Effect of temperature on the interactions between beet cyst nematodes (*Heterodera schachtii* and *Heterodera betae*) and sugar beet

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SUMMARY

Climate change is expected to cause a mean annual temperature increase in Germany of 2 °C by 2050 and up to 4 °C by 2100. This is likely to have effects on crop development and pathogen development as well. For this Ph.D. thesis, experiments were conducted to investigate the effect of increasing temperatures on the beet cyst nematodes *Heterodera schachtii* and *Heterodera betae* and thus study the changes of their interaction with their host plant the sugar beet (*Beta vulgaris* subsp.).

Differences in hatch between the beet cyst nematode species were assessed at constant temperatures in incubators as well as simulated temperature conditions set to be 4 °C higher than the standard temperature regime.

The optimal temperatures for hatch were found to be different for both cyst nematode species. The optimal temperature range for hatching of *H. schachtii* was found to be between 15 and 30 °C and for *H. betae* between 20 and 30 °C. Emergence of juveniles of both beet cyst nematode species began at 5 °C, however in very low percentages. For both beet cyst nematode species no significant differences were found in the final cumulative hatch percentages when comparing the standard temperature regime with the by 4°C increased temperature regime.

In the climate chambers, the influence of temperature on the interspecific competition between both beet cyst nematode species was studied. Both beet cyst nematode species performed better at higher temperatures. But there were no clear indications that under competition one of the two species will profit more from higher temperatures under the predicted climate change.

In conclusion, the damage done by cyst nematodes in sugar beet is expected to increase with global warming. *H. schachtii* and *H. betae* are likely to continue to cause damages. But in competition, neither of the two species will profit more from rising temperatures compared to the other.

The effect of experimental soil warming on *H. schachtii* population development and sugar beet performance was assessed for sugar beet cultivars that were susceptible, tolerant or resistant to *H.*

schachtii. In this study, soil heating lead to a significant increase in the final number of recovered cysts on the tolerant cultivar and susceptible cultivar. The resistant cultivar did not allow nematode reproduction at all. Therefore no effect of soil heating could be detected. Plant-nematode interaction varies greatly depending on the cultivar. Thus cultivar choice is an important element when trying to prevent nematode infestation or controlling nematode populations in the field. In case of high nematode population densities and with the expected increasing soil temperatures, planting nematode-resistant cultivars will become even more important in the future, as an effective tool to reduce nematode populations and prevent damages.

ZUSAMMENFASSUNG

Für Deutschland wird vorausgesagt, dass der Klimawandel eine Erhöhung der Jahresmitteltemperaturen um 2 °C bis zum Jahr 2050 und sogar um 4 °C bis zum Jahr 2100 bewirken wird. Dies bleibt nicht ohne Einfluss auf die Entwickelung von Kulturpflanzen und ihre Schaderreger. Ziel dieser Doktorarbeit war es, in verschiedenen Versuchen den Einfluss von erhöhten Temperaturen auf die Entwicklung der Rübenzystennematodenarten, *Heterodera schachtii* und *Heterodera betae* zu simulieren und Aussagen über die Veränderungen in der Interaktion mit ihrer Wirtspflanze, der Zuckerrübe (*Beta vulgaris* subsp.) zu treffen.

Unterschiede im Schlupfverhalten beider Rübenzystennematodenarten wurden ermittelt sowohl unter konstanten Temperaturbedingungen in Inkubatoren als auch in Klimakammern. In den Klimakammern wurde eine Erhöhung von 4 °C gegenüber den Standardtemperatur simuliert.

Die optimale Temperaturen für den Schlupf waren unterschiedlich für beiden Arten. Für *Heterodera schachtii* lagen sie zwischen 15 °C und 30 °C. Für *H. betae* wurden die höchsten Schlupfraten bei Temperaturen zwischen 20 °C und 30 °C beobachtet. Beide Arten begannen bei 5 °C zu schlüpfen, allerdings war der Prozentsatz der geschlüpften Tiere äußerst niedrig. Für beide Arten waren keine signifikanten Unterschiede in den finalen kumulativen Schlupfraten zwischen das um 4 °C erhöhte Temperaturregime und das Standardtemperaturregime in den Klimakammerversuchen zu erkennen.

In den Klimakammern wurde der Einfluss der Temperaturerhöhung auf die Konkurrenzfähigkeit beider Arten studiert. Es zeigte sich, dass die Populationsdichten beider Arten mit höheren Temperaturen anstiegen. Es ist zu erwarten, dass beide Rübenzystennematoden vom Klimawandel profitieren werden und daher verstärkt Schäden in Zuckerrüben verursachen werden. Die vorliegenden Ergebnisse zur Wettbewerbsfähigkeit der beiden Arten lassen keine Rückschlüsse darauf zu, dass eine der beiden Spezies stärker vom Klimawandel profitiert als die andere.

In den Experimenten wurde der Einfluss von erhöhten Bodentemperaturen auf die Populationsentwickelung von *Heterodera schachtii* an verschiedenen Zuckerrübensorten bewertet, nämlich an einer empfindlichen, einer toleranten und einer resistenten Sorte. An der toleranten und der empfindlichen Sorte führte die experimentelle Erwärmung des Bodens zu

deutlich gesteigerten Zystenzahlen an den Rübenwurzeln. Die resistente Sorte hingegen ließ unabhängig von der Bodenerwärmung keine Nematodenvermehrung zu. Im Fall eines hohen Infektionsdruckes, also bei hohen Nematodenzahlen im Boden und angesichts der prognostizierten steigenden Bodentemperaturen, wird der Anbau von resistenten Zuckerrübensorten zukünftig noch mehr an Bedeutung gewinnen. Die kluge Sortenwahl, also der Anbau resistenter Zuckerrübensorten ist und bleibt ein von die wichtigste Werkzeuge, um Nematodenpopulationen zu dezimieren und Nematodenschäden vorzubeugen.

Chapter 1:

General introduction

The European Union (EU) is the world's leading producer of sugar beet, with a production of 128.4 million tons in 2014, covering around 50 % of the global production (Eurostat, 2015). Twenty percent of the global sugar production is derived from sugar beet, while the remaining eighty percent is derived from sugar cane (Eurostat, 2015). The production and the price of sugar beet have recently decreased in the EU due to political decisions related to agricultural subsidies and due to the strong competition against sugar from sugar cane (Bruhns, 2009). Following the major reform of the sugar beet market in 2006, the EU sugar market is regulated by production quotas, minimum beet prices and trade mechanisms (Bruhns, 2009; Belboom & Léonard, 2012). Conversely, the use of sugar beet for the production of bio-ethanol could give sugar beet production upsurge (Von Blottnitz & Curran, 2007). The energy balance is very positive, with sugar beets producing 15-16 times more energy than is required to produce it (Řezbová, 2013). The energy output-input ratio for German commercial sugar beet farms is calculated at 15.4 (Reineke *et al.*, 2013). Germany is one of the main European producers of sugar beet with 358 000 hectares (Eurostat, 2015). Due to the high proportion of sugar beet in crop rotations, many leaf and soil-borne pathogens severely limit yield.

Beet cyst nematodes are considered one of the most important pests in sugar beet production worldwide and cause severe damage to sugar beet with yield losses of up to 25 % (Schlang, 1991). It is estimated that the annual yield loss in the EU countries on the world market sugar price level amounts to 90 million Euro (Müller, 1999). Affected plants show stunted growth, decreased chlorophyll content in leaves and symptoms of wilt late in the growing season especially when the plants are exposed to heat and/or water stress conditions (Hillnhütter). Belowground symptoms include the development of compensatory secondary roots, which can result in the typical "bearded" root symptom and an overall beet deformity (Cooke, 1987; Hilnhütter, 2010).

Beet cyst nematodes belong to the family Heteroderidae which is a group of sedentary biotrophic plant-parasitic nematodes characterized by the ability of the female to transform into a tough

brown cyst which protects the eggs which have been formed within her body (Bohlmann, 2015); see Figure 1 for details on the life cycle. Among the beet cyst nematodes, the sugar beet cyst nematode, *Heterodera schachtii* and the yellow beet cyst nematode, *Heterodera betae*, can be distinguished.

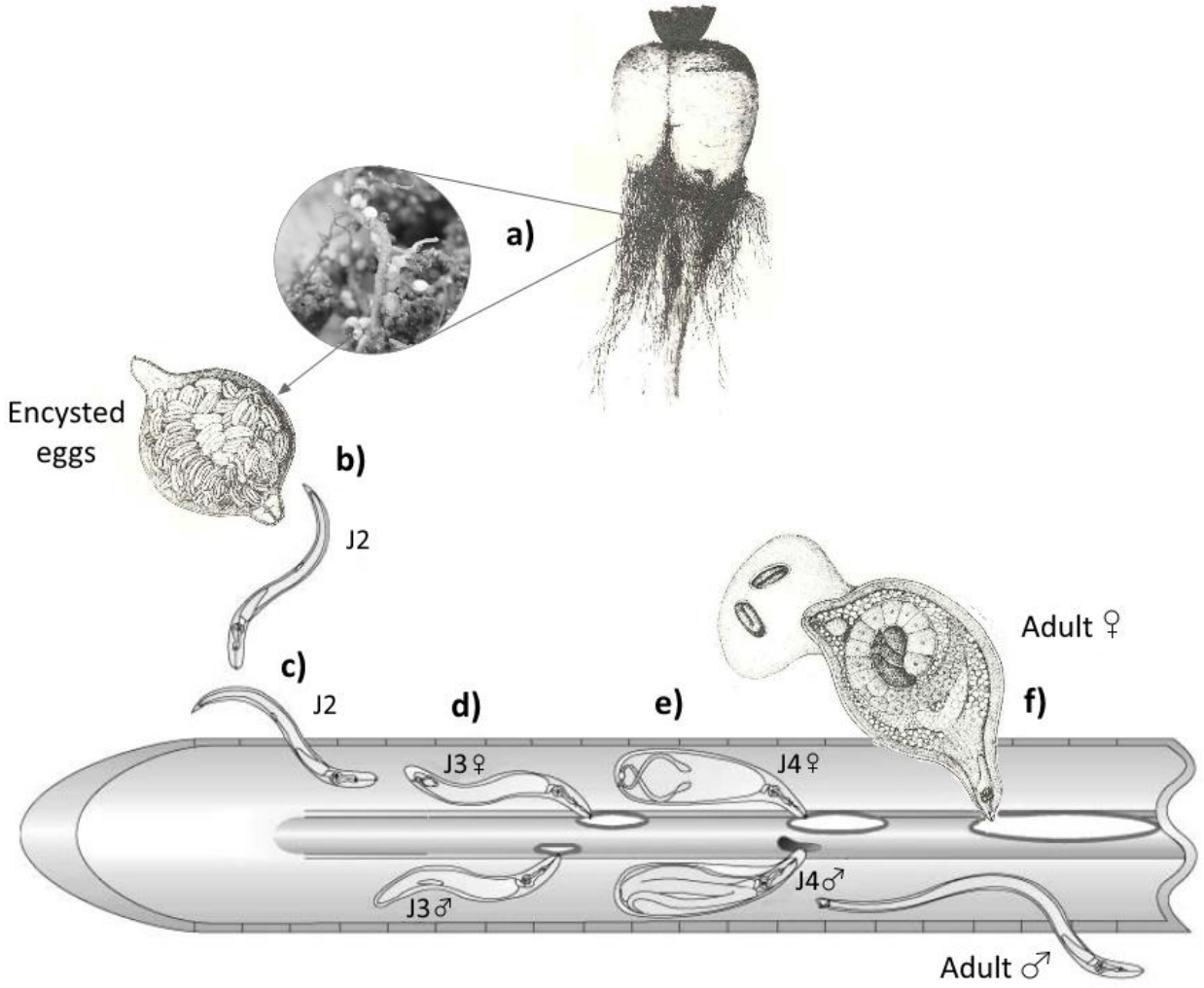

Figure. 1: Life cycle of *Heterodera schachtii* (a) Sugar beet with roots infested with cysts. (b) Eggs may remain dormant in the soil protected within the tanned cyst for many years. Under favourable conditions, the second-stage juvenile (J2) hatches and migrates towards a host root. (c) The J2 penetrates the root and migrates intracellularly through the cortex towards the vascular cylinder where it initiates formation of a feeding site. Sex is determined towards the end of the J2 stage. (d) A multinucleate feeding site (syncytium) is established by cell wall dissolution. (e) The female enlarges while the motile, vermiform adult male develops within the J4 cuticle. The male does not feed after the J3 stage and its syncytium begins to degrade. (f) The male leaves the root and fertilizes the adult female, which grows to rupture the root surface. Eggs develop within the female body wall, which tans to form the cyst. The life cycle of

Heterodera betae is similar with the exception that the occurrence of males is not known and reproduction is expected to occur via parthenogenetic mitosis. Adapted from Lilley *et al.*, 2005.

Until now research mostly focused on the biology and control of *H. schachtii*, which has been recognized as a plant pathogen since 1859 when it was associated with stunted and declining sugar beets in Germany (Schacht, 1859). Since then this nematode species was detected in most beet growing areas. Depending on soil type and temperature, the economic threshold of *H. schachtii* ranges from 500 - 1000 second stage juveniles (J2) and eggs 100 ml^{-1} soil (Müller, 1999). The yellow beet cyst nematode, *Heterodera betae*, was discovered in Dutch beet fields in 1975 and was first considered as a biotype of the clover cyst nematode, *H. trifolii*, able to parasitize sugar beet (Maas & Heijbroek, 1982). Later on, molecular and morphological characterisation established that *H. betae* was distinct from the *H. trifolii* complex and constituted a true species (Wouts *et al.*, 2001).

The yellow beet cyst nematode is less prevalent but has also been found damaging beet crops (Maas & Heijbroek, 1982). For *H. betae*, the damage threshold is estimated at 500 eggs and J2 100 ml^{-1} soil. The yield will be reduced by about 35% at 5000 eggs and J2 100 ml^{-1} soil (Maas & Heijbroek, 1982). The host ranges of *H. schachtii* and *H. betae* were shown to be very similar (Gracianne *et al.*, 2014; Maas & Heijbroek, 1982). Both species have been shown to co-occur in mixed populations in several locations in France, Spain, and Belgium (Gracianne *et al.*, 2014). However, knowledge on how both species interact in co-occurrence is very limited.

