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## Effects of temperature and duration of storage on the hatching behaviour of *Heterodera latipons* (Nematoda: Heteroderidae)

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**Abstract:** Mediterranean cereal cyst nematode (CCN) *Heterodera latipons* is considered as one of the main CCN that causes significant economic losses in the production of intolerant cereal crops in Turkey. Accordingly, this study aimed at evaluating the effect of different storing temperatures and durations on the emergence of second-stage juveniles (J2s) of the nematode populations obtained from the eastern Mediterranean (Adana and Hatay) and south-eastern (Gaziantep and Kilis) regions of Turkey. The obtained results revealed a variation in the J2s hatching profiles of the regional nematode populations. The eastern-Mediterranean and the south-eastern populations hatched at between 5 and 20 °C; however, considerable divergences in the J2 hatching were noticed. The highest emergence of J2s for the populations occurred at 10 °C, whereas the lowest level was observed at 5 °C. To simulate the preplanting soil temperatures in Turkey, the cysts were stored at 5 °C and 20 °C, before incubation at 10 and 15 °C, to stimulate hatching of the J2s. The highest cumulative hatching was obtained at a constant temperature of 10 °C, while the lowest cumulative hatching occurred at 5 °C. Moreover, storing cysts at 5 °C, before incubation at 10 and 15 °C, significantly stimulated hatching of the populations of *H. latipons* when compared to the control. However, storing the cysts at 20 °C, before incubation at 10 °C, substantially stimulated the emergence of J2s relative to the control. The obtained results about the hatching behaviour of *H. latipons* may help in the development of effective control strategies for this nematode in Turkey.

**Key words:** Hatching, *Heterodera latipons*, storage conditions, temperature

### 1. Introduction

Biotic and abiotic constraints have a major effect on the natural distribution of plant-parasitic nematode (PPN) species (Pearson and Dawson, 2003). Mediterranean cereal cyst nematode (CCN) *Heterodera latipons* is considered the most significant PPN species for the production of wheat and barley worldwide (Greco et al., 1992, 2002; Smiley et al., 2009). This species is globally distributed, living in highly contrasting environments (Subbotin et al., 2010). However, it is found mostly from the Mediterranean Basin, including Cyprus, Israel, Italy, Lebanon, Libya, Syria, Tunisia, Morocco, and Turkey (Saxena et al., 1988; Greco, 2002; Mokrini et al., 2014). The nematode is relatively scarce in Turkey, but common in provinces located along the southern coast, such as Adana, Osmaniye, Gaziantep, Kilis, and Mardin (İmren et al., 2015, 2018).

The life cycle of plant pathogens can be divided into 2 alternating phases: an epidemic phase that occurs in or on the host plants, and an interepidemic phase that commonly occurs outside of the host plants (Pariaud et al., 2009). The importance of PPN during an epidemic phase is to synchronize between the host phenology and the life cycle of the nematodes. The main abiotic factor is the temperature that influences the life cycles of parasitic nematodes (Scranton and Amarasekare, 2017). *H. latipons* has very limited potential for active dispersal ability into the soil (Scholz and Sikora, 2004). Temperature has the most significant impact on the major stages of a nematode biological cycle involved in embryonic development, hatching, postinfection activities, and survival in the soil (Trudgill et al., 2005; Kakaire et al., 2012; Kaczmarek et al., 2014; Vandenbossche et al., 2015).

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The effect of host plants with nematode emergence occurs mostly by the root exudates of plants. While most of the second-stage juveniles (J2s) hatch in water, without stimulation by the root exudates, the exudates from the host root cells have a specific effect on CCNs (Grundler et al., 1992). The J2s of CCNs are hatched if the physiological barrier is not an interepidemic factor and appropriate environmental factors, such as soil moisture, adequate temperature, and oxygen, are available. In particular, the temperature is likely to differ across locations, much more during the interepidemic periods than during the epidemic periods, because optimal temperatures for host growth are quite similar to that of the geographical locality. The timely hatching of J2s of CCNs is a crucial portion of this synchronization.

The effect of temperature on the hatching of J2s of CCN species, particularly on *H. avenae* and *H. filipjevi*, has been studied by many researchers under field and laboratory conditions (Mokabli et al., 2001; Sahin et al., 2010; Imren et al., 2012). The cyst nematode *H. latipons* was reported in cereal fields in Turkey (Imren et al., 2015, 2018); however, limited information is available about the influence of temperature and storage conditions on the hatching behaviour. Therefore, the main objective of the current study was to subject the Turkish populations of *H. latipons* from the eastern Mediterranean (Adana, Hatay) and south-eastern (Gaziantep and Kilis) provinces to different storage durations at various temperatures and evaluate their effects on the hatching of J2s.

