

## **Threshold Temperatures and Thermal Requirements of *Psyllaphycus diaphorinae* (Hymenoptera: Encyrtidae), a Hyperparasitoid of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) and *Tamarixia radiata* (Hymenoptera: Eulophidae)**

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# Threshold temperatures and thermal requirements of *Psyllaphycus diaphorinae* (Hymenoptera: Encyrtidae), a hyperparasitoid of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) and *Tamarixia radiata* (Hymenoptera: Eulophidae)

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## Abstract

The temperature-driven development rates and longevity times of *Psyllaphycus diaphorinae* Hayat (Hymenoptera: Encyrtidae), a hyperparasitoid of *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae), 2 primary parasitoids of Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), were quantified. Development times and longevity were investigated at 6 average daily temperatures (15, 18, 20, 25, and 32 °C) that either were constant or fluctuated over 24 h. Thermal performance curves for egg-to-adult development of *P. diaphorinae* reared on *D. aligarhensis* were characterized using 1 linear and 2 nonlinear degree-d models (i.e., Ratkowsky and Performance-2). Daily thermal fluctuations had significant effects on development rates and longevity of *P. diaphorinae* adults. *Psyllaphycus diaphorinae* reared under fluctuating profiles completed development more slowly (20, 25, and 32 °C), and survived longer (18–32 °C) than those reared under constant profiles with equivalent mean temperatures. In addition, *P. diaphorinae* emerged from fluctuating but not constant profiles at 15 °C. Estimates of thermal constants (i.e., degree-d to complete development) predicted by the linear model were 36% lower for the constant temperature regimen than the fluctuating temperature regimen. Nonlinear and linear model estimations of the theoretical lower and upper thermal thresholds ranged between –3.7 to 11.9 °C and 33.7 to 34 °C, respectively. The development rate of male and female *P. diaphorinae* was most favorable at 30 °C. These results demonstrate the significant effects of average temperatures and daily temperature variations on *P. diaphorinae* life history.

Key Words: biological control; development rate; *Diaphorina citri*; nonlinear regression model; thermal threshold

## Resumen

Se cuantificaron las tasas de desarrollo impulsadas por la temperatura y el tiempo de longevidad de *Psyllaphycus diaphorinae* Hayat (Hymenoptera: Encyrtidae), un hiperparasitoide de *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) y *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae) dos parasitoides primarios del psílido asiático de los cítricos, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). Se investigaron el tiempo de desarrollo y la longevidad a 6 temperaturas promedias diarias (15, 18, 20, 25 y 32 °C) que fueron constantes o fluctuaron durante 24 horas. Se caracterizaron las curvas de rendimiento térmico para el desarrollo de huevo a adulto de *P. diaphorinae* criados sobre *D. aligarhensis* utilizando 1 modelo lineal y 2 modelos no-lineales días-grados (Ratkowsky y Performance-2). Las fluctuaciones térmicas diarias tuvieron efectos significativos en las tasas de desarrollo y la longevidad de los adultos de *P. diaphorinae*. *Psyllaphycus diaphorinae* criado bajo perfiles fluctuantes completó el desarrollo más lentamente (20, 25 y 32 °C), y sobrevivió más tiempo (18–32 °C) que aquellos criados bajo perfiles constantes con temperaturas medias equivalentes. Además, *P. diaphorinae* emergió de perfiles fluctuantes pero no constantes a 15 °C. Las estimaciones de las constantes térmicas (días-grados para completar el desarrollo) predichas por el modelo lineal fueron 36% más bajas para el régimen de temperatura constante que el régimen de temperatura fluctuante. Las estimaciones de modelos lineales y no lineales de los umbrales térmicos inferiores y superiores teóricos oscilaron entre –3,7 y 11,9 °C y entre 33,7 y 34 °C, respectivamente. La tasa de desarrollo de los machos y hembras de *P. diaphorinae* fue más favorable a 30 °C. Estos resultados demuestran los efectos significativos de las temperaturas medias y las variaciones diarias de temperatura en la historia de vida de *P. diaphorinae*.

Palabras Clave: control biológico; tasa de desarrollo; *Diaphorina citri*; modelo de regresión no lineal; umbral termal

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Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), was first detected in California, USA, in 2008 in backyard citrus in San Diego County (Grafton-Cardwell et al. 2013). Populations of this invasive pest expanded rapidly across southern California. In 2014, *D. citri* had reached the San Joaquin Valley, where about 77% of California citrus is grown (USDA NASS 2017), with isolated finds being reported as far north as San Francisco (Milosavljević et al. 2017a; CDFA 2019; Milosavljević & Hoddle 2019). The invasion of *D. citri* in California is problematic because this psyllid vectors *Candidatus Liberibacter asiaticus* Jagoueix et al. (Rhizobiaceae), a phloem-limited bacterium which is the causative agent of a lethal citrus disease, huanglongbing (Gottwald 2010; Milosavljević et al. 2018). Acknowledged as one of the greatest threats to global citrus production (Grafton-Cardwell et al. 2013), *Candidatus Liberibacter asiaticus* causes irregular fruit ripening and premature fruit drop, which result in significant yield reductions (Durborow 2012; Spreen et al. 2014). To date there have been over 1,070 cases of confirmed *Candidatus Liberibacter asiaticus*-infected citrus in California, all of which have been restricted to backyard citrus trees in Los Angeles, Orange, Riverside, and San Bernardino counties (David Morgan, CDFA, personal communication).