Recently, it was hypothesised that *H. betae* derived from *H. schachtii* as a result of speciation by polyploidy and that *H. betae* is better adapted to warmer habitats (Gracianne *et al.*, 2014). Temperature is often the most important environmental factor affecting nematode biology (Trudgill, 1995). Different species have different optimum temperatures for feeding, hatching, reproduction and survival (Neilson & Boag, 1996), hence their development is strongly influenced by the soil climate (Trudgill, 1995; Trudgill *et al.*, 2005; Kaczmarek *et al.*, 2014).

Climate change has been accelerated by increased anthropogenic greenhouse gas emission in the last century and is associated with rising temperatures around the globe (Solomon *et al.*, 2007). Climate change is expected to cause a mean annual temperature increase in Germany of 2 °C by

2050 and up to 4 °C by 2100 (Jacob & Podzun, 1997; Werner & Gerstengarbe, 2007), which will have effects on both crop and pathogen development (Weigel, 2005; Racca *et al.*, 2015). Rising average temperatures may lead to changes in crop phenology, but also in the incidence of pathogens (Racca *et al.*, 2015). Global warming may either increase or decrease crop production in the future, depending on local conditions (Rosenzweig *et al.*, 2002). For example, climate impact studies predict that sugar beet yield will increase because of the positive effects of warmer springs and increased CO_2 concentration at northern temperate latitudes where the length of the growing season currently limits production (Donatelli *et al.*, 2002; Qi *et al.*, 2005). Sowing may then occur earlier because of warmer weather conditions (Qi *et al.*, 2005). In Mediterranean-type environments, where high summer temperatures and water stress already limit crop production, simulations with increased temperatures have shown either a negative (Rosenzweig *et al.*, 2002), positive (Bindi & Olesen, 2011) or no impact (Donatelli *et al.*, 2002). Predicted global warming may shorten the generation time of nematodes which may increase the population density and dominance of species better adapted to higher temperatures.

The interaction between the beet cyst nematodes and sugar beet remains relevant as this pest can lead to serious yield losses. Understanding these interactions and the climatic factors influencing them is important in order to adapt management strategies. The work presented here is part of the research framework "KLIFF – climate impact and adaptation research in Lower Saxony", funded by the Ministry for Science and Culture of Lower Saxony. It is one of the first projects in Germany to investigate and assess potential effects of climate change on crop production at a regional scale. The aim of this thesis is to expand current knowledge on the influence of temperature on interactions between sugar beet cyst nematode (*H. schachtii*), yellow beet cyst nematode (*H. betae*) and the sugar beet plants. Experiments were conducted in the laboratory, in climate chambers and outdoors in heated containers. In the following chapters, answers to several research questions will be presented.

Chapter 1 is an introductory chapter, highlighting the importance of sugar beet production. Beet cyst nematodes are discussed as part of major constraints to sugar beet production. The two different beet cyst nematode species, *H. schachtii* and *H. betae*, are introduced, describing their biology and their impact on sugar beet production. Potential influences of rising temperatures on nematode development are described.

In **chapter 2**, hatching experiments were set up to study the influence of temperature on the hatching behaviour of the two beet cyst nematode species, *H. schachtii* and *H. betae*. Differences in hatch between the beet cyst nematode species were assessed at constant temperatures in incubators as well as under simulated temperature conditions set to be 4 °C higher than the standard temperature regime.

In **chapter 3**, the effect of increased soil temperatures on the interaction between sugar beet cultivars and *H. schachtii* was investigated. An electric heating mat system was established outdoors to increase the soil temperature by 2.8 °C in heated compared to unheated soil containers. The effect of experimental soil warming on *H. schachtii* population development and sugar beet performance was assessed for sugar beet cultivars that were susceptible, tolerant or resistant to *H. schachtii*.

In **chapter 4**, it was investigated how the beet cyst nematode species, *H. schachtii* and *H. betae* interact and whether temperature affects the interspecific competition between both species. In two climate chambers differing in temperature by 4 °C, sugar beet seedlings were challenged with *Heterodera schachtii*, *H. betae*, or a mixture of both species. To determine the relative abundance of both species in mixed populations, quantitative PCR systems were developed for both species based on sequence differences in the cytochrome oxidase subunit 1 gene. The aim was to determine if higher temperature regimes will induce shifts in the relative abundance of both beet nematode species.

In **chapter 5**, the results presented in the previous chapters are placed in a broader context. The implications of higher temperature regimes on the future impact of beet cyst nematodes and their management are discussed.

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Chapter 2:

Effect of temperature on the hatch of two German populations of the beet cyst nematodes, *Heterodera schachtii* and *Heterodera betae*

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Abstract

Beet cyst nematodes, *Heterodera schachtii* and *H. betae*, cause damage to sugar beet production and could become even more important with increasing soil temperatures. In northern Germany, temperatures are expected to rise by 4 °C by 2100. In this study, we investigated the hatch of two beet cyst nematode species at constant temperatures as well as simulated temperature conditions set to be 4 °C higher than the standard temperature regime. The effect of different constant temperatures on the emergence of second-stage juveniles of *H. schachtii* and *H. betae* was investigated using six incubators set at 5, 10, 15, 20, 25 and 30 °C for a period of 6 weeks. In a second experiment, the effect of increased and standard temperature regimes on the emergence of second-stage juveniles of *H. schachtii* and *H. betae* was investigated in climate chambers for 12 weeks. The highest cumulative hatching rates for *H. schachtii* were observed at temperatures between 15 and 30 °C and for *H. betae* between 20 and 30 °C, suggesting that this can be considered as the optimal temperature range for hatch. The emergence of juveniles of both beet cyst nematode species started at 5 °C, but cumulative hatch percentages were less than 1%. Differences in final cumulative hatching rate of *H. schachtii* and *H. betae* between the increased and standard temperature regime were not significant.

Keywords: Global warming, juvenile emergence, life cycle, thermal time

1. Introduction

The beet cyst nematodes, *Heterodera schachtii* and *H. betae* are regarded as the most important pests in sugar beet production systems worldwide leading to yield losses of up to 25% (Amiri et al. 2002).

Temperature is a major factor regulating the development of beet cyst nematodes (Griffin 1981a, Trudgill 1995). Predicted rising temperatures through global warming may result in a faster nematode development, shorter life cycle with the potential of more generations per growing season (Curi & Zmoray 1966, Griffin 1981b, Kakaire et al. 2012). Furthermore, an earlier hatch of eggs and emergence of juveniles from the cyst could result in a population build-up at an earlier developmental stage of the sugar beet plants when they are most vulnerable to nematode damage (Griffin 1981a, Olthof 1983, Wrather & Anand 1988). *Heterodera schachtii*, the sugar beet cyst nematode, is considered the most important nematode pests of sugar beet and is present in most sugar-beet growing areas (Cooke 1991). The yellow beet cyst nematode, *H. betae*, is less prevalent but is also reported to cause damage to beet crops (Wouts et al. 2001). Limited information is available on the influence of temperature on the hatching of eggs and emergence of second-stage juveniles of *H. betae* compared with *H. schachtii.* Current knowledge of hatching behaviour of *H. betae* is based on research conducted in 1982 on a special race of *H. trifolii* from the Netherlands (Maas & Heijbroek 1982, Steele et al. 1982), later identified as *H. betae* (Wouts et al. 2001). Previous studies suggested that *H. betae* is more adapted to warmer conditions (Maas & Heijbroek 1982). According to model predictions, temperature in northern Germany is expected to rise by 4 °C by 2100 (Jacob & Podzun 1997, Werner & Gerstengarbe 2007). It is therefore of pivotal interest to understand the role of increasing temperatures on hatching behaviour of these nematode species.

The research objectives of this study were to investigate i) differences in hatch in water of the beet cyst nematode species *H. schachtii* and *H. betae* at constant temperatures, and ii) hatch of both beet cyst nematode species under fluctuating standard- and 4 °C increased temperature regimes.

2. Materials and Methods

2.1. Nematodes

The *H. schachtii* population used in this study was originally isolated from a sugar beet field in Germany and is used in standard resistance tests (Müller & Rumpenhorst 2000). The *H. betae* population was originally isolated from a sugar beet field in Goch, North Rhine-Westphalia, Germany. Pure cultures of these populations were maintained on oilseed rape cultivar Ladoga at the Julius Kühn-Institut, Braunschweig. Newly-formed cysts were extracted from the soil using a MEKU high-pressure elutriator (MEKU, Wennigsen/Deister, DE) with 40 s high pressure/5 s pause settings. Separation of cysts from soil debris on the paper filter and cysts counting were carried out under a stereoscopic binocular (Leica MZ8, Wetzlar, Germany).

2.2. Experimental design

Experiment 1 - Effect of temperature on spontaneous hatch in water

The effect of different constant temperatures on the emergence of second-stage juveniles of *H. schachtii* and *H. betae* was investigated using six incubators (Heraeus BK 5060 EL, Langenselbold, Germany) set at 5, 10, 15, 20, 25 and 30 °C. Temperature within each incubator was recorded every hour with data loggers (Testo T175 T3, Lenzkirch, Germany). Five replicates per treatment were used, with each replicate consisting of a 2 ml Eppendorf tube with five newly-formed cysts in 1 ml tap water. The closed tubes were arranged in boxes and placed into the six corresponding incubators for a total duration of six weeks.

Experiment 2 – Effect of temperature on hatch under stimulation with the hatching agent $ZnCl_2$

The effect of a standard and an increased temperature regime on the emergence of second-stage juveniles of *H. schachtii* and *H. betae* was investigated in climate chambers for 12 weeks. The standard climate regime was set for the first two weeks at 18 °C day/8 °C night and the following 10 weeks at 20 °C day/11 °C night. In the increased temperature regime temperature was set 4 °C higher than in the standard temperature: the first two weeks temperature was set at 22 °C day/12 °C night and the following 10 weeks at 24 °C day/15 °C night. Six replicates per treatment were used, with each replicate consisting of a 2.0 ml Eppendorf tube with five newly-formed cysts in

1.0 ml of a 3 mM $ZnCl_2$-solution. The closed tubes were arranged in boxes and placed at the standard or increase temperature regimes in the corresponding climate chambers.

2.3. Data collection and analysis

Data on the emergence of juveniles was recorded weekly for the duration of the experiments. From each tube, hatched second stage juveniles were removed and counted under a stereo microscope (Leica MZ8; Wetzlar, Germany). The tubes with cysts were immediately replenished with either 1.0 ml tap water (Experiment 1) or 1.0 ml 3 mM $ZnCl_2$-solution (Experiment 2) per tube and returned to their respective incubator. At the end of the experiment, all cysts were crushed to count the number of eggs and juveniles remaining inside and the total number of eggs and juveniles (hatched plus unhatched) per replicate were determined. Data are presented as cumulative hatching rates of viable juveniles at each counting date. Data were checked for normality using the capability procedure test in SAS software Version 9.3 (SAS Institute Inc., Cary, NC, USA). Proc Mixed procedures with repeated measures were used in SAS to check for the effect temperature and nematode species (independent variables) on the number of hatched juveniles (dependent variable). Tukey multiple range tests were performed to separate means at 5% ($P \leq 0.05$) confidence level. Non-transformed means are presented in figures for clarity.

3. Results

Experiment 1 – Effect of temperature on spontaneous hatch in water

Cumulative hatching rates of *H. schachtii* (Fig. 1) and *H. betae* (Fig. 2) populations were recorded over 6 weeks at constant temperatures of 5 °C, 10 °C, 15 °C, 20 °C, 25 °C and 30 °C. At 5 °C, juveniles of both beet cyst nematodes were only detected after week 3 and week 4. Only up to 3 juveniles emerged per replicate from the incubated cysts at temperatures of 5 °C. Cumulative hatching rates ranged from 0 to 0.18% for *H. schachtii* and from 0 to 0.22% for *H. betae*.

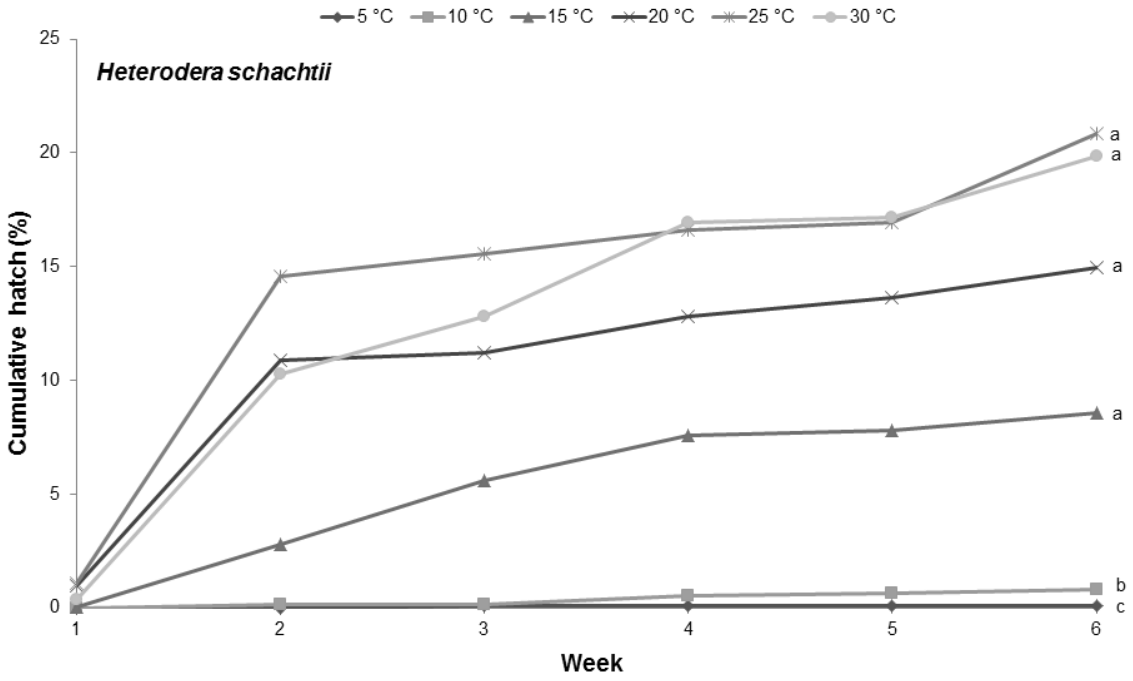

Fig. 1: Cumulative hatch of second-stage juveniles from five cysts of *Heterodera schachtii* in water at different temperatures (°C) over six weeks. Each point on the graph is a mean of five replicates. Data followed by the same letter are not significantly different at $P \leq 0.05$ with Tukey multiple range test.