## 2. Material and methods

### 2.1. Nematode populations

The populations of *H. latipons* were sampled in the 2016 growing season in wheat fields, which had been identified to be historically infested with a high density ( $>0.4$  cyst  $g^{-1}$  of soil) of *H. latipons* (Table 1) (Imren et al., 2012, 2018), located in 4 different provinces, representing slightly diverse climatic zones: Adana, Hatay in the eastern Mediterranean region, and Gaziantep and Kilis in the south-eastern region. At least 10 subsamples were randomly collected at depths of 0–20 cm to obtain a soil sample consisting of a minimum of 10 kg for each population. Cysts were extracted from the

soil samples using a modified sieving-decanting procedure (Fenwick, 1940). A total of 50 cysts were collected and examined using a Zeiss Stemi 508 V20 model stereobinocular microscope (Carl Zeiss GmbH, Oberkochen, Germany). Furthermore, the density of the nematodes  $g^{-1}$  of soil was estimated, considering the number of cysts in 250 g of soil used in the washing process. For each population, a minimum of 10 cysts were selected and maintained at 4 °C until use for molecular assays.

### 2.2. Molecular identification

The genomic DNA was extracted from a single mature cyst for each population using the method described by Subbotin et al. (2000). The internal transcribed spacer (ITS) regions of the ribosomal DNA of the populations were amplified with TW81 (5'-TCCTCCGCTAAATGATATG-3') and AB28 (5'-CGTAACAAGGTAGCTGTAG-3') primers using a Bio-Rad T100 model thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amplified ITS products were bidirectionally sequenced externally by Macrogen Inc. sequencing service (Seoul, South Korea). The resulting sequences were aligned with Clustal-W (Thompson et al., 1994) and analysed using BLAST sequence search in GenBank to identify the closest reference sequences in the complete GenBank database.

### 2.3. Effect of different temperatures on hatching of the second-stage juveniles

The effects of temperature on the emergence of the J2s of 4 *H. latipons* populations (Table 1) were examined at 20, 15, 10, and 5 °C. For each treatment, 12 replicates were used. For each replicate, 1 newly-formed cyst was arranged in 1.0 mL tap water, in a 2.0 mL Eppendorf tube. The tubes were placed in a randomized complete block design in storage boxes and incubated in a WTC Binder KBWF 240 growth chamber (Binder GmbH, Tuttlingen, Germany) adjusted at the particular temperatures. A data logger was used to record the temperature within each incubator every hour.

### 2.4. Hatching of the second-stage juveniles following the storage temperature

The influence of different periods at various storage temperatures on the subsequent hatching of J2s of 4 populations of *H. latipons* was examined. The cysts were stored at 20 and 5 °C for 8 weeks to simulate the climate

**Table 1.** Origin and cyst *H. latipons* populations used in this study.

Region of the country	Province	Location	Mean of number of cysts and eggs in g soil	GenBank accession No.
South-east Anatolia	Gaziantep	Karkamış	0.56	MT758684
	Kilis	Elbeyli	0.82	MT758687
Eastern Mediterranean	Adana	Sarıçam	0.67	MT758686
	Hatay	Mazmanlı	0.23	MT758685

conditions of summer, winter, and spring (or autumn). The experiment was set up in a completely randomized design in the growth chamber. At the end of the storage period, 1 mL of tap water was added to each Eppendorf tube and the tube was kept at a constant temperature of 15 and 10 °C for 12 weeks. These were compared to the control treatments, which were incubated directly at 15 or 10 °C, without prior storage.

### 2.5. Statistical analysis

The obtained datasets from the experiments were separately subjected to 1-way ANOVA using IBM SPSS Statistics for Windows 21.0 (IBM Corp., Armonk, NY, USA), and significant differences between the means were determined using the Tukey HSD test ( $P < 0.05$ ) for each temperature treatment. The Shapiro-Wilk and Levene tests were performed to check the normality and homogeneity of the variances, respectively.

