Fitness cost of *Rhagoletis cerasi* (Diptera: Tephritidae) adults emerged from pupae with different dormancy regimes: the case of prolonged chilling

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Abstract

The European cherry fruit fly, *Rhagoletis cerasi* (Diptera: Tephritidae), is a univoltine species that overwinters at pupal stage. Under optimum overwintering conditions pupae will develop into adults the next spring. Shorter or longer than optimum chilling periods induce prolonged pupae dormancy. Pupae that enter prolonged dormancy due to a short chilling period exhibit high emergence rates after a second cycle of cold/warm periods. Adults found to be larger and less fecund compared to their counterparts from pupae with annual diapause. On the other hand, extreme long chilling periods at pupal stage results in high mortality rates. However, for one Greek population, a substantial number of adults emerged following prolonged chilling of pupae (ca. 18 consecutive months). In this study, we used three *R. cerasi* populations in order to address possible geographical variation in fitness cost of adults from pupae with prolonged dormancy. In addition, the fitness traits of these adults emerging after prolonged pupae chilling were compared with that of their counterparts from pupae with annual diapause or prolonged dormancy. Our results reveal no population-specific variation in fitness cost of adults from pupae with prolonged dormancy. Within a population, lifetime fecundity did not differ between adults emerged from pupae with prolonged dormancy and those emerged after prolonged pupae chilling. Adults emerged from pupae exposed to prolonged chilling suffer an additional reduction in adult longevity compared to adults from pupae with prolonged dormancy. Hence, fitness of *R. cerasi* adults is regulated by diapause regimes of pupae.

Introduction

Under species-specific conditions, insects can enter prolonged dormancy, whose duration is determined by environmental cues, such as temperature and humidity (Higaki and Ando, 2000; Higaki, 2005; Higaki *et al.*, 2010), and/or internal cues related to the availability of metabolic resources (Menu and Desouhant, 2002). Repeated cycles of chilling and warming provide a reliable cue for terminating prolonged dormancy in most species, as opposed to prolonged chilling (Higaki, 2005, 2006). Remarkably, only a few studies have addressed the impact of prolonged diapause on adult fitness, and the results appear to vary in a species-specific manner. For instance, there was no cost of prolonged dormancy detected for the chestnut weevil (Menu and Desouhant, 2002; Soula and Menu, 2005) or the cabbage beetle (Wei *et al.*, 2010). In contrast, life-history trade-offs in fecundity rates associated with prolonged dormancy were detected in the sawfly *Neodiprion* sp. (Sullivan and Wallace, 1967; Lyons, 1970) and the European cherry fruit fly *Rhagoletis cerasi* L. (Moraiti *et al.*, 2012a). Despite any associated fitness costs, long life cycles increase the probability of population survival in spatially and/or temporally heterogeneous habitats based on bet-hedging mechanisms (Danforth, 1999) and/or genetic polymorphisms for life cycle length that can be segregated within populations (Hedrick, 1986). For univoltine insects with obligatory diapause, the expression of diversified bet-hedging strategies in response to unpredictable temperature regimes during diapause seems to be of great importance for population persistence under *in situ* environmental disturbance (e.g., insufficient chilling due to climate change) (Moraiti *et al.*, 2014; Moraiti and Papadopoulos, 2017). Bet-hedging seems to be particularly advantageous in natural populations that experience high seasonal variance in temperatures combined with a short breeding period (Akhund-Zade *et al.*, 2020). Understanding the ecological conditions that drive the expression of long life cycles in insect populations in relation to the fitness consequences within and among populations is an extremely challenging subject in evolutionary biology.

The European cherry fruit fly, *R. cerasi* (Diptera: Tephritidae), is a stenophagous, univoltine species with an obligatory diapause in the pupal stage (Boller and Prokopy, 1976). Diapause termination exhibits great plasticity within populations, including expression of bet-hedging

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(Moraiti *et al.*, 2014). In the wild, pupae are expected to terminate diapause as long as they have satisfied chilling requirements during the winter, and adults emerge in the next fruiting season in synchrony with the phenology of the local cherry cultivars (Papanastasiou *et al.*, 2011). Nevertheless, a proportion of pupae can follow an extended dormancy for more than 1 year under specific temperature regimes. Depending on the population, pupae subjected to 3–8°C for a period ranging from 4 to 8 months can successfully terminate annual diapause (Moraiti *et al.*, 2014). Shorter and longer than optimum chilling periods (within 3–8°C) as well as temperatures higher than 10°C (regardless of the duration of the chilling period) trigger prolonged dormancy responses that are concluded after a second cycle of exposure to cold/warm temperatures (Moraiti *et al.*, 2014; Moraiti and Papadopoulos, 2017). An extended chilling period of 12 consecutive months at 3–5°C resulted in low emergence rates for *R. cerasi* pupae (Vallo *et al.*, 1976). Similarly, our preliminary experiments using *R. cerasi* pupae from three Greek populations that were exposed to cold for approximately 18 consecutive months revealed extremely high pupae mortality rates (>80%) (Supplementary Material, Table S1). Because such a long cold period is unlikely to exist in the wild (as opposed to temperature regimes that allow expression of pupae prolonged dormancy), prolonged chilling is considered to be an extremely stressful conditions for diapause termination, highlighting the capacity of pupae to exhibit phenotypic plasticity in dormancy responses.