At 10 °C, no juveniles of both species emerged after one week of incubation. Similarly, low numbers of juveniles of *H. betae* emerged from the cyst with cumulative hatching rates from 0 to 0.2%, which were not significantly different from those at 5 °C (P = 1.00). Emergence of *H. betae* juveniles at 10 °C was not significantly different than at 5 °C, with cumulative hatching ranging from 0.3 to 2%. At 25 °C, final cumulative hatching rates of 20.8% for *H. schachtii* and 9.7% for *H. betae* were detected. For *H. schachtii* there was no significant difference in hatching rate between 15 and 30 °C.

The hatching rate of *H. betae* was significantly higher at 25 °C than at 5 °C (P = 0.006), 10 °C (P = 0.006) and 15 °C (P = 0.026). There was no significant difference in final hatching rates between 20 and 30 °C. *Heterodera betae* hatched more rapidly than *H. schachtii*, and after the first week hatching rates of around 50% of the final cumulative hatching rate were reached at temperatures of 20-25 °C. A high proportion of *H. schachtii* juveniles emerged already in the second week of incubation.

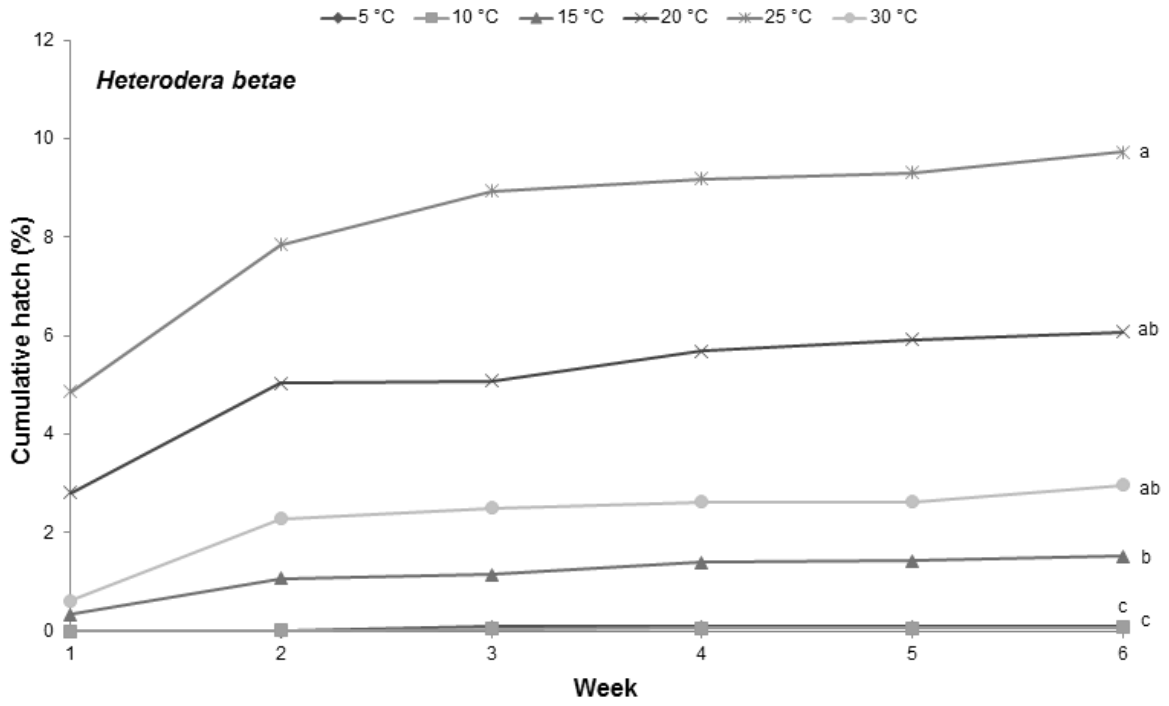

Fig. 2: Cumulative hatch of second-stage juveniles from five cysts of *Heterodera betae* in water at different temperatures (°C) over six weeks. Each point on the graph is a mean of five replicates. Data followed by the same letter are not significantly different at $P \leq 0.05$ with Tukey multiple range test.

Experiment 2 – Effect of temperature on hatch under stimulation with the hatching agent $ZnCl_2$

Percentages of cumulative hatched juveniles of *H. schachtii* and *H. betae* populations over 12 weeks at the standard temperature regime (Ts) and at the increased temperature regime (Ti) are shown in Fig.3. Cumulative hatch was higher for *H. schachtii* than for *H. betae* in both standard ($P = 0.001$) and increased temperature regimes ($P = 0.043$). About 92% of second-stage juveniles of *H. schachtii* emerged from the cysts. No difference of temperature treatment on the total hatch percentage was recorded for *H. schachtii* ($P = 0.99$). During the first two weeks, cumulative hatch percentages below 5% were recorded. After four weeks, 57% (Ts) and 60% (Ti) of *H. schachtii* juveniles emerged. About 77.6% juveniles of *H. betae* emerged at the increased temperature regime, which is more than in the standard temperature regime (69.6%); however, these differences were not statistically different ($P = 0.42$). After the first five weeks, more than

50% of juveniles emerged. The cumulative hatching curves indicate a delayed hatch of around 3 weeks for all treatments, except for *H. betae* at increased temperature regime. After the second week, the cumulative hatching rate of *H. betae* was significantly higher (P = 0.0002) in the increased temperature regime (24%), compared with the standard temperature regime (5.8%).

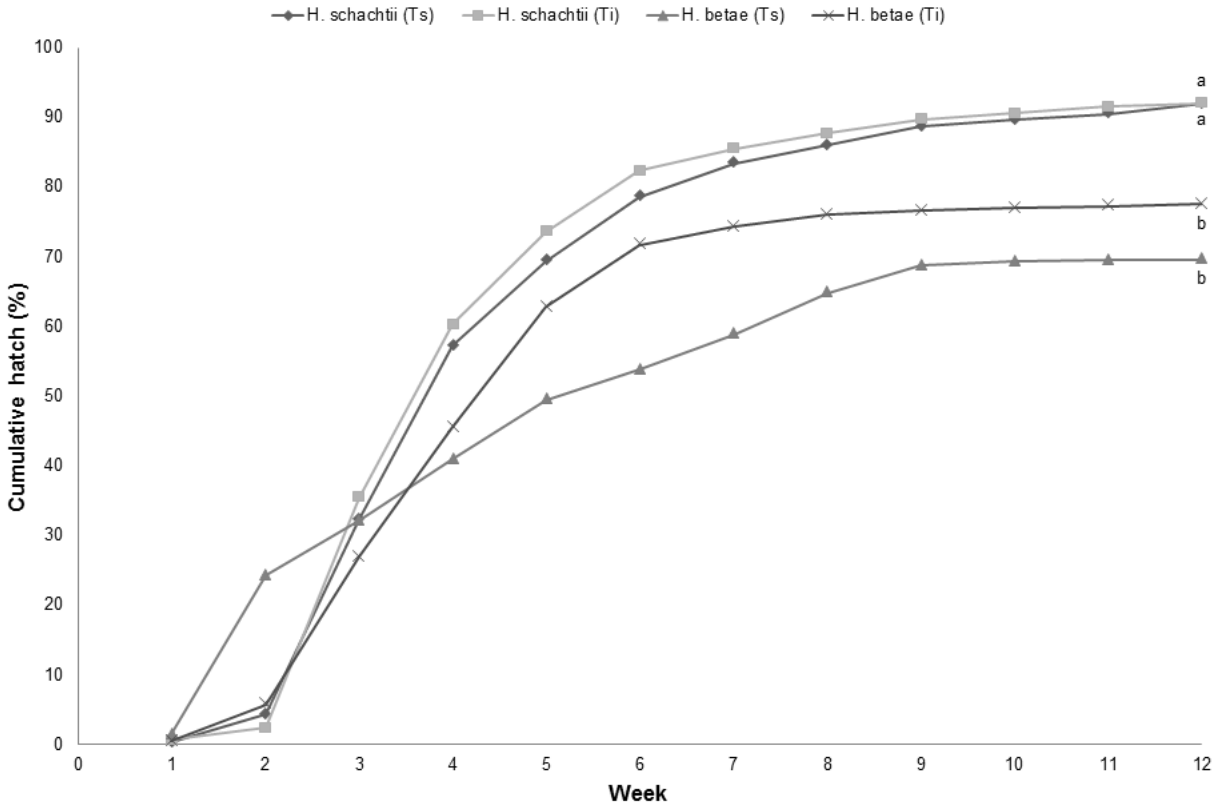

Fig. 3: Cumulative hatch of second-stage juveniles from five cysts of *Heterodera schachtii* and *H. betae* in 1.0 ml 3 mM $ZnCl_2$-solution at a standard and an increased temperature regime over twelve weeks. Each point on the graph is a mean of six replicates. Data followed by the same letter are not significantly different at $P \leq 0.05$ with Tukey multiple range test. Capital letters were used to show differences between weeks inside a treatment. Small letters were used to show differences between different treatments.

4. Discussion

The optimal temperatures for hatch were different for both beet cyst nematode species. The optimum temperature range is the temperature range within which most nematode development (e.g. hatching) occurs. With *H. schachtii* we found that there was a broader temperature range

over which similar cumulative hatching rates occurred in comparison with *H. betae*. The optimal temperature range for hatch of *H. schachtii* was found to be between 15 and 30 °C and for *H. betae* between 20 and 30 °C. Hatch of *H. schachtii* at 15 °C and 20 °C was, although not significantly, lower, than at 25 °C and 30 °C, suggesting that 15 till 20 °C is only a sub-optimal temperature range for hatch of the studied *H. schachtii* population. Previous comparative hatch tests reported 25 °C and 30 °C as optimal temperatures for hatch of both beet cyst nematode species (Maas & Heijbroek 1982).

Emergence of second-stage juveniles of *H. schachtii* began at a base temperature 5 °C; however, in very low percentages. This finding from our experiments concurs with results of hatch tests as reported by Kakaire et al (2012). The base temperature (Tb) is the temperature below which no measurable development occurs. By contrast, other studies investigating *H. schachtii* hatch reported higher base temperatures of 6.3 °C (Griffin 1988), 8 °C (Caswell & Thomason 1991) and 10 °C (Maas & Heijbroek 1982). These variations in Tb values could be due to several factors such as variations in their adaptability to temperature, as well on the methodology and accuracy of assessment (Kakaire et al. 2012). Emergence of second-stage juveniles of *H. betae* also began at 5 °C; however, similar to *H. schachtii* also in very low percentages. These results are in contrast to previous hatch studies where hatch of *H. betae* populations from the Netherlands began only at 15 °C (Maas & Heijbroek 1982, Steele et al. 1982).

Cumulative hatching rates recorded here for *H. schachtii* in water are in accordance with the mean hatch percentage of *H. schachtii* out of 44 tests in distilled water of 13% after 3 weeks at 25 °C (Clarke & Shepherd 1964) and close to hatching rates reported for both species in tap water (Maas & Heijbroek 1982). In contrast, other hatch studies reported that up to 50% of the second-stage juveniles of *H. schachtii* hatch from eggs spontaneously within the first week in water (Clarke & Perry 1977, Zheng & Ferris 1991).

For both beet cyst nematode species, no significant differences were found in the final cumulative hatch percentages when comparing the standard and the increased temperature regimes. Cumulative hatching percentages reached about 90% for *H. schachtii* in both temperature regimes. Final numbers of *H. betae* juvenile emergence were lower than those for *H. schachtii*, which is in accordance with previous studies (Maas & Heijbroek 1982, Steele et al. 1982). Rising soil temperatures may exert a selection pressure among beet cyst nematodes to adapt to changes in temperature (Kakaire et al. 2012). The direct effect of temperature on the development and

population growth of nematodes also explains the importance of thermal adaptation; the abilities to acclimatize, orient towards optimal temperatures and anticipate on the occurrence of extreme low and high temperatures are profoundly selected behaviours (Yeates et al. 2004). For example, the potato cyst nematodes, *Globodera pallida* and *G. rostochiensis* differ in their temperature responses. *Globodera pallida* generally hatches and reproduces at lower temperatures than *G. rostochiensis*, and *G. rostochiensis* is more competitive than *G. pallida* at temperatures above 20 °C (Franco 1979). We found that *H. schachtii* had a broader optimal temperature range and a higher cumulative hatching rate at higher temperatures than *H. betae*. Increasing temperatures are thus likely to cause higher levels of hatching of beet cyst nematodes, potentially resulting in higher infestation levels. Although increasing temperatures could have a positive effect on sugar beet yield due to faster phenological development, this effect can be mitigated due to the earlier incidence of beet cyst nematodes (Racca *et al.*, 2015). Temperature has also been reported to influence the pathogenicity of nematodes so that the damage threshold of *H. schachtii* on sugar beet grown in Germany declined from 20 eggs g^{-1} soil in April to 2.5 eggs g^{-1} soil in May as a result of an increase in soil temperature at planting (Steudel & Thielemann, 1970). A recent study on the spatial distribution of both beet cyst nematodes on sea beet, *Beta vulgaris* spp. *maritima* reported that *H. schachtii* mainly occurred in the colder environments of northern Europe, whereas *H. betae* was preferentially distributed in the warmer environments of southern Europe (Gracianne *et al.*, 2014).

