasitation was performed by subjecting a single *Hypera* larva to a single female parasitoid. The parasitoid and *Hypera* larva were separated as soon as parasitation was observed.

Collection of Hypera larvae in 2016

In the morning, *H. meles* larvae were collected from insect tents (2.5x2.5m). These tents were also used for the experiment trying to determine parasitoid influence on weevil populations across years. The tents contained a flowering white clover sward and *H. meles* weevils. Care had been taken to avoid parasitoids in the tent.

Flower heads were picked and examined for larvae belonging to larval stages 3 and 4. The collection was done as gently as possible in order not to disturb the experiment more than necessary. The retrieved larvae were gently transferred to 30ml plastic cups with a small brush for storage. The cups contained white clover flowers as a food source.

Before the start of the experiment, each larva was examined to determine the larval stage. Larval stages were evaluated based on measurements of the head, following Detwiler (1923). Collected larvae all belonged to either the second last of final instar stages i.e. stages three or four. All larvae were weighed to determine the start weight of the larvae. Handling the larvae

was done using small brushes. The first part of the experiment was performed on 25 larvae, part two utilized 21 larvae.

Parasitation tubes in 2016
Parasitation of *Hypera* weevil larvae was carried out in 6.5ml test tubes closed with a dental cotton roll with Ø1.5cm, length 2.0cm.

Collection of parasitoids 2016
B. curculionis cocoons were obtained by sorted debris from the DLF's seed processing facility. The sorting procedure was described previously. *B. curculionis* adults were hatched under laboratory conditions in transparent polycarbonate boxes (22.4x31x12cm [LxWxH]) with a central Ø6.4cm hole in the lid. Per hatching box, adult parasitoids were hatched from 600 grams of the sorted material. The cages were wrapped in black plastic to encourage hatched adults to move through the central hole in the lid. Collection of the adults was done by having a Ø8cm cone made of overhead plastic on top of the 6.4cm hole. The top of the cone was removed to facilitate access. On top of the cone, a Ø8.4cm 280ml or 520ml plastic container was used for parasitoid collection. The container, placed top down, was held in place with rubber bands. In the collection container, imagines were offered a 15 percent w/w sugar solution. The solution was administered by dripping the solution onto a dental cotton roll (Ø0.7cm, length 3.6cm). The dental roll was inserted through a Ø0.7 cm hole on the lower part of the top plastic container. As the imagines appeared in the collection container the sugar solution were administered one to two times a day, depending on the number of imagines in the collection container. As more and more imagines emerged, the collection container was replaced with another collection container. Male and female parasitoids were stored together to encourage mating.

B. curculionis females were selected randomly among newly hatched parasitoids which had spent a couple of days in the collection containers to ensure mating. Individual female parasitoids were assigned to an individual test tube.

The parasitation in 2016
Collected weevil larvae were transferred to a white clover flower head, serving as a natural feeding and hiding place for the larvae. The flower was modified to only having three to five florets. This modification enabled better observation of interaction between larva and parasitoid. The larva was left on the flower head for a minimum of five minutes to hide and start feeding. Then the flower head with larva was gently placed in the test tube containing a parasitoid. The setup was monitored and if parasitation occurred, the weevil larva was gently removed from the tube together with the white clover flower. If no parasitation occurred within the first 15 min of observation, the larva and flower head were transferred to another test tube with another parasitoid. The setup ensured that each larva was only parasitized once.

Monitoring of the larvae in 2016
After parasitation, the larvae were kept at constant temperatures in a climate cabinet, with the set points: 70% relative humidity, 25°C day and 15°C night with a D:N of 16:8 hours. Each larva was held on a white clover flower with three to five florets. Humidity was provided by planting the freshly cut white clover flower in 5ml 1.5% water agar in 30ml plastic cups with lids. The lids were punctured with 1mm holes to enable ventilation. The larvae were moved to a fresh flower in fresh media when needed. Fresh flowers were picked from an organically maintained white clover strip and prepared just prior to use.

The experiment was run two times, one where larva weight gain was measured every second day (part 1) or each day (part 2). Weighing the larva was done by gently removing the larva from the floret and transferring it on to filter paper and recording the larva weight on a 7-digit microbalance (Santorius MC5). Larval mortality and duration until pupation was recorded.

Parasitation experiments in 2017
Experiments in 2017 was carried out as in 2016, but with some modifications.

In the 2017 experiment, the number of weevil larvae was increased. In total 242 larvae were used in the experiment. Of these, 228 could be monitored throughout the experimental period, the remaining 14 either escaped or went into pupation within the first two days before the second weight measurement could be obtained.

For both larval stages three and four, 34 individuals were successfully parasitized. The control group consisted of 80 individuals for each larval stage. The number of larvae in the control group, i.e. non-parasitized, was deliberately greater than the treatment group. This was done as larvae were collected in open field plots. Rearing weevil larvae in cages had been tried; however, due to problems concerning pollination and survival of adult weevils this strategy was abandoned.

The experimental setup was changed from the previous experiments in regard to storage conditions. The relative humidity was increased (85%) and the temperature was decreased to 18°C. These changes would slow the development of the larvae and provide humidity for the system in a different way than using a water agar solution. Instead of providing the larva with white clover florets still attached to the flower stem, individual florets were given to the weevil larva when needed. This decreased the risk of the larva not capable of finding its food source. Supplying the larva with single florets meant that the experimental unit could be decreased. Instead of utilizing 30ml plastic cups, the larva could be kept in Eppendorf tubes, thereby securing the larva easier access to the food source. Larva weight was registered every second day. The decrease in storage temperature allowed the larvae to be followed for up to 14 days.

Hypera larvae were collected in an organically maintained experimental strip. Per collection, 200 white clover heads were picked and brought to the laboratory. Larvae were obtained by carefully examining the florets and brushing the flower heads by hand. During the experiment period, five collections were made.

B. curculionis females were picked from newly hatched parasitoids kept in collection containers together with male parasitoids to allow mating. To increase the high success rate for parasitation, active female parasitoids were selected. This was done by exposing the female parasitoids to fecal matter from weevil larvae. Only females who responded positively to the presence of feces were selected. The ability of *B. curculionis* to locate a host has previously been studied by McKinney and Pass (1977), who found the parasitoid to be attracted to feces from the larvae of *H. postica*.

After parasitation the larva was kept at constant temperatures in a climate cabinet. Climate set points were 85% relative humidity and 18°C with a D:N of 12:12 hours. Fresh florets were provided when handling the larvae.

Larval weight gain was recorded every second day by gently removing the larva from the floret and transferring it on filter-paper to a 7-digit microbalance (Santorius MC5). Larva mortality and duration until pupation was also recorded.

To obtain the number of weevils successfully emerging as imagines, weevil cocoons were kept separately at room temperature until the adult weevil had hatched, i.e. 37 days after the final weevil larvae had produced a cocoon or had died. At this time point, the number of parasitoid cocoons, adult parasitoids, weevil larvae dying before construction a weevil cocoon or weevil larvae not emerging from pupation were recorded.

Statistics

Data were analysed with the statistical software R (R Core Team, 2017) in which the built in function glm() for generalized linear models was used. The response variable as either: the number of weevil larvae developing into an adult weevil, a parasitoid cocoon or the weevil larva ending up either dead or dead in the pupation stage. A two-way ANOVA analysis was used to evaluate the influence of the fixed effects: parasitation and larva stage. Multiple comparisons were made by using glht() from the package multcomp (Hothorn et al., 2008). For multiple tests, P-values were adjusted to control Type-I error by using the multivariate T-distribution (Bretz et al., 2008). In cases of different significances occurring between the ANOVA analysis and the multiple comparisons boot strapping was applied on the final model to reevaluate the archived P-values.

The relative growth rate (RGR) was calculated as: ln(end weight) – ln(start weight) / time. Weight was calculated in mg and time was treated in either hours or days. In case of a larva not eating, the RGR would become negative. Such observations were removed from the data set.

Comparison of the variables: RGR, maximum weight of larvae and time to pupation was carried out with the function lm() in R. Model reduction was carried out with ANOVA. Model evaluation was done by residual plots and plotting the sample quantiles against the theoretical quantiles (qqplot). Multiple comparisons were made by using either glht() or emmeans() from the package emmeans (Lenth, 2018). Significant levels of $P<0.05$, $P<0.01$ and $P<0.002$ are indicated as *, ** and ***.

Results

Preliminary parasitation experiments in 2016

With the larvae developing at 25 degrees, the time to pupation was short. This resulted in few weight measurements in experimental part one and a few more measurements in part two of the experiment. However, part two might introduce a bias coupled to the decreased time between disturbing the larvae. Observations confirmed a tendency to larvae not feeding if handled too often. Furthermore, the experimental setup with a few florets attached to the white clover flower stem and water agar providing moisture for the system in 30ml cups made for an environment in which the larva could become disorientated and not able to finding its way back to the food source, the floret. It was observed that if a larva somehow was separated from the food source, the larva had a hard time finding its way back.

Part one gave weight measurements on two occasions: two and five days after parasitation. Hereafter the larva started pupation. No differences were seen between parasitized and non-parasitized larva. Data are not shown due to low number of replicates. The relative growth rate decreased slightly as the larvae started pupation. Observations suggested that in the time up to the starting of pupation, the larva stopped feeding resulting in a decrease in weight.

Part two of the experiment resembled the findings of part one. No weight differences and relative growth rates were seen between parasitized and non-parasitized larvae. As the weight of the larva was recorded every day following the parasitation, measurements could only be obtained on three occasions. Within these days, the relative growth rate decreases revealed a linear respiratorial energy consumption.

Table 20 summarizes the outcome of the parasitation experiment for experimental part one and two.

In the first part of the 2016 experiment non-parasitized weevil larvae either developed into an adult weevil (n=7), died (n=4) or had previously been parasitized by another wasp (n=1). For

the parasitized weevil larvae, 46 percent developed a parasitoid cocoon. Of these, two adult parasitoids hatched. The remaining cocoons, 62 percent, contained a parasitoid larva.

Table 21 divides the second part of the 2016 experiment based on larval instar stage. For the third instar, all non-parasitized larvae died. For the parasitized larvae 1/3 (n=1) developed into adult weevils and 2/3 (n=2) died. For the forth instar, parasitation succeeded in 71 percent (n=7) of the times. The remaining parasitized larvae either died or developed into adult weevils. For the non-parasitized fourth instar larvae, around 1/3 (n=3) died and the remaining developed into adult weevils (n=5).

TABLE 20. The outcome of the parasitation experiment parts one and two. In the table, the fate of weevil larvae parasitized and not parasitized is shown in percent. The outcome being: Diseased, development into an adult weevil, development of a parasitoid cocoon, a second unknown parasitic wasp. Also, the total number of weevil larvae are given.

Experiment	Treatment	Number larvae	Weevil	Parasitoid			Other	
			% adult	% cocoon	% adult	% total	% other wasp	% Dead
Part 1	Parasitized	13	23	46	15	62	8	8
	Non-parasitized	12	58	0	0	0	8	33
Part 2	Parasitized	10	20	0	50	50	0	20
	Non-parasitized	11	45	0	0	0	0	55

TABLE 21. Shows experiment part 2 subdivided into the third and fourth larvae developmental stage. For table description, see table 20.

Larval developmental stage	Treatment	Number larvae	Weevil	Parasitoid			Other	
			% adult	% cocoon	% adult	% total	% other wasp	% Dead
Third instar	Parasitized	3	33	0	0	0	0	67
	Non-parasitized	3	0	0	0	0	0	100
Forth instar	Parasitized	7	14	71	0	71	0	14
	Non-parasitized	8	63	0	0	0	0	38

Parasitation experiments in 2017

In the 2017 experiment, the number of weevil larvae was increased to a total of 228 weevil larvae. Table 22 summaries the outcome of the parasitation experiment. The data show that parasitized weevil larvae primarily develop into a parasitoid cocoon or die in the larval stages. Of the parasitized larvae only six and nine percent for larvae instars three and four respectively develop into an adult weevil, indicating that the parasitation did not succeed. The non-parasitized weevil larvae primarily developed into adult weevils or died prior to finalizing metamorphosis. The number of weevil larvae dying before reaching adulthood seems similar between parasitized and non-parasitized third instar larvae.

As the utilized larvae were collected from natural conditions, there was a possibility of the larvae being parasitized before collection. In table 22, the amount of prior parasitized weevil larvae is seen to be five percent for the third larvae instars and 15 percent for the fourth larvae instar.

TABLE 22. The outcome of the parasitation experiment. Percent larvae developing into an adult weevil, a parasitoid cocoon, an adult parasitoid or dying either in the larval stage or in the pupation stage are shown per treatment and larval development stage. Parasitoid cocoon and adult are summarized. Also shown is the number of larvae used in the different treatments.

Larvae development stage	Treatment	Number of larvae	Weevil	Parasitoid			Diseased	
			% adult	% cocoon	% adult	% total	% dead in the larvae stage	% dead at pupation
Third instar	Parasitized	34	6	47	0	47	41	6
	Non-parasitized	80	40	4	1	5	44	11
Fourth instar	Parasitized	34	9	44	0	44	44	3
	Non-parasitized	80	46	15	0	15	25	14

TABLE 23. The possible outcomes of the experiment are given on the odds-scale for the treatments. Differences between treatments are given based on pairwise comparisons. Number in brackets represents the 95 percent confidence interval. A, ANOVA analysis compiling the main effect of the treatment (parasitation), larval stage and the interactions of the mail effects are given (T x LS). NS denotes non-significant. *, ** and *** represent P-values <0.05, <0.01, <0.001 respectively.

Treatment		Risk of dying at weevil larval stage	Risk of dying at weevil pupal stage	Possibility of hatching as weevil adult	Possibility of development of a parasitoid cocoon
Parasitized		0.54 [0.34, 0.84]	0.55 [0.37, 0.83]	0.12 [0.04, 0.58]	0.44 [0.29, 0.67]
Non parasitized		0.65 [0.46, 0.91]	0.61 [0.43, 0.79]	0.44 [0.33, 0.59]	0.07 [0.05, 0.13]
Difference		0.83 NS	0.91 NS	0.12***	5.78***
ANOVA	Treatment (T)	***	NS	***	***
	Larval stage (LS)	NS	NS	NS	NS
	T x LS	**	NS	NS	NS

To further establish if differences occurred between the treatments, data were treated as binomial and converted to the log-odds scale; in the following table 23 estimates are given on the odds scale. In table 23, it can be seen that the risk of dying in the larval stage is influenced by the interaction between treatment and larval developmental stage. However, when comparing the 95 percent confidence interval and making pairwise comparison, the differences are no longer significant.

Table 24 depicts the number of larvae having a positive weight gain when weighing the larvae at 2 to 16 days after parasitation (DAP). The differences between the number of weevil larvae included per treatment and the number of larvae shown in table 24 are based on larvae dying, entering pupation or larvae having a negative weight gain, occurring just prior to the pupation or in situations where larvae show signs of diseases such as fungal infection.

TABLE 24. The number of weevil larvae after the parasitation event. The number of larvae having a positive weight gain at each measuring point, days after parasitation (DAP) are shown for the two larval stages and treatments.

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Treatment	Larval stage	Days after parasitation (DAP)							
		2	4	6	8	10	12	14	16
Parasitized	3	21	20	19	11	6	2	-	-
	4	32	24	9	3	1	-	-	-
Non-parasitized	3	41	38	37	22	10	3	5	1
	4	71	41	14	9	1	-	-	1

Based on the larval numbers in table 24 a relative growth rate (RGR) could be calculated. Figure 11 shows the average larval weight and RGR for the two larval stages.
Larvae of stage three gained weight until DAP eight to ten, after which the weight gain stopped as the larvae stopped feeding and entered pupation. For the fourth larval stage the weight increase stopped around DAP four to six. The RGR decreased for both larval stages three and four within the period. Although the larvae gained weight, the decrease in RGR would imply an increase in energy consumption (respiration) as the larvae grew. Differences between non-parasitized and parasitized were not observed for either larvae stages three or four.

Treating the decreasing RGR as a linear regression did not result in differences between the linear slopes. However, the time in DAP to pupation differed between larval stages, which would be expected based on larval development. Data are not shown.