In an effort to protect California's \$3-billion-per-yr citrus industry (USDA NASS 2017; Babcock 2018) from *Candidatus Liberibacter asiaticus* spread, programs to reduce vector densities in urban areas were undertaken (Hoddle & Pandey 2014). One mitigation approach was the initiation in 2010 of a classical biological control program targeting populations of *D. citri* in urban areas. The first step of this program was foreign exploration in Punjab, Pakistan, part of the home range of *D. citri*, in an effort to locate host specific natural enemies for potential use in California (Hoddle 2012). Collections of parasitized *D. citri* nymphs resulted in the return of 13 parasitoid species to the University of California-Riverside, Insectary and Quarantine Facility, Riverside, California, USA, including *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), *Diaphorencyrtus aligarhensis* (Shafee, Alam & Argarwal) (Hymenoptera: Encyrtidae), and *Psyllaphycus diaphorinae* Hayat (Hymenoptera: Encyrtidae) (Triapitsyn et al. 2013; Hoddle et al. 2014). *Tamarixia radiata* and *D. aligarhensis* are primary parasitoids of *D. citri* (Hoddle & Pandey 2014; Bistline-East et al. 2015), and both species have been used worldwide in classical biological control programs targeting *D. citri* (Halbert & Manjunath 2004; Milosavljević et al. 2017a). *Psyllaphycus diaphorinae*, initially described as an uncommon primary parasitoid of *D. citri* (Hayat 1972; Mehrnejad & Emami 2005), was evaluated in quarantine as a candidate natural enemy species to control *D. citri* in California. However, biology studies revealed that *P. diaphorinae* was an obligate hyperparasitoid of *D. aligarhensis* and *T. radiata* (Bistline-East & Hoddle 2016).

In quarantine, *D. aligarhensis* was identified as a preferred host for *P. diaphorinae* when compared to *T. radiata* (Bistline-East & Hoddle 2016). The Pakistani population of *D. aligarhensis* is a bi-parental solitary endoparasitoid that has been mass produced and released in California for classical biological control of *D. citri* (Milosavljević et al. 2017a, 2019a). *Diaphorencyrtus aligarhensis* parasitizes second through fourth instar *D. citri* nymphs, and provides additional mortality through host feeding (Rohrig 2010; Rohrig et al. 2011; Hoddle et al. 2014; Bistline-East et al. 2015; Vankosky & Hoddle 2017a, b). *Psyllaphycus diaphorinae* preferentially parasitizes the pupal stage of *D. aligarhensis* (Bistline-East & Hoddle 2016). Aspects of the basic biology and life history of *P. diaphorinae* have been studied in quarantine (Bistline-East & Hoddle 2016), but the effects of temperature on the development and adult longevity of this hyperparasitoid have not been previously reported.

Temperature profoundly affects insect growth, population dynamics, and species distributions (Gilbert & Raworth 1996; Obrycki & Kring

1998; Jalali et al. 2009; Koda & Nakamura 2010; Marchioro & Forester 2011; Nooten et al. 2014; Milosavljević et al. 2016, 2017b, 2019a, b; Schowalter 2016; Damos et al. 2018). For poikilotherms, increasing temperature can have significant effects on life history parameters such as development time, lifespan, and reproduction rates (Roy et al. 2002; Kontodimas et al. 2004, 2007; Broatch et al. 2006; Logan et al. 2006; Pilkington & Hoddle 2006a, 2007a; Zamani et al. 2007; Pilkington et al. 2014; Luhning & DeLong 2016). Life history parameters of many arthropod species have been quantified from studies performed at constant temperatures (Pilkington & Hoddle 2006b, 2007b; Quinn 2017). However, field temperatures fluctuate over time and the effects of fluctuating temperatures on immature development, and adult survivorship, and fecundity rates of arthropods are likely to be of more ecological significance when compared to the effects of constant temperatures (Lyons et al. 2013; Colinet et al. 2015; Wu et al. 2015).

To investigate the effects of thermal dynamics on *P. diaphorinae*, we compared the development rates and longevity of male and female parasitoids reared under 5 average daily temperatures (15, 18, 20, 25, and 32 °C) that were either constant or fluctuated over 24 h. The thermal thresholds for male and female *P. diaphorinae* males and females were estimated using 1 linear and 2 nonlinear (Performance-2 and Ratkowsky) temperature-dependent models. Models were evaluated based on the goodness-of-fit to observed development rates for constant and fluctuating temperatures, and biological relevance of the theoretical lower and upper development threshold estimations. The results of these studies are presented here.

## Materials and Methods

### MATERIAL SOURCES FOR EXPERIMENTS

*Diaphorina citri* nymphs used as hosts for *D. aligarhensis* development were obtained from *Candidatus Liberibacter asiaticus*-free colonies maintained at the University of California-Riverside, Insectary and Quarantine Facility, Riverside, California, USA (Hoddle & Pandey 2014; Bistline-East et al. 2015; Bistline-East & Hoddle 2016). The initial population of *Candidatus Liberibacter asiaticus*-free *D. citri* were collected from backyard citrus trees in Riverside and Los Angeles counties, California, USA (California Department of Food and Agriculture permit number 2870). *Diaphorina citri* colonies were reared on 1.5-yr-old *Citrus volkameriana* V. Tenore & Pasquale (Rutaceae) plants at 27 ± 2 °C, a 14:10 h (L:D) photoperiod, and 40 ± 5% relative humidity (RH), in riser cages (15 × 15 × 30 cm [W × D × H]) constructed from 2 U-shaped acrylic risers (S&W Plastics F2191, Riverside, California, USA) with the back covered with no-see-um netting (Skeeta, Bradenton, Florida, USA), and the front covered with a 30 cm long no-see-um sleeve to allow access into cages (Bistline-East et al. 2015). Plants were grown in greenhouses at the University of California-Riverside, Agricultural Operations (27 ± 2 °C, 50 ± 6% RH, and natural sunlight) in 0.5 L pots with University of California-Riverside Type III potting soil mix, with daily watering, and Osmocote Pro 19:6:12 granular fertilizer (The Scotts Company LLC, Marysville, Ohio, USA) applied every 3 mo. Plants were pruned regularly until new shoots (i.e., flush growth) emerged; *D. citri* requires newly developing young citrus foliage for oviposition and nymph development (Milosavljević et al. 2018).

