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# Developmental differences of local populations of alfalfa weevil (*Hypera postica*) (Coleoptera: Curculionidae)

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**Abstract:** Many invasive insect pests show phenotypic variation between local populations across their geographical range. Understanding and integrating such differences will help to improve pest management. Population differences are the result of local adaptation to environmental conditions and may be detected using ecological analyses. Immature stages may represent especially interesting study objects as they represent important stages in the life cycle of insects and in many cases may be the most destructive stage. The alfalfa weevil, *Hypera postica* (Gyllenhal, 1813), is a variable worldwide pest that heavily damages alfalfa fields and is widely distributed in Iran. The lack of information about Iranian populations of *H. postica* has motivated us to examine immature life stages of three western populations (Karaj, Hamedan, Tuyserkan) and one eastern population (Jovein) under laboratory conditions. We measured the percentage of survival and the developmental times of each immature stage. The results suggested that the mean duration of the pupa stage significantly differs among the eastern population and all western populations ($P < 0.005$). Egg survivorship was lower in the eastern population (72.5%). The importance of differences in development times between populations is discussed in regards of pest management strategies.

**Key words:** Alfalfa pests, larval instars, percentage survival, population divergence, pupa stage, Iran

## 1. Introduction

Every year several Iranian alfalfa (*Medicago sativa* L.) farms, as well as farms around the world, face huge damages from *Hypera postica*, commonly known as alfalfa weevil (Blodgett and Lenssen, 2004; Khanjani, 2012). In response to the huge genetic diversity in alfalfa (Chandra and Pandey, 2011; Živković et al., 2012) and in respect to differences in environmental and ecological conditions, *H. postica* itself shows large geographic variation with different local adaptations. Accordingly, the alfalfa weevil with its wide distribution in the world (Holarctic region) (Skuhrovec, 2013) can be separated into several genetic lineages (Böttger et al., 2013). Understanding these patterns of population divergence will help to understand the speciation status of *H. postica* populations. Moreover, understanding such differences is essential for establishing effective, locally adapted pest management strategies.

Unfortunately, only a few alfalfa weevil populations have been studied so far. Most current studies have focused on American and Japanese lineages. In less than a century after its introduction in America (Titus, 1909), populations have diverged and a detectable hybrid zone between lineages has been established (Hsaio, 1996; Bundy et al., 2005). Differences between alfalfa weevil populations have been detected for molecular (Hsiao, 1993; Kuwata et al., 2005; Böttger et al., 2013), morphological (Pienkowski et al., 1969; Bland, 1984), and ecological aspects (Armbrust et al., 1969; Davis, 1970; Hsiao and Hsiao, 1985; Skuhrovec et al., 2014). Some of the ecological variation that was observed in the American alfalfa weevil lineage was related to life history traits; for example, the location of pupae differed with some populations having pupae located on the ground and others located on plants (Bundy et al., 2005). Furthermore, the number of laid eggs differs among populations (Coles and Day, 1977). Besides these previously studied traits, the survival percentage and the mean developmental time of each life stage represent promising candidate traits that can be studied under laboratory conditions (Harcourt, 1969; Pöckl and Humpesch, 1990; Bentz et al., 2001; Milbrath et al., 2007). In one of the earliest comparative analyses of life history traits of alfalfa weevil populations, Schroder and Steinhauer (1976) compared three European populations with American populations. Their results indicated similarities between some American populations and European ones (Schroder and Steinhauer, 1976).

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Such information about life cycle aspects can be directly implemented in pest management strategies. For instance, any significant changes in mean development time may affect timing of parasitoid release, economic threshold, and insecticide applications (Tang and Cheke, 2008).