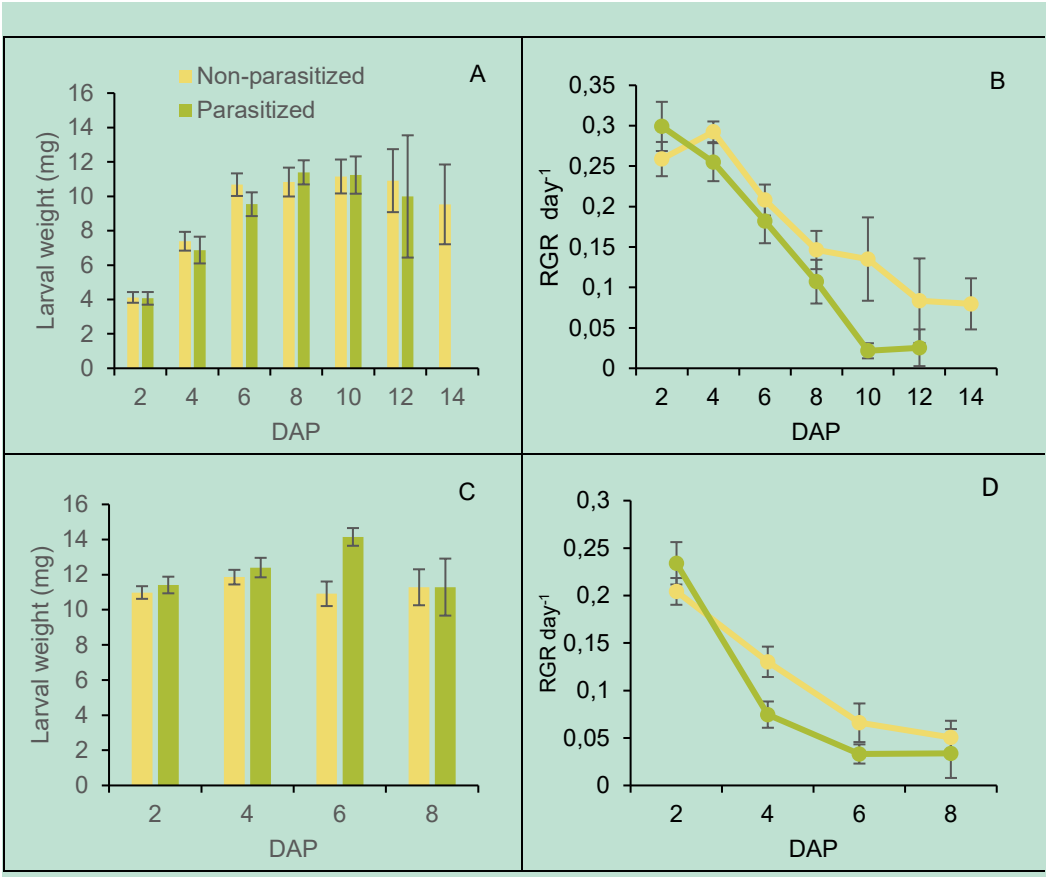

FIGURE 11. Larval stage 3 (A,B) and 4 (C,D) showing the average larval weight (A,C) and relative growth rate (RGR) (B,D) per day after parasitation (DAP). Non-parasitized depicted in light brown and parasitized larvae depicted in green. Standard errors (SE) are given.

In table 25 the maximum weight gain is not different between treatments or larval stages. Further, the ANOVA analysis shows that treatment was the only main factor influencing the maximum weight gain. Across the three parameters (table 25) treatment did seem to influence all parameters, whereas larval stage influenced the parameters RGR and time to pupation.

TABLE 25. The table summarizes response variables connected to the time just before the weevil larvae stops their weight gain. The table shows the average maximum weight, the relative growth rate (RGR) up to the time of the maximum weight and the time of parasitation to the time of pupation. An ANOVA analysis compiling the main effect of the treatment (parasitation), larval stage and the interactions of the main effects are given (T x LS). NS denotes non-significant. *, ** and *** represent P-values <0.05, <0.01, <0.001 respectively. Different capital letters denote differences between treatments and larval stages combined (interaction).

Treatment	Larval stage	Maximum achieved weight (mg)	RGR hour^{-1}	Time in experiment to pupation (hours)
Parasitized	3	10.9±0.7 A	0.008±0.001 AB	287±13 B
	4	12.8±0.5 A	0.006±0.001 A	233±10 B
Non-parasitized	3	11.9±0.6 A	0.011±0.002 B	268±12 B
	4	12.5±0.3 A	0.006±0.001 A	199±7 A
ANOVA	Treatment (T)	***	***	***
	Larval stage (LS)	NS	***	***
	T x LS	NS	NS	NS

For the fourth larval stage the relative growth rate was not significant different between parasitized and non-parasitized larvae. Differences was neither found for the third instar larvae. However, the relative growth rate for the non-parasitized third instar larvae was significantly higher than the growth rate of the fourth instar larvae.

The time from parasitation to pupation was significantly lower for fourth instar non-parasitized larvae compared to the other treatments (table 25).

Discussion

In 2016, the preliminary experiments revealed a high mortality rate for the weevil larvae. Highest was the percentage of dead larvae for the non-parasitized larvae for both parts one and two. This would suggest that the experimental setup had a flaw and introduced a bias when comparing parasitized and non-parasitized larvae. It indicates that larvae chosen for parasitation were more fit than the larvae chosen as the control group. Based on those observations it is speculated if the parasitoid can distinguish between healthy and unhealthy host larvae. When setting up the 2016 experiment, a number of weevil larvae intended for parasitation were not parasitized by a chosen individual parasitoid. At first, this was thought to indicate low fecundity of the parasitoid; however, it could also be a sign of low fitness of the *Hypera* larva. Moreover, as these larvae were not successfully parasitized, they were transferred to the control group of non-parasitized larvae.

In general, parasitized weevil larvae have a significantly higher probability of developing into a parasitoid cocoon when compared to non-parasitized larvae. This evidently means that the host defenses do not encapsulate the parasitoid egg. Therefore, the host *H. meles* is not capable of reducing the effectiveness of the parasitoid as described for *H. postica* and other *Hypera* weevils (Dysart & Day, 1976, Salt & van der Bosch, 1967).

According to prior observations, multiple punctures of the parasitoid *B. curculionis* increase mortality of the larvae of *H. postica* (Duodu & Davis, 1974b). With this in mind, the strategy of only allowing the parasitoid to punctuate the larvae of *H. meles* one time seems beneficial.

Previous studies of *B. curculionis* have shown that *H. postica* larvae have a decreased survival rate compared to non-parasitized larvae and that the parasitoid *B. curculionis* prefers younger larval developmental stages; that is, instar stages one to three (Duodu & Davis, 1974b). Premature mortality in young instar larvae of *H. postica* was 24 percent higher in parasitized larvae compared to non-parasitized (Bartell & Pass, 1978a); also parasitized larvae had an altered growth and development rate compared to control.

In the current study, no differences in survival were found between the larval developmental stages. In the current study, the utilized parasitoids were not given the opportunity of choosing between larval instars. Our observations of the parasitation event showed that fourth instar larvae defended themselves with greater force than the younger larvae. They wrangled the body violently when touched by the parasitoid. Evidently, it can be speculated that the preferences for younger hosts observed by Duodu and Davis (1974b) could be caused by the host's capability for physical defence.

Bartell & Pass (1978a) observed that parasitized larvae were smaller in respect to overall length, widths and head capsule widths. Moreover, the time until obtaining the maximum larval size differed between parasitized and non-parasitized with parasitized larvae needing between 14 and 21 days and non-parasitized needing 17 to 18 days. Interestingly, Bartell & Pass (1978a) saw differences in the physiological developmental speed of chronologically same age parasitoid larvae. According to the authors, this could point towards non-diapausing *B. curculionis* developing faster than diapausing larvae, meaning that entering diapause would be determined early in the parasitoid larval stage or maybe even earlier.

Previously, third and fourth instar larvae of *H. postica* have been shown to consume less feed when parasitized by *B. curculionis* (Duodu & Davis, 1974a, Armbrust et al., 1970). This could not be confirmed for *H. meles* larvae in the current study. Comparing mean fresh weight measurements by Doudu & Davis (1974a) with the present study, it could therefore be speculated that *H. meles* larvae are less affected by the parasitation events. However, there were indications that the respiration was higher for parasitized larvae.

This speculation would have to be re-tested as the precise larval age (days after eclosion from egg) is unknown in the present study. Bartell & Pass (1978a) point out that the differences in developmental time for the parasitoid larvae believed to be either a diapausing or a non-diapausing form could obscure the results from prior studies where differences in feed intake were found between parasitized and non-parasitized *H. postica* larvae. These findings could be due to reduced feeding time period and not a reduced feeding rate.

Doudu & Davis (1974a) utilized measurement techniques capable of a more accurate determination of the fate of the consumed feed, so they could show that the net efficiency of conversion of ingested food to body matter was higher for parasitized larvae when compared to non-parasitized larvae. Such measurements require establishing feed dry matter. As larvae of *H. postica* consume alfalfa/lucerne (*Medicago sativa* L.) leaves, dry matter can be obtained relatively simply. If such measurements were to be conducted in the present study, the dry matter of unripe white clover seeds or seed pods would have to be determined. The experimental complications bound to such measurements were discarded early on in the experimental planning phase.

Overall, it seems that parasitation does not have a marked effect on feed consumption or survival rates for host larvae and hence there does not seem to be a direct positive effect of parasitation on *H. mele*s larval damage on white clover seeds.

Appendix 4.3 Parasitoid release combined with pesticide application

Firstly, the two classes of active ingredient in two pesticides registered in white clover for seed production were tested on the parasitoid. Of these insecticides, one contains a pyrethroid and one contain a neonicotinoid. Following, the effectiveness of the insecticides was tested on the parasitoid

Testing different doses of two insecticides on *B. curculionis*

The aim of the bioassay were to test how lambda-cyhalothrin and thiacloprid influenced the survival of the parasitoid *B. curculionis*.

Materials and methods

In the bioassay two widely used active ingredients were tested, a pyrethoroid and a neonicotinoid respectively. Testing was done with the active ingredients thiacloprid and lambda-cyhalothrin. The experiments were carried out in small glass vials adjusting the guidelines from the Insecticide Resistance Action Committee (IRAC) (IRAC, 2018) test method number 011. The normal dose per ha for Karate 2.4 WG (a.i. lambda-cyhalothrin) is 7.5g/ha, and for Biscaya OD 240 (a.i. thiacloprid) the normal doses is 72g/ha. The dosage were based on the normal dosage (ND) in white clover seed production of either compounds, These were for Karate 2.5 WG (lambda-cyhalothrin) 0.3kg/ha and Biscaya OD 240 (thiacloprid) 0.3 l/ha. 12 dilutions of the ND were used: 0.00 (control), 0.002, 0.005, 0.02, 0.1, 0.25, 0.50, 0.75, 1.0, 1.25, 1.5 and 2.0xND. The compounds thiacloprid (purity 99.9 percent, Sigma Aldrich) and lambda-cyhalothrin (purity 98.7 percent, Sigma Aldrich) were made in 100xND stock solutions with acetone (chromasolv® purity 99.9 percent 34850, Sigma Aldrich). The stock solution were diluted into the used concentrations. Later thiacloprid concentrations of 3.0, 4.0 and 6.0xND were evaluated.

The bioassay were carried out in 20ml vials (5.7x2.75cm) (VWR, 548-0154) with fitting 2.4cm PP screw caps (VWR, 548-0161). 1ml of the concentrations were pipetted into the vials and left on a Movil-Rod (J. R. Selecta s.a.) until the acetone had evaporated, resulting in the dispersion of the active ingredient on the sides of the vials. Following visual inspection of the vials, the lids were screwed tight and vials were stored at 6°C in darkness.

B. curculionis cocoons were obtained by sorting debris from the DLF's seed processing facility. The sorting procedure was described previously. *B. curculionis* adults were hatched under laboratory conditions in transparent polycarbonate boxes (22.4x31x12cm WxDxH) with lids with a central Ø6.4cm hole. Up to six containers were used. Per hatching box, adult parasitoids were hatched from 600 grams of the sorted material. The cages were wrapped in black plastic to encourage hatched adult parasitoids to move through the central hole in the lid. Collection of the imagines were done by having a Ø8cm cone made of overhead plastic on top of the 6.4cm hole. The top of the cone was removed to facilitate access. On top of the cone a Ø8.4cm 280ml or 520ml plastic container was used for parasitoid collection. The container, placed top down, was held in place with rubber bands. In the collection container, imagines were offered a 15 percent w/w sugar solution. The solution was administered by dripping the solution onto a dental cotton roll (Ø0.7cm 3.6cm in length). The dental roll was inserted through a Ø0.7cm hole on the lower part of the plastic container. As the imagines appeared in the collection container, they fed freely on the cotton roll, on which the sugar solution were ad-

ministered one to two times a day, depending on the number of imagines in the collection container. As more and more imagines emerged, the collection container was replaced with another collection container.

Parasitoids were collected from the collection containers by suction sampling using a home-made aspirator contraption. On average 10 imagines were placed into the coated glass vials. Both substances were tested twice having per concentration 9 replicates in round one and 10 replicates in round two. The test rounds were separated in time. The later tested high concentrations 3, 4 and 6xND were only carried out once with five replicates.

As each test round required more than 1000 parasitoids, the parasitoids were collected from the beginning of the hatching and until enough individuals had emerged, thus the parasitoids did not have the same age. Care was taken to divide parasitoids of different ages between the concentrations and replicates. In total 2789, parasitoids were used to determine lambda-cyhalothrin influence and 2375 parasitoids were used in the case of thiacloprid.

After 24 hours, the numbers of dead, affected and not affected were determined visually.

Statistics
Data analysis was done in R (R Core Team, 2017) using a four-parametric log-logistic function in the drc package (Ritz et al., 2015). Prior to the selection of the four-parametric function, a model selection tool was used to compare different log logistic and Weibull models. Based on the selected model effective doses (ED) ED10, 50, 75 and 90 were calculated.

Results
The resulting model parameters can be seen in table 26 when the effect of the active ingredients were tested on adult individuals. The normal dose for Karate 2.4 WG (a.i. lambda-cyhalothrin) is 7.5g/ha and for Biscaya OD 240 (a.i. thiacloprid) 72g/ha. Comparing the normal dose values with the ED values in table 27 it is clearly seen, that the normal doses will affect the parasitoids significantly. For lambda-cyhalothrin the outcome is death within 24 hours and for thiacloprid the outcome is behaviour differences, i.e. shaking is seen after 24 hours. As thiacloprid did not result in death within 24 hours, the parasitoid was subjected to doses of 3, 4 and 6 times the normal dose. These higher doses did not result in immediate death within 24 hours. Including the high doses in the analysis of the dose response curve did not cause changes.
The setup did not allow for feeding the parasitoids subjected to especially thiacloprid; thus if the parasitoids were capable of overcoming the symptoms of the poisoning is not known.

TABLE 26. The model parameters for the four-parametric log-logistic function for the active ingredients (a.i.) lambda-cyhalothrin and thiacloprid found in the insecticides Karate 2.5 WG and Biscaya OD 240 respectively. Standard errors for the four parameters are given. For lambda-cyhalothrin the response is given as death, for thiacloprid the response is given as visible behavioural differences.

Model parameters	Concentration (g a.i./ ha)	
	lambda-cyhalothrin	thiacloprid
Slope at ED_{50}	-1.32±0.27	-1.06±0.23
Minimum	17.07±2.23	0.83±4.04
Maximum	84.71±2.69	72.18±2.71
ED_{50}	0.68±0.11	2.10±0.58

TABLE 27. Effective dose at 10, 50 75 and 90 percent for the active ingredients (a.i.) lambda-cyhalothrin and thiacloprid found in the insecticides Karate 2.5 WG and Biscaya OD 240 respectively. Standard errors for the four parameters are given.

Effective dose	Concentration (g a.i./ ha)	
	lambda-cyhalothrin	thiacloprid
ED_{10}	0.13±0.05	0.27±0.15
ED_{50}	0.68±0.11	2.10±0.58
ED_{75}	1.56±0.38	5.91±1.97
ED_{90}	3.59±1.39	16.61±8.29

Discussion

Previously, parasitoids' responses to insecticides were tested (IOBC-WPRS, 2017); however, the authors were not able to find information on whether parasitoids belonging to the family of Ichneumonidae or the genus of *Bathyplectes* were subjected to compounds such as the widely used pyrethroid, e.g. lambda-cyhalothrin, or neonicotinoid, e.g. thiacloprid. Studies with now banned insecticides such as methyl parathion and carbofuran influence parasitoid populations negatively (Bartell et al., 1976, Davis, 1970, Hower & Luke, 1979). This is of course also to be expected as the parasitoid is more exposed to direct contact due to its nature of host searching. Studies on parasitoids subjected to thiacloprid are few. According to IOBC-WPRS (2017) only one study has been recorded in the organizations database. In the mentioned study, thiacloprid was found to be highly lethal towards *Encarsia formosa* Gahan (Hymenoptera, Aphelinidae) (Van de Veire & Tirry, 2003). Studies on parasitoids subjected to lambda-cyhalothrin are more frequently; see for example (Sterk et al., 1999).

The half-life of lambda-cyhalothrin on foliage is reported to be between 5 and 6 days (Fan et al., 2013, GLEAMS, 2001). However, half-life in soil and water is much higher (42.5 – 53.7 days) (Laskowsik, 2002, NPIC, 2001) the differences seems to be due to the differences in absorption capacities (Boehncke et al., 1990). By using the half-life for foliage on the normal doses (7.5 g/ha), and comparing to table 27, more than 90 percent of the parasitoid will die after 5 to 6 days. The concentration of active compound remaining on the foliage will first be lower than ED_{50} after 40 to 48 days.