## 3. Results

### 3.1. Nematode populations

*H. latipons* populations were determined in the wheat-growing areas of 4 sampled sites in Adana, Hatay,

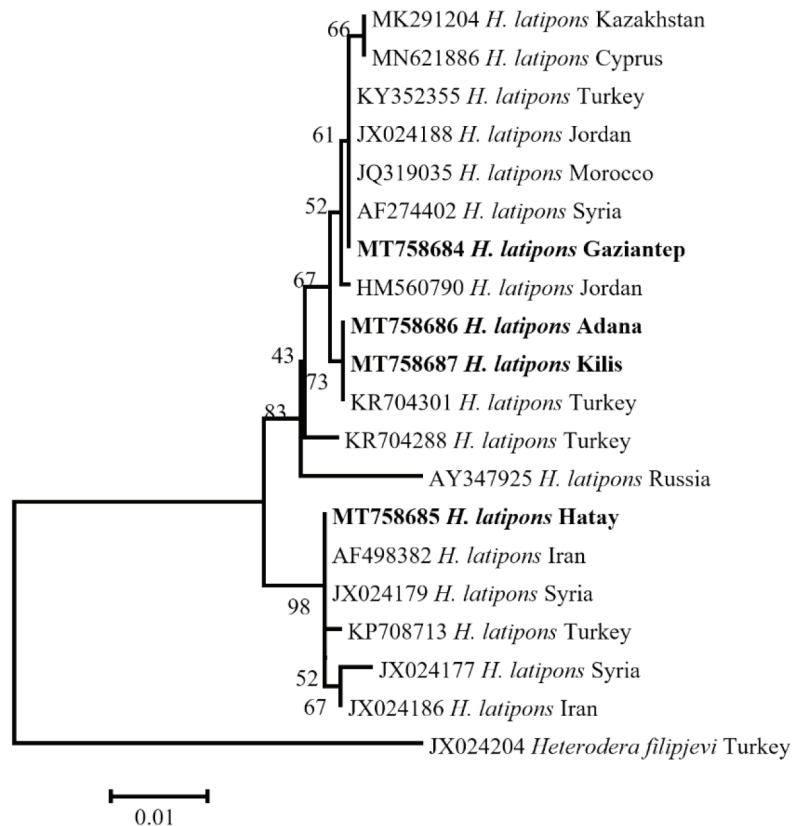
Gaziantep, and Kilis provinces, which were identified to be historically infested with *H. latipons* (Table 1). The average number of cysts per g of soil was estimated to determine the density of *H. latipons*. The highest density of nematodes was 0.82 cyst g<sup>-1</sup> of soil, obtained in samples from Kilis Province, while the lowest cyst density (0.23 cyst g<sup>-1</sup> of soil) was recorded in samples from Hatay Province.

### 3.2. Molecular identification

The amplification product using the TW81 and AB28 primers for the cyst populations were fragments of approximately 1040 bp. BLAST analysis of the ITS sequences confirmed the morphological identification of the populations. All sequences of the populations from this study were deposited in GenBank under the accession numbers listed in Table 1, and compared based on the ITS loci of the *Heterodera* populations, as shown in Figure 1.

### 3.3. Hatching of the *H. latipons* second-stage juveniles at different temperatures

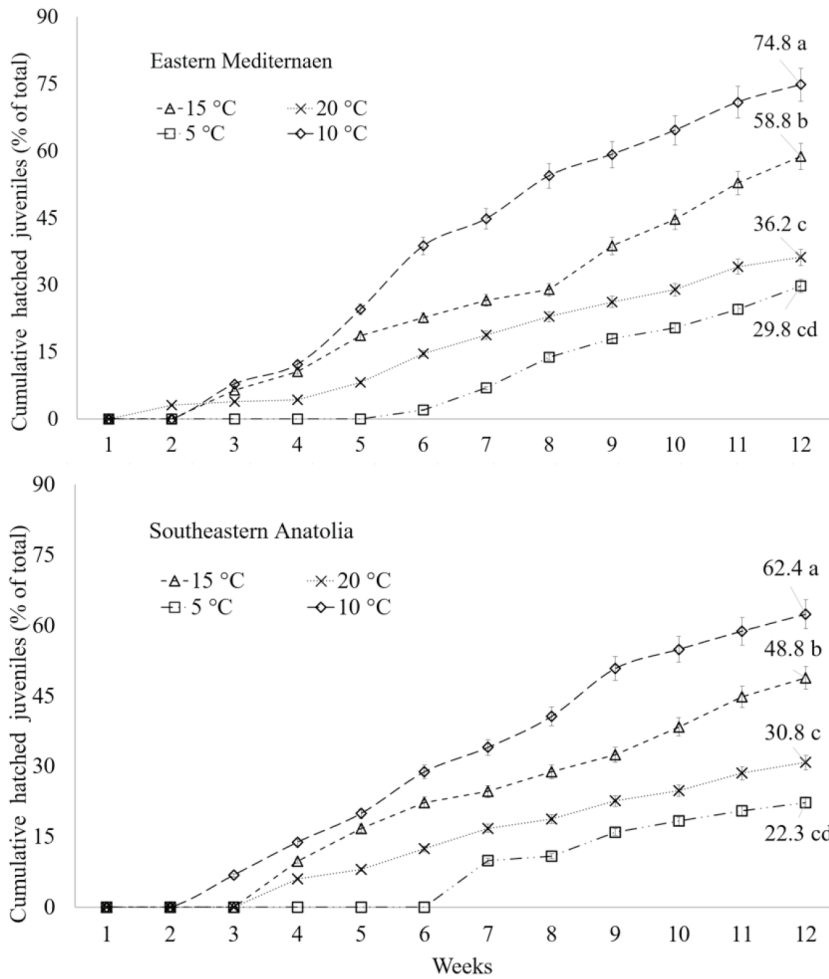
The hatching rate of the J2s of the eastern Mediterranean populations was significantly ( $P \leq 0.05$ ) different than that of the south-eastern populations, according to the incubation temperature for 12 weeks. In general, limited