To establish experimental cohorts of *D. citri* nymphs on which to raise *D. aligarhensis* (and subsequently *P. diaphorinae*), individual citrus plants were isolated in riser cages and transferred from greenhouses to University of California-Riverside, Insectary and Quarantine Facility for *D. citri* inoculation. Twenty non-sexed *D. citri* adults were introduced into each riser cage, and females were allowed to mate

and lay eggs for 8 to 10 d before removal. *Diaphorina citri* nymphs were left to develop to second through fourth instars, which required an additional 7 to 10 d at  $27 \pm 2$  °C,  $40 \pm 5\%$  RH, and a 14:10 h (L:D) photoperiod.

*Diaphorencyrtus aligarhensis* and *P. diaphorinae* adults used for experiments were taken from colonies established in University of California-Riverside, Insectary and Quarantine Facility. These colonies originally were established from specimens reared from parasitized *D. citri* nymphs collected from citrus orchards in Punjab, Pakistan (USDA-APHIS permit number P526P-11-00103; Hoddle 2012; Bistline-East & Hoddle 2016). *Diaphorencyrtus aligarhensis* were reared on potted *C. volkameriana* infested with second through fourth instar *D. citri* nymphs and kept in  $15 \times 15 \times 30$  cm (W  $\times$  D  $\times$  H) riser cages at  $27 \pm 2$  °C,  $40 \pm 5\%$  RH, and a 14:10 h (L:D) photoperiod following the procedure described above (Bistline-East et al. 2015). *Psyllaphycus diaphorinae* were reared on *D. aligarhensis* pupae developing inside parasitized *D. citri* nymphs, and *P. diaphorinae* colonies were maintained under similar conditions as described above for *D. aligarhensis* (Bistline-East & Hoddle 2016). Newly emerged *D. aligarhensis* and *P. diaphorinae* adults were collected daily from colonies, sexed, labeled, and held in individual 200  $\mu$ L O-ring centrifuge tubes (Microwtube brand, Simport Scientific Inc., Vancouver, British Columbia, Canada) provisioned with honey droplets and maintained at 16 °C and a 4:10 h (L:D) photoperiod until used for experiments. Single female *D. aligarhensis* and *P. diaphorinae* were confined with 2 males of the same species for 24 h within each vial to ensure they were mated before being used for experiments.

#### EFFECTS OF TEMPERATURE ON HYPERPARASITOID DEVELOPMENT AND LONGEVITY

The effects of 5 fluctuating temperatures that averaged 15, 18, 20, 25, and 32 °C, over 24 h on the development times, and longevity of male and female *P. diaphorinae* were quantified and compared to 5 corresponding temperatures that were held constant over 24 h. At the beginning of experiments, individual citrus plants infested with second through fourth instar *D. citri* nymphs were chosen randomly from the stock colony and placed inside riser cages at 27 °C, 40% RH, and a 14:10 h (L:D) photoperiod. To obtain a synchronous cohort of hosts for *P. diaphorinae* parasitism, 10 honey-fed male and 5 female *D. aligarhensis* were released from O-ring centrifuge tubes into each riser cage, and females were allowed to oviposit for 3 d. Thereafter, adults were removed, and *D. aligarhensis* larvae were left to develop into pupae, which required approximately 8 to 10 d (Bistline-East & Hoddle 2016), after which they were exposed to *P. diaphorinae*.

After 8 to 10 d, developing *D. aligarhensis* were exposed to 5 male and 15 female *P. diaphorinae* that were released into each riser cage, and females were allowed to oviposit for 24 h before removal. Immediately after removing *P. diaphorinae*, the cages with plants and *D. aligarhensis* pupae exposed to *P. diaphorinae* were placed into growth chambers (model I-30BLL; Percival Scientific, Perry, Indiana, USA) set to 1 of the 10 experimental temperature regimens described above (Table 1). For constant temperature regimens, growth chambers were programmed to hold the target temperature static ( $\pm 0.2$  °C) for the entirety of the study. For fluctuating temperature regimens, growth chambers were programmed with 9 temperature steps to incrementally increase and decrease temperatures around target temperature averages. The mean values of fluctuating temperatures were equivalent to the corresponding constant temperatures ( $\pm 0.2$  °C) over a 24 h period. Each fluctuating temperature profile was modeled after field recorded temperatures that had the desired average target temperature (Table 1; CIMIS 2018). Thus, with respect to fluctuating tempera-

**Table 1.** Stepwise settings used in temperature cabinets for fluctuating temperature regimens calculated from an average of 2 yr of data (Jan 2013–Dec 2014) from the California Irrigation Management Information System (station #44) weather station at the Riverside Citrus Experiment Station, Riverside, California, USA.

Hour	Mean temperature (°C)					Photoperiod
	15	18	20	25	32	
1:00 AM	13	13	15	20	23	Dark
2:00 AM	11	13	15	20	23	
3:00 AM	11	10	15	20	23	
4:00 AM	11	10	13	17	23	
5:00 AM	9	10	13	17	21	
6:00 AM	9	10	13	17	21	
7:00 AM	9	10	15	20	26	Light
8:00 AM	12	15	15	20	26	
9:00 AM	12	15	20	25	34	
10:00 AM	17	20	20	25	34	
11:00 AM	17	20	20	29	38	
12:00 PM	19	23	24	29	38	
1:00 PM	21	23	24	33	41	Dark
2:00 PM	21	26	27	33	41	
3:00 PM	21	26	27	33	41	
4:00 PM	21	26	27	33	41	
5:00 PM	19	24	27	33	40	
6:00 PM	19	24	25	29	40	
7:00 PM	19	24	25	29	40	
8:00 PM	15	22	25	26	35	
9:00 PM	15	22	20	26	35	
10:00 PM	13	17	20	23	28	
11:00 PM	13	17	18	23	28	
12:00 AM	13	13	18	20	28	

ture regimens, these temperatures represent realistic thermal exposure scenarios (McCalla et al. 2019; Milosavljević et al. 2019a).