The flower heads which contribute the most to the overall yield, are those with a fully reflexed head and a brown stem. These flowers are also the ones with the highest number of insect damage (Topbjerg & Ytting, 2009). See also supplementary data 1. These flower heads are pollinated during the main flowering period where insecticides are applied in crops, where pests are observed. If the parasitoids would be present in the seed field at the time of the main flowering, the use of lambda-cyhalothrin at normal doses would surely have killed the parasitoids. Found ED_{90} values would be expected when reducing the normal doses by half.

The bioassay with lambda-cyhalothrin shows that the insecticide cannot be applied if the parasitoid are intended as a biocontrol agent.

For thiacloprid on plant foliage the half-life is 3 to 5 days (Mukherjee & Gopal, 2000, Seenivasan & Muraleedharan, 2009); the major part of the compound taken up by the plant and translocated to shoots and later fruits (Alsayeda et al., 2008). It is uncertain if the parasitoid can utilize white clover flowers as a source of pollen and nectar. Studies have shown parasitoids to be more abundant on Cruciferous and Asteraceae (Hogg et al., 2011) and foraging of *B. curculionis* has been observed on dandelion flowers (*Taraxacum officinale* L.) (Jacob & Evans 2000), aphid honeydew has been seen to extend the life of *B. curculionis* (England & Evans, 1997). White clover flowers are therefore not perceived as a source of feed for the parasitoid. Therefore, contact with the insecticide must occur on foliage, though the risk of exposure through honeydew exists. Another neonicotinoid (imidacloprid) has been found to accumulate in flowering organs with grave impacts on the survival of parasitoids (Kischik et al., 2007) and

other non-target insects (Kischik, 2015) feeding on the floral organs. See also Pisa et al. (2015). For thiacloprid, direct contact have also been found lethal to parasitoids with 52 percent mortalities to occur within 24 hours (Willow et al., 2019).
In the current experiment *B. curculionis* was in direct contact with thiacloprid. Mortalities up to 18 percent was seen at the normal dose. At a 10 times dilution, mortality after 24 hours was still 1.5 times higher than the control. The parasitoid was evidently affected by the encounter. Recuperation time was not possible to investigate under the given setup.
Given a half-life of 3 to 5 days the foliage concentration of thiacloprid would still be higher than the ED_{50} value (table 27) after 22 to 40 days, suggesting the parasitoid population to be deeply affected by the compound. Had the parasitoid been in contact with foliage 6 to 10 days after application, more than 90 percent of the parasitoids would have been affected, and it is questionable if the parasitoid would be able to display normal behavior. Previously, behavior changes in foraging have been found in *B. terrestris* when subjected to safe concentrations of thiacloprid (Mommaerts et al., 2009).

Semi-field evaluations of the usage of insecticides in combination with the release of the parasitoid

The aim was to evaluate if common insecticides could be used in combination with the parasitoid *B. curculionis* to control *Hypera* weevils and how the introduction of the insecticide would affect seed yield. This was done in two similar setups in 2016 and in 2017.

Materials and methods

In 2016 and in 2017 two similar setups were used to study if the parasitoid could be used in combination with two commonly used insecticides to control *Hypera* weevils. The experimental setup comprised of tents in a flowering white clover crop, with *H. meles* present in the tents. In some treatments the tents were sprayed with pesticides and in some treatments, parasitoids were released. Different combinations of pesticides and parasitoids were tested.

Experimental setup in 2016

Tents used in the 2016 experiment

Soil emergence traps (BT2007, Bugdorm) measuring 60x60x60cm (LxWxH) were used as bottomless tents; in total 20 were used. These tents were placed in one row in an organically maintained experimental white clover strip. A shallow trench, 5-10cm in depth, was dug around the cages to allow burial of the flaps on the lower parts of all four sides. The tents were held down by four tent spikes in the corners. The spikes were likewise buried in the trench. Soil were compacted to prevent insects from escaping. After installation, the white clover within the tent was swathed to a height of 12-15 cm and treated with lambda-cyhalothrin by applying 0.3kg/ha Karate 2.5 WG (Syngenta Nordics A/S) diluted in 200 l water dispersed by using a backpack sprayer. The aim of swathing and spraying was to synchronize white clover flowering and clean the tents from potential white clover pests and parasitoids.

Collecting weevils for the 2016 experiment

In 2016, adult weevils were collected primarily by having five iron hatching traps placed in field borders of a previous year' white clover seed field. The hatching traps (Ø 39cm) were lined with weed cloth underneath a nonwoven cloth. Trapped adult weevils were collected weekly. Caught adult weevils were kept under shaded outdoor conditions in a polycarbonate box (22.4x31x12cm [LxWxH]) with a fitting lid, with a central Ø6.4cm hole covered by a fine meshed net. White clover leaves were regularly supplied as feed. To ensure enough weevils for the experiment additional weevils were caught using 28 hatching traps in a second year conventional white clover seed field.

Obtaining parasitoids for the 2016 experiment

B. curculionis cocoons were obtained by sorting debris from the seed processing facilities of DLF and DSV. The sorting procedure was described previously in section I. The process of hatching of *B. curculionis* adults was similar to what was described above. Male and female parasitoids were stored together to encourage mating.

To supply *H. meles* larvae, adult female and male weevils were in 2016 released in the tents at a density of six females and three male per m^2. Release occurred 20 days after the initial insecticide treatment as flower heads were abundant. Prior to release, male and female weevils had been stored together to encourage mating.

Treatments performed in 2016
The experiment consisted of five treatments with four replicates in 2016, and two insecticides tested on the parasitoid. In 2016 both insecticides were either applied before (treatments 1 and 3) or after the release of the parasitoids (treatments 2 and 4). Table 28 outlines the experimental setup for 2016. The insecticides were 1) Biscaya OD 240 (Bayer A/S) active ingredient 240g/l thiacloprid applied at the dosage of 0.3 l/ha, diluted in 200 l water and 2) Karate 2.5 WG (Syngenta Nordics A/S) active ingredient 25 g/kg lambda-cyhalothrin applied at the dosage of 0.3kg/ha, diluted in 200 l water. The insecticides were dispersed using a backpack sprayer, spraying inside the tents for 2 seconds and directing the nozzle in a zigzag motion.

Female parasitoids were set out in densities of six per m^2 with additional two male parasitoids per m^2 to ensure mating. Prior to release female and male had been kept together to encourage mating. The release of the parasitoids was done eight days after the release of the weevils and two days after the insecticide application in treatments 1 and 3. For treatments 2 and 4, the parasitoid release happened the day before the insecticide application.

The control treatment with only *H. meles* is not present in the current experimental lineup. However, for comparisons the treatment with only *H. meles* present in the parasitoid density experiment is utilised for comparisons with the treatment without insecticide application.

TABLE 28. Setup of the 2016 insecticide tent experiment. The treatments are shown per dates in 2016. Abbreviations *B.c.*, *B. curculionis. H. m., H. meles*.

Treatment	08-Jun	14-Jun	16-jun	17-Jun	18-Jun
Biscaya followed by *B.c.* release	*H. m* release	Biscaya OD 240 application	Parasitoid and bumblebee release		
Release of *B.c.* followed by Biscaya			Parasitoid release	Biscaya OD 240 application	Release of bumblebees
Karate followed by *B.c.* release		Karate 2.5 WG application	Parasitoid and bumblebee release		
Release of *B.c.* followed by Karate			Parasitoid release	Karate 2.5 WG application	Release of bumblebees
Release of *B.c.*			Parasitoid and bumblebee release		

In the experiments concerning the integration with parasitoids and insecticide, a control treatment with only *H. meles* is absent. However, in the parasitoid density experiments such a

treatment was present. Therefore, for comparison purposes the later treatment is shown when comparing yield components in the following.

To ensure feed for the parasitoids as it is uncertain if the parasitoid can feed on white clover flowers, the tents were supplied with a 15 percent w/w sugar solution in a 6.5ml test tube closed with a Ø1.5cm, length 2.0cm dental cotton roll. The test tube was placed at the same height as the white clover canopy and positioned horizontally to insure wetting of the cotton roll.

Pollination of the experimental tents was done by releasing bumblebees (*Brombus terrestris* L., Hymenoptera: Apidae) (cat.nr. 207, BioProduction EWH). Regular inspection secured that at least two bumblebees were present in each tent at all times.

Monitoring the effect of the treatment
In 2016, white clover flower heads were sampled in late July, 41 days after the release of the parasitoids. This harvest consisted of collection of flower heads at two growth stages: flower heads reflexed with a green stem and flower heads reflexed with a brown stem. Per growth stage, 25 flower heads were picked. The flower heads were kept in brown paper bags (8.0x26.5cm WxH) stored outside in cages measuring 75x68x83cm (LxWxH) with a fine mesh on three sides (mesh size 0.2x0.2mm) and a glass top and door. Storing lasted at least 8 weeks to allow weevil larvae to pupate and hatch as adult weevils or the development of the parasitoid cocoon. Registration of weevil larvae, cocoons, adult female and male together with parasitoid cocoons and adult females and males was done manually by gently threshing the flower heads by hand.

On the last collection date, 10 flower heads of the two growth stages were picked for analysis of yield components. From harvest to analysis, the flower heads were stored in brown paper bags (8.0x26.5cm WxH) at -20 °C. White clover flower heads per m^2 were assessed on an area of 10x10cm and divided into six categories: 1). Buds, 2). At least one floret open, 3). All florets open or senesced, 4). All flowers senesced but not harvestable, 5). Harvestable flower head reflexed with green stem and 6). Harvestable flower head reflexed with brown stem. Of the ten flower heads collected, five were randomly picket and the number of florets counted in these five flower heads. To lower workload, 20 florets per flower head were randomly selected and the number of intact florets, the number of insect damage florets and number of non-pollinated florets were counted. Then number of seeds in intact florets and the number of seeds in insect-damaged florets were counted in five florets selected from the pool of intact florets and insect-damaged florets respectively.

Yield components
The yield components were calculated on the same principles as used under the experiments with parasitoid density but also described in the following. The percentages of pollinated and intact florets, pollinated and damaged florets and non-pollinated florets were calculated based on the total number of florets evaluated. The number of seeds was counted for up to five florets, which enabled the calculation of the average number of seeds in intact and damaged florets.

The number of florets per head was registered, which enabled the estimation of the number of intact florets and damaged florets per flower head. Further, it also enabled the calculation of seeds per head from intact florets and seeds per head from damaged florets. In turn, this would be used to estimate the yield per flower head using a 1000 seed weight of 0.7g (Langer & Rohde, 2005) together with the estimated seed contribution from intact florets and from damaged florets.

Treatments performed in 2017

In 2017 slight differences to the treatment setup were performed. The difference to the 2016 experiment is described below.

In 2017 both insecticides were applied before release of the parasitoids (treatments 2 and 4) and in treatments without release of the parasitoid (treatments 1 and 3) Table 29 presents the experimental overview. The female parasitoids were released in the tents at densities of 12 per m^2 with additional six male parasitoids per m^2 to ensure mating. Parasitoids were released 12 days after the initial weevil release. This was 6 days after the insecticide treatment with either Biscaya OD 240 or Karate 2.5 WG.

TABLE 29. The setup of the 2017 insecticide tent experiment. Treatments are shown per date in 2017. Abbreviations *B.c.*, *B. curculionis*.

Treatment	15-Jun	21-Jun	27-jun
Biacaya	*Hypera* weevil and bumblebee release	Biscaya OD 240 application	Bumblebee release
Biscaya followed by *B.c.* release			Parasitoid and bumblebee release
Karate		Karate 2.5 WG application	Bumblebee release
Karate followed by *B.c.* release			Parasitoid and bumblebee release
Only release of *B.c.*			Parasitoid and bumblebee release

In 2017 two additional sampling time points were introduced: 9 days and 22 days after release of the parasitoids per sampeling date 20 flowerheads belonging to the previously described flower head stages were picked. The experiment lasted 30 days. An earlier harvest was necessary as an increase in aphids numbers threatened to destroy the experiment. Both years, release of field collected hoverflies (Diptera, Syrphidae) and green lacewings (*Chrysoperla carnea*, Stephens) (Neuroptera: Chrysopidae) (BioProduction EWH) was used in an attempt to control the buildup of aphid populations.

Statistics

Yield components were analyzed with the statistical software R (R Core Team, 2017) using the function lme() from the nlme package (Pinheiro et al., 2018) with tent as the random effect. The different yield components were analyzed as a two-way ANOVA having treatment and floret category as the fixed effects and tent number as a random effect. Multiple comparisons were made by using glht() from the package multcomp (Hothorn et al., 2008). For multiple tests, P-values were adjusted to control Type-I error by using the multivariate T-distribution (Bretz et al., 2008).

In cases of different significances occurring between the ANOVA analysis and the multi comparisons boot strapping was applied on the final model to reevaluate the archived P-values.

Model evaluation was done by residual plots and plotting the sample quantiles against the theoretical quantiles (qqplot). Evaluation of the residuals of the random effect was done by including the random effect in a linear model lm(). The qqplot of the random effect was evaluated as a plot of the predicted random effects (EBLUPs) of the end model.

Hatched adult weevils and found parasitoid cocoons were analyzed with the built in function glm() for generalized linear models. Pairwise comparisons was done using the emmeans package (Lenth, 2018)

Statistics on the flower head category data were analyzed with lm() in the statistical program R. Significant levels of P<0.05, P<0.01 and P<0.002 are indicated as *, ** and ***.

Results

The 2016 experiment tried to clarify if the release of the parasitoid could be done in combination with the usage of insecticides. Preceding the onset of the experiment, female weevils had seven days to oviposit. Before and after the insecticide application parasitoids were released. The overall assumption was that the beneficial influence of the parasitoid would be time limited. The effect would be from the time of release until the insecticide was administered. This would then be compared to the situation where the insecticide was applied prior to the release. In the first situation, parasitoids would only influence weevil larvae residing in the flower heads within this period. In the second scenario, weevil larvae surviving the insecticide application would be subjected the presence of a parasitoid.

Yield components 2016

The 2016 treatments consisted of five treatments. Adult parasitoids were released two days prior or after the application. No comparable treatment with only the introduction of the weevil pest was enclosed. However, the trials with parasitoid density included such a trial, therefore the comparison of treatments with release of parasitoids and no parasitoids can be carried out by comparison across experiments. Such comparisons will only include the control (parasitoid release alone) as issues with randomization and physical placement of the experimental units (tent) will otherwise occur.

Flower heads per m^2 and florets per flower head showed no differences between treatments and flower head categories. However, the portion of non-pollinated florets was found to differ as the treatment involving Karate either before or after the release of the parasitoid and bumblebees yielded a high percentage of non-pollinated florets. The overall average of non-pollinated florets was 17.6±5.7 percent. For the treatment in which Karate followed the release of the parasitoid, the percentage of non-pollinated flowers was more than double the average for both flower head categories. When Karate was administered prior to the release of the parasitoid and bumblebees, the percentage of non-pollinated florets was also high, although not as high as the earlier mentioned. For the application of Biscaya the percentage of non-pollinated florets lay in the area of 2 to 9.5 percent. Karate had a huge impact on the behavior of the bumblebees with Karate having a lasting effect on the bumblebees i.e. reduced visits in the flower heads reflexed with a green stem. This was not seen for Biscaya.

In figure 12A no added benefit was seen by releasing parasitoid prior to or after an Biscaya application. Compared to the control with only parasitoid release no differences were seen and so the Biscaya application could be seen as unnecessary. Comparing the application with Karate prior to the release of the parasitoids a similar pattern appeared. The drop in percent pollinated and intact florets for the treatment with Karate applied after the release of the parasitoid release can be connected to the low percentage of pollinated florets and might introduce a bias as the available weevil larval food source depend on the number of pollinated florets.