**Figure 1.** Neighbour-joining tree constructed based on the ITS sequence alignment of the *H. latipons* populations from this study and corresponding populations representing species from GenBank. Numbers on the branches represent bootstrap values obtained from 1000 bootstrap replications.

**Table 2.** Percentage of cumulative hatched J2s ( $\pm$ SD) of *H. latipons* populations over 12 weeks at different temperatures.

Week	Eastern Mediterranean population				Southeast Anatolia population			
	10 °C	15 °C	20 °C	5 °C	10 °C	15 °C	20 °C	5 °C
1	0	0	0	0	0	0	0	0
2	0	0	3	0	0	0	0	0
3	7.8	6.4	3.8	0	6.8	0	0	0
4	12.2	10.5	4.2	0	13.8	9.8	6	0
5	24.5	18.6	8.2	0	19.9	16.8	8	0
6	38.7	22.6	14.6	2	28.8	22.3	12.4	0
7	44.8	26.5	18.8	6.9	34	24.6	16.8	9.9
8	54.4	28.9	22.9	13.8	40.6	28.8	18.8	10.8
9	59.2	38.7	26.2	18	50.8	32.4	22.6	16
10	64.6	44.6	28.9	20.3	54.9	38.4	24.8	18.3
11	70.9	52.8	34.1	24.5	58.8	44.8	28.5	20.5
12	74.8	58.8	36.2	29.8	62.4	48.8	30.8	22.3



**Figure 2.** Effect of various temperatures on the emergence of J2s (J2s) of *H. latipons* populations. Cumulative percentages of hatched J2s followed by the same letter were not significantly different according to the Tukey HSD test at  $P \leq 0.05$ . Vertical bars indicate standard deviation of the means.

**Table 3.** Effect of storage temperature at 5 °C, for 8 prior incubations at 10 and 15 °C for 12 weeks, on the cumulative hatching of *H. latipons* populations.

Week	Eastern Mediterranean population		Southeast Anatolia population	
	10 °C	15 °C	10 °C	15 °C
1	0	2.3	0	2.3
	0	4.2	0	3.2
2	2.2	4	4.9	5.9
3	13.5	16.6	8.8	14.8
4	22.6	20.5	16.9	26.8
5	37.7	30	26.8	28.3
6	49.9	41.8	40	30.6
7	60.9	44.6	45.6	34.8
8	62.4	52.2	57.8	38.4
9	70.7	58.4	60.9	42.4
10	77.4	60.3	64.8	54.8
11	80.2	63.8	71.4	60.8
12	0	2.3	0	2.3

J2s emerged from the incubated cysts of the populations at 5 and 20 °C. However, hatching increased gradually and reached a maximum level at 10 and 15 °C for 12 weeks. The highest hatching from the incubated cysts for all of the tested populations occurred at a temperature of 10 °C, while the lowest emergence of J2s occurred at a temperature of 5 °C. The total cumulative percentages of hatching at 10 °C ranged from 74.8% to 62.4% for the eastern Mediterranean and the south-eastern populations, respectively. The second highest hatching appeared at 15 °C, and ranged from 58.8% to 48.4% for the eastern Mediterranean and the south-eastern populations, respectively. Moreover, they were statistically significantly different from those observed at 20 and 5 °C, which did not exceed 37%.