The immature stages of *H. postica* are as equally important as adult stages for pest management. The alfalfa weevil has four larval instars. The first and second instars cause less visible damage to the plant as they feed inside developing alfalfa terminal shoots. In contrast, the third and fourth instars are responsible for visible damage caused by feeding on leaves (Radcliffe and Flanders, 1998). Both instars, however, contribute to dramatic deceases in quality and quantity of the first alfalfa harvest in many regions (Radcliffe and Flanders, 1998) and sometimes lead to a complete loss of the harvest (Khanjani and Pourmirza, 2004). Larvae may further cause indirect damage by decreasing the digestible cell solute proteins of alfalfa and a higher amount of fibers that have lower value for feeding livestock (Summers, 1998).

Due to its high abundance, *H. postica* is the number one pest of alfalfa in Iran (Sanaei et al., 2015a). Many of the previous studies on *H. postica* in Iran focused on pest management in specific regions (Khanjani and Pourmirza, 2004; Moradi-Vajargah et al., 2011, 2013) without respect to population differences and so far life history traits have only been studied for Hamadan populations (Zahiri et al., 2010a, 2010b, 2014). However, very recently, some morphological variation among Iranian populations has been detected (Sanaei et al., 2015b, 2015c). In the current study, the immature life stages of Iranian populations of *H. postica* were investigated to document population differences. For this goal, we ran laboratory trials and investigated six immature stages for three western populations (Karaj, Hamedan, and Tuyserkan) and one eastern population (Jovein).

## 2. Materials and methods

### 2.1. Collecting of material

Between 27 and 29 April 2014, adults of *H. postica* were collected from four different alfalfa farms in Iran. Three of the locations were in the northwest of Iran (Tuyserkan: 34°31′16.2″N, 48°18′43.4″E; Hamadan: 35°7′6.28″N, 49°4′44.69″E, Karaj: 35°48′4.6″N, 50°57′39.6″E), whereas the last location was in the northeast of Iran (Jovein: 35°38′10.3″N, 57°24′14.2″E) (Figure 1).

### 2.2. Rearing

The adults from each location were reared in plastic boxes (16 × 14 × 5 cm) under laboratory conditions at 25 ± 1 °C, 40 ± 5% relative humidity (RH), and 12 L:12 D photoperiod. The adults were fed on fresh cuts of alfalfa leaves and steams. To obtain eggs that were 24 h old, the stems were taken from boxes every day. The eggs were carefully picked from the stems. Forty eggs from each population were transferred to dampened filter paper in 40 Plexiglas boxes (8 × 13.5 cm) with a camel hair brush (Zahiri et al., 2010b). The boxes were covered with mesh with less than 1-mm mesh size. The dishes were incubated in a germinator at 23 ± 1 °C and 75 ± 5% RH. Under natural conditions, *H. postica* lays eggs in alfalfa stems; hence, to mimic the natural environmental conditions, we kept the filter paper dampened by adding sterilized drip water daily and kept the germinator dark with no light period. After the first larvae hatched, the photoperiod was set to 8 L:16 D in order to prevent aestivation in adults (Dewitt and Armbrust, 1972; Zahiri et al., 2010b).

### 2.3. Feeding

Fresh alfalfa was provided every 3 days from the University of Tehran farm (35°48′04.6″N, 50°57′39.6″E). We carefully checked for any pests and larvae and stored the food plants in a refrigerator (7 ± 1 °C). Fresh alfalfa with developing terminal shoots (mainly to feed the two earliest larval instars) was replaced every 12 h and larvae were transferred carefully with a moist brush to new alfalfa. Experimental units were checked every 12 h for accurate identification of larval instars following the chaetotaxy method (Skuhrovec, 2006). However, it was not possible to distinguish prepupae and pupae stages precisely. Therefore, prepupae and pupae stages were considered as one stage in statistical analyses (Pienkowski et al., 1969).

### 2.4. Statistics

The percentage survival of each immature life stage was calculated for each population (Table 1). Durations of the immature development times were considered as variables in SPSS 19 (Brosius, 2011). Descriptive statistics (mean, variance, etc.) were calculated for each station (Table 2). For univariate analysis, normality tests were done for populations and sexes separately. Neither populations nor sex showed a normal distribution; hence, nonparametric tests were chosen for univariate analysis. For sex the Mann–Whitney test and for populations Kruskal–Wallis analysis were applied.