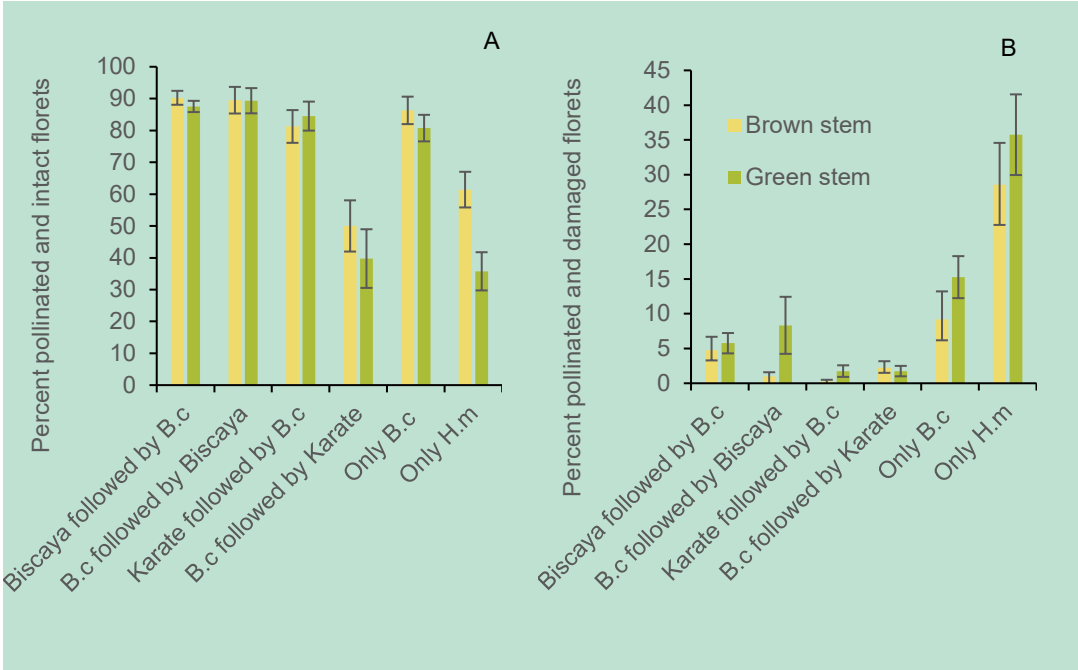

FIGURE 12. A. Percent florets pollinated and intact for the two flower head categories, fully reflexed with a brown stem (light brown) and fully reflexed with a green stem (green) for the five treatments plus the control from the parasitoid experiment (Only H.m.). B. percent pollinated and damaged. Abbreviations: B.c. *B. curculionis*, H.m. *H. meles*. Average values with standard errors are shown.

When looking at the percentage of pollinated and damaged florets (figure 12B) no significant difference was found between treatments in the flower head category with brown stems. However, for the flower heads with a green stem, there was a significant difference between the control (parasitoid only) and the two Karate treatments (supplementary date 2). This evidently would suggest an increase in pest control when applying the insecticide Karate to the parasitoid releases. Further, as the effect was seen in both Karate experiments, it is thought that the observed effect was solely an effect of the insecticide, i.e. the weevil adults and released parasitoids were killed.

No significant differences were found between the parasitoid only treatment and the control from the parasitoid density experiments a decrease in the number of pollinated and damaged florets were seen (figure 12B).

The seed yield per flower head (figure 13) did in general not show differences between treatments or flower head category. The only differences were found for fully reflexed flower heads in the treatment where *B. curculionis* release was followed by Karate application. Interestingly the seeds per floret seemed to be similar between the treatments with Karate applied after the parasitoid and the control treatment from the density experiment. A statistical comparison was not made as the results were derived from two different experiments.

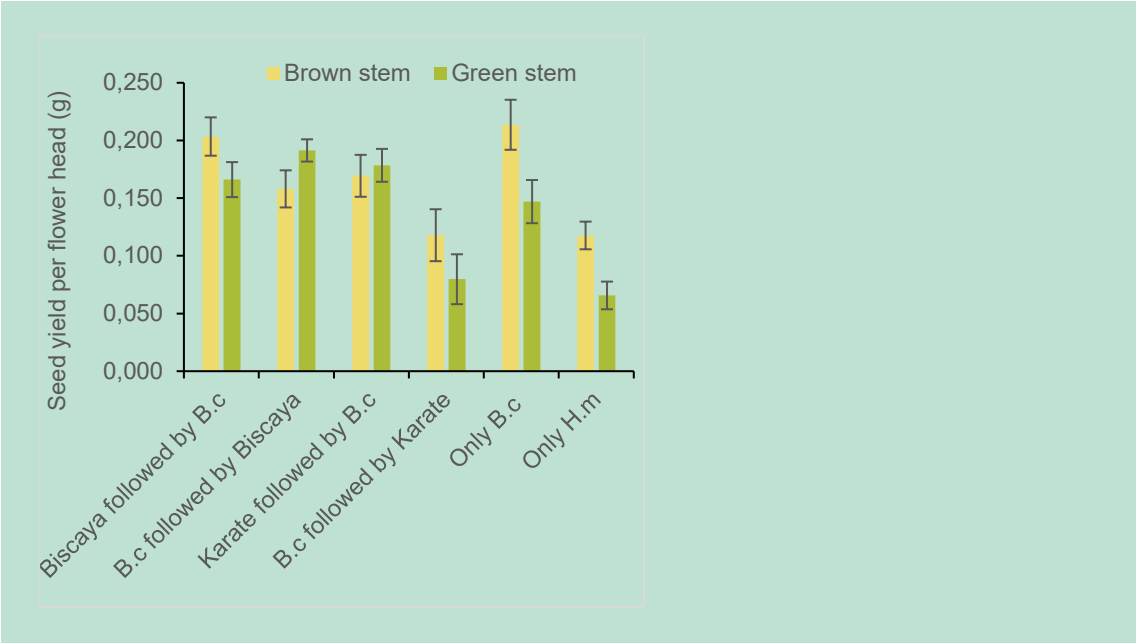

FIGURE 13. Seed yield per flower head for the four treatments and the control from the parasitoid experiment (Only H.m.). Results are given for the two flower head categories, reflexed with a brown stem (light brown) and reflexed with a green stem (green). Average values with standard errors are shown. Abbreviations: B.c. *B. curculionis*, H.m. *H. meles*.

In the situation with no parasitoids released, as seen in the density experiment, the added benefit was seen. The effect might be related to a prolonged exposure of the parasitoid on the weevil larvae or it could be a coincidence. As no direct influence can be seen in figure 13, the later might be the explanation. However, more knowledge on the interaction would be beneficial.

Yield components 2017

As for 2016, flower heads per m^2 and florets per flower head did not show differences comparing treatments and flower head categories. The only recognizable difference was found between the control treatment (only *B. curculionis* released) and treatment with *B. curculionis* released after the application of Karate. Comparing the two treatments, an increased number of flower heads in the control treatment for the reflexed flower heads with a brown stem was seen (supplementary data 2).

The application of Karate followed by the parasitoid yielded a high number of non-pollinated florets. However, this year only the flower head category consisting of reflexed florets with a green stem was influenced by a less than average pollination (41.8±6.6%) compared to an overall average of 16.8±2.4 percent non-pollinated florets. As seen in 2016 Karate apparently had an effect on the pollinators resulting in a decreased pollination.

For the percentage of pollinated and intact florets a general trend was not visible. Only the treatment with application of Karate followed by the release of the parasitoids showed a reduction in pollinated and intact florets when compared to the remaining treatments. This was further only found for the green stemmed floral category. Comparing the percentage of pollinated and damaged florets no clear differences were seen. Applying one of the insecticides, with or without the additional parasitoid, decreased the percentage of pollinated and damaged florets, evidently showing a beneficial effect of the insecticide on the pest, data not shown. The yield per flower head did not reveal a noticeable difference between treatments, neither did treatments with and without parasitoid release.

Collection of adult pests and parasitoid cocoons from collected flower heads
In 2016. Collected flower heads in the two categories: yielded in total 158 adult weevils together with only one cocoon of the parasitoid. The retrieved weevils showed no differences in relation to treatment or flower head category

Across all three sampling dates and treatments in 2017, a total of eight parasitoid cocoons and adult parasitoids were found together with 35 adult weevils. Of the found weevils 11 were found on one sampling occasion in one tent. Due to the low number of collected weevils and parasitoid no comparisons were made.

Density of flower heads in the six categories
Collectively over the two years, no differences were seen in the density of the six flower head categories.

Discussion
The utilization of insecticides in conventional white clover seed production is widespread with the insecticides Karate and Biscaya (Miljøstyrelsen, 2019). The active compound in Biscay has been shown to be harmful to a range of Hymenopterans including a range of parasitoids and bumblebees (Pisa et al., 2015, Ellis et al., 2017).
For lambda-cyhalothrin the active compound in Karate has been found to affect *B. terrestris* with strong sub-lethal effects which over time lead to mortality or workers showed signs of incoordination and convulsion, with workers gradually becoming apathetic (Ceuppens et al., 2015).
The active compound in Karate is effective on contact and can be applied numerous times. To protect pollinators application has to be carried out at times when the pollinators are not in the crop. However, as the product has a lasting effect (Miljøstyrelsen, 2019), the return of the pollinators will evidently expose them to the compound. Pollinators do however, not return to treated fields immediatly as pyrethroids have a repellent effect on pollinators, which is reversible within one to three days (Rieth & Levin, 1988, DLF, 2015). At the return, the active compounds are still potent as the half-life is between 5 to 6 days (Fan et al., 2013, GLEAMS 2001), thus the pollinators will at the return be subjected to the insecticide. For the active compound in Biascya, thiacloprid, on foliage the half-life is 3 to 5 days (Mukherjee & Gopal, 2000, Seenivasan & Muraleedharan, 2009). As the major parts of neonicotinoid insecticides is taken up by the plant and translocated to shoots (Alsayeda et al., 2008), the compound pose a risk of being found in bee-collected pollen and nectar as found by Ellis et al. (2017) for thiacloprid, resulting in premature death and decreased reproductively.

In the setup, bumblebees were used as pollinators. To secure feed for the weevil larvae, the white clover needed pollination, thus introduction of bees could not be avoided. Bumblebees were chosen as they can be bought in small colonies and are able to perform pollination in the small tents without access to a hive, as observed in a preliminary experiment indicating that up to 75 percent of florets could be pollinated (experiment not shown). As both insecticide groups are harmful for the pollinators (Sanchez-Bayo & Goka, 2014), activity in the tents was checked regularly, ensuring two active bumblebees present in the tents throughout the experiment. Direct and indirect contact with the pesticide was unavoidable given the setup.
For both years, the pollination for florets in treatments with thiacloprid was at the same level as treatments without insecticides (figure 12A, supplementary data 2). For treatments involving lambda-cyhalothrin, pollination was affected in both years in one of the treatments. The other treatment involving Karate did not show significantly decreased pollination, though a slight decrease was observed.

It has not been established that the parasitoid can use white clover flowers as a source of feed and other sources of food seems more obvious (England & Evans, 1997, Hogg et al. 2011, Jacob & Evans, 2000), therefore the parasitoid was fed sugar water. An added benefit of feeding

on sugar water was that the parasitoid would not encounter concentrations of thiacloprid in the feed. Direct contact with pesticides on foliage was thus planned to occur. As seen previously in the study, the normal utilized dose of thiacloprid affects the parasitoid without being lethal and the parasitoids' ability to recuperate is unknown. With a half-life of 3 to 5 days on foliage (Mukherjee & Gopal, 2000, Seenivasan & Muraleedharan, 2009) the ED_{50} value would first occur after more than 22 days. For the parasitoid to be active around the main flowering when crop protection is most needed, the parasitoid would encounter higher ED-values.
With releases within 6 days of the insecticide application more than 90 percent of the parasitoids would have been affected, and it is questionable whether the parasitoid would be able to display normal behavior. Previously, behavior changes have been found in bumblebees (Mommaerts et al., 2009, Ellis et al., 2017).

In 2016 the parasitoid was released 2 days after application of the insecticides and in 2017 the release was delayed to 6 days after application. In 2016 the parasitoid would have encountered the full doses and in 2017 the dose would have been halved due to the half-life of 3 to 5 days of thiacloprid. Parasitoid ovipositioning behavior is seen to be influenced by pesticides (Desneux et al., 2007) with general fewer ovipositioning attempts. In treatments with thiacloprid no differences in seed yields was found.

In both years, no differences were found when comparing seed yields per flower head of the flower head category with the brown stem in the two treatments with thiacloprid. Adding the parasitoid did not increase yields (2017), neither did the time of release (2016).
Compared to the treatment with only parasitoid release, the seeds per flower head was higher in 2016 compared to 2017 when insecticide and parasitoid were combined. In 2017, only utilizing the parasitoid gave the highest yields when focusing on the brown-stemmed flower head category, found previously to contribute the most to the overall yield (Topbjerg & Ytting, 2009).

Lambda-cyhalothrin influenced negatively on the parasitoid and as found in the previous section the pyrethroid is lethal to the parasitoid. This has also been reported for other parasitoids (Desneux et al., 2004). No differences were seen for the seed yield per flower head from the 2016 treatments containing the lambda-cyhalothrin and the parasitoid. This point towards no effect of the parasitoid. As the insecticide influences the pollinator as well, the percentage of non-pollinated florets should be higher in the treatments with Karate. However only in one treatment in both 2016 and 2017 did the percentage of non-pollinated florets stand out (supplementary data 2).

How the two insecticides affect the weevil is not known.
In the present study, the influence on the weevil larvae can be seen by comparing the percentage of pollinated and damaged florets. However, the percentage of pollination might be hampered by the insecticide influence on the pollinator as discussed earlier.
For both years the highest number of damaged florets was seen when the weevil was undisturbed by insecticide and parasitoids, which translated into the highest number of seeds eaten. Adding insecticides or parasitoids decreased the weevil damage and increased yields. When looking only at treatments including agents towards the weevil, damage to pollinated and damaged florets was highest when only the parasitoid was present (supplementary data 2). Therefore, the insecticides has a greater influence on the damage caused by the weevil larvae. In the experiment, testing the food intake of parasitized and non-parasitized weevil larvae, no differences was found. This also suggests a higher percentage of damaged florets.

The experimental design
To evaluate if the insecticide influence on the parasitoid, two levels of evaluation was used. First, a laboratory bioassay in glass vials and secondly a semi-field study in tents.
The laboratory setup produced a dose-response curve capable of estimating different effective doses (ED) on the parasitoid. These could be coupled to the half-life of the active compound,

thereby predicting the outcome where the parasitoid to be introduced in fields subjected to insecticide application.
The second semi field setup tried to evaluate how seed yield would be affected when active hosts were into the parasitoid-insecticide setup. To do so, weevil host larvae would have to be kept feeding until pupation resulting in hatching adult weevils or parasitoid cocoons. This required egg laying possibilities for the weevils and feed for the weevil larvae. As the weevil larva primarily feeds on seeds and seed pods, the white clover would have to be pollinated, which introduced a third party, a pollinator. Bumblebees were chosen as they are willing to pollinate in the tent setup, as seen in a pre-evaluation of the setup.
Having so many levels of interaction in one setup is clearly problematic as confounded variables can make interactions indistinguishable. However, if seed yield were to be estimated, the number of experiment parts seemed needed. If costs would have allowed, it would have been preferred to have more than the two levels of evaluation (laboratory and semi field). It would have been convenient to split up the levels of interaction as seen in for example Mommaerts & Smagghe (2011) when evaluating the toxicity of a pesticide.

The cage setup did not provide sufficient observations for treatment comparisons on number of cocoons and adult weevils per tent or flower heads. Therefore the seen treatment differences are only seen based on yield components which are secondary observations derived from the interactions of the treatments on the weevil and to an certain part on the pollinators.

Low numbers of cocoons and adult weevils were also seen in the other tent setups, i.e. assessment of the parasitoid density experiments and the later year to year quantification. For field releases zero encounters were also high, though collected weevil larvae still produced numerous adult weevils or parasitoid cocoons when kept for hatching. It is clear that treatment comparisons on hatching adult weevils or formation of parasitoid cocoons would greatly benefit from increasing the number of collected flower heads. This would in turn have demanded larger tents, which the project did not have access to, due to purchase costs.

Appendix 4.4 Parasitoid release reduces *Hypera* seed damage the following year

The aim was to evaluate if released *B. curculionis* year one (2016) would have an effect on seed damage caused by larvae of *Hypera* weevils the following year (2017). The experiment lasted two years. During the experiment, the number of *Hypera* weevils was evaluated in the autumn of the first year and in the spring the second year. In late July in the second year, the final registrations were done, with collection of flower heads 12 days before and at the final day of the experiment.

Materials and methods

Experimental setup

A total of eight tents measuring 2.5x2.5x2.0m (LxWxH) were set up in a one-year old white clover stand. A finely meshed (mesh size 1x1 mm) insect net (6.5x7 m WxL) with a 2.5m zip lock opening was stretched across three iron arches (2.2x3.13m WxH). The mesh was positioned with the opening on the longitudinal side of the tents. A 20-30cm deep trench was dug and the surplus mesh material was covered with soil. Additionally, sandbags were positioned on top of the covered trench. For stability, 1.2m wood planks were wedged in between the arches. After installation, the white clover within the tent were swathed to a height of 12-15cm. after removal of the swathed material, the white clover stand in the tents were treated with lambda-cyhalothrin with the intent of killing white clover seed pests enclosed in the tents. Lambda-cyhalothrin was applied in the form of 0.3kg/ha Karate 2.5 WG (Syngenta Nordics A/S) diluted in 200 l water and dispersed using a backpack sprayer.