At 20 °C, the J2s of the 2 populations were initiated to hatch at various times. For all of the eastern Mediterranean populations, the J2s began to hatch 2 weeks after the beginning of the experiment, while the J2s of the south-eastern populations only started to emerge after 4 weeks (Table 2, Figure 2). Despite the early onset of hatching in the J2s of the eastern Mediterranean populations, hatchability at 20 °C was the lowest among all of the populations when compared to the other temperatures (10 and 15 °C). After 12 weeks, the cumulative hatching of J2s was considerably lower (30.8%) in the south-eastern populations than those of the eastern Mediterranean populations (36.2%).

At 15 °C, the J2s of all of the *H. latipons* populations hatched from cysts at different times, and their cumulative hatching rate was higher than those noticed at 5 and 20 °C. Hatching of the J2s in the eastern Mediterranean populations began 3 weeks after the start of the experiment, while J2 hatching in the south-eastern populations started at 4 weeks after the beginning of the experiment. The final cumulative hatching rate of the J2s in the eastern Mediterranean populations was 58.8%, while the incidence of emergence of the J2s in the south-eastern populations was 48.8% after 12 weeks (Table 2, Figure 2).

All of the populations tested showed higher cumulative performance (74.8%) at 10 °C than at the other temperatures ( $P \leq 0.05$ ) after 12 weeks. Hatching of the J2s in the eastern Mediterranean populations began 3 weeks after the beginning of the experiment, while J2 emergence in the south-eastern populations began 4 weeks after the start of the experiment. The final cumulative hatching rate of J2s in the eastern Mediterranean populations was 74.8%, while the incidence of J2 emergence in the south-eastern populations was 62.4% after 12 weeks.

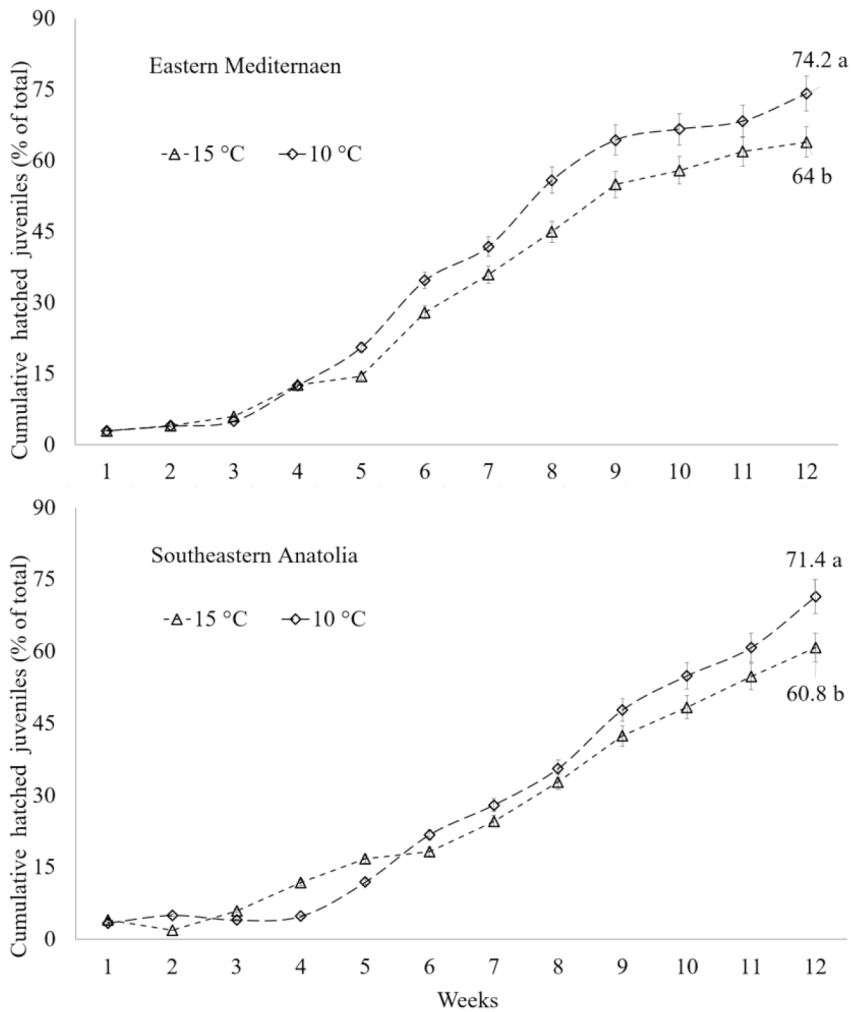
The number of hatched J2s at 5 °C was lower than those at 10, 15, and 20 °C ( $P \leq 0.05$ ) for all of the tested populations. In the south-eastern populations, the hatching of J2s began 7 weeks after the start of the experiment, while J2 hatching in the eastern Mediterranean region populations started only 6 weeks after the beginning of

the experiment. The final cumulative hatching rate of the J2s in the eastern Mediterranean populations was 29.8%, while the incidence of emergence of the J2s in the south-eastern populations was 22.3% after 12 weeks.