Raw data on survivorship were analyzed according to age-stage, two-sex life table analysis with the program TWOSEX-MSChart (http://140.120.197.173/ecology). The age-stage specific survival rate (sxj), where "x" is age and "j" is stage, was calculated and a graph was drawn with SigmaPlot 12 (Systat Software, San Jose, CA, USA) (Figure 2).

## 3. Results

According to statistical analysis, except for duration of pupa stage, there were no specific differences between immature stages among populations. The descriptive statistical analysis showed that pupa stage in the Jovein population was longer than in other populations: the mean of the pupa stage in the Jovein population was 8.635

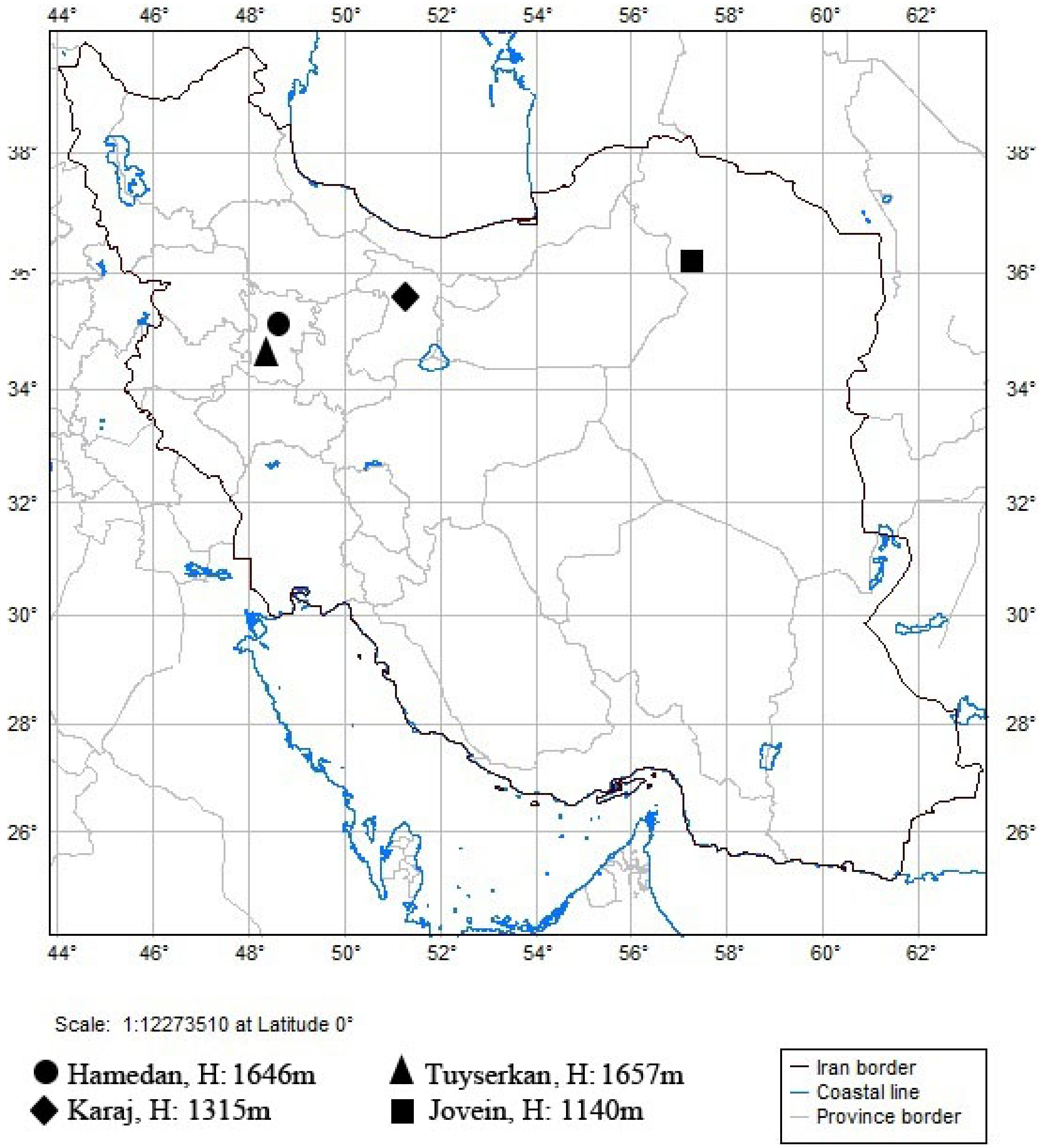


**Figure 1.** Location of four Iranian populations analyzed in this study.

**Table 1.** Survival percentage and number of dead specimens for immature life stages and entire immature life stage in each population.

| Station | Egg | L1 | L2 | L3 | L4 | Pupa | Development |
|---|---|---|---|---|---|---|---|
| Tuyserkan | 40 (95%)<br>2 dead | 38 (97.35%)<br>1 dead | 37 (94.59%)<br>2 dead | 35 (97.14%)<br>1 dead | 34 (100%) | 34 (94.12%)<br>2 dead | 100 (80%)<br>8 dead |
| Hamedan | 40 (97.5%)<br>1 dead | 39 (94.87%)<br>2 dead | 37 (97.30%)<br>1 dead | 36 (100%) | 36 (100%) | 36 (100%) | 100 (90%)<br>4 dead |
| Karaj | 40 (95%)<br>2 dead | 38 (97.35%)<br>1 dead | 37 (100%) | 36 (97.30%)<br>1 dead | 36 (100%) | 36 (97.22%)<br>1 dead | 100 (87.5%)<br>5 dead |
| Jovein | 40 (72.5%)<br>11 dead | 29 (96.55%)<br>1 dead | 28 (92.86%)<br>2 dead | 26 (100%) | 26 (100%) | 26 (100%) | 100 (65%)<br>14 dead |