Prolonged pupae dormancy comes at a fitness cost for *R. cerasi* adults, even though adults emerged from prolonged dormancy had larger body size than their counterparts emerged from pupae with annual diapause (Moraiti *et al.*, 2012a). Body size may affect the diapause termination pathway of *R. cerasi* pupae. In comparison with flies emerging from pupae following annual diapause, adults emerging after prolonged dormancy express decreased lifetime fecundity, shorter oviposition period and extended post-oviposition period (Moraiti *et al.*, 2012a). Females of two allopatric, genetically distant populations emerged after prolonged pupae dormancy expressed similar fecundity rates and oviposition patterns (Moraiti *et al.*, 2012a). In contrast, fecundity rates and reproductive periods of *R. cerasi* adults that emerged from pupae with annual diapause differed among allopatric populations with restricted gene flow rates (based on DNA microsatellites). In the face of gene flow, *R. cerasi* adults emerging from pupae with annual diapause were characterized by substantial variation in a suite of fitness traits (including lifetime fecundity and longevity), which were mainly attributed to ecological heterogeneity of their local habitats (Moraiti *et al.*, 2012b). Considering the geographic variation in fitness traits of adults emerging after annual pupae diapause (Moraiti *et al.*, 2012b), there could be substantial genetic divergence (among populations) in life-history trade-offs in adults emerging after prolonged pupae dormancy as well. Notably, the limited number of *R. cerasi* adults emerged from pupae subjected to prolonged chilling (ca.18 consecutive months), which is a major stress for pupae survival, bore no abnormalities (such as malformed wings). The adult life-history traits of such rare phenotypes remain completely unexplored. Whether the trade-offs between dormancy duration and adult fitness are genetically based is of outmost importance to understand adaptation to novel environments or to environmental changes associated with climate change, and for describing the evolutionary potential of a population.

In the current study, we adopted an integrated approach for testing the trade-off hypothesis between prolonged pupae dormancy and fitness costs of *R. cerasi* adults. Considering the geographical variation in life-history traits of adults from pupae with annual diapause (Moraiti *et al.*, 2012b), we hypothesize that fitness cost of *R. cerasi* adults emerging following prolonged pupae dormancy differ among populations (Feder and Filchak, 1999). To test our hypothesis, we conducted comparative demographic studies among adults emerged from pupae with annual diapause and prolonged dormancy from three geographically isolated, Greek populations of *R. cerasi* with various levels of gene flow rates. Moreover, we assessed the fitness of adults that emerged from pupae subjected to prolonged chilling. Given that only pupae from the Kamari population produced a substantial number of adults for assessing demographic parameters (Supplementary Material, Table S1), we quantify the relative fitness cost of cold stress for this population only. Life-history traits of these adults were compared with those of their counterparts emerging from pupae with either annual diapause or prolonged dormancy. We tested the hypothesis that adults emerged following prolonged pupae chilling exhibit higher fitness cost than their counterparts emerged from pupae with prolonged dormancy or annual diapause.

A suite of life-history traits of adults was used as a proxy of fitness in each experiment, including fecundity and adult longevity, both of which are closely associated with lifetime reproductive success (van Buskirk and Steiner, 2009). In addition, adult body size metrics were recorded for each individual. Along with head width that is considered as the most reliable metric for adult size of *R. cerasi* adults (Remund and Boller, 1976), thorax width and thorax length were also included due to their relatedness with adult longevity and/or the duration of reproductive periods (Moraiti *et al.*, 2012a, 2012b). In an attempt to determine if the observed body size differences between adults emerged from pupae with annual diapause and prolonged dormancy is the outcome of selection against smaller individuals in the group of individuals that followed prolonged dormancy, the distribution of body size traits were considered. We assume that both groups of adults would have shown similar ranges of body size but the distributions would have been strongly skewed towards larger individuals for those emerged following prolonged pupae dormancy. The distribution of body size traits of adults emerged from pupae subjected to prolonged chilling are included.

Material and methods

Rhagoletis cerasi *populations*

Even though the life-history traits of *R. cerasi* adults emerging from pupae with annual diapause and prolonged dormancy from Mikra (Thessaloniki, Macedonia) and Kernitsa (Achaia, Peloponnesus) populations have been published earlier (Moraiti *et al.*, 2012a), the effect of population on fitness costs has not been addressed. To test our hypothesis for geographical variation in fitness trade-offs of adults, we used the data from the above two populations along with those from Kamari (Magnesia, Thessaly) (Supplementary Material, Fig. S1, Table S2). Kamari is a coastal village in the Magnesia regional unit of Thessaly, and population genetic structure based on microsatellite markers revealed that the Mikra and Kamari populations belong to the same cluster with gene flow, while the Kernitsa population is genetically distinct (Augustinos *et al.*, 2014). It is noted that Mikra and Kernitsa populations were previously referred to as Macedonia and Peloponnesus, respectively (Moraiti *et al.*, 2012a), which are the

Regions wherein they belong (see Supplementary Material, Fig. S1).

Rhagoletis cerasi pupae were recovered from field-infested, wild-growing sweet cherries from Kamari, and from abandoned sweet cherry trees from the other two locations. Field-infested fruit were brought to the laboratory of Entomology and Agricultural Zoology at the University of Thessaly (Volos, Greece) and placed in plastic containers over a layer of dry sand (1 cm thick), allowing mature larvae to pupate under constant conditions (25 ± 1°C, 65 ± 5% relative humidity, and a photoperiod of L14:D10). Pupae were collected by sieving the sand for 2 weeks and kept in plastic Petri dishes weekly.

Experiment 1. geographical variation in body size and life-history traits of adults that emerged after annual diapause and prolonged dormancy

We used adults from the three Greek populations (Mikra, Kamari, Kernitsa). Adults emerged either from pupae with annual diapause after one cycle of warm−cold periods ('1-year' adults) or from prolonged dormancy that was terminated after a second cycle of warm−cold periods ('2-year' adults). The experimental process followed for producing both '1-year' and '2-year' adults was the same as that described in Moraiti *et al.* (2012*a*) (Supplementary Material, Fig. S2).