**3.4. Hatching of *H. latipons* second-stage juveniles following storage temperature**

Percentages of the cumulative hatched J2s of the eastern Mediterranean and the south-eastern populations were recorded weekly at 15 and 10 °C for 12 weeks, following prior exposure to storage temperatures 5 and 20 °C for 8 weeks. These treatments were compared to the control treatments, which were incubated directly at 10 and 15 °C, without an initial storage period. J2s that were incubated at 15 and 10 °C following prior exposure to storage periods showed higher hatching in all of the populations after 12 weeks than in the previous hatching experiment at the other temperatures.

Variations in the J2s hatching patterns between the eastern Mediterranean and south-eastern populations were observed after 8 weeks of cyst storage at 5 and 20 °C, followed by incubational periods at 10 and 15 °C for 12 weeks. The storage period at 5 °C, followed by incubation at 10 °C, delayed hatching in the eastern Mediterranean and south-eastern populations. Hatching of the J2s in the populations tested at 10 °C occurred 3 weeks after a storage period of 8 weeks at 5 °C, while hatching of the J2s in the eastern Mediterranean and south-eastern populations for at 15 °C occurred immediately after the 8-week storage period at 5 °C. The final cumulative hatching for the eastern Mediterranean populations ranged between 80.2 and 63.8%, for 10 and 15 °C, respectively. Moreover, the overall incidence of emergence of the J2s in the south-eastern populations was between 71.4 and 60.8% at 10 and 15 °C, respectively (Table 3, Figure 3).



**Figure 3.** Effect of storage temperature at 5 °C, for 8 prior incubations at 10 and 15 °C for 12 weeks, on the cumulative hatching of *H. latipons* populations. Cumulative percentages of hatched populations. Vertical bars indicate the standard deviation of the means.



**Table 4.** Effect of storage temperature at 20°C, for 8 prior incubations at 10 and 15 °C for 12 weeks, on the cumulative hatching of *H. latipons* populations.

Week	Eastern Mediterranean population		Southeast Anatolia population	
	10 °C	15 °C	10 °C	15 °C
1	3	3	3.4	4
	4	4	5	1.9
2	5	6	4	5.9
3	12.5	12.6	4.8	11.8
4	20.6	14.5	11.9	16.8
5	34.7	28	21.8	18.3
6	41.9	36	28	24.6
7	55.9	45	35.6	32.8
8	64.4	55	47.8	42.4
9	66.7	58	54.9	48.4
10	68.4	62	60.8	54.8
11	74.2	64	66.4	60.8
12	3	3	3.4	4