The incremental temperature increase and decrease steps for fluctuating temperature regimens were modeled over 2 yr (Jan 2013–Dec 2014) of daily temperature data from a meteorological station located at the Riverside Citrus Experiment Station, Riverside, California, USA (CIMIS 2018). Daily temperature averages within 0.2 °C of each of the target constant temperatures were selected for each respective time period and date for each yr. After initial selection, hourly air temperatures (compiled from CIMIS 2018) for each selected d were averaged per h for every target mean temperature. These data were used to program temperature fluctuations within chambers over a 24 h period that when averaged were equivalent to the target mean temperature (Table 1). Target temperature regimens and relative humidity in each of the chambers were confirmed using HOBO Pro V2 Temperature/RH loggers (Onset Computer, Pocasset, Massachusetts, USA) set to record temperature and humidity every 15 min. The photoperiod setting for all experiments was 14:10 h (L:D), with light intensity of  $100 \mu\text{E m}^{-2} \text{s}^{-1}$ . All growth chambers were maintained at  $45 \pm 5\%$  RH regardless of the experimental temperature regimen, which was the observed average relative humidity for selected time periods in Riverside, California, USA (CIMIS 2018).

Four riser cages were assigned to each of 10 temperature regimens to provide sufficient development data for a combined minimum of 40 emerged adult male and female *P. diaphorinae*. Cages were rotated daily within incubators to counteract potential thermal gradients (see Ashmore & Janzen 2003 for more details). Riser cages were inspected for adult *P. diaphorinae* that emerged from initial *D. aligarhensis* cohorts within each temperature profile, beginning 7 d

after experimental trials were initiated. Subsequent checks for adult emergence at every experimental temperature were performed daily, and inspections continued for 14 consecutive d after the last recorded emergence in each cage. Emerged hyperparasitoids were isolated daily into individual 200  $\mu$ L O-ring centrifuge tubes (Mircrowtube brand, Simport Scientific Inc., Vancouver, British Columbia, Canada), sexed, labeled, and maintained in their respective incubators. All vials were provisioned with raw honey droplets as a source of carbohydrates, and replenished on alternating d for the duration of the experiment. Emerged male and female *P. diaphorinae* were monitored at 24-h intervals until death to determine adult longevity under each temperature regimen. Adult *P. diaphorinae* that died prematurely (e.g., trapped in honey droplets) were removed from statistical analyses.

No development was observed from cages of *P. diaphorinae* maintained at constant 15 °C. Sixty d after exposure to *P. diaphorinae*, 10 *D. aligarhensis* pupae were harvested randomly from riser cages held at constant 15 °C and dissected under a dissecting microscope (Leica MZ12.5, Meyer Instruments, Houston, Texas, USA); 8 of the 10 pupae contained live larvae. An additional 10 *D. aligarhensis* mummies were then collected and reared at 27 °C, 40% RH, and a 14:10 h (L:D) photoperiod to identify the species (i.e., *D. aligarhensis* or *P. diaphorinae*). After 7 d at 27 °C, 8 adult *P. diaphorinae* emerged from the 10 harvested *D. aligarhensis* mummies. The remaining 2 mummies were considered dead when no emergence occurred 4 wk after the initiation of the species identity test. The remaining *D. aligarhensis* exposed to *P. diaphorinae* and held at constant 15 °C were maintained for 90 d post-exposure. No hyperparasitoids emerged, and replicates at this temperature were discontinued (Table 2).

## STATISTICAL ANALYSES

To analyze whether *P. diaphorinae* development and longevity were affected by temperature (treated as a continuous variable), each temperature profile (i.e., 15, 18, 20, 25, or 32 °C) was considered separately. For each temperature profile, a 1-way analysis of variance (ANOVA) was performed using the 4 treatment combinations (i.e., females constant, females fluctuating, males constant, and males fluctuating) in SAS (2009). Response variables were log-transformed as necessary prior to analysis of variance to meet assumptions of normality and homoscedasticity. If the analysis of variance was significant at the 0.01 alpha level, individual pairwise comparisons were made for gender within regimens (i.e., comparing females vs. males for fluctuating and constant regimens separately) as well as regimen within gender (i.e., comparing fluctuating vs. constant for females and males separately), a total of 4 comparisons were made, between treatment means using the DIFF option in the LSMEANS statement of the GLM procedure in SAS. For all experimental temperatures, there were no significant

differences between the development rates of male and female *P. diaphorinae*, and the values were pooled for subsequent model fitting (see Results; Tables 2 & 3).

## MATHEMATICAL MODELS

To assess the relationship between temperature and development rate ( $r = 1/d$ , where  $d$  is mean development time in d) of male and female *P. diaphorinae* under constant and fluctuating temperature regimens, 1 linear (Campbell et al. 1974) and 7 nonlinear (i.e., Beta [Yin et al. 2003; Auzanneau et al. 2011; Shi et al. 2015], Lactin-2 [Logan et al. 1976; Lactin et al. 1995], Brière-2 [Brière et al. 1999], Performance-2 [Shi et al. 2011; Wang et al. 2013], Lobry-Rosso-Flandrois [Lobry et al. 1991; Rosso et al. 1993], Weibull [Angilletta 2006], and Ratkowsky [Ratkowsky et al. 1983]) development rate models were fit to datasets. Temperatures for which no *P. diaphorinae* emergence was recorded were not used for model fitting.