Acquiring adult weevils and parasitoids
Adult weevils were collected by having five iron hatching traps, Ø39cm lined with weed cloth underneath a nonwoven cloth. The traps were placed in field borders of a previous year organic white clover seed field. Adult weevils were also collected by vacuum suction, utilizing a converted leaf blower (Husqvana 125BV). A cotton bag were custom fitted to the tip of the suction tube, easing the handling of collected insects. Collections were made in surrounding organic white clover strips and in commercial white clover seed fields. Until release, the weevils were kept under outdoor conditions in transparent polycarbonate cages (22.4x31x12cm LxWxH) with a ventilated lid. Weevils were fed fresh organic white clover leaves, which were replaced when needed.

B. curculionis cocoons were obtained by sorting debris from the DLF's seed processing facility. The sorting procedure was described previously. The process of hatching of *B. curculionis* adults was similar to what was described previously. Male and female parasitoids were stored together to encourage mating.

Experimental setup
Adult *H. meles* females were on the 8 of June 2016 Introduced into the experimental tents at a density of one per m^2, accompanied by 0.5 males per m^2. The adults were released 20 days after the insecticide treatment. Prior to release, male and female weevils had been stored together to encourage mating.

Two treatments, with or without introduced parasitoids, were set up in four replicates. Parasitoids were introduced eight days later than the weevils. Density of female parasitoids was set to one per m^2, accompanied by 0.5 males per m^2. Parasitoids were given a 15 percent w/w sugar solution in a 6.5ml test tube closed with a Ø1.5cm, length 2.0cm dental cotton roll. The test tube was placed at the same height as the white clover canopy and positioned horizontally to insure wetting of the cotton roll. In spring 2017, additional parasitoids were released in the eight tents: 1 female parasitoid per m^2 and 0.33 male parasitoid per m^2 were added to the existing parasitoid populations.

Pollination in the experimental tents were done by bumblebees (*Brombus terrestris* L., Hymenoptera: Aphidae) (cat.nr. 207, BioProduction EWH). Regular inspection secured that through the growing seasons around 10 bumblebees were present in each tent at all times.

Aphid populations were controlled by release of field collected hoverflies (Diptera: Syrphidae) and green lacewings (*Chrysoperla carnea*, Stephens) (Neuroptera: Chrysopidae) (BioProduction EWH).

Sampling
In 2016, triplet samples of 25 white clover flower heads were sampled from each tent in late July, 43 days after the release of the weevils. The flower heads were kept in brown paper bags (8.0x26.5 cm WxH) stored outside in 75x68x83cm cages (LxWxH) with a fine mesh on three sides (mesh size 0.2x0.2mm) and a glass top and door. The storage time lasted at least eight weeks to allow weevil larvae to pupate and hatch as adult weevils or the development of the parasitoids cocoon. For weevils the number of: larvae, cocoons, adult females and males were registered together with parasitoid: cocoons, adult females and males. Registrations were done manually by gently threshing the flower heads by hand.

In 2017 two iron emergence traps Ø39cm, lined with black weed cloth, were used per tent to evaluate the number of adult weevils within each tent. Traps were set out for a seven days period in mid-May and emptied every day. Caught *Hypera* weevils were counted and released. Traps were moved daily in a random pattern. Force was applied to secure contact of the traps with the soil surface.

At the end of the experiment, the number of weevils and parasoitoid cocoons were evaluated by collecting five samples of 20 white clover flower heads per tent. Collections were done twice 12 days apart. The flower heads were kept in brown paper bags until registration of weevils and parasitoids could be carried out.

At the last collection time point, flower heads were collected for yield component analysis. Three replicates of 10 flower heads were collected per tent and stored in brown paper bags at -20°C. The flower heads were equally mature and were all reflexed flower heads with a green stem.

Furthermore, the white clover flower heads per m^2 were assessed on an area of 10x10cm. The flower heads were grouped into five categories according to their maturity: 1) Buds, 2) At least one floret open, 3) All florets open or senesced, 4) All flowers senesced but not harvestable, 5) Harvestable flower head reflexed with green stem and 6) Harvestable flower head reflexed with brown stem. Of the ten flower heads collected, five were randomly picked and the number of florets counted. To lower workload, 20 florets per flower head were randomly selected and the number of intact florets, number of insect damage florets and the number on non-pollinated florets were counted. The number of seeds in intact florets and the number of seeds in insect-damaged florets were counted in five florets selected from the pool of intact florets and insect-damaged florets respectively.

Yield components
Yield components are calculated on the same principles as used under the experiments with parasitoid density and the usage of parasitoid release together with the usage of insecticide. In the following, the calculations will shortly be revisited. The percentages of pollinated and intact florets pollinated and damaged florets and non-pollinated florets were calculated based on the total number of florets evaluated. The number of seeds was counted for up to five florets, which enabled the calculation of the average number of seeds in intact and damaged florets.

Florets per head were registered, which enabled the estimation of the number intact florets and damaged florets per flower head. Further, it also enabled the calculation of seeds per head from intact florets and seeds per head from damaged florets. In turn, this would be used to estimate the yield per flower head using a 1000 seed weight of 0.7 (Langer & Rohde, 2005) together with the estimated seed contribution from intact florets and from damaged florets.

Statistics
The evaluation of autumn hatched weevils, overwintering weevils and other linear comparisons was analyzed using the function lm() for linear regression using the statistical software R (R Core Team, 2017). Found hatched weevils were analyzed by treating the data as Poisson distributed using the glm() function in R for a generalized linear model.

Yield components were analyzed with the statistical software R using the function lme() from the nlme package (Pinheiro et al., 2018) with tent as the random effect. The different yield components were analysed as a two-way ANOVA having treatment and floret category as the fixed effects and tent number as a random effect. Multiple comparisons were made by using glht() from the package multcomp (Hothorn et al., 2008). For multiple tests, P-values were adjusted to control Type-I error by using the multivariate T-distribution (Bretz et al., 2008).

In cases of different significances occurring between the ANOVA analysis and the multi comparisons, boot strapping was applied on the final model to re-evaluate the archived P-values.

Model evaluation was done by residual plots and plotting the sample quantiles against the theoretical quantiles (qqplot). Evaluation of the residuals of the random effect was done by including the random effect in a linear model lm(). The qqplot of the random effect was evaluated as a plot of the predicted random effects (EBLUPs) of the end model.

Hatched adult weevil and found parasitoid cocoons were analysed with the built in function glm() for generalized linear models. Pairwise comparisons was done using the emmeans package (Lenth, 2018).

Statistics on the flower head category data were analysed with lm() in the statistical program R. Treatment were set as the fixed variable and the flower head categories were set as the response variable. The data were analysed using a one way ANOVA and pairwise comparisons were calculated using the glht() function.

Results

Hatched weevils and parasitoid cocoons

At the end of the experimental period and 12 days prior to, only one *B. curculionis* cocoon was retrieved and a total 174 *H. meles* adults were collected. Therefore the calculation of a parasitation of *H. meles* larvae was not done. The number of adult *H. meles* was analysed for the two collection dates. Neither dates nor treatment had an influence on the number of weevils hatching from the collected flower heads.

At harvest, the number of flower heads in the category fully reflexed with a green stem was counted. Based on this, the number of *H. meles* weevils found in the tents averaged 38.4±7.0 per m^2. These numbers are evidentially less that what was found the year before (average 88.7±16.5 *H. meles* per m^2), for the same flower head category.

However, as treatments did not produce differences in the number of adult weevils the weevil per m^2 differences must reflect a difference in the number of flower heads between years, which is a common difference between second and first year seed crops.

Yield components

Overall, no differences were seen between treatments for the different yield components, only the amount of flowers per flower head category differed significantly. Treatments did not show differences between flower head categories.

Insect activity through 2016 and 2017 until harvest

In late summer of 2016, the newly hatched generation of *H. meles* was plentiful in all tents and the weevils were seen to assemble in large numbers at the lower edges of the tents. Collections of flower heads from the two treatments showed the treatment without release of the parasitoidl to contain an average of 74.5±28.8 (SE) *H. meles* adults per m^2 and the treatment with release contained 107.2±21.6 *H. meles* per m^2.

In spring 2017, *H. meles* activity was seen in the tents from the May 3. At this date mating and flight were observed. White clover flowers were present in all tents on June 1. On the July 7 *B. curculionis* was captured in hatching traps set out in the tents. The appearance of the parasitoid coincided with increased activity of *H. meles* noted in adjacent white clover areas.

The number of *H. meles* in the tents was estimated in mid May 2017. Over a period on seven days, hatching traps were emptied and moved daily. Per day, the captured number of *H. meles* in the tents without parasitoid release was 49.7±11.6 per m^2 and in the tents with parasitoid release 116.4±13.9 *H. meles* were caught per m^2 per day. In June of 2017, female *B. curculionis* was seen in the treatment tents prior to the release of additional parasitoids this year, showing that parasitation of weevil larvae had occurred in 2016.

Discussion
The experiment cannot with certainty establish whether the added parasitoid has a beneficial effect on the *H. meles* weevil population.

The winter survival for *H. meles* in the tents seemed good, as seen when comparing the numbers of individuals prior to and after the winter months. However, the differences in pest population between the treatments were still obvious. Leveling out the populations was not attempted due to the difficulties in obtaining the amount of weevils needed for release in the control tents. For the parasitoid, observations showed that it survived the winter month and hatched the next spring. Numbers were, however, miniscule. Most significant was the encounter of the parasitoid in hatching traps in the first week of July, which should represent the time of emergence of the parasitoid.

Problems encountered when maintaining the experiment
Although the experiment produced results, the difficulties in maintaining the experiment have undoubtedly influenced the results.

Shortly after start the experiment was mistakenly sprayed with Karate in the tents with no parasitoid release and Biscaya in the tents with parasitoid release. The late summer collection of flower heads from the two treatments showed the tents without release of the parasitoid to contain fewer *H. meles* adults per m^2 than the treatment with release. The differences between the trials were seen as a result of the two different insecticides sprayed in the different treatments. Karate had mistakenly been sprayed in the treatment without release of the parasitoid and Biscaya had been sprayed in the treatment with release of the parasitoid, Karate being a more effective insecticide than Biscaya.

In mid-summer 2016, white clover plants in three of the eight tents suffered from aphid population pressure. In these tents the white clover plants had to be replaced. This was done in late October when 25 19.5x19.5cm (WxL) white clover swards with a thickness of 5 to 10cm were transplanted to the tents in question. In the process of replacing the white clover swards, numerous lepidopteran pupae were found in the soil. By hatching the pupae, it was determined that *Noctua pronuba* L. (Lepidoptera: Noctuidae) larvae might also have harmed the white clover roots. In late spring of 2017, adult *N. pronuba* adults were seen flying around in several of the tents.

Appendix 4.5 Effects of field releases of the parasitoid

Field releases was used to evaluate how the parasitoid would spread after release and how the weevils were distributed in a field. In addition, the releases were used to investigate the occurrence of the two weevils *H. nigrirostris* and *H. meles*. The trials examine the distribution of the parasitoid *B. curculionis* at specific distances to a release point and how the host were distribution at these points.

Materials and methods
Field size
Available area with organic white clover seed production was in 2016 143 ha and in 208 ha in 2017. This was a significant drop from the previous ten-year average, 591ha (Brancheudvalget for Frø, 2019) and hampered areas suitable for experimental releases. In 2016, only four fields were available for experimental work covering 48.2 ha and having an average field size of 13.8±3.8 ha. Due to the low number of fields, releases were done in all four fields. In 2017, eight fields were available with a total area of 91.9ha. Parasitoid were released in four fields averaging 12.3±1.1ha. The remaining four fields averaging 10.7±1.9ha were used as controls with no release of the parasitoid.

The lack of control fields in 2016 meant that a semi-control within the four release fields would be needed. Two transects, each with seven collection points was laid out in either a zigzag pattern or in a straight line. The control transects were placed as far from the release point as possible.

Release of parasitoids 2016
The utilized cocoons had previously been obtained from a debris fraction acquired from the seed sorting process at the seed processing facilities at DSV and DLF. Cocoons were sorted from the debris fraction as mentioned earlier. The cocoons would be set out at one location and later sampling would then be made at different distances to this point.

In 2016, the fraction of white clover seed sorting debris containing the parasitoid cocoons weighed 208 kg in total. To account for the high weight of the material and to keep the layer of the material as low as possible, the release of the parasitoid cocoons was done by using Euro pallets (1200x800mm LxW) with fitting pallet sides. To prevent the cocoons from dropping out of the setup and to keep weed seeds from spreading, a weed cloth covered the surface of the pallets and parts of the pallet side. Depending on field size, two to four pallets with pallet sides were stacked on top of each other. This enabled a sufficient large surface area to keep the layer of material containing the parasitoid cocoons to a maximum of four cm. On top of the arrangement, an additional pallet covered with a weed cloth served as a lid and protection from rainfall. The sides of each pallet were lined with an insect net, net size 2.0x2.0mm. The net allowed the parasitoid exit of the setup and prevented major cocoon predators from entering the setup.

In 2016, the parasitoid cocoons were released in late March (week 13) approximately one month before defoliation of the white clover seed crop. According to the thermal needs adult parasitoids would hatched after 399 °CDD.

Estimation of possible number of parasitoids released
To survey the possible number of parasitoids capable of hatching, six samples of approximately 15g were taken from the material containing the parasitoid cocoons. Sampling was done as the material was placed in the pallet setup. Samples were scooped up, ensuring that material from the entire layer was present. Samples were stored at room temperature in 30 ml plastic cups with perforated lids to ensure ventilation. The number of hatched parasitoids were checked daily for the following month.

At harvest, samples were taken from the pallet setup to calculate the percentage of parasitoids hatched. Per field, four samples were taken: two from the bottom and two from the top of the material containing the parasitoid cocoons. Samples were stored in brown paper bags, 80x45x265mm (LxWxH) at room temperature. From each sample, 70 cocoons were randomly selected to determine if the parasitoid had hatched.

Insect sampling
In 2016, three sampling trips to the fields were planned: 1. Prior to defoliation (weeks 20 to 22), 2. Around full flowering (weeks 26 and 27) and 3. Just before harvest (week 31).
Adult weevil and parasitoids as well as weevil larvae were sampled by suction sampling. At the last visit flower heads were collected the estimate weevil adults and parasitoid cocoons.

Transect arrangement
Sampling and collection of flower heads were done following a fan-like transect (figure 14). From the release point seven transects were placed in a fan pattern. Transects were placed at a distance of 25-26° in an easterly direction as the dominant wind direction in Denmark is from a westerly direction. The transects were named A to G with transects A,D and G having sampling points 2, 10, 25, 50, 100 and 175m from the release point. Transects B, C, E and F did

not have the sampling point at 2m from the release point. This meant that the three samples 2m from the release point would not overlap. To account for the lack of control fields in 2016 two semi-control transects were placed as far from the release point as possible. Firstly a line transect consisting of seven suction points 10 paces apart and secondly a zigzag pattern consisting of seven suction points likewise 10 paces apart.

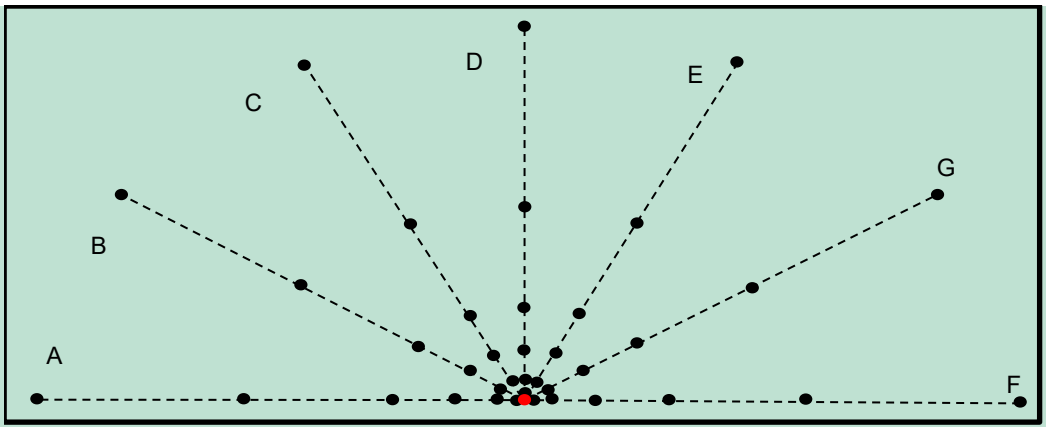

FIGURE 14. The utilized sampling strategy. Sampling points are displayed as filled circles. Parasitoid release point is represented by a red circle. Transects are labeled A to F. Samples for transects A, C, F taken at 2, 10, 25, 50, 100 and 175 meters from the parasitoid release point.

Suction sampling

Suction sampling had in preliminary testing shown to capture 27±7 percent weevils. Sampling was performed with modified leaf blowers Husqvana 125BVx (Husqvana AB, Sweden) or a Stihl SH86 C-E (Andreas Stihl AG and Co KG, Germany). A cotton bag, fitting the end of the suction tube, was used to collect weevil pests and parasitoids. For structure stability and easy removal of the collection bag, the bag was sandwiched between two plastic buckets (1180 ml, Ø133mm) (Unipack Superfoss, Denmark). The bottom of the plastic buckets were cut out. The setup was fitted into the end of the suction tube. To secure a tight fit between the outer bucket and the suction tube, two to three large rubber bands were put on the outer plastic bucket. Per field, 52 suction samples were taken, including control sampling.