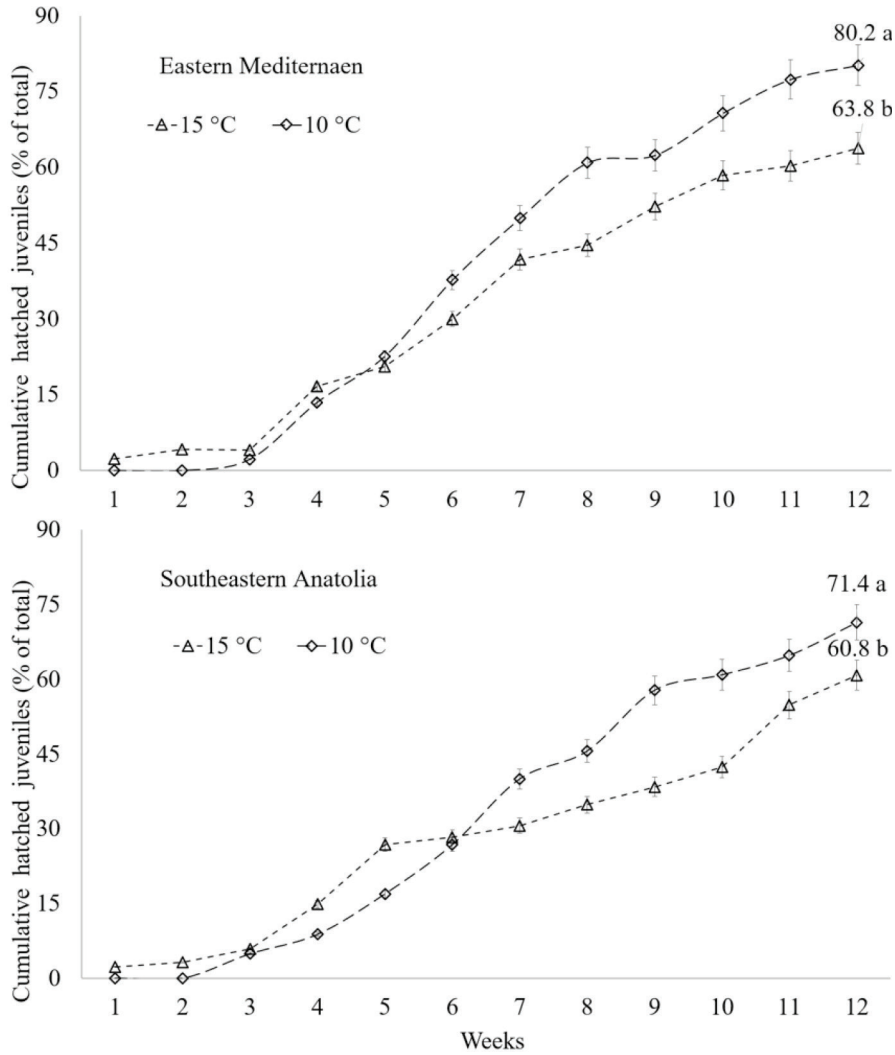
An increase in J2s hatching was observed in the eastern Mediterranean and south-eastern populations when stored at 20 °C for 8 weeks, and then incubated at 10 and 15 °C. Hatching of the J2s in the eastern Mediterranean populations after 8 weeks of prior exposure of the cysts to 20 °C for 10 and 15 °C started in the first week, at a hatching rate that ranged between 74.2% and 64%, respectively. Furthermore, J2s in south-eastern population hatched in the first week after prior exposure to 20 °C for 8 weeks and showed final cumulative hatching of 66.4%–60.8% (Table 4, Figure 4).

#### 4. Discussion

Mediterranean CCNH. *latipons* has been the most studied PPN on cereal cropping systems through the world (Cook and Noel, 2002; Nicol, 2002; Nicolet al., 2003). The cyst (dead female body containing eggs) can survive adverse conditions for many years, and therefore, its management becomes complicated (Dababat and Hendrika, 2018). Control of *H. latipons* populations requires an understanding of its development stages and biological features, such as hatching (Scholz and Sikora, 2004). This study was conducted to investigate the J2 hatching behaviours of 4 Turkish *H. latipons* populations obtained from the eastern Mediterranean region (Adana and Osmaniye) and south-eastern region (Gaziantep and Kilis) at different temperatures and storage durations. The populations of *H. latipons* showed an intraspecific polymorphism. The nematode populations were divided

into groups within the phylogenetic tree constructed by the neighbour-joining algorithm method based on the ITS sequences. Likewise, Madani et al. (2004) and Rivoal et al. (2003) demonstrated intraspecific variation among *H. latipons* populations based on molecular methods. İmren et al. (2012) observed genetic variations in some specimens of *H. latipons* in the Mediterranean region of Turkey. However, Toktay et al. (2015) did not detect any genetic variations in 3 *H. latipons* populations from the east Anatolian region of Turkey. Intraspecific phenotypic variation determined in the current study could be explained as a specific heritable adaptation to the different climates in which they evolved (Rivoal and Cook, 1993).