**Table 2.** Descriptive statistics of immature life stages for each population.

| Population | Stage | Minimum | Maximum | Mean | Std. error | Std. deviation | Variance |
|---|---|---|---|---|---|---|---|
| Tuyserkan | Egg | 6.0 | 8.0 | 6.984 | 0.0654 | 0.3699 | 0.137 |
| | Larva 1 | 1.0 | 4.5 | 2.094 | 0.1132 | 0.6405 | 0.410 |
| | Larva 2 | 1.0 | 6.0 | 2.484 | 0.1925 | 1.0887 | 1.185 |
| | Larva 3 | 1.0 | 4.0 | 2.344 | 0.1513 | 0.8561 | 0.733 |
| | Larva 4 | 3.0 | 6.0 | 3.719 | 0.1308 | 0.7399 | 0.547 |
| | Pupa | 6.5 | 8.5 | 7.375 | 0.1029 | 0.5820 | 0.339 |
| Hamedan | Egg | 6.0 | 7.5 | 6.875 | 0.0642 | 0.3850 | 0.148 |
| | Larva 1 | 1.0 | 4.5 | 1.972 | 0.1379 | 0.8276 | 0.685 |
| | Larva 2 | 1.0 | 5.0 | 2.500 | 0.1942 | 1.1650 | 1.357 |
| | Larva 3 | 1.0 | 4.5 | 2.444 | 0.1348 | 0.8087 | 0.654 |
| | Larva 4 | 3.0 | 5.5 | 3.819 | 0.1246 | 0.7479 | 0.559 |
| | Pupa | 6.5 | 9.0 | 7.528 | 0.1126 | 0.6755 | 0.456 |
| Karaj | Egg | 6.0 | 8.0 | 6.914 | 0.0780 | 0.4615 | 0.213 |
| | Larva 1 | .5 | 4.0 | 1.971 | 0.1194 | 0.7065 | 0.499 |
| | Larva 2 | 1.5 | 4.0 | 2.386 | 0.1067 | 0.6311 | 0.398 |
| | Larva 3 | 1.0 | 5.0 | 2.500 | 0.1478 | 0.8745 | 0.765 |
| | Larva 4 | 2.5 | 5.0 | 3.557 | 0.0934 | 0.5527 | 0.305 |
| | Pupa | 6.5 | 9.0 | 7.714 | 0.0923 | 0.5462 | 0.298 |
| Jovein | Egg | 6.0 | 7.5 | 6.788 | 0.0743 | 0.3788 | 0.143 |
| | Larva 1 | .5 | 2.5 | 1.846 | 0.1066 | 0.5435 | 0.295 |
| | Larva 2 | 1.0 | 4.0 | 2.173 | 0.1328 | .6774 | 0.459 |
| | Larva 3 | 1.0 | 3.5 | 2.250 | 0.1337 | .6819 | 0.465 |
| | Larva 4 | 3.0 | 5.0 | 3.981 | 0.1126 | .5741 | 0.330 |
| | Pupa | 7.0 | 9.5 | 8.635 | 0.1317 | .6717 | 0.451 |