Experiment 2. Effects of prolonged pupae chilling on body size and adult life-history traits

Pupae from the Kamari population were used to determine trade-offs of prolonged pupae chilling on adult body size and life-history traits. A cohort of 484 pupae kept at 25 ± 1°C for approximately 3 months and then transferred to 3 ± 1°C for approximately 18 consecutive months. Concluding the chilling period, pupae transferred back at 25 ± 1°C to facilitate the emergence of adults (Supplementary Material, Fig. S2). Only 13.4% of pupae produced adults ('prolonged chilling' adults). All remaining pupae were recorded dead three months after being transferred and kept at 25 ± 1°C. Demographic traits and body size of 'prolonged chilling' adults were compared to '1-year' and '2-year' counterparts (see Experiment 1). It should be noted that all pupae used in Experiment 2 (pupae producing the '1-year' and '2-year' adults and pupae subjected to prolonged chilling) were obtained from a unique wild cherry fruit collection and treated uniformly until assigned into two cohorts (for producing either both the '1-year' and '2-year' adults or the 'prolonged chilling' adults) in order to eliminate any possible environmental variability.

For both experiments, within 24 h of emergence, one male and one female from each population and type of dormancy were placed into an individual cage constructed from a transparent plastic cup of 400 ml capacity (6.5 × 9 × 12 cm) placed upside down on a 9-cm diameter plastic Petri dish. As oviposition substrates for females, we used five 18 mm, hollow, ceresin domes of black colour that were fitted into similar size holes perforated in the Petri dish. We ran 47–50 replicates (1 mating pair/replicate) for '1-year' and '2-year' adults, and 26 replicates for 'prolonged chilling' adults. Flies were provided *ad libitum* with adult food (a mixture of yeast hydrolysate, sugar and water, 1:4:5 by weight, respectively) and water. Daily egg production as well as female and male ages at death were recorded under constant laboratory conditions. The body size of all adults was estimated from three measures: thorax length, thorax width, and head width. Thorax length was measured from the anterior margin of thorax to posterior tip of scutellum, whereas thorax and head width were taken at the widest point of the thorax and head, respectively. All measurements were carried out under a stereomicroscope (Leica MZ12) fitted with an ocular micrometer (1 mm = 2 ocular units).

Statistical analysis

Kolmogorov−Smirnov tests were used to assess the normality of data distributions. Pearson's correlation between adult body size traits (thorax length, thorax width, head width) showed that all three traits are strongly associated with each other (Supplementary Material, Table S3). To handle these correlations, principal components analysis (PCA) was used to construct a composite score for the three body parameters controlling for dependencies among them. An index score variable was established, which was an optimally weighted combination of the three correlated variables (thorax length, thorax width, and head width) for each experiment. There were three body traits for each experiment so the first principal component for each of them was used to construct the composite index in a single dimension. For Experiment 1, the first principal component (PC1_Exp1) accounted for 82.18% of the total variability and was used to represent the overall body size. For Experiment 2, the first principal component (PC1_Exp2) accounted for 79.99% of the total variability and was used to represent the overall body size. Linear regression models were used to explore the relationship between body size (being represented by the first principal component for each Experiment) and population, type of dormancy, and sex. A Cox proportional hazard model was used to assess the effects of population, type of dormancy, sex, and body size on adult longevity, as well as the effects of population, type of dormancy and body size on the duration of pre-oviposition, oviposition, and post-oviposition periods of females (Collett, 2003). Negative-binomial regression analysis was used to examine the effects of type of dormancy, population and body size for Experiment 1 and for type of dormancy and body size for Experiment 2 on lifetime fecundity. Negative binomial regression was used for modeling over-dispersed count outcome variables. Parameter estimates were expressed as Incidence Rate Ratios (IRR) (and 95% confidence intervals), which is the ratio of the number of eggs produced by flies in a group of interest to the number of eggs produced by flies in the reference group. IRRs greater than 1 indicate greater number of eggs produced by flies in the group of interest while IRRs lower than 1 indicate greater number of eggs produced by flies in the reference group. Bonferroni corrected post-hoc comparisons were performed for the type of dormancy in Experiment 2. All two-way interactions were considered for all models but only the significant ones were included in the final models. *P*-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 26.0 (IBM Corp.Armonk, NY)). Violin plots were produced in R Version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria), using the package ggplot2 (Wickham, 2016).

Results

Experiment 1. Geographical variation in body size and life-history traits of adults emerging following annual and prolonged pupae dormancy

Morphometry

The '2-year' individuals were on average larger than the '1-year' individuals for all three body size traits across all three populations and both sexes (Supplementary Material, Fig. S3). The

distribution of the thorax length values of the '1-year' and '2-year' females seem to overlap. In contrast, the distribution of thorax length values for the '2-year' males is partly overlapped with that of the '1-year' males regardless of the population (Supplementary Material, Fig S3). The distribution of head width and thorax width values of the '2-year' adults was largely within the distribution range of the '1-year' adults, except for females from Kernitsa (head width) and Kamari (thorax width). Using the PC1_Exp1 as body size metric, linear regression analysis revealed significant interactions between sex with type of dormancy ($P = 0.003$) and sex with population ($P = 0.046$) (Supplementary Material, Tables S4). Pairwise comparisons of interactions are given in Table S5 (Supplementary Material).

Survival and longevity

The average longevity was 64 ± 2.1, 59.5 ± 2.2 and 56.3 ± 2.0 days for the '1-year' adults from the Mikra, Kamari and Kernitsa populations, respectively. Average longevity of the '2-year' cohorts of adults from Mikra, Kamari and Kernitsa was 55 ± 2.8, 53.5 ± 3.1 and 53.9 ± 2.8 days, respectively (Supplementary Material, Table S6). Cox regression analysis revealed both body size (PC1_Exp1) and the type of dormancy as significant predictors of adult longevity in contrast to population and sex (Table 1). An inverse relationship between body size and adult longevity was detected (Supplementary Material, Fig. S4). Mortality rates of the '2-year' adults gradually increased from 5 to 10 days of age. Survival rates of '1-year' adults remained high up to 50-days of age for all three populations (Supplementary Material, Fig. S5).