This study provides insight on the influence of temperature on the comparative hatch among beet cyst nematode species from Germany; however, these results cannot be directly extrapolated to determine the hatching behaviour under field conditions. Further studies investigating the effect of temperature on hatching rates, and the life cycle of both beet cyst nematode species during growing season in the field are necessary to better predict the influence of increasing temperatures on beet cyst nematode population dynamics.

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Chapter 3:

Influence of experimental soil warming on population density of *Heterodera schachtii* and the performance of different sugar beet cultivars

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Abstract

Under global warming, crop pests such as nematodes are expected to develop faster and expand their geographical range. Temperature is known as a crucial factor in nematode population dynamics. We hypothesized that rising soil temperature will positively influence population densities of the sugar beet cyst nematode (*Heterodera schachtii*). An electric heating mat system with a semi-automatic temperature control was established to increase the soil temperature by 2.8 °C in heated compared to unheated 96-litre soil containers placed outdoors. Temperature, sugar beet cultivar and the interactions between these two parameters significantly affected the final number of cysts of *H. schachtii* recovered. The resistant cultivar 'Nemata' did not allow nematode reproduction in all treatments. Soil heating resulted in higher fresh weight in the beet cultivar 'Belladonna'. However, sugar beet fresh weights were lower in treatments challenged with *H. schachtii*. Percentages of white sugar content were significantly higher in nematode-free treatments. Based on these data we predict that an increase in soil temperature by 2.8 °C and cultivation of non-resistant cultivars will result in higher *H. schachtii* infestation levels in the soil.

Keywords – cyst nematodes, global warming, soil temperature, resistant cultivars

1. Introduction

Global warming is projected to lead to an increase in mean air temperatures by +2 °C by the year 2050 and up to +4 °C by the year 2100 in northern Germany (Jacob and Podzun 1997; Werner and Gerstengarbe 2007). Several studies have shown that in a warmer climate, pests may become more active than they are currently and may expand their geographical range (Coakley et al. 1999; Garrett et al. 2006; Rosenzweig et al. 2001).

The sugar beet cyst nematode, *Heterodera schachtii*, is considered the most important nematode pest in sugar beet production causing an estimated yield loss of up to 25% (Cooke 1991; Schlang 1991). Temperature is a major factor regulating the developmental rate and the population dynamics of beet cyst nematodes (Kakaire et al. 2012; Trudgill 1995). Elevated temperature levels may affect beet cyst nematodes directly by influencing their developmental rate (Griffin 1988; Trudgill 1995) and indirectly by altering host plant physiology (Chakraborty 2005). The developmental rate of *H. schachtii* has been shown to be linearly related to the temperature (Trudgill 1995). Therefore, predicted rising temperatures through global warming, can result in a faster nematode developmental rate, shorter life cycle durations with the potential that more generations could be completed in a growing season (Kakaire et al. 2012). Beet cyst nematodes mostly produce 2 to 3 generations per year in central Europe (Kakaire et al. 2015). The thermal time relationship (i.e. summation of cumulative differences between daily mean temperature and a specified base temperature expressed in degree-days) has also been used for a model of egg production of *H. schachtii* (Caswell and Thomason, 1991).

Soil temperature in the surface layer is significantly affected by seasonal changes in the air temperature (Jacobs et al. 2011). Despite the importance of soil temperature for the biology of nematodes, experiments focusing on the effect of increasing soil temperatures on nematode development under outdoor conditions have received little attention. Most studies aimed at understanding responses of nematodes to global warming have been conducted in Antarctica (Convey and Wynn-Williams 2002; Simmons et al. 2009; Sinclair 2002) or in other natural ecosystems (Bakonyi et al. 2007; Briones et al. 2009; Kardol et al. 2010), but not in agricultural systems. Recently, a soil warming system based on infrared heaters was used to investigate the response of nematodes to elevated temperature in conventional and no-tillage cropland systems (Dong et al. 2013). Heating cables have been proven to be a stable and reliable method for

studying the effect of elevated soil temperatures on agricultural crops in soil ecosystems under field conditions (Patil et al. 2013; Siebold and von Tiedemann 2012). In this paper, we describe the application of an experimental soil heating system containing heating mats, to study warming effects on beet cyst nematode development on sugar beet. The aim of this study was to assess the effect of increased soil temperatures on the interaction between sugar beet cultivar and *H. schachtii*.

2. Materials and methods

Heating mat system and control unit

The heating system and the control unit method used in this study were adopted from a method used at the Institute for Sugar Beet Research (IfZ, Göttingen, Germany) to study the influence of soil warming on the development of beet necrotic yellow vein virus on sugar beet (Bornemann, pers comm.). The heating system consisted of a heating mat, a temperature controller, temperature sensors and a power supply. The heating mat type HMG (Hillesheim GmbH, Waghäusel, Germany) had a metal carrier and was directly plugged into the HTI 16 (Hillesheim GmbH Waghäusel, Germany) temperature controller. The temperature controller HTI 16 was set at 20 °C. The integral controller measured the average temperature over the entire surface of the heating system directly from the heating wire and registered a temperature change immediately. The heating system operates with 3600 W heating energy. A temperature sensor (PT 100) monitored the temperature in the soil in order to switch on or off the heating until the set maximum temperature of 27 °C of the heating mat was reached. The HTI 16 temperature controllers and two temperature data loggers (Testo 175 T3) were installed on a wooden board with a rain cover to avoid contact with water.

Experimental design

The experiments were located outdoors at the Julius Kühn-Institut, Braunschweig, Germany. The experimental set-up consisted of a two-factorial design with four unheated blocks (ambient temperature regime) and four heated blocks (increased temperature regime). Each block was a 96 l rectangular plastic container filled with a 30 cm thick soil layer and with six 2 l pots with a single beet plant. Each block consisted of 3 sugar beet cultivars and 2 nematode levels (0 and 10

eggs and juveniles of *H. schachtii* per cm^3soil). Sugar beet cultivars 'Alabama' (susceptible to *H. schachtii*; KWS GmbH, Einbeck, Germany), 'Belladonna' (tolerant to *H. schachtii*; KWS GmbH, Einbeck, Germany) and 'Nemata'(resistant to *H. schachtii*; Syngenta Seeds, Kleve, Germany) were seeded. Heating mats were placed on a layer of 20 l steam-sterilised field soil. Above the heating mat, containers were filled with another layer of 70 l steam-sterilised field soil. Soil medium had a pH of 5.3, organic matter 26%. The texture was clay 8.5%, silt 21.3% and sand 70.2%. The mineral content in the soil medium calculated in mg/100g of soil consisted of Potassium (K): 4mg/100g of soil, Phosphorous (P): 13mg/100g of soil and Magnesium (Mg): 3mg/100g of soil. Pots were filled with soil medium mixed with slow-release fertiliser (Osmocote Exact® Standard® 15% N, 9% P_2O_5, 12% K_2O and 2% MgO) at the rate of 2 g/kg. The average temperature difference between unheated and heated containers was set at ±2.8 °C. The total duration of the experiment was 19 weeks.

Nematode culture and determination of initial and final nematode population densities

The *H. schachtii* population used in this study was originally isolated from a sugar beet field in Germany and has been used in standard resistance tests (Müller and Rumpenhorst 2000). Pure cultures of this population were maintained on oilseed rape (*Brassica napus* L.) plants (cultivar Ladoga) in loess soil under greenhouse conditions at the Julius Kühn-Institut, Braunschweig.

Nematode inoculum in loess soil was mixed with the steam-sterilised loam soil, to obtain an initial population density of 10 eggs plus juveniles per cm^3 of soil. Control treatments consisted of steam-sterilised loam mixed with loess soil.

Cysts were extracted at the onset of the experiment and after the experimental run to determine initial and final nematode population densities. Three subsamples of 100 cm^3 soil were used to extract cysts using a MEKU high-pressure elutriator (MEKU, Wennigsen/Deister, Germany) with 40 s high pressure/5 s pause settings. Counting and separation of cysts from soil debris and other organic materials on the paper filter were carried out under a stereoscopic binocular (Leica MZ8). Cysts were crushed with a Janke and Kunkel homogenizer (IKA, Staufenberg, Germany) for 30 s at 1000 rpm in plastic tubes in 1 ml distilled water. The crushed sample was then washed into a beaker and topped up to 20 ml. Nematode suspensions were well mixed before aliquots of 1 ml were taken. Number of eggs and juveniles were counted in 1 ml counting slides under an

inverted microscope (Axiovert25). Reproduction rates were calculated using the formula Rf = Pf/Pi (Where Rf = reproduction factor, Pf = final nematode (eggs and juveniles) number per 100 cm^3 soil from each treatment and Pi = initial nematode (eggs and juveniles) number per 100 cm^3 soil from each treatment

Assessment of sugar beet performance

At harvest, pots were removed from the containers. Beet fresh weight was recorded after they were cleaned with a brush. Beets were cut, blended and the homogenous beet pulp was immediately shock frozen and stored at -20 °C. The beet pulp was clarified with 0.3% (w/v) $Al_2(SO_4)_3$-solution. In the filtrates, sugar content (sucrose) was assessed by polarimetry (ICUMSA 2003). White sugar content was calculated with the new `Braunschweig formula´ (Buchholz et al. 1995).

Data analysis

Data were analysed using SAS software Version 9.3 (SAS Institute Inc., Cary, NC, USA). Prior to analysis of variance (ANOVA), nematode counts were log transformed y = log(x + 1) to standardize variances. Effects of soil heating, sugar beet cultivar and their interaction (independent variables) were analysed with regard to final number of cysts, number of eggs and juveniles, reproduction factor of *H. schachtii*, fresh beet weight, sugar content and white sugar content (dependent variables). The SAS mixed model (Proc mixed) with repeated measures was used to analyse the data. Means were separated using the Tukey HSD tests at 5% confidence level.

3. Results

Performance of heating mat system

Soil temperature recordings (Fig. 1) showed that temperature of the heated containers was always above the temperature in the unheated containers, resulting in a positive temperature increase throughout the experiment. During the whole experimental period, the temperature regime in the heated containers was on average 2.8 °C higher compared to the control (Table 1).

Table 1: Soil temperature (°C) measured at 10 cm depth in the unheated and heated containers over the entire experimental duration.

	Unheated containers	**Heated containers**
Mean soil temperature	18.5 ± 0.2	21.3 ± 0.5
Maximum soil temperature	31.7	32.6
Minimum soil temperature	6.6	14.3

The heated treatment followed the daily natural temperature fluctuations as recorded in the natural outdoor unheated treatment (Fig. 1). The temperature difference between unheated and heated plots was lower when ambient temperatures (e.g. in unheated plots) approached the set maximum temperature of 27 °C, so that extremely high temperatures and overheating was avoided, and maximum temperatures in unheated and heated plots were in the same range. The temperature difference between the heated and unheated containers was higher at lower ambient temperatures especially during the night-time, leading to an increased night-time warming effect.

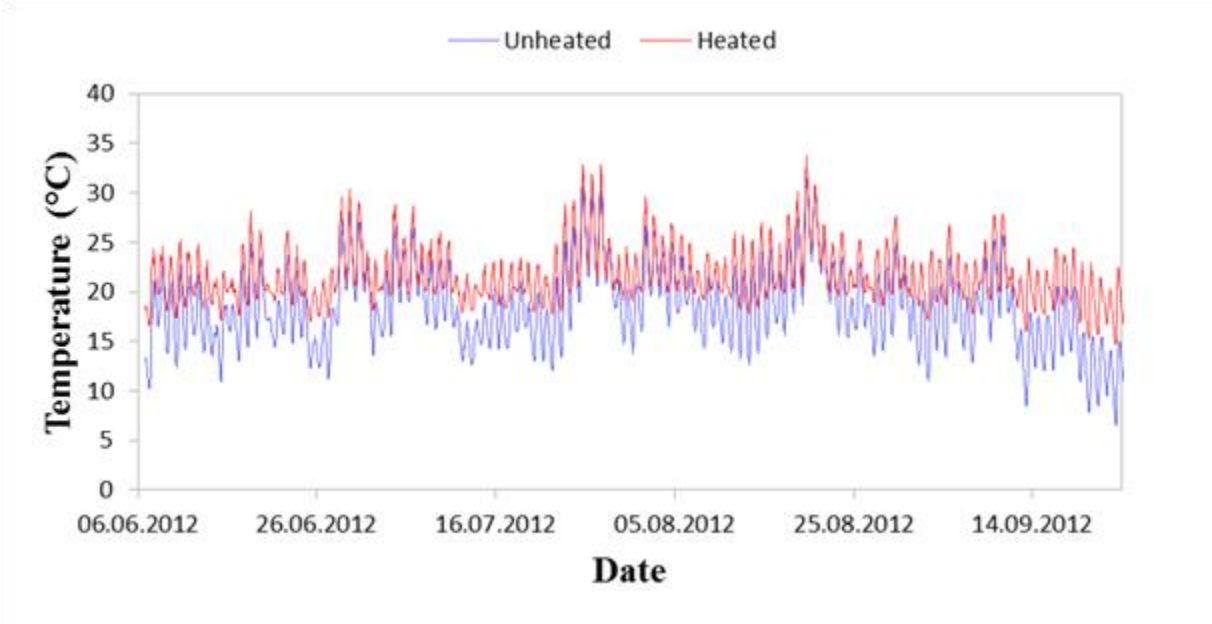

Fig. 1: Soil temperatures recorded in unheated (blue) and heated (red) blocks over the entire experimental duration from 6 June to 24 September 2012.

Effect of soil heating on *H. schachtii* population development on different sugar beet cultivars

Temperature, sugar beet cultivar, and their interaction significantly affected the final number of cysts of *H. schachtii* recovered (Table 2). The final number of eggs and second stage juveniles of *H. schachtii* was significantly affected by sugar beet cultivar (Table 2).