Linear regression was used to calculate thermal constants (i.e., degree-d necessary for completion of development), theoretical lower development thresholds (i.e.,  $T_{min}$ ), and adjusted coefficient of determination ( $R^2_{adj}$ ) was used as a measure of model goodness-of-fit. Linear models with high values of  $R^2_{adj}$  indicated a good fit to the data for each response variable (Table 3; Campbell et al. 1974). The linear relationship between development rate of *P. diaphorinae* and temperature was assessed for each response variable using the PROC REG procedure in SAS (2009). In each analysis, Cook's  $D$  metric was calculated to identify influential outliers with a critical value of Cook's  $D > 4/n$ , where  $n$  represents the number of observations (Bollen & Jackman 1990). Once identified, outliers exhibiting large externally studentized residuals and leverage (i.e., greater than 3 standard deviations away) were removed from analyses. For the linear model, temperatures equal to or less than 25 °C (i.e., 18, 20, and 25 °C) were used in analyses because these data points aligned with the linear portion of the dataset.

Development rate data of *P. diaphorinae* were used to parameterize nonlinear models (Table 3). Of the 7 nonlinear models evaluated, the Beta, Lactin-2, Brière-2, Lobry-Rosso-Flandrois, and Weibull models failed to fit to development rate data for *P. diaphorinae* (see Results section) regardless of temperature regimen, and were removed from further analyses. The performance of the remaining 2 nonlinear models, Performance-2 and Ratkowsky, were evaluated based on the goodness-of-fit to observed data (i.e., residual sum of squares [RSS]), and the biological relevance of model estimations (i.e.,  $T_{min}$  [lower theoretical temperature bound for development],  $T_{opt}$  [optimal temperature for development], and  $T_{max}$  [upper theoretical temperature bound for development]). The 2 nonlinear models tested have 4 parameters and thus, the same degrees of freedom [ $df$ ], and 2 'conceptual' lower (i.e.,  $T_{min}$ ) and upper development (i.e.,  $T_{max}$ ) thresholds (Mirhosseini et al. 2017; Ratkowsky & Reddy 2017).

**Table 2.** Mean development time in d ( $\pm$  SE) for female and male *Psyllaphycus diaphorinae* reared under constant and fluctuating temperatures.

	Development time (mean d $\pm$ SE)				
	15 °C	18 °C	20 °C	25 °C	32 °C
<b>Female</b>					
Constant	No emergence	29.94 $\pm$ 0.63 <sup>a</sup> (20)	22.62 $\pm$ 0.49 <sup>bc</sup> (74)	14.05 $\pm$ 0.05 <sup>b</sup> (24)	10.23 $\pm$ 0.23 <sup>b</sup> (31)
Fluctuating	37.19 $\pm$ 0.58 <sup>a</sup> (43)	31.30 $\pm$ 0.65 <sup>a</sup> (53)	24.77 $\pm$ 1.01 <sup>a</sup> (15)	19.25 $\pm$ 1.41 <sup>a</sup> (18)	14.46 $\pm$ 0.40 <sup>a</sup> (31)
<b>Male</b>					
Constant	No emergence	33.11 $\pm$ 0.73 <sup>a</sup> (36)	20.69 $\pm$ 0.36 <sup>c</sup> (55)	14.14 $\pm$ 0.14 <sup>b</sup> (19)	10.00 $\pm$ 0.26 <sup>b</sup> (29)
Fluctuating	35.31 $\pm$ 0.32 <sup>a</sup> (46)	29.94 $\pm$ 0.71 <sup>a</sup> (38)	22.64 $\pm$ 0.60 <sup>ab</sup> (25)	15.39 $\pm$ 0.94 <sup>a</sup> (31)	13.71 $\pm$ 0.35 <sup>a</sup> (21)

Means followed by the same letter within the same temperature are not significantly different at  $\alpha = 0.05$ ; (n) denotes number of individuals. SEs are based upon the pooled variance and the individual sample sizes.

**Table 3.** Mathematical models and parameter estimates for 3 performance functions describing the relationship between development rate ( $D_r$ ) and temperature for *Psyllaphycus diaphorinae* reared under constant and fluctuating temperature regimes.

Model	Model equation	Parameter	Parameter estimate		Reference
			Constant	Fluctuating	
Ordinary linear	$D_r = a + bT$ $K = 1/b$ $T_{min} = -a/b$	$a$	0.0055	0.0035	Campbell et al. 1974
		$b$	-0.0655	-0.0302	
		$K$	181.81	285.71	
		$T_{min}$	11.91	8.63	
		$R^2_{adj}$	0.9897	0.9898	
Performance-2	$D_r = b(T - T_{min})(1 - e^{c(T - T_{max})})$	$b$		0.0036	Shi et al. (2011);
		$c$		2.2256	
		$T_{min}$		8.03	Wang et al. (2013)
		$T_{max}$	No convergence	33.78	
		$T_{opt}$		30.01	
		residual sum of squares			0.00002
Ratkowsky	$\sqrt{D_r} = b(T - T_{min})(1 - e^{c(T - T_{min})})$	$b$		0.0087	Ratkowsky et al. (1983)
		$c$		2.0992	
		$T_{min}$		-3.74	No convergence
		$T_{max}$		33.95	
		$T_{opt}$		30.09	
		residual sum of squares			0.00008

See the corresponding references for a complete description of these models and their parameters.