Reproduction

Box plots showing the duration of reproductive periods (pre-oviposition, oviposition, and post-oviposition) for females from each population emerged from pupae with annual diapause and prolonged dormancy are illustrated in Fig. S6 (Supplementary Material). Cox regression analysis revealed that population was the only significant predictor of the mean duration of pre-oviposition period, whereas the duration of post-oviposition period was significantly affected only by the type of dormancy (Table 1). Females from Kamari initiated laying eggs later than those of Kernitsa. The '2-year' adults had a longer post-oviposition period (in line with published results given in Moraiti *et al.*, 2012*a*). None of the tested factors (population, body size, type of dormancy) was a significant predictor of the duration of oviposition period (Cox regression analysis, Table 1).

Lifetime fecundity of females emerged from pupae with annual diapause ('1- year') and prolonged dormancy ('2-year') for the three populations is shown in fig. 1. A negative-binomial regression analysis revealed the type of dormancy as the only significant predictor of the lifetime fecundity (Table 2). The number of eggs produced by the '1-year' females were approximately three times higher than those of the '2-year' females (IRR (95% CI) = 3.061 (2.265, 4.137), $P < 0.001$) (Table 2). In addition, there was an increase in the proportion of days with zero egg-laying within the oviposition period for the '2-year' females from Mikra and Kernitsa (26.9 and 27.8%, respectively), compared to their '1-year' counterparts (16.5 and 12.7%, respectively). No difference was detected in females from Kamari (ca. 20% of oviposition days for both female cohorts).

Experiment 2. Effects of prolonged pupae chilling on body size and adult life-history traits

Morphometry

The distribution of body size traits of males and females for the different types of dormancy in the Kamari population is shown in fig. 2. There is higher variability in distribution of thorax width values of 'prolonged chilling' males compared to both '1-year' and '2-year' counterparts. In contrast, differences in the distribution range of head width or thorax length values among the three groups of adults are limited. Considering the correlations between the three body size traits (Supplementary material, Table S3), we used the PC1_Exp2 as the dependent variable for body size in a linear regression analysis, which revealed that sex and type of dormancy were significant predictors of adult body size. Females were larger than males ($P < 0.001$). Adults emerged from pupae with prolonged chilling were smaller than the '2-year' adults ($P = 0.003$) but larger than the '1-year' adults ($P = 0.005$) (Table 3).

Survival and longevity

The average longevity of the '1-year', '2-year' and 'prolonged chilling' adults from the Kamari population was 59.5 ± 2.2, 53.5 ± 3.1 and 45.4 ± 2.9 days, respectively (see also Supplementary Material, Table S7). Cox regression analysis revealed that adult longevity was affected by the type of dormancy, but neither body size (PC1_Exp2) nor sex were significant predictors of adult longevity (Table 4).

The survival rates of the '1-year' adults were higher than those of the '2-year' and 'prolonged chilling' counterparts during the first 50 days of age (fig. 3). The 'prolonged chilling' adults exhibited increased age-specific mortality rates after the adult day 50 compared to both '1-year' and '2-year' counterparts (fig. 3).

Reproduction

The mean duration of pre-oviposition periods for the '1-year', '2-year' and 'prolonged chilling' females from the Kamari population were 9.7 ± 0.5, 9.4 ± 0.5 and 8.2 ± 0.6 days, respectively (fig. 4). A Cox regression analysis reveals that neither the type of dormancy nor body size (PC1_Exp2) were significant predictors of the duration of the pre-oviposition period (Table 4). The duration of the oviposition period of the '1-year' females (46 ± 2.9 days) was longer than that of the '2-year' (30.8 ± 3.7 days) and 'prolonged chilling' females (29.3 ± 3.9 days) (fig. 4). A Cox regression analysis revealed that only the type of dormancy was a significant predictor of the duration of oviposition period (Table 4). The average post-oviposition period was longer for the '2-year' females (10.3 ± 2.4 days) than their '1-year' (3.1 ± 0.8 days) and 'prolonged chilling' counterparts (5.7 ± 1.5 days) (fig. 4). The type of dormancy had a significant effect on the mean duration of post-oviposition period, as opposed to body size (Table 4).

The average lifetime fecundity was lower for the 'prolonged chilling' females (74.6 ± 14.9 eggs/female) than their '2-year' (90.4 ± 14.6 eggs/female) and '1-year' counterparts (259.7 ± 21.4 eggs/female). Negative-binomial distribution regression analysis revealed that the type of dormancy ($P < 0.001$) had a significant effect on lifetime fecundity of females from the Kamari population (Table 5). Lifetime fecundity differed between the '1-year' and the '2-year' females (Bonferroni test, $P < 0.001$), and the '1-year' females and the 'prolonged chilling' females (Bonferroni test, $P < 0.001$). No difference in lifetime fecundity was detected between the '2-year' and the 'prolonged chilling' females (Bonferroni test, $P = 1.000$). The 'prolonged chilling' females produced approximately 4 times fewer eggs compared to '1-year'

Table 1. Effects of explanatory variables of the Cox proportional hazard model on adult longevity and the duration of reproductive periods of females emerged from pupae with annual diapause (1-year adults) and pupae with prolonged dormancy (2-year adults)

Factor	Wald's χ^2 (df)	P	Exp (B) (95% CI)
Adult longevity			
Population (ref: Kernitsa)	3.808 (2)	0.149	
Mikra	3.252 (1)	0.071	0.829 (0.676, 1.016)
Kamari	0.075 (1)	0.784	0.972 (0.790, 1.194)
Sex (ref: male)	1.460 (1)	0.227	0.866 (0.685, 1.094)
Type of dormancy (ref: 2-year adults)	5.253 (1)	0.022	1.267 (1.035, 1.552)
Body size	14.422 (1)	<0.001	1.318 (1.143, 1.520)
Preoviposition period			
Population (ref: Kernitsa)	7.820 (2)	0.020	
Mikra	1.370 (1)	0.242	0.839 (0.625, 1.126)
Kamari	7.745 (1)	0.005	0.645 (0.474, 0.878)
Type of dormancy (ref: 2-year females)	2.141 (1)	0.143	1.264 (0.923, 1.731)
Body size	0.027 (1)	0.869	0.869 (0.822, 1.180)
Oviposition period			
Population (ref: Kernitsa)	4.142 (2)	0.126	
Mikra	0.937 (1)	0.333	0.865 (0.645, 1.160)
Kamari	1.075 (1)	0.300	1.173 (0.868, 1.585)
Type of dormancy (ref: 2-year females)	1.499 (1)	0.221	0.830 (0.616, 1.119)
Body size	3.607 (1)	0.058	1.201 (0.994, 1.450)
Post-oviposition period			
Population (ref: Kernitsa)	1.272 (2)	0.529	
Mikra	0.181 (1)	0.670	0.939 (0.701, 1.257)
Kamari	1.249 (1)	0.264	0.841 (0.621, 1.139)
Type of dormancy (ref: 2-year females)	26.740 (1)	<0.001	2.323 (1.688, 3.195)
Body size	2.793 (1)	0.095	1.166 (0.974, 1.395)