days, while in the western populations it was 7.375 days in Tuyserkan, 7.523 days in Hamedan, and 7.714 days in Karaj. On the other hand, the range of duration for pupa stage was longer in the Jovein population (7–9.5 days) than the western populations (Table 2). Nonparametric tests of immature stages for sexes showed no significant differences between sex (E: 0.461, L1: 0.644, L2: 0.925, L3: 0.919, L4: 0.072, $P = 0.053$). Univariate analysis of the development of immature stages done by nonparametric Mann–Whitney test showed no significant differences between sexes in any of the variables ($P > 0.05$). The nonparametric Kruskal–Wallis test showed significant differences in L4 and pupa stages among stations (L4: $P = 0.048$, pupa: $P < 0.005$). For comparing L4 and pupa stages in populations with two by two methods, we used the Mann–Whitney test (Table 3). Pupa stages showed significant differences between the Jovein population and all other populations ($P < 0.005$). For the L4 stage, the Mann–Whitney test indicated that there were significant differences between the Jovein and Tuyserkan populations ($P = 0.039$) and also between the Karaj and Jovein populations ($P = 0.003$). P-values of other populations for comparison of L4 and pupa stages are available in Table 3. According to the scatter plot (Figure 2), the L1 survival rate is more than 0.8 in western populations and 0.6 in the eastern population (Jovein). In the Hamedan population, L1 and pupa stages have the highest and L3 has the lowest survival rate (0.6–0.8) among all immature life stages (except eggs). The survival rate pattern in the Jovein population is different than that of the western populations. The most important differences in survival percentage of each immature life stage among populations were observed in egg survival percentage between western and eastern populations (Table 1). The Jovein population survival percentage was observed to be 72.5% in the egg stage. In the Jovein population 11 eggs were not hatched. On the other hand, the egg survival percentages in Tuyserkan, Hamedan, and Karaj were 95%, 97.5%, and 95%, respectively.