Table 2: *F*-values for effect of temperature (T) and sugar beet cultivar (C) on mean number of cysts and eggs plus second stage juveniles (J2) of *H. schachtii* from 100 cm^3 soil.

	T	C	T x C
Cyst	7.39*	317.45*	6.21*
Eggs + J2	2.16	308.79*	1.82

*P < 0.05

Final number of eggs and second stage juveniles of *H. schachtii* recovered on the resistant cultivar 'Nemata' were lower than on susceptible cultivar 'Alabama' and tolerant cultivar 'Belladonna'(Table 3).

Table 3: Effect of temperature and sugar beet cultivar on the mean number of cysts and eggs plus second stage juveniles (J2) of *H. schachtii* from 100 cm^3 soil. Values presented are means of four replicates (transformed means are in parentheses). Tukey test was used to separate the means at P < 0.05.

	Cysts	Eggs + J2
Temperature		
Unheated	360 (2.2)[b]	8972 (3.4)[a]
Heated	631 (2.4)[a]	13443 (3.6)[a]
Cultivar		
Alabama	748 (2.8)[a]	15275 (4.1)[a]
Belladonna	717 (2.9)[a]	18090 (4.2)[a]
Nemata	22 (1.3)[b]	258 (2.2)[b]

On the susceptible cultivar ‘Alabama’, *H. schachtii* reached a reproduction rate in the heated treatment about twice as high as in the unheated treatment (Fig. 2). The reproduction rate of *H. schachtii*, on the tolerant cultivar ‘Belladonna’ was not different from the susceptible cultivar Alabama. The resistant cultivar ‘Nemata’ did not allow nematode reproduction in both unheated and heated treatments.

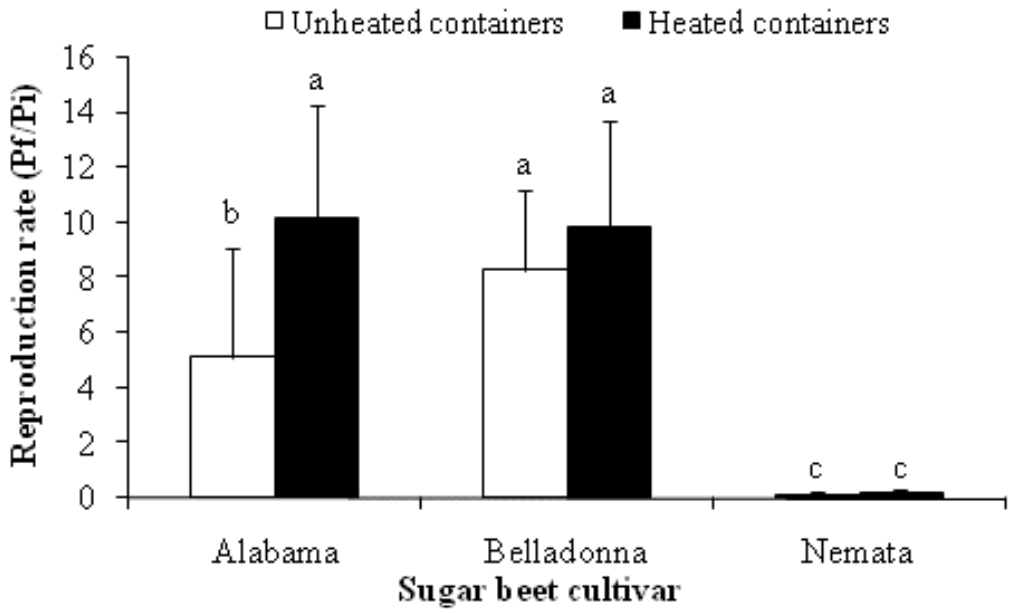

Fig. 2: Mean reproduction rates of *Heterodera schachtii* on sugar beet cultivars ‘Alabama’, ‘Belladonna’ and ‘Nemata’ in unheated and heated treatments after 19 weeks. Data shows mean ± standard deviation. Tukey test was used to separate the means at $P < 0.05$ level. Means separated by different letters were significantly different.

Effect of soil heating, sugar beet cultivar and nematodes on sugar beet performance

Nematodes had a significant effect on sugar beet fresh weight, in both unheated- and heated plots. In nematode treatments, beet fresh weights did not differ between unheated and heated plots. Soil heating effects on sugar beet fresh weight were only significant in nematode-free treatments and were dependent on sugar beet cultivar, with either a neutral, positive or negative effect for the susceptible, tolerant and resistant cultivar, respectively. Sugar beet fresh weights of the cultivars ‘Alabama’ and ‘Belladonna’ were significantly lower in the nematode treatments

than in the nematode-free treatments independent of soil heating (Fig. 3). Nematodes had a significant effect on sugar beet fresh weight of the resistant cultivar ‘Nemata’ in unheated blocks.

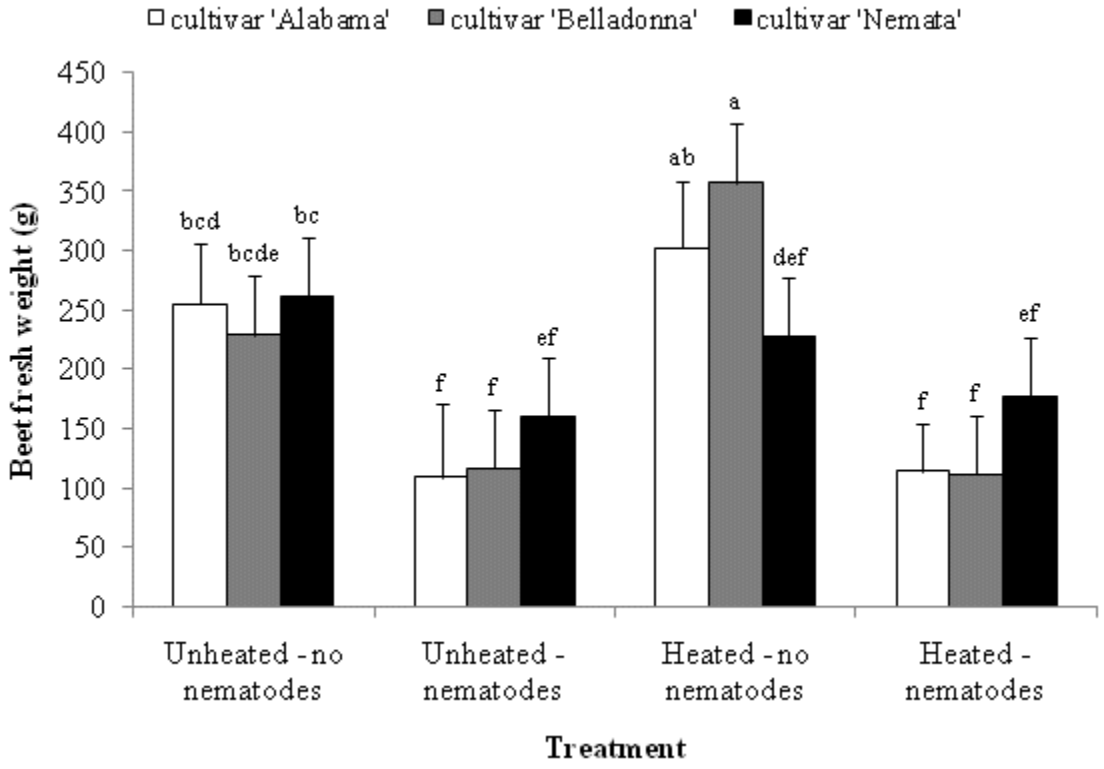

Fig. 3: Sugar beet fresh weights (g) of the sugar beet cultivars ‘Alabama’, ‘Belladonna’ and ‘Nemata’ across different heating- (unheated and heated) and nematode treatments (no nematodes and treated with beet cyst nematodes) after 19 weeks. Data show means ± standard deviation. Tukey test was used to separate the means at $P < 0.05$ level. Means with different letters are significantly different.

Sugar beet cultivar and nematodes had a significant effect on white sugar content, with higher values on resistant cultivar ‘Nemata’ than on the tolerant cultivar ‘Belladonna’ (Table 4).

Table 4: Effect of temperature, sugar beet cultivar and nematodes on mean white sugar content. Tukey test was used to separate the means at $P < 0.05$.

	White sugar content (%)
Temperature	
Unheated	11.052[a]
Heated	14.740[a]
Cultivar	
Alabama	12.862[ab]
Belladonna	12.080[b]
Nemata	13.745[a]
Nematode	
No nematodes	13.952[a]
Nematodes	11.839[b]

Percentages white sugar content were significantly lower in treatments challenged with nematodes (Table 5).

Table 5: F-values for effect of temperature (T), sugar beet cultivar (C) and nematodes (N) on white sugar content.

	T	**C**	**N**	**T x C**	**T x N**	**C x N**	**T x C x N**
White sugar content	3.75	5.59*	26.83*	1.93	1.95	1.77	2.47

*$P < 0.05$

4. Discussion

Performance of the heating mat system

The heating mat system used in our experiment was able to maintain the targeted temperature difference throughout the experiment. The increased temperature regime followed closely the natural temperature fluctuations as observed in the unheated plots, which is considered important for the reliability of soil warming experiments (Aronson and McNulty 2009). Throughout our experiment, an average temperature increase of 2.8 °C was attained concurring with observations made in other experiments using buried heating cables (Patil et al. 2013; Siebold and von Tiedemann 2012). Technically, heating was attained by a series of short heating pulses which were not long enough to allow the formation of extreme temperatures which could have lead to overheating and adverse effects on plant growth. Minimum soil temperatures differed more than maximum daily temperatures between unheated and heated treatments. This effect can be explained by the settings of the heating mat system, which is more effective (e.g. producing more heating pulses) at lower temperatures than at temperatures close to the set turn-off temperature of 27 °C. The temperature difference was higher at lower ambient temperatures especially occurring during the night-time, leading to an increased night-time warming effect. Temperature data over the past five decades also show faster warming during the night than during the day and this asymmetric warming can have effects on plant biology (Peng et al. 2013). Additionally, nematodes could also be affected by higher minimum temperatures, since penetration rate and development of *H. schachtii* larvae is accelerated at soil temperatures above 10 °C (Griffin, 1981). In the heated treatment, this temperature threshold of 10 °C has been reached for the entire period of the experiment.

Although, warming with infrared heaters has proven to be a very reliable and realistic warming method (Aronson and McNulty 2009; Kimball et al. 2008), it often fails to establish a substantial rise in soil temperature, especially at lower depths (Aronson and McNulty 2009; Dong et al. 2013; Harte et al. 1996). In a recent study, responses of soil nematodes to soil warming by infrared radiators was investigated, but only an increase in daily soil temperature of 0.62 °C to 1.20 °C at 5 cm depth was reached (Dong et al. 2013). The results of our study showed that soil warming with a heating mat system is useful to study climate warming effects on the nematode population density and crop performance under outdoor conditions. It should be considered that

heating mats unevenly distribute temperature through the soil profile as temperature is higher closer to the heating mat and effects on nematodes close to the heating mats cannot be excluded. However, temperature was set at a maximum of 27 °C in our experiment to avoid overheating and to account for this potential effect.

Effect of soil heating on *H. schachtii* population density on different sugar beet cultivars

Previous studies under laboratory or greenhouse conditions on the effect of temperature on the population density of *H. schachtii* indicated a clear temperature effect, i.e. the higher the soil temperature the greater was the nematode reproduction rate (Griffin 1981; 1988; Santo and Bolander 1979). In our study, soil heating lead to a significant increase in final number of recovered cysts on tolerant cultivar 'Belladonna' and susceptible cultivar 'Alabama'. The resistant cultivar 'Nemata' did not allow nematode reproduction in both unheated- and heated treatments. The reproduction rates of *H. schachtii* on the tolerant cultivar 'Belladonna' were high and were not statistically different from the rate on the susceptible cultivar 'Alabama' under heated conditions. However, under unheated conditions, the reproduction rate on the tolerant cultivar 'Belladonna' was significantly higher than for 'Alabama'. These results were unexpected as most studies report lower Pf/Pi values on tolerant than on susceptible varieties (Daub and Westphal 2012; Niere 2009). Tolerant varieties are occasionally regarded as partly resistant due to their genetic background originating from *Beta maritima*, but they are unable to reduce the population density of *H. schachtii* (Daub and Westphal 2012; Niere 2009). The tested resistant sugar beet cultivar remained effective in reducing *H. schachtii* at higher temperatures. In the case of high nematode population densities and increasing soil temperatures, planting of nematode-resistant cultivars aiming at reducing nematode populations might become more important in the future.

Effect of soil heating, sugar beet cultivar and nematodes on sugar beet performance

Nematodes had a significant negative effect on sugar beet fresh weight. This effect differed by sugar beet cultivar but not by soil heating. Sugar beet fresh weights of the susceptible and tolerant were significantly lower in the nematode treatments than in the nematode-free treatments independent of soil heating. In our study, soil heating had overall no significant effect on sugar beet performance. However, the effect of soil heating was dependent on the sugar beet cultivar

with either a neutral, positive or negative effect for the susceptible, tolerant and resistant cultivar, respectively

Sugar beet cultivar and nematodes had a significant effect on white sugar content, with higher values on resistant cultivar than on the tolerant cultivar. Percentages of white sugar content were significantly lower in treatments challenged with nematodes.

Accordingly, in previous studies, the main factors determining sugar yield were identified as nematode infestation and sugar beet cultivar (Hauer *et al.*, 2016; Hauer *et al.*, 2015). Sugar content of beets was shown to be negatively affected by root damage by beet cyst nematodes (Kenter et al. 2014). In contrast, Cooke and Thomason (1979) reported no effect of *H. schachtii* on sugar content. These contrasting effects could be related to the cultivars used, as there has been a significant breeding progress in sugar beets that might account for changes in plant responses (Loel et al., 2014). Susceptible cultivars evidenced a stronger decrease in sugar content than resistant cultivars, coinciding with an increase in *H. schachtii* population densities (Kenter et al. 2014). In the nematode treatments, white sugar content was higher for the resistant cultivar.