Pupae obtained from field-infested cherries from Mikra (Macedonia), Kamari (Thessaly) and Kernitsa (Peloponnesus). Body size refers to the first principal component (PC1_Exp1). Data for adults from Mikra and Kernitsa have been previously published (Moraiti *et al.*, 2012*a*).

females (IRR (95% CI) = 3.916 (2.354, 6.513), $P < 0.001$). The '1-year' females produced approximately 3.5 times more eggs than the '2-year' females ($P < 0.001$) (Table 5).

Age-specific egg-laying patterns of females were substantially different among cohorts with different types of dormancy (Supplementary Material, Fig. S7). In contrast to the '1-year' females, there were no well-defined peaks of oviposition rates for the '2-year' females or the 'prolonged chilling' females. The '1-year' females laid continuously high number of eggs compared to both '2-year' and 'prolonged chilling' females that expressed rather sporadic egg-laying events (Supplementary Material, Fig. S8).

Discussion

The fitness cost of extended dormancy strategies in overwintering insects with obligate diapause is poorly addressed. We determined whether population origin of *R. cerasi* adults emerged from pupae with prolonged dormancy is a significant predictor of their reduced performance (Moraiti *et al.*, 2012*a*). Our results reveal that the '2-year' females from different populations suffer a similar decrease in lifetime fecundity compared to their '1-year'

counterparts. The type of dormancy was the only significant predictor for both lifetime fecundity and the duration of post-oviposition period. The duration of pre-oviposition period was a population-specific trait that was not affected by the type of dormancy. Both the type of dormancy and adult size had an effect on adult longevity. Therefore, the hypothesis that there is geographic variability in fitness cost of adults with prolonged dormancy is rejected. Prolonged chilling at pupal stage shortens longevity but lifetime fecundity of females is not further decreased, compared to '2-year' counterparts. The duration of the oviposition period was similar for the 'prolonged chilling' and the '2-year' females, but it was shorter than that of their '1-year' counterparts. Hence, the second hypothesis that fitness cost of 'prolonged chilling' adults is higher compared to '1-year' or '2-year' counterparts is partially confirmed.

Adult body size and different dormancy regimes

The '2-year' adults were larger than the '1-year' ones, similar to what has been shown in previous studies (Danforth, 1999; Menu and Desouhant, 2002; Moraiti *et al.*, 2012*a*). Yet, the
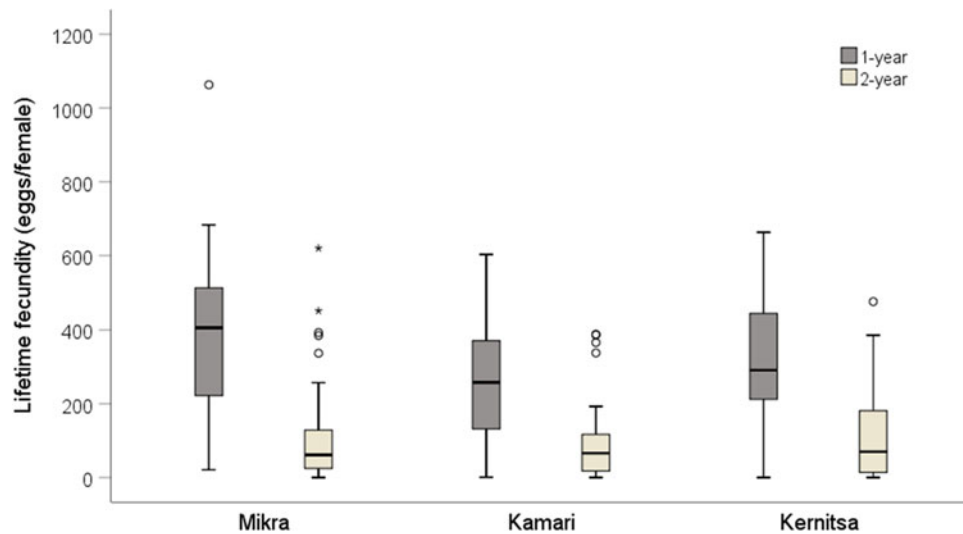
Figure 1. Box plots depicting the lifetime fecundity of females emerged from pupae with annual diapause (1-year) and prolonged dormancy (2-year). Pupae obtained from field-infested cherries from Mikra (Macedonia), Kamari (Thessaly) and Kernitsa (Peloponnesus). For 1-year females: $N = 50$, 50 and 43 females from Mikra, Kamari and Kernitsa population, respectively. For 2-year females: $N = 48$, 44 and 43 females from Mikra, Kamari and Kernitsa populations, respectively. Data for females from Mikra and Kernitsa have been previously published (Moraiti *et al.*, 2012*a*).