The coefficient of determination ( $R^2$ ) and its adjusted value ( $R^2_{adj}$ ), although commonly used in the literature to describe the fit of nonlinear models to development data, were not used to assess goodness-of-fit for nonlinear models because they do not describe accurately the validity of a nonlinear fit to data (see Spiess and Neumeier 2010 for further explanation). Instead, nonlinear model goodness-of-fit was assessed with the residual sum of squares (Shi et al. 2015; Ratkowsky & Reddy 2017; McCalla et al. 2019; Milosavljević et al. 2019a):

$$RSS = \sum_{i=1}^n (y_i - \hat{y}_i)^2$$

where  $n$  represents the number of observations and denote the observed and expected development rate at the  $i$ -th temperature, respectively. Nonlinear models with small values of the residual sum of squares (e.g.,  $RSS < 0.001$ ) indicated a good fit to the data.

The Performance-2 model was fit with development rate in its untransformed form as the response variable, because data satisfied homogeneity assumptions (see Ratkowsky 2004 for more details). The Ratkowsky model was fitted with the square root of development rate as the response variable, because no statistical comparisons were made with the Performance-2 model (Ratkowsky & Reddy 2017). All temperature-based development models were fit for each response variable using the PROC NLIN procedure in SAS (2009), and graphed in SigmaPlot vers. 12.0 (Systat 2008).

## Results

### EFFECT OF TEMPERATURE AND REGIMEN TYPE ON HYPERPARASITOID DEVELOPMENT

*Psyllaphycus diaphorinae* males and females completed egg-to-adult development under experimental temperature profiles from 18 to 32 °C in the constant regime and 15 to 32 °C in the fluctuating re-

gime (Table 2). Average temperature and regimen type (i.e., constant or fluctuating) significantly affected mean development time (regimen effects:  $P < 0.001$  for all temperature profiles; Table 2). Nevertheless, male and female hyperparasitoids responded equivalently to the temperature treatments (gender effects:  $P > 0.44$  for all temperature profiles; Table 2). Egg-to-adult development time of *P. diaphorinae* decreased with increasing temperature to a minimum of  $10.00 \pm 0.26$  d (males) and  $10.23 \pm 0.23$  d (females) at 32 °C constant and  $13.71 \pm 0.35$  d (males) and  $14.46 \pm 0.40$  d (females) at 32 °C fluctuating (Table 2). Hyperparasitoids completed development faster at constant 20 to 32 °C than the corresponding fluctuating temperatures (Table 2), and development times did not vary between constant and fluctuating temperature regimens at 18 °C (Table 2).

For the linear and 2 nonlinear (i.e., Ratkowsky and Performance-2) models fitted to *P. diaphorinae* development rate data parameter,  $R^2_{adj}$  (linear models), and residual sum of squares (nonlinear models) estimates are presented in Table 3. The linear model had  $R^2_{adj}$  values  $> 0.98$  for both constant and fluctuating temperature regimens (Table 3). The Beta, Lactin-2, and Brière-2, Lobry-Rosso-Flandrois, and Weibull nonlinear models failed to converge for both constant and fluctuating datasets, a finding which could be partially attributed to the limited number of experimental temperatures being evaluated. Therefore, these models were removed from further consideration. The remaining 2 nonlinear models, Ratkowsky and Performance-2, had small residual sum of squares values ( $< 0.00008$ ) for fluctuating temperature regimens, indicating a good fit to datasets (Table 3). For constant temperatures, however, Ratkowsky and Performance-2 both failed to converge, and again this may have been a consequence of too few experimental temperatures being evaluated.

Linear regression estimates indicated the total development time in degree d for immature *P. diaphorinae* was significantly lower (i.e., 36% lower) under the constant temperature regimen than the corresponding fluctuating regimen (Table 3; Fig. 1). The Ratkowsky and Performance-2 models fit to fluctuating temperature data estimated similar values for the optimum ( $[T_{opt}]$ : about 30 °C) and upper ( $[T_{max}]$ :

about 34 °C) development temperatures for *P. diaphorinae* females and males (Table 3). The theoretical critical lower temperatures ( $T_{min}$ ) above which development of female and male *P. diaphorinae* occurs were not estimated equally by the linear and 2 nonlinear models. For the fluctuating dataset, estimates of  $T_{min}$  for the Ratkowsky model (−3.74 °C) were lower than those estimated by the linear (8.63 °C) and Performance-2 (8 °C) models (Table 3; Fig. 1).

#### EFFECT OF TEMPERATURE AND REGIMEN TYPE ON HYPERPARASITOID LONGEVITY

Mean longevity of *P. diaphorinae* adults was significantly affected by average temperature and thermal variation (regimen effect:  $P < 0.001$  for all temperature profiles; Table 4). Maximum longevity of  $56.08 \pm 2.66$  d (males) and  $83.55 \pm 2.89$  d (females) was observed at 15 °C fluctuating and  $41.20 \pm 1.15$  d (males) and  $52.72 \pm 4.81$  d (females) at 18 °C constant (Table 4). Above these temperatures, longevity steadily decreased with rising temperature, reaching minimums of  $2.29 \pm 0.28$  d (males) and  $7.61 \pm 0.74$  d (females) at 32 °C fluctuating and  $11.57 \pm 1.63$  d (males) and  $14.15 \pm 1.58$  d (females) at 32 °C constant. Despite the similarity in overall trend, average lifespan duration was significantly different between the 2 genders (gender effect:  $P < 0.001$  for all temperature profiles) across all experimental temperatures except fluctuating 20 °C and constant and fluctuating 25 °C (Table 4). The magnitude of this effect was greatest near the lower and upper extremes of experimental temperatures (Table 4). No significant temperature effects were observed between genders at 25 °C. At intermediate temperatures, *P. diaphorinae* females and males reared under fluctuating profiles completed development more slowly (20 and 25 °C) and survived longer (18–25 °C) than those reared under constant temperatures with equivalent means (Table 4). In comparison with constant regimens, fluctuating regimens produced *P. diaphorinae* males with a lifespan approximately 5 times shorter and *P. diaphorinae* females with a lifespan approximately 2 times shorter at 32 °C.