In conclusion, the results show that a heating mat system allows studying the effects of soil warming attributed to climate change on nematode density and crop performance under outdoor conditions. However, this approach also comes with some limitations. Indeed, the temperature difference was more than the envisaged 3 °C difference at low ambient temperatures and less than 3 °C at high ambient temperatures. The temperature is higher closer to the heating mat, which could influence nematode development. The combination of belowground soil warming and aboveground heaters (Thakur et al. 2014) should therefore be explored for studying effects of soil warming on nematode development and crop performance. The results of the present study indicate that future global warming with temperature increases of up to ±3 °C and cultivation of non-resistant cultivars can result in higher *H. schachtii* populations in soil. Beet cyst nematodes negatively influenced sugar beet performance and effects of soil heating on sugar quality were dependent on sugar beet cultivar. Cultivar choice is an important element as the outcome of plant-nematode interactions varies greatly depending on the cultivar (Heijbroek et al., 2002; Kakaire et al., 2015). As in the last decades, there was a tremendous progress in breeding success, also breeding for resistance and tolerance to sugar beet pests, including beet

cyst nematodes, will increase in significance (Heijbroek et al., 2002; Loel et al., 2014). Although positive effects on yield could also be expected due to the advance of phenological stages of sugar beet with higher temperatures in spring, this effect can be mitigated due to the earlier incidence of sugar beet pests, including beet cyst nematodes (Racca et al., 2015). As the multiplication rate is temperature dependent, it is expected that more generations can be completed in a growing season under increased temperature regimes. In a recent study on the number of generations it was confirmed that temperature was crucial in determining the number of generations of *H. schachtii* in a growing season on oil seed rape, but also there were clear differences depending on the cultivar used (Kakaire et al., 2015). Temperature has also been reported to influence the pathogenicity of nematodes, so that the damage threshold initial population density (Pi) of *H. schachtii* on sugar beet grown in Germany declined from 20 eggs (g soil)$^{-1}$ in April to 2.5 eggs (g soil)$^{-1}$ in May as a result of an increase in soil temperature at planting (Steudel and Thielemann, 1970; Steudel and Thielemann, 1970). Further research using different sugar beet cultivars and different nematode species are needed to better understand the influence of soil warming on nematode development and crop performance.

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Chapter 4:

Influence of temperature on the interspecific competition between the beet cyst nematode species *Heterodera schachtii* and *Heterodera betae* on sugar beet

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Abstract

Beet cyst nematodes are one of the most important pests of sugar beet. The two beet cyst nematode species, *Heterodera schachtii* and *Heterodera betae*, can occur in mixed populations but information about how the species behave in co-occurrence is very limited. Temperature is an important factor shaping nematode development and interactions between related species. Predicted global warming may shorten the generation time of nematodes which may increase the population density and dominance of species better adapted to higher temperatures. We studied the effect of increased temperature on the interspecific competition between *H. schachtii* and *H. betae*. In two climate chambers differing in temperature by 4 °C, sugar beets were grown in loess infested with of 10 nematodes per ml of soil of either *H. schachtii*, or *H. betae*, or 50% mixtures of both. To determine the relative abundance of both species in mixed populations, a quantitative PCR system was developed for both species based on sequence differences in the cytochrome oxidase subunit 1 gene. Both beet cyst nematode species performed better at higher temperature. A higher number of cysts and juveniles of *H. schachtii* than from *H. betae* were recovered in pots with a single inoculated species. A significant difference in the competitive ability of both species under both temperature regimes could not be observed. However, the trend that *H. schachtii* multiplied slightly better than *H. betae* in competition was alleviated at higher temperature. In conclusion, the damage done by cyst nematodes in sugar beet is expected to increase with global warming. *H. schachtii* and *H. betae* are likely to continue to cause damages. But neither of the two species will profit more from rising temperatures compared to the other.

Keywords*:* global warming, soil temperature, real-time PCR

1. Introduction

Temperature is often the most important environmental factor affecting nematode biology (Trudgill, 1995). Different species have different optimum temperatures for feeding, hatching, reproduction and survival (Neilson & Boag, 1996). Predicted climate change may shorten the generation time of nematodes which may increase the population density and dominance of species better adapted to higher temperatures (Kakaire *et al.*, 2012; Kaczmarek *et al.*, 2014). For example, the two species of potato cyst nematodes, *Globodera pallida* and *Globodera rostochiensis,* differ in their temperature responses (Franco, 1979; Kaczmarek *et al.*, 2014). Low soil temperatures are likely to favour *G. pallida* whereas warmer temperatures are likely to favour *G. rostochiensis,* which hatches more quickly (Franco, 1979). This has implications for interspecific competition between the two cyst nematode species at different temperatures, when they occur as mixed populations in the field and lead to different host responses and final nematode populations (Kaczmarek *et al.*, 2014). In the case of two closely related species as *Heterodera schachtii* and *Heterodera betae,* interactions are expected to be mutually antagonistic as they compete for the available feeding sites (Den Nijs & Lock, 1990). *Heterodera schachtii* is considered as one of the most important nematode pests on sugar beet and is present in most sugar beet growing areas (Cooke, 1991). The yellow beet cyst nematode, *H. betae*, is less prevalent but has also been found damaging beet crops (Maas & Heijbroek, 1982). The host ranges of *H. schachtii* and *H. betae* were shown to be very similar, and both species have been shown to co-occur in mixed populations in several locations in France, Spain and Belgium (Gracianne *et al.*, 2014). In that study, it was hypothesised that *H. betae* derives from *H. schachtii* as a result of speciation by polyploidy and that *H. betae* is better adapted to warmer habitats (Gracianne *et al.*, 2014). However, knowledge on how both species behave in co-occurrence is very limited.

H. schachtii and *H. betae* have similar morphology but have minor differences in size (Amiri *et al.*, 2003). The traditional identification using morphological characteristics is time-consuming and requires specialized taxonomic skill, especially in the case of species mixtures (Fleming *et al.*, 1998). Alternatively, quantitative PCR can enable rapid detection and quantification of target species (Powers, 2004). Molecular quantification techniques have significantly evolved during

the last decade and can also be applied to explore ecological interactions (Torr *et al.*, 2007; Campos Herrera *et al.*, 2015). Despite the high potential of current PCR-based methods, they are until now only rarely used in nematode ecology. The combination of traditional and molecular detection methods was able to reveal intense competition of entomopathogenic nematodes with other free-living nematodes (Campos Herrera *et al.*, 2015) and study habitat associations between different entomopathogenic nematodes (Torr *et al.*, 2007).

In this study, we investigated how populations of both species develop in co-occurrence, and whether temperature affects the interspecific competition between the beet cyst nematode species. The objectives of this study were: 1) to determine the effect of temperature on the population dynamics of *H. schachtii* and *H. betae* in interspecific competition and 2) to design a real-time based PCR method for the detection of the relative proportions of *H. schachtii* and *H. betae.*

2. Materials and methods

Plant cultivation

The cultivar 'Alabama' was selected for this experiment due to its susceptibility to *H. schachtii* (KWS, Einbeck, Germany). Three sugar beet seeds of this variety were planted per pot in 500 ml volume plastic pots in pure underground loess (Müller & Rumpenhorst, 2000). Slow release fertiliser (Osmocote Exact® Standard® 15% N, 9% P2O5, 12% K2O and 2% MgO) was added to the soil at 2 g / kg of loess. Seedlings were allowed to germinate and grow at a 14/10 hours light/dark regime for three weeks till they reached the four-leaf stadium (BBCH 14) and were then thinned out to one plant per pot.

Nematodes

The *H. schachtii* population used in this study was originally isolated from a sugar beet field in Germany and is used in standard resistance tests (Müller & Rumpenhorst, 2000). The *H. betae* population was isolated from a sugar beet field in Elsdorf, North Rhine-Westphalia, Germany. Pure cultures of these populations were maintained on oilseed rape plants (cultivar Ladoga) at the Julius Kühn-Institut, Braunschweig (Germany).

Cysts out of the pure cultures were extracted using a MEKU high-pressure elutriator (MEKU, Wennigsen/Deister, Germany) with 40 s high pressure/5 s pause settings. Counting and separation of cysts from soil debris and other organic materials were carried out under a stereoscopic binocular (Leica MZ8). Cysts were placed on Bearmann funnels in a 3 mM $ZnCl_2$-solution to stimulate the emergence of second-stage juveniles (Müller & Rumpenhorst, 2000). The nematode suspension was tapped off every two days. Nematode stock was stored at approximately 4 °C until further use. To estimate the population density, the stock solutions were stirred and total nematode numbers determined using a 1 ml sub-sample from the stock, counted at 40X magnification using an Axiovert 25 (Carl Zeiss®) inverted microscope. The counting was replicated three times and the mean count calculated. The initial population density (P*i*) was adjusted to 10 nematodes/ml. Nematodes were applied one day after thinning out until one plant per pot.

Experimental design

Experiments were conducted in two climate chambers. In the first climate chamber, a standard climate regime was set to 18 °C day/8 °C night and in the second climate chamber the regime was set to 22 °C day/12 °C night. For both temperature regimes, the treatments were the following: control treatment (no nematodes), inoculation with *H. schachtii*, inoculation with *H. betae*, inoculation with both *H. schachtii* (50%) and *H. betae* (50%). Ten replicates per treatment were used. Initial population density of the nematode treatments was 10 second stage juveniles per ml soil. Control pots received the same amount of water. Pots in each climate chamber were placed in a complete randomized design. Two Testo T175A data loggers were installed with sensors at 5 cm depth inside the soil. The total duration of the experiment from inoculation till termination was 15 weeks.

Data collection

Experiments were terminated by turning the pots upside down through a sieve to a soil collection container after chopping off the beet leaves. Three subsamples of 100 ml were used to extract cysts using a MEKU high-pressure elutriator (MEKU, Wennigsen/Deister, Germany) with 40 s high pressure/5 s pause settings. Cysts were extracted as described above to determine final

nematode population densities. Cysts were crushed with a Janke and Kunkel homogenizer (IKA, Staufenberg, Germany) for 30 s at 1000 rpm in plastic tubes in 1 ml distilled water. The crushed sample was then washed into a beaker and topped till a concentration of around 400-500 juveniles per ml was attained, which was continuously agitated before pipetting off 1ml of the suspension into nematode counting slide and counting the number of eggs and juveniles in 1 ml counting slides (Shepherd, 1986).

Morphological quantification of nematodes

Nematode numbers (eggs and second stage juveniles) from 100 ml of soil were determined under an inverted microscope (Axiovert25 CarlZeiss®) at 40X magnification using a nematode counting slide chamber of 1 ml capacity.

Primer and probe sets: design and specificity of primers

H. schachtii and *H. betae* species-specific primers and probes were designed for the cytochrome oxidase subunit 1 (COI) gene (Table 1). For sequence determination COI was amplified by adding 1 µl DNA of the selected species to the PCR reaction mixture containing x 0,25 µl of TrueStart HotStart Taq DNA Polymerase (Thermo Scientific, Wilmington, DE, USA) 1 µM of forward primer JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) (Bowles et al. 1992), 1 µM of the reverse primer JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′) (Derycke et al. 2005), and ddH2O up to a final volume of 50 µl. The PCR program settings were as follows: initial denaturation step at 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 41 °C for 30 s and 72 °C for 45 s; and an additional amplification step at 72 °C for 8 min.

DNA extraction

DNA was extracted out of 1ml nematode suspension (with nematode numbers estimated before by microscopic counting as described above) using manufacturer's protocol for extraction out of animal tissue of the QIAMP DNA Mini Kit (Qiagen). The DNA was resuspended in 200 µL of elution buffer and stored at -20 °C until use. All DNA samples were analysed using 1 µL per duplicate in a Nanodrop ND–1000 v3.3.0 (Thermo Scientific) to estimate the quality and quantity.

Cloning and sequencing

PCR products from the COI were cloned using the vector pGEM®-T Easy and *Escherichia coli* JM109 high-efficiency competent cells (Promega, Madison, WI, USA) which were used for transformation of the ligation product. The resultant plasmid DNA obtained from the *Escherichia coli* culture was purified following the PureYield™ Plasmid Miniprep System (Promega GmbH, Mannheim, Germany) guidelines. Samples were sequenced on both strands byMacrogen Europe (Amsterdam, The Netherlands). Plasmid DNA concentrations were determined by measuring the optical density using a spectrophotometer (Nanodrop ND–1000 v3.3.0, Thermo Scientific).

Molecular quantification: TaqMan assay

The oligonucleotide sequences designed for use with *H. schachtii* from forward primer, reverse primer and TaqMan probe respectively are presented in Table 1. Gel electrophoresis for PCR products of both primer sets showed amplification of a product of the desired length. qPCR was performed using the CFX96TM Real-Time System (BioRad, Munich, Germany).

Real-time PCR reaction mixture contained 0.25 µl of TrueStart HotStart Taq DNA Polymerase (Thermo Scientific, Wilmington, DE, USA), 5 µl Truestart buffer, 5 µl 2 mM dNTP, 5 µl 25 mM MgCl2, 1.5 µl 10 µM forward primer, 1.5 µl of the 10 µM reverse primer, 1.5 µl of the 10 µM hydrolysis (Taqman®) probe and ddH2O up to a final volume of 50 µl. The PCR program settings were as follows: initial denaturation step at 95 °C for 5 min; 40 cycles of 94 °C for 15 s, 60 °C for 1 min with detection of released FAM. Pearson correlation analysis showed a significant correlation between microscopic counts and qPCR estimates of number of beet cyst nematodes in samples (R-Square = 0.615, P = 0.003). Linear regression analysis revealed a positive relationship between microscopic counts and number of genes in 1ml samples of y= 1.369x+2.133 for *H. schachtii* and y= 1.770x+0.133 for *H. betae* (with x is the log10 of microscopically counted nematodes per ml and y is the log10 of the number of genes per ml).