Table 2. Results of the negative-binomial regression model regarding the effects of type of dormancy, population and body size on lifetime fecundity of *Rhagoletis cerasi* females from Mikra (Macedonia), Kamari (Thessaly) and Kernitsa (Peloponnesus)

Factor	Wald χ^2 (df)	P	IRR (95% CI)
Type of dormancy (ref: 2-year females)	52.986 (1)	<0.001	3.061 (2.265, 4.137)
Population (ref: Kernitsa)	4.326 (2)	0.115	
Mikra	0.341 (1)	0.559	1.088 (0.819, 1.445)
Kamari	2.101 (1)	0.147	0.809 (0.607, 1.078)
Body Size	0.000 (1)	0.998	0.999 (0.834, 1.195)

Body size refers to the first principal component (PC1_Exp1). For 1-year females: $N = 50$, 50 and 43 females from Mikra, Kamari and Kernitsa population, respectively. For 2-year females: $N = 48$, 44 and 43 females from Mikra, Kamari and Kernitsa population, respectively. Data for females from Mikra and Kernitsa have been previously published (Moraiti *et al.*, 2012*a*).

body size of the '2-year' adults is a subset of the largest '1-year' individuals, implying that larger individuals within a cohort are more likely to successfully survive the energetically demanding period of prolonged dormancy of *R. cerasi* pupae. Nutrient utilization during pupae diapause is acknowledged as a dynamic process that affects energy reserves and could determine the diapause status (Hahn and Denlinger, 2007, 2011). Larger individuals are more likely to extend diapause for an additional year than smaller individuals due to size dependency in stored energy resources that can be metabolized during diapause (Menu and Desouhant, 2002). On the other hand, it is possible that body size could be decreased by delayed emergence since smaller individuals suffer higher metabolic costs resulting in greater resource consumption (Matsuo, 2006). For example, the rates of reduction in body size as a consequence of experimentally extended diapause were greater in smaller individuals of the beetle *Exechesops leucopis* (Matsuo, 2006). Adults emerging after three years were smaller than the

second-year group, but those emerged after 1 year were smaller than the '2-year' counterparts. Likewise, in our study, the 'prolonged chilling' adults were smaller than the '2-year' adults but larger than the '1-year' adults. With regard to the results of 'prolonged chilling' adults, further studies are needed both for getting a better insight on body size variability within a population and for providing a solid answer on whether larger *R. cerasi* pupae within a cohort are more likely to enter prolonged dormancy after being exposed to warm temperatures following insufficient chilling.

Effects of extended pupae dormancy on adult life history traits

Our results demonstrate that there is no variation at population-level in the trade-offs between prolonged pupae dormancy and fecundity for the three *R. cerasi* populations tested. It is therefore suggested that adults emerged after prolonged dormancy express a similar phenotype among the three populations that is characterized by large body size and a dramatically decreased lifetime fecundity estimated at approximately the 1/3 of the '1-year' counterparts. Temporal environmental variation is known to produce a type of generalist phenotype in insect populations that expresses moderately high fitness across environments, but not as high as specialist phenotypes that are matched to a particular environment (Condon *et al.*, 2014). The reduced lifetime fecundity could be attributed to physiological constraints due to accumulated metabolic resource loss during prolonged diapause (Sinclair, 2015). Indeed, the energy consumed during overwintering can be a proxy for the energy available for reproduction for insect species that have limited ability to replenish reserves post-winter, such as capital breeders, and/or adult body size is positively correlated (Sinclair, 2015). However, this does not seem to be the case for *R. cerasi* females that must feed to achieve reproductive maturity, and body size is not correlated to fecundity (in line with previous results Moraiti *et al.*, 2012*a*, 2012*b*). Another possible explanation could be the presence of genetic constraints because natural selection either acts antagonistically on pupae dormancy and adult life-histories simultaneously or acts only
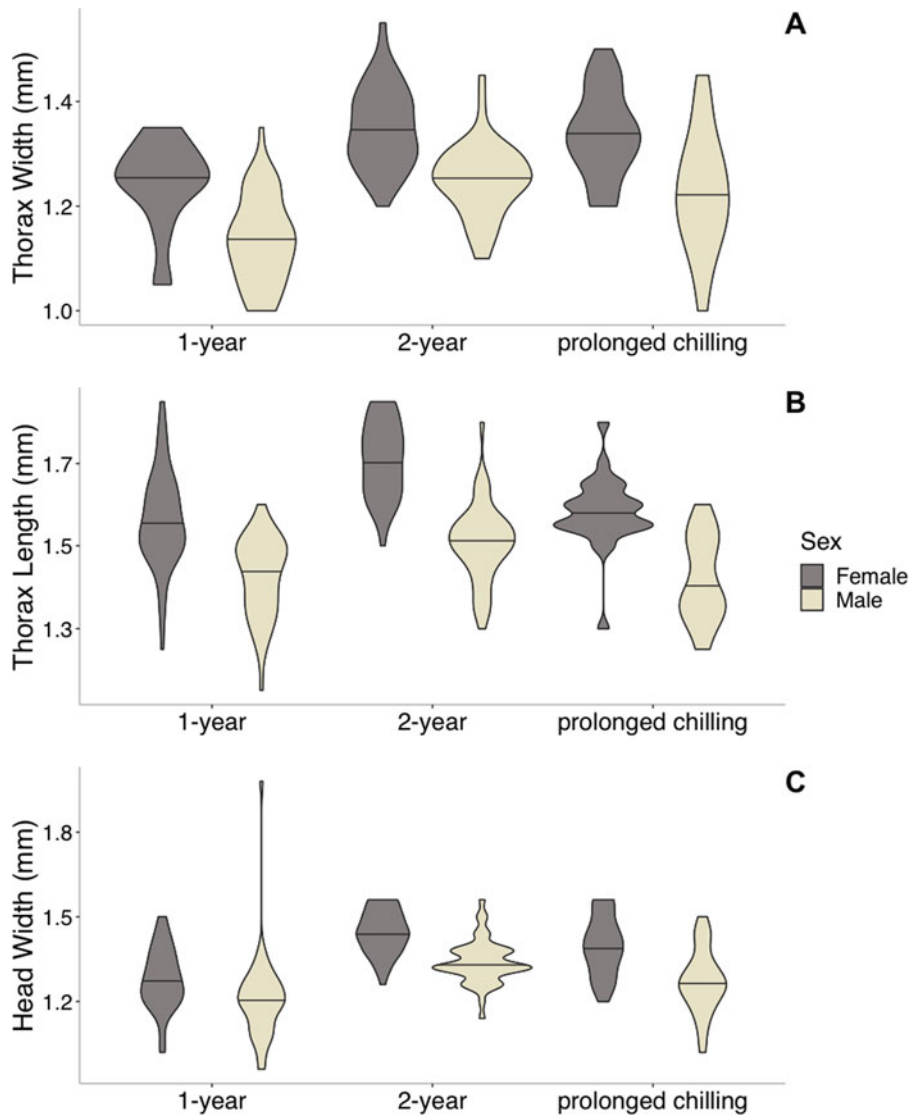
Figure 2. Violin plots of body size traits (a) thorax width, (b) thorax length, and (c) head width of *Rhagoletis cerasi* males and females emerged from pupae (a) with annual diapause (1-year), (b) prolonged dormancy (2-year), and (c) pupae that were exposed to cold for approximately 18 consecutive months (prolonged chilling). Pupae were obtained from Kamari (Thessaly). $N = 50$, 47 and 26 (males and females) for the 1-year, 2-year and prolonged chilling group, respectively.