## Discussion

Relatively few studies have examined the effects of temperature on the development of hyperparasitoids (Yeagan & Braman 1989 [Hymenoptera: Ichneumonidae]; Kfir et al. 1993 [Hymenoptera: Eulophidae]; Brodeur & McNeil 1994 [Hymenoptera: Pteromalidae]; Rice & Allen 2009 [Hymenoptera: Ichneumonidae]) because the majority of studies of importance to biological control programs focus on primary parasitoid species. Additionally, experiments with hyperparasitoids are costly in labor and time because up to 4 (i.e., plant, primary host, primary parasitoid, and hyperparasitoid) interacting communities across multiple trophic levels need rearing and management. The present study is the first to evaluate and demonstrate the significant effects of different temperature exposure regimens on development and longevity of *P. diaphorinae*, a hyperparasitoid of 2 primary parasitoids, *D. aligarhensis* (host used here) and *T. radiata*, both primary parasitoids of *D. citri* in Punjab Pakistan.

Development times for female and male *P. diaphorinae* reared under constant and fluctuating temperatures decreased with increasing temperatures, regardless of temperature regimen, and both genders responded equivalently to the temperature treatments. *Psyllaphycus diaphorinae* completed its development across all of experimental temperature profiles, except at constant 15 °C (i.e., lowest experimental temperature examined in this study) where no adult emergence occurred (Table 2). Therefore, fluctuating temperatures allowed successful development of both male and female hyperparasitoids over

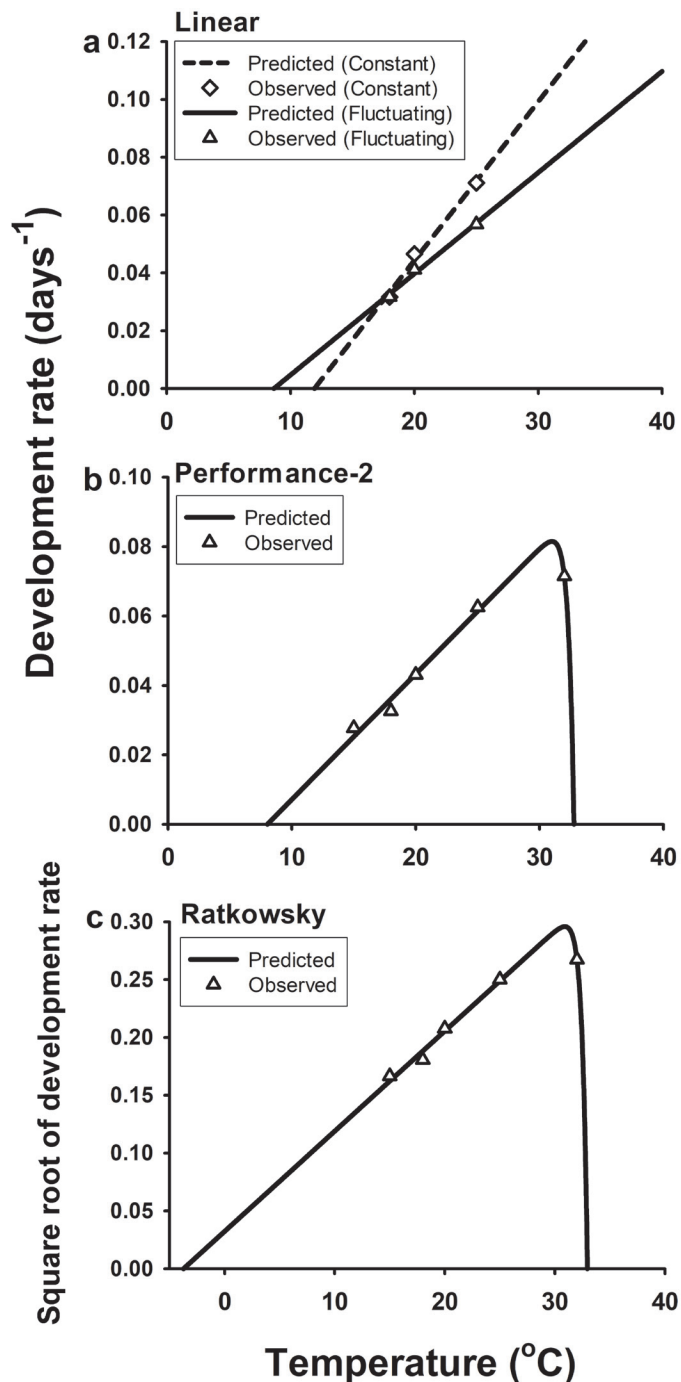


Fig. 1. Predicted rate of total development as a function of temperature for *Psyllaphycus diaphorinae* (pooled males and females) at different constant and fluctuating temperatures using linear (a), Performance-2 (b), and Ratkowsky (c) models. In the linear and Performance-2 charts, the ordinate is the rate of development ( $1/D$ , per d), and the abscissa is temperature (°C). In the Ratkowsky chart (c) the ordinate is the square root of development rate ( $\sqrt{1/D}$ , per d), and the abscissa is temperature (°C). Symbols represent mean observed data. Solid lines represent model predictions for fluctuating temperatures and dashed lines for constant temperatures. For linear regression (a), data values for 32 °C were omitted because of significant deviation from rectilinearity.

a wider temperature range (i.e., 15–32 °C), than did constant temperatures (i.e., 18–32 °C). These results support the hypothesis that thermal variation at low average temperatures, above and below the daily thermal mean, allows arthropod development to occur at temperatures outside the limits defined by constant regimens (Jensen

**Table 4.** Mean longevity in d ( $\pm$  SE) for female and male *Psyllaphycus diaphorinae* maintained under the constant and fluctuating temperatures at which they were reared.