Table 1: Specific primers and TaqMan probes for detecting *Heterodera schachtii* and *H. betae.*

Species	Name	Primer and probe (5´-3´)
Heterodera schachtii	HsCOI175f	F: GCCTATTTTAGAGCAGCTACT
	HsCOI255r	R: ACCATAAATCCTCATTAGTCAA
	HsCOI203tp	P: FAM-TTGCTATTCCAACTGGTATTAAGGTTT-3'-TAMRA
Heterodera betae	HbCOI110f	F: GTTTTATTGGCTGTTTGGT
	HbCOI194r	R: GTTGCTGCTCTAAAATAAGC
	HbCOI138tp	P: FAM-CCATATATTTGTGGTTGGAATAGATATGG-3'-TAMRA

Code: F forward primer, R reverse primer, P TaqMan® Probe

Data analysis

Data was analysed using SAS software Version 9.3 (SAS Institute Inc., Cary, NC, USA). Prior to analysis of variance, nematode counts were log transformed y=log(x + 1) to standardize variance. Effects of temperature, nematode and their interaction on final number of cysts and number of juveniles were evaluated. The mixed model (MIXED) procedure was used to analyse the data. Tukey studentized range test was used to separate means at 5% confidence level. Data on relative abundance was analysed with Kruskal-Wallis test and multiple comparisons with Dunn procedure. Significance level was corrected with Bonferroni procedure.

3. Results

Effect of temperature and species on final number of cysts and second stage juveniles

The effect of a 4 °C temperature increase on the population development of *H. schachtii* and *H. betae* was tested. The effect of temperature and nematode initial population densities on the final number of cysts and second stage juveniles (J2) of the respective beet cyst nematodes and interaction effects are shown in Table 2. Temperature had a significant effect on the final number of cysts ($F = 638.48$; $P < 0.0001$) and second stage juveniles ($F = 798.66$; $P < 0.001$). In the increased temperature regime (Ti), a significantly higher final amount of cysts and J2 were recovered than in the standard temperature regime (Ts) for both beet cyst nematode species. Beet cyst nematode species had a significant effect on the final number of cysts ($F = 15.13$; $P < 0.0001$) and juveniles ($F = 7$; $P < 0.001$).

Table 2: Effect of temperature, nematode treatment and their interaction on the final number of cysts and eggs plus second stage juveniles (J2) of *H. schachtii* from 100 ml soil.

	Cysts	Eggs + J2
Temperature		
Standard temperature regime (Ts)	87[b]	10761[b]
Increased temperature regime (Ti)	672[a]	84498[a]
F-value	638.48	798.66
P -value	< 0.0001	< 0.001
Nematode treatment		
H. schachtii	476[a]	60212[a]
H. betae	330[b]	39524[b]
H. schachtii + *H. betae*	331[b]	43153[b]
F-value	15.13	7,00
P-value	< 0.0001	< 0.002
Temperature x Nematode		
F-value	1.33	1.22
P-value	0.274	0.303

Statistical analysis was performed on log transformed data (y= log(x+1)).Values presented are means of ten replicates. Tukey studentized range test was used to separate the means at P<0.05 level. Means separated by different letters were significantly different.

Final number of nematodes recorded for *H. schachtii* was higher than for *H. betae* alone and in the treatment with both *H. schachtii* and *H. betae*, in the increased temperature regime (Fig. 1).

At the standard temperature regime, there was no difference in total nematode numbers between different nematode treatments.

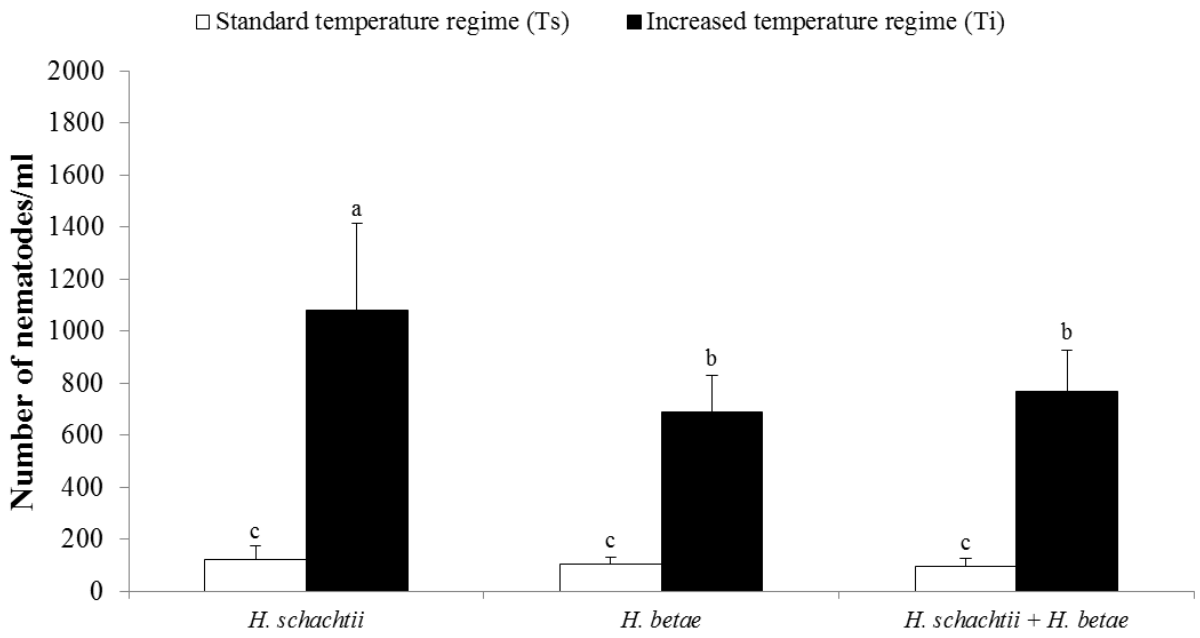

Fig. 1. Final nematode population density across the different temperature and nematode treatments as estimated by counting. Data shows means + standard deviation. Tukey studentized Range Test was used to separate the means at $P < 0.05$ level. Means separated by different letters are significantly different from each other.

Influence of temperature on relative proportions of *H. schachtii* and *H. betae*

Final percentages of *H. schachtii* signal in mixed treatments with an initial inoculation of 50% *Heterodera schachtii* and 50% *H. betae* were not significantly different between the temperature regimes (Fig. 2). In the standard temperature regime, the relative proportions were 59% *H. schachtii* and 41% *H. betae*. In the increased temperature regime, the proportions were 53% *H. schachtii* and 47% *H. betae*. Although these differences in relative proportions were not significant, the data suggest a slightly better performance of *H. schachtii* relative to *H. betae*.

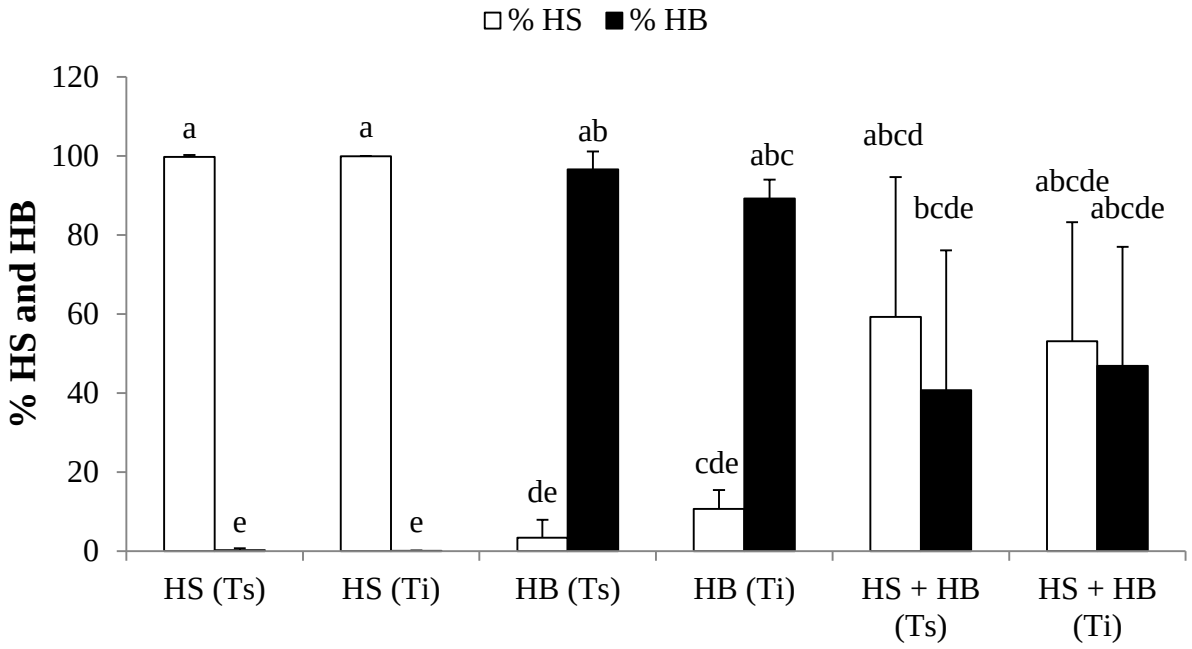

Fig. 2. Final percentual proportion of *H. schachtii* signal in treatments with *H. schachtii* (HS), *H. betae* (HB) and with 50% *H. schachtii* and 50% *H. betae* (HS + HB) in standard temperature regime (Ts) and increased temperature regime (Ti). Data shows mean + standard deviation. Data was analysed with Kruskal-Wallis test and multiple comparisons with Dunn procedure. Significance level was corrected with Bonferroni procedure. Means separated by different letters are significantly different from each other.

In these mixed treatments, the data range was large, meaning that among replicates there are samples containing more *H. schachtii* and others containing more *H. betae* (Fig 3.). No clear effect of competition between both beet cyst nematode species in species mixtures under elevated temperature could be detected. In case of single-species inoculations, it is expected that both species perform better under elevated temperatures. More specifically, *H. schachtii* performed better than *H. betae* under increased temperatures.

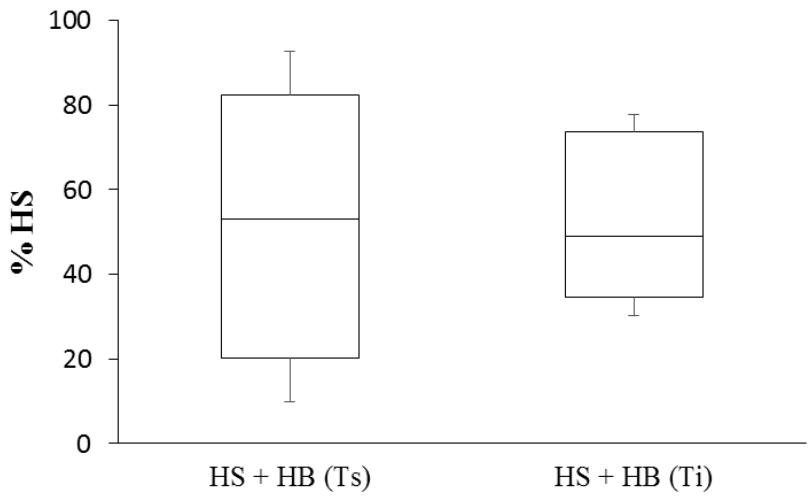

Fig. 3. Box plot of final percentages of *H. schachtii* signal raw data in treatments with 50% *H. schachtii* and 50% *H. betae* (HS + HB) in standard temperature regime (Ts) and increased temperature regime (Ti). The minimum and maximum percentages are depicted by the whiskers, the box signifies the upper and lower quartiles, and the median is represented by a short black line within the box.

Validation of a qPCR system for quantification of the beet cyst nematode species, *H. schachtii* and *H. betae*

A specific set of primers and probes (Table 1) was developed for both H. *schachtii* and *H. betae*. Pearson correlation analysis showed a significant correlation between microscopic counts and qPCR estimates of number of beet cyst nematodes in samples (R-Square = 0.615, P = 0.003). Linear regression analysis revealed a positive relationship between microscopic counts and number of genes in 1 ml samples of y= 1.369x+2.133 for *H. schachtii* and y= 1.770x+0.133 for *H. betae* (with x is the log10 of microscopically counted nematodes per ml and y is the log10 of the number of genes per ml). The qPCR assay was to a high degree species-specific in terms of the *H. schachtii* and *H. betae* populations used in this study. *H. schachtii* treatments, qPCR analysis resulted in 99.8% and 99.9% *H. schachtii* in standard and increased temperature regimes, respectively (Fig. 2). In the treatments containing *H. betae* also some signal for *H. betae* was detected (Fig. 2) In the *H. betae* treatments qPCR analysis resulted in 96,6% and 89,3% *H. betae* in standard and increased temperature regimes, respectively.

4. Discussion

In our study, increased temperature led to a significant increase in cyst nematode population numbers. Eight times more cysts, as well as second stage juveniles, were retrieved from pots cultivated at an increase in temperature of 4 °C. The final nematode populations were different depending on the beet cyst nematode species present. *H. schachtii* showed the highest population density which was significantly higher than that of *H. betae*. Differences in population development at the increased temperature regime were much higher than at the standard temperature regime for all nematode treatments. Previous studies under laboratory or greenhouse conditions on the effect of temperature on the population dynamics of *H. schachtii* indicated a clear direct temperature effect, i.e. the higher the soil temperature the greater was the nematode reproduction and pathogenicity (Santo & Bolander, 1979; Genet, 1981; Griffin, 1981, 1988).