Per sampling point 20 suctions of 10 seconds were performed. Care was taken not to overlap the suctions and secure an equal suction velocity. For the first measurement at grower 2, 10 suctions were taken per sampling point.

When encountering a parasitoid, the specimen was collected using a aspirator contraption fitting a 30ml plastic cup. The plastic cup was then removed from the aspirator and stored for later confirmation of the parasitoid. To ensure ventilation at storage, the plastic cup was closed with a lid having a Ø1cm hole covered by a finely meshed iron net.

Collection of flower heads at harvest

White clover flower heads were collected as close to the white clover seed harvest as possible. In 2016, 25 flower heads were collected at each of the 38 transect points together with seven collection points in the two separate semi-control layouts. In total 52 samples of 25 flower heads were taken per field. The collected flow heads were completely reflexed and had a green stem.

Collected flower heads were stored in brown paper bags, 80x45x265cm (LxWxH) and stored in large outdoor insect cages, 75x68x83cm cages (LxWxH). The cages had a fine mesh on three sides (mesh size 0.2x0.2mm) and a glass top and door. The cages were protected from direct sunlight by a west to east facing wall with a roof overhang. Furthermore, the position

was shaded by trees. Storage lasted at least 8 weeks to allow weevil larvae to pupate and hatch as adult weevils or the development of the parasitoids cocoon. After storage, the flower heads were gently threshed by hand and registration of weevils and parasitoid cocoons was done. For the weevils, the number of larvae, cocoons, adult females and males was counted. For the parasitoid the number of cocoons, adult females and males was counted.

Per field, the density of flower head and development was estimated at 10 sites in 2016. The sites were positions within the laid out fan-shaped transect grids including the transect acting as a semi-control.

Experimental setup of the 2017 field release trials
In 2017, the field release experiments resembled the experimental setup of 2016 though with some alterations described in the following. In table 30 the distanced to the previous year release are shown

Release of parasitoids 2017
The sorting of the white clover seed harvest debris had improved. The total weight of the debris material, containing the parasitoid cocoons, weighed 23.6 kg. Of these, a sub-fraction of 11.7kg contained by far the major portion of cocoons capable of hatching. This sub-fraction was used for the release of the cocoons. This also meant that the release setup could be changed as the pallet system was heavy and impractical when inspecting the status of the material. Instead, square plastic containers with fitting lids were chosen. The containers had a capacity of 55l and were made from clear plastic. On each of the four sides a rectangular hole was cut out and an insect net (mesh size 2x2mm) was glued over the hole. The holes were made as large as possible. In the bottom, a drainage hole was made and covered with the insect netting. The material was laid out in a layer of up to 2 cm.

TABLE 30. Distanced between 2017 fields to previous year release (km)

Field	Treatment	Distance to closest previous release (km)
1701	Control	18.7
1702		71.1
1703		51.6
1704		32.5
1705	Release	32.5
1706		9.0
1707		1.2
1708		2.0

Two visits to the fields were made: 1. Within the growing period (weeks 24 to 27) and 2. Just before harvest (weeks 29 to 32). On the visit just before harvest, samples were taken to calculate the percentage of parasitoids hatched. Per field, six samples of approximately 8g were taken, scooping material from the entire layer of the material. From each sample, 100 cocoons were randomly selected to determine if the parasitoid had hatched. From the time of sampling to analysis, the samples were stored at -20°C.

Insect sampling
The parasitoid cocoons were introduced after the defoliation. Insect sampling was done by sweep netting and collection of flower heads. The number of white clover flower heads was estimated per m^2. The utilized sampling techniques and sampling strategies are described in the following.

Transect arrangement
The fan-shaped transect layout used to survey the spread of the hatching adult parasitoids was repeated in 2017. As control fields were introduced, six line-transects were laid out in these fields. This was done to survey the natural population of the weevil and the parasitoid. To compare between control and release fields the line transects were used in all fields. The line transects were 100m long and positioned either 10m (n=3) or 50m (n=3) from a field border opposite the release point. The line transects were laid out 20m apart. Per line transect, samples were taken for each 10m resulting in 10 samples per transect. In fields with the release of parasitoid cocoons, the line transects were positioned opposite the release point. Depending on field shape, the two transect layouts, i.e. fan-shaped and line-shaped, sometimes overlapped. Field shape also meant that the position of the line-transect sometimes was compromised.

Sweep netting of parasitoids and weevils
Collection of weevils and parasitoids through the season was not carried out in 2017 as the suction sampling had shown disappointing results the previous year. To confirm the presence of active adult parasitoids sweep netting was carried out in control and release fields in 2017 at the time when adult parasitoids were expected to have emerged from the introduced cocoons. Sweep netting was performed in the week 24 to 27. The line transects with six transects were used. Per line transect, samples were taken for every 10m resulting in 10 samples per transect. Each sample consisted of 20 sweeps with a Ø38cm collection net. Care was taken to evenly distribute the sweeping on the 10m sample area.

Collection of flower heads at harvest
20 flower heads were collected per sampling point of the fan-shaped transect. In addition to the 2016 collection an additional sampling point 5m from the release point was introduced, giving 45 points in the fan-shaped transect layout. Collection of flower heads was in 2017 also done in six line transects of 100m as described earlier, each line transect had 10 sampling points each. In fields with the release of parasitoid cocoons, the line transects were positioned opposite the release point.

Statistics
The statistical program R (R Core Team, 2017) was used in for the statistical calculations. The built in functions lm() and glm() were used for normal distributed and binomially distributed data respectively. Pairwise comparisons were done using the emmeans package (Lenth, 2018).

Data displayed large numbers of zeroes, thus data analysis was tried on models capable of accounting for these large number of zeroes, i.e. hurdle and zero-inflated models compared to binomial, Poisson and negative binomial models. However, model evaluation did not yield an increase in model precision. Significant levels of $P<0.05$, $P<0.01$ and $P<0.001$ are indicated as *, ** and ***.

Results
Hypera species present in organic grown white clover
The vast majority of collected *Hypera* weevils belonged to the species *H. meles*. Field collected flower heads in 2016, 2017 and initial collections in 2015 resulted in registration of 1970 *H. meles* adults and nine *H. nigrirostris*.

Suction sampling in 2016
A total of 13 *B. curculionis* adults were caught using the suction method. The adult *B. curculionis* were caught in two of the four fields. In one field on the first sampling date, eight were caught. Of these eight, six were caught in one sample taken two meters from the release

point, reflecting that the parasitic wasp had begun to emerge and spread. On the second sampling date (late June, early July) three were caught and two were caught on the third sampling date (early August). Overall, no differences in adult *B. curculionis* were seen either between growers or when comparing between distances including the two controls.
For *H. meles,* a total of 453 was collected by suction sampling. Of these, 89.2 percent was collected just prior to harvest of which 83.4 percent was collected in one field. Prior to defoliation 6.6 percent of the total number of adults were collected and in second sampling 4.2 percent of the total were found.

Collection of flower heads in 2016
The collected flower heads were stored until all emerged adult weevils in the material were dead. Afterwards all flower heads were looked through for parasitoids and pests. In total 5139 flower heads were collected yielding 223 adult *H. meles* with an observed 91.5:8.5 female to male ratio. Of *B. curculionis* cocoons a total of 378 were found. Of these, 16 cocoons were opened and 6 adult parasitoids were retrieved, hence adult parasitoids would have been overlooked.

The probability of finding a parasitoid cocoon was not different between either the distances to the release point or the two semi control transects. When analysing for the probability of finding an adult *H. meles* weevil differences were only seen between the linear control transect, placed as far as possible from the release point and at the distance of 175m to the release point.

The probability of finding a *H. meles* adult was in 2016 0.39±0.008 and 0.26±0.008 of finding a parasitoid cocoon, which per flower head converts to 0.04±0.004 *H. meles* and 0.075±0.007 parasitoid cocoons. The percentage of parasitation was calculated to 62.3±2.9 percent.

For comparisons, in 2015, 700 flower heads were collected in seven fields. From these flower heads 277 *H. meles* adults hatched and 22 *B. curculionis* cocoons were found. For *H. meles* 137 were gender determined giving a 85.7:15.3 female:male ratio. The probability of finding *H. meles* adults was in 2015 0.48±0.021 and 0.07±0.014 for finding a parasitoid cocoon, which per flower head converts to 0.39±0.057 *H. meles* and 0.031±0.006 parasitoid cocoons. The percentage of parasitation was calculated to 7.09±1.6 percent.

In an effort to determine if proximity to field borders influenced the number of *Hypera* larvae and adults the fan-shaped transects were divided into two groups consisting of points closer than 30m to the field borders or more than 30m from the field borders. No differences in the number of found weevil adult or larvae were found between the two categories.

As can be seen in table 31 the probability of finding either an adult *H. meles* or a parasitoid cocoons varied between fields.

TABLE 31. The probability of finding adult *H. meles* and *B curculionis* cocoons at the four release fields in 2016. Numbers are given on the odds scale and standard errors are shown. Capital letters display differences within treatment. Differences are based on the logit scale P<0.05.

Field	Probability of finding adult *H. meles*	Probability of finding a *B. curculionis* cocoon
1601	0.29±0.04 AB	0.37±0.04 AB
1602	0.33±0.02 A	0.32±0.02 B
1603	0.21±0.01 B	0.42±0.01 A
1604	0.30±0.03 A	0.36±0.03 A

The 2017 field release trials

After the introduction of the cocoons to the white clover fields, netting was performed to estimate the size of the weevil and parasitoid population in the fields. At the time of netting, the release boxes were inspected visually. All four boxes showed signs of adult parasitoids hatching. Figure 15 shows the possibility of capturing a parasitoid (A) and a weevil (B) across all fields.

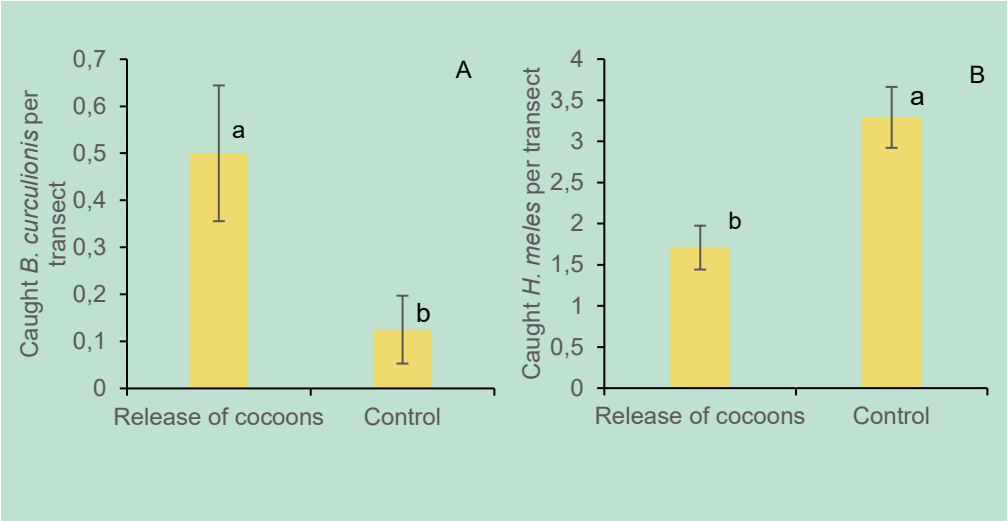

FIGURE 15. Result of netting in four fields where cocoons of the parasitoid *B. curculionis* were introduced compared with four control fields. Netting was carried out as the parasitoids began to emerge. A. Number of *B. curculionis* captured by netting. B. Number of *H. meles* captured by netting. Letters indicate significant differences P<0.05 for A and B, respectively.

In figure 15A the number of caught *B. curculionis* are seen to be higher in the release fields, which would suggest that the introduction of the cocoons to the white clover field increases the number of parasitoids in the field. In figure 15B the number of weevil *H. meles* is highest in the control fields. This would imply that later seed damage by the weevil larvae would be highest in the control fields.

The average number of weevils caught per field ranged in the control fields from 1.5±0.6 to 6.8±1.0, and for the treatment fields with the introduction of parasitoid cocoons the span of caught weevils were between 0.0 and 3.3±0.8.

For the parasitoid *B. curculionis* the captured average numbers spanned from 0.0 to 0.3±0.3 in the control treatments and from 0.2±0.2 to 0.8±0.5 in the treatment fields.

Collection of flower heads in 2017

The collected flower heads were stored until all emerged adult weevils in the material were dead. Afterwards all collected flower heads were looked through for parasitoids and pests. In total 13.221 flower heads were collected yielding 1470 adult *H. meles* and 360 *B. curculionis* cocoons of which 60 were opened. A total of 36 adult parasitoids were retrieved from the flower heads. The female to male ratio of the hatched adult *H. meles* was 90.5:9.5.

When, comparing the number of weevils, parasitoids and the parasitation frequency, no differences were found between the transect layouts. This suggests an even distribution of the pest and parasitoid across the fields where the cocoons were released.

As seen in table 32 no differences between treatments were found for the number of *H. meles* and the parasitoids found per flower head. However, the percentage of parasitation did display differences between control and release fields. A pairwise comparison of the calculated fraction of parasitation between the control field and the release point showed a 14.4 percent increase in parasitation (P-value <0.001 ***) at the release point.

TABLE 32. The number of *H. meles* and *B curculionis* found per flower head and the percentage of parasitation in four fields in 2017 for the two treatments: Introduction of cocoons and control with no introduction of parasitoid cocoons. For the fields with introduction calculations based on both transect setups are displayed. Numbers are given on the response scale with standard errors. Capital letters displays differences P<0.05.

	Treatment	*H. meles* / flower head	*B. curculionis* / flower head	Percent parasitation / flower head
Release	Around release point	0.076±0.006 B	0.029±0.004 A	27.1±2.8 A
	Away from release	0.098±0.007 B	0.027±0.003 A	22.3±1.9 A
Control		0.169±0.007 A	0.029±0.003 A	12.7±1.7 B

The possibility of finding a weevil or parasitoid cocoon was analysed as binomial observations. The analysis showed that the possibility of encountering a parasitoid was highest around the release point and lowest in the control fields (table 33).

TABLE 33. The probability of finding adult *H. meles* and *B. curculionis* cocoons in the release field and the control field. Numbers are given on the odds scale and standard errors are shown. Capital letters display difference based on the logit scale P<0.05.

	Treatment	Probability of finding adult *H. meles*	Probability of finding *B. curculionis* cocoon
Release	Around release point	0.44±0.006 B	0.22±0.006 A
	Away from release	0.42±0.010 B	0.18±0.010 B
Control		0.46±0.005 A	0.13±0.004 C

Analysis of the different distances in the fan-like transect did not display differences for the parasitation frequency, number of adult *H. meles* and cocoons of *B. curculionis*. Neither did the linear transect layouts. Separation of response variables between growers can be seen in table 34, which displays the probabilities of finding an adult *H. meles* or parasitoid cocoon for the eight fields used in 2017.

TABLE 34. The probability of finding adult *H. meles* and *B curculionis* cocoons at the four release fields and four control fields (2017). Numbers are given on the odds scale and standard errors are shown. Capital letters display differences within treatments. Differences are based on the logit scale P<0.05.

Field	Treatment	Probability of finding *H. meles* adult	Probability of finding *B. curculionis* cocoon
1701	Control	0.49±0.01 A	0.04±0.01 C
1702		0.46±0.01 AB	0.13±0.01 BC
1703		0.46±0.01 AB	0.13±0.01 BC
1704		0.45±0.01 B	0.16±0.01 A
1705	Release, around release point	0.36±0.03 B	0.31±0.03 A
1706		0.45±0.02 A	0.16±0.02 C

1707		0.41±0.01 AB	0.23±0.01 B
1708		0.44±0.03 AB	0.17±0.02 BC
1705	Release, away from release point	0.40±0.02 A	0.25±0.02 A
1706		0.46±0.01 AB	0.13±0.01 C
1707		0.43±0.01 A	0.20±0.01 B
1708		0.46±0.02 A	0.13±0.02 C

Discussion

For the three years, it is likely that the introduction of the cocoons did increase parasitation. Prior to implementing releases, the parasitation of *H. meles* by *B. curculionis* was 7.09±1.6 percent. The next year parasitation was on average 62.3±2.9 percent across the four fields in the study. The third year, in which there were sufficient organic white clover seed fields to allow both control and introduction of the parasitoid cocoons, the positive effect of the introduction was again observed with release of cocoons resulting in approximately 24.7 percent parasitation and with no release showing a parasitation rate of 12.7 percent (table 32). Previously, parasitation rates are known to vary and values between 5 and 95 percent have been reported (Dysart & Day, 1976, Flanders et al., 1994, Pike & Burkhardt, 1974a, Schroder & Metterhours, 1980). The large span in parasitation covers the registered maximum rate of parasitation found in 2016, which, however, is equivalent to findings by Schroder & Metterhours (1980) on *H. postica*.