	Longevity (mean d $\pm$ SE)				
	15 °C	18 °C	20 °C	25 °C	32 °C
<b>Female</b>					
Constant	No emergence	52.72 $\pm$ 4.81 <sup>a</sup> (18)	39.68 $\pm$ 1.93 <sup>a</sup> (50)	21.35 $\pm$ 1.17 <sup>b</sup> (26)	14.15 $\pm$ 1.58 <sup>c</sup> (20)
Fluctuating	83.55 $\pm$ 2.89 <sup>a</sup> (40)	61.61 $\pm$ 1.85 <sup>a</sup> (13)	42.13 $\pm$ 3.16 <sup>a</sup> (47)	28.69 $\pm$ 1.38 <sup>a</sup> (16)	7.61 $\pm$ 0.74 <sup>c</sup> (26)
<b>Male</b>					
Constant	No emergence	41.20 $\pm$ 1.15 <sup>c</sup> (39)	33.49 $\pm$ 2.66 <sup>b</sup> (35)	18.60 $\pm$ 1.13 <sup>b</sup> (25)	11.57 $\pm$ 1.63 <sup>b</sup> (14)
Fluctuating	56.08 $\pm$ 2.66 <sup>b</sup> (39)	52.05 $\pm$ 1.40 <sup>b</sup> (22)	41.29 $\pm$ 2.63 <sup>a</sup> (34)	30.78 $\pm$ 0.74 <sup>a</sup> (23)	2.29 $\pm$ 0.28 <sup>d</sup> (21)

Means followed by the same letter within the same temperature are not significantly different at  $\alpha = 0.05$ ; (n) denotes number of individuals. SEs are based upon the pooled variance and the individual sample size.

1906; Packard & Packard 1993; Gilbert & Raworth 1996; Kontodimas et al. 2004; Hall et al. 2011; Colinet et al. 2015; McCalla et al. 2019; Milosavljević et al. 2019a, c).

The duration of adult life span of *P. diaphorinae* increased, however, with decreasing temperature regardless of the temperature regimen, and females lived longer than males on average (Table 4). Interestingly, *P. diaphorinae* reared under high fluctuating temperatures had greatly reduced longevity in comparison with hyperparasitoids reared under constant temperature counterparts (Table 4), a finding that aligns with the results of previous studies showing a narrow range of thermotolerance for many insect taxa (Neven 2000; Colinet et al. 2015; McCalla et al. 2019; Milosavljević et al. 2019a, c). This is notable, because hyperparasitoids reared under high fluctuating temperature regimens appear to be at risk of accumulation of detrimental effects due to exposure to temperature extremes (Colinet et al. 2015), and these may be amplified further by adverse temperature effects on the host upon which they are developing.

There also were observed differences in estimated values of thermal constants (and lower development thresholds [see below]) for *P. diaphorinae* hyperparasitoids between the models and experimental temperature regimens. Linear regression estimates, for example, indicated that fewer degree d were required for completion of development for *P. diaphorinae* under constant temperature regimens when compared with fluctuating temperatures (Table 3; Fig. 1). These results are supported by other studies suggesting that phenology predictions using models fit to constant temperature data could produce unrealistic estimates of generation times for field populations that are exposed to fluctuating temperature cycles (Liu et al. 2002; Warren & Anderson 2013; Colinet et al. 2015; McCalla et al. 2019; Milosavljević et al. 2019a, c).

The theoretical lower thermal thresholds for development of *T. radiata* and *D. aligarhensis*, 2 primary parasitoids *P. diaphorinae* attacks, range from  $-6$  to  $12$  °C (McCalla et al. 2019; Milosavljević et al. 2019a). In this study, thermal thresholds, estimated by the linear and 2 non-linear (i.e., Ratkowsky and Performance-2) models ranged from  $-4$  to  $12$  °C for *P. diaphorinae* (Table 3), which falls within the previously reported range for these 2 primary parasitoids of *D. citri*. The Ratkowsky and Performance-2 models fit to fluctuating temperature data also produced similar estimates for the optimum (i.e.,  $T_{opt}$ ) and upper (i.e.,  $T_{max}$ ) development temperatures of *P. diaphorinae* with the maximum development rate being observed between  $30$  and  $32$  °C. As with low temperatures, our findings tentatively suggest that *P. diaphorinae* may be able to tolerate high temperatures (i.e.,  $> 32$  °C for  $> 13$  h; Table 1) in the field. However, these  $T_{min}$  and  $T_{max}$  values are 'conceptual' model estimates that represent the theoretical minimum and maximum temperatures at which development may occur (McMeekin et al. 2013; Ratkowsky & Reddy 2017). It is highly unlikely, however, that devel-

opment occurs at temperatures below  $0$  °C as predicted by models. Nevertheless, these theoretical values (i.e.,  $T_{min}$  and  $T_{max}$ ) could provide approximations of the actual lower and upper development limits (i.e.,  $MINt$  and  $MAXt$ ) which probably lie within these conceptual thermal bounds predicted by fitted models. To more accurately define  $T_{min}$  and  $T_{max}$  for *P. diaphorinae*, additional development studies at smaller temperature increments close to predicted values would be needed.

In summary, this study demonstrates the significant effects of different temperature exposure regimens on life history parameters of *P. diaphorinae*. Significantly, this hyperparasitoid attacks immature *D. aligarhensis* and *T. radiata*, 2 important primary parasitoids of *D. citri*, in part of its home range of Punjab, Pakistan. Results presented here indicate that realistic fluctuating temperature scenarios significantly influenced development rates and longevity of *P. diaphorinae*, resulting in significant deviations from observations for constant temperature regimens. This finding is in alignment with similar studies on *D. aligarhensis* (Milosavljević et al. 2019a) and *T. radiata* (McCalla et al. 2019), the 2 dominant primary parasitoids that attack *D. citri*.

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