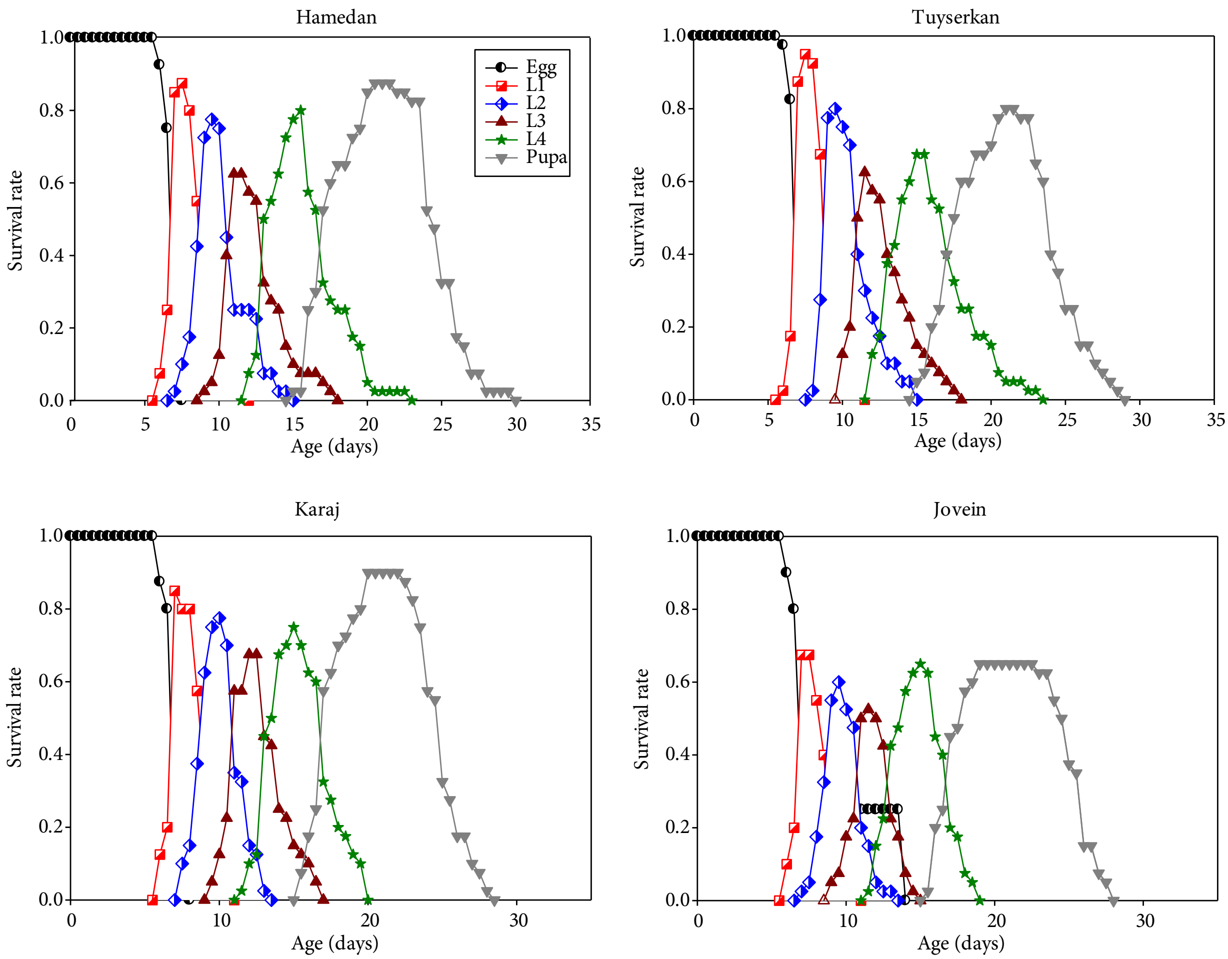


**Figure 2.** Age vs. immature stage survival rates of Iranian population of *Hypera postica* (Hamedan, Karaj, Tuyserkan, Jovein).

## 4. Discussion

A variety of biotic and abiotic factors are responsible for local adaptation of *H. postica* and also for a degree of plasticity in different regions (Bundy et al., 2005). Such factors may also include changes in life history traits such as the survival percentage and the development time of each immature life stage. The main finding in this study is certain differences in immature life stages among western and eastern Iranian populations of alfalfa weevil.