Table 3. Results of the linear regression analysis for the effects of the type of dormancy and sex on body size of *Rhagoletis cerasi* adults from the Kamari population (Thessaly)

Factor	Wald χ^2 (df)	P	B (95% CI)
Type of dormancy (ref: prolonged chilling)	122.304 (2)	<0.001	
1-year	8.012 (1)	0.005	−0.475 (−0.804, −0.146)
2-year	8.737 (1)	0.003	0.501 (0.169, 0.834)
Sex (ref: male)	138.094 (1)	<0.001	1.157 (0.777, 1.537)
Type of dormancy × sex	1.598 (2)	0.450	
1-year × females	0.888 (1)	0.346	−0.224 (−0.690, 0.242)
1-year × males	0.000 (1)	0.989	0.003 (−0.467, 0.474)

Body size refers to the first principal component (PC1_Exp2). $N = 100$, 94 and 52 adults for the 1-year, 2-year and prolonged chilling group, respectively.

on one trait and there is a correlated response in the other trait because the two traits might be genetically linked (Schmidt *et al.*, 2005). Even though the genetic basis of this trade-off is less addressed, negative genetic correlation between diapause duration and post-diapause fecundity have been found in a spider

mite (Ito, 2007). Adult longevity, along with the type of dormancy, is affected by body size, but the correlations between body size and longevity was negative and weak (Supplementary Material, Fig. S4). Despite the fact that negative correlations themselves are difficult to interpret (Agrawal *et al.*, 2010), the lack of a

Table 4. Effects of the explanatory variables of the Cox proportional hazard model on adult longevity and the duration of reproductive periods of females emerged from pupae with annual diapause (1-year adults), prolonged dormancy (2-year adults) and after prolonged pupae chilling (prolonged chilling adults) from Kamari population (Thessaly)

Factor	Wald's χ^2 (df)	P	Exp (B) (95% CI)
Adult longevity			
Type of dormancy (ref: prolonged chilling)	10.974 (2)	0.004	
1-year	6.817 (1)	0.009	0.606 (0.416, 0.883)
2-year	9.884 (1)	0.002	0.558 (0.388, 0.803)
Sex (ref: male)	0.377 (1)	0.539	1.111 (0.794, 1.554)
Body size	0.414 (1)	0.520	1.068 (0.873, 1.307)
Preoviposition period			
Type of dormancy (ref: prolonged chilling)	2.137 (2)	0.344	
1-year	2.087 (1)	0.149	0.661(0.377, 1.160)
2-year	0.686 (1)	0.408	0.796 (0.463, 1.367)
Body size	0.343 (1)	0.558	0.919 (0.691, 1.221)
Oviposition period			
Type of dormancy (ref: prolonged chilling)	6.878 (2)	0.032	
1-year	6.553 (1)	0.010	0.492 (0.285, 0.847)
2-year	0.940 (1)	0.332	0.769 (0.452, 1.308)
Body size	0.132 (1)	0.717	0.949 (0.717, 1.257)
Post-oviposition period			
Type of dormancy (ref: prolonged chilling)	10.061 (2)	0.007	
1-year	3.716 (1)	0.054	1.703 (0.991, 2.927)
2-year	2.035 (1)	0.154	0.668 (0.384, 1.162)
Body size	0.941 (1)	0.332	1.151 (0.866, 1.531)

Body size refers to the first principal component (PC1_Exp2).
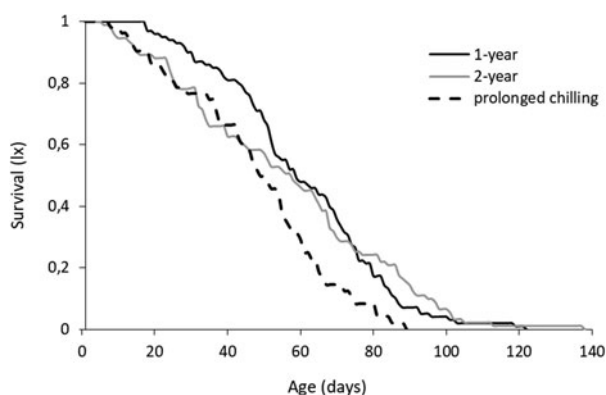

Figure 3. Age specific survival for *Rhagoletis cerasi* adults (males and females) emerged from pupae with annual diapause (1-year adults), (b) prolonged dormancy (2-year), and (c) pupae that were exposed to cold for approximately 18 consecutive months (prolonged chilling). Pupae were obtained from Kamari (Thessaly). *N* = 100, 94 and 52 adults for 1-year, 2-year and prolonged chilling group, respectively.

strong correlation implies that body size is weakly related to adult fitness, and hence is not a good proxy for *R. cerasi* longevity.