In this study, the effect of the inoculation of the beet cyst nematode species, *Heterodera schachtii* and *Heterodera betae*, on their development was tested. The interactions between both beet cyst nematode species were expected to be mutually antagonistic as they compete for the available feeding sites (Den Nijs & Lock, 1990). We found a higher number of cysts and juveniles of *H. schachtii* alone at the end of the experiment than from *H. betae* alone or both beet cyst nematode species mixed. Temperature has also been shown to influence the interspecific competition between cyst nematodes (Foot, 1978; Kaczmarek *et al.*, 2014). Several competition studies report that low population densities of one nematode species were associated with the largest population densities of the other species (Umesh *et al.*, 1994; Herve *et al.*, 2005; Avelino *et al.*, 2009). Soil temperature at the time of primary infection has been shown to be important in determining the dominant species in interspecific competition (Lawn, 1990). At cooler starting temperature *Heterodera glycines* was dominating *Pratylenchus scribneri*, whereas at 32 °C *H. glycines* reproduction was suppressed by *P. scribneri* (Lawn, 1990). In our study, both beet cyst nematode species performed better at higher temperatures, but there was no significant difference in the relative abundance of both species under both temperature regimes. *H. betae* reproduces via parthenogenetic mitosis and it could be expected that populations of *H. schachtii* which reproduces amphimictically should initially develop slower than those of *H. betae* because

of the presumed necessity for copulation prior to reproduction (Acosta, 1980; Maas & Heijbroek, 1982). In our study, *H. schachtii* showed the highest population density which was significantly higher than that of *H. betae*. Hatching studies on the tested beet cyst populations have shown that *H. schachtii* hatched within a broader temperature range and had a higher hatching rate than *H. betae* (Vandenbossche *et al*., 2015). However, the difference in population development at the increased temperature regime was much higher than at the standard temperature regime for all nematode treatments.

In this study, we describe a qPCR assay for detection and quantification of the two beet cyst nematode species, *H. schachtii* and *H. betae*, based on hydrolysis (TaqMan®) probes targeting the COI region. Currently, there is a range of qPCR methods that can be applied to differentiate between genera and species of plant parasitic nematodes (Berry *et al.*, 2008; Nowaczyk *et al.*, 2008; Toyota *et al.*, 2008; Toumi *et al.*, 2013). The traditional identification of *Heterodera* species using morphological characteristics is time-consuming and requires specialized skill, especially in the case of species mixtures. A molecular detection system for beet cyst nematodes based on the ITS-region was developed for use in a simple PCR-reaction (Amiri *et al.*, 2002) and qPCR (Madani *et al.*, 2005). However, prior tests with the qPCR primer system for *H. schachtii* resulted in a non-specific reaction (data not shown). The mitochondrial cytochrome oxidase subunit 1 (COI) gene was successfully used to discriminate between many species of free-living marine nematodes (Derycke *et al.*, 2010) and cereal cyst nematodes (Toumi *et al.*, 2013). A major problem that must be overcome with any qPCR assay is the design of primers and probe sets to ensure that no cross-reactions occur. We could verify that our assay was to a high degree species-specific in terms of the *H. schachtii* and *H. betae* populations used in this study. Although the qPCR analysis of the samples containing either *H. schachtii* or *H. betae* confirmed the relative proportions, we detected small percentages of *H. schachtii* signal in some *H. betae* samples, respectively. Possibly, a slight non-specific reaction of *H. betae* samples with *H. schachtii* primers might have occurred.

In conclusion, we found in our study that both beet cyst nematode species performed better at higher temperatures, but there was no significant difference in the relative abundance of both species under both temperature regimes. The final nematode populations were also different depending on the beet cyst nematode species present and were highest when only *H. schachtii*

was present. Further studies investigating the effect of temperature on the development of both beet cyst nematodes are necessary to better understand the interactions between both cyst nematode species and to better predict the influence of increasing temperatures on beet cyst nematode population dynamics.

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Chapter 5:

General discussion

Differences in temperature effects on the development of *Heterodera schachtii* and *Heterodera betae*

Different nematode species have different optimum temperatures for feeding, hatching, reproduction and survival (Neilson & Boag, 1996), hence their development is strongly influenced by the microclimate.

In chapter 2, the optimal temperatures for hatch were found to be different for both cyst nematode species. The optimal temperature range for hatch of *H. schachtii* was found to be between 15 and 30 °C and for *H. betae* between 20 and 30 °C. Hatch of *H. schachtii* at 15 °C and 20 °C was, although not significantly, lower, than at 25 °C and 30 °C, suggesting that 15 till 20 °C is only a sub-optimal temperature range for hatch of the studied *H. schachtii* population. Emergence of juveniles of both beet species began at 5 °C, however in very low percentages. *H. schachtii* displayed a broader temperature range over which similar cumulative hatching rates occurred in comparison with *H. betae*. For both beet cyst nematode species no significant differences were found in the final cumulative hatch percentages when comparing the standard and the 4 °C increased temperature regime.

In chapter 4, increased temperature led to a significant increase in beet cyst nematode numbers. The final nematode populations were also different depending on the beet cyst nematode species present. *H. schachtii* showed the highest population density which was significantly higher than that of *H. betae*. Further, the effect of co-infection of the beet cyst nematode species, *H. schachtii* and *H. betae*, on their population development was tested. The interactions between both beet cyst nematode species were expected to be mutually antagonistic as they compete for the available feeding sites (Den Nijs & Lock, 1990). Temperature has been shown to influence the competitive dominance of cyst nematodes (Foot, 1978; Kaczmarek *et al.*, 2014) as soil temperature at the time of primary infection has been shown to be important in determining the dominant species in interspecific competition (Lawn, 1990). In this study, both beet cyst

nematode species developed better at higher temperatures, but there was no significant difference in the relative abundance of both species under both temperature regimes. We found a higher number of cysts and juveniles of *H. schachtii* alone at the end of the experiment than from *H. betae* alone or both beet cyst nematode species mixed.

Experimental methods in climate change studies

A variety of experimental approaches, such as infrared heaters, open top chambers, and heating mats have been used to study climate change effects under field conditions in different environments. Our results (chapter 3) show that a heating mat system allows studying the effects of soil warming attributed to climate change on nematode density and crop performance under outdoor conditions. Experimental soil heating led to an increased nematode development on the susceptible beet cultivar, but not on the resistant cultivar. However, the applied approach also comes with some limitations. Indeed, the temperature difference was higher at low ambient temperatures and lower at high ambient temperatures. The temperature is higher closer to the heating mat, which could influence nematode development. The combination of belowground soil warming and aboveground heaters (Thakur *et al.*, 2014) should therefore be explored for studying effects of soil warming on nematode development and crop performance. Alternatively, free air CO_2 enrichment studies offer a lot of opportunities as climate change is simulated with minimal impacts on the study system (Long *et al.*, 2004; Ainsworth & Long, 2005)

Other climatic factors influencing host-parasite interactions

This work focused on elevated temperatures as a result of climate change. However, other climatic factors such as increased CO_2 increased surface ozone, and soil moisture may influence nematode populations.

Carbon dioxide

Crop sense and respond directly to rising CO_2 through photosynthesis and stomatal conductance, and this forms the basis for the CO2 fertilization effect on yield (Long *et al.*, 2004). Specifically for sugar beet, an increase in storage root yield of 12.1% can be expected (Weigel & Manderscheid, 2012). Due to observations that elevated CO_2 increases root production (Rogers *et al.*, 1994; Kimball & Sparks, 2001), it can be expected that this will also affect plant parasitic

nematodes. Effects of increased carbon dioxide on nematode abundance range from being positive (Yeates *et al.*, 1997; Yeates *et al.*, 1999), neutral, or negative (Hoeksema *et al.*, 2000). However, most studies report neutral effects (Hungate *et al.*, 2000; Sonnemann & Wolters, 2005)

Surface ozone

Increased combustion of fuels will increase not only atmospheric CO_2 but also atmospheric nitrogen oxide concentrations, which, when coupled with climate change, will result in a continuous increase in surface ozone (O_3) concentration (Long, 2006). Contrastingly, the stratospheric O_3 layer is getting depleted by man-made chloride and bromide compounds which lead to an increment solar radiation approaching the earth (Bao *et al.*, 2014). Surface ozone can have negative effects on the reproduction of and development of cyst nematodes (Weber *et al.*, 1979) and on the community structure and functional diversity of soil nematodes (Bao *et al.*, 2013). The application of ozone gas has also been tested as a nematode control strategy (Qiu *et al.*, 2009; Msayleb & Ibrahim, 2011)

Solar radiation

The effect of UV-B on plant performance varies by species and cultivar (Heisler *et al.*, 2003), but generally rising UV-B are expected to have detrimental effects on crop yield (Kakani *et al.*, 2003). Reports on the influence of enhanced UV-B radiation on nematode abundance in soil are contradictory with either negative (Koti *et al.*, 2007) or positive (Bao *et al.*, 2014) effects.

Soil moisture

Nematodes are basically aquatic organisms and require water for their activity. Either too high or too low moisture levels in soil affects the nematodes (Jones *et al.*, 1969). Decreases in summer rainfall and soil moisture projected by climate models for temperate regions are likely to adversely affect plant parasitic nematodes (Garrett *et al.*, 2006). However, the independence of moisture stress and nematode stress on crops is fairly common (Davis *et al.*, 2014).

Interactions between climatic factors

Climate change can rarely be described by single parameter changes as different climatic factors interact, so ideally large multifactorial experiments are required to unravel their combined effect (Garrett *et al.*, 2006).

Global warming and beet cyst nematode management

The interaction between the beet cyst nematodes and sugar beet remains relevant due as this pest can lead to serious yield losses. Understanding these interactions and the climatic factors influencing these interactions is vital towards the development of management strategies. Farmers have been dealing with climatic variability between years successfully in the past and they are likely to cope well with future climatic change (Siebold, 2013). Rising temperatures may be beneficial for farmers in Germany, since the extended growing season may allow more flexibility in sowing and harvesting dates. Although increasing temperatures could have a positive effect on sugar beet yield due to faster development, this effect can be mitigated due to earlier incidence of beet cyst nematodes (Racca *et al.*, 2015).

Temperature has also been reported to influence the pathogenicity of beet cyst nematodes, so that the damage threshold of *H. schachtii* on sugar beet grown in Germany decreased from 20 eggs g^{-1} soil in April to 2.5 eggs g^{-1} soil in May as a result of an increase in soil temperature at planting (Steudel & Thielemann, 1970). Furthermore, increased nematode damage can produce entry ports for fungi to enter inside the plants (Hilnhütter, 2010).

Besides global warming, political frameworks may influence the future importance of nematodes in sugar beet production (Belboom & Léonard, 2012). For example, the EU directive 2009/28/EC for the promotion of the use of energy from renewable sources, will lead to an increasing cultivation of renewable resources like oilseed rape and sugar beet (Belboom & Léonard, 2012; Siebold, 2013). Interestingly, oilseed rape is also a good host for *H. schachtii*. Increased sugar beet and oilseed rape cultivation, however, will lead to enhanced pathogen pressure.

Climate change will also affect pest management with regards to timing, preference, and efficacy of the different measures (Juroszek & von Tiedemann, 2011). Possible effects of climate change on selected beet cyst nematode management measures are listed below:

Crop rotation

Crop species better adapted to local climatic conditions may be required (Juroszek & von Tiedemann, 2011).

Sowing and harvesting date

Climate change will presumably change the optimal choice of sowing/planting date, which is a simple a cheap method to escape biotic and abiotic stress (Juroszek & von Tiedemann, 2011). Harvest date has a high influence on population dynamics of *H. schachtii* (Hauer *et al.*, 2016). As such, a strong decrease in field nematode populations is possible when the host plants are removed before nematodes could fulfill their life cycle (Hauer *et al.*, 2016).

Cultivar choice

The use of tolerant and resistant cultivars is a cheap, environmentally sound and effective method in beet cyst nematode management (Niere, 2009; Juroszek & von Tiedemann, 2011). As climate change induced increased temperatures can reduce host plant resistance (Barbetti *et al.*, 2012), it is relevant that cultivar performances are tested under climate change scenarios. In chapter 3, we found that the plant-nematode interaction can vary considerably depending on the planted cultivar. We found that increased soil temperatures lead to a significant increase in the final number of recovered cysts on the tolerant cultivar and susceptible cultivar, but not on the resistant cultivar. The resistant cultivar did not allow nematode reproduction at all. Thus cultivar choice, and especially the use of resistant cultivars, will continue to be an important element when trying to prevent nematode infestation or controlling nematode populations in the field.

Trap crops

Trap crops are cultivated before sugar beet on more than 40% of the German sugar beet cropping areas, often to reduce nematode population, but also to improve yield (Hauer *et al.*, 2016). Under favourable conditions, reduction of up to 70% of the population can be achieved while maintaining the sugar beet yield on a high level (Heinrichs, 2011). However, the nematode reduction effect depends on high degree of the trap crop variety and environmental conditions (Niere, 2009). An early sowing date immediate after harvesting is a prerequisite to establish trap crop stands producing sufficiently high biomass and rooting density (Hauer *et al.*, 2016). In the

case that climate changes allows earlier sowing, this could positively influence the efficacy of nematode trap crops.

Chemical control

In Europe, nematicides are not registered for use in sugar beets (Niere, 2009; Hauer *et al.*, 2016). Recent test trials of a nematicide development product based on Abamectin did not show efficacy against beet cyst nematodes (Hauer *et al.*, 2016).

Biologic control

Natural antagonists of *H. schachtii* are diverse and their suppressive effect is important in the natural decline of field populations (Westphal & Becker, 1999, 2001; Niere, 2009). Transferring of the suppressiveness was successful under experimental conditions, but is still not established in practice (Niere, 2009). The practical establishment of the entomopathogenic fungus *Hirsutella rhossiliensis* as a biological control agent was not successful (Gutberlet, 2000; Slaats, 2007). Climate changes may differentially affect the physical distribution and/or phenology of host, agent, and target, which can influence biocontrol efficacy (Ziska & McConnell, 2015).

To conclude, rising temperature is one among multiple climatic factors that can stimulate the incidence and impact of beet cyst nematodes on sugar beet production. Hereby, relative contributions of *H. schachtii* and *H. betae* will probably not drastically change. In case of high nematode population densities and with the expected increasing soil temperatures, planting nematode-resistant cultivars will become even more important in the future, as an effective tool to reduce nematode populations and prevent damages.

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DECLARATIONS

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form. Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen,

(Signature)

Bart Vandenbossche

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen,

(Signature)

Bart Vandenbossche