### 4.1. Egg development and survival

Zahiri et al. (2010b) determined the survival percentage and immature stage development time of Hamedan populations at different temperatures but similar RH and photoperiod to those in our study. The results of Zahiri et al. (2010 b) at 24 °C are comparable to those of our study of the Hamedan population and other western populations (our temperature regime was 23 ± 1 °C). However, the mean time of egg development was 7.89 days in their study, whereas we determined a mean egg development time of 6.87 days for Hamedan and an average of 6.92 days for all western populations. This result is not surprising as generally developmental times decrease with increasing temperatures. Schroder and Steinhauer (1976) illustrated 73.5% percentage egg survival for some European and American populations in a photoperiod and temperature similar to those of our study, but they did not report RH. The low egg survival of American and European populations compared to Iranian populations (Tuyserkan and Karaj: 95%, Hamedan: 97.5%, Jovein: 72.5%) might be related to different laboratory conditions in the mentioned studies. In addition, with respect to the fast evolutionary rate in most agricultural insect pests, and especially *H. postica* (Hsiao, 1993), population divergence between the Iranian lineage and American and European lineages is more reasonable.

With 72.5% egg survivorship, the Jovein population differed from other populations (more than 95% egg survivorship in the western populations). The normal unhatched eggs in western populations showed deformations or wizened shape. However, in the eastern

**Table 3.** P-values of nonparametric analysis (Mann–Whitney) for comparing (A) pupa stage and (B) fourth instar larva stage in four populations.

| A | Tuyserkan | Hamedan | Karaj | Jovein |
|---|---|---|---|---|
| Tuyserkan | | 0.429 P-value (2-tailed) | 0.018 P-value (2-tailed) | P < 0.005 |
| Hamedan | | | 0.118 P-value (2-tailed) | P < 0.005 |
| Karaj | | | | P < 0.005 |
| B | Tuyserkan | Hamedan | Karaj | Jovein |
| Tuyserkan | | 0.546 P-value (2-tailed) | 0.488 P-value (2-tailed) | 0.039 P-value (2-tailed) |
| Hamedan | | | 0.176 P-value (2-tailed) | 0.163 P-value (2-tailed) |
| Karaj | | | | 0.003 P-value (2-tailed) |

population (Jovein), 10 eggs remained unhatched for a long time with an unusual feature. The black head capsule clearly showed embryo movement in these 10 eggs (Figure 3). No deformation, change in color, or wizening was observed for about 14 days (similar to the movement when larvae going to hatch). Only 1 egg hatched normally like in the western populations in 8 days. This abnormality may be related to a genetic disorder or pathogens that had infected only the Jovein population. Although it is possible that this inefficient hatching of eggs resulted from environmental pathogens, all specimens were kept in the same conditions with the same feeding habits in sterile germinators, so we assume a genetic disorder for the Jovein population. For finding a more logical explanation, more research is suggested.

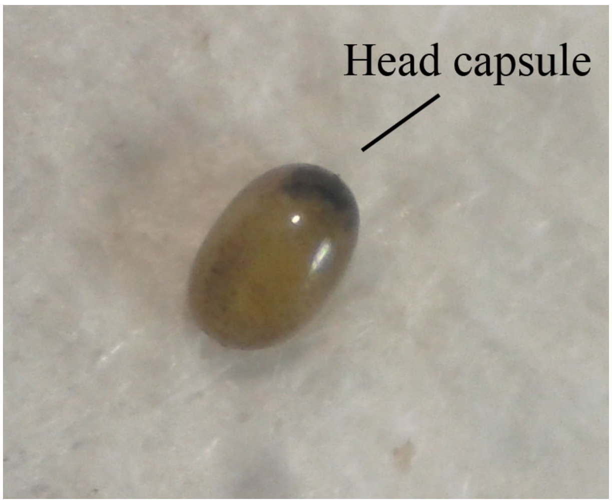


**Figure 3.** Unhatched egg from Jovein population. Head capsule showed embryo movement until day 14.

### 4.2. Larval and pupae stages

Analysis of immature stages of Iranian populations of *H. postica* indicated that the duration of the pupa stage of the Jovein population was significantly different from all other populations. Furthermore, the pupa stage has significant differences between the Tuyserkan and Karaj populations, too, yet the pupa stage was about 1 day longer on average in the Jovein population. In addition, the fourth instar larva showed significant differences among some populations. In the L4 stage, there was a significant difference between the Jovein and Tuyserkan populations and also the Jovein and Karaj populations. However, there was no significant difference between the Jovein and Hamedan populations in the L4 stage. This means that we cannot distinguish the divergence pattern among western and eastern populations by duration of the L4 stage.