The longevity of adults emerged after prolonged pupae chilling was shorter than the '2-year' counterparts, and females expressed similar lifetime fecundity and reproductive periods. Considering all adults emerging from pupae with different dormancy pathways had *ad libitum* access to food (protein and sugar), increased protein intake may allow flies to compensate for resource depletion during extended pupae dormancy, resulting in an absence of variability in lifetime fecundity between the two groups. Despite the fact that physiological and molecular studies are needed to properly explain the cross stages effects of pupae diapause subjected to stressful conditions (such as prolonged chilling) on fitness traits of adults, this study is, to the best of our knowledge, the first that characterizes rare adult phenotypes in insect species with obligate diapause.

Population persistence under spatiotemporal environmental variation

Organisms follow a combination of different life-history strategies (specialization, generalization, phenotypic plasticity, bet-hedging, and dispersal) for coping with spatiotemporal variation in their habitats, instead of uniformly adopting a single strategy. Diversifying and conservative bet-hedging strategies can co-exist in the same trait in fluctuating environments (Haaland *et al.*, 2020), while plastic bet-hedging has been reported for an amphicarpic annual plant (Sedeh *et al.*, 2009) and a wolf spider (Rádai, 2021). We suggest that *R. cerasi* follow an adaptive integrated strategy for terminating diapause, with bet-hedging to be 'fine-tuned' by plasticity in response to environmental stimuli for
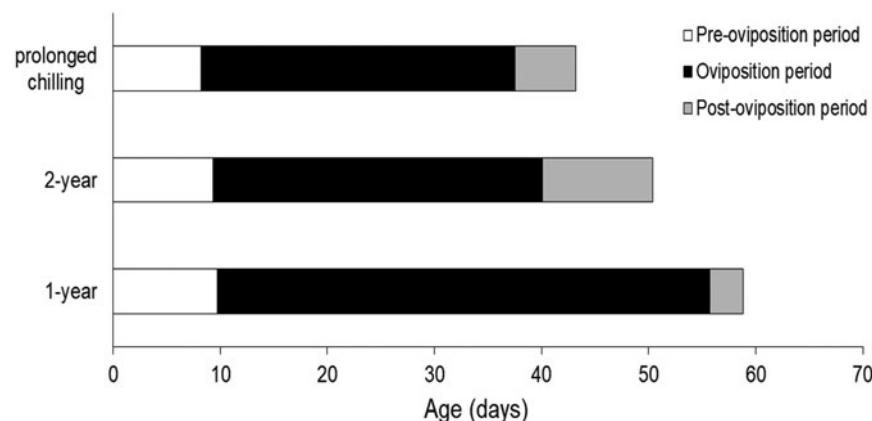
Figure 4. Average duration of reproductive periods of *Rhagoletis cerasi* females emerged from pupae (a) with annual diapause (1-year), (b) prolonged dormancy (2-year), and (c) pupae that were exposed to cold for approximately 18 consecutive months (prolonged chilling). Pupae were obtained from Kamari (Thessaly). N = 50, 44 and 25 for the 1-year, 2-year and prolonged chilling females, respectively.

Table 5. Results of the negative-binomial regression regarding the effects of the type of dormancy and body size on fecundity of *Rhagoletis cerasi* females from Kamari population (Thessaly)

Factor	Wald's χ^2 (df)	P	IRR (95% CI)
Type of dormancy (ref: prolonged chilling)	33.046 (2)	<0.001	
1-year	27.652 (1)	<0.001	3.916 (2.354, 6.513)
2-year	0.165 (1)	0.685	1.115 (0.659, 1.887)
Body size	1.107 (1)	0.293	1.167 (0.875, 1.556)

Body size refers to the first principal component (PC1_Exp2). N = 50, 44 and 25 females for the 1-year, 2-year and prolonged chilling group, respectively.

diapause termination. Different levels of plasticity in pupae development seems to be optimized under different environmental conditions. Pupae survival is facilitated by the repeated warm/cold periods (reliable cue) for terminating extended diapause, as opposed to prolonged chilling (unreliable cue). From a physiological perspective, we expected higher pupae mortality rates for the '2-year' than the 'prolonged chilling' individuals because low temperatures (0–5°C range) reduce the energetic cost of diapause (Hahn and Denlinger, 2007; Sinclair, 2015). Nonetheless, warm winters (>10°C constant temperature) (Moraiti *et al.*, 2012*a*) or warm spells (25°C) during cold winters (Moraiti & Papadopoulos, unpublished data) drive *R. cerasi* pupae to prolonged dormancy, instead of death. *Rhagoletis cerasi* might have developed physiological mechanisms to cope with warm conditions during dormancy, but not to deal with a possible accumulation of cold injuries and/or resource depletion under prolonged chilling conditions (Koštál *et al.*, 2006; Sinclair, 2015).

Adults emerging from *R. cerasi* pupae with prolonged dormancy expressed a similar phenotype (generalists) among populations, in contrast to those emerging from pupae with annual diapause that are tightly adapted to their local habitats (specialists) (Moraiti *et al.*, 2012*b*). The fundamental fitness components of the '2-year' adults are similar to adults emerging from pupae exposed to extreme environmental stress (prolonged chilling). Accordingly, a generalist phenotype is likely expressed by adults emerging from pupae with extended dormancy that determines adult performance, regardless of population and environmental conditions. Overall, persistence of *R. cerasi* populations in unpredictable environments is facilitated by a complex but flexible life history strategy.

Future studies on the physiological mitigation mechanisms for overwintering energy drain will provide much needed data to link prolonged dormancy, winter energetics, and pupae mortality with adult performance in *R. cerasi*. Even though uncovering the genomic basis of the life-history strategies of a non-model species remains challenging, performing assays from the omics spectrum will be useful for determining sub-organismal *R. cerasi* phenotypes under different temperature regimes (Dillon and Lozier, 2019). Linking physiology and functional genomics can thus be a valuable approach for teasing apart how *R. cerasi* populations expressing high levels of variability in diapause termination have adapted to local environmental heterogeneity.

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