The differences in parasitation could be year to year variation, however, as the probability of finding a hatched weevil adult seems within the same range for the two years (2015 and 2016), though the probability of finding a parasitoid cocoons had increased almost four fold. This suggests the presence of an increased population of adult parasitoids as an outcome of the release of the cocoons.

Comparing the number of hatched weevils in 2017, the differences between release fields and control were also seen for the weevil per flower head. This would indicate that the release would lower the overwintering adult weevil population. The estimated number of parasitoids hatching was in 2016 0.3 parasitoid per m^2 and in 2017 0.11 per m^2. Initially, the expected number of parasitoids capable of hatching was much higher. The 0.3 parasitoid per m^2 resulted in a parasitation percentage of 62.3 of the found weevil larvae. For the 0.11 parasitoid per m^2 in 2017 the parasitation was between 22.3 and 27.1 percent depending on distances to the release point. Comparing the parasitation percentage per released parasitoid per m^2 between years, the parasitation percentage is consistent. According to the calculation, 0.5 parasitoid per m^2 would be able to parasitize all *H. meles* larvae present, though, as the ovipositioning period of the weevil is longer than the lifespan of the parasitoid, coverage would have to obtained by numerous releases or a continuous hatching of the parasitoid. Evidently from the experiments with releasing different numbers of the parasitoids, parasitation of all weevil larvae was not achieved, even when introducing 80 times as many parasitoids as calculated above.

B. curculionis has been found to disperse at astonishing rates (Chamberlin, 1926, Dowell & Horn, 1977, Dysart & Puttler, 1965), and the distances between the first and second year of release seems within achievable distances of the parasitoid. The search pattern (Dowell & Horn, 1977) and the non-density dependent host parasitation (Barney et al., 1977, Yeargan & Latheef, 1976) support the findings of the parasitation occurring in whole fields (table 32), a tendency also found for *B. anurus* (Moore, 2014). However, differences between release and control fields in 2017 must originate from either the release or local dense populations of the parasitoid.

Field 1705 displays the highest probability of finding the parasitoid. Of the fields with releases, this field is the one furthest away from the previous years releases (table 30). If a local population of the parasitoid would exist, the probability of finding an adult weevil would be expected to be low. However, this seems not to be the case and therefore the release must have increased the probability of finding a parasitoid cocoon.

It is highly possible that hatched parasitoids will leave the white clover field as they require carbohydrates as feed. Whether the parasitoid would be able to find its way back to the white clover is uncertain.
White clover flowers do not seem suited for parasitoid feeding. Flowers of the Cruciferous and Asteraceae family have been found to contain higher numbers of parasitoids, which would suggest flowers to be accessible for parasitoids (Hogg et al., 2011). *B. curculionis* has been found to be attracted to dandelion flowers (*Taraxacum officinale* L.) (Jacob & Evans, 2000), and aphid honeydew has been seen to extend the life of *B. curculionis* (England & Evans, 1997). Aphid infestation in the white clover field and weeds in the white clover seed field could be seen as feeding opportunities for the parasitoid.

Release and defoliation
Defoliation could influence the number of adult parasitoids present in the white clover field as the management practice if leaf material and developed flower heads are removed. By removing the material, parasitoids residing within the crop would likewise be removed. Furthermore, early opened weed florets could act as a food source for the parasitoid. By removing the food source, the parasitoid would potentially have to exit the field to find alternative food sources. The material may be removed, but most often it is left in the field.

The choice of setting out the parasitoid cocoons after defoliation meant that it was plausible that the diapausing larvae would not accumulate enough thermal time to emerge at the period of main flowering, which again would suggest that the occurrence of the parasitoid would be displaced in relation to the activity of the weevil larvae. Had the parasitoids been released earlier the chance of them being killed or removed by the defoliation practice could also be envisioned. The period of the main flowering is here thought to coincide with the highest egg laying frequency of the weevil because of the availability of pollinated florets.

When utilizing late defoliation, which is used in organic white clover seed crops with a high incidence of pests, the emergence of the parasitoid might be displaced in regard to the main flowering and weevil larvae activity. Hence, the cocoons were primed by storing them at 25°C and 51%RH for two days prior to the release. Prior to the priming, the cocoons was kept at 5°C. Previously, in the storage experiment in 2017, it had been seen that the cocoons responded positively to temperature priming and cocoons could be taken directly from a temperature of 5°C and transferred to 25°C.

Number of needed parasitoids per m^2
In the attempt to influence the damage caused by larvae of *H. meles*, the number of needed parasitoids was estimated based on results from collected weevils in 2015 and results from Langer & Rohde (2005) and Hansen & Boelt (2008). To estimate the number of parasitoids that should be released, first the number of host weevil larvae has to be estimated.

The observations by Langer & Rohde (2005) and Hansen & Boelt (2008) showed the number of adult *H. nigrirostris* weevils hatched from collected white clover flower heads to range from 3.4 to 9 egg-laying female per m^2. Suction sampling of *H. meles* in 2015 showed a density of one female per m^2 after correction of the efficiency of the suction device. Based on the former, a rough guess in the number of adult weevils per m^2 has been established. The precise number of eggs positioned by *H. meles* is unknown, and at the best a qualified guess can be established. In literature, numbers from 200 to 294 eggs per female are reported (Markkula &

Tinnila, 1956, Detwiler, 1923); however, it needs to be stressed that these numbers are based on observations of both *H. meles* and *H. nigrirostris*. As the fraction of female and male weevils is not known at the time of both mating and ovipositioning, a reasonable estimate would be a 50:50 percent fraction female:male (Hansen & Boelt, 2008), though this estimate is very uncertain. Evaluation of the number of hatched females and males from the field collected white clover heads showed a distribution of 90:10 (female:male). There are no data on the percentage of females to males dying in the cold season and at the time of ovipositioning. It can be speculated if males die following mating and that females should be the only sex present, i.e. the suction sampling might display the correct number of adults weevils per m^2. Following this, one female per m^2 could be considered likely.

The change in female to male ratio would evidently almost double the number of egg-laying weevils per m^2 and thus increase the portion of yield loss owed to *Hypera* weevils in the calculations by Hansen & Boelt (2008) and in turn result in a lowering of the treshold for economic damage (*Hypera* weevils per m^2).
In doing so the percentage female to male, the percentage of survival and the average number of eggs would be appropriate to consider. Determining the number of hatched females and males, an average of 70:30 percent was observed, the winter survival was evaluated in 2017 prior to the field introduction of the cocoons. Large variations were found between subsamples, and numerous subsamples produced no hatched individuals. Based on the subsamples in which adult parasitoids hatched, a survival rate of 3.6 percent was seen. The total number of cocoons provided could be estimated based on number of tonnes of debris delivered. In 2015 a total around 23,500,000 cocoons were provided, of which 854,000 cocoons were estimated to contain a viable parasitoid larvae. If all possible *Hypera* weevils larvae were to be parasitized, one female capable of ovipositioning would have to be released per $2m^2$, yielding a total area of 48ha. Considering that 30 to 40 percent would be incapable of ovipositioning (Salt & van den Bosch, 1967, Yeargan & Pass,1978), the number of parasitoids would be three times as high. This is if female weevils oviposit 200 eggs and parasitoid females oviposit 400 eggs.

Sampling from the actually released cocoons in 2017 at a time when the majority of the living larvae should have hatched revealed that only 0.11 adult parasitoids per m^2 had hatched. Calculations had earlier estimated that 0.28 parasitoids per m^2 could be expected. In 2016, the expected number of hatched adult parasitoids was 1.33 per m^2, however only 0.3 parasitoids per m^2 was obtained. As in 2017 the actual number of hatched parasitoids was much lower than expected. In 2016 an estimated 23 percent of the set out cocoons hatched, in 2017 this was only 3.9 percent. As seen in the hatching part of the storage experiments there seems to be a significant decrease in the number of individuals capable of exiting diapause and completing the transmission to adulthood with similar observations seen previously (Pike & Burkhardt, 1974a).

The cocoons available in 2017 were collected from a total of 208ha. The average area for white clover seed production within the last ten years is 591ha (Brancheudvalget for Frø, 2019). Thus 2017 was an exceptional year concerning the production of organic white clover seeds. The parasitoid is also present in conventional white clover seed producing systems, thus increasing the potential amount of parasitoid cocoons. The area of conventional white clover production is on a ten-year average 3,586ha (Brancheudvalget for frø, 2019). Based on the available area for producing the parasitoid cocoons, there is no concern over material shortcomings and thus, even though a better functional response of the parasitoid could be desired, the sheer numbers possible for release would without doubt make an impact on the *Hypera* weevils in organically grown white clover seed production.

Criticisms of the release conditions

As invertebrate predation has been shown to be the primary mortality reason of diapausing larvae (Cherry & Armbust, 1975), the parasitoid was released in shielded setups with netting. This enabled the adult parasitoids to spread after hatching and hindered predators access to the cocoons.
The first setup was not constructed to keep out rain. Evidently, this encourage fungal growth which could have influenced the survival of the parasitoid, though hypha growth in the released material was uncommon even though the setup would restrict air flow.

For the second setup, a rain tight lid was tried. This increased temperature and on one occasion increased temperature to above 40°C for a time span of about 60 minutes. As the parasitoid is known not to tolerate high temperatures (Hama & Davis, 1983), a predator safe rainout shelter capable of providing air circulation would have been preferable for the releases.

Also, management practices in the white clover crop do not in particular account for the presence of beneficial parasitoids. Releasing the parasitoids can be obstructed by defoliation, if material is removed. Harvest and later drying of the harvested material seem to influence the number of living parasitoids, as more than 95 percent of the larvae die in the days following harvest. Given such obstacles, it could be suggested to have areas of the white clover crop not harvested for seeds but for parasitoid cocoons. Obtaining the cocoons could still be done as a traditional harvest though without drying and followed by a sorting of the cocoons.

Suction sampling had in preliminary tests shown a capacity for recapturing 27±7 percent of released weevils. Under field conditions this related to capturing almost no insects and for the second year the sampling method was abandoned.

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Supplementary data 1. Release of different densities of the parasitoid

Per collected flower head, 20 florets are randomly selected for the estimation of the yield components.
Average values with standard errors are shown. Capital letters depicts differenced of the pairwise comparisons.
Abbreviations: FC, flower head category, T, Treatment (Parasitoid m^{-2})
Two-way ANOVA with treatment and flower head category are shown. Significant levels of P<0.05, P<0.01 and P<0.001 are indicated as *, ** and ***.

Yield components from the 2016 experiment

Flower head category	Treatment (Parasitoid m^{-2})	Percent pollinated and intact florets	Percent pollinated and damaged florets	Percent Non-pollinated florets
BB	0	61.4±5.6 DE	28.6±6.0 A	10±2.3 AB
	3	70.5±6.5 BE	24.0±5.6 A	5.5±2.1 A
	6	88.5±3.0 CE	5.5±1.7 A	6.0±1.6 AB
	9	89.3± 2.6 EF	6.5±1.8 A	4.3±2.2 AB
	12	84.0±3.5 DE	6.5±2.1 A	9.5±2.9 AB
GB	0	35.8±6.0 AB	35.8±5.8 A	28.5±5.2 C
	3	48.3±7.6 ACB	31.5±6.4 A	20.3±5.1 BC
	6	68.5±4.5 BDF	20.0±4.8 A	11.5±2.1 AB
	9	78.5±6.3 BDE	13.3±5.4 A	8.3±2.0 AB
	12	70.3±6.6 BDE	16.8±5.9 A	13±2.6 BC
Average		69.6±4.6	18.2±15.8	11.6±1.6
ANOVA				
T * FC		0.616 NS	0.818 NS	0.028 *
Flower head category		<0.001 ***	<0.001 ***	<0.001 ***
Treatment		0.013 NS	0.160 NS	0.024 *

Flower head category	Treatment (Parasitoid m^{-2})	Intact seeds per intact floret	Intact seeds per damaged floret
BB	0	2.69±0.17 AB	1.38±0.19 A
	3	3.29±0.14 AB	0.90±0.26 A
	6	3.21± 0.20 AB	0.54±0.22 A
	9	2.96±0.15	1.18±0.26 A
	12	3.13±0.24 AB	0.99±0.35 A
GB	0	2.46±0.29 AB	0.802±0.15 A
	3	2.23±0.17 AB	0.70±0.21 A
	6	2.53±0.16 AB	1.06±0.20 A

	9	2.29±0.19 AB	1.05±0.33 A
	12	2.40±.25 AB	0.74±0.18 A
Average		2.73±0.09	0.95±0.7
ANOVA			
T * FC		0.321 NS	0.158 NS
Flower head category		<0.001 ***	0.220 NS
Treatment		0.807 NS	0.550 NS

Flower head category	Treatment (Parasitoid m^{-2})	Florets per flower head	Seeds per heads from non-damaged florets	Seeds per heads from damaged florets
BB	0	84.8±6.0 A	140.8±20.0 ABC	32.1±7.7 A
	3	82.1±5.1 A	186.5±23.0 ACD	31.7±11.3 A
	6	90.7±4.7 A	254.9±18.9 CF	5.9±3.4 A
	9	98.8±7.2 A	253.5±19.7 AF	16.7±5.4 A
	12	87.1±6.2 A	223.4±23.6 ACE	9.3±3.7 A
GB	0	78.3±4.2 A	80±18.0 BD	18.8±3.3 A
	3	83.9±8.1 A	94.2±16.2 BE	17.3±6.2 A
	6	78.0±4.0 A	140.2±16.3 AB	16.4±3.4 A
	9	84.6±5.3 A	160.2±27.9 BC	14.7±4.8 A
	12	85.5±5.6 A	149.4±23.6 ABC	22.7± 7.1 A
Average		85.5±2.3	170.5±14.4	18.4±3.0 A
ANOVA				
T * FC		0.571 NS	0.700 NS	0.0844 NS
Flower head category		0.069 NS	<0.001 ***	0.330 NS
Treatment		0.548 NS	0.028 *	0.533 NS

Flower head category	Treatment (Parasitoid m^{-2})	Flower heads per m^2	Yield per flower head intact floret (g)	Yield per flower head damaged floret (g)	Yield per flower head (g)
BB	0	800±173 A	0.1±0.01 AB	0.019±0.005 A	0.12±0.01 BCD
	3	450±206 A	0.13±0.02 A	0.016±0.006 A	0.15±0.02 DE
	6	425±118 A	0.18±0.01 AC	0.002±0.001 A	0.18±0.01 AC
	9	925±111 A	0.18±0.01 AD	0.0060.002 A	0.18±0.02 CD
	12	800±147 A	0.16±0.02 ACD	0.003±0.001 A	0.16±0.02 CD
GB	0	125±95 A	0.05±0.01 ACD	0.012±0.002 A	0.07±0.01 A
	3	650±330 A	0.06±0.01 BCD	0.009±0.003 A	0.07±0.01 AC
	6	300±108 A	0.1±0.01 DE	0.009±0.002 A	0.11±0.01 AD
	9	725±278 A	0.11±0.02 CE	0.005±0.002 A	0.12±0.02 ACD
	12	325±111 A	0.1±0.02 CD	0.007±0.003A	0.11±0.02 AB
Average		552±65	0.12±0.01	0.009±0.002	0.13±0.01
ANOVA					
T * FC		0.189 NS	0.650 NS	0.053 NS	0.705 NS
Flower head category		0.0426 *	<0.001 ***	0.783 NS	<0.001 ***
Treatment		0.192 NS	0.027 *	0.203 NS	0.044 *

Yield components from 2017 experiment

Flower head category	Treatment (Parasitoid m^{-2})	Percent pollinated and intact florets	Percent pollinated and damaged florets	Percent Non-pollinated florets
BB	0	64.3±5.02 A	18.5±2.8 A	17.3±3.9 A
	6	67.5±5.36 A	13.5±3.5 A	19.0±3.0 A
	12	76.5±2.69 A	14.5±2.5 A	9.0±1.4 A
	22	75.0±4.74 A	11.8±4.2 A	13.3±2.9 A
	44	80.3±3.19 A	8.0±1.8 A	11.8±2.0 A
GB	0	63.0±7.05 A	26.3±5.7 A	10.7±2.7 A
	6	73.3±4.83 A	19.0±3.2 A	7.8±2.2 A
	12	79.3±4.01 A	14.5±3.7 A	6.3±1.8 A
	22	71.8±5.32 A	20.3±4.6 A	8.0±1.5 A
	44	69.8±6.14 A	18.5±4.7 S	11.8±4.6 A
Average		71.9±2.46	16.3±1.6	11.7±1.4
ANOVA				
T * FC		0.399 NS	0.605 NS	0.297 NS
Flower head category		0.531 NS	0.004 **	0.005 **
Treatment		0.245 NS	0.359 NS	0.288 NS