### 4.3. Iranian population status

The Hamedan and Tuyserkan populations are very close to each other and they both are affected mostly by mountainous weather conditions (Zehzad et al., 2002). It is not surprising that there is no significant difference in their immature life stages. The Karaj population also is affected by the Alborz Mountain in the north of Iran (Zehzad et al., 2002). However, the mean of temperature and annual precipitation is quite different due to high-elevation land in Tuyserkan and Hamedan (National Geoscience Dataset of Iran, 2015). On the other hand, the Jovein population is located in the northeast of Iran and experienced semiarid weather conditions (Zehzad et al., 2002). The annual precipitation in Jovein is less than that of the northwest of Iran, where the Karaj, Hamedan, and Tuyserkan populations are located (National Geoscience Dataset of Iran, 2015). We suggest that the large geographic distance decreases the chance of gene flow between populations. Geographic barriers and environmental differences may be responsible for the divergence between western and eastern populations. For most agricultural pests, the mixture of populations by human activity is undeniable (Loxdale and Brookes, 1990; Sakai et al., 2001; Sanaei et al., 2015c). Hamedan Province (the locality of the Hamedan and Tuyserkan populations) is one of the most important alfalfa-exporting sites that provide the feeding crop for livestock in many provinces of Iran. This huge amount of alfalfa trade could increase gene flow between western populations (Sanaei et al., 2015b, 2015c). The produced alfalfa in Jovein agroindustry companies can cover the needs of alfalfa in the west of Iran. Therefore, the chance of human-mediated translocation of alfalfa weevils between western and eastern populations is limited.

### 4.4. Implications for pest management

For controlling *H. postica* in Iranian alfalfa fields, several pest management strategies have been suggested (Khanjani and Pourmirza, 2004; Khanjani, 2012; Moradi-Vajargah et al., 2013). However, as population differences so far have largely been ignored, these strategies were neither successful nor applicable in the long term (Tohidfar et al., 2013) in other regions. Most of the strategies completely depended on the development time of immature life stages of the alfalfa weevil. Early harvesting of the first alfalfa crop is strongly recommended in many regions (Khanjani, 2012). It is very important that the first harvest be done when alfalfa weevil larvae are within the economic threshold (Casagrande and Stehr, 1973; Blodgett et al., 2000). Sheep grazing of the first crop is another strategy among Iranian traditional farmers (Ardakani and Emadi, 2008) that again should be applied at the economic threshold of alfalfa weevil (Goosey et al., 2004). In addition, the duration of larval stages can directly be used in degree-day model analysis (Goosey, 2012) and flaming strategies (Khanjani, 2012).

However, nowadays biological control of alfalfa weevils is mostly done with Hymenoptera parasitoids. *Bathyplectes curculionis* (Thomson, 1887) (Hymenoptera: Ichneumonoidae), *Bathyplectes anurus* (Thomson, 1887) (Hymenoptera: Ichneumonoidae), and *Tetrastichus incertus* (Ratzeburg, 1844) (Hymenoptera: Eulophidae) are the main established parasitoids in Iran preying on *H. postica* (Tohidfar et al., 2013). In integrated pest management, the release time of parasitoids needs to be estimated precisely. In this case, considering the variation in developmental times of immature stages among populations is essential. The duration of immature stages in eastern population is relatively longer than in western populations of alfalfa weevil, with at least 1 more day in the pupa stage. As has been discussed in this paper, these subtle differences in immature life stages are crucial in pest management strategies and also increase our knowledge about population divergence of alfalfa weevils in the west and east of Iran.

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