The hatching and development of *H. latipons* can be affected by the host and environmental conditions, and in particular, temperatures (Philis, 1999; Scholz, 2001). *H. latipons* population structure studies from different regions have suggested the presence of ecotypes that cause differentiation in hatching cycles, ultimately resulting in the induction or suppression of diapause under diverse temperature conditions (Scholz and Sikora, 2004; Hajihasani et al., 2011). Several studies have investigated the hatching process of cyst nematodes, *H. avenae* and *H. filipjevi* using populations from different regions of the world (Rivoal, 1986; Mokabli et al., 2001; Sahin et al., 2010). However, there are limited reports on the hatching behaviour of *H. latipons* (Scholz and Sikora, 2004). This study revealed that hatching experiments with J2s of the eastern Mediterranean and south-eastern populations



**Figure 4.** Effect of storage temperature at 20 °C, for 8 prior incubations at 10 and 15 °C for 12 weeks, on the cumulative hatching of *H. latipons* populations. Cumulative percentages of hatched populations. Vertical bars indicate the standard deviation of the means.

of *H. latipons* showed clear variations in the hatching patterns. In addition, this study was the first examination on this topic regarding *H. latipons* populations collected from the eastern Mediterranean and south-eastern regions of Turkey. Hatching increased gradually to reach a maximum level during an incubation period of 12 weeks at 10 and 15 °C. The highest hatching rate from the incubated cysts of all of the examined populations was observed at 10 °C, while the lowest rate of J2s emerged at 5 °C. Moreover, only a limited number of J2s emerged from the incubated cysts of all of the investigated populations at 20 °C. Rivoal (1986) reported that populations of *H. avenae* hatched in a wide range of temperatures according to their geographical location, which was below 10 °C. Similarly, Mokabli et al. (2001) stated the emergence of *H. avenae* starting from 3 to 25 °C in Algeria. İmren et al. (2012) reported that the highest cumulative hatching (82.3%) was obtained at a

constant 10 °C for cyst nematode *H. avenae*. Sahin et al. (2010) reported that cyst nematode species *H. filipjevi*, in the central Anatolian population in Turkey, hatched primarily between 5 and 15 °C, the most rapid emergence being at 10 and 15 °C. In a similar study, Scholz and Sikora (2004) underscored that hatching of populations of *H. latipons* in Syria occurred at about 10 °C. In the present study, the highest hatching occurred at 10 °C, which had great consistency with previous studies conducted with Heterodera species.

The use of different temperatures to simulate seasonal fluctuations helped to explain the hatching processes of CCNs (Rivoal, 1983; Mokabli et al., 2001). Storage of cysts at low and high temperatures before the J2 hatching assays was used to simulate normal winter temperature changes in the eastern Mediterranean and south-eastern regions. Storage of *H. latipons* cysts at 5 and 20 °C for 12 weeks,

before incubation at 10 and 15 °C, significantly induced hatching of the eastern Mediterranean and south-eastern populations when compared to cysts that had not been stored at this temperature. This explained the transition from the winter season (5 °C pretreatment) to the hot spring (20 °C incubation), which led to quick and short hatching of the J2s. Similar results were noted in the study conducted by Grabbert and Berger (1987), who recorded an increase in the incidence of hatching of *H. avenae* J2s in Germany after 16 days of exposure at 5 °C. These results were in agreement with the results of Fisher (1981), who found that the highest level of J2 emergence among Australian *H. avenae* populations ranged from 20 to 10°C. In addition, hatching of J2s was present in 77%–95% of the cysts of Spanish *H. avenae* populations when transferred from 20 to 10 °C (Valdeolivas, 1991). Moreover, Mokabli et al. (2001) and İmren et al. (2012) reported that exposure by cysts to incubation at 3 and 5 °C for 2 months caused the stimulation of J2 hatching of *H. avenae* populations from northern France and Turkey, respectively. Baklawa (2017) also recorded the stimulation of hatching of *H. avenae* after exposure at 5 and 20 °C.

The eastern Mediterranean (Adana, Osmaniye) and south-eastern (Gaziantep, Kilis) provinces of Turkey have subtropical or arid climates, respectively. Winters are suitable for wheat cultivation, but summers are completely

warm and dry, with minimal rainfall. The optimum temperature for J2 hatching in a Turkish population is between 10 and 15 °C, and in particular, 10 °C. Based on these temperatures, the highest J2 hatching rate may easily occur for *H. latipons* cysts in nature at these temperatures frequently appeared in the cereal fields of those provinces in each region.

The results of this study could help to develop control strategies to reduce the population density of *H. latipons* in the soils of Turkey. The synchrony between the J2 emergence of *H. latipons* and planting of wheat under Mediterranean conditions leads to severe early infestation and crop loss. Adjusting the planting dates to avoid simultaneity between the emergence of J2s and the more sensitive stages of the crops can optimize the final yield of wheat. Therefore, management methods, such as crop rotation, minimal tillage, and early planting, which were practical against the ecotypes of *H. latipons* in southern Syria and Iran (Hajihassani et al., 2011; Scholz and Sikora, 2004), should be employed against the Turkish populations of *H. latipons*.

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