Flower head category	Treatment (Parasitoid m^{-2})	Intact seeds per intact floret	Intact seeds per damaged floret
BB	0	2.82±0.19 A	0.82±0.13 A
	6	2.65±0.19 A	1.02±0.29 A
	12	2.92±0.16 A	1.16±0.23 A
	22	3.13±0.16 A	1.75±0.40
	44	3.02±0.15 A	1.73±0.31 A
GB	0	2.43±0.20 A	0.49±0.14 A
	6	2.59±0.17 A	0.91±0.23 A
	12	2.92±0.16 A	0.69±0.20 A
	22	2.61±0.15 A	0.92±0.14 A
	44	2.93±0.13 A	1.25±0.26 A
Average		2.81±0.07	1.06±0.11
ANOVA			
T * FC		0.373 NS	0.570 NS
Flower head category		0.041 *	0.020 **
Treatment		0.220 NS	0.073 NS

Flower head category	Treatment (Parasitoid m^{-2})	Florets per flower head	Seeds per heads from non-damaged florets	Seeds per heads from damaged florets
BB	0	68.4±6.1 A	118.0±13.0 A	11.2±2.7 A
	6	80.4±5.7 A	139.7±14.7 A	10.4±2.8 A
	12	75±4.7 A	169.1±20.0 A	18.9±5.3 A
	22	69.4±4.8 A	163.3±15.0 A	12.9±2.8 A

	44	71.9±3.9 A	171.9±13.4 A	10.5±1.9 A
GB	0	74.8±6.3 A	119.1±22.4 A	7.5±1.6 A
	6	80.9±4.5 A	152.2±14.9 A	9.9±2.8 A
	12	75.4±3.9 A	173.2±15.0 A	9.5±2.7 A
	22	77.9±4.0 A	144.5±14.9	14.8±3.9 A
	44	71.3±4.4 A	146.5±16.6	12.2±2.8 A
Average		74.5±1.5	149.6±7.8	11.8±1.2 A
ANOVA				
T * FC		0.824 NS	0.700 NS	0.305 NS
Flower head category		0.334 NS	0.498 NS	0.249 NS
Treatment		0.284 NS	0.144 NS	0.624 NS

Flower head category	Treatment (Parasitoid m^{-2})	Flower heads per m^2	Yield per flower head intact floret (g)	Yield per flower head damaged floret (g)	Yield per flower head (g)
BB	0	1375±180 A	0.083±0.009 A	0.008±0.002 A	0.09±0.009 A
	6	1200±183 A	0.098±0.01 A	0.007±0.002 A	0.103±0.01 A
	12	1375±149 A	0.118±0.014 A	0.013±0.004 A	0.13±0.015 A
	22	1300±311 A	0.114±0.01 A	0.009±0.002 A	0.12±0.01 A
	44	1500±324 A	0.12±0.009 A	0.007±0.001 A	0.125±0.009 A
GB	0	225±102 B	0.083±0.016 A	0.005±0.001 A	0.087±0.015 A
	6	200±0 B	0.107±0.01 A	0.007±0.002 A	0.113±0.011 A
	12	250±155 B	0.121±0.01 A	0.007±0.002 A	0.127±0.01 A
	22	275±75 B	0.101±0.01 A	0.010±0.003 A	0.109±0.01 A
	44	200±141 B	0.103±0.012 A	0.009±0.002 A	0.109±0.012 A
Average		790±104	0.105±0.005	0.008±0.001	0.111±0.006
ANOVA					
T * FC		0.935 NS	0.688 NS	0.284 NS	0.749 NA
Flower head category		<0.001 ***	0.502 NS	0.257 NS	0.428 NS
Treatment		0.940 NS	0.144 NS	0.630 NS	0.153 NS

Supplementary data 2. Parasitoid release combined with pesticide application

Data is shown for the two flower head categories: fully reflexed with a brown stem (BB) and fully reflexed with a green stem (GB) for the five treatments.
Abbreviations: B.c. *B. curculionis*. FC, flower head category, T, Treatment. NA, not available. Average values with standard errors are shown. Capital letters depicts differenced of the pair-wise comparisons. Per collected flower hear, 20 florets are randomly selected for the estimation of the yield components.

Two-way ANOVA with treatment and flower head category are shown. Significant levels of P<0.05, P<0.01 and P<0.001 are indicated as *, ** and ***.

Yield components from the 2016 experiment

Flower head category	Treatment	Percent pollinated and intact florets	Percent pollinated and damaged florets	Percent Non-pollinated florets
BB	Biscaya followed by B.c	90.3±2.2 A	4.8±1.9 AB	5.0±1.7 A
	B.c followed by Biscaya	89.5±4.2 A	1.0±0.6 A	9.5±4.2 AB
	Karate followed by B.c	81.3±5.1 AB	0.3±0.3 A	18.5±5.2 AC
	B.c followed by Karate	50.0±8.0 AB	2.3±0.9 A	47.8±8.4 BC
	Only B.c	86.3±4.2 A	9.2±4.0 AB	4.5±1.4 A
GB	Biscaya then B.c	87.5±1.8 A	5.8±1.5 AB	6.7±1.4 AB
	B.c then Biscaya	89.3±4.0 A	8.3±4.1 AB	2.3±1.3 AB
	Karate then B.c	84.5±4.6 A	1.8±0.8 A	13.8±4.5 AB
	B.c then Karate	39.8±9.2 B	1.8±0.8 A	58.5±9.6 C
	Only B.c - Control	80.7±4.2 AB	15.3±3.0 B	4.0±1.9 A
Average		77.6±5.4	4.9±1.4	17.6±5.7
ANOVA				
T * FC		0.478 NS	0.215 NS	0.215 NS
Flower head category		0.118 NS	0.021 NS	0.021 *
Treatment		0.008 **	0.021 NS	0.021 *

Flower head category	Treatment	Intact seeds per intact floret	Intact seeds per damaged floret
BB	Biscaya followed by B.c	3.32±0.18 A	2.58±0.73 A
	B.c followed by Biscaya	2.96±0.23 A	2.00±2.00 A
	Karate followed by B.c	2.97±0.22 A	6.00± NA
	B.c followed by Karate	2.93±0.29 A	1.7±0.85 A
	Only B.c	3.28±0.18 A	2.42±0.47 A
GB	Biscaya then B.c	2.85±0.14 A	2.47±0.37 A
	B.c then Biscaya	3.53±0.17 A	2.58±0.45 A
	Karate then B.c	3.30±0.22 A	2.13±0.98 A

	B.c then Karate	2.83±0.31 A	3.77±0.23 A
	Only B.c - Control	2.60±0.20 A	1.83±0.30 A
Average		3.04±0.09	2.39±0.18
ANOVA			
T * FC		0.014 *	0.013 *
Flower head category		0.400 NS	0.809 NS
Treatment		0.822 NS	0.698 NS

Flower head category	Treatment	Florets per flower head	Seeds per heads from non-damaged florets	Seeds per heads from damaged florets
BB	Biscaya followed by B.c	95.4±6.3 A	281.1±23.0 A	31.5±6.0 A
	B.c followed by Biscaya	86.0±5.1 A	224.3±22.9 A	14.6±14.6 A
	Karate followed by B.c	100.6±7.3 A	240.0±24.9 A	38.4±NA
	B.c followed by Karate	111.2±9.2 A	204.0±30.2 A	20.7±11.5
	Only B.c	98.2±6.6 A	287.3±31.5 A	32.4±9.3 A
GB	Biscaya then B.c	89.8±7.0 A	227.2±22.5 A	15.5±2.5 A
	B.c then Biscaya	83.6±4.6 A	257.9±16.6 A	38.7±12.9 A
	Karate then B.c	94.7±6.1 ±	250.3±19.7 A	18.6±14.1 A
	B.c then Karate	96.1±6.0 A	182.1±35.6 A	18.6±7.2 A
	Only B.c - Control	87.7±6.1 A	188.6±24.8 A	28.6±5.2 A
Average		94.6±2.8	233.8±11.3	25.2±3.1 A
ANOVA				
T * FC		0.867 NS	0.059 NS	0.361 NS
Flower head category		0.485 NS	0.046 *	0.371 NS
Treatment		0.214 NS	0.363 NS	0.465 NS

Flower head category	Treatment	Flower heads per m^2	Yield per flower head intact floret (g)	Yield per flower head damaged floret (g)	Yield per flower head (g)
BB	Biscaya followed by B.c	1075±327 A	0.20±0.01 A	0.007±0.003 A	0.20±0.02 A
	B.c followed by Biscaya	1275±327 A	0.16±0.02 AB	0.001±0.001 A	0.16±0.02 AB
	Karate followed by B.c	775±189 A	0.17±0.02 AB	0.001±0.001 A	0.17±0.02 AB
	B.c followed by Karate	775±193 A	0.11±0.02 AB	0.004±0.002 A	0.12±0.02 AB
	Only B.c	700±248 A	0.20±0.02 A	0.012±0.004 A	0.21±0.02 A
GB	Biscaya then B.c	300±100 A	0.16±0.02 AB	0.007±0.002 A	0.17±0.02
	B.c then Biscaya	375±165 A	0.18±0.02 AB	0.011±0.005 A	0.19±0.01 AB
	Karate then B.c	275±63 A	0.18±0.01 AB	0.003±0.003 A	0.18±0.01 AB
	B.c then Karate	325±170 A	0.08±0.02 B	0.003±0.002 A	0.08±0.02 B
	Only B.c - Control	775±295 A	0.13±0.02 AB	0.015±0.003 A	0.15±0.02 AB
Average		665±81	0.16±0.01	0.006±0.002	0.16±0.01
ANOVA					
T * FC		0.257 NS	0.055 NS	0.353 NS	0.041 *
Flower head category		0.001 **	0.012 *	0.113 NS	0.027 *
Treatment		0.662 NS	0.061 NS	0.069 NS	0.052 *

Yield components from the 2017 experiment

Flower head category	Treatment	Percent pollinated and intact florets	Percent pollinated and damaged florets	Percent Non-pollinated florets
BB	Biscaya	82.8±2.9 A	3.3±1.0 A	14.0±2.5 A
	Biscaya then B.c	76.8±4.7 AB	9.0±2.0 AC	14.3±3.5 A
	Karate	66.8±4.2 AB	9.0±1.6 AC	24.3±4.3 AB
	Karate then B.c	72.3±5.1 AB	8.8±1.5 AC	19.0±4.9 A
	Only B.c	70.5±3.6 AB	12.8±2.7 BC	16.8±3.1 A
GB	Biscaya	90.0±2.0 A	4.3±1.6 AB	5.8±1.4 A
	Biscaya then B.c	89.3±2.1 A	3.5±1.4 A	7.3±1.9 A
	Karate	80.0±5.2 A	5.0±2.0 AB	15.0±5.1 A
	Karate then B.c	54.5±6.4 B	3.8±1.0 A	41.8±6.6 B
	Only B.c	73.5±4.3 AB	15.8±3.4	10.8±2.2 A
Average		75.6±2.6	7.5±1.0	16.9±2.5
ANOVA				
T * FC		<0.001 ***	0.094 NS	<0.001 ***
Flower head category		0.172 NS	0.090 NS	0.530 NS
Treatment		0.004 **	<0.001 ***	0.005 **

Flower head category	Treatment	Intact seeds per intact floret	Intact seeds per damaged floret
BB	Biscaya	2.8±0.2 AB	1.9±0.6 A
	Biscaya then B.c	3.2±0.2 AB	1.0±0.3 A
	Karate	3.4±0.2 AB	1.6±0.3 A
	Karate then B.c	3.0±0.2 A	0.9±0.2 A
	Only B.c - Control	3.1±0.2 AB	1.3±0.3 A
GB	Biscaya	3.2±0.1 AB	1.8±0.4 A
	Biscaya then B.c	3.0±0.1 B	1.4±0.4 A
	Karate	3.0±0.1 AB	0.8±0.3 A
	Karate then B.c	2.6±0.1 A	0.4±0.3 A
	Only B.c - Control	2.8±0.2 AB	0.5±0.2 A
Average		3.0±0.1	1.1±0.2
ANOVA			
T * FC		0.102 NS	0.415 NS
Flower head category		0.083 NS	0.010 *
Treatment		0.239 NS	0.193 NS

Flower head category	Treatment	Florets per flower head	Seeds per heads from non- damaged florets	Seeds per heads from damaged florets
BB	Biscaya	69.4±4.5 A	164.0±16.8 AB	9.6±3.7 AB
	Biscaya then B.c	68.9±4.6 A	164.5±15.0 AB	7.9±2.2 AB
	Karate	75.5±4.7 A	170.5±16.6 AB	15.9±4.5 AB
	Karate then B.c	60.3±3.9 A	128.1±13.7 AB	4.6±1.0 AB
	Only B.c - Control	72.2±4.4 A	159.9±18.8 AB	16.8±6.4 A
GB	Biscaya	71.5±3.6 A	203.2±12.9 B	10.0±3.1 AB
	Biscaya then B.c	78.1±5.3 A	209.3±16.3 B	9.3±2.7 AB
	Karate	70.1±2.6 A	176.5±20.2 AB	8.6±4.2 AB

	Karate then B.c	73.2±4.4 A	108.1±17.7 A	0.7±0.5 AB
	Only B.c - Control	73.5±4.7 A	151.8±14.5 AB	5.1±1.4 B
Average		71.2±1.7	163.6±9.3	9.0±1.6
ANOVA				
T * FC		0.197 NS	0.133 NS	0.194 NS
Flower head category		0.131 NS	0.204 NS	0.016 *
Treatment		0.674 NS	0.044 *	0.191 NS

Flower head cate-gory	Treatment	Flower heads per m^2	Yield per flower head intact floret (g)	Yield per flower head damaged floret (g)	Yield per flower-head (g)
BB	Biscaya	850±132 AC	0.115±0.012 AB	0.007±0.003 AB	0.118±0.012 AB
	Biscaya then B.c	1125±225 BC	0.115±0.11 AB	0.006±0.002 AB	0.119±0.011 AB
	Karate	1100±129 BC	0.119±0.012 AB	0.011±0.003 AB	0.128±0.013 AB
	Karate then B.c	675±232 AB	0.09±0.01 AB	0.003±0.001 AB	0.093±0.010 AB
	Only B.c - Con-trol	1575±160 C	0.112±0.013 A	0.012±0.004 A	0.12±0.012 AB
GB	Biscaya	825±193 AC	0.142±0.01 B	0.007±0.002 AB	0.146±0.009 B
	Biscaya then B.c	750±104 AB	0.147±0.011 B	0.006±0.002 AB	0.149±0.011 B
	Karate	400±196 AB	0.124±0.14 AB	0.006±0.003 AB	0.126±0.014 AB
	Karate then B.c	650±185 AB	0.076±0.012 A	0.001±0.000 AB	0.076±0.012 A
	Only B.c - Con-trol	125±69 A	0.106±0.01 AB	0.004±0.0001 B	0.109±0.01 AB
Average		808±77	0.114±0.007	0.006±0.001	0.118±0.007
ANOVA					
T * FC		0.001 **	0.133 NS	0.194 NS	0.075 NS
Flower head category		<0.001 ***	0.204 NS	0.016 *	0.408 NS
Treatment		0.753 NS	0.044 *	0.191 NS	0.053 NS

[Tekst - Slet ikke efterfølgende linje, sektionsskifte]

Alternative Management Strategy Towards Weevils in White Clover Seed Production - Utilization of a Natural Enemy

In Denmark, the most important *Hypera* species are the lesser clover leaf weevil *Hypera nigrirostris* (Fabricius) and clover head weevil *Hypera meles* (Fabricius). This project documents that both species are parasitized by the parasitoid *Bathyplectes curculionis* (Thomson), parasitizing the young weevil larvae in the clover heads.

The current project studied if the parasitoid can be conserved and isolated from harvested white clover crops and hereafter be used to regulate Hypera weevils in consecutive clover crops. The prerequisites would, thus, be 1) that the cocoons can be separated from the harvested raw material, 2) that the cocoons can be stored throughout the winter season, 3) that the cocoons can be released in the new clover seed field, 4) that adequate numbers of the adult parasitoid can hatch at the right time related to the development of the weevil larvae and 5) that the released parasitoids reduce the damage by the weevil within the year of release or reduce the number of weevils hatching, which constitutes the weevil population in the following year.

The results revealed that the number of intact and pollinated florets was significantly positively influenced by the number of parasitoids and increased from 60% to 85-85% in both years with an increase in the number of released parasitoids.

The number of clover head weevils hatching from the white clover flower heads, which would constitute the following year's population was, however, not affected by the number of parasitoids released.

The results from the project show that the parasitoid *B. curculionis* of the clover head weevil has the potential of being part of a future strategy to increase the seed yield and reducing the application of insecticides in white clover seed production.

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